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The Biology of the Germ line in Echinoderms

Gary M. Wessel^{*}, Lynae Brayboy, Tara Fresques, Eric A. Gustafson, Nathalie Oulhen, Isabela Ramos, Adrian Reich, S. Zachary Swartz, Mamiko Yajima, and Vanessa Zazueta

Department of Molecular Biology, Cellular Biology, and Biochemistry, Brown University, Providence, Rhode Island

SUMMARY

The formation of the germ line in an embryo marks a fresh round of reproductive potential. The developmental stage and location within the embryo where the primordial germ cells (PGCs) form, however, differs markedly among species. In many animals, the germ line is formed by an inherited mechanism, in which molecules made and selectively partitioned within the oocyte drive the early development of cells that acquire this material to a germ-line fate. In contrast, the germ line of other animals is fated by an inductive mechanism that involves signaling between cells that directs this specialized fate. In this review, we explore the mechanisms of germ-line determination in echinoderms, an early-branching sister group to the chordates. One member of the phylum, sea urchins, appears to use an inherited mechanism of germ-line formation, whereas their relatives, the sea stars, appear to use an inductive mechanism. We first integrate the experimental results currently available for germ line determination in the sea urchin, for which considerable new information is available, and then broaden the investigation to the lesser-known mechanisms in sea stars and other echinoderms. Even with this limited insight, it appears that sea stars, and perhaps the majority of the echinoderm taxon, rely on inductive mechanisms for germ-line fate determination. This enables a strongly contrasted picture for germ-line determination in this phylum, but one for which transitions between different modes of germ-line determination might now be experimentally addressed.

INTRODUCTION

Diversity in Development

As remarkable as the mechanism of fate acquisition is in early development, perhaps even more striking is the diversity by which this task is accomplished. Syncytial early development (e.g., insects), invariant cleavage (e.g., ascidians), variable cleavage (e.g., mammals), discoidal (partial) cleavage (e.g., birds), etcetera all contribute to the early embryogenesis—and it gets even more variable and complex after formation of the various cellular layers (germ layers) that give rise to the tissues outside (ectoderm), inside (endoderm), and in between (mesoderm). Not only is the final product diverse, but the mechanisms of getting there are too. Certainly animals have many commonalities—the

phylotypic stage, originally popularized by von Baer, is one good example (see e.g., Gilbert, 2013), yet diversity is more the rule than the exception.

Diversity of Germ Cell Development

Germ cells are not exempt from this developmental diversity—in fact they absolutely excel at this trait. The germ line is the lineage of cells that leads to construction of eggs and sperm. While many tissues and cells are essential for this development, for example, the gonads (ovary and testis), the germ line is distinct from those because it is capable of passing hereditary information (genome) from one generation to the next, whereas all other cells (the so-called somatic cells, or soma) are programmed to terminate with the adult.

The first-popularized publication documenting the use of the term "germ" for the reproductive lineage is a comprehensive monograph on the origin of germ cells in 38 species of marine hydroids, by August Weismann in (1883). In it, this giant in the field of developmental biology detailed the anatomy of various stages in the life cycle of these organisms, including the migratory precursors of germ cells (*Keimzellen*). The term *stem cells* (*Stammzellen*) was used in this monograph for the first time to point out putative migratory sperm progenitors. The terms *primordial germ cells* (*Urkeimzellen*) and *germ line* (*Keimbahn*) appear throughout the work, which charts the life cycle of the *Keimzellen*. The concept of *germ plasm* (*Keimplasma*) was discussed in a later publication, from an 1885 lecture given at the University of Freiburg, Weismann (1889), in reference to a distinct area of cytoplasm in the egg and early embryo that was always associated with the germ-line lineage. Here "germ" is used not to describe a pathogenic microbe, instead signifying the rudiment of a living organism, the initial stage in development, or the source for subsequent development (germ from the French *germe*, and the Latin *germen* meaning sprig, bud, or seed).

An important concept of the germ line is continuity. Following successful fertilization of functional gametes, the developing embryo will eventually make primordial germ cells (PGCs), a lineage that will commit largely if not exclusively to the germ cells. At some later point in development, the PGC population will expand through mitosis as germ-line stem cells and eventually begin gametogenesis (development of eggs and/or sperm). Successful fertilization of the next generation completes the cycle. This scheme is generally true for the germ line of most metazoans, although the details are markedly variable between even closely related species. For excellent perspectives on diversity in reproduction, consider the monographs by Birkhead (2002) and by Judson (2003).

Unique PGC Features

PGCs are precursors, formed in the embryo, that give rise to eggs or sperm. In many ways, they are the mother of all stem cells, and as such, their formation and development may have embellishments. Without a functional germ line, sexually reproducing organisms cannot reproduce, and the unique genetic composition of that individual is lost. While many cells of an organism are necessary for the germ line to develop and to be maintained, the germ-line lineage is distinct from the supporting cells (the soma) of the organism.

PGCs usually form in one portion of the embryo, sometimes even in association with the extra-embryonic tissues, and in a space distinct from the somatic portion of the gonad. The PGCs almost universally migrate extensively from their birthplace to the developing gonadal tissues, and do so in a defined period of embryogenesis; if they fail to reach the developing gonad by the end of this time window, the PGCs usually die by apoptosis. It is feasible that this PGC migration is a test that has been retained to select for PGCs bearing intact mechanisms for signal transduction, migration, homing, etcetera—characteristics essential for development of the next generation and reflective of an intact, and functional genome. Failure of the PGCs to apoptose may result in their conversion to teratomas (from the Greek *teras* for monster, or prodigy), which contain a variety of fetal-like tissues from all three germ layers. Although potentially dangerous for the individual harboring these growths, it does show the complete developmental potential retained by the PGCs.

Formation of PGCs in Animals

PGC fates may be acquired by a variety of contrasting mechanisms in animals, from a multipotent cell in adults, by *inherited* molecules, or predominantly by *inductive* cellular interactions. These developmental strategies are likely overlapping in mechanism with many shared intermediates.

The inherited mechanism, in which maternal instruction segregated within the oocyte drives development of PGCs in the cells that have acquired it. This mechanism has been widely studied in diverse organisms such as *Drosophila*, *Caenorhabditis elegans*, and *Danio rerio* (see e.g., Gao and Arkov, 2013; Lai and King, 2013; Voronina, 2013). Given their short generation time, the genetic screening approaches available, and their effective adaptation for laboratory research of these organisms, it is not coincidental that research on these organisms has led the field in elucidating mechanisms for germ-line specification. The PGCs in this group of organisms are segregated early during embryogenesis and contain electron-dense cytoplasmic organelles, called germ plasm (Geigy, 1933; Bounoure, 1934; Jazdowska-Zagrodzinska, 1966; Smith, 1966; Buehr and Blacklar, 1970; Illmensee and Mahowald, 1974; Okada et al., 1974; Eddy, 1975; Warn, 1975; Frohnhof et al., 1986; Ikenishi et al., 1986; Niki, 1986). The germ plasm in these animals was found to contain many unique RNAs and proteins that are conserved among diverse organisms, molecules such as Vasa (Seydoux and Braun, 2006; although see also Kassmer and Krause, 2013). These molecules are now often conveniently used as markers for researchers to identify PGCs in many organisms because of their conserved expression pattern in the germ line. Formation of these cells early in development means they are often insulated from normal developmental activities and are relatively quiescent for much of embryogenesis.

The inductive mechanism of PGC specification (fate determination by cell interactions) is less well understood, and is studied primarily in the mouse (Surani et al., 2008; Lesch and Page, 2012). This mechanism of germ-line determination appears to rely on cell-to-cell signaling molecules, which impinge upon changes in the epigenetic landscape in cells that are otherwise destined to become somatic mesoderm. Inductive-derived PGCs generally form later in embryos than in the inherited strategy. Multiple epigenetic modifiers, including *Prdm* family members, appear necessary in the early mouse germ-line precursors to repress

genes involved in a somatic mesoderm fate, including *Hox* genes. The frequency and distribution of animals utilizing inductive determination suggest that it is the basal or ancestral strategy of germ-line determination; animals using the inherited mechanism instead appear to have independently acquired this mechanism. This apparent transition in developmental mode is prevalent throughout phylogeny (e.g., Extavour and Akam, 2003), and may result in a continuum of mechanistic change between organisms (Juliano and Wessel, 2010; Seervai and Wessel, 2013).

The third general mechanism of germ-line determination, the adult-derived mechanism, is probably also reliant heavily on inductive interactions, if only because it is difficult to image a prolonged, segregated portion of maternal cytoplasm remaining in adults. Significant research is devoted to this topic in planaria and hydra (e.g., Chong et al., 2011; Rouhana et al., 2012) as it is associated with the vast potential for understanding totipotent adult stem cells.

Echinoderms have recently become popular for studying germ-line determination. The same properties that have made these animals amenable to understanding fertilization, calcium regulation, gene regulatory networks, sperm activation, chemotaxis (e.g., Ettensohn et al., 2004; Davidson, 2010; McClay, 2011; Peter and Davidson, 2011) make these embryos useful to understand germ-line determination. Large numbers of embryos ($>10^6$ per adult) can be obtained that are mostly transparent; develop predictably and synchronously; are facile to manipulation; and have a rich, classical literature describing them. Further, these animals represent a sister group to chordates in the deuterostome clade, and the research community has generated large amounts of genomic knowledge and resources (e.g., spbase.org; Weinstock et al., 2006).

Early Development of the Sea Urchin

Sea urchin embryos develop rapidly (15–60 min cell cycles depending on the species), synchronously, and predictably. Large cultures can be cultivated, treated, and dissociated, while individual embryos can be injected or manipulated to test important features of their developmental mechanisms. The first three cell divisions in the sea urchin are equal and yield a symmetrical eight-cell embryo (Fig. 1). The fourth cell division, however, is uneven—the four embryonic cells (blastomeres) that form on the animal hemisphere divide equally yielding eight uniform cells of intermediate size (mesomeres), whereas cells of the vegetal-tiered hemisphere divide unequally, yielding the four largest cells (macromeres), and the four smallest cells at this stage (micromeres). The mesomeres and macromeres of the early embryo will form the majority of the ectoderm and endomesoderm, respectively, whereas the micromere lineage quickly diverges to extremes in cell fates and function.

The micromeres are an inductive signaling center of the early embryo. Classic experimental approaches by Horstadius found that micromeres were capable of inducing other embryonic cells to develop into endomesodermal fates (Horstadius, 1973). This property was most apparent when transplanted to the animal pole (opposite their normal site), resulting in a host embryo with a second, new endomesodermal axis (Ransick and Davidson, 1993). The mechanism behind this induction is not understood, but the micromeres do acquire nuclear

beta-catenin shortly after their formation, and evidence suggests Notch-Delta, and Wnt8 signaling as candidates for this inductive process (e.g., McClay, 2011).

Further division of the micromeres yields the large micromeres (LMics) and the small micromeres (sMics)—two developmental extremes. LMics are fated to the skeletogenic lineage, and ingress into the blastocoel several hours later as primary mesenchyme cells. This ingression (individual cells detaching from an epithelium and moving into a new space) is associated with their loss of adhesion to other cells, secretion of enzymes associated with dissolution of the basal lamina, followed by an increase in adhesion for the extracellular matrix on the opposite side of the basal lamina (Fink and McClay, 1985; McClay, 2011). A fixed number of primary mesenchymal cells survive ingression, depending on the species, and this population will migrate throughout the vegetal hemisphere of the blastocoel. They eventually aggregate and begin depositing a calcite-based internal skeleton consisting of various rods, connections, and barbs; skeleton morphologies are species-specific (Ettensohn, 2009; McClay, 2011; Rafiq et al., 2012). While many treatments may inhibit developmental progression to actually forming skeletons, no reported experimental approaches, tests, or perturbations have diverted the fate of LMics from a skeletogenic one to a different cell/tissue type. Further, when cells are placed in culture, in isolation from all other cells of the embryo, they still replicate the primary mesenchymal cell phenotype and behavior, and even synthesize skeletal elements (e.g., Okazaki, 1975). The sMics, on the other hand, appear to contribute to the germ line (see below). Thus, within one cell division, the two progeny gain extremes of developmental fate: the LMics become the singular, somatic, skeletogenic lineage that will terminate with metamorphosis of the larvae, whereas the sMics contribute to the germ line and are thus gene rationally immortal.

Another distinction between the micromere descendants is their subsequent impact on the embryo. Micromeres are the major signaling center of the embryo (Horstadius, 1973; see also Ransick and Davidson 1993). After cell division, however, this inductive capability is retained by LMics but is essentially lost in the sMics. For example, the sMics in *Hemicentrotus pulcherrimus* have only 1/10th the inducing strength of one LMic (Kurihara and Amemiya, 2005). Horstadius 1973 documented the inducing capacity of micromeres by a series of transplants, either moving donor micromeres to ectopic sites in an entire host animal, or to various tiers of early embryos. When the animal-most tier (mesomere lineage) was opposed to the micromeres, a normal looking (though smaller) embryo formed with all the characteristics of a normal gastrula and larvae. This result showed both the strong inducing capacity of the micromeres, and the total plasticity of early embryonic cells to receive and respond to such signals by changing their developmental fates to become cell types, even different germ layer derivatives, that they would normally not acquire. Remarkably, a competent larva can form and metamorphose when derived from only mesomeres and micromeres; Minokawa and Amemiya (1999) showed that the larvae formed by these animal cap inductions, devoid of macromeres, were capable of metamorphosing to form a normal adult, and that the sMics would form from the micromeres and exhibit normal developmental programing. Development of these juveniles to reproductive age, however, was not reported.

The sMics Contribute to the Germ Line of Sea Urchins

Several lines of reasoning and experimentation suggest that the sMics contribute to the germ line. These include their cellular behavior, the molecules they harbor selectively, and results from perturbation studies—that is, in their absence, the embryo is not gravid (do not make gametes).

The behavior of the sMics first suggested their role in the germ line. These classically defined behaviors of PGCs include: (1) formation following (two) asymmetric divisions (Fig. 2); (2) a large nuclear-to-cytoplasmic volume ratio, with a cytoplasm depleted in many organelles, especially yolk (Pehrson and Cohen, 1986); (3) overly condensed chromatin relative to their siblings beginning at their formation (Tanaka and Dan, 1990); (4) a slow cell cycle relative to their neighbors, dividing only twice through development to the late gastrula stage (Fig. 3; Pehrson and Cohen, 1986; Tanaka and Dan, 1990); (5) transcriptional repression, as seen by chromatin modifications and RNA polymerase activity (Swartz et al., 2013); and (6) migratory pattern, reaching the tip of the gut during gastrulation and integrating into the coelomic pouches, the site of adult rudiment formation (see Cameron et al., 1987; Cameron, 1991, for discussion of lineage tracing). Each of these sMic features is consistent with the hypothesis that they are, or at least contribute to, the germ line in the sea urchin. It should be noted that long-term labeling of the sMics has not been accomplished, so it is not possible to conclude what various fates the lineage may contribute to, but it appears that the sMics do not contribute to any functional structures of the embryo (Cameron et al., 1987; Cameron et al., 1991) and instead is devoted to the germ line (Yajima and Wessel, 2011). Ideally the cells should be traced with a marker gene (fluorescent protein gene embedded in the genome) so that the fate can be traced longitudinally, throughout its development, particularly in the rudiment. This might be accomplished by identification of a sMic-unique promoter that can be used to drive GFP expression, or using a constitutive promoter driving GFP to mark transplanted sMics in a host embryo that is unlabeled or labeled with a different fluorescent protein. Certainly integrating genes into a select site of the genome now should be possible in this organism, especially with newly established zinc finger nuclease constructs or TALENs, as reported for a variety of other cell types and organisms (e.g., Xiao et al., 2013). One drawback for this approach becoming routine is the prolonged period needed to derive a subsequent, reproductively competent generation, which can take more than several months to multiple years, depending on the species, and limits long-term and repetitive analysis on lines of such genetically manipulated animals.

A screen for genes that, in other organisms, are involved in germ-line determination was undertaken to identify orthologous sequences involved in germ-line determination in the sea urchin, and its results also suggested that the sMics contribute to the germ line (Juliano et al., 2006). Fourteen selected genes were identified in the genome of *Strongylocentrotus purpuratus*, and were tested by in situ hybridization and qPCR. These included Boule (RNA binding protein), Vasa (DEAD-box containing RNA binding proteins), Seawi (piwi ortholog involved in piRNA function; see e.g., Mani and Juliano, 2013), germ cell-less (nuclear pore-associated protein; see e.g., Voronina, 2013), and Ovo (the zinc-finger containing transcription factor). The transcripts of almost all of the genes identified were found to accumulate uniformly in oocytes throughout oogenesis—no evidence was apparent to

suggest that these oocytes compartmentalized, or segregated the conserved mRNAs into a germ plasm. This does not eliminate the possibility that proteins derived from these transcripts are produced and aggregate distinctly in the cytoplasm, but at least the mRNAs inform us of the absence of transcript segregation and that the mechanism of germ-line acquisition may not be a strict inheritance mechanism in sea urchins. The transcripts encoding Pumilio (RNA binding protein), Tudor (scaffolding protein), MSY (RNA binding protein), and CPEB1 (translational regulator through polyadenylation) were also found to be uniformly distributed during embryonic development. In contrast, the transcripts for Vasa, Seawi, and Ovo became enriched in the vegetal plate of the mesenchyme blastula stage while Vasa, Nanos2, and Seawi localized in the descendants of the sMics at the tip of the archenteron during gastrulation, and were then enriched in cells of the left coelomic pouch in larvae (Juliano et al., 2006; Fujii et al., 2009). These results suggested that (1) the sea urchin does not use a germ-plasm mechanism of germ-cell determination and (2) the sMics are candidates that contribute to the germ line.

Ransick et al. (1996) indirectly tested if the sMics constitute a definitive PGC lineage during embryonic development. They removed the micromeres, the parent cells of the sMics formed at the fourth cleavage (Fig. 1), and cultured the remaining embryo. Remarkably, the animals developed rather normally, and even metamorphosed on time. More-over, most of the adults made gametes. They concluded that the germ-cell lineage is not formed obligatorily by the fourth cell division, and instead, that it must segregate during postembryonic development, within the adult rudiment, or even following metamorphosis (Ransick et al., 1996). Support was garnered for this conclusion from the literature (McBride, 1903) in which cells with germ-cell cytology were described initially in the coelomic pouches of metamorphosing animals. This led to the conclusion that the germ line is not definitively established until the basic body plan of the adult is formed.

The adult body plan of sea urchins and other echinoderms is pentaradially symmetric starting with the adult rudiment, which forms within a bilaterally symmetric larva prior to metamorphosis. The sea urchin embryo is also a maximally indirect developer—that is, the adult has a distinct body plan that forms only from the rudiment formed in the larvae (Peterson et al., 1997; Davidson and Erwin, 2006). Thus, were germ lines not formed until the adult body plan was established, then the germ line would not be specified until after rudiment formation (Dixon, 1994; Ransick et al., 1996). Other animals used for studies in germ-line determination, such as flies, nematodes, and frogs, are more direct, indirect developing organisms in terms of body axis formation. Each of these animals establishes an axis in early development that is basically the same as the adult axis, and they specify their germ lines early in development, whereas sea urchins, molluscs, and sea stars, among other organisms, do not form their adult body axis until late in larval development and their germ line is argued to be specified only following this period (Dixon, 1994; Ransick et al., 1996). A caveat to these conclusions for maximally indirect developers is that, with the exception of the sea urchin undergoing micromere removal (Ransick et al., 1996), assessment of the germ line has been made only cytologically, not functionally. It should be emphasized here also that no definitive germ plasm has been reported in sea urchins during embryonic development.

Removal of the micromeres at the 16-cell stage results in significant compensatory development and fate transitions within the embryo (Ransick and Davidson, 1993). Further, removal of the LMics (the primary mesenchymal cells) just prior to gastrulation results in trans-fating of pigment cells to primary mesenchymal cells, whereby other mesodermal cells change fate to become skeletogenic cells, a fate they would not normally display (Ettensohn and McClay, 1988). In the early embryo, micromeres have a capacity to induce new axial development when transplanted to ectopic positions, and their removal results in a delay of development in the mesendodermal tissues of the embryo even though the embryos form normal larvae (Horstadius, 1950; Ransick and Davidson, 1995). Removal of the micromeres also results in a significant up-regulation of Vasa, the RNA helicase, in all remaining cells of the embryo from *Strongylocentrotus purpuratus* (Voronina et al., 2008). This is significant in that Vasa is believed to be involved in germ-line specification and maintenance (Lasko and Ashburner, 1988; Lasko and Ashburner, 1990; Tomancak et al., 1998; Tanaka et al., 2000; Styhler et al., 2002; Salinas et al., 2007). Indeed, the micromeres and then the sMics are the sole cells that retain substantial Vasa in a normal embryo, and its up-regulation throughout the embryo following micromere removal may have a compensatory function in re-specifying the multipotent cell line or another germ-line lineage (Voronina et al., 2008).

Given the hypothesis that the sMics contribute to the germ line in adult sea urchins, this population was depleted at the 32-cell stage and the resultant embryos were raised to adulthood (Yajima and Wessel, 2011; Figs. 4 and 5). These embryos developed normally, on schedule with their control siblings and with all embryonic structures, and the larvae developed adult rudiments that metamorphosed. The adults, however, were sterile with gonads that lacked gametes, whereas adults from control and micromere-deleted embryos developed gonads containing gametes, suggesting that the sMics do indeed contribute to the germ line. (Recall that lineage analysis of the sMics has not been extended beyond gastrulation, so strictly referring to them as PGCs has not been resolved. It is possible that their lineage diverges in the adult rudiment to also contribute to somatic lineages that may enjoy compensatory development upon sMic removal earlier in development.) These results suggest that timing for an initial PGC-like specification might occur after the 16-cell stage, but that the PGC precursors have segregated by the 32-cell stage. The mechanism of specification of these cells is not understood, yet some interesting observations have been found. sMicdepleted embryos developed normally but did not express compensatory levels of Vasa, as did embryos from a micromere deletion, implying that the compensatory gene regulatory network was not activated in sMic-deleted embryos (Fig. 5). This is compatible with the finding that sMics have lost inductive or cell signaling capability, which was held by their precursors, the micromeres. Thus, the sMics may begin insulating their cellular interactions soon after their establishment, which is a feature similar to PGCs of other organisms (Seydoux and Braun, 2006). While direct lineage tracing experiments certainly need to be accomplished in the future, we can conclude from the results so far that germ-line specification in the sea urchin depends on the sMics, either directly as lineage products or indirectly by a later signaling mechanism that directs neighboring cells to this fate.

Asymmetric Cell Divisions in Formation of sMics

The sea urchin embryo undergoes two sequential, asymmetric cell divisions of micromeres at the 4th and 5th cleavages to yield the LMics and the sMics. This cleavage pattern is invariant, is the first observable break in symmetry, and leads to differential cell fates. The mitotic spindles of these unequally dividing micromeres move toward the vegetal pole prior to cytokinesis and are peripherally located to the vegetal cortex; the aster of the micromere pole appears to be anchored to the vegetal cortex and presents a characteristic flattened shape. Quantitation of immunofluorescence signals of centrosomal materials demonstrated that micromere centrosomes are smaller than the macromere centrosomes (Holy and Schatten, 1991). These observations suggest that centrosomes of the micromere are different than other centrosomes, perhaps in content, density, or at least in distribution of components, thus providing a unique, microtubular environment at the vegetal pole. In addition to resultant cell-size differences, this asymmetric mitotic matrix possibly contributes to asymmetric distribution of germ-line components such as Vasa protein during the process of unequal cleavage at the 4th and 5th cell divisions (Figs. 6 and 7; Yajima and Wessel, 2011).

Although detailed mechanisms of these unequal cell divisions at the 16- and 32-cell stages is understudied in the sea urchin, several lines of functional evidence suggests a conserved mechanism of asymmetric cell division. This evidence includes pharmacological interference and dominant-negative protein expression, both indicating that the heterotrimeric G protein G_i and its interaction partner, activator of G-protein signaling (AGS), are necessary for the asymmetric cell divisions in many organisms such as *Drosophila*, *C. elegans*, and mammals (Morin and Bellache, 2011). The AGS/Pins (activator of G-protein signaling/partner of invertebrate scute) family of proteins are conserved, multi-domain molecular scaffolds found in many organisms from yeast to humans, and are reported to be involved in asymmetric cell divisions so far in *Drosophila*, *C. elegans*, and sea urchins (Schaefer et al., 2000; Betschinger and Knoblich, 2004; Voronina and Wessel, 2006; Park and Rose, 2008; Krueger et al., 2010; Morin and Bellache, 2011). In the sea urchin, inhibition of $G_{\alpha i}$ signaling by pertussis toxin interfered with micromere formation and led to defects in embryogenesis (Voronina and Wessel, 2006). Introduction of exogenous, dominant-negative AGS proteins, containing only the G-protein regulatory (GPR) domains, selectively prevented the asymmetric division during normal micromere formation (Voronina and Wessel, 2006). These results imply that cortical proteins located at the vegetal pole, where these proteins accumulate, regulate the asymmetric cell divisions in this embryo, and thus may be involved in cell-fate specification of the sMics to that of the germ-cell lineage.

Another pathway involving the Wnt-signaling mechanism may also be involved in these processes of asymmetric cell division and cell fate specification. Earlier studies of the testis in *Drosophila* found that germ-line stem cells (GSCs) normally divide asymmetrically, giving rise to one germ-line stem cell and one gonoblast (Yamashita et al., 2003). These germ-line stem cells use intracellular mechanisms involving centrosome function and a cortically localized Wnt-pathway member, Adenomatous Polyposis Coli (APC), to orient their mitotic spindles perpendicular to the stem-cell niche within the testis. This spindle directionality provides an asymmetric outcome in which one daughter cell remains in the

niche and self-renews its stem cell identity, whereas the other cell becomes displaced from the niche and initiates differentiation into spermatocytes. In *C. elegans*, extrinsic Wnt signaling modulates spindle structures through APR-1 (the *C. elegans* APC-related gene)/APC during telophase of the asymmetric division of EMS cells at the four-cell stage, which in turn promotes asymmetrical nuclear localization of WRM-1/beta-catenin and POP-1 (the *C. elegans* TCF/LEF transcription factor)/TCF (Sugioka et al., 2011). APR-1 is asymmetric along the cortex, and can cause an asymmetric distribution of astral microtubules. Perturbation of the Wnt signaling pathway altered this microtubule asymmetry and led to changes in nuclear WRM-1 (*C. elegans* beta-catenin) asymmetry, gene expression, and cell-fate determination, suggesting that the nuclear localization of proteins is regulated through the modulation of asymmetric microtubules and that this regulates cell fate (Sugioka et al., 2011).

The sea urchin embryo may have incorporated similar mechanisms for asymmetric cell division. Although still untested, these conserved Wnt pathway molecules, such as Wnt8 (Croce and McClay, 2006; Kumburegama and Wikramanayake, 2008), Dishevelled (Wikramanayake et al., 1998; Ettensohn, 2006), and nuclear beta-catenin, are localized at the vegetal pole and/or in the micromeres and sMics (Emily-Fenouil et al., 1998; Wikramanayake et al., 1998; Logan et al., 1999; Vonica et al., 2000). These Wnt-pathway molecules are thought to establish an initial animal-vegetal (A-V) polarity in this embryo, but may also participate in the asymmetric cell division of micromeres. Wnt and Delta, another micromere-enriched signaling molecule (Oliveri et al., 2002; Oliveri and Davidson, 2004), could be involved in signaling between adjacent cells to establish a differential signal between micromeres and macromeres at the 16-cell stage, leading to cell-fated asymmetry in the micromeres that gives rise to the LMics and sMics at the next cell cycle. Importantly, a recent report demonstrated the involvement of Wnt3a in expansion of the early PGC population in the mouse embryo (Bialecka et al., 2012), implying that the Wnt pathway may be involved in PGC specification in many organisms. Testing direct contributions of Wnt pathway molecules to the asymmetric cell division for LMic and sMic formation and to the cell specification of PGCs in the sea urchin would be very useful, especially as it relates to the asymmetric distribution of germ-line determinants or factors. Directly linking a well-known signaling pathway to the distribution of a germ-line factor (Vasa) would provide important mechanistic insight for stem-cell determination in general, especially since germ-line factors are found broadly in stem cells and multipotent cells throughout phylogeny.

Calcium in Specification of sMics

Calcium dynamics in the micromeres—The ion calcium is a versatile intracellular messenger in eukaryotic cells. Calcium is the most ubiquitous of the intracellular messengers, and most cell types contain conserved calcium signaling machinery (Carafoli et al., 2001). Its concentration within the cytoplasm is spatially and temporally controlled by ion channels, exchangers, and pumps, which coordinately are able to elevate calcium levels over 1,000-fold (to several micromolar amounts) in less than a second, and then promptly restore their low, resting levels (nano-molar concentrations). Calcium-transporting proteins are located in the plasma membrane and in the membranes of organelles such as the endoplasmic reticulum, the mitochondria, and the lysosomes, where they each play specific

roles in the cellular homeostasis of calcium. In general, the transduction of the calcium signals is mediated by reversible binding to specific classes of proteins that act as calcium sensors.

Historically, fertilization calcium waves and local calcium signals, as well as their mechanisms of propagation and many of their fundamental components, were discovered and heavily studied using sea urchin eggs and sperm. It is known that calcium signaling at fertilization is accomplished by the coordinate action of various mechanisms, including different messengers (e.g., cADPR, NAADP, and IP₃), different intracellular stores (e.g., the endoplasmic reticulum and endolysosomes), and different calcium channels and receptors (e.g., plasma membrane voltage-gated ion channels), which have all been remarkably conserved through the course of evolution (Carafoli, 2001; Whitaker, 2006). Cell divisions utilize calcium during mitosis, so the question becomes: Do the distinct cells that are rapidly generated after the early cleavages in an embryo utilize calcium signals to accomplish coordinated tasks such as the asymmetrical mitotic divisions, axis patterning, and cell fate?

In sea urchin embryos, many large-scale cell fate decisions are made along the A–V axis. By the 60-cell stage, the A–V axis is further patterned to produce five distinct territories that are specified by a signal transduction cascade initiated by the micromeres at the 16-cell stage (McClay, 2011). The micromeres are characterized not only by their small size, but also by their distinct plasma membrane, cytoplasmic inclusions, and nucleus. The plasma membrane of the micromere is mainly composed of a smooth, non-villar membrane (Yazaki, 2001). The smooth membrane is more adhesive than the villous membrane originally seen in other blastomeres (Kuraishi and Osanai, 1989), and lacks a specific egg surface protein (ES-1) (Yazaki, 1993). Intercellular ion communication remains between the micromeres and the macromeres during early development (Yazaki et al., 1999). The micromeres are also characterized by nuclear accumulation of beta-catenin, a cofactor of the TCF transcription factor that is required to specify the vegetal-cell fate in the sea urchin embryo (Logan et al., 1999).

The endoplasmic reticulum in the micromeres—Staining the endoplasmic reticulum was accomplished by microinjecting the egg with a saturated solution of the fluorescent dye dicarbo-cyanide DiLC16 (according to the method of Terasaki and Jaffe (1991) The dye spreads from an oil drop into endoplasmic reticular membranes, but not into other organelles) and shows an apparent tubular membrane network in the cortex of the blastomeres without any obvious polarity until the two-cell stage. From the four-cell stage, however, the endoplasmic reticulum gradually accumulates in the vegetal half of the embryo, and finally becomes enriched in the vegetal-pole cortex of micromeres at the 16-cell stage (Fig. 8; Yazaki, 2001; Yazaki et al., 2004).

Plasma membrane currents and L-type voltage-gated calcium channels in the micromeres—Calcium-dependent currents and steady state conductance in early sea urchin blastomeres were measured using whole-cell patch-clamp techniques, showing that L-type calcium channels are asymmetrically distributed along the A–V axis and very few are found in the micromeres (Dale et al., 1997). The calcium currents (all occurring during metaphase of the cell cycle) decreased from 8.5 $\mu\text{A}/\text{cm}^2$ at the four-cell stage to 5.4 pA/cm^2

at the eight-cell stage. In 16-cell stage embryos, calcium currents were $7.4 \mu\text{A}/\text{cm}^2$ in the mesomeres, $2.3 \text{ pA}/\text{cm}^2$ in the macromeres, and were not detectable in the micromeres (Table 1; Dale et al., 1997). Whether or not blocking any of these ion fluxes alters the asymmetric phenotype in the embryo is not known, but would be an important addition to the studies demonstrating that protein and organelle asymmetry contribute to this special cell division.

High resting calcium concentrations and characteristic global calcium oscillations in the micromeres

The intracellular concentration of calcium was measured directly in embryos at the 16-cell stage using the calcium indicator Fura-2/AM and a mixture of Dextran-conjugated Oregon green-BAPTA 488 and Rhodamine red injected originally into the egg (Yazaki, 2001). These injected embryos then were removed from their fertilization envelopes, and the blastomeres were separated at the four-cell stage. Following two more cell divisions, this procedure results in the formation of many quarter embryos consisting of two mesomeres, one macromere, and one micromere. Calcium fluxes in the cells were then assessed with the calcium indicators, which revealed that resting calcium concentrations are characteristically elevated in the micromeres during the 4th cleavage. Within a quarter embryo, the fluorescence-intensity ratio of micromere-to-macromere for two sea urchin species (*H. pulcherrimus* and *Pseudocentrotus depressus*) is significantly higher than 1.0, and is significantly different from the ratios in mesomere/macromere and mesomere/mesomere pairs ($P < 0.05$); all blastomeres other than the micromeres essentially have the same intracellular calcium concentration (Table 2) (Yazaki, 2001). Calcium levels subsequently oscillated for about 10 min in the micromeres. Repeated elevations of calcium were measured until the levels stabilized in later stages of the mitotic cycle (Fig. 9).

Oscillations do not occur in calcium-deficient sea water, and are evoked by the addition of calcium ions into the bathing medium, suggesting that calcium influx initiates the calcium transients. Since L-type voltage-gated calcium channels seem to be absent from the micromere plasma membrane, other types of calcium channels must be involved in the initiation of these oscillations. Pharmacological studies, using a blocker of IP3-mediated calcium release (Xestospongol), a store-operated calcium entry inhibitor (2-aminoethoxydiphenyl borate (2-APB)), and an inhibitor of stretch-dependent ion channels (gadolinium), suggest that the high calcium concentrations and oscillations in the micromeres are triggered by calcium influx caused by the activation of stretch-dependent calcium channels, followed by the release of calcium ions from the endoplasmic reticulum (Yazaki, 2001; Yazaki et al., 2004). It is not clear if this calcium behavior is a common feature of asymmetric divisions, where the products are so disparate in size, yet a physiological explanation to the asymmetric cell division in this embryo seems important to consider for future studies. It is also important to consider how asymmetric signaling pathways (Wnts, G-proteins, as discussed above) may also impact physiological behaviors such as calcium flux in different cells.

Micromeres—Possible Role of Calcium as a Signaling Messenger

Embryos come in all shapes and sizes. In sea urchins, the specification of the micromeres and subsequent LMics and sMics is a direct consequence of the variation in the topography of cytokinesis during the very early divisions. The location of the mitotic spindle determines the point of the cleavage furrow constriction and membrane addition and, therefore, the resultant size of the daughter cells (Shuster and Burgess, 2002). In early sea urchin embryos, it is known that separation of sister chromatids is accompanied by a brief, sharp, small calcium transient, an order of magnitude smaller than those measured at fertilization (Groigno and Whitaker, 1998). The transient occurs ~12 min before spindle elongation and always precedes it. Thus, it is reasonable to suppose that calcium is involved in controlling the formation of the furrow during cytokinesis. Whether or not calcium signals are directly involved in positioning the cleavage furrow to generate the embryos asymmetrical cells is less certain, however. The position of the furrow is determined by the disposition of the spindle's astral microtubules, and it appears that the spindle forms in the region of lowest microtubule density (Yoshigaki, 2001). It is known also that the contractile ring in the furrow is an actomyosin-based motility system (Kiehart et al., 1982). In smooth muscle, contraction is regulated by calcium via calmodulin activation of myosin light-chain kinase (MLCK). It has been shown in sea urchin that calmodulin is activated at the cleavage furrow, and that cleavage is inhibited by an anti-MLCK peptide (Torok et al., 1998). Thus, one can speculate that localized calcium signals and calmodulin activation may be involved not just in the control of the furrow but in determination of the position of the furrow (Wilding et al., 1995). It is also important to note that sea urchin embryos can divide and develop normally in seawater lacking calcium, so the inference is that release from internal stores is sufficient for proper signals, as happens at fertilization, but contributing influx from the external media is likely to occur in normal seawater.

Endoplasmic reticular accumulation, high resting calcium concentrations, and repetitive calcium transients markedly occur in the micromeres starting at the time that their fate is determined, right before the 4th division, together indirectly implicating a role for active calcium signaling during vegetal specification in sea urchin embryos (Yazaki, 2001; Yazaki et al., 2004). In sea urchins, it is known that Wnt proteins signal through multiple pathways that regulate developmental events, including cell polarity, cell fate, and body-axis specification (Logan and Nusse, 2004). The primary outcome is the stabilization of cytosolic beta-catenin; in fact, specification of the micromeres depends on the nuclear localization of beta-catenin by the late 16-cell stage (Logan et al., 1999; McClay, 2000), which will activate vegetal-specific genes by binding to a specific transcriptional regulator (TCF). Beta-catenin initially accumulates in the cytoplasm, and is then transferred to the nucleus only in the micromeres in the late phase of the 16-cell stage (20 min after the 4th cleavage) (Logan et al., 1999). Its nuclear localization is regulated by the activity of casein kinase I (CKI) and glycogen synthase kinase 3 β (GSK3 β) (Amit et al., 2002; Leonard and Etensohn, 2007). Calcium gradients along the A–V axis begin before the 4th cleavage, and are likely initiated by stretch-dependent calcium influx, not by Wnt signals themselves. Since the increase in calcium apparently precedes nuclearization of beta-catenin, it is possible that calcium regulates protein kinase C (PKC) activity, thereby interfering with GSK3 β activity. This raises the suggestion that the local accumulation of endoplasmic reticulum in the vegetal

half of four-cell stage embryos is the first step in the process that gives rise to the micromeres.

The majority of data linking calcium signals and pattern formation comes from vertebrate embryos. It is known, for example, that in *Xenopus* and zebrafish, the canonical Wnt pathway does not involve calcium at all. In the alternative, planar cell polarity Wnt/calcium pathway, however, a second class of Wnts signals maintains ventral cell fate (Kuhl et al., 2000a), and these Wnts can activate PKC and calmodulin kinase II (CaMKII) and can interact with an exclusive subset of Frizzled receptors (a family of G-protein receptors) (Kuhl et al., 2000b). Wnt/calcium signaling occurs through Dishevelled (Dsh), a multivalent protein that interacts with the GSK3 β /beta-catenin pathway via a so-called DIX domain. Although Dsh was one of the first components described in Wnt pathways, its function still remains puzzling. It is known that a Dsh DIX construct is able to activate both PKC and CaMKII (Sheldahl et al., 2003), and that a morpholino oligonucleotide directed against endogenous *Xenopus Dsh* blocks activation of PKC, as measured enzymatically (Kuhl et al., 2000b). Nevertheless, although sea urchin Dsh localizes to the vegetal cortex of early embryos, no links between Dsh and calcium effectors/signals have been reported so far (Leonard and Etensohn, 2007).

Post-Translational Gene Regulation in the Determination of PGCs

Despite vast mechanistic variation, sexually reproducing metazoans employ a common group of molecules for PGC specification and development. One of the most ubiquitous and extensively conserved factor is the DEAD-box RNA helicase Vasa. In all animals observed to date, Vasa protein is ultimately enriched in PGCs or multipotent PGC progenitor cells during embryo development (e.g., Raz, 2000; Gustafson and Wessel, 2010a; Lasko, 2011). Several animals accomplish this selectivity not by transcriptional mechanisms, but by post-translational activity regulating Vasa protein stability and turnover.

Protein Turnover Is a Conserved Mechanism for PGC Protein Enrichment

In *Drosophila*, Vasa protein localizes to the pole plasm in the developing embryo. In vitro protein interactions as well as genetic data suggest that Oskar may anchor Vasa to polar granules in the oocyte pole plasm, thereby contributing to its eventual incorporation into the pole cells of the developing embryo (Hay et al., 1990; Lasko and Ashburner, 1990; Breitwieser et al., 1996). *Drosophila* Vasa is ubiquitinated in the oocyte, and Gustavus and Fsn are two paralogous E3 ubiquitin ligases required for Vasa ubiquitination, thereby removing Vasa from regions outside the pole plasm (Styhler et al., 2002; Kugler et al., 2010). The deubiquitylating enzyme Fat facets is required for Vasa pole-plasm accumulation; thus, the persistence of Vasa is presumably in a war between ubiquitylating and de-ubiquitylating activities (Liu et al., 2003). Protein turnover also regulates the Vasa ortholog GLH-1 in *C. elegans*. Phosphorylation of GLH-1 by KGB1 (Jun N-terminal kinase) targets it for proteolysis, whereas association with the CSN-5 (COP9 signalosome subunit 5) enhances GLH-1 stability in the germ line (Orsborn et al., 2007). In addition, the oocyte-to-embryo transition in *C. elegans* relies heavily on selective maternal protein turnover (DeRenzo and Seydoux, 2004). In the embryonic germ line (P-lineage), PIE-1, MEX-1, and POS-1 proteins are stable, whereas they are selectively degraded in the developing somatic

blastomeres (Reese et al., 2000; DeRenzo et al., 2003). Conversely, MEX-5 and MEX-6 proteins are selectively degraded in the P-lineage but are retained in the somatic blastomeres (Guo and Kemphues, 1995; Schubert et al., 2000). During zebrafish embryonic development, Vasa protein is uniformly distributed at the time of PGC specification (Yoon et al., 1997; Knaut et al., 2000). PGC-specific Vasa protein enrichment occurs, in part, through soma-specific Vasa proteolysis (Wolke et al., 2002). Thus, post-translational regulation of Vasa appears to be an important level of regulation that ultimately directs PGC specification.

Post-Translational Modifications Regulate Vasa Function in Germ Cells

Although it is currently unclear how protein turnover contributes to Vasa protein localization in mammals, both mouse (Mvh) and human (Ddx4) Vasa orthologs are subject to several post-translational modifications. These include serine, threonine, and tyrosine phosphorylation, as well as arginine methylation and lysine acetylation (Gustafson and Wessel, 2010b; Lasko, 2013; <http://www.phosphosite.org>). Recent data in mice have identified Hat1 as the acetyltransferase responsible for Mvh acetylation at Lys405, and found that this post-translational modification is developmentally regulated and required for Mvh RNA-binding activity (Nagamori et al., 2011). It will be important to test how small-molecule inhibitors of the acetylation process, such as histone deacetylase (HDAC) inhibitors, influence the post-translational regulation, and function of Vasa and other germ cell-specific acetylated proteins. Vasa arginine methylation is also found in *Drosophila*, *Xenopus*, and mice. The arginine methyltransferase Capsuleen (dPRMT5/csl/dart5) is required for symmetric dimethylarginine modifications of Vasa in vivo (Kirino et al., 2010). Although the functional significance of these Vasa arginine methylations is currently unclear, they may facilitate its interaction with Tudor proteins and its localization to perinuclear nuage structures in germ cells, as occurs with several Piwi/Argonaut proteins (Liu et al., 2010; Chen et al., 2011; Mathioudakis et al., 2012).

Vasa Protein Enrichment in the sMics

In the sea urchin *S. purpuratus*, Vasa protein present uniformly during early embryogenesis, but eventually becomes enriched in the 16-cell stage micromeres and subsequent sMics (Fig. 10) (Juliano et al., 2006; Voronina et al., 2008). Several pieces of evidence suggest that cell type-specific protein turnover is the major regulatory mechanism behind this sMic protein enrichment in the midst of uniform Vasa mRNA. First, although the Vasa untranslated regions (UTRs) are unable to direct sMic protein accumulation, the Vasa coding sequence alone is sufficient for sMic protein enrichment (Fig. 10c) (Gustafson et al., 2011). Second, Vasa protein accumulates throughout the embryo following proteasome inhibition (Fig. 10d) (Gustafson et al., 2011). As with *Drosophila*, sea urchin embryos express a Gustavus ortholog (and E3 ubiquitin ligase involved in target proteins for proteasomal degradation) that interacts with Vasa in vitro. However, knockdown of endogenous Gustavus protein reduces endogenous Vasa protein and *reduces* the frequency of Vasa protein enrichment in the sMics, whereas overexpression of the Vasa-interacting domain of Gustavus results in ectopic Vasa protein accumulation throughout the embryo (Gustafson et al., 2011). Together, these data suggest that Gustavus may have two opposing functions: a degrading function outside of the future germ cells and a positive regulatory function that stabilizes

Vasa protein in the sMics. Despite several protein domains indicative of an E3 ubiquitin ligase presumed to be involved in protein degradation, similar evidence was reported for a positive regulatory role of Gustavus in *Drosophila* (Kugler et al., 2010). To our knowledge, these are the only examples where a putative E3 ligase *promotes* the stability of its target ligand. We speculate, however, that this phenomenon of positive and negative modulation of protein stability may be more widespread, and may be a common developmental process underscoring the complex regulatory nature of protein ubiquitylation and turnover.

This post-translational, regulatory conservation of Gustavus may also extend to vertebrate PGCs and germ cell development. Two Gustavus orthologs, SSB-1 and SSB-4, have been identified in the mouse and are expressed in several adult tissues, including the testis and ovary. In particular, their expression was found in somatic ovarian granulosa cells during folliculogenesis, but was not detected in the developing oocytes (Xing et al., 2006). While this work did not investigate the Gustavus orthologs during mouse embryonic development, these expression data may reflect contextual differences between their functions in embryo and adult gonads. Indeed, one of the zebrafish Gustavus orthologs is expressed in the PGCs during embryonic development, while both are enriched in the testis and ovary (Li et al., 2009).

Protein Turnover Is an Essential Mechanism in an Embryonic Gene Regulatory Network

While extensive data on transcriptional regulation comprises most of our current knowledge of gene regulation during sea urchin embryonic development, it is clear that post-translational regulatory mechanisms are essential for germ cell formation during embryogenesis. In addition to Vasa, other factors may be subject to similar post-translational gene regulation within the sMics. The transcript *seawi* (the sea urchin *piwi* ortholog) also is uniformly distributed throughout the early embryo, yet Seawi protein becomes enriched in the micromeres at the 16-cell stage just as seen with Vasa (Juliano et al., 2006; Rodriguez et al., 2005). Although Vasa and Seawi primarily function in a post-transcriptional regulatory context, the sea urchin embryo also utilizes selective protein turnover to adjust transcription factor abundance both within and outside the germ-cell lineage. For example, the transcription factor SoxB1 is degraded specifically in blastula vegetal cells comprising the endomesoderm (Angerer et al., 2005). This preferential SoxB1 protein turnover is dependent upon nuclear beta-catenin function, which is necessary for normal SoxB1 turnover and can cause ectopic degradation in animal blastomeres (Angerer et al., 2005). Spatial and temporal transcriptional regulation by beta-catenin is also dependent upon its differential protein stability and post-translational regulation within the developing embryo. In the 16-cell embryo, unphosphorylated beta-catenin translocates to the micromere nuclei, whereas beta-catenin in mesomeres and in macromeres is phosphorylated by GSK3 β and degraded. This intricate, post-translational regulation of beta-catenin is essential for proper A–V axis patterning in the early embryo (Emily-Fenouil et al., 1998; Wikramanayake et al., 1998; Logan et al., 1999; Sherwood and McClay, 1999; Angerer and Angerer, 2000) and documents the shared mechanistic utility of somatic and germ-line functions.

Transcriptional Regulation

Many in the sea urchin research community have sought to understand the transcriptional control of embryogenesis. Since the *S. purpuratus* genome sequence became available in 2006 (Weinstock et al., 2006), the work of many labs has identified a large set of transcription factors that are expressed lineage-specifically during development (e.g., Spbase.org). Despite these efforts, few genes are known to be selectively expressed in the sMics at any stage; further, it is not known how these few sMic enriched genes are transcriptionally regulated. The dearth of transcription factors therein may suggest that the sMics are a transcriptionally quiet territory.

The chromatin state of the sMic indeed suggests broad transcriptional repression relative to the somatic cells of the embryo. Antibodies that distinguish between catalytic states of RNA Polymerase II (RNAPII) have proven useful for visualizing the transcriptional activity of cells. RNAPII is regulated by phosphorylation on its C-terminal domain (CTD), which contains numerous copies of the heptapeptide repeat YPTSPS. Phosphorylation on Ser5 and Ser2 are associated with initiation and elongation, respectively (Nakamura and Seydoux, 2008). By immunofluorescence, sMic nuclei appear depleted for the Ser2 phosphoepitope by the blastula stage, which persists through gastrulation (Swartz et al., 2014). This observation suggests that the sMics are transcriptionally repressed during their migratory phase. A consequence of broad repression might be to insulate the sMics from differentiation as they translocate from the vegetal plate to the coelomic pouches, a time when they are exposed to numerous inductive signals as the surrounding endomesoderm differentiates (Swartz et al., 2014). The mechanism by which the sMics become depleted for RNAPII phospho-Ser2 (pSer2) remains unknown, although this depletion appears to be a hugely conserved feature of germ cells, shared by the PGCs of *Drosophila*, *C. elegans*, mice, and ascidians (Seydoux and Dunn, 1997; Seki et al., 2007; Shirae-Kurabayashi et al., 2011). Surprisingly, though, each of these organisms achieve RNAPII repression via distinct pathways involving genes specific to their species (Mello et al., 1996; Batchelder et al., 1999; Zhang et al., 2003; Ghosh and Seydoux, 2008; Hanyu-Nakamura et al., 2008). As these genes are not conserved in the sea urchin, it is likely that at least one other mechanism of repression evolved in the echinoderm clade. One possible player is the RNAPII phosphatase Ctdspl2/SCP2, whose transcript was found to be enriched in the sMics (Swartz et al., 2014). The timing of sMic pSer2 depletion differs from *Drosophila* and *C. elegans*, where it occurs before migration. In this regard, the sMics are more similar to mouse PGCs, where pSer2 is depleted during PGC migration to the genital ridge (Seki et al., 2007). It remains unknown, however, how mouse PGCs become depleted for pSer2.

Transcriptional activity is intimately linked with epigenetic modification to the DNA and surrounding nucleosomes. A faithful marker of repressive heterochromatin is histone 3 lysine 9 trimethylation (H3K9me3). Upon their creation at the 5th cleavage, the sMics are highly enriched for H3K9me3 compared to somatic cell nuclei, which persists to blastula stage. Thus, there appear to be at least two separate phases of sMic transcriptional repression: H3K9me3-positivity followed by pSer2 depletion as the sMics translocate. Histone modification has also been observed in the PGCs of *Drosophila* and *C. elegans*, which both display a loss of H3K4me, a marker of active euchromatin (Schaner et al., 2003).

In *Drosophila*, this depletion is mediated by SU(VAR)3–3 (ortholog of the LSD-1 amine oxidase) (Rudolph et al., 2007), and occurs concomitantly with pSer2 depletion. Mice also display methylation transitions: during migration to the presumptive gonad, the PGCs lose H3K9me3 and acquire the repressive mark H3K27me (Seki et al., 2007). The transcriptional repressor Blimp1 is responsible for repressing the majority of inactivated genes in mouse PGCs (Ohinata et al., 2005), potentially by recruiting histone methyltransferase machinery (Ancelin et al., 2006). Blimp1 is conserved in the sea urchin, but it does not have a known role in the sMics; rather, it appears to function during specification of the endome-soderm, where it is required to activate Wnt8 (Minokawa et al., 2005; Livi and Davidson, 2006). In addition to specific histone modification, the nucleosome remodeling complex Swi/SNF has also been implicated in transcriptional repression; mutation of the ARID-containing factor Osa in *Drosophila* leads to derepression of the somatic transcript *zen* (Martinho et al., 2004). The SWI/SNF related factors BAF250, Brg1, and ISWI were each identified as sMic-enriched in sea urchins, but their involvement in regulating the transcriptome remains untested (Yajima and Wessel, 2012; Swartz et al., 2013).

Perhaps reflective of their transcriptional repression, only one transcription factor is known to be uniquely expressed in the sMics to date: the forkhead factor FoxY (Fig. 11) (Ransick et al., 2002). FoxY is only transiently activated in the sMics during blastula stage, and subsequently shifts into the non-skeletogenic mesoderm upon gastrulation. The early sMic phase of FoxY expression is dependent on Delta/Notch signaling from presumptive mesoderm, and *cis*-regulatory analysis of its promoter has revealed a functional Suppressor of Hairless site, the transcriptional effector of Delta/Notch signaling. All known FoxY targets are specific to the non-skeletogenic mesoderm cells, however; therefore, its function in the sMics is unclear. FoxY knockdown in its sMic expression phase has no effect on *nanos* transcript levels (Materna et al., 2013). Nanos is modestly reduced in larval stages with FoxY knockdown, although this is likely an indirect effect due to failure to form the coelomic pouches as a consequence of non-skeletogenic mesoderm defects (Song and Wessel, 2012). What happens to the sMics with FoxY knockdown has also not been determined. Still, germ cells in many species require a permissive niche environment, so failure to organize coelomic pouches could have profound effects on sMic gene expression.

RNAs Are Selectively Retained in the sMics

Several lines of evidence suggest that the sMics are transcriptionally repressed. How the sMics acquire germ-line RNAs is, therefore, an important question. The sMics appear to initially inherit their RNAs rather than transcribe them *de novo*, consistent with the broad distribution of RNAs encoding Vasa and Seawi in early embryos that is later refined to the sMics during gastrulation (Juliano et al., 2006; Voronina et al., 2008). In other organisms such as *Drosophila*, mRNA localization is an important mechanism during early development (Becalska and Gavis, 2009) and localization of *nanos* RNA in the germ line depends on sites in its 3'-UTR (Gavis et al., 1996; Andrews et al., 2011).

One of the strengths of the sea urchin as a model organism is the ease and diversity of manipulations possible for studying developmental features. Investigators of this and many animals often inject mRNAs into eggs and early embryos to test the function or the

regulation of proteins, and *Xenopus* β -globin UTRs are often used to direct constitutive translation throughout the embryos. This sequence of UTRs was shown to be innocuous to normal mRNA translational regulation in a variety of cases. Surprisingly though, injection of an mRNA coding for mCherry flanked by *Xenopus* β -globin UTRs showed a selective fluorescence signal in the sMics of gastrulae (Fig. 12). In situ hybridizations, using a mCherry probe, of the injected embryos indicate that this selective protein accumulation resulted from the prolonged retention of its RNA in the sMics (Fig. 1B) (Gustafson and Wessel, 2010c; Oulhen and Wessel, 2013). After microinjection at the one-cell stage, the RNA is strongly retained uniformly in embryos during early development until blastula. During gastrulation, however, the RNA becomes detectable only in the sMics (Oulhen and Wessel, 2013).

Retention of exogenous mRNA selectively in the sMics could result from either selective RNA decay in the non-sMic cells, and/or selective RNA protection in the sMics. mRNA decay can depend on *cis*-acting elements within each RNA but also on the mRNA degradation machinery present at a specific time, in specific cells. RNA turnover often begins by deadenylation, followed by the action of exonucleases that degrade RNAs from 5'-to-3' or 3'-to-5' (Garneau et al., 2007). In eukaryotic cells, the 5'-to-3' degradation pathway depends on removal of the 5'-cap by the decapping proteins Dcp1 and Dcp2 (Scherer et al., 2006). The resulting uncapped RNA is then degraded by the 5' exoribonuclease, Xrn1 (Larimer et al., 1992; Hsu and Stevens, 1993). The 3'-to-5' degradation pathway is directed by a multi-subunit exonuclease complex, the RNA exosome (Garneau et al., 2007; Schmid and Jensen, 2008; Lykke-Andersen et al., 2011). mRNA can also be degraded by endonucleases, followed by degradation of decay products by exonucleases (Otsuka and Schoenberg, 2008). Finally, mRNAs can also be degraded by piRNAs and miRNAs (Selbach et al., 2008; Rouget et al., 2010). In zebrafish, miR-430 is the most abundant miRNA family expressed during early development, and accumulates during the maternal-to-zygotic transition. miR-430 facilitates the deadenylation and clearance of maternal mRNA during early embryogenesis (Giraldez et al., 2006). In *Drosophila*, a cluster of zygotically expressed miRNAs targets maternal mRNAs for turnover as part of the zygotic degradation pathway (Bushati et al., 2008). Remarkably in the sea urchins, neither the m⁷GTP cap nor the polyadenylation tail is required for sMic RNA retention of mCherry and GFP reporters containing *Xenopus* β -globin UTRs (Oulhen and Wessel, 2013). Interestingly, an RNA containing only the GFP open reading frame without a 3'-UTR is not selectively retained in the sMics (Fig. 13).

While the mechanisms of somatic-cell mRNA turnover have been explored in several organisms, how those RNAs remain protected in the PGCs is poorly understood. The best evidence comes from work in zebrafish, where it was first recognized that germ-line RNAs targeted by miR-430, including *nanos*, were differentially degraded in the soma versus the germ line (Koprunner et al., 2001; Mishima et al., 2006). That is, while miR-430 is present in the PGCs, its targets are protected within these cells. It was subsequently found that the germ-line RNA binding protein Dead end 1 (Dnd1) binds near and occludes miR-430 sites from Argonaute machinery (Kedde et al., 2007). Yet, Dnd1 is not conserved outside of vertebrates. Still, miRNAs are expressed in echinoderms during embryonic development

(Kadri et al., 2011; Song et al., 2012), and could be involved in selective RNA retention, that is, degradation of mRNAs outside of the sMics but retention of mRNAs in the sMics due to protection of the target transcripts from the miRNAs.

In the sea urchin, the deadenylase CNOT6 is present in somatic cells but is specifically depleted in the sMics by Nanos, likely with its binding partner Pumilio (Fig. 3). CNOT6 is a core deadenylase of the conserved CCR4-NOT complex, a major cytoplasmic regulator of RNA stability (Wiederhold and Passmore, 2010). Reduced dead-enzylase activity provides the sMics with a generally stable environment for inherited RNA. Misexpression of CNOT6 in the sMics results in their failure to retain RNAs and accumulate Vasa protein, and possibly leads to their apoptosis. Conversely, global knockdown of CNOT6 expands the domain of RNA retention beyond the sMics and into the entire endomesoderm. These results indicate that differential CNOT6 regulation is central to the RNA retention phenomenon (Swartz et al., 2014). This selective retention could also be explained by a differential RNA storage mechanism. Germ cells usually contain granules related to P bodies (processing bodies), called germ granules, which play a role in RNA accumulation and decay. They regulate maternal RNA levels required for germ-cell specification (Anderson and Kedersha, 2006). RNA injected at the one-cell stage might be subject to regulation as a maternal RNA by the embryo. Depending on the proteins present in the sMics, specific granules could protect this RNA from degradation.

In contrast to this selective RNA retention observed with non-specific *Xenopus* β -globin UTRs in gastrula, sMics also specifically retain reporter RNAs containing endogenous sequences. Injection of an RNA containing the GFP open reading frame surrounded by *nanos2* UTRs is exclusively retained in sMics of blastula whereas the RNA containing *Xenopus* β -globin UTRs is still detected in all the cells at this stage (Oulhen et al., 2013). *nanos2* UTRs accelerate the selective retention in the sMics, that is, these transcripts are more rapidly degraded outside of the sMics. Data obtained from two sea urchin species, *S. purpuratus* (*Sp*) and *H. pulcherrimus* (*Hp*), indicate that the *nanos2* 3'-UTR directs this specific and selective RNA retention in the sMics. A sequence composed of 357 nucleotides in *Hp* and 388 nucleotides in *Sp*, is required for this selective RNA retention. This sequence, present in *nanos2* 3'-UTR and defined as GNARLE for Global Nanos Associated RNA Lability Element, is required for the specific retention of the RNA in the sMics. GNARLE is sufficient for high protein enrichment in the sMics, suggesting a role for GNARLE in inhibiting the translation in the non-sMics and/or stimulating translation in the sMics (Fig. 14). Therefore, GNARLE plays a role in both promoting selective RNA retention and translational activity of nanos in the sMics. *Hp* and *Sp* GNARLE are functionally conserved: injection of either *Sp* or *Hp* GNARLE RNA in *Sp* fertilized eggs give similar RNA retention and protein accumulation. *S. purpuratus* and *H. pulcherrimus* diverged between 7.2 and 14 million years ago (Lee, 2003). In contrast, no similar GNARLE was found in the sea urchin *Lytechinus variegatus*, which diverged from *S. purpuratus* between 30 and 50 million years ago (Smith et al., 2006), nor in the starfish *Patiria miniata*, which diverged from sea urchins approximately 500 million years ago (Hinman et al., 2003; Smith et al., 2006).

A combination of degradation in the non-sMics and protection in the sMics seems to be required for strong, selective RNA retention. How GNARLE regulates RNA stability is not

well understood yet. Between 50 and 55 miRNAs have been identified in the sea urchin (Campo-Paysaa et al., 2011; Song et al., 2012). By sequence analysis, we found that GNARLE could potentially be targeted by three of these miRNAs in both *S. purpuratus* and *H. pulcherrimus*. Yet, perturbation of Dicer, an enzyme essential for miRNA biogenesis, does not affect the level of *nanos2* transcript significantly in *S. purpuratus* embryos, indicating that miRNAs are not or are not solely responsible for the decay of *nanos2* RNA outside of the sMics (Oulhen et al., 2013). In *Drosophila*, the *nanos* 3'-UTR contains a 547-nucleotide element mediating its RNA localization at the posterior pole of the syncytial embryo (Gavis et al., 1996). The protein rumpelstiltskin (rump), a heterogeneous nuclear ribonucleoprotein (hnRNP) M homolog, binds *nanos* RNA directly to regulate its localization. More recently, the Argonaute family member Aubergine (Aub) was also found to be a *nanos* RNA localization factor, independent of its function in RNA silencing. Aub interacts with *nanos* mRNA in vivo and co-purifies with rump in an RNA-dependent manner (Becalska et al., 2011). The piRNA pathway was also shown to be required for *nanos* mRNA deadenylation and decay as well as translational repression in *Drosophila* embryos (Rouget et al., 2010). Aub is present in a complex with the RNA-binding protein Smaug, the deadenylase CCR4, *nanos* mRNA, and piRNAs that target the *nanos* 3'-UTR. In the sea urchin, preliminary data using a gel-shift assay, suggests that one or several proteins from mesenchyme blastula can bind to *Sp* GNARLE, and might be involved in RNA stability and or translational control (Figure 15) (Oulhen and Wessel, unpublished data).

Left-Right Asymmetry in Germ Line Formation

The sea urchin develops as a bilaterally symmetrical embryo, but shows signs of left-right (L/R) asymmetry as the larval form takes shape. The first morphological indication of symmetry break occurs following gastrulation, when the eight sMic descendants sort into the nascent coelomic pouches. The morphogenetic process of pouch formation requires Hedgehog signaling, based on inhibitor experiments, but perturbing this pathway does not affect coelomic pouch gene expression (Materna et al., 2013). Similarly, inhibiting BMP2/4 prevents bilateral sorting of the sMics—they instead remain centrally located at the foregut tip. It does not, however, alter their accumulation of *vasa*, *nanos*, or *seawi* RNAs (Luo and Su, 2012), possibly because those transcripts are inherited by the sMics rather than actively transcribed. The non-skeletogenic mesoderm constituents of the pouches, however, lose expression of numerous transcriptional regulators (e.g., SoxE, Pax6), indicating disruption to the sMic niche environment (Luo and Su, 2012).

Although some slight embryo-to-embryo variance is seen, the sMics usually split so that there are five in the left pouch, and three in the right (Pehrson and Cohen, 1986; Tanaka and Dan, 1990). The precise mechanism of this assortment is unresolved, but conserved L/R asymmetry genes are required. In late gastrula/early larval stages, *nodal* transcript is detectable asymmetrically on the right side ectoderm (Duboc et al., 2005). Pharmacological inhibition of Nodal during pouch formation causes symmetric assortment of sMics such that there are four on either side (Luo and Su, 2012). Multi-drug transporter (MDR) activity is also involved since treatment with MDR-inhibiting drugs randomizes sMic L/R sorting (Campanale and Hamdoun, 2012). This phenotype may point to a failure in chemoattraction

—in *Drosophila*, the ABC transporter *mdr49* is required for secreting a lipid-modified peptide that attracts PGCs to the gonad (Ricardo and Lehmann, 2009; Fig. 16).

Nodal signaling also appears to impinge upon the survival of sMics in the right coelomic pouch, which have been proposed to apoptose due to loss of *Nanos* expression (Fujii et al., 2009; Juliano et al., 2010). By the pluteus stage, the right sMics normally become TUNEL-positive, indicative of apoptosis; inhibition of Nodal prevented this apoptosis whereas elevating Nodal signaling with exogenous Activin induced apoptosis in left side sMics. Consistent with these results, Nodal inhibition also causes the persistence of *vasa*, *nanos*, and *seawi* RNAs in the right coelomic pouch (Luo and Su, 2012). Nodal acts through the homeodomain repressor Pitx2 (Duboc et al., 2005), which is the likely repressor of FoxY in the right coelomic pouch cells (Materna et al., 2013). Therefore, one possibility is that *vasa*, *nanos*, and *seawi* transcripts are direct targets of this pathway. Alternatively, the loss of detectable germ-line transcripts in the right coelomic pouch could simply be due to Nodal-dependent apoptosis of the sMics.

The upstream-most signal for L/R asymmetric gene expression in the urchin embryo remains enigmatic. A potential organizing center has been recently discovered in the sea urchin even before coelomic pouch formation. In mid-to-late gastrula-stage embryos, a mesendodermal patch of *nodal*-positive cells appears on the right side of the archenteron. This *nodal* transcript accumulation requires FGF and BMP signaling, and is repressed on the left side by Delta/Notch signaling. Perturbation of the mesendodermal organizer prevents subsequent accumulation of *nodal* transcript in the left-side ectoderm, which appears to involve mesendodermal/ectodermal crosstalk via Univin (TGF- β) signaling, and randomizes the placement of the adult rudiment (Bessodes et al., 2012). Since rudiment formation can still occur, it suggests that coelomic pouch development occurs largely normally. An interesting open question is whether or not the sMics properly home under these conditions, and if the resultant sea urchins are fertile. Further, although the L/R asymmetry is seen earlier in these embryos, the mechanism for this process is unknown.

Multi-Drug Resistant/ABC Transporters in Germ-Cell Development

ATP-binding cassette (ABC) transporters are trans-membrane proteins found in nearly all cells that help control the flow of information between cells and their environment. These transporters provide energy-dependent shuttling of a variety of compounds across the plasma membrane to eliminate metabolites and secondarily remove drugs intended to work inside the cell (Higgins et al., 1990; Dean et al., 2001). Members of this transport family also provide protection in the export of toxins and xenobiotics (Deeley et al., 2006; Sarkadi et al., 2006; Dean et al., 2001). ABC transporters are highly conserved and present in all free-living organisms, from bacteria to humans. Their function is essential to the protection and normal development of the embryo. ABC efflux transporters are large membrane-bound proteins, with membrane-spanning domains and a cytoplasmic ATP-binding domain that is required to shuttle compounds in an ATP-dependent manner. In addition to transportation and protection, ABC effluxers actively influence the fate of cells by modulation of cell differentiation, lysosomal and mitochondrial homeostasis, potassium channel regulation, pigmentation, and migration (Shipp and Hamdoun, 2012; Gökirmak et al., 2012). The ABC

efflux transporter family is the parent family of multi-drug resistant transporters, which participate in chemotherapy resistance in cancer cells and antibiotic resistance in bacteria.

Remarkably, ABC transporters are implicated in the migration of PGCs. In *Drosophila*, *mdr49*, an ABCB efflux transporter, is essential for the appropriate migration of PGCs. Ricardo and Lehmann (2009) generated a loss-of-function allele for this transporter and demonstrated that mutant embryos showed germ cell-migration defects, which included migration through the posterior hind gut and lack of association with the somatic gonad. The PGCs formed, but during their migratory phase, moved to aberrant positions. In *C. elegans* and *Drosophila* embryos ABCC and ABCB members of the ATP-dependent transporters, respectively, efflux prenylated peptides from mesodermal cells to attract germ cells during their migration (Yabe et al., 2005; Ricardo and Lehmann, 2009). This finding highlights the functional diversity that multi-drug resistant transporters have during normal development, although exactly how these transporters are regulated in normal development is unknown. Data from the oncogene literature suggests that miRNAs are also involved in the regulation of ABC efflux transporters (Haenisch et al., 2008), specifically that ABCC2 is down-regulated in mature mouse testis by mir-379 (Haenisch et al., 2011). Yet, our understanding is limited in how all the diverse functions are intertwined to form a reproductively competent organism. Hamdoun et al. (2004) observed that the ABCC- and ABCB-type transporters were present in eggs and cleavage-stage sea urchin embryos. The authors demonstrated that the fertilization response involves upregulation of multi-drug efflux transport activity as part of egg activation, and that this activity protects the embryo from exogenous toxins. Inhibition of the efflux activity further affects cell division, suggesting that the transporter activity also has some role in passage through mitosis; indeed, *S. purpuratus* embryos express ~20 ABC efflux transporters over the first three days of development (Shipp and Hamdoun, 2012; Gökirmak et al., 2012). *ABCB1a* mRNA is 10–100 times more abundant than mRNA of other ABC transporters in the developing embryo, and the ABCB1a protein localizes to the apical membrane of the embryo while ABCC5a is expressed on the basolateral membrane of polarized cells (Shipp and Hamdoun, 2012; Gökirmak et al., 2012).

It was recently found that the ABCB- and ABCC-type transport activity is downregulated in the sMics (Campanale and Hamdoun, 2012). This is unexpected given that ABC efflux transporters function to protect cells. In fact, the sMics retain significantly more calcein, a reporter of ABCB- and ABCC-type efflux from cells, than any other cell in the embryo (Campanale and Hamdoun, 2012). According to Campanale and Hamdoun (2012), this phenomenon is true in the sMics of other echinoid species as well, including the sea urchin *Lytechinus pictus* and the sand dollar *Dendraster excentricus*, indicating a conserved sMic activity from a common ancestor that diverged at least 250 million years ago (Smith et al., 2006). Logically, it seems rational that ABC efflux transporters function to protect stem cells from harmful toxins and differentiation (Zhou et al., 2001; Bunting, 2002), and hematopoietic stem cells express more efflux pumps than differentiated cells (Bunting et al., 2000). The loss of some ABC transporters in the sMics is contrary to what might be expected, so it will be important to learn if this observation simply represents a decrease in one family of ABC transporters or if overall activity actually decreases in these cells.

Perhaps the absence of efflux normally ascribed to the ABCB- and ABCC-type transport activity contributes to the downregulation of metabolic activity detected in the sMics, which globally protects the cell. Another possibility is that sMics may transiently lose their ability to efflux in order to acquire other germ line-specific modifications.

Diversity of Germ-Line Development in Echinoderms

Echinoderms are a very diverse and ancient lineage, first appearing in the fossil record approximately 550 million years ago (Porter, 1977; Greenstein, 1993; Kroh, 2007; Kroh and Smith, 2010; Erwin et al., 2011; Smith et al., 2013). The ancient echinoderms diversified very rapidly, with four distinct body plans within 10–15 million years (Smith et al., 2013). Due to the rapid diversification of echinoderms over the subsequent 500 million years, it has been difficult to accurately determine the phylogenetic relationship between extant echinoderms. Part of the difficulty is that different results are obtained depending on the methods for determining the phylogenetic relationships (rRNA sequences, mitochondrial sequences, morphology, larva, etcetera), which yields slightly different phylogenetic trees. Near unanimous agreement exists that the crinoids (feather stars and sea lilies) are the root of the echinoderm tree (Littlewood et al., 1997; Janies, 2001; Mallatt and Winchell, 2007; Janies et al., 2010; Mah and Foltz, 2011; Pisani et al., 2012), and there is little conflict over the close relationship between Holothuridea (sea cucumbers) and Echinoids (sea urchins and sand dollars). The majority of the conflicts arise from the uncertain position of Ophioids (brittle stars) within echinoderms (Okanishi et al., 2011; Perseke et al., 2012), but several studies suggest that Asterozoa (brittle stars and sea stars) are the sister group to Echinozoa (sea urchins, sand dollars, and sea cucumbers) (Fig. 17). A more comprehensive method for accurately determining the phylogenetic tree of echinoderms is needed.

However the phylogenetic relationships of echinoderms are resolved, though, it is clear that only the euechinoids (sea urchins and sand dollars) have sMics (Tanaka and Dan, 1990; Juliano and Wessel, 2009; Vellutini and Migotto, 2010). Therefore, when considering germ-line determination mechanisms and identifying which cells are responsible for this character in other echinoderms, the context will change dramatically. The cidaroids (pencil urchins) do have micromeres, or at the very least, asymmetrically dividing micromere-like cells at the 4th cell cleavage (Wray and McClay, 1988; Bennett et al., 2012). The micromeres and sMics are likely a derived trait unique to Echinoidea, as a single gain of micromeres, and subsequently sMics, in a single branch is more parsimonious than micromeres being the ancestral state and then lost independently at least three times. sMics are precursors of the PGCs of the sea urchin and therefore are protected in the embryo in a variety of ways discussed above. One hypothesis in their appearance in sea urchins might be their co-evolution with the hyaline layer. Cidaroids do not make sMics (at the 5th cell cleavage at the very least), do not have a hyaline layer, and instead depend on the fertilization envelope to constrain the blastomeres. Similarly, most echinoderms do not make hyalin, with the notable example of some brittle stars (Yamashita, 1985). Many developing embryos (e.g., sea stars) depend on the fertilization envelope to constrain the blastomeres, but the relatively small sMics may require an extra layer of constraint in the embryo to aid in their retention. The loss of a blastomere to even an eight-cell embryo is easily surmountable, but the loss of the PGCs at an early stage could lead to sterility (Yajima and Wessel, 2011).

It is important to note that echinoderms undergo both indirect and direct development, and different species from the Asterozoa, Ophiurozoa, Echinozoa, and Holothurozoa show this diversity. Direct-developing species do not form a feeding larval structure, which likely it really is dramatic changes their PGC biogenesis. Some of the characteristics used to distinguish direct developers from indirect developers include egg, larval and post-larval size, rate of development, gonadal volume measurements, and numbers and volume of eggs. Although some of these characters are of questionable reliability, a feeding larval-development stage is most likely the primitive mode of development in echinoderms. The variability between direct developmental modes also suggests that they emerged independently in several lineages (Holland 1991). As all of the crinoids described so far undergo direct development (Holland 1991; Nakano et al., 2003), our analyses of coelomogenesis and PGC specification will focus on indirect developers. The only other echinoderm phylogenetic clade in which the specification of the PGCs has been functionally tested is that of the Asterozoa (sea stars). For this reason we will outline what is known about germ-cell specification in sea stars first.

Asterozoa (Sea Stars)

The origin of germ cells in the sea star *Asterina pectinifera* was traced back to the posterior enterocoel of 2-day-old bipinnaria larvae. Inoue et al. (1992) removed the posterior enterocoel from 2-day-old bipinnaria larvae and observed a decrease in the number of germ cells in the cellular cluster of 6-week-old brachiolariae juveniles. Germ cells in this case were identified through morphological characteristics: large cells, large nuclei, mitochondrial aggregations, and electron-dense, nuage-like structures (Inoue et al., 1992). Furthermore, the germ-line factor Vasa accumulates in the posterior enterocoel of the sea stars *P. miniata* and *Asterias forbesi* (Juliano and Wessel, 2009). Older morphological studies that identified PGCs with classical germ-cell characteristics are consistent with these recent data because the origin of the germ line was described to be in the dorsal horn of the left somatocoel of the bipinnaria larvae, which we interpret to be derived from cells in the posterior enterocoel (Gemmell, 1914; Chia and Walker 1991). Together, these observations suggest that the posterior enterocoel is the origin of PGCs in the Asterozoa clade.

So what is the left posterior somatocoel/enterocoel? Coeloms are fluid-filled cavities of mesoderm that, in the case of echinoderms, arise from the archenteron. Chia and Walker (1991) outlined that asterozoa use three general strategies to develop their coeloms. In the first type, two coelomic pouches arise from the anterior of the archenteron, and then grow posteriorly, the left much faster than the right. The left pouch also gives rise to the water-vascular system. In the second type, a single coelom arises from the anterior of the archenteron and grows posteriorly on both sides as a U-shaped coelom. Later in development, two septa form on each side of the U to form the anterior coelom and two posterior coeloms (left and right). In the third type, the coeloms arise from both the anterior and posterior ends of the archenteron (Chia and Walker 1991). Recently, Morris et al. (2009, 2011) found the second mode of coelomogenesis to be used for the development of *Parvulastra exigua* larvae whereas the third mode of coelomogenesis seems to be used for the development of *P. miniata*, *Asterina pectinifera*, *Pteraster tesselatus*, and *Asterina glacialis* (*Marthasterias glacialis*) because the left posterior coelomic pouch in these

animals arises by budding off the left posterior side of the archenteron (Fig. 18; Gemmill, 1914; Heath, 1917; Kuraishi and Osanai, 1992; David et al., 1994; Hinman et al., 2003). Deviations from this mode of coelomogenesis were noted in earlier descriptions of other Asteroidea larval development. For example, in 1914, Gemmill noticed that the posterior enterocoel in the sea star *Asterias rubens*, although usually developing from the dorsal left side of the stomach, was also rarely seen to arise from the right side of the gut and also medially (Gemmill, 1914). He also noticed the posterior enterocoel in *Porania pulvillus* usually developed on both sides of the dorsal stomach (Gemmill, 1915).

Because the left posterior coelom is believed to be important for PGC specification in sea stars, we will consider larval coelomogenesis in our analysis of what is known about PGC specification in the other echinoderm clades to determine if a similar structure to the Asteroidea left posterior coelom is also found in other echinoderm clades. This analysis is important because the literature reveals a marked lack of knowledge of PGC specification in other echinoderm clades.

Crinoids (Feather Stars, Sea Fans, Sea Lillies)

The only studies of crinoid germ-line specification that we are aware of were outlined nicely by Holland (1991). In this article, the author states that the most detailed morphological study of the origin of crinoid germ line, although incomplete and controversial, was accomplished by Achille Russo (Russo, 1902). In Russo's studies of the developing *Antedon mediterranea* larvae, he found that PGCs were first detected morphologically in the cystidean larval stage from the mesentery separating the left and right somatocoels in the anal interradius. Then these cells accumulated in the mesenchymal layer of the mesentery to make up the primary gonad. Russo states that this is homologous to the development of the Holothuroid gonad, yet, in crinoids, this primary gonad then vanishes! Later in development, Russo detected secondary PGCs in the pentacrinoid larval stage from the coelomic epithelial cells of the ventral mesentery crossing the right somatocoel, and these cells form the secondary gonad near the oral end of the axial organ. (Russo, 1900) Holland (1991) outlines serious doubts of a vanishing gonad in crinoid development, and concludes by saying the identification of PGCs in crinoids begs serious reinvestigation. A recent study of the development of *Antedon bifida* by Sabine Engle reinstates this confusion (Engle, 2013). In this animal, morphologically defined germ cells form in the horizontal mesentery. Yet, morphological germ cells also form in the left extension of the left somatocoel. Again, Engle concludes by looking to future studies for clarification of PGC specification.

The larval development of many stalked crinoids has been studied. Because these descriptions are similar and *Oxycomanthu japonicas* is the most extensively studied species for larval development, we will use Holland's (1991) description of *O. japonicas* larval development to outline the development of stalked crinoid larval coeloms. Gastrulation produces two concentric spheres, the outer ectodermal layer, and the inner endo-mesodermal with little blastocoelar space. The endo-mesodermal archenteron sphere then constricts in the center, which yields a dumb-bell-shaped mesenteric sac. The posterior lobe of the dumbbell buds off first, which later buds into the left and right somatocoels. The anterior lobe of the dumbbell then buds to form the enteric sac, axocoel, and hydrocoel. While other

echinoderms have right and left axocoels and hydrocoels, only crinoids have the equivalent of the left axocoels and hydrocoels; the right are absent (Chia and Walker 1991).

The early larval development of the stalkless crinoid, *Metacrinus rotundus*, has recently been described by Nakano et al. (2003) and Omori et al. (2011). After gastrulation the archenteron is similar to *O. japonicas* gastrulation. The archenteron begins by constricting between the anterior and middle wall to yield a dumbbell-shaped sac. The anterior lobe is the first to bud from the dumbbell, which corresponds to the axo-hydrocoel. The posterior lobe and middle part of the dumbbell then separate from each other. The posterior lobe, or somatocoel, then separates into left and right somatocoels. Mean while, the middle part, or enteric sac, elongates towards the posterior and separates the left and right somatocoels (Nakano et al., 2003; Omori et al., 2011).

Ophiuroidea (Brittle Stars)

The development of morphologically distinct PGCs in Ophiuroidea is reported to occur during the final stage of metamorphosis, in a cluster in the wall of the left posterior coelomic sac (Cuenot 1891; Russo, 1893, 1903 a, b; Bury, 1896; Hamann, 1901; McBride, 1903; Narasimhamurti, 1932, 1933; Smith, 1940; Olsen, 1942a; Austin, 1966; Hendler 1991). Embryogenesis in Ophiuroidea species is also varied. Hendler (1991) summarizes that one coelomic cavity forms from the tip of the archenteron. This original sac then splits into left and right coelomic sacs, and these sacs divide a second time to form two left coelomic sacs (anterior and posterior) and two right coelomic sacs (anterior and posterior). From there, hydrocoels form between the anterior and posterior sacs, and in different cases either arise from anterior or posterior sacs. Deviations from this general scheme of Ophiuroid early development are common (Hendler 1991). In some cases, the right anterior coelomic sac does not develop or disappears during metamorphosis (Müller, 1846a, b, 1851, 1852a; McBride, 1903; Mortensen, 1921; Narasimhamurti, 1933; Olsen, 1942a; Hendler 1991). In others, the left posterior sac grows directly from the archenteron rather than from the anterior sac (Grave, 1900, 1916; Hendler 1991).

In our analysis of the literature, Ophiuroid embryos generally contain two anterior coeloms and two posterior coeloms. The left anterior coelom becomes the axocoel while the left posterior coelom buds to form the hydrocoel and somatocoel (Bury, 1889, 1896; McBride, 1903; Narasimhamurti, 1933; Mortensen, 1937; Yamashita, 1985; Hendler 1991). The confusion and lack of knowledge come in regards to the origin of each coelom. In a report of *Amphipholis kochii* development, the author was unsure how the left and right posterior coeloms were formed, especially because the right posterior coelom formed later in development than the left posterior coelom (Yamashita, 1985). In a report of *Amphiodia occidentalis* development, the archenteron was split into a left pouch and an anterior/medial pouch. The left pouch gave rise to the left coelom while the medial pouch definitively gave rise to the left axocoel and the anterior region of the gut. Also, the origin of the right coelom and posterior gut was uncertain and there was only an account of one coelom on the right side (Emlet, 2006). In a report of *Ophiodaphne for-mata* development, the left and right anterior coelomic pouches divided to form anterior and posterior coeloms on each side (Tominaga et al., 2004).

Holothuroidea (Sea Cucumbers)

Information regarding germ-cell specification in holothuroids is very scarce. Smiley states that, given the available evidence, PGC's have not been identified in holothuroids prior to metamorphosis (Smiley, 1986). Coelomogenesis in *Parastichopus californicus* starts from a single out-pocket on the dorsal aspect of the archenteron. This sac then divides, with the anterior portion becoming the future hydrocoel while the posterior portion will divide once more to become the left and right somatocoels. Further-more, development of the coeloms is similar in direct developers, except for the time of appearance of the structures (Ohshima, 1921; Hyman, 1955; Smiley, 1986; Smiley et al 1991). In other studies, such as that for *Holothuria grisea*, coelomogenesis starts from a single out-pocket on the apex of the archenteron; the rest is similar to *P. californicus* in that the somatocoel precursor buds from the left posterior of the anterior sac and later gives rise to both the left and right somatocoel (Balser et al., 1993). Recently, McCauley et al. (2012) described the development of *Parastichopus parvimensis* and found only a posterior enterocoel, but no obvious anterior enterocoels.

Echinoids (Sea Urchins, Sand Dollars, Pencil Urchins)

We refer the reader to the text above for the most modern view of PGC specification in echinoids; here we only mention coelomogenesis. In the most recent account of sea urchin development, paired enteric sacs form from the tip of the archenteron. They then extend posteriorly on both sides of the esophagus where they give rise to the left and right axocoel, hydrocoel, and somatocoel, which extend anterior to posterior, respectively (the tooth sacs in the adult arise from evagination of the left posterior somatocoel (Smith et al., 2008). In older accounts of sea urchin coelomogenesis, the left and right enteric sacs divided into anterior and posterior sacs, and the left anterior sac was reported to give rise to the axocoel and hydrocoel. Whether the differences between these two accounts are a result of the analysis of different species or simply a modern technique remains uncertain.

Overall Conclusions

We hypothesize that the left posterior enterocoel represents the ancestral mode of germ-line specification in the echinoderm clade because the sMic lineage is not conserved among echinoderms (the location of PGC specification in sea urchins) although the posterior coelomic pouches (the location of PGC specification in sea stars) are conserved in all echinoderm clades. As mentioned above, germ-line molecular markers accumulate in the posterior enterocoel of sea stars, and its removal decreases the number of germ cells in older larvae (Inoue et al., 1992; Juliano and Wessel, 2009). In order to test this hypothesis, more experimental analyses are required, including posterior enterocoel explants or photoablation studies in species from several clades, to test if this structure is the origin of germ cells in these other echinoderm clades. In addition, molecular analyses needs to be performed to determine if conserved germ-line determinants start to accumulate in the left posterior enterocoel in the early larvae stage of development in these other echinoderm clades. Future studies should include lineage-tracing analyses where cells from each coelom are labeled early in development to determine if they take on germ-cell characteristics later in development and to test which cells of each coelom the PGCs originate from.

It is important to note that the ancestral mode of germ-line specification in all metazoans is hypothesized to be inductive (cell interactions) instead of inherited (acquisition of maternal determinants) (Extavour and Akam, 2003). If the posterior enterocoels represent the ancestral mode of germ-line specification within the echinoderm clade, it is possible to postulate that the sea urchin sMic lineage represents a transition from the specification of the germ line in later larval development to an earlier embryonic time point, the 32-cell stage. The sMics are cell-autonomous in their ability to express *nanos* in culture (Yajima and Wessel, 2012). Sea urchins have many derived features in development, including micromeres, hyaline layers, and endoskeletons in larvae. As such, the Echinoidea lineage within the echinoderm phylum may represent a transition from an inductive mechanism for germ-line specification to a more inherited-like mechanism. If this is the case, we can compare germ-line specification mechanisms between echinoderm clades to determine what is required for this shift from one mechanism to another.

Johnson et al. (2011) hypothesized that a benefit of the inherited mode of PGC specification is the relinquishing of restrictions on signaling mechanisms (formerly required for germ-cell specification) so that the embryo can develop more derived larval characters. Coincidentally with the birth of the sMic lineage in sea urchins, a variety of factors in sea stars localize instead to the larval skeleton in sea urchins (Hinman et al., 2003; Koga et al., 2010). Did the shift allow for diversification of gene regulatory networks and transcription factors at earlier time points in the sea urchin larvae, resulting in more diversity in larval form and formation of a larval skeleton? And do the holothuroids, animals without a sMic lineage but with a less morphologically complex larval skeleton, represent another point on the continuum between sea stars and sea urchin mechanisms of PGC specification? Future experiments that test the timing of PGC specification in sea cucumbers are necessary—for example, an accumulation of germ-line markers at a developmental time between those of the sMic in sea urchins and the posterior enterocoels in sea stars would support this hypothesis.

The adult rudiment forms in the anterior left side of sea urchins but in the entire posterior of sea star larvae. Are L/R signaling molecules restricted to PGC specification in sea stars? In order to address these questions, the inductive signals required for PGC specification in sea stars need to be elucidated. Only then we can analyze the function of the same signaling molecules in sea urchins, testing if they have become unrestricted and contribute to diversification of body structures in early development. Also, we can test if inductive signals required for PGC specification in sea stars are conserved in a broader sense with other metazoans that use inductive mechanisms for PGC specification, such as in the mouse.

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Abbreviations

ABC transporter

ATP	binding cassette transporter
A–V	animal–vegetal
L/R	left–right [asymmetry]
LMics	large micromeres
PGC	primordial germ cell
sMic	small micromeres
UTR	untranslated region

Glossary

Adult rudiment	The larval structure that contains the adult primordial tissues. It will develop throughout the larval stage and eventually evert at metamorphosis to form the juvenile sea urchin.
Large micromeres (LMics)	Sister cells to the small micromeres that give rise solely to the larval skeleton.
Micromeres	The parent cells of the large and small micromeres, formed by an asymmetric cell division at the 4th cell cycle of the sea urchin embryo.
Primordial germ cells (PGCs)	The lineage of an embryo/larva that is committed to producing the germ line in animals. These cells migrate to the gonad and multiply to eventually form the eggs or sperm in the adult.
Small micromeres (sMics)	Cells formed at the 5th cell division of embryogenesis by an asymmetric cell division that contribute to the germ line of the adult sea urchin.

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Figure 1.

Diagram of the development of a sea urchin. Early development yields Vasa-positive cells (shown in red, beginning with a uniform Vasa positive egg and early embryo). At the 32-cell stage, the sMics are uniquely Vasa-positive. These cells move into the coelom during gastrulation, segregate into the left and right coelomic pouches, and expand to contribute to the germ cells of the adult, and likely also to some somatic cells of the rudiment.

Figure 2.

The morphology of the sMics. The early embryo of *Lytechinus variegates* was imaged in a variety of ways to expose the various cells. **A**: DAPI-labeled 32-cell stage viewed from the vegetal pole. **B–D**: lateral view of 32-cell stage embryos viewed with differential interference contrast (B), scanning electron microscopy (SEM) (C), and pseudocoloring (D) to identify the various tiers of cells. **E–H**: DAPI-stained progressions, viewed from the vegetal pole, of the formation of the four sMics (centered) followed by cell division of the large micromeres adjacent to them. The cell cycle of the sMics is shown relative to their neighboring and sibling cells. **K**: Spli 32-cell embryo fixed for SEM showing the various tiers of cells and the LMics and sMics at the bottom. **M**: A stereo view of a 32-cell stage embryo viewed from the vegetal pole. Stereo view glasses (B/W) or slightly crossed eyes are needed to view this pair. For more detailed information on the other cell types, see, for example, Horstadius (1976), McClay (2011), Gilbert (2013). Figure courtesy of John Morrill, personal communication.

Figure 3.

Sea urchin sMics exhibit slow cell-cycling, as revealed by bromo-deoxyuridine (BrdU) pulse-chase studies. The sMics divide much slower relative to the somatic cells. Late gastrula (**A**, side view; **B**, top view) showing sMics selectively labeled with bromo-deoxyuridine (BrdU). BrdU was pulsed in the embryo following fertilization, washed, and the embryos were incubated until various time points. The sMics retain the BrdU relative to other cells of the embryo because they divide much slower than other cells (soma) and thereby do not dilute the BrdU in their genome (Tanaka and Dan, 1990; similar results were first shown by Pehrson and Cohen, 1986). Scale bar in A. 10 μ m; in B 20 μ m.

Figure 4.

The sMics are dispersible for development. Diagram at top pointing out the sMic lineage (red). **A**: Method of sMic removal with a glass pipette. The resulting embryos develop into a larva (**B–E**), form an adult rudiment (**D, E**, arrow in **E**), and metamorphos into an adult (**F**) that does not produce gametes. Scale bars, $__ \mu\text{m}$.

Figure 5.

Summary of the Vasa-compensatory mechanism. Vasa mRNA is abundant in *S. purpuratus* 16-cell stage embryos, but not in *L. variegates* embryos of the same stage. When micromeres are removed from embryos of each species, Vasa protein is upregulated in the blastulae in proportionl to the amount of *vasa* mRNA present. Adults are obtained from each species, at a frequency proportional to the amount of Vasa protein upregulated. When sMics are removed from either species at the 32-cell stage, no Vasa up-regulation is detectable and no germ cell production is seen.

Figure 6.

Vasa associates with the mitotic spindle in early cleavage divisions. Vasa is present throughout the cytoplasm in early embryos, but becomes enriched in the spindle. during mitosis. Scale bars, μm . Reprinted with permission from Yajima and Wessel (2011).

Figure 7.

Vasa is asymmetrically partitioned into the micromeres. During anaphase of the 4th cell division in the vegetal tier of blastomeres, Vasa becomes enriched in the future micromere region of the spindle and concentrates in the vegetal-most region of the embryo. This phenomenon is repeated again to form the sMics, which retain the highest concentration of Vasa. Whole embryos are shown in A and B and are each ~90 microns in diameter, whereas the micromeres shown in C, D, and E are each ~10 microns in diameter. in the embryo. Scale bars, ___ μm . Reprinted with permission from Yajima and Wessel (2011).

Figure 8.

The endoplasmic reticulum is enriched at the vegetal pole in early development. Scale bar, μm . scale bar is 50 microns Reprinted with permission from Yazaki et al. (2004).

Figure 9.

Calcium fluxes (red circle in photos, and red line in graph) are unique in the sMics. Scale bar, $__\mu m$. Scale bar - 50 microns Reprinted with permission from Yazaki (2001).

Figure 10.

Post-translational Vasa regulation during sea urchin embryogenesis. **A:** Whole-mount in situ RNA hybridization of endogenous *vasa* transcript at the indicated developmental stages. **B:** Immunofluorescence localization of endogenous Vasa protein at the indicated embryonic stages. **C:** Two synthetic mRNAs were co-injected into fertilized eggs, then imaged at the blastula stage. One mRNA contained the Vasa open reading frame with a C-terminal GFP tag flanked by β -globin 5'- and 3'-UTRs. The control mRNA contained only the mCherry open reading frame flanked by β -globin 5'- and 3'-UTRs. **D:** Immunofluorescence

localization of endogenous Vasa protein in embryos cultured in artificial seawater (ASW) alone, ASW containing 0.5% dimethyl sulfoxide (DMSO), or ASW containing 25 or 50 μ M MG132. Scale bars, μ m. "control blastulae are 100 microns in diameter."

Figure 11.
Temporal dynamics of sMic transcriptional activity. Sea urchin embryonic stages are schematized with the sMics indicated in green. Colored bars below indicate timing of events in the sMics

Figure 12.

sMic retention of an exogenous RNA containing the *mCherry* open reading frame surrounded by the *Xenopus* β -globin UTRs. **A:** mCherry fluorescence at the gastrula stage after RNA injection. **B:** In situ RNA hybridization at the gastrula stage after injection of the RNA.

Figure 13.

Summary of the RNA constructs tested for sMic RNA retention. The open reading frame is surrounded by *Xenopus β-globin* UTRs. The black circle represents the m⁷GTP cap, and the polyadenylation tail is symbolized by A₃₀. In the second construct, the "x" indicates the mutation of the polyadenylation signal in the 3'-UTR from *β-globin*.

Figure 14.

Sp GNARLE is necessary for selective RNA retention and protein accumulation in the sMics. **A:** Schematic representation of *nanos2* RNA, indicating the location of GNARLE in the 3'-UTR. **B:** RNAs containing the *Sp nanos2* 5'-UTR followed by a GFP open reading frame and by either GNARLE or Δ GNARLE 3'-UTR were injected in *S. purpuratus* fertilized eggs. At blastula stage, RNA retention was tested by in situ hybridization and protein accumulation was followed by GFP fluorescence.

Figure 15.

Differential accumulation of CNOT6 in somatic and germ-line blastomeres. **A:** *Cnot6* transcript (green) is detectable in all cells of an 18-hr blastula by fluorescence in situ hybridization, except in the sMics, which are labeled by Vasa immunofluorescence (red). **B,C:** Schematization of differential RNA retention in somatic cells versus sMics. **B:** *Cnot6* RNA is present in all somatic cells, and is translated and likely incorporated into a CCR4-NOT related complex, where it affects the degradation of germ-line RNAs. **C:** Nanos/Pumilio targets the *Cnot6* transcript for degradation, leading to a reduction in de-adenylase

activity in the sMics. This reduction creates a generally stable environment for both endogenous germ-line RNAs and microinjected reporters.

Figure 16.

ABC transporters are involved in PGC success. **A:** In *Drosophila*, the ABC transporter *mdr49* is required for germ-cell migration to the mesoderm. An *mdr49*^{D3.16} embryo (bottom) shows lost germ cells. Reprinted with permission from Ricardo and Lehmann (2009). **B:** The fluorescent indicator calcein indicates ABC activity the absence of fluorescence indicates active efflux, whereas the presence indicates a lack of ABC efflux activity. Remarkably, the sMics have low ABC-effluxing activity and, as a result, retain calcein in the cytoplasm. 10 microns Reprinted with permission from Campanale and Hamdoun (2012)

Figure 17.

Members of the phylum Echinodermata. There are five extant classes of echinoderms: Crinoidea, Ophiuroidea, Asteroidea, Holothuridea, and Echinoidea. Representatives of these classes: sea lily *Metacrinus rotundus*; brittle star *Amphipholis kochii*; sea star *P. miniata*; sea cucumber *Holothuria grisea*; sea urchin *S. purpuratus*. Schematic drawings of larval stages indicating different tissues for each organism. **A**: A 3.5-day, non-feeding auricularia larva. **(B)** A 6-day, eight-armed ophiopluteus. **(C)** A 5-day bipinnaria larva. **(D)** A 4-day aspidochirotid auricularia. **(E)** A 4-armed pluteus larva. Dorsal views of the larvae are shown. Members of the class Echinoidea, which have a micromere lineage, are split into the subclasses Euechinoids (sea urchins and sand dollars) and Cidaroids (pencil urchins).

Figure 18.

Late gastrula stage from *P. miniata*. This dynamic sequence shows mesenchyme migrating from the apical plate (*ap*) and coming in contact with the anterior vesicle (*av*). **1:** The archenteron bears the usual two enterocele pouches and a single, left posterior one. **2:** Anterior end of bipinnaria showing the completion of the mesenchyme formation at the apical pole and its migration to the esophageal region. The left hydrocele is provided with a pore canal and hydropore. *lp*, left posterior vesicle. **3:** Section of gastrula showing the apical plate with signs of mesenchyme formation. **4:** Bipinnaria showing mesenchyme migrating

past the anterior vesicle (*av*) to the esophagus. The left hydrocele has severed its connection with the archenteron. *lp*, left posterior vesicle in process of formation. **5**: Surface overview of gastrula immediately before mesenchyme development from the apical plate. Reprinted with permission from Heath (1917).

TABLE 1

Electrophysiological Properties of the 16-Cell Embryo (From Dale et al., 1997)

Cell	Cell diameter (microns)	Resting potential (mV)	Calcium current (microAmps/cm ²)
Mesomere	31	-25 ± 3	7.4 ± 1.7
Macromere	37	-27 ± 4	2.3 ± 1.2
Micromere	20	-9 ± 1	0

TABLE 2

Relative Calcium Concentration in the 16-Cell Stage Blastomeres

Species	Micromere/ macromere	Mesomere/ macromere
<i>Hemicentrotus pulcherrimus</i>	1.14 (± 0.018)	1.02 (± 0.004)
<i>Pseudocentrotus depressus</i>	1.10 (± 0.010)	1.00 (± 0.004)