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Germline Cell Formation and Gonad Regeneration in Solitary and Colonial Ascidians

Kaz Kawamura,[1]* Stefano Tiozzo,[2,3] Lucia Manni,[4] Takeshi Sunanaga,[1] Paolo Burighel,[4] and Anthony W. De Tomaso[3]

The morphology of ascidian gonad is very similar among species. The testis consists of variable number of testicular follicles; the ovary consists of ovarian tubes that are thickened forming the germinal epithelium with stem cells for female germ cells with the exception of botryllid ascidians. Peculiar accessory cells that would be germline in origin accompany the oocytes. Using *vasa* homologues as a molecular marker, germline precursor cells can be traced back to the embryonic posterior-most blastomeres and are found in the tail of tailbud embryo in some solitary and colonial ascidians. In *Ciona*, they are subsequently located in the larval tail, while in colonial botryllid ascidians *vasa*-expressing cells become obscure in the tail. Recent evidence suggests that ascidian germ cells can regenerate from cells other than embryonic germline. An ensemble of the embryonic stringency of germ cell lineage and the postembryonic flexibility of gonad formation is discussed. *Developmental Dynamics 240:299–308, 2011.* © 2011 Wiley-Liss, Inc.

Key words: ascidian; germline; accessory cell; gonad; PGC; stem cell; Vasa

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INTRODUCTION

In sexually reproducing organisms, the germline is defined as the sequence of cells which develop into gametes and are devoted to transfer the genome information from generation to generation. The germline is one of the earliest lineages to be determined in many animal embryos. Its segregation from somatic lineage occurs in at least two distinct modes: in model organisms like *Caenorhabditis* (Strome and Wood, 1983), *Drosophila* (Lehmann and Ephrussi, 1994), and *Xenopus* (Ikenishi and Tanaka, 1997), the primordial germ cells (PGCs) segregate early in embryonic development, and their specification is due to maternally localized determinants (*preformation*; for review, see Extavour and Akam, 2003; Strome and Lehmann, 2007). The future germ cells appear only from the progeny of the PGCs, as shown by depletion and transplantation experiments (e.g., Illmensee and Mahowald, 1974). In other taxa such as mammals, germ cells are also determined early in development, while they arise as a consequence of inductive signals from surrounding tissues (*epigenesis*; De Sousa Lopes et al., 2004). The manner by which germ cells are committed and specified has evoked much interest from viewpoints of not only cell and developmental biology but also comparative and evolutionary biology.

In both preformation and epigenesis, the germline, once determined, is strictly separated from soma. But, is this true for all metazoans? In adult hydras, interstitial multipotent cells

[1]Laboratory of Cellular and Molecular Biotechnology, Faculty of Science, Kochi University, Kochi, Japan
[2]Université Pierre et Marie Curie (Univ Paris 06) and CNRS, UMR7009 "Biologie du Développement", Observatoire Océanologique,Villefranche-sur-mer, France
[3]Molecular Cellular and Developmental Biology, University of California Santa Barbara, Santa Barbara, California
[4]Department of Biology, University of Padova, Padova, Italy
All authors contributed equally to this work.
Grant sponsor: JSPS; Grant number: 21570227.
***Correspondence to: Kaz Kawamura, Laboratory of Cellular and Molecular Biotechnology, Faculty of Science, Kochi University, Kochi 780-8520, Japan. E-mail: kazuk@kochi-u.ac.jp**

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can give off various kinds of somatic lineage cells such as nerve and nematocyte, while they can also express a germ cell-specific molecular marker when hydras attain to sexual maturity (Mochizuki et al., 2001). In planarians, a population of multipotent cells called neoblasts strongly express a *Vasa* homologue, an RNA helicase gene found in germ cells across the metazoans, in both sexually mature and sexually immature individuals, suggesting a role of neoblasts in both regeneration and gametogenesis (Shibata et al., 1999). These results suggest that, at least in platyhelminths and hydrozoans, adult animals contain the totipotent cells.

Ascidiacea is a class of marine organisms in the chordate subphylum Tunicata and represent modern-day descendants of the chordate ancestor (Delsuc et al., 2006). They share some features with Vertebrata, including a notochord, dorsal hollow nerve tube, and a post-anal tail at the larval stage and including the adult pharynx, gill slits, and endostyle, a related organ of the vertebrate thyroid gland (Berrill, 1950, Burighel and Cloney, 1997). The ascidian embryo is a typical example of determinative or mosaic development (Satoh, 1994). However, after metamorphosis somatic cells in some species show the remarkable developmental flexibility such as regeneration (Brien, 1968, Carnevali and Burighel, 2010) and asexual development (Manni et al., 2007, Kawamura et al., 2008a). Due to their phylogenetic position and well characterized embryonic development, ascidians represent excellent models to study the germline segregation from soma.

In this manuscript, we review classical studies and recent findings in an attempt to provide a general understanding of germ cell anatomy and development in solitary and colonial ascidians. First, we present a comparative morphological description of the gonads in three sub-orders of Ascidiacea: Aplousobranchiata and Phlebobranchiata of the order Enterogona, and Stolidobranchiata of the order Pleurogona. In this section, particular attention will be dedicated not only to germ cells but also to germline accessory cells, because recent studies have insight into the soma/germ origin of the accessory cells. Second, we introduce the recent progress in embryonic origin of germline cells and the subsequent behavior of germ cells during metamorphosis. Findings from several different species are compared with one another. Finally, we review the postembryonic germline formation and development in colonial ascidians, where sexual reproduction and a continuous asexual propagation coexist in the same organism.

MORPHOLOGY OF ASCIDIAN GONADS

a. Structure of the Mature Sexual Organs

All ascidian species are hermaphroditic, and some species carry male and female gonads clustered in the same structure. In *Styela plicata*, for example, zooids have several gonads on each side of the body, and a single sausage-like gonad consists of the ovary and numerous testes (Tucker, 1942). In other species, the ovary is separated from the testis. In *Distomus variolosus*, ovaries and testes are located on the right and left sides of the body, respectively (Berrill, 1948; Newberry, 1968).

In ascidians, the gonads are located in the major mesenchymal space of the adult zooid (Kawamura et al., 2008a). In the order Enterogona, this mesenchymal space is situated in the abdomen and delimited by the epicardium and the epidermis (see Fig. 1a,b in Kawamura et al., 2008a). In *Ciona*, the epicardium develops into the perivisceral epithelium to encircle the digestive tract, so that the mesenchymal space is mainly confined to the perivisceral region. Consequently, the gonad becomes closely associated with the viscera. In contrast, in the order Pleurogona, zooids do not have the epicardium. The gonad is located in the major mesenchymal space that is bordered by the epidermis and the peribranchial epithelium (see Fig. 1c in Kawamura et al., 2008a).

In both the Enterogona and Pleurogona, the ovary consists of a branched ovarian tube and female germ cells of various developmental stages (Fig. 1). The ovarian tubes gather to form the oviduct that opens to the cloacal cavity. In *Clavelina* (Aplousobranchiata), one side of the ovarian tube is thickened, where small undifferentiated cells, oogonia and juvenile oocytes are buried (Fig. 1A; Mukai, 1977a). In *Ciona* (Phlebobranchiata), on the other hand, the ovarian tube is thickened all around the circumference (Fig. 1B; Sugino et al., 1990; Okada and Yamamoto, 1993). In the Pleurogona such as *Styela* (Stolidobranchiata), the ovarian tube takes the inverted T-shape (Fig. 1D; Tucker, 1942). Many oocytes are associated with the thickened wall of tube. This wall has been named the germinal epithelium (Tucker, 1942). In *Polyandrocarpa*, a single gonad consists of the testes and the ovary (Fig. 1E). The germinal epithelium of ovarian tube is situated on the side opposite to the testes. Botryllid ascidians show an atypical female gonad, because it lacks the germinal epithelium (Fig. 1F). Instead, each ovary is provided with its own oocyte surrounded by accessory cells continuous to the small oviduct (follicle stalk; see below). At maturity, the oviduct opens directly to the atrial chamber.

In *Ciona intestinalis*, the germinal epithelium is separated into three parts; the stratified region, the follicular region, and the terminal region (Fig. 1B; Sugino et al., 1990; Okada and Yamamoto, 1993). The stratified region is where oogonia proliferate actively. The oogonia are classified into 3 types, and the juvenile oocytes into 4 types (Fig. 1C). Cytoplasmic electron-dense materials can be seen in types G1 and G2 oogonia, and after disappearing from type G3 oogonia they reappear at the pachytene stage of type C3 oocytes (Okada and Yamamoto, 1993). The oocytes develop in the follicular region. As they grow, they are detached from the germinal epithelium but are still connected with the ovarian tube by the follicle stalk (Fig. 1B).

In all ascidian species, the testis branches into multiple lobes named the testicular follicles (Berrill, 1950). Each testicular follicle extends into narrow efferent tubules which come together to form the spermiduct opening into the cloacal cavity. In *Boltenia villosa*, spermatogonia are polygonal in shape, having a high nucleus cytoplasm ratio with a prominent nucleolus (Cavey, 1994). They form clonal clusters named spermatocysts, in

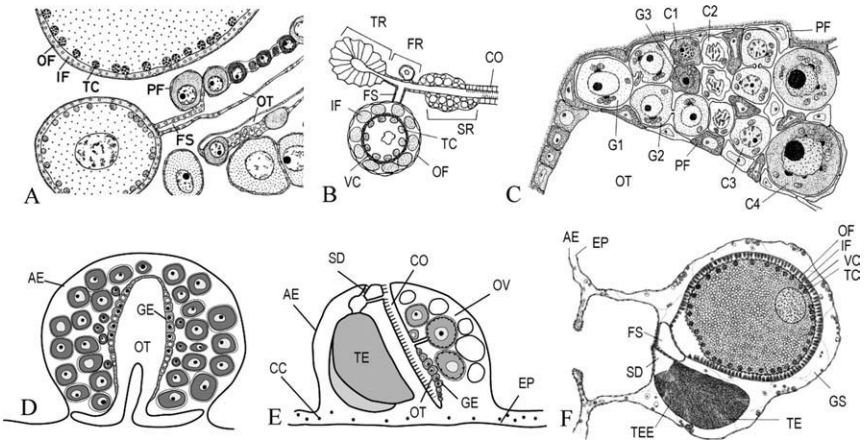

Fig. 1. Anatomy of ascidian gonads. **A:** Ovary of *Clavelina* (order, Enterogona; suborder, Aplousobranchiata; modified from Mukai, 1977a). **B:** Ovary of *Ciona* (order, Enterogona; suborder, Phlebobranchiata; modified from Sugino et al., 1990). **C:** Fine anatomy of the *Ciona* germinal epithelium (modified from Okada and Yamamoto, 1993). G1–G3, developmental stages of oogonium. C1–C4, developmental stages of oocytes. **D:** Ovary of *Styela* (order, Pleurogona; suborder, Stolidobranchiata). **E:** Gonad of *Polyandrocarpa* (order, Pleurogona; suborder, Stolidobranchiata; modified from Sunanaga et al., 2007). **F:** Gonad of *Botryllus* (order, Pleurogona; suborder, Stolidobranchiata; modified from Mukai and Watanabe, 1976). AE, atrial epithelium; CC, coelomic cell; CO, ciliated oviduct; EP, epidermis; FR, follicular region; FS, follicle stalk; GE, germinal epithelium; GS, gonadal space; IF, inner follicle cell; OF, outer follicle cell; OT, ovarian tube; OV, ovary; PF, primary follicle cell; SD, spermiduct; SR, stratified region; TC, test cell; TE, testis; TEE, testicular epithelium; TR, terminal region; VC, vitelline coat.

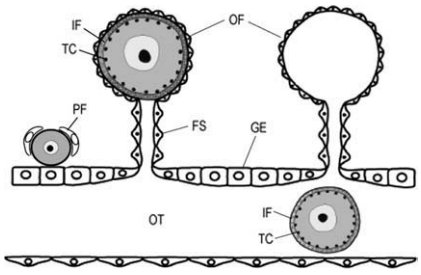

Fig. 2. Oocytes before and after ovulation. FS, follicle stalk; GE, germinal epithelium; IF, inner follicle cell; PF, primary follicle cell; OF, outer follicle cell; OT, ovarian tube; TC, Test cell.

which germ cells are interconnected with each other by cytoplasmic bridge. Consistent with this ultrastructural finding in *Boltenia* (Cavey, 1994), bromodeoxyuridine (BrdU) -labeled germ cells are observable in clusters in the testis of *Botryllus primigenus* (see Fig. 6D in Kawamura et al. 2008b). During spermiogenesis in *Boltenia* the numerous mitochondria fuse each other to make a single large mitochondrion flanked by the nucleus that undergoes elongation and chromatin condensation (Cavey, 1994). Mature spermatozoans are flagellate and can accumulate along the spermiduct before spawning.

b. Accessory Cells

Much attention has been paid to the gonad accessory cells of ascidians because of their peculiar structure and possible function. Since the classical study in *Styela plicata* (Tucker, 1942), the following nomenclature has been generally adopted. Oocytes before vitellogenesis stages are surrounded by a single layer of accessory cells, called primary follicle cell (Fig. 2). Then, isolated cells appear in the space between primary follicle cells layer and oolemma. They are further separated into two cell layers by the vitelline coat. As a result, three cellular envelopes of the oocytes are recognizable: outer follicle cells, inner follicle cells, and test cells (Fig. 2). In *Botryllus schlosseri*, the inner follicle cells contribute with the oocyte in synthesizing the vitelline coat components (Manni et al., 1993). The squamous outer follicle cells connect the egg to the follicle stalk (Fig. 2; Lambert, 2009). Test cells are progressively encased in superficial depression of the oocyte, whereas inner follicle cells remain on the vitelline coat. During ovulation, the oocyte is shed into the ovarian duct through the follicle stalk, accompanied by inner follicle cells, while the outer follicle cells remain in situ (Fig. 2; Mukai, 1977a,b).

The origin of the primary follicle cells has been the focus of many studies. Some investigators have observed that they derive from the germinal epithelium in *Styela plicata* (Tucker, 1942), *Styela clava* (Ermak, 1976), *Ciona savignyi* (Sugino et al., 1987, 1990), and *C. intestinalis* (Okada and Yamamoto, 1993). Other investigators have suggested that the primary follicle cells come from free hemoblasts of the hemocoel (Peres, 1954; De Vincentiis, 1962; Mancuso, 1965; Newberry, 1968; Mukai and Watanabe, 1976; Manni et al., 1993, 1995; Sunanaga et al., 2006). In *B. primigenus*, the primary follicle cells appear to have the same origin as the PGCs, because they express a PGC-specific molecular marker at the earliest stage of differentiation (Sunanaga et al., 2006).

The origin of the three cellular egg envelopes (outer follicle, inner follicles, and test cells) is also debated. Mukai and Watanabe (1976) suggested that in *B. primigenus* the inner follicle cells and the test cells come from the primary follicle cells, and that the outer follicle cells come directly from blood cells. Other studies indicated that the test cells originate from blood cells independently from the outer and inner follicle cells (De Vincentiis, 1962; Mancuso, 1965). Tucker (1942), on the basis of histological observations on *Styela plicata*, and Ermak (1976), using autoradiographic studies on *Styela clava*, have pointed out that the primary follicle cells actively proliferate and serve as the progenitor of all three kinds of accessory cells. Ultrastructural studies in *B. schlosseri* (Manni et al., 1993) support this third opinion, since no evidence indicates that external elements infiltrate through the primary follicle cells.

In comparison with female accessory cells, male accessory cells have been object of less attention (reviewed in Burighel and Cloney, 1997; Burighel and Martinucci, 2000). In botryllids, the testis originates in early buds from the loose aggregate of cells (Mukai and Watanabe, 1976; Manni et al., 1995). In *B. primigenus*, each cell in the aggregate expresses a PGC-specific marker (Sunanaga et al., 2006). A portion of the cell aggregate develops into a compact cell

mass that consists of male germ cells and somatic cells (Sunanaga et al., 2006). In *B. schlosseri*, the somatic accessory cells attach to the basal lamina of the prospective peribranchial wall and begin to take the epithelial configuration (Burighel and Cloney, 1997). Then they enclose the male germ cells to form the testicular epithelium that separates them from oocytes exposed to the blood flow. In *Boltenia villosa*, the male accessory cells do not have the characteristics of Sertoli cells in mammals and other vertebrates (Cavey, 1994) where they are involved in hormone production, blood–testis barrier, and testis compartments. Instead, they are only involved in phagocytic activity during testis development and, especially in *Botryllus*, during zooidal regression (Cavey, 1994; Jorgensen and Luetzen, 1997; Burighel and Martinucci, 2000).

EMBRYONIC SOURCE OF THE GERMLINE

a. Ontogeny

In the first half of 20th century, several hypotheses were proposed concerning the origin of ascidian germ cells, including the embryonic dorsal cord, mesenchymal mass, blood cells, etc (for review, Berrill, 1950). However, it has been only in the past 2 decades that substantial progress has been made in our understanding of embryonic origin and further development of ascidian germline.

Following a pioneering study on the ascidian embryonic cell lineage (Nishida, 1987), Hirano and Nishida (2000) first suggested that in *Halocynthia roretzi* the B7.6 blastomeres (at 64-cell stage) might be the germ cell precursors. Further evidence for this hypothesis was provided by Fujimura and Takamura (2000), using the *Ciona* Vasa homologue (*CiVH*) as probe. The *vasa* gene encodes ATP-dependent RNA helicase belonging to the DEAD box family. It is expressed by germ cells and PGCs in all animals examined so far (Schupbach and Wieshaus, 1986; Hay et al., 1988; Komiya et al., 1994; Yoon et al., 1997; Kuznicki et al., 2000; Noce et al., 2001; Sagawa et al., 2005; Rebscher et al., 2007), although in some metazoans, *vasa* appears to play roles in both germline and somatic stem cell identities during embryogenesis (Ikenishi and Tanaka, 1997; Dill and Seaver, 2008; Brown et al., 2009) or throughout the adult life (Mochizuki et al., 2001; Extavour et al., 2005; Gustafson and Wessel, 2010). Therefore, *vasa* and its homologues have been widely used as one of the most reliable molecular markers for identifying germ cells and PGCs (Raz, 2000).

In both *Halocynthia* and *Ciona*, maternal *vasa* mRNA are uniformly distributed during oocyte maturation, and begins to accumulate at the posterior cortex right after fertilization (Shirae-Kurabayashi et al., 2006; Prodon et al., 2009). Thereafter, *CiVH* is inherited to posterior-most blastomeres such as B3 (4-cell stage), B4.1 (8-cell stage), B5.2 (16-cell stage), B6.3 (32-cell stage), and B7.6 (64-cell stage; Fig. 3A–E; Fujimura and Takamura, 2000; Shirae-Kurabayashi et al., 2006). The posterior-most blastomeres are also enriched in postplasmic components such as *PEM* and *macho-1* RNA (Yoshida et al., 1996; Nishida and Sawada, 2001). *PEM* is associated with the centrosome attracting body (CAB; Nishikata et al., 1999). The CAB remains localized to the cortex of the vegetal blastomeres until the gastrula stage, when the unequal cleavage forms B7.6 blastomeres. Like PGCs in many animal species, ascidian germline blastomeres are characterized by the *vasa* expression, abundant mitochondria, and a nuage in the cytoplasm (Iseto and Nishida, 1999; Shirae-Kurabayashi et al., 2006).

In general, germline precursor cells are quiescent mitotically and somatic genes are resting. In *Drosophila* and *Caenorhabditis*, this is because RNA polymerase II has lost enzymatic activity. In ascidian B7.6 blastomeres, gene expression is suppressed genome-wide (Tomioka et al., 2002). However, gene suppression seems to occur in the manner different from those of *Drosophila* and *Caenorhabditis*, because in *Halocynthia* embryos the carboxy-terminal region of RNA polymerase II remains phosphorylated (Tomioka et al., 2002).

In *C. intestinalis* embryos unlike *H. roretzi*, the B7.6 cells divide once during gastrulation, giving rise to B8.11 and B8.12. The postplasmic components such as PEM are partitioned exclusively into B8.11 cells, while most of *CiVH* signal is segregated into B8.12 cells (Shirae-Kurabayashi et al., 2006). B8.11 and B8.12 are arranged in tandem at first but separate from each other during tail elongation. The B8.11 blastomere shows a weak *CiVH* signal, but the cell is no more discernible at larval stage, while the descendant of B8.12 develops normally into germ cells (Shirae-Kurabayashi et al., 2006). They reside in the elongating tail by the late tail-bud stage and proliferate in situ (Fig. 3F). Finally, in the larva of *C. intestinalis* it is possible to recognize eight Vasa-positive PGCs in the endodermal strand of the tail at the distal (posterior) area (Fig. 3G; Takamura et al., 2002; Shirae-Kurabayashi et al., 2006). In *C. savignyi*, on the other hand, the number of immunopositive PGCs is coupled (8 vs. 4 in C. intestinalis; cf., Takamura et al., 2002). In *H. roretzi*, B7.6 blastomeres do not divide further during embryogenesis (Nishida, 1987), suggesting that the larva has, if any, a smaller number of PGCs in the tail.

In the colonial ascidian, *B. schlosseri vasa* mRNA is distributed in posterior blastomeres during early embryogenesis, reminiscent of results in *Ciona* transcript, but in the larvae mRNA and protein are found exclusively in the trunk region (Brown et al., 2009; Rosner et al., 2009). In *Ciona*, the CiVH signal appears in the trunk of tailbud embryo, while it becomes faint or remarkably reduced in the late tadpole larvae (Takamura et al., 2002; Shirae-Kurabayashi et al., 2006). In *B. primigenus*, both vasa mRNA and protein are evident until the tailbud stage (Fig. 3J), but no signals can be found in the tail nor the trunk of tadpole larva (Fig. 3K).

Taken together, these results suggest that in both solitary and colonial ascidians the number of germline blastomeres and their descendant is more or less constant during embryogenesis to the tailbud stage, but size and distribution of cells in the tail and trunk of larvae are variable between different species.

b. PGCs During Metamorphosis

Ascidian gonad formation and development after metamorphosis have been described ultrastructurally in *C.*

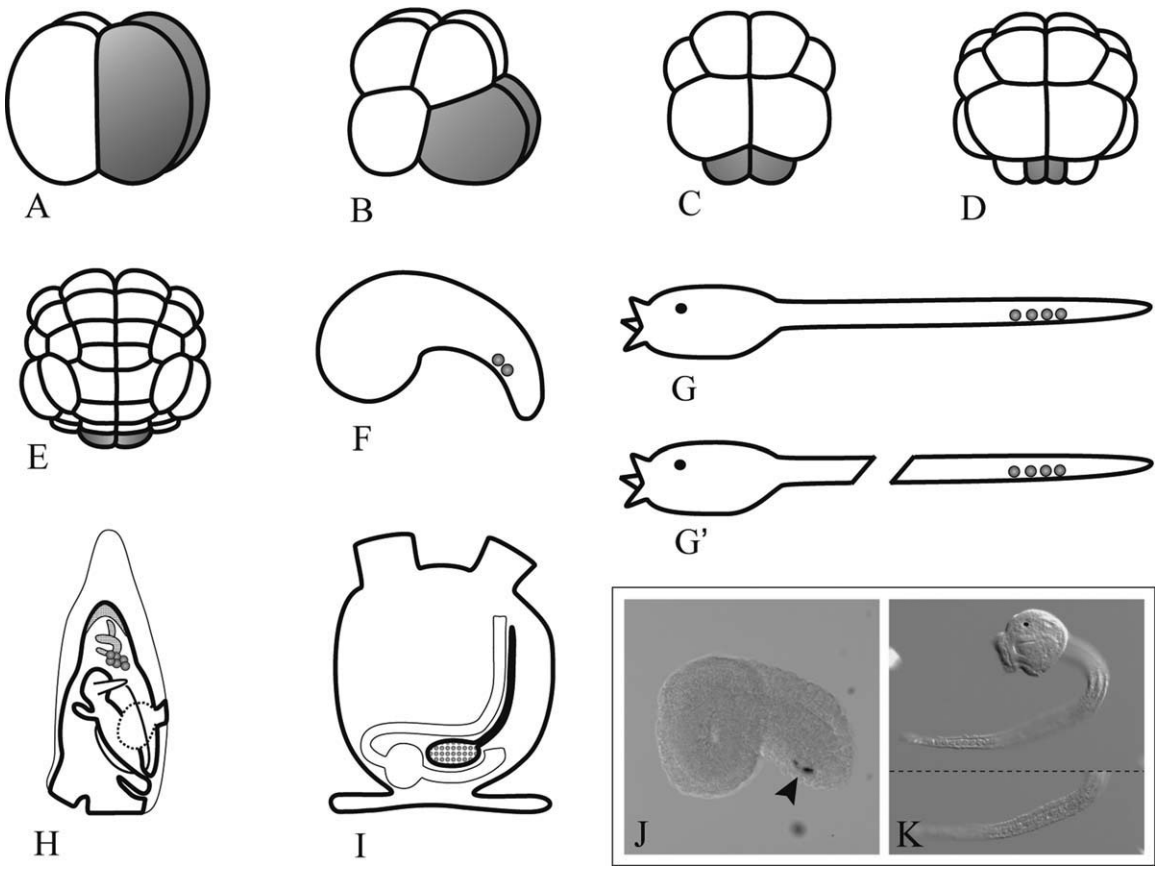

Fig. 3. Germline cell lineage from embryo to juvenile through metamorphosis. **A:** At the 4-cell stage. **B:** At the 8-cell stage. **C:** At the 16-cell stage. **D:** At the 32-cell stage. **E:** At the 64-cell stage. **F:** Tail-bud stage. **G:** Tadpole larva. **G′:** Tail-cut tadpole larva. **H:** Metamorphosing zooid. **I:** Juvenile zooid. In case of the tail-cut tadpole larva in *Ciona intestinalis*, *vasa*-expressing cells are not found in the metamorphosing zooid (H) but reappear in the juvenile zooid (I). **J,K:** In situ hybridization of vasa in tailbud embryo (J) and tadpole larva (K) in *Botryllus primigenus*. Note that a few cells are vasa-positive in a tailbud embryo (arrowhead), but they disappear from the tail of a larva. (Photographs courtesy of Ms. M. Tashiro, Kochi University).

intestinalis (Yamamoto and Okada, 1999, Okada and Yamamoto, 1999). One to 2 days after metamorphosis, the absorbed tail tissues are located in the body trunk between the esophagus and stomach where a wide mesenchymal space is formed by the epidermis and epicardium (Yamamoto and Okada, 1999). The PGCs are found solely on the surface of the regressing tail tissue (Fig. 3H). They can be visualized by distinctive nuage in the cytoplasm around the nuclear membrane and they also express high levels of vasa protein (Takamura et al., 2002). In 2–3 days after metamorphosis the gonadal rudiment, consisting of PGCs and somatic cells, appears as a transparent solid cell mass in the vicinity of the digestive tract. According to Takamura et al. (2002), the somatic part of the gonad rudiment originates from the derivative of the larval dorsal strand. As mentioned, *C. intestinalis* larvae have eight CiVH-positive cells (PGCs) in the tail (Takamura et al., 2002; Shirae-Kurabayashi et al., 2006). In 4–5 days after metamorphosis, approximately 10 presumptive germ cells are observable in a single gonad. The gonadal rudiment forms a lumen in 7 days later, and differentiates into the lobe of the testicular rudiment and the tubular ovarian rudiment 11–12 days later (Fig. 3I; Okada and Yamamoto, 1999; Takamura et al., 2002; Shirae-Kurabayashi et al., 2006).

To examine whether *Ciona* germ cells are derived only from the vasa$^+$ cells in the larval tail, 60–75% of the tail was excised and individuals monitored (Fig. 3G′; Takamura et al., 2002). Despite the missing tail, tadpoles underwent metamorphosis and soon became juvenile zooids. In these manipulated adults, vasa expression was not detected until 9 days after metamorphosis, but was recognizable by 15 days in the presumptive gonad. This result indicated two significant points: First, in the absence of tail, a vasa$^+$ gonad does not develop with the appropriate timing; Second, the vasa signal can regenerate de novo after embryogenesis. The former supports a direct relationship between the embryonic vasa expression and germ cell development in juvenile zooids. The latter suggests that vasa$^-$ precursors exist, revealing that regulative events also occur during *Ciona* germ cell formation (Takamura et al., 2002). The relationship between vasa$^+$ and vasa$^-$ precursors is unknown at the present time.

POSTEMBRYONIC GERMLINE DEVELOPMENT IN COLONIAL ASCIDIANS

a. Gonad Formation

The anatomy of the gonads in *Polyandrocarpa misakiensis* is similar to

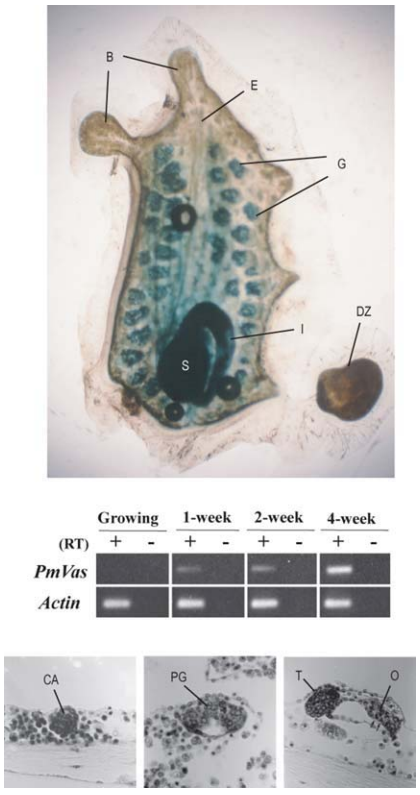

Fig. 4. Gonad formation and *vasa* expression during asexual reproduction of *Polyandrocarpa misakiensis*. Upper: Adult zooid, ventral view. A few rows of gonads (blue) are arranged longitudinally on each side of the body. They do not participate in the gonad formation of growing buds (new asexual zooids). B, bud; DZ, developing zooid; E, endostyle; G, gonad; I, intestine; S, stomach. Middle: RT-PCR of *vasa* transcripts. Note that growing buds have no *vasa* signals. A weak signal first appears in 1-week-old zooids, and thereafter it becomes strong gradually. Lower: Gonad formation. The gonad arises as a small cell aggregate in the ventral coelomic space. It becomes large and separates into two lobes to form the ovary and testis, respectively. CA, cell aggregate; O, ovary; PG, primordial gonad; T, testis.

gonads of solitary ascidians. In adult zooids, several rows of gonads are arranged longitudinally in the ventral mesenchymal space (Fig. 4 upper). A single gonad consists of two lobes of testis and a straightforward ovarian tube with the germinal epithelium (Fig. 1E; Sunanaga et al., 2007).

P. misakiensis, like *Stolonica* and *Distomus* (Berrill, 1948), propagates by budding (Fig. 4 upper). Budding occurs by the protrusion of the epidermis and the peribranchial epithelium between which mesenchymal cells are located (Kawamura and Nakauchi, 1986). Buds do not have any preexisting organs of the adult, but they renew all tissues and organs including the gonad after separated from the parent. Of interest, *Polyandrocarpa vasa* signals cannot be found at all in protruding buds but they are first detectable in developing zooids (Fig. 4 middle), suggesting that the germ cells regenerate de novo in each asexual reproduction (Sunanaga et al., 2007). It is noteworthy that in *Polyandrocarpa* each zooid lives a solitary life and does not have functional connection with the budding parent zooid. This feature is far different from botryllid ascidians, in which each zooid and buds are interconnected by the vascular network (Manni et al., 2007). As discussed later, germ cells in *Botryllus* can be transferred through this vascular network (Sabbadin and Zaniolo, 1979).

In *P. misakiensis*, the gonadal rudiment arises as a coelomic cell aggregate in developing zooids (Fig. 4 bottom; Sunanaga et al., 2007). It is discernible from surrounding coelomic cells and epithelial tissues by means of *vasa* expression. The cell aggregate becomes a compact and solid clump, which later separates into two parts; one is the testicular rudiment, and the other the ovarian rudiment with the lumen (Fig. 4 bottom). In the developing ovary, the lumen becomes the ovarian tube, and a portion of the tube becomes thickened to form the germinal epithelium. Small diverticula extend from each of the developing testis and ovary. They are the spermiduct and the oviduct, respectively.

By contrast, in the genera *Botryllus* and *Botrylloides*, gametogenesis is complicated by the constant asexual growth of the colony and by the fact that the new generations are directly connected with the parent (Manni et al., 2007). The colony reaches sexual maturity only after several successions of blastogenic generations (Mukai and Watanabe, 1976; Brown et al., 2009; Rosner et al., 2009). Mature colonies usually produce male organs at first and thereafter form both male and female organs. Even at this hermaphroditic stage, they will shift resources between production of sperm vs. both sperm and egg dependent on environmental conditions, with sperm being predominant when colonies are under stress, and both sexes produced under optimal conditions (Sabbadin, 1960; Sabbadin and Zaniolo, 1979; Newlon et al., 2003).

In colonial ascidians, the germline development is not necessarily synchronized with the blastogenic development. In the botyllid ascidians, morphological and genetic studies have suggested that both primordial testes (compact cell mass) and ovaries (oocytes) take several asexual generation to complete development, and are transported from one asexual generation to another until maturity is finally reached (Mukai and Watanabe, 1976, Sabbadin and Zaniolo, 1979). Thus individual zooids can harbor multiple stages of germline development for both male and female gonads.

As mentioned earlier, the botryllid female gonad lacks the germinal epithelium (Berrill, 1941; Manni et al., 1993, 1994). Instead, loose cell aggregates with undifferentiated configuration are located in the wide mesenchymal space (gonadal space) between the peribranchial epithelium and the epidermis (Mukai and Watanabe, 1976; Manni et al., 1994). In *B. primigenus*, they proliferate slowly (Kawamura et al., 2008b). They separate into paired cells in the ovary (Mukai and Watanabe, 1976), one of which differentiates into the oogonium, and the other into the primary follicle cell (Sunanaga et al., 2006). Developing oocytes make contact with the peribranchial epithelium by means of the follicle stalk (Fig. 1F). The loose cell aggregate also forms the compact cell mass that proliferate very rapidly (Kawamura et al., 2008b). The compact cell mass differentiates into spermatogonia and the testicular epithelium. Developing testes are connected with the peribranchial epithelium by means of a short spermiduct, a diverticulum of the testicular epithelium. The *vasa* homologue is expressed most strongly by loose cell mass, paired cells, and compact cell mass, of which the loose cell mass is in the most primitive and undifferentiated state (Sunanaga et al., 2006).

b. Origin of Germline Precursor Cells

In *Botrylloides violaceus* and *B. schlosseri*, *vasa*-expressing coelomic

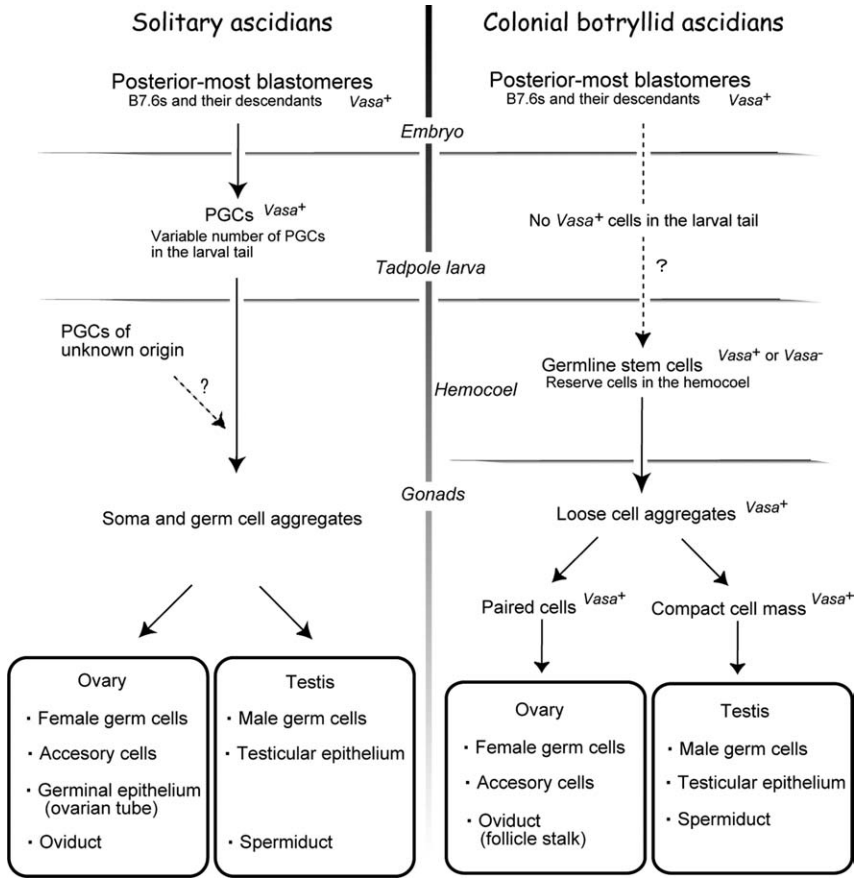

Fig. 5. Comparison of the gonadal cell formation between solitary and colonial ascidians. In both, *vasa* is segregated similarly in the posterior-most blastomeres during embryogenesis. In *Ciona*, the posterior-most blastomeres become *vasa*-expressing PGCs and form the gonadal rudiment together with somatic cells. It is doubtful if PGCs in the larval tail are the only progenitor of germ cells. In colonial botryllids, on the other hand, vasa-expressing cells disappear during larval formation. The germline competent cells reappear in the hemocoel of adult colonies. They should be responsible for the flexibility of gonad formation.

cells (hemoblasts) exist in both the body cavity and test vessels outside the gonad (Brown and Swalla, 2007; Brown et al., 2009), although in *B. primigenus vasa*-positive hemoblasts are very few in number in the hemocoel (Sunanaga et al., 2006). Therefore, *vasa*-positive hemoblasts in the hemocoel are likely PGCs (Fig. 5). However, this conclusion is based on correlative data and needs to be confirmed using a direct lineage tracing and/or transplantation assays.

As mentioned earlier, in *P. misakiensis vasa*-expressing germline precursor cells appear de novo in developing zooids. When buds were treated with double-stranded *vasa* RNA, zooids lacked *vasa*-positive cell aggregates and gonads (Sunanaga et al., 2007), indicating that the de novo *vasa* expression is essential for gonad formation. In *B. primigenus*, germline precursor cells disappear completely from colonies when the vascularization has been made by extirpating zooids and buds from the colonies (Sunanaga et al., 2006). However, both *vasa* signal and germ cells can regenerate in such colonies in approximately 2 weeks (Sunanaga et al., 2006). These results strongly suggest that in *Polyandrocarpa* and *Botryllus* germ cells come not only from *vasa*-positive hemoblasts but also from *vasa*-negative coelomic cells in the hemocoel.

Recently, genes encoding *nanos* and *piwi* have been cloned from *B. primigenus* (Sunanaga et al., 2008, 2010). The gene *nanos* is expressed widely by somatic multipotent cells, coelomic undifferentiated cells, and germ cells in the gonadal space. In the gonad, male germ cells express *nanos* most extensively. Consistent with this expression pattern, the gene knockdown of *nanos* by siRNA had effect most severely on spermatogenesis (Sunanaga et al., 2008). The gene *piwi* is expressed by germ cells and coelomic undifferentiated cells (Sunanaga et al., 2010). In germ cells, the expression pattern was completely overlapped with that of *vasa*, while in the hemocoel *piwi*-expressing cells were distributed more widely than *vasa*-expressing cells (Sunanaga et al., 2010). The *piwi* knockdown resulted in the defect of germline precursor cells (Sunanaga et al., 2010). These results suggest that in *B. primigenus piwi*$^{+}$/*vasa*$^{-}$ cells also serve as the germline stem cells reserved in the hemocoel (Kawamura and Sunanaga, 2010).

c. Long-Lived Germline and Germline Parasitism

In *B. schlosseri* it has been demonstrated that when two colonies come into close contact, terminal projections of the vasculature, called ampullae, reach out from each individual and come into contact. The ampullae can fuse together and form a single chimeric colony. This fusion results in a concomitant exchange of blood cells between individuals including circulating oocytes and spermatogonial cells (or their progenitors). As expected, a parabiont colony shed genetically distinct gametes (Sabbadin and Zaniolo, 1979). Complete germ cell replacement has even been demonstrated in some pairs of colonies, a situation that can persist for the remaining lifespan of the "parasitized" individual, even when two colonies are surgically separated a few days after fusion (Stoner et al., 1997; Brown et al., 2009). This finding supports the hypothesis that a population of mobile cells can retain germline competence. In fact, cells from newly metamorphosed individuals, far before sexual maturity, are competent to reconstitute the germline following transplantation into fertile colonies (Brown et al., 2009; Carpenter et al., unpublished). *Vasa* positive cells were observed in the gonads, as well as a population of mobile cells scattered throughout the open circulatory system in both fertile and non fertile colonies. Vasa was also expressed in cell

populations with high levels of ALDH activity that have been shown to contain functional germline precursors (Laird et al., 2005). These results indicate that circulating long-lived germline precursor cells exist and contribute to gametogenesis throughout the life of the colony. In turn, fertility is likely based on developmental cues that cause these progenitors to move from the blood stream to a niche in the forming bud and initiate germline formation (Brown et al., 2009).

CONCLUSION AND PERSPECTIVE

In solitary ascidians the *vasa*-expressing blastomeres segregate accurately from somatic cell lineages during early stages of embryogenesis, occupying the posterior-most positions. Subsequently, their descendant cells behave as PGCs in the tail of tadpole larva (Fig. 5; Fujimura and Takamura, 2000; Shirae-Kurabayashi et al., 2006). In *Ciona*, these cells form the gonad rudiment immediately after metamorphosis (Yamamoto and Okada, 1999; Takamura et al., 2002). In colonial botryllids, the embryonic segregation of *vasa* transcript appears to take place in the same manner as *Ciona* until the tailbud stage. However, it is still uncertain whether the *vasa*-expressing embryonic cells in botryllids are genuine PGCs that differentiate into germ cells in the adult. In *B. primigenus vasa* signal becomes vague temporarily before and after metamorphosis and it reappears later in the gonad of adult colonies (Fig. 5). In *B. schlosseri*, the signal is dispersed in the larval trunk rather than the tail and, immediately after metamorphosis, it is localized in the hemocytes of juvenile colonies (Brown et al., 2009).

It is uncertain at present whether the difference between *Ciona* and botryllid ascidians comes from taxonomic distance among animals or from solitary and colonial lives. To solve the question, embryogenesis of *Diazona* and *Styela* would be available. The former is a colonial ascidian belonging to Cionidae, and the latter is a solitary ascidian relative to Botryllidae.

As mentioned, the germline segregation is well characterized in unmanipulated *Ciona* embryos, but tail extirpation studies also suggest another source of progenitors (Fig. 5; Takamura et al., 2002). In *Polyandrocarpa* and *Botryllus*, both germ cells and gonads can regenerate from coelomic cells (Sunanaga et al., 2006, 2007), suggesting that the hemocoel of colonial ascidians could be a reservoir of germline stem cells (Fig. 5). In *Botrylloides violaceus*, *vasa*-expressing cells in the test vessel may represent such germline stem cells (Brown and Swalla, 2007). In *B. primigenus*, cells expressing *piwi* instead of *vasa* may serve as reserve cells (Sunanaga et al., 2010). According to Laird et al. (2005), it seems likely that the presumptive germline stem cells preserve the unipotency toward gonadal cells. Further purification of these cells is underway, which may allow in vitro approaches, and further transplantation, and lineage tracing.

Gametogenesis in both solitary and colonial ascidians begins with aggregates of undifferentiated cells (Fig. 5). In some cases, they develop into hermaphroditic gonads, and in other cases such as *Distomus* they become either testis or ovary. It remains to be solved how genes and environment influence male and female gonad formation.

The relationship between germ cells and their accessory cells is also interesting. In *Ciona*, the gonad rudiment arises as soma and germ cell aggregates, although the destination of somatic cells is unknown at present. If the primary follicle cells in *Ciona* come from the germinal epithelium as argued by Sugino et al. (1990) and Okada and Yamamoto (1993), they might be also germline cells. According to Sunanaga et al. (2006, and unpublished data), female germ cells and primary follicle cells in *B. primigenus* would be equivalent in origin. When two undifferentiated cells come into contact with each other as paired cells in the gonadal space (Fig. 5), the cell fate toward soma or germ might be determined alternatively. It remains to be an open question how these cells communicate.

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