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Autonomy in specification of primordial germ cells and their passive translocation in the sea urchin

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Abstract

The process of germ line determination involves many conserved genes, yet is highly variable. Echinoderms are positioned at the base of Deuterostomia and are crucial to understanding these evolutionary transitions, yet the mechanism of germ line specification is not known in any member of the phyla. Here we demonstrate that small micromeres (SMics), which are formed at the fifth cell division of the sea urchin embryo, illustrate many typical features of primordial germ cell (PGC) specification. SMics autonomously express germ line genes in isolated culture, including selective Vasa protein accumulation and transcriptional activation of *nanos*; their descendants are passively displaced towards the animal pole by secondary mesenchyme cells and the elongating archenteron during gastrulation; Cadherin (G form) has an important role in their development and clustering phenotype; and a left/right integration into the future adult anlagen appears to be controlled by a late developmental mechanism. These results suggest that sea urchin SMics share many more characteristics typical of PGCs than previously thought, and imply a more widely conserved system of germ line development among metazoans.

Keywords: Vasa, PGC, Germ line, Cadherin, Sea urchin, *Strongylocentrotus purpuratus*

INTRODUCTION

Germ line specification is essential for an organism to produce offspring. Despite its importance in species propagation, the process appears highly variable among organisms even within the same phylum, and probably maximizes the diversity of reproductive niches. Two major germ line specification mechanisms have been proposed: autonomous and conditional specification. Autonomous, or inherited, specification includes several model organisms such as *Drosophila* (Williamson and Lehmann, 1996), *C. elegans* (Kimble and White, 1981) and zebrafish (Yoon et al., 1997). Primordial germ cells (PGCs) in these organisms are specified early in development by inherited cytoplasmic determinants from maternal stores (Gao and Arkov, 2012). By contrast, conditional, or inductive, processes specify PGCs later in development by intercellular communication; this form of specification is primarily responsible for PGCs in mouse (Lawson and Hage, 1994; Tam and Zhou, 1996), axolotl (Nieuwkoop, 1947), cnidarians, sponges (Extavour and Akam, 2003) and ascidians (Shirae-Kurabayashi, 2006).

Although the mechanisms of germ line specification vary in each organism, PGCs share many characteristics. For example, regardless of the mechanism of specification, autonomous or conditional, conserved genes are important in this process and include the RNA helicase *vasa*, the translational repressor *nanos*, and the small RNA regulator *piwi*. These genes and, depending on the organisms, many others are responsible for PGC specification, differentiation and/or maintenance in the germ line among metazoans (for reviews, see Ewen-Campen et al., 2010; Extavour and Akam, 2003; Raz, 2000). During specification, the PGCs initially cluster with each other and then later in development migrate collectively to specific regions where the gonads will form. E-cadherin appears to be essential for regulating these events, and E-cadherin-deficient PGCs fail to cluster, migrate or even specify themselves as PGCs in mouse, zebrafish and *Drosophila* (Cano et al., 2000; Okamura et al., 2003; Marthiens et al., 2010; Kardash et al., 2010; Matsui, 2010; Saga, 2010; Tarbashevich and Raz, 2010).

Echinoderms are a sister group to the Chordates and are an early branching group in Deuterostomia. Although the molecular mechanisms of germ line specification in this phylum are not known, their eggs do not have obvious preformed germ line components (Ransick et al., 1996; Juliano et al., 2006) and thus its germ line is considered to be conditionally specified during late development. Recent studies, however, suggest that small micromeres (SMics) formed at the fifth cellular division have PGC features. The SMics are located at the vegetal tier in association with endomesodermal precursors, divide more slowly than their adjacent cells (Tanaka and Dan, 1990), express germ line-related genes such *vasa, nanos* and *piwi* (Juliano et al., 2006), and are involved in germ cell formation in the adult (Yajima and Wessel, 2011a). SMics are not necessary for larval development and contribute only to subregions of the coelomic pouches (Pehrson and Cohen, 1986), the precursor of the adult rudiment (see Fig. 7B). After early larval stages, the left coelomic pouch expands and becomes the major contributor to the adult rudiment and will develop most adult structures before metamorphosis. The nascent coelomic pouches formed at the tip of the archenteron by prism stage consist of 40% cells from the SMic lineage and 60% from the macromere lineage (Cameron et al., 1987; Cameron et al., 1991), which is a distinct lineage derived from the second vegetal tier of the blastomeres (Veg2) formed at the fifth cell division. Therefore, it is intriguing to hypothesize that SMics are PGCs and Veg2 macromeres are a somatic multipotent cell lineage.

We tested here several molecular and morphological features of SMics that are typical of PGCs, such as autonomous expression of germ line-related molecules, passive de-epithelialization and translocation behavior during gastrulation, Cadherin-dependent cell specification and clustering, and we conclude that several overarching mechanisms appear conserved between the SMic lineage and the more widely studied PGCs, such as *Drosophila* pole cells.

MATERIALS AND METHODS

Animals, embryos and larval culture

S. purpuratus were collected in Long Beach, CA, USA, and housed in aquaria containing artificial seawater (ASW; Coral Life Scientific Grade Marine Salt; Energy Savers Unlimited, Carson, CA, USA) at 16°C. Gametes were acquired by 0.5 M KCl injection. Eggs were collected in ASW and sperm were collected dry. To obtain embryos, fertilized eggs were cultured in ASW or Millipore-filtered seawater (MFSW) at 16°C. When early stage embryos were required for blastomere labeling, fertilization was performed in the presence of 1 mM 3-aminotriazol (Sigma, St Louis, MO, USA) to inhibit cross-linking of the fertilization envelope. Before labeling, envelopes were removed by gentle pipetting.

Chemical treatment and immunolabeling

Whole-mount immunostaining was conducted as described previously (Yajima, 2007b; Yajima and Kiyomoto, 2006). Briefly, late larvae were fixed with methanol at −20°C for 1 hour and rinsed twice in PBS saturated with calcite (PBSC). Washed specimens were immunostained with 1:300 SpVasa antibody

(Voronina et al., 2008) for 3 hours at room temperature, and after rinsing with PBSC they were reacted with Cy3 goat anti-rabbit immunoglobulin G (IgG) antibody (Invitrogen) for 3 hours at room temperature. After further rinsing with PBSC under the same conditions, larvae were mounted on a glass slide and observed by confocal laser microscopy (Zeiss LMS510). FM1-43 (McNeil et al., 2003) was used at a final concentration of 4 nM and embryos were treated immediately before imaging. FM1-43 is a lipophilic, membrane-impermeant fluorescent dye and only cells exposed to the outside of the embryo will fluoresce in media containing FM1-43 (Covian-Nares et al., 2008).

Injection, blastomere labeling and micromere isolation

mRNA was transcribed from Vasa-GFP (Gustafson et al., 2011) and membrane-mCherry (Megason and Fraser, 2003) constructs using the SP6 mMessage mMachine (Ambion). SpG-cadherin morpholino (G-cad MO) was prepared by Gene Tools (Philomath, Oregon, USA) and was designed against the 5′ UTR of *SpG-cadherin*: 5′-TCCACCTCGGATTTACAGCCATCGT-3′. Injection into fertilized eggs was performed as described (Yajima et al., 2007) using ~6 pl of injection mix (mRNA, MO, dye). The blastomere injection for the Mic, SMic, mesomere or macromere was performed iontophoretically with an Axoporator 800A (Molecular Devices, Sunnyvale, CA, USA) either at the 16-cell or 32-cell stage as follows: embryos were placed in a glass chamber as previously described (Yajima, 2007b) and the fluorescent dye (Fluoro-ruby, Sigma) and/or 0.1 mM G-cad MO was injected into each blastomere while being observed by fluorescence microscopy (Zeiss Axioplan). Mics were isolated using a glass needle at the 16-cell stage as described (Yajima, 2007a) and were cultured in plastic Petri dishes with MFSW at 16°C. The distance of PMC migration and the number of cell divisions in the culture were manually calculated from images selected randomly from five clusters of isolated Mic descendants.

Time-lapse recording

Embryos were injected with Vasa-GFP mRNA, and the resulting Vasa-GFP protein became enriched in the SMic lineage (Gustafson et al., 2011). Membrane-mCherry mRNA was co-injected to better visualize the membrane movements of the SMics and other cells in the embryo. For prolonged visualization, embryos were embedded in a 0.5% soft agar plate to immobilize them during the recording. For time-lapse recordings, images were taken every 25 minutes for 10-15 hours, and five to seven *z*-stacks were collected with a depth of 35 μm for each time point using a Zeiss 710-2 photon confocal microscope at MBL, Woods Hole, MA, USA. The resultant *z*-stack images were projected using Zen 200 software (Zeiss) and movies were made using ImageJ software (NIH).

RESULTS

Small micromeres autonomously express and maintain germ line determinants

Small micromeres (SMics) are formed at the fifth division in the *S. purpuratus* embryo and contribute to germ cell formation in the adult (Yajima and Wessel, 2011a). To delineate the mechanism of SMic specification, Vasa-GFP mRNA was injected into fertilized eggs, and the micromeres (Mics), which are parent blastomeres of SMics, were isolated at the fourth cell division using a glass needle and were cultured (*n*=25; Fig. 1A) in a plastic Petri dish with Millipore-filtered natural seawater (MFSW), which is the same medium in which the embryos were grown and the SMics were exposed. This culture approach was previously used to document that the large micromeres (LMics) develop exclusively and autonomously into the skeletogenic lineage (Endo, 1966; Okazaki, 1975a; Okazaki, 1975b). The LMics divide several times in culture and migrate to form spicules in the presence of serum, just as programmed in vivo. The fate of the sibling cells of LMics, the SMics, in these culture conditions is unknown. Depending on the condition of the dissection, all four Mics were isolated together (Fig. 1B) or one or two of the four were isolated and cultured (Fig. 1C). In both cases, 45-60 minutes after the beginning of the culture procedure, these cells asymmetrically divided and formed the sibling LMics and SMics. Vasa

protein selectively accumulated asymmetrically into the SMics, just as it does in vivo (Fig. 1B,C, arrows) (Yajima and Wessel, 2011b), such that the skeletogenic LMics only have background levels of Vasa whereas the SMics possess strong Vasa signal. Vasa accumulation increased in the SMics following this unequal division and Vasa accumulated in its typical, perinuclear and granular-like structure (Fig. 1B,C, right two panels). This post-translational mechanism of Vasa regulation as seen in vivo (Gustafson et al., 2011) was faithfully replicated in these isolated culture conditions.

We cultured isolated Mics up to 5 days postfertilization in MFSW. At 48 hours postfertilization (hPF) (day 2), the LMic lineage demonstrated weak (background level) Vasa-GFP expression and migrated extensively with extended filopodia (in five explants the minimum migration distance was 12±1 μm and maximum was 66±6 μm), whereas the SMic lineage cells remained adherent to each other in their original aggregate, did not divide, and maintained the typical (perinuclear and granular) Vasa expression (Fig. 1D). From day 3 to day 5, the LMic descendants underwent further cell divisions, reaching 14-36 cells per cluster (up to five cell divisions, n=5) with a corresponding decrease in size, whereas the SMic rarely underwent a cell division (once, maximally), remained within their original site, and retained perinuclear Vasa expression (Fig. 1E). This in vitro phenotype of no migration and slow cell division of the SMics further replicated the in vivo phenotype. Additionally, we tested the ability of these cells to undergo new transcriptional activation. *nanos* is a translational repressor and is usually found in PGCs. In the sea urchin, *nanos* is expressed and maintained specifically within the SMic lineage (Juliano et al., 2006; Juliano et al., 2010; Fujii et al., 2009). We tested the ability of SMics to activate *nanos* transcription in culture by qPCR. *nanos* mRNA accumulation was quantified in isolated Mic descendants after 1 day in culture and immediately after isolation at the 16-cell stage. These values were then compared with embryos both before nanos expression (2-cell stage) and at the late blastula stage when *nanos* accumulation is maximal. We found that *nanos* transcriptional activation in vitro closely replicates its activation in embryos (Fig. 1F) (Juliano et al., 2006).

Overall, these experiments show faithful replication in vitro of several SMic lineage-specific features that occur in embryos: strong cell-cell adhesion, low cell division, no cell migration, post-translational regulation of Vasa, and transcriptional activation of *nanos*. We conclude from these results that initial specification of the SMics occurs autonomously in the embryo following the 16-cell stage. These conclusions also support previous findings suggesting that the initial specification of the SMic lineage occurs at this stage in early embryogenesis (Yajima and Wessel, 2011a). Furthermore, since SMics are capable of maintaining Vasa and *nanos* expression and their morphological features even after the LMics have migrated away from them, these results suggest that an initial step in SMic specification is completed at this stage and that their subsequent translocation to the coelomic pouches might be passive during gastrulation in vivo.

De-epithelialization of SMic descendants at the beginning of gastrulation

Movement of the SMics and their descendants was examined during gastrulation by two different methods: labeling in static sections with Vasa antibody and time-lapse recordings with Vasa-GFP and membrane-mCherry. The membrane-bound mCherry provides contrast to the cytoplasmic Vasa and reveals cellular dynamics, which is especially important for the SMics examined here in order to characterize cellular extensions and migration. The Vasa-GFP mRNA injected into eggs expresses fluorescent Vasa protein throughout the early embryo, but it accumulates selectively within the SMics and becomes more selective for SMic descendants during gastrulation (Voronina et al., 2008; Gustafson et al., 2011). Thus, SMics can be readily traced during development with either of these experimental approaches and the results were perfectly complementary. Vasa-positive SMic descendants were present in the epithelial layer of the blastula (Fig. 2A-D), even following ingression of the LMic descendants [or primary mesenchyme cells (PMCs); Fig. 2A,C, arrows] into the blastocoel. Only at the beginning of invagination of the vegetal plate to form the archenteron, did the SMic descendants de-epithelialize into the blastocoel and lose contact with

the extra-embryonic environment (Fig. 2B-F, Fig. 3A). This de-epithelialization contrasts with the ingression of PMCs or secondary mesenchyme cells (SMCs; Fig. 2G,H, arrows) that penetrate through the basal lamina by their own migratory force via a classic epithelial-mesenchymal transition (EMT) (Katow and Solursh, 1981; Wu and McClay, 2007). PMCs ingress at the blastula stage and become skeletogenic cells, whereas SMCs ingress from the elongating archenteron during gastrulation and become several different cell types, including pigment cells, blastocoelar cells and muscle cells (Ruffins and Ettensohn, 1993; Ruffins and Ettensohn, 1996). Unlike PMCs and SMCs, SMic descendants remain together with minimal shape changes and appear to be passively translocated in response to epithelial movements (supplementary material Movies 1-3). In the absence of a more specific mechanistic understanding, we refer to this change in SMic positioning as de-epithelialization.

The de-epithelialization process was further tested using FM1-43 (Fig. 4). Vasa-GFP mRNA was injected into embryos to label SMic descendants and embryos were bathed in media containing FM1-43 immediately before imaging to label cells exposed to the extra-embryonic environment (Fig. 4). From 20-25 hPF, SMic descendants showed both the Vasa-GFP (green) and FM1-43 (red) signals, indicating that they are exposed directly to the extra-embryonic environment (the medium) at this time. The vegetal plate, however, changes after 27 hPF, and FM1-43 signal is found irregularly within the thickening vegetal plate as a result of the reorganization of cells in this region. At 29 and 30 hPF, the thickening vegetal plate displaced the bulk of SMic descendants into the blastocoel, which were then FM1-43 negative. This internalization is coincident with ingression of the SMCs and before the endodermal invagination (Endo, 1966). Thus, the cells surrounding the SMic descendants are SMCs (Ruffins and Ettensohn, 1993; Ruffins and Ettensohn, 1996) (Fig. 4, 29 hPF, arrows). By 33 hPF, it became clear that the Vasa-positive SMic descendants had lost contact with the extra-embryonic environment, and the FM1-43 signal became more linearly organized in the apical region of the embryo, a likely result of the junctional complexes that form at this time (Fig. 4, 33 hPF, arrowheads), indicating that this reorganization of the vegetal plate is completed. The cells remaining in contact with the SMic descendants appeared to continue to be SMCs (Fig. 2G,H, arrows): SMCs started extending pseudopodia to migrate and the SMic descendants moved towards the animal pole within clusters of those SMCs (Fig. 2I,J, Fig. 3B,C). These results further suggest that SMic descendants are unlikely to be migratory, but are moved passively by the contraction of the epithelia and the migratory force of SMCs.

Passive movement of SMics along the extending archenteron

Early in gastrulation, the SMCs, as derived from the Veg2 lineage (Horstadius, 1950; Logan and McClay, 1997; Sherwood and McClay, 1999; Sweet et al., 2002), formed a cluster of cells in the vegetal region of the blastocoel, which encompassed the SMic descendants during gastrulation (Fig. 3B,D, arrows). SMCs formed a variety of shapes during their extension, and the SMic descendants adhering to the SMCs also demonstrated a variety of displacement organizations, yet they consistently stayed together (Fig. 2I-P). Unlike migratory cells, such as the PMCs (Fig. 3B,C, red arrows) and SMCs (Fig. 3B,C, blue arrows), SMics never formed blebs or pseudopodia, which are typical devices for cell migration. Instead, it appeared that the SMic descendants were passively translocated, carried by SMCs and the elongating archenteron (Fig. 3C,D; supplementary material Movies 3-5).

Once endodermal invagination was completed (Fig. 2P), the SMic descendants formed a fine line at the tip of the archenteron (Fig. 2Q) before moving into either the left or right coelomic pouch anlagen (Fig. 2R-T, Fig. 3E; supplementary material Movie 6). The SMC-derived coelomic pouches were created in advance of SMic descendant migration (Fig. 2R, arrows). We speculate that SMic descendants become migratory after this point in development in order to enter into the forming coelomic pouches. Until the end of gastrulation, however, the results here (Figs 1, 2, 3 and 4) suggest that SMics are unlikely to be motile but

are adherent to each other and to SMCs. SMics thus utilize the SMC migratory force and the elongation force of the archenteron to reach the tip of the archenteron, in close apposition to the forming coelomic pouches (as summarized in Fig. 7A).

Left/right determination is not completed at the fifth division

The first major morphological change during sea urchin embryogenesis occurs at the asymmetric fourth cell division into Mics, and subsequently into LMics and SMics at the 32-cell stage. The SMics are the smallest cells at this point in development, are Vasa-protein positive, and are responsible for the germ cells in the adult (Yajima and Wessel, 2011a). They are destined for the right and left coelomic pouches of the larvae, which will give rise to the adult rudiment. Routinely, each developing pouch will acquire three to five SMic descendants and a total of eight after gastrulation. The cells in the left pouch proliferate significantly more than those on the right at the late larval stage, and a majority of the germ cells are thought to come from the left coelomic pouch, which is also the side that makes the major contribution to the adult rudiment cells (Cameron et al., 1991; Aihara and Amemiya, 2001).

To test whether SMics at the fifth division are predetermined to a particular side, one of the four SMics was labeled with dye at the 32-cell stage (Fig. 5A), just after its formation. Its lineage was traced to the prism/pluteus stage, by which time the SMic descendants go through at least two cell divisions (Tanaka and Dan, 1990; Cameron et al., 1987). As a result, several labeled SMic descendants from a single SMic were found in the nascent coelomic pouches. Left/right (L/R) selection of labeled cells was random, even though they shared a common origin at the 32-cell stage; they translocated into both pouches equally (Fig. 5B; ratio of 1:1; n=3), more into the left or right side (Fig. 5C; ratio of 1:3; n=7), or into just one of the pouches (ratio of 0:4; n=1). These results suggest that the L/R decision has not been made at least at the fifth division and that a subsequent step of specification must occur, perhaps under the influence of lefty/nodal after the blastula stage (Duboc et al., 2005).

G-cadherin is essential for SMic specification

Cadherins function in a variety of processes in addition to their roles in cell adhesion. In the context of germ cell development, recent evidence suggests that these gene products might participate in germ cell specification, migration, and even in asymmetric cell division. Here, we tested whether G-cadherin functions in SMic development. Although sea urchins have several Cadherin genes, *G-cadherin* mRNA preferentially accumulates in the SMic descendants during gastrulation and becomes even more enriched in cells of the left coelomic pouch in early larvae (Miller and McClay, 1997). The left coelomic pouch is where most SMic descendants accumulate and are thought to contribute to the majority of adult structures, including the germ line; thus, this asymmetric expression of *G-cadherin* is intriguing. G-cadherin shares a number of similarities with the classic vertebrate E- and N-cadherins, including its Cadherin-specific repeats and a highly conserved cytoplasmic domain that is essential for catenin binding (Miller and McClay, 1997).

A G-cadherin morpholino antisense oligonucleotide (G-cad MO) was used to determine functionality in the SMics. First, it was injected into fertilized eggs to block zygotic translation of G-cadherin. High doses (1 mM) of G-cad MO caused severe developmental defects, including (1) loss of adhesive strength between blastomeres; (2) loss of asymmetric division and of the enrichment of Vasa-GFP in Mics at the 16-cell stage, with failure of the embryo in gastrulation; and (3) loss of SMic descendants in gastrulae (Fig. 6A, middle panels). These phenotypes were never observed in control embryos injected with dye and other gene-specific MOs, suggesting a specific knockdown phenotype driven by G-cad MO (supplementary material Fig. S3, control embryos) (Juliano et al., 2010; Yajima et al., 2012; Yajima and Wessel, 2011b). Further, because the different Cadherins of *S. purpuratus* have unique 5′UTR and signal sequences, the knockdown is specific to G-cadherin.

Eggs injected with a lower dose (0.2 mM) of G-cad MO formed an irregular number of Mics, with inconsistent localization of Vasa. These embryos were delayed in gastrulation and the specific localization of Vasa in SMic descendants was often unclear, both with Vasa-GFP and with endogenous Vasa protein (Fig. 6A, lower panels; supplementary material Fig. S3). Vasa-specific localization in Mics at the 16-cell stage was also lost in embryos treated with SDS, a classical treatment that interrupts asymmetric divisions resulting instead in symmetric divisions at the 16-cell stage (supplementary material Fig. S3) (Langelan and Whiteley, 1985). The results suggest that asymmetry and/or a physically smaller cell might be important for specific localization of Vasa in Mics and SMics, rather than a specific function of G-cadherin.

To further test the hypothesis that G-cadherin has a role in SMic specification, we injected G-cad MO (0.1 mM) mixed with red fluorescent dye into one Mic at the 16-cell stage (Fig. 6B). A Mic divides into two groups: the LMic lineage that becomes the PMCs (the skeletogenic cell lineage); and the SMic lineage. In G-cadherin-deficient (Cad-deficient) Mics, the LMic lineage successfully differentiated into PMCs and ingressed into the blastocoel at the blastula stage, as normal (sibling) cells do, and the SMic lineage was located in the vegetal plate as normal (Fig. 6B, 30 hPF). After gastrulation, Cad-deficient PMC descendants successfully differentiated into skeletogenic cells to form larval spicules. However, Cad-deficient SMic descendants (n=18) were never found in the coelomic pouches, in contrast to the wild-type sibling SMic descendants (Fig. 6B, 60 hPF). These results suggest that newly synthesized G-cadherin is essential for SMic descendant cell specification and/or movement.

We next tested whether Cad-deficient SMic descendants lose Vasa-GFP either by cell death or by transdifferentiation into other lineages such as PMCs. G-cad MO was injected into only one of the SMics at the 32-cell stage (Fig. 6C). Although injected SMics often died shortly after injection owing to technical difficulties, five out of ten embryos demonstrated SMic descendants labeled with dye that ingressed into the blastocoel and integrated into a group of skeletogenic cells derived from the PMCs. G-cad MO had no effect on the fate of the macromere and mesomere lineages (Fig. 6D), suggesting that only SMics are significantly dependent on G-cadherin for their phenotype. Although it is not clear whether these Cad-deficient SMic descendants fully transfated into PMCs and functioned as skeletogenic cells, it is apparent that they acquired migratory activities and lost their germ line lineage characteristics, such as Vasa expression. These results suggest that G-cadherin contributes significantly to SMic specification during embryogenesis.

DISCUSSION

Within one cell division of the Mics, the resultant siblings are separated by the most extreme developmental potentials. One lineage, the LMics, is irreversibly committed to a singular fate: the larval skeletogenic lineage. These cells actively ingress into the blastocoel following a proscribed series of cell shape changes. Once in the blastocoel, they extensively and individually migrate along the wall of the blastocoel and, based on interactions with the ectoderm, undergo a species-specific pattern of biomineralization (for reviews, see Ettensohn, 1992; Wilt, 2002; Wu et al., 2007). The fate of their siblings, the SMics, is completely different. The SMics contribute to adult structures, including the germ line, and therefore provide the genetic basis for species propagation and the developmental potency necessary for subsequent generations of new eggs, sperm and totipotent embryos. The basis for this immediate developmental divergence between LMics and SMics is not clear, but experiments described herein show several key developmental features of SMics that are relevant to early germ line determination in this organism.

The developmental origin of the germ line and the specification mechanism of sea urchins have long been controversial. Sea urchin SMics have several PGC-like features, such as early segregation at the fifth division, a transcriptionally quiescent state (Pehrson and Cohen, 1986), a slower cell cycle (Tanaka and Dan, 1990), expression of germ line markers (Juliano et al., 2006), and a contribution to germ cell

formation in the adult (Yajima and Wessel, 2011a). Yet, when the precursors of these cells, the Mics, are removed from the embryo, the resultant adults are still gravid and form eggs and sperm in the absence of the Mics (Ransick et al., 1996). One explanation for this is a reassignment of germ cell fate by Vasa upregulation in the Mic-deficient embryo via post-translational mechanisms (Voronina et al., 2008; Gustafson et al., 2011; Yajima and Wessel, 2011a). In this study, we further ascertained that SMics are autonomously specified at least partially as early as the fifth cell division, clustered together in a G-cadherin-dependent manner, are transferred passively by SMCs and the developing endoderm during gastrulation, and that specific numbers of SMics consistently migrate into the L/R coelomic pouches (summarized in Fig. 7). All of the features indicated here are widely shared characteristics of PGCs, such as *Drosophila* pole cells. Pole cells are autonomously specified immediately after cellularization, have E-cadherin-dependent migration and specification mechanisms, are passively translocated during gastrulation, and have a mechanism later in development to regulate the final number of pole cells in the L/R gonads (Huettner, 1923; Illmensee and Mahowald, 1974; Technau, 1987). Taken together, these two widely divergent organisms have remarkable similarities in PGC specification.

However, the sea urchin embryo employs a system distinct from that of the *Drosophila* embryo for germ line specification. The sea urchin egg does not have localized maternal germ line components, and adults are fertile when Mics, the parent blastomeres of SMics, are removed at the fourth division (Ransick et al., 1996). Furthermore, recent findings have demonstrated that Vasa, a well-conserved germ line marker originally found in *Drosophila* pole cells, is expressed in every blastomere of the sea urchin embryo and even in somatic adult rudiment cells (Yajima and Wessel, 2011b; Yajima and Wessel, 2011c) (our unpublished results). This type of broad expression of Vasa protein has been found in many animals, including planarians (Shibata et al., 1999), hydra (Bosch and David, 1987; Mochizuki et al., 2001; Rebscher et al., 2008), annelids (Carre et al., 2002; Oyama and Shimizu, 2007; Rebscher et al., 2007; Rebscher et al., 2012) and urochordates (Rosner et al., 2009), and suggests that Vasa might have much broader functional roles than originally anticipated, possibly including compensatory activities for the germ line.

Among metazoans, conditional (inductive) specification and late segregation of the germ line are more prevalent and widespread than autonomous (inherited) specification and early segregation. Indeed, conditional specification is the mechanism found in representative basal metazoans [e.g. cnidarians and sponges (reviewed by Extavour and Akam, 2003)]. For these reasons, conditional specification and late segregation are proposed to be the ancestral mode of germ cell specification in the Metazoa. Sea urchins are a relatively advanced group within echinoderms, and other echinoderms do not have SMics or restricted germ line gene expression until later in their development (Juliano and Wessel, 2010). Therefore, we hypothesize that the sea urchin embryo has recently acquired changes to adopt a more autonomous manner of PGC specification. This evolutionarily distinct feature of sea urchin embryos among echinoderms will be a useful tool to reveal mechanisms of germ line evolution and the multipotent cell specification program that might be widely conserved throughout the animal kingdom. Further lineage analysis of the SMic descendants, their specification and migration mechanisms, especially during adult rudiment formation in the late larva, will be important in elucidating these germ cell mechanisms more effectively.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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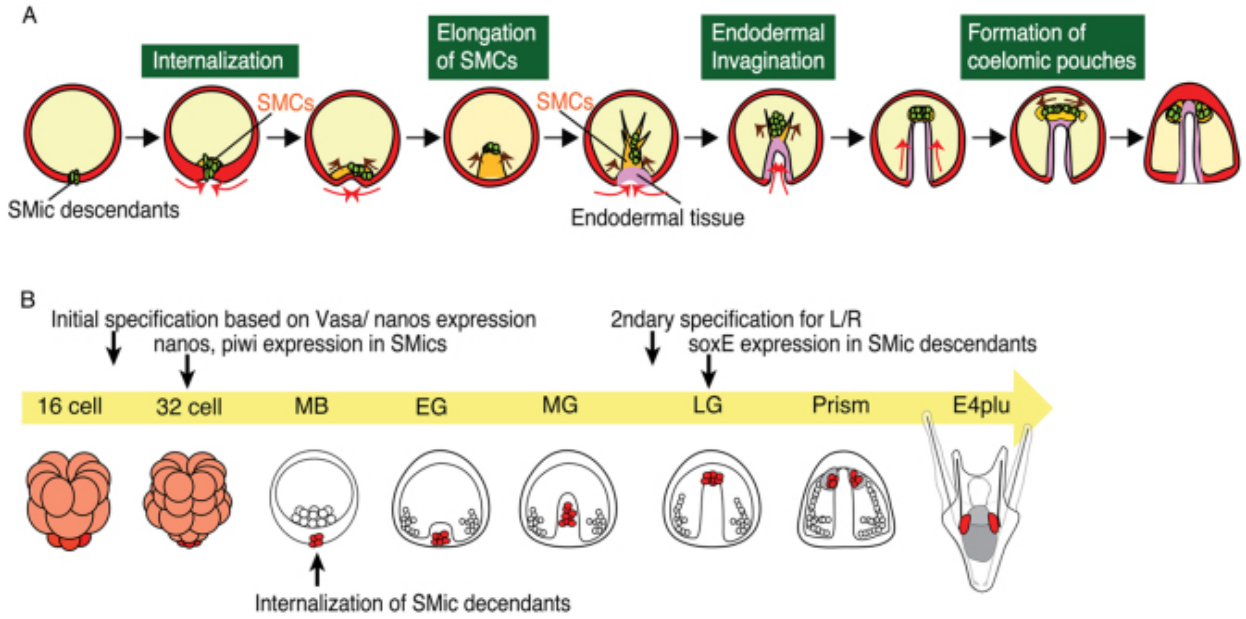
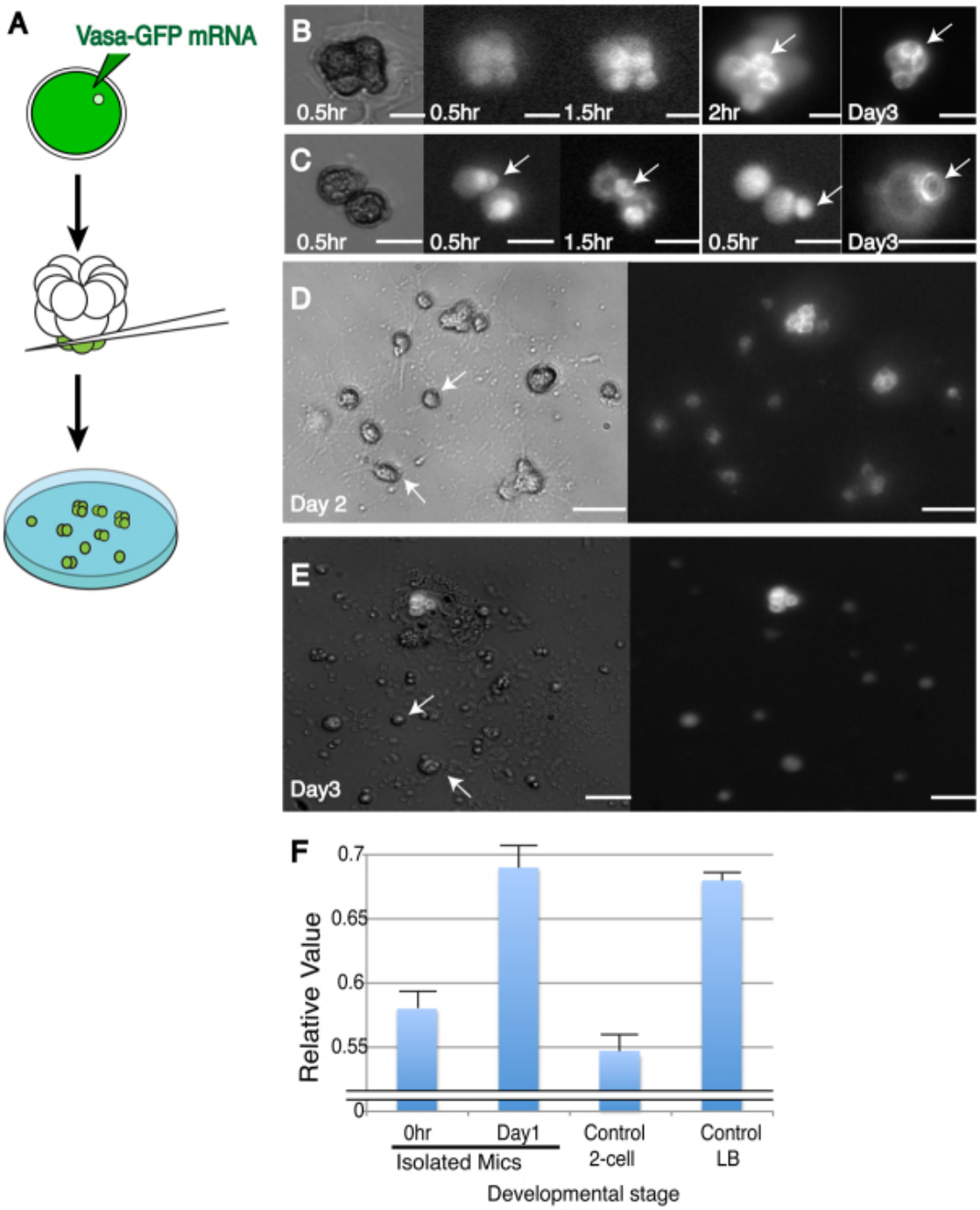
Figures and Tables

Fig. 7.



Summary of initial SMic specification. (**A**) SMic descendants (green) are mechanically de-epithelialized into the blastocoel by the contraction of the epithelia and reorganization of the vegetal plate, and are passively carried up towards the animal pole by SMCs (orange). (**B**) SMic specification during early development. Vasa-positive cells are indicated in red. MB, mid-blastula; EG, early gastrula; MG, mid-gastrula; LG, late gastrula; E4plu, pluteus stage; SMC, secondary mesenchyme cell; SMic, small micromere; L/R. left/right.

Fig. 1.



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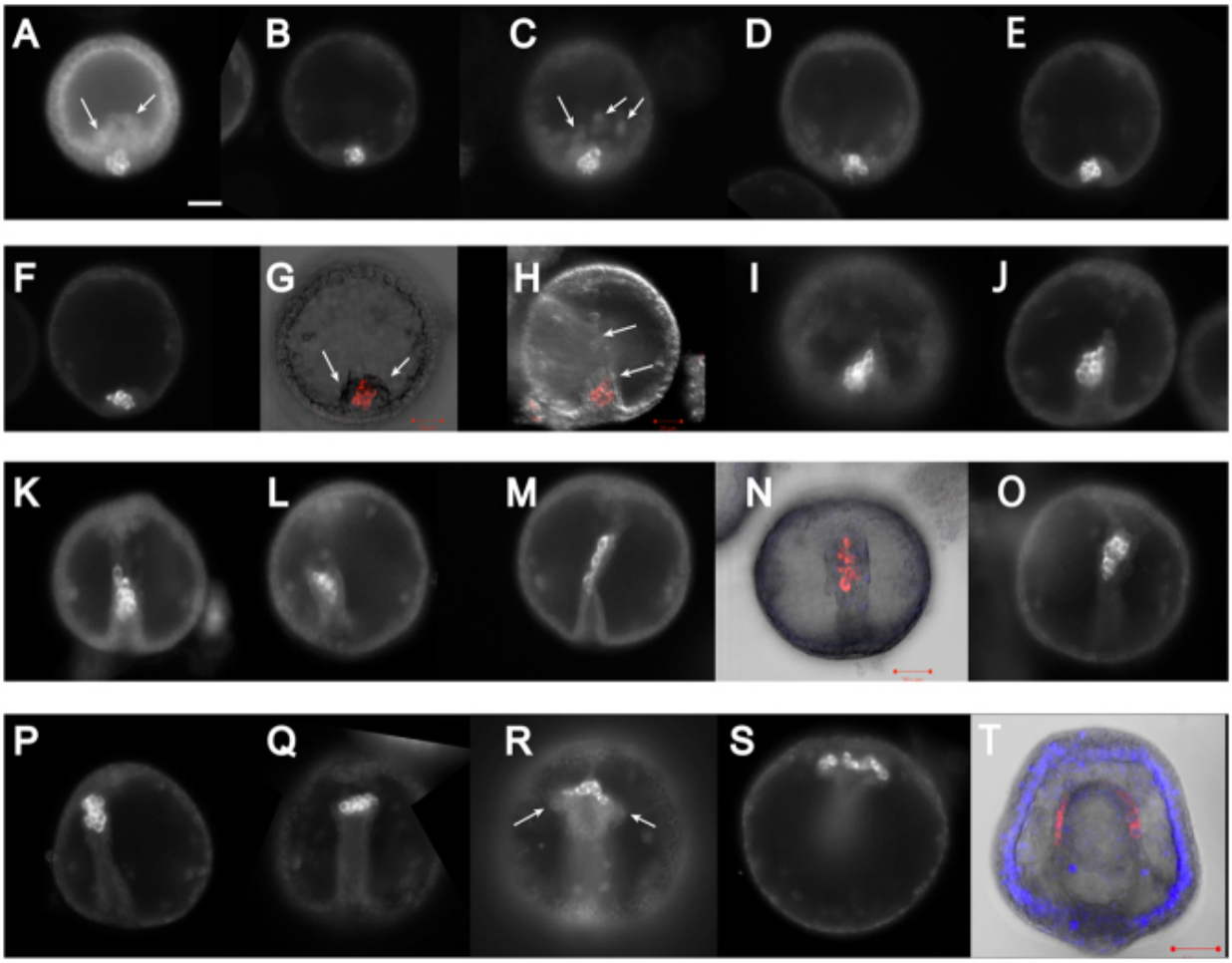
Small micromeres (SMics) autonomously accumulate Vasa and *nanos* in isolated culture. (**A**) Experimental procedure for isolation and culture of micromeres (Mics) from *S. purpuratus*. (**B**,**C**) A set of four Mics (B) or two Mics (C), each isolated from a single embryo, and its Vasa-GFP expression after isolation. Time after isolation indicated bottom left; 0 hours corresponds to the 16-cell stage, 2 hours to the 128-cell stage, day 2 to gastrula and day 3 to pluteus larval stage in siblings raised in vivo. Perinuclear localization of Vasa became apparent in the SMics in vitro (arrows), similar to in vivo embryos. (**D**,**E**) A cluster of Mic descendants at day 2 or day 3. Vasa-positive SMic descendants remained in the same position, whereas LMic descendants (arrows) went through several cell divisions, extended pseudopodia, and migrated extensively in the culture dish. (**F**) *nanos* expression levels in the isolated Mic population increased in line with the schedule of normal in vivo development. Expression was normalized to an internal control, *ubiquitin*. Error bars indicate s.d. Scale bars: 10 μm in B,C; 20 μm in D,E.

Fig. 2.



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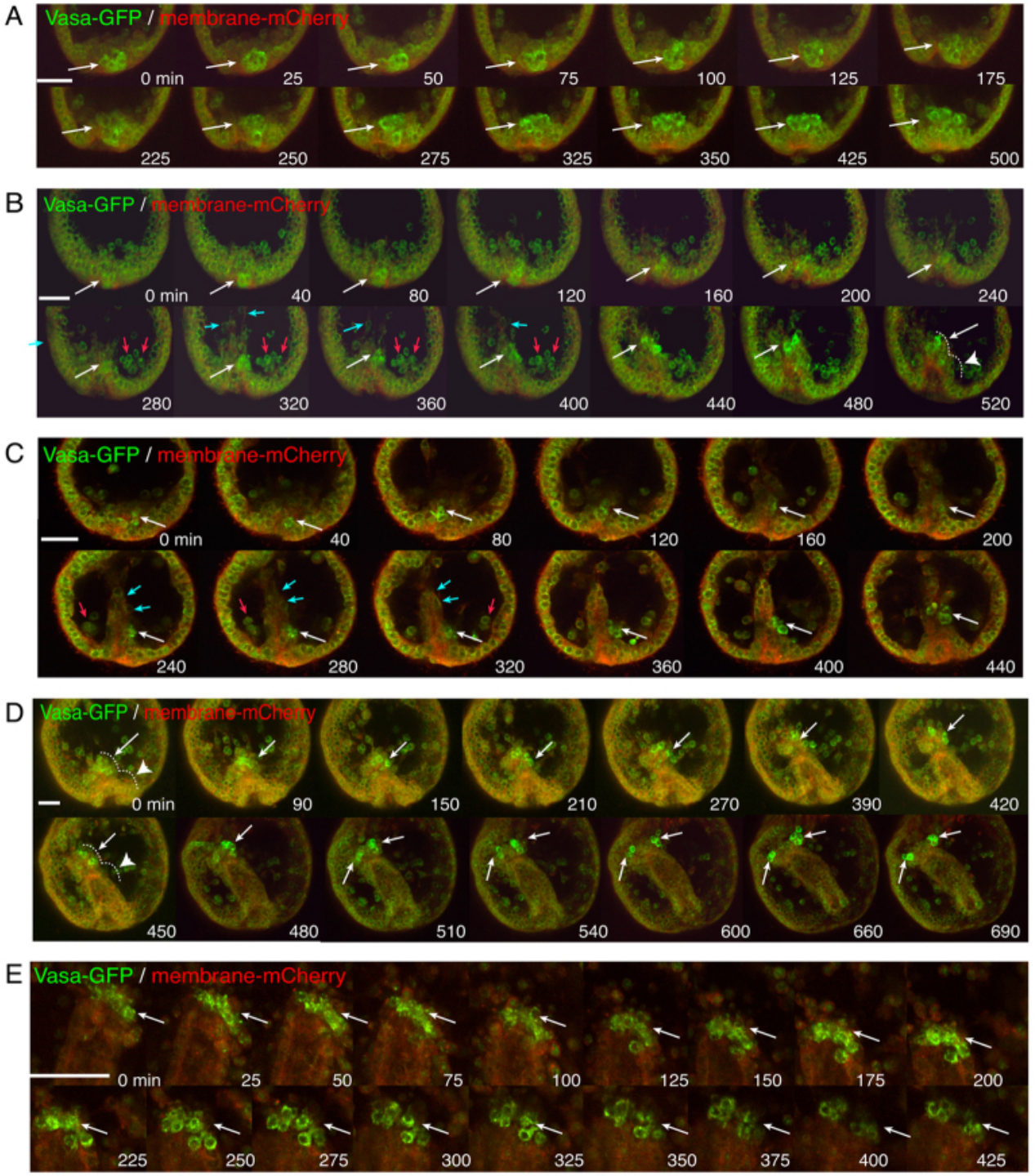
SMic translocation during gastrulation. (**A-T**) SMics were tracked by Vasa immunolabeling. (G,H,N,T) Vasa, red; Hoechst, blue. Arrows indicate PMCs (A,C) or SMCs (G,H,R). Scale bars: 20 μm.

Fig. 3.



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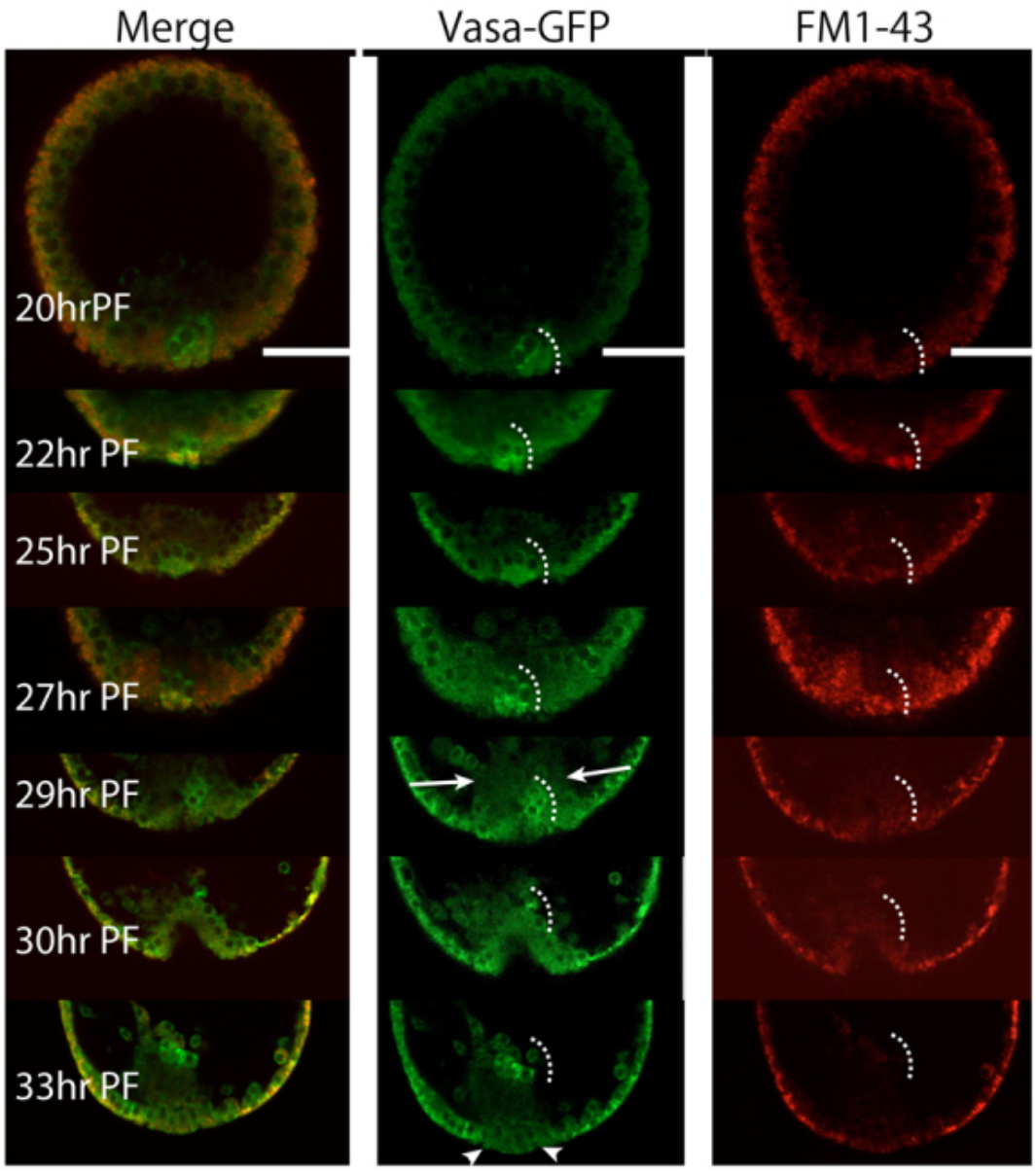
Time-lapse imaging of gastrulation. A subset of the time-lapse series is displayed. Elapsed time (minutes) is indicated. SMic descendants were labeled by Vasa-GFP (green, white arrows) and the entire embryo was labeled by membrane-mCherry (red). For the full time series, see supplementary material Figs S1 and S2 and Movies 1-6. (**A**) De-epithelialization of SMic descendants. (**B**) De-epithelialization and SMCs elongation. (**C**) SMC elongation before endodermal invagination. (**D**) Endodermal invagination and formation of coelomic pouches. (**E**) SMic descendants formed a fine line at the tip of archenteron, became less adherent and translocated into either the left or right coelomic pouch after gastrulation. Red arrows indicate PMCs and blue arrows indicate SMCs. Dotted lines (B,D) indicate a clump of SMCs /SMics (white arrows) and an endodermal region (arrowheads). Scale bars: 20 μm.

Fig. 4.



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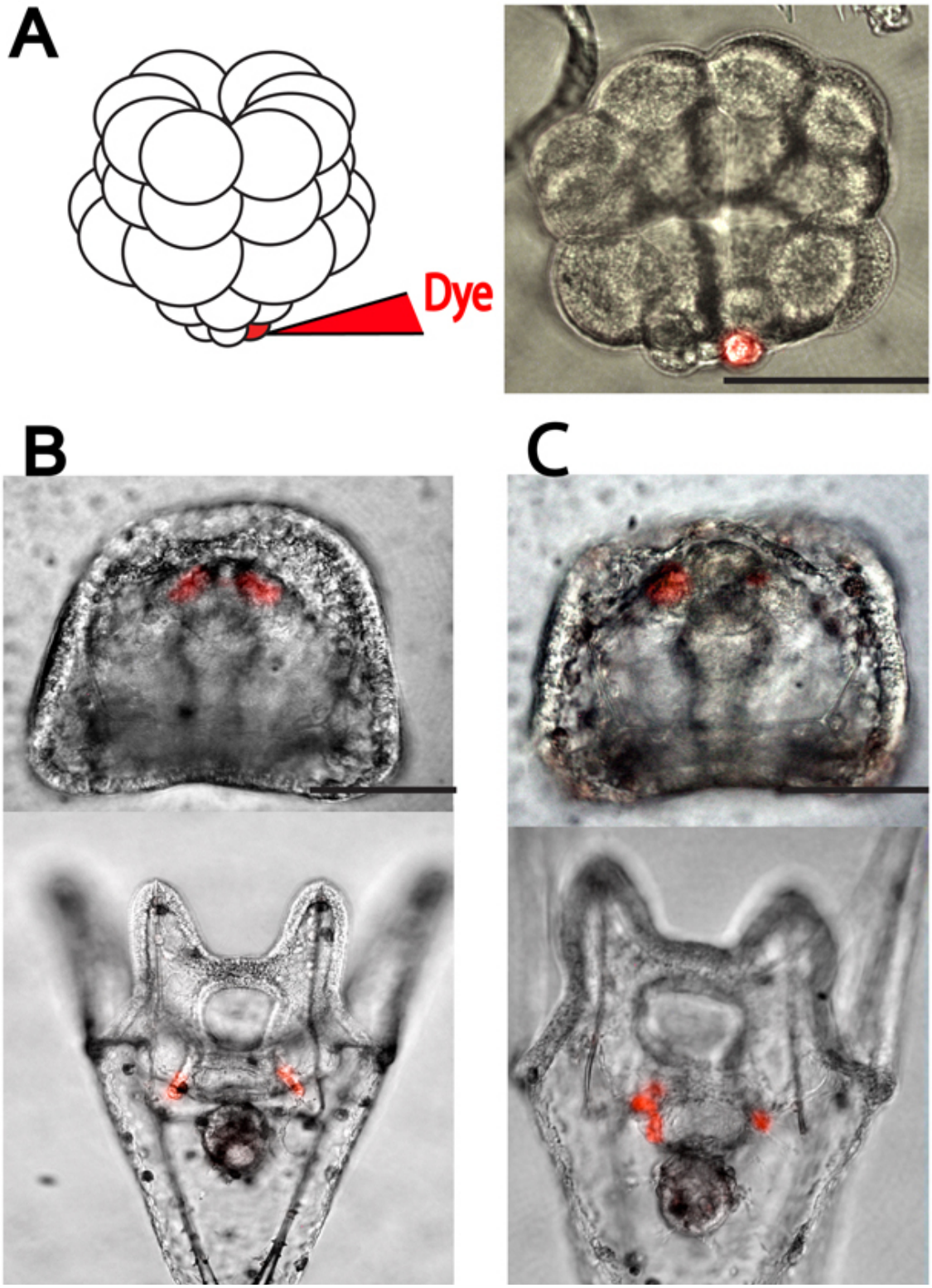
Initial de-epithelialization of SMic descendants. SMic descendants were labeled with Vasa-GFP and cells of the blastula exposed to the outside were labeled with FM1-43 (red). Dotted lines indicate a cluster of SMic descendants. Arrows at 29 hPF indicate SMCs and arrowheads in 33 hPF indicate a smoothly closed vegetal plate. hPF, hours postfertilization. Scale bars: 20 μm.

Fig. 5.



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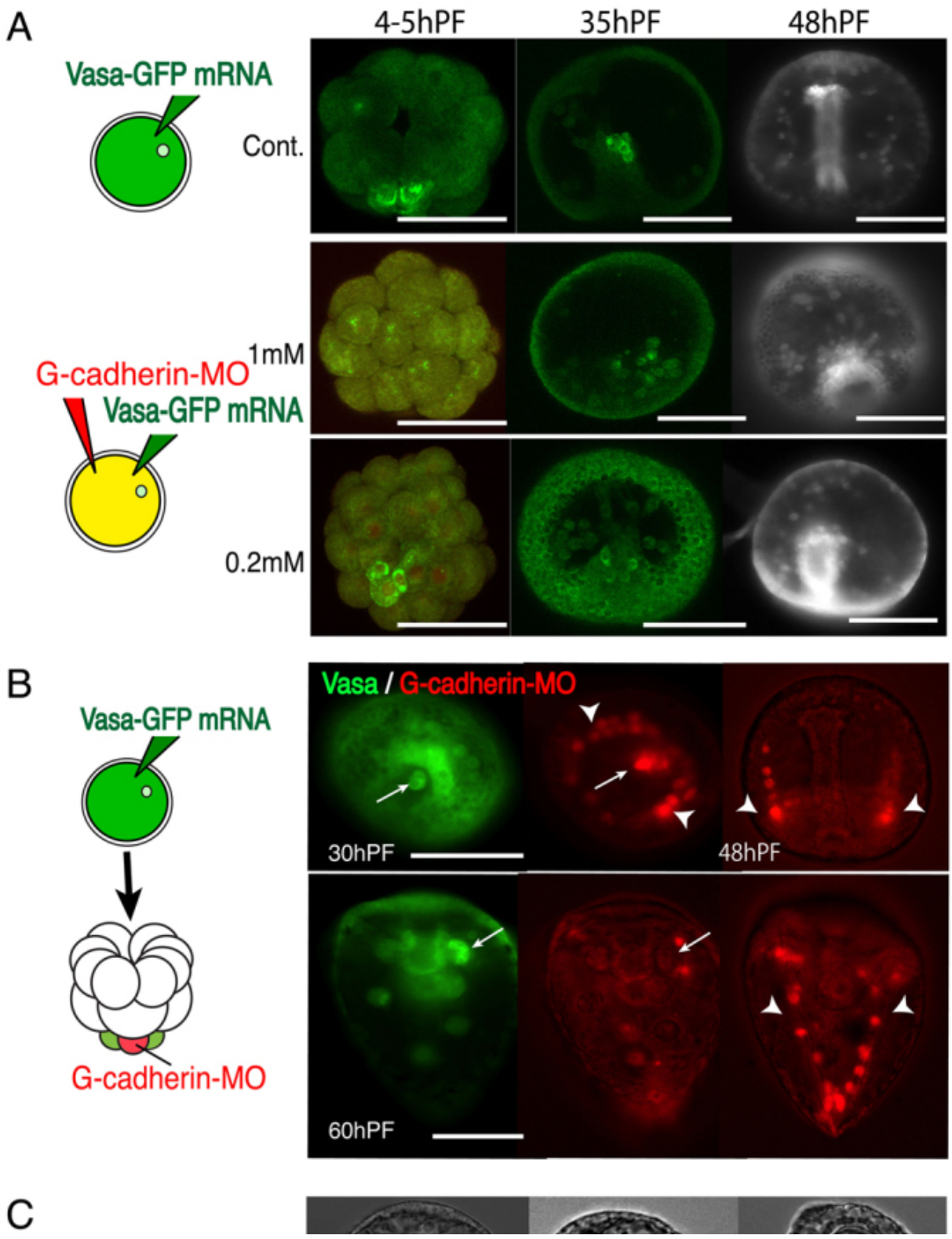
Left/right fate decisions have not been made by the 32-cell stage in SMics. (**A**) A single SMic was labeled with fluorescent dye and tracked in larvae. (**B**,**C**) Symmetrical (B) or asymmetrical (C) distribution of the single SMic descendants. Scale bars: 50 μm.

Fig. 6.



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G-cadherin-dependent translocation of SMic descendants during gastrulation. (**A**) Vasa-GFP and either 1 mM or 0.2 mM G-cad MO were co-injected into fertilized eggs. In G-cad MO embryos, specific Vasa localization in Mics and SMic was lost (4-5 hPF), embryos failed in gastrulation (35 hPF), and showed significant developmental defects (48 hPF). (**B**) Vasa-GFP (green) was injected into eggs and 0.1 mM G-cad MO (red) was injected into one of the Mics at the 16-cell stage. G-cad MO-injected SMic (arrows) and LMic (arrowheads) descendants were properly located in the vegetal plate

and in the blastocoel, respectively, at mesenchyme blastula stage (30 hPF), yet the SMics never reached the tip of the archenteron following gastrulation (48 hPF) nor the Vasa-positive coelomic pouches in larvae (60 hPF, arrow). (**C**) G-cad MO was injected into one of the SMics at the 32-cell stage. G-cad MO-injected SMic descendants (arrows) moved into the blastocoel with the PMCs. (**D**) G-cad MO was injected into one of the mesomeres or macromeres. Injected descendants successfully differentiated into ectoderm or endoderm (arrows). Scale bars: 50 μm.