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ORIGINAL ARTICLE

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Primordial germ cells originate from the endodermal strand cells in the ascidian *Ciona intestinalis*

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Abstract The origin of germ cells in the ascidian is still unknown. Previously, we cloned a *vasa* homologue (*CiVH*) of *Ciona intestinalis* from the cDNA library of ovarian tissue by polymerase chain reaction and showed that its expression was specific to germ cells in adult and juvenile gonads. In the present study, we prepared a monoclonal antibody against CiVH protein and traced the staining for this antibody from the middle tailbud stage to young adulthood. Results showed that positive cells are present in the endodermal strand in middle tailbud embryos and larvae. When the larval tail was absorbed into the trunk during metamorphosis, the CiVH-positive cells migrated from the debris of the tail into the developing gonad rudiment, and appeared to give rise to a primordial germ cell (PGC) in the young juvenile. The testis rudiment separated from the gonad rudiment, the remainder of which differentiated into the ovary. PGCs of the testis rudiment and the ovary rudiment differentiated into spermatogenic and oogenic cells, respectively. When the larval tail containing the antibody-positive cells was removed, the juveniles did not contain any CiVH-positive cells after metamorphosis, indicating that the PGCs in the juvenile originated from part of the larval tail. However, even in such juveniles, positive cells newly appeared in the gonad rudiment at a later stage. This observation suggests that a compensatory mechanism regulates germline formation in *C. intestinalis*.

Keywords Ascidian · Vasa protein · Primordial germ cells · Germ cell origin · Gonad development

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Introduction

Ascidians belong to the phylum Chordata together with cephalochordates and vertebrates. They have many of the features of the prototype of the ancestral Chordata, such as a notochord, a dorsal nerve cord and pharyngeal gill slits (Satoh 1994; Satoh and Jeffery 1995). They are hermaphrodites with adults having both an ovary and a testis. After fertilization, the zygotes divide bilateral-symmetrically and the cleavage pattern is invariant. Eventually, the process gives rise to a typical tadpole larva with a simple tissue organization. The metamorphosis of the larva begins when it settles, after which the larval tail is absorbed into the trunk within hours. Several days after settlement, the larvae metamorphose into juveniles and start feeding.

Most of somatic cell lineages during embryogenesis have been described (Conklin 1905; Nishida 1987). Recently, Hirano and Nishida (1997, 2000) have expanded this analysis up to 1-month-old juveniles in the ascidian *Halocynthia roretzi*. However, they were unable to trace the germ cell lineage, since the germ cells and gonad rudiment of this species could not be distinguished in 1-month-old juveniles. On the other hand, in other ascidian species belonging to the family Cioninae, such as *Ciona intestinalis* and *C. savignyi*, the gonad rudiments are detectable several days after metamorphosis. In these species, animals mature sexually within 2–3 months, and the early development of the gonad and early oogenesis have been well studied by electron microscopy (Sugino et al. 1987, 1990; Okada and Yamamoto 1993, 1999; Yamamoto and Okada 1999). Using *C. intestinalis*, Yamamoto and Okada (1999) traced germ cell precursors back to the debris of the larval tail of juveniles 12 h after settlement, but did not identify the origin of germ cells at an earlier stage.

Several molecules specific to germ line cells have been identified in other animals (Wei and Mahowald 1994), but only Vasa protein or its homologue is detected in germ line cells or germ cells throughout the life cycle of an animal (Ikenishi 1998). Vasa and its homologue be-

long to the DEAD-box protein family, which functions putatively as an RNA helicase (Schmid and Linder 1992; Lüking et al. 1998). Genes coding these proteins have been cloned in *Drosophila* (*vasa*; Schüpbach and Wieschaus 1986; Hay et al. 1988; Lasko and Ashburner 1988), *Caenorhabditis elegans* (*glh-1* and *glh-2*; Roussell and Bennett 1993; Gruidl et al. 1996), zebrafish (*vas*; Yoon et al. 1997), *Xenopus* (*XVLG1*; Komiya et al. 1994), mouse (*mvh*; Fujiwara et al. 1994), and rat (*rvh*; Komiya and Tanigawa 1995).

Previously, we isolated several cDNA clones for DEAD-box protein genes from a cDNA library of *C. intestinalis* ovary (Fujimura and Takamura 2000). One of them, *Ci-DEAD1*, was similar in amino acid sequence to other *vasa* homologues. *Ci-DEAD1* is expressed specifically in germ cells of adult and juvenile gonads. Therefore, we named the gene *CiVH* (*C. intestinalis vasa* homologue, formerly *Ci-DEAD1*). In situ hybridization revealed that, during embryogenesis, maternal *CiVH* mRNA is distributed uniformly in the cytoplasm of one- and two-cell embryos, localized also to the most-posterior blastomeres after the four-cell stage, and finally partitioned into several cells of the endodermal strand at the tailbud stage. However, we could not trace the signal at the larval stage and during metamorphosis, because of nonspecific staining in the tunic that covers the larvae and juveniles.

In the present study, we prepared an anti-CiVH monoclonal antibody using GST-CiVH fusion protein as immunogen, and investigated the behavior of the cells positive for this antibody from the middle tailbud stage to young adulthood.

Materials and methods

Biological materials

C. intestinalis specimens were collected near the Education and Research Center of Marine Bio-Resources of Tohoku University, Onagawa Bay, Japan and the Mukaishima Marine Biological Station of Hiroshima University, the Inland Sea of Japan. Eggs and sperm were obtained by dissection of the gonoducts. After artificial insemination, they were incubated for 16–18 h until hatching at 18–20°C. Larvae were allowed to settle and metamorphose in plastic dishes at 20°C. Resultant juveniles were transferred into 100-l tanks, raised at 18–25°C and fed with the *Phaeodactylum sp.*, *Nannochloropsis oculata* and a formulated feed of *Artemia salina* (Nichiku Yakuhin Kogyo). These samples were fixed with 100% methanol or 10% formalin in filtered sea water overnight at 4°C for immunostaining, or 4% paraformaldehyde/0.5 M NaCl-0.1 M MOPS buffer (pH 7.5) overnight at 4°C for in situ hybridization, and stored at –30°C in 100% ethanol until used. The age of juveniles is designated in terms of days after settlement according to Yamamoto and Okada (1999), although the growth rate of juveniles was not always synchronous between experimental series.

Preparation of anti-CiVH monoclonal antibody

The full-length *CiVH* cDNA was digested with *Bam*HI and cloned in-frame into *Escherichia coli* expression vector pGEX4T-1 (Pharmacia Biotech, Piscataway, N.J.). CiVH protein was expressed as a construct fused with a glutathione S-transferase (GST) and was purified using the Bulk GST Purification Module (Pharmacia Biotech). The purified protein solution was mixed well with Freund's complete adjuvant and the emulsion was injected into 7-week-old mice 3 times at 4- or 2-week intervals. Following cell fusion, 446 hybridoma cells were screened by ELISA using the immunogen. The monoclonal cell line, which showed the strongest reaction, line no. 613, was used for subsequent experiments.

Western blot analysis

Total protein was extracted from *E. coli* expressing GST or GST-CiVH fusion protein, and from adult ovary, testis and unfertilized eggs of *C. intestinalis*. Each protein was separated by SDS-polyacrylamide gel electrophoresis, stained with Coomassie Brilliant Blue (CBB) and blotted onto a PVDF membrane (Bio-Rad Laboratories, New York, N.Y.). The membrane was first immersed in 5% skim milk/phosphate-buffered saline (PBS) containing 0.01% thimerosal for 1 h to block nonspecific reactions, and then cultured in the anti-CiVH monoclonal antibodies for 1 h at room temperature. After being washed 6 times in PBS containing 0.05% Tween-20, the membrane was incubated for 1 h at room temperature with horseradish peroxidase-conjugated rabbit anti-mouse IgG serum (Zymed Laboratories, San Francisco, Calif.) diluted 1:1,000 with 5% fetal bovine serum in PBS. After being washed as before, the proteins were visualized with DAB substrate (0.06% w/v diaminobenzidine, 0.015% v/v hydrogen peroxide in PBS).

In situ hybridization and immunostaining

Whole-mount in situ hybridization and whole-mount immunostaining were carried out as described previously (Takamura 1998; Fujimura and Takamura 2000). Some of the stained samples were embedded in paraffin to be sectioned. Fixed adult ovaries and testes were embedded in polyester wax (BDH Chemicals, Poole, UK), sectioned, and stained immunohistologically with the antibody as described previously (Takamura et al. 1996). They were then observed with a differential interference microscope (Axiophot 2, Carl Zeiss).

Tail-cutting experiment

Part of the larval tail was removed manually with a scalpel under a dissecting microscope (see also Results). Tail-cut larvae were cultured in plastic dishes until they had settled naturally, and raised as described above.

Results

Specificity of anti-CiVH monoclonal antibody

To ascertain the specificity of the antibody against CiVH protein, western blot analysis was performed using total protein extracted from *E. coli* expressing GST or GST-CiVH fusion protein (Fig. 1A). A single band was detected only in the latter case with this antibody (lane 4 in Fig. 1A, black arrowhead). Its molecular mass (*Mr*), at about 100 kDa, corresponded to that predicted from the DNA of the GST-CiVH construct. Although GST protein was detected by CBB staining (lane 1 in Fig. 1A, white arrowhead), the antibody did not react with it (lane 3 in Fig. 1A).

Two major bands were detected by western blot analysis using adult gonads (lane 4, 5 in Fig. 1B, arrowheads) with lower molecular masses (about 66 kDa and

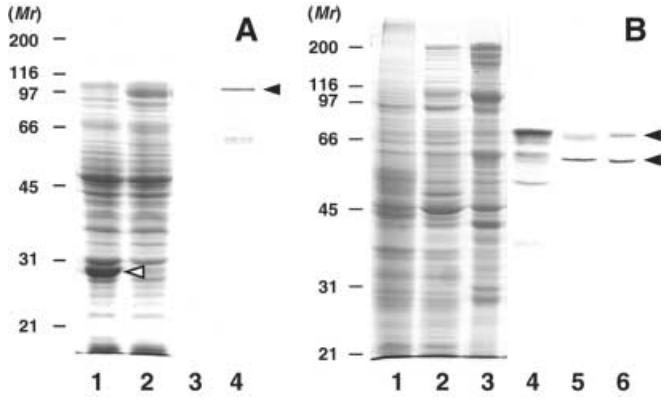

Fig. 1A, B Immunoblot analysis with anti-*Ciona intestinalis vasa* homologue (anti-CiVH) monoclonal antibody. **A** Total protein was extracted from *Escherichia coli* expressing glutathione S-transferase (GST; *lanes 1* and *3*) and GST-CiVH fusion protein (*lanes 2* and *4*). The extracts were stained with Coomassie Brilliant Blue (CBB; *lanes 1* and *2*) or incubated with anti-CiVH monoclonal antibody (*lanes 3* and *4*). GST-CiVH fusion protein was detected with this antibody (lane 4, *black arrowhead*), while GST protein (lane 1, *white arrowhead*) was not. **B** Total protein was extracted from adult testis (*lanes 1* and *4*), ovary (*lanes 2* and *5*) and unfertilized eggs (*lanes 3* and *6*) of *C. intestinalis*. The extracts were stained with CBB (*lanes 1–3*) or treated with anti-CiVH monoclonal antibody (*lanes 4–6*). Two major bands were detected in all these samples (lanes 4–6, *black arrowheads*)

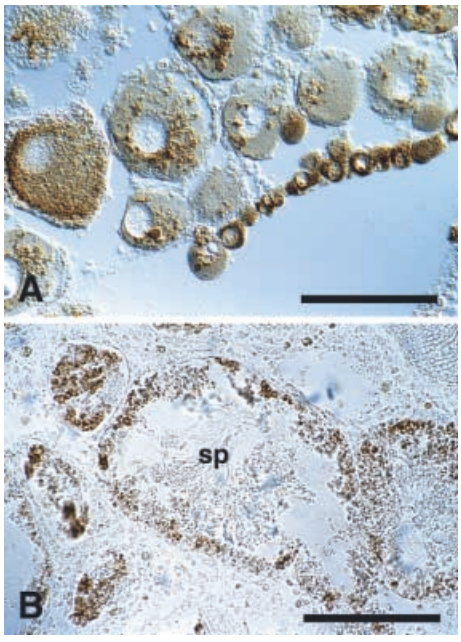

Fig. 2 Immunohistostaining of adult ovary (**A**) and testis (**B**) of *C. intestinalis* with anti-CiVH monoclonal antibody. *Scale bar* represents 100 µm. In the ovary, the signal is distributed throughout the cytoplasm of smaller oocytes and in the perinuclear cytoplasm of larger oocytes. In the testis, large spermatogenous cells localized in the peripheral region of tubulus seminiferus were strongly stained by the antibody, while sperm (*sp*) was not

55 kDa, respectively) than that predicted from the *CiVH* cDNA sequence (about 72 kDa). Both bands were also detected in unfertilized eggs (lane 6 in Fig. 1B).

To investigate the specificity of this antibody, we performed immunohistostaining on sections of the adult ovary and testis (Fig. 2). In the ovary, this antibody reacted specifically with the developing oocytes, but not other cell types (e.g. follicle cells and test cells). The signal was distributed uniformly in the cytoplasm of the smaller oocyte, while it was concentrated around the nucleus of the larger oocyte (Fig. 2A). In the testis, a strong signal was present in the spermatogenous cells localized to the peripheral region of the tubulus seminiferus, but not in the more mature cells such as the sperm (Fig. 2B). This distribution of CiVH protein corresponded to that of its mRNA determined by in situ hybridization (Fujimura and Takamura 2000).

Gonad development after metamorphosis

The development of the gonads was investigated using live individuals. After the larvae had settled, most of their tail was absorbed into the trunk, and the body axis rotated through 90 . Finally, the oral siphon, endostyle and cerebral ganglion came to be localized on the upper side (anterior), ventral side and dorsal side, respectively (Fig. 3A, C). Immediately after metamorphosis, part of the larval tail remained as cell debris in the space between the esophagus and the stomach. It is noted that the posterior end of the dorsal strand is connected to this debris (Fig. 3B, D and E, black arrowhead). A teardrop-shaped cell mass is found to be protruding from the tail debris (Fig. 3D, asterisk). However, this cell mass is not the precursor of the gonad rudiment, as it remains when the gonad rudiment becomes evident elsewhere (data not shown). Okada and Yamamoto (1999) referred to this structure as a periesophagial body. They suggested that it controls gonadal development through an endocrine function. In the 4-day-old juveniles, a cell with a large nucleus is detectable at the posterior end of the dorsal strand (Fig. 3E, black arrowhead). In the 6-day-old juveniles, a slender cell mass is evident in the space between the debris and the pyloric gland (Fig. 3F, white arrowhead). In the 10-day-old individuals, this cell mass is larger and rounder (Fig. 3G, white arrowhead), and still connected to the dorsal strand. In the 19-day-old individuals, the cell mass has become a sac-shaped structure with a large lumen (Fig. 3H). This structure is the gonad rudiment, its morphological features being consistent with those described in recent studies on gonad development (Okada and Yamamoto 1999; Yamamoto and Okada 1999).

Behavior of CiVH-positive cells during and after metamorphosis

Previously, we revealed the distribution of *CiVH* mRNA using in situ hybridization (Fujimura and Takamura 2000).

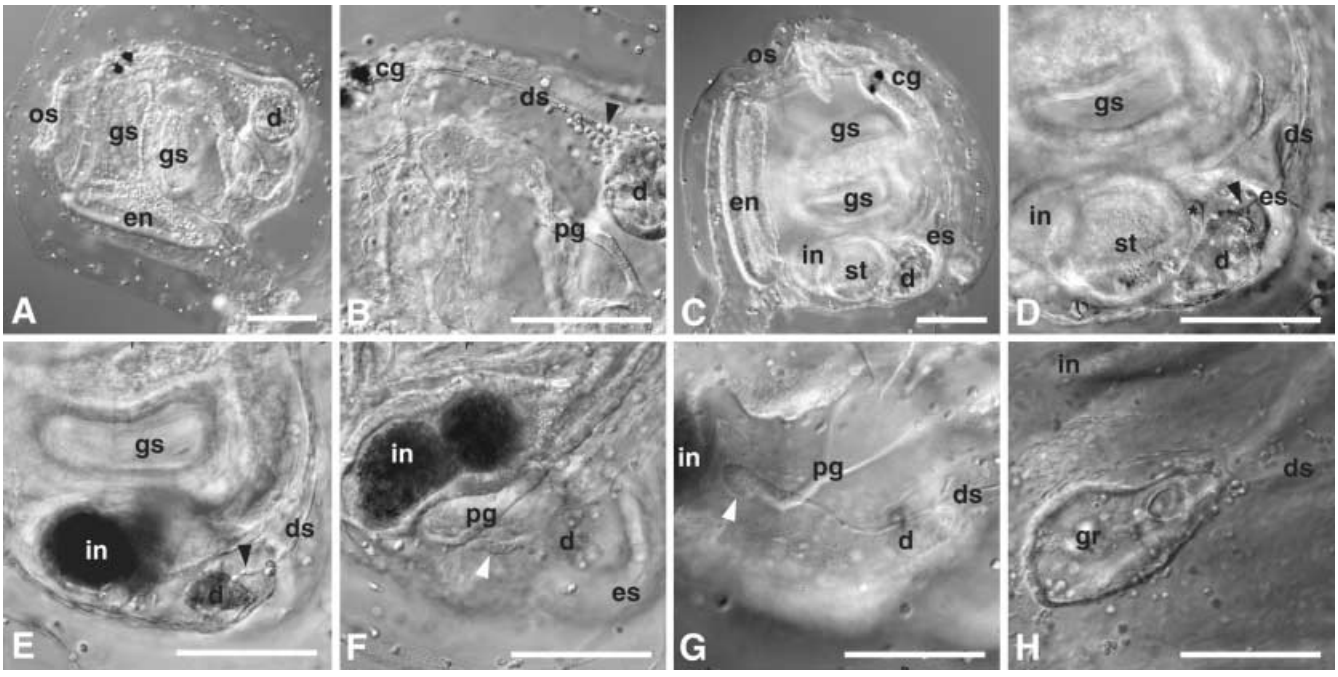

Fig. 3A–H Development of the gonad rudiment after metamorphosis in *C. intestinalis*. *Scale bar* represents 100 µm. Individuals were photographed at 2 days (**A**, **B**), 3 days (**C**, **D**), 4 days (**E**), 6 days (**F**), 10 days (**G**), and 19 days (**H**) after settlement. **B** and **D** are magnified views of **A** and **C**, respectively. Most of the larval tail is absorbed into the larval trunk during metamorphosis with only debris (*d*) remaining in the space between the stomach and esophagus of the juvenile (**A**). This debris is connected with the posterior end of the dorsal strand (*black arrowhead* in **B**, **D** and **E**). In the 3-day-old juveniles (**C**, **D**), in which the rotation of the body axis has finished, a teardrop-shaped cell mass has protruded from the tail debris (*asterisk* in **D**), but it is not a gonad rudiment. In the 4-day-old juveniles, a cell with a large nucleus is evident at the posterior end of the dorsal strand (*black arrowhead* in **E**). In the 6-day-old juveniles, a slender cell mass (*white arrowhead* in **F**) is located between the debris and the pyloric gland. In the 10-day-old individuals, this cell mass is larger, rounder (*white arrowhead* in **G**) and attached to the dorsal strand. In the 19-day-old animals, this structure has become a sac with a large lumen, representing a gonad rudiment (*cg* cerebral ganglion, *d* debris of larval tail, *ds* dorsal strand, *en* endostyle, *gr* gonad rudiment, *gs* gill slit, *es* esophagus, *in* intestine, *os* oral siphon, *pg* pyloric gland, *st* stomach)

We traced the mRNA back to the late tailbud stage but could not detect it at the larval stage or during metamorphosis due to nonspecific staining of the larval tunic. Therefore, we decided to investigate the distribution pattern of CiVH protein with anti-CiVH monoclonal antibody.

At the middle tailbud stage, this antibody reacted with the anterior portion of the neural tube, endoderm and a few cells of the endodermal strand (Fig. 4B). This distribution almost corresponded to that detected by in situ hybridization with *CiVH* antisense riboprobe (Fig. 4A). The position and number of positive cells in the endodermal strand, however, differed slightly (compare Fig. 4A and B, arrowheads). In the larva, the signal in the trunk became faint, while positive cells in the endodermal strand were located in the posterior half of the tail (Fig. 4C) and increased in number, typically to eight cells (Fig. 4D, arrowheads). During metamorphosis, positive cells moved into the trunk region with other tail tissues, and finally localized to the debris of the larval tail (Fig. 4E, F, arrowhead). When this debris had almost disappeared, eight positive cells aligned in the space between the intestine and stomach (Fig. 4G, arrowhead). In the 15-day-old animals, the positive cells increased in number and formed a mass positioned laterally to the pyloric gland (Fig. 4H, arrowhead). This position appeared to correspond to that of the developing gonad rudiment (compare Fig. 3F and G).

In the 19-day-old individuals, there was a distinct gonad rudiment with a large lumen containing numerous positive cells in the periphery (Fig. 5A). With growth, the gonad rudiment invaginated to form folds, and positive cells were localized to its peripheral region (Fig. 5B, C). Concurrently, another structure containing positive cells appeared around the gonad rudiment. This structure was connected to the gonad rudiment by a thin tubular structure (Fig. 5B, arrowheads). This positive cell mass increased in size and then scattered in the peritoneum of the intestine (Fig. 5C, arrowheads). In the young adults, many positive cell masses were distributed in the peritoneum of the intestine and the stomach (Fig. 5E). This structure was the testis, because it contained mature sperm (Fig. 5G, H). On the other hand, the original gonad rudiment developed morphologically into a typical ovary (Fig. 5D, E) containing many developing oocytes (Fig. 5F).

Development of the gonad in the tail-cut experiment

To determine whether the CiVH-positive cells in the developing gonad rudiment originate from the positive cells in the larval tail, we removed some of the tail and raised the larvae for the same period in which intact controls de-

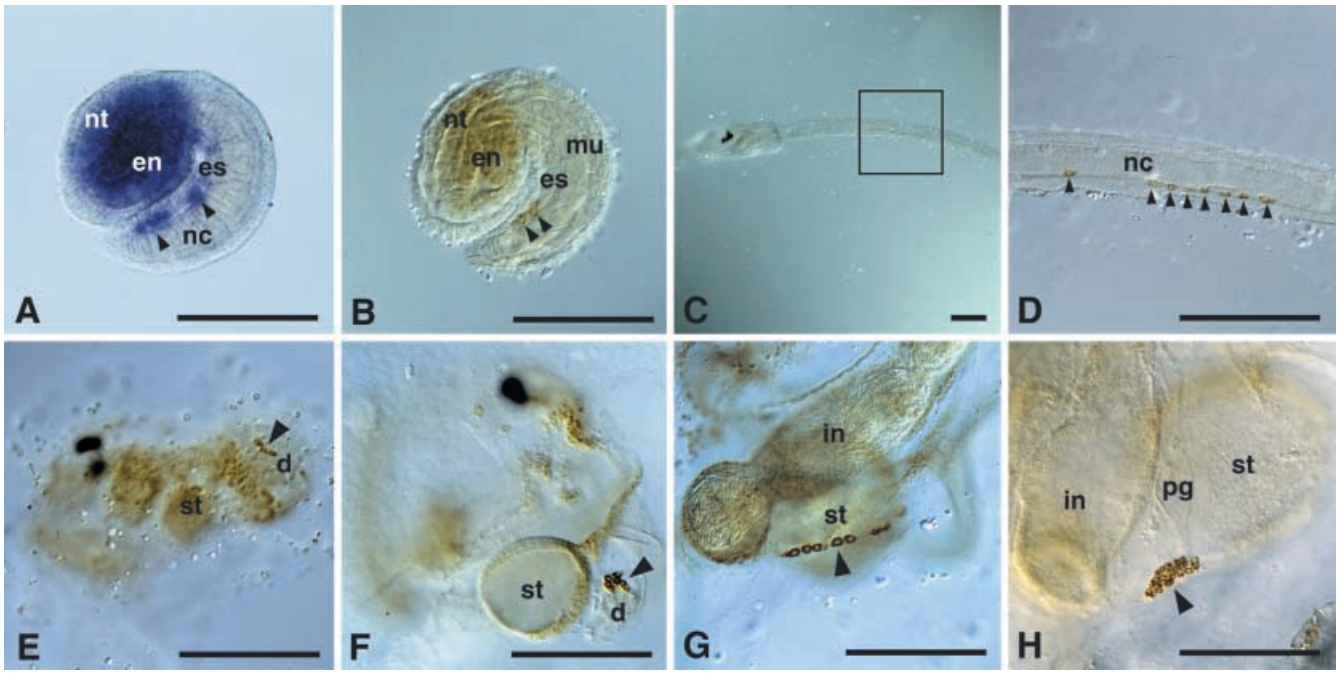

Fig. 4A–H Localization of *CiVH* mRNA and CiVH protein in *C. intestinalis*. *Scale bar* represents 100 µm. **A** Whole-mount in situ hybridization of a middle tailbud probed for *CiVH* antisense riboprobe. Strong signal is evident in the anterior neural tube and the endoderm regions, as well as in a few endodermal strand cells (*arrowheads*). **B–H** Immunostaining with anti-CiVH antibody in a middle tailbud embryo (**B**), a larva (**C**, **D**), and 1-day- (**E**), 3-day- (**F**), 9-day- (**G**) and 15-day-old animals (**H**). **D** is a magnified view of the area *boxed* in **C**. **B** The positive region in the middle tailbud embryo is very similar to that found by in situ hybridization (compared with **A**). In the tail, several round cells of the endodermal strand are stained (*arrowheads*). **C**, **D** In larvae, positive cells are located in the endodermal strand of the posterior half of the tail (*arrowheads* in **D**). **E**, **F** During metamorphosis, the larval tail was absorbed into the trunk region and only debris remains in the space between the esophagus and stomach. Positive cells are localized with this debris (*arrowheads*). **G** At 9 days after settlement, most of the debris has disappeared and positive cells are aligned towards the ventral side (*arrowhead*). **H** At 15 days after settlement, positive cells have increased in number and formed a cluster (*arrowhead*). The morphology of this cell mass is very similar to that of the developing gonad rudiment shown in Fig. 3 (*d* debris of larval tail, *en* endoderm, *es* endodermal strand, *in* intestine, *mu* muscle, *nc* notochord, *nt* neural tube, *pg* pyloric gland, *st* stomach)

veloped distinct gonads. When almost all of the larval tail was removed, most of the tail-cut larvae failed to settle and mature. The few metamorphosed individuals were malformed and could not grow normally. Thus, we allowed tail-cut larvae to retain 25–40% of the tail, since positive cells were located in the posterior half of the tail (Fig. 6B, C; arrowheads). Most of the treated larvae (Fig. 6A) settled on the plastic dish and metamorphosed normally, though they grew more slowly than the control. We observed the distribution of CiVH-positive cells in from two to ten specimens every 3 days after settlement. Until 9 days after settlement, no positive cell was found in any of the specimens examined (Fig. 6D). A few positive cells, however, appeared in the 15-day-old individuals (Fig. 6E, white arrowhead). With growth, the number and position of positive cells became similar to those in normal gonad rudiments (Fig. 6F, white arrowhead) or ovary rudiment (Fig. 6G, white arrowhead). Additionally, several scattered positive cell masses appeared around the intestine (Fig. 6F, G; black arrowheads), and in older individuals, mature sperm was observed in the vas deferens (data not shown). This experiment was carried out twice and the same results were obtained.

Discussion

Specificity of anti-CiVH monoclonal antibody

Since the anti-CiVH monoclonal antibody strongly reacted with only the GST-CiVH construct, it is specific to CiVH protein. In addition, the pattern of antigen distribution in the ovary, testis and middle tailbud embryos detected by this antibody was very similar to that of the *CiVH* mRNA. Western blot analysis using total protein of adult gonads, however, showed that this antibody reacted with two major antigens of lower molecular weight than expected from the *CiVH* cDNA sequence. It should be determined in future whether either or both of the bands constitute true CiVH proteins. We will attempt to purify these antigens by affinity chromatography and analyze the amino acid sequences. However, this ambiguity does not necessarily prevent tracing of the cells positive for this antibody during metamorphosis.

Differentiation of gonads

Several reports have described the early development of gonads and oogenesis in the ascidians *C. savignyi* and *C.*

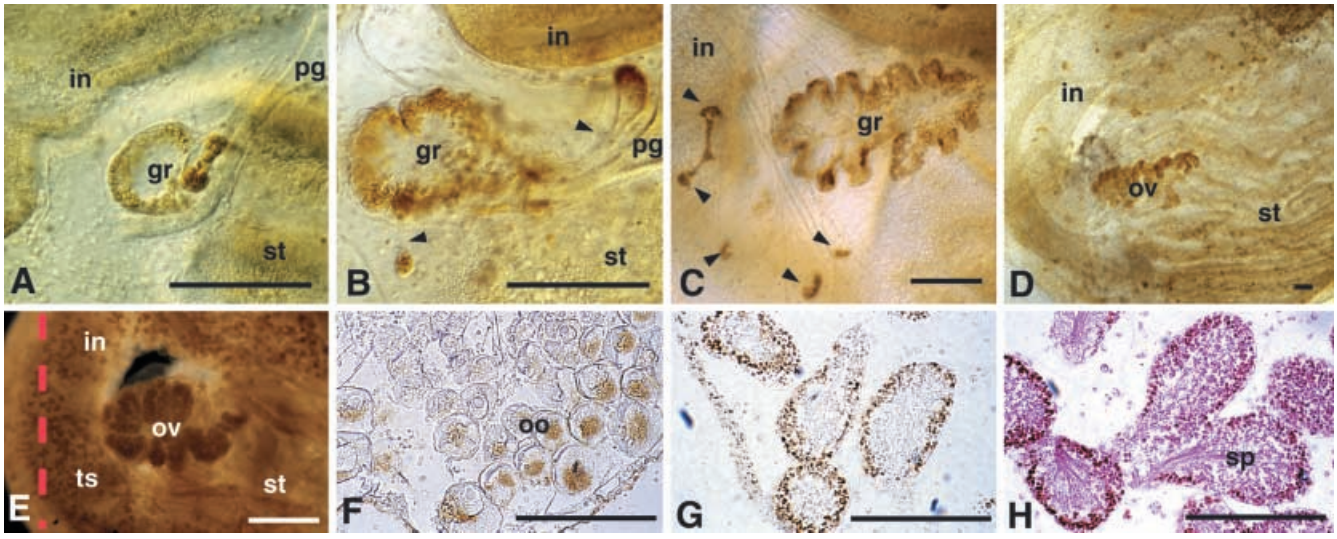

Fig. 5A–H Localization of CiVH protein during differentiation of the gonad in *C. intestinalis*. *Scale bars* represent 100 µm except in **E** (1 mm). Animals are 19 days old (**A**), 25 days old (**B**, **C**), 36 days old (**D**), and 63 days old (**E**), and are immunostained with anti-CiVH monoclonal antibody. **F** is a sectioned ovary of the same individual shown in **E**. **G** and **H** are sections at the level indicated by a *red line* in **E**. **H** is counter-stained with eosin Y. **A** At 19 days after settlement, a sac-shaped structure, which contains many positive cells, is seen near the pyloric gland and is judged to be a gonad rudiment. **B**, **C** At 25 days, the gonad rudiment has enlarged and invaginated to form numerous folds. In one individual, two positive-cell clusters appeared to be protruding from the gonad rudiment (*arrowheads* in **B**), while in another, several positive-cell clusters were scattered around the gonad rudiment, perhaps in the peritoneum of the intestine (*arrowheads* in **C**). **D**, **E** In older animals, these scattered positive-cell clusters increased in number and were present in the peritoneum not only of the intestine but also of the stomach. **F–H** At 63 days after settlement, the gonad rudiment had apparently differentiated into an ovary containing many small oocytes (**F**), while the scattered positive-cell cluster differentiated into the testis containing mature sperm (**G**, **H;** *gr* gonad rudiment, *in* intestine, *oo* oocyte, *ov* ovary, *pg* pyloric gland, *sp* sperm, *st* stomach, *ts* testis)

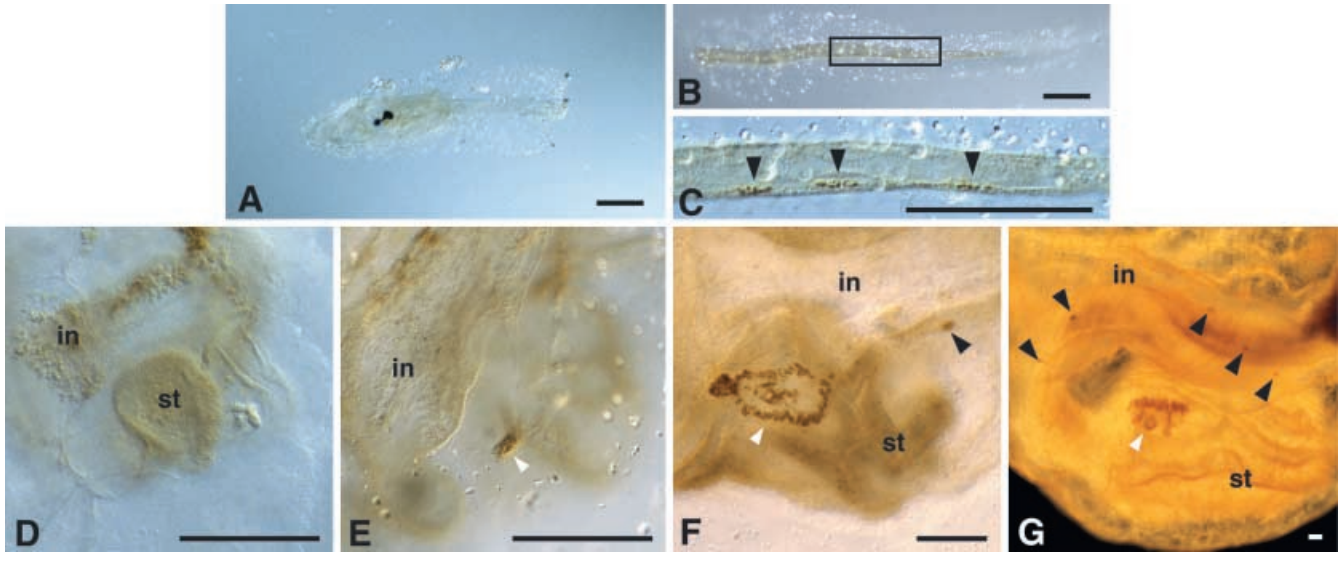

Fig. 6A–G Staining of CiVH-positive cells in tail-cut larvae. All *scale bars* represent 100 µm. The samples are immunostained with anti-CiVH monoclonal antibody. **A** A tail-cut larva. **B, C** A removed larval tail. **C** is a magnified view of the region *boxed* in **B**, and several positive cells are present in the endodermal strand (*arrowheads*). **D** A 9-day-old individual. At this stage, no positive cells were found in the space between the esophagus and stomach. **E** A 15-day-old individual. A small positive-cell cluster (*white arrowhead*) is seen in the space between the stomach and the intestine. **F** A 32-day-old individual. Positive cells have increased in number and appear to exist in a sac-shaped structure (*white arrowhead*) resembling the gonad rudiment shown in Fig. 5B. A single cluster of positive cells is also evident in the peritoneum of the intestine (*black arrowhead*). **G** A 53-day-old adult. Many positive cells are detected in the structure (*white arrowhead*) similar to the developing ovary shown in Fig. 5D, E. Several clusters of positive cells are also scattered in the peritoneum of the digestive organ (*black arrowheads*; *in* intestine, *st* stomach)

intestinalis (Sugino et al. 1987, 1990; Okada and Yamamoto 1993; Yamamoto and Okada 1999). Our results are consistent with previous observations, and provide new insight into the mechanism of gonad differentiation.

First, the gonad rudiment was found to be connected to the dorsal strand in juveniles. The dorsal strand is the duct extending posteriorly from the neural gland in the adult ascidian (Goodbody 1974). The nerve cord from the cerebral ganglion also extends posteriorly along the dorsal strand in the juveniles (Takamura, unpublished data). These observations suggest that the dorsal strand and the nerve cord along it, play an important role during the early phase of somatic gonad formation in juveniles, perhaps through the secretion of gonadotropic hormone.

Second, the somatic gonad rudiment seems to be generated from the posterior end of the dorsal strand and/or a part of the tail debris. In the 4-day-old juveniles (Fig. 3E), a cell with a large nucleus was observed at the posterior end of the dorsal strand. As the juveniles grew, the cell mass connected to the posterior end of the dorsal strand became larger, extending ventrally and finally leading to the formation of the somatic gonad rudiment (Fig. 3F, G).

Third, primordial germ cells of *C. intestinalis* did not migrate as far into the gonad rudiment as in other animals (for a review see Wei and Mahowald 1994), but PGCs associated with the developing somatic gonad rudiment.

Finally, we demonstrated the separation of the testis rudiment from the gonad rudiment, followed by the subsequent differentiation of the remaining gonad rudiment into ovary. Okada and Yamamoto (1999) have also reported that the gonad rudiment differentiates into both ovary and testis. These observations suggest that the testis rudiment protrudes from the gonad rudiment. Because the testis rudiment increased in number around the digestive organs even after the ovary had differentiated morphologically, the early formed testis rudiment might divide further at a later stage. The sex determination of germ cells in each rudiment may also occur at this time.

Origin of the germ cells

Yamamoto and Okada (1999) described the development of the gonads and origin of germ cells in *C. intestinalis*. They traced the germ cell lineage electron microscopically back to germ cell precursors, which associated with the debris of the larval tail in juveniles just after the initiation of metamorphosis. The results of the present study are consistent with their findings, that is, CiVH-positive cells were located in this debris, migrated into the gonad rudiment, and finally differentiated into both oocytes and sperm. Moreover, we speculated that the positive cells originated from the larval endodermal strand. The basis for this speculation is as follows: (1) most of the larval tail remains as cell debris in juveniles; (2) the number of positive cells is almost the same (typically eight) in larva and juveniles (compare Fig. 4D and G); and (3) juveniles that developed from tail-cut larvae showed no positive cells at least until 9 days after settlement.

The earliest stage examined was the middle tailbud stage. At this stage, the anterior portion of the neural tube and the endoderm in the trunk are also stained by anti-CiVH antibody, but the signal in these regions becomes faint in larvae and during metamorphosis. In *Xenopus*, somatic cells of embryos also contained XVLG1 (*Xenopus* vasa-like gene 1) protein until stage 42, while germline cells contain this protein throughout their life cycle as seen in *C. elegans* and *Drosophila* (Ikenishi et al. 1996; for review see Ikenishi 1998). Therefore, although all germline cells contain Vasa protein or its homologues, not all cells containing Vasa protein are germline cells. Thus, the neural tube and endoderm are not likely to have germline cells. Our observation of hatched larvae suggests that the CiVH-positive cells in the endodermal strand at the middle tailbud stage are a good candidate for the germ cell precursor. The fact that the cell number (two) at the middle tailbud stage is not the same as that (eight) in the larval endodermal strand suggests that either two positive cells at the middle tailbud stage divide at least twice by the larval stage or other cells in the endodermal strand newly synthesize CiVH protein.

In other animals such as *C. elegans* and *D. melanogaster*, the germ cell lineage is inherited by the most posterior blastomeres during early embryogenesis, whose cytoplasm contains germline-specific granules, P granules and polar granules, respectively (Mahowald 1962; Wolf et al. 1983). Recently, a novel structure designated the centrosome-attracting body (CAB) was reported in the most posterior blastomeres of ascidian embryos (Hibino et al. 1998). Iseto and Nishida (1999) observed that the ultrastructure of the CAB resembled germ plasm reported in other animals and suggested that the CAB-containing posterior-most blastomeres are germline precursors. The posterior-most blastomeres at the 64-cell stage, B7.6 blastomeres, are the precursor cells of the posterior part of the larval endodermal strand (Nishida 1987). This fact and the distribution of *CiVH* mRNA in the posterior-most blastomeres during embryogenesis (Fujimura and Takamura 2000) suggest that B7.6 blastomeres are the precursors of the cells positive for anti-CiVH antibody in the larval endodermal strand.

However, Hirano and Nishida (2000) reported that labeled B7.6 blastomeres of another ascidian, *Halocynthia roretzi*, do not divide through the larval stage and are located near the intestine of juveniles after metamorphosis, but it is unclear which tissues these cells belong to. Additionally, in a different ascidian species, *Ciona savignyi*, the number of cells positive for anti-CiVH antibody in the larval endodermal strand was typically four (data not shown). On the other hand, there are ascidian species with anural larvae such as *Molugla occulta* (Satoh 1994). These findings indicate a species-specific modification in the formation of the primordial germ cell.

Compensation of germline cells

Because CiVH-positive cells do not exist in the juveniles that have developed from tail-cut larvae, it is plausible

that germ cells normally originate from CiVH-positive cells in the larval endodermal strand. However, even the juveniles that developed from tail-cut larvae had almost normal gonads and germ cells at a later stage. This indicates a mechanism of compensation in germline cell formation in *C. intestinalis*.

Currently, we have no information about the origin of these CiVH-positive cells. However, we would make two points. First, the CiVH-positive cells may have originated from the cells remaining in the anterior part of the tail, as we did not completely remove the tail region from larvae. In fact, the signals detected by in situ hybridization with *CiVH* antisense probe were frequently found in the anterior and middle parts of the endodermal strand at the tailbud stage (Fig. 4A; Fujimura and Takamura 2000). This observation suggests that cells in the endodermal strand may have the potential to become germ cells. Second, the possibility cannot be excluded that other cell types such as coelomic compensate for CiVH-positive cells in the absence of the original germ cells. Additionally, even in normal development, cells other than endodermal strand cells may participate in germ cell formation at a later stage. To solve these problems, we will attempt to track the fate of CiVH-positive cells in detail after removal of the larval tail using light- and electron-microscopy.

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References

Conklin EG (1905) The organization and cell lineage of the ascidian egg. J Acad Nat Sci (Phila) 13:1–119

Fujimura M, Takamura K (2000) Characterization of an ascidian DEAD-box gene, *Ci-DEAD1*; specific expression in the germ cells and its mRNA localization in the posterior-most blastomeres in early embryos. Dev Genes Evol 210:64–72

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

Goodbody I (1974) The physiology of ascidians. Adv Mar Biol 12:1–149

Gruidl ME, Smith PA, Kuznicki KA, McCrone JS, Kirchner J, Roussell DL, Strome S, Bennett KL (1996) Multiple potential germ-line helicases are components of the germ-line-specific P granules of *Caenorhabditis elegans*. Proc Natl Acad Sci USA 93:13837–13842

Hay B, Jan LY, Jan LY (1988) A protein component of *Drosophila* polar granules is encoded by *vasa* and has extensive sequence similarity to ATP-dependent RNA helicase. Cell 55:577–587

Hibino T, Nishikata T, Nishida H (1998) Centrosome-attracting body: a novel structure closely related to unequal cleavages in the ascidian embryo. Dev Growth Differ 40:85–95

Hirano T, Nishida H (1997) Developmental fates of larval tissues after metamorphosis in ascidian *Halocynthia roretzi*. I. Origin of mesodermal tissues of the juvenile. Dev Biol 192:199–210

Hirano T, Nishida H (2000) Developmental fates of larval tissues after metamorphosis in the ascidian, *Halocynthia roretzi*. II. Origin of endodermal tissues of the juvenile. Dev Genes Evol 210:55–63

Ikenishi K (1998) Germ plasm in *Caenorhabditis elegans*, *Drosophila* and *Xenopus*. Dev Growth Differ 40:1–10

Ikenishi K, Tanaka TS, Komiya T (1996) Spatio-temporal distribution of the protein of *Xenopus vasa* homologue (*Xenopus vasa*-like gene 1, *XVLG1*) in embryos. Dev Growth Differ 38:527–535

Iseto T, Nishida N (1999) Ultrastructural studies on the centrosome-attracting body: electron-dense matrix and its role in unequal cleavages in ascidian embryos. Dev Growth Differ 41:601–609

Komiya T, Tanigawa Y (1995) Cloning of a gene of the DEAD box protein family which is specifically expressed in germ cells in rats. Biochem Biophys Res Commun 207:405–410

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

Lasko PF, Ashburner M (1988) The product of the *Drosophila* gene *vasa* is very similar to eukaryotic initiation factor-4A. Nature 335:611–617

Lüking A, Stahl U, Schmidt U (1998) The protein family of RNA helicases. Crit Rev Biochem Mol Biol 33:259–296

Mahowald AP (1962) Fine structure of pole cells and polar granules in *Drosophila melanogaster*. J Exp Zool 151:201–215

Nishida H (1987) Cell lineage analysis in ascidian embryos by intracellular injection of tracer enzyme. III. Up to the tissue restricted stage. Dev Biol 121:526–541

Okada T, Yamamoto M (1993) Identification of early oogenetic cells in the solitary ascidians, *Ciona savignyi* and *Ciona intestinalis*: an immunoelectron microscopic study. Dev Growth Differ 35:495–506

Okada T, Yamamoto M (1999) Differentiation of the gonad rudiment into ovary and testis in the solitary ascidian, *Ciona intestinalis*. Dev Growth Differ 41:759–768

Roussell DL, Bennett KL (1993) *glh-1*, a germ-line putative RNA helicase from *Caenorhabditis*, has four zinc fingers. Proc Natl Acad Sci USA 90:9300–9304

Satoh N (1994) Developmental biology of ascidians. Cambridge University Press, New York

Satoh N, Jeffery WR (1995) Chasing tails in ascidians: developmental insights into the origin and evolution of chordates. Trends Genet 11:354–359

Schmid SR, Linder P (1992) D-E-A-D protein family of putative RNA helicases. Mol Microbiol 6:283–292

Schüpbach T, Wieschaus E (1986) Maternal-effect mutations altering the anterior-posterior pattern of the *Drosophila* embryo. Roux's Arch Dev Biol 195:302–317

Sugino YM, Tominaga A, Takashima Y (1987) Differentiation of the accessory cells and structural regionalization of the oocyte in the ascidian *Ciona savignyi* during early oogenesis. J Exp Zool 242:205–214

Sugino YM, Tominaga A, Takashima Y, Ishikawa M (1990) Early oogenesis in the ascidian, *Ciona savignyi*. Invert Reprod Dev 17:165–170

Takamura K (1998) Nervous network in larvae of the ascidian *Ciona intestinalis*. Dev Genes Evol 208:1–8

Takamura K, Ueda Y, Irie U, Yamaguchi Y (1996) Immunohistology with antibodies specific to test cells in the ascidian *Ciona intestinalis* suggests their role in larval tunic formation. Zool Sci 13:241–251

Wei G, Mahowald AP (1994) The germline: familiar and newly uncovered properties. Annu Rev Genet 28:309–324

Wolf N, Priess J, Hirsh D (1983) Segregation of germline granules in early embryos of *Caenorhabditis elegans*: an electron microscopic analysis. J Embryol Exp Morphol 73:297–306

Yamamoto M, Okada T (1999) Origin of the gonad in the juvenile of a solitary ascidian, *Ciona intestinealis*. Dev Growth Differ 41:73–79

Yoon C, Kawakami K, Hopkins N (1997) Zebrafish *vasa* homologue RNA is localized to the cleavage planes of 2- and 4-cell-stage embryos and is expressed in the primordial germ cells. Development 124:3157–3166