Diversity and Commonality in Animals

Kazuya Kobayashi
Takeshi Kitano
Yasuhiro Iwao
Mariko Kondo *Editors*

Reproductive and Developmental Strategies

The Continuity of Life

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Diversity and Commonality in Animals

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Preface

When organisms become multicellular, the specialization of cell types is established, which results in the acquisition of a variety of biological functions. During the specialization of cell types, organisms achieve the production of germ cells in which their genetic material is recombined by meiosis. To achieve effective "sex", animals further develop male (spermatozoa) and female (eggs) germ cells. Fertilization, the fusion between a spermatozoon and an egg, requires self/non-self-recognition mechanisms and begins the process of embryogenesis. Animals accomplish genetic diversity through meiosis and fertilization. During embryogenesis, animals must produce specialized cell types in accordance with their body plan. This series of phenomena is essential to the continuity of life in the animal kingdom. This book reviews the diversity of the animal kingdom, including reproductive strategies and germ cell differentiation mechanisms, sex determination and differentiation, the mechanisms of fertilization, and body axis formation. Of particular interest is the diversity of molecules and mechanisms used to achieve the same biological purpose in different animals. This raises the question of whether or not each mechanism is conserved at a taxonomic classification level. The answer to this question will not be obvious until we examine a variety of animals: the mechanism might be the result of specialization within a certain classification level; alternatively, the mechanism identified in one animal species might be an important mechanism common to all animals. In other words, scientists may find a new common principle hidden in the diversity of molecules and mechanisms. In this book, our aim is to motivate readers to understand the universality and diversity of biological systems involved in animal reproduction and development. A brief introduction to the four parts of the book (reproductive strategies and germ cell differentiation mechanisms, sex determination and differentiation, mechanisms of fertilization, and body axis formation) is presented in the following four paragraphs.

Metazoans have achieved sexual reproduction through the production of germ cells. In sexual reproduction, offspring are produced by a new combination of parental genes. This has led to an explosion of diversity in metazoans. The mechanisms leading to the differences between somatic cells and germ cells and the methods of germline stem cell (GSC) regulation are expected to be closely associated

with reproductive strategies. In Part I (11 chapters), the diversity associated with the mechanisms of metazoan germ cell differentiation and reproductive strategies is introduced. The separation of somatic and germ cells, referred to as the determination of primordial germ cells (PGCs), occurs via three mechanisms: preformation, epigenesis, and postembryonic germ cell development. The mechanisms associated with preformation and epigenesis have been well studied in the fly and mouse, respectively. Interestingly, in ascidians, both preformation and epigenesis occur during embryogenesis. The biological significance of these mechanisms is discussed. Gamete formation through GSC regulatory mechanisms is unique among animals. These mechanisms are well studied in the fly, medaka, and mouse. It has been reported that GSC regulation in *Caenorhabditis elegans* and the quail is controlled by nutritional status and seasonal changes, respectively. Some metazoans that possess pluripotent stem cells undergo postembryonic germ cell development. Typically, they reproduce asexually but develop PGCs or germ cells from pluripotent stem cells when they reproduce sexually. These organisms may switch between asexual and sexual reproduction, depending on environmental conditions and/or life cycle stage. The reproductive switching mechanisms and phenomena in hydra, jellyfish, planarians, and annelids are introduced in Part I. The reproductive switching phenomenon is also observed in the social amoeba *Dictyostelium discoideum*. The reproductive strategy of switching between asexual and sexual reproduction confers advantages with respect to offspring fitness.

Part II (9 chapters) pertains to sex determination and differentiation in crustaceans, insects, fish, amphibians, reptiles, birds, and mammals. The sex determination system is a biological system that directs the undifferentiated embryo into a sexually dimorphic individual. Sex determination sets the stage for sex differentiation, which is established by multiple molecular events that form either a testis or an ovary. Male heterogamety (XY) is conserved in mammals and the fly; female heterogamety (ZW) is ubiquitous in birds and silkworms; and poikilothermic vertebrates (fish, amphibians, and reptiles) and crustaceans exhibit environmental sex determination systems in addition to genetic sex determination. In tropical fish, sex is completely controlled by environmental or social factors. Thus, significant diversity exists in the sex determination and differentiation mechanisms of animals. Part II summarizes the general information and recent knowledge regarding sex determination and differentiation in animals and presents current perspectives on these research fields.

Sexual reproduction in animals and plants requires fertilization. Fertilization is a unidirectional chain of events leading to important changes for embryonic development, including the restoration of male and female diploid genomes and the induction of egg activation to elicit polyspermy block and to initiate cell cycles for early embryonic development. Animals have evolved a variety of elaborate molecular and cellular mechanisms to accomplish fertilization. In Part III (7 chapters), we describe the diversity of fertilization mechanisms and provide insight into the universal and key systems conserved during evolutionary processes. The following subjects are included: sperm motility and function prior to fertilization, post-copulatory reproductive strategies in sperm, sperm and egg interactions and self-sterility, and

polyspermy block during animal fertilization. In addition, special topics involved in the establishment of fertilization are included, such as intercellular signals for oocyte maturation, sperm–egg fusion at the plasma membrane, and protein–tyrosine kinase signaling during fertilization.

When an animal is observed, what is the first thing that catches the eye? It may be the way it moves, how it behaves, the color of the body, and of course, the shape and structure of the animal. Animals can be grouped according to general body shape; among metazoans, the shapes include asymmetrical, radial, and bilateral. Asymmetry is also found in symmetrical animals. There are even animals that change their body plan during development. In Part IV (6 chapters), we focus on body axis formation and investigate how bodies are formed. To encompass this enormous diversity, we cover a broad range of taxa, from cnidarians to vertebrates, and introduce the recent understanding of body axis development. For years, biologists have been fascinated by the mechanisms for body axis development. The axes are defined by maternal and zygotic determinants at different times during development. Comparative studies have shown that there are key molecules involved in the determination of axes; furthermore, these molecules are shared among animals. This highlights the evolutionary conservation of mechanisms underlying the axis development process, a crucial concept of several chapters. Although axis determination is a conserved process, related animals do not necessarily look similar in structure. There are some unique body axes that appear to be contrary to their phylogenetic position. For example, echinoderms are classified in a sister clade to chordates and ascidians are chordates, like humans and other vertebrates; however, their body axes are significantly different. Although Part IV is not all-encompassing, we hope that readers will gain some insight into the formation of body axes and share our fascination with this process, which incorporates both conservation and diversity.

This book provides new understanding of the universality of biological systems through the comparison of a variety of reproductive and developmental mechanisms. We hope that the book is useful for undergraduates, graduate students, and professional scientists who seek a greater awareness of animal reproduction and development.

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Contents

Part I
Diversity in Reproductive Strategies and Germ Cell Differentiation Mechanisms

Chapter 1
Germ-Cell Formation in Solitary Ascidians: Coexistence of Preformation and Epigenesis

Maki Shirae-Kurabayashi and Akira Nakamura

Abstract In metazoans, primordial germ cells (PGCs) are the only type of cells that transmit genetic material into the next generation and are therefore vital for species preservation. PCGs are formed in two ways: they originate from cells that inherit maternal determinants in the germ plasm (preformation), or arise epigenetically in the early embryonic stages or the adult stage through cell-cell interaction (epigenesis). The epigenetic mode of PGC formation has been proposed to be ancient, but it can change dramatically during evolution. Several groups of animals have independently evolved the preformation mode, which is therefore polyphyletic. Although several conserved mechanisms and molecules involved in the maintenance and differentiation (gametogenesis) of germ cells have been identified, the principles and evolutionary paths of PGC specification remain largely unknown.

In ascidians, which are chordate siblings of vertebrates, the embryos contain post-plasm, a specific cytoplasm that accumulates a series of specific maternal components including germ-cell determinants, and is thus the equivalent of the germ plasm. Our previous studies showed that in the *Ciona robusta (Ciona intestinalis* type A) embryo, PGCs originate from the descendants of the posterior-most blastomeres that inherit the post-plasm at the ~110-cell stage. However, PGCs are also reported to form epigenetically in this species. When preformed PGCs are surgically removed from tadpole larvae, PGCs re-appear in the gonads after metamorphosis and can develop into functional gametes. Therefore, *C. robusta* appears to have an epigenetic mode of PGC formation, in addition to the better-known preformation mechanism. Because of this unique feature, *Ciona* is an ideal system for investigating two modes of PGC formation in a single chordate species.

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We previously analyzed the molecular functions of evolutionarily conserved germline-related genes in *C. robusta* during early development, and found that they have conserved roles in germ-cell maintenance. Furthermore, recent advances in genome-editing technology will enable us to perform comparative analyses of the molecular mechanisms involved in two modes of PGC formation in *C. robusta*. Here, we introduce this unique and fascinating system for PGC formation in solitary ascidians, and provide future perspectives to further elucidate its evolutionary path in ascidians and other metazoans.

Keywords Primordial germ cells · Germ plasm · Ascidian · *Ciona intestinalis* type A · *Ciona robusta* · predetermination · Epigenesis

1.1 Introduction

In animal developmental biology, the formation of primordial germ cells (PGCs) has been classified into two modes: by the incorporation of maternal determinants in the germ plasm (preformation) (e.g., flies, nematodes, and fish), or by cellular interactions during early embryogenesis (e.g., mice) and in adulthood (e.g., sponges, planarians, and cnidarians) (epigenesis). Phylogenetic studies suggest that epigenesis from pluripotent stem cells is the ancestral method of PGC specification (Extavour and Akam 2003; Juliano et al. 2010; Johnson and Alberio 2015), and that two modes appear polyphyletic even within small taxonomic groups (Extavour and Akam 2003; Johnson et al. 2003). Consistent with this idea, recent studies on primitive metazoans (e.g., sponges, planarians, and cnidarians) have indicated that the specific cytoplasmic components in the pluripotent stem cells, such as the chromatoid bodies in the planarian neoblasts, contain evolutionally conserved molecules such as Vasa, Nanos, and Piwi, which are generally expressed at high levels in germ cells in many animal groups (Newmark et al. 2008; Juliano et al. 2010; Rink 2013; Wolfswinkel 2014). In the case of the cnidarian *Clytia*, there is no germ plasm, but the specific cytoplasmic area in eggs and early embryos where the determinants for pluripotent stem cells accumulates has been reported (Leclère et al. 2012). Furthermore, recent studies on vertebrate embryology suggest that the germ plasm-dependent PGC determination is advantageous to maintain animal species and accelerate species diversification (Evans et al. 2014; Johnson and Alberio 2015). Several fundamental principles in PGC specification and gametogenesis, such as the repression of somatic differentiation programs (Nakamura and Seydoux 2008) and silencing of transposable elements (Siomi and Kuramochi-Miyagawa 2009) have been revealed. However, since the molecular mechanisms of PGC specification have been drastically modified during evolution, the original mechanisms and the evolutionary paths of the diversification of PGC specification are largely unknown.

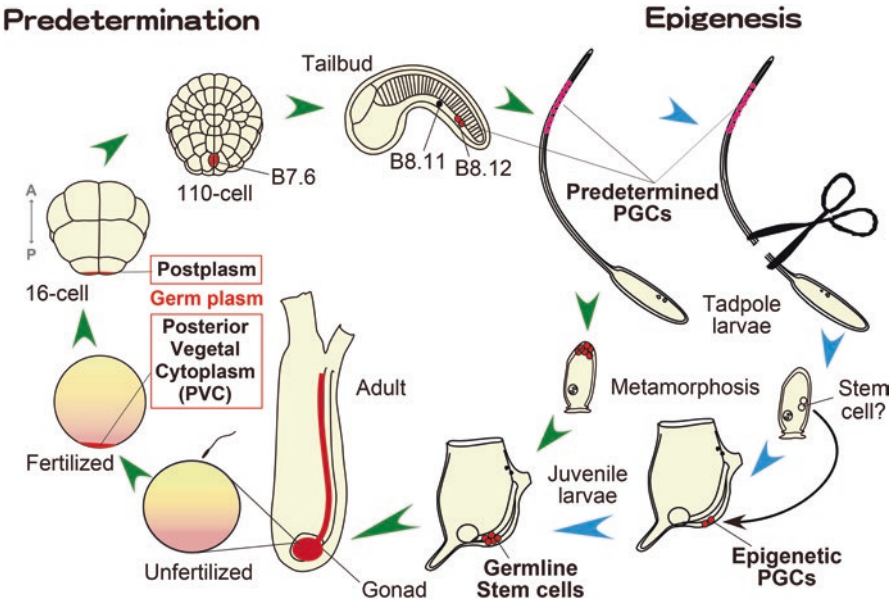

Fig. 1.1 The life cycle and dual-mode PGC formation in *C. robusta* (recently renamed from *Ciona intestinalis* type A (Brunetti et al. 2015)). *C. robusta* embryos contain a specific type of cytoplasm called the post-plasm, which accumulates the conserved germline marker Vasa homolog (CiVH). The posterior-most blastomeres of the last cleavage stage, known as B7.6 cells, undergo asymmetric cell division. Post-plasmic *Vasa* RNA is incorporated to form perinuclear germ granules in the posterior daughter cells, which are called B8.12 cells and are regarded as predetermined PGCs (Shirae-Kurabayashi et al. 2006). However, when these predetermined PGCs, which localize to the larval tail, are removed, Vasa-positive PGCs appear in the future gonadal area after metamorphosis (Fujimura and Takamura 2000; Takamura et al. 2002) and become functional gametes in the adult (Shirae-Kurabayashi and Sasakura, in preparation). Thus, these Vasa-positive cells are thought to be epigenetic PGCs.

Ascidian embryos contain the post-plasm, a specific type of cytoplasm in the posterior pole (Yoshida et al. 1996; Shirae-Kurabayashi et al. 2006; Fig. 1.1). The post-plasm is thought to be the equivalent of germ plasm in other animals. After the eight-cell stage, the pair of post-plasm-containing blastomeres undergo three unequal cleavages to form small blastomeres in the posterior pole. In ~110-cell embryos, the post-plasm remains in the pair of posterior-most blastomeres, called B7.6, of which descendants become PGCs. Thus, the germ-cell specification in ascidians occurs via the preformed mode (Shirae-Kurabayashi et al. 2006). However, experimental evidence has indicated that the solitary ascidian *Ciona* also has the epigenetic modes of PGC specification (Takamura et al. 2002). Thus, *Ciona* presents an ideal system for investigating the two modes of PGC specification in a single chordate species.

In this chapter, we describe what is presently known about the mechanisms of PGC formation in solitary ascidians, primarily in *Ciona*, and discuss prospects for further research.

1.2 Preformed (Germ Plasm–Dependent) PGC Formation in Solitary Ascidians

1.2.1 The Centrosome-Attracting Body (CAB) Is Crucial for Unequal Cleavage and Somatic-Cell Fate Determination in Cleavage-Stage Embryos

Ascidians are one of the most popular experimental animals in classical embryology. At the beginning of the twentieth century, Conklin (1905) described the "cap of deeply stained protoplasm at posterior pole of cells" in the eight- to 16-cell stage *C. intestinalis* (probably type B) embryos. Subsequently, in *C. robusta* and *Halocynthia roretzi*, the centrosome-attracting body (CAB) was described as a specific cytoplasmic structure in the posterior pole of early-stage embryos (Hibino et al. 1998; Nishikata et al. 1999). The CAB structure, which is relatively resistant to detergent treatment that extracts cytoplasmic materials, is assembled de novo during the eight- to 16-cell stages and associates with one of the centromeres in the posterior-most blastomeres via a thick microtubule bundle. Ultramicroscopic observations show that the CAB contains an electron-dense matrix in which endoplasmic reticulum (ER) and ribosome-like granules accumulate (Iseto and Nishida 1999; Sardet et al. 2003; Prodon et al. 2005). The CAB is a hard and inflexible structure, and attempting to remove or transplant the CAB causes the embryo to break down. When the posterior vegetal cytoplasm (PVC) containing the CAB precursor was removed from one-cell embryos, the embryos did not form the CAB and failed to undergo unequal cleavage. Furthermore, transplanting the PVC into the anterior side of another one-cell stage caused ectopic CAB assembly and unequal cleavage in the anterior blastomeres. These results suggest that the CAB contributes to the unequal cleavage patterning of the posterior blastomeres (reviewed by Nishida et al. 1999). After the eight-cell stage, the cytoplasmic region, where the CAB is present, is called the post-plasm. The post-plasm accumulates a series of specific maternal mRNAs, including that of the ascidian-specific gene *posterior end mark-1* (*Pem-1*) (Yoshida et al. 1996; Negishi et al. 2007; Kumano and Nishida 2009; Prodon et al. 2010). These maternal RNAs that accumulate in the post-plasm are called post-plasmic/PEM RNAs (Prodon et al. 2010). In addition, the cortical region adjacent to the post-plasmic membrane enriches in the PKC-Par3/Par6 complex (Patalano et al. 2006), which plays conserved roles in centrosome orientation in metazoans (Munro 2006). Whether the CAB components interact directly with the Par complex is currently unclear.

Not only do the CAB structure and its components associate with the centrosome to organize unequal cleavage patterns in the posterior blastomeres, but they also control the morphogenic gradient along the anterior-posterior (AP) axis and somatic-cell differentiation by spatially and temporally regulating the timing of protein expression of post-plasmic/PEM RNAs. These include the muscle-differentiation transcription factor Macho1 and the cell-signaling factor Wnt5 (reviewed by

Lemaire et al. 2008; Kumano and Nishida 2009; Makabe and Nishida 2012). It is highly likely that these proteins are translated from maternal post-plasmic RNAs tethered to the ER in the CAB to establish protein accumulation or concentration gradients in the cleavage embryos. Therefore, the acquisition of the CAB structure, which accumulates and stabilizes specific maternal molecules (such as somatic and germline determinants) at the posterior pole, may be a key event in ascidian evolution for the rapid determination of both the somatic and germ-cell fates during embryogenesis.

1.2.2 The CAB Maintains the Germ Plasm and Partitions it to PGC Progenitors

Ultramicroscopic observations have revealed that the CAB contains an electron-dense matrix with a structure similar to that of the germ plasm in other animals, implying that PGCs are formed through the preformation mode. It has been suggested that maternal components involved in germ-cell formation are accumulated to the CAB (Iseto and Nishida 1999), and that the posterior-most blastomeres that inherit the CAB are the germline in ascidians. Nishida (1987) traced the cell lineage of *Halocynthia* and *Ciona* embryos and found that the posterior-most blastomeres at the last cleavage stage, termed B7.6 cells, are located in the mid-region of the endodermal strand during the tailbud stage. Therefore, they were long thought to be PGCs that will develop into gametes after metamorphosis. However, this idea was partially revised by detailed B7.6 cell-tracing experiments in *Ciona* (Shirae-Kurabayashi et al. 2006).

Ninety years after Conklin's description, a maternal transcript that accumulates in the post-plasm of cleavage-stage *Ciona savigni* embryos, named *Pem* (*Posterior end mark*), was isolated using differential screening with biased egg fragments prepared by centrifugation (Yoshida et al. 1996). Subsequently, a homolog of the evolutionarily conserved germline gene *Vasa* (previously called *CiVH*) was isolated in *C. robusta. Vasa* RNA is enriched in the post-plasm in cleavage-stage embryos, incorporated into B7.6 cells, and inherited by the endodermal strand cells in the tailbud embryo (Takamura et al. 2002). The incorporation of *Vasa* RNA into the B7.6 cells strongly supports the hypothesis that B7.6 cells are PGCs. However, the detailed examination of *Pem-1* and *Vasa* RNA distributions in *C. robusta* revealed that they are not distributed identically in the endodermal strand; the *Vasa* RNA signals have additional locations (Shirae-Kurabayashi et al. 2006). This observation raised two possibilities: that several endodermal-strand cells other than B7.6 cells start to express *Vasa* mRNA and differentiate into PGCs, or that B7.6 cells undergo asymmetric cell division to form *Pem-1* RNA-containing and -free cells (Shirae-Kurabayashi et al. 2006; Fig. 1.2).

To better understand PGC formation and maintenance in early development, we traced the fate of B7.6 cells and their descendants using anti-Vasa antibodies and the

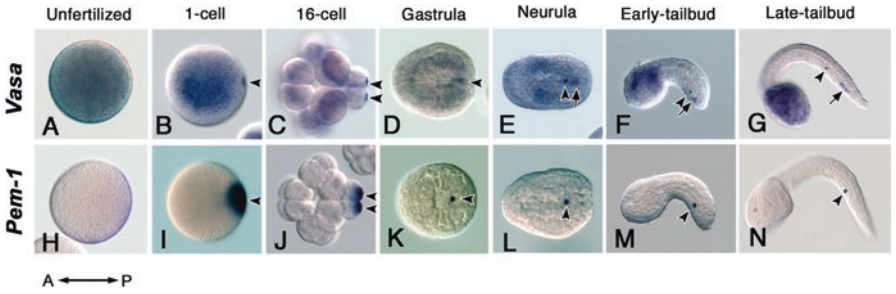

Fig. 1.2 Localization of the post-plasmic/PEM RNAs *Vasa* and *Pem-1* during embryogenesis. Both *Pem-1* RNA and *Vasa* RNA localize to the post-plasm and to the anterior B7.6-descendants in the tail at the tailbud stage (B8.11, arrowheads). However, *Vasa* RNA has a different specific distribution in the posterior cells of the tail in tailbud embryos (B8.12, *arrows*) (Photographs are reproduced from Shirae-Kurabayashi et al. 2006 with permission)

fluorescent dye CM-DiI. We found that the B7.6 cells divide asymmetrically during the gastrula stage to produce two distinct daughter cells: the smaller anterior B8.11 cells and the larger posterior B8.12 cells (Shirae-Kurabayashi et al. 2006; Fig. 1.1). In tailbud embryos, B8.11 cells contain an actin-rich mass, and the nucleus appeared to be lost. B8.11 cells were then detected on the surface of the intestine 14 days after metamorphosis, and never contributed to the primordial gonad formation (Shirae-Kurabayashi et al. 2006). Considering that B7.6 cells inherit the actin-rich CAB structure, the actin-rich mass in B8.11 cells is probably a remnant of the CAB. In the tailbud embryos, the B8.11 cells resided adjacent to B7.2 descendants that moved from the tail to the trunk prior to tail absorption and formed the intestine in juveniles after metamorphosis (Nakazawa et al. 2013; Kawai et al. 2015). These observations suggest that B8.11 cells attach to B7.2 descendants and are passively carried to the intestine.

In contrast, the pair of B8.12 cells formed Vasa-positive perinuclear granules and became mitotically active to proliferate 8–16 Vasa-positive cells. These B8.12 descendants were passively carried to the larval trunk by the contraction of other tail cells, including the notochord and nerve cells, during metamorphosis. Nine to 10 days after metamorphosis, when juvenile larvae started filter feeding, the PGCs actively escaped from the tail debris and were incorporated into the primordial gonads. These observations support the idea that the B8.12 rather than B8.11 cells are the PGCs that will produce gametes in adults, and suggest that CAB remnants are cleared from the PGCs during the asymmetric division of the B7.6 cells (Shirae-Kurabayashi et al. 2006).

Our experiments showed that *Vasa* RNA was incorporated into both B8.11 and B8.12 cells, whereas *Pem-1* RNA was partitioned only into B8.11 cells. How do these two post-plasmic/PEM RNAs behave differently? Sardet et al. (2003) has shown that *Pem-1* RNA is tightly attached to the CAB structure via the cortical ER (cER). However, after a detailed analysis of the distribution of a series of post-plasmic/PEM RNAs, Paix et al. (2009) reported that, unlike cER-tethered,

electron-dense materials that included *Pem-1* RNA, *Vasa* RNA-containing materials were localized to the gap between cERs and were not directly tethered to the cER in the CAB. They propose that *Vasa* mRNA is released into the cytoplasm by the breakdown of CAB structure during B7.6-cell division, although it is partially captured by the CAB remnants in B8.11 cells. In addition to *Vasa*, many other postplasmic/PEM RNAs are incorporated into B8.12 PGCs (Yamada 2006; Prodon et al. 2007; Makabe and Nishida 2012), suggesting that their protein products are expressed in the B8.12 cells. In ascidians, therefore, the germ plasm, which is incorporated into PGCs, is arranged in the gaps between cERs of the CAB structure. In contrast to *Ciona* and *Phallusia mammilata*, B8.11 cells (and specific *Pem-1* mRNA signals in the B8.11 cells) are undetectable in *Halocynthia roretzi* tailbud embryos, probably because the *H. roretzi* CAB is rapidly degraded after gastrulation.

1.2.3 Two Critical Functions of Pem-1 Protein in Germline Blastomeres

Because the *Pem-1* RNAs in *Ciona* and *Phallusia mammilata* are incorporated only into B8.11 cells, which seem to have no function after gastrulation, we hypothesized that the Pem-1 protein plays important roles during the cleavage stages. Consistent with this idea, morpholino oligonucleotide-mediated knockdown of *Pem-1* in three ascidian species revealed that Pem-1 is involved in unequal cleavage in germline blastomeres during the cleavage stage (Negishi et al. 2007; Prodon et al. 2010; Shirae-Kurabayashi et al. 2011). Although the Pem-1 function in asymmetric division appears to be operated by its presence in the CAB, Pem-1 was also found to accumulate in the nucleus of *C. robusta* and *H. roretzi* germline blastomeres (Shirae-Kurabayashi et al. 2011; Kumano et al. 2011). The nuclear Pem-1 functions to maintain the transcriptionally quiescent state in the germline blastomeres during the cleavage stages (Shirae-Kurabayashi et al. 2011; Kumano et al. 2011; Fig. 1.3). In *C. robusta* and *H. roretzi*, upon the

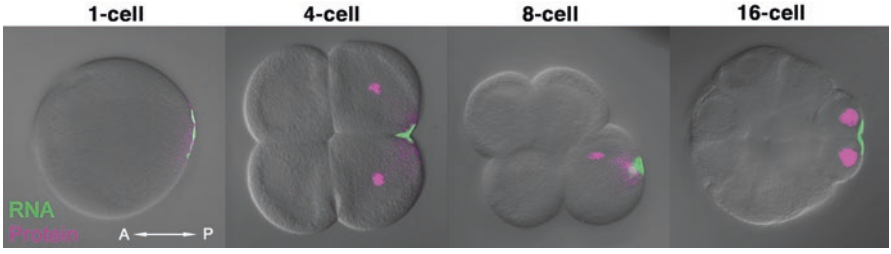

Fig. 1.3 Nuclear localization of Pem-1 protein in cleavage-stage embryos. *C. robusta* embryos with 1, 4, 8, or 16 cells were probed for -*Pem-1* mRNA (green) and Pem-1 protein (magenta). In the pair of germline blastomeres, *Pem-1* mRNA is highly concentrated in the post-plasm at the posterior cortex, while the protein products are concentrated in the nuclei

cleavage of a parental germline blastomere in 4- to 110-cell stage embryos, the post-plasm-free daughter cells begin the zygotic transcription of somatic genes, but the post-plasm-inheriting daughter cells do not. For example, in *C. robusta*, *Foxa.a* and *Soxb1* are expressed in this manner from the 8-cell stage; beginning in the 16-cell stage, *Fgf9/16/20* and *Admp* are transcribed in the post-plasm-free somatic daughter cells. The *Not*, *Foxa*, *Foxd.a*, and *Soxb1* mRNAs have similar expression patterns in *H. roretzi*. Intriguingly, when the *Pem-1* RNA was knocked-down, these somatic genes were ectopically transcribed in germline blastomeres even in the presence of the post-plasm (Shirae-Kurabayashi et al. 2011; Kumano et al. 2011). Given that all of these genes encode essential transcription factors for somatic-cell fate determination in the cleavage stage, these data support the idea that Pem-1 acts as the transcriptional repressor that prevents germline blastomeres from undergoing somatic differentiation.

Transcriptional repression in germ cells during embryogenesis has also been reported in other animals that have the germ plasm, such as *Drosophila* and *Caenorhabditis elegans*. In these embryos, species-specific proteins (e.g., PIE-1 in *C. elegans* and Pgc in *Drosophila*) globally repress mRNA transcription in PGCs by inhibiting the phosphorylation of the C-terminal domain (CTD) of RNA polymerase II (RNAPII), a critical modification for active transcription (reviewed in Nakamura and Seydoux 2008). Although *Pem-1* is an ascidian-specific gene and its protein product has no known protein domains, the short sequence at its C-terminal end (WRPW) matches the binding motif for the transcriptional co-repressor, Groucho (Negishi et al. 2007). In the *Ciona* genome, two Groucho genes are encoded, and these protein products were co-immunoprecipitated with Pem-1 in a mammalian cell-culture assay (Shirae-Kurabayashi et al. 2011). Intriguingly, immunohistochemical studies have shown that the phosphorylation of RNAPII CTD is weaker in the *C. robusta* germline than in neighboring somatic cells but is not totally eliminated. Therefore, the transcriptional repression in germline blastomeres by Pem-1 appears not to be global (Shirae-Kurabayashi et al. 2011). In contrast, the *H. roretzi* Pem-1 (PEM), similar to PIE-1 and PGC, is known to bind P-TEFb, which phosphorylates RNAPII CTD Ser2 to promote transcriptional elongation (Kumano et al. 2011). Notably, the amino-acid sequences in ascidian Pem-1 orthologs shows only 40 % identity (Negishi et al. 2007). Thus, Pem-1 in *C. robusta* and *H. roretzi* may have adopted discrete strategies to repress mRNA transcription in the germline.

In mice, which use the epigenetic mode of PGC formation, the transcription factor Blimp1 is critical for PGC formation. Blimp1 exerts its function, at least in part, by repressing the expression of somatic genes (Ohinata et al. 2005; reviewed by Saitou and Yamaji 2012). Taken together, our data support the hypothesis that the repression of somatic transcriptional programs is a fundamental hallmark of PGC specification in animal development (reviewed in Nakamura and Seydoux 2008; Nakamura et al. 2010).

1.2.4 The Initiation of Zygotic Expression in Ascidian PGCs

An important and as-yet unanswered issue in the study of the ascidian germline is when and how zygotic transcription is initiated in the germ cells. In *C. robusta*, Pem-1 signals remain in the nucleus of B8.12 cells (regarded as PGCs) after B7.6-cell division, but disappear after B8.12 cells begin to divide in the neurula stage, suggesting that these B8.12 descendants escape from the Pem-1-dependent transcriptional repression.

However, our preliminary data suggest that the zygotic expression of post-plasmic/PEM RNA genes in the PGCs may be initiated in much later stages. In *C. robusta*, Vasa protein expression in the B8.12 cells was rapidly upregulated even in the presence of the transcriptional inhibitor actinomycin D, suggesting that the release of maternal *Vasa* mRNA from the CAB contributes to the production of the protein during these stages (Shirae-Kurabayashi et al. 2006). In contrast, exogenous reporter assays for promoter regions of germline-related genes such as *Vasa* have so far failed, and zygotic *Vasa* expression in PGCs using its intron sequence probe has not been detected in the tailbud stage (Shirae-Kurabayashi, in preparation). In tadpole larvae, B8.12 cells start to divide to form 8–16 PGCs. These PGCs never move away from the tail region, even though the tail shrinks and most other tail cells, such as endodermal strand, notochord, and epithelial cells, dramatically change shapes and undergo cell death from the onset of metamorphosis (Shirae-Kurabayashi et al. 2006). Our preliminary data suggest that the post-plasmic/PEM RNA gene, *Tdrd7*, a homolog of an evolutionally conserved germline-specific gene, starts its zygotic expression in PGCs in juvenile larva 9–10 days after metamorphosis (Shirae-Kurabayashi, in preparation). This observation implies that the zygotic expression of other germline-related genes would start from the juvenile stage after metamorphosis, when the animals start feeding and the PGCs become migratory to move toward future gonads.

1.3 Epigenetic (Germ Plasm-Independent) PGC Formation in Solitary Ascidians

1.3.1 Epigenetic PGCs Appear After Tail-Cut Experiments

In ascidians, the regeneration of adult somatic tissues and the existence of stem cells have been reported (reviewed by Jeffery 2015). In the solitary ascidian *C. robusta*, not only somatic cells, but also germ cells appear to be capable of being regenerated or newly formed from pluripotent cells in young adults. Takamura et al. (2002) first detected epigenetic PGCs by tail-cut experiments. When predetermined PGCs, which are located in the tail of tadpole larvae, were removed by tail cutting, these tail-cut larvae were able to grow into normal juveniles without Vasa-positive PGCs formed by the preformation mode. Surprisingly, several days after metamorphosis,

a few Vasa-positive cells were detected at the future gonadal area, and they subsequently formed the primordial gonad with somatic gonadal cells (Takamura et al. 2002; Fig. 1.4). These epigenetic PGCs are functional, because they can produce gametes (Shirae-Kurabayashi and Sasakura, unpublished data).

The coexistence of preformed and epigenetic PGCs in a single species appears to be rare in metazoans, although other unusual modes of PGC formation were reported recently. In the sea urchin *Strongylocentrotus purpuratus*, small micromeres in the vegetal pole that contain germ plasm-like cytoplasm become PGCs (Yajima and Wessel 2011). Interestingly, artificial removal of the small micromeres at the 24-cell stage promotes the reconstruction of the concentration gradient of *Vasa* gene products in the embryo and the formation of new PGCs. In contrast, removing the micromeres at the 28-cell stage resulted in an animal that grew to adulthood without gametes. These results suggest that sea urchin embryos in the early cleavage stage have the potential to regenerate the germ plasm, and that the regeneration mechanism seems to occur at the post-transcriptional level (Yajima and Wessel 2011). In another case, the cnidarian *Clytia*, appears to use maternally provided germ plasm-like cytoplasm to determine the pluripotent stem cell fate (Leclère et al. 2012). These findings provide further implications that the preformation mode of germ cell formation had evolved from the mechanisms used to form and maintain pluripotent stem cells in primitive metazoans (Juliano et al. 2010). In the case of *C. robusta*, PGCs can be epigenetically produced in a germ plasm-independent manner. We proposed that this mechanism is frequently used in natural conditions. Under laboratory culture conditions, the tails of tadpole larvae sometimes fail to shrink because of trivial tail bending or delayed stimulation for metamorphosis. In these cases, the tail tissues are left behind in the tunic or pinched off from the trunk. However, these unusual larvae often successfully form the adult body, although this takes longer than for usual growth. Furthermore, *C. robusta* post-metamorphic juveniles can survive for about 20 days in filtered seawater without food, and resume growth when they obtain food, although these animals have smaller bodies and fewer PGCs than those with a sufficient food supply (Shirae-Kurabayashi, in preparation). Thus, the post-metamorphic juveniles of solitary ascidians can adapt to drastic changes in environmental conditions, analogous to the L1 arrest in *C. elegans* (Baugh 2013). We propose that the cell plasticity to produce epigenetic PGCs would be beneficial to maintain species in the natural environment.

In contrast to solitary ascidians, germline progenitors appear to be determined at the very early stage of life in colonial ascidians, which grow by an asexual reproduction (Laird et al. 2005; Brown et al. 2009; Rinkevich et al. 2013; Voskoboynik and Weissman 2015), although the germline also appears to be derived from pluripotent stem cells (Weissman 2015). Notably, a series of recent studies in species of Botryllidae (*Botryllus primigenus*, *Botryllus schlosseri*, and *Botrylloides violaceus*) failed to answer whether PGCs originate from the B8.12 cells, or only from other lineages by epigenetic mechanisms (Laid et al. 2005; Kawamura and Sunanaga 2011; Rosner et al. 2013; Voskoboynik and Weissman 2015). Interestingly, in the colonial ascidian *Botryllus primigenus*, *Vasa* mRNA accumulates in the post-plasm in cleavage embryos, and in the presumptive B8.12 PGCs in the tail of tailbud

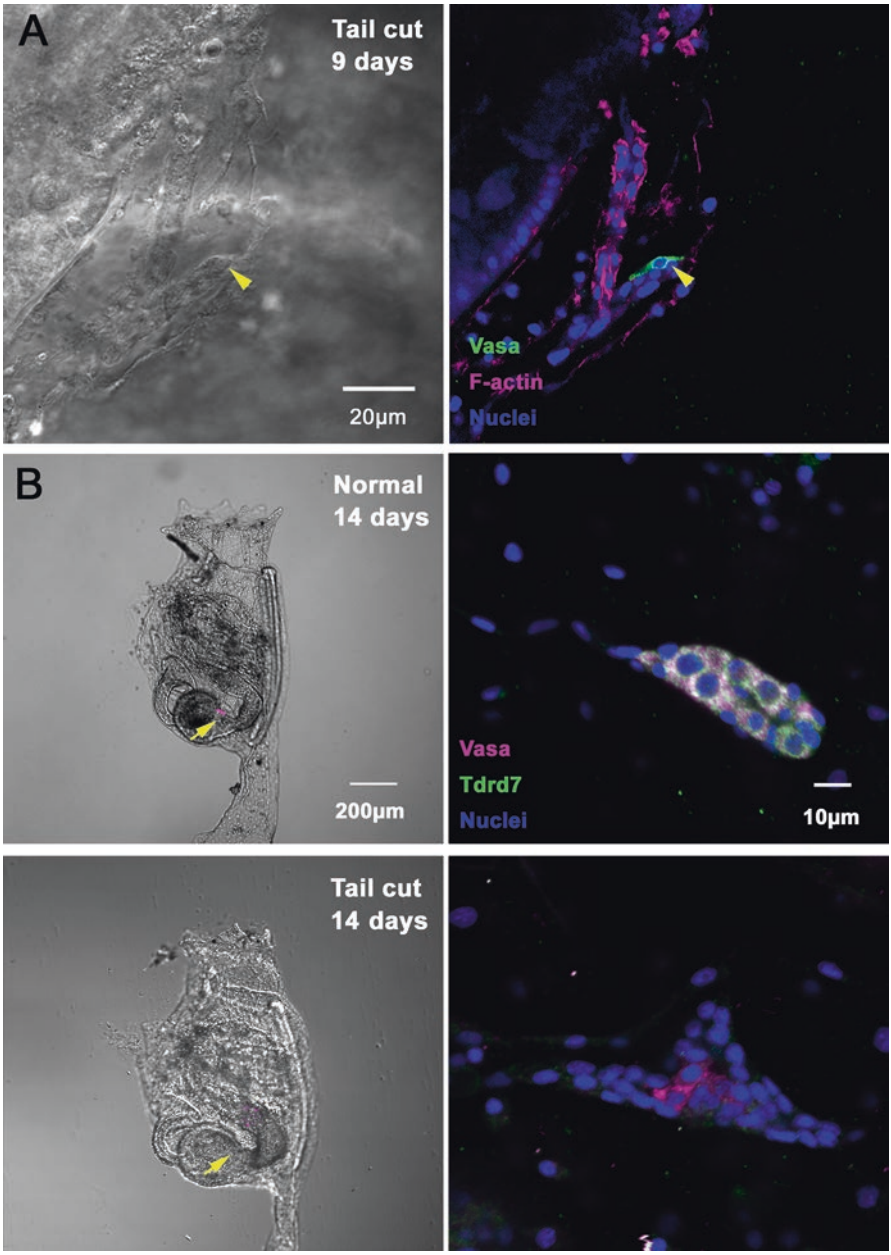

Fig. 1.4 Epigenetic PGCs appeared after tail-cut experiments. (**a**) When predetermined PGCs were removed from tadpole larvae by tail cutting, Vasa-positive cells (green) appeared in the future gonadal area in juveniles 9 days after metamorphosis. (**b**) Normal and tail-cut larvae 14 days after metamorphosis. In normal development, the primordial gonad includes PGCs that express both Vasa (magenta) and Tdrd7 (green), which is required for germ granule formation in predetermined PGCs. However, tail-cut larvae have fewer Vasa-expressing PGCs. These PGCs are scattered in the primordial gonad and do not express Tdrd7

embryos (Kawamura et al. 2011). However, *Vasa* expression is undetectable in tadpole larvae (Kawamura et al. 2011). Furthermore, Sunanaga et al. (2010) has shown that, in *B. primigenus*, germline stem cells in the adult coelom do not express *Vasa* but express *Piwi*, which is reportedly expressed in germline and pluripotent stem cells, especially in primitive metazoans. Since colonial and compound ascidians are viviparous or ovoviviparous, these tadpole larvae possess metamorphosed zooids in their trunks (e.g., Berrill 1950; Brewin 1959; Millar 1971). Therefore, these ascidians achieve rapid colony formation within several hours after settling to substrates by an asexual reproduction. They can also degenerate and dedifferentiate somatic tissues to endure environmental changes even as adults. Because of their advanced cellular plasticity, colonial botryllid ascidians may lose the preformed mode of PGC formation or promote dedifferentiation of preformed germ cells before metamorphosis. Thus, further characterization and comparison of the mechanisms underlying PGC formation between solitary and colonial ascidians will be an interesting future issue.

It remains unclear whether the epigenetic PGCs in *C. robusta* originate from pluripotent stem cells, reprogrammed somatic stem cells, or dedifferentiated somatic cells. In *C. robusta* larvae, zygotic *Vasa* expression is upregulated in the trunk cells of newly hatched tadpoles and is rapidly downregulated prior to metamorphosis (Shirae-Kurabayashi et al. 2006). This implies that Vasa in trunk cells might play a role in somatic cells. Furthermore, our tail-cut experiments have revealed that epigenetic PGCs originate from one or a few Vasa-positive cells, and that these Vasa-positive cells can be found only in the future gonadal area in juveniles (Fig. 1.4). This observation suggests that epigenetic germ cells may be born *de novo* in the region near the primitive gonads. Taking all of these data together, we hypothesize that somatic stem cells are present in the future gonadal area in post-metamorphic juveniles, and that they may receive an inductive signal, resulting in their dedifferentiation to change their fate into the germline.

1.4 Future Perspectives

It has been suggested that the epigenetic mode of PGC formation is ancient, and the preformation mode has evolved independently among different taxonomic groups (reviewed by Extavour and Akam 2003; Johnson et al. 2003). Recent studies also suggest that rapid germ plasm–dependent PGC determination is advantageous for species survival and accelerating species diversity (Evans et al. 2014; Johnson and Alberio 2015). However, *C. robusta* seems to retain the epigenetic mechanism of PGC formation, probably because it enables the animal to adapt to rapid changes in environmental condition in shallow seacoasts. We expect that studies investigating the mechanisms of PGC formation in this fascinating species will shed light on the primitive mechanism for epigenetic PGC formation in chordates and the evolutionary path by which the modes of PGC formation have changed.

To date, comparative analyses of the molecular mechanisms of cell differentiation in ascidians have been conducted based on the conservation of structures and functions of given factors with other species. However, these traditional approaches may be unable to reveal the core mechanisms of PGC formation in ascidians, because the critical factors that determine the PGC fate appear to be species-specific, not only within predetermined groups but also within closely related epigenetic groups, including mammals (Irie et al. 2015; Sugawa et al. 2015). Furthermore, the factor may be associated with species-specific characteristics involved in stemness. For instance, patterns of DNA methylation in the ascidian genome differ from those in other metazoans: low methylation of transposable elements and the hypermethylation of the gene body in housekeeping and maternal genes (Suzuki et al. 2007; Okamura et al. 2010). We favor the idea that these differences probably affect the state of stem cells, including PGCs. Therefore, in ascidians, comprehensive and comparative analyses of gene expression profiles in the germline and other stem cells will provide important clues to elucidate the molecular cascade by which two modes of PGC formation operate in a single species. Furthermore, recent developments of genome-editing technology will enable gene knockout (and probably knock-in) approaches in this animal (Sasaki et al. 2014; Treen et al. 2014). The application of these state-of-the-art technologies in ascidians will dramatically accelerate the research on PGC formation and maintenance, particularly regarding the mechanism by which epigenetic PGCs are induced after metamorphosis, when stem cells are likely to be free from the restrictions controlled by maternal factors.

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