Diversity and Commonality in Animals

Kazuya Kobayashi
Takeshi Kitano
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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.

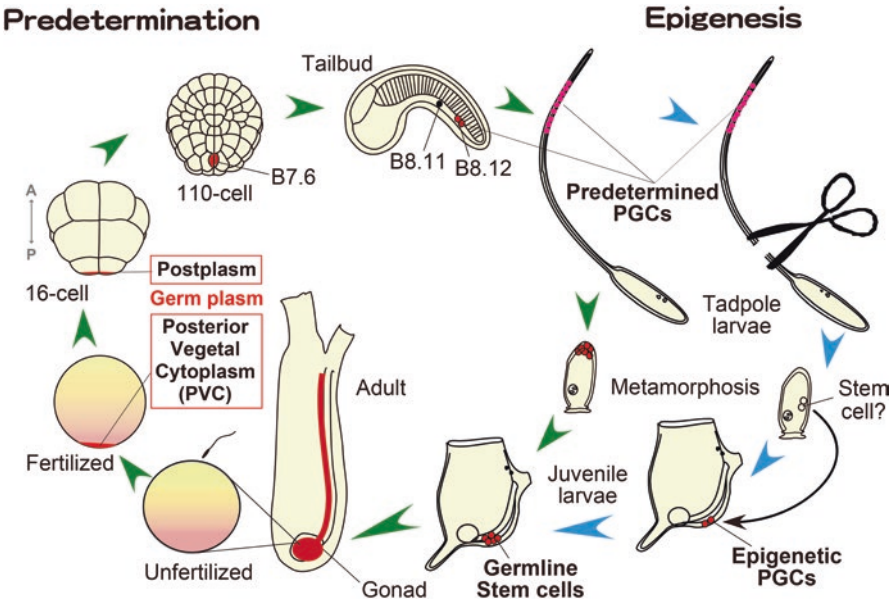

Fig. 1.1 The life cycle and dual-mode PGC formation in *C. robusta* (recently renamed from *Ciona intestinalis* type A (Brunetti et al. 2015)). *C. robusta* embryos contain a specific type of cytoplasm called the post-plasm, which accumulates the conserved germline marker Vasa homolog (CiVH). The posterior-most 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

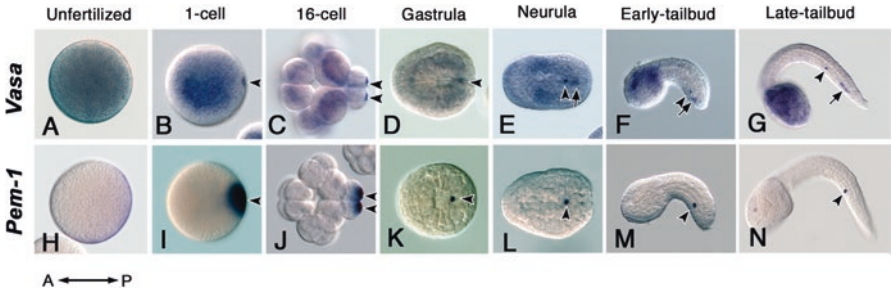

Fig. 1.2 Localization of the post-plasmic/PEM RNAs *Vasa* and *Pem-1* during embryogenesis. Both *Pem-1* RNA and *Vasa* RNA localize to the post-plasm and to the anterior B7.6-descendants in the tail at the tailbud stage (B8.11, arrowheads). However, *Vasa* RNA has a different specific distribution in 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

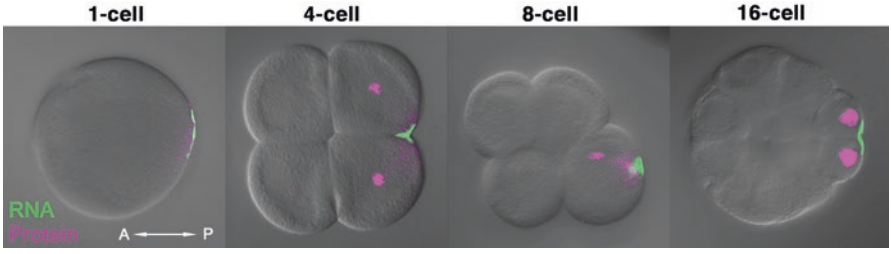

Fig. 1.3 Nuclear localization of Pem-1 protein in cleavage-stage embryos. *C. robusta* embryos with 1, 4, 8, or 16 cells were probed for -*Pem-1* mRNA (green) and Pem-1 protein (magenta). In the pair of germline blastomeres, *Pem-1* mRNA is highly concentrated in the post-plasm at the posterior cortex,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

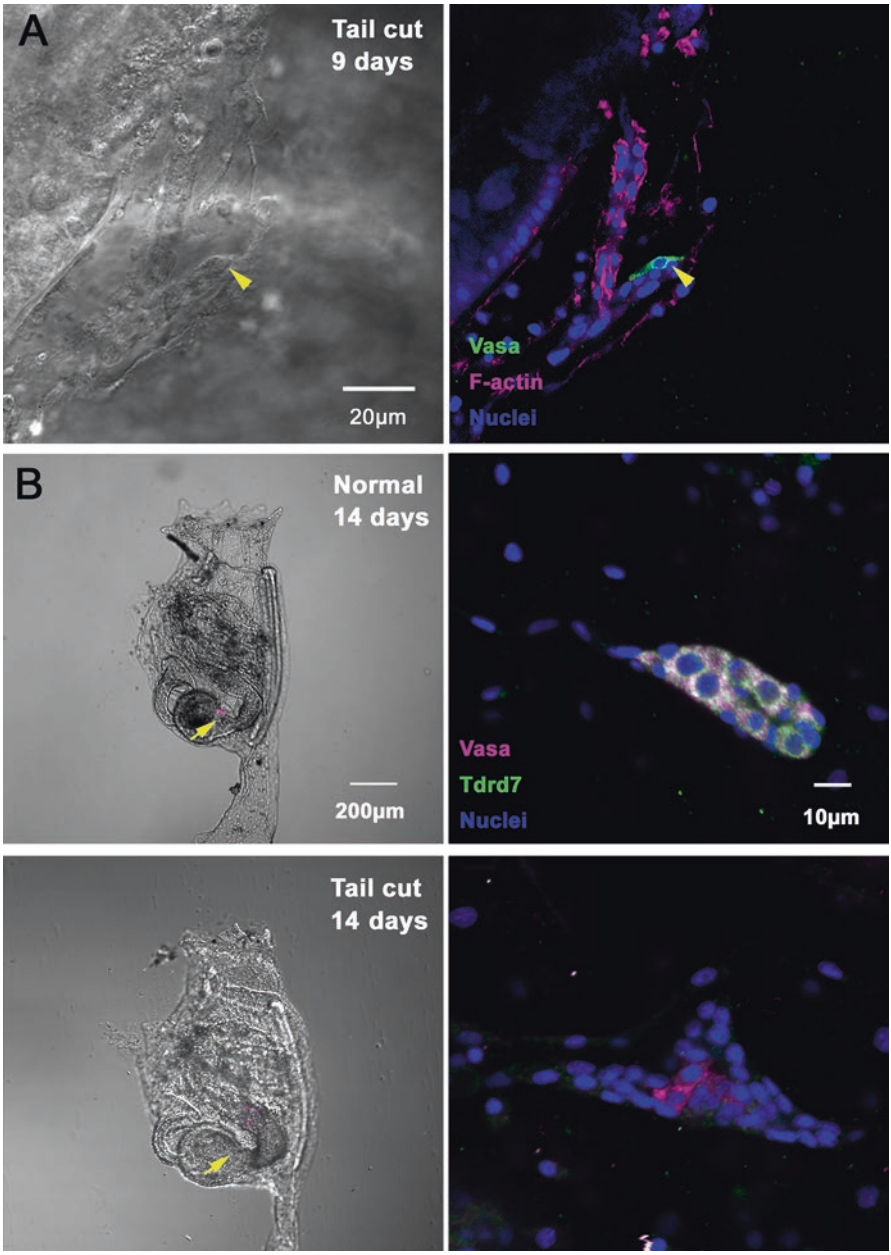

Fig. 1.4 Epigenetic PGCs appeared after tail-cut experiments. (**a**) When predetermined PGCs were removed from tadpole larvae by tail cutting, Vasa-positive cells (green) appeared in the future gonadal area in juveniles 9 days after metamorphosis. (**b**) Normal and tail-cut larvae 14 days after metamorphosis. 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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Chapter 2
Regulatory Mechanisms of the Germline Stem Cell Niche in *Drosophila melanogaster*

Yoshiki Hayashi and Satoru Kobayashi

Abstract Stem cells possess the unique ability to produce both self-renewing and differentiating daughter cells continuously throughout an animal's lifespan. Understanding the mechanisms that regulate proper stem cell maintenance is a central issue in basic biology and medical science. Since the stem cell niche hypothesis arose in the late 1970s, it has been widely believed that stem cells are maintained within a specialized extracellular microenvironment known as the niche. Germline stem cells (GSCs) in the fruit fly, *Drosophila melanogaster*, provide an excellent model for study of the stem cell niche in vivo. The first molecular components constituting the *Drosophila* ovarian GSC niche were identified in 1998. Since that time, identification of niche components and our understanding of how the niche maintains stem cells have continued to progress. In this review, we introduce how the niche maintains GSCs, as well as how the niche itself is precisely formed in the tissue. In addition, we discuss recent findings showing that the state of the host organism, including nutrient status and aging, affects niche function and stem cell maintenance.

Keywords Germline stem cell · *Drosophila melanogaster* · Stem cell niche · Extracellular matrix · Heparan sulfate proteoglycan · Glypican · Aging · Nutrient condition

2.1 Germline Stem Cells and Their Niche in *Drosophila melanogaster*

How stem cells are maintained in an animal's body has been a long-time enigma in life science. In 1978, Schofield proposed that hematopoietic stem cells are maintained within the specialized extracellular space in bone marrow, and named the

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space as “niche” (Schofield 1978). The niche is supposed to consist of the extracellular space constituted by surrounding stromal cells (niche cells) and an accumulation of local signaling molecules, which work together to maintain stem cells. Although the stem cell niche hypothesis is widely accepted among the stem cell researchers, in vivo evidence showing the identification of niche cells and signaling molecules responsible for stem cell maintenance has long been unclear.

The *Drosophila* ovarian GSC niche was the first niche in which key components were identified. Since that discovery, our understanding of how the niche maintains stem cells has rapidly progressed in a variety of tissues. Before we introduce the molecular mechanisms that regulate the GSC niche, we introduce the morphology and development of GSCs and their niche cells in both sexes.

2.1.1 Morphology and Development of the Drosophila *Ovarian GSC Niche*

The *Drosophila* adult female has one pair of ovaries, located in the abdomen. Each ovary consists of approximately 16 ovarioles, which are chains of developing oocytes (Fig. 2.1). At the anterior tip of each ovariole is a region called the germarium, each of which has two or three GSCs at its own anterior tip (Fig. 2.1). Each GSC division generates a GSC and a differentiating daughter cell. GSC division occurs horizontally relative to the ovariole axis, and the daughter located posteriorly (now called the cystoblast) follows the differentiation fate. The cystoblast undergoes four synchronous cell divisions with incomplete cytokinesis, giving rise to a cyst of 16 interconnected cells called the germline cyst. Among these 16 cells, one of the two cells with four cytoplasmic bridges follows the oocyte fate, whereas the other 15 cells eventually differentiate into nurse cells that provide maternal factors to the growing oocyte. At the posterior region of the germarium, each germline cyst is surrounded by somatic follicle cells, and is pinched off from the germarium as an egg chamber. Because GSCs divide and generate differentiating daughter cells continuously, the ovariole looks like a chain of developing egg chambers, with younger chambers on the anterior side and older chambers on the posterior side.

The ovarian GSC niche is composed of somatic gonadal cells located at the anterior part of germarium. At the anterior tip of germarium, there are eight to ten disc-shaped somatic gonadal cells, named as terminal filament cells (Fig. 2.1). The posterior-most terminal filament cells are attached to five to seven cap cells, which associate with GSCs and other types of somatic gonadal cells called escort cells (Fig. 2.1). Cap cells are known to play a role as a signaling center for maintenance of both GSCs and escort cells (Xie and Spradling 1998; Decotto and Spradling 2005; Rojas-Rios et al. 2012). Escort cells, also called inner germarium sheath cells, are the third type of somatic gonadal cells constituting the anterior part of germarium. Escort cells tightly associate with GSCs and their descendants, or cystoblats and developing cysts, and their role is required to help the cysts to move posteriorly

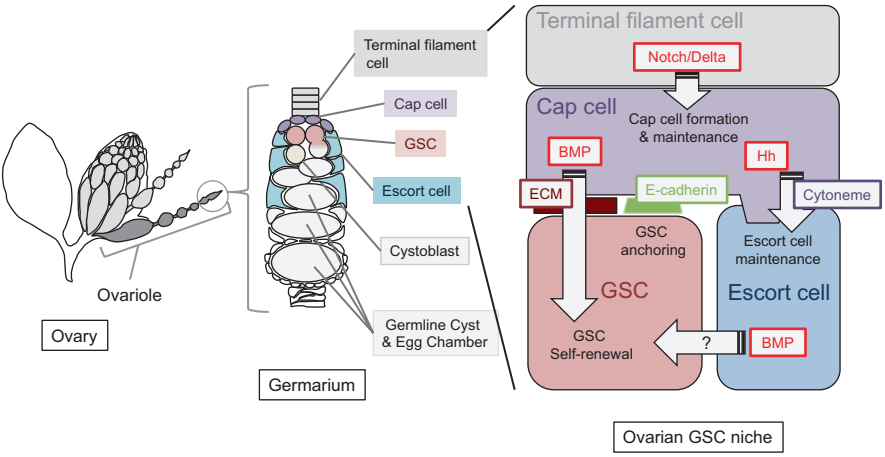

Fig. 2.1 Morphology and molecular components of the ovarian GSC niche. The ovary consists of approximately 16 ovarioles, each of which has a germarium at its anterior tip (left). GSCs and their niche cells (terminal filament cells, cap cells, and escort cells) are located at the most anterior region of the germarium. Each GSC divides asymmetrically to produce a new GSC and a cystoblast that undergoes oogenesis (middle). Within the GSC niche, cap cells are maintained by Notch signal induced by terminal filament cells, and they maintain GSCs by providing BMP signal. Cap cells also maintain function of escort cells to maintain GSCs by providing Hh signal with cytonems. Escort cells are another potential source of the BMP signal for GSC maintenance (Rojas-Rios et al. 2012) (right)

within the germarium. Furthermore, escort cells are involved in the maintenance of GSCs (Morris and Spradling 2011; Rojas-Rios et al. 2012). Among these somatic cell types, cap cells are known to be the major components of the GSC niche.

Cap cells first appear in the ovary of the late third instar larva, when they differentiate from a group of somatic cells called intermingled cells. Differentiation of cap cells is induced by Notch signaling from newly formed terminal filament cells (Song et al. 2007). Once they form, they provide growth factor signals to neighboring primordial germ cells (PGCs), and the PGCs associated with newly formed cap cells adopt a GSC fate (Song et al. 2004). When ectopic formation of cap cells is induced by overexpression of Notch signal, the resultant ectopic cap cells can maintain GSCs even in the absence of terminal filament cells and escort cells (Song et al. 2007). Furthermore, PGCs that are not able to associate with cap cells cannot maintain their undifferentiated status, and consequently directly undergo differentiation (Asaoka and Lin 2004). These findings indicate that establishment and maintenance of GSCs are dependent on cap cell functions, and show that cap cells are essential components of the female GSC niche.

Additional evidence emphasizes the importance of cap cells in the GSC niche function. First, cap cells provide signaling molecules that are essential for GSC maintenance (Xie and Spradling 1998). Second, the number of cap cells is closely related to the number of GSCs that can be maintained (Xie and Spradling 2000). Consistent with this, in order to be maintained, GSCs must be directly anchored to cap cells via E-cadherin-mediated adhesion (Song and Xie 2002). Finally, if one

GSC is removed experimentally, other GSCs undergo symmetric division to occupy the empty space, indicating that the number of GSCs is determined by the size of the space formed by cap cells (Xie and Spradling 1998, 2000). This finding clearly indicates that cap cells constitute the "physical space" to be occupied by GSCs.

2.1.2 *Morphology and Development of* Drosophila *Testicular GSC Niche*

Drosophila males have a pair of testes, and GSC niches are located at the anterior tips of each testis. The male GSC niche consists of approximately nine GSCs and two types of associated somatic gonadal cells: hub cells and cyst stem cells (CySCs). Hub cells, the most anteriorly located somatic gonadal cells in testis, associate directly with GSCs and CySCs on their posterior side (Fig. 2.2). GSCs undergo asymmetric division to generate one daughter cell that remains associated with hub cells and another daughter cell that is displaced posteriorly from the hub cells. The daughter cell that remains associated with the hub cells maintains stem cell character, whereas the other follows the differentiation fate. The differentiating daughters, called gonialblasts, undergo four divisions with incomplete cytokinesis to form a cluster of 16 interconnected germline cells in a single cyst. These germline cysts then enter premeiotic S-phase, undergo spermatogenesis, and eventually give rise to 64 sperm.

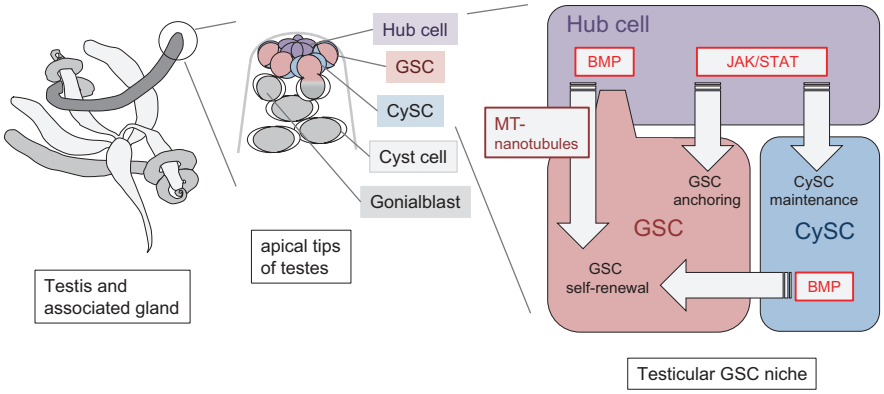

Fig. 2.2 Morphology and molecular components of the testicular GSC niche. The testicular GSC niche is located in the most anterior region of each testis (left). The testicular GSC niche consists of GSCs and two types of somatic gonadal cells, hub cells and CySCs (middle). In the testicular GSC niche, hub cells are thought to serve as the master signaling center. Hub cells maintain GSCs by providing JAK/STAT and BMP signals. Hub cells also maintain CySCs by providing JAK/STAT signal, and CySCs provide BMP signal to associated GSCs, thereby contributing to their maintenance (right)

In the testicular GSC niche, hub cells and CySCs play essential roles in GSC maintenance. Specifically, hub cells act as the master signaling center in the male GSC niche (Kiger et al. 2001; Tulina and Matunis 2001; Kawase et al. 2004). Hub cells provide growth factor signals that contribute to the maintenance of both GSCs and CySCs (Fig. 2.2). Hub cells first appear in male gonads at a late embryonic stage (Le Bras and Van Doren 2006), when they arise from the anterior subset of the male somatic gonadal precursors (SGPs). Once they form, they secrete growth factor signals to neighboring PGCs, thereby inducing them to adopt a GSC fate (Sheng et al. 2009).

Formation of hub cells is induced by Notch signaling from SGPs (Kitadate and Kobayashi 2010). Notch signaling is necessary and sufficient for hub cell formation, and the ligand (Serrate) and receptor (Notch) are expressed in almost all SGPs. These findings are consistent with the idea that potentially all SGPs have the ability to differentiate into hub cells, but this is repressed in the posterior region. Indeed, hub cell fate in posterior SGPs is repressed by epidermal growth factor (EGF) and Boss/Sevenless signaling (Kitadate et al. 2007; Kitadate and Kobayashi 2010). Interestingly, the ligands for these signaling pathways, Spitz and Boss, are both expressed in PGCs, and decreasing the number of PGCs induces ectopic formation of hub cells. Thus, SGPs sense PGC number via these signals that modulate hub cell number, and this acts as a mechanism for securing GSCs from a small number of PGCs (Kitadate and Kobayashi 2010).

CySCs, the stem cells for somatic gonadal cells known as cyst cells, are located at the anterior tip of the testis in association with hub cells (Fig. 2.2). Two CySCs flank each GSC, and both of these cell types associate directly with hub cells. CySCs divide asymmetrically, generating a CySC and a cyst cell. Two cyst cells envelope each gonialblast; cyst cells also surround developing germline cysts and are essential for germline cyst differentiation (Kiger et al. 2000). CySCs, another important signaling center of the GSC niche, provide growth factor signals required for GSC maintenance (Fig. 2.2). It remains unclear when and how CySCs are formed, but they are considered closely related to hub cells: CySCs and hub cells share common precursors in embryonic gonads (Dinardo et al. 2011), and CySCs can transdifferentiate into hub cells in adult testis (Voog et al. 2008).

2.2 Molecular Mechanisms Involved in Formation of the GSC Niche

The stem cell niche is thought to be formed by local accumulation of signal molecules that regulate stem cell maintenance; however, the identities of these molecules have remained mysterious. The *Drosophila* male and female GSC niches provide excellent models for studying how stem cells are maintained in vivo. Below, we

describe how GSCs are maintained by local signals from niche cells, and how the GSC niche area is determined at a molecular level.

2.2.1 Molecular Mechanisms Constituting the Ovarian GSC Niche

The ovarian GSC niche was the first model system used to study how stem cells are maintained in vivo. In the late 1990s, Xie and Spradling screened for genes responsible for ovarian GSC maintenance, and identified *decapentaplegic* (*dpp*) as an essential gene (Xie and Spradling 1998). *dpp*, the orthologue of mammalian bone morphogenetic protein (BMP) 2/4, is one of the ligands of the *Drosophila* BMP signaling pathway. Once *dpp* function is lost, GSCs cannot be maintained, resulting in drastic loss of GSCs. Conversely, overexpression of *dpp* causes over-proliferation of GSCs, resulting in a tumorous phenotype (Xie and Spradling 1998). These results clearly show that Dpp is an essential molecular constituent of the ovarian GSC niche.

The molecular mechanisms by which Dpp maintains GSCs have also been revealed. When Dpp secreted from cap cells is received by neighboring GSCs, it activates the BMP signaling pathway within the GSCs. This in turn leads to phosphorylation of Mother against dpp (Mad), a downstream transcriptional regulator of BMP signaling. Phosphorylated Mad (pMad) then binds to a discrete silencer sequence of *bag of marbles* (*bam*), thereby repressing transcription of this gene (Chen and McKearin 2003), which promotes differentiation of cystoblasts. Thus, Dpp-mediated BMP signaling activity maintains GSCs as undifferentiated stem cells by suppressing *bam* transcription.

2.2.2 Molecular Mechanisms Constituting the Testicular GSC Niche

The testicular GSC niche was the second model niche in which molecules responsible for stem cell maintenance were identified. In this system, Unpaired (Upd), a secreted cytokine that activates the Janus kinase/signal transducer and activator of transcription (JAK-STAT) pathway, was identified as a signal responsible for GSC maintenance (Kiger et al. 2001; Tulina and Matunis 2001). *upd* is highly expressed in hub cells, and loss of *upd* function prevents GSC maintenance. Conversely, ectopic expression of *upd* causes over-proliferation of GSCs, resulting in a tumorous phenotype (Kiger et al. 2001; Tulina and Matunis 2001). It remains unclear how JAK/STAT signaling maintains GSCs, but at a minimum it promotes GSC adhesion to hub cells (Fig. 2.2) (Leatherman and Dinardo 2010).

The BMP signaling pathway also contributes to the male GSC niche. In testis, two BMP ligands, Dpp and Glass bottom boat (Gbb: a *Drosophila* orthologue of mammalian BMP 5/8), function in GSC maintenance (Kawase et al. 2004). These two ligands are thought to function cooperatively, but Gbb plays the predominant role in male GSC maintenance. Once *gbb* function is lost, GSCs cannot be maintained, resulting in loss of GSCs; by contrast, the *dpp* mutant has a less severe phenotype. In both of these cases, expression of the differentiation marker *bam* is elevated in GSCs, although the function of *bam* in the testis is thought to be dispensable for GSC differentiation. Interestingly, in contrast to the case of *upd*, forced expression of neither *gbb* nor *dpp* induces over-proliferation of GSCs. Another difference from the ovarian GSC niche is that the BMP signaling pathway is required, but may not be sufficient for male GSC maintenance (Kawase et al. 2004).

It remains unclear which somatic cell type in the testicular GSC niche is responsible for GSC maintenance. Hub cells are the primary candidate for this role because *upd* and both BMP ligands are highly expressed in this cell type (Fig. 2.2). However, a competing model contends that CySCs play the essential role in GSC maintenance. In this model, Upd from hub cells activates the JAK/STAT signaling pathway in both GSCs and CySCs, but the primary role of the JAK/STAT signal in GSCs is to anchor GSCs to hub cells rather than to maintain their stem cell character (Leatherman and Dinardo 2010). According to this model, the primary role of JAK/STAT signaling in the male GSC niche is maintenance of CySCs, rather than GSCs. The JAK/STAT signal in CySCs maintains these cells by up-regulating transcription of *zfh1*, a transcriptional factor essential for CySC maintenance. CySCs provide BMP ligands to neighboring GSCs; BMP signaling within GSCs maintains the stem cell character of GSCs (Fig. 2.2) (Leatherman and Dinardo 2010). This model is based on the testis phenotype observed when JAK/STAT signaling activity is lost only in the germline. Under these conditions, GSCs are no longer anchored to hub cells and are consequently displaced away. Nonetheless, GSCs can still be maintained if they are adjacent to CySCs (Leatherman and Dinardo 2010). This phenotype suggests that GSCs can be maintained even if they lose attachment to hub cells, and that CySCs are the niche components essential for GSC maintenance. Although these data are suggestive, there is also evidence showing that GSCs can be maintained even in testis lacking CySCs (Lim and Fuller 2012). Further studies are needed to determine which cell plays the predominant role in male GSC maintenance.

2.2.3 Molecular Mechanisms That Define GSC Niche Size

Maintenance of proper GSC number is essential not only for organismal homeostasis, but also for continuity of life. Consequently, the size of the niche in which GSCs reside must be defined precisely. Because secreted growth factors play pivotal roles in GSC maintenance, the area of the niche is thought to be defined as the space in which growth factor signals distribute. Although the area of the niche should be

strictly defined, the molecular mechanisms that regulate this area are not well understood. Below, we introduce two recent models that seek to explain how the signaling area is regulated in the GSC niche.

2.2.3.1 Role of Extracellular Molecules in Niche Size Regulation

In general, ligands of growth factor signaling pathways are regulated by the factors in the extracellular environment, such as cell surface molecules and extracellular matrix. One of the major components of the matrix is heparan sulfate proteoglycan (HSPG) (Hacker et al. 2005), a family of sugar-modified proteins conjugated to heparan sulfate (HS) chains. HSPGs are located on the cell surface and in extracellular matrix such as the basement membrane. The important role of HSPGs in vivo is regulation of growth factor signaling in the extracellular space by regulating ligand stability and distribution, and/or serving as co-repressors of signaling complexes via direct interactions with ligands (Hacker et al. 2005).

Drosophila has at least three evolutionally conserved HSPG families: the glypican, syndecan, and perlecan families. Among these, glypicans are well studied in regard to their regulation of growth factor signaling. For example, one *Drosophila* glypican, encoded by *division abnormally delayed* (*dally*), regulates BMP signaling activity by stabilizing Dpp in the extracellular space and contributes to generation of a proper morphogen gradients during imaginal disc development (Fujise et al. 2003; Akiyama et al. 2008).

In the ovarian GSC niche, Dpp is the essential molecular constituent of the niche area, which is limited to within a single cell diameter of the cap cells; therefore, the distribution of Dpp must be tightly controlled. Studies from our group and others showed that Dally can define the ovarian GSC niche area by regulating BMP signal activity (Guo and Wang 2009; Hayashi et al. 2009). In the ovarian GSC niche, *dally* is highly expressed in cap cells. In *dally* mutants, BMP signal activity is lost from the GSCs, which can no longer be maintained. In addition, *bam* expression is observed in *dally* mutant GSCs. Finally, forced expression of *dally* in somatic gonadal cells induces ectopic formation of GSC-like cells, which are positive for BMP signal activity, negative for *bam* expression, and have the morphological characteristics of GSCs. These data indicate that Dally defines the ovarian GSC niche area by regulating the range of BMP signaling activity. Because Dally can stabilize Dpp protein in the extracellular space (Akiyama et al. 2008), it may stabilize Dpp on the surface of cap cells to limit the distribution of Dpp exclusively to these cells.

HSPGs also have a function in the testicular GSC niche. In this system, two *Drosophila* glypicans, *dally* and *dally-like*, are required for maintenance of GSCs (Hayashi et al. 2009). It remains unclear how glypicans regulate GSC maintenance. Given that these two glypicans regulate JAK/STAT signal by regulating Upd distribution in the egg chambers (Hayashi et al. 2012), one possibility is that they

also regulate JAK/STAT signaling activity in the male GSC niche. Further studies are required to determine how glypicans are involved in the male GSC niche.

In addition to the controversies described above, several unsolved questions remain. Although *dally* is sufficient to maintain ovarian GSCs, loss of all types of HSPGs causes a more severe phenotype than a single mutation of *dally* (Hayashi et al. 2009). These facts strongly suggest that other types of HSPGs, such as syndecan and perlecan, are also involved in the regulation of GSC niche formation. Studying the individual functions of these other classes of HSPGs is crucial to achieving a comprehensive understanding of HSPG function in the stem cell niche.

Another example of how extracellular molecules regulate the stem cell niche is the function of Type IV collagen in the female GSC niche (Wang et al. 2008). Type IV collagen, the major component of the basement membrane, is localized between cap cells and GSCs, where extracellular matrix is thought to exist. In vitro assays revealed that Type IV collagen directly binds Dpp via its C-terminal domain, and thus regulates Dpp-dependent BMP signaling activity during embryogenesis. Interestingly, loss of Type IV collagen caused an increase in the number of GSCs. Together, these observations suggest that Type IV collagen regulates GSC number by controlling the range of Dpp-mediated BMP signaling. In contrast to glypican, which is thought to stabilize Dpp on the surface of signal-producing cells, Type IV collagen is thought to limit the BMP signal range by trapping Dpp in the extracellular matrix between cap cells and GSCs. Thus, extracellular molecules regulate Dpp distribution in several ways, including stabilization and trapping. Stabilization of Dpp could contribute to maintaining the total quantity of Dpp held in the niche. Once glypican is lost, the amount of Dpp within the niche area declines, and GSCs may not be maintained. On the other hand, Type IV collagen may contribute only to the trapping of Dpp, ensuring its proper localization. Thus, loss of Type IV collagen may change Dpp localization without affecting the total amount of Dpp within the niche. Leaked Dpp may be received by differentiating germ cells, such as cystoblasts, to inhibit their differentiation. This phenotype suggests that an extracellular molecule other than glypican also serves to regulate the stability of Dpp.

2.2.3.2 Short-Range Signal Transduction Via Microprotrusions

Recent studies proposed that juxtacrine signals are transduced via direct interactions with microprotrusions such as primary cilia and cytonemes. For example, during *Drosophila* larval development, Dpp and fibroblast growth factor (FGF) transduce their signals between the wing imaginal disc and the associated tracheal air sac primordium in a juxtacrine manner, via the cytoneme (Roy et al. 2014).

In the GSC niche, signal transduction is also achieved in a juxtacrine fashion, and BMPs play important roles in this process. Surprisingly, Inaba et al. reported that a novel type of microprotrusion, termed microtubule-dependent nanotubules (MT-nanotubules), is essential for BMP signal transduction in the male GSC niche (Inaba et al. 2015).

MT-nanotubules, which are approximately 3.3 μm in length and 0.4 μm in width, are morphologically and molecularly distinct from the other microprotrusions. Each GSC has about seven MT-nanotubules, each of which is oriented toward a hub cell. Dpp emanating from hub cells is received by a BMP type I receptor, Thickvein (Tkv), which is localized on MT-nanotubules, and thus activates BMP signaling in GSCs. This MT-nanotubule-mediated BMP signal transduction is essential for GSC maintenance. Interestingly, formation of MT-nanotubules is specific for GSCs, and the formation process itself is dependent on the activation of BMP signaling. Therefore, formation of MT-nanotubules and the detection of the BMP signal in GSCs establish a positive-feedback loop, ensuring that GSCs are maintained only near signal producing cells.

In females, there is no evidence that microprotrusions contribute to signal transduction between cap cells and GSCs. However, there is one example showing that Hedgehog (Hh) signal transduction between cap cells and escort cells is achieved via a microprotrusion (Fig. 2.2) (Rojas-Rios et al. 2012). In that case, a microprotrusion called the cytoneme orients from cap cells toward escort cells in order to transduce the Hh signal to the latter cells. This cytoneme-dependent Hh signal transduction is required for maintenance of escort cell function to maintain GSCs. Thus, cytoneme-dependent Hh signal transduction is indirectly involved in GSC maintenance.

2.2.4 Influence of Nutrient Status and Aging on the GSC Niche

In animal tissue, the ability of stem cells to produce daughter cells is affected not only by local signals within the niche, but also by the condition of the host organism, including such factors as nutrient status and aging. Thanks to the successful identification of niche components, the *Drosophila* GSC niche is the ideal model for studying how the stem cell niche is influenced by, and responds to, those factors. Here we discuss how the condition of the organism, specifically in regard to nutrient status and aging, affects the stem cell niche.

2.2.5 Nutrient Status and the GSC Niche

The nutrient status of the host animal is thought to be one of the most important environmental cues for the GSC niche. However, it is not completely clear how nutrient status affects reproductive ability. Studies of the ovarian GSC niche system have shed light on this matter.

Poor nutrient conditions, such as shortage of amino acids, severely affects the reproductive ability of female *Drosophila* (Drummond-Barbosa and Spradling

2001). Nutrient status affects several steps during oogenesis, including niche maintenance, GSC proliferation, and survival of daughter cells. When flies are fed a low-protein diet, the rate of GSC proliferation decreases to one-fourth of the rate under protein-rich conditions. In addition, the survival rates of the cellular descendants of GSCs (egg chambers) are severely decreased. In total, reproductive ability under normal conditions is 60-fold higher than under protein-limited conditions, suggesting that nutrient condition is a crucial external cue that governs reproductive ability (Drummond-Barbosa and Spradling 2001).

Insulin signaling is an evolutionarily conserved pathway that connects external nutrient status to cellular responses. In the ovarian GSC niche, insulin signaling also plays a central role in sensing nutrient status. In *Drosophila*, nutrient status is perceived in the central nervous system (CNS), which contains two clusters of neurosecretory cells. Under nutrient-rich conditions, these cells secrete the *Drosophila* orthologue of insulin (Drosophila insulin-like peptide: Dilp) (LaFever and Drummond-Barbosa 2005). Dilp is received by GSCs and cap cells, in which it stimulates various responses (Fig. 2.3). In GSCs, the insulin signaling pathway is autonomously required for proliferation, although the underlying mechanism remains unclear. In cap cells, the insulin pathway plays dual roles. Dilp regulates cap cell and GSC maintenance in a cell-autonomous and non-autonomous manner, respectively (Hsu and Drummond-Barbosa 2009, 2011). First, the insulin signal regulates cap cell maintenance by regulating cap cell competency to respond to the Notch signaling pathway, an essential signaling pathway for cap cell maintenance (Hsu and Drummond-Barbosa 2011). Second, insulin signaling within cap cells regulates GSC maintenance in a non-autonomous manner by regulating attachment between cap cells and GSCs. Insulin signaling regulates expression of E-cadherin, which is essential for anchoring of GSCs to cap cells. Because the number of the cap cells directly affects the number of GSCs they maintain, and a sufficient level of E-cadherin is essential for GSC maintenance, insulin-mediated nutrient sensing

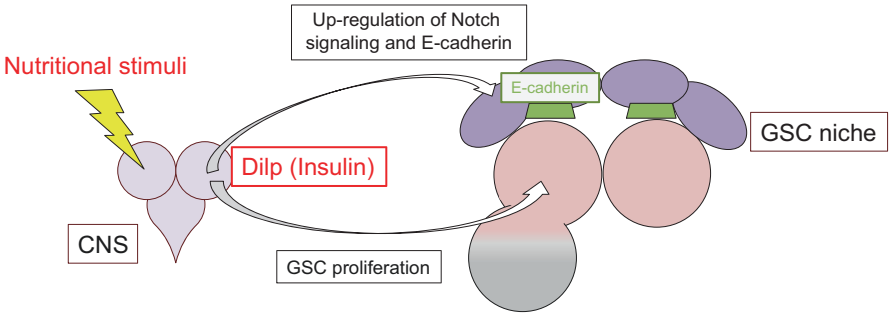

Fig. 2.3 Influence of nutritional stimuli on the GSC niche. Nutrient stimuli, such as a protein-rich diet, induce expression of *dilps* in *Drosophila* CNS (left). Dilps are then received in both cap cells and GSCs, where they activate insulin signaling. Insulin signaling in GSCs promotes GSC proliferation, and thus egg production. Insulin signaling in cap cells plays a dual role: activation of Notch signaling for maintenance, and expression of E-cadherin for GSC anchoring (right)

contributes to reproductive ability by regulating both the competence of niche cells to maintain GSCs and the ability of GSCs to proliferate.

Similar to female GSCs, poor nutrient conditions affect the proliferation of male GSCs. It has been shown that poor nutrient conditions slow down cell cycle progression of male GSCs, and amino acid is a key ingredient that affects the cell cycle of male GSCs (Roth et al. 2012). This slowing down of the GSC cell cycle results from misorientation of the centrosome within male GSCs. Poor nutrient condition reduces the Insulin pathway activity within GSCs, which in turn causes misorientation of the centrosome. This abnormality causes activation of the centrosome orientation check point, and cell cycle progression is slowed down (Roth et al. 2012). In the absence of the check point activity, GSCs normally proliferate even in poor nutrient conditions. However, these GSCs lacking the check point activity cannot be maintained in poor nutrient conditions. These findings suggest that centrosome-mediated slowdown of GSC proliferation is essential for GSC maintenance in poor nutrient conditions (Roth et al. 2012).

2.2.6 Aging and GSC Niche Ability for Reproduction

Aging has a major impact on an organism's reproductive ability. Although aging affects reproduction at multiple levels, its effects are thought to be mediated, at least in part, by the ability of GSCs to reproduce.

The effect of aging on the GSC niche has been studied in male and female *Drosophila*. In both cases, the numbers of GSCs and niche cells, including cap and hub cells, decrease with aging (Wallenfang et al. 2006; Boyle et al. 2007; Pan et al. 2007). In the female GSC niche, the numbers of GSCs and cap cells decrease approximately 70 % and 90 % in 2-month-old females, respectively, relative to those of 1-week-old females. In addition, the proliferation rate of GSCs in aged females declines to about 80 % of that in young females. This is caused partly by a decrease in BMP signaling activity in GSCs. Partial loss of the BMP pathway ligands Dpp and Gbb promotes loss of GSCs in aged females; consistent with this, the expression of BMP activity markers in GSCs also declines with age. Conversely, either overexpression of BMP ligands in cap cells or forced expression of BMP receptor in GSCs can rescue age-dependent GSC loss, suggesting that aging affects both GSCs and their niche cells (Pan et al. 2007).

Besides BMPs, aging affects another important molecular component of the GSC niche, the adherens junction that anchors GSCs to cap cells. In the GSC niche of 2-month-old females, the expression level of Cadherin between cap cells and GSCs declines to about 20 % of that in 1-week-old females. Because Cadherin-mediated cell adhesion is essential for anchoring GSCs to cap cells, and hence for their maintenance, this is another important mediator of the effect of aging on reproduction. Indeed, overexpression of Cadherin in GSCs can rescue age-dependent GSC loss (Pan et al. 2007).

Interestingly, age-dependent decline in GSC niche function is rescued by forced expression of superoxide dismutase (SOD), which is essential for removal of reactive oxygen species (ROS), the main source of oxidative damage in the tissue (Pan et al. 2007). Overexpression of SOD in cap cells restores the numbers of cap cells and GSCs, as well as the proliferation rate of GSCs, even in 2.5-month-old females. Forced expression of SOD in GSCs also effectively rescues the age-dependent loss of GSC functions such as maintenance and proliferation. These findings suggest that oxidative stress is a major cause of age-dependent decline of GSC niche function, and that this stress causes damage to both GSCs and their niche cells.

The influence of aging is also observed in the male GSC niche. As in females, age-dependent decline in the numbers of GSCs and niche cells is also observed in males (Wallenfang et al. 2006; Boyle et al. 2007). In this case, age seems to impact hub cell quality. In the aged testicular GSC niche, expression of several hub cell markers, such as E-cadherin and Fasciclin III, is affected (Boyle et al. 2007). In addition, expression of *upd*, an essential signaling molecule in the male GSC niche, is significantly reduced in aged males. Conversely, forced expression of *upd* in hub cells in aged males can rescue the age-related decline in GSC number (Boyle et al. 2007). These findings suggest that aging damages hub cell function, and in particular decreases production of the niche signaling molecule Upd.

2.3 Concluding Remarks

In this review, we introduced the classical conception of the molecular mechanisms regulating the stem cell niche, and then discussed newer ideas regarding the formation of the stem cell niche in a limited area of the tissue. Finally, we discussed how the condition of the organism, including nutrient condition and aging, affects the stem cell niche.

Although some of the work described in this review was made possible by the relatively simple and distinct morphology of the *Drosophila* GSC niche, some of these concepts could be applied to other stem cell niches with fewer distinct morphological characteristics. For example, the role of ECM in defining niche area provides insight into stem cell niches without morphological distinction, e.g., the gut epithelial stem cell niche of *Drosophila*. In this stem cell niche, the growth factor responsible for stem cell maintenance is Wingless (*Drosophila* orthologue of *wnt*). Wingless is expressed in all muscle cells arranged uniformly beneath the gut epithelium; however, stem cells are not located on all muscle cells, but instead are localized sporadically on the muscle cell layer (Lin et al. 2008). Although there is neither a morphologically distinct niche nor a detectable pattern of growth factor expression in this tissue, stem cells are not ubiquitously localized. This observation raises the possibility that some mechanism may regulate growth factor signals after secretion. Interestingly, Wingless is a regulatory target of HSPGs, and ECM is present between the epithelium and muscle layer. It may be worth investigating

whether specialized ECM is capable of accumulating ligands within a limited area for the purpose of stem cell maintenance. Because stem cell niches without morphological distinction are known to exist in various tissues, e.g., the mouse testicular gamete stem cell niche (Yoshida et al. 2007), the concept that ECM defines the niche area may aid our understanding of a variety of stem cell niches that do not have distinct tissue morphologies.

The GSC niche of *Drosophila* has also been exploited to investigate how the condition of the organism affects the stem cell niche. Although several researchers have speculated that the condition of the host exerts a crucial influence on stem cell maintenance, it has been difficult to determine how the status of the whole organism affects the stem cell niche. In part, this is because we had not yet precisely identified niche components in many tissues; as a consequence, researchers could not assess the molecular changes related to alteration of the host condition. The *Drosophila* GSC niche is an ideal model in which to assess these influences precisely. This is in large part because many molecular markers indicate the niche condition, including cell adhesion markers and signaling molecules responsible for stem cell maintenance. Furthermore, genetic manipulation can easily be applied to test the biological significance of the molecular change described above.

To more completely understand the influence of the environment surrounding the GSC niche, it could be interesting to use an ex vivo culture system of *Drosophila* gonads. *Drosophila* gonads can be cultured in vitro, and this culture system has been a powerful option to observe the stem cell behavior with live imaging (Prasad et al. 2007; Morris and Spradling 2011; Sheng and Matunis 2011). With this culture system, it may also be possible to mimic the in vivo environment and identify specific chemical compounds that affect niche function by adding or removing them from the culture media. For example, it would be interesting to assess the influence of certain neurotransmitters or chemicals related to oxidative stress, such as H_2O_2, on the stem cell niche. A recent study of mammalian cultured stem cells revealed that factors such as a hypoxic environment, or metabolites such as acetyl-CoA or methionine, affect the character of stem cells (Takubo et al. 2010; Shiraki et al. 2014; Moussaieff et al. 2015). It also may be worth investigating the influence of these factors in this ex vivo culture system and comparing their effects among different stem cell systems.

Although efforts to understand how the organism's condition affect the stem cell niche were initiated recently, identification of environmental factors that affect the stem cell niche could progress rapidly by combining several experimental strategies described above. In this review, we focused only on aging and nutrient condition as tissue-extrinsic factors, as these two factors were long thought to affect an animals' reproductive ability. The influence of other factors, such as steroid hormones, have been discussed in a recent excellent review (Uryu et al. 2015).

It has been just two decades since the first molecular evidence pertaining to stem cell maintenance was obtained in the *Drosophila* ovarian GSC niche. Since that time, our understanding of stem cells and their niches has progressed rapidly, both in the GSC niche and in other systems. However, studies addressing some newer concepts, such as the role of ECM or the influence of the organism's condition, have

started very recently, and many unsolved questions persist. We believe that a total understanding of the stem cell niche can be achieved using this simple stem cell niche as a model. The resultant knowledge will provide insight into basic stem cell biology as well as contribute to medical research involving stem cells.

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Chapter 3
Regulation of Germline Stem Cells in the Teleost: Gametogenesis, Sex, and Fecundity

Minoru Tanaka

Abstract Stem cells are generally defined as the cells that retain themselves while they keep producing differentiating cells. Presence and regulation of stem cells are, therefore, essential for maintaining homeostasis of the tissue. In the gonad, germline stem cells are responsible for producing a tremendous number of gametes at the appropriate time during a reproductive period. In addition, it was recently revealed in teleost fish Medaka that regulation of germline stem cells is related to some other aspects of reproduction. The intrinsic mechanism that determines whether to produce sperm or eggs begins to act in germline stem cells, and the proliferation of germline stem cells causes sex reversal against the direction of the sex determination gene. Furthermore, germline stem cells seem to be involved in fecundity through regulation of the germ cell number and size of regulation. This chapter provides an overall picture of germline cells and discusses their novel roles, which have been mainly revealed through the analysis of the Medaka.

Keywords Sex determination · Sex reversal · Germ cells · Medaka

3.1 Introduction

Whether germline stem cells are present or absent in the mammalian ovary has been a long-standing issue in terms of not only scientific interest but also increasing demand by regenerative medicine. Based on extensive research in mammalian species, especially in mice, there seem to be no typical germline stem cells in the ovary

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(Lei and Spradling 2013; Tanaka 2013). With the onset of ovary development, all the germ cells commit oogenesis and form primordial follicles. The initial number of follicles in the fetus would probably be large enough to sustain reproduction. This number, however, hardly explains the reproduction in females of other species, like amphibia and fish, which produce more eggs. The question then arises regarding the presence or absence of germline stem cells in both testes and ovaries, and, if present, how germline stem cells are regulated in a species-specific manner in reproduction.

3.2 Presence of Germline Stem Cells in the Ovary: Location and Oogenic Process

The teleost fish Medaka spawn dozens of eggs every day during a reproductive period of approximately 3 months in summer, and was the first vertebrate in which the existence of germline stem cells was experimentally proved by clonal analysis (Nakamura et al. 2010). Since it was previously found that *nos2* is a marker of oogonia (Aoki et al. 2009), Nakamura et al. successfully labeled the *nos2*-expressing cells with EGFP and showed that the labeled cells keep producing eggs that are capable of expression of EGFP. This result indicates that a population of *nos2*-expressing oogonia harbors germline stem cells.

The clonal analysis also reveals a temporal process of oogenesis. *Nos2*-expressing germ cells undergo three to five rounds of mitotic division and form germ clusters (Gcys: cystic germ cells). The Gcys cells in the single cluster are connected to each other by an intercellular bridge. They then enter meiosis to form an early diplotene stage of oocytes (Gdip).

The *nos2*-expressing cells are not a homogenous population. The population includes a mitotically quiescent population and an active population, but they are not morphologically distinguishable. Unlike Gcys germ cells, all the *nos2*-expressing germ cells are present in isolation, enclosed one by one by *sox9b*-expressing supporting cells. Therefore *nos2*-expressing germ cells are often called Gs germ cells (single isolated germ cells). In contrast, the interconnected Gcys cells are surrounded by *sox9b*-expressing cells as a unit of single cluster.

The Medaka ovary is suspended from the coelomic epithelium and is located just above the gut. The ovary is anatomically divided into two regions, the ventral stromal compartment, where follicles grow and maturate, and the dorsal ovarian cavity, into which mature oocytes are ovulated (Tanaka 2009). The germinal epithelium, a thin multicellular layer of epithelium, separates the stromal compartment from the ovarian cavity. Germ cells of Gs, Gcys, and Gdip cells, embedded in *sox9b*-expressing cells, are located within the germinal epithelium. The Gs cells harboring germline stem cells are scattered within the germinal epithelium. The regions where Gs cells with *sox9b*-expressing cells are present are called “germinal cradles,” and represent the niche regions. The germinal cradles are connected to each other by the

cellular processes of *sox9b*-expressing cells (ovarian cords) within the germinal epithelium. Thus, ovarian cords are a network of *sox9b*-expressing tubules with germinal cradles that harbor Gs, Gcys, and Gdip. The Gdip exit from the germinal cradles into the stromal compartment as they form the follicle (Nakamura et al. 2010).

3.3 The Presence of Germline Stem Cells in the Testis: Location and Spermatogenic Process

Medaka testes are composed of an array of tubule structures that are irradiated from an efferent duct in the center of the testis. Spermatogonia are located at the peripheral end of tubule structures, and spermatogenesis proceeds in the tubules proximally towards the efferent duct.

Like oogenesis, spermatogonia have *nos2*-expression. Therefore, these cells are likely to include a population of germline stem cells. Consistent with this, Medaka produce EGFP-labeled eggs that are derived from transplanted spermatogonia. These germ cells are relatively large and occupy most distal (peripheral) positions in the tubules, and are often called type A spermatogonia. Spermatogonia give rise to relatively small and cystic germ cells called a type B spermatogonia and spermatocytes, which are positioned in more proximal areas near the efferent duct (Tanaka 2009).

Nos2-expressing spermatogonia are enclosed with *sox9b*-expressing supporting cells (*sox9b*-expressing Sertoli cells). *Sox9b* expression is more intense in the Sertoli cells surrounding spermatogonia and is hardly detectable in the Sertoli cells with spermatocytes (Tanaka 2013). This situation is again very analogous to that observed in the oogenic process.

3.4 Regulation of Germline Stem Cells: Commitment to Gametogenesis and Homeostasis

3.4.1 Nos2 *and 3*/Vasa

As mentioned above, *nos2* expression in germ cells represents a population of oogonia and spermatogonia that include germline stem cells. The first expression of *nos2* in Medaka is observed in germ cells with the onset of testicular morphogenesis and tubular formation approximately 1 month after fertilization. During the larval stage before the development of male secondary sex characteristics, the testis remains as a gonadal primordium and does not exhibit any structural characteristics of a testis. Germ cells in the larval testis do not express *nos2*, which is the same as those found in developing oogenesis (Nishimura and Tanaka 2014).

This observation suggests that *nos2* expression is related to stem cell establishment or maintenance, which is also consistent with developmentally late expression of *nos2* (at the stage of sex differentiation) in zebrafish (Beer and Draper 2013) and *nos2* induction in mice by GFRa1/GDNF, which are known to be crucial for stem cell maintenance (Sada et al. 2012). In other words, in the larval stage of fish, germ cells may be under way to developing into germline stem cells.

In contrast, *nos3* are continuously expressed in a lineage of germline. First *nos3* is supplied maternally, with zygotic expression beginning at the timing of germline specification at the early gastrulation stage in Medaka (Kurokawa et al. 2006; Aoki et al. 2008, 2009). In zebrafish, *nos3* is described as a pan-marker of germline (Köprunner et al. 2001) and functions in the maintenance of germline stem cells (Beer and Draper 2013).

Vasa expression may also be suggestive in that the expression holds for a character of germline stem cells. Unlike *nos2*, *vasa* is detected in the cells capable of developing into gametes. Primordial germ cells migrating in the early embryos, gonocytes in the gonadal primordium, and a population of germline stem cells in the adult testis and ovary, all express *vasa* (Tanaka et al. 2001; Nóbrega et al. 2010). However, the germ cells committed to gametogenesis, like Gcys in ovary and type B spermatogonia, dramatically decrease its expression. Since regulation by 3'UTR of *vasa* is essential for germ cell-specific expression of *vasa*, the mechanism might be linked to the establishment or maintenance of germline stem cells.

3.4.2 AMH/MIS System

Anti-Müllerian Hormone (AMH)/Müllerian Inhibiting Substance (MIS) belongs to a BMP superfamily and is a secreting factor responsible for regression of female reproductive organs (upper vagina and oviduct) during male development in mammals. Accordingly, AMH/MIS is specifically expressed in male supporting cells (Sertoli cells). However, this factor is expressed in both male and female developing gonads of teleost fish.

A Medaka mutant, *hotei*, with a defect in a type II receptor of AMH/MIS, reveals an unexpected role of this factor. The defect of AMH/MIS signaling promotes proliferation of germline stem cells and causes a huge functional gonad with an excessive number of gametes (Morinaga et al. 2007). Interestingly, a quiescent type of germline stem cell does not seem affected by this signaling, but a mitotically active type of germline stem cell proliferates to produce the gametes when the signal is impaired. Interestingly, the receptor and the ligand (AMH/MIS) are both expressed in the supporting cells. The chimeric analysis between mutant and wildtype cells supports an intersupporting cell action of AMH/MIS signaling. Collectively, AMH/MIS signaling adjusts the proper number of germ cells through regulating the status of supporting cells, which affects proliferation of an active type of germline stem cell (Nakamura et al. 2012).

3.4.3 *Sox9b*

During gonadal development in mice, *sox9* shows male-specific expression in Sertoli cells and is a direct effector of male-determining gene, *Sry*, on the Y chromosome. Therefore, loss of *sox9* function displays a female-like phenotype. There are two orthologues in Medaka, *sox9a* and *sox9b* (Klüver et al. 2005; Nakamura et al. 2012). Since only *sox9b* is expressed in the supporting cells, *sox9b* seems to correspond to mammalian *sox9* in the gonad. But *sox9b* is expressed in both female and male supporting cells in Medaka (see above).

Consistent with both female and male expression, Medaka *sox9b* does not directly involve male development, which is in contrast to its role in mammals. A Medaka *sox9b* mutant forms gonads, suggesting that the identity of supporting cells are retained in the mutant. However, the mutant loses germ cells during development. The mutant supporting cells develop a disorganized basement membrane that is important for the separation of supporting cells from others and often displays abnormal cellular blebs. Chimeric analysis between mutant and wildtype supporting cells in the gonad demonstrates that *sox9b* heterozygous mutant cells have less ability to hold germ cells than wildtype cells. Homozygous mutant cells still retain the ability to surround the germ cells but are expelled from the wildtype cell population in the gonad. This suggests that cellular interaction between supporting cells and others is impaired, which may be reason the germ cells are gradually lost (Tanaka 2013). Supporting this, qPCR analyses shows aberrant expression of components of extracellular matrix like cadherin and metalloproteases. Regulation of the extracellular matrix is an important aspect of germline stem cell maintenance (Nakamura et al. 2012).

3.4.4 *Pluripotency*

Several factors have been identified in maintaining the pluripotency of germline stem cells in mammals. Although solid evidence has not been provided, some experiments and expression patterns suggest that these factors are involved in germline stem cell regulation.

Oct4/*Pou5f1* and *nanog* are intriguing since they are expressed in Medaka spermatogonia and Medaka ES cells but not in the somatic cells. Interestingly, ES cell derivation from blastula cells is blocked in Medaka when *oct4* is abolished (Wang et al. 2011), and the expression identifies stem cell populations in the adult gonads (Froschauer et al. 2013; Sánchez- Sánchez et al. 2010), suggesting involvement of these factors in the pluripotency. In zebrafish, *soxB1* in addition to the other two factors are essential in mid-blastula cells for transition from maternal to zygotic expression (Lee et al. 2013).

Plzf1 is a transcriptional factor that is also known to be involved in the maintenance of germline stem cells in mammalian testis. TypeA and possibly some popu-

lations of type B spermatogonia express Plzf protein in adult zebrafish (Ozaki et al. 2011). The transcripts are detected in trout undifferentiated spermatogonia together with *oct4* and *nos2* (Bellaiche et al. 2014). It is interesting to note that transplants of fluorescent germ cells driven by *vasa* promoter or *sox17*, which had been cultured in vitro, maintain *plzf* expression and generate sperm in zebrafish subcutaneous tissues (Kawasaki et al. 2012). Although there has been no solid evidence, these observations suggest an analogous role of mammalian *plzf* in the germline stem cells of teleost fish.

3.5 Sex Determination of Germline Stem Cells

Germline stem cells are the unique cells producing both sexes of gametes, eggs, and sperm. The sexual status of germline stem cells in mammals has not, however, been examined, possibly because of the presence of germline stem cells only in the ovary. In the teleost fish both ovary and testis possess germline stem cells (Tanaka 2013). Reciprocal transplantation of germ cells from adult trout gonad into the fish larva of the opposite sex indicates that some fraction of germ cells develop into the gametes of the opposite sex (e.g., germ cells from the testis become oocytes in the ovary) (Okutsu et al. 2006). This is also true with germ cells in Medaka. This observation suggests that germline stem cells are sexually indifferent or are not sexually determined.

The question then arises how the decision about sperm or egg is regulated in the germline stem cells. One possible explanation is that gametogenesis is controlled by the surrounding cellular environment one by one. In this scenario, the intrinsic mechanism of germ cell sex determination is not postulated. Rather gametogenesis is perceived as two different developmental processes, and every step of the critical events is regulated by the somatic cells. However, the important issue of germ cell sex determination has not been evaluated experimentally before.

Contrary to this general perception, a recent study has shown the presence of an intrinsic mechanism of germ cells underlying their sexual fate. Disruption of *foxl3* causes germ cells to fill the ovary with functional sperm in Medaka (Nishimura et al. 2015). In female embryos, *foxl3* is expressed in an active type of germline stem cell and subsequent Gcys (cystic) germ cells, but not in the germ cells that enter meiosis (Gdip) and in the gonadal somatic cells. In males, *foxl3* is first expressed in stem type germ cells just after the gonadal primordium is formed, but is soon diminished, long before testicular structures develop. The phenotype clearly indicates that *foxl3* functions to repress the initiation of spermatogenesis. Interestingly, a quiescent type of germline stem cell does not express *foxl3*. This suggests that the sex is determined in germ cells during the course of commitment of germline stem cells to gametogenesis.

This result also shows another important point. Because the mutant with disrupted *foxl3* produces functional sperm in the ovary, the mechanism for the initia-

tion of gametogenesis can be separated from that for germ cell sex determination. The germ cell sex determination is an independent and essential mechanism that is initiated in germline stem cells.

3.6 Fecundity and Germline Stem Cells

There seem to be several developmentally important points that critically determine the number of gametes during gametogenesis. The regulation of the number is directly linked to the potential capability of individuals to generate offspring.

Hotei mutants exhibit numerous germ cells tht result from an elevated activity of proliferation in the active type of germline stem cells. Interestingly, however, no apparent fluctuation of AMH/MIS expression (responsible for this phenotype; see above) is observed in the wildtype Medaka. Therefore, there should be some regulation that modulates the downstream activity of AMH/MIS signaling in the supporting cells to properly regulate the number of gametes in the wildtype gonad.

It may be interesting to note here that, in zebrafish, the mutation in *alk6b*, a gene for the type Ib BMP receptor, causes germline tumors (Neumann et al. 2011). Also, the inhibitor of TGFb/BMP signaling suppresses differentiation of spermatogonia into sperm in the culture medium (Wong and Collodi 2013). Although the detailed mechanism is not known, these results suggest the importance of TGFb/BMP signaling beyond the species.

Regulation of the mitotic activity of germline stem cells affects the ability for fecundity. In the wildtype Medaka the mitotic activity is sexually different during gonadal formation (Saito et al. 2008). This difference is regulated downstream of the sex determination gene and is essential for the sex differentiation of the gonad and the whole body. In this context, the most important point is that if the germ cell number is changed artificially the difference causes sex reversal in opposition to the direction of the sex determination gene. The increased mitotic activity in an active type of germline stem cell causes male-to-female sex reversal (Morinaga et al. 2007; Nakamura et al. 2012a). The Medaka with the lower number of germ cells develops functional testes (Kurokawa et al. 2007). In zebrafish, the sex is also affected by the initial number of germ cells, and production of more oocytes favors female development (Rodriguez-Mari et al. 2010). Therefore, regulation of the mitotic activity of germline stem cells is closely linked to the proper sex determination by the signal downstream of the sex determination gene.

Consistent with this finding, a promoted mitotic activity of an active type of germline stem cell in *hotei* mutants displays a female-biased sex differentiation. Approximately half of XY Medaka exhibit a female phenotype with functional ovaries.

3.7 Conclusion

Studies with teleost fish reveal the importance of germline stem cells, which have not been discovered in mammals. Loss of typical germline stem cells in the ovaries is not common among vertebrates. It is important to understand that absence or presence of germline stem cells simply represents one of the possible strategies that females may adopt and take. A great advantage in using teleost fish is that the female and male germline stem cells that are present in teleost fish provide an essential and deep insight into their roles. We understand the core mechanism underlying the maintenance of germline stem cells and the initiation into gametogenesis. In addition, studies with teleost fish are exploring a way to analyze novel functions of germline stem cells that nobody expected, involving sex and fecundity. Germline stem cells are not merely the cells that produce gametes, but also have the ability to regulate the internal environment for gametogenesis.

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Chapter 4
Regulatory Mechanism of Spermatogenic Stem Cells in Mice: Their Dynamic and Context-Dependent Behavior

Shosei Yoshida

Abstract The germline is the only cell type that is inherited by the next generation in many multicellular animals. For the purposes of successful reproduction, animals need to produce enough gametes for a sufficient duration of time. It is also crucial to adjust the production of gametes according to the reproduction strategy that each species uniquely develops. In many animals, these features of germ cells are provided by the function of stem cells. Stem cells by definition continually produce differentiating cells (e.g., spermatozoa) while maintaining their own population, viz. the stem cell pool. Mammalian spermatogenic stem cells (also termed spermatogonial stem cells or SSCs) represent the most studied stem cell types, and have been providing important insights into not only the biology of reproduction but also for stem cell research in general. This chapter describes the current position of mammalian (mostly mouse) spermatogenic stem cell research, as well as its future directions. First, in contrast to a general thought that stem cell division always gives rise to one self-renewing and one differentiating daughter cell, the spermatogenic stem cell of each mouse follows a variable fate. Their self-renewal and differentiation is balanced at the level of population; such stem cell dynamics are designated as "population asymmetry." Second, the current knowledge regarding the identity of "actual" stem cells (cells that support homeostatic spermatogenesis) and their *in vivo* dynamics will be discussed. Third, our focus will move on to the flexible change of the stem cell behavior depending on tissue contexts; some spermatogonia act as "potential" stem cells which differentiate under homeostasis but contribute to post-insult regeneration or post-transplantation colony formation. Finally, our current knowledge and upcoming questions about the "facultative" or "open" stem cell niche for mouse spermatogenesis will be discussed.

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4.1 Introduction

Mammalian spermatogenic stem cells represent a typical example of *tissue stem cell*s (or *adult stem cell*s) (Spradling et al. 2011; Morrison and Spradling 2008). Undifferentiated and proliferative cells are so named in vivo when they support the processes of tissue homoeostasis and/or repair by producing differentiating cells while maintaining their own population. Spermatogenic stem cells provide the foundation of continual spermatogenesis that continues in the long term, as well as the transmission of genetic and epigenetic information to the next generation. Therefore, they have attracted special interest from the fields of reproductive biology and genetics. In addition, mammalian spermatogenetic stem cells have provided a unique opportunity to investigate the common properties of tissue stem cells (Yoshida 2012; Spradling et al. 2011).

Despite decades of intensive studies, the cell-biological nature of mammalian spermatogenic stem cells remains to be fully elucidated (de Rooij and Russell 2000; Meistrich and van Beek 1993; Yoshida 2012). This is at least partly because the anatomically defined stem cell niche has not been found in seminiferous tubules, the spermatogenic center in mammalian testis (Russell et al. 1990) (Fig. 4.1). In some tissues (e.g., *Drosophila* testis and ovary, mammalian intestinal epithelium), stem cells are exclusively localized to an anatomically defined stem cell niche, which provides invaluable cues to investigate the dynamics and regulation of stem cells (Morrison and Spradling 2008; Stine and Matunis 2013; Barker 2014; Losick et al. 2011). In the seminiferous tubules of mice, on the other hand, stem cells are scattered and intermingle with differentiating progenies as described below (Hess and Franca 2005; Russell et al. 1990; Yoshida 2016; Skinner and Griswold 2005) (see also Fig. 4.5).

The mammalian seminiferous tubules (Fig. 4.1) are 150–200 μm in diameter across species, while their total length per testis is up to 2 m in mice and over 200 m in humans. In most instances, seminiferous tubules show a uniform architecture of *seminiferous epithelium* (a composite of Sertoli cells' epithelium and different stages of germ cells) both around the circumference and along the longitudinal (Fig. 4.1b, c). Epithelia of tall and polarized Sertoli cells develop a prominent network of tight junctions, the anatomical basis of the *blood-testis barrier* that separates the *basal* and *adluminal compartments* of the tubules. The basal compartment is the gap between the tight junctions and the basement membrane, where spermatogonia (viz. the mitotic germ cells including the stem cells and differentiation-destined amplifying cells) are located. Upon entering meiosis, these cells (now called spermatocytes) translocate to the adluminal compartment without disrupting

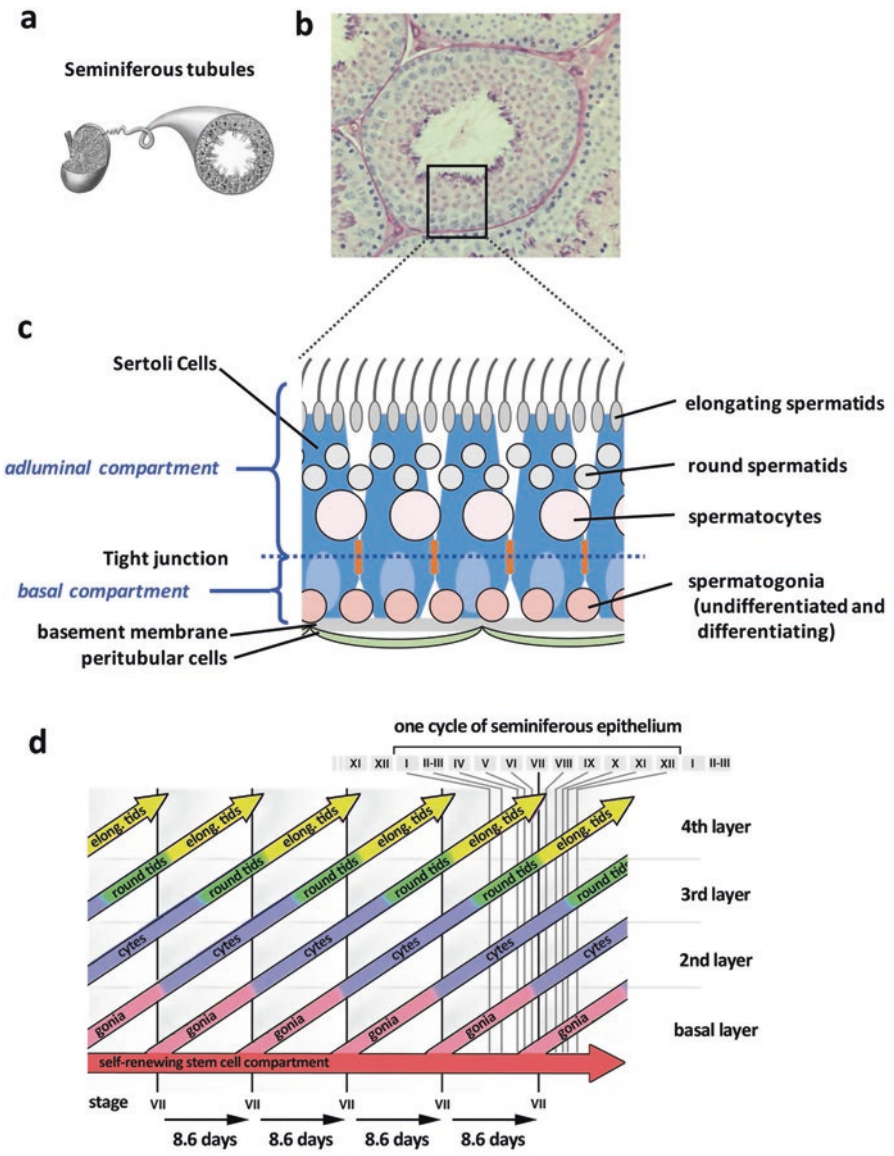

Fig. 4.1 Anatomy of the mouse seminiferous tubules. (**a**) Schematic representation of a mouse testis and constituent seminiferous tubules, which are convoluted and tightly packed in the testicular capsule. (**b**) PAS-stained mouse testis showing a cross section of a seminiferous tubule. (**c**) Schematic drawing showing the architecture of seminiferous epithelium, corresponding to the rectangular region in (**b**). In seminiferous tubules, different stages of germ cells are found among Sertoli cells that comprise single-layered epithelia inside the tubules. Each Sertoli cell simultaneously nourishes different (typically four) stages of germ cells. Germ cells turn over as they mature on the surface of Sertoli cells. See text for more details. (**d**) A schematic explanation of the seminiferous epithelial cycle in mice. The self-renewing pool of undifferentiated spermatogonia gives rise to the differentiating cells periodically with a regular interval of 8.6 days. The differentiation process to mature sperm takes 35 days, which is four times of 8.6 days. As a result, four different steps of germ cells are always found as four layers. A cycle is divided into stages I to XII, on the basis of the combination of germ cell types. Duration of a cycle and the number of stages is species-specific. See text for details (adapted from Yoshida (2016))

the function of the blood-testis barrier, through an ingenious dynamism of tight junctions (Smith and Braun 2012). In the adluminal compartment, spermatocytes undergo two meiotic divisions to form haploid spermatids, which are eventually released into the lumen after their maturation into spermatozoa. The entire process of mouse spermatogenic differentiation takes about 35 days to complete. Because this process proceeds with regular intervals (8.6 days in case of mice), the combination of differentiating germ cells observed in seminiferous epithelium changes periodically, which is referred to as the *seminiferous epithelial cycle* (Fig. 4.1d) (Leblond and Clermont 1952; Oakberg 1956; Russell et al. 1990; Hogarth and Griswold 2010; Yoshida 2016). The structure of seminiferous tubules and the seminiferous epithelial cycle are commonly observed among amniotes (i.e., mammals, birds, and so-called reptiles) (Yoshida 2016; Lombardi 1998).

Most if not all of the stem cell functions reside in a small subpopulation of spermatogonia termed *undifferentiated spermatogonia* (Yoshida 2010, 2012; de Rooij and Russell 2000; Meistrich and van Beek 1993; Oatley and Brinster 2008) (Fig. 4.2a). However, it remains to be elucidated how these cells accomplish the stem cell functions and support tissue homeostasis, post-insult regeneration, and post-transplantation repopulation (Yoshida 2012; Yoshida et al. 2007a). The population of undifferentiated spermatogonia are morphologically heterogeneous, comprising isolated cells (A_s or A_{single}) and syncytia of two (A_{pr} or A_{paired}) or more (A_{al} or $A_{aligned}$) cells connected via intercellular bridges (de Rooij and Russell 2000; Huckins 1971; Russell et al. 1990) (Fig. 4.2a). Most syncytia of undifferentiated spermatogonia (A_{pr} and A_{al}) have 2^n (viz. 4, 8, or 16) cells, reflecting the incomplete cytokinesis in the telophase of their mitotic divisions (Russell et al. 1990), while a small fraction of A_{al} contain other numbers (3, 5, 6, etc.) of cells. It was originally proposed by Huckins in 1971 that in homeostasis A_s cells act as the stem cells while A_{pr} and A_{al} are irreversibly committed for differentiation and unidirectionally extend their syncytial length (Huckins 1971; Oakberg 1971) (Fig. 4.2b). This theory became prevailing and is known as the "A_s model" (de Rooij and Russell 2000; Meistrich and van Beek 1993; Russell et al. 1990).

In 1994, Brinster and colleagues established a stem cell transplantation technique (Brinster and Avarbock 1994; Brinster and Zimmermann 1994; Brinster 2007). In this experiment, a testicular cell suspension is injected into the lumen of emptied host seminiferous tubules, whose residential germ cells have been previ-

Fig. 4.2 (continued) (**b**) Schematic representation of the "A_s model", indicating that A_s are the only self-renewing cells and all the syncytia including A_{pr} and A_{al} are irreversibly committed for differentiation. Modified from Aloisio et al. (2014). (**c**) Summary of GFRα1+/Nanos2+ and Ngn3+/Rarg+ compartments of undifferentiated spermatogonia. While the former primarily act as the actual stem cells and self-renew in undisturbed situations, the latter largely differentiate. However, the Ngn3+ cells retain the capacity to revert to the GFRα1+ cells (shown by an arrow with asterisk), which become prominent during post-insult regeneration or post-transplantation repopulation. Note that both compartments contain A_s, A_{pr}, and A_{al} spermatogonia, although their ratio is different, indicating that morphology (number of cells within a syncytia) is not a primary determinant of cell behavior. See text for details

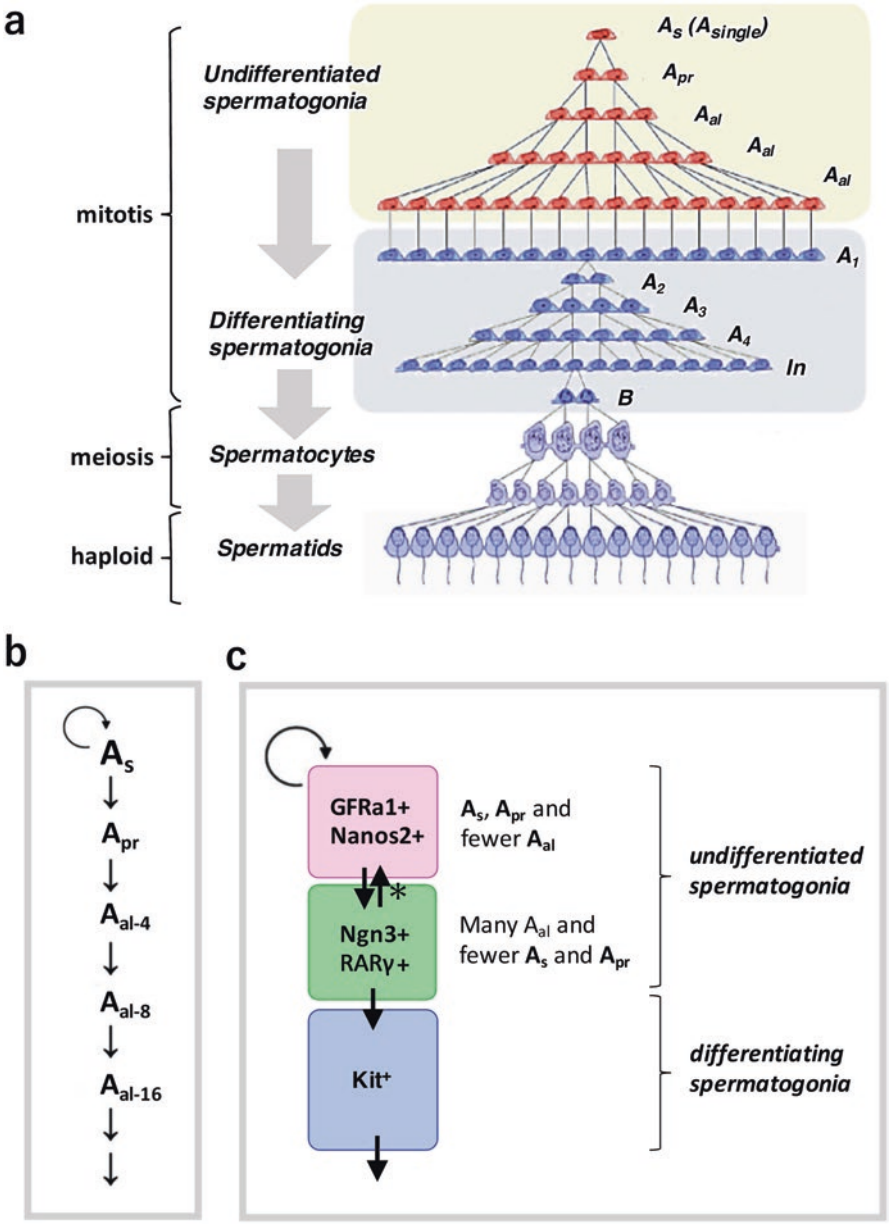

Fig. 4.2 Heterogeneous properties of spermatogonia and the stem cell models. (**a**) Scheme of the spermatogenic cell types observed in the mouse testis, including spermatogonia (mitotic cells), spermatocytes (meiotic cells) and spermatids (haploid cells). Note the syncytial formation due to the interconnection of sister cells. Spermatogonia are classified into "undifferentiated" and "differentiating" populations; the former is the primary responsive population for stem cell functions. See text for more details. Modified from the original drawing by (Bloom and Fawcett 1975).

ously depleted (by chemicals, radiation, or mutations) while the structure of the seminiferous tubules including the Sertoli cells remains intact. The injected "stem cells" then settle in the basal compartment and form colonies, which can subsequently restore the entire spermatogenetic population. This breakthrough enabled functional and quantitative detection of "stem cells" as *colony-forming units*, with a similar principle to that described for hematopoietic stem cells. As a natural consequence, "A_s cells" have been often considered to be a synonym of "stem cells that support homeostasis," "stem cells that support regeneration after tissue damage," and "stem cells that repopulate after transplantation."

In this decade, the development of new techniques for research in this field has provided a number of observations that appear to be inconsistent with the "A_s model", at least in its original form. These techniques also challenge the aforementioned theory that A_s cells always serve as the stem cells regardless of the context (Yoshida et al. 2007a; Yoshida 2012) (Fig. 4.2c). First, an intravital live imaging technique captured the fragmentation of syncytial spermatogonia to form A_s cells as a result of intercellular bridge breakdown, indicating that A_s and syncytia are interconvertible (Nakagawa et al. 2010; Hara et al. 2014). It was also discovered that some A_s cells are destined for differentiation rather than self-renewal (Nakagawa et al. 2010). Furthermore, some spermatogonia that usually differentiate in undisturbed conditions actively contribute to the self-renewing pool upon post-insult regeneration and post-transplantation colony formation (Nakagawa et al. 2007, 2010; Yoshida et al. 2007a; Barroca et al. 2009). These properties of Ngn3+ cells are consistent with the conceptual proposal of "potential stem cells" made by Potten and colleague (Potten and Loeffler 1990).

The mouse spermatogenic stem cell system thus appears to be not as simple as previously thought; rather, it consists of an intricate internal functional structure. Moreover, this system behaves differently under different contexts (viz. homeostasis *vs*. regeneration/transplantation). Such elaborate and flexible features should ensure a robust production of sperm for a long reproductive period, and should be underlain by heterogeneous gene expression among undifferentiated spermatogonia. The present is an exciting era to re-think the dynamics of spermatogenic stem cells, because emerging experimental methods make it possible to analyze the cell behavior over time in vivo, and at higher resolutions than have ever been achieved before. In this chapter, the current understanding and future avenues of research into the mammalian (mostly mouse) spermatogenic stem cells will be described.

4.2 Population Asymmetry of the "Actual" Stem Cell Dynamics

By definition, the dynamics of "actual" stem cells (i.e., cells that support homeostasis of adult spermatogenesis) can only be assessed through long-term cell fate analyses that do not destroy the tissue architecture. These analyses have been enabled by pulse-labeling experiments, using animals expressing tamoxifen-inducible Cre proteins

(CreER) under the control of regulatory sequences of genes which show specific expression in particular cells of interest (Hayashi and McMahon 2002; Yoshida 2012).

In mouse spermatogenesis, such pulse-labeling experiments have been performed using a number of CreER lines based on different gene expression patterns. The identity of actual stem cells is one of the most important questions that these experiments seek to answer; however, before going into this question, I will first discuss an intriguing aspect of the dynamics of actual stem cells which has been clarified by such pulse-labeling experiments, namely, *population asymmetry* (Yoshida 2012; Klein and Simons 2011) (Fig. 4.3a). When actual stem cells maintain tissue homeostasis (Fig. 4.3a, left panel), it has often been considered to be true that each stem cell divides asymmetrically to produce one stem cell and one differentiating cell (Fig. 4.3a, middle panel). Such dynamics, termed *division asymmetry*, have been established in large part by elegant studies on *Drosophila* germline stem cells (Fuller and Spradling 2007; Spradling et al. 2011). However, division asymmetry is not a prerequisite for the balanced behavior of a stem cell population (Fig. 4.3a, left panel); as shown in the right panel of Fig. 4.3a, this balance can also be achieved at a population level, even if individual stem cells behave differently (i.e., a pair of daughter cells derived from a single stem cell may both differentiate, both self-renew, or show different fates). Such stem cell dynamics is referred to as the *population asymmetry*. I emphasize here that the original "A_s model" did not discuss internal dynamics within the A_s compartment, although division asymmetry has been postulated to occur in subsequent discussions (Huckins 1971; Meistrich and van Beek 1993; de Rooij and Russell 2000).

Whether the stem cells follow the dynamics of division asymmetry or population asymmetry cannot be distinguished, if their fate is traced at the population level. Rather, this question must be addressed by analyzing the clonal fate of individual stem cells (Yoshida 2012; Klein and Simons 2011) (Fig. 4.3b). Division asymmetry predicts that, when the fate of individual stem cell clones is traced over the long term (maybe on the scale of several months, provided that reproduction period of the mouse lasts for up to a couple of years), the size of each clone should not change (Fig. 4.3b left panel). If population asymmetry takes place, on the other hand, the clone size will change and become variable over time (Fig. 4.3b right panel). In fact, the results of such experiments using CreER lines indicate that population asymmetry underlies the dynamics of mouse spermatogenic stem cells (Nakagawa et al. 2007; Klein et al. 2010; Hara et al. 2014; Komai et al. 2014; Sada et al. 2009) (Fig. 4.3c). Further, statistical and mathematical analyses suggest that the observed population asymmetry reflects a *neutral competition* among stem cells following simple stochastic rules (Klein et al. 2010; Hara et al. 2014) (Fig. 4.3c). Such a stochastic population asymmetry has also been observed in other stem cell-supported tissues, and is now considered to be a universal rule of tissue stem cell dynamics (Klein and Simons 2011).

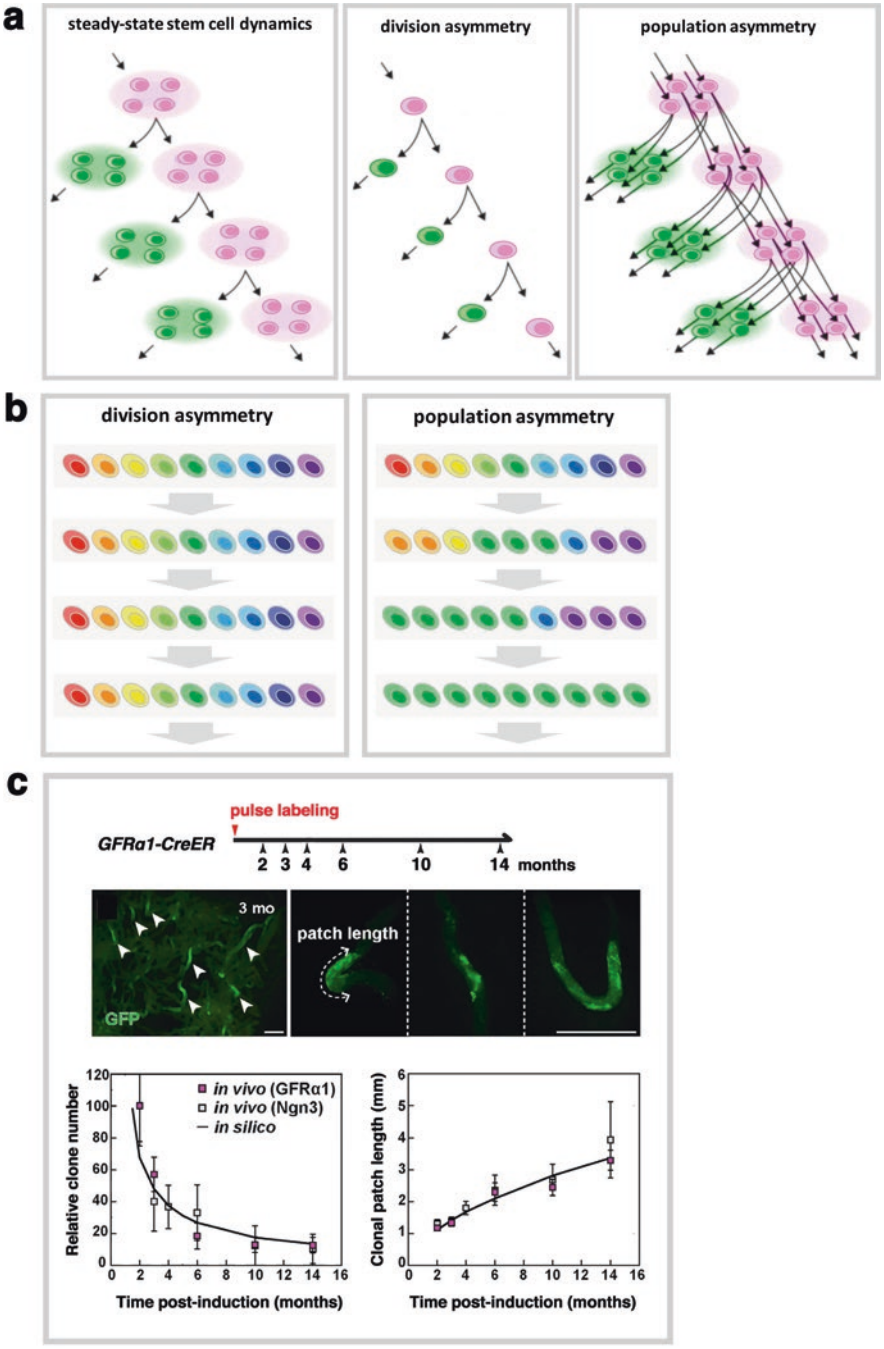

Fig. 4.3 Stem cell dynamics: division asymmetry and population asymmetry. (**a**) Concepts of tissue stem cell dynamics (left panel). The generalized view of stem cell dynamics, which maintain the undifferentiated population while constantly giving rise to the differentiating progeny. The number (pool size) of stem cells needs to be kept constant in order to maintain homeostasis (middle panel). Stem cell dynamics according to the "division asymmetry" model, in which stem cells always

4.3 Deciphering the "Actual" Stem Cell Identity and Behavior

Consider now the cellular identity of "actual" stem cells that show the dynamics of population asymmetry and support steady-state spermatogenesis. To the best of the author's knowledge, genes which have been shown to identify sub-populations of undifferentiated spermatogonia that exhibit long-term actual stem cell behavior by CreER-mediated pulse-labeling analysis include *Gfrα1*, *Nanos2*,1 *Id4*, *Pax7*, and *Bmi1* (Hofmann et al. 2005; Hara et al. 2014; Sada et al. 2009; Aloisio et al. 2014; Komai et al. 2014; Oatley et al. 2011a; Sun et al. 2015). Among these, *Gfrα1* (encoding a component of receptor tyrosine kinase for GDNF [glial cell line-derived neurotropic factor]), *Nanos2* (encoding an evolutionally conserved germline-specific RNA binding protein), and *Id4* (encoding a negative regulator for basic-helix loop helix [bHLH] transcription factors) have been shown to be essential for the process of establishment and/or long-term maintenance of stem cells (Sada et al. 2009; Oatley et al. 2011a; Naughton et al. 2006).

Gfrα1, *Nanos2*, *Id4*, *Pax7*, and *Bmi1* are all expressed in subpopulations of undifferentiated spermatogonia, but their expression patterns are not identical. Among these genes, the broadest expression is observed for *Gfrα1* and *Nanos2*, whose expressions are roughly overlapped with each other (Nakagawa et al. 2010; Suzuki et al. 2009; Sada et al. 2009). Of note is that *Gfrα1/Nanos2* expression is largely

Fig 4.3 (continued) divide asymmetrically, to produce one self-renewing and one differentiating daughter cell. Here, the tissue-level balance between self-renewal and differentiation is caused by the asymmetric fate behavior of individual stem cells (right panel). Stem cell dynamics according to the "population asymmetry" model, in which self-renewal and differentiation are balanced at the population level, while individual stem cells show variable fates. See text for more details. (**b**) Predicted patterns of clonal evolution of stem cells over time, assuming the dynamics of division asymmetry and population asymmetry. Cohorts derived from different stem cells (on the top) are shown in different colors in the following time points (below) (left panel). According to the division asymmetry model, each stem cell always produces one stem cell, so the number of surviving stem cell clones and their size will remain unchanged over time (right panel). According to the population asymmetry model, some stem cell clones are lost (through differentiation without self-renewal) and are replaced by the expansion of other clones. As a result, the number of surviving stem cell clones decreases, while their size (average number of stem cells within a single surviving clone) increases, over time. (**c**) An example of experimental evaluations of the number and the size of surviving spermatogenic stem cell clones over time (top panel). Experimental schedule of clonal fate analysis of pulse-labeled spermatogonia using GFRα1-CreER (or Ngn3-CreER) over 14 months during adulthood (middle panel). Images of single pulse-labeled stem cell-derived clones (GFP-positive), 3 months after induction by tamoxifen administration. The length of patches of GFP-positive cells reflects the size of the particular clones (bottom panel). Summarized data indicating that the surviving clones decrease in number while increasing in size over the 14-month period. Clones induced by GFRα1-Cre and those induced by Ngn3-CreER show indistinguishable long-term behavior, indicating their long-term equipotency. Lines are the results of in silico simulation on the assumption of neutral competition between stem cells (adapted from Hara et al. (2014))

reciprocal with that of *Ngn3* (*neurogenin3* or *neurog3*, a bHLH transcriptional regulator) in the population of undifferentiated spermatogonia, and that pulse-labeling experiments revealed that Ngn3+ cells show a detectable but far smaller actual stem cell activity compared to that of GFRα1-positive cells (Yoshida et al. 2004; Nakagawa et al. 2007; Hara et al. 2014). Therefore, the vast majority of actual stem cell activity resides within GFRα1+/Nanos2+ cells, as shown schematically in Fig. 4.2c. Intriguingly, *Id4*, *Bmi1*, and *Pax7* are expressed in subpopulations of GFRα1+ cells (predominantly A_s cells, with a few syncytia that are mostly A_{pr}). Although direct comparisons of their expression patterns have not been performed, *Pax7* appears to be expressed in the fewest cells among them (Aloisio et al. 2014; Komai et al. 2014; Oatley et al. 2011a).

How can we interpret these results, if different populations all show actual stem cell activity in pulse-labeling experiments (Fig. 4.4)? One possibility is that the actual stem cell activity is restricted to a single small population, and that any population that shows actual stem cell behavior includes these small number of cells (Fig.4.4a). However, this is not the only possibility, as the actual stem cells may dynamically interconvert between multiple states showing different gene expression and/or morphology, thus comprising a heterogeneous stem cell pool (Fig. 4.4b). Furthermore, there may be long-term stem cells that slowly replenish the short-term stem cells, necessitating careful cell-fate analyses over a wide range of timescales (Fig. 4.4c). In addition, a mixture of these models could be the case.

In accordance with the model shown in Fig. 4.4a, the expression of *Id4*, *Pax7,* or *Bmi1* in restricted subpopulations of GFRα1+ spermatogonia (a majority of which are A_s cells) led to the proposal that these populations are the actual stem cells (Aloisio et al. 2014; Komai et al. 2014; Oatley et al. 2011a; Chan et al. 2014) (Fig. 4.4d, e). This proposal requires careful evaluation given that the expression patterns of these genes seem not to be identical to one another. Therefore, precise comparison of these genes' expression profiles is of primary importance. In addition, it is also essential to know whether the actual stem cell activity is restricted to the cells of interest (as shown for GFRα1+ cells, because GFRα1– cells have only a trace of actual stem cell activity), or whether the activity is also detected in other fractions. In in vitro-cultured spermatogonia, an Id4+ fraction predominantly exhibits transplantable stem cell activity over an Id4– fraction (Chan et al. 2014). Although this observation indicates a strong correlation between *Id4* expression and transplantable stem cell potential, one should be careful in extrapolating this result to in vivo actual stem cells. This is not only because gene expression can change between in vitro and in vivo. More importantly, it has also been demonstrated that actual stem cell activity is distinguishable from that of transplantable stem cells, since Ngn3+ undifferentiated spermatogonia – which shows only low levels of actual stem cell activity – makes a significant contribution to the post-transplantation repopulation (Nakagawa et al. 2007; Yoshida et al. 2007a). Further considerations are necessary for these proposals regarding the morphological reversibility of GFRα1+ spermatogonia. Although they assume that, at least in an undisturbed situation, syncytial spermatogonia (viz. A_{pr} and A_{al}) are irreversibly committed and never fragment into A_s or shorter chains (Fig. 4.4d, e) (Aloisio et al. 2014; Chan et al.

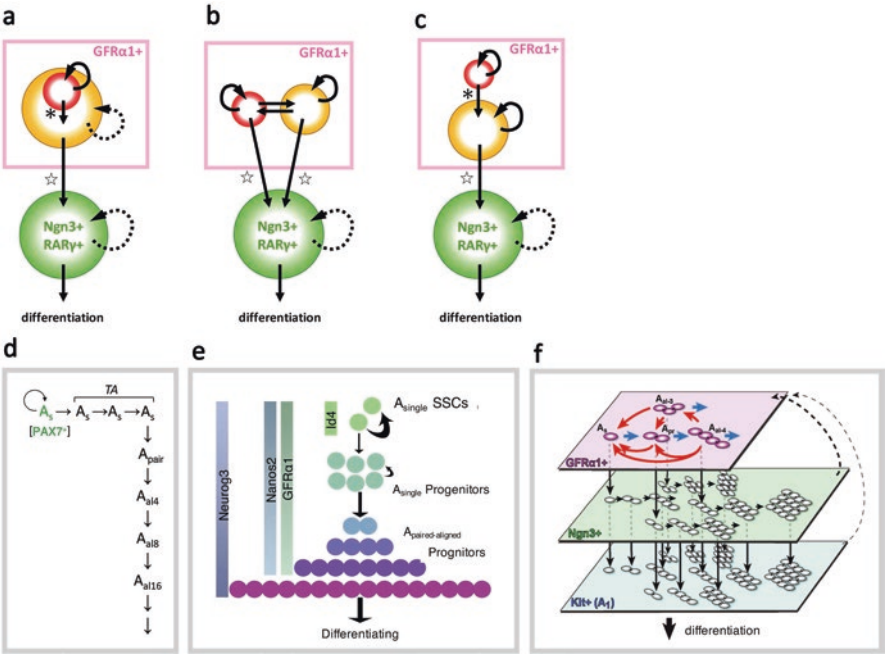

Fig. 4.4 Possible functional structures and proposed models of actual stem cell compartment. (**a**–**c**) Typical examples of possible functional structures of a stem cell system, in which multiple (two in these instances) subpopulations of GFRα1+ spermatogonia (shown in red and yellow) represent actual stem cell behavior. The round arrows indicate self-renewal, while dotted round arrows indicate transit amplification. The arrow with asterisks may be reversible. Reversibility of arrows with stars can be prominent in some contexts (such as regeneration and repopulation), although they are restricted in homeostasis. (**a**) The actual stem cell function may be restricted to a single defined population (red), and any population that includes this will show the actual stem cell function. (**b**) The actual stem cells may interconvert between multiple states with different gene expression and/or morphology ("dynamic heterogeneity"). (**c**) The "short-term" stem cells (yellow) may be replenished by "long-term" stem cell population (red). In this scheme, the latter should be very small in number and/or very slow in proliferation. Otherwise, the turnover of "short-term" stem cells will be too fast and may be better designated as "progenitors" or a "transient-amplifying population", although this might be an issue of semantics. Investigations across short and long time-scales (viz. days to months) are necessary to evaluate this possibility. In this scheme, the short-term clonal dynamics will eventually be shifted to that of long-term stem cells. The experiments shown in Fig. 4.3c do not support this scheme (although they cannot be ruled out), since a single minimal model can predict both short and long-term behaviors (see Hara et al. (2014) for details). (**d**–**f**) Recent proposals of stem cell dynamics. (**d**) Proposal by Aloisio et al. (2014), in which a small population of Pax7+ (also GFRα1+) A_s cells exclusively act as actual stem cells. (**e**) Proposal by Chan et al. (2014), in which an Id4+ (also GFRα1+) population of A_s cells act as actual stem cells. (**f**) Proposal by (Hara et al. 2014), in which actual stem cells interconvert between multiple states of A_s and syncytia (A_{pr} and A_{al}). (**d**) and (**e**) are on the line of Fig. 4.4a; (**f**) suggests the dynamic heterogeneity shown by Fig. 4.4b. See text for more details

2014), it has recently been observed (using intravital live imaging) that such fragmentation does indeed frequently occur (Hara et al. 2014).

The second model of actual stem cell dynamics shown in Fig. 4.4b is supported by data from intravital live imaging and detailed pulse-labeling analysis of the GFRα1+ population (Hara et al. 2014). Intravital live imaging of fluorescent protein-labeled cells in intact tissues provides a unique and powerful technique to assess the behavior of stem cells (and other cell types) over time at single-cell resolution (Brown and Greco 2014). So far, using this system, the behavior of GFRα1+ and Ngn3+ spermatogonia have been continually filmed for over 3 days (Yoshida et al. 2007b; Hara et al. 2014). Intriguingly, GFRα1+ (*gfr*α1-EGFP) cells actively migrate in the basal compartment between the Sertoli cells and the differentiating progeny (with some bias to vasculature) (Hara et al. 2014) (see later in Fig. 4.5d). Intravital live imaging also provides quantitative in vivo data including the frequencies of cell division (either compete or incomplete) and fragmentation of syncytial spermatogonia. In parallel, single-cell-resolution fate analysis of the GFRα1+ population has been performed using CreER-mediated pulse-labeling (Hara et al. 2014). On the basis of these quantitative data, a minimal biophysical model was synthesized, assuming that all GFRα1+ cells follow a common, simple, and stochastic rule, depending only on a couple of measured parameters (viz. the rates of cell division and syncytial fragmentation). This mathematical model was able to capture the wide range of observed data over a wide range of time scales (from days to over a year), and described the dynamics of population asymmetry. This lead to the hypothesis that the entirety of the GFRα1+ cell population comprises a single stem cell pool, in which cells stochastically interconvert between different states of A_s and syncytia through incomplete division and fragmentation (Fig. 4.4f).

However, this model does not take into account the subpopulations (or substates) of GFRα1+ cells that are positive for *Id4*, *Pax7*, etc. Therefore, a link between such a simple mathematical model describing the overall population dynamics of GFRα1+ cells, and the details of the actual behavior of its subpopulations will be of particular interest. Cells may interconvert between states that are positive and negative for such genes as *Id4* and/or *Pax7* along with the morphological interconversion, or may follow more complex dynamics. Live-imaging analysis of these subpopulations (ideally by means of simultaneous observation of multiple populations) will provide fundamentally important information, and detailed analyses of pulse-labeled cells' behavior will be important as well. In this regard, it is interesting to note that pulse-labeled *Pax7* clones may display dynamics that are inconsistent with the theory of stochastic population asymmetry; these could be distinct characteristics of this small population, although more in-depth analysis will be necessary to reach any conclusions in this matter (Aloisio et al. 2014).

To summarize, the cellular identity of actual stem cells and their in vivo dynamics is currently surrounded by some controversy. Clearly, we need more experimental data before reaching a full understanding of the functional structure of the actual stem cell compartment. In any case, cells may transit between multiple states that show different morphologies and patterns of gene expression, and per-

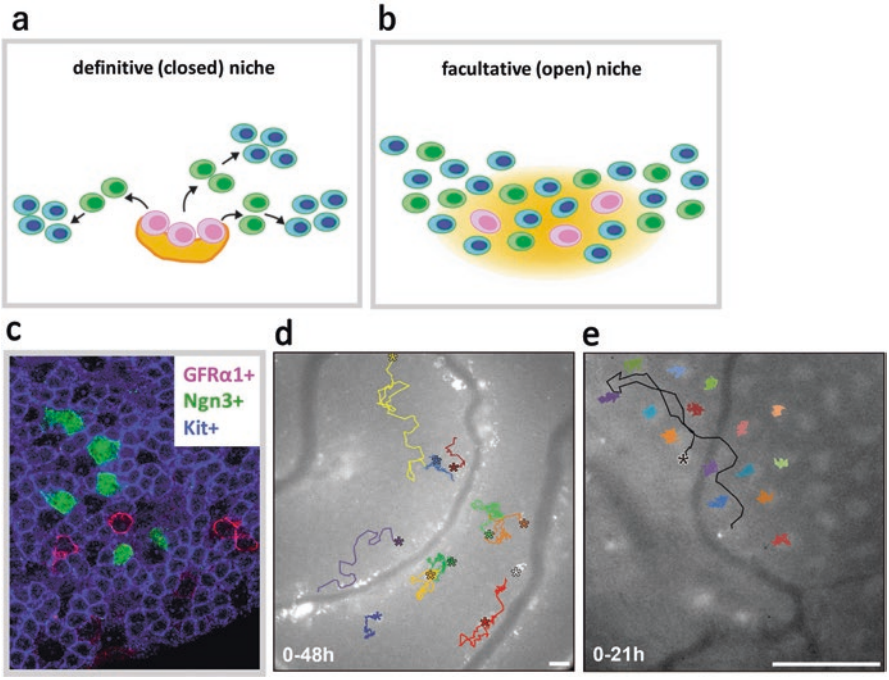

Fig. 4.5 "Defined (closed)" versus "facultative (open)" stem cell niche. (**a**) Schematic representation of a definitive (or closed) stem cell niche. Stem cells (magenta) are clustered to a discrete niche region (orange), while differentiating progeny (green and then blue) occur outside of the niche. The spatial arrangement of cells may recapitulate the process of differentiation. (**b**) Schematic representation of a facultative (or open) stem cell niche, where stem cells (magenta) are intermingled with differentiation progeny (green and blue). (**c**–**e**) Facultative (open) niche features of mouse seminiferous tubules. (**c**) In the basal compartment of seminiferous tubules, GFRα1+ (magenta) cells are intermingled with Ngn3+ (green) and Kit+ (blue) spermatogonia. (**d**) Active migration of GFRα1+ spermatogonia in seminiferous tubules, revealed by intravital live imaging. Trajectories of individual GFRα1 cells over 48 h are shown in different colors, with asterisks indicating the starting points. (**e**) Migration of a GFRα1+ spermatogonium (black trajectory) between Sertoli cells that are not motile (colored trajectories), traced from 21 h of filming. Scale bar, 50 μm. (**c**) Modified from Ikami et al. (2015). (**d**–**e**) Reproduced from Hara et al. (2014)

haps different degrees of self-renewal/differentiation activities, either uni-directionally or bi-directionally with different frequencies (Yoshida et al. 2007a; Yoshida 2016; Krieger and Simons 2015). Therefore, detailed pulse-labeling and intravital live-imaging experiments should be performed on different subpopulations so that one can discriminate between the alternative possibilities shown in Fig. 4.4a–c. In combination with these experiments, single-cell transcriptome analyses (e.g. the pioneering study performed for developing spermatogonia (Hermann et al. 2015)) as well as interdisciplinary studies involving statistical analyses of such multi-dimensional and large-scale data and theoretical modeling will be particularly important.

4.4 Context-Dependent Behavior of "Potential" Stem Cells

Within the population of undifferentiated spermatogonia, *Ngn3* is expressed in a largely reciprocal manner with *Gfrα1* and *Nanos2* (Yoshida et al. 2004; Suzuki et al. 2009; Nakagawa et al. 2010). In undisturbed testes, GFRα1+ cells give rise to Ngn3+ cells, a majority of which differentiate but rarely contribute to the long-term stem cell pool; however, Ngn3+ populations retain the potential to revert back to self-renewing actual stem cells, and this potential becomes apparent during post-insult regeneration and post-transplantation repopulation (Fig. 4.2c) (Nakagawa et al. 2007, 2010; Yoshida et al. 2007a). The aforementioned findings clearly indicate that "stem cells that actually support homeostasis for a long term" and "stem cells that contribute to regeneration or post-transplantation repopulation" are different. In other words, "stem cells" are context-dependent entities.

Potential stem cells may be important for the long-term continuity of spermatogenesis during adulthood: small perturbations to the actual stem cell pool would be rapidly countered by the genesis of actual stem cells from potential stem cells (Nakagawa et al. 2007). As previously mentioned, Ngn3+ cells show the typical properties of potential stem cells (Fig. 4.2c); these cells differentiate into Kit+ differentiating spermatogonia in response to retinoic acid (RA), which occurs in a temporally periodical manner along the seminiferous epithelial cycle (Hogarth et al. 2015; Sugimoto et al. 2012; Vernet et al. 2006). Indeed, most of the stem cell potential of Ngn3+ cells is lost upon transition to Kit+ cells, while a weak post-transplantation repopulation activity of Kit+ cells is reported (Ohbo et al. 2003; Barroca et al. 2009).

Potential stem cells are of particular interests due to their hybrid properties, being capable of both self-renewal and differentiation. Interesting findings have been reported recently, regarding the molecular mechanism underlying this hybrid property of Ngn3+ cells. Consistent with the shared self-renewing potential, Ngn3+ and GFRα1+ cells, but not Kit+ cells, show common characteristics in their global epigenetic status, viz. low level of repressive histone modification (dimethylated Lys9 of histone H3) detected by immunofluorescence, and DNA hypo-methylation. Such global epigenetic changes may be a result of differential expression of DNA methylation enzymes and related factors (Shirakawa et al. 2013). Because the loss of the vast self-renewing potential (to become Kit+) accompanies a dynamic epigenetic change, it is referred to as an "epigenetic checkpoint" (Shirakawa et al. 2013). Thorough investigation will throw light on the molecular mechanisms determining whether cells retain or lose their self-renewing potential.

On the other hand, the differentiation-primed character of Ngn3+ cells can be explained, at least in part, by their differentiation competence in response to RA. An in vivo study using mice deficient of vitamin A, the dietary precursor of RA, demonstrated that exposure to RA causes the Ngn3+ (but not GFRα1+) cells to differentiate to Kit+ cells (Ikami et al. 2015). Here, restricted expression of *RARγ* (a RA receptor family member) in Ngn3+ (but not GFRα1+) cells is critical: disruption of *RARγ* affects the normal differentiation to Kit+ cells, while ectopic expression of

RARγ in GFRα1+ cells confers the differentiation competence to become Kit+ in response to RA, probably skipping the Ngn3+ state (Ikami et al. 2015; Gely-Pernot et al. 2012). Therefore, the Ngn3+ potential stem cells may be considered "stem cells with a high competence of differentiation," rather than "differentiating cells retaining a potential of self-renewal."

In other stem cell-supported systems, Ngn3+ spermatogonia-like cells (viz. those retaining the self-renewing potential but destined for differentiation) have been revealed to play roles in supporting tissue integrity, in particular, during regeneration after insult. These include, among many instances, *Drosophila* male and female germline and mammalian intestinal epithelium (Kai and Spradling 2004; Brawley and Matunis 2004; van Es et al. 2012). Recently, fate analysis of hematopoietic stem cells in an undisturbed situation has been achieved using a pulse-labeling strategy (Sun et al. 2014; Busch et al. 2015). In particular, a novel method of clonal labeling called *barcoding* – a clever combination of induced random integration of a transposable element into the genome and next generation sequencing – enabled an analysis of the clonal evolution of hematopoietic cells, despite their cohorts not being clustered. Intriguingly, the results appear to show different behavior of stem cells in normal homeostasis from the established post-transplantation stem cell behavior (Sun et al. 2014). Therefore, context-dependent flexible change of cell fate selection may be a feature widely shared among tissue stem cells.

4.5 Stem Cell Regulation in Facultative (Open) Niche

Niche is an important factor in the regulation of tissue stem cells. In typical examples of tissues harboring "defined" or "closed" stem cell niches (i.e., mammalian intestinal crypts, bulge of hair follicles, and tips of *Drosophila* testis and ovary), stem cells are clustered to a particular region and sometimes tethered to specialized *niche cells* (Morrison and Spradling 2008; Stine and Matunis 2013) (Fig. 4.5a). In mouse seminiferous tubules, the area of basal compartment proximal to the vasculature network appears to serve as the stem cell niche, to which GFRα1+ and Ngn3+ undifferentiated spermatogonia show biased localizations (Chiarini-Garcia et al. 2001, 2003; Yoshida et al. 2007b; Hara et al. 2014). However, this region represents quite different properties from that of defined niches: GFRα1+ cells are scattered and intermingled with their differentiating progenies (viz. Ngn3+ and Kit+ cells) (Fig. 4.5c). Moreover, GFRα1+ cells are actively migrating over this area, rather than staying in particular positions (Hara et al. 2014) (Fig. 4.5d). Therefore, seminiferous tubules represent "facultative" or "open" niches, characterized by the intermingling of stem cells with differentiating progeny, and the absence of distinct structures that cluster or tether the stem cells (Morrison and Spradling 2008; Stine and Matunis 2013) (Fig. 4.5b). The prominent migration of stem cells may also be a common feature of facultative niche-supported systems, although to the best of the author's knowledge this has (so far) only been observed in mouse seminiferous tubules.

Regardless of being defined or facultative, the ultimate function of stem cell niches is to keep the number of stem cells stable. In the case of a defined niche, its physical size (spatial extension) determines the number of stem cells (or the pool size); cells remain undifferentiated inside the niche, yet they differentiate outside of it. If the niche expands or shrinks, the stem cell number changes proportionally (Kitadate et al. 2007; Kitadate and Kobayashi 2010). This is achieved at least in part by restricted localization of differentiation-inhibiting factor(s) and/or exclusion of differentiation-inducing factor(s) (Morrison and Spradling 2008; Fuller and Spradling 2007).

In facultative niches like mouse seminiferous tubules, it is unlikely that the] localization of differentiation-inhibiting and/or inducing factors governs the stem cell fate, given that stem cells are migrating and intermingle with differentiating cells in a "salt-and-pepper" pattern. Indeed, the concentration of RA, a differentiation-inducing factor, is suggested to be increased over the basal compartment in a spatially uniform manner in particular stages of the seminiferous epithelial cycle (i.e., VII and VIII) (Sugimoto et al. 2012; Hogarth et al. 2015; Vernet et al. 2006). Although both GFRα1+ and Ngn3+ spermatogonia are subjected to a high concentration of RA during these stages of the cycle, only Ngn3+ cells differentiate, while GFRα1+ cells remain undifferentiated and maintain the stem cell pool. As described above, this may be due, at least in part, to the differential responsiveness of GFRα1+ and Ngn3+ cells to RA, which in turn reflects the differential expression of *RARγ* (Ikami et al. 2015). This clarifies a key question: how is the number of GFRα1+ cells kept constant while producing differentiation-primed Ngn3+ cells? The answer is not immediately obvious at this stage.

The facultative niche environment of seminiferous tubules involves many components, which may affect the stem cell dynamics, e.g., basement membrane, Sertoli cells, peritubular myoid cells, macrophages, differentiating germ cells, etc. The crucial role of Sertoli cells in this process is suggested by the observations that a change in Sertoli cell number alters the number of (transplantable) stem cells (Oatley et al. 2011b), and that elevation and suppression of Notch signaling in Sertoli cells causes the decrease and increase of GFRα1+ spermatogonia, respectively (Garcia et al. 2014). A crucial role of macrophages in the regulation of stem cell number has also been suggested (DeFalco et al. 2015). The GDNF protein acts on GFRα1+ spermatogonia and is known as a critical factor for the continuity of spermatogenesis in vivo; in addition, this protein is important for the long-term spermatogonial culture in vitro, maintaining the activity of self-renewal and production of sperm following transplantation (Meng et al. 2000; Kanatsu-Shinohara et al. 2003; Kubota et al. 2004; Hofmann 2008). While Sertoli cells are the major source of GDNF production, the essential role of GDNF expressed by myoid cells has recently been demonstrated (Chen et al. 2014, 2016; Sato et al. 2011). Fibroblast growth factor (FGF) signaling, another essential factor for efficient in vitro cultivation, has also been suggested to be involved in the regulation of GFRα1+ cell number (in particular, that mediated by FGF8 and FGFR1) (Hasegawa and Saga 2014). These extracellular factors should interplay with germ cell-intrinsic factors, which regulate self-renewal and differentiation, such as *Plzf*, *Id4*, and *Etv5* (reviewed by (Song and Wilkinson 2014)).

Elucidation of the detailed mechanisms of regulation by individual factors (on molecular to cellular scales), as well as the overall concept of stem cell pool regulation in seminiferous tubules (on cellular to tissue scales), warrants further investigation. For example, stem cell regulation during the seminiferous epithelial cycle is of particular interest.

4.6 Perspectives

The status of the study of mammalian (mostly mouse) spermatogenic stem cells has been overviewed with an emphasis on the dynamics and context-dependent flexibility in their fate selections. Ongoing and emerging questions have also been pointed out. Mouse spermatogenesis exhibits many interesting properties that may not be so apparent in other well-studied tissues, and therefore provides an invaluable opportunity to capture fundamental features of stem cell dynamics. Interestingly, that which has been observed in this system is often observed in other stem cell systems. It is hoped that the study of spermatogenesis will continue to provide insightful observations and contribute to the general understanding of in vivo stem cell dynamics.

Finally, this chapter is concluded by emphasizing the unique aspect of germline stem cells compared to other tissue stem cells. Gametes are dispensable for the individuals' development or survival, although essential for the genesis of the next generation. This is why animals can modulate their modes of gamete production, so that they can evolve unique strategies of reproduction. For example, seasonally breeding animals dynamically change their activity of gametogenesis in response to environmental cues (e.g., day length or temperature). Although laboratory mice are continual breeders like humans, many mammalian, avian, and reptile species (whose spermatogenesis occurs in seminiferous tubules) breed seasonally in the wild. Regulation of the stem cell modes in these animals warrants future investigations. Knowledge from mice and other continually breeding model animals will provide the foundation to answer this interesting question.

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Chapter 5
Nutritional Control of the Germline Development in *Caenorhabditis elegans*

Masamitsu Fukuyama

Abstract Food is an ultimate regulator of animal reproduction. Because of its invariant cell lineage, ease of synchronized culture, and powerful genetics, the nematode *Caenorhabditis elegans* (*C. elegans*) has served as an excellent model system for delineating the genetic pathways that mediate the nutritional regulation of germline development. *C. elegans* possesses multiple nutritional checkpoints during post-embryonic development that temporally arrest developmental programs in the germline and somatic stem/progenitor cells. The insulin/insulin-like growth factor (IGF) signaling (IIS) pathway and other factors such as adenosine monophosphate (AMP)-activated kinase (AMPK) and mechanistic (or mammalian) Target of Rapamycin Complex 1 (mTORC1) constitute the signaling network dedicated to regulating developmental quiescence of the germline. Furthermore, additional nutrient-responsive pathways adjust the size of the germline stem/progenitor cell pool by altering the balance between self-renewal and differentiation, as well as the balance between cellular survival and death. These findings illustrate the molecular mechanisms that coordinate germline development with the dietary environment by altering the behavior of its stem/progenitor cells.

Keywords *Caenorhabditis elegans* · Germline · Nutrition · Insulin/insulin-like growth factor signaling pathway · Quiescence · Diapause

5.1 Introduction

In the wild, animals routinely experience undernourishment. Food is typically distributed in patches, and its amount and quality not only fluctuate seasonally but can also be limited by inter- and intra-species competition. Thus, animals have clearly

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developed adaptive mechanisms that permit successful reproduction despite fluctuations in food availability. For example, many mammalian species living in the temperate zone enter hibernation during winter to withstand the lack of food and to give birth to live young in the spring as a means of maximizing their chance of encountering ample food resources. In addition to the capacity to anticipate such seasonal changes in food availability, animals including nematodes, insects, and mammals can temporally arrest reproductive development when unexpectedly faced with nutritionally poor environments (Cassada and Russell 1975; Johnson et al. 1984; Bronson and Rissman 1986; Ruaud and Bessereau 2006; Angelo and Van Gilst 2009; Tennessen and Thummel 2011; Schindler et al. 2014). Although this apparently common starvation response has long been documented, its cellular and molecular mechanisms still remain unclear. The nematode *Caenorhabditis elegans* (*C. elegans*) is a powerful model for understanding the nutritional regulation of development because large populations of the nematode larvae can be grown synchronously under the nutritionally optimal condition, and reversible growth arrest can be induced in a robust manner by simply transferring worms to the nutritionally poor condition. In this review, I focus on the genetic mechanisms that link the progression of germline development in *C. elegans* with the dietary environment.

5.2 *C. elegans* Germline Development Under the Nutritionally Replete Condition

Caenorhabditis elegans is an approximately 1-mm long, free-living nematode that feeds on bacteria proliferating in rotten fruits and stems (Frézal and Félix 2015). The sexes of *C. elegans* include hermaphrodites and males, which are determined by the presence of two or one sex chromosome(s) X, respectively. XX hermaphrodites can reproduce by self-fertilization as well as by mating with XO males. Males spontaneously arise among the progeny of self-fertilized hermaphrodites under the standard laboratory culture condition at a frequency of less than 1% (Hodgkin et al. 1979). Recent studies have shown that males are rarely found in natural populations (Félix and Duveau 2012). Thus, almost all of the studies that have explored the nutritional regulation of germline development focused on hermaphrodites. Accordingly, only the germline development of the hermaphrodite is summarized below.

C. elegans has a largely invariant somatic cell lineage and its life cycle is about 3 days under the standard laboratory culture condition. The fertilized zygote undergoes embryogenesis and hatches as a first stage (L1) larva. After hatching out of the eggshell, L1 larvae initiate post-embryonic development and go through four larval (L1–L4) stages, each of which is separated by a molt, and become adults (Fig. 5.1).

During early embryogenesis, the P4 blastomere, which is fated to produce only germ cells, is segregated from the somatic lineage (Sulston et al. 1983). The P4

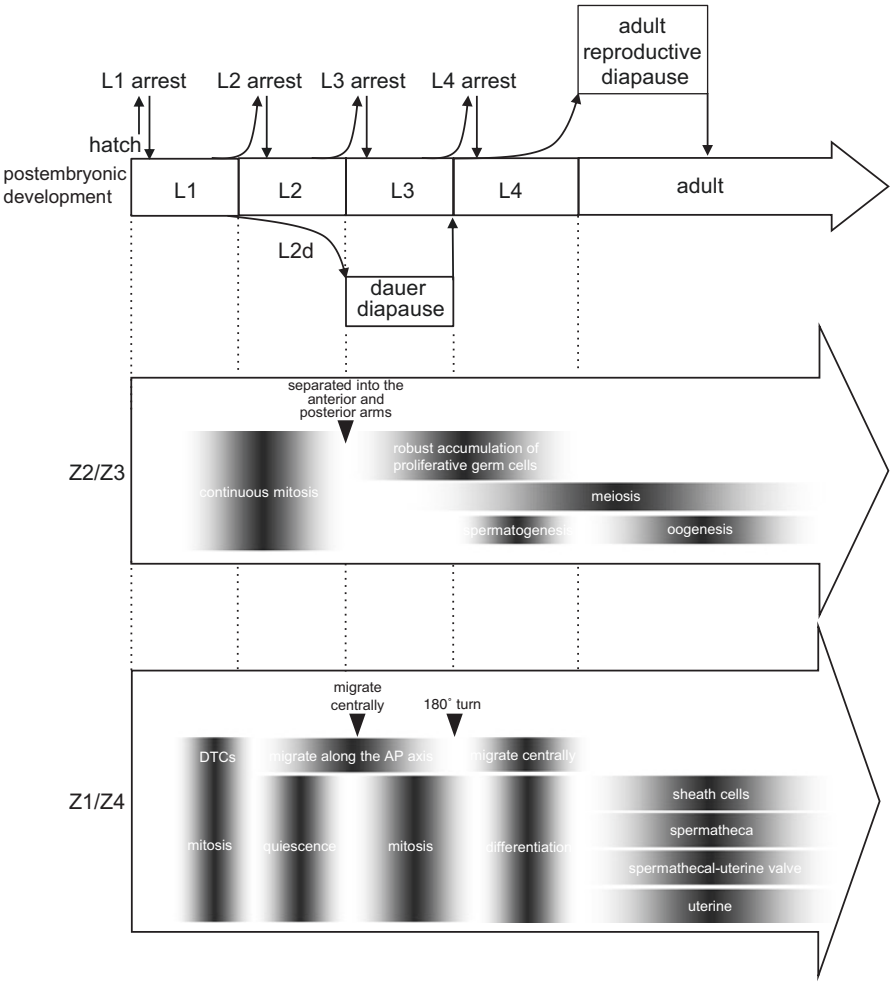

Fig. 5.1 Postembryonic development of the *C. elegans* germline and somatic gonad. The postembryonic development of *C. elegans* is separated into four larval (L1–L4) and adult stages. Nutritional deprivation can induce developmental arrest at each larval stage (L1–L4 arrest) and the suppression of reproductive activity, called adult reproductive diapause (ARD). An increase in the ratio of dauer pheromone/food signals leads to dauer diapause. The Z2/Z3 and Z1/Z4 cells are the precursors of germ cells and the somatic gonad, respectively. Note that the length of shaded boxes does not indicate the duration of one round of the indicated cellular processes such as "mitosis" and "oogenesis." These events are repeated multiple times during the indicated period. See Fig. 5.2 for the anatomy of the somatic gonad and the germline of the adult hermaphrodite. Also see text for details

blastomere undergoes another round of cell division to give rise to the Z2 and Z3 primordial germ cells by mid-embryogenesis, and these cells remain quiescent until newly hatched L1 larvae start feeding on sufficient amounts of bacterial food (Sulston et al. 1983). In the newly hatched larva, Z2 and Z3 cells are sandwiched

between the Z1 and Z4 somatic gonadal precursors. All four cells are encased in a pod comprised of the basal lamina. Post-embryonic development of the germ line and somatic gonad has been described in detail (Hirsh et al. 1976; Kimble and Hirsh 1979; Kimble and White 1981) and is depicted in Fig. 5.1. Under nutritionally favorable conditions, quiescent Z2/Z3 and Z1/Z4 cells become "reactivated" to initiate post-embryonic development during the L1 stage. Z1 and Z4 cells and their descendants undergo a spatiotemporally stereotyped pattern of cell division, migration, and differentiation. In contrast, Z2 and Z3 cells and their descendants continue to proliferate and exhibit variable axes and timing of cell divisions.

The distal tip cells (DTCs) arise from the anterior-most Z1 descendant and the posterior-most Z4 descendant cells at the L1 stage. Located at the distal tips of anterior and posterior gonadal arms, DTCs guide gonadal elongation. The Z1- and Z4-derived DTCs first migrate along the ventral body wall muscles in the anterior and posterior directions, respectively, until L3 larvae molt to become L4 (L3/L4 molt). The DTCs then make a 180° turn and migrate centrally along the dorsal body wall muscles until the L4/adult molt, eventually forming the U-shaped gonad (Fig. 5.2a; Hirsh et al. 1976; Hedgecock et al. 1987). In addition to guiding gonadal elongation, DTCs also prevent differentiation (meiosis) through the GLP-1/Notch signaling pathway (Kimble and White 1981; Austin and Kimble 1987; Henderson et al. 1994; Berry et al. 1997; Nadarajan et al. 2009).

The Z1/Z4 descendants other than the DTCs migrate into the center of the gonad, separating the germ cells into the anterior and posterior arms of the gonad just before the L2/L3 molt. These gonadal progenitor cells later undergo additional cell divisions and eventually differentiate into the sheath cells, the spermathecae (which store sperm), the spermathecal-uterine valve, and the uterus (Fig. 5.2a).

During the L3 and L4 stages, germline stem/progenitor cells undergo robust proliferation. The average number of germline stem/progenitor cells per gonadal arm increases from approximately 30 cells in early L3 larvae, peaks during the late L4 and early adult stages at 200–250 cells, and then subsequently decreases (Killian and Hubbard 2005; Michaelson et al. 2010; Luo et al. 2010; Korta et al. 2012; Dalfó et al. 2012; Gerhold et al. 2015). The distal population of germ cells in both of the gonadal arms remains mitotically active through adulthood, forming the "mitotic zone" or "proliferative zone" (Fig. 5.2b). Conversely, as the germ cells move proximally away from the mitotic zone, they enter meiosis. The most proximal population of about 40 germline stem/progenitor cells per gonad arm initiate meiosis at the mid-L3 stage, and produce approximately 140–160 sperm during the L4 stage (Kimble and White 1981).

After progression to the adult stage, proximal, meiotic germ cells differentiate exclusively into oocytes, and the vulva connection to the uterus opens to the exterior in the middle of the body. Differentiated oocytes are fertilized one by one when passing from the proximal end of the oviduct through the spermatheca to the uterus via the spermathecal-uterine valve. Fertilized embryos from both of the gonadal arms are laid through the vulva. Thus, one can see the developmental sequence of

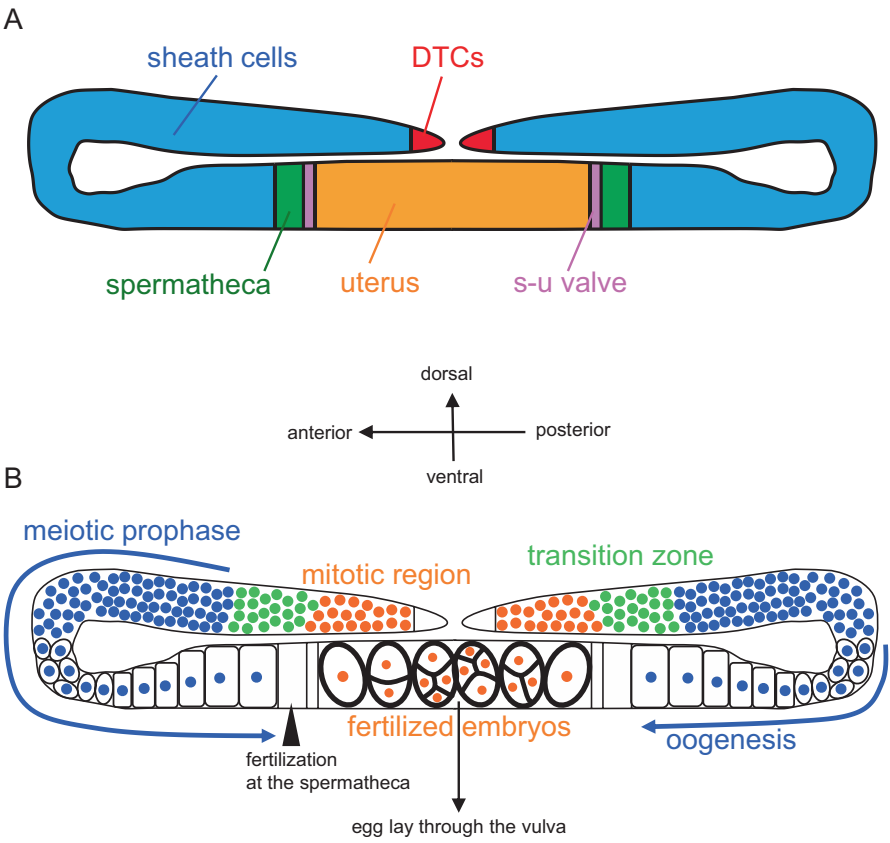

Fig. 5.2 The somatic gonad and germ cells of the adult hermaphrodite. The cartoons indicate the somatic gonad (**a**) and germ cells inside the gonad (**b**). See text for details. s-u valve: spermathecal-uterine valve

the germline all along the distal-proximal axis in the *C. elegans* gonad, providing a powerful experimental model to study the mechanisms that control stem/progenitor cell maintenance and differentiation (Kershner et al. 2013).

The germline is a syncytium by the L2 stage, with a layer of nuclei surrounding the inner wall of the gonad (Hirsh et al. 1976; Kimble and Crittenden 2005). However, each nucleus occupies a membranous alcove-like structure and does not divide synchronously (Hirsh et al. 1976; Kimble and Hirsh 1979); thus, they are referred to as germ "cells" in this review.

5.3 *C. elegans* Germline Development Under the Nutritionally Deprived Condition

C. elegans appears to possess at least one nutritional checkpoint at each larval stage that arrests subsequent developmental processes in response to nutritionally unfavorable conditions (Fig. 5.1). It has been established that when hatched under nutritionally poor conditions, *C. elegans* L1 larvae can suspend larval development for over a week until ample food is supplied (Johnson et al. 1984). This developmental dormancy has often been dubbed "L1 diapause," and has been utilized to prepare synchronized populations of larvae. Recently, it was proposed that L1 diapause be termed "L1 arrest" (Baugh 2013). Thus, "L1 arrest" is used to indicate this developmental dormancy in this review. During L1 arrest, Z2 and Z3 cells, as well as somatic progenitor cells, remain developmentally quiescent (Johnson et al. 1984; Hong et al. 1998; Baugh and Sternberg 2006; Fukuyama et al. 2006). The dormant Z2 and Z3 cells contain a pair of centrosomes, condensed chromosomes, and 4N DNA content (Schaner et al. 2003; Fukuyama et al. 2006). Furthermore, they can undergo mitosis even when fed in the presence of hydroxyurea, which blocks passage through the S phase (Fukuyama et al. 2006). These observations indicate that Z2 and Z3 cells arrest at the G2 phase during L1 arrest.

In addition to L1 arrest, recent studies have shown that nutritional deprivation during the late L1, L2, and L3 stages results in developmentally reversible arrest at the early L2, L3, and L4 stages, called L2, L3, and L4 arrest, respectively (Ruaud and Bessereau 2006; Schindler et al. 2014). However, the behavior of germ cells during these developmentally dormant periods has not yet been elucidated.

During the L4 stage, all of the somatic progenitor cells withdraw from mitosis. When exposed to starvation, some L4 larvae arrest at the L4 stage, whereas others molt and enter adult reproductive diapause (ARD), which involves the extreme suppression of reproductive activity and shrinkage of the germline (Angelo and Van Gilst 2009; Seidel and Kimble 2011; Schindler et al. 2014).

In addition to the developmental arrest induced by food restriction, larvae become morphologically distinct L2 larvae, called L2d larvae, when they are continuously exposed to a high population density with less food from the late L1 stage. Instead of becoming L3 larvae, L2d larvae eventually enter dauer diapause after molting (Fig. 5.1; Popham and Webster 1979; Golden and Riddle 1982; Golden and Riddle 1984; Albert and Riddle 1988). Dauer larvae are morphologically distinct from L3, do not feed, and can survive for months (Cassada and Russell 1975; Klass et al. 1976). Wild-type dauer larvae contain approximately 40 germ cells/gonadal arm (Narbonne and Roy 2006a). Studies using temperature-sensitive mutants, which constitutively enter the dauer diapause at the restrictive temperature, suggest that germline proliferation progressively slows down during L2d and eventually arrests at G2 during dauer diapause (Narbonne and Roy 2006a).

Population density is monitored by the concentration of "dauer pheromone," which is a mixture of ascarosides, derivatives of the dideoxysugar ascarylose (Jeong et al. 2005; Butcher et al. 2007, 2008, 2009a, b; Pungaliya et al. 2009; Srinivasan

et al. 2012). Dauer pheromone promotes dauer formation and inhibits exit from the dauer diapause, and its activity is opposed by an elusive cue termed "food signal," which is found in yeast extract and bacterial cultures (Golden and Riddle 1982, 1984).

Because the *C. elegans* cell lineage is mostly invariant, the stereotypical modes of developmental arrest at multiple developmental time points have been defined at a single-cell resolution for many tissues (Liu and Ambros 1991; Euling and Ambros 1996; Hong et al. 1998; Baugh and Sternberg 2006; Fukuyama et al. 2006; Narbonne and Roy 2006a; Ruaud and Bessereau 2006; Angelo and Van Gilst 2009; Seidel and Kimble 2011; Schindler et al. 2014). This feature provides unique experimental models to explore the nutritional regulation of developmental processes, including germline development. I will overview the emerging mechanisms engaged in coupling germline development to the dietary environment below.

5.4 L1 Arrest (L1 Diapause)

Some components of the highly conserved insulin/insulin-like growth factor (IGF) signaling (IIS) pathway (Fig. 5.3) play a key role in regulating the nutrient response of Z2 and Z3 cells. The strong, temperature sensitive loss-of-function allele of *daf-2/insulin receptor, daf-2(e979)* results in reversible developmental arrest at the L1 stage even in the presence of ample bacterial food at the restrictive temperature (Vowels and Thomas 1992; Gems et al. 1998; Baugh and Sternberg 2006). In such

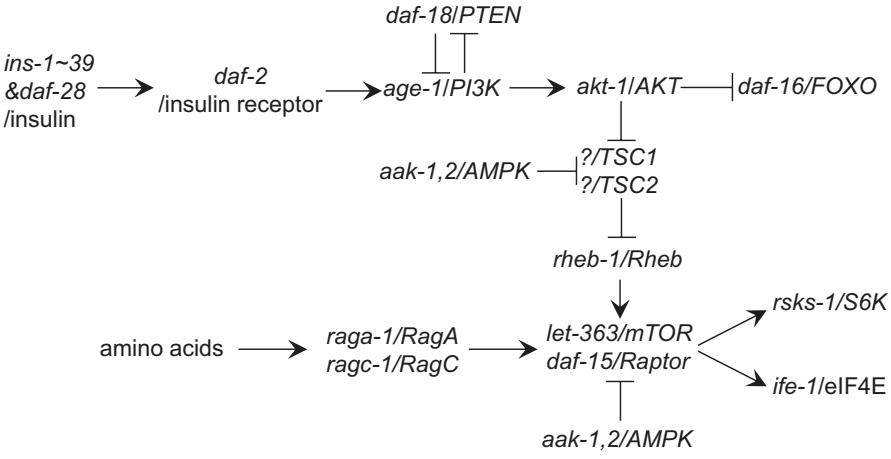

Fig. 5.3 Model of the *C. elegans* IIS and mTORC1 pathways. *C. elegans* gene names/mammalian proteins encoded by the orthologous genes are indicated. Some of the genes in these pathways are omitted. Note that part of the genetic hierarchy is deduced from studies of other organisms and phenotypic similarities in *C. elegans*. Because the *C. elegans* ortholog of TSC1 and TSC2 have not been identified, whether or not *akt-1* regulates the activity of *rheb-1* remains unknown

daf-2/insulin receptor arrested mutant larvae, Z2 and Z3 cells, as well as somatic progenitor cells, have failed to exit from quiescence (Fukuyama et al. 2015).

Conversely, *daf-18/phosphatase and tensin homolog (Pten)*, which negatively regulates the IIS pathway (Fig. 5.3), is required for maintaining the developmental arrest of both Z2/Z3 and somatic progenitor cells during L1 arrest. Newly hatched *daf-18/pten* mutant larvae resume germline proliferation and initiate somatic development even under the nutritionally poor condition (Fukuyama et al. 2006, 2015). The inappropriate germline proliferation in starved *daf-18/Pten* L1 larvae is dependent on *age-1/phosphoinositide 3-kinase (Pi3k)* and *akt-1/Akt (protein kinase B)*, both of which act upstream of *daf-16/forkhead box O (Foxo)* in the IIS pathway to regulate dauer formation and lifespan (Fig. 5.3; Murphy and Hu 2013). However, starved *daf-16/Foxo* null mutant L1 larvae only show inappropriate reactivation of somatic progenitor cells, but not the germline stem/progenitor cells, which suggests that the developmental arrest of somatic and germline stem/progenitor cells is regulated in distinct manners (Baugh and Sternberg 2006; Fukuyama et al. 2006, 2015). Despite this observation, *daf-16/Foxo* might mediate the IIS pathway to maintain quiescence of Z2 and Z3 cells, because *daf-2(e979)* mutant larvae have been reported to bypass L1 arrest and develop into adults even at the restrictive temperature when *daf-16/Foxo* is knocked down by RNAi (Baugh and Sternberg 2006). Although data regarding the fertility status of *daf-2(e979)* and *daf-16(RNAi)* adults are unavailable, the possibility exists that Z2 and Z3 cells were released from mitotic arrest in the experiments, which would suggest that the *daf-2/insulin receptor* modulates Z2/Z3 quiescence via *daf-16/Foxo*.

The IIS pathway in the hypodermis seems to play a critical role in reactivating quiescent Z2 and Z3 cells, because transformed *daf-2(e979)* mutant animals expressing *daf-2/insulin receptor* only in the hypodermis can develop into apparently fertile adults at the restrictive temperature (Fukuyama et al. 2015; Fukuyama, unpublished observation). However, hypodermal expression of *daf-18/Pten* in its starved mutant larvae only suppresses the defect in arresting the somatic, but not the germline development (Fukuyama et al. 2015, Fukuyama, unpublished observation). Furthermore, expression of *daf-18/Pten* in the nervous system, body wall muscles, and the intestine does not suppress the germline defect in mutant animals (Fukuyama et al. 2015; Fukuyama, unpublished observation). Consistent with these findings, expression of a constitutively active form of AKT-1/AKT in the hypodermis, the nervous system, and the intestine does not have any influence on the mitotic arrest of Z2 and Z3 cells in starved, newly hatched wild type larvae. However, its hypodermal expression can efficiently cause the neural and mesodermal progenitors to initiate post-embryonic development (Fukuyama et al. 2015). These observations suggest that in addition to the hypodermis, *daf-18/Pten* and *akt-1/Akt* in a tissue other than the hypodermis, the nervous system, body wall muscles, and the intestine, contribute significantly to the nutritional response of Z2 and Z3 cells. Considering that introducing *daf-2/insulin receptor* activity only in the hypodermis of its strong loss-of-function mutants is sufficient to reactivate quiescent Z2 and Z3 cells, the activity of *daf-18/Pten* and *akt-1/Akt* in a tissue other than the hypodermis may act in the non-IIS pathway (Fig. 5.4).

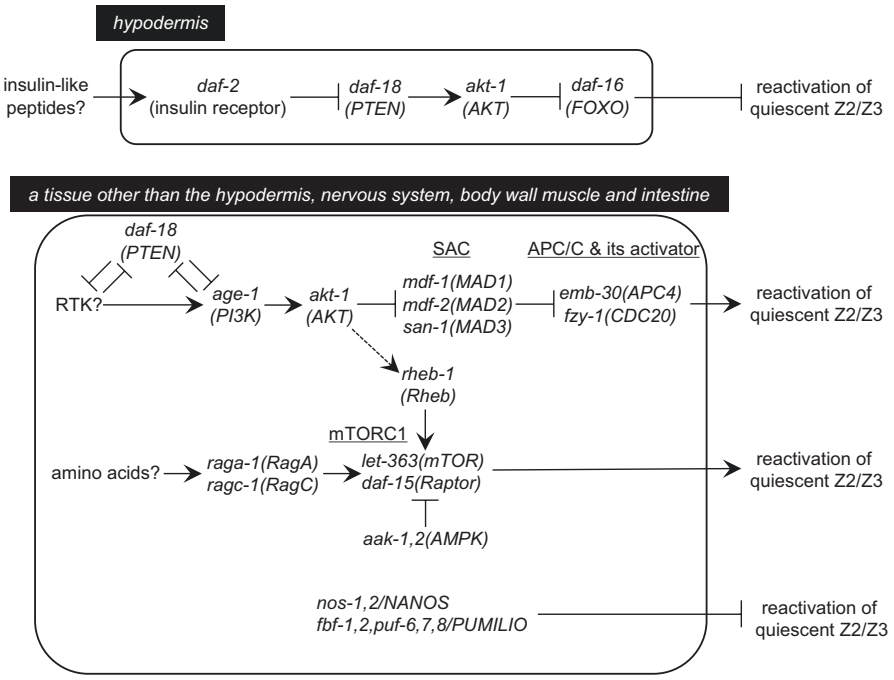

Fig. 5.4 Speculative model of the regulation of Z2/Z3 reactivation. *C. elegans* gene names are followed by their mammalian orthologs (parentheses). This model predicts that the canonical IIS pathway in the hypodermis and the non-IIS pathway that involves *daf-18/Pten* and *akt-1/Akt* in an unidentified tissue regulate reactivation of quiescent Z2 and Z3 cells. Because both NOS-1 and NOS-2 are enriched in the germline during embryogenesis (Subramaniam and Seydoux 1999), these proteins and their putative binding partners, the *fem-3* binding factor (FBF) and Pumilio and FBF (PUF) proteins may act in the germline. Conversely, whether or not the site of action of the non-IIS and TORC1 pathways is the germline remains to be examined. Some of the conserved genes engaged in regulation of the IIS pathway, mTORC1, SAC, and APC/C are omitted because their involvement in the control of Z2/Z3 reactivation has not been established

Because *daf-18/Pten* regulates the quiescence of Z2 and Z3 independently of *daf-16/foxo*, several genes that potentially act downstream of *akt-1/Akt* have been proposed. The Spindle Assembly Components (SAC) is a conserved protein complex that delays mitosis by inhibiting the activity of a ubiquitin ligase complex known as Anaphase Promoting Complex/Cyclosome (APC/C) when the mitotic spindles fail to attach every chromosome (reviewed in Foley and Kapoor 2013). Watanabe et al. reported several lines of genetic and biochemical evidence that *akt-1/Akt* promotes the transition from the G2 phase to mitosis by its inhibitory phosphorylation of the SAC component MDF-1/MAD1 (Watanabe et al. 2008).

Similar to loss-of-function *daf-18/Pten* mutations, inhibition of *mdf-1/mad1* as well as other SAC components, including the *C. elegans* orthologs of human MAD2 and MAD3 encoded by the *mdf-2* and *san-1* genes (Kitagawa and Rose 1999; Stein et al. 2007), results in inappropriate proliferation of both Z2/Z3 germline precursors

and epidermal progenitor V cells during L1 arrest. Ectopic proliferation caused by impairment of *mdf-1/MAD1* requires activity of *emb-30* and *fzy-1*, which encode the APC/C component APC4 and APC/C activator CDC20, respectively (Furuta et al. 2000; Kitagawa et al. 2002). However, both the *fzy-1/cdc20* mutation and expression of a non-phosphorylatable form of MDF-1 can only partially suppress the overproliferation in *daf-18/Pten* mutant animals. These observations led Watanabe et al. to propose that there may be other AKT-1/AKT targets whose phosphorylation contributes to inappropriate germ cell proliferation in *daf-18/Pten* mutant animals (Watanabe et al. 2008).

Consistent with this idea, mechanistic (or mammalian) Target of Rapamycin Complex 1 (mTORC1), whose mammalian counterpart acts downstream of AKT (Fig. 5.3), has been implicated in the regulation of the germline quiescence during L1 arrest (Fig. 5.4; Fukuyama et al. 2012). mTORC1 is a conserved serine/threonine protein kinase complex that includes the catalytic subunit mTOR and its scaffold Raptor, which recruits substrates to the complex (reviewed in Sengupta et al. 2010; Laplante and Sabatini 2012). In mammalian cultured cells, the activation of AKT kinase by insulin and insulin-like growth factor 1 (IGF1) stimulates the small GTPase Rheb by downregulating its GTPase Activating Protein (GAP) complex, which consists of tuberous sclerosis 1 (TSC1) and TSC2 tumor suppressors (Inoki et al. 2002; Potter et al. 2002); further, GTP-loaded Rheb activates mTORC1 (Long et al. 2005; Sancak et al. 2007). In addition to insulin and IGF1, full activation of mTORC1 requires input from amino acids through Rag GTPases (Sancak et al. 2008; Kim et al. 2008). Rag GTPases are highly conserved from yeast to mammals, and can be divided into two subfamilies that form obligate heterodimers, one composed of orthologs of mammalian RagA and RagB and the other containing RagC and RagD orthologs.

Although the process is not effective, amino acids can release Z2 and Z3 cells from quiescence in the presence of ethanol, which is utilized by *C. elegans* to synthesize fatty acids (Castro et al. 2012; Fukuyama et al. 2015). This raises the possibility that mTORC1 promotes proliferation of Z2 and Z3 cells in response to dietary amino acids. *C. elegans* possesses singular orthologs of mTOR, Raptor, Rheb, RagA/B, and RagC/D, which are encoded by *let-363*, *daf-15*, *rheb-1*, *raga-1*, and *ragc-1*, respectively (Fig. 5.3; Hara et al. 2002; Long et al. 2002; Jia et al. 2004; Li et al. 2004). Inhibition of each of these genes can partially suppress the inappropriate proliferation of germline stem/progenitor cells in *daf-18/Pten* mutant animals during L1 arrest (Fukuyama et al. 2012). In addition to insulin/IGF1 and amino acids, mammalian AMP-activated kinases (AMPKs), which are activated by an increased cellular AMP/ATP ratio, have been shown to suppress mTORC1 activity through its inhibitory phosphorylation of Raptor and stimulatory phosphorylation of TSC1/2 (Fig. 5.3; Inoki et al. 2003; Gwinn et al. 2008). Similar to *daf-18/Pten*, simultaneous loss of *aak-1* and *aak-2*, both of which encode the α-catalytic subunit of AMPK, causes defects in maintaining the quiescence of Z2 and Z3 cells during L1 arrest (Fukuyama et al. 2012). This phenotype also at least partially requires *let-363/mTOR*, *daf-15/Raptor*, *rheb-1/Rheb*, *raga-1/RagA*, and *ragc-1/RagC*. These findings imply that *daf-18/Pten* and *aak-1,2/Ampk* control starvation-induced quiescence in Z2 and Z3 cells by suppressing mTORC1 activity (Fig. 5.4). Neither

the TSC1 nor the TSC2 ortholog is readily identifiable by sequence homology in the *C. elegans* genome. However, this does not preclude the existence of a similarly functioning complex. Thus, whether the *C. elegans* IIS pathway directly controls mTORC1 activity remains to be demonstrated.

Although almost all of the wild type animals can grow into fertile adults when brought back to the nutritionally optimum condition after several days of L1 arrest, significant populations of both *daf-18/Pten* and *aak-1,2/Ampk* mutant larvae become sterile after the same experience (Watanabe et al. 2008; Fukuyama et al. 2012). Because most of the *daf-18/Pten* and *aak-1,2/Ampk* mutant animals that are grown continuously in the nutritionally replete condition after hatching are fertile, undergoing L1 arrest causes this sterility. Nonetheless, whether failure to maintain the germline quiescence directly triggers the sterility, or if other cellular mechanisms cause the sterility, has not yet been rigorously investigated. However, these findings clearly illustrate the significance of nutritional control of germline development for successful reproductive growth.

In addition to the aforementioned genes, *nos-1* and *nos-2*, both of which encode orthologs of the *Drosophila* translational repressor *nanos*, are reportedly required for preventing Z2 and Z3 cells from precocious proliferation in unfed, newly hatched larvae (Subramaniam and Seydoux 1999). Z2 and Z3 cells lose immunoreactivity against di- and tri-methylated lysine 4 in histone H3 (H3meK4) and acetylated lysine 8 in histone H4 (H4acetyleK8) during embryogenesis. This immunoreactivity is undetectable during L1 arrest and only becomes detectable once L1 larvae are fed and initiate post-embryonic development (Schaner et al. 2003). Although the physiological significance of these changes in histone modification remains elusive, *nos-1* and *nos-2* are also required for this reduction in the immunoreactivity against H3meK4 during embryogenesis (Schaner et al. 2003).

In *Drosophila*, Nanos is recruited to the Nanos response elements (NREs) in the 3′ untranslated region of *hunchback* mRNA by Pumilio, which directly binds to the NREs (Murata and Wharton 1995; Sonoda and Wharton 1999). Simultaneous inhibition of five *C. elegans pumilio*-like genes, *fbf-1*, *fbf-2*, *puf-6*, *puf-7*, and *puf-8* also results in inappropriate proliferation of Z2 and Z3 cells during L1 arrest, implying that *nos-1* and *nos-2* function with these *pumilio*-like genes to maintain the germline quiescence (Subramaniam and Seydoux 1999). Inhibition of *nos* and *pumilio*-like genes also interferes with the proper migration of Z1 and Z4 cells to Z2 and Z3 cells during embryogenesis and causes ectopic cell death in their descendants. Strikingly, *Drosophila* primordial germline progenitors lacking *nanos* and *pumilio* also exhibit similar phenotypes (Kobayashi et al. 1996; Asaoka-Taguchi et al. 1999; Schaner et al. 2003), suggesting that the physiological roles of the Nanos-Pumilio complex in germ cells are conserved between these two species. However, whether the IIS pathway, SAC, and the dietary environment regulate the activity of the protein complex remains to be elucidated in *C. elegans* and *Drosophila*.

In parallel with the IIS pathway, d17iso-glucosylceramide, a sphingolipid derived from monomethyl branched-chain fatty acids (mmBCFAs), has been shown to promote post-embryonic development in several somatic lineages via the activity of mTORC1 in the intestine (Kniazeva et al. 2004, 2008; Zhu et al. 2013). Whether

post-embryonic proliferation of germ cells requires mmBCFAs remains to be examined. Previous studies have shown that mTORC1 primarily acts autonomously in the germ line to promote its post-embryonic development (Korta et al. 2012). However, it would still be interesting to test whether the intestinal activity of mTORC1 contributes to the exit of Z2 and Z3 cells from mitotic quiescence in the future.

5.5 Dauer Diapause

Approximately 40 *dauer larvae formation* (*daf*) genes are required for either promoting or preventing dauer formation (reviewed in Fielenbach and Antebi 2008 and Ludewig and Schroeder 2013). As well, loss-of-function mutations in the former *daf* genes result in defects in dauer formation, called the Dauer-defective, or Daf-d phenotype, whereas those in the latter *daf* genes induce constitutive formation of dauer, called the Dauer-constitutive, or Daf-c phenotype. *daf* genes encode components involved in the biosynthesis of dauer pheromone, proper formation of ciliated sensory neurons, cyclic Guanine Monophosphate (cGMP) signaling, IIS (Fig. 5.3), Transforming Growth Factor (TGF)-β signaling (Fig. 5.5), and steroid hormone signaling pathways. An ortholog of the human sterol carrier protein (SCPx) involved in the biosynthesis of dauer pheromone is encoded by *daf-22*, which acts far upstream in the genetic pathway that regulates dauer formation (Golden and Riddle 1985; Butcher et al. 2009a, b). The ciliated head sensory neurons, called amphid neurons, express not only multiple dauer pheromone receptors, but also components of the cGMP signaling pathway, *daf-7/TGF-β* and *daf-28/insulin-like peptide* (Coburn and Bargmann 1996; Komatsu et al. 1996; Birnby et al. 2000; Murakami et al. 2001; Li et al. 2003; Kim et al. 2009; Park et al. 2010; McGrath et al. 2011). Genetic studies led to the establishment of a model that indicates that *daf-6*, which is required for proper formation of ciliated sensory neurons, acts upstream of the cGMP signaling, IIS, and TGF-β signaling pathways and that these pathways function in parallel to prevent entry to the dauer diapause and/or to promote progression to the L3 stage (Albert et al. 1981; Vowels and Thomas 1992; Thomas et al. 1993; Gottlieb and Ruvkun 1994; Larsen et al. 1995; Riddle and Albert 1997). However, subsequent studies have shown that inhibition of *daf-11/transmembrane guanylyl cyclase* reduces expression of the reporter gene containing the *daf-7/TGF-β promoter* fused to the *green fluorescent protein (gfp)* gene (Murakami et al. 2001). In addition,

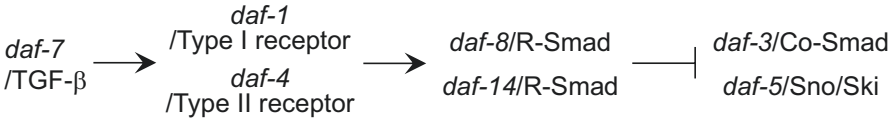

Fig. 5.5 Regulation of dauer formation in *C. elegans* by the TGF-β signaling pathway. The *C. elegans* gene names and mammalian proteins encoded by the orthologous genes are indicated

expression of the *daf-28/insulin-like peptide gfp* reporter gene is downregulated in *daf-11/transmembrane guanylyl cyclase* and *daf-7/TGF-β* single loss-of-function mutants (Murakami et al. 2001; Li et al. 2003). These findings suggest that the cGMP and TGF-β signaling and IIS pathways interact with each other.

The steroid hormone signaling pathway is mediated by *daf-12*, which encodes a nuclear receptor similar to the vertebrate vitamin D and liver-X receptors, and by its ligands, the bile acid-like steroids called dafachronic acids (Antebi et al. 2000; Snow and Larsen 2000; Motola et al. 2006). Genetic analysis suggests that the steroid hormone signaling pathway acts downstream of the cGMP and TGF-β signaling and IIS pathways (Riddle et al. 1981; Vowels and Thomas 1992; Thomas et al. 1993; Gottlieb and Ruvkun 1994; Larsen et al. 1995; Gems et al. 1998; Gerisch and Antebi 2004). Consistent with this indication, *gfp* reporter genes of *daf-9*, which encodes a functional homolog of mammalian cytochrome P450, CYP27A1 and is required for the biosynthesis of dafachronic acids (Motola et al. 2006), are upregulated in *daf-11/transmembrane guanylyl cyclase*, *daf-7/TGF-β*, and *daf-28/insulin-like peptide* loss-of-function mutants (Gerisch and Antebi 2004; Mak and Ruvkun 2004). Furthermore, the IIS pathway likely acts both upstream of and in parallel with or downstream of the steroid hormone signaling pathway, because the administration of a dafachronic acid to loss-of-function *daf-2/insulin receptor* mutant animals circumvents dauer diapause, but still results in developmental arrest at the L3 stage (Motola et al. 2006).

Experiments using a temperature-sensitive, *daf-c daf-2/insulin receptor* mutant animals suggest that, as larvae pass through L2d and become dauer larvae, germ cells progressively slow their division rate and finally undergo mitotic arrest during the diapause (Narbonne and Roy 2006a). Larvae harboring a *daf-c* mutation in the *daf-2/insulin receptor* and *daf-7/TGF-β* develop into dauer larvae with supernumerary germ cells when the activity of *daf-18/Pten* and *aak-1,2/Ampk* is inhibited (Narbonne and Roy 2006a; Narbonne et al. 2010). Examination of the germ cell number in *daf-2/insulin receptor; aak-2/Ampk* double mutant animals during L2d and dauer formation suggested that they fail to appropriately slow the rate of germline proliferation. Despite this, their germ cells become quiescent after the mutant larvae enter the dauer diapause (Narbonne and Roy 2006a). Tumor suppressor Liver Kinase B1 (LKB1) is a serine/threonine kinase whose mutations are responsible for Peutz-Jeghers syndrome, a hereditary disorder that predisposes patients to many types of cancer (Jenne et al. 1998; Hemminki et al. 1998). Mammalian LBK1, which is complexed with STE20 related adaptor (STRAD) and morula 25 (MO25), is a major upstream kinase that activates AMPK through direct phosphorylation of the conserved threonine (Hong et al. 2003; Hawley et al. 2003; Woods et al. 2003; Shaw et al. 2004). Amino acid residues surrounding the threonine in AAK-2/AMPK are conserved extensively enough to allow its phosphorylation to be detected through the use of an antibody against the phospho-Thr^{172} of human AMPKα (Lee et al. 2008). Similar to loss of *aak-1,2/Ampk*, inhibition of *par-4* and *strd-1*, which encode the *C. elegans* orthologs of LKB1 and STRAD, respectively, also results in defects in limiting the proliferation of germ cells to prepare for dauer diapause and apparent elimination of AAK-2/AMPK phosphorylation on the conserved threonine,

underscoring the physiological importance of the phosphorylation (Lee et al. 2008; Narbonne et al. 2010). Experiments using *rrf-1* mutant animals in which RNAi was primarily effective in the germline (Sijen et al. 2001; Kumsta and Hansen 2012) implied that *aak-2/Ampk* acts in the germline to coordinate its proliferation with entry into dauer diapause (Narbonne and Roy 2006a).

It should be emphasized that simultaneous inhibition of *aak-1* and *aak-2* apparently does not interfere with overt dauer formation in *daf-2/insulin receptor* and *daf-7/TGF-β* mutant animals (Narbonne and Roy 2006a; Narbonne et al. 2010), which suggests that *aak-1,2/Ampk* are engaged in the developmental arrest of only germline and not somatic tissues. In contrast, *daf-18/Pten*, as its name suggests, acts in the IIS pathway to regulate multiple events of dauer formation, which includes not only developmental quiescence in both germline and somatic lineages, but also physiological changes and remodeling of several tissues (Riddle et al. 1981; Vowels and Thomas 1992; Gottlieb and Ruvkun 1994; Larsen et al. 1995; Morris et al. 1996; Kimura et al. 1997; Ogg et al. 1997; Gems et al. 1998; Ogg and Ruvkun 1998).

Several observations suggest that *daf-18/Pten* probably mediates both the canonical IIS and non-IIS pathways in separate tissues to control germline quiescence, which is reminiscent of the regulation of Z2/Z3 quiescence during L1 arrest (Fig. 5.4). The canonical IIS pathway likely acts primarily in the nervous system and/or the intestine to regulate dauer formation at the organismal level. Previous studies have shown that expression of *daf-2/insulin receptor* and *age-1/Pi3k* in the nervous system allows their corresponding mutant animals to efficiently bypass dauer arrest and develop into fertile adults (Apfeld and Kenyon 1998; Wolkow et al. 2000). In accord with this observation, neuronal expression of *daf-16/Foxo* enables *daf-2/insulin receptor; daf-16/Foxo* double mutant animals to restore dauer formation (Libina et al. 2003; Narbonne and Roy 2006b). However, recent studies have reported that expression of exogenous *age-1* in the intestine also enables its null mutant animals to develop into fertile adults as neuronal expression does (Iser et al. 2007). Furthermore, other studies have shown that expression of both *daf-2/insulin receptor* and *daf-16/Foxo* in the intestine can rescue the Daf-c phenotype in *daf-2/insulin receptor*, and the Daf-d phenotype in *daf-2/insulin receptor; daf-16/Foxo* mutant animals, respectively, whereas expression in the nervous system cannot (Hung et al. 2014). What causes this discrepancy remains to be determined. However, these observations suggest that the canonical *daf-2-daf-16* IIS pathway in somatic tissues makes significant contributions to developmental arrest in both germline and somatic lineages upon entry into dauer diapause. In contrast, neither neuronal nor intestinal expression of *daf-18/Pten* can suppress the gonadal growth (and presumably the proliferation of germline stem/progenitor cells) in *daf-2/insulin receptor; daf-18/PTEN* animals although it can induce several dauer-specific somatic events such as growth arrest, high intestinal fat, alae formation, and radial constriction (Masse et al. 2005). Despite this, *daf-18/Pten* in the nervous system and/or the intestine likely contributes to the suppression of gonadal growth and germline proliferation, because the gene mediates the IIS pathway downstream of

daf-2/insulin receptor and upstream of *daf-16/Foxo*. Moreover, the aforementioned observations suggest that *daf-18/Pten* also acts in a tissue distinct from the nervous system and the intestine to modulate the gonadal and germline growth, possibly via the non-IIS pathway. In addition to the IIS pathway, *daf-18/Pten* reportedly antagonizes *vab-1*, which encodes a *C. elegans* ortholog of the Eph receptor tyrosine kinase, through its protein phosphatase activity (Brisbin et al. 2009). Thus, VAV-1/Eph or other receptor tyrosine kinases might be involved in the developmental arrest of somatic gonad and germline during dauer diapause.

Similar to the IIS pathway, the TGF-β signaling pathway acts in the nervous system to regulate dauer diapause (Inoue and Thomas 2000; da Graca et al. 2004). In the soma, these pathways might control the biosynthesis of dafachronic acids, which ultimately modulates the activity of *daf-12*(see Fielenbach and Antebi 2008). In addition to *daf-18/Pten*, *daf-12* also appears to regulate somatic and germline events of dauer formation in distinct manners. Genetic studies have suggested that *daf-12* acts downstream of both the IIS and TGF-β signaling pathways to regulate dauer formation (Riddle et al. 1981; Vowels and Thomas 1992; Thomas et al. 1993; Gottlieb and Ruvkun 1994; Larsen et al. 1995; Gems et al. 1998). Phenotypic analysis of various *daf-12* alleles suggests that the ligand-bound form of the DAF-12 protein prevents dauer formation and promotes reproductive growth under favorable conditions, whereas its unliganded form induces dauer formation under unfavorable conditions (Riddle et al. 1981; Antebi et al. 1998; Ludewig and Schroeder 2013). Mutations that impair only ligand-bound DAF-12 result in "partial-dauer" larvae, which contain supernumerary germ cells (Antebi et al. 1998; Antebi et al. 2000). Similarly, a null mutation in *daf-9* likewise causes partial dauer phenotypes (Albert and Riddle 1988; Gerisch et al. 2001; Jia et al. 2002). These observations suggest that ligand-bound DAF-12 only prevents somatic events of dauer formation under favorable conditions. In contrast, dauer-defective *daf-12* mutations, which reduce the activity of both liganded and unliganded DAF-12, can suppress constitutive formation of dauer larvae caused by the inhibition of the IIS and TGF-β signaling pathways (Riddle et al. 1981; Vowels and Thomas 1992; Thomas et al. 1993; Gottlieb and Ruvkun 1994; Larsen et al. 1995; Gems et al. 1998), which implies that unliganded DAF-12 promotes developmental arrest of both somatic tissues and germline under unfavorable conditions. The *gfp* reporter assay suggests that *daf-12* is ubiquitously expressed (Ludewig and Schroeder 2013). Because of this and phenotypic mosaicism, *daf-12* is thought to regulate dauer formation in a cell-autonomous manner (Antebi et al. 2000; Fielenbach and Antebi 2008). However, whether *daf-12* acts autonomously to modulate germline proliferation remains unclear.

5.6 The Physiological Significance of G2 Arrest

Somatic cells in animals including *C. elegans* generally become quiescent at the G0/G1phase (Hedgecock and White 1985; Hong et al. 1998; Cheung and Rando 2013). In contrast, during development in *C. elegans*, *Drosophila*, and mice, germline stem

and progenitor cells have been found to undergo G2 arrest (Su et al. 1998; Narbonne and Roy 2006a; Fukuyama et al. 2006; Clejan et al. 2006; Seki et al. 2007). Why do somatic and germ cells arrest the cell cycle at distinct phases? One of the most devastating threats to the genome integrity is a DNA double-strand break (DSB). The majority of spontaneous DSBs occur during DNA replication (Pfeiffer et al. 2000; Cox 2001). However, mechanical stresses and reactive oxygen species from cellular metabolism can generate DSBs even in quiescent cells (Lieber 2010). There are two major pathways for the repair of DSBs: error-prone nonhomologous end-joining (NHEJ) and error-free homologous recombination (HR; Symington and Gautier 2011). NHEJ directly joins the broken ends of DNA, and frequently results in small deletions and insertions. Conversely, HR uses an intact homologous sequence primarily in a sister chromatid as a template for the repair of the broken ends. Yeast and cultured vertebrate cells during the late S and G2 phases have been shown to be more resistant to ionizing radiation, which causes DSBs, than cells during the G1 and early S phases (Terasima and Tolmach 1961; Hatzfeld and Williamson 1974; Brunborg and Williamson 1978; Giaccia et al. 1985; Jeggo 1990; Kadyk and Hartwell 1992; Takata et al. 1998). In cultured vertebrate cells, this heightened resistance during the late S and G2 phases is mainly attributable to enhanced HR activity that is restricted during this period of the cell cycle (Takata et al. 1998; Rothkamm et al. 2003; Hinz et al. 2005; Jazayeri et al. 2005). Although it is currently unknown whether HR is also regulated in a cell-cycle dependent manner in *C. elegans*, Ahmed and colleagues have shown that HR is primarily used to repair radiation-induced DNA damage in germ cells (Clejan et al. 2006). Thus, the G2 arrest might ensure error-free HR for the repair of DSBs in germ cell progenitors (Clejan et al. 2006).

The G2 arrest may also allow germ cells to be refractory to cues that trigger differentiation. In other words, somatic stem and progenitor cells might have to stay at G0/G1 to be responsive to multiple signals that instruct cell fate at the risk of suffering mutations caused by NHEJ. To this end, several findings support the latter notion. Human embryonic stem cells (hESCs) at G1 are reportedly more competent to differentiation than those in S and G2 (Sela et al. 2012). Furthermore, hESCs can only commit to endoderm and neuroectoderm differentiation in early and late G1, respectively (Pauklin and Vallier 2013). Similarly, separate cell fate decisions in *C. elegans* vulval precursor cells take place sequentially in the late G1/early S phase and upon completion of the S phase (Ambros 1999).

5.7 Robust Accumulation of Germline Stem/Progenitor Cells During Later Larval Stages

The robust germline growth during the late larval stages is significantly impaired by the temperature-sensitive, reduction-of-function mutation in the *daf-2/insulin receptor* gene at the semi-permissive temperature (Michaelson et al. 2010). Comparison of the M and S phase indices and DNA content measurements between wild type and the *daf-2/insulin receptor* mutant animals suggest that the gene contributes to the rapid accumulation of germ cells primarily by regulating the cell cycle of stem/progenitor cells.

The reduction in the number of germline stem/progenitor cells in *daf-2/insulin receptor* mutant animals can be suppressed by knocking down *daf-18/Pten* and *daf-16/Foxo*. Rescue and knockdown experiments involving the manipulation of the genetic activity of these genes in tissue-specific fashions suggest that the canonical IIS pathway consisting of *daf-2/insulin receptor*, *daf-18/Pten*, and *daf-16/Foxo* acts primarily in the germline. Moreover, similar to dauer formation, the germline defect in *daf-2/insulin receptor* mutants is also suppressed by knockdown of *daf-12* (Dalfó et al. 2012).

C. elegans possesses 40 *ins* genes encoding insulin-like peptides (Duret et al. 1998; Kawano et al. 2000; Pierce et al. 2001; Li et al. 2003). RNAi targeting two of these genes, *ins-3* and *ins-33*, has been found to interfere with the robust germline proliferation after the mid-L3 stage in a manner dependent on both *daf-18/Pten* and *daf-16/Foxo*, suggesting that the IIS pathway acts downstream of these *ins* genes. Genetic analysis suggests that both *ins-3* and *ins-33* act in the soma, and that the reporter genes for *ins-3* and *ins-33* are expressed in various types of cells and tissues (Michaelson et al. 2010; Ritter et al. 2013). Although expression of both of the *ins* genes are reportedly induced during L1 development (Chen and Baugh 2014), whether their expression, secretion, and/or activity are regulated by the dietary environment during later larval development remains unknown.

In addition to the IIS pathway, the TGF-β signaling pathway also promotes the robust accumulation of germline stem/progenitor cells (Dalfó et al. 2012). Adult animals carrying loss-of-function mutations in genes that mediate the TGF-β signaling pathway including *daf-7/TGF-β*, *daf-1/Type I receptor*, and *daf-8*,*14*/*R-Smad*, show a significant decrease in the number of germ cell nuclei in the mitotic zone. The defect observed in all of these mutant animals can be suppressed by reducing the activity of *daf-3/Co-Smad* and *daf-5/Sno/Ski*, conserved downstream components of the canonical TGF-β signaling pathway. In contrast, loss of *daf-12* does not suppress the germline defect in *daf-7/TGF-β* mutants, suggesting that *daf-7/TGF-β* controls the size of the stem/progenitor cell pool through a pathway that is distinct from the pathway that regulates dauer formation.

Inhibition of the TGF-β signaling pathway does not slow down the cell cycle or induce ectopic activation of apoptosis in the mitotic zone. Instead, compared to wild type, reduced activity of *daf-7/TGF-β* causes stem/progenitor cells to enter meiosis

at the point closer to the DTCs, which is reminiscent of the phenotypes observed in *glp-1/Notch* mutants and suggests that the TGF-β signaling pathway either promotes self-renewal and/or suppresses differentiation into oocytes (Michaelson et al. 2010). Rescue and knockdown experiments and expression analyses have suggested that the site of action of the pathway is DTCs (see Fig. 5.2a), which also expresses *glp-1/Notch* ligands, *lag-2* and *apx-1* (Henderson et al. 1994; Nadarajan et al. 2009). This raises the hypothesis that the TGF-β signaling pathway regulates germ cell behaviors via the *glp-1/Notch* pathway. Although this possibility has not been excluded experimentally, genetic analysis suggests that the TGF-β signaling pathway works through a mechanism that acts in parallel with the *glp-1/Notch* pathway (Dalfó et al. 2012).

Previous studies have demonstrated that the *gfp* reporter gene of *daf-7/TGF-β* is primarily expressed in the pair of ASI chemosensory neurons in the head (Ren et al. 1996; Schackwitz et al. 1996). Food and the lower temperature (15 °C) promote expression of the *gfp* reporter genes in the ASI neurons, whereas dauer pheromone and the higher temperature (25 °C) downregulate the expression (Ren et al. 1996; Schackwitz et al. 1996). Expression of *daf-7/TGF-β* only in the ASI neurons of its loss-of-function mutant animals can suppress the reduction in the number of germ cells in the mitotic zones (Dalfó et al. 2012). As well, laser ablation of ASI neurons, exposure to dauer pheromone, and a lower concentration of bacterial food all lead to a reduction in the number of germline stem/progenitor cells in the proliferation zone in a *daf-5/Sno/Ski*-dependent manner. These observations suggest that environmental chemosensory inputs regulate differentiation of germline stem/progenitor cells via the TGF-β signaling pathway.

Well-characterized substrates of mTORC1 include the p70 ribosomal S6 kinases (S6Ks; reviewed in Ma and Blenis 2009; Fig. 5.3). Mammalian S6K1 makes critical contributions to the ability of mTORC1 to promote proliferation (Fingar et al. 2004). A null mutation of *rsks-1*, which encodes the sole *C. elegans* ortholog of S6K, results in a decline in the rate of proliferation in the mitotic zone during the late larval stages, which is reminiscent of the phenotypes caused by inhibition of the IIS pathway (Michaelson et al. 2010; Korta et al. 2012). The distributions of nuclear DNA content in the germline progenitors, however, are not as markedly different between wild type and *rsks-1/S6k* null mutants as between wild type and *daf-2insulin receptor* mutants (Korta et al. 2012). These observations differ from the finding that reduced activity of the IIS pathway results in a greater proportion of nuclei with higher DNA content (Michaelson et al. 2010). In addition to the role in promoting cell proliferation, *rsks-1/S6k* also inhibits differentiation of the germline progenitors. The loss of the gene causes the border between the mitotic zone and the proximally adjacent region where cells enter the early meiotic prophase, termed the transition zone (Fig. 5.2b), to shift significantly towards DTCs, as observed when *glp-1* and the TGF-β signaling pathway are impaired (Michaelson et al. 2010; Dalfó et al. 2012; Korta et al. 2012; reviewed in Hubbard et al. 2013). The analysis of genetic interactions between *rsks-1/S6k* and *glp-1* suggests that *rsks-1/S6k* acts in parallel with or downstream of *glp-1* in the germline (Korta et al. 2012). Furthermore, a *rsks-1/S6k* null mutation also results in a reduction in germ cell size. In contrast,

animals carrying a *daf-2/insulin receptor* loss-of-function mutation do not exhibit this phenotype, suggesting that *rsks-1/S6k* and *daf-2/insulin receptor* regulate the rapid accumulation of the germline stem/progenitor cells by distinct mechanisms.

RNAi targeting against *let-363/mTOR* and *daf-15/Raptor* in *rrf-1* mutant animals (see above) results in fewer numbers of mitotic zone nuclei than corresponding numbers in *rsks-1/S6k* null mutants (Korta et al. 2012). Furthermore, introduction of a mutation that substitutes the conserved threonine residue, which is phosphorylated by mTORC1 in both humans and *Drosophila*, with alanine, impairs the capability of the *rsks-1/S6k* transgene to alleviate the germline phenotype in its null mutant animals. This suggests that *let-363/mTOR* and *daf-15/Raptor* promote the accumulation of the germline progenitors via *rsks-1/S6k*. The RNAi targeting against *ife-1*, which encodes eukaryotic Initiation Factor 4E (eIF4E), another conserved downstream effector (Fig. 5.3), not only results in fewer germline stem/progenitor cells, but also enhances the germline phenotype in the *rsks-1/S6k* null mutants. These findings suggest that mTORC1 promotes robust larval proliferation via both *rsks-1/S6k* and *ife-1/eIF4E*.

In cultured mammalian and *Drosophila* cells, mTORC1 mediates amino acid signaling to activate S6K (Hara et al. 1998; Radimerski et al. 2002). Dietary restriction in *C. elegans* can be employed by either feeding diluted bacterial food or by a feeding-defective mutation (Lakowski and Hekimi 1998; Greer et al. 2007), both of which result in a reduced germline progenitor pool (Korta et al. 2012). Similarly, inhibition of an intestinal peptide transporter encoded by *pept-1*, which is required for incorporating peptides from the gut lumen (Meister 2013), also leads to the germline phenotype, suggesting that dietary amino acids significantly contribute to the accumulation of the larval germline stem/progenitor cells (Korta et al. 2012). Loss of *rsks-1/S6k* attenuates the effects of both the dietary restriction and loss of *pept-1*. These findings are consistent with the model that mTORC1–*rsks-1/S6k* signaling mediates the robust larval germline proliferation promoted by dietary amino acids. Conversely, the mechanism by which *rsks-1/S6k* inhibits differentiation of the germline progenitors remains obscure. In contrast to loss of *rsks-1/S6k*, neither dietary restriction nor loss of *pept-1* induce a striking effect on differentiation of the germline, and whether or not depletion of mTORC1 activity affects the proliferative cell fate remains to be determined.

At the single cell level, dietary restriction in L4 and adult animals has been shown to slow down the progression of metaphase and anaphase (Gerhold et al. 2015). This mitotic delay requires activity of SAC. Loss of SAC activity results in embryonic and larval lethality due to defects in chromosome segregation. Dietary restriction apparently enhances these phenotypes, which underscores an enhanced requirement of SAC for preventing chromosome segregation errors upon dietary restriction. Because AKT-1/AKT kinase negatively regulates SAC activity in L1 larvae (Watanabe et al. 2008), dietary restriction during the L4 and adult stages may activate SAC by downregulating the IIS pathway. Hence, it would be interesting to test if hyperactivation of the IIS pathway in the germline during dietary restriction induces an defect in chromosome segregation.

5.8 The Adult Reproductive Diapause/Oogenic Germline Starvation Response

When early/mid-L4 larvae are removed from food, a fraction of the animals develop into adults that shrink their germlines and suppress their reproductive activity (Angelo and Van Gilst 2009; Seidel and Kimble 2011). This starvation-induced state was termed "adult reproductive diapause (ARD)" (Angelo and Van Gilst 2009).

During ARD, oocyte growth and embryonic production are suppressed, and each gonadal arm contains at most one embryo (Angelo and Van Gilst 2009; Seidel and Kimble 2011). In addition, the germline, which has initiated oogenesis, gradually reduces in size due to apoptotic death of differentiated germ cells, leaving a small pool of germline stem/progenitor cells. After a 30-day ARD period, almost all of the functional sperm in the spermatheca are depleted (Angelo and Van Gilst 2009). However, if subsequently returned to the nutritionally favorable condition, the reserved population of germline stem/progenitor cells initiates production of viable oocytes and regenerates a functional germline (Angelo and Van Gilst 2009; Seidel and Kimble 2011). Furthermore, animals recovered from up to 30 days of ARD still exhibit a mean adult lifespan comparable to that of continuously fed adults, which suggests that ARD suppresses the aging process, similar to the L1 arrest and dauer diapause (Klass and Hirsh 1976; Johnson et al. 1984; Angelo and Van Gilst 2009). Angelo and Van Gilst also reported that induction of ARD requires relatively high population densities and that ARD induces embryonic arrest in the uterus (Angelo and Van Gilst 2009). However, these findings were not supported experimentally by subsequent studies (Seidel and Kimble 2011).

Many of the genetic mechanisms that regulate entry into, maintenance of, and recovery from ARD remain to be delineated. The maintenance of sperm within the spermatheca during ARD requires activity of *nhr-49*, which encodes an ortholog of mammalian hepatocyte nuclear factor-4α (HNF4α; Van Gilst et al. 2005a; Angelo and Van Gilst 2009). Furthermore, loss of *nhr-49* also severely impairs the ability to produce progeny after recovery from the diapause. Animals carrying a strong loss-of-function mutation of *ced-3*, which encodes a caspase essential for apoptosis, display defects in oocyte formation and embryonic production after exiting ARD, resulting in much fewer progeny compared to wild type animals. This suggests that the appropriate removal of germ cells by apoptosis during diapause is required for maintaining reproductive ability. Although *nhr-49* is not required for triggering cell death upon entry into ARD, *nhr-49* mutant animals accumulate cellular debris in the uterus after the recovery, raising the possibility that autophagy or phagocytic mechanisms that keep the gonad clean are impaired. Because *nhr-49* has been shown to regulate expression of genes involved in mitochondrial β-oxidation and fatty acid desaturation (Van Gilst et al. 2005b), a shift in fatty acid metabolism might play a significant role in ARD.

In addition to inducing apoptosis, neither the maintenance of the stem/progenitor cell pool during the ARD nor the regeneration of the germline after exiting ARD

requires *nhr-49* activity. These observations suggest that some ARD processes are operated by a mechanism that is distinct from the mechanism mediated by *nhr-49*. L4 larvae that have failed to enter ARD and develop into adults end up with multiple embryos that develop in utero and eventually hatch inside and consume their mother, which is dubbed "bagging" or "bag of worms." Angelo and Van Gilst first noted that inhibition of this facultative vivipary was a feature that distinguished ARD (Angelo and Van Gilst 2009). However, Seidel and Kimble found that adult worms fated to bag had the capacity to shrink their germlines during starvation and to regenerate them upon re-feeding, both of which have been described as striking features of ARD (Angelo and Van Gilst 2009; Seidel and Kimble 2011). This finding led Seidel and Kimble to propose the term, "oogenic germline starvation response (OGSR)," which is defined by shrinkage of the germline that coincides with oogenesis, and the capacity for germline regeneration upon re-feeding (Seidel and Kimble 2011). Similar to OGSR, young adult worms have been shown to trigger cell death in the germline in response to a 6 h period of starvation (Salinas et al. 2006). The retinoblastoma (Rb) orthologous gene *lin-35* reportedly promotes this starvation-induced apoptosis by repressing the gonadal expression of the anti-apoptotic gene *ced-9*, which encodes a *C. elegans* ortholog of human BCL-2 and suppresses activity of *ced-3* (Láscarez-Lagunas et al. 2014). However, whether *lin-35/Rb* also mediates execution of ARD/OGSR remains to be examined.

5.9 Future Perspectives

Studies on the starvation-induced arrest of *C. elegans* development have begun to uncover genetic pathways that play a key role in the nutritional regulation of germline development. The IIS pathway not only mediates the decision between quiescence and reactivation of germline stem/progenitor cells at the nutritional checkpoints, but also promotes the robust accumulation of stem/progenitor cells during late larval stages. Previous studies have suggested that the IIS pathway in the hypodermis, the nervous system, and the intestine contributes to the developmental arrest of the germline during L1 arrest and dauer diapause (Apfeld and Kenyon 1998; Wolkow et al. 2000; Libina et al. 2003; Narbonne and Roy 2006b; Iser et al. 2007; Hung et al. 2014; Fukuyama et al. 2015), respectively. Conversely, the robust accumulation of stem/progenitor cells is controlled by the activity of the pathway primarily in the germline (Michaelson et al. 2010). In the case of the developmental quiescence of the germline, the possibility that *daf-18/Pten* also functions in an additional site, such as germline stem/progenitor cells and somatic gonadal precursors, remains to be rigorously examined.

Among the 40 *ins* genes identified in the *C. elegans* genome, approximately half have been shown to contribute to the regulation of dauer diapause (Pierce et al. 2001; Li et al. 2003; Cornils et al. 2011; Matsunaga et al. 2012; Fernandes de Abreu et al. 2014; Hung et al. 2014). Most of these insulin-like peptides promote entry into and/or inhibit exit from dauer diapause, and likely act as agonists to DAF-2/insulin

receptor. However, the remaining insulin-like peptides apparently serve as antagonists that inhibit entry into and/or promote exit from the diapause. What is the molecular basis of this antagonism? Do all of these insulin-like peptides really act on DAF-2/insulin receptor as the receptor? Which *ins* gene monitors the fluctuations in the dietary environment and dauer pheromone? Is there any subset of *ins* genes that are engaged in the regulation of germline quiescence? These questions should be addressed in order to delineate the mechanism by which the *ins* genes coordinate germline development with dauer diapause. Similar questions should also be answered to elucidate the mechanism by which the IIS pathway regulates the reactivation of quiescent Z2 and Z3 cells upon recovery from L1 arrest. Although it has recently been shown that overexpression of *ins-4*, *ins-6*, and *daf-28* can release mesodermal progenitor cells from quiescence in newly hatched starved larvae (Chen and Baugh 2014), whether these genes can also reactivate Z2 and Z3 cells remains unclear. Simultaneous inhibition of these *ins* genes (and *ins-5*) induces the dauer arrest instead of L1 arrest (Hung et al. 2014). Because a strong *daf-2/insulin receptor* loss-of-function allele results in L1 arrest, there may be additional *ins* genes that regulate the exit from L1 arrest. The IIS pathway also regulates starvation-induced arrest at the later larval stages (Schindler et al. 2014), but *ins* genes that allow developing larvae to pass through the later nutritional checkpoints have not yet been identified.

Genes that act downstream of the IIS, mTORC1, and TGF-β signaling pathways to enable the germline development to respond to a shift in the dietary environment have also yet to be identified. The IIS pathway regulates the exit of germ cells from the G2 phase during L1 arrest and upon entry into dauer diapause, and possibly the robust proliferation of germline stem/progenitor cells (Fukuyama et al. 2006; Narbonne and Roy 2006a; Michaelson et al. 2010), raising the possibility that the pathway ultimately utilizes the same cell cycle regulator as a common downstream effector in these distinct physiological contexts. SAC and APC/C might mediate the IIS pathway to control not only the quiescence of Z2 and Z3 cells, but also germline proliferation upon entry into dauer diapause, as well as during later larval stages (Watanabe et al. 2008). However, whether SAC and APC/C and their upstream regulators *daf-18/Pten* and *akt-1/Akt* act cell-autonomously in Z2 and Z3 cells remains to be confirmed, introducing the likelihood that the somatic activity of these factors indirectly affects the G2 arrest. Moreover, genetic analysis has suggested that in addition to SAC, *akt-1/AKT* promotes the exit from G2 through a different cell cycle regulator (Watanabe et al. 2008) that might also be impinged upon by *aak-1,2/Ampk* and mTORC1 signaling (Fukuyama et al. 2012).

Recent advances in stem cell biology have revealed unexpected roles of metabolism in stem cell maintenance (reviewed in Shyh-Chang et al. 2013). In addition to metabolism, changes in the nutritional status have been known to modulate many cell- and molecular-biological processes, such as autophagy and global translational regulation, partly via the IIS and mTORC1 pathways (Laplante and Sabatini 2012). As well, nutrient-responsive, cellular, and biochemical events remain largely unexplored in *C. elegans* germline stem/progenitor cells. Once such nutrient responses are discovered, their significance on the behaviors of germline stem/progenitor cells

and their relationships to the known regulatory pathways such as the IIS, mTORC1, and TGF-β signaling pathways can be readily investigated.

Finally, dietary nutrients and metabolites that coordinately and directly promote germline development have not been revealed. Amino acids play a key role in reactivating quiescent Z2 and Z3 cells and in promoting the accumulation of germline stem/progenitor cells, potentially through the mTORC1 pathway (Korta et al. 2012; Fukuyama et al. 2015). However, amino acids are not sufficient to reverse the quiescence of Z2 and Z3 cells (Fukuyama et al. 2015). Feeding L1-arrested *C. elegans* a miniscule amount of ethanol (0.08 %) can induce expression of several *ins* genes and accumulation of lipid droplets in the intestine, which is consistent with the finding that ethanol is assimilated into fatty acids (Castro et al. 2012; Fukuyama et al. 2015). Notably, the ethanol-induced *ins* genes include *daf-28* and *ins-33*, which facilitate recovery from L1 arrest and inhibit dauer formation, and promote the robust accumulation of germline stem/progenitor cells, respectively (Li et al. 2003; Michaelson et al. 2010; Fernandes de Abreu et al. 2014; Hung et al. 2014; Chen and Baugh 2014). Thus, these findings elicit questions, such as what is the genetic pathway that mediates the induction of *daf-28* and *ins-33* expression by ethanol? Do fatty acids also upregulate ethanol-inducible *ins* genes? It would be interesting to test whether ethanol and amino acids can antagonize dauer pheromone, or block entry into L3, L4 arrest, and ARD. Because the combination of ethanol and amino acids is rather inefficient at reactivating Z2 and Z3 cells in liquid culture (Fukuyama et al. 2015), there may be an additional nutrient that needs to be sensed and/or ingested. Alternatively, chemical or mechanical stresses concomitant with the liquid culture might inhibit germline reactivation.

Future elucidation of the regulatory network that couples germline development in *C. elegans* to the dietary environment would also help shed light on the mechanisms that control stem/progenitor cell behaviors in other animals. In addition to *C. elegans*, recent studies have demonstrated that components of the IIS pathway and mTORC1 also regulate stem/progenitor cell behaviors in mice and *Drosophila* (LaFever and Drummond-Barbosa 2005; Groszer et al. 2006; Yilmaz et al. 2006; Tothova et al. 2007; Miyamoto et al. 2007; Hsu et al. 2008; Hsu and Drummond-Barbosa 2009; Renault et al. 2009; LaFever et al. 2010; Sun et al. 2010; Chell and Brand 2010; Hsu and Drummond-Barbosa 2011; Sousa-Nunes et al. 2011; Yilmaz et al. 2012). For example, loss of *Pten* in hematopoietic stem cells leads not only to failure to maintain quiescence, but also to depletion of the stem cell pool in mice (Yilmaz et al. 2006), which is reminiscent of the finding that starved *daf-18/Pten* mutant larvae fail to maintain quiescence in Z2 and Z3 cells and become sterile adults after release from L1 arrest (Fukuyama et al. 2006; Watanabe et al. 2008; Fukuyama et al. 2012). Similar to *Pten*, murine *Foxo* genes play a critical role in maintaining quiescence and survival of hematopoietic and neural stem cells (Tothova et al. 2007; Miyamoto et al. 2007; Renault et al. 2009). Furthermore, recent studies have demonstrated that *Foxo3a* is also essential for maintaining leukemia initiating cells (leukemia stem cells), and contributes significantly to the resistance to treatment with the anti-cancer drug imatinib (Naka et al. 2010). Historically, studies on diapause and aging in *C. elegans* led to the identification of *daf-16/Foxo* as a key,

conserved downstream target of the IIS pathway (Ogg et al. 1997; Lin et al. 1997; Brunet et al. 1999; Kops et al. 1999; Nakae et al. 1999; Rena et al. 1999; Tang et al. 1999; Biggs et al. 1999). Thus, the insights gained from studies on the nutritional regulation of the *C. elegans* germline might provide clues to form hypotheses regarding the genetic mechanisms that maintain somatic and cancer stem cells in mammals.

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Chapter 6
Seasonal Regulation of Reproduction in Vertebrates: Special Focus on Avian Strategy

Ai Shinomiya and Takashi Yoshimura

Abstract Temperate zones are characterized by seasonal changes in the environment; therefore, reproduction in most temperate animal species is restricted to a specific season to maximize the survival of their offspring. Among vertebrates, birds have evolved highly sophisticated mechanisms for seasonal reproduction to accommodate their adaptation for flight. For example, the mass of Japanese quail (*Coturnix japonica*) testes increases more than 100-fold within a few weeks. Under short-day conditions, gonadal development is suppressed and testes retain their immature size. However, when quail are transferred to long-day conditions, germ cell differentiation begins immediately. Conversely, when they are transferred to winter conditions (short day and low temperature), arrested meiosis and germ cell apoptosis cause rapid testicular regression. Recent molecular analysis revealed the signal transduction pathway regulating seasonal reproduction. Comparative analysis of seasonal reproduction in various species also revealed both the similarity (i.e., signal transduction machineries) and diversity (i.e., responsible cells or organs) of these mechanisms among various vertebrate species.

Keywords Seasonal reproduction · Photoperiodism · Thyroid hormone · Thyrotropin

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6.1 Introduction

To adapt to seasonal changes in environment, species alter their physiology and behavior. Responses to changing seasons include alterations in growth rate, metabolism, and immune function, as well as the initiation of molting, migration, nesting, hibernation, and reproductive activity. Photoperiod is the primary cue for the initiation of seasonal responses for most species. Other environmental factors, such as temperature and precipitation, vary over years and are thus unreliable compared with changes in day length. The term "photoperiodism" encompasses these seasonal changes in physiology and behaviors caused by photoperiodic changes (Garner and Allard 1920). Among seasonally regulated changes, the mechanism triggering seasonal reproduction has been extensively studied. Most birds and small to medium mammals breed during the spring and summer. Incubation or gestation periods of these species last only for a few weeks. These species are known as long-day (LD) breeders. In contrast, larger mammals, such as goats, sheep, and Japanese macaques, breed during the fall, and have gestation periods of approximately 6 months. Thus, their offspring are born during the following spring and summer. These species are known as short-day (SD) breeders. The offspring of both LD and SD breeders are born and mature when the climate is moderate and food is abundant (Fig. 6.1).

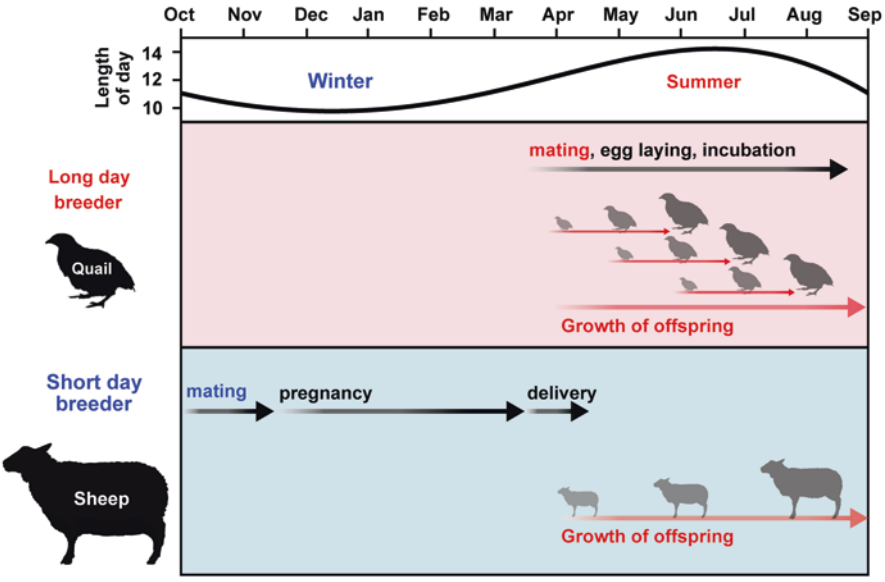

Fig. 6.1 Calendar of long-day and short-day breeders. Small species with short gestation or incubation periods mate in spring and summer. In contrast, relatively large species with approximately 6-month gestation periods mate in fall. Both long-day and short-day breeders give birth in spring (modified from Shinomiya et al. 2014)

Seasonal reproduction in vertebrates is controlled by the hypothalamic-pituitary-gonadal (HPG) axis. Gonadotropin-releasing hormone (GnRH) synthesized mainly in the preoptic area of the hypothalamus is secreted from the median eminence into the hypophyseal portal vessels. Secreted GnRH activates the secretion of gonadotropins (luteinizing hormone [LH] and follicle-stimulating hormone [FSH]) from the pars distalis of the anterior pituitary gland. LH and FSH regulate development of the gonads. Among vertebrate species, birds show the most dramatic changes in gonadal size (typically more than 100-fold) (Dawson et al. 2001), thereby making them excellent study organisms to contribute to our understanding of photoperiodic mechanisms. Among mammals, photoperiodism has been extensively studied in hamsters and sheep because of their marked photoperiodic responses, although their seasonal gonadal changes are less dramatic than those of birds. The substantial seasonal responses in birds may be related to adaptations for flight. In addition to dramatic gonadal responses, most birds have very short breeding seasons, and the HPG axis is automatically switched off and their gonads start to regress even though the day length is still increasing. This phenomenon is known as photorefractoriness (Hahn and MacDougall-Shackleton 2008). In this section, we discuss the current understanding of the mechanisms regulating seasonal reproduction in birds, mammals, and fish.

6.2 Identification of Key Genes Regulating Seasonal Reproduction

The Japanese quail (*Coturnix japonica*) is an excellent model for studying photoperiodism, because of its rapid and dramatic responses to changing photoperiods (Follett et al. 1998). Local illumination of the mediobasal hypothalamus (MBH) by radioluminous-painted beads induced testicular growth (Homma et al. 1979). Lesions on the MBH blocked the photoperiodic responses of LH secretion and gonadal development (Sharp and Follett 1969; Juss et al. 1993). In addition, expression of c-Fos, a marker of neuronal activation, is induced in the MBH by LD stimulus (Meddle and Follett 1997). The MBH is, therefore, considered central to seasonal reproduction in quail. By using differential subtractive hybridization analysis, LD-induction of the type 2 deiodinase gene (*DIO2*) and LD-suppression of the type 3 deiodinase gene (*DIO3*) were observed in the ependymal cells (also known as tanycytes). These cells line the ventrolateral walls of the third ventricle within the MBH (Yoshimura et al. 2003; Yasuo et al. 2005, Fig. 6.2). *DIO2* encodes for the thyroid hormone-activating enzyme that converts the precursor T_4 to the bioactive triiodothyronine (T_3) (Bernal 2002), whereas *DIO3* encodes for the thyroid hormone-inactivating enzyme that metabolizes T_4 and T_3 to inactive reverse T_3 (rT_3) and 3,3′-diiodothyronine (T_2), respectively (Fig. 6.3). The reciprocal switching of *DIO2* and *DIO3* appears to regulate the local thyroid hormone concentration within the MBH. Moreover, T_3 concentration within the MBH is about tenfold higher

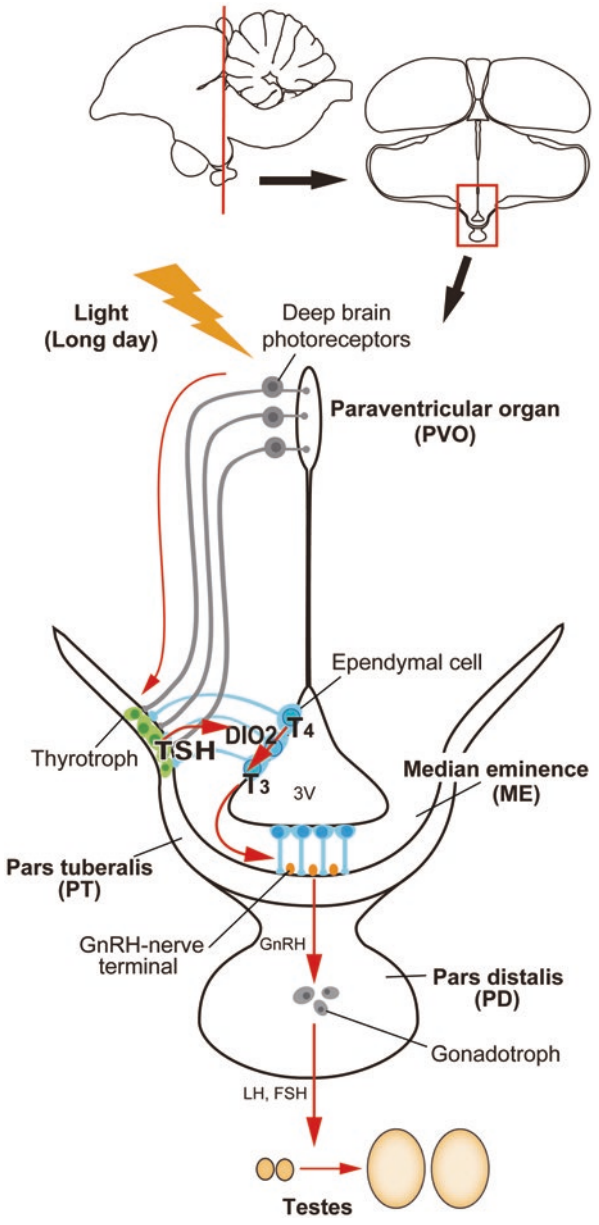

Fig. 6.2 Signal transduction pathway regulating avian seasonal reproduction. Light information is received by deep brain photoreceptors. When this information is transmitted to the pars tuberalis (PT) of the pituitary gland, TSH is secreted to the hypothalamus to induce DIO2 expression. DIO2 activates thyroid hormone to regulate seasonal morphological changes in GnRH nerve terminals and glial processes and thereby regulate GnRH secretion (modified from Yoshimura 2013)

under LD than under SD conditions, even though plasma concentrations are similar during both photoperiod conditions (Yoshimura et al. 2003). Functional significance of this locally-activated thyroid hormone has been demonstrated by pharmacological analyses. Intracerebroventricular (i.c.v.) infusion of T_3 under SD conditions induced testicular development, whereas infusion of a DIO2 inhibitor under LD

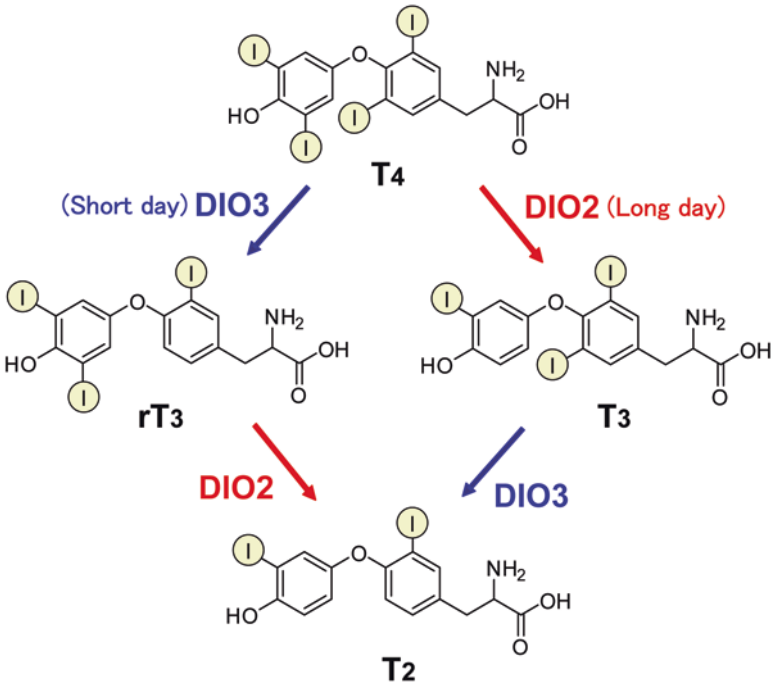

Fig. 6.3 Metabolic pathway of thyroid hormones. Type 2 iodothyronine deiodinase (DIO2) converts precursor T_4 to bioactive T_3 under long-day conditions, whereas type 3 iodothyronine deiodinase (DIO3) metabolizes both T_4 and T_3 to an inactive form under short-day conditions

conditions attenuated testicular development (Yoshimura et al. 2003). Photoperiodic regulation of *DIO2* and/or *DIO3* has also been confirmed in a number of other avian species, such as the tree sparrow (Watanabe et al. 2007), chicken (Ono et al. 2009), great tit (Perfito et al. 2012), and canary (Stevenson and Ball 2012).

6.3 Thyrotropin in the Pars Tuberalis Is the Springtime Hormone

When quail were transferred from SD to LD conditions, an increase in plasma gonadotropin (LH) was observed 22 h after the dawn of the first day of LD treatment (Nicholls et al. 1983; Follett et al. 1998; Nakao et al. 2008). As previously discussed, reciprocal switching of *DIO2* and *DIO3* plays a critical role in the regulation of seasonal reproduction and this switching precedes photoperiodic induction of gonadotropin release by approximately 4 h (Yasuo et al. 2005). Genome-wide gene expression analysis during the transition from SD to LD conditions in Japanese quail (Nakao et al. 2008) revealed the induction of two genes 4 h prior to *DIO2/*

DIO3 switching (i.e., 14 h after dawn) in the pars tuberalis of the pituitary gland. The pars tuberalis consists of thin layers of cells surrounding the median eminence (Fig. 6.2). One of the two genes encodes for the thyroid-stimulating hormone β subunit (*TSHB*) and the other encodes for the transcriptional co-activator eyes absent 3 (*EYA3*). Although EYA3 is a transcriptional co-activator, the expression sites of *EYA3*, and *DIO2* and *DIO3* were distinct. That is, *EYA3* is expressed in the pars tuberalis, whereas *DIO2* and *DIO3* are expressed in the ependymal cells. Therefore, it appears that EYA3 is not involved in the regulation of *DIO2*/*DIO3* switching. In contrast, the expression of the TSH receptor (TSHR) and binding of ^{125}I-labeled TSH were observed in the ependymal cells where *DIO2* and *DIO3* are expressed. Furthermore, i.c.v. TSH administration induced *DIO2* expression and reduced *DIO3* expression in the ependymal cells even under SD conditions, whereas passive immunization against TSH attenuated LD induction of *DIO2* expression (Nakao et al. 2008). The involvement of the TSHR-Gsα-cAMP signaling pathway in the TSH regulation of *DIO2* expression was further demonstrated by promoter analysis. Because the magnitude of testicular growth induced by i.c.v. TSH infusion was similar to that observed in birds exposed to LD stimulus, it was concluded that the LD-induced pars tuberalis TSH is a major factor regulating seasonal reproduction in birds (Fig. 6.2).

6.4 Local Thyroid Hormone Regulates Neuro-glial Plasticity Within the Median Eminence

Due to their lipophilic nature, thyroid hormones were believed to traverse plasma membranes only by passive diffusion. However, involvement of a membrane transport system has been reported. Some members of the organic anion transporting polypeptide (Oatp) family transport thyroid hormones in mammals (Abe et al. 2002: Hagenbuch and Meier 2004) and the involvement of a member of this family in transporting T_4 into the quail brain has been reported (Nakao et al. 2006). Oatp1c1, which is expressed in the ependymal cells within the MBH and choroid plexus, is a highly specific transporter of T_4. In addition to Oatp1c1, another thyroid hormone transporter, monocarboxylate transporter 8 (MCT8), was found in the ependymal cells within the hamster MBH (Herwig et al. 2009). Although MCT8 appears to be involved in the regulation of photoperiodism, its expression is upregulated under SD conditions, when thyroid hormone is not necessary.

Thyroid hormone is involved in the development and plasticity of the central nervous system (Bernal 2002). Target sites for the photo-induced T_3 in the quail MBH are the thyroid hormone receptors (*THRα*, *THRβ*, and *RXRα*) localized in the median eminence (Yoshimura et al. 2003). To understand the activity of thyroid hormone within the MBH, an ultrastructure of the median eminence was examined using electron microscopy. Dynamic morphological changes were reported between the GnRH nerve terminals and glial end-feet (Yamamura et al. 2004). Under SD

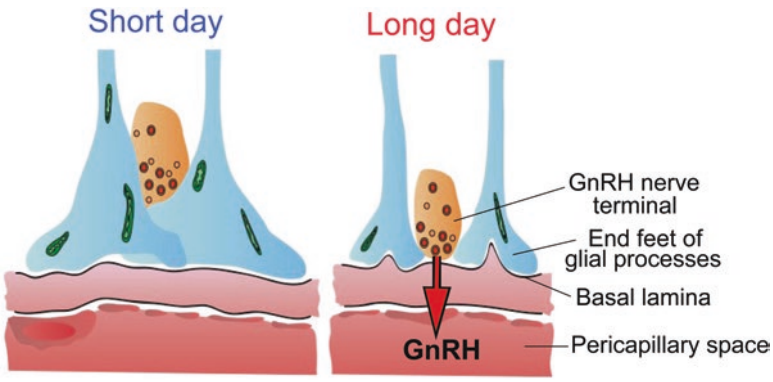

Fig. 6.4 Morphological changes between GnRH nerve terminals and glial end-feet. Long day activated thyroid hormone within the MBH regulates neuro-glial interaction in the median eminence. These morphological interactions appear to regulate or modulate seasonal GnRH secretion from the hypothalamus to portal capillary (modified from Yoshimura 2004)

conditions, many GnRH nerve terminals were encased by the end-feet of glial processes and did not contact the basal lamina. In contrast, many GnRH nerve terminals were in close proximity to the basal lamina under LD conditions (Fig. 6.4). It has been proposed that the nerve terminals of hypothalamic neurons must directly contact the pericapillary space for the secretion of the hypothalamic neurohormone into the portal capillary (Prevot et al. 1999). Therefore, it is suggested that these morphological changes regulate or modulate seasonal GnRH secretion from the median eminence. It is also interesting to note that seasonal plasticity within the GnRH system is reported in ewes (Jansen et al. 2003).

6.5 Involvement of Deep Brain Photoreceptors in Avian Seasonal Reproduction

Although the eye is the only photoreceptor organ in mammals, photoreceptive organs are localized in eyes, pineal organs, and the deep brain in non-mammalian vertebrates. Photo-capability in the deep brain was first demonstrated in European minnows, in which it controls changes in skin color (von Frisch 1911). Subsequently, evidence for the existence of a deep brain photoreceptor that regulates seasonal reproduction was reported for ducks. Blind ducks continue to show photoperiodic responses, whereas covering the heads of ducks with black caps blocks testicular responses (Benoit 1935). Moreover, injection of India ink under the scalp in pinealectomized sparrows abolishes the photoperiodic response (Menaker et al. 1970). Both pinealectomized and blinded quail undergo gonadal development in response to light cues (Siopes and Wilson 1974). In addition, photostimulation of the hypothalamus using light fibers and light-emitting beads induces testicular development

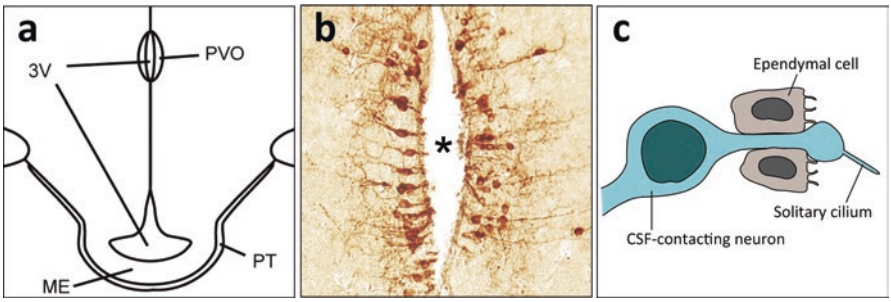

Fig. 6.5 OPN5-positive cerebrospinal fluid (CSF)-contacting neurons are deep brain photoreceptors. (**a**) Schematic drawing of the quail mediobasal hypothalamus. (**b**) Representative photomicrograph of OPN5-positive neurons in the paraventricular organ (PVO) (*asterisk*: cerebrospinal fluid). (**c**) Schematic drawing of the CSF-contacting neurons (modified from Yoshimura 2013)

in sparrows (Yokoyama et al. 1978) and Japanese quail (Homma et al. 1979). It has been confirmed that broad-spectrum light penetrates into the brains of various vertebrate species (Hartwig and vanVeen 1979; Foster and Follett 1985; Oishi and Ohashi 1993).

Many researchers have attempted to identify deep brain photoreceptors. Several rhodopsin family proteins (e.g., rhodopsin [RH], melanopsin [OPN4] and vertebrate ancient [VA]-opsin) are reported to be localized in the avian deep brain region (Silver et al. 1988; Wada et al. 1998; Chaurasia et al. 2005; Halford et al. 2009). In addition, a novel opsin called Opsin 5 (OPN5) was recently found localized in the paraventricular organ (PVO) with in the MBH (Nakane et al. 2010; Yamashita et al. 2010). This is intriguing because lesions around the PVO block the photoperiodic responses of gonads in Japanese quail (Sharp and Follett 1969). Immunohistochemical analysis of OPN5 revealed its presence in the cerebrospinal fluid (CSF)-contacting neurons (Fig. 6.5). The CSF-contacting neurons in the PVO have long been candidate for a deep brain photoreceptor because the retina and pineal organ evaginate from the diencephalon around the third ventricle where the PVO is located. Furthermore, the morphology of CSF-contacting neurons resembles that of the photoreceptor cells in the developing retina (Vigh-Teichmann et al. 1980). Functional analysis of OPN5 protein using *Xenopus* oocyte demonstrated that OPN5 is a short-wavelength sensitive photopigment (Nakane et al. 2010; Yamashita et al. 2010). In addition, intrinsic photosensitivity of OPN5 positive CSF-contacting neuron was demonstrated by slice patch-clamp analysis (Nakane et al. 2014). Therefore, OPN5-expressing CSF-contacting neurons in the PVO are the deep brain photoreceptors and are important for seasonal reproduction in birds (Fig. 6.2). Long wavelength light is generally thought to penetrate into the brain more effectively than short wavelength light. However, feathers, skin, bone, and brain tissue are known to autofluoresce in the range of UV to blue light and short wavelength light has been demonstrated to penetrate into the quail hypothalamus. In addition, LD stimulation with short-wavelength light indeed triggered testicular growth in eye-patched and pinealectomized blind quail (Nakane et al. 2010). Because UV and blue light are known

to regulate photoperiodism in mammals (Brainard et al. 1986) and insects (Lees 1981), results observed in quail are not surprising. However, it is possible that multiple opsins are involved in the deep brain photoreception to cover wide range wavelength light.

6.6 Seasonal Changes in Testicular Size

As previously mentioned, seasonal changes in testicular mass typically increases 100-fold in birds. When quail were raised under LD conditions, they reached puberty at 6–7 weeks of age (Marin and Satterlee 2004). However, when they were raised under SD conditions, their gonadal development was suppressed and testes remained small and immature (Ikegami et al. 2015). The diameter of seminiferous tubules was small and contained only Sertoli cells and gonocytes. Once quail were transferred from SD to LD conditions, meiosis and spermatid were detected by 5 and 14 days after transfer, respectively. Thus, testicular development was accomplished within a few weeks (Follett and Maung 1978; Lin et al. 1990; Follett et al. 1998; Nakao et al. 2008; Ikegami et al. 2015, Fig. 6.6).

When quail were transferred from LD to SD conditions or SD and low temperature (SL) conditions, meiosis continued for a few weeks and subsequently testicular mass decreased (Ikegami et al. 2015). The slow initiation of SD/SL-induced testicular regression was in contrast to the rapid rate of photoinduction. It appeared that repeated exposure to SD or SL conditions is required to initiate testicular regression and this mechanism may enable birds to avoid misinterpreting several consecutive cloudy or cold days as the autumnal stimuli.

Under SD conditions, arrest of meiosis is the primary cause of gradual testicular regression. In marked contrast, substantial germ cell apoptosis was observed under SL conditions (Fig. 6.6). This extensive germ cell death is rarely observed in mammalian species and seemed to account for the rapid and dramatic testicular regression observed in avian species. Thus, seasonal testicular regression caused by SL stimuli in quail is mediated by the arrest of germ cell differentiation and apoptosis.

6.7 Underlying Mechanism of Seasonal Testicular Development and Regression

Vertebrate reproductive activity is regulated by the HPG axis. When quail are raised under SD conditions, their HPG axis is suppressed. However, once they are transferred to LD conditions, the photoperiodic signaling pathway is immediately switched on and the HPG axis is activated. The activation of the LH-dependent steroidogenesis pathway in the testes results in increased testosterone production. Because removal of testosterone increases germ cell apoptosis, whereas

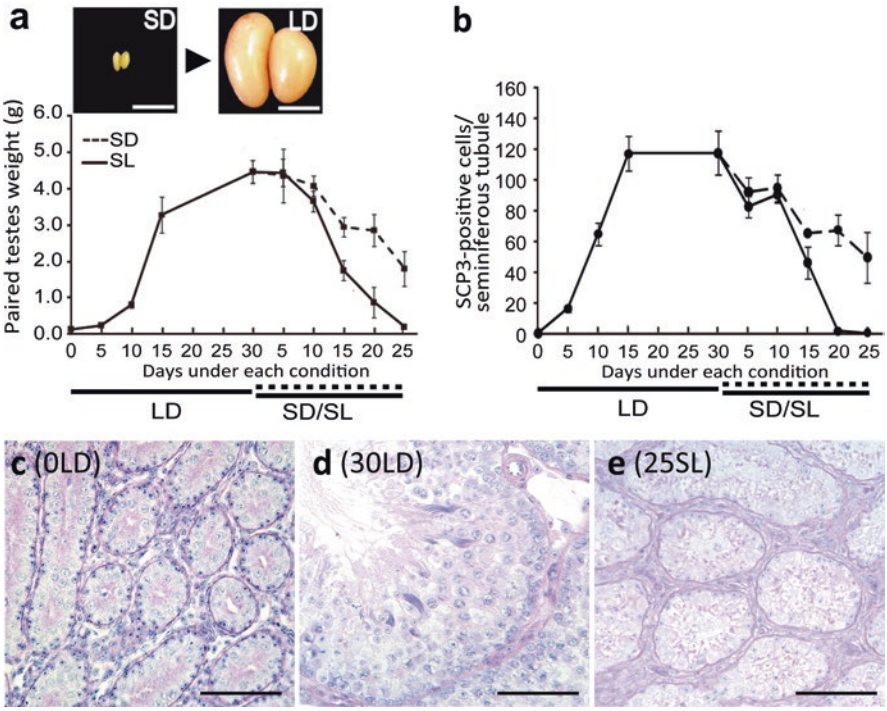

Fig. 6.6 Effect of changing day length and temperature on testicular weight and meiosis. (**a**) Testes of quail kept under short-day (SD) and long-day (LD) conditions. Scale bars: 1 cm. Changes in testicular mass in quail transferred from SD to LD conditions and then to short day/low temperature (SL solid line) or SD (dashed line) conditions. (**b**) Changes in number of SCP3-positive cells. (**c**–**e**) Representative histological images of a quail testis under SD condition (0LD) (**c**), 30 days after transferred to LD condition (30LD) (**d**), and 25 days transferred back to SD and low temperature condition (25SL) (**e**). Scale bars, 50 μm (modified from Ikegami et al. 2015)

reintroduction decreases it, testosterone is considered a survival factor for germ cells (Nandi et al. 1999). Accordingly, activation of the LH-dependent steroidogenesis pathway induces germ cell differentiation and inhibits germ cell apoptosis under LD conditions (Fig. 6.7).

When quail were transferred from LD to SD conditions, the photoperiodic signaling pathway was switched off, and serum LH gradually decreased (Ikegami et al. 2015). However, because serum LH and testosterone did not return to basal levels under SD conditions, the arrest of meiosis was partial and apoptotic cells were rarely observed. On the other hand, when quail were transferred to SL conditions, serum LH rapidly decreased (Ikegami et al. 2015). The shut-down of the LH-dependent steroidogenesis pathway appeared to be a component of the mechanism regulating seasonal testicular regression (Ikegami et al. 2015, Fig. 6.7). In addition, a significant increase in the serum T_3 level and induction of thyroid hormone receptors, deiodinases (*DIO2* and *DIO3*), and apoptosis-related genes were observed in the testes during the climax of testicular regression under SL conditions.

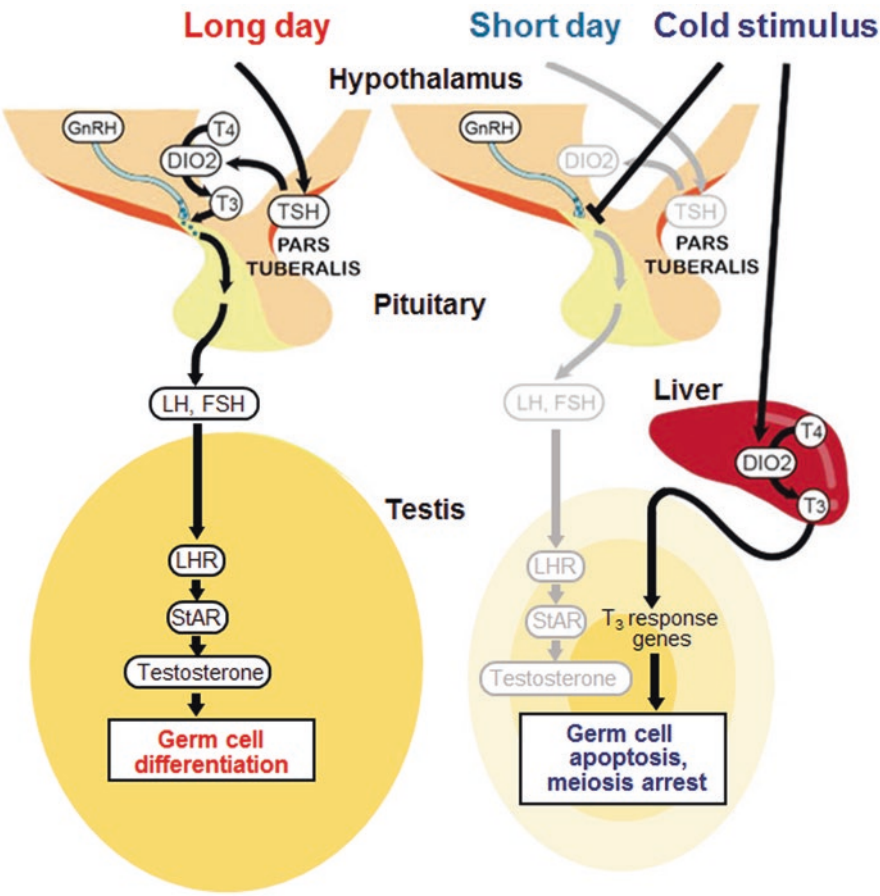

Fig. 6.7 Mechanisms regulating seasonal testicular development and regression. The HPG axis is activated under LD conditions. LH induces testosterone production through LHR and StAR induction. Testosterone acts as a germ cell-survival factor and inhibits apoptosis. Low-temperature stimulus attenuates GnRH secretion and acts in concert with SD stimulus to shut down the HPG axis. Low temperature-induced serum T_3 acts on TH receptors to regulate T_3 response genes involved in metamorphosis and in promoting apoptosis (modified from Ikegami et al. 2015)

Furthermore, daily T_3 administration mimicked the effects of low temperature on germ-cell apoptosis and testicular regression (Ikegami et al. 2015). When low temperature-induced expression of *DIO2* was examined in various tissues, it was observed in the liver (Ikegami et al. 2015). Therefore, it was concluded that low temperature-induced circulating T_3 from the liver acts on the testes to activate gene cascades similar to the ones involved in amphibian metamorphosis and causes germ cell apoptosis in quail testes (Ikegami et al. 2015, Fig. 6.7). Metamorphosis is an irreversible biological process that involves considerable changes in body structure. As such, it is interesting that genes involved in the metamorphosis of amphibians are also activated during the annual reproductive cycle in quail.

The reported effects of thyroidectomy or thyroid hormone treatment on seasonal breeding have often been contradictory (Dawson et al. 2001). However, recent studies have demonstrated that thyroid hormone plays dual roles in the regulation of seasonal reproduction. Central action induces seasonal testicular development, whereas peripheral action mediates seasonal testicular regression (Ikegami et al. 2015). It has been suggested that thyroid hormone is also involved in photorefractoriness (Dawson et al. 2001). However, its underlying mechanism remains to be clarified in future studies.

6.8 Signal Transduction Cascade for Seasonal Reproduction in Mammals

Thyroidectomy blocks the transition to the seasonal reproductive state in sheep (Moenter et al. 1991). Involvement of thyroid hormone in the regulation of mammalian seasonality has been suggested for several decades (Nicholls et al. 1988). However, its precise mode of action has been unknown. After the discovery of photoperiodic *DIO2/DIO3* switching in birds, photoperiodic regulation of *DIO2* and/or *DIO3* with in the MBH was reported in a number of mammalian species, such as hamsters (Watanabe et al. 2004, 2007; Revel et al. 2006; Barrett et al. 2007; Freeman et al. 2007; Yasuo et al. 2007a), rats (Yasuo et al. 2007b), mice (Ono et al. 2008), and even in SD breeding sheep (Hanon et al. 2008) and goats (Yasuo et al. 2006). Therefore, local thyroid hormone activation within the MBH is considered central to the regulation of seasonal reproduction in mammals (Fig. 6.8). However, in marked contrast with birds and other non-mammalian species, the eye is the only photoreceptor organ in mammals. Light information is transmitted to the pineal gland through the circadian pacemaker, the suprachiasmatic nucleus (SCN). In mammals, photoperiodic information is decoded based on the duration of melatonin secretion by the pineal gland (Reiter 1980; Yamazaki et al. 1999). Because melatonin plays a deterministic role in mammalian seasonal reproduction, pinealectomy abolishes seasonal responses and melatonin administration mimics the effect of short photoperiod in mammals (Reiter 1980). Although melatonin controls *DIO2/DIO3* switching, melatonin receptors are absent in the ependymal cells where *DIO2/DIO3* are expressed (Schuster et al. 2000; Song and Bartness 2001). In contrast, melatonin receptors are densely expressed in the mammalian pars tuberalis (Williams and Morgan 1988; Wittkowski et al. 1988; Reppert et al. 1994; Klosen et al. 2002; Dardente et al. 2003). Therefore, it was predicted that TSH secreted from the PT may mediate melatonin action on the regulation of *DIO2/DIO3* switching in mammals. This hypothesis was tested using TSHR and melatonin receptor knockout mice (Ono et al. 2008; Yasuo et al. 2009). Melatonin administration had no effect on *DIO2/DIO3* switching in the TSHR and MT1 melatonin receptor null mice, whereas melatonin affected *DIO2/DIO3* switching in MT2 null mice. These results suggest that melatonin acts on the MT1 melatonin receptor to regulate *DIO2/DIO3* switching through the TSH-TSHR signaling pathway in mammals.

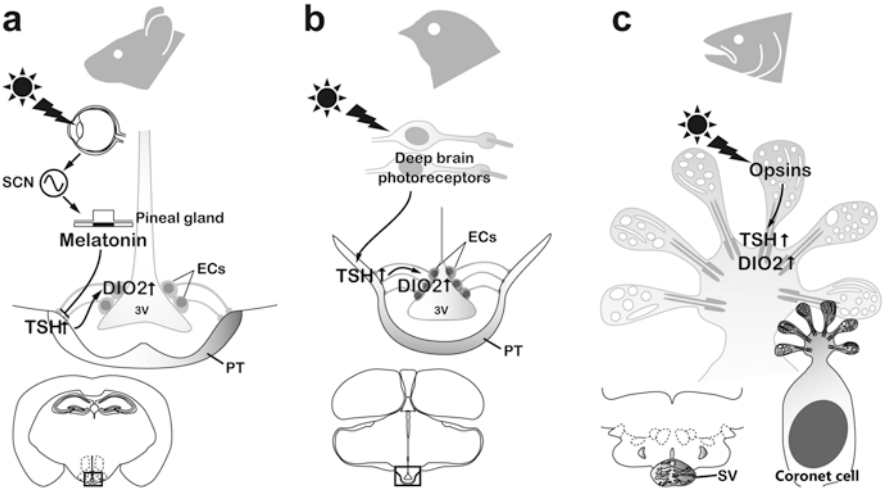

Fig. 6.8 Signal transduction pathways that regulate vertebrate seasonal reproduction. (**a**) In mammals, light information received by the eyes is transmitted to the pineal gland via the suprachiasmatic nucleus (SCN). Photoperiodic information is translated into the melatonin secretion profile. Melatonin suppresses TSH in the pars tuberalis (PT) of the pituitary gland. (**b**) In birds, light information is directly received by deep brain photoreceptors and melatonin is not involved. (**c**) In fish, input pathway, clock, and output pathway are located in the saccus vasculosus (SV) (modified from Nakane and Yoshimura 2014)

The RF-amides such as kisspeptin, a ligand for the G protein-coupled receptor, GPR54, and RFamide-related peptide 3 (RFRP-3) are involved in the regulation of GnRH secretion (Clements et al. 2001; Kotani et al. 2001; Muir et al. 2001; Ohtaki et al. 2001; Clarke et al. 2008). Seasonal regulation of kisspeptin and RFRP-3 occurs in hamsters (Revel et al. 2006, 2008). Administration of TSH to Siberian (*Phodopus sungorus*) and Syrian hamsters (*Mesocricetus auratus*) induces the expression of kisspeptin and RFRP-3 as well as gonadal development under SD conditions (Klosen et al. 2013). T_3 also provoked significant testicular growth and kisspeptin expression in Siberian hamsters under SD conditions (Henson et al. 2013). This suggests that LD induces TSH and, following the activation of thyroid hormone by DIO2, regulates kisspeptin, RFRP-3, and the HPG-axis in mammalian species.

6.9 Functional Targeting of Thyrotropin by Tissue-Specific Posttranslational Modification

TSH generated in the pars tuberalis of the pituitary gland is the springtime hormone in birds and mammals. However, a well-known function of TSH derived from the pars distalis of the pituitary gland was stimulation of the thyroid gland (i.e., production and secretion of thyroid hormones). It was not clear how these two TSHs avoid

functional crosstalk within the body. TSH in the pars distalis is the under the control of the hypothalamic-pituitary-thyroid (HPT) axis (Szkudlinski et al. 2002). Therefore, TSH in the pars distalis is positively regulated by thyrotropin-releasing hormone (TRH) and negatively regulated by T_3 with a negative feedback loop. In contrast, TSH in the mammalian pars tuberalis is under the control of melatonin and independent of the HPT axis (Ono et al. 2008). Pars tuberalis-derived TSH is released into peripheral circulation, but it does not stimulate the thyroid gland. TSH is a glycoprotein hormone and TSH produced from the pars distalis has a sulfated biantennary *N*-glycans. This *N*-glycan is recognized and rapidly metabolized by the liver. In contrast, pars tuberalis-derived TSH had a sialylated multibranched *N*-glycans. This large *N*-glycan was recognized by immunoglobulin (IgG) and albumin in the peripheral circulation and formed the macro-TSH complex and lost its bioactivity (Ikegami et al. 2014). Thus, tissue-specific glycosylation appears to regulate the functional diversification of the two TSHs.

6.10 Signal Transduction Cascade for Seasonal Reproduction in Fish

Most fish living outside the tropical regions also show marked seasonal changes in physiology and behavior. Medaka (*Oryzias latipes*) are LD breeders, and their gonads develop in response to elongated day-length (Koger et al. 1999). Salmonids, SD seasonal breeders, show distinct photoperiodic responses, such as migration and parr-smolt transformation. Smoltification is closely linked to thyroid hormone (Robertson 1949; Nishikawa et al. 1979). Although all fishes examined had higher circulating levels of melatonin during the night than during the day, there are few reliable data consistent with a major physiological role for melatonin in the seasonal reproduction of fish (Urasaki 1976; Garg 1989; Masuda et al. 2005; Borg 2010). This is in marked contrast to mammals, but is similar to birds. Fish do not have an anatomically distinct pars tuberalis, a regulatory hub of seasonal reproduction in birds and mammals. Thus, the signal transduction pathway for seasonal reproduction in fish remained unknown.

A recent study of masu salmon (*Oncorhynchus masou masou*) revealed that key elements for vertebrate seasonal reproduction, such as rhodopsin family proteins, TSH, TSHR, and DIO2, are all expressed in the saccus vasculosus (SV). The SV is an organ only observed in fish and is located on the floor of the hypothalamus, posterior to the pituitary gland. Although its existence was first described in the seventeenth century (Collins 1685), its physiological function remained unknown for several centuries. The SV consists of coronet cells, supporting cells, and CSF-contacting neurons (Sueiro et al. 2007). The coronet cells have morphologically specialized features; globules occupy the apical cellular structures of these cells. Each globule has cilia, as do photoreceptors in the retina and CSF-contacting neurons in the PVO (Jansen et al. 1982; Vigh and Vigh-Teichmann 1998). Therefore, the coronet cells are considered to be a family of CSF-contacting neurons.

Immunohistochemical analysis has revealed localization of photopigments (OPN4 and SWS1), TSH, and DIO2 in coronet cells (Nakane et al. 2013, Fig. 6.8). The expression of these photoperiodic regulatory mechanisms within the SV implies that the SV plays a pivotal role as a seasonal sensor in fish. Indeed, isolated SVs respond to photoperiodic changes *in vitro* and ablation of the SV prevents photoperiodically-induced gonadal development (Nakane et al. 2013). These results suggest that coronet cells within the SV have multiple functions, including photoreception and neuroendocrine output.

6.11 Conclusions

In summary, studies have uncovered the signal transduction cascade that regulates seasonal reproduction, from photoreceptors to neuroendocrine output, in birds (Fig. 6.2). Light information received by deep brain photoreceptors (e.g., OPN5, RH, OPN4, and VA-opsin) is transmitted to the pars tuberalis and LD induced TSH secreted from the pars tuberalis acts on TSHR to regulate DIO2/DIO3 switching in the ependymal cells. Bioactive T_3 converted from T_4 by DIO2 causes morphological changes in GnRH nerve terminals and glial processes in the median eminence, thereby regulating seasonal changes in GnRH secretion (Fig. 6.2). Involvement of thyroid hormones in the regulation of seasonal reproduction has been suggested for several decades. Recent comparative studies clearly revealed that the local activation of thyroid hormone within the hypothalamus is a key factor in the regulation of seasonal reproduction in a number of avian and mammalian species. It is important to note that this mechanism is also conserved in fish (Nakane et al. 2013) and is universal among various vertebrate species.

Rapid and dramatic seasonal changes in testicular size are intriguing. When birds are transferred from LD to SD conditions, gonads regress to immature size. Spermatogonial stem cells reside in a microenvironment called a niche. Thus, further studies are required to elucidate the regulation of spermatogonial stem cells and their niche under SD and LD.

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Chapter 7
Roles of Germline Stem Cells and Somatic Multipotent Stem Cells in *Hydra* Sexual Reproduction

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Abstract In *Hydra*, there are distinct germline stem cells (GSCs). The GSCs are cell-autonomously sex determined in *Hydra*: there are egg-restricted stem cells (EgSCs) and sperm-restricted stem cells (SpSCs). The sex of the individual polyp is determined by the sex of the GSCs present in the body: polyps that have EgSCs are female and produce eggs; while polyps that have SpSCs are male and produce sperm. Occasionally an EgSC transdetermines into an SpSC. The newly emerged SpSC proliferates vigorously and then differentiates into sperm, while EgSCs are eliminated in the presence of SpSCs. Thus, the animal changes sex from female to male. This process is called masculinization. The third stem cell type in *Hydra* is referred to as multipotent stem cells (MPSCs). MPSCs are somatic stem cells in normal polyps and differentiate exclusively into somatic cells such as nerve cells and nematocytes (cnidarian stinging cells). However, if GSCs are lost during asexual reproduction by budding or regeneration, new ones are regenerated from MPSCs. Thus, sexual reproduction is guaranteed for every polyp. In the rest of this chapter we further discuss the nature of MPSCs found in other lower metazoans and the absence of MPSCs in cnidarians other than hydrozoans.

Keywords Germline stem cells · Multipotent stem cells · Sex determination · Masculinization · Cell plasticity · *Hydra* · Cnidaria

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7.1 Introduction

Germline cells are the cell lineage that contributes the continuity of life. In several model animals such as *Drosophila* and *Caenorhabditis elegans*, germline cells are set aside in early embryogenesis. On the other hand, in many nonmodel animals, it has been revealed that germline cells are generated from multipotent cells (MPCs) (Juliano and Wessel 2010). In most of these animals, such MPCs are lost by differentiation during embryogenesis. However in *Hydra*, MPCs are still present in adult polyps as multipotent stem cells (MPSCs) and retain the ability to generate germline stem cells (GSCs). Thus, *Hydra* is an ideal system to examine the relationship between MPCs/MPSCs and GSCs.

Germline cells have one of the two sexes. In many higher animals, the sex of the germline cells is determined by the signals produced by the gonads, which originate from the mesoderm. However the mechanisms of the germline sex determination differ depending on the animal, probably because these systems developed independently in different bilaterian clades. *Hydra*, as a member of the Diploblastica, have no mesoderm and no gonads, and the sex of the GSCs is determined cell autonomously: there are both egg-restricted stem cells (EgSCs) and sperm-restricted stem cells (SpSCs) in *Hydra*. The autonomous sex determination of germline cells is the ancestral condition before mesoderm development. Thus, *Hydra* is one of the ideal systems to study ancestral mechanisms of sex determination of germline cells.

In the first part of this chapter, we introduce *Hydra* and its stem cell system. In this part, the properties of three kinds of stem cells— MPSCs, EgSCs, and SpSCs— and their interaction are explained. On the basis of their properties and interactions, the sex of individual polyps is determined.

In the rest of the chapter, we discuss the properties of MPSCs in general and then introduce dedifferentiation and transdifferentiation, which are widely observed in cnidarian species that have no MPSCs. The dedifferentiation and transdifferentiation are an alternative mechanism to the MPSC system for producing necessary cells such as nerve cells.

7.2 Body Plan and Cell Lineages in *Hydra*

7.2.1 Hydra *Has a Simple Body Plan*

Hydra is a solitary freshwater cnidarian polyp with a simple body plan (Fig. 7.1). The body is a hollow cylinder. At the oral end of the body, there is a hypostome (a mouth) surrounded by several tentacles, while at the aboral terminus, the cylinder forms a slender stalk, or peduncle, that ends with a sticky basal disk, with which the animal attaches to substrates, such as waterweeds, or to the water surface to float around. Prey is caught by the tentacles, brought into the gastric cavity through the mouth, and digested. The fecal pellet is excreted from the mouth.

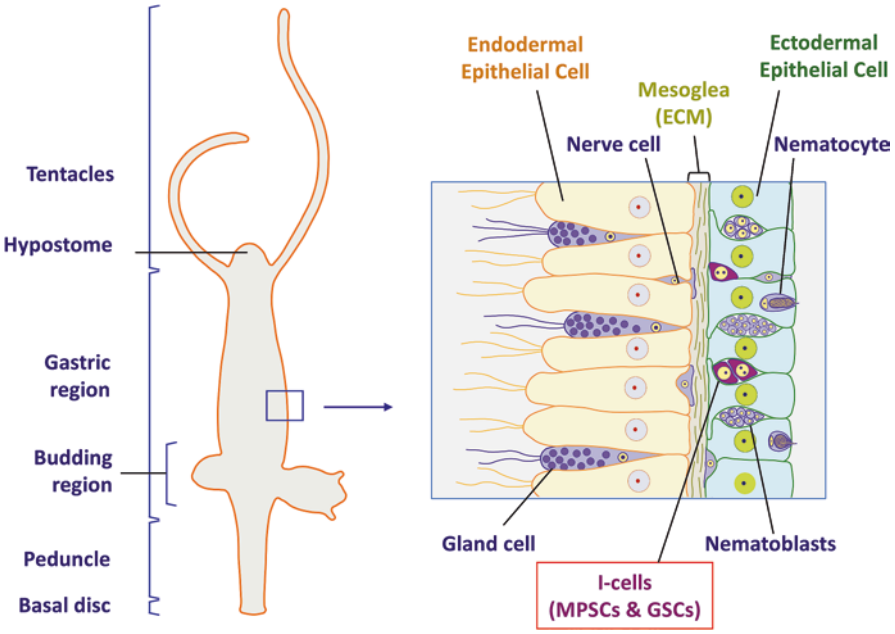

Fig. 7.1 Schematic drawing of an asexually budding *Hydra*, showing the different regions of the polyp and an enlargement of the body wall. The tissue consists of ectodermal and endodermal epithelial layers throughout the body. The mesoglea is the extracellular matrix (ECM) located between the two epithelial cell layers. Multipotent stem cells (MPSCs) and germline stem cells (GSCs) are lodged in the interstices between ectodermal epithelial cells. Nerve cells, gland cells, and nematocytes are the differentiation products of the MPSCs. Nematoblasts are differentiating precursors to nematocytes and occur in clusters connected by cytoplasmic bridges

The hollow cylindrical body column consists of two germ layers (diploblasty): the outer layer consists of ectodermal epithelial cells, and the inner layer consists of endodermal epithelial cells (or digestive cells). Both types of epithelial cell are epitheliomuscular cells. In the ectodermal epithelial cells, the muscle fibers run longitudinally along the body axis on the basal side, while those in endodermal epithelial cells extend around the axis. Between the two cell layers there is an extracellular matrix called the mesoglea, which gives strength and elasticity to the body wall.

In the interstitial spaces between the epithelial cells, there are several differentiated cell types: nerve cells, nematocytes (cnidarian stinging cells) for mainly capturing prey, and gland cells for producing digestive enzymes. In a sexual polyp, gametes (eggs and sperm) are also formed in the ectodermal interstices (not shown in Fig. 7.1, see Fig. 7.2 and Sect. 7.7.2, 3). In addition to these differentiated cell types, there are three types of undifferentiated cells, collectively referred to as interstitial stem cells (I-cells) (see Sect. 7.2.2).

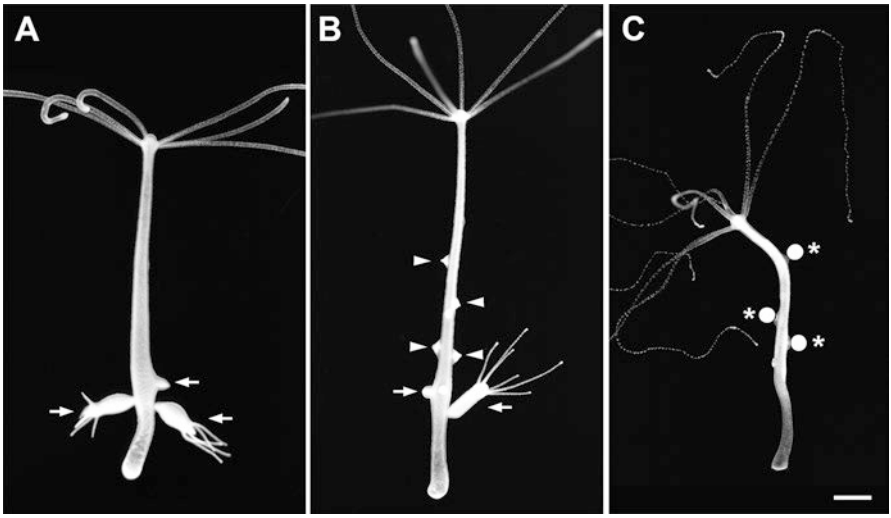

Fig. 7.2 Asexual and sexual polyps of *Hydra magnipapillata.* (**A**) Asexual polyp with a bud protrusion and two young buds (arrow). (**B**) Male polyp with several testes (arrowhead). (**C**) Female polyps with three eggs (asterisk)

Since there are only a few cell types in *Hydra* tissue, each of them can be easily identified under a phase-contrast microscope with the maceration procedure, where the tissues are dissociated into single cells in a semifixed state (David 1973). The cell composition of the tissue is kept constant by homeostatic control in the asexual state (Bode et al. 1976).

7.2.2 Hydra *Tissue Consists of Three Independent Cell Lineages*

All cell types present in *Hydra* belong to one of the three independent stem cell lineages: the ectodermal and endodermal epithelial cells, and the I-cells. These three lineages proliferate independently, and cell type conversion among them is never observed (Sugiyama and Fujisawa 1978a, b; Wanek et al. 1980).

The ectodermal and endodermal epithelial cells proliferate and, with the expansion of their numbers, are displaced along the body axis toward the budding region to produce new polyps, or toward the two extremities, i.e., the hypostome and basal disk. At the base of the tentacles and in the basal disk, both epithelial cell types are arrested at G2 of the cell cycle (Dübel 1989; Dübel and Schaller 1990). While endodermal epithelial cells show no major functional changes at these sites, ectodermal epithelial cells differentiate into specialized cell types. At the base of the tentacles, they differentiate into battery cells, which take up numerous mature nematocytes migrated from the body column, where they are differentiated. In the basal disk,

ectodermal epithelial cells differentiate into glandular epithelial cells, which secrete mucus substances so that polyps can attach to substrates. Both epithelial cells continue movement toward the tips of the tentacles or the center of the basal disk, and then are sloughed off.

I-cells are undifferentiated stem cells in *Hydra*. They are 12–20 μm in size and angular in shape. They possess a granular cytoplasm and a large nucleus (8–12 μm) with one or two conspicuous nucleoli (David 1973). I-cells in *Hydra* differentiate into all cell types other than epithelial cells, i.e., nerve cells, nematocytes, gland cells, and gametes. Thus, the I-cells of *Hydra* are different from planarian neoblasts, which are pluripotent stem cells and give rise to all cell types spanning three germ layers, including epithelial cells (Wagner et al. 2011; Wolfswinkel et al. 2014; Zhu et al. 2015).

Although I-cells are histologically indistinguishable from each other, several I-cell cloning experiments have revealed that they consist of three distinct subpopulations, which have different differentiation capabilities: one is multipotent stem cells (MPSCs), which give rise to nerve cells, nematocytes, and gland cells; the other two are germline stem cells (GSCs)—egg-restricted stem cells (EgSCs) and sperm-restricted stem cells (SpSCs), which give rise to eggs or sperm, respectively. Figure 7.3 shows the interactions between these cell types, which are described in detail in section 7.3–7.6.

7.3 Multipotent Stem Cells in *Hydra*

7.3.1 Cloning of Multipotent Stem Cells: A Single Multipotent Stem Cell in Hydra *Can Give Rise to Nerve Cells, Nematocytes, and Germ Cells*

The multipotency of I-cells was first shown in *Hydra magnipapillata* by David and Murphy (1977). They treated host tissue with nitrogen mustard (NM) to eliminate all of the I-cells (Diehl and Burnett 1964). This NM-treated tissue was dissociated into single cells and then combined with a small amount of dissociated normal tissue to make aggregates such that only one clone-forming I-cell was included per aggregate (Fig. 7.4). In *Hydra*, a reaggregated cell clump can regenerate into a polyp (Noda 1970, 1971; Gierer et al. 1972). In all of the regenerated animals that had I-cells, both nerve cells and nematocytes were also present, indicating that a single I-cell proliferated and gave rise to both nematocytes and nerve cells. No aggregates that had only nerve cells or only nematocytes were obtained. In this manner, the multipotency of a single I-cell was demonstrated (Fig. 7.3 [1]). However, NM-treated tissue did not live long enough for cloned stem cells to develop a sexual polyp. To overcome this problem, Bosch and David (1986, 1987) carried out a similar experiment using a mutant strain that has temperature-sensitive I-cells (Sugiyama and Fujisawa 1978a, b) as the host, and then showed that individual I-cells can differentiate not only into somatic cells but also into germ cells (eggs and sperm).

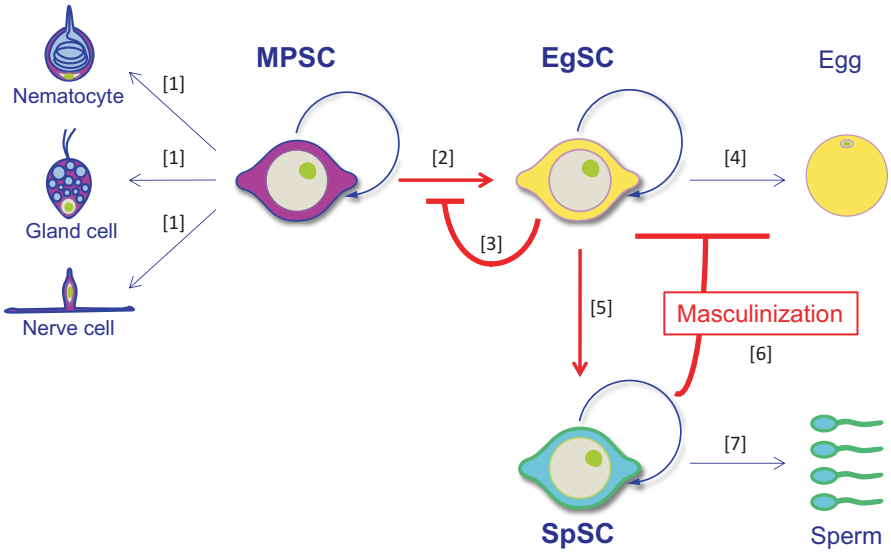

Fig. 7.3 Differentiation pathway of multipotent stem cells (MPSCs), egg-restricted stem cells (EgSCs), and sperm-restricted stem cells (SpSCs), and the interaction among these cells. MPSCs self-renew and differentiate into three kinds of somatic cells [1]. An MPSC can differentiate into an EgSC [2], but in the presence of EgSCs the differentiation from an MPSC into an EgSC is suppressed [3]. EgSCs differentiate only into eggs [4]. An EgSC occasionally transdetermines into an SpSC [5]. A newly appeared SpSC proliferates and subsequently masculinizes the polyp [6]. While EgSCs are eliminated from the tissue by masculinization [6], SpSCs differentiate only into sperm [7]

Multipotency of an I-cell was directly observed in vivo (Teragawa and Bode 1995; Hager and David 1997; David 2012). By injection of the fluorescent carbocyanin dye DiI into the body wall to make small patches of stained tissue, the fate of the I-cells migrated from the patches was traced. It was observed that single I-cells proliferated and then gave rise to both nerve cells and nematocytes. It was also observed that differentiation always takes place after an asymmetric cell division in which an I-cell divides to yield one committed cell and one stem cell; occasionally one I-cell divides to yield two differently committed cells. When both daughter cells remain stem cells, they always stay as pairs. Single stem cells were observed only when one of the two daughter cells initiated differentiation (David 2012). Hereafter, we refer to these stem cells as multipotent stem cells (MPSCs).

7.3.2 Somatic Cell Differentiation from Multipotent Stem Cells

When an MPSC is committed to differentiate into nematocytes, the cell divides synchronously several times to form a “nest” of 4, 8, 16, and 32 cells, which are connected by intercellular bridges as a result of incomplete cytokinesis. All of the

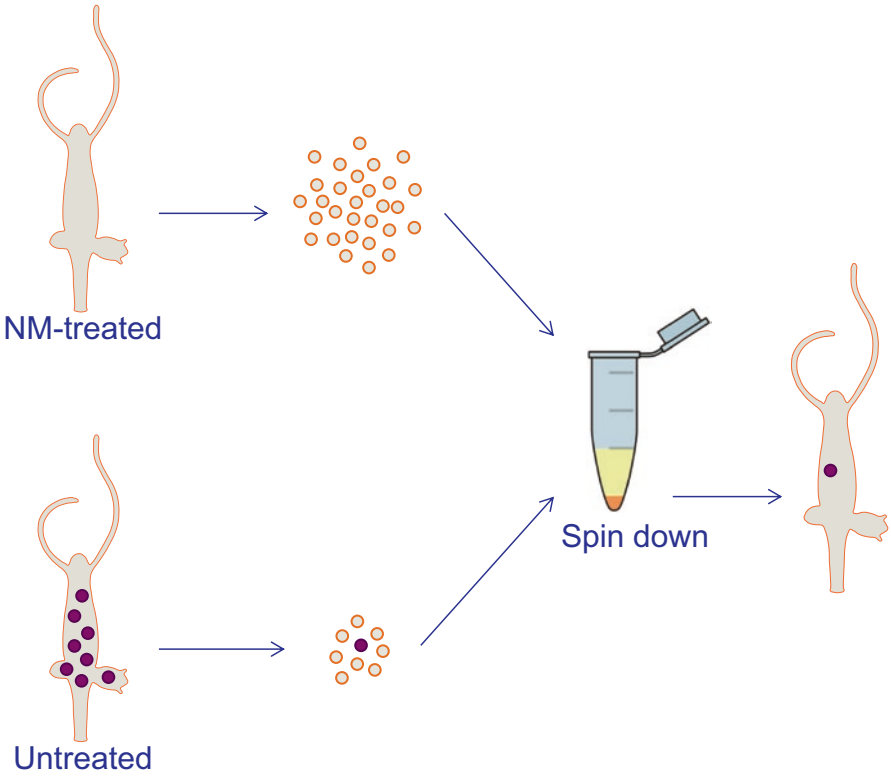

Fig. 7.4 Interstitial stem cell (I-cell) cloning method of David and Murphy (1977). Nitrogen mustard (NM)–treated polyps and untreated polyps are dissociated into single cells. A large number of the former and a small number of the latter cells are mixed and spun down to make a cell aggregate. Aggregates can regenerate into polyps. By controlling the relative amounts of NM-treated and untreated cells, aggregates can be formed, which have only one I-cell, and hence the differentiation capability of a single I-cell can be analyzed

cells in a nest give rise to the same type of nematocyte, i.e., any one of four types (David and Gierer 1974; Tardent 1995; Beckmann and Özbek 2012). In nerve cell differentiation, commitment occurs during the S-phase. The precursors often migrate toward the head or basal disk, where the nerve cell density is high, and divide to yield two or four nerve cells (Hager and David 1997). Gland cells secrete digestive enzymes into the gastric cavity. Although gland cells are capable of self-renewal, they are also differentiated continuously from MPSCs. Gland cell differentiation occurs after a committed MPSC moves from the ectoderm to the endoderm by passing through the mesoglea (Smid and Tardent 1986; Schmidt and David 1986; Bode et al. 1987).

7.4 Germline Stem Cells in *Hydra*

7.4.1 Identification of Germline Stem Cells

There are two kinds of GSCs in *Hydra*—EgSCs and SpSCs—which self-renew in a polyp and give rise to egg lineage cells (oocytes and nurse cells) and sperm, respectively, but not to somatic cells. GSCs were not found in the cloning experiments done by Bosch and David (1986, 1987), presumably because of the small number of GSCs in comparison with MPSCs in the tissue. To isolate GSCs, different methods were required.

The existence of SpSCs was first suggested by an analysis with monoclonal antibody AC2 (Mab-AC2) in *Hydra oligactis* (Littlefield et al. 1985). Mab-AC2 recognizes subpopulations of I-cells in asexual males and all sperm lineage cells in sexual male polyps. Then polyps containing GSCs (EgSCs or SpSCs) but no MPSCs were obtained either by treatment of *Hydra* with hydroxyurea (HU) or by culture of mutant animals with temperature-sensitive I-cells at the restrictive temperature (Littlefield 1985, 1991; Nishimiya-Fujisawa and Sugiyama 1993, 1995).

Although it is not clear why the GSCs of the mutant strain, which has temperature-sensitive I-cells, are resistant to high temperature, they survived 4- to 8-day culture at a nonpermissive temperature (25 °C) in our experiments. In the case of HU, it is known to specifically kill cells in the S-phase of the cell cycle (Sinclair 1965). Since the cell cycle times of MPSCs and GSCs are about 24 h and 2.5–4 days, respectively (Campbell and David 1974; Holstein and David 1990; Littlefield 1991), HU treatment beyond 4 days abolishes both MPSCs and GSCs. Therefore, these animals become "epithelial *Hydra*," which have only epithelial cells and lack all cells of the I-cell lineages except for self-renewing gland cells. When the treatment time is shortened to 2–3 days, some GSCs survive, while all MPSCs are eliminated. In this way, animals that have GSCs but no MPSCs are generated.

Animals that have lost MPSCs gradually lose the ability to move or catch prey, since no nerve cells and nematocytes are newly produced. Nevertheless, they can digest food if they are hand fed, and they can grow and produce buds as normal animals do. When sexual differentiation is induced, they produce eggs or sperm, depending on which type of GSCs they have. These animals can be maintained for years, during which time their GSCs retain the ability to differentiate into gametes, indicating that they are real stem cells.

7.4.2 Evolutionarily Conserved Properties of Hydra *Germline Stem Cells*

Until recently, the dominant idea was that the sex of germline cells in higher animals is determined by the gonad in which the germline cells are located. However, recent data from *Drosophila*, mice, and medaka fish indicate that intrinsic factors

in germline cells are also important in determining their sex (McLaren 1981; Burgoyne 1987; Hashiyama et al. 2011; Nishimura et al. 2015), as in earlier reports on *Hydra* GSCs (Littlefield 1985, 1991; Nishimiya-Fujisawa and Sugiyama 1993, 1995). We propose that autonomous sex determination of GSCs is the ancestral condition and that somatic involvement in sex determination evolved later when body plans became more complex.

Another evolutionarily conserved trait of germline cells in animal species is cyst formation. It is observed not only in spermatogenesis (Iwamori et al. 2010) but also in female germline cells in prenatal mice (Pepling and Spradling 1998); in the germinal cradle in female medaka fish (Nakamura et al. 2010); in female *Xenopus* (Kloc et al. 2004); and in female *Drosophila* (Lin and Spradling 1993). Germline cyst formation is also observed in *Hydra*, and the cyst is usually referred to as a nest (Littlefield 1985, 1991: Nishimiya-Fujisawa and Sugiyama 1993, 1995). When male and female polyps containing only GSCs (SpSCs or EgSCs) and no MPSCs were stained with monoclonal antibody C41 (Mab-C41) (David et al. 1991), SpSCs/sperm lineage cells appeared in longitudinal filaments in the male body (Fig. 7.5C), while EgSCs/egg lineage cells showed a patchy appearance in the female body (Fig. 7.5D) (Nishimiya-Fujisawa and Sugiyama 1995). When these animals were dissociated into single cells by the maceration procedure (David 1973), it was confirmed that both the egg and sperm lineage cells form syncytial clusters connected by intercellular bridges. The microtubule bundles that span the cytoplasmic bridges in germline nests are distinctly thicker than those in MPSC pairs (Lim et al. 2014). In *Hydra*, the cell number in germline nests is usually smaller than 32 but in some instances larger than 100. The number is not always a power of 2 (Littlefield 1994), in contrast to that in nematoblast nests, which mostly form nests of 4, 8, 16, or 32. This suggests that nests of germline cells often break. In mouse testes, such fragmented spermatogonial cells also serve as stem cells (Hara et al. 2014). We expect the same system to work in *Hydra*.

Spermatogonial stem cells in mice are scattered throughout seminiferous tubules. In such cases, the niche is described as open or facultative, and it is not restricted to anatomically specialized cells, e.g., testicular hub and ovarian cap cells in *Drosophila*, and gonadal distal tip cells in *C. elegans* (Morrison and Spradling 2008). Similarly, in *Hydra*, EgSCs and SpSCs, as well as MPSCs, are distributed throughout the body column except in the head, peduncle, and basal disc (Bode et al. 1973; Nishimiya-Fujisawa and Sugiyama 1995), suggesting that GSCs and MPSCs are stably maintained within an open niche. In open-niche systems, the mechanisms that determine the homeostatic balance between self-renewal and differentiation are not known. Recently, in mouse spermatogenesis, it was revealed that the balance is achieved by the differential sensitivity of spermatogonia to the differentiation-inducing signal retinoic acid (Ikami et al. 2015). However it has remained unclear how this different competence is attained. In *Hydra*, some EgSCs and SpSCs are also retained after gametogenesis, but the mechanism preventing their differentiation is not known.

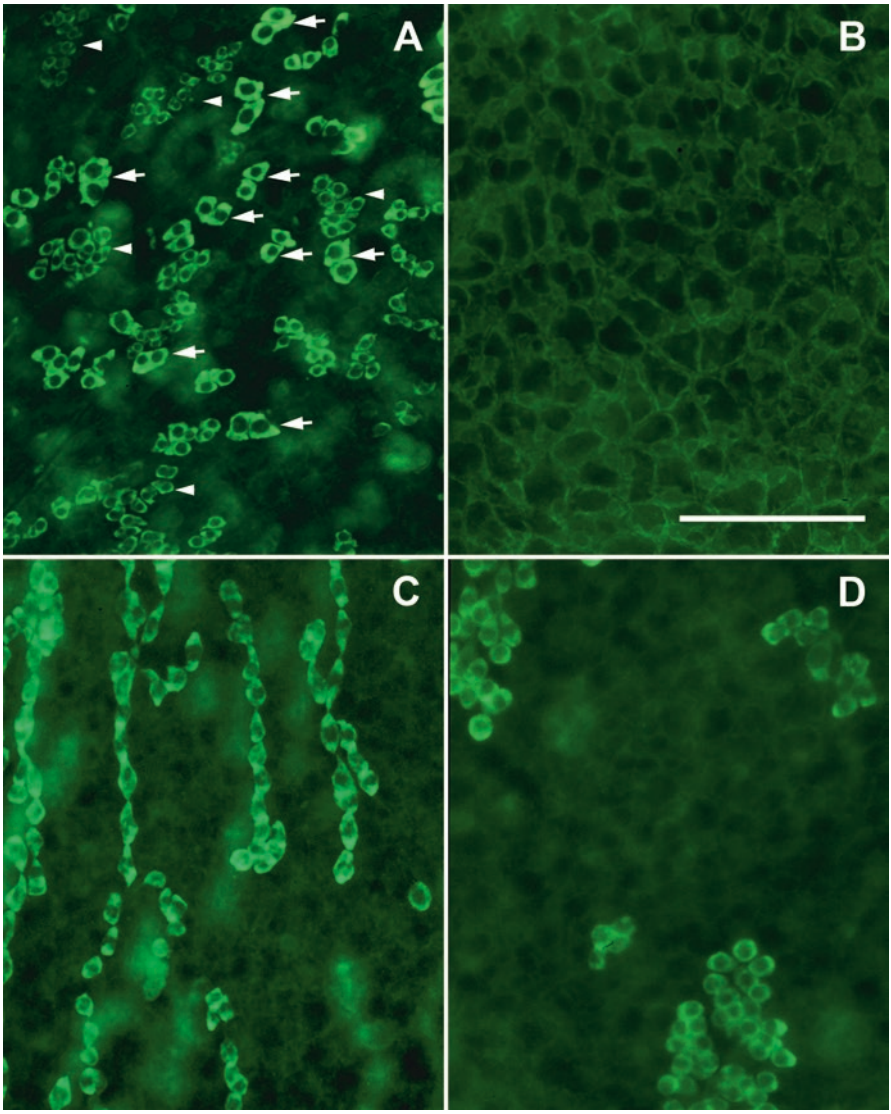

Fig. 7.5 Immunostaining of interstitial stem cell (I-cell) lineage cells with monoclonal antibody C41 (Mab-C41). (**A**) Normal male tissue. Both I-cells (arrows) and nematoblasts (arrowheads) are stained. (**B**) Epithelial *Hydra* with no I-cell lineage cells. (**C**) Male tissue containing only sperm lineage cells, which are arranged in strings. (**D**) Female tissue containing only egg lineage cells, which are arranged in patchy nests. Scale bar, 100 μm. (The Mab-C41 was obtained courtesy of Prof. Charles David, Munich)

7.5 Multipotent Stem Cells Differentiate into Egg-Restricted Stem Cells Only When the Latter Are Absent

7.5.1 In Normal Polyps, Multipotent Stem Cells Function as Somatic Stem Cells and Do Not Give Rise to Germline Stem Cells or Gametes

Because a single MPSC can differentiate into gametes, in addition to somatic cells (Bosch and David 1986, 1987), it was assumed that EgSCs and SpSCs in *Hydra* are only intermediate precursors between MPSCs and gametes, and that MPSCs continuously give rise to GSCs and/or directly to gametes in cooperation with GSCs in a sexual polyp. However, recent experiments, described below, have shown that MPSCs do not differentiate into gametes directly, nor do they form GSCs in a normal polyp, which already has GSCs in its tissue (Chiemi Nishimiya-Fujisawa, manuscript in preparation).

We created several transgenic animals that expressed green fluorescent protein (GFP) under the control of the promoter of *Cnnos1*, one of the two *Drosophila nanos* homologues in *Hydra*. One freshly hatched animal from a microinjected egg was chimeric-transgenic: it expressed GFP only in a small number of MPSCs (transgenic), while most of the MPSCs were GFP negative (nontransgenic). During growth of the polyp, the GFP-positive MPSCs increased clonally and formed large patches in the tissue. During further asexual reproduction, we selected buds containing larger numbers of GFP-positive MPSCs. By repeating such a selection several times, we finally obtained animals full of GFP-positive MPSCs. Nevertheless, when sexual differentiation was induced, these transgenic animals did not express GFP in the eggs (Fig. 7.6A), although the *Cnnos1* promoter is active in MPSCs, EgSCs, and eggs. The polyps of this *Hydra* clone have never expressed GFP in eggs.

To explain these results, we hypothesized that during embryogenesis, GFP-negative/nontransgenic EgSCs developed from GFP-negative/nontransgenic MPSCs, which were the dominant population of MPSCs at the embryonic period. These GFP-negative EgSCs proliferated and were maintained during the period of selection for polyps containing larger numbers of GFP-positive/transgenic MPSCs. The absence of GFP-positive/transgenic EgSCs in these animals suggested that MPSCs do not differentiate into EgSCs when EgSCs are already present.

To test this idea directly, we removed all pre-existing GFP-negative/nontransgenic EgSCs and looked for the formation of new GFP-positive/transgenic EgSCs from GFP-positive transgenic MPSCs. To do this, we cut the transgenic animals into small pieces so that some of the tissue pieces included only transgenic MPSCs and no EgSCs, while others included both transgenic MPSCs and nontransgenic EgSCs. These tissue fragments were allowed to regenerate, and then sexual differentiation was induced. GFP was detected in eggs in several regenerates (Fig. 7.6B), while most of the regenerates produced GFP-negative eggs, as before the regeneration.

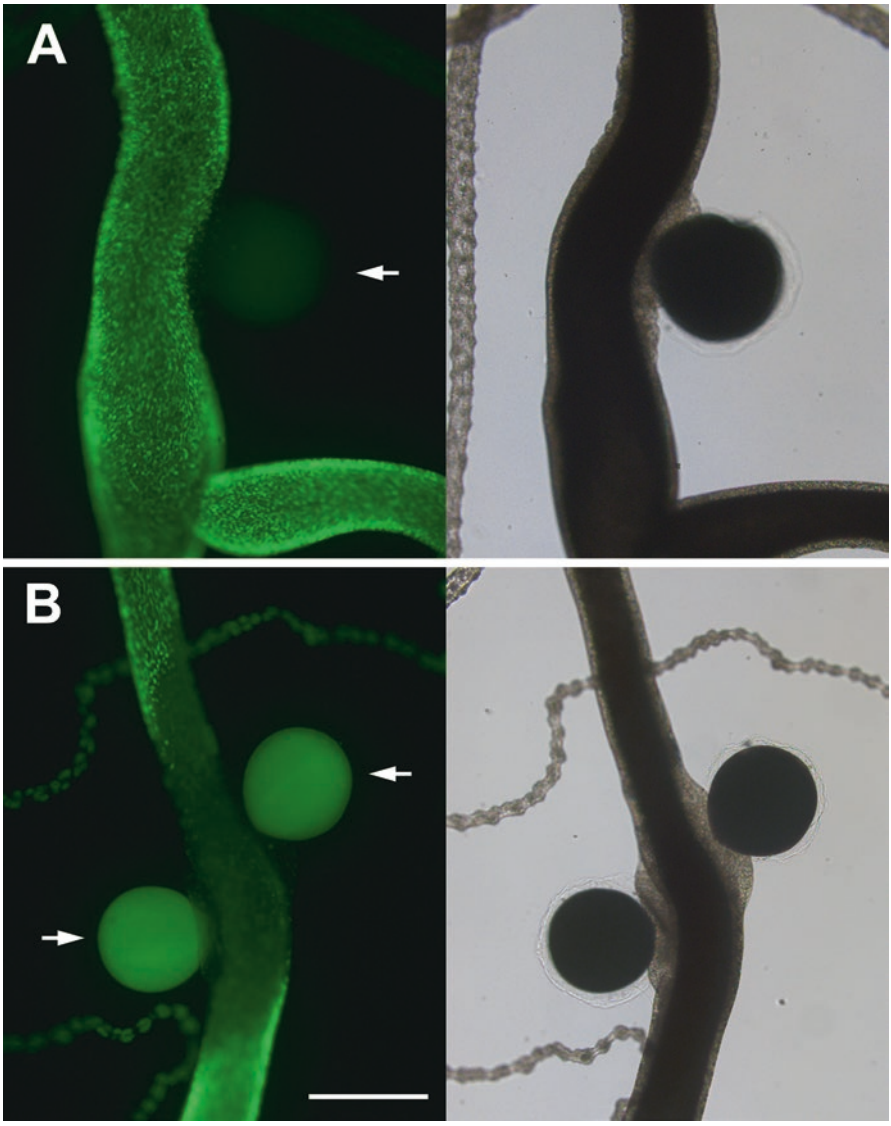

Fig. 7.6 Sexual female transgenic animals expressing green fluorescent protein (GFP) under the control of a *Cnnos1* promoter. Both animals originate from the same clone. (**A**) The left panel shows a fluorescent microscopic image of a original female polyp having a transgene (*Cnnos1*::GFP) in multipotent stem cells (MPSCs) but not in egg-restricted stem cells (EgSCs), thereby expressing no GFP in the egg. The right panel shows a differential interference contrast (DIC) view of the same polyp. (**B**) The left panel shows a fluorescent microscopic image of a polyp having the transgene both in MPSCs and in EgSCs, and accordingly expressing GFP in the eggs. The right panel shows a DIC view of the same polyp. This animal is a subclone of a *Cnnos1*::GFP transgenic *Hydra* (A), which regenerated from a small piece of transgenic tissue and was propagated by asexual budding. Scale bars, 500 μm

This result indicates that MPSCs can give rise to EgSCs but not when EgSCs are already present in the tissue (Fig. 7.3 [2], [3]). Thus, it can be concluded that MPSCs are functionally somatic stem cells dedicated to production of somatic cells during normal growth. However, when GSCs are lost—e.g., in MPSC cloning experiments (Bosch and David 1987) or in small regenerating tissue fragments as described above—MPSCs give rise to GSCs.

7.5.2 Multipotent Stem Cells Give Rise to Egg-Restricted Stem Cells But Not Sperm-Restricted Stem Cells

Bosch and David (1987) used the mutant male strain ms-1, which produces immotile sperm, as the I-cell donor for their first MPSC cloning experiment and obtained both male and female clones. Then, to examine the stability of the sex of MPSCs, they recloned the MPSCs of these males and females 6–8 months later (Bosch and David 1986). When they used female clones as the MPSC donors, only female polyps were obtained, while both male and female polyps were obtained when male clones were used. Their conclusion was as follows: in *Hydra*, there are male and female MPSCs, which give rise to sperm and eggs, respectively; the female MPSCs are sexually stable, while male MPSCs are labile and often change into female MPSCs.

In contrast to their findings that MPSCs themselves have separate sexes, we unexpectedly found that MPSCs give rise only to EgSCs and not to SpSCs, on the basis of the following experimental results (Fig. 7.3 [2]). By in situ hybridization using cell type–specific probes (Chiemi Nishimiya-Fujisawa, manuscript in preparation), we showed that EgSCs and SpSCs are absent but MPSCs are present in the peduncle region (the lower part of the polyp under the budding region) of both male and female polyps. To examine the sex of these MPSCs, we excised the peduncle regions from male and female polyps, and we allowed them regenerate (Nishimiya-Fujisawa and Sugiyama 1995). While all of the regenerates from female strains became female as expected, essentially all of the regenerates from three male strains also became female (Table 7.1). Actually, these three male strains were originally female, but a spontaneously emerged male polyp had been kept clonally thereafter. We refer to such sex-reversed males as phenotypic males. In addition, we also obtained one female regenerate from another male strain, Stable Sex strain E (SSE). These observations support the idea that MPSCs give rise only to EgSCs even in a stable born male, and that somehow SpSCs may secondarily appear. Although males contain SpSCs, they were not differentiated from MPSCs but from EgSCs, as will be mentioned in Section 7.6.3. The difference between phenotypic males and born males may be that the conversion rate of EgSCs to SpSCs is high in the latter strain.

In contrast to the above results, we did not get any female regenerates from peduncle tissue of strain ms-1, which was used as the I-cell donor in the cloning experiment done by Bosch and David (1986, 1987). This result may suggest that there are male MPSCs, as shown by Bosch and David (1986). However, the possibility of SpSC contamination in the peduncle region of this strain was not completely ruled out.

Table 7.1 Sexual differentiation of peduncle tissue regenerates

Strain[a]	Sex	No. examined	No. of regenerates forming			
			Testes	Eggs	Both	Neither
nem-1.m	♂	31	1	29	1[b]	0
SSC.m	♂	13	0	13	0	0
nB-2	♂	22	0	22	0	0
ms-1	♂	8	8	0	0	0
SSE	♂	24	23	1	0	0
nem-1	♀	19	0	19	0	0
SSC	♀	28	0	28	0	0
SSB	♀	22	0	21	0	1[c]

Induction of sexual differentiation was started when each regenerate had produced several mature offspring.

[a]nem-1, SSC, and SSB were originally female. nem-1.m and SSC.m arose by spontaneous sex reversal. ms-1 and SSE were originally male. nB-2 was obtained by a sexual cross of nem-1.m and SSC. See details in Nishimiya-Fujisawa and Sugiyama (1995).

[b]One offspring produced eggs, while four others produced testes.

[c]This clone of regenerates all turned into epithelial *Hydra*.

7.6 Relationship Between Sperm-Restricted Stem Cells and Egg-Restricted Stem Cells

7.6.1 Sperm-Restricted Stem Cells Can Masculinize Female Polyps

In gonochoristic species of *Hydra*, when a piece of male tissue is grafted onto a female polyp, the female turns into a male and produces sperm even if the male tissue is removed 2–3 days after grafting (Goetsch 1922; Tardent 1966, 1968). This phenomenon has been termed "masculinization." As the cause, a masculinizing hormone from male epithelial tissue was once suspected (Brien 1962, 1963). This idea has now been disproved by analysis of chimeric polyps containing different epithelial cell and I-cell lineages. When I-cells from a female animal (in this case, MPSCs and EgSCs) were introduced to an epithelial *Hydra*, which was a male before elimination of I-cells (in this case, MPSCs and SpSCs), the polyp turned into a female: the sex of such chimeras is always the same as that of the I-cell donor (Littlefield 1984; Campbell 1985), indicating that I-cells (MPSCs and/or GSCs), and not epithelial cells, determine the sex of polyps.

Subsequently, it was shown that SpSCs alone can masculinize a female polyp. When a male polyp, containing only SpSCs and no MPSCs (Fig. 7.5C), was grafted onto a normal female, the female turned into a male (Fig. 7.3 [6]) (Littlefield 1986; Nishimiya-Fujisawa and Sugiyama 1995). Furthermore, when a BrdU-labeled male polyp containing only SpSCs was grafted onto a normal female polyp, the BrdU-labeled sperm lineage cells were observed to massively migrate into the female tissue (Nishimiya-Fujisawa and Sugiyama 1995; Nishimiya-Fujisawa and Kobayashi 2012).

Back in 1985, Sugiyama and Sugimoto grafted a mutant male that produced immotile sperm onto a female that produced deformed nematocytes. Even though the male tissue was removed 2–3 days later, the female turned into a male, which produced only immotile sperm, while this polyp continued to produce deformed nematocytes. This result can be interpreted as follows: SpSCs of the mutant male migrated to the female tissue, proliferated, and gave rise to immotile sperm, while the MPSCs of the female polyp remained in the masculinized polyp and continued to produce deformed nematocytes. It appears that MPSCs are not involved in the masculinizing process.

7.6.2 Egg-Restricted Stem Cells Are Eliminated During Masculinization

When a BrdU-labeled female polyp containing only EgSCs (Fig. 7.5D) was grafted onto a normal male, migration of EgSCs into the host tissue was never observed, although EgSCs showed migratory capability when the same type of female polyp was grafted onto an epithelial *Hydra* (Fig. 7.5B), i.e., in the absence of SpSCs in the host polyp (Nishimiya-Fujisawa and Kobayashi 2012).

To investigate the fate of EgSCs during masculinization, a female polyp containing only EgSCs and no MPSCs was labeled long enough with BrdU so that all of the egg lineage cells were labeled, and it was then grafted onto an unlabeled male polyp containing only SpSCs (Fig. 7.5C). Several days after grafting, the grafted polyps were macerated, and the BrdU-labeled egg lineage cells and unlabeled sperm lineage cells were counted. Most of the egg lineage cells (labeled) disappeared within several days (Fig. 7.3 [6]). The remaining egg lineage cells were all postmitotic differentiating cells. Thus, EgSCs are eliminated during masculinization (Chiemi Nishimiya-Fujisawa, manuscript in preparation). This experiment also showed that conversion of EgSCs to SpSCs does not occur during masculinization. On the other hand, differentiating egg lineage cells are resistant to the masculinizing effect and survive. This may explain the transient hermaphroditism occasionally observed when a polyp changes sex.

In contrast to the behavior of EgSCs, the number of SpSCs (unlabeled) increased rapidly, as expected from the whole-mount BrdU staining of heterosexual grafts. Thus, SpSCs proliferate rapidly and migrate to the place where other SpSCs are absent during masculinization (Chiemi Nishimiya-Fujisawa, manuscript in preparation). However, when the ratio of SpSCs to epithelial cells reached the original value before grafting, the rapid increase stopped. This behavior of SpSCs is almost the same as the recovery process of MPSCs when their number was experimentally reduced (Bode et al. 1976), suggesting that the SpSC population is homeostatically maintained even during masculinization. Combining the findings from Sects. 7.6.1 and 7.6.2, it can be concluded that masculinization is the process in which SpSCs proliferate, migrate into empty spaces (female tissue), and differentiate into sperm, while EgSCs are completely eliminated. Because of the masculinizing effect,

polyps have either EgSCs or SpSCs, and the sex of the polyp is directly determined by the sex of the GSCs (EgSCs or SpSCs) present in the body.

7.6.3 Transdetermination of Egg-Restricted Stem Cells into Sperm-Restricted Stem Cells

Masculinization is not only induced experimentally by grafting, but is expected to occur during spontaneous sex reversal from female to male. We observed several cases of spontaneous female-to-male sex reversal in several independent clones of females, which were obtained by HU treatment and had only EgSCs and no MPSCs (Fig. 7.5D; Nishimiya-Fujisawa and Sugiyama 1995). These female clones had been maintained for several years by hand feeding. Since SpSCs have strong masculinizing ability (see above), it is unlikely that the female polyps contained any SpSCs. Thus, only EgSCs were present in these females before the sex reversal. Thus, on the basis of the occurrence of sex reversal, we conclude that an EgSC transdetermined into an SpSC (Fig. 7.3 [5]). The newly emerged SpSC proliferated and subsequently masculinized the female.

The following observation also supports the idea that even in a normal polyp, SpSCs arise only from EgSCs and not from MPSCs. When the previously mentioned transgenic female polyp—which expressed GFP only in MPSCs and not in egg lineage cells (Fig. 7.6A)—turned into a male, sperm lineage cells were also GFP negative. Since GFP-positive MPSCs are the dominant population in such animals, if SpSCs were formed from MPSCs, the probability of the occurrence of GFP-positive SpSCs should be high. As a result, GFP-positive SpSCs should be observed in testes. However, this has never been observed (Chiemi Nishimiya-Fujisawa, manuscript in preparation).

7.7 Reproduction in *Hydra*

7.7.1 Asexual and Sexual Reproduction

Under favorable conditions, *Hydra* reproduce asexually by budding (Fig. 7.1; Fig. 7.2A). When a polyp grows to a certain size (approximately 1 cm long), a bud always develops on the lower part of the body column. As the bud evaginates, a head, including tentacles and a mouth, is formed. Then a basal disk develops at the base of the bud, and the bud is finally pinched off from the parental body. The pinching-off of the bud is induced by neuropeptide GLW-amides (Takahashi et al. 1997), inducing constriction of ectodermal sphincter muscle fibers, which run circularly at the base of the basal disk of the bud (Campbell 1997). The whole budding process takes about 3 days.

Under unfavorable conditions (e.g., low temperature for *H. oligactis*; starvation for *H. magnipapillata*), animals initiate sexual differentiation (Fig. 7.2B, C) (Hyman 1928; Sugiyama and Fujisawa 1977). The cues for inducing sexual reproduction seem to be species specific in *Hydra*. When sexual differentiation starts, many hydrozoans develop medusae. Although *Hydra* belong to the Medusozoa in the Cnidaria, they do not develop medusae. The medusa stage is thought to have been lost during the evolution of *Hydra*. *Hydractinia echinata*, which is closely related to *Hydra* and is also commonly used in laboratories, develops partly reduced medusae called gonophores, which do not detach from the polyps. Since *Hydra* have no medusae or gonads, both spermatogenesis and oogenesis occur directly in the ectodermal cell layer.

7.7.2 Spermatogenesis

When spermatogenesis begins in *Hydra*, SpSCs proliferate and strings of sperm lineage cells are formed (Littlefield 1991; Littlefield et al. 1985; Munck and David 1985; Kuznetsov et al. 2001; Fig. 7.5C). These cells then gather together at the base of several presumptive testes on the gastric region of the polyp. What we call testes in *Hydra* are only sacs of ectodermal epithelial cells. During further development, the testes become nipple-shaped, with the more differentiated cells located near the tip. Sperm are released from the hole at the top of the testis (Fig. 7.2B).

Spermatogonia and spermatocytes in the basal region of testes express two components of polycomb repression complex 2 (PRC2): *HyEED* (*Embryonic Ectoderm Development*) and *HyEZH2* (*Enhancer of zeste* in *Drosophila*) (Genikhovich et al. 2006). PRC2 methylates histone H3 and with an anti-H3 di-/trimethyl K27 antibody, a signal was detected in nuclei of spermatogonia and spermatocytes, suggesting the existence of a PRC2 complex and possible epigenetic control of sex differentiation in *Hydra*.

7.7.3 Oogenesis

In the beginning of oogenesis in *Hydra*, EgSCs proliferate massively to make patchy nests (Fig. 7.5D), and the nests fuse to make an large egg patch, which consists of approximately 4000 egg lineage cells (Zihler 1972; Honegger et al. 1989; Littlefield 1991; Miller et al. 2000; Alexandrova et al. 2005). Most of these cells terminate their cell cycle and pass through a premeiotic S-phase to achieve 4N DNA content. About 50 larger cells enter prophase I of meiosis (leptotene to pachytene), and two or three cells become diplotene oocytes (Alexandrova et al. 2005). Finally, through competition between these candidates, only one or two cells develop into mature oocytes (Miller et al. 2000).

The mass of egg lineage cells in the egg patch, other than growing oocytes, turn into nurse cells and transfer cytoplasm to the developing oocytes, which consequently grow dramatically in size. The nurse cells then undergo apoptosis and are phagocytized by the developing oocytes, which have branched pseudopodia at this stage. Interestingly, the apoptotic program of the phagocytized nurse cells is arrested and apoptotic bodies persist in the cells of the embryo for 2–3 months until hatching (Technau et al. 2003). In the final stage of oogenesis, the oocyte retracts its pseudopodia and rounds up. After undergoing meiosis, the oocyte breaks through the ectodermal epithelium (Fig. 7.2C), and fertilization occurs immediately if sperm are present.

7.8 Sex Phenotypes in *Hydra*

7.8.1 *Hermaphroditism and Gonochorism in* Hydra

Hydra are hermaphroditic or gonochoristic (with separate sexes), depending on the species. *H. viridissima* (green *Hydra*), *H. circumcincta*, and *H. hymanae* are hermaphroditic; *H. vulgaris*, *H. magnipapillata*, and *H. oligactis* are gonochoristic. In gonochoristic species, the ratio of male to female hatchlings is reported to be about 1:1 (Littlefield 1994), suggesting some sort of genetic control. However, there is no evidence of sex chromosomes (Anokhin et al. 2010), or no report of sex-determining genes so far. It is also known that in gonochoristic species of *Hydra*, the sexual phenotype of polyps is labile, and individual polyps or buds formed by asexual reproduction can change sex from female to male or from male to female (Tardent 1966, 1968) with a strain-specific frequency. This means gonochoristic *Hydra* are actually chronological hermaphrodites. Simultaneous hermaphroditism is a rare event in these species, probably because of the masculinizing effect.

7.8.2 *Sex Determination Model in Gonochoristic* Hydra *Polyps*

The sex of polyps of gonochoristic *Hydra* strains is directly determined by the sex of GSCs present in the polyps: a female possesses EgSCs, while a male possesses SpSCs. Sex reversal from male to female occurs when SpSCs are lost and EgSCs are newly generated from MPSCs. Littlefield (1994) noted that in *H. oligactis*, SpSCs are susceptible to high temperature; thus, sex reversal from male to female could be induced by a temperature shift up. Sex reversal in the opposite direction occurs when an EgSC turns into an SpSC and subsequent masculinization takes place (Nishimiya-Fujisawa and Sugiyama 1995; Nishimiya-Fujisawa and Kobayashi 2012; Fig. 7.3). The conversion rates from EgSC to SpSC may differ depending on the strain. However, it is not known how the rates are determined genetically, epigenetically, and perhaps environmentally. The mechanisms of sex determination and sex reversal have not been fully elucidated yet.

7.9 What Are Multipotent Stem Cells?

7.9.1 Multipotent Stem Cells Are a Cell Lineage That Contributes to the Continuity of Life

While in some model animals such as *Drosophila* and *C. elegans*, germline cells are sequestered during early embryogenesis, in *Hydra*, GSC loss can induce regeneration of new GSCs from MPSCs at any time during adulthood. This mode of GSC generation from MPSCs or multipotent cells occurs not only in *Hydra* but also in a wide variety of animals on the phylogenetic tree. For instance, in an adult planarian, GSCs that express *Djnos* (the orthologue of *Drosophila nanos*) can be regenerated from *Djnos*-negative multipotent neoblasts (Sato et al. 2006; Wagner et al. 2011). In demosponges, although no GSCs have been reported yet, archeocytes (migratory adult pluripotent stem cells) can give rise to oocytes, while choanocytes (flagellated food-entrapping cells) can transform into archeocytes and then differentiate into sperm (Funayama et al. 2010). In these lower animals, as in *Hydra*, cells with MPSC-like properties persist in adulthood and contribute to somatic cell production, asexual reproduction, and regeneration of missing parts of the body, as well as to germline generation. In the sea urchin, GSCs arise after embryogenesis from multipotent adult rudiment cells in a coelomic pouch, which are descended from small micromeres and develop into an adult body during metamorphosis (Ransick et al. 1996). In the Annelida and Mollusca, 4d lineage multipotent mesoblasts (or their descendant blast cells) are the source of GSCs (Rebscher et al. 2012; Rebscher 2014). Moreover, in most plants, the shoot apical meristem supports both plant growth and germline production: cells of the shoot apical meristem constitute MPSCs. Thus, this mode of GSC (or germline cell) generation is shared among many different phyla and can be considered the more ancestral mode (Juliano and Wessel 2010).

MPSCs are neither somatic stem cells nor GSCs in a strict sense. However, since GSCs are responsible for producing the next generation, they contribute to the continuity of life. Furthermore, since GSCs arise from MPSCs in many animals, MPSCs are definitely one of the cell lineages that contribute to the continuity of life, and are an immortal cell lineage, as are GSCs.

7.9.2 Multipotent Stem Cells Express Germline-Specific Genes and Have Properties Similar to Those of Germline Stem Cells

In *Hydra*, MPSCs express so-called germline genes, as do GSCs; both cells strongly express *Cnnos1*, *2* (the orthologues of *Drosophila nanos*); *Cnvas1*, *2* (the orthologues of *Drosophila vasa*); *CnPL10* (the orthologous gene for mouse DEAD box protein Ddx3); and *hywi*, *hyli* (the orthologues of *Drosophila piwi*) (Mochizuki et al. 2000, 2001; Lim et al. 2014; Juliano et al. 2014). A set of these genes is also

expressed in MPSCs/multipotent cells in other animals (Shibata et al. 1999; Sato et al. 2006; Juliano et al. 2006; Funayama et al. 2010; Rebscher et al. 2012); in some cases it has been shown that these genes are indispensable for MPSC maintenance and/or function (Rabinowitz et al. 2008: Palakodeti et al. 2008; Juliano et al. 2010a). Juliano and colleagues (2010b) have referred to the genetic regulatory program that operates in both MPSCs and GSCs as the germline multipotency program (GMP).

In addition, it has been reported that I-cells (MPSCs and GSCs), as well as differentiating germline cells in *Hydra*, have electron-dense bodies in the cytoplasm (Noda and Kanai 1977), which look like the germinal granules ("nuage" is the general term) in *Drosophila* and in *Rana* (Mahowald 1962, 1972; Mahowald and Hennen 1971). Nuage was originally identified as cytoplasmic electron-dense material in germline cells and later found to be an RNA and protein complex containing Vasa and Piwi proteins (Voronina et al. 2011; Lim and Kai 2007). Recently, in *Hydra* it has been shown that Hywi, Hyli proteins and other nuage components (i.e., orthologues of mouse Maelstrom and TDRD9) are localized in nuage of MPSCs, GSCs, and egg lineage cells (Lim et al. 2014; Juliano et al. 2014). Sequencing of the small RNAs bound to Hywi—so-called PIWI-interacting RNAs (piRNAs)—has revealed large numbers of sequences complementary to transposons in *Hydra*. Thus, the activity of transposons in MPSCs and GSCs may be suppressed similarly to germline cells in *Drosophila* and mice (Lim and Kai 2007; Siomi et al. 2011).

7.9.3 New Concepts Regarding Multipotent Stem Cells Proposed by Others

Considering the potential importance of the GMP and nuage components in both MPSCs and GSCs, and the ability of MPSCs to generate/regenerate germline cells/GSCs, Solana (2013) revised the germline concept to include MPSCs and called these MPSCs primordial stem cells (priSCs). priSCs correspond to the adult pluripotent stem cells (MPSCs in this chapter) mentioned by Agata and colleagues (2006), who distinguished these stem cells from lineage-specific stem cells such as neural and hematopoietic stem cells, which have an evolutionarily different origin. Woodland (2016) further developed Solana's idea and pointed out the possibility that MPSCs are immortal cell lineages, as are GSCs.

7.9.4 Unresolved Issues with Multipotent Stem Cells, Germline Stem Cells, and Germline Multipotency Program Component Genes

The multipotent differentiation capability of MPSCs/multipotent cells into both GSCs and somatic cells is shared among many different phyla; however, it is not clear whether the origins of these MPSCs/multipotent cells during embryogenesis

are evolutionarily conserved or not. There is a possibility that they have arisen independently in different phyla and represent an example of convergent evolution (see 7.10.2). Even though the origin of MPSCs may be different, their gene expression may still be similar. Önal et al. (2012) compared gene expression between planarian neoblasts and mouse embryonic stem cells (ESCs). They found that important genes for pluripotency in ESCs—including genes upstream and downstream from Oct4, Nanog, and Sox2—are well conserved and upregulated in neoblasts.

So far, it is not known what kinds of genes differ between MPSCs and GSCs. It will be interesting to find genes that are differentially expressed in MPSCs and GSCs; some genes enable multipotency in MPSCs, whereas others suppress somatic differentiation in GSCs and permit latent multipotency, which is achieved after fertilization in embryos. It is also challenging to identify the genes that are expressed in both MPSCs and GSCs and are involved in the continuity of life.

Recently, Alié et al. (2015) compared the gene expression profile of archeocytes in Porifera with that of MPSCs in *Hydra* (Hemmrick et al. 2012) and of neoblasts in *Planaria* (Solana et al. 2012), and identified a conserved gene set composed of 180 genes. It was poor in transcription factors but enriched in RNA regulators, including GMP component genes. It was also shown by phylostratigraphy analysis that most of the genes predated the origin of opisthokonts, i.e., they were present in the last common eukaryotic ancestor. This phylostratigraphy result is acceptable since both multipotent and germ cells are the two oldest cell types; the unicellular ancestors of animals are supposed to have alternated between asexual and sexual reproduction. Immortality may be governed by genes in this conserved set.

Interestingly, in *Hydra*, *Cnnos2*; *Cnvas1*, *2*; *CnPL10*; and *hywi*, *hyli* are all expressed in epithelial cells, as well as in MPSCs and GSCs (Mochizuki et al. 2000, 2001; Lim et al. 2014, Juliano et al. 2014). Furthermore, it was shown by RNAi inhibition that *hyli* activity is required to maintain the integrity of epithelial cells (Juliano et al. 2014). Nuage has also been found in *Hydra* epithelial cells (Hobmayer et al. 2012). If these GMP component genes and nuage are involved in maintaining the continuity of life, epithelial cells in *Hydra* should also exhibit immortality similarly to MPSCs and GSCs, as has been suggested by Martínez and Bridge (2012) and Woodland (2016).

7.10 The Cnidarian World

7.10.1 Phylogeny and Relationship to Other Metazoans

Cnidarians are generally regarded as diploblastic (see Seipel and Schmid 2006; Technau and Steele 2011) and constitute the sister clade to triploblastic bilaterians, which further diverged to the Protostomia and Deuterostomia (Technau and Steele 2011; Steele et al. 2011; Collins et al. 2006). Because of the simple architecture of the body, and the basal placement on the phylogenetic tree, cnidarians are expected to share ancestral developmental mechanisms with both the Protostomia and

Deuterostomia. In particular, several cnidarian whole-genome sequences have revealed that they have most of the major classes of signaling molecules and transcription factors (Putnam et al. 2007; Chapman et al. 2010). Cnidarians are expected to retain fundamental mechanisms for cell differentiation and morphogenesis, which have been further developed and modified in higher metazoans.

The phylum Cnidaria includes highly divergent animals, and their phylogenetic relationships are not fully resolved yet. For example, the Myxozoa—a group of simple endoparasitic animals, which once were classified as protozoans—are now placed near the Medusozoa in the Cnidaria on the basis of genome and transcriptome analyses (Siddall et al. 1995; Jiménez-Guri et al. 2007; Foox and Siddall 2015).

In recent phylogenetic analyses with mitogenomics (Park et al. 2012; Kayal et al. 2013), the generally accepted class Anthozoa (the Hexacorallia and Octocorallia) and the class Scyphozoa (the Discomedusae and Coronatae) were revealed not to be monophyletic, and closer relationships of the Octocorallia to the Medusozoa and of the Discomedusae to the Hydrozoa have been suggested (Fig. 7.7) (Kayal et al. 2013). The advantage of analyses with mitogenomics over those with nuclear DNA is that the orthology is assured in the former. These analyses provided evidence that the polyp emerged before the medusa in cnidarian evolution, and strongly supported the idea that ancestral cnidarians had bilateral symmetry (Finnerty et al. 2004), from which the radial tetrameral symmetry of most medusozoans evolved.

7.10.2 Have the Multipotent Stem Cells of Hydra *Evolved Independently from Those of Other Metazoans?*

On the basis of phylogenetic analyses, the Hydrozoa are established as a distal branch in the Medusozoa (Fig. 7.7) (Collins et al. 2006; Kayal et al. 2012). In addition, hydrozoans are the only clade that is reported to have undifferentiated I-cells among cnidarians (Steele et al. 2011). These two facts suggest that the MPSC systems in hydrozoans evolved independently from those in bilaterians (Chapman et al. 2010; Steele et al. 2011).

One possible alternative scenario is that the origin of MPSCs/multipotent cells is common to both the Cnidaria and Bilateria, and that multipotent cells generated during embryogenesis in the Hexacorallia—the ancestral branch of the Cnidaria—were lost secondarily after GSC formation. Indeed, GSCs are found in the Hexacorallia, although MPSCs have not been detected yet. In a stony coral, *Euphyllia ancora*, GSCs expressing *Eapiwi*—a *piwi* homologue of *Euphyllia*—were detected in mesenterial filaments throughout the year, irrespective of the spawning season (Shikina et al. 2015). The mesenteries are the folds of endodermal gastrodermis running along the body axes, and are the sites of gametogenesis. Likewise, in *Nematostella*, by the use of anti-Vasa antibody, it was shown that GSCs are located in the mesenteries of primary and adult polyps (Extavour et al. 2005). When expression of the GMP component genes *Nvvas1, −2*; *NvPL10*; and *Nvnos2* was examined during embryogenesis, it was initially detected in invaginating

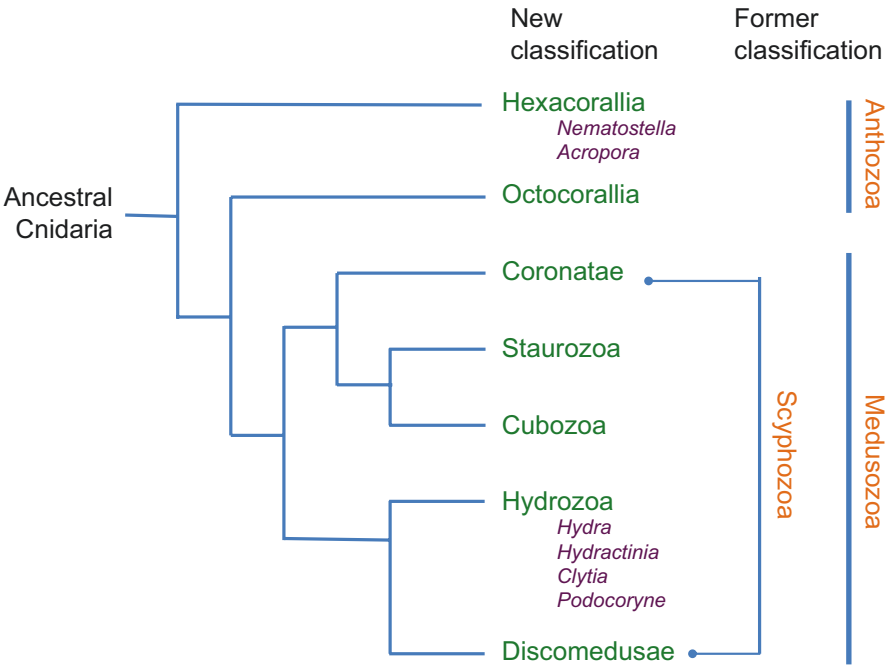

Fig. 7.7 Cnidarian phylogenetic tree based on mitogenomics (modified from Kayal et al. 2013). The analysis denied the monophyly of Anthozoa and Scyphozoa, supporting that ancestral cnidarians had bilateral symmetry. In addition, the analysis provided further evidence for the polyp-first hypothesis. This phylogenetic relationship may support that the interstitial stem cells (I-cells) found only in Hydrozoa but not in other cnidarians have evolved independently from multipoint (stem) cells (MPSCs/MPCs) found in Bilaterians (see also the Sects 7.10.2 and 7.10.3)

(ingressing) endodermal cells and then gradually restricted to mesenterial cells. In mature polyps the expression was detected in putative germline cells (Extavour et al. 2005). Since GSCs are segregated gradually from a larger population of cells that express GMP component genes, it will be interesting to see whether these cells are multipotent like MPSCs in *Hydra*, even though they are not stem cells but are lost by differentiation.

7.10.3 Multipotent Stem Cells Versus Cell Plasticity: Two Different Modes of Cell Differentiation

How do cnidarians that have no MPSCs differentiate nerve cells and nematocytes during asexual reproduction? Actually, cnidarian cells exhibit strong plasticity: they are capable of dedifferentiation and transdifferentiation from ectodermal epithelial cells in both *Nematostella* and *Acropora* (Shinzato et al. 2008; Nakanishi et al.

2012; Marlow et al. 2012; Wolenski et al. 2013), and partly from endodermal epithelial cells (Nakanishi et al. 2012). Recently, neural progenitor cells have been found in *Nematostella*. However, these cells are constantly formed from ectodermal cells by transdifferentiation and have only limited proliferating ability (Richards and Rentzsch 2014). The progenitor cells express *NvsoxB(2)* (an orthologue of bilaterian *SoxB*), which has a conserved role in neurogenesis.

Transdifferentiation has also been shown in the hydrozoan jellyfish *Podocoryne carnea* (Schmid 1992). Although this species is phylogenetically very close to *Hydractinia* and *Hydra*, neither I-cells nor GSCs have been reported in the polyp. Nevertheless, pieces of striated muscle isolated from the medusa can dedifferentiate and start DNA replication. They become flagellated tissue clumps, and then the cells give rise to smooth muscle and nerve cells. Occasionally, endodermal cells are also formed and the tissue becomes bilayered to form a manubrium with tentacles—the sexual organ of a medusa. In such cases, nematocytes and gland cells are also formed (Schmid et al. 1982; Schmid and Alder 1984; Galliot and Schmid 2002). During the initial steps of transdifferentiation, muscle cell specific genes are inactivated (Yanze et al. 1999), indicating that dedifferentiation has occurred.

GMP component genes are also upregulated during transdifferentiation. For example, in *Nematostella*, *Nvnos1* (one of the two *nanos* homologues) is expressed in scattered cells in the ectodermal epithelial tissue, which are presumably nematocyte precursors transdifferentiating from epithelial cells (Extavour et al. 2005). In *P. carnea*, expression of *Cniwi* (the *piwi* homologue) is upregulated during transdifferentiation of striated muscle cells to proliferating smooth muscle cells, and to terminally differentiated RFamide-positive nerve cells (Seipel et al. 2004).

In *P. carnea*, germline cells appear to differentiate de novo from the entocodon (*entos*: within; *kodon*: bell) of a developing medusa, which originates from ectodermal epithelial cells of the medusa bud. The entocodon differentiates into a manubrium, where gametogenesis takes place in adult medusa. Three GMP component genes—*Cniwi* and *Pcnos1*, *−2* (the *nanos* homologues)—are initially expressed widely in the entocodon, gradually becoming restricted to germline cells in the manubrium (Seipel et al. 2004: Torras et al. 2004). Strong expression of these genes is never observed in the polyp, which is consistent with the absence of I-cells in polyps in this species.

Thus, self-renewal and transdifferentiation of epithelial cells seem to have similar roles to MPSCs in the Cnidaria. There is a view that cell plasticity and transdifferentiation is more ancestral than the MPSC system (Gold and Jacobs 2013). However, both transdifferentiation and the MPSC system occur in the closely related hydrozoans *Podocoryne* and *Hydra*, implying that both systems may easily interconvert in the Cnidaria. Since GMP component genes are upregulated during dedifferentiation as in MPSCs, dedifferentiation may be a reprogramming process to an undifferentiated multipotent state in the Cnidaria.

7.10.4 *Origin of Interstitial Stem Cells in* Hydra *and Other Hydrozoans*

I-cells differentiate during embryogenesis. Studies with transmission electron microscopy (TEM) have shown that I-cells are mainly generated by asymmetric cell division from presumptive ectodermal cells but can also arise in an endodermal inner cell mass in *Hydra* (Noda and Kanai 1980; Martin et al. 1997). If there are two kinds of I-cells—i.e., of ectodermal origin and of endodermal inner cell mass origin in *Hydra*—it is interesting to know whether there is some functional difference between them, e.g., the former are MPSCs and the latter are GSCs. On the other hand, with use of in situ hybridization with *HyEED*, although I-cells were detected at the same locations as those found in the TEM studies described above, the observation was interpreted as evidence that I-cells are generated in the endodermal inner cell mass and migrate to the ectodermal cell layer (Genikhovich et al. 2006). Since I-cell generation takes place before mesoglea formation in *Hydra*, it may be difficult to determine the origin of I-cells with certainty. Further molecular analysis to detect asymmetric cell division may be needed to determine the origin of the I-cells in *Hydra*.

In contrast, the endodermal origin of I-cells is well established in *H. echinata* and *Clytia hemisphaerica*, and it has been shown that they migrate into the ectodermal layer (Rebscher et al. 2008; Leclère et al. 2012).

7.10.5 *In* Hydractinia echinata*, Interstitial Stem Cells Can Give Rise to Epithelial Cells*

In *Hydra*, MPSCs do not give rise to epithelial cells; in contrast, in *H. echinata*, I-cells have been shown to differentiate into all cells in the body, including epithelial cells (Müller et al. 2004; Künzel et al. 2010). I-cells were eliminated from a wild-type host colony by treatment with mitomycin. I-cells were then introduced from a mutant strain, which had a different morphology and sex from those of the host. Over time, the phenotype of the host changed into that of the donor. It was concluded that I-cells of *Hydractinia* are totipotent, but it was not determined whether there are distinct GSCs and MPSCs.

In *Hydractinia*, *polynem (pln)*—a POU domain–containing gene—is expressed in I-cells. When *pln* was ectopically expressed in epithelial cells by a transgene, *myc*, *vasa*, *nanos*, and *piwi* were upregulated, and the epithelial cells turned into an I-cell-like neoplasm (Millane et al. 2011), suggesting that *pln* is a stem cell regulator in *Hydractinia*.

pln is closely related to class III POU family genes, while *Oct4* (one of four Yamanaka reprograming factors) belongs to class V, which is a vertebrate novelty (Gold et al. 2014). Paralogous genes may have similar functions in phylogenetically

distant animals, but an evolutionary relationship between MPSCs and stem cells in the mouse embryo (e.g., an inner cell mass) has not been revealed so far. It will be interesting to examine to what extent the MPSCs in lower animals and mammalian stem cells (e.g., induced pluripotent stem cells, epiblast cells, or primordial germ cells) have similar gene expression profiles.

7.11 Conclusions

All extant animals have arisen from hundreds of millions of years of evolution. This continuity is maintained by the germline, and it is this function of the germline that has attracted our interest. During the past two decades, GSC formation has been analyzed in a wide variety of animals, and it has been shown that GSCs in *Hydra* have several evolutionarily conserved properties: they are formed from MPSCs, like many other nonmodel animals (Juliano et al. 2010b); cyst/nest formation by EgSCs in *Hydra* is also observed in mice and fish (Pepling and Spradling 1998; Nakamura et al. 2010); in *Hydra*, there are two kinds of cell-autonomously sex-determined GSCs (i.e., EgSCs and SpSCs), and such intrinsic sex determination in GSCs is a more ancestral mechanism than extrinsic sex determination by instructive signals in gonads, which vary from one species to another; and GSCs and MPSCs in *Hydra* are maintained under open-niche conditions similar to those of spermatogonia in mouse. Thus, by identifying the genes that produce the differences between MPSCs, EgSCs, and SpSCs in *Hydra*, we have a good chance of understanding the basic evolutionarily conserved properties of GSCs, MPSCs, and hopefully other stem cells, such as ESCs and induced pluripotent stem cells, in mice.

MPSCs (I-cells) are found only in the Hydrozoa. Other cnidarians use an alternative system of dedifferentiation and transdifferentiation to produce differentiated somatic cells. In these cnidarians, epithelial cells are highly plastic. During the dedifferentiation of epithelial cells, upregulation of GMP component genes has been reported. Investigation of the epigenetic control governing dedifferentiation is another interesting subject in the Cnidaria.

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Chapter 8
Reproductive Strategies in Marine Hydrozoan Jellyfish: Sexual Medusae and Asexual Polyps

Noriyo Takeda, Ryusaku Deguchi, and Takeshi Itabashi

Abstract Hydrozoan jellyfish belong to the phylum Cnidaria and generally have radial symmetry with diploblastic layers. Many species of hydrozoan jellyfish have two characteristic forms: a sessile polyp and a free-swimming medusa. Sessile polyps multiply through asexual reproduction, whereas free-swimming medusae undergo sexual reproduction by releasing eggs and sperm. Although these reproductive systems are known to be adaptations to seasonal changes in the physical environment of the sea, little has been written about when and exactly how hydrozoan jellyfish carry out asexual and sexual reproduction in nature. Here, we describe the life cycle of hydrozoan jellyfish, including polyp reproduction, medusa formation, oocyte maturation, spawning, fertilization, and metamorphosis. We discuss current research on the asexual and sexual reproductive systems of three marine hydrozoan species: *Cytaeis uchidae*, *Cladonema pacificum*, and *Clytia hemisphaerica*.

Keywords Reproduction · Life cycle · Oocyte maturation · Spawning · Fertilization · Metamorphosis

8.1 Introduction

The phylum Cnidaria is composed of five main classes: Hydrozoa, Cubozoa, Staurozoa, Scyphozoa, and Anthozoa (Daly et al. 2007; Houliston et al. 2010). A characteristic feature of cnidarians is the cnidocyte, a cell that releases a nematocyst in response to chemical or mechanical stimulation (Babonis and Martindale 2014).

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As cnidarians are radially symmetric and have a diploblastic body with two germ layers (an outer ectoderm and an inner endoderm), they are generally considered to have branched off early from bilaterians, the animals with bilateral symmetry (Philippe et al. 2009; Houliston et al. 2010; Leclère et al. 2016).

Hydrozoa is the most diverse group of Cnidaria and contains several species that have contributed to the promotion of basic science, e.g., *Hydra* spp. for understanding of regeneration (Bosch 2007) and *Aequorea victoria* for the discovery of green fluorescent protein (GFP) (Shimomura 2008). Many hydrozoan species have separate sexes and form sessile polyps for asexual reproduction and medusae for sexual reproduction during different life stages (Leclère et al. 2016). The structural features and the size of the medusa vary considerably from species to species, e.g., well-grown free-swimming medusae in *Aequorea* (Shimomura 2008) and degenerative sessile medusae in *Stylactaria* and *Hydractinia* (Namikawa 1991; Walther et al. 1996). The species that cycle through the two forms, asexual polyps and sexual medusae, demonstrate the complex reproductive strategy of hydrozoan jellyfish. Various questions relating to the transition from asexual to sexual reproduction in such hydrozoans remain. Here, we focus on the reproductive system in three marine hydrozoans (*Cytaeis uchidae*, *Cladonema pacificum*, and *Clytia hemisphaerica*), all of which demonstrate sessile polyps for asexual reproduction and free-swimming medusae for sexual reproduction.

8.2 Life Cycle in Hydrozoans

Figure 8.1a shows the life cycle of *Cytaeis uchidae* in nature. Polyps of this species settle on the shell surface of the sea snail *Nassarius (Niotha) livescens*, with which they are symbiotic (Hirai and Kakinuma 1971, 1973; Inoue and Kakinuma 1992). Natural products derived from the host gastropod contribute to the induction of *Cytaeis* to metamorphose into a polyp. The primary polyp forms a stolon and subsequently generates polyps, and polyps connected to one another through stolons grow on the shell surface by asexual reproduction (Fig. 8.1b) (see Sect. 8.3). The polyps are dioecious, and therefore, the sexual phenotype of medusae derived from a single colony is also defined as either male or female. A polyp colony of *Cytaeis* on a single shell of a host gastropod is thought to be a single clone with one sex type.

In nature, the medusa buds of *Cytaeis uchidae* that form on a stolon grow into baby medusae that detach during the summer months (June–August, in Asamushi, Japan) (Hirai and Kakinuma 1973). Young medusae grow until they are able to produce a number of oocytes in the ovaries, or sperm in the testes. They thereafter spawn gametes daily in response to changes in light condition (e.g., from dark to light) (Takeda et al. 2006), while increasing the number of oocytes or sperm (see Sect. 8.4). After fertilization occurs (see Sect. 8.5), fertilized eggs develop into swimming planula larvae within 1–3 days. Planulae become attached to a host gastropod shell and initiate metamorphosis into polyps that engage in asexual reproduction (Inoue and Kakinuma 1992) (see Sect. 8.6). The other two species,

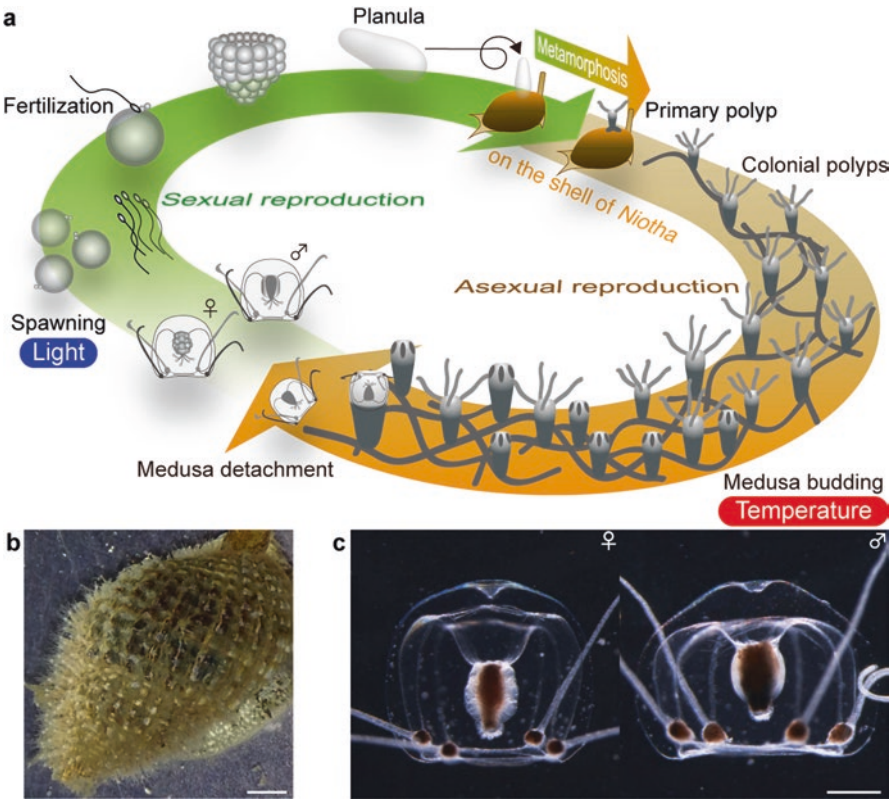

Fig. 8.1 Life cycle of hydrozoan jellyfish. (**a**) Schematic illustration of the life cycle of *Cytaeis uchidae* in nature. (**b**) *Cytaeis* colonial polyps on the *Niotha* shell. Scale bar represents 2 mm. (**c**) *Cytaeis* female (left) and male (right) medusa. Scale bar represents 500 μm

Cladonema pacificum and *Clytia hemisphaerica*, exhibit a similar life cycle, although their polyp colonies are formed on various substrates including rocks, algae, and the surface of other animals (Hirai and Kakinuma 1957; Migotto 1996; Calder 2012).

8.3 Formation of Sexual Medusa from Asexual Polyp

8.3.1 Cytaeis uchidae (Hydrozoa: Anthoathecata)

Cytaeis medusae (Fig. 8.1c) are found during the summer season in nature, and medusa buds are observed in polyp colonies on the shells of *Nassarius (Niotha) livescens* collected during this season (Inoue and Kakinuma 1992). However, a polyp transplanted from the shell surface of the gastropod to a new substrate (e.g.,

a plastic dish) and maintained at 20–23 °C can regenerate a polyp colony and then bud into medusae throughout the year, regardless of the season. Interestingly, heteromorphic polyps possessing nematocysts of basitrichous isorhizas have been observed along the apertures of gastropod shells (Namikawa 2005), whereas colonies of transplanted polyps have morphologically uniform tentacles. Although the colonial polyps have the potential to generate medusae over the course of several months or years as they form new polyps and medusa buds, they abruptly reduce asexual reproduction and die out after entering full production of medusae (Hirai and Kakinuma 1973). However, when such a polyp is transplanted to a new dish before starting the decline in asexual reproduction, it can reproduce itself sufficiently to carry out medusa production, in the same way as a polyp transplanted from the host gastropod shell can.

The chemical and/or environmental cues that modulate the balance between asexual and sexual reproduction are still poorly understood. One hypothesis is that in nature, the host gastropod somehow controls signaling pathways to prevent needless reproduction (Hirai and Kakinuma 1973; Inoue and Kakinuma 1992). Consequently, polyps separated from the gastropod may lose access to the proper signals controlling the transition from asexual to sexual reproduction, entering continuous production of medusae year-round in the laboratory.

8.3.2 Cladonema pacificum (Hydrozoa: Anthoathecata)

Cladonema medusae (Fig. 8.2a) are also observed in the summer season (June–August, Tohoku region, Japan) (Deguchi and Itoh 2005). Under constant light conditions, fluctuation of water temperature every other day from 5 °C to 25 °C or from 15 °C to 25 °C accelerates medusa bud formation in polyp colonies cultured in the laboratory (Kakinuma 1962). Likewise, a simple temperature rise reliably induces polyp formation and medusa production in this species (Fig. 8.2b) (Deguchi and Itoh 2005). When colonial polyps are stored at 5 °C for at least 2 months, their polyps degenerate, but their stolons remain. At that point, an increase in temperature up to 20 °C initiates polyp production within 1 week. Shortly thereafter, medusa buds are formed on the side surfaces of polyp bodies and then released as baby medusae. Medusa production continues for about 1 month and then stops, even if the temperature remains constant at 20 °C. Although sexual reproduction does not occur, polyps maintain asexual reproduction; each isolated polyp regrows into a colony with newly formed polyps that lack the ability to produce medusa buds. Therefore, once colonial polyps have produced medusae, they cannot regain the ability to reproduce sexually without experiencing a period of low-temperature (Deguchi and Itoh 2005). Both polyps and stolons may be partly composed of stem cells that possess totipotency, which confers the ability to differentiate into germline cell types under low-temperature stimulus. In short, *Cladonema* undergo asexual and sexual induction after experiencing long-term low-temperature conditions, such as that which occurs in nature during the winter (Fig. 8.2b). Sexual induction in several species of

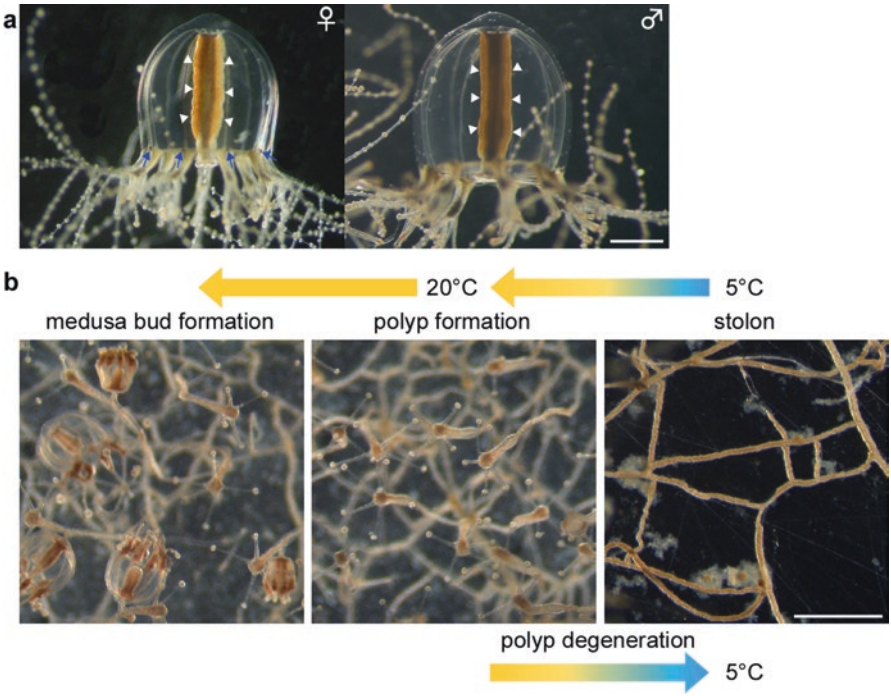

Fig. 8.2 Transition from asexual to sexual reproduction in response to changes in temperature in *Cladonema*. (**a**) Mature female (left) and male (right) medusa of *Cladonema pacificum*. Arrowheads indicate the ovary and testis. Arrows (blue) indicate ocelli. (**b**) Sexual induction regulated by environmental temperature. Images are not identical fields. Scale bar represents 1 mm

Hydra is also sensitive to low-temperature conditions (Park et al. 1965; Davison 1976; Martinez and Bridge 2012), suggesting that *Cladonema* and *Hydra* have the same or similar molecular mechanisms of sexual induction regulated by environmental temperature (see Chap. 7 for details on *Hydra*).

Kakinuma (1969, 1988) examined the fate of medusa buds cut from mother polyps at different stages of development (stages 1–10, Fig. 8.3a, b) in *Cladonema*. A medusa bud cut during the early stages of development (stages 1–4) is transformed into a stolon, and then it becomes a polyp. A medusa bud cut before the formation of radial canals (stages 5 and 6) also returns to a polyp, over a longer time. When the bud is separated before the pigmented light-detecting structure (called the ocellus) is completely formed at the tip of the radial canals (stage 7), it forms a relatively small baby medusa when exposed to light but degenerates into a cell cluster and forms neither a stolon nor a polyp when maintained in the dark. However, once the medusa bud finishes ocellus formation (stage 8), it develops into a complete medusa regardless of light or dark conditions. After the tentacles are differentiated (stages 9 and 10), a medusa bud cut from the mother polyp follows the same developmental path. Interestingly, the transition between polyp and young medusa bud is reversible,

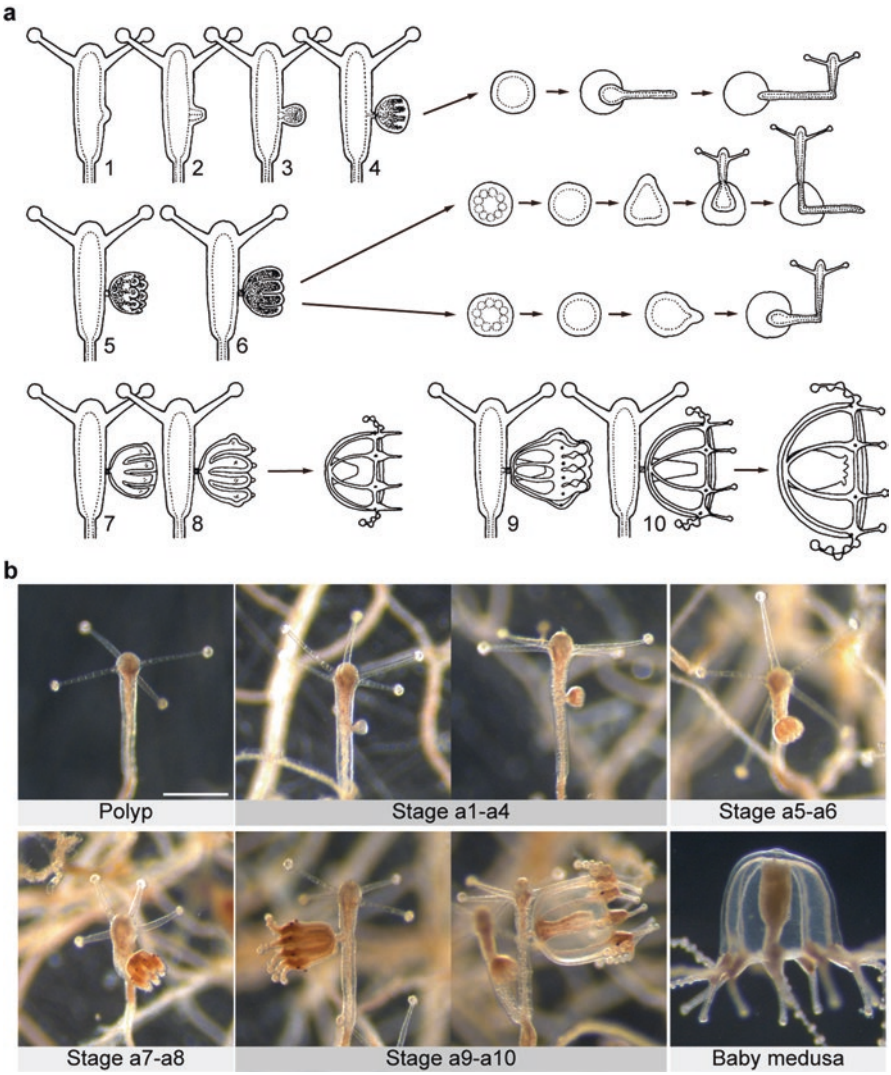

Fig. 8.3 Medusa formation in *Cladonema*. (**a**) Regeneration of medusa bud separated from the mother polyp at different stages of development (Stages 1–10) (Reproduced with permission from Kakinuma 1988). (**b**) Images of polyp of *Cladonema pacificum* corresponding to each developmental stage. Images are not identical polyps. Scale bar represents 500 μm

but the formation of the ocellus that receives the light signal as environmental input determines the definitive timing of irreversible development toward sexual reproduction. Thus, the seasonal sexual induction of *Cladonema* appears to depend on changes in light as well as in temperature.

8.3.3 *Clytia hemisphaerica (Hydrozoa: Leptothecata)*

The timing and period of medusa production in *Clytia* is not controlled solely by environmental temperature (Houliston et al. 2010). In this species, however, sex determination during the polyp stage is sensitive to and reversible by changes in temperature (Carré and Carré 2000; Houliston et al. 2010). The great majority of medusae are female when the polyps are maintained at a temperature of 24 °C, but they are male at 15 °C. After liberation at 24 °C, medusae that have not yet formed gonads and that are exposed to a low temperature of 15 °C for 1 day become male. However, medusae liberated at 15 °C are always male, even if the temperature is raised to 24 °C. Carré and Carré (2000) suggested that polyps and young medusae of *Clytia* initially have both sexual germline cells, and irreversibly become male in response to a low-temperature environment. Although every hydrozoan species seems to possess a mechanism to determine the sexual phenotype in response to the sex being genetically determined, there may be species-specific temperature ranges that alter the ratio of female to male medusae in nature.

8.4 Gamete Production for Sexual Reproduction

8.4.1 *Oocyte Growth Before Oocyte Maturation*

Ovaries or testes in medusae are covered with a single thin layer of ectodermal epithelium (Houliston et al. 2010; Takeda et al. 2013). Within the order Anthoathecata (which includes *Cytaeis* and *Cladonema*), ectodermal epithelium cells cover the eggs or sperm, which surround the manubrium. In contrast, within the order Leptothecata (which includes *Clytia*), the ovaries or testes are formed along the radial canals, but the eggs or sperm are sandwiched between the endoderm and ectoderm layers in the same way as in Anthoathecata. Although baby medusae have the ability to release some oocytes shortly after detachment (a few days in *Cytaeis* and about 10 days in *Cladonema*), their ovaries are not yet fully developed enough to produce a lot of gametes. The full production of gametes in *Cytaeis* and *Cladonema* needs about 2 weeks after detachment, whereas *Clytia* require 3–4 weeks for gonad formation and maturation. Adult *Cytaeis* medusae release about 50 eggs per spawning each day, for the remainder of the organism's lifetime (2–3 months in the laboratory). This capacity for gamete release implies that oocytes may be derived from stem cells in order to generate so many eggs (Nishimiya-Fujisawa and Kobayashi 2012).

In *Cytaeis* ovaries, which encircle the stomach, a number of fully developed oocytes of approximately 110 μm in diameter are observed just before spawning (Takeda et al. 2006), whereas immature oocytes with smaller diameters remain in the ovaries after spawning. Oocytes that remain in the ovary 6 h after the previous ovulation have diameters of about 80 μm, gradually increasing in size until the next

spawning (12 h after the previous spawning, diameter = 85 μm; 18 h, 95 μm; 24 h, 110 μm) (Takeda et al. unpublished data). Oocytes that develop for more than 16 h after the previous spawning can initiate meiosis in an intact ovary in response to light stimulus, but those that develop for fewer than 14 h never progress to meiotic maturation (Takeda et al. 2006).

In the *Clytia* gonad, oocyte growth occurs in three main stages, distinguished on the basis of size and nuclear features (e.g., position, chromosomes, nucleolus) (Amiel and Houliston 2009). Fully-grown oocytes, in which the germinal vesicle (GV) with dispersed nucleoli has reached the cell cortex, are 160–180 μm in diameter (Stage III oocyte) at spawning. Immediately after their release, the ovary contains mainly Stage I oocytes (less than 120 μm in diameter) that have a single, large nucleolus in the center of the GV. These oocytes grow to Stage III over time through Stage II (oocytes with a diameter of 120–140 μm have a fragmented nucleolus and semi-dispersed chromosomes), while the GV moves to the animal pole to establish oocyte polarity. The number of Stage III oocytes increases gradually, although about half of the oocytes in the ovary remain at Stage I 20 h after the previous spawning. At spawning, the ovary possesses oocytes of all stages, suggesting that both the differentiation of germline stem cells into gametes and their growth operate continuously.

8.4.2 Oocyte Maturation and Spawning

The release of gametes from medusae is generally controlled by changes in light conditions. Both light stimulus after a period of darkness and darkness after a period of light trigger the initiation of meiotic maturation. Ovarian oocytes, which finally reach the pronucleus stage after the completion of meiosis, are spawned and await fertilization from mature sperm, released by male medusae. Whether the stimulus is light or darkness depends on the species (Miller 1979; Freeman and Ridgway 1988). The trigger of meiotic maturation is light for *Cytaeis* and *Clytia*, whereas it is darkness for *Cladonema.* In *Cytaeis*, exposure to light for 1 s is enough to induce oocyte maturation and subsequent spawning, but effective induction by light stimulus requires pre-incubation in darkness for more than 1 h. An ocellus specialized for light reception is not necessary for the spawning pathway, and the oocyte itself, when isolated from the ovary, never initiates meiosis in response to light stimulus (Takeda et al. 2006). In addition, oocytes must remain inside the ovary for more than four min after receiving the light stimulus, suggesting that the ovarian ectodermal epithelium is responsible for the light reception and subsequent signal transduction to the oocytes. To activate the signal transduction cascade initiating meiosis, it is suggested that the light stimulus induces the release of a spawning-inducing substance (Ikegami et al. 1978) and/or a maturation-inducing hormone (MIH) (Freeman 1987). In *Cytaeis*, the application of some kinds of neuropeptides derived from *Hydra magnipapillata* induces oocyte maturation and spawning, and mesh-like

networks of neural cells recognized by the antibodies against the neuropeptides appear in ovarian ectodermal epithelium (Takeda et al. 2013). In *Clytia* and *Cladonema*, the main components of MIH have been identified as amidated tetrapeptides (W/RPRPamides), which are synthesized by neural cells in the ovarian ectoderm and released in response to light cues (Takeda et al. 2018). These peptides are also produced by male testes to trigger sperm release. The light-sensitive protein Opsin 9 is coexpressed with MIH within neural cells and plays an essential role in light-triggered spawning (Quiroga Artigas et al. 2018). Thus, the regulatory mechanisms linking light reception with the initiation of oocyte maturation may be very simple in hydrozoan jellyfish.

It is well known that cyclic AMP (cAMP) is involved in the initiation of oocyte maturation in several kinds of hydrozoan jellyfish (Freeman and Ridgway 1988). The measurement of intracellular cAMP in oocytes of *Cytaeis* demonstrates that it is necessary for cAMP to rise for a certain period of time in order to induce oocyte maturation (Takeda et al. 2006). The cAMP rise induces the activation of protein kinase A (PKA) (Deguchi et al. 2011). Inhibition of PKA activity blocks the cAMP-induced meiotic maturation of oocytes, in which the cAMP concentration dramatically increases (Takeda et al. 2006), indicating that the activation of the cAMP/PKA pathway is required for the initiation of oocyte maturation in *Cytaeis*.

Meanwhile, oocyte maturation triggered by either light stimulus or a microinjection of a cAMP analog is prevented by the presence of cyclin-dependent kinase inhibitors (e.g., roscovitine) (Deguchi et al. 2011). The activity of histone H1 kinase is greatly enhanced at metaphase I of meiosis, suggesting that maturation-promoting factor could begin to be active before or after germinal vesicle breakdown (GVBD) (Takeda et al. unpublished data). Mitogen-activated protein kinase (MAPK) is also activated after GVBD, followed by its inactivation after fertilization (described below) (Kondoh et al. 2006; Amiel et al. 2009; Arakawa et al. 2014). Although MAPK activation is not necessary for the early events of meiosis, including GVBD (Amiel et al. 2009; Deguchi et al. 2011), Mos, as a MAPK kinase kinase, regulates assembly and positioning of the meiotic spindle (Amiel et al. 2009), postmeiotic G_1-arrest of cell cycle progression (see Sect. 8.6), and polyspermy block (see Sect. 8.5).

In *Cytaeis*, gap junction-like structures connecting oocytes and ectodermal epithelial cells exist at the Pro-I stage (Deguchi et al. 2011). These junctions disappear after the completion of meiosis I, enabling oocytes to escape the ovary by breaking through the ectodermal epithelium (Fig. 8.4). Morpholino knockdown of Mos in oocytes of *Clytia* prevents spawning, while oocytes themselves complete meiotic maturation and then undergo irregular cytokinesis (pseudocleavage) without the arrest at the pronucleus stage (Amiel et al. 2009). Therefore, the breakdown of the ectodermal layer, which leads to spawning, could be caused by the activation of the intracellular Mos/MAPK pathway (Fig. 8.4). This suggests that a molecular mechanism controls the interaction between oocytes and ectodermal epithelium cells to ensure the appropriate timing of spawning.

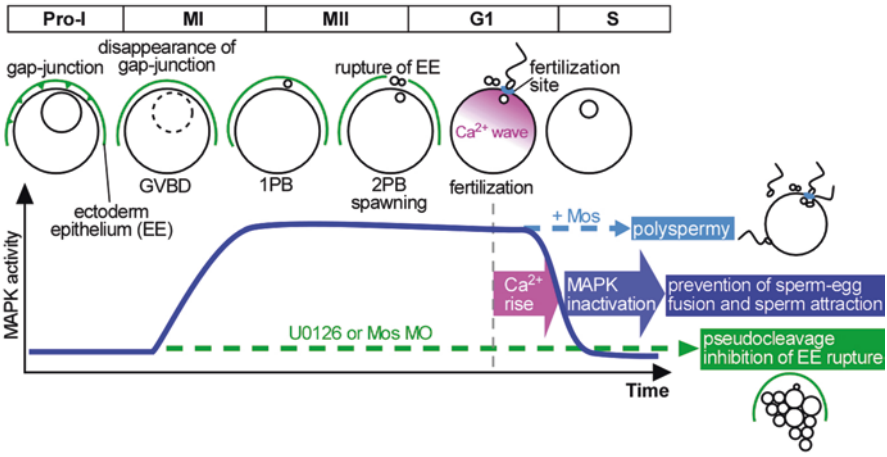

Fig. 8.4 MAPK regulation in hydrozoan jellyfish eggs. MAPK is activated after GVBD, followed by its inactivation after fertilization. The inhibition of MAPK activation by morpholino knockdown of Mos or U0126 treatment in oocytes prevents spawning and induces pseudocleavage without the arrest at G_1. On the other hand, permanent MAPK activation by Mos mRNA injection causes polyspermy. Thus, the sperm-induced Ca^{2+} rise and its downstream MAPK inactivation are responsible for proper fertilization in hydrozoan jellyfish eggs

8.5 Fertilization in Sexual Reproduction

8.5.1 Surface Functional Property of Eggs

Mature eggs of hydrozoan jellyfish, including *Cytaeis*, *Cladonema*, and *Clytia*, remain arrested at the G_1-like pronucleus stage until fertilization (Freeman and Ridgway 1993; Kondoh et al. 2006; Arakawa et al. 2014). A thin jelly coat is the only extracellular matrix surrounding the unfertilized eggs in most hydrozoan species (Freeman and Miller 1982; Yamashita 1988). In spite of the lack of a firm extracellular structure such as the vitelline envelope, the sperm fusion site is restricted to the plasma membrane of the animal pole (Freeman and Miller 1982; Deguchi et al. 2005), which is located just above the female pronucleus and is devoid of microvilli (Yamashita 1988; Deguchi and Itoh 2005). The molecules responsible for sperm binding and fusion have not yet been identified, although specific glycoproteins located on the animal pole region are believed to be responsible for sperm binding or fusion (Freeman 1996).

Another striking feature of unfertilized eggs of hydrozoan jellyfish is their superior ability to attract sperm (Miller 1985; Kondoh et al. 2006; Arakawa et al. 2014). The attractants, which are released from the eggs themselves rather than from the surrounding jelly coats (Kondoh et al. 2006), are assumed to be species-specific peptides (Miller 1985). The sperm-egg fusion site is not formed until the completion

of meiosis II, whereas the sperm-attracting ability is acquired as early as the end of meiosis I in hydrozoan jellyfish oocytes (Freeman 1987).

In some hydrozoan species, such as *Cladonema*, fertilized eggs become sticky and adhere to each other or to various substrates (Hirai and Kakinuma 1957; Yamashita 1988; Kondoh et al. 2006). The increase in the surface adhesive property in *Cladonema* eggs occurs within 10 min of fertilization. This change is not observed in *Cytaeis* and *Clytia* eggs after fertilization. The increase in the surface adhesive property in *Cladonema* eggs may be controlled by MAPK inactivation; eggs treated with U0126, an inhibitor of MEK (MAPK kinase), become sticky without fertilization, and, conversely, Mos-expressing eggs do not show the phenomenon even after fertilization (Kondoh et al. 2006). As *Cladonema* eggs contain no obvious cortical granules (Yamashita 1988), adhesive substance(s) are secreted or transported onto the egg surface by an unknown mechanism, different from cortical granule exocytosis. The surface adhesive property may enable the fertilized *Cladonema* eggs to remain in the appropriate habitat (Hirai and Kakinuma 1957).

8.5.2 Intracellular Ca^{2+} Rise and Ca^{2+}-Dependent MAPK Inactivation in Fertilized Eggs

The entry of a fertilizing sperm into an egg in hydrozoan jellyfish, including *Cytaeis*, *Cladonema*, and *Clytia*, causes a rise in the cytosolic free Ca^{2+} in the egg, which spreads from the animal pole to the vegetal pole in a wave-like fashion (Fig. 8.4) (Deguchi et al. 2005; Kondoh et al. 2006; Arakawa et al. 2014). Detailed observations in *Cytaeis* eggs, which are perfectly transparent and suitable for tracing the behavior of fertilizing sperm, demonstrated that the Ca^{2+} wave originates at the sperm fusion site and starts at the moment of sperm fusion (Arakawa et al. 2014). The Ca^{2+} wave in hydrozoan jellyfish appears to depend mainly on Ca^{2+} release through IP_3-dependent pathways (Deguchi et al. 2005).

Within a few minutes of sperm fusion, MAPK in *Cytaeis* and *Cladonema* eggs is abruptly dephosphorylated and inactivated (Fig. 8.4) (Kondoh et al. 2006; Arakawa et al. 2014). The MAPK inactivation seems to be triggered by a Ca^{2+} rise at fertilization, as induction of Ca^{2+} release by IP_3 injection inactivates MAPK without insemination, and, conversely, inhibition of sperm-induced Ca^{2+} rise by BAPTA or EGTA injection inhibits MAPK inactivation (Kondoh et al. 2006; Arakawa et al. 2014). Hydrozoan jellyfish eggs are expected to have a more direct MAPK inactivation mechanism (e.g., involvement of Ca^{2+}-dependent MAPK phosphatase), given the short time interval between the onset of Ca^{2+} rise and the completion of MAPK dephosphorylation.

8.5.3 *Sperm Fusion and Attraction, Regulated by Ca^{2+} Rise and MAPK Inactivation*

It has long been known that *Clytia* eggs treated with the Ca^{2+} ionophore A23187 lose the ability to be fertilized (Freeman and Miller 1982; Freeman and Ridgway 1993). Our recent work using *Cytaeis* eggs showed that sperm fusion is immediately (within 10 s) precluded by an IP_3-induced Ca^{2+} rise, and that suppression of the Ca^{2+} rise by EGTA allows successive sperm fusions after insemination, resulting in polyspermy (Arakawa et al. 2014). It is, therefore, likely that a Ca^{2+} rise is not only sufficient but also necessary for the prevention of sperm fusion in hydrozoan jellyfish eggs. When *Cytaeis* eggs are injected with Mos mRNA in order to suppress MAPK inactivation and are then inseminated, the first sperm fusion is accompanied by a Ca^{2+} rise that renders the egg unable to be fertilized, although it continues to attract sperm (Arakawa et al. 2014). After the completion of the Ca^{2+} rise, however, the egg accepts the next sperm and exhibits another Ca^{2+} rise. In addition, MAPK inactivation by U0126 blocks sperm fusion without fertilization or a Ca^{2+} rise (Arakawa et al. 2014). These data suggest that MAPK inactivation is also a prerequisite for blocking additional sperm fusion, independently of the mechanism that prevents subsequent sperm fusions by a Ca^{2+} rise. The mechanisms underlying the prevention of sperm fusion at the plasma membrane of hydrozoan jellyfish eggs remain unknown; at least, it seems unlikely that depolarization of the plasma membrane is responsible (Berg et al. 1986; Arakawa et al. 2014). Considering the timing of the two events in fertilized eggs, the Ca^{2+} rise and MAPK inactivation may be responsible for a fast but transient block and a late but permanent block to polyspermy, respectively (Fig. 8.4).

Downregulation of the sperm-attracting ability after fertilization is reported to occur in eggs of various hydrozoans, including *Cytaeis*, *Cladonema*, and *Clytia* (Freeman and Miller 1982; Kondoh et al. 2006; Arakawa et al. 2014). This phenomenon is not pronounced immediately after fertilization; it takes several minutes for the sperm-attracting ability to decrease to the minimum level (Kondoh et al. 2006; Arakawa et al. 2014). The classical understanding is that the Ca^{2+} rise is responsible for the downregulation of sperm attraction (Freeman and Miller 1982), but recent analyses further revealed that MAPK inactivation downstream of the Ca^{2+} rise is a necessary and sufficient step for this event. In fact, MAPK inactivation in unfertilized eggs induces downregulation of sperm attraction without a Ca^{2+} rise, and, as described previously, Mos-expressing eggs, which contain active MAPK, continue to attract sperm even after fertilization or a Ca^{2+} rise (Kondoh et al. 2006; Arakawa et al. 2014). In addition, reactivation of MAPK in fertilized eggs restores the sperm-attracting ability (Arakawa et al. 2014). As the cytoplasm extracted from fertilized eggs still attracts sperm (Miura and Deguchi unpublished data), downregulation of sperm attraction may be due to a shutdown in the release of sperm attractants, rather than a reduction of the substances in eggs. In addition, an egg fragment that contains no nucleus also loses its sperm-attracting ability when treated with A23187 or U0126 (Miura and Deguchi unpublished data), indicating that MAPK is distributed

throughout the egg and can be inactivated even in the absence of a nucleus. It is possible that the downregulation of sperm attraction by MAPK inactivation may help to block polyspermy, in cooperation with the inhibition of sperm fusion at the plasma membrane in hydrozoan jellyfish eggs (Fig. 8.4).

Thus, the sperm-induced Ca^{2+} rise and its downstream MAPK inactivation are responsible for various post-fertilization events in hydrozoan jellyfish eggs. These events are thought to help the fertilized eggs initiate and undergo normal development.

8.6 Steps Toward Asexual Reproduction

8.6.1 Cell Cycle Progression

Fertilized eggs of hydrozoan jellyfish are released from a G_1-arrest and undergo DNA synthesis before becoming two-cell embryos (Freeman and Ridgway 1993). An A23187- or IP_3-induced Ca^{2+} rise in unfertilized eggs also causes the G_1/S transition (Freeman and Ridgway 1993; Kondoh et al. 2006; Arakawa et al. 2014), which is followed by pseudocleavage (Freeman and Ridgway 1993; Kondoh et al. 2006). In *Cytaeis* and *Cladonema*, Ca^{2+}-induced MAPK inactivation is a prerequisite step for the G_1/S transition; a Ca^{2+} rise cannot initiate the G_1/S transition unless MAPK is inactivated, and MAPK inactivation induces this event (Kondoh et al. 2006; Arakawa et al. 2014). In addition, injection of mRNA encoding Mos1 and Mos2 into a blastomere of *Clytia*, a two-cell embryo, causes MAPK activation and the arrest of the cell cycle at G_1, whereas knockdown of Mos1/Mos2 during oocyte maturation prevents the oocytes from being arrested at G_1 (Amiel et al. 2009). Nevertheless, it is possible that slightly elevated Ca^{2+} levels are also required for full DNA replication, as *Cytaeis* eggs injected with EGTA and then treated with U0126 cannot enter the S phase (Arakawa et al. 2014).

8.6.2 Metamorphosis from Planula Larva to Sessile Polyp

Fertilized eggs of hydrozoan jellyfish undergo cleavage and embryogenesis, developing into swimming planula larvae within 1–3 days (Freeman 1981; Deguchi et al. 2005). They are then stimulated to metamorphose into sessile primary polyps after encountering appropriate substrates (e.g., *Nassarius* (*Niotha) livescens* for larvae of *Cytaeis uchidae*). Intensive investigations using *Hydractinia echinata* (Müller and Leitz 2002; Guo et al. 2017), the larvae of which metamorphose into polyps on snail shells inhabited by hermit crabs, have suggested that molecules produced by bacteria of the genera such as *Alteromonas* and *Pseudoalteromonas* functions as natural inducers of metamorphosis. These molecules stimulate the sensory neurons to

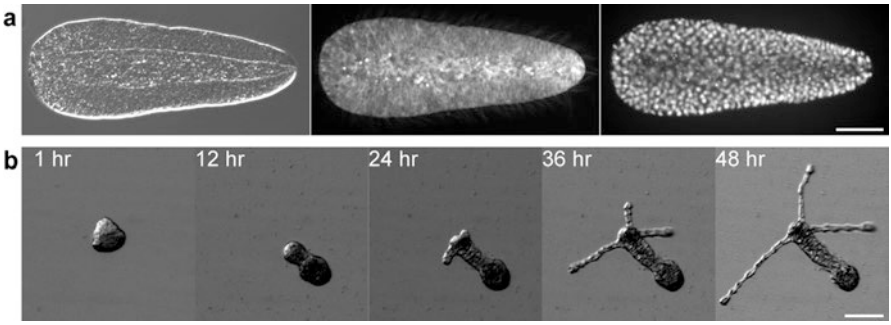

Fig. 8.5 Metamorphosis in *Cytaeis* planula larvae. (**a**) Planula larvae 3 days after fertilization. Left, differential interference contrast (DIC); center, mCherry-tubulin expressed from the microinjected mRNA; right, DNA stained with Hoechst33342. Scale bar represents 50 μm. (**b**) Sequential images of transformation from planula to sessile polyp. In each image, the time after removal of PMA (phorbol 12-myristate 13-acetate) is shown. Scale bar represents 100 μm

release GLWamide-family peptides that trigger the intracellular downstream pathways leading to metamorphosis (Hassel et al. 1996; Katsukura et al. 2003). Metamorphosis is also triggered by cesium chloride (CsCl), which is believed to stimulate the sensory neurons containing GLWamides (Katsukura et al. 2003). The downstream mediator of GLWamides in the metamorphic pathway is protein kinase C (PKC), which requires metamorphosis in some hydrozoan species for activation (Müller 1985; Freeman and Ridgway 1990).

Similar mechanisms may be involved in other hydrozoan species, including *Cytaeis*, *Cladonema*, and *Clytia*. Indeed, *Cytaeis* larvae (Fig. 8.5a) respond to CsCl, although the rate of metamorphosis is low (Arakawa and Deguchi unpublished data). However, treatment with a PKC activator (e.g., phorbol 12-myristate 13-acetate) triggers metamorphosis in a remarkably efficient manner (Fig. 8.5b) (Itabashi et al. unpublished data). *Clytia* planula larvae initiate metamorphosis in natural seawater but not in sterile seawater (Freeman 1981), and CsCl as well as GLWamides induces metamorphosis at high rates (Freeman 1981, 2005). During the metamorphosis of *Clytia*, the posterior region of the planula larva, which corresponds to the egg's animal pole, forms the upper part of the polyp (hydranth), and the anterior region forms the stolon(s) (Freeman 1981, 2005). The animal-vegetal polarity may be determined by the distributions of mRNA encoding two Frizzled family Wnt receptors, CheFz1 and CheFz3, which are localized at the animal pole and the vegetal pole, respectively (Momose and Houliston 2007).

8.7 Future Research Needs

As described, hydrozoan jellyfish are sensitive to cues in the physical environment (e.g., temperature, light) that guide various processes during reproduction. To discover the crucial substances involved in the respective events, it is necessary to identify the mechanisms for sensing temperature and light conditions that arbitrarily oscillate over short and long time-scales and for converting the input information to reproductive processes. Temperature and light conditions may activate and modulate various endogenous downstream pathways of gene expression leading to sex determination (Pen et al. 2010; Yatsu et al. 2015), circadian clock regulation (Dunlap et al. 2004; Chen et al. 2015), and other important biological processes. As a variety of hydrozoan jellyfish are found around Japan throughout the year (Kakinuma 1988), different species may have particular physiological responses to the seasonal changes in temperature and light in the sea. Future work using quantitative micro/macroscopic techniques (e.g., optical micro-heater (Oyama et al. 2015) and microthermography (Arai et al. 2015)) will increase our understanding of the intricate life cycle of hydrozoan jellyfish in nature.

The *Hydra* Peptide Project (1993–2007) systematically screened low molecular-weight peptides in *Hydra magnipapillata* to reveal the sequence, distribution, and function of various peptides (Fujisawa 2008). Some of these or related peptides are also distributed in hydrozoan jellyfish and may regulate the "turning points" during reproduction, such as induction of spawning and metamorphosis, as mentioned above. Despite past efforts, however, the distribution and function of many peptides remain unknown. Utilization of the information provided by the *Hydra* Peptide Project as well as genome and expressed sequence tag (EST) projects for other hydrozoans will accelerate future studies to identify the molecules responsible for important phases, such as the transition from asexual to sexual reproduction (induction of medusa formation).

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Chapter 9
Reproductive Strategies in Planarians: Insights Gained from the Bioassay System for Sexual Induction in Asexual *Dugesia ryukyuensis* Worms

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Abstract Some freshwater planarians (Platyhelminthes, Turbellaria, Seriata, and Tricladida) reproduce asexually by transverse fission and subsequent regeneration. Depending on environmental conditions, some asexual worms also develop complex hermaphroditic reproductive organs from planarian pluripotent stem cells, or neoblasts. Acquired sexual worms then mate, and eventually lay a cocoon filled with several fertilized eggs and a large number of yolk gland cells. The mechanisms underlying the switch from an asexual to a sexual state and the differentiation of germ cells from adult stem cells are of interest because they represent fundamental aspects of both reproductive biology and developmental biology. To study this mechanism, an experimental system was established in a triploid asexual strain, the OH strain of *Dugesia ryukyuensis* (Kobayashi et al. 1999). In this assay system, asexual worms acquire sexuality and cease asexual reproduction by transverse fission when experimentally dosed with sex-inducing substances produced by sexually mature planarians. Acquired sexual worms are then able to produce sex-inducing substances by themselves to maintain a sexual state, without the intake of sex-inducing substances. Interestingly, triploid acquired sexual worms sexually produce four types of offspring, namely, diploid asexual worms, diploid innate sexual

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worms, triploid asexual worms, and triploid innate sexual worms. Although the relationship between sexuality and ploidy has not yet been clarified, innate sexuality noticeably differs to acquired sexuality because worms never convert to an asexual state. This review discusses insights obtained from the study of these intricate biological phenomena to help elucidate the mechanisms used for reproductive strategies in planarians, including that of switching from an asexual to a sexual state.

Keywords Germ cells · Planarian · *Dugesia ryukyuensis* · Asexual reproduction · Sexual reproduction · Sexual induction · Sex-inducing substance

9.1 Introduction

Freshwater planarians (Platyhelminthes, Turbellaria, Seriata, and Tricladida) exhibit highly diverse reproductive strategies (Pearse et al. 1987). Here, we describe four types of reproductive strategies, namely (i) sexual reproduction, (ii) sperm-dependent parthenogenesis, (iii) asexual reproduction, (iv) simultaneous asexual and sexual reproduction, and (v) switching between asexual and sexual reproduction.

9.1.1 Sexual Reproduction in Planarians

Sexual planarians have hermaphroditic organs (Fig. 9.1). Male reproductive organs consist of a large number of testes, while female reproductive organs consist of a pair or pairs of ovaries that are connected to a large number of yolk glands (an organ unique to planarians) via a pair of oviducts (Fig. 9.1a, b). The copulatory apparatus is complex, containing both male genital organs (including a penis) and female genital organs (including a vagina) (Fig. 9.1c–i) (Kawakatsu et al. 1976). Copulation is reciprocal in these species, with allosperm received in the vagina being pooled within a copulatory bursa through a bursa stalk (Fig. 9.1c–i). Allosperm move

Fig. 9.1 (continued) (**b**) Ventral view of a live sexual worm: *gp* genital pore, *mo* mouth, *ov* ovary, *ph* pharynx. The image is positioned with the anterior aspect to the top. Scale bar: 2 mm. (**c**–**i**) Complex morphology of the copulatory apparatus: *bs* bursa stalk, *ca* common antrum, *cb* copulatory bursa, *cg* cement gland, *gp* genital pore, *ma* male antrum, *ph* pharynx, *pp* penis papilla, *sv* spermiducal vesicle, *va* vagina. *Body axes* are indicated in each figure: *A* anterior, *D* dorsal, *L* left, *P* posterior, *R* right, *V* ventral. (**c**) Dorsal view of a worm prepared by in situ hybridization (Kobayashi et al. 2012) without a probe, to produce a clear view on whole mount preparation of the copulatory apparatus, particularly the bursa stalk and cement glands. Positions of the aqua blue and green lines correspond approximately to similar lines in (**d**–**f**). Scale bar: 2 mm. (**d**, **e**) Images of HE stained sagittal sections around the copulatory apparatus. Colored areas indicate the sexual organs: orange, bursa stalk; pink, copulatory bursa; green, genital pore; blue, male antrum; yellow, vagina. (**f**–**i**) 3D image of the copulatory apparatus was constructed from the colored portions of serial HE images using the program Delta Viewer 2.1.1 (Movie1, Movie2). Four screen shots (dorsal view [F], ventral view [G], left view [H], and right view [I]) were captured from the 3D image

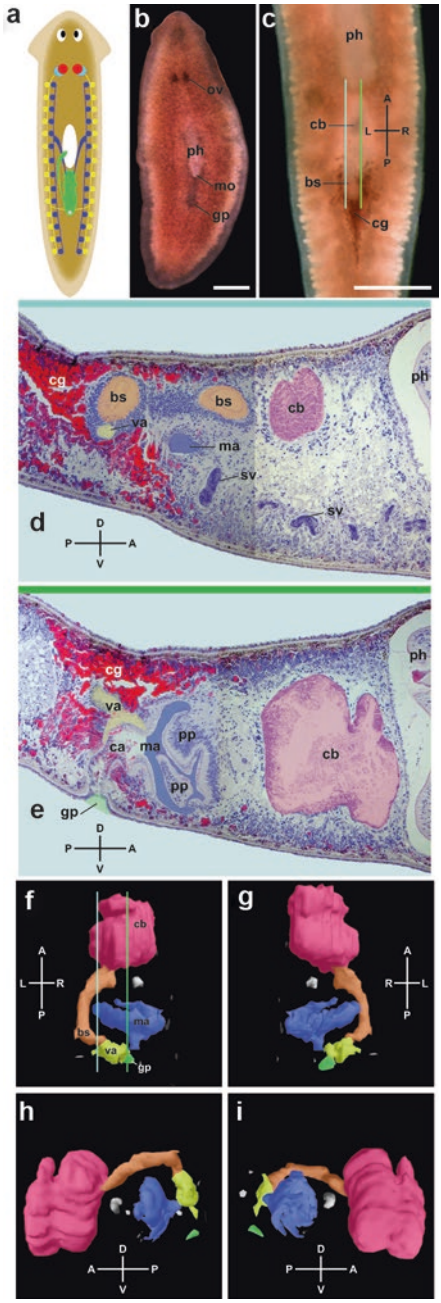

Fig. 9.1 Reproductive system of *Dugesia ryukyuensis*. (**a**) Topological position of the reproductive organs. Colored regions indicate the sexual organs: red, ovary; aqua blue, seminal receptacle; blue, testes; yellow, yolk gland; green, copulatory apparatus with a genital pore. The white region within the body is the pharynx. The image is positioned with the anterior aspect to the top.

through oviducts, and are received by a pair of seminal receptacles that are adjacent to the pair of ovaries (Fig. 9.1a; Kobayashi et al. 2002a). Oocytes released from the ovaries are cross-fertilized with the allosperm within the seminal receptacles. When fertilized eggs are transferred to the copulatory apparatus via the oviducts, yolk gland cells are also released to the oviducts. Several fertilized eggs and a large number of yolk gland cells then form a composite egg (referred to as a cocoon) within the copulatory apparatus (Fig. 9.2a). Cocoons are laid on a substrate, such as a stone. Embryonic development in a cocoon proceeds, with nutrients being obtained by ingesting components of the yolk gland cells (Fig. 9.2b) and by cannibalizing siblings (Sakurai 1991; Cardona et al. 2005; Harrath et al. 2009). Several juveniles eventually hatch from a single cocoon.

9.1.2 Sperm-Dependent Parthenogenesis in Planarians

The exact definition of sexual and asexual reproduction remains controversial. In sexual reproduction, offspring are produced "with new combinations of the parental genes," as defined by Williams (1966). According to this definition, parthenogenesis should be categorized as asexual reproduction. In *Schmidtea polychroa*, both sexual and parthenogenetic reproductive types are present. Parthenogenetic worms are also simultaneous hermaphrodites with internal fertilization as sexual worms. Parthenogenetic reproduction in *S. polychroa* requires copulation (sperm), i.e., sperm-dependent parthenogenesis or pseudogamy (Beukeboom et al. 1996; Beukeboom and Vrijenhoek 1998; D'Souza et al. 2004, 2005, 2006; D'Souza and Michiels 2008, 2010). After copulation, allosperm trigger zygote division and embryogenesis. However, the paternal alleles conveyed by allosperm either degenerate within the zygote or are expelled with one of the polar bodies (Benazzi 1970).

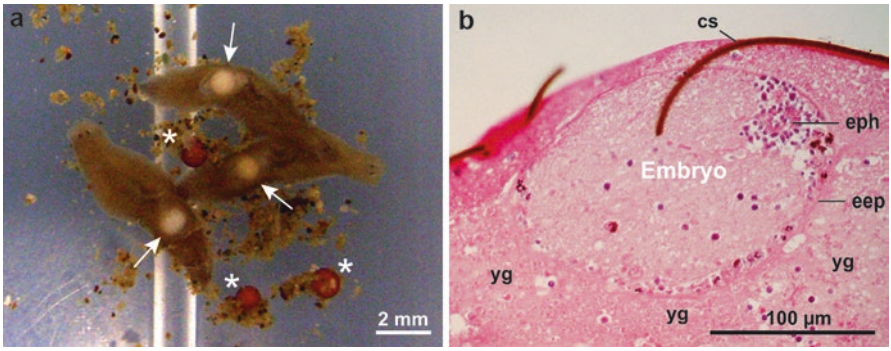

Fig. 9.2 Cocoon deposition and an embryo of the species *Dugesia ryukyuensis*. (**a**) Three separate worms laid a cocoon formed within the copulatory apparatus (arrows). Asterisks indicate cocoons. (**b**) Image of an HE stained embryo: *cs* cocoon shell, *eep* embryonic epithelium, *eph* embryonic pharynx, *yg* yolk gland cells. The embryo assimilates a large number of yolk gland cells through the embryonic pharynx

The reproductive types in *S. polychroa* differ depending on ploidy level. For instance, sexual worms are diploid, whereas parthenogenetic worms are triploid and tetraploid (Benazzi 1982). Triploid *S. polychroa* worms develop a hexaploid female germ line and a diploid male germ line, which are produced from pluripotent stem cells that are called neoblasts. Neoblasts exist and proliferate within the mesenchymal space of the entire body of planarians, and differentiate to support the regeneration and maintenance of all cell types (Wolff and Dubois 1948; Lange and Gilbert 1968; Saló and Baguñà 1985, 2002; Newmark and Sánchez Alvarado 2000; Orii et al. 2005; Sánchez Alvarado and Tsonis 2006; Wenemoser and Reddien 2010; Wagner et al. 2011; Shibata et al. 2012). In triploid *S. polychroa* worms, diploid primordial male germ cells and hexaploid primordial female germ cells are produced by the elimination of a haploid set from the triploid neoblast and the doubling of the triploid sets of neoblasts, respectively (Benazzi 1966a, b; Gremigni et al. 1980a, b, 1982). Regular meiosis subsequently occurs in both germ lines, resulting in triploid oocytes and haploid spermatozoa (Benazzi 1966b). Triploid oocytes are parthenogenetically activated by haploid sperm.

9.1.3 Asexual Reproduction in Planarians

Asexual reproduction is achieved by successive biological processes from transverse fission to regeneration (Morita 1990). However, the capacity for fission and regeneration seems to be independent. For example, asexual reproduction by fission has not been reported in *Bdellocephala brunnea* (Fig. 9.3a), even though this species has the capacity for regeneration following surgical amputation. In general, the term "asexual planarians" (or asexual worms) refers to asexually reproductive worms lacking reproductive organs.

9.1.4 Simultaneous Asexual and Sexual Reproduction in Planarians

The sexual planarians of *Seidlia auriculata* (Fig. 9.3b) appear to undergo fission at a level that is posterior to the copulatory apparatus along the anterior- posterior (AP) axis. This simultaneous asexual and sexual reproduction may contribute to fitness, because it allows the worms to gain the benefit of both asexual and sexual reproduction. Although the mechanism to control asexual and sexual reproduction within a single individual is of interest, it has been technically difficult to study and, thus, remains poorly understood, particularly in comparison to species that simply switch between asexual and sexual states.

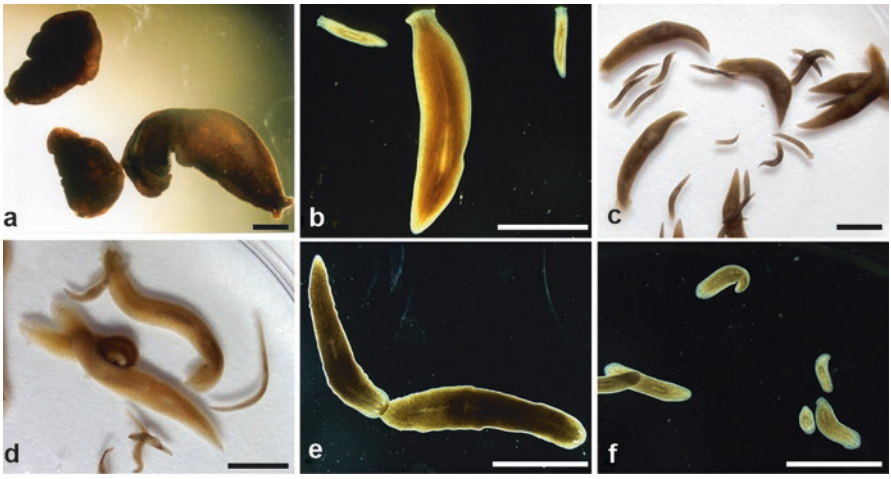

Fig. 9.3 Diversity of planarian reproductive strategies (**a**) *Bdellocephala brunnea*, an oviparous species, collected from a natural habitat. (**b**) *Seidlia auriculata* worms derived from a clonal population by fission. Under laboratory conditions, some worms develop reproductive organs. Even sexually mature worms (the larger worm) undergo fission at a point posterior to the copulatory apparatus. The smaller worms will develop reproductive organs, again. (**c**) *Dugesia japonica*, (**d**) *Girardia dorotoceplala* worms derived from a clonal population by fission. On exposure to a lower temperature, some worms cease to undergo fission and develop reproductive organs. The smaller worms in the images are asexual, whereas the larger worms are sexual. (**e**) *Schmidtea mediterranea* sexual specimens. (**f**) *S. mediterranea* asexual specimens. Scale bar: 5 mm

9.1.5 Switch Between Asexual and Sexual Reproductive States in Planarians

According to a classical definition, some planarian species have at least two physiological races that differ in their manner of reproduction; namely, an asexual and a sexual race (Kenk 1937; Okugawa and Kawakatsu 1954). In such species, the asexual race reproduces exclusively by fission, and does not develop reproductive organs. In contrast, the sexual race is able to switch between an asexual state without reproductive organs and a sexual state. The mechanism by which this reproductive switch is achieved is not yet clear, even though the phenomenon was first described more than a century ago (Curtis 1902; Curtis and Schulze 1924). Under laboratory conditions, asexual worms of the sexual race develop reproductive organs from planarian pluripotent stem cells (or neoblasts) when exposed to environmental stimuli (i.e., temperature changes), and then commence sexual reproduction (instead of asexual reproduction by transverse fission) (Fig. 9.3c, d; Jenkins 1967; Vowinckel 1970; Vowinckel and Marsden 1971a, b; Benazzi 1974). The switch from an asexual state without reproductive organs to a sexual state is termed sexual induction. Understanding the process by which adult stem cells (neoblasts) differentiate into complicated hermaphroditic reproductive organs is important to the fields of reproductive biology and developmental biology. However, an experimental strategy

with a sophisticated control is required to investigate this mechanism. This review discusses recent discoveries in the complex reproductive strategies of planarians, drawing on insights gained from a bioassay system for sexual induction that was established using triploid *D. ryukyuensis* worms by Kobayashi et al. (1999).

9.2 A Biological Assay System for Sexual Induction in *D. ryukyuensis*

9.2.1 Establishment of the Biological Assay System

Sexual induction in asexual worms is triggered by certain environmental stimuli, such as temperature change under laboratory conditions. However, sexual induction by environmental stimulation is unstable, and does not seem feasible as a relatively quick and reliable assay system to study the mechanism. In contrast, Kenk (1941) successfully induced the differentiation of reproductive organs in the asexual body by transplanting the anterior third portion of the body of a sexual worm to the posterior two-thirds of the body of an asexual worm in *Girardia tigrina*. These results suggest that hormone-like chemicals derived from the sexual body induce the development of reproductive organs in the asexual body. Grasso and Benazzi (1973) also successfully achieved sexual induction by feeding sexually mature *Polycelis nigra* worms to asexual *Dugesia gonocephala* worms. Many scientists have since demonstrated that asexual worms are sexualized if fed with sexual worms of the same or a different species (Benazzi and Grasso 1977; Sakurai 1981; Teshirogi 1986; Hauser 1987). These results clearly demonstrate that sexual planarians contain putative hormone-like chemicals (referred to as sex-inducing substances) that are not species-specific.

To isolate and identify sex-inducing substances, Kobayashi et al. (1999) established a bioassay system for sexual induction using the triploid asexual race of *D. ryukyuensis* (the OH strain). The OH strain was derived from one asexual worm that was collected in 1984 by Dr. Sachiko Ishida, Hirosaki University. Asexual worms of the OH strain have only reproduced asexually and have not shown any evidence of sexuality under laboratory conditions so far. However, all asexual worms are sexualized within approximately 1 month, if fed daily with *B. brunnea* (Fig. 9.3a; Kobayashi et al. 1999). In such cases, worms consistently develop a pair of ovaries, testes, yolk glands, and a copulatory apparatus, in that order. The process of sexual induction is divided into five distinct stages based on morphological characteristics (Fig. 9.4a). At stage 1, the ovaries become sufficiently large to be externally apparent behind the head, although no oocytes are evident. At stage 2, oocytes appear within the ovaries; however, other reproductive organs remain undetectable. At stage 3, the primordial testes and the yolk gland primordia emerge, and the copulatory apparatus becomes visible in the post-pharyngeal region. At stage 4, spermatocytes appear within the testes. At stage 5, mature yolk glands are formed, and many

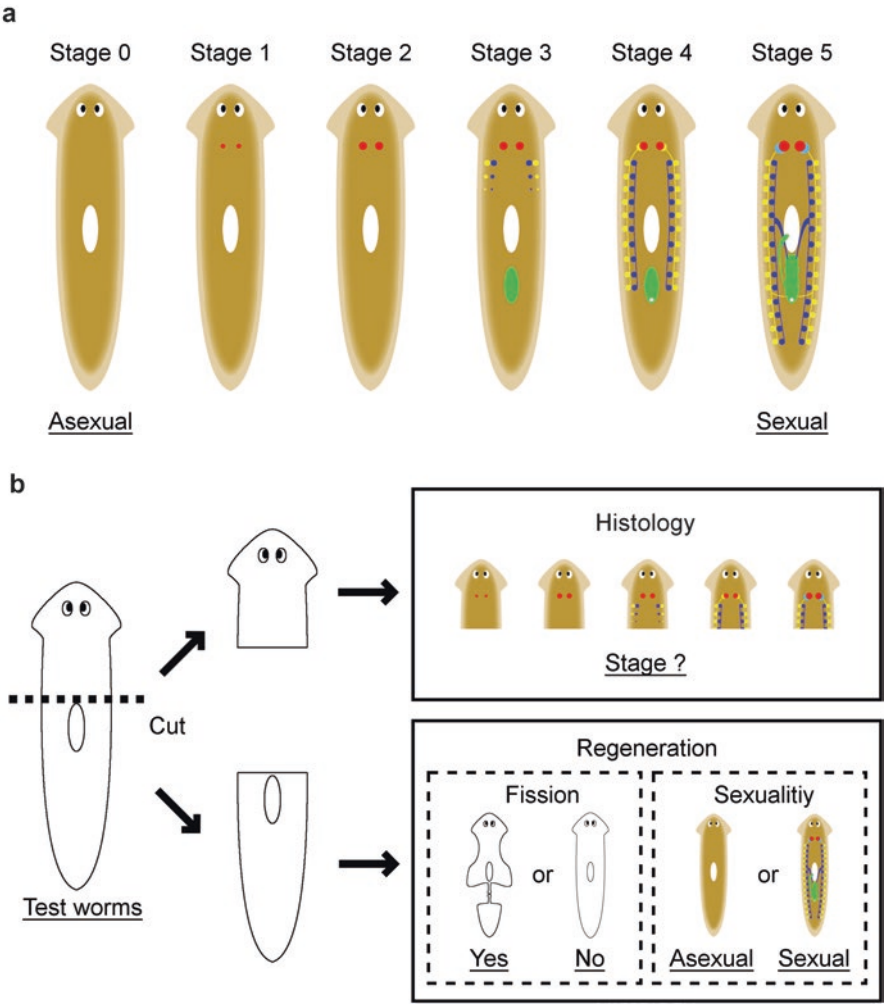

Fig. 9.4 (**a**) Five distinct stages during sexual induction. Developmental and topological positions of the reproductive organs. Colored regions indicate the sexual organs: red, ovary; aqua blue, seminal receptacle; blue, testes; yellow, yolk gland; green, copulatory apparatus with a genital pore (Adapted from Kobayashi and Hoshi 2002). (**b**) Scheme for the amputation-regeneration test. Worms were cut at the prepharyngeal level during sexual induction. Head fragments were used for histological analysis to determine the stage of sexual induction. Tail fragments were allowed to regenerate without the stimulus of sex-inducing substances, to evaluate whether the worms acquire sexuality and lose fissiparous capacity

mature spermatozoa are detectable within the testes. The fully sexualized worms in this assay system are termed acquired sexual worms (Hoshi et al. 2003).

9.2.2 Maintenance of Acquired Sexuality and Disappearance of Fissiparous Capacity

The process of sexual induction has a "point-of-no-return", after which acquired sexuality is maintained autonomously. If feeding on sexual planarians is stopped at the early stage of sexual induction, test worms tend to revert to the asexual state. In contrast, if feeding on sexual planarians is continued until the late stage of sexual induction, test worms continue developing sexual organs, even when feeding with sexual planarians is stopped. To determine the specific stage of this point-of-no-return, an amputation-regeneration test was performed (Fig. 9.4b; Kobayashi et al. 1999; Kobayashi and Hoshi 2002). Test worms were cut at a prepharyngeal level during sexual induction. The head fragments were used for histological research to determine the various stages of sexual induction, whereas the tail fragments were fed with a common diet of chicken liver, which contained no sex-inducing activity, and were allowed to regenerate. If the test worms had already acquired sexuality, the tail fragments were expected to regenerate to become sexual worms without being fed sexual worms. Because acquired sexual worms do not undergo transverse fission anymore, fissiparous capacity was expected to be lost at some point during sexual induction. The amputation-regeneration test is also useful to evaluate the potential capacity for fission in worms, because decapitation facilitates fission in the tails of regenerants (Best et al. 1969, 1975) (Fig. 9.4b).

Figure 9.5 presents the results of the amputation-regeneration test. Worms decapitated at stages 1–4 underwent fission, whereas those at stage 5 did not. This result suggests that the fissiparous capacity disappears between stages 4 and 5. In contrast, the regenerants derived from worms decapitated at stages 1 and 2 became asexual, whereas those derived from worms decapitated at stages 3–5 became sexual worms. This result suggests that the point-of-no-return for sexuality exists between stages 2 and 3, and the loss of fissiparous capacity occurs after the acquisition of sexuality in the OH strain of *D. ryukyuensis*. Thus, the processes of fission and development of reproductive organs may be independent of each other.

9.3 Sex-Inducing Substances in Planarians

Test worms were not sexualized by being fed with conspecific asexual worms under the conditions of the assay system (Kobayashi et al. 1999). Thus, sex-inducing substances may be absent or occur in very small quantities in asexual worms. The test worms were fully sexualized by being fed with the sexually mature worms of *B.*

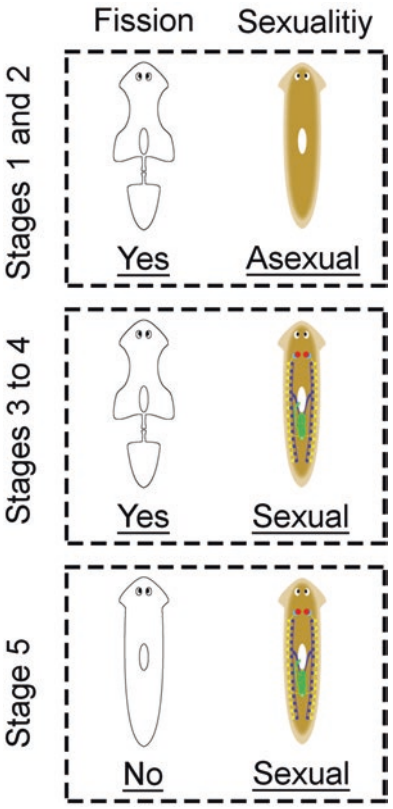

Fig. 9.5 Different fates of test worms during sexual induction in the amputation-regeneration test. Worms from stage 3 onwards consistently produced sexual regenerants. Thus, acquisition of sexuality may occur between stages 2 and 3. In addition, the worms may lose fissiparous capacity between stages 4 and 5 (adapted from Kobayashi and Hoshi 2002)

brunnea, which were collected from a natural habitat. The acquired sexual worms were maintained for over a year on a common diet of chicken liver that has no sex-inducing activity. Subsequently, test worms were fully sexualized by being fed with the acquired sexual worms (Kobayashi et al. 2002b). Thus, sex-inducing substances may be de novo-synthesized and/or specifically concentrated compounds derived from chicken liver in acquired sexual worms. Sex-inducing substances are expected to help reproductive organs to continue developing after the point-of-no-return (Kobayashi and Hoshi 2002; Kobayashi et al. 2002b). Therefore, the identification of sex-inducing substances would provide important clues regarding the mechanisms underlying the maintenance of sexuality in planarians.

Using this assay system, Kobayashi and Hoshi (2011) obtained a hydrophilic fraction with strong sex-inducing activity from *B. brunnea* and conspecific sexual worms, referred to as Fr. M0 + M10. The authors suggested that sex-inducing substances in Fr. M0 + M10 are papain-resistant and putative low-molecular weight (MW) (<500) compounds. The heat stability of these compounds remains inconclusive; however, we confirmed that sex-inducing substances in Fr. M0 + M10 are heat stable in this review (Fig. 9.6). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of a testis marker *Dr-Y1* (Ishizuka et al. 2007)

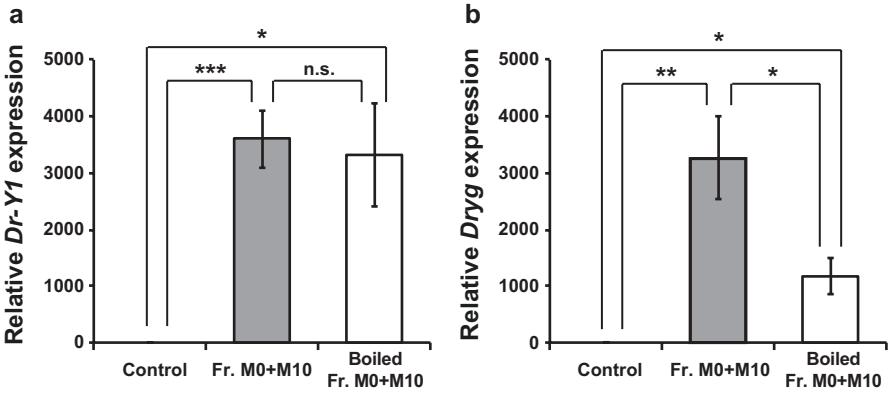

Fig. 9.6 Thermal stability of sex-inducing substances in Fr. M0 + M10. Fr. M0 + M10 derived from 4 g wet weight of *Bdellocephala brunnea* was prepared as described in Kobayashi and Hoshi (2011). Fr. M0 + M10 derived from 2 g of *B. brunnea* (wet weight) was boiled at 100 °C for 15 min. Boiled Fr. M0 + M10 and Fr. M0 + M10 were both dried, mixed with 150 μL of chicken liver homogenate, freeze-dried, and used as test food for sexual induction. Chicken liver homogenate was used as a negative control. Test worms (25) were fed daily on a piece of food over 4 weeks. RNA from boiled Fr. M0 + M10 (n = 7), Fr. M0 + M10 (n = 7), and the control (n = 7) were used in qRT-PCR analyses. (**a**) Expression of a testis marker, *Dr-Y1*. (**b**) Expression of a yolk gland marker *Dryg*. Measurements were normalized to the expression levels of *Dr-gapdh*. Mean values ± SEM (error bars) are shown. Statistical significance was calculated using *t*-tests (**P* < 0.05, ***P* < 0.01, and ****P* < 0.001); *n.s.* indicates no significance

and a yolk gland marker *Dryg* (Hase et al. 2003) showed that the heat treatment of Fr. M0 + M10 did not affect sex-inducing activity, with most worms successfully developing the testes (Fig. 9.6a) and yolk glands (Fig. 9.6b) compared to the control treatment. However, unexpectedly, the expression of *Dryg* was lower in the heat treatment than in the non-heat treatment, suggesting a delay in the development of yolk glands (Fig. 9.6b). Therefore, sex-inducing substances in Fr. M0 + M10 are heat-stable, but heat-unstable co-factors might be present that enhance the maturation of yolk glands, with which sexual induction would proceed more efficiently.

To examine the distribution of the sex-inducing substances in Fr. M0 + M10, acquired sexual worms were cut into three pieces: the head (H) fragment containing no sexual organs; the middle (M) fragment containing a pair of ovaries, testes, and yolk glands; and the tail (T) fragment containing testes, yolk glands, and copulatory apparatus (Fig. 9.7a). The test worms were fed Fr. M0 + M10 derived from the three fragments. Sex-inducing activity was observed in the M and T fragments, but not in the H fragments (Kobayashi and Hoshi 2011). Moreover, when the three fragments were allowed to regenerate via feeding with a common diet of chicken liver, the H fragments regenerated to become asexual, whereas the M and T fragments regenerated to become sexual (Fig. 9.7a; Kobayashi et al. 2012). These results strongly suggest that the sex-inducing activity of Fr. M0 + M10 is also associated with the maintenance of sexuality. We previously stated that test worms from stage 3 onwards (i.e., after the point-of-no-return) retain acquired sexuality. In the OH

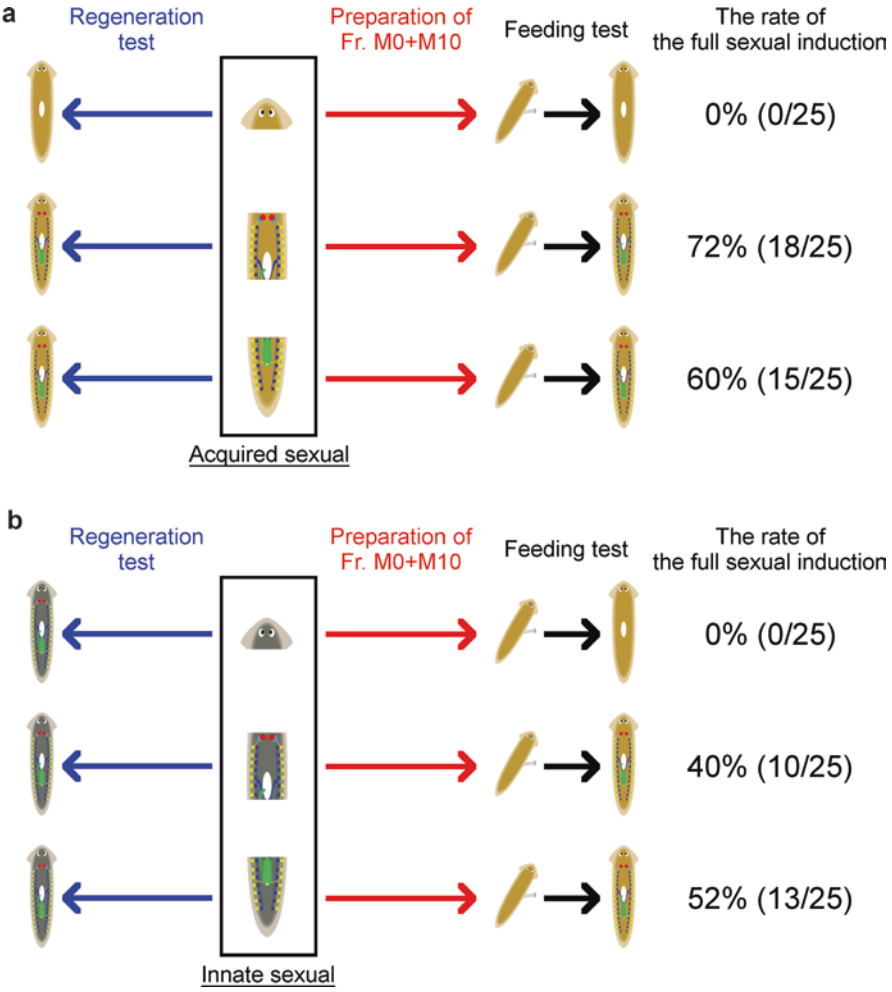

Fig. 9.7 Three fragments obtained based on the topological position of the reproductive organs: relationship between reproductive mode of regenerants and sex-inducing activity (**a**) Acquired sexual worms. (**b**) Innate sexual worms. In the regeneration test, each of three fragments was allowed to regenerate for 3 months and then the reproductive mode of the regenerants was examined (The result was adapted from Kobayashi et al. 2012). In the feeding test, Fr. M0 + M10 derived from each of three fragments was prepared as described in Kobayashi and Hoshi (2011). Sex-inducing activity in each of the three fragments of acquired sexual worms was adapted from Kobayashi and Hoshi (2011)

worms, a sufficient quantity of sex-inducing substances may have been produced in a body part equivalent to the M and T fragments from stage 3 onwards, which facilitated the maintenance of acquired sexuality. The M and T fragments contain numerous testes and yolk glands. Thus, these organs may be candidate organs producing sex-inducing substances; however, further studies are needed.

9.4 Regulation of Sexual Induction by Planarian D-Amino Acid Oxidase

Recently, using electrospray ionization-mass spectrometry (ESI-MS), circular dichroism (CD) spectroscopy, and nuclear magnetic resonance spectroscopy (NMR), we discovered that Fr. M0 + M10 contains tryptophan (Trp) (Kobayashi et al. 2017). We also found that both chiral forms of Trp exhibit ovary-inducing activity, with D-Trp activity being stronger than that of L-Trp in *D. ryukyuensis* (Kobayashi et al. 2017). Moreover, additional studies revealed that, besides D-Trp, four D-amino acids (D-arginine, D-asparagine, D-leucine, and D-phenylalanine) also possess ovary-inducing activity, suggesting that D-amino acids are important for sexual induction in planarians (Maezawa et al. 2014).

Most amino acids exist as L-isomers within living organisms (Hamase 2015). Natural proteins are exclusively built from L-amino acids. However, the development of enantioselective and other highly sensitive analytical methods has revealed that various D-amino acids naturally exist both as free and protein-bound forms. D-amino acids play a key role in the regulation of many biological processes in living organisms (Khoronenkova and Tishkov 2008). For example, D-serine (D-Ser) is the endogenous coagonist of *N*-methyl-D-aspartate (NMDA) receptors that are important for excitatory synaptic transmission, and are involved in various processes, such as learning and memory retention (Nishikawa 2005). The regional distribution of D-amino acids is dependent on the expression of amino acid racemases and D-amino acid degrading enzymes. In other words, the activity of these enzymes may be correlated with the physiological activity of D-amino acids. In mammalian brains, glial cells produce D-Ser from L-Ser via the action of serine racemase, and degrade D-Ser by D-amino acid oxidase (DAO) (Tanaka et al. 2007). The distribution of serine racemase in the brain is similar to that of D-Ser (Panatier et al. 2006). In addition, free D-Ser content is lower in the cerebellum compared to the cerebrum of mice in which DAO activity was much higher in the cerebellum compared to the cerebrum (Nagata 1992). Although a tryptophan-specific racemase has not yet been isolated in animals, DAO orthologs that degrade neutral and basic D-amino acids (including D-Trp) have been found to be highly conserved in several species, ranging from yeast to humans (Pollegioni et al. 2007).

A *DAO* homolog gene, *Dr-DAO*, was isolated from *D. ryukyuensis*. Dr-DAO recombinant protein degrades neutral, basic, and acidic D-amino acids in vitro (Maezawa et al. 2014). Because acidic D-amino acids are primarily degraded by D-aspartate oxidase (DDO) in many animals (Yamamoto et al. 2010), *Dr-DAO* may be a common ancestral gene of *DAO* and *DDO*. In *D. ryukyuensis*, homogenates of asexual worm bodies had higher DAO activity than those of sexual worms. Thus, the concentration of D-amino acids in asexual worms may be maintained at comparatively lower levels. However, analyses of expression and function showed that *Dr-DAO* might have different roles depending on the reproductive modes of *D. ryukyuensis* (Maezawa et al. 2014). First, in asexual worms, *Dr-DAO* is highly expressed in the parenchyma. RNAi knockdown of *Dr-DAO* in asexual worms results in the formation of immature ovaries in the absence of D-amino acid administration. Thus, Dr-DAO protein may repress early ovarian development through the degradation of D-amino acids in asexual worms, with increased concentrations of D-amino acids due to *Dr-DAO* knockdown inducing ovarian development. However, surprisingly, RNAi knockdown of *Dr-DAO* during sexual induction resulted in delayed ovarian maturation. These contradictory results may be explained by differences in gene expression patterns during sexual induction. At stage 3/4, strong transient expression of *Dr-DAO* was detected in the oogonia of developing ovaries, as well as the parenchyma throughout the body. However, at stage 5, *Dr-DAO* expression disappeared in the ovaries, and was limited to the parenchyma of the head region. These results suggest that overall *Dr-DAO* expression throughout the body gradually decreases with the progression of sexual induction. In contrast, transient *Dr-DAO* expression in the ovaries may be required to complete the establishment of functional ovaries. Further research is required to clarify the role of *Dr-DAO* in the ovaries and parenchyma, along with details on the molecular mechanisms involved.

Interestingly, although the expression of *Dr-DAO* has not been identified in other reproductive organs (such as the copulatory apparatus, testes, and yolk glands), RNAi knockdown of *Dr-DAO* during sexual induction also results in concomitant delay in the differentiation of other reproductive organs. These results suggest that Dr-DAO protein promotes the establishment of functional ovaries with oogonia and oocytes, and ovarian maturation seems to be associated with the development of other reproductive organs. Thus, there may be a developmental interrelationship between reproductive organs, in which the maturation of one organ affects the subsequent differentiation of other organs, and, maybe, vice versa. Sexual induction probably needs to be orchestrated in a controlled manner via feedback signaling among organs. This model is also supported by RNAi knockdown experiments of other genes required for germ line development (as described in Sect. 9.5).

9.5 Developmental Inter-relationship Among Reproductive Organs and the Maintenance of Sexuality

Previous studies using RNAi have suggested that the testis is responsible for retaining sexuality in sexual worms (Collins et al. 2010; Nakagawa et al. 2012a, b). For example, transcripts of *npy-8* that encode a neuropeptide Y (NPY) superfamily member in *S. mediterranea* have been detected in a variety of cells within the central and peripheral nervous system, and within a dorsal population of cells associated with the lobes of the testes in sexual worms (Collins et al. 2010). Because the RNAi knockdown of *npy-8* in sexually mature worms results in the regression of the testes, the NPY-8 peptide in cells surrounding the lobes of the testes may act in a neuroendocrine fashion to influence the development of the testes and to maintain its proper function. Interestingly, RNAi knockdown of *npy-8* also triggered the regression of the copulatory apparatus. Thus, maturation of the testes may be associated with the functions and development of the copulatory apparatus. However, it could not be determined whether *npy-8* knockdown sexual worms completely lost sexuality and became asexual, because *S. mediterranea* worms seem to lack the capacity to switch reproductive modes in the first place (discussed in Sect. 9.6).

In contrast, *D. ryukyuensis* is able to switch between asexual and sexual states, making it an ideal model organism to investigate the reproductive organ(s) that are responsible for the acquisition and maintenance of sexuality. In many animals, *nanos* homologs play important roles in germ cell development (Kobayashi et al. 1996; Maezawa et al. 2009). Nakagawa et al. (2012a) showed that the *nanos* homolog in *D. ryukyuensis*, referred to as *Dr-nanos*, is expressed in the ovarian primordia of asexual worms, and in the oogonia and spermatogonia of sexual worms. RNAi knockdown of *Dr-nanos* during long-term (40-day) sexual induction resulted in the inhibition of ovarian and testicular development, with many *Dr-nanos* knockdown worms possessing no testis and small ovaries with few oogonia. In contrast, knockdown of *Dr-nanos* did not influence the development of other reproductive organs, such as the copulatory apparatus and yolk glands. The authors briefly mentioned that most worms appeared to return to an asexual state when fed with a common diet of chicken liver for 5 months after the RNAi treatment finished (Nakagawa et al. 2012a). This study seems to indicate that the testis is required to retain acquired sexuality, supporting the suggestion made by Collins et al. (2010). Yet, unlike *S. mediterranea*, somatic reproductive organs (such as a copulatory apparatus and yolk glands) were not affected by the absence of testis in *D. ryukyuensis*. However, these results need to be interpreted with caution. First, because the authors looked at RNAi-treated worms as a single mass, the fate of individuals (returning to asexual or becoming sexual) could not be attributed to their RNAi phenotypes (e.g., existence of the testis) with sufficient precision. Second, the authors performed RNAi knockdown treatment for a long time (40 days) while feeding sexual worms with substances containing sex-inducing substances. An

overdose of sex-inducing substances might have had a cumulative effect, leading to the formation of additional reproductive organs, overwhelming the effect of RNAi knockdown treatment.

Therefore, in this review, we again carefully investigated the phenotypes of *Dr-nanos* knockdown worms to verify the reproductive organ(s) responsible for sexuality. We performed *Dr-nanos* knockdown treatment with short-term (28-day) sexual induction and an amputation-regeneration test (as described in Sect. 9.2.2, with minor modifications). In this approach, we aimed to minimize the possible overdose effect of sex-inducing substances during the knockdown experiment. Moreover, the amputation-regeneration test enabled us to associate the acquisition (or not) of sexuality precisely with the degree of differentiation of each reproductive organ. First, we confirmed that short-term sexual induction was sufficient to sexualize control worms, with many worms being found to possess copulatory apparatus with a genital pore (Table 9.1). However, in *Dr-nanos* knockdown worms, ovaries and copulatory apparatus were only detected in two-thirds of worms, and only a few worms had a genital pore based on external observation (Table 9.1). Because *Dr-nanos* seems to be expressed in male and female primordial germ cells, knockdown probably had an indirect effect on copulatory apparatus, resulting in inhibition or delay in the development of the copulatory apparatus. Next, focusing on the two-thirds of *Dr-nanos* knockdown worms that developed copulatory apparatus, we performed an amputation-regeneration test (as described in Sect. 9.2.2, with minor modifications). The control and *Dr-nanos* knockdown worms were cut into two pieces at the prepharyngeal level. The head fragments were allowed to regenerate for 1 month by being fed a common diet of chicken liver. We found that most of the head fragments from the knockdown worms regenerated to become asexual, whereas all control head fragments regenerated to become sexual (Table 9.2). This result was surprising, because copulatory apparatus usually forms after the point-of-no-return in sexual induction (the acquisition of sexuality);

Table 9.1 External morphology of *Dr-nanos* knockdown worms during sexual induction

Target gene of RNAi[a]	Number of test worms that developed a pair of ovaries (%)[b]	Number of test worms that developed a copulatory apparatus (%)[b]	Number of test worms that developed a genital pore (%)[b]
Control (water)	25/25 (100.0)	24/25 (96.0)	12/25 (48.0)
Dr-nanos	20/30 (66.7)**	20/30 (66.7)*	2/30 (6.7)**

[a]Worms were fed twice at 3-day intervals with chicken liver containing about 580 ng of *Dr-nanos* dsRNA, or an equal volume of water (Control). One day after the second feeding, posterior and anterior regions of the worms were amputated, and the trunk fragments were allowed to regenerate for 10 days. The regenerated worms were then fed chicken liver containing about 580 ng of the dsRNAs of the respective genes twice, at 3-day intervals. The test worms were then fed minced *Bdellocephala brunnea* bodies supplemented with dsRNAs (aliquots of about 110 ng of dsRNA mixed with the minced bodies) of the respective genes daily for 4 weeks

[b]Development of a pair of ovaries and copulatory apparatus was examined using a stereomicroscope after 4 weeks of the feeding treatment. Asterisks indicate statistically significant differences (**$P < 0.005$, *$P < 0.01$), determined using the chi-square test

Table 9.2 Amputation-regeneration test after *Dr-nanos* knockdown during sexual induction

Target gene of RNAi[a]	Sexual worms (%)[b]
Control (water)	16/16 (100.0)
Dr-nanos	1/14 (7.1)*

[a]After 28 days of sexual induction, *Dr-nanos* knockdown worms with a copulatory apparatus were cut at the prepharyngeal level and head fragments were allowed to regenerate by feeding on a diet of chicken liver

[b]Regenerants that developed a genital pore 1 month after regeneration, were judged as sexual worms. Asterisks indicate statistically significant differences (*$P < 0.001$), determined using the chi-square test

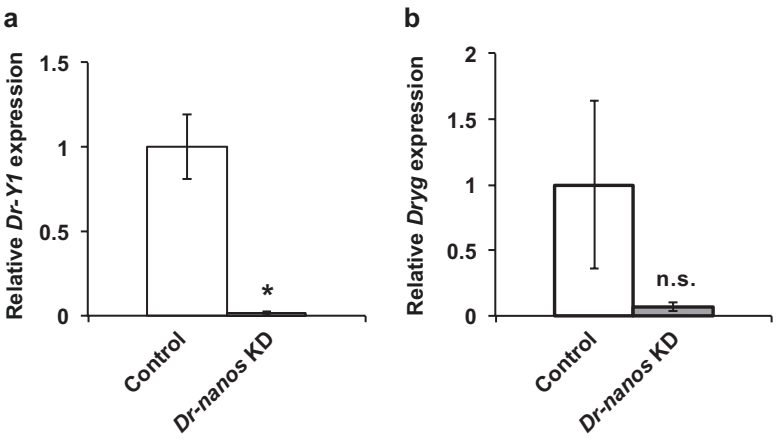

Fig. 9.8 Effect of *Dr-nanos* knockdown on *Dr-Y1* and *Dryg* mRNA levels. (**a**) *Dr-Y1* qRT-PCR using RNA from tail fragments of the control (n = 15) or *Dr-nanos* knockdown (KD) worms (n = 6). (**b**) *Dryg* qRT-PCR using RNA from tail fragments of the control (n = 15) or *Dr-nanos* KD worms (n = 14). Measurements were normalized to the expression levels of *Dr-ef1*. Mean values ± SEM (error bars) are shown. Statistical significance was calculated using the Student's *t*-test (*$P < 0.005$); *n.s.* indicates no significance

specifically, the head fragments with this organ normally do not return to an asexual state. The corresponding tail fragments were homogenized immediately after amputation to obtain RNA for qRT-PCR analysis, to check the degree of sexual differentiation. We found that the development of the testis was severely affected in *Dr-nanos* knockdown worms, with significantly lower expression of the testis marker gene *Dr-Y1* (Ishizuka et al. 2007) (Fig. 9.8a). In addition, the knockdown worms had lower expression of the yolk gland marker *Dryg* (Hase et al. 2003) than the controls (although this effect was not statistically significant, due to large variation in the controls) (Fig. 9.8b). One possible explanation for the suppression of yolk glands is that a developmental inter-relationship might exist between reproductive organs, with yolk glands formation being delayed because of a smaller (immature) ovary and/or the absence of testis in knockdown worms. This would also explain the absence of copulatory apparatus in one-third of knockdown worms.

In summary, our results indicate that the acquisition of sexuality is associated with the development of the testis, supporting the results of Nakagawa et al. (2012a). However, our research also suggests that the yolk glands are associated with sexuality. As there may be a developmental interrelationship between the testis and the yolk glands, at this stage we could not determine which organs are truly responsible for sexuality. Further research is required to address these issues. For example, it is necessary to identify the organs responsible for autonomously producing the sex-inducing substances that retain sexuality.

9.6 Production of Diploid and Triploid Offspring by Breeding of Triploid Acquired Sexual Worms

Although asexual worms in an asexual race of *D. gonocephala* became fully sexualized after being fed *P. nigra*, the sexualized worms (termed ex-fissiparous worms) were sterile (Grasso and Benazzi 1973). The cause of sterility appeared to be an ovarian abnormality termed hyperplasic ovary (Gremigni and Banchetti 1972a, b; Harrath et al. 2014). One possible explanation for such defects in sexual reproduction is, if asexual reproduction is adaptive in some environments, asexual races may not have switched to the sexual state over a long period of time. In this case, mutations may accumulate in the genetic system for sexual reproduction, due to lack of natural selection acting on sexual phenotype, leading to defects in the differentiation of reproductive organs. A similar example is seen in *S. mediterranea*, in which there are two races: sexual and asexual races (Fig. 9.3e, f). The sexual race only reproduces sexually, and never undergoes transverse fission. In contrast, the asexual race only reproduces asexually by fission and regeneration. We fed *B. brunnea* (Fig. 9.3a) to asexual worms (Fig. 9.3f); however, there was no occurrence of sexual induction. The asexual race has a translocation chromosomal abnormality (Newmark and Sánchez Alvarado 2002) that probably causes defects in the differentiation of reproductive organs.

In contrast, acquired sexual worms of *D. ryukyuensis* are fertile, which is a useful advantage (although the OH strain is a triploid asexual race with a translocation abnormality) (Sato et al. 2005; Kobayashi et al. 2008a, 2009). In terms of fertility, the assay system for sexual induction in the OH strain is, in a sense, analogous to natural sexual induction in the sexual race. There are at least three types of ploidy in *D. ryukyuensis* (Kawakatsu et al. 1976, 1995): diploidy (2×), triploidy (3×), and mixoploidy (2× + 3×) (Oki et al. 1981). It was believed that triploid sexual *D. ryukyuensis* worms undergo pseudogamy (sperm-dependent parthenogenesis) (Tamura et al. 1995, 1998), as occurs in *S. polychroa* (Beukeboom et al. 1996; Beukeboom and Vrijenhoek 1998; D'Souza et al. 2004, 2005, 2006; D'Souza and Michiels 2008, 2010). However, the breeding of triploid acquired sexual worms produced both triploid and diploid offspring in a ratio of 1:2–1:3, with a significance level of 5 % (Chi-square test) (Kobayashi et al. 2008a). The emergence of diploid offspring is quite complex, and cannot be explained by pseudogamy. Chinone et al.

(2014) clearly demonstrated that both diploid and triploid offspring are sexually produced by breeding triploid acquired sexual worms. In the OH strain of *D. ryukyuensis*, diploid primordial male germ cells are produced via the elimination of a haploid set from the triploid neoblast. Regular meiosis subsequently occurs in male germ lines to yield haploid spermatozoa. In contrast, triploid primordial female germ cells differentiate from triploid neoblasts. Thus, both haploid and diploid oocytes are produced within the ovaries by a unique process of meiosis. The resultant offspring include diploid and triploid individuals (Chinone et al. 2014).

In general, triploidy is considered an evolutionary dead end because of the problems associated with chromosomal pairing and segregation during meiosis. Therefore, the formation of tetraploid or diploid individuals from triploid individuals is a rare phenomenon. However, triploid asexual *D. ryukyuensis* worms have the potential to be sexualized, and triploid sexual worms could sexually produce both diploid and triploid offspring. Another example is seen in a population of green frogs, *Bufo pseudoraddei baturae*. Triploid individuals produce diploid primordial male germ cells via the elimination of a haploid set from triploid somatic cells, and tetraploid primordial female germ cells via the elimination and duplication of triploid somatic cells, to yield haploid sperm and diploid oocytes via meiosis. The resultant offspring are triploid (Stöck et al. 2002). In triploid animals, sexual reproduction may be more widespread than generally assumed.

9.7 Existence of Two Sexual Races

The reproductive races of planarians were previously classified based on morphological observations in natural habitats and under laboratory conditions (Kenk 1937). Based on this classical definition, a sexual race contains exclusively sexual worms that do not express asexual potential (innate sexual worms), as well as sexual worms that are able to switch to the asexual state (acquired sexual worms). However, the assertion of these two sexual races is subject to controversy, because it was difficult to show that innate sexual worms are not the result of asexual worms that have acquired sexuality.

Recently, Kobayashi et al. (2012) showed that these two sexual worms are distinguishable by experimental examination. In brief, the inbreeding of acquired sexual worms produces both asexual and sexual offspring at a ratio of 2:1, with a significance level of 5 % (Kobayashi et al. 2009). Asexual offspring become acquired sexual worms after being fed *B. brunnea*, just as the worms of the OH strain did. In contrast, sexual offspring, termed innate sexual worms, become sexually mature without being fed *B. brunnea* (Fig. 9.9; Kobayashi et al. 2012). Here, we demonstrated that the distribution pattern of sex-inducing activity is similar between acquired sexual worms and innate sexual worms. As already shown in Fig. 9.7a, the sex-inducing activity of acquired sexual worms only occurs in the M and T fragments, not the H fragments (Fig. 9.7a; Kobayashi and Hoshi 2011). In innate sexual worms, sex-inducing activity also occurs in the M and T fragments only, not the H

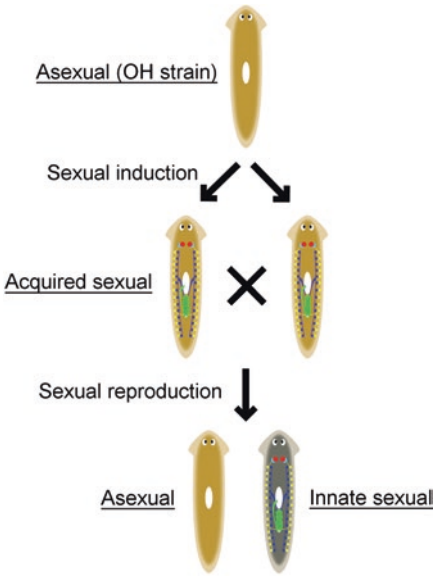

Fig. 9.9 Illustration of experimental animals derived from the OH strain of *Dugesia ryukyuensis*. Acquired sexual worms required the administration of sex-inducing substances to develop reproductive organs, whereas innate sexual worms developed reproductive organs without the need for sex-inducing substances (adapted from Kobayashi et al. 2012)

fragments (Fig. 9.7b). However, the regeneration pattern was surprisingly different between acquired sexual worms and innate sexual worms. As shown previously, the H fragments of acquired sexual worms regenerated to become asexual (Fig. 9.7a), probably due to the lack of sex-inducing substances in that fragment. In comparison, the H fragments of innate sexual worms regenerated to become sexual, despite the lack of a sufficient amount of sex-inducing substances (Fig. 9.7b). These findings indicate the constitutive production of sex-inducing substances in innate sexual worms. This result is consistent with observations that innate sexual juveniles could develop sexual organs without being fed *B. brunnea*, suggesting that innate sexual worms cannot switch to the asexual state. Acquired sexual worms and innate sexual worms are clearly distinguishable by the results of the head regeneration experiment. Thus, Kobayashi et al. (2012) demonstrated the existence of both innate and acquired sexual races.

These differences may be attributed to differences in neoblasts between the acquired sexual worms and the innate sexual worms. Recently, this hypothesis was tested by Nodono et al. (2012), using a rescue experimental system. The rescue experimental system was originally established by Baguñà et al. (1989). The authors rescued the X-ray irradiated sexual worms in *S. mediterranea* (which would otherwise eventually die after irradiation) by injecting neoblasts from the asexual race, and showed that the worms became asexual. Conversely, the X-ray-irradiated asexual race was rescued by injecting neoblasts from the sexual race, and developed reproductive organs. This experimental system demonstrated the pluripotency of neoblasts (Kobayashi et al. 2008b; Wagner et al. 2011). Baguñà et al. (1989) described this system as "transformation by replacement of neoblasts" in *S. mediterranea* from the asexual race to the sexual race and vice versa. Similarly,

Nodono (2012) injected the neoblasts of acquired sexual worms and innate sexual worms of *D. ryukyuensis* into X-ray-irradiated asexual worms. As expected, the rescued asexual hosts that had been injected with the neoblasts of the acquired sexual worms became asexual, whereas those that had been injected with the neoblasts of innate sexual worms became sexual. These findings confirm that the neoblasts of acquired sexual and innate sexual worms are quite different.

Innate sexual worms tend to be less prolific than acquired sexual worms (Kobayashi et al. 2012). However, innate sexual worms undoubtedly transmit sexuality to their offspring (Kobayashi et al. 2012). Innate sexual races may contribute to the reproductive success of future offspring by guaranteeing sexual reproduction. Whether the offspring become asexual or innate sexual is probably controlled genetically. However, at present, we cannot adequately address this issue. As mentioned in the previous section, the inbreeding of acquired sexual worms produces both diploid and triploid offspring at a ratio of 1:2 or 1:3. One may expect that this enables us to predict the relationship between polyploidy and innate sexuality (or asexuality). However, offspring produced by the inbreeding of acquired sexual worms contained all possible combinations: diploid asexual worms, diploid innate sexual worms, triploid asexual worms, and triploid innate sexual worms. Most of the diploid offspring were innate sexual worms, whereas two-thirds of the triploid offspring were asexual worms (Kobayashi et al. 2009). In addition to the complex process of sexual reproduction in the OH strain of *D. ryukyuensis* (as described in the previous section), competition amongst embryos for survival within the cocoons prevents classical (Mendlian) genetic analysis. Further studies are required to determine the mechanisms underlying innate sexuality and asexuality.

9.8 Perspectives

In general, temperature change is viewed as the most important factor regulating the switch between asexual and sexual states. Even oviparous planarian species like *B. brunnea* undergo the full development of reproductive organs in colder seasons, with subsequent degeneration occurring in warmer seasons (Kobayashi et al. 2002a). Interestingly, *B. brunnea* collected in colder seasons tend to have stronger sex-inducing activity that are able to fully sexualize the test worms of the OH strain, whereas those collected in warmer seasons tend to have weaker sex-inducing activity that only induce immature ovaries (Kobayashi et al. 2002a). Thus, under natural conditions, freshwater planarians are expected to control sex-inducing substances for the development of their reproductive organs across seasons, probably producing endogenous substances or utilizing externally taken substances. Once the signaling pathway for sexual induction is activated, various types of molecules are expected to work, such as transcription factors, neurotransmitters, chemical substances, and so on. The sex-inducing substances discussed here might represent a subset of these molecules, and may not necessarily be the very first factor to initiate the sexual induction in planarians. However, the sex-inducing substances we

have targeted so far are able to activate all necessary pathways to induce sexual reproduction, with identification being required by future studies.

So far, several studies have identified molecules involved in the sexual reproduction of planarians. The neuropeptide NPY-8, which is specifically associated with testicular differentiation in *S. mediterranea* (Collins et al. 2010), is, indeed, a notable discovery. Because planarians probably receive various environmental stimuli through the central nervous system (Inoue et al. 2004, 2014, 2015), the nervous system should be the most important for planarians lacking a blood circulatory system to initiate the development of reproductive organs in response to environmental stimuli. Meanwhile, the involvement of mammalian sex steroid-like compounds in planarians has also been proposed (Fukushima et al. 2008; Miyashita et al. 2011). Such chemical compounds produced within a reproductive organ may induce the development of other reproductive organs, and provide positive feedback to the nervous system on the maintenance of the sexual state. However, the molecules described here are probably not our candidates. Sex-inducing substances in Fr. M0 + M10 are hydrophilic, heat-stable, papain-resistant, and putative low-MW compounds (<500). NPYs and mammalian sex steroids are not consistent with these features, and are not likely to be the substances that act as sex-inducing substances in our experimental sexual induction.

Sex-inducing substances seem to be common and/or specific compounds at least within the suborder Continenticola, because substances sourced from *B. brunnea*, which belongs to the family Dendrocoelidae, induce sexual reproduction in *D. ryukyuensis*, which belongs to the family Dugesiidae. We recently discovered that Fr. M0 + M10 from acquired sexual worms contain much higher levels of Trp (about 25-fold higher) than that obtained from asexual *D. ryukyuensis* worms (Kobayashi et al. 2017). Trp induces the development of immature ovaries, but cannot induce that of other reproductive organs. Other compounds contained in Fr. M0 + M10 are required to trigger full sexual induction. In the future, sex-inducing substances will be isolated and purified via high performance liquid chromatography (HPLC) in the bioassay system introduced in the present review, and identified by liquid chromatography/mass spectrometry (LC/MS) and nuclear magnetic resonance (NMR) analysis.

Finally, a wide variety of reproductive modes in planarians were briefly described in this review, including exclusive sexual reproduction, simultaneous asexual and sexual reproduction, and the switch between asexual and sexual reproduction. Interestingly, asexual reproduction by transverse fission does not seem to be directly associated with sexual induction. At least in the OH strain of *D. ryukyuensis*, the cessation of asexual reproduction does not trigger sexual induction. Rather, following acquisition of the sexual state (specifically characterized by the production of sex-inducing substances), asexual reproduction seems to be inhibited. In terms of the switch between asexual and sexual states, the most important points to understand include how planarians prevent, initiate, and maintain the development of reproductive organs. These factors are all involved in the mechanism by which the production of the sex-inducing substances is regulated. We expect that there may be a common mechanism to regulate sex-inducing substances within the order

Tricladida. Furthermore, the way that this mechanism is controlled may vary among planarians in different environmental situations, leading to the expression of different types of sexuality. In this chapter, we introduced the asexual, acquired sexual, and innate sexual worms of the OH strain of *D. ryukyuensis*. Ongoing transcriptome analysis of these worms is expected to provide additional clues on the mechanism(s) by which the production of sex-inducing substances is regulated. We believe that our future findings on *D. ryukyuensis* will provide important insights on the variety of reproductive strategies in planarians.

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Chapter 10
Reproductive Strategies in Annelida: Germ Cell Formation and Regeneration

Ryosuke Tadokoro

Abstract Annelida are metameric, eucoelomate bilaterian worms belonging to Lophotrochozoa, which is a major group of protostomes. This phylum includes Polychaeta, Oligochaeta, Hirudinea (leeches), and Archiannelida, and it is an important link in the evolution of body plan, regeneration, and reproduction. Although annelids generally reproduce sexually, many species can switch to asexual reproduction, proliferating exponentially, as seen in planarians, hydras, and some other lower invertebrates. Asexual reproduction is achieved through dedifferentiation and through stem cells regeneration, instead of being based on germ cells as in the case of sexual reproduction. Thus, studies on regeneration mechanisms and germ cells are essential for understanding annelid reproduction. In this chapter, Annelida's reproduction and germ cell formation and regeneration are reviewed. Based on our research on the oligochaete Enchytraeus japonensis, a unique process of germ cell regeneration during asexual reproduction is proposed.

Keywords Annelida · Germ cells · PGCs · Stem cells · Regeneration · Reproduction

10.1 Introduction

Since the Cambrian age, annelids have survived, selecting the most favorable mode of reproduction depending on circumstances and the environment. Almost all oligochaetes, leeches, and polychaetes can proliferate through sexual reproduction, although all groups also have hermaphroditic species. Oligochaetes and leeches are generally hermaphroditic, laying a few eggs wrapped in a cocoon (Brusca and Brusca 1990), which increases fertility, protecting eggs from environmental changes and bacteria. Hermaphroditic and dioecious species are typically found within

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polychaetes; many species lay a small number of eggs, whereas others lay a larger number of eggs as a strategy to increase their survival rate (Izuka 1903; Okada 1941; Hauenschild 1960; Bentley et al. 2001). The sexual reproduction and germ cell formation in annelids are described in this chapter.

Unlike leeches, oligochaetes and polychaetes generally possess a high regeneration ability (Bely 2006), allowing some species to reproduce asexually and proliferate exponentially. Some of these species divide their body into several fragments, each of which regenerates a new individual, whereas other species bud new generations from growth zones (Berrill 1952; Bell 1959; Christensen 1959; Bouguenec and Giani 1989; Nakamura 1993; Schmelz et al. 2000; Bely and Wray 2001). In asexual reproduction, stem cells, instead of germ cells, and dedifferentiation systems play a central role in reproduction. To date, regeneration processes have been well described in both oligochaetes and polychaetes based on histological analyses. Here, the regeneration process of oligochaetes is briefly described, focusing on *Enchytraeus japonensis* as an example (Nakamura 1993; Myohara et al. 1999). Interestingly, these worms can regenerate germ cells that are often lost by fission or accidental cutting (Tadokoro et al. 2006). Thus, in addition to the commonly known animal germ cell system, *E. japonensis*, uses the regeneration of somatic tissues and germ cells as a reproductive strategy. Although the origin of regenerating germ cells has been investigated in other oligochaetes and in polychaetes (Iwanoff 1928; Gates 1943; Herlant-Meewis 1964b; Vannini 1947; Dorsett 1961), it is still largely unknown. Previous and recent studies on germ cell regeneration will also be reviewed here.

10.2 Sexual Reproduction in Annelids

Oligochaetes and leeches are generally hermaphroditic, and their male and female gonads and germ cells are located around the coelomic cavity in special segments of the head region termed genital segments. These segments are characterized by a thickened epidermis called the clitellum (Fig. 10.1a) (Stephenson 1930; Brusca and Brusca 1990), and oligochaetes and leeches are therefore collectively classified as Clitellata (Stephenson 1930; Jamieson 1992). Most clitellate species have several pairs of ovaries and testes, and their location and number are used as taxonomic characters (Michaelsen 1929; Jamieson 2006).

Male reproductive organs comprise the testes, seminal vesicles, male funnel, seminiferous tube, and penis (Fig. 10.1a). During spermatogenesis, sperm stem cells (spermatogonia) within the testes are released into seminal vesicles, where they undergo repeated incomplete cell divisions with maintaining cytoplasmic connections. As a result, the spermatogonia develop a morula-like morphology (Fig. 10.1a) (Stephenson 1930; Jamieson 1992) that later differentiates into spermatids and sperm. Thereafter, mature sperm enter the male funnel and are transported to the penis via the seminiferous tube (Fig. 10.1a) (Brusca and Brusca 1990; Stephenson 1930; Jamieson 1992). The histological characterization of

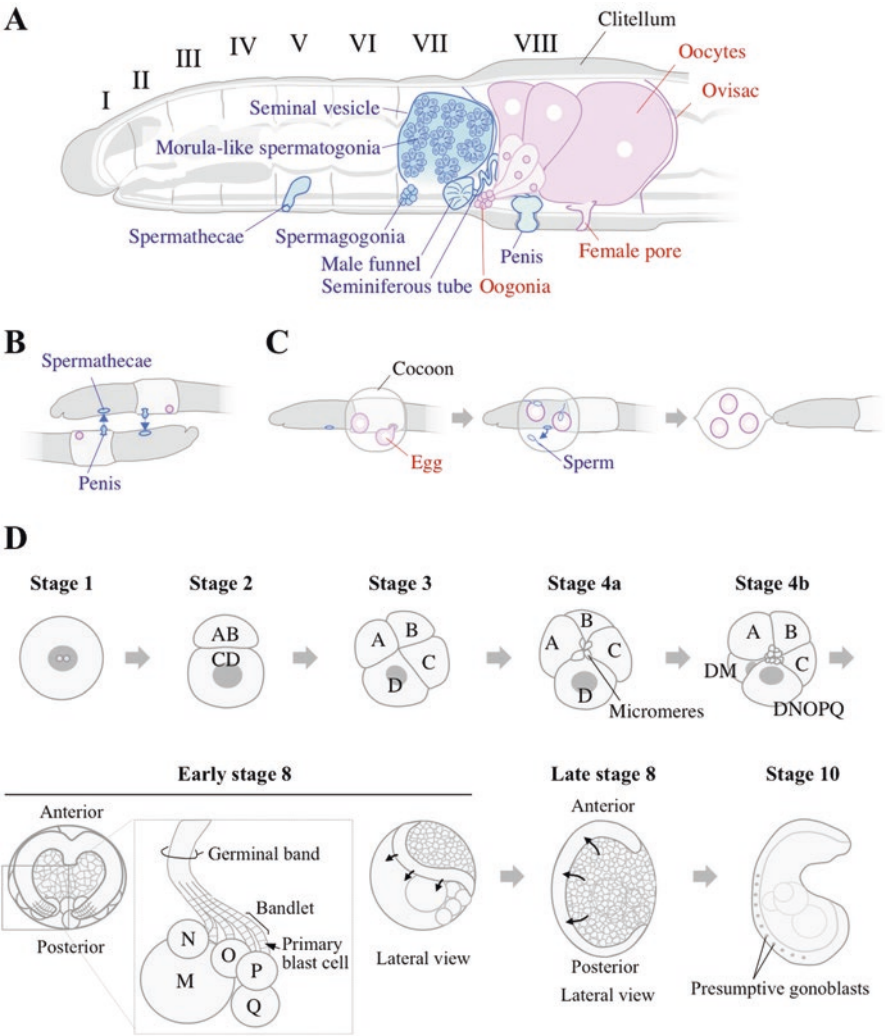

Fig. 10.1 Sexual reproduction in Clitellates. (**a**) Male and female reproductive organs and germline cells of oligochaete, *Enchytraeus japonensis*, colored in blue and pink, respectively. (**b**) Mating behavior of oligochaetes; Blue and pink circles indicate spermathecae and female genitalia, respectively. (**c**) Process of egg deposition and fertilization in an oligochaete. (**d**) Schematic illustration of embryogenesis in the leech *Helobdella robusta*; Darker gray areas indicate the teloplasm. N, O, P, and Q are ectodermal teloblasts and M is a mesodermal teloblast (redrawn from Weisblat et al. (1980))

Spermatogonia and reproductive organs is a straightforward process. Oocyte stem cells (oogonia) are harbored in the ovary and mature within the ovisac, which is a vesicle-like structure derived from the septum (Fig. 10.1a) (Stephenson 1930; Jamieson 1992; Brusca and Brusca 1990). Oocytes are transported along the

oviduct and deposited in the clitellum through the female pore (Stephenson 1930; Jamieson 1992).

At the onset of breeding, two worms join their head regions aligned in opposite directions and each releases sperm from the penis and deposits it into the partner's spermatheca (Fig. 10.1b) (Stephenson 1930; Michaelsen 1929; Avel 1959; Brusca and Brusca 1990). After mating, the worms secrete cocoon material from the clitellum, surround the eggs with it, and displace the cocoon toward the anterior region by vermicular movements (Fig. 10.1c) (Edwards and Lofty 1972; Brusca and Brusca 1990). Eggs are fertilized by the partner's sperm within the cocoon when it passes on the spermatheca's pore (Fig. 10.1c) (Edwards and Lofty 1972; Brusca and Brusca 1990) and the cocoon is finally shed from the head of worm (Fig. 10.1c) (Edwards and Lofty 1972; Brusca and Brusca 1990).

Reproduction in polychaetes is more diverse than that in oligochaetes and leeches. Although most polychaetes are dioecious, some species are hermaphroditic; some individuals are born either as male or female and then change sex (Bacci and Bortesi 1961). Stem cells of both male and female germ cells are harbored in the gonads or in the coelomic epithelium and mature in the coelomic fluid (Olive and Clark 1978; Fischer 1974, 1975; Sawada 1975). Although in most species germ cells are located in specific segments, in primitive species these cells float in the coelomic cavity of all segments (Olive and Clark 1978; Fischer 1974, 1975; Sawada 1975). Mature oocytes are released through the nephridium or by rupturing the body wall (Goodrich 1945), as male and female ducts develop poorly compared with those of oligochaetes. In many cases, eggs are deposited in masses enclosed by a gel, and fertilization occurs after egg deposition (only a few species mate before egg laying) (Brusca and Brusca 1990). In some species of Nereididae, Syllidae, and Eunicidae, sexually mature worms, or parts of the body filled with germ cells (epitokes), simultaneously swim toward the sea surface, releasing numerous eggs and sperm on specific full moon nights (Izuka 1903; Okada 1941; Hauenschild 1960; Bentley et al. 2001). This swarming behavior has attracted the attention of biologists, particularly ecologists and ethologists. Although it has been shown that swarming behavior is activated by brain-controlled hormones (Hauenschild 1960), the molecular mechanisms regulating this periodic behavior remain unknown.

10.3 Germ Cell Formation During Embryogenesis

The origin of annelid germ cells has long been debated based on the traditional histological observations performed over the last 50 years. As a result of recent gene expression analyses and cell tracing experiments more details on the origin of annelid germ cells have emerged. Here, previous and current studies on the embryonic origin of germ cells are reviewed, mainly performed on the clitellates *Helobdella robusta* (leech) and *Tubifex tubifex* (oligochaete).

As background information for the observations and experiments described below, I present a brief review of *H. robusta* development (for details, see Weisblat

et al. 1980). After fertilization,the *H. robusta* zygote divides into four blastomeres via two cleavage divisions (A, B, C, and D quadrants in Stage 3, Fig. 10.1d) (Weisblat et al. 1980). Almost synchronously, each blastomere produces micromeres in alternating directions (clockwise/counter-clockwise), which are spirally accumulated around the animal pole (spiral cleavage) (Fig. 10.1d, stages 4a and 4b). These micromeres contribute to the head's ectoderm and mesoderm (Shankland and Savage 1997). The D quadrant divides into four pairs of ectodermal stem cells (N, O, P, and Q teloblasts) and a single pair of mesodermal stem cells (M teloblasts) on both sides of the blastomere (Fig. 10.1d, stages 4b–8) (Weisblat et al. 1980). The teloblasts then undergo asymmetric cell division, continuously generating primary blast cells in bandlets, which fasciculate together on each side, producing germinal bands (Fig. 10.1d, early stage 8) (Weisblat et al. 1980). The germinal bands on each side are displaced ventrally as they elongate and finally merge on the ventral midline, forming the tubular body of the worm (Fig. 10.1d, early stage 8 to late stage 8) (Weisblat et al. 1980). Embryogenesis of the oligochaete, *Tubifex*, is similar to that of *H. robusta*, despite the different timing of cleavage (Shimizu 1980).

Meyer (1929) reported that in *Tubifex* primordial germ cells (PGCs) emerge in situ around the presumptive genital segments during embryogenesis. Penners and Stäblein (1930) postulated that *Tubifex* PGCs derive from M teloblasts and migrate to the genital segments through amoeboidal movements. On the other hand, it has been suggested that in the oligochaete *Eisenia fetida* the first primary blast cells produced by M teloblasts do not contribute to PGCs (Devries 1971). Thus, it is difficult to determine the origin of PGCs based only on histological observation. Studies on the origin of germ cells have recently made substantial progress, as germ cell gene markers (for example, *nanos*, *vasa*, *piwi*) allow the unambiguous identification of germline cells.

In the twenty-first century, the research team of David Weisblat reported the expression pattern of the *nanos* gene in *H. robusta* (Kang et al. 2002). This gene encodes a translational repressor protein that is specifically expressed in the germline cells of almost all organisms (Kobayashi et al. 1996; Tsuda et al. 2003; Koprunner et al. 2001; Sato et al. 2006). During *H. robusta* early development, maternal transcripts of *nanos* are first accumulated in the yolk-free cytoplasm (teloplasm) of zygotes and then inherited by the D quadrant and its descendants DM and DNOPQ (Fig. 10.1d, stages 1–4b) (Kang et al. 2002). Until this stage, *nanos* mRNA behavior is identical to that displayed in the teloplasm (Fig. 10.1d, dark gray areas). Maternal expression of *nanos* decreases by stage 7 and zygotic transcripts are expressed in the ectodermal and mesodermal teloblasts, in their descendants (i.e., primary blast cells), and in germinal bands (Fig. 10.1d, early stage 8) (Kang et al. 2002). At this stage, *nanos* expression in the germinal bands almost disappears, except in the presumptive gonoblasts located in the 11 mid-body segments (Fig. 10.1d, stage 10, spots), which will develop into germ cells (Kang et al. 2002). Cell tracing experiments confirmed that these *nanos*-expressing gonoblasts derive from M primary blast cells (Kang et al. 2002) and might correspond to cells previously proposed to be PGCs.

Recently, the expression patterns of the conserved germline makers DEAD-box RNA helicase *vasa* and the ncRNA binding protein *piwi* (Shibata et al. 1999; Yoon et al. 1997; Fujiwara et al. 1994; Mochizuki et al. 2001; Cox et al. 1998, 2000; Lau et al. 2006; Kuramochi-Miyagawa et al. 2001; Deng and Lin 2002), were shown to be similarly distributed (Cho et al. 2014). Interestingly, during *Helobdella* embryogenesis, *nanos* and *vasa/piwi* mRNAs are preferentially expressed in male and female PGCs, respectively (Cho et al. 2014). Oyama and Shimizu (2007) showed that *vasa* is broadly expressed in *Tubifex* embryonic cells, including mesodermal cells, in patterns similar to those of *nanos* and *vasa* in *Helobdella*. Furthermore, based on cell ablation experiments, these authors determined that PGCs are generated from primary m cells (m10 and m11), corresponding to the presumptive genital segments, but not from cells deposited by M teloblasts before blast cell generation (Kato et al. 2013). As PGCs do not disappear when blast cells adjacent to m10 and m11 are ablated (Kato et al. 2013), the destiny of these blast cells might be determined at birth. However, it is still largely unknown how PGCs are determined in specific primary blast cells.

Previous studies on polychaetes have suggested that germ cells might also derive from the mesodermal linage corresponding to M teloblasts and/or bandlets (Malaquin 1925, 1934; Iwanoff 1928). Rebscher and co-authors (Rebscher et al. 2007, 2012) reported the expression pattern of *vasa* mRNA and its encoded protein in *Platynereis dumerilii*, showing that mesoblasts (4 d) deposit four *vasa*-positive cells that become PGCs after migration. These four *vasa*-positive cells appear to be identical to the cells that Schneider and Bowerman (2007) designate as "prospective PGCs." In *Capitella teleta*, germline cells appear to emerge near mesodermal bands, although the details are still unclear (Giani et al. 2011; Dill and Seaver 2008). Thus, almost all studies suggest that annelids' PGCs derive from mesodermal lineages generated in early embryogenesis, with the timing of segregation differing among species. It has often been debated whether PGCs are specified by preformation or by epigenesis: whereas in preformation the germline is specified by maternal cytoplasmic determinants, as is the case in fruit flies and nematodes, in epigenesis germ cell segregation is induced from the somatic lineage (Eddy 1975; Extavour and Akam 2003). In *Helobdella*, PGCs are specified around the 20th round of cell division, when most *nanos* maternal transcripts have disappeared, although PGCs' specification by preformation generally occurs at earlier stages of embryogenesis. In *Tubifex* species, inheritable determinants explaining germline specification by preformation were not identified, despite the thorough assessment of *vasa*-expression from embryogenesis to juveniles. Thus, germline specification seems to be regulated by epigenesis in annelids, although it has not been experimentally verified at the molecular level.

10.4 Asexual Reproduction in Annelids

Agamic asexual reproduction has the advantage of producing a large number of offspring at a low cost; however, it rarely produces individuals able to adapt to a wide variety of environments, as is the case of sexual reproduction. Asexual reproduction is typically found among oligochaetes, but not among leeches. In the asexual phase, oligochaetes divide into several fragments, producing entire worms by regeneration and/or by growing head and tail structures, i.e., by autotomy or paratomy (Fig. 10.2a) (Brusca and Brusca 1990). In paratomy, which is typically observed in Naididae and Aeolosomatidae, fission occurs after the formation of head and tail structures in prospective cutting planes (budding zones) (Fig. 10.2a, right) (Bely and Wray 2001; Herlant-Meewis 1954). In contrast, species dividing by autotomy (e.g., those in Enchytraeidae) split without preforming a budding zone (Fig. 10.2a, left) (Bell 1959; Christensen 1959, 1964; Bouguenec and Giani 1989; Nakamura 1993; Schmelz et al. 2000). Asexually reproducing worms are able to completely regenerate head and tail structures, whereas sexually reproducing species can only regenerate their anterior region (Bely 2006).

In annelids, regeneration proceeds through a series of phases (Fig. 10.2b). Here I will describe the course of *Enchytraeus japonensis* regeneration, reviewing some previous studies (Nakamura 1993; Myohara et al. 1999; Yoshida-Noro and Tochinai 2010). After amputation, circular muscles adjacent to the wound immediately contract to prevent the discharge of fluid (Herlant-Meewis 1964a, b; Bilej 1994). Epidermal cells cover anterior stumps, whereas the intestinal wall and epidermis interconnect to cover posterior stumps (Herlant-Meewis 1964a, b; Myohara et al. 1999). This process is conserved amongst almost all annelids that are able to regenerate. Five hours post amputation, epidermal cells and stem-like cells begin to proliferate, resulting in the accumulation of a large number of undifferentiated cells around the stump. A regeneration blastema is formed during the first 24 h following amputation, i.e., 1 day post amputation (dpa) (Fig. 10.2, 1 dpa). During the next 24 h, the anterior blastema elongates and tiny cell aggregates form on the dorsal and ventral sides of the blastema (Fig. 10.2, 2 dpa). These aggregates are thought to correspond to primordial brain and ventral nerve cord. As the blastema grows further, major tissues and organs regenerate and become morphologically recognizable. Morphological segmentation is also visible in the epidermis on the third day after amputation (Fig. 10.2, 3 dpa). Subsequently, each organ and tissue grows, enlarges, and regeneration is complete at the fourth dpa (Fig. 10.2, 4 dpa). In the posterior blastema, the growth zone and pygidium are regenerated by the second dpa, and segments are newly formed via the growth zone (Fig. 10.2). The process of regeneration following amputation is more or less similar in asexual reproducing worms, regardless of species.

The regeneration processes that take place after blastema formation are mediated by genes involved in body patterning, morphogenesis, and organogenesis during embryogenesis (Akimenko et al. 1995; Bely and Wray 2001; Gardiner et al. 1995; Khan et al. 2002). In contrast, blastema formation is unique to the regeneration

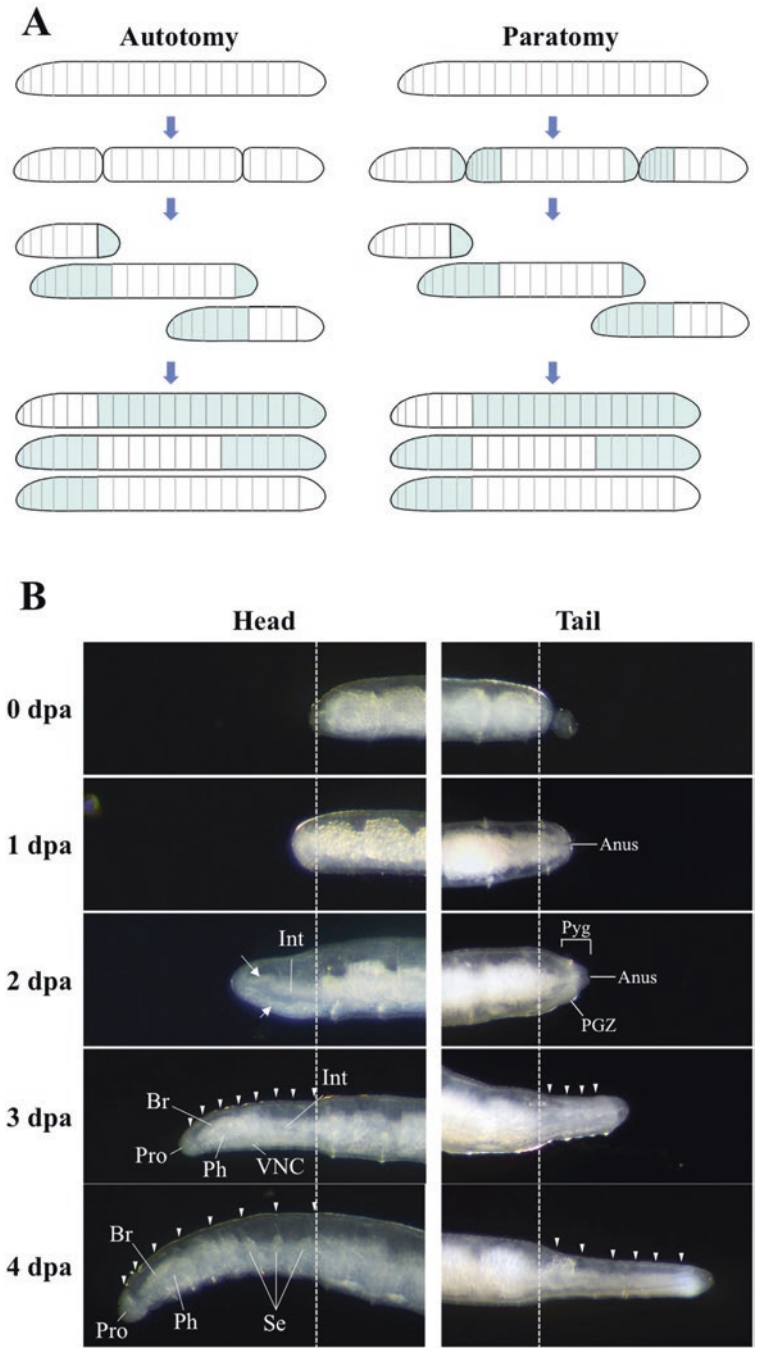

Fig. 10.2 Asexual reproduction in oligochaetes: Fission and regeneration. (**a**) Types of asexual reproduction in oligochaetes – autotomy and paratomy; Blue regions indicate newly formed body portions. (**b**) Anterior and posterior regeneration of the oligochaete *Enchytraeus japonensis*

process, i.e., it is not found in embryogenesis, and the origin of blastema cells has long been debated. Histological analyses of annelid regeneration have been performed since the nineteenth century in parallel with those of hydras, planarians, and salamanders. Hydras and planarians regenerate all body parts from pluripotent stem cells scattered throughout their body (Baguna 2012), whereas blastema formation in salamanders depends on cell dedifferentiation (Lentz 1969; Lo et al. 1993).

Dedifferentiation and stem cells referred to as neoblasts are characteristic of oligochaete asexual reproduction (Randolph 1892). Neoblasts typically have a large nucleus, ovoid shape, and basophilic cytoplasm, and are located on both sides of each septum of the trunk segments, across the ventral nerve cord (Randolph 1892). Although it has long been debated if neoblasts are pluripotent stem cells similar to planarian neoblasts, several experiments, including 5′-bromo-2-deoxyuridine (BrdU) pulse-chase, have suggested that neoblasts migrate to the stump and mainly contribute to regeneration of mesodermal tissues (Tadokoro et al. 2006; Sugio et al. 2012). Nerve axons extend from the pre-existing ventral nerve cord into the blastema during the early phase of regeneration in *E. japonensis* (Yoshida-Noro et al. 2000; Muller 2004), and neurons of the ventral nerve cord and brain appear to be newly formed from the epidermis in *Limnodrilus* (Cornec et al. 1987). Pre-existing nerve cords must play an important role in regeneration as ablation of the nerve cord inhibits head regeneration (Avel 1959, 1961). Endodermal tissues, such as the esophagus, pharynx, and digestive tract, are derived from preexisting endodermal tissues (Tweeten and Reiner 2012). In *E. japonensis* regeneration of the digestive tract is also achieved by dedifferentiation and re-differentiation (Takeo et al. 2008). However, studies on annelid regeneration lag behind that of planarians and other model animals, and experimental demonstrations of the mechanisms underlying annelid regeneration have rarely been performed.

10.5 Early Studies on Annelid Germ Cell Regeneration

The abovementioned fission and accidental cutting frequently yield worm fragments that lack the original genital segments containing germ cells. However, these fragments might propagate sexually after anterior regeneration, suggesting that germ cells regenerate along with somatic cells. Based on histological observations, some researchers have postulated germ cell regeneration. For example, Iwanoff (1928) observed germ cells during the regeneration of spionid polychaetes, proposing that germ cells were regenerated from the peritoneum. Gates (1943) and

Fig. 10.2 (continued) body parts, after amputation; Images were captured using a bright field stereomicroscope (Leica MZ10F with a coupled CCD Nikon DS-Ri1 camera); Dashed lines indicate amputation sites and arrowheads point out segmental borders; White arrows indicate cell aggregates in the blastema. *Br* brain, *dpa* days post amputation, *Int* intestinal tube, *Pgz* posterior growth zone, *Ph* pharynx, *Pro* prostomium, *Pyg* pygidium, *VNC* ventral nerve cord (Tadokoro, original figures)

Herlant-Meewis (1964b) suggested that *Perionyx* and *Lumbriculus* germ cells arose in the inter-segmental septum. During the regeneration of the polychaete *Spirirbis*, monogenital segments appear to be able to generate germ cells from stem-like cells located around the muscle and blood vessels (Vannini 1947). In other polychaete species, it has been hypothesized that germ cells formed from the ventral epithelium migrate to the genital segments (Dorsett 1961). Overall, these classic histological observations suggest that PGCs can regenerate from somatic cells or from stem cells although the origin of such PGCs might differ between species. However, these observations have not provided conclusive evidence, as the origin of regenerated PGCs is even more difficult to trace in histological studies than in embryogenesis when molecular markers are not used.

10.6 Germ Cell Regeneration in *Enchytraeus japonensis*

We previously addressed the question of germ cells origin using the oligochaete *E japonensis* found at the Agricultural Research Center for Tohoku Region (Fukushima Prefecture, Japan) (Nakamura 1993). As described in Sect. 10.5, this worm has one or two pairs of neoblasts in each segment and a strong regeneration capability, both anteriorly and posteriorly (Tadokoro et al. 2006; Yoshida-Noro and Tochinai 2010; Sugio et al. 2012). This species fragments into ~10 pieces by autotomy and within 5 days these fragments regenerate head and tail structures, producing individuals that propagate asexually under normal culture conditions (i.e., in high density) (Nakamura 1993). However, in worms cultured at extremely low densities after starvation and subsequent feeding, male and female germ cells, as well as other reproductive organs, develop in the seventh (sometimes in the sixth and seventh) and eighth segments, respectively, by the tenth day after fragmentation (Myohara et al. 1999). Thus, *E. japonensis* sexualization is controlled under experimental conditions, never occurring in high-density conditions. After autotomy and amputation, germ cell regeneration occurs as in other species (Tadokoro et al. 2006). The course of germ cell regeneration in *E. japonensis* was identified through expression patterns of *piwi* mRNA (Tadokoro et al. 2006). *piwi* mRNA, encodes a small RNA-associated protein (piwi interacting RNA; piRNA) (Lau et al. 2006), well known due to its involvement in *Drosophila* germline stem cell maintenance (Cox et al. 1998, 2000). *Piwi* expression pattern and function have recently been investigated in a wide variety of animals. For example, murine *piwi* homologs expressed in male germ cells regulate their differentiation to spermatocytes (Kuramochi-Miyagawa et al. 2001, 2004; Deng and Lin 2002). In planarians and jellyfish, *piwi* is expressed in germ cells and stem cells that differentiate to somatic and germ cells (Seipel et al. 2004; Reddien et al. 2005; Rossi et al. 2006). We isolated *piwi* from *E. japonensis* (*Ej-piwi*) and assessed its expression patterns in germ and stem cells (Tadokoro et al. 2006). Similar to what has been found in other animals, *Ej-piwi* mRNA is expressed in male germ cells (spermatogonia and differentiated spermatids) and female germ cells (oogonia and oocytes) but not in the somatic gonads of sexually

mature worms (Tadokoro et al. 2006). In asexually reproducing worms, *Ej-piwi*-positive cells are found in tiny masses in the seventh and eighth segments, and dispersed single cells along the trunk region, dorsally to the ventral nerve cord (Fig. 10.3a); these *Ej-piwi*-positive cells are morphologically and positionally distinct from neoblasts (*piwi*-negative cells) (Tadokoro et al. 2006). These *piwi*-positive cells also express the *vasa-related genes* (*vgl1* and *vgl2*) whereas neoblasts only express *vgl2* (Sugio et al. 2008). The cell masses in the seventh and eighth segments are male and female germ cells or their precursors and remain silent during asexual reproduction. During the sexualization process, these cells expand markedly and differentiate to mature germ cells, thereby increasing *piwi* expression in the seventh and eighth segments (Fig. 10.3b) (Tadokoro et al. 2006). In addition, *piwi*-expressing cells in the trunk region change during head regeneration, but not during sexualization (Tadokoro et al. 2006). These cells start to proliferate near the anterior stump only 2 days after amputation, when the blastema has already derived from neoblasts and other cells (Fig. 10.3b) (Tadokoro et al. 2006). From the third to the fifth day after amputation, the *piwi*-expressing cells found in the blastema progressively change their distribution toward the seventh and eighth genital segments (Fig. 10.3b) (Tadokoro et al. 2006). Based on these data and on other evidence, *E. japonensis* germ cell regeneration appears to proceed as follows (Tadokoro et al. 2006): In preparation for incipient self-fragmentation, *piwi*-positive germline stem cells are stored in the ventral nerve cord of the trunk region; after the blastema is formed from somatic stem cells and others, germline stem cells migrate to the genital segments, regenerating male and female germ cells or their germ cell precursors. This mechanism ensures the maintenance of germ cells during repeated asexual reproduction and supports this reproductive strategy. Ozpolat and Bely (2015) have recently investigated *piwi* expression patterns during *Pristina leidyi* paratomy, and found that. *piwi* was expressed in newly formed tissues in the fission zone, although *piwi* signals were not found in *E. japonensis* regeneration blastema. However, *piwi*-expressing cells were distributed dorsally to the ventral nerve cord in an irregular pattern, which was very similar to the pattern found for *piwi*-expressing cells in *E. japonensis*, and seem to contribute to the germ cell regeneration. Statistical analyses and live imaging analyses revealed that *piwi*-expressing cells migrate toward the newly formed tissues in the fission zone, but not to the regeneration zones (Ozpolat and Bely 2015). Thus, *piwi*-expressing cells on the ventral nerve cord might be a conserved origin of germ cells, at least in asexual reproducing oligochaetes.

10.7 Embryonic Origin of *E. japonensis* Germline Stem Cells

During *E. japonensis* anterior regeneration, germ cells in the seventh and eighth segments are regenerated from germline stem cells, but not from neoblasts. This raises the question of whether germline stem cells generate *de novo* from neoblasts when needed or are segregated during embryogenesis. To address this issue, our collaborators investigated the expression of *piwi* and *vasa-related genes* (*Ej-vlg1*

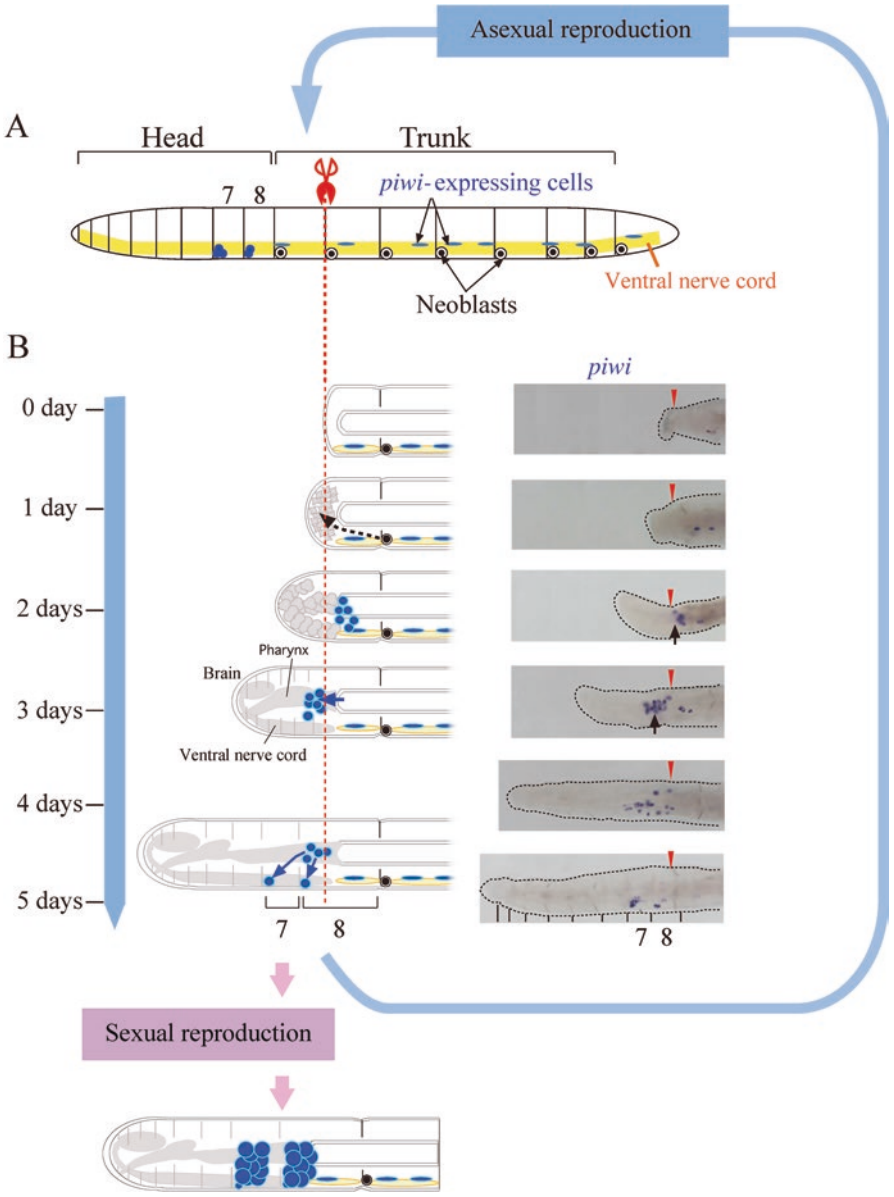

Fig. 10.3 Germ cell regeneration in *Enchytraeus japonensis*. (**a**) *piwi* mRNA expression in an intact worm; masses of *piwi*-expressing cells are located on the ventral side of the 7th and 8th segments, which correspond to genital segments, and single *piwi*-expressing cells are sparsely distributed on the dorsal side of the ventral nerve cord; (**a**) pair of neoblasts is attached to the ventral side of each septum in the trunk region. (**b**) The process of germ cell regeneration during asexual reproduction; The dashed red line and red arrowheads indicate amputation sites; pictures on the right show the expression patterns of *piwi* mRNA during regeneration, and illustrations on the left summarize the model of germ cell regeneration on the 5 days following amputation (redrawn from Tadokoro et al. (2006, 2009)

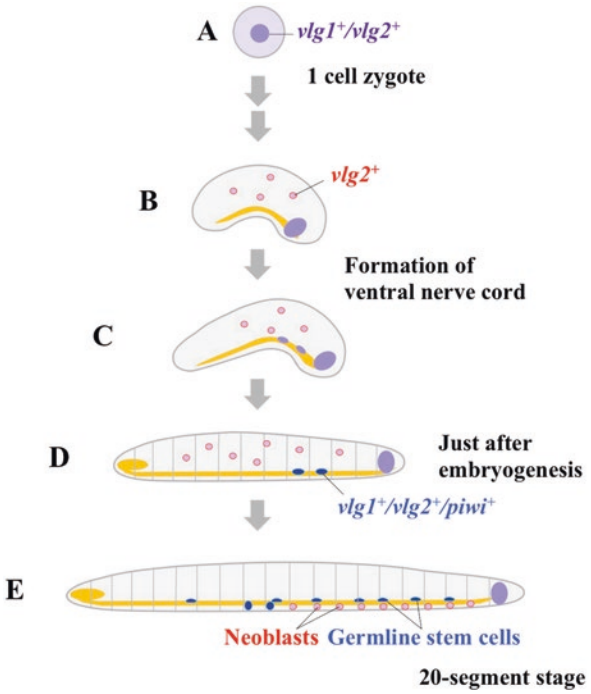

Fig. 10.4 Embryonic origin of *Enchytraeus japonensis* germline stem cells. Schematic illustrations showing the expression pattern of *vasa-related gene 1* (*vlg1*), *vasa-related gene 2* (*vlg2*), and *piwi* transcripts from the zygote to the 20-segment stage worm. (**a**) *vlg1* and *vlg2* are expressed in the perinuclear cytoplasm. (**b**–**c**) When the ventral nerve cord (yellow) is formed, *vlg1*$^+$*/vgl2*$^+$ (purple) *and vgl2*$^+$ (*red*) cells emerge, and then *vlg1*$^+$*/vgl2*$^+$ increase. (**d**) Just after embryogenesis, *vlg1*$^+$*/vgl2*$^+$*/piwi*$^+$ (blue) cells corresponding to germline cells appear on the ventral nerve cord. E. At the 20-segment stage, *vgl2*$^+$-positive cells corresponding to neoblasts are located in each segment, behind the ventral side of the septum (redrawn from Sugio et al. (2008))

and *Ej-vlg2*), from embryogenesis to adult worm (Sugio et al. 2008). In adult and 20-segment worms, *piwi*-positive germline stem cells and germ cell precursors in the seventh and eighth segments also express *Ej-vlg1* and *Ej-vlg2*, whereas neoblasts and cells in the posterior growth zone express *Ej-vlg2* and *Ej-vlg1/Ej-vlg2*, respectively (Fig. 10.4e) (Sugio et al. 2008). These cell populations, which are characterized by different combinations of gene expression patterns, emerge in a stepwise fashion during embryogenesis (Sugio et al. 2008): *Ej-vlg1* and *Ej-vlg2* transcripts, which are initially detected in the zygote (Fig. 10.4a), are maternally supplied as they are also expressed in unfertilized oocytes; *Ej-vlg1 and Ej-vlg2* are still expressed in M teloblasts and other blastomeres, but after nerve cord formation in late embryogenesis, these transcripts mainly accumulate in the posterior region, which might correspond to the presumptive posterior growth zone, although single *Ej-vlg2*-positive cells can be found scattered throughout the body (Fig. 10.4b, c); *Ej-piwi-/Ej-vlg1-/Ej-vlg2*-positive cells corresponding to germline cells emerge by

the end of embryogenesis (Fig. 10.4b, c). These expression patterns suggest that neoblasts (*Ej-vlg2*-positive) and *Ej-vlg1-/Ej-vlg2*-positive cells are segregated during embryogenesis (Fig. 10.4b), and that germline stem cells (*Ej-piwi-/Ej-vlg1-/Ej-vlg2*-positive) are generated from the *Ej-vlg1-/Ej-vlg2*-positive cells in the posterior region by the end of embryogenesis (Fig. 10.4d) (Sugio et al. 2008). Recently, it has been reported that germline cells regenerate *de novo* around the posterior growth zone in the polychaete *C. teleta*, although PGCs are found in the anterior region (Giani et al. 2011). Although these results suggest that somatic cells are able to generate germline cells, the possible supply of germline stem cells by the posterior growth zone of *E. japonensis* adults remains to be explored. In addition, *piwi*-positive cells often proliferate in the trunk region of intact worms, implying that they might maintain themselves during posterior growth, without any supply from other cells (Tadokoro unpublished). Comparative analysis of germline and somatic stem cells lineages between asexually reproducing *E. japonensis* and sexual reproducing species might allow an improved understanding of how germline cells and stem cells arose during evolution.

10.8 Conclusions

In this chapter, annelids' reproduction has been described with special reference to the germ cell formation and somatic and germline cell regeneration. Asexually reproducing annelids, particularly oligochaetes in which germ cells are limited to the head region, continuously face the high risk of losing germ cells during repeated fission. To avoid this risk, all tissues containing germ cells are completely regenerated in *E. japonensis* using a dedifferentiation system and two types of stem cells: somatic and germline. In contrast, most sexually reproducing species do not have these stem cells and/or have less ability to dedifferentiate. As suggested from several studies, an organism's stem cell and dedifferentiation ability, as well as their regeneration capacity, is highly correlated with their mode of reproduction. Oligochaetes and polychaetes, which are the ancestors of oligochaetes, are thus good models for understanding how stem cells and germ cells are established and the factors contributing to different regeneration abilities among species. Despite the histological, cell trace, and gene expression pattern analyses performed for annelids, these worms still lag behind other model animals in terms of gene manipulation techniques. Recently, the research group of Dr. Florian Raible and Dr. Kristin Tessmar-Raible established sophisticated technologies in *Platynereis dumerilii*, including transgenic lines, cell ablation, and gene targeting by genome editing (Backfisch et al. 2013, 2014; Bannister et al. 2014; Zantke et al. 2014). These innovations represent a significant advance in the study of annelid biology. By adapting these technologies to other annelid species, it might be possible to comprehensively elucidate on the reproduction, regeneration, reproductive behavior, and many biological issues of annelids within larger contexts such as evolution and environment.

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Chapter 11
Reproductive Strategies in Social Amoeba

Masashi Fukuzawa

Abstract The social amoebozoans live solitarily in the soil and have a unique life history, involving three resistant stages: asexual multicellular development to form fruits with spores supported on a stalk, sexual multicellular development to generate macrocysts, and unicellular formation of a microcyst. Depending on the environmental cues, the social amoeba adopts one of the reproductive modes in order to survive under unfavorable conditions. The model organism, *Dictyostelium discoideum*, is the most widely studied social amoeba and has three mating types. Macrocysts are the sexual version of spores, and contain recombinant offspring derived from gamete fusion of two mating types followed by meiosis. The mating-type locus was recently identified; single *mat* genes determine two mating types, and the third type is specified by composite *mat* homologs. In addition to the heterothallic sexual pathway, some other species, as well as a few known wild-type isolates of *D. discoideum*, exhibit self-fertile, homothallic behavior. Volatile sex pheromones, including the gaseous plant hormone, ethylene, are known to influence macrocyst production. Thus, the sexual pathway of the social amoeba is interesting, and studies on dictyostelids will provide evolutionary insights into reproductive strategies adapted by simple multicellular organisms. In this chapter, after introducing the unique features of the social amoeba, their sexual development leading to macrocyst formation and the known molecular mechanisms that regulate this process are reviewed.

Keywords Dictyostelium · Social amoeba · Gametes · Zygote · Macrocyst · Cell fusion · Chemotaxis · Cyclic AMP · Sexual pheromones

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11.1 Introduction

Living organisms exhibit a variety of reproductive strategies. In general, most organisms, whether diploid or haploid, have two sexes. Even lower eukaryotes such as yeast or unicellular algae undergo sexual reproduction by isogametes (i.e., plus/minus gametes). However, the social amoebae, which have a unique life cycle that begins with the solitary amoeba and then forms multicellular fruiting bodies by cell aggregation, are an exception with at least three sexes (Hurst and Hamilton 1992; Hurst 1996; Bloomfield 2011). This organism is an interesting model to investigate the development of multiple sexes.

In this chapter, I discuss the *Dictyostelium* system as a model organism, as this will help readers to better understand this unusual species located at the root of the animal/plant boundary in the phylogenetic tree (in fact, *Dictyostelium* is closer to animals than to plants: Fig. 11.1). Next, I describe the process of sexual development and macrocyst formation, as well as recent studies on the reproductive strategy adopted by the social amoeba possessing multiple sexes.

The social amoebozoans, or dictyostelids, live in the soil as solitary amoebae where they feed on bacteria. They were first reported nearly 150 years ago by a German microbiologist (Brefeld 1869). They are haploid eukaryotic cells, which proliferate upon feeding on bacteria; however, if they are deprived of food they enter a social stage. During starvation, amoebae start to express the molecules necessary for aggregation and obtain the ability to synthesize, release, detect, and degrade cAMP (Kessin 2001). The molecular basis of *Dictyostelium* chemotaxis has been extensively studied since it is highly informative for understanding the process in mammalian cells. Periodic cAMP pulses released from the center of each aggregation territory attract cells from the surrounding area, and multicellular aggregates assemble from seven (Bonner and Dodd 1962) to one million cells. Once aggregated, the cells form a mound and subsequently differentiate into two cell types; prestalk and prespore cells. These cells exist randomly in the mound, and begin to organize when a tip emerges at the apex. As cell-sorting continues, the tip elongates to form a 3-D structure called a migrating slug, in which the sorted cells make

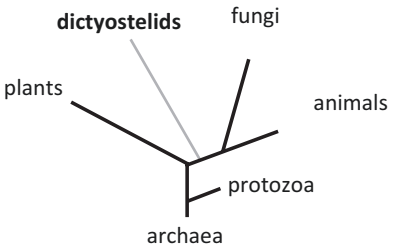

Fig. 11.1 Phylogenic tree of the selected phyla showing the evolutionary position of the social amoebozoans, dictyostelids. This is based on comparisons using the *Dictyostelium discoideum* genomic sequence (Eichinger et al. 2005)

patterns; the prestalk cells occupy the anterior third and the prespore cells occupy the remaining posterior region (Williams et al. 1989; Thompson et al. 2004).

The type species *Dictyostelium discoideum* was first described in 1935 (Raper 1935), and over the past three decades, due to its ease of handling and advanced molecular genetics tools, this species has established its position as an important model amoebozoan for the study of fundamental cell and developmental biology (Kessin 2001).

11.2 Dictyostelid Lineage

The genome of *D. discoideum* is approximately 34 Mb in size and is divided across six haploid chromosomes. The whole genome sequence was published in 2005 (Eichinger et al. 2005), and has since been annotated and maintained by researchers at dictyBase (http://dictybase.org/) (Basu et al. 2013). Currently, the genomes of three other dictyostelids (*D. purpureum*, *D. fasciculatum*, and *Polysphondylium pallidum*) have been sequenced and published on dictyBase. The genome of *Acytostelium subglobosum* has also been sequenced (Urushihara et al. 2015), along with those of several other wild-type species, thus expanding the genomic information on inter-species relationships.

The molecular phylogeny based on genomic data revealed that *D. discoideum* is more closely related to animals than to plants (Fig. 11.1), with a higher number of orthologous genes common in animals than in yeast (Eichinger et al. 2005; Williams 2010). This implies that genes/signaling pathways that were originally thought to exist only in the animal kingdom are often present in dictyostelids. Indeed, Src homology 2 (SH2) domains, which are confined to animals and are involved in the regulation of protein-protein interactions via tyrosine phosphorylation, are found in 13 homolog genes in *D. discoideum* (Eichinger et al. 2005). Among these, four signal transducer and activator of transcription (STAT) proteins, which have roles in regulating various cellular signaling pathways in mammalian cells, have been well characterized (Williams 2003). *Dictyostelium* CblA, which is another SH2-containing protein, has domain architecture similar to that of metazoan Cbl proteins (Langenick et al. 2008), and interacts with one of the four STAT proteins (STATc).

Amoebae are highly motile, and divide like cultured mammalian cells. Functions of the tumor suppressor, phosphatase and tensin homolog (PTEN), in cell motility and chemotaxis was first revealed by studies using *Dictyostelium* amoebae; the reciprocal pattern of PTEN and phosphatidylinositol 3-kinase (PI3K) localization determines cell polarization (Funamoto et al. 2002; Iijima and Devreotes 2002).

Conversely, plant-specific systems regulate the final morphogenesis in the life cycle, i.e., stalk and spore formation, both of which exhibit plant-like characteristics. Differentiation of stalk and spore cells requires cellulose deposition, and *dcsA*, which encodes a plant-specific cellulose synthase, results in the formation of a thick resistant wall. *DcsA* exists in the genome as a single gene (i.e., plants have multiple copies), and *Dictyostelium* became the first living organism in which cellulose

synthase was totally eliminated by gene disruption (Blanton et al. 2000). The plant hormone cytokinin is involved in spore differentiation (Anjard and Loomis 2008). Another gaseous plant hormone, ethylene, induces sexual development of *Dictyostelium mucoroides* as described below (Amagai 1989). A plant-type, single-domain myb protein (mammalian myb has three myb domains) controls the process of stalk differentiation (Fukuzawa et al. 2006).

Therefore, the functional study of *Dictyostelium* orthologs facilitates the analysis of factors that are often directly relevant to their animal/plant counterparts, and provides a model for lower multicellular eukaryotes, most of which is clearly not applicable in unicellular yeasts.

11.3 Three Survival Strategies

Dictyostelids have a unique life history enabling them to survive under severe conditions in nature, involving spores, microcysts, and macrocysts (Fig. 11.2). The existence of these resistant, alternative forms may be one of the reasons they have thrived since they diverged from the plant lineage.

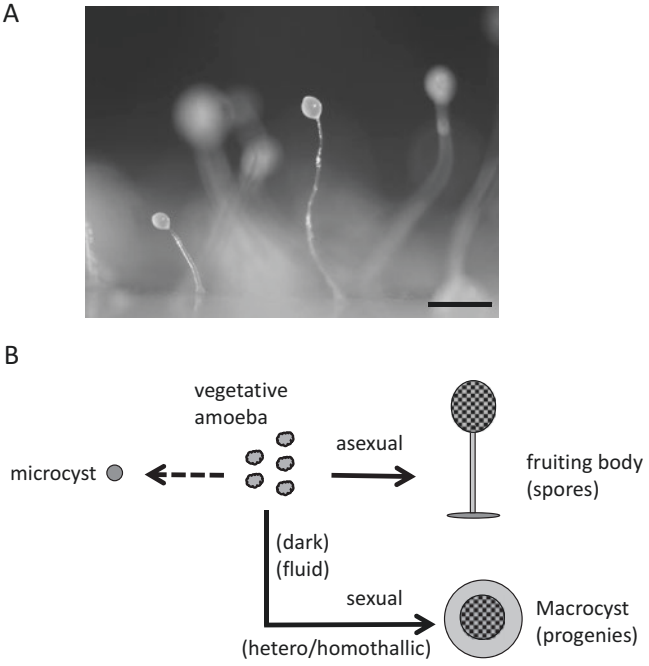

Fig. 11.2 Developmental forms of the social amoeba. (**a**) Fruiting bodies of *Dictyostelium discoideum*. Bar; 1 mm. (**b**) Schematic drawing of the three resistant stages of the social amoebozoans. The dotted arrow shows the microcyst pathway, which is not present in *D. discoideum* but has been found in some species. The objects are not drawn to scale

Resistant spores result from asexual development, which requires a multicellular, aggregative stage. *D. discoideum* utilizes a signaling pathway for spore maturation that is homologous to that used in mammalian neurons; GABA (gamma-aminobutyric acid) signaling induces the release of neuro-transmitting peptide (SDF-2), leading to the activation of protein kinase A during the final step of spore maturation from progenitor cells (prespore cells) (Loomis 2014). Spores can survive under extreme environmental conditions.

Encystation is often detected in solitary protists as a major stress response, and this is also observed in several early diverging social amoeba species, such as *Polysphondylium pallidum*, which is able to differentiate into a resistant form called a microcyst. This process is not sexual development because a solitary amoeba changes into a unicellular microcyst without development, and there is no cell fusion during the process. The microcyst pathway has not been fully studied since the most diverged group of dictyostelids, group 1, which includes the model *D. discoideum*, does not possess the pathway. However, a previous study using a group 2 species, *P. pallidum*, suggested the involvement of cAMP signaling (Kawabe et al. 2009).

Macrocysts are multicellular, resistant forms observed in a wide range of dictyostelids, and are formed during the sexual cycle. Dictyostelids have polymorphic mating types, with heterothallic and/or homothallic mating systems, to produce macrocysts (Table 11.1). The presence of one mating system in some species does not necessarily indicate the absence of the other mating system; it is possible that the mating behavior has not yet been identified in the wild. Evidence that macrocysts arise through sexual development has been shown in ultrastructural studies (Erdos et al. 1972; Okada et al. 1986), demonstrating that a synaptonemal complex, an indicator of meiosis, appears in the nuclei of a sufficiently matured macrocyst.

Further evidence of meiosis during macrocyst formation is shown in some previous studies. Using wild-type and mutant strains (to discriminate each progeny from wild-type based on phenotypic observation), Wallace and Raper (Wallace and Raper 1979) showed that under specific culture conditions, the heterothallic mixture of the two cell-types fused to generate an aggregative structure surrounded by a thick cellulose wall. In addition, some of the germinated cells from that structure exhibited the mutant phenotype (hence a genetically heterogenic population derived from the macrocyst), which indicates that the offspring were presumably derived through meiosis. However, owing to the low frequencies of recombinant offspring and the difficulty of macrocyst germination (which remains unresolved), further segregation analysis has not been performed successfully. Further evidence is established from the fusion of haploid cells between the two mating-type strains during the initial stage of macrocyst formation, yielding a zygotic cell with a single diploid nucleus, in which the DNA content is double that of a haploid nucleus (Okada et al. 1986; Ishida et al. 2005). Physical and genetic evidence of meiosis in homothallic strains has also been shown in some species (Erdos et al. 1972; Macinnes and Francis 1974). Meiosis should occur sometime after macrocyst formation; however, the exact timing of meiosis during maturation and dormant periods of macrocysts has not been demonstrated to date.

Table 11.1 Mating types of representative social amoeba species

Species	Taxon group	Het/Hom (Number of mating type known)	References
Dictyostelium discoideum	Group 4	Both Bloomfield (2011)	Nickerson and Raper (1973), Clark et al. (1973), and Erdos et al. (1973)
Dictyostelium purpureum	Group 4	Both (>3)	Mehdiabadi et al. (2009), Hagiwara et al. (2004), and (2005)
Dictyostelium rosarium	Group 4	Het Bloomfield, Bloomfield (2011)	Chang and Raper (1981)
Dictyostelium giganteum	Group 4	Het Bloomfield, Bloomfield (2011)	Erdos et al. (1975) and Mehdiabadi et al. (2010)
Dictyostelium mucoroides	Group 4	Both	Blaskovics and Raper (1957), Nickerson and Raper (1973), and Cavender and Kawabe (1989)
Dictyostelium lacteum	Group 3	Het	Anderson (1986))
Polysphondylium album	Group 2	Het, no mat homolog	Kawakami and Hagiwara (2008), and Bloomfield (2011)
Polysphondylium pallidum	Group 2	Het	Kawakami and Hagiwara (2008)
Acytostelium subglobosum	Group 2	(Hom)	Bloomfield (2011)
Dictyostelium fasciculatum	Group 1	Not known, no mat homolog	Bloomfield (2011)

Species shown in bold have published genome sequences (Basu et al. 2013; Fey et al. 2009; Heidel et al. 2011). Taxonic groups are based on phylogenic analyses of ribosomal RNA sequences (Fey et al. 2009). In *Dictyostelium fasciculatum*, macrocyst formation has not been observed. Parentheses in *Acytostelium subglobosum* represent predicted homothallism (this table is based on Bloomfield 2011)

11.4 Sexual Development Through Heterothallic Cell Fusion in *Dictyostelium discoideum*

Details of sexual development in the most-studied, heterothallic cycle of *D. discoideum* are shown in Fig. 11.3.

Macrocyst formation is strictly controlled by environmental conditions; light inhibits and water enhances gametogenesis (acquisition of fusion competence). Calcium is necessary for zygote formation through macrocyst formation (Lydan and O'Day 1988a, b), and phosphate ions inhibit the process (Lydan and O'Day 1988c). During the early stages of the sexual cycle, calcium is required for cell fusion rather than for the induction of gametes (McConachie and O'Day 1986).

The first step of the sexual cycle involves the formation of gametes, which are ultimately defined as fusion competent (FC) cells, derived from different mating types (e.g., strain NC4 [type I] and strain V12 [type II]). Under experimental conditions, approximately 16 % of the mixed cell population is induced to form gametes (McConachie and O'Day 1986). Morphologically, these gametes can be distin-

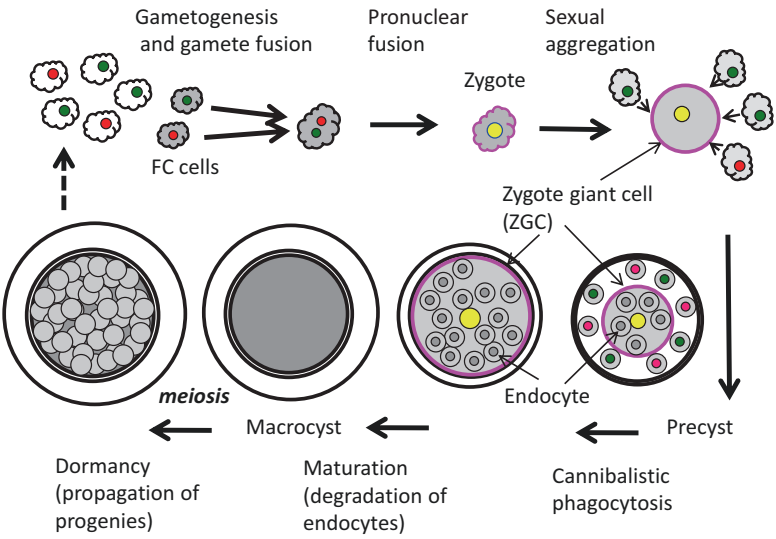

Fig. 11.3 Sexual cycle of heterothallic mating in *Dictyostelium discoideum.* Under dark and flooded conditions, gametogenesis is induced and a pair of fusion-competent (FC) cells of different mating types (shown as grey cells with a green or red nucleus) fuse to produce a diploid zygote, in which the two nuclei eventually fuse (shown as a yellow nucleus). The zygote then becomes a zygote giant cell (ZGC; shown in the pink circle) and attracts the surrounding cells, which are ingested as endocytes thus increasing its volume as it engulfs the prey cells. During maturation, the endocytes are digested by the ZGC and haploid progenies originated from the fused nucleus occupy the whole mass of the macrocyst. Under laboratory conditions, the process from gamete fusion to the formation of a macrocyst takes approximately 48 h. This figure was modified from O'Day and Keszei (2012)

guished from others based on their smaller size, rapid movements, and frequent contact with nearby cells (O'Day et al. 1987).

FCs can be experimentally induced into each mating type by starving under dark conditions, without the heterothallic mixing of cells (Ishida et al. 2005; Saga and Yanagisawa 1982). This is particularly useful for analyzing the early events of sexual development as most of the cells are synchronously induced to form gametes, which fuse immediately when the two mating-types are mixed. The only limitation is that this may not represent the natural mode of zygote formation; the high fusion activity of the gametes results in multiple cycles of cell fusion followed by the formation of a multinucleate syncytium. The formation of syncytium can be demonstrated using nuclear-labeled cells (Fig. 11.4; Fukuzawa, unpublished). The syncytium eventually dissociates into individual zygotes with diploid nuclei within 8 hours of incubation (Ishida et al. 2005). During homothallic mating of *D. mucoroides* Dm7, gametogenesis seems to occur after the cells have formed aggregates rather than at the unicellular stage (Filosa and Dengler 1972; Amagai 1989).

Gamete fusion generates a larger, binucleate cell, which then differentiates into a zygote with cytoplasmic swelling and pronuclear fusion (Szabo et al. 1982;

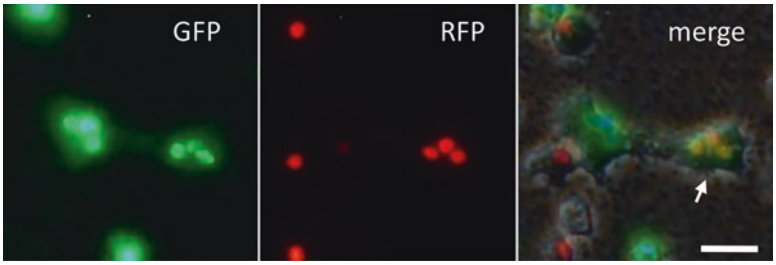

Fig. 11.4 Pronuclear fusion. Strain Ax2 (type I) was labeled with GFP (green fluorescent protein) fused to a putative nucleoli-localization sequence (NoLS-GFP: Fukuzawa, unpublished) and strain V12 M12 (type II) with Histone 2B-RFP (red fluorescent protein). These strains were individually induced to become fusion-competent, and were then mixed to promote cell fusion. Images were obtained by epifluorescence microscopy after 0.5 h of incubation. The merged image was created from GFP, RFP, and phase-contrast channels. The arrow indicates a giant cell (as a form of syncytium) with three fused nuclei. Bar; 20 μm

McConachie and O'Day 1987). The resultant diploid cell is called a zygote giant cell (ZGC). Interestingly, once formed, the ZGC directs the further process of macrocyst formation without being influenced by the environmental conditions required for macrocyst formation (i.e., darkness and humidity) and forces the surrounding, starved haploid cells to contribute to the macrocyst (Saga and Yanagisawa 1982). The macrocyst provides a safe and nutritious environment for the ZGC, which is the seed for the next generation.

In mammals, zygotes undergo a series of mitotic divisions (cleavage) during the development of a multicellular organism, and primordial germ cells arise as a separate population from the somatic cells early in development. However, *D. discoideum* zygotes immediately undergo meiosis, followed by mitosis, to produce a number of haploid offspring within a macrocyst. In this respect, the diploid zygote of *Dictyostelium* might be analogous to the primordial germ cell in animals, since it produces haploid cells via meiosis. However, haploid cells developed from the macrocyst are not gametes at this time because they do not fuse until gametogenesis is induced, and they can proliferate and live as solitary amoebae.

To generate sexual aggregates, induced haploid cells respond to cAMP by chemotaxis and move towards mated cell clumps (O'Day and Durston 1979) or to the ZGC disaggregated from clumps (O'Day 1979), suggesting that sexual development utilizes similar mechanisms of chemotaxis as asexual development. Sexual aggregation results in a much lower cell yield within an aggregate (~200 cells) (O'Day and Keszei 2012) compared with asexual aggregation (~100,000 cells). This may be due to the low level of membrane-bound cAMP phosphodiesterase (PDE) and excess levels of a PDE inhibitor produced by the ZGCs, both of which would weaken cAMP wave formation resulting in the subsequent low cell yield contributing to the sexual aggregate (Abe et al. 1984).

Once sexual aggregates are formed, the ZGC and aggregated cells are surrounded by a secreted sheath material, which includes cellulose, and are isolated from the outside environment. At this time, the aggregated cells are vulnerable, and sacrifice

themselves for the ZGC. The ZGC exhibits cannibalistic behavior, which is not observed during asexual development. It begins to engulf the surrounding prey cells, which in turn become phagosomes called endocytes (Figs. 11.3 and 11.5). Thus, the ZGC increases in size without cell division, and finally occupies the entire mass of the sexual aggregate, which contains the engulfed prey cells. Following their degradation during macrocyst maturation, endocytes become a source of energy (Fig. 11.5) and are used by progenies originating from the ZGC nucleus. The phagocytic mechanism of self-eating in sexual aggregates seems to be different from that exhibited by vegetative cells that phagocytose bacteria (Lewis and O'Day 1985); however, this mechanism has not been investigated at a molecular level.

The existence of cannibalism in the life cycle of *Dictyostelium* raises an interesting question in fundamental biology: how do cells recognize self/non-self, particularly within the same species (heterothallic), or even within the same strain (homothallic)? This issue of allorecognition has been well-studied in asexual development. When two *Dictyostelium* species are mixed they eventually organize into separate aggregates containing pure populations (Raper and Thom 1941). Recently,

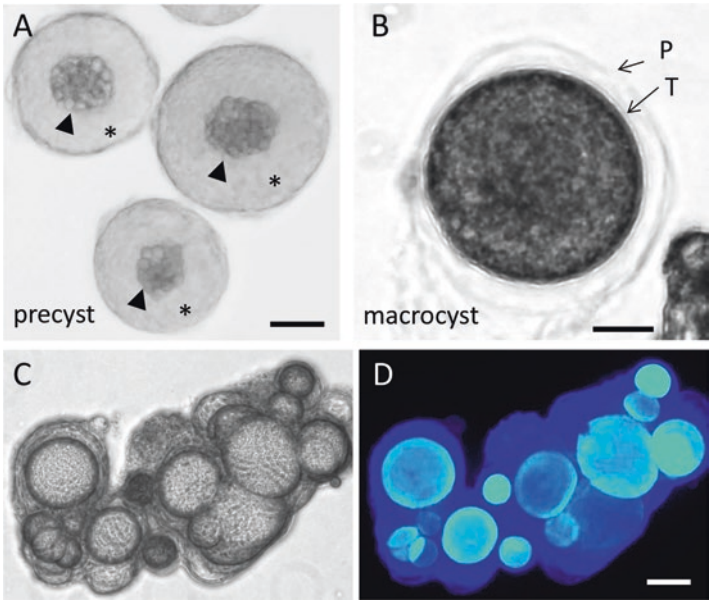

Fig. 11.5 Precysts and macrocysts. (**a**) Precysts. Within each sexual aggregate, a developing ZGC (arrowhead) containing endocytes is clearly seen in the center of the surrounding layers of prey cells (asterisk). Bar; 25 μm. (**b**) A dormant macrocyst. Note that endocytes have been broken down into small granules and the cytoplasm of the ZGC is almost uniform. *P* primary wall, *T* tertiary wall. The secondary wall subtends the inner primary wall. Bar; 10 μm. (**c**, **d**) Mature macrocysts stained for cellulose deposition. (**c**) Phase contrast. (**d**) Calcofluor white staining. The tertiary wall showed intense staining. The image was obtained by epifluorescence microscopy. Scale bar; 50 μm. During several weeks of dormancy, meiosis should occur in the ZGC followed by mitosis in order to propagate progenies; however, these maturation steps are not well understood

the molecular basis of kin discrimination within *D. discoideum* strains was revealed (Benabentos et al. 2009; Hirose et al. 2011). The self-recognition system needed to cooperate with relatives involves two polymorphic genes, *tgrB1* and *tgrC1*, which encode cell-adhesion proteins that are present on the cell surface. During cell sorting, TgrB1 recognizes TgrC1, and vice versa, suggesting that self-recognition is mediated by heterotypic interactions between the two cell-surface proteins.

Kin discrimination seems to occur during sexual phagocytosis; sexual zygotes of *D. discoideum* strains contain prey cells within the cell mass (Figs. 11.3 and 11.5) and prefer to ingest amoebae of the same species (hence cannibalism). Amoebae of other species such as *D. mucoroides* or *P. pallidum* are not efficiently ingested (Lewis and O'Day 1986). Although sophisticated mating-type determination should be associated with allorecognition mechanisms, the molecular basis of selective cannibalistic phagocytosis, including the involvement of TgrB1/TgrC1 or other sex-specific adhesion molecules, remain unknown.

After ingesting surrounding cells as endocytes, the ZGC matures as a macrocyst with the complete formation of three wall layers (Fig. 11.5) (Erdos et al. 1972). The outer-most wall is derived from the primary wall formed as a sheath material during sexual aggregation. The secondary wall resembles the wall of the microcyst (Hohl et al. 1970) and is located underneath and adjacent to the primary wall. The tertiary wall is the thickest structure, and resembles the spore coat observed during asexual development. These walls contain cellulose (Bloomfield 2011) and form a transparent gap between the pair of primary/secondary walls and the tertiary walls. Following the development of the macrocyst, it remains quiescent for weeks. Despite a few reports describing macrocyst germination in *D. mucoroides* (Erdos et al. 1972; Nickerson and Raper 1973), it is very difficult to obtain germinated progenies from *D. discoideum* macrocysts under experimental conditions; therefore, sexual genetic techniques have not yet been established (Wallace and Raper 1979). During dormancy, the endocytes gradually disappear as they are degraded into granular fragments, and the diploid nucleus of the ZGC initiates meiosis, presumably within a few days of macrocyst formation (Erdos et al. 1972; Okada et al. 1986). The progenies obtain resources derived from prey cells and increase their cell number by mitosis before they leave the shell.

11.5 Molecular Genetics of the Sexual Cycle

As in most protists, the molecular events underlying sexual reproduction in *Dictyostelium* remain poorly understood. Fertilization is an essential part of initiating multicellular development and includes cell-cell interaction via allorecognition. In mammals, many proteins are involved in sperm-egg interactions (Le Naour et al. 2000; Miyado et al. 2000; Inoue et al. 2005; Ikawa et al. 2008). In plants, generative cell specific 1 protein (HAP2/GCS1) is sperm-specific and is known to be essential for angiosperm fertilization (Mori et al. 2006; von Besser et al. 2006; Wong and Johnson 2010). In addition, the HAP2/GCS1 ortholog determines male fertility in

malaria parasites (Hirai et al. 2008). Of note, the *D. discoideum* genome possesses a HAP2/GCS1 ortholog, although its function in sexual-cell fusion remains to be elucidated (Araki et al. 2012).

Early studies aimed to identify cell-surface glycoproteins potentially involved in sexual-cell fusion. Plant lectins—concanavalin A (ConA), wheat-germ agglutinin (WGA), and lentil lectin (LCA)—have an inhibitory effect on cell fusion (O'Day and Rivera 1987; Ishikawa et al. 1991). Using fusion-blocking antibodies produced against "cell ghosts" of a type II strain HM1, a glycoprotein, gp70, was identified (Urushihara et al. 1988; Ishikawa et al. 1991). Gp70 binds ConA and is only present on type II mating type cells (Urushihara 1992); however, its encoding gene has not been identified. The fusion-blocking antibody recognized another candidate protein, GP138, which is expressed on gametes of both mating types (type I and type II) (Urushihara et al. 1988; Suzuki and Yanagisawa 1989). GP138 families are encoded by four genes (*gp138A-D*). Antisense knockdown of *gp138A* and *gp138B* resulted in reduced cell fusion (Fang et al. 1993). In contrast, a quadruple mutant with disruption of all four genes still contained low levels of GP138 proteins and exhibited sexual competency (Hata et al. 2001). Considering the presence of various truncated forms of *gp138* family genes on chromosome 5, these results suggest further redundancy of candidate molecules.

Many attempts have been made to isolate genes involved in sexual development, based on the view that a subset of genes unique to sexual reproduction should exist. However, although this approach has resulted in the characterization of several genes, it has not been very successful. In order to identify sexual genes, a cDNA pool was constructed for gamete-specific subtraction mRNA using type I FCs (Muramoto et al. 2003). Overall, 24 genes were selected and their functions were analyzed using gene knockout and RNAi (Muramoto et al. 2005). Interestingly, although extensive attempts were made to create mutants, most (16/24 of knockouts and 20/24 of RNAi) exhibited normal phenotypes for the sexual cycle. In addition to the previously identified "tiny macrocyst" mutant (*tmcA*) (Shimizu et al. 1997), cDNA analysis further yielded *tmcB* and *tmcC* genes. Both genes are mutual homologs involved in cell adhesion during sexual aggregation; therefore, poor macrocyst formation is attributed to a defect in cell adhesion. cAMP signaling was altered in *tmcB* mutants while it was normal in *tmcC* mutants, suggesting that other processes are also defective in the latter (Muramoto et al. 2005). These *tmc* genes are not involved in the early steps of sexual reproduction, such as cell fusion (Urushihara and Muramoto 2006).

For genes involved in earlier stages of the sexual cycle, a gamete-specific mating type A (*gmsA*) gene was isolated from the gamete-enriched cDNA library (Muramoto et al. 2003). The gene product shared homology with *mta2* from *Chlamydomonas reinhardtii*, which is a gamete-specific gene with homology in the *a* region of the mating-type plus (female) locus (Ferris et al. 2002). Inactivation of *gmsA* resulted in reduced macrocyst formation, although macrocyst development itself and asexual morphogenesis were unaffected.

Another gene from the library significantly involved in mating was macrocyst formation A (*macA*) (Araki et al. 2012). An insertional mutant of this gene in the

type I strain developed a normal asexual cycle, but showed almost no cell fusion with the opposite mating type (type II strain) during the sexual cycle. MacA possesses LamGL and discoidin domains, which are associated with adhesion. MacA is highly glycosylated and possesses a transmembrane domain, suggesting that this protein is localized to the membrane. Because the MacA protein sequence is identical between type I and type II strains, and cell-adhesion domains are present within the molecule, this protein may function in cell-cell interactions during gamete fusion rather than in the recognition of mating types. Interestingly, *macA* is mainly expressed in FCs of the type I strain, and its knockout in type I strains resulted in significant defects in cell fusion compared with the wild-type type II partner, suggesting that the molecular interaction of MacA is heterothallic.

The existence of multiple steps in the regulation of the sexual cycle, even in such a simple organism, makes it difficult to isolate key sexual genes (Fig. 11.3) (Urushihara and Muramoto 2006). Therefore, most gamete-specific genes do not necessarily regulate sexual reproduction and their phenotypic defects in mutants may not be visible. In addition, since sexual aggregation shares components of cAMP signaling with asexual development, many genes involved in cell aggregation could be shared by both pathways. For instance, disruption of the cAMP receptor A (*carA*) gene, which is indispensable for asexual aggregation, suppresses macrocyst formation (Shimizu et al. 1997). Although several important mutants have been reported, they are not sufficient and most of the molecular mechanisms involved in the sexual pathway remain elusive.

11.6 Mating-Type Locus

The number of different sexes in dictyostelids depends on the species (Table 11.1). The presence of mating types in social amoebae was first demonstrated in "paring" studies, in which *Dictyostelium* strains isolated from the wild were paired in various combinations to form macrocysts (Clark et al. 1973; Erdos et al. 1973). Most strains tested had been grouped into three mating types, and each required either of the remaining two types to form macrocysts (heterothallic), except for one strain with self-compatibility (strain AC4; homothallic). In the model species *D. discoideum*, the three mating types (types I, II, and III) have been determined (and most likely, no more sexes exist). These mating types can fuse in an equivalent manner, and self-fusion of each type is strictly inhibited. Despite earlier studies suggesting the existence of a single mating-type locus with two or more alleles (Wallace and Raper 1979; Robson and Williams 1979, 1980), until recently, the genetics of sex determination have been an enigma. As gamete-enriched, functional sex genes like *gmsA* or *macA* (see above) are present in both sexes, it was believed that sex might be determined in some way at the level of transcription of sex genes. However, this hypothesis was incorrect, and the fundamentals of sex determination in *Dictyostelium* have recently been discovered.

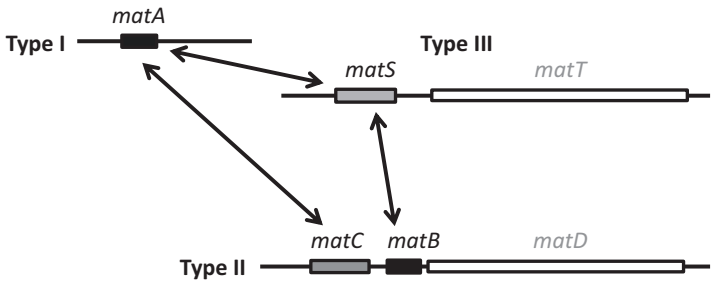

Fig. 11.6 Schematic drawing of the mating type locus in *Dictyostelium discoideum* and three combinations of *mat* genes required for the heterothallic formation of macrocysts. Each *mat* locus within the single mating-type is found between similar flanking sequences on chromosome 5. The *mat* genes with homology are represented by boxes of the same color: *matA* and *matB* (black), *matS* and *matC* (gray). *matT* and *matD* (white) are homologous, but are not involved in mating-type determination. The three possible compatibilities for heterothallic mating are indicated by double-headed arrows (this figure is modified from Bloomfield 2011)

Bloomfield et al. (2010) took a comparative genomic hybridization approach to identify genetic differences between two mating types (types I and II) of *D. discoideum*. Using micro-arrays prepared from the sequenced genome of the Ax4 strain (type I) (Bloomfield et al. 2008), the genomic DNA of ten independent type I or type II strains was analyzed by cross-hybridization. The micro-array contained around 8500 DNA probes of the 10,500 deduced genes, and interestingly, only a single gene was identified, which is only present in type I strains.

This gene (mating type A; *matA*) encodes a small protein of 107 amino acids, with no significant homology in the database. The cognate locus of *matA* was cloned from type II and III strains, and the organization of the mating-type locus was revealed (Fig. 11.6). The two genes (*matT* and *matD*) are homologous, but not to *matA*; they are dispensable for sex determination. However, they show weak homology to members of the HAP2/GCS1 family, which localize to the membrane and have a role in gamete fusion in plants (Wong and Johnson 2010), suggesting a supportive role during sexual fusion. The type III strain possesses *matS* in the locus; however, it is not homologous to *matA*.

Knockout of *matA* in type I strains completely abolished sexual development with opposite mating types (type II or type III). Using this type I mutant, single or various combinations of *mat* genes were re-introduced into the *matA* null background (mating-type "congenic" strains) and macrocyst formation was investigated by crossing with wild-type strains. For example, introduction of *matS* (derived from type III) into *matA* null cells with a type I background was sufficient to convert the sex from type I to type III. Complementation experiments investigating various combinations of *mat* genes introduced into the congenic strains have shown that the *mat* genes are necessary and sufficient for sex determination.

Those experiments further revealed the composite nature of type II mating strains (namely the third sex), in which two genes (*matC* and *matB*) are present at the single mating locus. *matC* is homologous to *matS* in type III, while *matB* is homologous to *matA* in type I (Fig. 11.6).

Therefore, based on the gene structure, the existence of the third sex as type II could derive from anomalous cell fusion between ancestral type I and type II cells followed by gene rearrangements. The opposite is also possible, such that mating type II might be ancestral to the other mating types. Evolutionary insights into the relationship between *mat* genes and multiple sexes in dictyostelids may be elucidated when the organization of the *mat* locus in other species has been investigated.

11.7 Homothallic Cell Fusion and Sex Pheromones in *D. discoideum*

During the sexual development of *D. discoideum*, "selfing" within the same mating type (homothallism) is prevented by unknown mechanisms. However, some of the known homothallic strains of type III, or heterothallic type I or II strains under suitable conditions as described below, are able to undergo homothallism. Therefore, the molecular mechanisms that regulate self-compatibility seem to be highly complex. Two *D. discoideum* strains, AC4 and ZA3A, have been identified as naturally homothallic. The mating-type locus in these strains has been investigated, and interestingly, both isolates displayed a type III heterothallic pattern, with diverged *matS* and *matT* (Bloomfield et al. 2010). Since most known type III strains are not self-fertile, the diverged *mat* coding sequences in AC4 and ZA3A may be attributed to their homothallic ability.

The ability to undergo homothallism resulted in the identification of volatile sexual pheromone(s), which control gametogenesis in the opposite sex. The identified pheromone seems to act specifically on the opposite mating type. Under sexual conditions, induced gametes from NC4 (type I) cells produce a volatile substance (Morris et al. 1982; O'Day et al. 1987), which acts to induce macrocyst formation with the opposite V12 (type II) responder cells placed distantly from NC4 cells (inducer) through the airspace. In this situation, there is no cell contact between the two strains; however, the homothallic formation of a macrocyst in type II cells was observed, a process that never occurs under normal conditions (O'Day and Lewis 1975) (Lewis and O'Day 1977). Another sex hormone was investigated using zygotes (made of type I and II) as the inducer and NC4 (type I) cells as the responder; a macrocyst was induced in type I alone with a diffusible molecule under 2 kDa in size secreted by the zygotes (Lamphier and Yanagisawa 1983).

These results suggest that both type I and II strains have the intrinsic ability to undergo homothallism when the opposite mating-type stimulates the signaling pathway via type-specific sex pheromones. Although pheromone-regulated mating has been observed in a few other species (Lewis and O'Day 1976, 1979; O'Day and Lewis 1981), the chemical nature of these and other molecular mechanisms that permit homothallism have yet to be determined.

11.8 Homothallic Cell Fusion in *D. mucoroides* and Ethylene

D. discoideum cells adapted to laboratory conditions are the most suitable for molecular genetic studies. However, the mating type of the representative axenic strains is type I, which usually employs a heterothallic pathway with another mating type (that is usually not adapted to laboratory conditions: non-axenic wild-types) to form a macrocyst. Conversely, early genetic studies demonstrated that meiosis occurs in *D. mucoroides* homothallic macrocysts (Macinnes and Francis 1974). Therefore, homothallic development has mostly been studied in *D. mucoroides* rather than *D. discoideum*, highlighting that appropriate molecular genetic techniques and whole-genome information, such as the presence of *mat* genes, are lacking in this species. Although a heterothallic strain was reported in *D. mucoroides* (Table 11.1), homothallic mating has been extensively studied using strain 7 (Dm7). Dm7 develops normally to form fruiting bodies under light conditions, but under dark or submerged conditions, it efficiently forms a macrocyst by itself. Ethylene and ethylene-inducible *zyg1* gene have been demonstrated to be key components for homothallic sexual development in this species.

The plant hormone ethylene is a readily diffusible gas hormone that acts in many ways to modulate plant growth and development, including wounding, pathogenic attack, seed germination, sprout growth, senescence, and fruit ripening (Johnson and Ecker 1998). Ethylene production in Dm7 cells was confirmed by gas chromatography (Amagai 1984). Further genetic analyses in which genes for the ethylene synthetic pathway were cloned, revealed that *Dictyostelium* possess the same enzymes in this pathway as higher plants (Fig. 11.7). ACC oxidase (*aco*) genes were cloned from *D. discoideum* and *D. mucoroides* (*Dd-aco* and *Dm-aco*, respectively). Dd-ACO shows homology to plant-type ACO, with 24.3 % identity and 40.0 % similarity to *Arabidopsis* ACO (accession number AAC27484) (Amagai 2012).

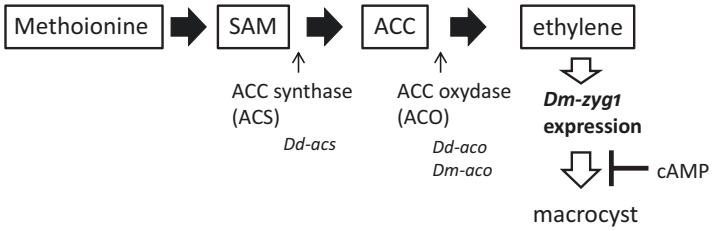

Fig. 11.7 Biosynthetic pathway and regulation of ethylene in *Dictyostelium*. The ethylene biosynthetic pathway in *Dictyostelium* utilizes the same enzymes as higher plants. Methionine is converted to S-adenosyl-L-methionine (SAM) by SAM synthase in the presence of ATP. ACC synthase catalyzes the production of 1-aminocyclopropane-1-carboxylic acid (ACC), which is the immediate precursor of ethylene, from SAM. ACC is then catalyzed by ACC oxidase to produce ethylene during the final step of the pathway. *Dictyostelium* homologs of these enzymes are shown. Ethylene induces *zyg1* expression in *D. mucoroides* to enhance homothallic cell fusion, but the effect of ethylene in the sexual pathway is unclear in *D. discoideum*. The amount of cAMP generated in the light is higher than that generated in the dark, and has an inhibitory effect on macrocyst formation in *D. mucoroides* (this figure is modified from Amagai 2012)

Despite the low homology, Dd-ACO has characteristic domains responsible for catalyzing the oxidation of ACC. When overexpressed in Dm7, *Dd-aco* (and also *Dm-aco*)-transformed cells produced approximately 70 % more ethylene than untransformed cells (Amagai et al. 2007; Amagai 2011). When the expression of endogenous *Dm-aco* mRNA was down regulated by *Dd-aco* RNAi, ethylene production was reduced to 83 % of that observed in untransformed cells. In these Dm7 transformants, the rate of homothallic macrocyst formation correlates well with the amount of ethylene produced (Amagai et al. 2007; Amagai 2011).

Ethylene receptors in plants are histidine kinases that are involved in a two-component signaling pathway (Wang et al. 2002). *D. discoideum* possesses 15 histidine kinase genes in its genome (Singleton and Xiong 2013), although a homology search against the whole genome sequence does not specify any plant ethylene receptor orthologs among these or other genes in the ethylene-response pathway. In *D. mucoroides*, the presence of a functional ethylene receptor(s) has been suggested using 1-methylcyclopropene (1-MCP), which inhibits ethylene receptors; ethylene gas inhibits macrocyst formation in Dm7 (Amagai et al. 2007). Ethylene receptor orthologs should be identified at the molecular level until the whole genome of *D. mucoroides* is available.

In *D. mucoroides*, the ethylene signal has been shown to regulate the expression of *zyg1* (Amagai et al. 2007). This gene was isolated from the differential screening of a cDNA library comparing mRNA expression between unfused cells and early macrocysts (Amagai 2002). *zyg1* encodes a protein of 268 amino acids with no homology to known proteins, and is enriched in the early macrocyst. Interestingly, this gene is only found in *D. mucoroides*, and no orthologs have been detected in *D. discoideum* or in the published genomes of five dictyostelid species. Although it has not been proven that *zyg1* is necessary for homothallic formation of macrocysts (a gene knockout strain has not been created), a gain-of-function mutation leads to efficient homothallic cell fusion in *D. mucoroides* (Amagai 2002). Furthermore, ectopic expression of *zyg1* in a type I strain of *D. discoideum* enhances homothallic cell fusion followed by macrocyst formation, which does not occur without a matching mating type under normal conditions (Amagai et al. 2014). Surprisingly, ectopic expression of *zyg1* in mammalian cells (mouse myoblasts) causes efficient cell fusion (Fig. 11.8) (Amagai et al. 2012). It is clear that ethylene is not involved in this instance. This may suggest that the biological function of Zyg1 is not specific for sexual development in *D. mucoroides* but includes general cell-fusion events.

The presence of enzymes responsible for ethylene synthesis in *D. discoideum* implies that ethylene is also produced in this species. An inhibitor of ethylene production, ethionine, inhibits zygote formation in *D. discoideum*, and this effect is nullified following the addition of ACC, which is the immediate precursor of ethylene (Amagai 1992). Since the effects of ethylene on the sexual cycle have only been shown in *D. mucoroides*, in which ethylene signaling seems to be utilized in a limited manner during the regulation of *zyg1* expression, further analysis is needed to clarify the biological significance of ethylene in dictyostelids.

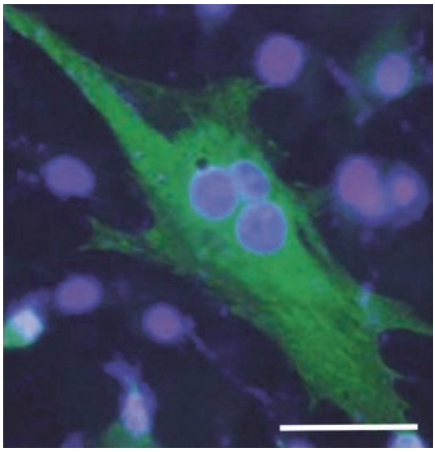

Fig. 11.8 Ectopic cell fusion of mouse myoblasts (C2C12) induced following the transfection of a *mzyg1* (humanized version of *Dictyostelium mucoroides zyg1*) gene. The product of the transgene is hemagglutinin (HA)-tagged and visualized using an anti-HA antibody followed by a FITC (fluorescein isothiocyanate)-conjugated secondary antibody (green). Nuclei are labeled with DAPI (4′,6-diamidino-2-phenylindole: purple) (reprinted from Amagai et al. 2012). Bar; 50 μm

11.9 Prospects

Multi-allelic mating systems are observed in some lower eukaryotes. A ciliate, Tetrahymena, has seven mating types encoded in two nuclei; both a somatic and germline nuclei contain the DNA of every mating type. However, later during sexual reproduction, the DNA-winnowing process stochastically operates on germline nuclei to delete six genes until one mating type remains in the progeny (Cervantes et al. 2013). In basidiomycete fungi, a two-locus system regulates mating, with various combinations of pheromone/receptor genes and homeodomain transcription factors (Raudaskoski and Kothe 2010). The mating-type locus of *D. discoideum* involves the threefold organization of homologous *mat* sequences. This suggests that the mating system adapted by *Dictyostelium* is not similar to that in other known multi-allelic systems, and that it has evolved independently from an ancestral set of *mat* genes. The organization of the *mat* locus may suggest that type I exists as one sex and that type III exists as the other (the gender has not yet been fixed), with the third composite sex of type II suggested to be equivalent to self-infertile hermaphrodites (Bloomfield 2011).

Despite the observation that mating and sexual recombination in the social amoeba occur frequently in the wild (Flowers et al. 2010), genetics associated with the sexual cycle have not been sufficiently analyzed due to the very low efficiency of macrocyst germination. However, recent progress has made it possible to use the mating type “congenic” strains with a common genetic background of a single host

strain (type I); this should provide an experimental platform and open up comparable omics studies to permit the identification of molecules and their interactions involved in sexual development. The outcome would help to answer many of the questions raised, such as how the *mat* gene(s) regulate self/non-self-recognition and production of sex pheromones, and which genes encode gamete recognition molecules present on the cell surface. In addition, the accumulation of genome information for other dictyostelid species would provide evolutionary insight to account for the number of sexes, and whether homothallism or heterothallism is ancestral. Future studies on dictyostelids promise to yield important biological insights into the molecular mechanisms of sexual reproduction in social amoebozoans.

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Part II
Diversity in Sex Determination and Differentiation

Chapter 12
Environmental Control of Sex Differentiation in *Daphnia*

Kenji Toyota, Norihisa Tatarazako, and Taisen Iguchi

Abstract The cladoceran crustacean genus *Daphnia* (daphnids) exhibits a unique reproductive strategy in response to changes in environmental conditions of their habitat. This is known as environmental sex determination (ESD). Under favorable environmental conditions, daphnids produce clonal female offspring by parthenogenesis. Under unfavorable environmental conditions (e.g., short day length, low temperature, food shortage, overpopulation, and/or a combination thereof), daphnids produce male offspring by parthenogenesis and then switch reproductive methods to undergo sexual reproduction. Based on pharmacological and molecular experiments, the juvenile hormone pathway and *doublesex1* gene have been identified as responsible factors in the process of ESD and subsequent sexual differentiation in daphnids. Furthermore, morphological observations of the detailed developmental processes that occur during female and male embryogenesis have been made. Despite substantial efforts in studies of the regulatory mechanisms governing sexual differentiation, there remains a large knowledge gap. In this chapter,

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we provide background information and recent progress in these research fields and present an overview of current knowledge regarding ESD and sexual differentiation in daphnids, mainly focusing on *Daphnia pulex* and *D. magna*.

Keywords Environmental sex determination (ESD) · *Daphnia pulex* · *Daphnia magna* · Juvenile hormone · Doublesex1 gene

12.1 Introduction

The Cladoceran order (commonly called water fleas) is one of the dominant organisms in freshwater zooplankton communities and belongs to the subclass Branchiopoda, bearing flattened leaf-like legs used to generate water currents for the filtering apparatus (Hebert 1977). The Cladocera is an ancient clade of branchiopod crustaceans comprising 16 or 18 family lineages (Olesen 1998; Stenderup et al. 2006). Among them, one of the most well-studied species are in the family Daphniidae (family lineage: Crustacea, Branchiopoda, Cladocera, Daphniidae), particularly genus *Daphnia*. To date, approximately 200 *Daphnia* species, consisting of three subgenera (*Daphnia*, *Ctenodaphnia*, and *Australodaphnia*), have been reported (Adamowicz et al. 2009; Kotov et al. 2013). All age classes of *Daphnia* are primarily pelagic (open ocean) and are found in most freshwater environments except for extreme habitats such as hot springs. Some species may frequently be found attached to waterweeds and the bottom sediments of shallow ponds.

Approximately 100 years ago, it was reported in cladocera species (including daphnids) that the sex of offspring is determined by environmental conditions (environmental sex determination (ESD)) (Grosvenor and Smith 1913; Smith 1915). ESD is regarded as one of the most important traits underpinning their complicated and sophisticated life history, attracting many scientists engaged in ecological, developmental, and evolutionary biology (Smith 1915; Banta and Brown 1929; Hobæk and Larsson 1990; Kleiven et al. 1992; Eads et al. 2008; Gilbert and Epel 2009; Colbourne et al. 2011).

In this chapter, we discuss ESD and sexual differentiation of daphnids, mainly focusing on *Daphnia pulex* and *D. magna*. Daphnids shift from production of female offspring to male offspring in response to certain external environmental stimuli (e.g., day-length (i.e., photoperiod), temperature, food shortage, and overcrowding). Adult female and male daphnids show apparent sexual dimorphism (female- or male-specific morphological change) and are distinguishable based on morphological features in the first instar juvenile. Little is known about the molecular mechanisms underlying sexual determination and differentiation. In the following sections, based on previous reports and our current findings, we summarize the developmental processes of sexually dimorphic traits observed in *D. pulex* and *D. magna*.

12.1.1 Life History of Daphnia

Daphnids employ cyclical parthenogenesis, in which parthenogenesis and sexual reproduction are rotated in response to environmental conditions such as day-length, temperature, food shortage, overcrowding, and presence of predators (Smith 1915; Banta and Brown 1929; Hobæk and Larsson 1990; Kleiven et al. 1992; Pijanowska and Stolpe 1996) (Fig. 12.1). Within the Branchiopoda, cyclical parthenogenesis began during the Permian period (Taylor et al. 1999). Most cladoceran species use cyclical parthenogenesis. Daphnids are important zooplankton in freshwater ecosystems. They have a short generation time (approximately 1 week in laboratory conditions) and their lifespan range is from 2 months to 1 year when reared under colder temperatures (Gliwicz et al. 2001). During favorable growing conditions, daphnids parthenogenetically produce female offspring and this results in the exponential growth of clonal populations. However, when unfavorable conditions arise, males are produced by parthenogenesis and these males are genetically identical (i.e., a clone) to their sisters and mothers. This suggests that mating a male with a female of the same clone is genetically equivalent to selfing. In these unfavorable conditions, a female can mate with a male that is exhibiting sexual eggs and resting eggs (protected by ephippium (modified carapace)) are formed. Resting eggs tolerate extreme conditions (e.g., drying and freezing) and can be viable for over 100 years (Caceres 1998). These resting eggs can hatch and develop as females when favorable conditions are restored. In this way, daphnids use cyclical

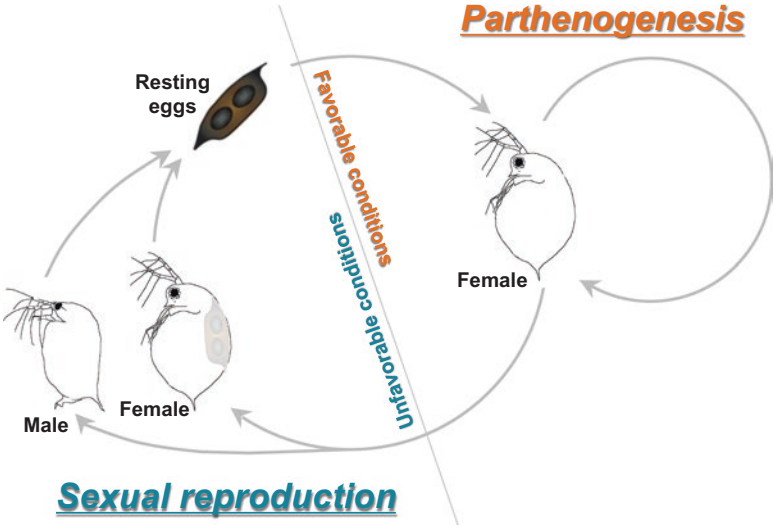

Fig. 12.1 Schematic illustration of cyclical parthenogenesis in daphnids. Daphnids produce female offspring by parthenogenesis during favorable conditions, and produce males under unfavorable conditions. Males and females mate and produce resting eggs, which are able to tolerate extreme conditions (e.g., drying and freezing) and hatch as females when favorable environmental conditions are restored

parthenogenesis to take advantage of changing habitat environmental conditions. Parthenogenesis allows for rapid propagation during favorable growing seasons and sexual reproduction contributes to an increase in genetic variation and survival rate (Barton and Charlesworth 1998). Therefore, the development of sexually dimorphic traits depending on external environmental conditions is a fundamental phenomenon of daphnids. This allows daphnids to appropriately employ their reproductive strategies to accommodate seasonally changing environments (Kleiven et al. 1992) (Fig. 12.1).

12.1.2 Daphnia as a Model Organism

Daphnids are a representative and historically well-used model organism for laboratory studies. There has been an accumulation of information on *D. pulex* and *D. magna* in terms of evolutionary, ecological, physiological, developmental, ecotoxicological, and genetic properties. The benefits of using these two species are as follows: they are easy to breed and maintain under laboratory conditions, they propagate rapidly because of their short reproductive cycle, and embryogenesis can be observed in vitro. Additionally, due to the diploidy nature of the parthenogenetic egg being maintained by mitosis-like meiosis (which skips a part of the first meiosis) individuals within a single strain are most likely to be genetically identical (Hiruta et al. 2010). This allows scientists to analyze environmental impacts on physiological and developmental processes in individuals with identical genetic backgrounds.

In addition to the aforementioned advantages, useful experimental tools have been established for *D. pulex* and *D. magna* (e.g., whole-mount *in situ* hybridization and immunostaining using developing embryos (Sagawa et al. 2005), immunofluorescence and fluorescence *in situ* hybridization (FISH) (Tsuchiya et al. 2009), expressed sequence tags (Watanabe et al. 2005), genetic linkage maps (Cristescu et al. 2006; Routtu et al. 2010), DNA micro-arrays (Shaw et al. 2007; Watanabe et al. 2007), electroporation (Kato et al. 2010b), RNA interference (RNAi) (Kato et al. 2011b; Hiruta et al. 2013), transgenesis (Kato et al. 2012), and genome editing (TALEN and CRISPR/Cas9) (Hiruta et al. 2014a; Nakanishi et al. 2014, 2015; Naitou et al. 2015)). Establishment of cell lines and virally-induced transgenic lines is still ongoing (Robinson et al. 2006; Hiruta et al. 2018). Furthermore, the whole-genome sequencing project for *D. pulex* is complete (Colbourne et al. 2011). Combined with the recent progresses in bio-informatics and high-throughput omics technologies, these experimental tools enable scientists to study the cells, tissues, and organs within daphnids from multifaceted viewpoints such as transcriptomics (Colbourne et al. 2011; Eads et al. 2007; Toyota et al. 2014, 2015b), proteomics (Fröhlich et al. 2009), and metabolomics (Poynton et al. 2011). These tools allow for the analysis and accumulation of large amounts of data and this makes *D. pulex* and *D. magna* attractive model organisms for studying the underlying molecular mechanisms of phenotypic alterations in response to environmental stimuli.

12.2 Male Induction

It has been demonstrated that several environmental cues (*e.g.*, low photoperiod, low temperatures, food shortage, and overpopulation) trigger the production of male offspring (Smith 1915; Banta and Brown 1929; Hobæk and Larsson 1990; Kleiven et al. 1992). Despite many studies on male induction in daphnids, a reproducible induction system for male offspring has not been established. This is because multiple environmental cues are involved in the sex determination of daphnids and this is difficult to reproduce in the laboratory.

12.2.1 Male Induction by the Juvenile Hormone and Its Analogs

Over the last decade, several studies have demonstrated that the production of male offspring is induced by exposure to the juvenile hormone (JH; a representative endocrine factor among arthropods) or its artificial analogs (used as pesticides) (Fig. 12.2) in *D. magna* in a dose-dependent manner, even when the offspring are reared under female-producing conditions (Olmstead and LeBlanc 2002; Tatarazako et al. 2003). To date, this phenomenon has also been reported in other cladocera

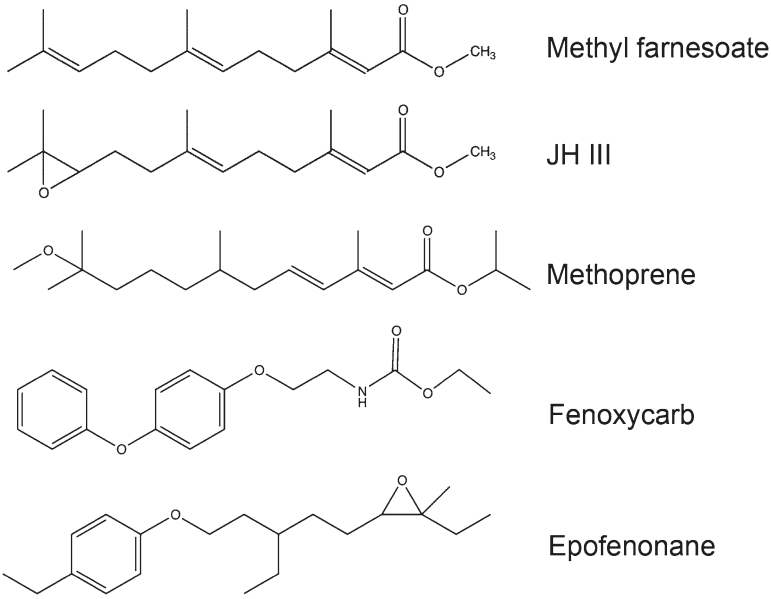

Fig. 12.2 Chemical structures of methyl farnesoate (innate juvenile hormone, JH, in daphnids), JH III (representative JH in insects), and methoprene, fenoxycarb and epofenonane (artificial JH agonists used as pesticides)

genera such as *Ceriodaphnia*, *Moina*, *Bosmina*, *Oxyurella*, *Leberis*, *Leydigia*, and *Disparalona* (Oda et al. 2005; Kim et al. 2006; Sinev and Sanoamuang 2011; Toyota et al. 2013). In addition, the genetic differences in the male offspring induced by JH treatment are substantial (i.e., the offspring are not genetically identical) (Oda et al. 2006).

The stage at which the daphnid was sensitive to JH was calculated using pulse treatments of JH on various embryonic stages and reproductive cycles of the mother. Male sex determination occurred during the oocyte maturation period (20–30 h before ovulation) in the mother's ovary in *D. magna* and *D. pulex* (Olmstead and LeBlanc 2002; Ignace et al. 2011; Toyota et al. 2015a). Unfortunately, no studies have been able to quantify the innate JH level from extracts of daphnid species, even using liquid chromatography (LC)- or gas chromatography-mass spectrometry (GC-MS) methods. Quantification of the innate JH level of daphnids during the sex determination period is essential for understanding the physiological role of the JH as a male sex determinant.

12.2.2 Male Induction by Environmental Change

We have recently established a reliable induction process for male and female offspring in response to day-length differences (i.e., photoperiod) in the WTN6 strain of *D. pulex* (Toyota et al. 2015a, b). In this strain, offspring sex can be controlled by modifying the day-length. A mother produces female offspring under long-day conditions (14 h light: 10 h dark). Male offspring develop under short-day conditions (10 h light: 14 h dark). This relationship enables the evaluation of factors involved in the primary signal cascades governing ESD in daphnids.

12.3 Regulatory Pathways for Male Sex Determination in Daphnids

The JHs are a family of sesquiterpenoid compounds and have primary roles in the regulation of metamorphosis and reproduction in insects and crustaceans (Nijhout 1994). JHs also regulate the generation of alternative phenotypes such as caste differentiations in social insects (Miura 2005), male ornament development in several coleopterans (Emlen and Nijhout 1999; Gotoh et al. 2014), phase polyphenisms in locusts (Tanaka 2001), diapauses in mosquitoes (Denlinger 2002), and inducible defenses when co-treated with predatory kairomones (chemical cues released by predators) in daphnids (Oda et al. 2011; Miyakawa et al. 2013a).

12.3.1 JH Synthesis and Degradation Pathways

Organs that synthesize JH in several insects and decapod crustaceans (e.g., crab and shrimp) have been identified as the corpora allata (a pair of small endocrine organs with nervous connections to the brain) and mandibular organs (a pair of glandular organs located in the mandibular region), respectively (Cassier 1979; Laufer et al. 1987). It is unknown where JH synthesis occurs in daphnids. Methyl farnesoate (MF), a natural precursor of insect JH, has been detected in the hemolymph of various decapod and non-decapod crustaceans (*e.g.*, *Artemia salina*, a Branchiopod) (Laufer et al. 1987; Laufer and Biggers 2001). MF is now regarded as an innate JH in crustaceans (Laufer et al. 1987).

The presence or absence of an epoxide ring is the only difference between juvenile hormone III (JH III, a representative JH molecule in insects) and MF (Fig. 12.2), suggesting that the biosynthesis pathway of MF in crustaceans is similar to that of JH III in insects (Miyakawa et al. 2014). The JH III biosynthesis pathway has two distinct parts: the mevalonate pathway, which is highly conserved in bilaterians, and the JH III-specific biosynthesis pathway, which is comprised of enzymatic steps unique to JH-producing organisms. Farnesyl pyrophosphate, an intermediate in the mevalonate pathway, is converted to MF by four enzymes: farnesyl phosphatase, farnesol dehydrogenase, farnesal dehydrogenase, and juvenile hormone acid O-methyltransferase (JHAMT) (Miyakawa et al. 2014). MF is further converted to JH III by CYP15A1 (MF epoxidase) in insects (except for Lepidoptera). Recent studies have shown that *D. pulex* has the *JHAMT* gene, although it lacks *CYP15A1* orthologs in its genome (Daimon and Shinoda 2013; Miyakawa et al. 2014; Toyota et al. 2015a). Furthermore, it has been demonstrated that the *D. pulex* JHAMT protein generates MF by catalyzing farnesoic acid. However, unlike insects, it does not produce JH III from JH III acid (Toyota et al. 2015a), suggesting that MF is a candidate for innate JH in daphnids as well as other crustaceans.

Generally speaking, the JH concentration in the hemolymph is maintained at equilibrium by continuous synthesis and degradation. JH is principally degraded by the enzymes JH esterase (JHE) or JH epoxide hydrolase (JHEH). The primary products of these reactions (JH acid and JH diol, respectively) are further metabolized by either ester hydrolysis or epoxide hydration and are subsequently conjugated and excreted (de Kort and Granger 1981). Although no degradation enzymes of MF has been found in crustaceans, genes homologous to *JHE* and *JHEH* were identified in some species such as the shrimp *Neocaridina denticulate* (Sin et al. 2015) and *D. pulex* (Toyota et al. 2015a). Surprisingly, *D. pulex* has more than ten copies of *JHE*-like genes in its genome, although their physiological function remains largely unknown (Toyota et al. 2015a).

12.3.2 Regulatory Mechanisms of MF During the Sex-Determining Period

Although MF has been reported to induce male production in daphnids (Olmstead and LeBlanc 2002), its endogenous physiological role as a male sex determiner remains unknown. This knowledge gap is largely due to a lack of suitable daphnid strains in which offspring sex can be experimentally modified without MF treatment. To shed light on this matter, we established a reliable induction system of male and female offspring responding to day-length differences in the WTN6 strain of *D. pulex* (Toyota et al. 2015a). Using this relationship, it was suggested that in response to the short-day condition, increasing levels of MF during the sex-determining period caused male induction . The evidence for this is as follows: (1) the administration of MK-801 (an antagonist of the ionotropic glutamate receptors and known as a JH-biosynthesis inhibitor in several insects (Chiang et al. 2002; Begum et al. 2004; Geister et al. 2008)) suppresses male offspring production in the WTN6 strain under the short-day (i.e., male-producing) condition, and this inhibition is reversible when treated with an exogenous MF, (2) JHAMT generates MF by catalyzing farnesoic acid; however, unlike insects, *D. pulex* JHAMT does not convert JH III from JH III acid, and (3) *JHAMT* gene expression is significantly increased prior to the MF-sensitive period for male production under the short-day condition (Toyota et al. 2015a).

Furthermore, it has been demonstrated that N-methyl-D-aspartic acid receptors (NMDARs; a type of ionotropic glutamate receptor) are essential for male offspring production, acting as upstream regulators of MF signaling, by transcriptome analysis and pharmacological manipulation using the aforementioned experimental system of *D. pulex* WTN6 strain (Toyota et al. 2015b) (Fig. 12.3).

12.3.3 Reception Mechanisms and Downstream Factors of MF

Recent studies have shown that methoprene-tolerant (Met) and steroid receptor coactivator (SRC) are the main components of the JH receptor complex in insects (Charles et al. 2011; Li et al. 2011; Zhang et al. 2011). Recently, we discovered *Met* and *SRC* homologs in *D. pulex* and *D. magna* and reported that, as in insects, Met and SRC act as components of the MF/JH receptors in *Daphnia* species (Miyakawa et al. 2013b). We further showed that male induction by exogenous chemicals with MF/JH activity is likely to be caused by the MF signaling pathway via these MF receptor complexes (Miyakawa et al. 2013b).

In addition to MF receptor systems, we have identified *D. pulex* orthologs of *Krüppel homolog 1* (*kr-h1*) and *broad* genes, which are known as JH-responsive elements in insects (Suzuki et al. 2008; Kayukawa et al. 2012). However, the expression level of *kr-h1* gene, the primary target of Met in several insects (Kayukawa et al. 2012, 2013; Song et al. 2014; Cui et al. 2014), did not change under the short-day (male-inducing) condition, although the MF concentration was predicted

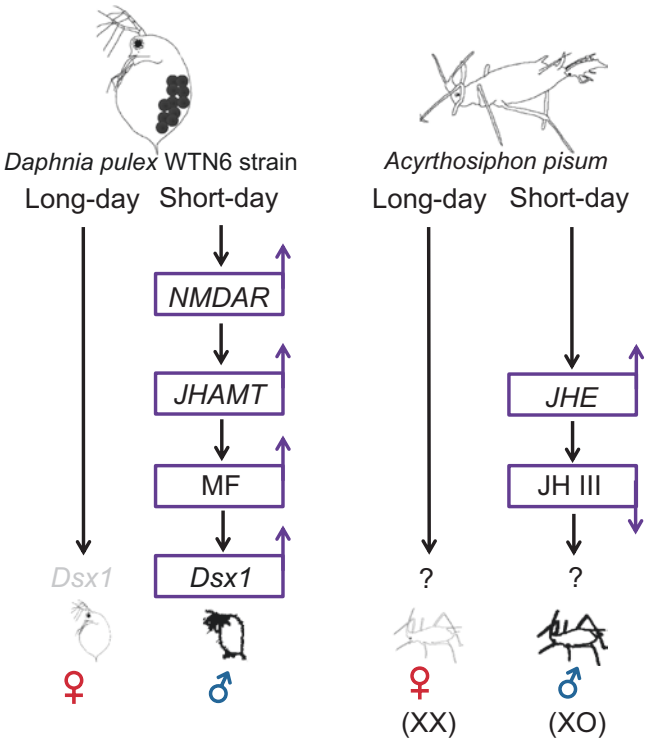

Fig. 12.3 Comparative schematic diagram of molecular signaling cascades regulating male offspring production in response to the short day stimulus in *D. pulex* WTN6 strain (left) and *Acyrthosiphon pisum* (right). *NMDAR* N-methyl-D-aspartic acid receptor, *JHAMT* juvenile hormone acid O-methyltransferase, *MF* methyl farnesoate, *Dsx1* doublesex1, *JHE* juvenile hormone esterase

to be high in the *D. pulex* WTN6 strain. These data suggest that the *D. pulex kr-h1* gene is not MF-responsive and the factors involved in the MF-signaling cascades for sex determination in *D. pulex* are different to the recognized JH signaling pathway in insects. Moreover, we identified some candidate genes responsible for signaling downstream of MF (e.g., protein kinase C pathway-related genes) by transcriptome analysis (Toyota et al. 2015b). These functional analyses are ongoing.

As discussed above, the study of MF signaling pathways provides important hints for explanation of the molecular mechanisms underlying male sex determination in daphnids. Previously, non-male producing genotypes have been reported in *D. pulex* (Innes and Dunbrack 1993) and *D. magna* (Yampolsky 1992). To identify quantitative trait loci (QTL) for male production, one of the current research approaches, QTL mapping analyses, is progressing. This approach uses crosses between wild-type females that produce female and male offspring (male producer) and females that produce only female offspring (non-male producer; unresponsive to MF) in *D. magna* (Eads et al. 2008). These studies can contribute to understanding the physiological regulations in the production of male offspring.

12.4 Reception Mechanisms of Environmental Stimuli

Although this section has focused mainly on MF/JH signaling pathways as a key element regulating ESD in daphnids, the reception mechanism of primary environmental cues has remained elusive. Here, we review the studies investigating photoreceptors as candidates for primary factors of ESD. Previous studies have suggested that several photoreceptors are located in the ommatidia of the compound eye of daphnids and these photoreceptors are receptive to light signals around 340 nm (ultra violet: UV), 450 nm, 510 nm, and 590 nm (Smith and Macagno 1990). According to a recent morphological study of the *Daphnia* nervous system, the compound eye is directly connected to the protocerebral scaffold by the optic neuropil (Weiss et al. 2012). Interestingly, in the cyclically parthenogenic aphid *Megoura viciae*, the photoperiodic signal triggering the switch from parthenogenesis to sexual reproduction (including male induction) is activated in a cluster of neurosecretory cells, which are located on the dorso-anterior region of the protocerebrum (pars intercerebralis). These neurosecretory cells play an important role in the measurement of scotophase (Lees 1964, 1981; Gao et al. 1999). Therefore, it is postulated that these neurosecretory cells secrete molecules that alter the developmental fate (viviparous female, oviparous female, or male) of the oocytes in the aphids. Based on these findings, photoreceptors localized in ommatidia of the compound eye might be responsible for the short-day-induced male production in *D. pulex* WTN6 strain.

In addition to photoreceptors, various environmental stimuli can trigger male induction in *D. pulex*: overpopulation and low temperature (Smith 1915) and *D. magna*: overpopulation and short day length (Hobæk and Larsson 1990) and overpopulation, short day length, and oligotrophy (Kleiven et al. 1992). Accordingly, primary environmental cues regulating the sex determination in daphnids are different among strains with different genetic backgrounds. It would be useful to clarify the establishment of stable male-inducing conditions in each strain of daphnids in response to rearing conditions.

12.5 Transduction of Maternal Signals During Parthenogenetic Oogenesis

The sexual fate of daphnids is determined during the oocyte maturation period, prior to ovulation from the mother's ovary (Olmstead and LeBlanc 2002; Toyota et al. 2015a). It is hypothesized that one or multiple maternal sex determiners are produced in the developing oocytes and these determiners act as downstream factors for MF signaling. In the case of *D. pulex* WTN6 strain reared at 18 °C, once the oocytes develop, they ovulate in approximately 70 h. During oogenesis, a cell cluster is organized, consisting of an oocyte and three nurse cells, which are morphologically indistinguishable after ovulation and grow in a similar manner during the early stages (Rossi 1980). Only the oocyte accumulates yolk granules and oil

droplets (24 h after ovulation), whereas the nurse cells degenerate 60–70 h after ovulation (Rossi 1980; Hiruta and Tochinai 2012). Tight and continuous association between oocytes, nurse cells, and ovarian tissues makes it possible for the mother to transmit environmental cues into developing oocytes. It is intriguing that a critical MF-sensitive period for sex determination is the middle to late stage of oogenesis (40–60 h after ovulation), when the degeneration of nurse cells is accelerated. Several studies have reported that maternal effects (e.g., food condition, photoperiod, and kairomones) are associated with the regulation of growth rate and fecundity (Tollrian 1995; LaMontagne and McCauley 2001; Alekseev and Lajus 2009; Gorbi et al. 2011), inducibility of resting eggs (Alekseev and Lampert 2001), and acquisition of an immune system (Little et al. 2003) in the offspring. However, the maternal factors responsible are still largely unknown. Recent studies revealed that maternal mRNAs in the ovulated eggs possibly regulate early embryonic development in daphnids (Kato et al. 2011a; Asada et al. 2014). These findings suggest that external environmental cues associated with sex determination could also be transferred to developing oocytes as maternal factors and these cues might control the sexual fate of the offspring in the next generation, although such maternal factors remain elusive.

12.6 Developmental Processes for Male Sexual Differentiation

Various male-inducing conditions have been reported in daphnids (oligotrophy, overpopulation, short day-lengths, and temperature). Although previous reports have described embryogenesis and the morphological landmarks in detail (Threlkeld 1979; Mittmann et al. 2014), the experimental conditions and/or observation methods differed among the studies and all the studies focused on female embryogenesis. This focus was due to lack of a stable male inducing system. However, male-inducing systems using JH treatment on *D. magna* (Olmstead and LeBlanc 2002; Tatarazako et al. 2003) and using short-day conditions on the *D. pulex* WTN6 strain (Toyota et al. 2015a) have been established. These male-inducing systems will contribute to the elucidation of male offspring induction in daphnids. We recently re-defined embryonic stages of the female and male in *D. pulex* and *D. magna* based on our time-lapse observation of daphnids reared under constant conditions (Hiruta et al. 2014b; Toyota et al. 2016).

12.6.1 Doublesex1 Gene as a Factor Responsible for Development of Male Traits

Although JH-induced male offspring production has enabled further studies concerning the molecular mechanisms underlying male sex determination processes in daphnids (Eads et al. 2007; Colbourne et al. 2011), factors responsible for the

development of male traits are not well understood. Recently, we identified the *D. magna doublesex1* (*DapmaDsx1*) gene, which exhibits male-specific expression patterns from early embryonic to adult stages (Kato et al. 2011a). A *dsx* gene was originally identified in the fruit fly *Drosophila melanogaster* as a critical and terminal transcription factor in the sex-determining cascade. It is spatiotemporally transcribed into sex-specific splicing isoforms by *transformer* genes and thus contributes to the development of sexually dimorphic traits (Burtis and Baker 1989; Robinett et al. 2010). However, the *DapmaDsx1* gene showed no sex-specific splicing isoforms by *transformer* gene (Kato et al. 2010a). It is also transcriptionally upregulated dominantly in male daphnids. Further analyses revealed that knock-down of *DapmaDsx1* in male embryos resulted in the production of female traits, including ovarian development, whereas overexpression of *DapmaDsx1* in female-destined embryos resulted in the development of male-like phenotypes such as elongation of the first antennae. This suggests that DapmaDsx1 acts as a key factor responsible for male trait development (Kato et al. 2011a). Also, it contains two conserved domains: the Dsx/Mab-3 (DM) domain at the N-terminus and the oligomerization domain at the C-terminus (Bayrer et al. 2005). Genes encoding the DM-domain have been reported to play similar roles among members of the animal kingdom such as the nematode *Caenorhabditis elegans* (Shen and Hodgkin 1988; Raymond et al. 1998) and multiple vertebrate species (Raymond et al. 2000; Kopp 2012; Matson and Zarkower 2012). We also previously analyzed other DM-domain genes (*DMRT11E*, *DMRT93B*, and *DMRT99B*) in *D. magna* displaying sexually dimorphic gene expression patterns in adult gonads (Kato et al. 2008). However, none of these genes exhibited sexually dimorphic expression patterns during embryonic development. This suggests that they are not involved in sex determination (Kato et al. 2008).

Moreover, we recently identified the *dsx* genes from the broader taxonomic group of cladocerans: *D. pulex*, *D. galeata*, *Ceriodaphnia dubia*, and *Moina macrocopa*. It was revealed that their dsx genes were expressed exclusively in males from embryonic to adult stage much like *D. magna*. This suggests that these genes may have similar functions in sex determination among cladocerans (Toyota et al. 2013) (Fig. 12.3). In addition to the *dsx1* gene, we identified the *dsx2* gene as its paralog from all aforementioned species except for *M. macrocopa* (Kato et al. 2011a; Toyota et al. 2013) and revealed that the *dsx2* gene also shows male-specific expression patterns, although its specific function remains unknown. Phylogenetic analysis revealed that the *dsx* gene duplication occurred prior to the divergence of these cladoceran species. These data suggest that there might be some kind of relationship between acquisition of the ESD system and *dsx* gene duplication among cladoceran species.

The extent of dsx's role as the main factor for male trait development in other crustaceans is unclear. Interestingly, in other crustaceans such as decapods, amphipods, and isopods, which belong to Malacostraca crustaceans, male sexual differentiation and development of secondary male-sex characteristics are regulated by insulin-like androgenic gland hormones released from androgenic glands, which are unique endocrine organs of Malacostraca crustaceans. However, their sexual fate is

primarily determined by their genotype (*e.g.*, XX/XY or ZW/ZZ) (reviewed in more detail in Ventura et al. 2015). Moreover, recent studies identified the crustacean female sex hormone, which is involved with the development of the female reproductive system, from the Blue Crab, *Callinectes sapidus*. This hormone is released from distinct neurosecretory cells in the eyestalk ganglia and is categorized into a peptide hormone (Zmora and Chung 2014). However, similar orthologs or analogical endocrine mechanisms involved in the sexual differentiation in daphnids have not been reported so far.

12.7 Common Principles of the Molecular Mechanism of ESD

Along with daphnids, the agricultural pest insects aphids have been reported as undergoing cyclical parthenogenesis (Simon et al. 2011). Although their sexes are determined by a genotypic XX/XO system, whether the X chromosome is released or not is regulated by environmental cues (short day length and low temperature) (Lees 1973). Moreover, several studies revealed that a low JH III (innate JH in aphids) concentration is necessary for the induction of sexual morphs (producing males or oviparous females) (Hardie 1981; Corbitt and Hardie 1985). In addition, a recent study using the Pea Aphid, *Acyrthosiphon pisum*, identified *JH esterase* (*JHE*; gene coding the JH degradation enzyme) as a key regulator responsible for decreasing the JH III concentration in response to sexual morph-inducing conditions (Ishikawa et al. 2012) (Fig. 12.3). Nevertheless, the ESD systems of daphnids and Pea Aphids are considered to have been acquired independently through evolution. It is interesting that the MF/JH system is designated as a sex-determining factor in both species, although in opposite concentrations. The integration of findings from these species sheds light on the variation in the common principles underlying the survival strategies of organisms living in changing natural environments.

12.8 Conclusions

Sex determination is the most fundamental developmental process contributing to the establishment of sexually dimorphic traits and has fascinated scientists for a long time. In this chapter, we provided an overview of studies concerning ESD and sexual differentiation in daphnids, mainly in *D. pulex* and *D. magna*, revealed by numerous important observations and experimental evidence. However, there is a lack of supporting evidence for the factors responsible and their molecular regulatory mechanisms. Recently, several new high-throughput omics technologies (e.g., genomics, transcriptomics, proteomics, metabolomics, epigenetics, etc.) and genetic tools (e.g., RNAi and genome editing methods) have become available. Further studies using these approaches can lead to further understanding of the establishment of sexual dimorphism in daphnids.

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Chapter 13
Sex Determination Cascade in Insects: A Great Treasure House of Alternative Splicing

Masataka G. Suzuki

Abstract Cytological and genetic studies using insects, performed in the first decades of the twentieth century, greatly contributed to establishing the notion that genotypic factors determine sexual fate. Since then, excellent studies of *Drosophila* have provided important clues to answering the question of how sex is determined. In *Drosophila melanogaster*, somatic sexual differentiation is regulated by a well-characterized genetic hierarchy composed of a primary genetic signal (X:A ratio), master regulator (*Sex-lethal*), subordinate regulator (*transformer*/*transformer-2*), and double-switch (*dsx* and *fru*). On the basis of the knowledge obtained from studies with *Drosophila*, scientists have gained understanding of molecular mechanisms of sex determination in a variety of insect species. Recent studies have revealed that several insect species, such as the silkworm and the mosquito, have a unique sex determination cascade, which is surprisingly different from that in *Drosophila*. The most characteristic feature of the sex-determining genes in insects so far identified is that their sex-specific expressions are controlled by alternative splicing. In this chapter, we give an overview of the sex-determining genes revealed thorough the studies of *D. melanogaster* and those homologues identified in either nondrosophilid insects or animal species other than insects. In particular, we provide a detailed description of the novel sex-determining genes identified in the silkworm on the basis of our recent studies.

Keywords Sex determination · Sexual differentiation · Sex-determining gene · Alternative splicing · Insects · Sex determination cascade · Silkworm

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13.1 Introduction

How is sex determined? Aristotle once answered this question as follows: "The heat of semen at the time of copulation determines the sexual fate." According to his hypothesis, hot semen produces males, whereas cold semen yields females (Jacqart and Thomasset 1988). Similar hypotheses, where environmental conditions such as temperature and nutritional status are major factors in determining sexual fate, were believed until the end of nineteenth century. The first indication relevant to a sex chromosome came from cytological studies conducted by Hermann Henking in 1891. In the course of studying sperm formation in *Pyrrhocoris* fire wasps, Henking noticed that one chromosome did not have a counterpart, unlike all other chromosomes, during the stages of meiosis in sperm cells. He designated this curious chromosome as the "X element" because of its strange nature (Henking 1891). The X element could be found in males of a number of different species. On the basis of his findings, Henking speculated that the X element played a crucial role in sex determination, but he could not provide direct evidence to support his idea. Later the X element became known as the X chromosome after it was established to indeed be a chromosome. In 1902, as a consequence of his cytological studies using the long-horned grasshopper, *Xiphidium fasciatum*, Walter Sutton proposed the hypothesis that the Mendelian laws of inheritance could be applied to chromosomes at the cellular level of living organisms (Sutton 1903). More than a decade after Henking's work, Nettie Stevens set out to investigate gamete formation in many insects—from termites to sand crickets to aphids and mealworms. In this last species, *Tenebrio molitor*, she noticed that fully formed spermatids showed something odd in the chromosome number and its size: half of them had ten large chromosomes, all of a similar size, while the other half had nine large chromosomes and one small one. On the other hand, the eggs had ten large chromosomes, without exception. She also found that somatic cells from the male always had a total of 19 large chromosomes and always one small "accessory" chromosome, while somatic cells from female mealworms always contained 20 large chromosomes. In 1905, she correctly concluded that the accessory chromosome determines male sex (Stevens 1905). The accessory chromosome is now known as the Y chromosome. In contrast to the male-specific Y chromosome, which determines male sex, Yoshimaro Tanaka identified a female-specific chromosome in the silkworm, *Bombyx mori*, and demonstrated by genetic analysis that the presence of the female-specific chromosome determines female sex (Tanaka 1916). This female-specific chromosome was designated as the W chromosome, which has also been found in frogs, snakes, and birds. In 1921, Calvin Bridges found that the primary genetic signal for sex determination in *Drosophila melanogaster* was the ratio of X chromosomes to sets of autosomes (Bridges 1921). A ratio of 1.0 leads to female development, while a ratio of 0.5 leads to male development. Thus, cytological and genetic studies using insects greatly contributed to establishing the notion that genotypic factors determine the sexual fate.

The primary genetic signal for sex determination observed in insects is manifested in a wide variety of ways. In *Megaselia scalaris* (Traut 1994; Sievert et al. 2000), *Ceratitis capitata* (Willhoeft and Franz 1996), *Bactorocera tryoni* (Shearman and Formmer 1998), *Lucilia cuprina* (Bedo and Foster 1985), and *Chironomus thummi* (Hägele 1985), an epistatic maleness factor is found on Y chromosomes. The mosquito *Culex tritaeniorhynchus* has no sex chromosome, and its male sex is determined by a dominant gene on an autosome (Baker and Sakai 1976). The diploid/haploid sex determination system is well known in Hymenoptera (Beukeboom 1995). In Lepidoptera, *Lymantria disper* has a Z-linked male determinant (M) and a maternally inherited female determinant factor (F) (Goldschmidt 1955). Thus, insects have continued to provide a deep understanding of the genotypic sex determination system. In particular, much has been learned about the sex determination cascade from molecular and genetic analyses of *D. melanogaster*. On the basis of the knowledge obtained from studies of *Drosophila*, scientists have gained understanding of molecular mechanisms of sex determination in a variety of insect species such as the Mediterranean fruit fly (medfly), *C. capitata*; the yellow fever mosquito, *Aedes aegypti*; the silkmoth, *B. mori*; the honeybee, *Apis mellifera*; and the jewel wasp *Nasonia vitripennis*. These insects are highly diverged from *Drosophila*; for example, *A. aegypti* and *N. vitripennis* are separated from *D. melanogaster* by about 250 and 300 million years, respectively (Gailey et al. 2006; Hasselmann et al. 2008). Therefore, comparisons of genes constituting the sex determination cascade in insects will be very helpful to gain insight into the evolutionary dynamics of the regulatory mechanisms that give rise to sexual dimorphisms. In this chapter, we give an overview of sex-determining genes revealed through studies of *D. melanogaster* and those homologues identified in either non-drosophilid insects or animal species other than insects. In particular, we provide a detailed description of the novel sex-determining genes identified in the silkworm on the basis of our recent studies.

13.2 Sex Determination Cascade in *Drosophila melanogaster*

Sex determination in *D. melanogaster* is organized as a hierarchical order of genes designated as a sex determination cascade (Fig. 13.1). As described above, the first signal of sex determination is the ratio of X chromosomes to sets of autosomes (A) (Bridges 1921). The X:A ratio, a balance mechanism in which X chromosomal gene products are titrated against autosomal gene products, governs sex determination. Recent findings have indicated that the X:A ratio predicts the sexual fate but does not actively specify it. Instead, the instructive X chromosome signal is more appropriately seen as collective concentrations of several X-encoded signal element (XES) proteins in the early embryo (Erickson and Quintero 2007). The concentration of XES proteins determines the activity state of the *Sex-lethal* (*Sxl*) gene, which sits at the top of the sex determination cascade. A sufficient amount of XSE is supplied only when the animal has two X chromosomes (namely, XX female), leading

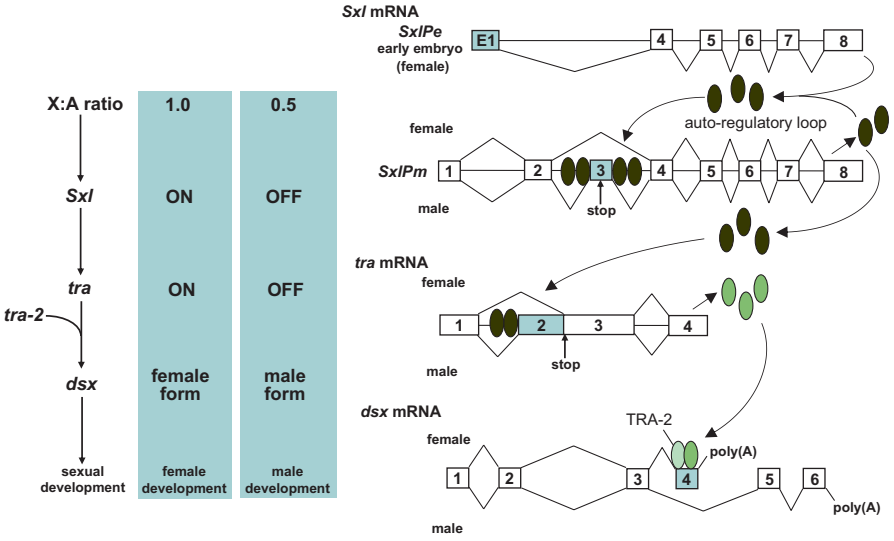

Fig. 13.1 Sex determination cascade and sex-determining genes in *Drosophila melanogaster*. The left panel shows the sex determination cascade, composed of *Sxl*, *tra*/*tra-2*, and *dsx* genes. The right panel shows sex-specific alternative splicing patterns in each gene. Transcripts from *tra-2* do not undergo sex-specific splicing. The primary genetic signal for sex determination is the ratio of X chromosomes to sets of autosomes (X:A ratio). Transcription from the *Sxl* establishment promoter (SxlPe) in the early embryonic stage is activated when the animal has two X chromosomes (namely, XX female). The protein encoded by transcripts from SxlPe induces female-specific splicing of *SxlPm* precursor messenger RNA (pre-mRNA), yielding the functional SXL protein. SXL protein binds to its own pre-mRNA to maintain the female-specific mode of splicing (an autoregulatory loop). SXL directs the female-specific splicing of *tra*, giving rise to functional TRA protein. TRA, together with TRA-2, binds to an exonic splicing enhancer (ESE) element located within the female-specific exon of *dsx*. The TRA and TRA-2 complex activates the weak 3′ splicing site preceding the female-specific exon to generate the female-type DSX protein (DSX^F). SxlPe is inactive when the animal has only one X chromosome (namely, XY male). In the absence of the protein products from SxlPe, *SxlPm* pre-mRNA is spliced by default, yielding transcripts including an intervening exon that contains a stop codon. A lack of functional SXL protein causes male-specific splicing of *tra*, making its encoded product nonfunctional because of a premature stop codon. In the absence of functional TRA protein, *dsx* pre-mRNA is spliced by default to generate the male-type DSX protein (DSX^M). The DSX^F and DSX^M proteins regulate the sex-specific transcription of target genes that encode the sexual phenotype of the body

to activation of transcription from the *Sxl* establishment promoter (SxlPe) at the early embryonic stage (Cline 1988; Keys et al. 1992; Estes et al. 1995; Erickson and Quintero 2007). The protein encoded by transcripts from SxlPe induces female-specific splicing of precursor messenger RNA (pre-mRNA) transcribed from the maintenance promoter, SxlPm, which is active in both sexes (Fig. 13.1). The female-specific splicing product from SxlPm encodes a functional SXL protein. The resulting SXL protein binds to its own pre-mRNA to maintain the female-specific mode of splicing (Fig. 13.1). The concentration of XSE is insufficient to activate SxlPe when the animal has only one X chromosome (namely, XY male).

In the absence of protein products from SxlPe, pre-mRNA from SxlPm is spliced by default, yielding transcripts including an intervening exon that contains a stop codon (Fig. 13.1). As a result, SxlPm transcripts in males do not encode a full-length protein. Thus, *Sxl* acts as the memory device for female sexual development via its autoregulatory function (Cline 1984; Bell et al. 1991). SXL directs female-specific splicing of its downstream gene *transformer* (*tra*), giving rise to functional TRA protein (Boggs et al. 1987) (Fig. 13.1). TRA, together with the protein product of *transformer-2* (*tra-2*), binds to an exonic splicing enhancer (ESE) element located within the female-specific exon of its downstream gene *doublesex* (*dsx*). The *dsx* ESE contains six copies of a 13-nucleotide (R1–R6) repeat sequence and is designated as dsxRE (Lynch and Maniatis 1995, 1996). TRA and TRA-2 are splicing factors belonging to the family of SR-type proteins whose amino acid sequences contain high frequencies of arginine (R) and serine (S) residues. The arginine- and serine-rich domain, which is known as the RS domain, mediates protein–protein interactions and regulates the recognition of specific splice sites (Manley and Tacke 1996). Proteins harboring the RS domain constitute a well-studied family of splicing regulators. An RS tetrapeptide (RSRS or SRSR) represents a functional unit in the RS domain of splicing activators (Graveley et al. 1998). Unlike other SR-type splicing factors, TRA lacks an RNA recognition motif (RRM) required for RNA binding. Therefore, TRA functions cooperatively with a factor such as TRA-2, which can bind to the cis-element in target pre-mRNAs. The TRA and TRA-2 (and one or more SR proteins) (Lynch and Maniatis 1995, 1996) complex binds to dsxRE functions to activate a weak 3′ splicing site preceding the female-specific exon, most likely by facilitating interactions of other general splicing factors with the RNA, to generate the female-type DSX protein (DSX^F) (Kan and Green 1999; Li and Blencowe 1999). A lack of functional SXL protein causes male-specific splicing of *tra*, making its encoded product nonfunctional because of a premature stop codon (Fig. 13.1). In the absence of functional TRA protein, *dsx* pre-mRNA is spliced by default to generate the male-type DSX protein (DSX^M). DSX^F and DSX^M regulate the sex-specific transcription of target genes that encode the sexual phenotype of the body (Baker and Wolfner 1988; Burtis and Baker 1989; Cline and Meyer 1996). To date, there are only a few validated direct gene targets for *dsx*: *Yolk Proteins* (*yp-1* and *yp-2*), *bric-à-brac 1*, *desatF* (also known as *Fad2*), and *Flavin-containing monooxygenase-2* (*Fmos-2*) (Burtis et al. 1991; Coschigano and Wensink 1993; Williams et al. 2008; Shirangi et al. 2009; Luo and Baker 2015). The regulation of yolk protein gene expression is by far the best characterized DSX function at the molecular level. DSX^F and DSX^M bind directly to several specific sites in an enhancer sequence designated as fat body enhancer (Burtis et al. 1991; Coschigano and Wensink 1993), which plays a role in directing the adult female fat body–specific transcription characteristic of the adjacent yolk protein genes, *yp-1* and *yp-2* (Garabedian et al. 1986). Recent genome-wide analyses by chromatin immunoprecipitation followed by sequencing (ChIP-seq) have revealed that DSX^F and DSX^M bind thousands of the same targets in multiple tissues in males and females, indicating that DSX action is regulated downstream from DSX binding (Clough et al. 2014).

Another known downstream target of *tra* is *fruitless* (*fru*). In females, transcripts from the *fru* gene undergo female-specific splicing by TRA and TRA-2; in addition, these two factors prevent translation of the female-specific *fru* transcripts (Ryner et al. 1996; Heinrichs et al. 1998; Usui-Aoki et al. 2000). In males, because of the absence of TRA, default splicing produces male-specific *fru* transcripts, yielding male-specific protein isoforms (FRU^M). Mutations in sex-specific *fru* transcripts disrupt both male courtship behavior and sexual orientation but have no effect on female behavior (Ito et al. 1996; Ryner et al. 1996; Anand et al. 2001; Lee and Hall 2001; Lee et al. 2001; Demir and Dickson 2005). The FRU^M proteins play central roles in producing sexual differences in the mAL interneuron cluster containing a male-specific ipsilateral neurite in the central nervous system (CNS) (Kimura et al. 2005). In females, a subset of mAL neurons dies by programmed cell death. In males, FRU^M proteins prevent the male counterparts from dying, allowing the development of mAL neurons. FRU^M proteins are also necessary for the development of the muscle of Lawrence (MOL), an abdominal muscle present only in males (Usui-Aoki et al. 2000; Anand et al. 2001). All FRU isoforms contain a BTB (Broad-complex, Tramtrack, and Bric-a-brac) domain and a C-terminal C2H2 zinc finger domain for the DNA-binding function (Zollman et al. 1994). Recently, the binding sequences for different FRU isoforms have been identified (Dalton et al. 2013; Neville et al. 2014). However, to date, no direct target gene has been validated for *fru*.

13.3 *Sxl* Orthologues and Their Sex Determination Role in Nondrosophilid Species

Is *Sxl*, the master regulatory switch for sex determination in *D. melanogaster*, conserved among insects? Several *Sxl* orthologues thus far characterized in dipteran species other than *D. melanogaster* have no sex-determining role. For example, the *Sxl* orthologues in the medfly (*C. capitata*), housefly (*Musca domestica*), and scuttlefly *M. scalaris* encode proteins similar to the SXL of *D. melanogaster* but do not show sexual differences in those expression patterns (Saccone et al. 1998; Meise et al. 1998; Sievert et al. 2000). Furthermore, transgenic expressions of these orthologues in *D. melanogaster* failed to induce feminization in males (Saccone et al. 1998; Meise et al. 1998), suggesting an alteration of protein functions. In nondipteran insects, *Sxl* orthologues identified from the silkworm, *B. mori*, were expressed in both sexes equally (Niimi et al. 2006). Taken together with these findings, sex-specific expression and the protein role of *Sxl* in *Drosophila* are not conserved among insects.

Hu antigen B (HuB), HuC, HuD, and HuR proteins belong to the ELAV/Hu protein family (Antic and Keene 1997; Wakamatsu and Weston 1997). HuR is expressed ubiquitously in various tissues, while the other ELAV/Hu proteins are expressed predominantly in neuronal cells and are involved in neuronal differentiation (Good

1995; Ma et al. 1996; Antic et al. 1999). The ELAV/Hu proteins have three copies of RNA-binding domains (RBDs). Interestingly, the amino acid sequences of the N-terminal two RBDs (RBD1 and RBD2) of ELAV/Hu proteins are significantly homologous (53% identity) to those of SXL (Szabo et al. 1991). NMR studies on RBDs of mouse HuC demonstrated that the two N-terminal RBD (RBD1 and RBD2) structures are quite similar to those of SXL (Inoue et al. 2000). In addition, RBD1-RBD2 binds specifically to a longer ARE RNA, UAUUUAUUUU, which is highly similar to the RNA sequence UGUUUUUUUU, which is preferentially bound by SXL RBD1-RBD2 (Handa et al. 1999). These findings indicate that the RNA-binding properties of the vertebrate HuC and SXL are similar, even though the target genes and the biological functions of the proteins are different. With regard to *Sxl* in *Drosophila*, it is postulated that not only the gene expression but also an altered protein function may have contributed to the gain of the sex determination function in *Drosophila*.

13.4 *tra* Orthologues and Their Sex Determination Role in Nondrosophilid Species

tra was considered to be a *Drosophila* genome–specific gene because *tra* orthologues showed an unusually high degree of evolutionary divergence when compared within *Drosophila* subgenus species (O'Neil and Belote 1992). The identification of *tra* orthologues from the medfly (*C. capitata*) and the olive fruit fly (*Bactrocera oleae*) changed this notion (Pane et al. 2002; Lagos et al. 2007). These *tra* homologues (*Botra* and *Cctra*) have a female-determining master function (Pane et al. 2002; Lagos et al. 2007). To date, the *tra* gene has been identified not only in dipteran and coleopteran species but also in more than a dozen hymenopteran species (ants and bees), which are the most basal order of the holometabolous insects (Schmieder et al. 2012; Geuverink and Beukeboom 2014). Six of seven sequenced ants have two copies of *tra*, resulting from an ancestral duplication rather than independent duplications in each of the six species (Privman et al. 2013). In three bee species where whole-genome information is available, the *tra* gene is also duplicated (Privman et al. 2013). One copy is named *complementary sex-determiner* (*csd*), which is the primary signal for sex determination in the honeybee, *A. mellifera* (Beye et al. 2003). It activates the second copy, designated as *feminizer* (*fem*), which is more conserved and retains the ancestral function to regulate its downstream target, *dsx* (Hasselmann et al. 2008). *csd* is considered to have arisen from duplication of the *fem* gene (Schmieder et al. 2012).

RNA interference (RNAi)–mediated knockdown of *tra* in *Musca* (Hediger et al. 2010), *Ceratitis* (Pane et al. 2002), *Anastrepha* (Ruiz et al. 2007), *Lucilia* (Concha and Scott 2009), *Apis csd* (Beye et al. 2003), *Apis fem* (Hasselmann et al. 2008), *Nasonia* (Verhulst et al. 2010), and *Tribolium castaneum* (Shukla and Palli 2012) caused disruption of endogenous *tra* function in these species and subsequent

male-specific splicing of the endogenous *dsx* pre-mRNAs, leading to the transformation of chromosomally female embryos into adult pseudomales. On the other hand, several previous studies and recent genome-wide analysis in a wide range of insect species revealed that *tra* shows a distinctly patchy distribution among insects (Geuverink and Beukeboom 2014). For example, no *tra* homologues have been found in lepidopteran insects, including *B. mori*, *Danaus plexippus*, and *Heliconius melpomene* (Mita et al. 2004; Geuverink and Beukeboom 2014) (Fig. 13.2). The most interesting pattern of *tra* distribution is seen in the Diptera genus. The *tra* gene is found in brachycera species, including *D. melanogaster*, while several mosquito species, which belong to a basal dipteran lineage, do not possess *tra* (Geuverink and Beukeboom 2014) (Fig. 13.2). The situation is the same in Coleoptera. One of the coleopteran species, *T. castaneum*, carries a *tra* orthologue (Shukla and Palli 2012), while the *tra* gene has not to date been found in the genome of the coleopteran *Dendroctonus ponderosae* (Geuverink and Beukeboom 2014). *Mengenilla moldrzyki*, which belongs to Strepsiptera—a sister order to Coleoptera—appears to lack a *tra* homologue (Geuverink and Beukeboom 2014) (Fig. 13.2).

TRA protein contains several conserved domains; one is shared only among Diptera (the "dipteran domain"), and another domain is present only in Hymenoptera (the "hymenopteran domain"). The most conserved part of the TRA protein is the TRA-CAM (C, *Ceratitis*; A, *Apis*; and M, *Musca*) domain (Hediger et al. 2010). This domain is found in all TRA proteins, except for those in *D. melanogaster*. Another conserved feature of TRA is a proline-rich region located near the C-terminus. This region is also present in all *tra* genes so far characterized in insects (Geuverink and Beukeboom 2014). However, the functional importance of the TRA-CAM domain and the proline-rich region remains unknown. The splicing pattern of all insect *tra* transcripts shows sexual dimorphism, and the female-specific splice variant only encodes a functional protein (Bopp et al. 2014).

The *tra* orthologue has also been isolated from two noninsect species, *Daphnia magna* and *Daphnia pulex*. They belong to the subphylum Crustacea, which is regarded as a sister group to insects (Kato et al. 2010; Chen et al. 2014) (Fig. 13.2). Recently, we have isolated a *tra* homologue in the acorn worm *Saccoglossus kowalevskii*, which is a hemichordate belonging to the superphylum Deuterostomia (Suzuki et al. 2015). *D. magna transformer* (*DmagTra*), *D. pulex transformer* (*Dptra*), and *S. kowalevskii tra* (*Sktra*) also contain the highly conserved TRA-CAM domain and the arginine/serine-rich region near the N-terminus. Unlike insect *tra* genes, all of these *tra* orthologues show no sexual dimorphism in their splicing patterns (Kato et al. 2010; Chen et al. 2014; Suzuki et al. 2015). However, the expression level of *Dptra* has been shown to be significantly higher in sexually mature males than in ephippial females, implying that *Dptra* might play an important role in switching between reproduction modes and sexual differentiation in *D. pulex*, but this requires further confirmation (Chen et al. 2014). Similarly, the mRNA level of *Sktra* was approximately 7.5-fold greater in the testes than in the ovaries, suggesting that *Sktra* might be involved in sexual differentiation of *S. kowalevskii* (Suzuki et al. 2015).

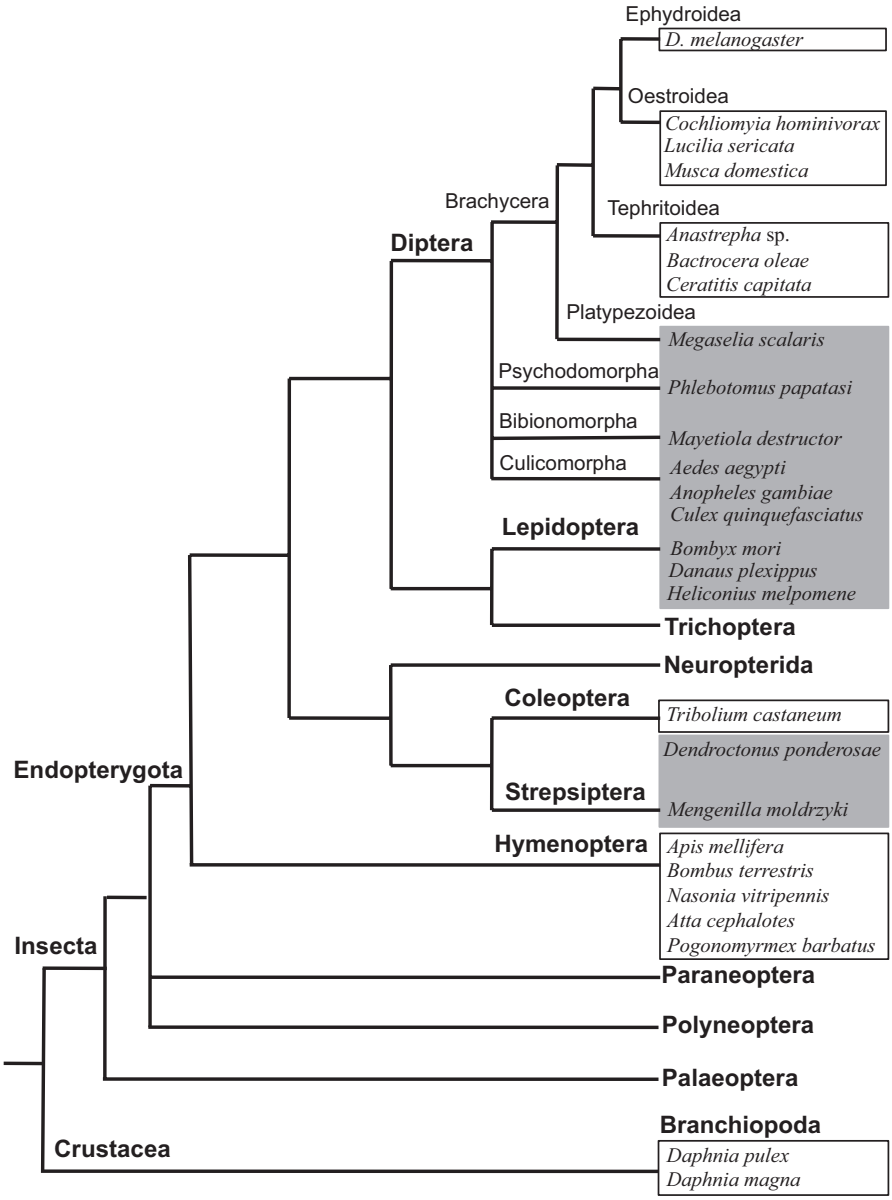

Fig. 13.2 Schematic diagram of the phylogenetic distribution of *tra*. The name of each species is noted on the *right side*. Species with *tra* are *squared*, while species lacking *tra* are in the *gray box*. The phylogenetic tree simply indicates the comparative relationship between each species, thus the branch length in the tree is not precisely indicated

These findings support the idea that the *tra* gene evolved independently in insect species and acquired a sex-determining gene function, especially as a *dsx*-splicing regulator. However, as described above, *tra* is present in the orders Hymenoptera, Coleotpera, and Diptera but, thus far, has not been found in Lepidoptera or in the basal lineages of Diptera (Geuverink and Beukeboom 2014), implying multiple independent losses or recruitment of *tra* into the sex determination cascade. Vertebrates and multiple metazoan phyla, including arthropods and nematodes, lack *tra*, which may be dispensable for embryogenesis, developmental processes, and some other biological processes. Interestingly, *dsx* genes in five Cladocera species (including *D. magna* and *D. pulex*), which are the closest relatives to the insects, are also expressed dominantly in males and do not exhibit the sex-specific splicing typical of insect *dsx* (Toyota et al. 2013). Unlike insect *tra* genes, both *D. magna tra* and *D. pulex tra* show no sexual dimorphism in their splicing patterns (Kato et al. 2010; Chen et al. 2014). These findings have led to the inference that the *tra–dsx* regulatory axis evolved independently in insect species.

13.5 *tra* as a Memory Device for Female Sexual Development

In tephritid fruit flies—such as *C. capitata*, *B. oleae*, and 12 *Anastrepha* species—the *Sxl* gene is not regulated in a sex-specific manner and does not appear to play the key discriminating role (memory device) in sex determination that it plays in *Drosophila* (Saccone et al. 1998; Lagos et al. 2007). As in the drosophilids, the tephritid *tra* gene is constitutively expressed in both sexes and its pre-mRNA undergoes sex-specific alternative splicing. In contrast to the *Drosophila* situation in which *Sxl* regulates *tra*, in the tephritids the gene *tra* acts as the memory device for sex determination via its autoregulatory function, i.e., through the contribution of the TRA protein to the female-specific splicing of its own pre-mRNA. The *tra* gene in the tephritids has male-specific exons that contain translation stop codons. The inclusion of these exons in mature *tra* mRNA in males results in the production of a truncated, nonfunctional TRA protein. In females, the male-specific exons are spliced out because of the presence of TRA protein (Pane et al. 2002; Lagos et al. 2007; Ruiz et al. 2007). The presence of putative TRA-TRA2 binding sites in the male-specific exons and in the surrounding introns may suggest that the TRA interacts with its own pre-mRNA through TRA-2, resulting in skipping of the male-specific exons. It has been found that TRA-2 proteins in *M. domestica* and *C. capitata* are required for female-specific splicing of *tra* pre-mRNAs (Burghardt et al. 2005; Salvemini et al. 2009; Hediger et al. 2010). In *Ceratitis* and *Musca*, it is postulated that maternal *tra* mRNAs trigger the autoregulatory loop in XX (female) embryos. The zygotically produced TRA protein controls the maintenance of *tra* autoregulation and female-specific splicing of *dsx* pre-mRNA. The resulting DSXF protein induces female development. In XY (male) embryos, the male-determining *M* factor located on the Y chromosome prevents *tra* autoregulation through unknown mechanisms. Therefore, TRA protein is not produced in XY embryos, and thus the

autoregulatory loop cannot initiate. Because of the absence of TRA protein, *dsx* pre-mRNA is spliced by default to yield DSXM protein, which induces male development (Pane et al. 2002; Hediger et al. 2010). A similar autoregulatory function is also thought to occur in the beetle *T. castaneum* (Shukla and Palli 2012).

The *fem* gene—a *tra* homologue in the honeybee, *A. mellifera*—needs its protein product to splice its own pre-mRNA into the productive female form, which establishes an autoregulatory feedback loop to maintain the female state throughout the bee's life (Gempe et al. 2009). The *csd* gene, which is a paralogue of *fem*, initiates the autoregulatory loop (Hasselmann et al. 2008). The female mode of *fem* splicing occurs only when the *csd* presents in the heteroallelic condition. The female form of Fem protein is active and induces female splicing of *A. mellifera dsx* (*Am-dsx*) pre-mRNA, resulting in the production of Am-DSXF. In the absence of Csd protein activity in males, where the *csd* gene is homo- or hemiallelic, *fem* pre-mRNA is spliced into the male form, which includes the male-specific exons containing an intervening stop codon. The male form of Fem protein is inactive; therefore, *Am-dsx* pre-mRNA is spliced by default to produce Am-DSXM protein. As in the dipteran insects *M. domestica* and *C. capitata*, Am-TRA2 protein acts together with heteroallelic Csd proteins and/or Fem proteins to mediate female *fem* splicing by binding to *fem* pre-mRNAs (Nissen et al. 2012).

The jewel wasp *N. vitripennis* employs a similar but slightly different autoregulatory function in the regulation of *tra* gene (*Nvtra*) expression. In this species, sex is determined on the basis of the ploidy of embryos (Heimpel and de Boer 2008); males are haploid, developing from unfertilized eggs, whereas diploid females develop from fertilized eggs. In diploid eggs, maternally provided *Nvtra* mRNA initiates the female-specific autoregulatory loop required to maintain the female state throughout the wasp's life (Verhulst et al. 2010). In haploid (unfertilized) eggs, it is postulated that zygotic transcription from the maternal *Nvtra* allele is prevented as a result of maternal imprinting, leading to male development.

13.6 *dsx*, the Most Conserved Regulatory Switch in Sex Determination

Orthologues of *dsx* have been found in each insect species examined, including Diptera, Lepidoptera, Coleoptera, and Hymenoptera species (Bopp et al. 2014). Primary transcripts from *dsx* genes thus far identified undergo sex-specific alternative splicing, producing either a male-specific (DSXM) or female-specific (DSXF) isoform (Schütt and Nöthiger 2000; Geuverink and Beukeboom 2014). The female-specific splicing of *dsx* transcripts in Diptera and Coleoptera is induced by TRA together with TRA-2 through canonical TRA-TRA2 binding sites, which are consistently present in the female-specific exon (Burghardt et al. 2005; Salvemini et al. 2009; Sarno et al. 2010; Shukla and Palli 2012). Interestingly, *dsx* pre-mRNAs in the honeybee, *A. mellifera*, lack the canonical binding sites of TRA-TRA2 proteins, even though its female-specific splicing needs Am-TRA2 proteins (Nissen et al.

2012). This finding suggests that the TRA-TRA2 protein-binding sites have evolved independently in the hymenopteran species. Consistent with this hypothesis, Am-TRA2 appears to have several amino acid replacements in the RNA-binding domain (RBD), one of which is a critical amino acid residue for female processing of *dsx* pre-mRNAs in *D. melanogaster* (Amrein et al. 1994). The *dsx* gene in the silkworm, *B. mori*—designated as *Bmdsx*—also lacks the canonical TRA-TRA2 binding sites (Suzuki et al. 2001). In contrast to *dsx* in other insect species, female-specific *Bmdsx* transcripts are produced by default splicing (Suzuki et al. 2001). TRA-2 proteins in this species are not relevant to sex-specific splicing of *Bmdsx* (Suzuki et al. 2012).

Geuverink and Beukeboom (2014) recently identified putative *dsx* orthologues in a number of primitive insect groups. For example, the human body louse, *Pediculus humanus corporis*, has a hypothetical protein composed of both a DM (Dsx/Mab-3 DNA-binding motif) domain and a dimerization domain (OD2). The DM domain is found in multiple genes of the DM superfamily group consisting of *dsx*, *mab3*, and related transcription factors (*DMRT*), while the dimerization domain is exclusively found in *dsx*. Five Cladocera species (*Daphnia magna*, *Daphnia pulex*, *Daphnia galeata*, *Ceriodaphnia dubia*, and *Moina macrocopa*), which are the closest relatives to the insects examined, also possess *dsx* genes (Kato et al. 2011; Toyota et al. 2013).

The *DMRT* genes appear to play a role in sex determination or sexual differentiation in all Metazoa; therefore, this gene family may represent the first example of sexual regulatory genes conserved across phyla (Hodgkin 2002; Kopp 2012). In vertebrates, orthologues of *DMRT1* are expressed dominantly in males and are required only to guide male sex determination (Matson and Zarkower 2012). Therefore, it is conceivable that animal species in which *DMRT1* is responsible for maleness do not necessarily need a *dsx*-splicing regulator such as an insect TRA. Interestingly, *dsx* genes in five Cladocera species (including *D. magna* and *D. pulex*), which are the closest relatives to the insects, are also expressed dominantly in males and do not exhibit the sex-specific splicing typical of insect *dsx* (Toyota et al. 2013). Unlike insect *tra* genes, both *D. magna* and *D. pulex tra* show no sexual dimorphism in their splicing patterns (Kato et al. 2010; Chen et al. 2014). These findings lead to the inference that the sex-specific splicing feature in *dsx* was acquired early in the evolution of insects and that the *tra–dsx* regulatory axis evolved independently in insect species.

13.7 *fru*, Another Downstream Regulator in the Sex Determination Cascade

In *D. melanogaster*, the male-specific splice isoform of the *fru* gene (FruM) encodes a set of transcription factors involved in the regulation of male courtship and copulation, as described above. Recent insights from nondrosophilid insects suggest a conserved evolutionary role for the transcription factor Fruitless. In the housefly, *M. domestica*, male-specific transcripts from the *fru* orthologue *Md-tra* are generated

by a conserved mechanism of sex-specific splicing (Meier et al. 2013). As in *Drosophila*, *Md-fru* is similarly involved in controlling male courtship behavior. A male courtship behavioral function for *Md-fru* was revealed by behavioral and neuroanatomic analyses with a hypomorphic allele of *Md-tra*, which specifically disrupts the expression of *Md-fru* in males, leading to severely impaired male courtship behavior. In addition, expression of *Md-fru* is confined to neural tissues in the brain, most prominently in the optic neuropil and in peripheral sensory organs (Meier et al. 2013). In the mosquitoes *Anopheles gambieae* and *A. aegypti*, *fru* orthologues show conservation of sex-specific alternative splicing and male-specific protein expression in neural tissues (Gailey et al. 2006; Salvemini et al. 2013). The female-specific exons of both genes each contain short sequences resembling the TRA-TRA2 binding sites but showing degeneration and lack of a consensus. Therefore, it is postulated that sex-specific splicing of *fru* transcripts in the mosquitoes is most likely under the control of splicing regulatory factors, which are different from TRA and TRA-2 found in other dipteran insects. This is consistent with the fact that sex determination in *A. aegypti* is different from that in *Drosophila*, where the key male determiner *M*, located on one of a pair of homomorphic sex chromosomes, controls sex-specific splicing of the *dsx* and *fru* orthologue (Hall et al. 2015). Moreover, a *tra* orthologue seems to be absent from both *Anopheles* and *Aedes* genomes (Gailey et al. 2006; Nene et al. 2007). In the hymenopteran *N. vitripennis*, the *fru* architecture is essentially identical to that in *Drosophila*, and the P1 transcripts, derived from the most distal *fru* promoter, undergo a conserved sex-specific splicing regulation (Bertossa et al. 2009). These findings suggest that conserved *fru* sex-specific splicing evolved prior to the divergence between Hymenoptera and Diptera (250–300 million years ago) rather than being acquired independently in both lineages.

In Orthoptera, which belongs to a phylogenetically more basal insect lineage, *fru* orthologues have also been isolated, but the situation is different from that of the *fru* genes in Diptera (Salvemini et al. 2010). For example, *fru* orthologues thus far identified in several *Chorthippus* grasshopper species, the desert locust *Schistocerca gregaria*, and the cockroach *Blatella germanica* show no sexual dimorphism in those splicing patterns (Ustinova and Mayer 2006; Boerjan et al. 2011; Clynen et al. 2011). In spite of this, RNAi knockdown experiments have demonstrated that *fru* orthologues in *S. gregaria* and *B. germanica* play important roles in regulation of successful copulation in the adult male and in male sexual behavior, respectively (Boerjan et al. 2011; Clynen et al. 2011). In addition, the spermatheca of female copulation partners of *fru* knockdown males in both species contained fewer or no spermatozoids, resulting in a reduction in progeny numbers in their naïve female copulation partners. This seemed likely due to smaller numbers of spermatozoa in the male seminal vesicles, caused by *fru* knockdown (Boerjan et al. 2012). These findings suggest that the role of fruitless as a master regulator of male sexual behavior has been conserved in insect evolution, at least from cockroaches to flies. This raises the question of whether the *fru* gene exists not only in insect genomes but also in vertebrate genomes, like its counterpart *dsx*, which is at the bottom of the sex determination cascade. Homology searches of reported sequences using *fru* BTB and ZnF domains as virtual probes resulted in no hits outside insects (Gailey et al. 2006).

13.8 Sex Determination Mechanisms in Insects Lacking the *tra* Gene

As described above, the *tra* gene acts as a memory device for female sexual development and thus plays a central role in the sex determination cascade. Conservation of the *tra* orthologue in more than a dozen hymenopteran species, which are the most basal order of the holometabolous insects, strengthens the importance of this gene in insect sex determination. On the other hand, considerable numbers of insect species appear to lack a *tra* orthologue, even though these species have sex-specifically spliced *dsx* and *fru* orthologues, both of which are well known as *tra*-downstream targets (Suzuki et al. 2001; Gailey et al. 2006; Nene et al. 2007; Salvemini et al. 2013). How do they control sex-specific splicing of *dsx* and *fru*, and determine their sexual fate, without a *tra* gene?

Maleness in the mosquito *A. aegypti* is determined by an *M* factor located on the homomorphic sex-determining chromosome within a Y chromosome–like region called the M locus (Newton et al. 1974). To date, no *tra* orthologues have been found in the *A. aegypti* genome. Consistent with this, there are no well-conserved TRA-TRA2 binding sites in both *fru* and *dsx* pre-mRNAs (Salvemini et al. 2011, 2013). *A. aegypti* has four *tra-2* paralogues, which phylogenetically form a single clade, apart from the other known dipteran and even nondipteran orthologues (Salvemini et al. 2013). It is therefore postulated that these *tra-2* paralogues evolved to have different sequence binding specificity and novel functions under selective pressure relaxation after gene duplication. Recently, Hall et al. (2015) reported that an M locus gene, *Nix*, which is a distant homologue of *tra-2*, functions as an *M* factor in *A. aegypti*. *Nix* knockout resulted in largely feminized genetic males and the production of female forms of *dsx* and *fru*. These findings suggest the possibility that *tra-2* paralogues may have acquired the function of a primary sex-determining gene during evolution in insects that lack a *tra* orthologue.

The silkworm, *B. mori*, also lacks a *tra* gene (Mita et al. 2004; Geuverink and Beukeboom 2014). Moreover, TRA-2 proteins in this species are not relevant to the sex-specific splicing of *Bmdsx* (Suzuki et al. 2012). In contrast to *dsx* in other insect species, female-specific *Bmdsx* transcripts are produced by default splicing (Suzuki et al. 2001). In accordance with these findings, *Bmdsx* lacks the canonical TRA-TRA2 binding sites (Suzuki et al. 2001). In this species, male splicing of *Bmdsx* transcripts requires the splicing inhibitor (BmPSI) and the male-specific isoform of IMP (IMP^M) proteins. These proteins form a complex that binds to a cis-regulatory element called CE1, located in the female-specific exon, and inhibits the female mode of splicing in males (Suzuki et al. 2008, 2010). Our previous data suggested that Imp^M maintains its male-specific mode of expression by an autoregulatory function and thus may function as a memory device for male development (Suzuki et al. 2014). Alternative splicing regulation by an autoregulatory function is not restricted to the female-specific splicing of *tra* and is also seen in common splicing regulators. For example, SRp20 and ASF/SF2, both of which belong to a highly conserved SR family of splicing factors, autoregulate the alternative splicing of

their own pre-mRNAs (Ge et al. 1991; Jumaa and Nielsen 1997). It could therefore be expected that the autoregulatory function originally present in the *Imp* gene may have been recruited to the male-determining pathway and utilized as a memory device for male development during evolution. *Imp* is a Z chromosome–linked gene whose dosage is twice as high in males as in females (Suzuki et al. 2010). The *Imp* orthologue is located on the Z chromosome in another lepidopteran species, *Samia cynthia ricini*, which lacks a W chromosome (Yoshido et al. 2011). If the *Imp* orthologue in this species also produces a male-specific splice isoform, then it is highly possible that such a male isoform of *Imp* may function as a memory device for male sexual development.

It has been shown genetically that female sex in *B. mori* is determined by the presence of a dominant feminizing factor (*Fem*) on the W chromosome (Hashimoto 1933). The W chromosome in *B. mori* lacks protein-coding genes and is almost completely occupied by selfish repetitive elements such as transposons (Abe et al. 2005). The only transcripts produced from the W chromosome are PIWI-interacting RNAs (piRNAs) (Kawaoka et al. 2011). piRNAs are 23–30 nucleotides of small RNAs acting as sequence-specific guides for PIWI proteins that cleave target RNAs mainly to disrupt the activity of transposons in the gonads (Garabedian et al. 1986; Malone and Hannon 2009). Recently, one of the piRNAs originating from the sex-determining region of the W chromosome appeared to function as *Fem* (Kiuchi et al. 2014). Inhibiting the expression of *Fem* piRNA in female embryos not only induced Imp^M expression (Sakai et al. 2015) but also shifted the splicing pattern of *Bmdsx* from the female to the male forms, suggesting that this piRNA is required for femaleness (Kiuchi et al. 2014; Sakai et al. 2015). *Fem* piRNA targets and cleaves mRNAs transcribed from the Z chromosome–linked gene that encodes a CCCH-tandem zinc finger protein. Knockdown of this gene expression in male embryos decreased the Imp^M expression (Sakai et al. 2015) and led to the production of the female-type *Bmdsx* transcripts (Kiuchi et al. 2014; Sakai et al. 2015), indicating that this gene is essential for silkworm masculinization, and it was therefore named *Masculinizer* (*Masc*). Recently our studies using transgenic silkworms have demonstrated that forced expression of *Masc* in females inhibits the normal development of ovaries and induces the formation of spermatids in the abnormal tissues [manuscript in preparation].

On the basis of these findings, we have proposed a genetic cascade regulating sex determination in *B. mori*, as follows (Fig. 13.3). In females (ZW), *Fem* piRNA disrupts the expression of *Masc*. Lower expression of *Masc* fails to induce expression of Imp^M. In the absence of Imp^M, *Bmdsx* pre-mRNA is spliced by default to generate the female-type BmDSX protein, which promotes female development. In males (ZZ), *Masc* expression remains at a higher level than in ZW individuals. This boosts the expression of *Imp* to a level sufficient to impose the male splicing mode on its own RNA. Once IMP^M protein is produced, its male-specific expression is maintained by an autoregulatory function. IMP^M protein, together with BmPSI, is altered to produce the male-specific splicing of *Bmdsx*. The male-type BmDSX protein promotes male development.

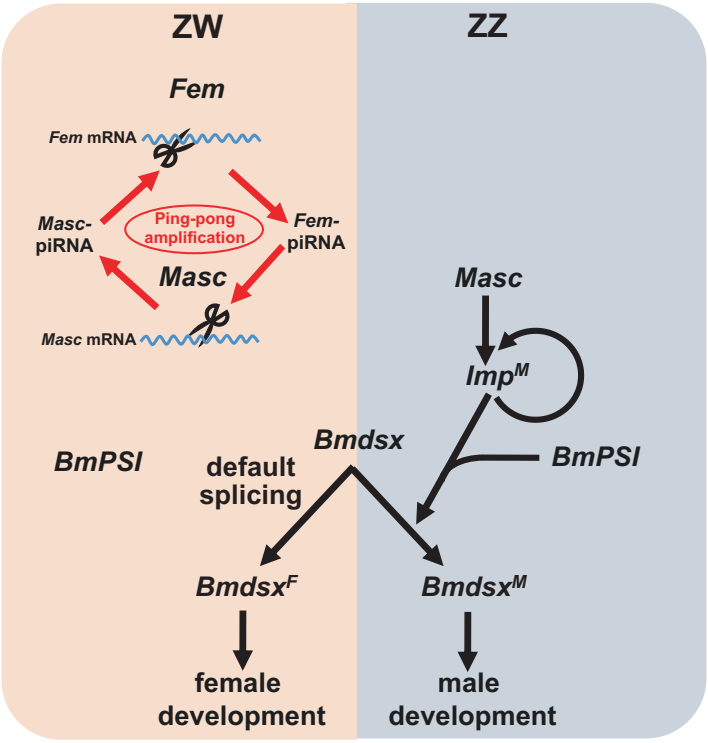

Fig. 13.3 Proposed genetic cascade regulating sex determination in *Bombyx mori*. In females (ZW), transcripts from the *Fem* locus are cleaved by the maternally transmitted *Masc* PIWI-interacting RNA (piRNA)–BmAgo3 complex. The *Fem* piRNA–Siwi complex cleaves *Masc* mRNA, resulting in the accumulation of *Masc* piRNA and further enhancement of feminization (for more details, refer to the review described by Katsuma et al. in 2015). Lower expression of *Masc* fails to induce expression of Imp^M. In the absence of Imp^M, *Bmdsx* precursor messenger RNA is spliced by default to generate the female-type BmDSX protein ($BmDSX^F$), which promotes female development. In males (ZZ), *Masc* expression remains at a higher level than in ZW individuals. This initiates the expression of Imp^M. Once the IMP^M protein is produced, its male-specific expression is maintained by an autoregulatory function. IMP^M protein, together with BmPSI, is altered to produce the male-specific splicing of *Bmdsx*. The male-type BmDSX protein ($BmDSX^M$) promotes male development

To date, *Fem* piRNA has been found only in *Bombyx* genomes, while its downstream target *Masc* is conserved among several lepidopteran species, some of which lack a W chromosome (Kiuchi et al. 2014). In such species, a Z chromosome–counting mechanism, such as XES factors in *Drosophila*, might regulate *Masc* expression, or a difference in the gene dosage of *Masc* itself may determine the sexual fate: ZZ animals with two genes develop into males, while ZO animals with only one *Masc* gene develop into females. A similar sex-determining mechanism has been reported in the chicken, which has a Z-linked *dmrt1* gene. In this species, where the homogametic sex is male (ZZ) and the heterogametic sex is female (ZW),

it is considered that two copies of the *dmrt1* gene promote male differentiation, while a single copy of the *dmrt1* gene promotes female differentiation (Smith et al. 2009).

B. mori presents the first known example of a sex-determining pathway controlled by the presence or absence of a piRNA. This *tra*-lacking insect also provides the first known example of a sex-specific splicing regulatory mechanism of *dsx* controlled by PSI and IMP orthologues. Whether this pathway is evolutionarily conserved among lepidopteran species will certainly be the subject of future research.

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Chapter 14
Genetic Control of Sex Determination and Differentiation in Fish

Masaru Matsuda

Abstract The sex of an individual is determined by the type of reproductive organs that they have, i.e., testes or ovaries. These reproductive organs originate from bipotential gonads. Therefore, sex is determined by the direction of gonadal development of a bipotential gonad, which can develop into either a testis or an ovary. Various strategies to determine the sex of a gonad are found in fish, and these strategies range from gonochorism, in which ovaries or testes are developed in different individuals, to sequential hermaphroditism including protogyny, protandry, and bidirectional sex change, and synchronous hermaphroditism. In gonochoristic species, the direction of gonadal development must be decided at a specific time. Regarding the timing, a cue establishes the bifurcation point, and the cue is either a genetic or an environmental factor. To describe how these cues control sex differentiation, this chapter focuses on gonochoristic fish. This chapter first describes the sex-determining genes (i.e., genetic cues) in fish and then summarizes gonadal development including sex reversals induced by non-genomic factors (environmental cues) and genomic factors.

Keywords *Oryzias* · Medaka · Master sex-determining gene · Gonadal development · Sex reversal

14.1 Introduction

Sexual reproduction is an important strategy to maintain genetic diversity in a species. For sexual reproduction, a species must make one of two types of gametes in different gonads, i.e., eggs in the ovary and sperm in the testis. Although this is common among animals, there is variation in the strategies to develop two types of reproductive organs. A common strategy is gonochorism, in which an individual

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either develops ovaries or testes. The other less common strategies are sequential hermaphroditism (e.g., protogyny, protandry, and bidirectional sex change) and synchronous hermaphroditism.

Mammals and birds are solely gonochoristic. In mammals, the sex is determined by the genetic male heterogametic system (XX–XY). In birds, the female heterogametic system (ZZ–ZW) is used. The sex-determining gene in mammals (*SRY/Sry*), which is located on the Y chromosome, was identified in 1999 (Sinclair et al. 1990; Koopman et al. 1991). This gene is common in all eutherians. In mammals undergoing normal development, there are no other sexual development options, such as hermaphroditic gonadal development and female heterogametic sex determination. However, fish species exhibit wide variation in sexual development options, e.g., gonochoristic or hermaphroditic gonadal development. Furthermore, sex determination in fish is not only influenced by genes but also the environment. Therefore, the fish model allows us to understand the mechanisms of sex determination and gonadal development in vertebrates.

The sex of a gonochoristic organism is decided at the moment of fertilization (i.e., the bifurcation point). In the male heterogametic XX–XY sex-determination system, the sex of an individual is decided by what chromosome the sperm is carrying (i.e., an X or Y chromosome) when it fertilizes an egg carrying the X chromosome. The gonochoristic organism must develop a reproductive organ, i.e. an ovary or testis. These organs originate from a bipotential gonad, which can develop into either an ovary or testis (but not both). A cue is received at the bifurcation point. When the cue is due to gene expression, the process is referred to as the genetic sex-determination system. When the cue is caused by environmental factors, the process is referred to as the environmental sex-determination system.

14.2 Sex Determination in Fish

In the genetic sex-determination system, a gene works as a cue to determine the direction of gonadal development. This gene is referred to as the master sex-determining gene. This gene is located on the top of the gene cascade for gonadal development. To date, six master sex-determining genes have been identified in fish.

14.2.1 The Dmy Gene in Oryzias latipes

After identifying the master sex-determining gene in mammals, the second sex-determining gene in vertebrates was identified in a fish species (Matsuda et al. 2002). In Medaka (*O. latipes*), a gene that encodes the DM (*Dsx* and *Mab-3*) domain was named as *dmy* (i.e., the DM domain gene on the Y chromosome). This gene was also identified as a strong candidate for the sex-determining gene in Medaka via a genomic survey for homologs of *dmrt1*, and the authors named the gene *dmrt1b*(Y) (Nanda

et al. 2002; Volff et al. 2003). The DM domain is a zinc finger-like DNA-binding motif that is found in both the *Caenorhabditis elegans mab-3* gene (Shen and Hodgkin 1988) and the *Drosophila dsx* gene (Baker and Ridge 1980). This gene was found in the sex-determining region of the Y chromosome of Medaka by the positional cloning method, which is a method based on information of the chromosomal region that is responsible for sex determination. *Dmy* mRNA and DMY protein were detected in somatic cells surrounding germ cells from stage 36 (2–3 days before hatching) to formation of the adult testis (Kobayashi et al. 2004).

The frame-shift type mutation in the *dmy* coding region causes sex-reversal (Matsuda et al. 2002). Although a Medaka carrying *dmy* exhibits an XY sex chromosome type, the organism carrying the mutated *dmy* develops as a female and lays normal eggs. Moreover, transgenic *dmy* demonstrated that *dmy* is sufficient for male development. When XX individuals have exogenous *dmy*, the individuals develop as normal males (Otake et al. 2010).

Closely related species of *O. latipes* include *O. sakaizumii*, *O. sinensis*, *O. curvinotus*, *O. luzonensis*, *O. mekongensis*, and *O. dancena* (Takehana 2011; Takehana et al. 2005) (Fig. 14.1). *Dmy* was found in *O. sakaizumii*, *O. sinensis*, and *O. curvinotus*. In these species, *dmy* is located on a syntenic chromosome harboring *dmy* in *O. latipes* (Matsuda et al. 2003). Conversely, it was not found in *O. luzonensis* and *O. mekongensis* (Kondo et al. 2003). Furthermore, the sex chromosomes of *O. luzonensis* and *O. mekongensis* have conserved synteny with linkage group 12 (LG12) (Hamaguchi et al. 2004) and 2 (LG2) (Tanaka et al. 2007) of *O. latipes*, respectively. Given that *dmy* on LG1 is originally a gene duplicated from *dmrt1* on LG9 (Kondo et al. 2006), the duplication should occur in a common ancestor of *O. latipes*, *O. sakaizumii*, *O. sinensis*, *O. curvinotus*, and *O. luzonensis* or more ancestral species.

14.2.2 *Gsdf^{Y} (Gonadal Soma-Derived Growth Factor on the Y Chromosome) in O. luzonensis*

O. luzonensis is the closest species to *O. curvinotus*, and consists of a monophyletic group including *O. latipes* (Fig. 14.1). Therefore, it was assumed that *O. luzonensis*, *O. curvinotus*, and *O. latipes* share a common ancestor and the sex-determining gene of that common ancestor was *dmy*. However, *dmy* was not found in *O. luzonensis*.

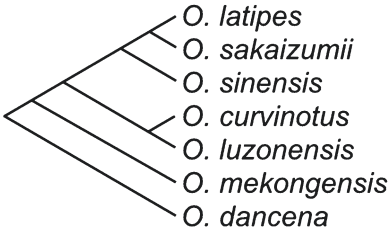

Fig. 14.1 Phylogenetic relationships among *Oryzias* species. The phylogenetic tree was derived from Takehana et al. (2005)

The leading candidate of the master sex-determining gene is *gsdf*^{Y}, which has been identified by positional cloning (Myosho et al. 2012). This gene is not related to *dmy*. *Gsdf*^{Y} contains an amino acid motif that is categorized as part of the TGF beta family.

Single-nucleotide polymorphism (SNP)-based genotyping was performed to map the sex-determining region on the Y chromosome (Tanaka et al. 2007). The sex-determining region on the *O. luzonensis* Y chromosome was narrowed by chromosome walking using the bacterial artificial chromosome (BAC) genomic clone (Myosho et al. 2012). Within the region that was narrowed to approximately 180 kb, one predicted gene was expressed differently between XY and XX embryos. This gene was a homolog of *gsdf*, which was identified in *O. latipes* (Shibata et al. 2010) and originally identified in *Oncorhynchus mykiss* (Sawatari et al. 2007).

In *O. luzonensis*, full-length mRNA of *gsdf* on the Y chromosome (*gsdf*^{Y}) had 12 base pairs different to those on the X chromosome (*gsdf*^{X}), including two synonymous substitutions in the coding region (Myosho et al. 2012). However, the amino acid sequences of *gsdf*^{X} and *gsdf*^{Y} were the same. Both *gsdf*^{Y} and *gsdf*^{X} were expressed in somatic cells surrounding the germ cells. However, the initiation of the expression mRNAs differed. In XY (*O. luzonensis*), both *gsdf*^{Y} and *gsdf*^{X} mRNAs were expressed at the same level at 5 days after hatching, whereas *gsdf*^{Y} mRNA expression was increased sixfold compared with *gsdf*^{X} at 5 days after hatching. The *cis*-regulatory region contained 13 nucleotide differences between *gsdf*^{Y} and *gsdf*^{X}. Given that the *gsdf*^{Y} mutation was one site that contributed to the upregulation of the promoter activity in the mammalian cell line (Myosho et al. 2012), the mutation site would cause increased *gsdf*^{Y} expression at an initial stage of gonadal development and function as a genetic cue for sex determination. In addition, a transgenic experiment demonstrated that XX individuals carrying exogenous *gsdf*^{Y} developed as males (Myosho et al. 2012).

14.2.3 *Sox3*^{Y} (SRY-Related HMG Box on the Y Chromosome) in *O. dancena*

In another *Oryzias* species, *O. dancena*, the master sex-determining gene *sox3*^{Y} was identified as allelic and arose from a mutation in the *cis*-regulatory region (Takehana et al. 2014). *Sox3* is a transcription factor that contains a conserved high-mobility group domain, which is characteristic of the *Sox* transcription factor family. *Sox3* expression is conserved in the central nervous system of vertebrate embryos (Koster et al. 2000; Bylund et al. 2003; Rizzoti et al. 2004; Dee et al. 2008). *Sox* genes are thought to function in testis development and male fertility maintenance (Jiang et al. 2013).

Sox3^{y} of *O. dancena* was identified in a homologous region of chromosome 10 in *O. latipes*. By BAC transgenesis, Takehana et al. (2014) identified a distant *cis*-regulatory element of *sox3* and demonstrated that the region was required for male

determination. In *O. dancena*, *sox3* was expressed in neural tubes and neuromasts in both XX and XY embryos at 5 days after hatching. Furthermore, additional signals were observed in somatic cells surrounding germ cells in XY gonads (Takehana et al. 2014). Conversely, *sox3* mRNA expression was not detected in adult gonads. Moreover, mutations induced by zinc-finger nuclease (ZFN) technology in *sox3* on the Y chromosome ($sox3^{Y}$) caused XY sex reversal (Takehana et al. 2014).

14.2.4 Amhr2 in Takifugu

The *anti-Müllerian hormone receptor type II* (*amhr2*) gene is a leading candidate of the master sex-determining gene in three *Takifugu* species (Kamiya et al. 2012). Kamiya et al. (2012) found that an SNP of *amhr2* is perfectly associated with phenotypic sex in *Takifugu rubripes*. All males were heterozygous for this SNP, whereas all females were homozygous for this SNP in *T. rubripes.* High-resolution genetic mapping and association mapping demonstrated that a polymorphism (C/G) that changes an amino acid (His/Asp384) in the kinase domain of *amhr2* was associated with sex. Although the SNP was absent in the wild population of *Tetraodon nigroviridis*, the SNP was completely correlated with sexual phenotypes in a wild population of two *Takifugu* species (*T. pardalis* and *T. poecilonotus*) (Kamiya et al. 2012).

The authors introduced comparable mutations in human *AMHR2* and found that SmadI activation was significantly reduced in P19 cells, a mice embryonic carcinoma cell line. This result suggested that in *Takifugu* species, $amhr2^{H384}$ on the Y chromosome is an allele with reduced function compared with $amhr2^{D384}$ on the X chromosome (Kamiya et al. 2012).

14.2.5 Amhy (Y Chromosome-Specific Anti-Müllerian Hormone) in Patagonian Pejerrey

The sex-determining locus of the Patagonian Pejerrey (*Odontesthes hatcheri*) has been identified (Hattori et al. 2010; Koshimizu et al. 2010). *O. hatcheri* is a gonochoristic fish from South American with an XX–XY sex-determination system. A duplicated copy of the *anti-Müllerian hormone* gene was found in male Patagonian Pejerrey (Hattori et al. 2012). Metaphase chromosome spreads of XX demonstrated two signals in a pair of acrocentric/telocentric chromosomes (*amha*) by fluorescence in situ hybridization using an *amhy* probe containing 7.3 kb of the 5′ flanking genomic region. Conversely, XY demonstrated signals of this pair (*amha*) and an additional strong signal in a single metacentric/submetacentric chromosome (*amhy*). Morpholino-mediated *amhy* knockdown inhibited testicular development, resulting in ovarian development in XY fish. Therefore, in the Patagonian Pejerrey, *amhy* is a leading candidate for the master sex-determining gene.

14.2.6 Sdy (Sexually Dimorphic on the Y Chromosome) in Salmonids

In salmonids, different Y chromosomes potentially evolved in each species (Woram et al. 2003). For example, among closely-related Pacific salmonids (*Oncorhynchus* species), the sex chromosomes are different from one another (Phillips et al. 2007; Phillips et al. 2005). Recently, an immune-related gene, *sdy*, was identified as the master sex-determining gene in Rainbow Trout, *Oncorhynchus mykiss* (Yano et al. 2014; Yano et al. 2012). The authors compared cDNA from XY gonads with that from XX gonads using next generation sequencing and found that a gene was expressed 155-fold higher in XY than XX gonads (Yano et al. 2012). This gene was named *sdy* and encoded a putative protein of 192 amino acids that displayed sequence homology with the C-terminal domain of *interferon regulatory factor 9* (*irf9*). The *sdy* gene was expressed during early testicular development (around the time of hatching) in epithelial cells of the dorsal side of the gonads and in some somatic cells surrounding the germ cells (Yano et al. 2012).

In a gain of function experiment, nine of the 73 genetic females (XX) carrying a transgene with 5 kb of the *sdy* promoter fused with the *sdy* cDNA exhibited a testis structure, and four of the nine transgenic XX females phenotypically appeared as males that produced sperm (Yano et al. 2012). Conversely, a loss of function experiment of the *sdy* using zinc-finger nuclease (ZFN)-induced mutagenesis revealed that a frame-shift or a premature stop codon mutation caused ovary development in XY Rainbow Trout (Yano et al. 2014).

The *sdy* sequence is highly conserved in 15 different species across seven genera of salmonids and is a male-specific Y-chromosome gene in the majority of these species (Yano et al. 2013). Although all species share conserved linkage groups in the three genera of the Salmoninae subfamily found in North America, the major sex-determining locus is located on a different linkage group in almost every species (Phillips 2013). These results suggest that this gene was translocated to different ancestral autosomes during salmonid evolution. Therefore, a new Y chromosome arises with every new translocation in salmonid species.

14.2.7 The Master Sex-Determining Genes in Fish

In contrast to mammals and birds, a diverse range of master sex-determining genes are observed in fish. The genes encode not only transcriptional factors but also growth factors or their receptors. The genes are not functionally conserved. However, they work as a genetic cue and trigger genes associated with gonadal development.

Furthermore, analysis of the sex-determining genes revealed minimal differences between X and Y chromosomes in the genome. In Medaka, the male-specific region is only 260 kb (Kondo et al. 2004). Although *dmy*, *amhy*, and *sdy* are Y-chromosome specific genes, $gsdf^{Y}$, $sox3^{Y}$, and *amhr2* are allelic. In *Takifugu*

amhr2, a single nucleotide difference was noted between the X and Y alleles. These observations suggest that the Y chromosome is functionally equal to the X chromosome with the exception of one function, which can decide the direction of gonadal development.

14.2.8 The XX–XY System Converts to the ZZ–ZW System

A slight genomic change in a species that has a male heterogametic sex-determination system, can cause the female heterogametic sex-determination system to be introduced. Well-calculated mating experiments using wild-derived sex reversal mutants in Medaka demonstrated an example of conversion of a male heterogametic into a female heterogametic sex-determination system (Otake et al. 2006). When a mutation in the *dmy* promoter region causes a decrease in the *dmy* mRNA level, some XY fish develop into females. Conversely, when YY fish were produced from the mutant, two Y chromosomes harboring the mutated *dmy* resulted in male development. That is, one mutation in one Y chromosome stops male development and results in female development, whereas two mutations in two Y chromosomes are sufficient to promote male development. In this situation, XY (heterogametic) is female, whereas YY (homogametic) is male; additionally, ZW (heterogametic) is female, whereas ZZ (homogametic) is male.

Master sex-determining genes in fish have been identified only in certain species that have a male heterogametic sex-determination system. Because these two systems exhibit minimal differences, master sex-determining genes identified in the future will be found in a species that has a female heterogametic system.

14.3 Sex Differentiation of Gonads

After the master sex-determining gene determines the direction of gonadal development, gene cascades of testicular or ovarian development are activated. In normal development, a bipotential gonad develops into a testis or an ovary following the first decision. Two types of gonadal development are noted in gonochoristic fish (Saito and Tanaka 2009). One type is found in two Medaka species and Tilapia (Fig. 14.2a), which both use male heterogametic (XX–XY) sex-determination systems. In these species, the morphologic sexual dimorphism during gonadal differentiation is first noted in the germ cell number (Kobayashi et al. 2004, 2008; Kobayashi and Nagahama 2009; Nakamoto et al. 2009). The patterns of germ cell proliferation in these three species are similar. The germ cell number first increases in XX gonads, whereas the number does not increase in XY gonads during this period. The other type of gonadal development is observed in Zebrafish, which is also thought to use an XX–XY sex-determination system. In this species, all gonads have oocytes at 6 weeks post-fertilization. However, after 7 weeks post-fertilization, gonads,

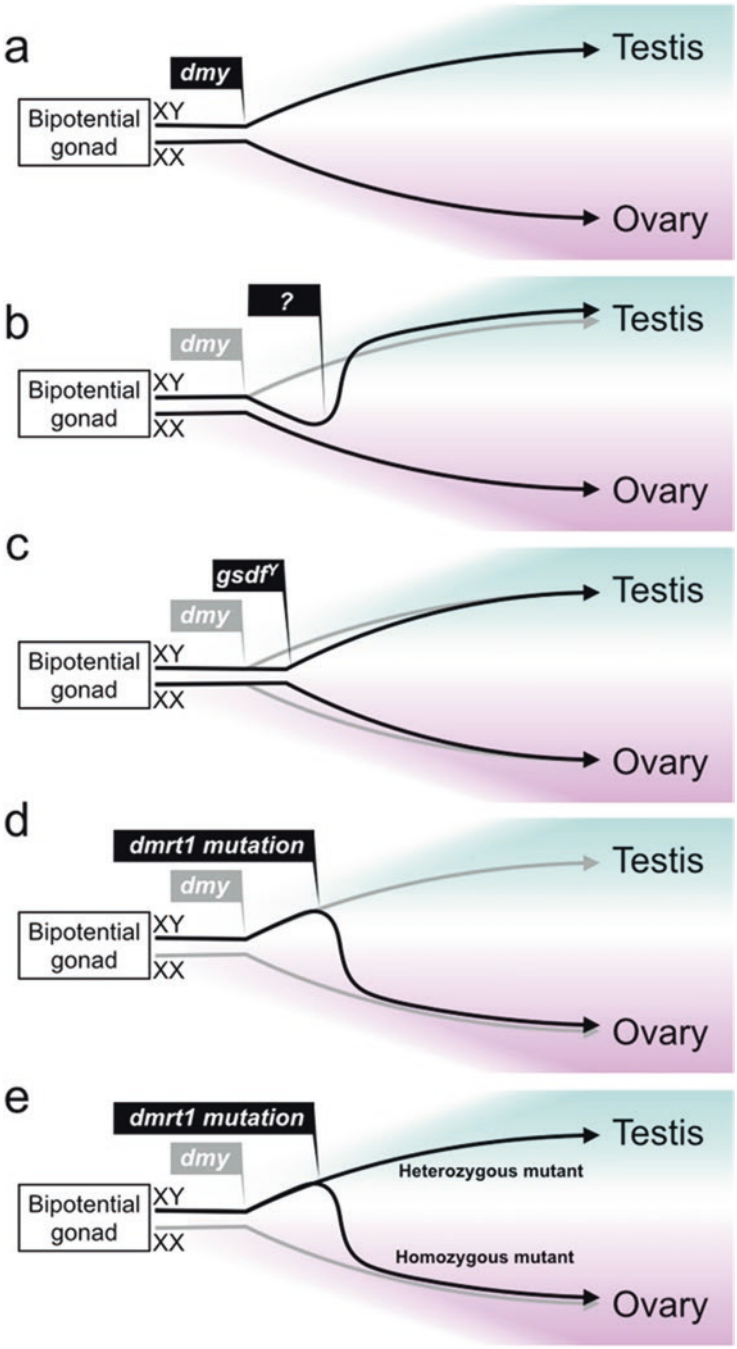

Fig. 14.2 The master sex-determining gene and gonadal development in gonochoristic species. The testis and ovary originate from a bipotential gonad in the embryo. (**a**) In normal development of Medaka, an XY gonad develops into a testis, whereas an XX gonad develops in to an ovary.

including spermatocytes, were found, and the number of the gonads developing into testis subsequently increased. Finally, half of the individuals had testes in the adult stage (Fig. 14.2b) (Maack and Segner 2004). These two types of gonadal development in gonochoristic fish result from differences in the timing of the genetic cues that activate the master sex-determining gene.

Therefore, when a new sex-determining gene has emerged, the pattern of gonadal development becomes modified. Comparisons between two *Oryzias* species provide a concrete example of the modification. In an evolutionary process, a common ancestor among *Oryzias latipes* and *O. luzonensis* used *dmy* as the master sex-determining gene. Then, $gsdf^{Y}$ emerged as the master sex-determining gene in an ancestor of the *O. luzonensis* lineage (Myosho et al. 2012). In *O. latipes*, *dmy* mRNA was detected in the gonadal somatic cells surrounding the germ cells at stage 36 when germ cell migration was concluded (Kobayashi et al. 2004). *Gsdf* was detected in the same cells at stage 37 (Shibata et al. 2010). Conversely, in *O. luzonensis,* $gsdf^{Y}$ was expressed in somatic cells surrounding germ cells at stage 37 (Myosho et al. 2012). The stage at which morphologic sex differences were observed in *O. luzonensis* was later than that noted in *O. latipes*. The germ cell numbers of XX gonads were significantly increased compared with XY gonads at stage 38, which occurred 1 day before hatching in *O. latipes*. In contrast, no differences were noted at hatching in *O. luzonensis* (Nakamoto et al. 2009). The differences became significant at 3 days after hatching. The timing of the expression of the master sex-determining gene in *O. latipes* (*dmy*) moved to the late stage in *O. luzonensis* ($gsdf^{Y}$), thereby delaying the proliferation of germ cells. These facts suggested that the delay of the sex-determining genetic cue resulted in a delay of morphological sexual development (Fig. 14.2c).

Dmrt1 is a critical gene for testicular development. *Dmrt1* expression is correlated to testicular development in many vertebrates (Bellefroid et al. 2013). *Dmrt1* mutants were analyzed in Medaka and it was demonstrated that gonads of XY mutants developed ovaries and laid normal eggs (Masuyama et al. 2012). Gonadal development of XY mutants first occurs as a testis and then switches to an ovary (Fig. 14.2d). The numbers of germ cells were similar to normal XY individuals at 5 days after hatching. The increase in germ cells was arrested and no oocytes were noted. However, oocytes were observed in the gonads of XY mutants at 10 days after hatching. Thereafter, gonadal development was similar to normal XX individuals. Many oocytes were found at 20 days after hatching and were found in the

Fig. 14.2 (continued) (**b**) In Zebrafish, all gonads have oocytes in the early developmental stages. However, half of the individuals had testes in an adult stage. (**c**) The delay of the sex-determining cue (the expression of the master sex-determining gene) resulted in a delay of morphologic sexual development. (**d**) In normal development, the first cue in the gonadal development of *dmrt1* mutant is the master sex-determining gene, *dmy*. However, the XY gonad of homozygous *dmrt1* mutant develops into an ovary. (**e**) XY gonads that were *dmrt1* heterozygous developed as testes, whereas *dmrt1* homozygous mutants developed as ovaries

ovarian cavity at 30 days after hatching. Conversely, gonadal development of heterozygous mutants is normal and follows the genetic sex. XY gonads that were *dmrt1* heterozygous developed as testes, whereas *dmrt1* homozygous mutants developed ovaries. Therefore, the *dmrt1* allele determines the direction of gonadal development in the XY mutant; that is, *dmrt1* functions as the master sex-determining gene and serves as a genetic cue (Fig. 14.2e). Because the action of the genetic cue is delayed, sex differences are delayed and are only noticeable at 10 days after hatching.

14.4 Sex Reversal

As the direction of gonadal development of *dmrt1* XY mutants was altered to ovaries, modification of downstream genes in the gene cascade of gonadal development caused sex reversal. Such sex reversal can induce not only genetic modifications but also environmental cues such as steroid hormones or temperature.

14.4.1 Male to Female Sex Reversal

The first study on complete XY females induced by estrogens has been reported using Medaka (Yamamoto 1953). Yamamoto administered a diet including estrone to newly hatched fry at 8 months and found that all fish differentiated into females regardless of sex chromosome types and exhibited normal ovaries. Given that sex control is important in aquaculture, induction of sex reversal using administration of steroid hormones has been studied in various fish species (Piferrer 2001). As a result, numerous reports on the successful induction of sex reversal have been published. However, the mechanisms are not well understood. Specifically, two reports state that gene mutations cause sex reversal to female.

A mutation in the anti-Müllerian hormone receptor of Medaka caused the dysregulation of germ cells and sexual development (Morinaga et al. 2007). Phenotype-driven Medaka *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis screens revealed 13 mutations affecting gonadal development in the Medaka (Morinaga et al. 2004). Three of the 13 mutations increased germ cell numbers. One of these three exhibited gonadal hypertrophy. The responsible gene mutation was identified as a disrupted *anti-Müllerian hormone receptor type II* (*amhrII*) gene (Morinaga et al. 2007). Gonads of half of the XY mutants developed with hypertrophic ovaries, whereas the remaining half developed enlarged testes. Hyperplasia of germ cells in the gonad by dysfunction of the *amh/amhrII* system may initiate ovarian development.

As previously mentioned, XY gonads with dysfunctional *dmrt1* develop into complete ovaries (Masuyama et al. 2012). Conversely, in the Nile Tilapia, *Oreochromis niloticus*, transcription activator-like effector nucleases (TALENs)

were used for targeted genome editing of *dmrt1* (Li et al. 2013). In fish with a high mutation rate (approximately 80%), a *dmrt1*-deficient testis exhibited a round shape similar to the ovary and a large cavity in the middle of the testis with no germ cells. The authors did not observe male to female sex reversal.

In addition, mutation of *gsdf* induces Medaka XY gonads to undergo ovarian development. All XY gonads with a homozygous mutation in *gsdf* developed into an ovary at early developmental stages, whereas two-thirds of *gsdf* mutant XY gonads developed into testes in the adult stages, demonstrating that *gsdf* function is critical for directing the bipotential gonad at early developmental stages (Imai et al. 2015).

14.4.2 Female to Male Sex Reversal

The first study on complete XX males induced by androgen used Medaka (Yamamoto 1958). The methods used were the same as those employed in estrogen-induced sex reversal. The success of sex reversals induced by androgen has been reported in numerous fish species. However, androgens do not appear to be a natural inducer for male development.

The *scl* (*sex character-less*) mutant of Medaka supports this hypothesis. This natural occurring mutant was found in a laboratory stack and exhibits no secondary sex characters in both males (XY) and females (XX) (Sato et al. 2008). The responsible gene for the mutant was the *p450c17* gene, which is a steroidogenic enzyme mediating both 17 alpha-hydroxylase (steroid 17 alpha-monooxygenase, EC 1.14.99.9) and 17,20 lyase activities. This gene is responsible for both androgen and estrogen synthesis. Therefore, it was expected that both androgen and estrogen would be very low in the mutants. XY mutants did not exhibit male-type large anal fins with a papillary process. XX mutants did not have urinogenital papilla and the ovaries did not have an ovarian cavity. Although Medaka typically mature at 3 months, at 6 months after hatching the XX gonads of the mutants had oocytes and spermatozoa and the XY gonads had mature sperm. These results suggested that androgen is not necessary for spermatogenesis but is required for male secondary sex characteristics.

Another method to induce female to male sex reversal involves impairing the estrogen-signaling pathway. This impairment can be achieved by treatment with an aromatase inhibitor or specific estrogen receptor antagonists, such as tamoxifen. Aromatase is a steroid genic enzyme (EC 1.14.14.1) that converts androgens to estrogens. These treatments decrease circulating estrogen and cause male sex reversal (Guiguen et al. 2010).

Environmental temperature-dependent sex determination has been reported in some fish species, i.e., the atherinid Atlantic Silverside *Menidia menidia*, (Conover and Heins 1987; Conover and Fleisher 1986) and the Pejerrey *Odontesthes bonariensis* (Strüssmann et al. 1996). The effect of temperature on the direction of gonadal

development after genetic sex has been characterized in species that has a genetic sex-determination system (Ospina-Alvarez and Piferrer 2008; Martinez et al. 2014; Fernandino et al. 2013). Recently, some of the mechanisms were revealed. High water temperature causes female-to-male sex reversal in Medaka (Hattori et al. 2007; Sato et al. 2005). Hayashi et al. (2010) presented evidence of female-to-male sex reversal in increased cortisol levels. High temperature treatment (33 °C) during 0 days post-fertilization to 5 days after hatching caused 25% of XX males to be produced. This effect was compared with the effects of a control temperature (26 °C), cortisol treatment (26 °C) and metyrapone, which is an inhibitor of cortisol synthesis. Increasing whole-body levels of cortisol with cortisol and high temperature treatments in hatching-stage embryos were trialed; however, increases were not noted with control and metyrapone treatments. (Hayashi et al. 2010). Furthermore, sex reversal caused by a high temperature and cortisol treatments was stopped by the administration of estrogen (Kitano et al. 2012). Actually, the male ratio was increased in all XX Southern Flounders (*Paralichthys lethostigma*) treated with blue tank color (i.e., the control) or cortisol (Mankiewicz et al. 2013). These facts suggest that cortisol induced by various types of stresses inhibits the ovarian developmental pathway.

Germ cells can affect the direction of gonadal development decided by the master sex-determining gene. XX Medaka subjected to high temperatures and cortisol exhibit decreased germ cell numbers, suggesting that a gonad with a decreasing number of germ cells develop into a testis (Hayashi et al. 2010). In Zebrafish (*Danio rerio*), gonads that are deficient in germ cells due to morpholino antisense oligonucleotides to the *dead end* (*dnd*) gene demonstrated an adopted testis fate with Sertoli and Leydig cells (Siegfried and Nusslein-Volhard 2008). Temporal specific germ cell ablation also exhibited masculinization (Dai et al. 2015). The authors utilized the metrodinazole (MTZ)/bacterial nitroreductase (NTR) system. Bacterial NTR is an enzyme that can convert the innocuous pro-drug MTZ to a cytotoxic product, which induces cell death. In this report, the authors established a transgenic line in which NTR was expressed in the germ cells and driven by the Zebrafish *dnd* promoter. When transgenic fish were treated with MTZ at 18 days post-fertilization for 1 week, the gonad was indifferent in the initial stage, and contained only gonial cells before histological sex differences were observed (Maack and Segner 2004). Almost all of the MTZ-treated transgenic males were still fertile, with significantly reduced levels of fertilization rates compared with control transgenic males (Dai et al. 2015). Given that all gonads contain oocytes at 6 weeks post-fertilization in Zebrafish, the decrease in the number of germ cells may directly change the direction of gonadal development to the testis pathway. In Medaka, germ cell-deficient gonads were induced by injection of morpholino antisense oligonucleotides to the *cxcr4* gene, which is a critical molecule for primordial germ cell migration (Kurokawa et al. 2006). Gonads of the germ cell-deficient Medaka developed into testes with increased expression of testis-specific genes (*p45011ß*, *11ßhsd2* and *dmrt1*) and without expression of ovary-specific genes (*aromatase* and *foxl2*) (Kurokawa et al. 2007). This result also demonstrates that germ cell deficient gonads develop into testes.

Overexpression of exogenous genes using transgenics can also induce female to male sex reversal. When the transgene is integrated into a chromosome, which is an autosome in most cases, the chromosome appears as a new heritable artificial sex chromosome (Myosho et al. 2012; Takehana et al. 2014; Otake et al. 2010). Transgenic *Oryzias latipes*, *O. luzonensis*, and *O. dancena* harboring inheritable *dmy*, *gsdf*^{Y}, and *sox3*^{Y} transgenes, respectively, develop as males. This gain of function experiment is required to determine whether these genes act as master sex-determining genes. Furthermore, in *O. dancena*, inheritable *gsdf*^{Y}, which is a leading candidate for a master sex-determining gene in *O. luzonensis*, functions as a genetic cue for testicular development (Takehana et al. 2014). *Gsdf* has a conserved role in testicular development in fish species, suggesting that modification of a downstream gene can affect the direction of gonadal development.

Many XX males that did not have the master sex-determining gene *dmy* were observed in laboratory stocks and wild populations of Medaka (Nanda et al. 2003; Shinomiya et al. 2004). Two cases are of note. One case involves sex reversal induced by environmental factors, such as high water temperature or stress. The other case involves gene mutation. This second case involves the advent of a new sex-determining gene rather than sex reversal caused by a gene mutation. One spontaneous mutant Medaka was isolated from the wild population of Japan (Shinomiya et al. 2010). This *dmy*-negative XX male trait is inheritable in a Mendelian manner. The authors succeeded in narrowing the responsible gene to a locus on LG8 that was designated as *sex-determining autosomal factor-1* (*sda-1*). Phenotypic sex contradicts the existence or non-existence of a master sex-determining gene in many individual fish from wild Medaka populations (Shinomiya et al. 2004). This fact implies that many genes can influence the sexual development of gonads and can act as genetic cues for determining the direction of gonadal development.

14.5 Future Directions

Even in an environmental sex-determination system, genomic information controls the sex of an individual. Therefore, to explain the genetic control of sex differentiation, the cue of sex determination must be associated with the downstream gene cascade for sexual development of the gonads. Recently, the cost of analyzing genomic information using next generation sequencing has been reduced. Therefore, many master sex-determining genes are expected to be identified in the near future. These master genes will be located in the gene cascade for gonadal development. As in the case of mice, functional analyses using reverse genetics have been a powerful tool for clarifying molecular mechanisms. This approach has been difficult until recently. Genome editing methods are now available, such as TALENs and CRISPR/Cas9 (Clustered Regularly Interspersed Palindromic Repeats/CRISPR associated protein 9) systems. Using these systems, we can easily obtain mutants that harbor a mutation in a specific genomic region. Analyses using these mutants will contribute to the explanation of molecular mechanisms for the genetic control of sex differentiation in fish.

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Chapter 15
Endocrine and Environmental Control of Sex Differentiation in Gonochoristic Fish

Takeshi Kitano

Abstract Sex in vertebrates, including fish, is usually determined by genotype. In medaka (*Oryzias latipes*), a gonochoristic fish with the XX/XY sex determination system, a gene that encodes the DM domain on the Y chromosome is identified as the master sex-determining gene. However, the sex-determining genes in many non-mammalian vertebrates remain unclarified. In contrast, sex determination in some reptiles, amphibians, and fish is influenced greatly by environmental factors. For example, although the genotypic sex determination mechanism in Japanese flounder (*Paralichthys olivaceus*) is basically the XX/XY type, genotypic females can be sex reversed to phenotypic males by rearing the larvae at high or low water temperatures during gonadal sex differentiation. In addition, the phenotypic sex of many teleost fish, including flounder, can be experimentally altered by treatment with sex steroid hormones, suggesting an important role for sex steroid hormones in gonadal sex differentiation in fish. In this chapter, we review general information and recent knowledge on the basic mechanisms of sex determination and gonadal sex differentiation, and present the effects of sex steroid hormones and water temperature on gonadal sex differentiation in gonochoristic fish.

Keywords Gonadal sex differentiation · Temperature-dependent sex determination · Estrogen · Androgen · Medaka · Japanese flounder · Gonochoristic fish

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15.1 Introduction

Sex in vertebrates, including fish, is usually determined by genotype. However, various environmental factors such as temperature also greatly influence sex determination in some nonmammalian species such as reptiles, amphibians, and fish (Adkins-Regan 1987). Pioneering medaka (*Oryzias latipes*) experiments by Yamamoto (1969) demonstrated that androgens and estrogens induce complete masculinization and feminization, respectively, leading to the hypothesis that estrogens and androgens are endogenous female and male inducers, respectively. Since then, many studies have investigated the effects of steroids on gonadal sex differentiation in gonochoristic fish.

Here, we focus primarily on several good fish models such as medaka, Nile tilapia (*Oreochromis niloticus*), and Japanese flounder (*Paralichthys olivaceus*) to study the mechanisms of sex determination and differentiation in gonochoristic fish. In medaka, which has the XX/XY sex determination system (Aida 1921), the DM domain gene on the Y chromosome (*dmy*) is identified as the master sex-determining gene localized on the Y chromosome (Matsuda et al. 2002, 2007). This species has several advantages such as small body size, short generation time, small genome size, and several useful strains (Ishikawa 2000). Therefore, the medaka is an excellent molecular genetic model of vertebrates for analyzing various biological phenomena, including embryonic development and sex determination. Nile tilapia, rainbow trout (*Oncorhynchus mykiss*), and common carp (*Cyprinus carpio*) are also useful models for studying sex differentiation because genetically all-male (XY) and all-female (XX) populations can be produced by fertilizing eggs from genotypic females (XX) with sperm from homogametic males (YY) and sex-reversed males (XX), respectively (Gimeno et al. 1996; Guiguen et al. 1999). Moreover, sex in Japanese flounder is genetically determined by the male heterogametic (XX/XY) system (Tabata 1991), and all-phenotypic female and male populations can be produced by rearing XX broods at 18 °C and 27 °C, respectively (Kitano et al. 1999), suggesting that this species is an excellent model to investigate temperature-dependent sex determination (TSD) mechanisms in fish.

In this chapter, we review general information and recent findings on the basic mechanisms of sex determination and differentiation and discuss the effects of sex steroid hormones and water temperature on gonadal sex differentiation in gonochoristic fish.

15.2 Gonadal Sex Differentiation in Gonochoristic Fish

The gonadal sex differentiation process in gonochoristic fish varies according to species, but in many species, undifferentiated gonads appear to change gradually to testes or ovaries after germ cell numbers becomes different between the sexes (three-spined stickleback—*Gasterosteus aculeatus* (Shimizu and Takahashi 1980;

Lewis et al. 2008); pejerrey—*Odontesthes bonariensis* (Strüssmann et al. 1996); chum salmon—*Oncorhynchus keta* (Robertson 1953); rainbow trout (Lebrun et al. 1982); medaka (Satoh and Egami 1972; Kobayashi et al. 2004); rosy barb—*Puntius conchonius* (Çek 2006); brown trout—*Salmo trutta* (Ashby 1957); black swordtail—*Xiphophorus helleri* (Essenberg 1923; Vallowe 1957); southern platyfish—*Xiphophorus maculatus* (Wolf 1931); and tilapia (Kobayashi et al. 2008)). Sex is first determined in gonadal somatic cells where the sex-determining genes are expressed and the sex difference in germ cell numbers occurs; subsequently, a sex difference in stromal tissue that represents formation of the ovarian cavity or efferent duct occurs.

Genotypic female (XX) medaka have more germ cells than genotypic males (XY) before hatching, and germ cells in XY gonads enter mitotic arrest, whereas they initiate meiosis in XX gonads (Fig. 15.1) (Kobayashi et al. 2004). *dmy* is expressed in somatic cells surrounding the germ cells during gonadal sex differentiation and inhibits germ cell proliferation in a male-specific manner because knockdown of *dmy* function increases germ cell numbers in XY fry at hatching (Paul-Prasanth et al. 2006). The gonadal soma-derived growth factor (*gsdf*), a member of the transforming growth factor-β (TGF-β) superfamily, is a candidate downstream *dmy* gene because its messenger RNA (mRNA) is expressed specifically by males beginning at the same stage as *dmy* in somatic cells and it appears to be involved in suppressing germ cell proliferation (Shibata et al. 2010). Actually, loss of *gsdf* function in medaka increases germ cell numbers in XY fry at hatching and causes male-to-female sex reversal in some cases (Imai et al. 2015). After hatching, the germ cells in XY gonads stop proliferating about 10 days after hatching (dah). Proliferation restarts and spermatogenesis progresses thereafter. Then, the efferent duct—which carries mature sperm, forms, and functional testis—develops. When germ cell proliferation restarts in XY medaka, DM-related transcription factor 1 (*dmrt1*) is expressed male specifically in the gonadal somatic cells (Sertoli cells) and appears to play essential roles in testicular differentiation in medaka (Masuyama et al. 2012).

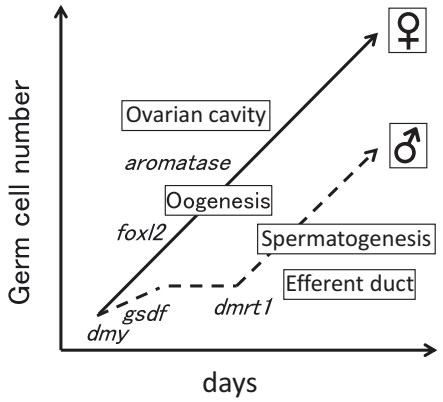

Fig. 15.1 Sexually dimorphic proliferation of germ cells and timing of transcription initiation of sex-related genes in medaka. XX medaka (solid lines) have more germ cells than XY males (dashed lines) before hatching, and subsequently germ cells in XY gonads enter mitotic arrest, whereas they initiate meiosis in XX gonads

In contrast, the molecular mechanism of germ cell proliferation during differentiation in the XX medaka ovary remains unclear. Forkhead box L2 (FOXL2) is a transcription factor that induces expression of *ovary-type aromatase (aromatase)*, which converts androgens to estrogens, in teleost fish (Wang et al. 2007; Yamaguchi et al. 2007), and *foxl2* mRNA is expressed female specifically in gonadal somatic cells of XX medaka beginning at hatching (Nakamoto et al. 2006), suggesting an important role for FOXL2 during ovarian differentiation in teleosts. After hatching, *aromatase* is expressed specifically in the somatic cells of XX gonads, the ovarian cavity forms, and the gonad changes into a functional ovary.

Follicle-stimulating hormone-*β (fshβ)* expression is detected equally in the pituitaries of both sexes of medaka (Hayashi et al. 2010), tilapia (Yan et al. 2012), and flounder (Yamaguchi et al. 2007) during gonadal sex differentiation, but follicle-stimulating hormone receptor (*fshr*) expression is much higher in the differentiating ovary than in the differentiating testis, suggesting that FSH signaling may induce gonadal *aromatase* expression in teleosts. Actually, loss of FSHR function inhibits ovarian development and causes female-to-male sex reversal in XX medaka by suppressing *aromatase* expression and the resultant estrogen biosynthesis (Murozumi et al. 2014). These findings strongly suggest that FSH regulates ovarian development and maintenance primarily through increased estrogen levels.

The germ cells of Nile tilapia enter into gonadal anlagen at 3 dah and do not change in either sex at 5–8 dah (Kobayashi et al. 2008). *gsdf* and *dmrt1* are expressed at higher levels in XY gonads beginning at 5 and 6 dah, respectively (Kaneko et al. 2015), whereas *foxl2* and *aromatase* are expressed at higher levels in XX gonads beginning at 5 dah (Ijiri et al. 2008). XX germ cells continue to proliferate at 8 dah, whereas XY germ cells do not change in number at 9–14 dah and subsequently restart proliferating, but spermatogenesis is not observed until 70 dah. The ovarian cavity and intratesticular efferent duct form at about 25 dah in XX and XY gonads, respectively, and the gonads change into a functional ovary or testis (Nakamura et al. 1998). Thus, although the process and expression of sex-specific genes during gonadal sex differentiation in tilapia and medaka are similar, the difference is that the ovarian cavity forms before and after initiating oogenesis in tilapia and medaka, respectively.

15.3 Involvement of Sex Steroid Hormones in Gonadal Sex Differentiation

15.3.1 Involvement of Estrogens

Exogenous estrogens feminize genotypic males of many species (reviewed by Hunter and Donaldson 1987). Estrogens such as estrone, 17*β*-estradiol (E2), and 17*α*-ethinyl estradiol (EE2) induce complete male-to-female sex reversal in medaka (Yamamoto 1969). Moreover, E2 downregulates *gsdf* expression and upregulates

aromatase expression during sex differentiation (Shibata et al. 2010; Kitano et al. 2012), indicating regulation of sex-specific genes by E2 in medaka. Exposure of an all-male common carp population to E2 for 90 days results in regular phenotypic female gonads containing oviducts and oocytes at various developmental stages, but no testicular tissue (Gimeno et al. 1996). Gonadal *dmrt1* expression during sex differentiation decreases during short-term E2 treatment of an all-male rainbow trout population obtained by fertilizing normal eggs (XX) with sperm from phenotypic males (YY), suggesting that the E2 feminizing treatment downregulates *dmrt1* expression in trout (Marchand et al. 2000). EE2 causes complete male-to-female sex reversal, upregulates *aromatase* expression, and downregulates *dmrt1* expression in tilapia (Kobayashi et al. 2003, 2008). Therefore, exogenous estrogens induce male-to-female sex reversal in teleosts by regulating the expression of sex-specific genes such as *gsdf*, *dmrt1*, and *aromatase*.

Brief treatment of Chinook salmon (*Oncorhynchus tshawytscha*) with the aromatase inhibitor fadrozole during sex differentiation causes genotypic females to develop into normal phenotypic males (Piferrer et al. 1994). Guiguen et al. (1999) reported that treatment of broods of rainbow trout and tilapia with an aromatase inhibitor (1,4,6-androstatriene-3-17-dione) results in a high percentage of masculinization of an all-female (XX) population. Moreover, fadrozole and the antiestrogen tamoxifen induce female-to-male sex reversal in XX flounder (Kitano et al. 2000, 2007), suggesting essential roles for endogenous estrogens during ovarian differentiation in many fish species. In contrast, treatment of medaka with fadrozole after hatching inhibits formation of the ovarian cavity and does not induce female-to-male sex reversal in XX fish (Suzuki et al. 2004). Moreover, loss-of-function of 17α-hydroxylase/17,20 lyase (P450c17), which helps synthesize androgens and estrogens, results in many diplotene-stage oocytes and spermatozoa in XX gonads (Sato et al. 2008). Therefore, endogenous estrogens may be indispensable for ovarian differentiation in many teleost fish, but not necessary in others such as medaka.

15.3.2 Involvement of Androgens

Exogenous androgens masculinize genotypic females in many species (reviewed by Hunter and Donaldson 1987). Androgens such as 17α-methyltestosterone (MT), 17α-ethinyl testosterone, and androstenedione induce complete female-to-male sex reversal in medaka (Yamamoto 1969). Treatment of tilapia with MT masculinizes genotypic females and increases gonadal expression of *dmrt1* (Kobayashi et al. 2008). MT and 11-ketotestosterone are fish-specific androgens that induce complete masculinization of Japanese flounder genotypic females, upregulate expression of anti-Müllerian hormone (*amh*)—which is a member of the TGF-β superfamily—and downregulate *aromatase* expression (Kitano et al. 2000; Yoshinaga et al. 2004). Hence, exogenous androgens likely induce female-to-male sex reversal in teleosts by regulating the expression of sex-specific genes such as *amh*, *dmrt1*, and *aromatase*.

In contrast, treatment of XY tilapia with the antiandrogen flutamide does not induce male-to-female sex reversal. Moreover, loss of P450c17 function in XY medaka results in a lack of secondary sex characteristics, but spermatogenesis proceeds as in wild-type XY fish (Sato et al. 2008). Therefore, endogenous androgens may not be required for testicular differentiation in teleosts. A functional analysis of androgen-related genes involved with testicular differentiation in gonochoristic fish is needed.

15.4 Effect of Temperature on Gonadal Sex Differentiation

Sex determination in some reptiles, amphibians, and fish is markedly influenced by environmental factors (Adkins-Regan 1987). Japanese flounder have the XX/XY sex determination system (Tabata 1991) and exhibit TSD. The details of temperature sensitivity and gonadal differentiation have been documented in this species, making it useful for studies investigating the physiology and molecular biology of TSD (Yamamoto 1995; Kitano et al. 1999). The percentages of females in normal (XX + XY) and genotypic female (all XX) larvae reared at various water temperatures from days 30 to 100 after hatching—a critical period of gonadal sex differentiation in flounder—are shown in Fig. 15.2. The percentages of females in normal and genotypic female broods reared at 18 °C were 53% and 100%, respectively. However, they decreased rapidly in both broods when the fish were reared at higher (25 °C) or lower (15 °C) water temperatures. In particular, the genotypic female broods reared at 27 °C were completely masculinized. Thus, 18 °C and 27 °C are

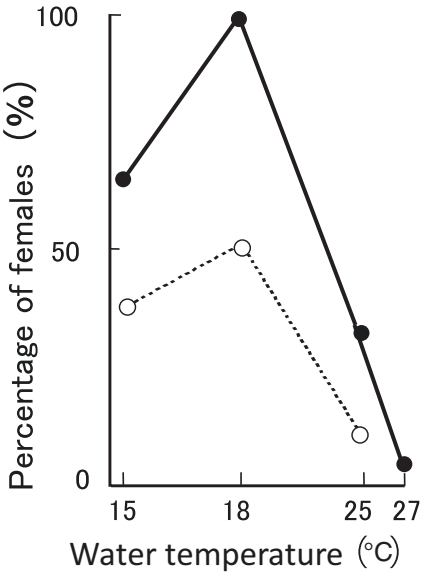

Fig. 15.2 Percentage of females in normal flounder (XX + XY, open circles) and genotypic female flounder (all XX, solid circles) reared at various water temperatures during gonadal sex differentiation. Phenotypic sex was determined at 10 months of age by histological observation of the gonad

the proper temperature conditions to change genotypic female broods to all-phenotypic females or males, respectively, from 30 to 100 days after hatching (Kitano et al. 1999). A similar thermal influence on fish has been demonstrated in studies on atherinids (Conover and Kynard 1981; Strüssmann et al. 1996, 1997), cichlids (Baroiller et al. 1995; Romer and Beisensherz 1996; Desprez and Mélard 1998), and channel catfish, *Ictalurus punctatus* (Patiño et al. 1996). Low-temperature treatment of atherinids during sex determination results in a higher percentage of females, and high-temperature treatment results in a higher percentage of males. In the cichlid *O. niloticus*, the percentage of males in all genotypic female progeny increases during rearing at high temperatures (32–37 °C) during the thermosensitive period (Baroiller et al. 1995). In contrast, the sex ratio of channel catfish is skewed toward females at 34 °C, but no effects occur at 20 °C or 27 °C (Patiño et al. 1996). These results suggest that both genotypic and TSD mechanisms are functional in some fish and that this phenomenon is more widespread in fish than previously believed.

According to current evolutionary theory, this TSD mechanism is adaptive to environments with moderate to large fluctuations in environmental conditions (Bulmer 1987). Environmental sex determination is favorable when environmental effects have different consequences for the two sexes. Conover and Heins (1987) showed that natural *Menidia menidia* populations at different latitudes, whose sex is determined by interactions between genotype and temperature during a specific critical larval development period (Conover and Kynard 1981), compensate for differences in the thermal environment and seasonality by adjusting the sex ratio response to temperature. Therefore, TSD may be a maintenance strategy for natural populations of some species.

15.5 TSD Molecular Mechanism

Phenotypic male and female Japanese flounder are produced by rearing XX larvae at 18 °C and 27 °C, respectively, during sex differentiation. The ovarian cavity and intratesticular efferent duct form at about 60 dah in the gonads of females reared at 18 °C and in the gonads of males reared at 27 °C, respectively, and the gonad changes into a functional ovary or testis (Fig. 15.3) (Yamamoto 1995; Kitano et al. 1999). *foxl2* and *aromatase* are expressed strongly in gonads of females reared at 18 °C at 50 dah when the gonad is sexually undifferentiated, whereas they are barely detected in gonads of males reared at 27 °C. In contrast, *amh* is expressed strongly in male gonads at 50 dah, but not in female gonads. Moreover, E2 treatment at a masculinizing temperature induces complete feminization of XX flounder, induces *foxl2* and *aromatase* expression, and suppresses *amh* expression (Kitano et al. 2007), suggesting that estrogens counteract masculinization in XX flounder at high temperature by regulating the expression of sex-specific genes such as *foxl2*, *aromatase*, and *amh*. To investigate whether XX gonads are temperature sensitive during sex differentiation, a gonadal organ culture experiment was performed using 55 dah

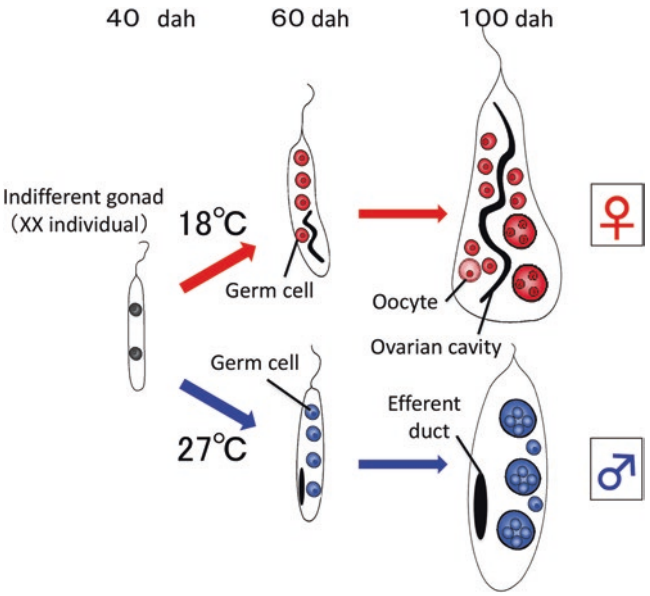

Fig. 15.3 Gonadal sex differentiation in XX flounder. The ovarian cavity and efferent duct form at about 60 days after hatching in the gonads of females reared at 18 °C and in the gonads of males reared at 27 °C, respectively

XX gonads. XX gonads cultured at 18 °C or 27 °C for 2 weeks expressed *foxl2* and *aromatase* but not *amh*, suggesting that the gonads are not temperature sensitive during sex differentiation.

Cortisol is the major glucocorticoid produced by inter-renal cells in teleosts, and production increases in response to various stressors such as heat shock. The hypothalamus–pituitary–inter-renal axis, which controls circulating cortisol levels, is highly conserved across vertebrates (Wendelaar Bonga 1997). The effects of cortisol on reproductive performance have been reported in many fish species (Wendelaar Bonga 1997). For example, cortisol suppresses E2 and testosterone secretion in rainbow trout ovarian follicles (Carragher and Sumpter 1990). Furthermore, cortisol inhibits FSH-induced estrogen production in cultured rat granulosa cells (Hsueh and Erickson 1978). Thus, a high concentration of cortisol appears to inhibit production of gonadal hormones essential for vertebrate ovarian development.

Another gonadal organ culture was performed to investigate whether cortisol directly induces masculinization of XX gonads. All XX gonads cultured with cortisol at 18 °C expressed *amh* but not *foxl2* or *aromatase*, demonstrating the masculinizing action of cortisol during sex differentiation (Yamaguchi et al. 2010). However, treatment of XX flounder with cortisol induces female-to-male sex reversal, and metyrapone (cortisol synthesis inhibitor) inhibits 27 °C–induced masculinization of XX flounder. Moreover, cortisol levels in flounder juveniles reared at 27 °C are significantly higher than in those reared at 18 °C during sexual differentiation.

Therefore, masculinization of flounder in response to high water temperature increases cortisol levels during gonadal sex differentiation.

XX medaka fry can be sex reversed to phenotypic males by high temperature (30–34 °C) during sex differentiation (Sato et al. 2005; Hattori et al. 2007). XX medaka fry were treated with cortisol and E2 at 26 °C or 33 °C during sex differentiation to investigate whether cortisol induces masculinization of XX fish. Cortisol treatment at 26 °C causes female-to-male sex reversal, and metyrapone inhibits masculinization of XX medaka at 33 °C (Hayashi et al. 2010). Moreover, the 33 °C treatment increased cortisol levels, whereas metyrapone suppressed the increased cortisol levels in the 33 °C treatment during sexual differentiation. However, high temperature or cortisol can inhibit proliferation of female-type germ cells and suppress *aromatase* expression while increasing *gsdf* expression in XX gonads during sexual differentiation. Exposure to E2 combined with either cortisol or high temperature prevents these effects (Kitano et al. 2012). Moreover, E2 completely rescues 33 °C–induced and cortisol-induced masculinization of XX medaka. These results strongly suggest that high temperature and cortisol induce female-to-male sex reversal in medaka by enhancing *gsdf* expression and suppressing *aromatase* expression, followed by estrogen biosynthesis.

Taken together, these results suggest that high temperature elevates cortisol levels, which causes female-to-male sex reversal by regulating the expression of the sex-specific genes such as *amh*, *gsdf*, and *aromatase*, and inhibiting estrogen biosynthesis during gonadal sex differentiation in teleosts with TSD. This mechanism may be common among animals with TSD.

15.6 Conclusions

This chapter has demonstrated that endogenous estrogens are indispensable for female sexual differentiation in many fish species such as tilapia, rainbow trout, and Japanese flounder, but may not be necessary in other fish such as medaka. This may be due to whether the ovarian cavity forms before or after oogenesis is initiated. Estrogens may impact early oogenesis and be involved in ovarian differentiation if the ovarian cavity forms before oogenesis is initiated. Estrogens may not affect early oogenesis and may only be involved in forming the ovarian cavity after oogenesis is initiated. The involvement of endogenous androgens in testicular differentiation remains unclear in teleosts. Therefore, a functional analysis of estrogen- and androgen-related genes needs to be performed using new technology such as genome editing.

This chapter has presented novel evidence of the involvement of cortisol in TSD of Japanese flounder and medaka. Cortisol induces female-to-male sex reversal, and metyrapone inhibits high-temperature-induced masculinization of XX fish. Moreover, high temperature increases cortisol levels, whereas metyrapone suppresses this increase during sexual differentiation, strongly suggesting that masculinization of XX fish induced by high temperature is mediated by increased cortisol.

Cortisol reportedly induces a sex change from ovary to testis through a decrease of E2 levels in the protogynous wrasse (*Halichoeres trimaculatus*) (Nozu and Nakamura 2015). Therefore, cortisol appears to have the capacity to induce masculinization not only in gonochoristic fish but also in hermaphrodites. Future studies should focus on the molecular mechanisms of masculinization and how cortisol causes female-to-male sex reversal in teleosts with TSD.

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Chapter 16
Variety of Sex Change in Tropical Fish

Yasuhisa Kobayashi, Ryo Nozu, Ryo Horiguchi, and Masaru Nakamura

Abstract Among vertebrates, teleost fish are of particular interest for their sexual diversity and plasticity. During ontogenesis in gonochoristic fish, the undifferentiated gonadal primordium develops into an ovary or testis, a process referred to as sex determination or sexual differentiation. After primary sex determination, the sexes of gonochoristic fish remain fixed for the remainder of their lives. Other fish are hermaphrodites, however, and can change their sex in adulthood, as either simultaneous hermaphrodites, which possess both functional ovarian and testicular tissue, or sequential hermaphrodites. Sequential hermaphrodite species can be divided into three groups. The first are protogynous (female-first) hermaphrodites; the fish begins its life as a female but later becomes a male. The reverse is the case for protandrous (male-first) species (the second group). A third group is composed of those few species that can change their sex serially (bidirectional sex change). This diversity of sexual plasticity is unique to fish and, as such, provides excellent model systems with which to investigate the mechanisms of sex determination and differentiation in vertebrates. In this chapter, we first discuss gonadal sex differentiation in hermaphroditic fish in comparison with gonochoristic fish and then describe various types of sex change in fish from the viewpoints of morphology and physiology.

Keywords Sex change · Gonad · Testis · Ovary · Protandry · Protogyny · Bidirectional sex change · Estrogen · Androgen · Sex determination · Sex differentiation · Wrasse · Grouper · Anemonefish · Gobiid fish · Tilapia

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16.1 Introduction

Among vertebrates, teleost fish show the greatest range of sexuality. Fish are categorized mainly as either gonochoristic or hermaphroditic (Atz 1964; Yamamoto 1969). In gonochoristic fish—the more common type—sexes are determined genetically or by environmental factors, as in other vertebrates (see chapters 14 and 15). After sex determination, undifferentiated gonad primordia (germ and somatic cells) differentiate into an ovary (female) or testis (male), with the sex remaining fixed thereafter. In contrast, hermaphroditic fish can change sex during their lives. Among vertebrates, sex change in fish is a rare and fascinating phenomenon. Since sex change commonly occurs after pubertal maturity, it is possible to investigate the changes that occur in the sex of germ and somatic cells in the adult gonad. Research into sex change in fish makes a major contribution to the field of sex determination, differentiation, and sexual plasticity in vertebrates.

With respect to sex change, hermaphroditic fish are either simultaneous or sequential hermaphrodites (Atz 1964; Yamamoto 1969). Simultaneous hermaphrodites possess both functional ovarian and testicular tissue. In contrast, species that are sequential hermaphrodites first possess functional ovarian tissue and then functional testicular tissue, or vice versa. In this chapter, we focus on sequential hermaphrodites, which are broadly divided into three types: protogynous species, which change from female to male; protandrous species, which change from male to female; and species that can change sex in both directions, from male or female and back again multiple times (Devlin and Nagahama 2002).

Our major interest is in understanding the physiological mechanisms, especially the endocrinology, of sex change in hermaphroditic fish. We believe that studies of sex change in hermaphrodites provide a valuable complement to knowledge of sex differentiation in well-studied gonochoristic fish, such as tilapia and medaka, and thereby deepen understanding of the complicated mechanisms of sex determination and sexual plasticity in vertebrates. Thus, in thre first Sect. (16.2.1), we describe the process of gonadal sex differentiation in several types of hermaphrodites in comparison with gonochoristic species. Then we use five different species as examples to describe recent work on sex change. Finally we discuss four important conclusions arising from this work: (1) ovarian differentiation may be the primary state in all sequentially hermaphroditic species; (2) sex change is controlled mainly by estrogen; (3) gonadotropin hormone (GTH) is involved in sex change; and (4) adult ovaries of gonochoristic species exhibit sexual plasticity, as shown for hermaphroditic species.

16.2 Gonadal Sex Differentiation in Gonochoristic and Hermaphroditic Fish

Sequential hermaphroditism in fish has evolved independently multiple times; over 350 species in 23 teleost families are sequential hermaphrodites (Frisch 2004). As described in 16.3, the gonads of these fish exhibit a high degree of sexual plasticity,

in some cases undergoing serial sex changes. In contrast, the gonads of gonochoristic fish remained fixed after sex determination, indicating a loss of sexual plasticity. To understand sexual plasticity in fish, it is essential to investigate the process of gonadal differentiation and formation during ontogeny. However, to date, information about gonadal differentiation in sex-changing fish remains limited. This limitation is due to the fact that the larvae of most of these fish—with the exceptions of groupers, anemonefish, and black porgy—are not robust and are consequently difficult to raise. We therefore first describe gonadal sex differentiation in a gonochoristic fish—the Nile tilapia—and the involvement of steroid sex hormones in gonadal differentiation. We then describe the process of gonadal sex differentiation in a protogynous grouper, in a protandrous anemonefish, in a protogynous wrasse and, finally, in a bidirectional sex-changing gobiid fish.

16.2.1 Gonadal Differentiation in the Gonochoristic Nile Tilapia

As seen in mammals, the sexes of the Nile tilapia, *Oreochromis niloticus*, are genetically determined XX/XY systems. All females (XX) and males (XY) may potentially be produced using sperm obtained from sex-reversed males (XX) or supermales (YY), respectively. Gonadal sex differentiation in the tilapia is summarized in Fig. 16.1 (Nakamura et al. 1998; Nakamura 2000, 2013). Until about 20 days posthatch (dph), the gonads of this fish are in an undifferentiated state; neither ovaries nor testes can be distinguished morphologically. Ovarian differentiation begins at

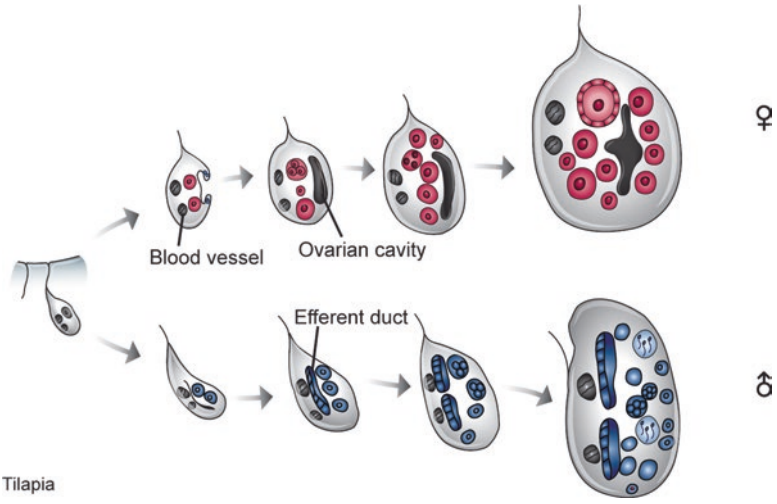

Fig. 16.1 Gonadal sex differentiation in the gonochoristic Nile tilapia, *Oreochromis niloticus*. The gonads of the fish differentiate into ovaries or testes at 20–25 days posthatch

20–24 dph, as determined by changes in both germ cells and somatic cells. During this phase, a sudden proliferation in germ cells and cysts of oogonia occurs. The oogonia then immediately transition into meiotic prophase oocytes. At about the same time, differentiating somatic cells begin to form the ovarian cavity (from which future ova will be discharged). First, elongations of somatic cells form at the base and tip of the ovaries on the lateral walls. Then two elongations form and extend in the direction of the lateral wall; eventually the ends of the elongations join, forming a space enclosed by somatic cell tissue on the lateral wall. Next, from 50 to 70 dph, the oocytes in the ovaries develop rapidly up to the perinucleolus stage. Division and proliferation of germ cells during the formation of spermatozoa in the testes is sometimes quiescent, making it difficult to determine when testicular differentiation begins during the differentiation of germ cells. However, differentiation of somatic cells for sperm duct formation begins at about the same time as ovarian differentiation (Nakamura et al. 1998; Nakamura 2000). Active changes in germ cells—such as the transition to division, proliferation, and meiosis of spermatogonium to form spermatozoa—occur much later than in the ovaries, starting at about 70 dph.

Next we outline the physiological framework, including the activities of endogenous sex hormones, by which undifferentiated gonads differentiate into ovaries or testes. First, to determine directly whether endogenous steroid sex hormones play an essential role in the initiation of gonadal sex differentiation in fish, we examined the differentiation of steroid-producing cells (SPCs)—the site of steroid hormone production—during this process. A notable finding is that SPCs are already differentiated in the gonads at around the time when sex differentiation begins (Nakamura and Nagahama 1985). This timing suggests that endogenous steroid hormones contribute to sex differentiation of the gonads in the Nile tilapia. Next, to define the relationship between steroid hormones and sex differentiation, we used immunohistochemistry with antibodies against the cholesterol side-chain cleavage enzyme (P450scc), 17α-hydroxylase (P450c17), 3β-hydroxysteroid dehydrogenase (3β-HSD), and aromatase (P450arom); the expression and localization of these enzymes was examined during gonadal sex differentiation (Nakamura 2013; Strüssmann and Nakamura 2002). In genetic females, the expression of all of these enzymes occurred in undifferentiated gonads before ovarian differentiation. Furthermore, the intensity of the immunopositive reactions and the number of cells exhibiting reactivity increased as ovarian differentiation progressed. A particularly notable finding was that P450arom, an enzyme required for estrogen synthesis, is already detectable in the gonads before sex differentiation. This observation indicates that endogenous estrogen is involved in inducing ovarian differentiation in undifferentiated gonads. In genetic males, however, undifferentiated gonads did not exhibit immunopositive reactions to the tested enzyme antibodies until after testicular differentiation. Once testicular differentiation began, weak positive responses to enzymes P450scc, P450c17, and 3β-HSD (but not to P450arom) were detected, but strong responses were not observed until immediately before spermatozoon formation began. Thus, unlike ovarian differentiation, steroid sex hormones are not directly involved in testicular differentiation.

In addition, various experimental findings suggest that endogenous estrogens are essential for gonadal sex differentiation. Administration of an aromatase inhibitor (AI)—which inhibits the synthesis of estrogen—to genetically female Nile tilapia during sex differentiation caused all individuals to change into males with testes. Simultaneous administration of an AI and estradiol-17β (E2) suppressed sex change and caused undifferentiated gonads to differentiate into ovaries (Nakamura 2000; Nakamura et al. 2000). Conversely, when a synthetic androgen, 17α-methyl testosterone (MT), was administered to genetically female Nile tilapia, the ovaries were transformed into testes. The expression of steroid-metabolizing enzymes during this MT-induced sex reversal was also examined immunohistochemically (Bhandari et al. 2006b). Almost no immunopositive reactions for any of the tested steroid hormone–metabolizing enzymes were observed in the MT-treated group. This result suggests that exogenous androgen can suppress the expression of several of these enzymes, thus inhibiting endogenous synthesis of estrogen during the sex differentiation. Taken together, these findings indicate that estrogens are inducers of ovarian differentiation and that the absence of estrogen is important for testicular differentiation. Therefore, the presence or absence of estrogen determines whether a fish becomes female or male.

16.2.2 Gonadal Differentiation in a Protogynous Grouper

Groupers (genus *Epinephelus*), which are protogynous, are important in fisheries throughout the world (Nakamura et al. 2005), and substantial efforts have been made to establish methods for their artificial breeding. As a part of this effort, we investigated the process of gonadal sex differentiation in the Malabar grouper (*Epinephelus malabaricus*) for 1 year after hatching (Fig. 16.2) (Murata et al. 2009). In fish at 11 dph, large primordial germ cells (PGCs) were evident in the primordial gonad tissue located below the mesonephric ducts on the dorsal side of the intestine. Up until 47 dph, neither the germ cells nor the somatic tissue showed any of the morphological characteristics of sexual differentiation. By 47 dph, the gonads had changed substantially, however, through an increase in the number of somatic cells. By 74 dph, the elongations indicating ovarian cavity formation (like those in tilapia ovarian differentiation) had developed further and were evident in the ovaries of all fish. However, ovarian germ cells had not yet begun active division. By 360 dph, oocytes were distributed within somatic tissue along the inner periphery of the ovarian cavity. These observations suggest that morphological changes associated with ovarian differentiation in the Malabar grouper begin at approximately 75 dph and that in all individuals the gonads differentiate directly into ovaries. No testicular differentiation was evident at the time of primary sex determination during ontogenesis.

To clarify the role of endogenous steroid hormones in sex differentiation in the grouper, we used immunohistochemistry to examine the expression of several steroidogenic enzymes—P450scc, P450arom, and P450 11β hydroxylase (P45011β),

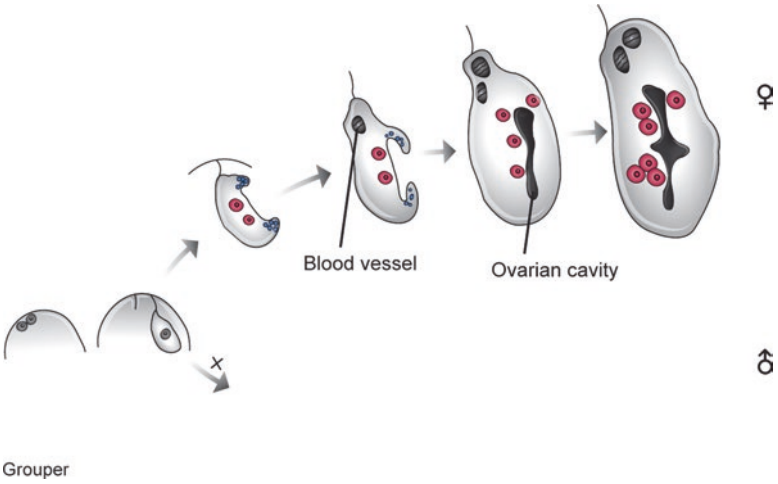

Fig. 16.2 Gonadal sex differentiation in the protogynus Malabar grouper, *Epinephelus malabaricus*. By 47 days posthatch, the gonads of all of the fish differentiate into ovaries. Direct differentiation of the gonads into testes is not observed in this fish

an important enzyme for androgen production in fish—during ovarian differentiation (Murata et al. 2011). P450scc and P450arom appeared first in the somatic cells surrounding the germ cells in undifferentiated gonads and were present throughout ovarian differentiation; in contrast, P45011β first appeared in the cluster of somatic cells in the ovary tunica near the dorsal blood vessels after ovarian differentiation. These observations suggest that endogenous estrogen is involved in ovarian differentiation. Treatment with androgen induced precocious spermatogenesis in the gonads of juveniles after ovarian differentiation (Murata et al. 2010). However, these testes reverted to ovaries upon withdrawal of androgen treatment (Murata et al. 2014).

16.2.3 Gonadal Sex Differentiation in a Protandrous Anemonefish

Anemonefishes (genus *Amphiprion*) are protandrous (male-first) fish. Histological analysis of the gonads of anemonefish has revealed that males and nonbreeding individuals possess bisexual gonads in which both mature testicular tissue and immature ovarian tissue coexist, whereas the gonads of females contain only ovarian tissues (Godwin 1994; Nakamura et al. 1994).

Gonadal sex differentiation in the anemonefish *Amphiprion clarkii* is shown in Fig. 16.3 (Miura et al. 2003, 2008a, b; Miura 2007) From hatching to 30 dph, the gonads are in a sexually undifferentiated state. Interestingly, the gonads of the fish differentiate into ovaries by 60 dph, and oocytes gradually develop and increase in

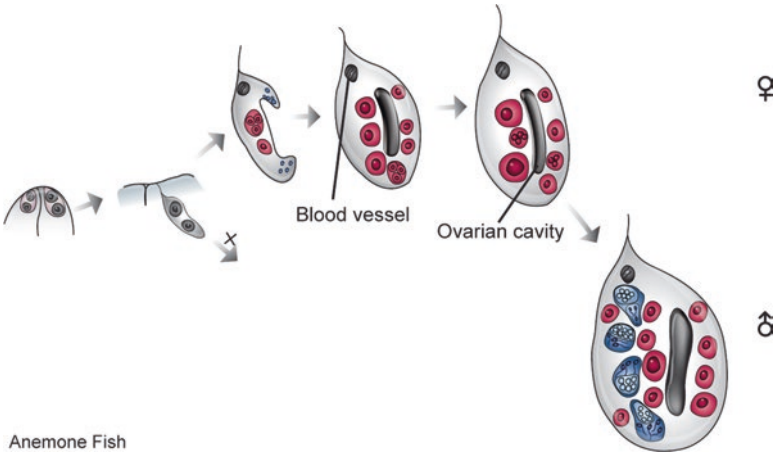

Fig. 16.3 Gonadal sex differentiation in the protandrous anemonefish *Amphiprion clarkii*. The gonads of all of the fish differentiate into ovaries by 60 days posthatch (dph). Between 60 and 183 dph the gonads of all of the fish contain oocytes at the perinucleolus stage, cysts of oocytes in the meiotic phase, and an ovarian cavity. Initial testicular differentiation, which occurs in the developed ovaries from 214 to 273 dph, is characterized by the appearance of spermatogenic germ cells among the oocytes. Direct differentiation of the gonads into testes is not observed in this fish

number as the ovaries grow, through 183 dph. Then, by 214 dph, cysts of differentiated spermatogenic germ cells become evident in the ovaries, and by 273 dph, ambisexual gonads with both ovarian and testicular tissues have formed. Thus, no primary males exist, and all males derive from females in the anemonefish, as previously thought (Shapiro 1992).

We have also examined the relationship between steroid hormones and gonadal differentiation in anemonefish (Miura et al. 2008a, b, 2013). Immunopositive cells reactive against P450scc, P45011β, and P450arom are present in sexually undifferentiated gonads by 30 dph, and they increase in activity around the time of ovarian differentiation at 60 dph. These results support the view that immature gonads around the time of sex differentiation have the potential to produce both androgen and estrogen. However, treatment with an AI and an estrogen antagonist (tamoxifen) around the time of primary ovarian differentiation had no effect on ovarian differentiation (e.g., differentiation and development of oocytes) and had no effect on ovarian cavity formation and induction of a seminal duct–like structure in the ovaries. These findings indicate that endogenous estrogen may not be required for primary ovarian differentiation in anemonefish. Androgen treatment during primary ovarian differentiation did not reverse differentiation of the ovary to testes. However, ovarian cavity development and differentiation of the seminal duct–like structure were delayed in the presence of androgen. The strength of immunoreactivity against P450scc and P45011β gradually increases, and testicular differentiation continues in the ovary until 210 dph; ambisexual gonads form by 270 dph. In contrast, P450arom-immunopositive cells with a weak signal intensity were seen in ambi-

sexual gonads just after testicular differentiation. Moreover, cells positive against P450arom were not present in ambisexual gonads, either in testicular or ovarian tissue at 270 dph. Production of E2 was high in the ovaries before the appearance of any testicular tissue, and it decreased along with the differentiation of testicular tissue. Production of 11-keto testosterone (11KT) in the gonads gradually increased with testicular differentiation. E2 treatment suppressed the naturally occurring differentiation of testicular cells such that only ovarian tissues formed in the gonad in vivo. These results suggest that a shift from estrogen to androgen production occurs during testicular differentiation; this shift may induce testicular differentiation in the ovary (Miura et al. 2013).

16.2.4 Gonadal Sex Differentiation in a Protogynous Wrasse and in a Bidirectional Sex-Changing Gobiid Fish

As mentioned above, many of the fish that undergo sex change are difficult to raise. To circumvent this problem, specimens of various sizes of a protogynous wrasse and a bidirectional sex-changing gobiid fish were collected from the wild so the gonadal status of juveniles of these fish could be examined histologically (Kobayashi et al. 2013).

Protogynous Wrasse The process of gonadal sex differentiation in the protogynous three-spot wrasse, *Halichoeres trimaculatus*, is shown in Fig. 16.4. Many of the smaller specimens, 10–30 mm in total length (TL), had undifferentiated gonads. Approximately 70–80% of specimens 30–40 mm in TL were observed to have ovaries containing oocytes in the perinucleolus stage. Approximately 20–25% of these larger fish were observed to have bisexual gonads, possessing both ovarian and testicular tissue. The remaining fish (4%) had fully differentiated testes. Among specimens of 40 mm in TL and greater, none was observed to have undifferentiated gonads; all of these specimens had begun to undergo sexual differentiation. Although 10–30% of the observed specimens possessed bisexual gonads, no such specimens were found among fish of 80 mm in TL and greater. Spermatids and sperm were observed within the bisexual gonads, and perinucleolus stage oocytes were distributed throughout the testicular tissue. Many specimens with bisexual gonads showed signs of oocyte degeneration. The bisexual organs of specimens of the large-size group were composed primarily of testicular tissue. These observations suggest that during the period of sexual differentiation, bisexual gonads differentiate into primary testes. Specifically, almost all primary males pass halfway through a female phase once. However, the relationship between gonadal sex differentiation and endogenous sex hormones in the wrasse remains unknown.

Bidirectional Sex-Changing Gobiid Fish The gobiid fish *Trimma okinawae*—the adults of which undergo bidirectional sex change—possess an ovary and testis simultaneously (Kobayashi et al. 2005). However, the gonad of juveniles before

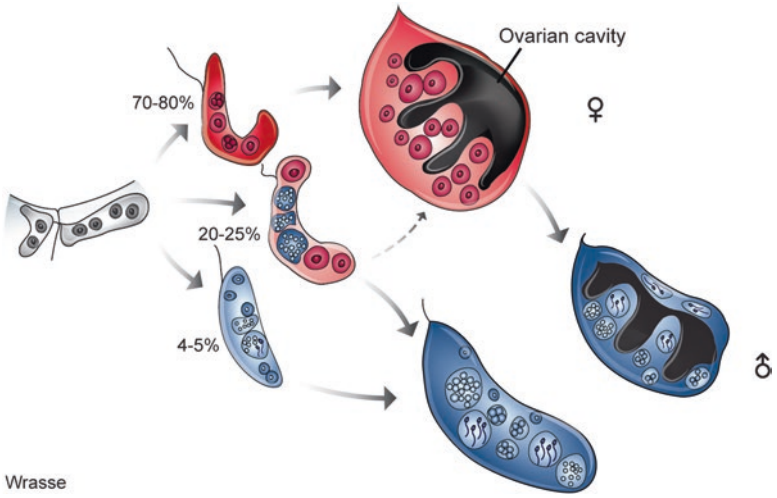

Fig. 16.4 Gonadal sex differentiation in the protogynus three-spot wrasse, *Halichoeres trimaculatus*. The gonads of fish 30–40 mm in total length differentiate into three types. The gonads of 70–80% of the fish differentiate into normal ovaries with oocytes, 20–35% have ambisexual gonads with both oocytes and spermatogenic germ cells, and 4% differentiate directly into testes. Ambisexual gonads later differentiate into defined ovaries or testes

puberty (less than 20 mm in TL) has no testicular tissue and consists only of ovarian tissue. It therefore seems likely that testicular tissue appears in the ovary after ovarian differentiation.

16.3 Morphology and Physiology of Sex Change in Hermaphroditic Fish

Fascination with the phenomenon of sex change in fish has led to numerous studies in the past. However, most of these studies have addressed sex change primarily from ecological, behavioral, or phylogenic perspectives (Erisman et al. 2013). Because most fish that undergo sex change are difficult to obtain or raise, the physiological and endocrinological mechanisms of their sex change remain unclear. Recently, observations of sex change in mature individuals after sex determination/differentiation have indicated that these fish would be good models for research into ovarian and testicular differentiation in vertebrates.

At an observational level, sex change in fish is triggered by social cues—typically loss of a larger male or larger female from a group or a harem. Upon receipt of the cue, responsive individuals quickly begin behaving like the opposite sex. Then, their gonads change to those of the opposite sex, and their body color and shape similarly become modified. In the 3.1–3.4.2, we describe the physiological changes during sex change in protogynous, protandrous, and bidirectional sex-changing fish.

16.3.1 Sex Change in Protogynous Wrasses

16.3.1.1 Visual Social Cues Affecting Sex Change

Wrasses exhibit diandric protogyny, with populations consisting of small initial-phase males (primary males), initial-phase females (primary females), and large terminal-phase males, which arise either from females that have undergone sex change to become males (secondary males) or from initial-phase males (terminal-phase primary males) (Ross 1982, 1983). Sex change from female to male in the saddleback wrasse, *Thalassoma duperrey*, occurs naturally in nature and can be induced by social factors under controlled experimental conditions. When a single large female is kept with a smaller conspecific of either sex, the large female will change sex, becoming a functional male within 8–12 weeks (Nakamura et al. 1989; Ross 1981, 1982, 1983). Social cues also induce sex change, from female to male, in the three-spot wrasse under captive conditions, as seen in the saddleback wrasse (Nozu et al. 2013).

16.3.1.2 Morphological and Physiological Changes During Sex Change in Wrasses

Histological analysis of gonadal transformations during sex change have been reported for many protogynous fish. These transformations follow a common process. First, ovarian tissue, including mature and immature oocytes, degenerates. Then, testicular tissue appears and develops. In the saddleback wrasse and three-spot wrasse, the histological process of gonadal sex change, which is similar in the two fish, is well described (Nakamura et al. 1989; Nozu et al. 2009). The process has been classified into six stages (Fig. 16.5).

Regardless of the direction of sex change, steroid hormones are known to play an important role in sex change (Devlin and Nagahama 2002; Frisch 2004). Upon the initiation of sex change, estrogen levels in the saddleback wrasse drop sharply, whereas during the second half of the process, androgen levels rise (Fig. 16.6) (Nakamura et al. 1989). This timing suggests that the fall in estrogen levels triggers the sex change. In support of this hypothesis, sex change has been induced in several varieties of protogynous fish via administration of an AI, which causes endogenous estrogen levels to decrease (Alam et al. 2006; Bhandari et al. 2004a, b; Higa et al. 2003; Nozu et al. 2009). Recently, sex change accompanied by falling estrogen levels was induced by administration of the stress hormone cortisol (Nozu and Nakamura 2015). This result suggests that cortisol plays a role in induction of sex change by regulating production of estrogen. On the other hand, administration of androgen also induces a female-to-male sex change (Bhandari et al. 2006a; Higa et al. 2003; Murata et al. 2014). It has also been reported for wrasses (Labridae) that a rise in androgen levels triggers sex change (Ohta et al. 2008a). From these kinds

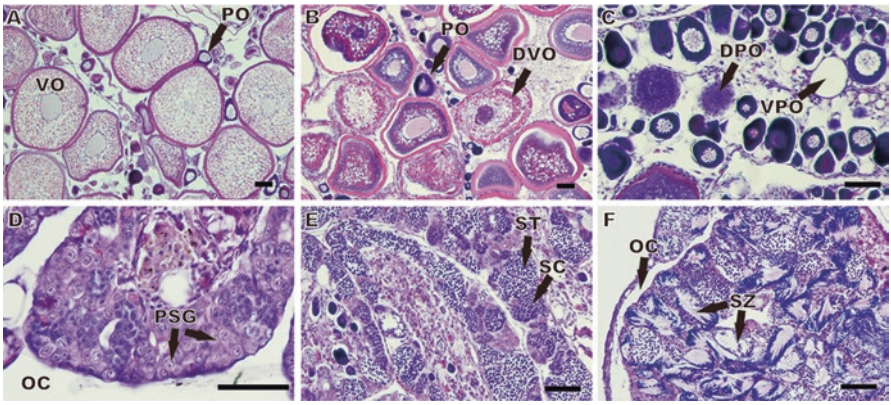

Fig. 16.5 Histology of gonadal sex change in the three-spot wrasse, *Halichoeres trimaculatus*. (**A**) Stage 1: ovary with previtellogenic oocytes (POs) and vitellogenic oocytes (VOs). (**B**) Stage 2: gonad including degenerate vitellogenic oocytes (DVOs), indicating the onset of sex change. (**C**) Stage 3: occurrence of degenerate previtellogenic oocytes (DPOs). (**D**) Stage 4: presumed spermatogonia (PSGs) proliferating actively in the peripheral region of the ovigerous lamella. (**E**) Stage 5: spermatogenesis occurring from the periphery of the ovigerous lamella. (**F**) Stage 6: mature secondary testis with an ovarian cavity (OC). *SC* spermatocyte, *ST* spermatid, *SZ* spermatozoon, *VPO* vestige of previtellogenic oocyte. Scale bars, 20 μm

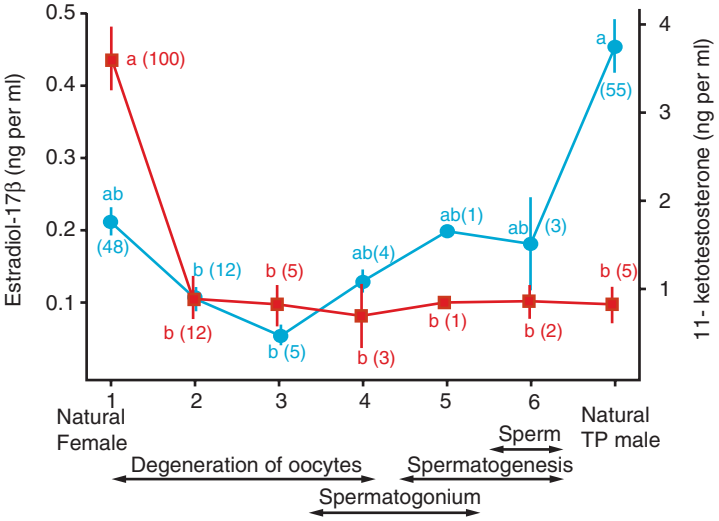

Fig. 16.6 Changes in plasma estradiol-17β (E2) and 11-keto testosterone (11KT) levels during a sex change in *Thalassoma duperrey*. E2 levels are highest in sexually mature females but drop rapidly at the onset of the sex change. 11KT levels increase gradually after the midstage of the sex change

of findings, it is now thought that although the key steroid sex hormones may vary depending on the type of fish, disruption of the steroid sex hormone balance (estrogen/androgen) is essential for the initiation of sex change. In fact, it has been demonstrated that disruption of this balance via administration of exogenous sex hormones leads to opposite-directional sex changes not observed in the wild (Kojima et al. 2008; Miyake et al. 2008). Thus, because an individual's sex is maintained through a balance of steroid hormones, fish that undergo sex change are endowed with an unusually high degree of sexual plasticity.

With the process of gonadal sex change defined, attention next focused on the expression of sex-related genes during sex change. Specifically, we examined the variation in the expression of sex-related genes in the three-spot wrasse. The genes examined include R-spondin 1 (*rspo1*) and Forkhead box protein L2 (*foxl2*), which are involved in ovarian differentiation and maintenance in vertebrates, and gonadal soma-derived factor (*gsdf*), doublesex and mab-3 related transcription factor 1 (*dmrt1*), anti-Müllerian hormone (*amh*), and sex-determining region Y box 9 (*sox9*), which play important roles in the differentiation and maintenance of testes. Changes in the expression of each of these genes during sex change were observed (Fig. 16.7). When sex change begins, the expression levels of *rspo1* decline. Of great interest, during degeneration of ovarian tissue (stages 2–3), oocytes degenerate and disappear via apoptosis; apparently, however, granulosa cells surrounding the oocyte survive throughout the gonadal transformation (Nozu et al. 2013). Consistent with this result, expression levels of *foxl2*, a granulosa cell molecular marker, did not decline during sex change (Kobayashi et al. 2010c). Next, a distinct increase in spermatogonia-like cells occurs and spermatogenesis begins (stages 4–5). Before and after this period, the expression levels of *gsdf*, *dmrt1*, *amh*, and *sox9* rise (Horiguchi et al. 2013; Nozu et al. 2015). These findings suggest that these genes contribute to testis formation during gonadal sex change. In particular, the expression levels of *gsdf*, a testis differentiation marker, rise early in the process (Horiguchi et al. 2013). With respect to messenger RNA (mRNA) localization, *gsdf* is localized to the ovary; weak expression can be observed in supporting cells adjacent to the gonial germ cells, and the level of expression increases with the progression of the sex change. At the next stage, *gsdf* is specifically expressed in cells of the Sertoli cell lineage. Apparently, therefore, Sertoli cells originate from the supporting cells adjacent to the gonial germ cells during sex change, i.e., the supporting cells seen in the ovary differentiate into Sertoli cells. Furthermore, although the rise in expression of *dmrt1* is delayed in comparison with *gsdf*, changes in the expression localization of these genes support this hypothesis (Nozu et al. 2015). However, the specific origins of many of the types of cells that contribute to gonadal tissue during sex change are still unclear. We anticipate that through detailed descriptions of cellular behavior, the mechanisms governing properties such as individual cell sexual plasticity and irreversibility will be defined.

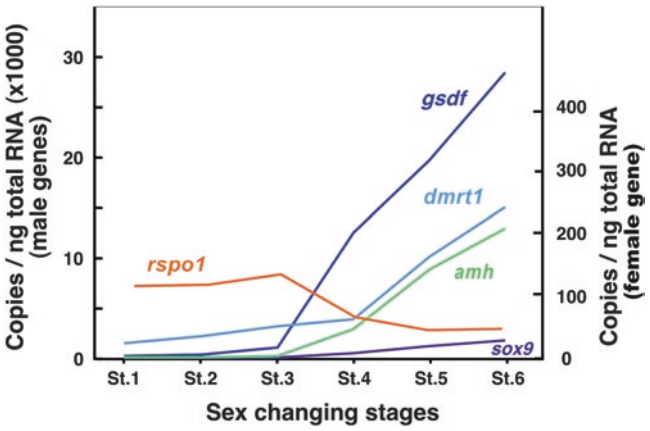

Fig. 16.7 Changes in expression levels of sex-related genes during a sex change in *Halichoeres trimaculatus*. Female-related gene: *rspo1*; male-related genes: *gsdf*, *dmrt1*, *amh*, and *sox9*. The expression of *rspo1* drops during the sex change. The expression of male-related genes increases from the midstage of the sex change

16.3.2 Sex Change in a Protogynous Grouper

Studies of groupers can be difficult because of the large size of the fish. We therefore chose a relatively small-sized species, the honeycomb grouper (*E. merra*), which is available in the wild, as an experimental model (Nakamura et al. 2005).

16.3.2.1 Morphology and Physiology of Sex Change

Sex change in groupers depends on age and body size, not on social factors (Murata et al. 2012; Nakamura et al. 2005). Therefore, to obtain baseline information on the sexuality of the honeycomb grouper, specimens were captured from the wild over a 1-year time span (Bhandari et al. 2003). Histological observations revealed that female-phase gonads consisted of oocytes in several developmental stages but lacked testicular tissue. At the beginning of the sex change, oocytes begin degenerating, followed by a proliferation of spermatogonia. Finally, no ovarian cells were observed, and the testis consisted of germ cells undergoing active spermatogenesis. Female and male fish were mostly smaller and larger, respectively, whereas fish undergoing sexual transition were in the intermediate size and weight ranges (Bhandari et al. 2003). During the breeding season, no specimens undergoing sex change were observed. During the nonbreeding season, in contrast, an overlap was present in the sex distribution of transitional individuals, indicating that sex change occurred during this time. Taken together, these observations indicate that sex change in the honeycomb grouper usually occurs in nonbreeding larger females (over 200 mm in TL).

As in the case of the protogynous wrasse, serum levels of E2 in the grouper are high in females but low in males and in individuals undergoing sex change (Bhandari et al. 2003, 2005). In contrast, 11KT levels are low in females and gradually increase in the transitional phase and in males (Bhandari et al. 2006a). These results suggest that low serum E2 levels and degeneration of oocytes, accompanied by a concomitant increase in levels of 11KT and a proliferation in spermatogenic germ cells, are the events mediating protogynous sex change in this species.

16.3.2.2 Involvement of Gonadotropin in Gonadal Sex Change

Since the signals for controlling gonadal sex change come from the brain, the hypothalamus–pituitary–gonad (HPG) axis is involved in this process (Godwin 2010). In teleosts, as in other vertebrates, gonadal steroidogenesis is largely controlled by two GTHs produced in the pituitary gland: follicle-stimulating hormone (FSH) and luteinizing hormone (LH). These GTHs contain a common glycoprotein hormone α-subunit (GPα), which forms a heterodimer with unique β-subunits (FSHβ and LHβ, respectively) (Swanson et al. 2003). In well-studied salmonids, FSH plays a significant role in puberty and gametogenesis, whereas LH is primarily involved in the final maturation of gametes in both sexes (Swanson et al. 2003). Variations in the expression profiles and roles of GTHs have been reported in teleosts, and several reports have suggested that GTHs are factors that control gonadal sex change (Godwin 2010). For example, sexually dimorphic expression of GTH subunit genes has been observed in the protogynous wrasse *Pseudolabrus sieboldi* (Ohta et al. 2008b). In the black porgy (*Acanthopagrus schlegelii*), which is protandrous, plasma LH levels were higher in males than in fish undergoing sex change (Lee et al. 2001). Furthermore, treatment with exogenous human chorionic gonadotropin (hCG) induced sex change in the protogynous wrasse *Coris julis* (Reinboth and Brusle-Sicard 1997). However, information on GTH expression patterns during the sex change process is lacking; as a consequence the specific biological roles of GTHs in sex change remain unresolved. This situation is beginning to change, however, as data indicating a role of GTHs in gonadal sex change have been reported recently, as described below.

The most comprehensive studies of the role of GTHs in sex change have been carried out in the protogynous honeycomb grouper (Kobayashi et al. 2010a). Although the control of sex change in this fish by social manipulation has not been possible, work on the different sexual phases has progressed using specimens captured from the wild (Bhandari et al. 2003). Specifically, transcripts of GTH subunit genes in the pituitary in different sexual phases of the honeycomb grouper have been quantified (Fig. 16.8). The relative levels of *gpα* and *lhβ* mRNA were higher in the breeding season than in the nonbreeding season. However, the levels did not differ significantly between different sexual phases during the nonbreeding season. In contrast, the expression pattern of *fshβ* transcripts showed marked sexual dimorphism. Transcripts of the *fshβ* subunit were low in breeding and nonbreeding female phases, and they substantially increased during female-to-male sex change, espe-

cially in the early transitional (ET) stage. Immunohistochemical analysis using antisera to the GTH subunit confirmed these results. Similarly, upregulation of the gonadal FSH receptor occurred during sex change (Alam et al. 2010). In addition, to identify the role of GTHs in gonadal sex change in this fish, in vivo treatments with bovine FSH and LH were carried out. After 3 weeks, FSH treatment had induced female-to-male sex change and had upregulated endogenous androgen levels and *fshβ* transcripts, whereas LH treatment had no effect on sex change. Taken together, these results strongly suggest that FSH triggers female-to-male gonadal sex change in the honeycomb grouper.

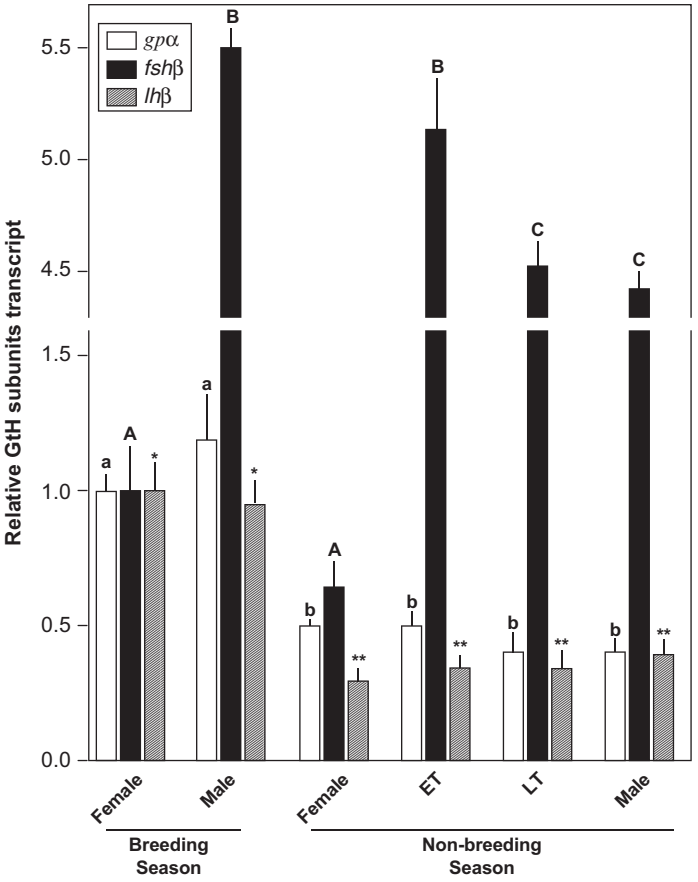

Fig. 16.8 Changes in gonadotropin hormone (GTH) subunit transcripts in pituitaries in different sexual phases in the honeycomb grouper, *Epinephelus merra*. Quantification of the transcript abundances of the *gpα*, *fshβ*, and *lhβ* genes in the pituitaries is determined by quantitative polymerase chain reaction (qPCR) and normalized to the abundance of glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) transcripts. The values are expressed as fold changes in abundance relative to the means in the breeding female. The data are shown as means ± standard errors of the means (SEMs). Data points not sharing a letter differ significantly, according to a Tukey–Kramer multiple comparison test. *ET* early transitional, *LT* late transitional (Redrawn from Kobayashi et al. 2010a)

16.3.3 Protandrous Sex Change in Anemonefish

16.3.3.1 Visual Social Cues Effecting Sex Change

Anemonefish are hermaphrodites, which usually live in small social groups with isolated anemones. Each group consists of one breeding pair—a functional female and male—together with several subadults or juveniles (Fricke and Fricke 1977; Fricke 1979; Moyer and Nakazono 1978; Ross 1978). When the largest individual—which is always female—disappears, the largest remaining male changes its sex to female, and the largest subadult becomes a functional male. This pattern strongly suggests that a social factor, specifically the disappearance of the functional female from a group, triggers sex change in male anemonefish.

16.3.3.2 Morphological and Physiological Changes During Sex Change

Histological analysis of the gonads of anemonefish has revealed that males and nonbreeders possess bisexual gonads, with coexisting mature testicular and immature ovarian tissues, whereas the gonads of females contain only ovarian tissues (Fig. 16.9) (Godwin 1994; Nakamura et al. 1994). During sex change, the ovarian tissues develop and the testicular tissues regress in the bisexual gonads (Godwin 1994). The mechanisms of this process have been studied extensively. As in other sex-changing species (Nakamura et al. 2005), gonadal steroid sex hormones—especially estrogen—are the key regulators of sex change in anemonefish (Godwin and Thomas 1993; Kobayashi et al. 2010b). However, the upstream mechanisms controlling the production and activity of gonadal steroid hormones during sex change in anemonefish remain largely unknown.

16.3.4 Bidirectional Sex Change in a Gobiid Fish

In protandrous and protogynous species, sex change is generally not reversible, occurring only once in the fish's life. In 1993, however, Sunobe and Nakazono first described bidirectional sex change in the Okinawa rubble goby, *T. okinawae* (family: Gobiidae) (Sunobe and Nakazono 1993). This gobiid fish has a polygynous mating system in which a group of individuals—a harem—normally consists of one dominant male and one or more females (Sunobe and Nakazono 1999). Removal of the dominant male from the harem results in female-to-male sex change by the largest female (protogyny). If the dominant male is returned to the harem, the fish that underwent the sex change transforms back into a female (protandry) (Fig. 16.10).

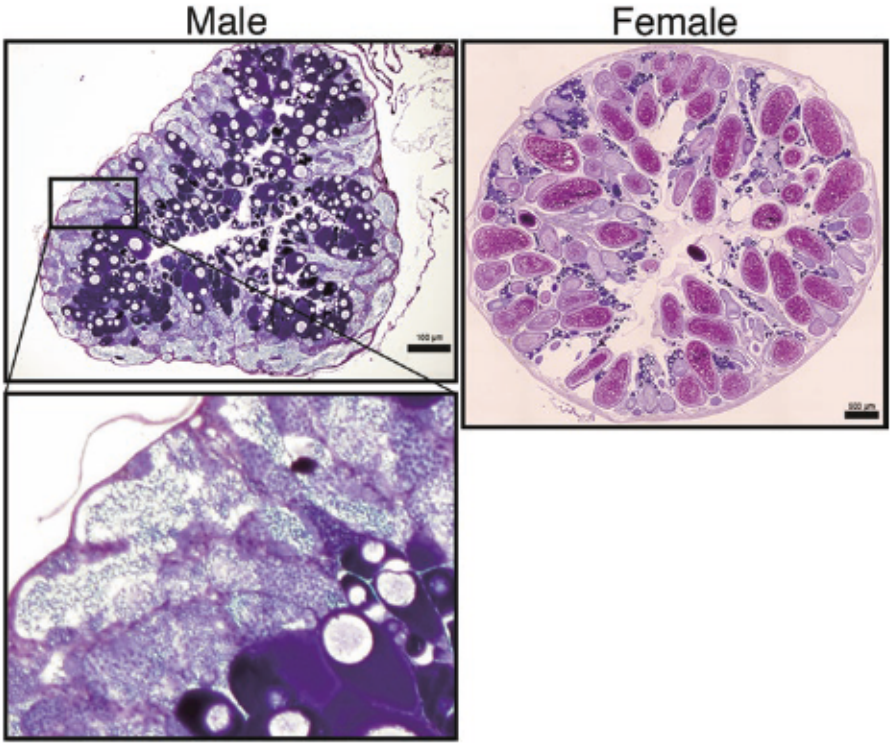

Fig. 16.9 Male and female gonadal structure in the protandrous anemonefish *Amphiprion clarkii*. Males possess an ovotestis gonad in which mature testicular tissue and immature ovarian tissue are both present and incompletely delimited. The cysts of spermatogenic tissue are separated by a thin cellular barrier. In contrast, females show no testicular tissues in the gonad

16.3.4.1 Visual Social Cues Affecting Bidirectional Sex Change

In *T. okinawae*, social interactions with conspecifics mediate bidirectional sex change. However, it is not clear how fish recognize their own social rank; changes in behavior at the beginning of a sex change have not been described in detail.

To investigate the temporal aspect of sex change in *T. okinawae*, we carried out laboratory experiments. When two males were kept together in an aquarium, the smaller male changed its sex to female (protandry). Conversely, when two females were kept together, the larger female changed its sex to male (protogyny). Protogynous sex change occurred within just 5 days, and protandrous sex change occurred within 10 days. These rates of sex change in *T. okinawae* are faster than those in other sex-changing fish species.

These observations indicate that the larger member of a pair is always male and the smaller member is always female (Fig. 16.11). However, it is not clear how the fish recognizes the body sizes of other fish or whether chemical cues might be

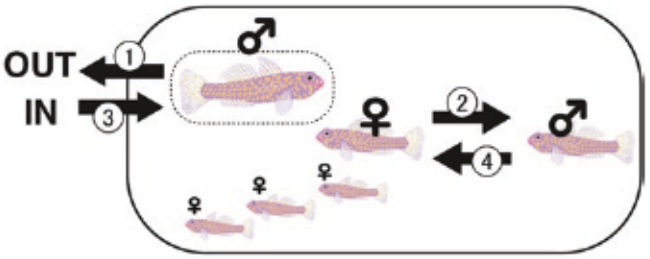

Fig. 16.10 Bidirectional sex change in the gobiid fish *Trimma okinawae*. The mating system of *T. okinawae* is a harem consisting of a dominant male and two or three females. Removal of the dominant male (1) results in a protogynous sex change in the largest female in the harem (2). If placed back into the harem with a larger male (3), the male reverts back to a female (protandrous sex change) (4).

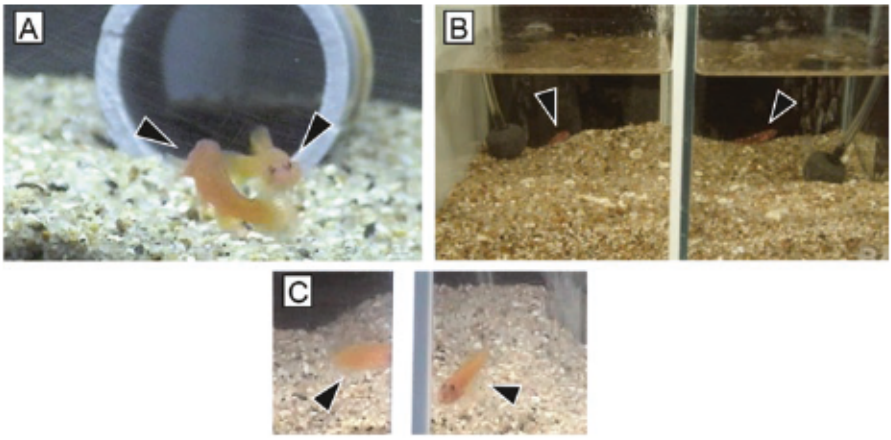

Fig. 16.11 Induction of a bidirectional sex change in the goby *Trimma okinawae*. (**A**) Two male or female fish are placed together in an aquarium, after which an intense competitive interaction occurs. (**B**) Two fish (males or females) are isolated in a glass-separated aquarium. The *arrowheads* indicate the experimental fish. (**C**) After pairing in the glass-separated aquarium, an intense competitive interaction occurs between the fish

involved. To address this question, we prepared a glass-separated aquarium (Fig. 16.11a) in which fish could see but not make physical contact with each other. As in the experiments described above, pairs of males or pairs of females were kept together in an aquarium but separated from each other by the glass. After 2 weeks, protandrous and protogynous sex changes were observed in pairs of females and pairs of males, respectively. This result indicates that sex change is induced by visual cues, not chemical or tactile cues. Interestingly, behavioral changes occurred within 30 min of social manipulation. After being placed together, the larger male or female attacked the smaller fish, which fled and often hid in a nest (Fig. 16.11b, c). After 30 min, however, the larger fish began to court the smaller fish. It is well

known that sexual behaviors are dependent on brain sex (Godwin 2010). Thus, the rapid behavioral changes in *T. okinawae* after social manipulation suggest that, regardless of gonadal sex, visual cues quickly alter the brain sex of the fish.

16.3.4.2 The Physiological Mechanism of Bidirectional Sex Change

The structure of the gonad of *T. okinawae* differs fundamentally from that of other fish that undergo sex change (Fig. 16.12) (Kobayashi et al. 2005). All gonads that were examined, including those from female- and male-phase fish, unambiguously contained both ovaries and testes attached to an accessory gonadal structure. In addition, the oviduct and sperm duct are separate in this fish. In short, this fish, by having both ovaries and testes, is equipped to rapidly respond to its social status, even though only one gonad is active at a given time. This unique gonadal structure facilitates sex change in either direction in this species.

As mentioned above, GTHs are involved in gonadal sex change. GTHs, produced by the pituitary, are secreted into the blood and act primarily at the level of the gonads (Kobayashi et al. 2004). The actions of GTHs are mediated by GTH receptors (GTHRs) on the surface of the target cells in the gonads (Oba et al. 2001). The genes for two GTHRs—FSHR and LHR, which specifically bind FSH and LH, respectively—have been cloned from various vertebrates, including fish (Oba et al. 2001). Analysis of GTHR activity in the gonads of sex-changing fish presumably therefore would help reveal the mechanism by which GTHs function. This analysis has proven difficult, however, because the active and inactive regions of the gonads in sex-changing fish typically are difficult to separate. In this regard, we have found that *T. okinawae*, which can undergo bidirectional sex change, is a good model with which to investigate the activity of GTHRs in the gonad. In this fish, active and inactive gonad tissue can be separated and collected easily (Kobayashi et al. 2005). We have found that the earliest change in the gonads in this fish is a change in the

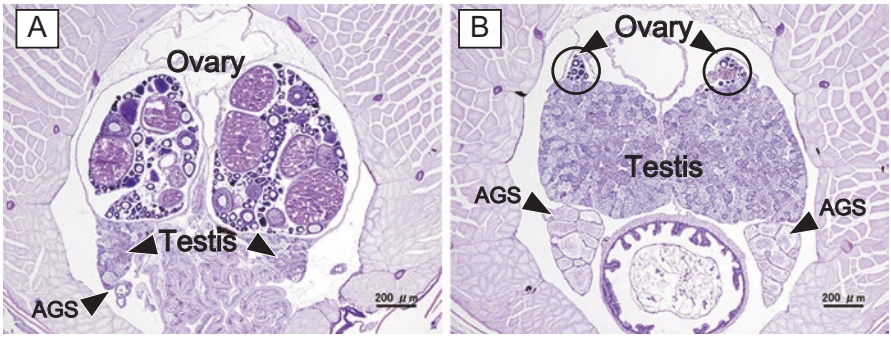

Fig. 16.12 Gonadal structure of **A** functional female and **B** male *Trimma okinawae*. The female gonad contains vitellogenic oocytes but no spermatozoa in the testicular region. The male gonad has spermatozoa in the testicular region but no vitellogenic oocytes. *AGS* accessary gonadal structure. Bars, 200 μm (Redrawn from Kobayashi et al. 2005)

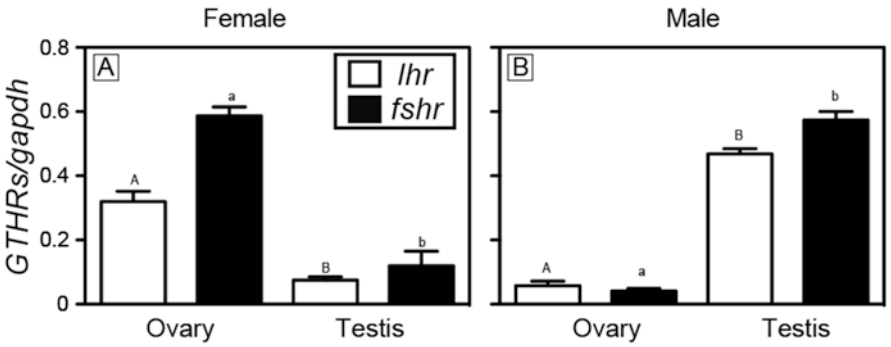

Fig. 16.13 Transcripts of both gonadotropin hormone receptors (GTHRs) (lhr: *white bar*; fshr: *black bar*) in the gonads of **A** female-phase and **B** male-phase *Trimma okinawae*. The values are means ± standard errors of the means (SEMs). The different *superscript letters* indicate significant differences ($P < 0.05$)

expression levels of the GTHRs. The expression of both receptors was found to be confined to the active gonad of the corresponding sexual phase (Fig. 16.13). During a sex change from female to male, the ovary initially had high levels of *fshr* and *lhr* and eventually was transformed into testicular tissue. The gonads started to change with the switching on of GTHR expression, which was discernible within 8–12 h of a visual social cue (Kobayashi et al. 2009). These findings suggest that sex change is initiated through rapid changes in the brain and behavior, followed by changes, mediated by GTHRs, in the gonad.

16.4 Sexual Plasticity in Gonochoristic Fish

It has been assumed for some time that sexual plasticity of the ovaries in vertebrates is lost after sexual differentiation, except in some hermaphroditic species. In a contradiction of that view, however, we demonstrated recently that at least some gonochoristic fish maintain their sexual plasticity into adulthood. Specifically, depletion of estrogen by AI treatment induced functional testicular differentiation in the ovaries of tilapia, medaka, and zebrafish (Fig. 16.14) (Paul-Prasanth et al. 2013; Takatsu et al. 2013). We also induced testicular differentiation in the ovaries of adult specimens of other gonochoristic fish—the carp (*Cyprinus carpio*) and the golden rabbitfish (*Siganus guttatus*)—by AI treatment (Nakamura et al., unpublished data). It is evident from these results that E2 plays a critical role in maintaining the female phenotype. In addition, estrogen treatment induced a complete change from a testis to a mature ovary in males of the wrasse *H. trimaculatus* (Kojima et al. 2008). As mentioned above, AI treatment to artificially deplete estrogen brought about a complete change of ovaries into mature testes in the grouper *E. merra* and the wrasse

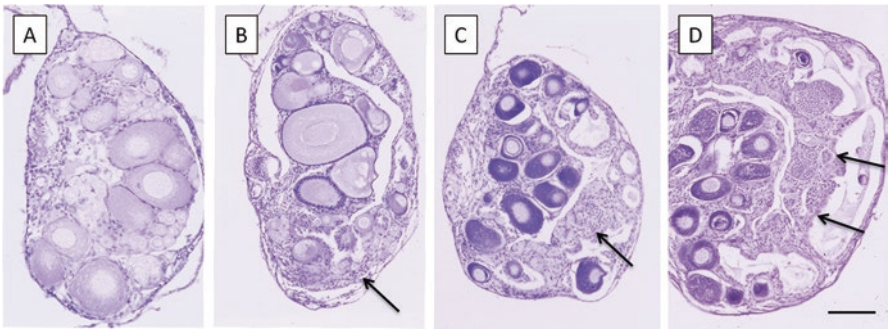

Fig. 16.14 Sexual plasticity of the ovaries in the gonochoristic Nile tilapia, *Oreochromis niloticus*. Differentiation and development of testicular tissues in the ovaries by treatment with an aromatase inhibitor (AI). (**A**) Normal immature ovary. (**B**) Immature ovary of a fish treated with an AI for more than 1 month; note the tissue structure changes near the edge of the ovarian cavity (OC) after the onset of AI treatment (arrow). (**C**) Initial testicular differentiation in the ovary; testicular tissue, including spermatogonia and spermatocytes (arrow), appears near the OC. (**D**) Development of spermatogenic tissues in the ovary; active spermatogenic tissue (arrows) spreads along the wall of the OC

H. trimaculatus, which are protogynous (Bhandari et al. 2004a, b; Nozu et al. 2009). The same treatment also induced the opposite sex change, from female to male, in the anemonefish *A. clarkii* (Nakamura et al. 2015). These results demonstrate clearly that some germ cells and somatic cells in the gonads of gonochoristic fish retain plasticity after sexual differentiation and maturation.

16.5 Conclusion

Although the relationship between sex change and sex-determining genes has attracted considerable interest recently, the nature of this relationship remains poorly understood. Sex-changing fish undergo secondary sex determination after sexual maturation, so the same individual experiences life as both a female (with ovaries) and a male (with testes). Thus, it is evident that both sexes have the same genetic makeup. Previously for gonochoristic fish, it was believed that males possess sex-determining genes and that females do not; during primary sex determination, these genes are expressed in the somatic cells that surround the germ cells and cause them to differentiate into testes. In contrast to that view, however, we have shown—through detailed observation of the sexual differentiation processes in the grouper *E. malabaricus*, the anemone fish *A. clarkii*, and the gobiid fish *T. okinawae*—that primary males are not present. All males in these species arise only as secondary males that have originated from females. Experimentally, in gonochoristic fish such as tilapia, medaka, and zebrafish, females can be changed into males (artificial secondary males) by AI treatment (Paul-Prasanth et al. 2013; Takatsu et al. 2013). Offspring that arise by fertilization of normal female eggs with the

sperm from artificial secondary male individuals (XX) are all female. Thus, in *E. malabaricus* and *A. clarkii*, which consist only of secondary males, the offspring should all be female. For these reasons, it is highly likely that monoandrous species of sex-changing fish do not possess sex-determining genes or that these genes have become dysfunctional. These species therefore depend on a physiological mechanism of sex determination to enable the change from female to male, the subsequent production of males, and the creation of female/male gonochorism—and to achieve sexual reproduction. The existence of physiological mechanisms of sex determination indicates that sex-determining genes are not essential.

Undifferentiated gonads in the protogynus wrasse *H. trimaculatus* differentiate into ambisexual gonads and into ovaries at the time of sex differentiation. In addition, direct differentiation into testes (primary sex differentiation) has been observed (unpublished data). Mature females later change into males. These findings strongly suggest that sex determination in the wrasse involves both sex-determining genes and a physiological mechanism. However, the observed percentage of direct testicular differentiation was extremely low (less than 5%). This fact suggests that functional sex-determining genes in this fish might be undergoing evolutionary loss. To examine this possibility, it will be necessary to define sex ratios through progeny tests such as mating of primary males with normal females or mating of secondary males with normal females.

Changes in the levels of endogenous steroid hormones provide a physiological mechanism for regulating sex change. Through research in saddleback wrasses, which undergo sex change in response to social factors, we have shown that endogenous estrogen is deeply involved in the transition from mature ovaries to functional testes. In wrasses, the levels of estrogen drop precipitously at the start of the sex change process. These fish have also been shown to transition from ovaries to testes when the levels of estrogen are artificially lowered with an AI (Higa et al. 2003; Nozu et al. 2009). Additionally, sex change was inhibited when estrogen was supplemented at the same time (Higa et al. 2003). A decrease in the levels of estrogen promotes the expression of genes involved in testicular differentiation (Horiguchi et al. 2013; Nozu et al. 2015). Artificial lowering of estrogen induces testicular differentiation not only in wrasses but also in the protogynous grouper *E. merra* and the protandrous anemonefish *A. clarkii* (Bhandari et al. 2004a, b; Nakamura et al. 2015). Although the processes of sex change vary in different fish, a commonality is the regulation of sex change through alterations in the levels of estrogen. This physiological mechanism of sex change—regulation by control of estrogen levels—is shared by the aforementioned fish species, which use only a physiological mechanism for primary sex determination, i.e., physiological sex differentiation.

By manipulating estrogen levels we have demonstrated that even mature ovaries can transform into testes in gonochoristic fish (e.g., tilapia, medaka, zebrafish, rabbit fish, and carp). This capability—previously considered impossible—confirms the hypothesis that a decrease in estrogen triggers sex change, and it shows that the ovaries of gonochoristic fish, like those of sex-changing fish, remain sexually plastic even after sex determination. In sex-changing fish, social stimuli induce a decrease in the production of estrogen. In contrast, ovaries of gonochoristic fish—

despite their retention of sexual plasticity—do not transform into testes, because these fish lack the regulatory mechanism that lowers estrogen to the level necessary for induction of a sex change. These considerations suggest that a certain concentration of estrogen, which is synthesized and secreted in the follicular tissue surrounding the eggs in fish ovaries in gonochoristic and hermaphroditic species alike, is responsible for modulating the transformation of ovaries into testes. Sex-changing fish therefore are able to undergo sex change through a regulatory mechanism for modulating levels of estrogen. Gonochoristic fish, despite having apparently lost sex-determining genes, retain the ability to form males and carry out sexual reproduction by means of this physiological mechanism sex determination, thereby retaining the sexual plasticity of their ovaries.

16.6 Future Directions

Sex change in many kinds of fish is initiated by social factors. For example, the behavior of *T. okinawae* changes dramatically in response to visual cues well before gonadal transformation begins (Kobayashi et al. 2009). The presence of different-sized individuals serves as a social stimulus in this fish, one that reaches the brain via visual perception and subsequently influences the gonads. However, it remains unclear what types of changes are triggered in the brain and how those changes mediate gonadal transformation (Kobayashi et al. 2013). As discussed in 16.3.2.2, FSH from the pituitary gland plays a dominant role in grouper sex change (Kobayashi et al. 2010a). In *T. okinawae* as well, sex change is regulated by rapid and dramatic changes in the expression of GTHRs in the gonads (Kobayashi et al. 2009). Stimuli from the brain therefore are clearly involved in the sex change process. We therefore anticipate that pinning down the details of sex change will require an understanding of the relevant processes and mechanisms occurring within the brain.

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Chapter 17
Sex Determination and Differentiation in Frogs

Michihiko Ito

Abstract In amphibians, it is believed that sex is genetically determined. The genetic sex-determining systems of amphibians include female (ZW) and male (XY) heterogamety. Interestingly, the Japanese Wrinkled Frog (*Glandirana* (*Rana*) *rugosa*) has both types of heterogamety, which was caused by geographic variation. Although almost all mammalian and avian species have heteromorphic sex chromosomes, the majority of amphibians, including the African Clawed Frog *Xenopus laevis*, possess homomorphic sex chromosomes. Thus, there should be a variety of sex-determining genes in amphibians. However, little is known about the molecular mechanisms underlying sex determination, although a W chromosome-linked gene *dm-w* in *X. laevis* was reported in 2008 to be responsible for a case of female sex determination. In contrast to the heterogamety, gonadal sexual differentiation follows a more conservative system. In many frog species, exposure of tadpoles with undifferentiated gonads to estrogen or androgen can induce male-to-female or female-to-male sex reversal, respectively. These findings suggest that sex steroid hormones have important roles in early sex differentiation. Estrogen- and androgen-synthesizing genes *cyp19a1* and *cyp17a1* show sexually dimorphic expression in early differentiating gonads in some frog species. In *X. laevis*, the structure called 'mass-in-line,' consisting of *cyp17a1*/*cyp19a1*-expressing cells, is involved in ovarian cavity formation. This chapter describes these situations in detail, and co-evolution between sex-determining genes and sex chromosomes is discussed. Germ cell development including gametogenesis and its endocrine control are also described.

Keywords Sex chromosome · Sex steroid · Sex-determining gene · Heterogamety · Gonadal development · Transcription factor · Sexual dimorphism

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17.1 Introduction

In vertebrates, sex is genetically or environmentally determined. Amphibians appear to adopt only genetic sex-determining systems with female (ZW) and male (XY) heterogametic sex chromosomes. In some frog species, it is speculated that there are a variety of XX/XY-type and ZZ/ZW-type sex-determining systems with different sex-determining genes. This chapter first describes the diversity of sex-determining systems from the viewpoint of homomorphic (morphologically indistinguishable) and heteromorphic (morphologically distinguishable) sex chromosomes, and discuss the relationships between sex-determining genes and systems as an evolutionary model for the sex-determining systems (Mawaribuchi et al. 2012).

In amphibians, no sex-determining genes have been verified except for a W chromosome-linked gene *dm-w* in the African Clawed Frog (*Xenopus laevis*) (Yoshimoto et al. 2008). *dm-w* could direct female sex as an anti-testis gene for the ZZ/ZW-type system. Section 17.3 describes the *dm-w* gene and its function and discusses the sex-determining mechanism in *X. laevis*. In the next section, the findings of a unique frog, the Japanese Wrinkled Frog (*Glandirana* (*Rana*) *rugosa*), are presented. This frog has ZZ/ZW- and XX/XY-types, which was caused by geographic variation (Miura 2007).

Sex steroids, including estrogen and androgen, play important roles in gonadal differentiation. Sections 17.5 and 17.6 describe sex reversal induced by sex steroids and sex steroid-synthesizing *cyp17a1/cyp19a1* genes during early gonadal differentiation. Recently Mawaribuchi et al. (2014) discovered several cell masses consisting of the *cyp17a1*-expressing cells on the anteroposterior axis of genetically female and male gonads soon after sex determination in *X. laevis*. Because the cell mass is connected to ovarian cavity formation, the default sex is discussed in Sect. 17.7. The next section describes several frog genes, whose orthologs are involved in mammalian testis and ovary formation and differentiation.

Finally, findings from research on frog and toad oocytes are presented. This research has greatly contributed to the understanding of not only oocyte maturation in vertebrates, but also cell cycle in eukaryotic cells. Lastly, an overview of endocrine control of gametogenesis, including frog oocyte maturation, is presented.

17.2 Sex-Determining Systems and Sex Chromosomes in Frogs and Toads

All of the amphibian species examined by this research, including frogs, display genetic sex determination, which includes both female and male heterogamety. The female heterogametic ZZ/ZW-type sex-determining systems are adopted in many species such as the Cane Toad *Bufo marinus* (Abramyan et al. 2009), the African Bullfrog *Pyxicephalus adspersus* (Schmid and Bachmann 1981), and the African Clawed Frog *Xenopus laevis* (Chang and Witschi 1956; Yoshimoto et al. 2008). In

Table 17.1 Sex-determining (SD) systems in frogs and toads

Species	SD-type	Morphology of sex chromosomes	Notes
Gastrotheca riobambae (Marsupial frog)	XX/XY	Heteromorphic	
Pyxicephalus adspersus (African Bullfrog)	ZZ/ZW	Heteromorphic	
Glandirana (*Rana*) *rugosa* (Japanese Wrinkled Frog)	XX/XY	Heteromorphic	*sox3*, *ar*, snd *sf1*
	or ZZ/ZW	Homomorphic	On sex chromosomes
Hyperolius viridiflavus (African Reed Frog)	XX/XY	Homomorphic	
Xenopus laevis	ZZ/ZW	Homomorphic	W-specific *dm-w*
(African Clawed Frog)			For sex determination

contrast, the XX/XY-type frog species include the African Reed Frog *Hyperolius viridiflavus* (De Almeida et al. 1990) and the Marsupial Frog *Gastrotheca riobambae* (Schmid et al. 1983) (Table 17.1). Interestingly, there are geographic variations in sex chromosome morphologies supported by both XX/XY-type and ZZ/ZW-type systems in the Japanese Wrinkled Frog *Glandirana* (*Rana*) *rugosa* (Miura 2007) (see Sect. 17.4).

The genotypic systems that determine sex in birds and mammals have remained stable for more than a hundred million years, resulting in the highly differentiated W and Y chromosomes from Z and X chromosomes, respectively. There are heteromorphic sex chromosomes in almost all mammalian and avian species (Graves 2008). However, more than 90% of frog species examined (including *H. viridiflavus* and the current model frogs *X. laevis* and *X. (Silurana) tropicalis*) possess homomorphic sex chromosomes, which could not be morphologically distinguishable (Eggert 2004; Schmid et al. 2010; Malcom et al. 2014). Why is sex chromosome homomorphism maintained in most frog species? This question applies also to reptiles or fish. Mawaribuchi et al. (2012) proposed a co-evolution model of sex-determining genes and sex chromosomes, in which undifferentiated and/or homomorphic sex chromosomes easily allow replacement of a sex-determining gene with a new gene, while highly differentiated and/or heteromorphic sex chromosomes are restricted to a particular sex-determining gene (Fig. 17.1). Examples of the latter are seen in therian mammals (i.e., marsupials and placentals) and birds. Monophyletic evolution of the Y or Z sex chromosomes is closely related to maintainability of sex-determining gene Y-linked *Sry* or Z-linked *dmrt1*, respectively. In this context, there could be as many kinds of sex-determining genes in frogs as in teleost fish (Ito and Mawaribuchi 2013), although little is known about amphibian sex-determining genes except for *dm-w* in *X. laevis* (Yoshimoto and Ito 2011). A recent report showed that its related species, *X. tropicalis*, possesses three different sex chromosomes: W, Y, and Z: ZW and WW females and YZ, YW, and ZZ males, although the authors presented no data about its sex-determining gene(s) (Roco et al. 2015).

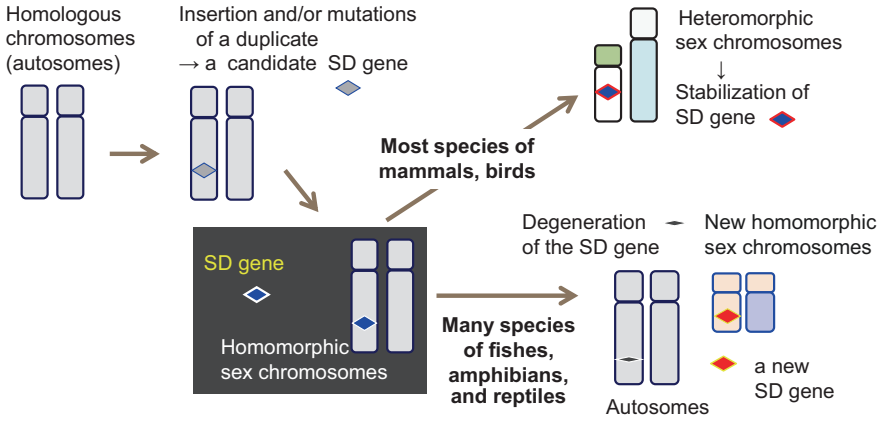

Fig. 17.1 Co-evolution between sex chromosomes and sex-determining (SD) genes in vertebrates. A candidate SD gene arose from a sex-related gene on one chromosome of a pair of autosomes, and then was established as a SD gene during species diversity. When a new candidate SD gene evolved as a stronger regulator for sex determination, the established SD gene should have been degenerated. In contrast, sex chromosome differentiation might lead to stabilization of the SD gene. In summary, a homomorphic state of sex chromosomes in many species of fishes, amphibians, and reptiles might allow an SD gene to change, while a heteromorphic state of sex chromosomes in most species of mammalians and birds might cause stabilization of SD genes (Mawaribuchi et al. 2012)

17.3 A W-Linked Sex-Determining Gene *dm-w* for a ZZ/ZW-Type Sex Determination in the African Clawed Frog *Xenopus laevis*

In *X. laevis*, W and Z sex chromosomes were only identified about 10 years ago. However, a female genome-specific gene, *dm-w* (*DM domain-containing W-link*), was discovered as a paralog of the *Dmrt1* gene in the species several years ago (Yoshimoto et al. 2008). Fluorescence in situ hybridization analysis for *dm-w* indicates that the W chromosome corresponds to one of the homologous chromosomes (Chromosome 2 L) in females. Chromosome 2 L has recently been named by a new chromosome nomenclature for *X. laevis* based on the phylogenetic relationship and chromosome length, because the species has an allotetraploid genome with 36 chromosomes consisting of two sets of 18 chromosomes (1L-9L and 1S-9S) (Matsuda et al. 2015). Accordingly, the Z chromosome corresponds to the other of the 2 L chromosomes in females and to a pair of them in males (Fig. 17.2a).

dm-w was generated through a partial duplication of *dmrt1*, which encodes a transcription factor characterized by the presence of a DNA-binding domain called the DM domain. *dm-w* shares high sequence identity with the DM domain of *dmrt1*, but *dm-w* has no corresponding region for the transactivation domain of DMRT1

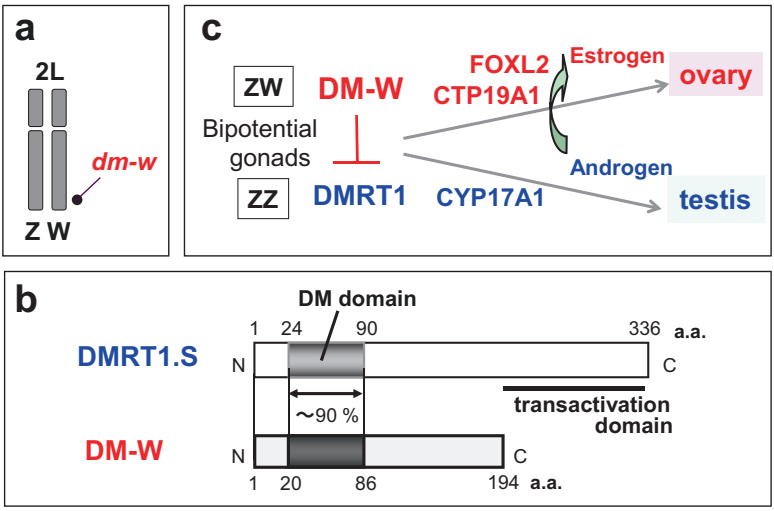

Fig. 17.2 Sex-determining gene *dm-w* in the African Clawed Frog *Xenopus laevis* (**a**) Schematic drawing of the Z and W chromosomes, which correspond to chromosome 2L. *dm-w* is located on the terminal region of the long arm on the W chromosome. The Z and W chromosomes are homomorphic: they are morphologically indistinguishable from each other by conventional chromosomal staining. (**b**) Schematic drawing of DM-W and DMRT1. The DM domain regions of the two proteins have high similarity (−90%) in the amino acid sequences. (**c**) A model for sex determination and differentiation in ZW and ZZ gonads. DM-W antagonizes DMRT1 as a transcription factor, resulting in high expression of *cyp19a1* and *foxl2*. CYP19A1 (aromatase) produces estrogen, leading to feminization mediated through estrogen receptors

(Fig. 17.2b; Yoshimoto et al. 2008). Overexpression of *dm-w* or *dmrt1* can induce female or male development of ZZ or ZW gonads, respectively, while the *dm-w* knockdown can cause female-to-male sex reversal (Yoshimoto et al. 2008; Yoshimoto et al. 2010). In addition, *dm-w* shows gonad-specific expression at a sex-determining stage and DM-W represses transcriptional activity by DMRT1 in vitro (Yoshimoto et al. 2008; Yoshimoto et al. 2010). Therefore *dm-w* is believed to direct female sex as a sex-determining gene. DM-W could antagonize testis formation induced by DMRT1, suggesting that *dm-w* might diverge from *dmrt1* as a dominant-negative type gene, i.e., as a 'neofunctionalization' gene for the ZZ/ZW-type system (Fig. 17.2c). *dm-w* is the only W-linked gene among sex-determining genes in vertebrates reported until now. Interestingly, it was recently reported that a female-specific piRNA, which is transcribed from its W-linked sequence, acts as a sex determinant by repressing masculinization in the silkworm (Kiuchi et al. 2014).

Dmrt1 induces testes formation in avian chicken (Smith et al. 2009; Lambeth et al. 2014), teleost fish Medaka (Masuyama et al. 2012), and mammalian mice (Zhao et al. 2015) as well as *X. laevis* (Yoshimoto et al. 2010). This relationship is likely to be conserved across vertebrate species. Importantly, the chicken's Z-linked

dmrt1 gene is required for male sex determination in the ZZ/ZW-type system (Smith et al. 2009). The Medaka sex-determining gene *dmy/dmrt1by* evolved from duplication of *dmrt1* (Matsuda et al. 2002; Nanda et al. 2002), similar to *dm-w* in *X. laevis*. In addition, *dmrt1* plays an important role in somatic cell masculinization in mice (Matson et al. 2011). These findings suggest that the *dmrt1*-driven masculinizing system has been conserved during vertebrate evolution (Yoshimoto et al. 2010). Thus, *X. laevis* and Medaka sex-determining genes (*dm-w* and *dmy*) evolved as negative and positive regulator genes, respectively, on this conserved *dmrt1*-driven masculinizing system.

How evolutionarily conserved is the *dm-w/dmrt1* system of sex determination? Bewick et al. (2011) reported that *dm-w* arose after divergence of a polyploid group including *X. laevis* and a diploid group including *X. tropicalis*, but before divergence of *X. laevis* and *X. cilivii*. Thus, the *dm-w/dmrt1* system could be used for sex determination in some species of the genus *Xenopus*. As described in Sect. 17.2, homomorphic sex chromosomes allow for the replacement of a sex-determining gene (Mawaribuchi et al. 2012). Because these frog species have homomorphic sex chromosomes, it is reasonable to hypothesize that *dm-w* emerged in the genus *Xenopus* during species diversity. In addition, we recently reported that *dm-w* might have been generated after allotetraploidization (Mawaribuchi et al. 2017b).

17.4 Geographic Variations of Sex-Determining Systems in the Japanese Wrinkled Frog *Glandirana* (*Rana*) *rugosa*

The Japanese Wrinkled Frog species (*Glandirana (Rana) rugosa*) can be divided into five genetic groups: Western Japan, Eastern Japan, XY, ZW, and Neo-ZW. The Western Japan, Eastern Japan, and XY groups have XX/XY systems, while in the ZW and Neo-ZW groups, sex is determined by ZZ/ZW systems (Miura 2007; Table 17.2). It is rare to find different sex-determining systems within a species. It is likely that the two different groups split apart just before species diversity occurred. From their chromosomal structures, artificial crossing between the groups, and phylogeny among the groups in this species, it is assumed that the XY and ZW

Table 17.2 Sex-determining (SD) systems of five groups of *Glandirana* (*Rana*) *rugosa* in Japan

Group	SD-type	Morphology of sex chromosomes
Western Japan	XX/XY	Homomorphic
Eastern Japan	XX/XY	Homomorphic
XY	XX/XY	Heteromorphic
ZW	ZZ/ZW	Heteromorphic
Neo-ZW	ZZ/ZW	Heteromorphic

groups were produced by hybridization between the ancestors of the Western Japan and Eastern Japan groups, and that the Y and Z or X and W sex chromosomes share their origins with chromosome 7 of the Western Japan and Eastern Japan groups, respectively (Miura et al. 1998; Miura 2007; Ogata et al. 2008). Another hybridization between the ancestors of the Western Japan and XY groups might have caused the emergence of the Neo-ZW group (Ogata et al. 2008).

Interestingly, three sex-related genes (*ar* for androgen receptor, *sf-1/ad4bp* for steroidogenic factor-1/adrenal 4-binding protein, and *sox3* (*Sry-type HMG box 3*)) are located on both the Z and W chromosomes or the X and Y chromosomes in each genetic group. *Ar* and *sf-1/ad4bp* exist on the short arms of the W and X chromosomes and the long arms of the Z and Y chromosomes. In contrast, the *sox3* localization is different from *ar* and *sf-1/ad4bp*. *Sox3* resides on the long arms of all four sex chromosomes (Uno et al. 2008). Multiple rearrangements should have occurred during the process of sex chromosome differentiation. To date, it remains unclear what gene is required for sex determination in the species. The *sox3* or *ar* gene might take part in female or male sex determination, respectively (Oshima et al. 2009; Miura et al. 2011; Fujii et al. 2014). It is possible that each of the three sex-determining genes in present in each of the five groups. This frog species will be very useful to clarify the evolutional relationships among sex-determining genes, sex-determining systems, and sex chromosomes in view of population and species diversity.

17.5 Sex Reversal

Heteromorphic sex chromosomes are uncommon among ectothermic (i.e., cold-blooded) vertebrates as mentioned in Sect. 17.2. Most ectothermic vertebrate species carrying homomorphic sex chromosomes that have the potential to undergo environmentally-induced sex reversal. Such a reversal might occur in ectotherms due to the general dependence of physiological processes on temperature (Perrin 2009). However, all species of amphibians investigated so far present genetic sex determinations. Additional effects of temperature have been known in a few species, most cases of which are masculinization of XX individuals at high temperatures (Eggert 2004). Many reptilian and some fish species adopt temperature sex determination (TSD). Why then do no amphibian species use the TSD system? It is possible that extinct amphibian species might have adopted the system. The author believes that TSD is not suitable for amphibian sex determination because gonadal sex is determined at the aquatic larval stage and this stage occurs in pools of fresh water.

Gonadal formation in many amphibian species, including frogs, is sensitive to sex steroids as in many species of reptiles, birds, and fish (Hayes 1998). In amphibians, it was previously believed that androgens or estrogens could cause sex reversal in genetically female XX or male ZZ individuals, respectively. In *X. laevis* with the ZZ/ZW-type, sex reversal is induced by the administration of estradiol benzoate in undifferentiated gonads before primordial germ cells migrate into the medullary region (Villalpando and Merchant-Larios, 1990). In the Japanese Wrinkled Frog

G. rugosa, estrogens or androgens can induce male-to-female or female-to-male sex reversal in ZZ or XX tadpoles, respectively (Nishioka et al. 1993; Shibata et al. 2002; Ohtani et al. 2003). Piprek et al. (2012b) reported that testosterone or estradiol exposure induced sex reversal as well as intersex and undifferentiated gonads in two closely related species of the Fire-Bellied Toads, *Bombina bombina* and *B. variegate*. Similarly, female-to-male or male-to-female sex reversal by androgen or estrogen administration was observed in an XX/XY-type frog species, *Hyla arborea*. In the case of Palearctic Green Toad *Bufo viridis*, which also has an XX/XY-type system, each female or male steroid caused a developmental delay in gonads and Bidder's organs (Piprek et al. 2012b). These findings suggest that susceptibility to hormonal sex reversal does not strongly depend on female or male heterogamety (ZZ/ZW or XX/XY). It is likely that higher differentiation of sex chromosomes is closely related to sex steroid insensitivity.

17.6 Sex Steroid Synthesis During Early Gonadal Development

Sex steroids, including estrogens and androgens, play important roles in sexual development and maturation of gonads, the brain, and other tissues in most species of vertebrates including frogs. In general, estrogens and androgens are considered to be female and male sex hormones for feminization and masculinization, respectively, but both types are present in each sex, albeit at different levels.

Estrogens are synthesized by aromatase, which is a member of the cytochrome P450 superfamily (Fig. 17.3). This estrogen-synthesizing enzyme, which is encoded by *cyp19a1*, converts androstenedione and testosterone into estrone and estradiol, respectively (Fig. 17.3). Thus, aromatase is a key enzyme in estrogen synthesis. In many non-mammalian vertebrates, *cyp19a1* mRNA shows high expression in the early stage of ovarian development, suggesting that signaling mediated through estrogen and its receptor is important for ovary formation after sex determination. For instance, in *X. laevis*, *cyp19a1* displays vastly higher expression in the ZW gonads than in the ZZ gonads soon after sex determination (Okada et al. 2009; Mawaribuchi et al. 2014; Fig. 17.2c). Expression of the *cyp19a1* mRNA gradually increases from the point of sex determination to an adult stage in the ZW gonads, but is hardly detected in the ZZ gonads during testicular development. Because the estrogen receptor α gene is expressed in both the ZZ and ZW gonads during early gonadal differentiation, aromatase expression should be critical for early ovarian formation in *X. laevis*. Analysis of the *dm-w*-expressing transgenic ZZ gonads suggests that DM-W upregulates *cyp19a1* expression to guide primary ovary development in ZW gonads in *X. laevis* (Okada et al. 2009). Moreover, Mawaribuchi et al. (2014) indicated that estrogen produced by aromatase could lead to ovarian cavities, which are derived from cell-mass structures in the species. The cell-mass structures are described in detail in the next section. *Cyp19a1* expression also shows higher expression in females than in males of indifferent gonads in the frog *G. rugosa* (Maruo et al. 2008; Miura et al. 2011).

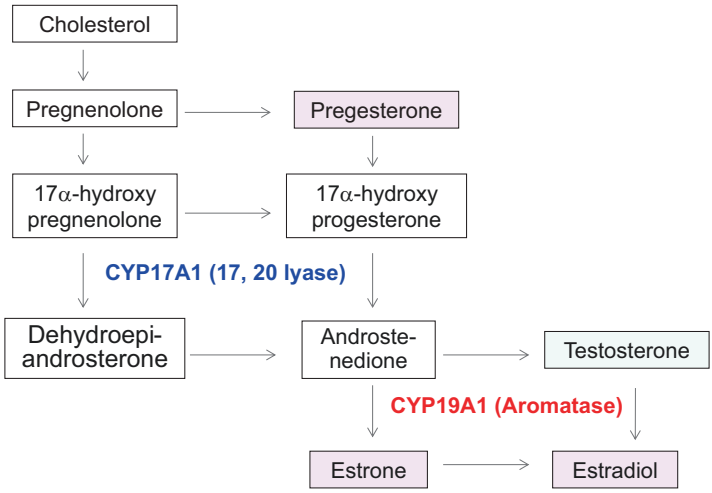

Fig. 17.3 Synthesis of sex steroid hormones in vertebrates. All steroid hormones are synthesized from cholesterol. The pathways outlined here are common to gonads and adrenals. The first committed step is the conversion of cholesterol to pregnenolone. CYP17A1 synthesizes dehydroepiandrosterone and androstenedione. Estrogens are formed from androgens (androstenedione and testosterone) by CYP19A1 (aromatase)

Another member of cytochrome P450 superfamily, CYP17A1, which is encoded by *cyp17a1*, has both 17α-hydroxylase and 17, 20-lyase activities. The latter enzyme activity directly produces two androgens: dehydroepiandrosterone and androstenedione (Fig. 17.3). The two androgens are subsequently transformed to other androgens: androstenedione and testosterone, respectively, by 17β-hydroxysteroid dehydrogenases (HSD17β3/β5). Therefore, CYP17A1 is a key enzyme involved in production of male sex steroids. In comparison to the *cyp19a1* expression, *cyp17a1* shows vastly higher expression in the ZZ gonads than in the ZW gonads soon after sex determination in *X. laevis*. The male gonad-enriched expression of *cyp17a1* was also observed in other frog species including *G. rugosa* (Maruo et al. 2008; Miura et al. 2011).

17.7 Germ Cells and Gonadal Structures During Tadpole Development

In many animals, primordial germ cells (PGCs) migrate into developing gonads. There, they proliferate and differentiate into oogonia and spermatogonia, and finally into oocytes and sperm. Although sex-determining genes are believed to be expressed in somatic cells of undifferentiated gonads in vertebrates, it was reported that the loss of germ cells induced gonadal masculinization in mammals and teleost fish (Guigon et al. 2005; Slanchev et al. 2005; Kurokawa et al. 2007). Piprek et al. (2012a) reported effects of busulfan, which can eliminate germ cells, on gonadal

development in *X. laevis*. Expectedly, germ cells are not necessary for the formation of the testes, but play important roles for development of the ovaries and the maintenance of the ovarian structures. In fact, the specific knockdown of *dmrt1* expresion in germ cells induced a decrease of the number, resulting in testicular development in genetically female gonads in *X. laevis* (Mawaribuchi et al. 2017a).

During early gonadal development, male germline stem cells (GSCs), also called primary spermatogonia, migrate into the medulla of male gonads, while female GSCs, also called primary oogonia, remain in the cortical region of female gonads. At this stage in *X. laevis*, the nuclei of the ZW and ZZ GSCs are large and unstructured, similar to their morphologies in the PGCs of the ZW and ZZ gonads before sex determination (Fujitani et al. 2016). At the prometamorphic stage, there are several cysts consisting of secondary oogonia, which are proliferating or entering their first meiosis, and primary oocytes around the ovarian cavities, which is a hallmark of developing ovarian gonads. Such cavity formation in differentiating ovaries is observed in many frogs and toads.

In *G. rugosa*, the first sexual dimorphism between female and male gonads appears as a structural change in the basement membranes for ovarian cavity or testicular cord formation (Saotome et al. 2010). Recently, Mawaribuchi et al. (2014) reported a similar observation in *X. laevis*. Moreover, they discovered several basement membrane-surrounded cell masses consisting of the *cyp17a1*-positive cells in line along the anteroposterior axis of genetically female and male gonads soon after sex determination. This "mass-in-line" structure has not been reported in species other than *X. laevis*, maybe because it is difficult to recognize such a structure using transverse sections. Importantly, at the prometamorphic stage, ovarian cavities form inside these masses of *cyp17a1*- and *cyp19a1*-positive, estrogen-producing cells in the ZW female gonads, while the "mass-in-line" pattern disappeared in the ZZ male gonads during testicular development (Fig. 17.4). Estrogen produced by CYP19A1 (aromatase) in the cell masses might lead to ovarian cavity formation. Or it could be assumed that the "mass-in-line" structure sectioned by basement membrane forms before the creation of ovarian cavities in both female and male gonads soon after sex determination. In other words, the default sex might be morphologically female in *X. laevis*. Agenesis of ovarian cavities in some androgen-treated ZW tadpoles could support this theory (Mawaribuchi et al. 2014). More recently, we indicated that TGF-β signaling is involved in the destruction of the mass-in-line structure, which may be maintained by estrogen (Wada et al. 2017).

17.8 Sex-Related Genes During Gonadal Development

In vertebrates, several genes showing sexually dimorphic expression have been implicated in sex determination and differentiation. They include: transcription factor-encoding genes (*dmrt1, sox9* (*Sry-type HMG box 9*), *sox3*, and *foxl2* (*forkhead box protein L2*)) and secreted protein-encoding genes (*amh* (*anti-Müllerian hormone*), *rspo1* (*R-spondin 1*), and *wnt4* (*Wingless-type MMTV integration site family, member 4*)).

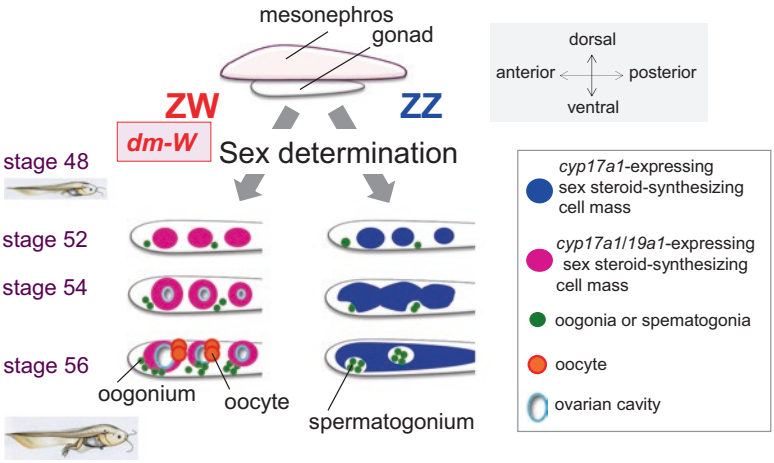

Fig. 17.4 An early sex differentiation mediated through masses consisting of sex steroid-synthesizing cells in *Xenopus laevis*. At stage 52, there are few morphological differences between the ZW and ZZ gonads. At this stage, CYP17A1 and/or CYP19A1 are expressed in several cell masses that are aligned along the anteroposterior axis (called 'mass-in-line') of both ZZ and ZW gonads. The 'mass-in-line' pattern is disrupted during testicular development in the ZZ gonads, while maintained in the ZW gonads, and ovarian cavities develop inside the cell masses

Dmrt1 participates in male sex determination in birds, and induces testis formation in various vertebrates including the frog *X. laevis*, as mentioned in Sect. 17.3. *Dmrt1* shows expression in pre-Sertoli and Sertoli cells in the male gonads of several vertebrate species. It also contributes to the development of both female and male germ cells in mice: *Dmrt1* negatively controls meiosis in male germ cells, but promotes meiosis in female germ cells (Matson et al. 2010; Krentz et al. 2011). In six frog and toad species (*X. laevis*, *Bombina bombina*, *Bufo viridis*, *H. arborea*, *R. arvalis* and *R. temporaria*), *dmrt1* is highly expressed in the developing testes, in both the somatic and germ cells (Piprek et al. 2013; Fujitani et al. 2016). In *G. rugosa* (the Western Japan group with the XX/XY-type), *dmrt1* shows higher expression in XY male gonads than in XX female gonads, prior to the onset of the sexually dimorphic expression of *cyp17a1* and *cyp19a1* (Miura et al. 2011).

Sox9 is a transcription factor gene that is a member of the *Sry-type HMG box* (*sox*) superfamily of genes. In mice, the sex-determining gene product SRY directly enhances transcription of *Sox9* in pre-Sertoli cells in undifferentiated XY gonads. SRY plays a pivotal role in male development: SOX9 could activate *Amh* and *Fgf9* (*fibroblast growth factor 9*) (Barrionuevo et al. 2012). However, it is not clear how SOX9 functions in sexual development in amphibians. *amh* does not appear to be a target of SOX9 in *G. rugosa* (Kodama et al. 2015). Interestingly, in the teleost fish Medaka, mutants that lack SOX9 function showed a seemingly paradoxical phenotype of female-to-male sex reversal, owing to a reduction of the germ cell numbers (Nakamura et al. 2012). It is possible that SOX9 also plays a role in germ cell maintenance in amphibians.

Like *sox9, sox3* belongs to the *sox* superfamily of genes. In therian mammals, *sox3* is located on the X-chromosome, because *Sry* was generated from *Sox3* by allelic mutation and evolved as a testis-determining gene in the XX/XY-type system. Therefore, *sox3* has the potential to induce testis formation as does SRY in mice and humans (Sutton et al. 2011). In the fish species *Oryzias dancena*, *sox3* was identified as a sex-determining gene on the Y chromosome (Takehana et al. 2014). In contrast, it remains unclear whether *sox3* contributes to female and/or male gonadal development in amphibians. In *X. laevis*, *sox3* is expressed in oocytes of early stages (Koyano et al. 1997). As mentioned in Sect. 17.4, in *G. rugosa*, *sox3* on the Z and W or X and Y chromosomes might be required for sex determination.

In mice, a fork-head transcriptional factor FOXL2 is required to prevent transdifferentiation of an adult ovary to a testis (Uhlenhaut et al. 2009). FOXL2 or DMRT1 might maintain feminization or masculinization in somatic cells of gonads, and determine identity of granulosa or Sertoli cells, respectively. FOXL2 and DMRT1 might play antagonistic roles in somatic sex determination as transcription factors (Matson et al. 2011). In both *G. rugosa* and *X. laevis, foxl2* shows vastly higher expression in females than in males during gonadal development (Oshima et al. 2008; Okada et al. 2009). Because female gonad-specific FOXL2 transcription follows high expression of *cyp19a1* in *X. laevis* (Okada et al. 2009), signaling mediated through estrogen and its receptor might activate *foxl2* expression. Clarification is needed with regard to the relationships beteween *foxl2*, *dmrt1*, and sex steroid signaling in non-mammalian vertebrates (Fig. 17.5).

AMH is a member of the tumor growth factor (TGF)-β superfamily. The hormone is responsible for the regression of Müllerian ducts during early development of testes in mammals, birds, and reptiles. In teleost fish, the orthologs of *amh* are involved in testicular development, although these fish lack Müllerian ducts. Interestingly, a male genome-specific (Y-linked) *amhy* evolved from duplication of *amh* as a male sex-determining gene in the teleost fish Patagonian Pejerrey (Hattori et al. 2012). In frogs, limited research on AMH has been reported. The anti-AMH antibodies reacted with differentiating Sertoli cells in developing testes of several frogs including *X. laevis* and *G. rugosa* (Piprek et al. 2013; Kodama et al. 2015).

In mammals, early ovary formation is involved in the WNT signaling pathway: the canonical WNT/β-catenin signaling is activated by WNT4 and RSPO1, which binds to the leucine-rich repeat containing G protein-coupled receptors LGR4-6

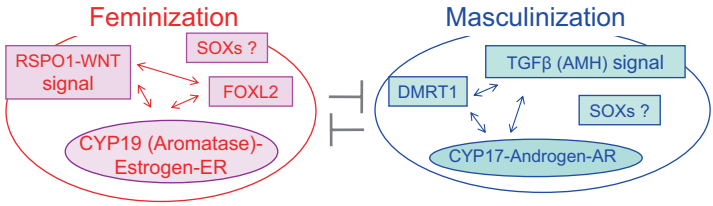

Fig. 17.5 A model of a cross-talk between/among feminization and/or masculinization signals during amphibian gonadal development. *ER* estrogen receptor, *AR* androgen receptor

(Chassot et al. 2014). In the frog *G. rugosa*, *wnt4* mRNA shows no sexually dimorphic expression (Oshima et al. 2005), while *rspo1* mRNA exhibits higher expression in genetically female gonads than in male gonads at an early stage before the *cyp17a1* and *cyp19a1* transcripts show sexual dimorphism (Miura et al. 2011). Immunohistochemical analysis using an anti-RSPO1 antibody revealed that the reactive protein displays significantly higher expression in developing ovaries of several frog species, and localization in oogonial cells and somatic cells in developing ovaries in *X. laevis* (Piprek et al. 2013).

As a summary of this section, a cross-talk between feminization and masculinization signals is proposed in Fig. 17.5. Although antagonistic effects between these signals might enhance and maintain feminization or masculinization in gonads, their molecular mechanisms remain unclear.

17.9 Endocrine Control of Gametogenesis

The hypothalamic-pituitary-gonadal (HPG) axis controls reproduction. In amphibians, as in other vertebrates, gonadotropin-releasing hormone (GnRH) is secreted from the hypothalamus by GnRH-expressing neurons. GnRH stimulates the synthesis and secretion of two gonadotropins: luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in the anterior portion of the pituitary gland (Fig. 17.6). LH and FSH stimulate the synthesis and secretion of sex steroids(estrogen and androgen) in gonadal somatic cells (Dagklis et al. 2015). In contrast, gonadotropin-inhibitory hormone (GnIH) negatively regulates the production and release of GnRH and gonadotropins in many vertebrate species (Tsutsui and Ubuka 2014). In amphibians, such inhibitory roles of GnIH to the HPG axis have not been demonstrated.

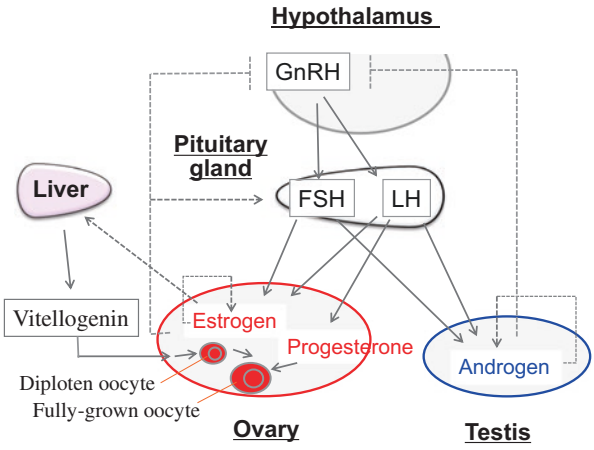

Fig. 17.6 Hypothalamic-pituitary-gonadal axis in frogs. *GnRH* gonadotropin-releasing hormone, *GnIH* gonadotropin-inhibitory hormone, *FSH* follicle-stimulating hormone, *LH* luteinizing hormone

In oviparous organisms, including amphibians, the HPG-liver axis is important for oocyte maturation. Vitellogenin is the precursor of the yolk proteins that represent a major source of nutrients for the developing embryos. Its liver-specific expression is enhanced by estrogen in adult females through its receptor, which interacts with the estrogen-responsive element in the promoter of the vitellogenin genes in *X. laevis* and the chicken (Klinge 2001). In frogs, the fully-grown oocytes where the yolk proteins are sufficiently stored are arrested at the first meiotic metaphase until progesterone triggers meiotic maturation (Fig. 17.6). The G2-M transition is regulated by maturation-promoting factor (MPF, also called M phase-promoting factor), which was discovered first in the Leopard Frog *Rana pipiens* oocytes as a cytoplasmic factor for oocyte maturation (Masui and Markert 1971; Smith and Ecker 1971). About 20 years after this discovery, MPF was molecularly identified as a protein kinase consisting of two subunits: CDC2 and CYCLIN B in *X. laevis* (Nurse 1990). LH secreted from pituitary glands induces production of progesterone in follicle cells around oocytes, which activates MPF through its receptor in the oocytes, and then germinal vesicle breakdown is performed. In the next step, the oocytes emit the first polar body, enter meiosis II without an intervening interphase, and then arrest at metaphase of the second meiotic division until fertilization (Masui, 2001; Philpott and Yew 2008).

Spermatogenesis in amphibians occurs in cysts composed of Sertoli cells enveloping germ cells at synchronous stages. In the cysts, spermatogonia proliferate and differentiate into meiotic spermatocytes, spermatids and mature spermatozoa. Androgen induced by gonadotropins enhances spermatogenesis in the toad *Anaxyrus fowleri* and induces spermatid formation and spermatogonial mitosis in the edible frog *Pelophylax esculentus*. In addition, during the reproductive season of the species, there is a strong relationship between high levels of androgen and the expression of c-kit, which is indispensable for spermatogenesis and spermatogonial proliferation (Raucci and di Fiore 2007). Interestingly, estrogen also induces an acceleration of spermatogenesis in juvenile *Xenopus laevis* (Hu et al. 2008), but stimulates testicular apoptosis during the reproductive season, mostly in spermatocytes in the toad *Rhinella arenarum* (Scaia et al. 2015). These findings suggest that androgen and estrogen could control the dynamic balance between cell proliferation, differentiation, and apoptosis in amphibian spermatogenesis.

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Chapter 18
Environmental Control of Sex Determination and Differentiation in Reptiles

Shinichi Miyagawa, Ryohei Yatsu, and Taisen Iguchi

Abstract Most vertebrates use a genetic sex determination system, whereas a diverse set of reptile taxa use an environmental sex determination system – more specifically, a temperature-dependent sex determination (TSD) system. The TSD system is where sex is established by the incubation temperature during a critical stage of embryonic development. It has been almost a half century since the first TSD system was found in a lizard species. Thermal effects on sex determination have been described in many other reptile species since then. TSD has been found in all crocodilians and tuataras examined and in most turtles and some lizards. However, clarification is needed about this unique mode of sex determination in reptiles, in particular, factors triggering the intrinsic genetic cascade, which leads either to development of a testis or ovary. In several instances, TSD has evolved separately in reptilian lineage and, therefore, the adaptive significance of TSD is an attractive topic from an evolutionary view. In this chapter, the general background and recent advancements for TSD research in reptiles is discussed.

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18.1 Introduction

In many vertebrates, sex is determined genetically (i.e., composition of sex chromosomes or sex-determining genes present). Mammals have male heterogamety (XX females/XY males) and in eutherians (placental mammals), maleness is determined by the master gene *Sry* on the distinct Y chromosome. Birds have female heterogamety (ZZ males/ZW females) and, for example, a double gene dosage of *dmrt1* on Z chromosome causes male development in offspring. Fish, amphibians, and reptiles have male and female heterogametic genetic sex determination with or without strongly differentiated sex chromosomes. In such genetic sex determination (GSD) systems, the sex of the embryo is regulated by genes inherited from its parents during conception. Thus, commitment of a specific sex is predetermined and it cannot normally be reversed at a later stage in higher vertebrates.

Conversely, some species, particularly reptiles, use an environmental sex determination (ESD) system, most commonly in the form of temperature-dependent sex determination (TSD). In the TSD system, the sex is determined not by its chromosomal component but by the temperature that the embryos experience during a certain stage of embryonic development. Different temperatures correspond to gonadal development of testis or ovary. The sex of the embryo cannot be determined in advance. The TSD system illustrates a direct relationship between a specific environmental temperature and offspring sex. TSD is not limited to reptiles. Extremely high temperatures can cause some degree of sexual phenotype reversal and can over-ride GSD systems in several fish and amphibian species. However, "pure TSD" (i.e., offspring sex is determined by temperature only, without apparent genetic effects) has not been confirmed in these species.

Reptiles are generally divided into four extant orders: Crocodylia (crocodiles, gavials, caimans, and alligators; ~25 species), Testudines or Chelonii (turtles; ~300 species), Squamata (lizards and snakes; over 9000 species), and Sphenodontia (tuatara; one species). Since the discovery of TSD in Agamid Lizards (*Agama agama*) in 1966 (Charnier 1966), it has been revealed that TSD occurs in all the crocodilian species examined, most turtle species, some lizard species, and tuatara, but is not found in snakes (Fig. 18.1). TSD is of interest in terms of diversity of sex determination systems within vertebrates and adaptive evolution. In this chapter, a brief background of TSD is presented, along with commonly used terminology in this field. The TSD patterns seen in each reptile species are also described. Unlike the GSD, the sex in TSD may be determined by many factors. Possible factors that affect offspring sex in TSD reptiles are discussed in this chapter. It is generally considered that TSD is representative example of "phenotypic plasticity", which is defined as the ability of an organism to change its phenotype in response to changes in the

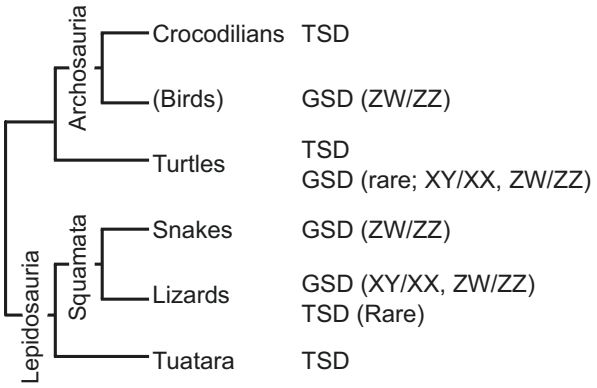

Fig. 18.1 Sex determination systems in reptiles. Phylogeny of amniotes with differential modes of sex determination is illustrated

environment conditions (Gilbert and Epel 2009). From this viewpoint, there are expected to be some adaptive advantages in TSD. Recent research on the adaptive significance of TSD are also discussed in this chapter.

18.2 General Background of TSD

In 1966, Charnier reported the first TSD in the Agamid Lizard (*A. agama*) (Charnier 1966). Her peculiar but important finding did not attract much attention due to it being published in a small paper in French. However, TSD has now attracted many scientists' attention. Three modes of TSD are categorized according to the sex ratios produced by temperature changes: (1) low temperatures produce Male and high temperatures produce Female (type Ia; MF pattern), (2) low temperatures produce Female and high temperatures produce Male (type Ib; FM pattern), and (3) low and high temperatures produce Females and intermediate temperatures produce Male (type II; FMF pattern) (Fig. 18.2). Most reptiles originally thought to have the MF or FM pattern, have been proven to have the FMF pattern when a wider range of incubation temperatures was examined. A clear MFM (low and high temperatures produce Males and intermediate temperatures produce Female) pattern has not been reported in any reptile species. Current TSD patterns are defined by sex ratio data obtained under constant temperature conditions in the laboratory. This may not reflect natural conditions, where temperatures fluctuate on a day-by-day basis. This effect should be taken into account.

The temperature that yields a 1:1 ratio of males and females is defined as the pivotal temperature (Fig. 18.2). Embryos incubated at a pivotal temperature develop either testes or ovaries. No intersex individuals develop. The mechanism that determines sexual fate at the pivotal temperature has not been identified. When undifferentiated gonadal pairs were isolated from turtle embryos and separately cultured at the pivotal temperature, both gonads shared a strong predisposition for one sex or

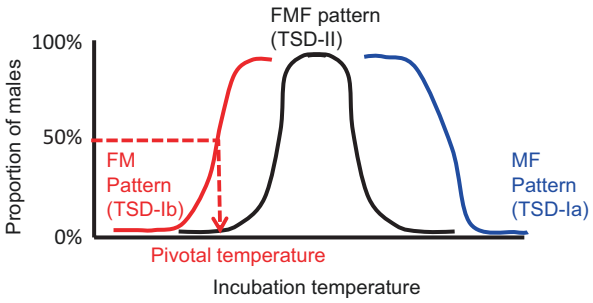

Fig. 18.2 Patterns of TSD defined by the sex ratio produced by different temperatures. Three differential patterns of TSD are illustrated: FM (female-male; TSD-Ib)-, FMF (female-male-female; TSD-II),- and MF (male-female; TSD-Ia) patterns. A pivotal temperature, defined as a temperature that induces a 1:1 sex ratio, is shown as an example

the other. Thus, the sex would be predetermined rather randomly, at least in the Red-eared Slider Turtle, *Trachemys scripta* (Mork et al. 2014).

The effects of temperature on sex determination are limited to a specific window during embryonic development called the temperature-sensitive period (TSP). This period can be defined as the critical stage that embryonic sex can be shifted to the same sex when eggs were transferred at either male- or female-producing conditions. For example, the TSP of the American Alligator (*Alligator mississippiensis*) has been defined as 10 days in the middle third of the embryonic development [developmental stages 21–24 (Ferguson 1985)]. However, a recent study showed earlier thermos-sensitivity in the American Alligator TSD (McCoy et al. 2015). In general, TSP corresponds to the morphologically undifferentiated and bipotential stage of developing gonads (Pieau and Dorizzi 2004).

18.3 TSD in Reptiles

18.3.1 Crocodylia

The order Crocodylia occupies an important position in the evolution of vertebrates as an ancestral reptile and amniotes. TSD in Crocodylia was first reported in the American Alligator (*A. mississippiensis*) (Ferguson and Joanen 1982). Subsequently, TSD has been found in 12 crocodilian species examined (Deeming 2004). Therefore, all crocodilians are thought to exhibit the TSD system (Lang and Andrews 1994). As members of the Archosaurian lineage (a clade that includes dinosaurs and pterosaurs), crocodilians are closer to birds both anatomically and biochemically than to other reptiles. Interestingly, all birds exhibit the GSD system with a Z and W sex chromosome heterogeneity. There is no evidence of temperature-dependency in sex determination in birds. In the chicken, gene dosage of *dmrt1* located on the

Z chromosome is responsible for male development (ZZ for males and ZW for females). Genome and FISH (fluorescent in situ hybridization) analysis revealed that alligator *dmrt1* is located on chromosome 3 of the Chinese Alligator (*A. sinensis*) with conserved synteny. This suggests that chromosome 3 in alligators and chromosome Z in chickens share an ancestral chromosome (Wan et al. 2013). Crocodilians are not thought to have sex chromosomes (Cohen and Gans 1970; St John et al. 2012).

The thermal ranges for crocodilian egg incubation are well-defined and are much narrower compared to other reptile species (Table 18.1). It is likely that crocodilians exhibit the FMF pattern, where females are produced at both low and high temperatures and males are produced intermediate temperatures, although female production at high temperatures is not supported in the literature (Ferguson and Joanen 1982; Lang and Andrews 1994). Sex-specific mortality at high temperature should also be taken into account, as the higher incubation temperature decreased hatching success. In the case of the American Alligator, constant incubation temperatures of 30 °C or 33 °C resulted in 100% female hatchlings or 100% male hatchlings, respectively. In general, 32.0–33.0 °C temperatures produce 90–100% male hatchlings among crocodilians. In the Chinese Alligator, ~90% of eggs are male at 33–35 °C, whereas most eggs are female at ~28 °C, although the total number of eggs tested was limited (cited in Lang and Andrews 1994).

18.3.2 Testudines

Testudines (turtles, tortoises and terrapins) is an early-diverged reptilian group closely related to the Archosaurian lineage (Wang et al. 2013; Shaffer et al. 2013). More than 300 species of turtles are alive today. Of these, 79 species have been tested for mode of sex determination and 64 use the TSD system (Ewert et al. 2004). The Testudines encompasses two suborders: the Cryptodira and the Pleurodira. There are two extant families in the Pleurodira: Pelomedusidae exhibit TSD (4 out of 4 species examined) but Chelidae do not (0/8) (cited in Ewert et al. 2004). The rest of the families belong to the Cryptodira suborder. Many of them showed TSD: Carettochelyidae (1/1), Cheloniidae (sea turtle, 6/6), Chelydridae (2/2), Dermatemydidae (1/1), Dermochelyidae (1/1), Emydidae (24/25), Geoemydidae (8/8), Kinosternidae (13/16), and Testudinidae (4/4). TSD was not found in Trionychidae, which encompasses the species that are commonly referred to as soft-shelled turtles (0/3) (cited in Ewert et al. 2004).

Compared with crocodilians, high temperatures produce only females in most turtles. For example, the Red-eared Slider Turtle (*Trachemys scripta*) produces 100% female hatchlings in warmer temperatures (31 °C) and 100% male hatchlings in cooler temperatures (26 °C). The Painted Turtle (*Chrysemys picta*) produces males at cooler temperatures and females at warmer temperatures (predominantly males at 21.5 °C and all females above 29.5 °C). In contrast, the Snapping Turtle (*Chelydra serpentine*) showed the FMF pattern, where intermediate temperatures

Table 18.1 Proportion of male offspring produced from eggs incubated at various temperatures in crocodilians

Family	Species	Temperature (°C)																	References
		26	28	28.5	29	29.5	30	30.5	31	31.5	32	32.5	33	33.5	34	34.5	35	36	
Alligatoridae	American Alligator (*Alligator mississippiensis*)	0	0				0				13				100			100[a]	a
Alligatoridae	American Alligator (*Alligator mississippiensis*)		0		0		0	0	0	0	66	100	100	84	35	7	0		b
Alligatoridae	Spectacled Caiman (*Caiman crocodilus crocodilus*)			0		0	0		0	52	95	90	85	75	50				c
Alligatoridae	Yacare Caiman (*Caiman yacare*)				0		0		0		13		69		93				d
Alligatoridae	Smooth-fronted Caiman (*Paleosuchus trigonatus*)		0			0	0		8		100								e
Crocodylidae	Mugger Crocodile (*Crocodylus palustris*)		0	0	0	0	0	0	0	22	59	93	31						c
Crocodylidae	Salt-water Crocodile (*Crocodylus porosus*)		0		0		0		16		86		17						f
Crocodylidae	Morelet's Crocodile (*Crocodylus moreletii*)								0	5	33		55	5					g
Crocodylidae	Freshwater Crocodile (*Crocodylus johnsoni*)		0		0		0		0		39		7						g
Crocodylidae	Nile Crocodile (*Crocodylus niloticus*)		0						0		91				87				h
Crocodylidae	Siamese Crocodile (*Crocodylus siamensis*)								0		0	100	60						d
Gavialidae	Indian Gharial (*Gavialis gangeticus*)									0[a]	89	20	15						g

References are: (a) Ferguson and Joanen (1982), (b) Lang and Andrews (1994), (c) Lang et al. (1989), Lang and Andrews (1994), (d) cited in Deeming (2004), (e) Magnusson et al. (1990), (f) Lang and Andrews (1994), Webb and Cooper-Preston 1989), (g) cited in Lang and Andrews (1994) and (h) Hutton (1987)

[a]Survival rate is very low

(23–27 °C) produce males, while higher or lower temperatures produce females (i.e., like the crocodilians). A comprehensive list of sex ratios and incubation temperatures has been reviewed in Ewert et al. 2004 and Ewert and Nelason 1991.

18.3.3 Lepidosauria (Squamata and Tuatara)

The subclass Lepidosauria includes Squamata (historically subdivided as lizards, snakes, and amphisbaenia) and Rhynchocephalia (tuatara). Squamata is the most diverged reptilian order but TSD is restricted to only Lacertilia (lizards). No TSD has been found in Serpentes (snakes) and Amphisbaenia (worm lizards). Lacertilia encompasses a widespread group, and TSD has been widely investigated in the following families: Agamidae (agamid lizards), Eublepharinae (eyelid geckos), Gekkonidae (geckos), and Scincidae (skinks). Most species with TSD show a similar sex determination pattern (FMF): only females are produced at high and low temperatures and intermediate temperatures produce varying proportions of male and female hatchlings (Table 18.2). However, the Oriental Garden Lizard (*Calotes versicolor*) has a unique FMFM pattern of TSD (Inamdar Doddamani et al. 2012).

Squamates have high variability in reproductive and sex determining modes. Viviparity (live-bearing) has evolved several times in squamate reptiles (Blackburn 2000). Although thermoregulation in the mother seems to be stable, temperature has an effect on offspring sex ratio in viviparous species, including the Southern Water Skink (*Eulamprus tympanum*), Spotted Skink (*Niveoscincus ocellatus*), and Indian Forest Skink (*Sphenomorphus indicus*) (Ji et al. 2006; Robert and Thompson 2001; Wapstra et al. 2004). These species are all phylogenetically distinct, therefore TSD in viviparity has evolved separately.

Tuatara is one of the most primitive amniotes, with their closest relation being the squamates. The Tuatara is biologically significant as the only extant animals of the order Rhynchocephalia. Living species (*Sphenodon punctatus* and *S. guntheri*) are restricted to offshore islands of New Zealand. They show low fecundity and sexual maturity takes at least 10 years. The reproductive cycle is very slow: females mate and lay eggs once every 2–5 years and incubation takes about 1 year to complete (Cree et al. 1992; Thompson et al. 1992). Tuatara showed an FM pattern of TSD at constant incubation temperatures (Table 18.3): males were produced above a pivotal temperature of 21.6 °C in *S. guntheri*, and 22.0 °C in *S. punctatus* (unnamed subspecies on Stephens Island, Cook Strait) (Mitchell et al. 2006). Hatchling survival is very low at 15 °C or below and at 25 °C or above (Thompson 1990). Despite this, an FMF pattern is plausible for this species. In the wild, the warmest nests during the TSP produce predominantly males in *S. punctatus* (Mitchell et al. 2006). Wild Tuataras have male-biased sex ratios due to compound effects (decreased female body condition, reduced survival, and a male-biased hatchling sex ratio) and current patterns of global warming could further skew the sex ratio towards males (Grayson et al. 2014). In terms of climate change, the Tuatara is considered one of the most threatened animals (Benson and Niederkorn 1991).

Table 18.2 Proportion of male offspring produced from eggs incubated at various temperatures in

Family	Species	Temperature (°C)											
		22	22.5	23	23.5	24	24.5	25	25.5	26	26.5	27	27.5
Eublepharidae	Leopard Gecko (*Eublepharis macularius*)									0			
Eublepharidae	African Fat-tailed Gecko (*Hemitheconyx caudicinctus*)												
Gekkonidae	Japanese Gecko (*Gekko japonicus*) (Japan)[a]					7							
Gekkonidae	Japanese Gecko (*Gekko japonicus*) (Eastern China)					17							
Gekkonidae	Madagascar Day Gecko (*Phelsuma madagascariensis grandis*)	0								0			
Agamidae	Rainbow Agama (*Agama agama*)									2.2 (26–27 °C)			
Agamidae	Jacky Dragon (*Amphibolurus muricatus*)			8				0		22			
Agamidae	Oriental Garden Lizard (*Calotes versicolor*)		0		0		50		100		67		80
Agamidae	Central Bearded Dragon[a] (*Pogona vitticeps*)	~50				~50				~60			
Scincidae	Southern Water Skink[a] (*Eulamprus tympanum*)							55					

References are: (a) Viets et al. (1994), (b) Tokunaga (1985), (c) Ding et al. (2012), (g) Quinn et al. (2007) and (h) Robert and Thompson (2001)

[a]TSD and GSD co-occur

selected squamata

28	28.5	29	29.5	30	30.5	31	31.5	32	32.5	33	33.5	34	34.5	35	36	37	References
2		19		29		83	88	90	74	80		7					a
0		3		0		80		50				7					a
75								21									b
49								17									c
33								0									a
		100															d
30		40		33		67		27									e
	50		30		25		0		5	50		100					f
~50				~50				~55				~75	~85	100	~95	100	g
				75				100									h

(d) Charnier (1966), (e) Harlow and Taylor (2000), (f) Inamdar Doddamani et al. (2012),

Table 18.3 Proportion of male offspring produced from eggs incubated at various temperatures in Tuatara

Species	Temperature (°C)										References
	16	17	18	19	20	21	22	23	24	25	
Sphenodon guntheri			3			25	74	100			a
Sphenodon punctatus punctatus			0				21	100			b
Sphenodon punctatus unnamed subspecies			0		7	4	30	100 (22.3 °C)	100		a

References are: (a) Mitchell et al. (2006) and (b) Mitchell et al. (2006), Nelson et al. (2004)

18.4 Factors that Affect TSD

In GSD, sexually dimorphic expression (e.g. *Sry* in mammals, *dmrt1* in chickens and *dmy* in Medaka) is responsible for triggering the gonadal gene cascade towards testicular development. Unlike GSD animals, however, it is unlikely that TSD animals have such a triggering gene that would result in sexually dimorphic expression. Rather, TSD may reflect regulation of non-genetic factors controlled by different temperature conditions or their combination. The mechanism that transduces temperature into a biological signal for testicular versus ovarian development is not known in any TSD animals so far, but possible factors that contribute to TSD have been investigated and these are further discussed below.

18.4.1 Contribution of Yolk

Yolk steroids play important roles in the development and growth of oviparous animals. Therefore, different levels of estrogens, androgens and/or their precursors may play a role in TSD. In the Snapping Turtle, the estradiol concentration in eggs at the female-producing temperature declines at a slower rate than at mixed sex and male-producing temperatures during TSP (Elf et al. 2002). There seems to be no association between yolk steroid levels and sex determination in alligators and lizards with TSD (Conley et al. 1997; Shine et al. 2007). Thus, it remains unknown whether yolk steroids directly contribute to initial sex determination in TSD. There can be large variations in the sex ratios among clutches of eggs laid at the pivotal temperature, even when incubated at a constant temperature (Lang and Andrews 1994). Maternal factor including yolk steroids may be one of the causative mechanism of such clutch effects, resulting in a skewed sex ratio (Elf 2003).

The Eastern Three-lined Skink (*Bassiana duperreyi*), a species of Scincidae family, exhibits the GSD system, but it can be over-ridden by temperature within the natural range (this "Mix" sex determination system is described the Chapter 18.7.2) (Shine et al. 2002). In low temperatures, removing yolk from a newly laid egg

causes the offspring to change to a male whereas adding yolk from a larger egg causes the recipient egg's offspring to change to a female (Radder et al. 2009). Thus, offspring sex in this species can be partly determined by yolk allocation. Similarly, a correlation between steroid hormones in yolk and offspring sex is suggested in the Japanese Gecko (Ding et al. 2012).

18.4.2 Estrogen and Enzymatic Activity

Estrogens play a central role in ovarian development and are produced from androgen substrates by cytochrome p450 aromatase. One may expect an optimum temperature for this enzyme activity that is near the temperature that produces female offspring. However, there were no differences in aromatase activity in the gonad-adrenal-mesonephric kidney complex (GAM) of developing alligator embryos between male- and female-producing temperatures during the TSP. After the TSP, there is a dramatic increase in aromatase activity in the female GAM, but activity at the male temperatures remained low (Smith et al. 1995). This is due to the differential gene expression levels between male and female GAMs. Aromatase transcripts can be detected in the brain, as well as in the gonads during TSP. But there are no significant sexual dimorphic expressions in the alligator during TSP (Gabriel et al. 2001; Parrott et al. 2014). In the European Pond Turtle (*Emys orbicularis*), another species with TSD, differential aromatase activity at male- and female-producing temperatures was detected towards the end of the TSP (Desvages and Pieau 1992). Taken together, these results suggest that aromatase (and therefore estrogens) does not play a role in the initial determination of sex in the alligator, but is indispensable later in ovarian differentiation.

Another refutation of the contribution of extragonadal estrogen (e.g. brain estrogen) to sex determination was observed in an organ culture experiment. In the Red-eared Slider Turtle, an isolated gonad endogenously retained the ability to sense changes in environmental temperature and responded similarly to estrogen-exposure by male or female temperature-dependent gene expression (Shoemaker-Daly et al. 2010; Matsumoto et al. 2013a, b). In the Olive Ridley Turtle (*Lepidochelys olivacea*), gonadal expression of *sox9*, a typical testis marker gene (see below), is maintained at male-producing temperatures but gradually disappears at female-producing temperatures in a common cultured condition (Moreno-Mendoza et al. 2001).

18.4.3 Germ Cells

Germ cell number and distribution could be one of the critical factors for the morphological differentiation in the gonads in some animals. In the teleost fish Medaka (*Oryzias latipes*), although they are GSD animals, female-biased germ cell

numbers during embryonic development is observed. Mutants showing abundant germ cells show male-to-female sex reversal (i.e. XY-female) (Morinaga et al. 2007), whereas loss of germ cells induces female-to-male sex reversal phenotypes (Kurokawa et al. 2007). In reptiles, however, there is no clear evidence of the contribution of germ cells to sex determination. Germ cell depletion by busulfan (a well-characterized germ cell toxin) treatment on the egg shell does not alter the morphological development of the fetal testis or ovary in *T. scripta* (DiNapoli and Capel 2007).

18.4.4 Chromatin Modification and Epigenetic Effects

The thermosensitive gene network can interact with chromatin regulators through heat shock proteins (HSPs) (Gibert et al. 2007). Temperature can thus modulate the chromatin structure and can directly be involved in transcriptional gene activation or inhibition by opening or condensing the chromatin. The HSP genes are highly conserved in all eukaryotes and can be divided into stress-inducible and constitutively expressed genes. Stress-inducible HSPs are expressed at low levels under non-stress conditions, but their expression increases rapidly in response to various stressors, including temperature stresses (heat or cold). In contrast, basal levels of constitutive genes are high and show relatively little change in the response to such stress. Furthermore, several HSPs can modify the trans-activational potency of the steroid hormone receptors as a molecular chaperone involving protein folding, stability, translocation, and competition with ligand binding. Thus, HSP function may affect cellular conditions in response to thermal effects. However, in the alligator, none of the HSPs (as well as cold-inducible RNA binding protein (*cirbp*) and HSP-binding protein (*hspbp*)) were differentially expressed in the GAM during TSP (Kohno et al. 2010).

DNA methylation represents the best-studied epigenetic modification and plays an important role in the regulation of genes involved in sex determination. Generally, DNA methylation at the regulatory regions is negatively correlated with associated gene expression. The *aromatase* promoter is hyper-methylated in the gonads of American Alligator embryos incubated at the male-producing temperature relative to embryos at the female-producing temperature (Parrott et al. 2014). On the contrary, the gonadal *sox9* promoter is hyper-methylated at the female-producing temperature in *A. mississippiensis*. Similar DNA methylation patterns have been observed in *T. scripta* (Matsumoto et al. 2013a, b). Thus, DNA methylation may be a key mediator involving temperature-dependent gene expression in TSD reptiles.

18.5 Gonadal Development and Differentiation in TSD Reptiles

18.5.1 Genes Involved in Sex Determination and/or Differentiation

Testicular and ovarian morphogenesis during the TSP has suggested the use of TSD in American Alligators (Smith and Joss 1994, Smith et al. 2009). The earliest morphological sign of testicular development at the male-producing temperature (33 °C) is forming pre-Sertoli cells in the medullar region of the gonad. After that, the Sertoli cells arrange into testis cords and the cortex is severely reduced. In contrast, ovarian differentiation at the female-producing temperature (30 °C) begins slightly later. The first indication of ovarian differentiation is fragmentation of the medulla of the gonad and thickening of the outer cortex and then proliferative germs cells in the thickened cortex region of the gonad.

Details on the thermal trigger of (pre-) Sertoli cell differentiation in testis are not known. Several studies have revealed that mammalian orthologous genes play important roles in sex determination and gonadal development in reptilian gonads, suggesting their evolutionarily conserved roles, although some disparities are also reported. In mammals, *Sox9* is a key transcriptional factor responsible for Sertoli cell-fate determination by triggering and activating the male-specific genes such as *anti-Müllerian hormone* (*Amh*). In support of this, *Sox9* expression precedes and activates the *Amh* expression in mice (Koopman et al. 2001). However, the expression of *amh* precedes *sox9* in the developing testis of the alligator (Western et al. 1999), which is similar to the case for the developing chick testis (Oreal et al. 1998). This implies that the initiation of *amh* expression is independent of *sox9* in alligators. In addition, *sox9* expression does not occur until late in the TSP, also implying Sertoli cell differentiation begins prior to *sox9* expression. It is nonetheless plausible that *sox9* may contribute to maintenance of *amh* expression as the *amh* promoters have several SOX9-binding sites and overexpression of SOX9 can activate the *amh* promoter in in vitro reporter gene assay (Urushitani et al. 2011). In the Red-eared Slider Turtle, sexually dimorphic expression of *sox9* was not established until late in the TSP as demonstrated by quantitative RT-PCR analysis (Barske and Capel 2010; Spotila et al. 1998). However, spatial gene expression analysis resulted in the sexually dimorphic expression of *sox9* early in the TSP and before the onset of *amh* expression (Shoemaker et al. 2007). In alligators and turtles, gonads form a GAM complex. Consequently, surgical removal of a gonad in the early developmental stage is difficult and contamination of non-gonadal tissue may mask expressed genes in the gonad (Shoemaker et al. 2007).

Intriguingly, many transforming growth factor beta (TGFβ) signaling genes (including *amh*, *gonadal soma derived factor* (*gsdf*), *amh receptor* (*amhr*)) have been identified as sex-determining genes in several fish species (Patagonian Pejerrey *Odontesthes hatcheri*, Luzon Medaka *Oryzias luzonensis*, Japanese Pufferfish *Takifugu rubripes*) (Kamiya et al. 2012; Myosho et al. 2012; Hattori et al. 2012).

It seems that TGFβ signaling is commonly responsible for sex determination in vertebrates and it is, therefore, possible that *amh* directly affects alligator sex determination. In addition to *sox9* and *amh*, DM domain genes including *dmrt1* control sex determination and differentiation in diverse metazoans (Matson and Zarkower 2012). *Dmrt1* expression shows dimorphic patterns during the TSP in many reptiles with TSD, including *T. scripta* and *L. olivacea* (Shoemaker et al. 2007; Torres Maldonado et al. 2002), although its roles in sex determination in reptiles is not fully understood.

Genes responsible for ovarian development and differentiation in mammals also show dimorphic patterns between male- and female-producing temperatures in reptiles. These genes include *forkhead box protein L2* (*foxl2*) and *wnt* signaling-related genes (e.g. *wnt4*, *R-spondin 1*, and *β-catenin*) in turtles (Matsumoto and Crews 2012; Shoemaker and Crews 2009). *Foxl2* plays a critical role in ovarian development and its loss leads to female-to-male sex reversal in goats (Boulanger et al. 2014). Although complete null mutation harbors ovaries (though primary follicles are depleted), conditional removal of *foxl2* in adult mice fails to maintain ovarian structure and transforms into testis-like structure (Uhlenhaut et al. 2009). Several *wnt* signaling-related genes are also critical for the ovarian pathway. In *T. scripta*, ectopic activation of the canonical *wnt* signaling pathway results in male-to-female partial sex reversal phenotypes, although inhibition of this pathway was not sufficient for female-to-male differentiation, similar to the case observed in mice (Mork and Capel 2013).

18.5.2 Hormonal Effects

As in many vertebrates, steroid hormones are essential for reptile development, reproduction, and health. Although steroid hormones are not likely a direct trigger of sex determination, estrogens and androgens play important roles in the development and expression of sexual characteristics. Indeed, administration of estrogens during the TSP over-rides temperature effects on eggs at the male-producing temperature in many reptiles. Estrogen treatment of alligator and turtles eggs incubated at a temperature that normally produces 100% males resulted in 100% female hatchlings (Bull et al. 1988; Crews et al. 1989; Lang and Andrews 1994) [Unexpectedly, male induction of estrogen-treated eggs in turtles has also been reported (Warner et al. 2014)]. Although estrogen function during ovarian development remains elusive, estrogen can repress SOX9 expression during sex determination in *T. scripta.* Exposure to estrogen during the early stages of gonad development induces down-regulation of SOX9 (Barske and Capel 2010).

It is not clear whether testicular differentiation in reptiles with TSD requires androgen signaling. In ovo exposure of alligator or turtle embryos to either non-aromatizable androgen (5α-dihydrotestosterone; DHT) or anti-androgens (cyproterone acetate and hydroxyflutamide) has no effect on gonadal differentiation both at the male- and female-producing temperature (Lance and Bogart 1992; Crews et al.

1989). In contrast, at the pivotal temperature, androgens induced the production of male-biased hatchlings in turtles and reduced the feminizing effects of estrogens when both were administered to embryos (Wibbels and Crews 1995; Wibbels et al. 1992). These results suggest that androgens may play a role in male sexual development. 5α-Reductase (enzyme that catalyzes testosterone to DHT) inhibitors produce female-biased embryos when eggs are incubated at a temperature likely to produce mostly males but this is probably due to accumulation of estrogen (Crews and Bergeron 1994).

The Leopard Gecko (*Eublepharis macularius*) has a similar pattern to the crocodilians (FMF pattern) and both sexes are produced over a broad range of incubation temperatures. They also exhibit relatively early sexual maturation (~45 weeks of age) and thus are useful for evaluating the temperature effects on animals hatched from eggs at different temperatures. For example, adult males hatched from eggs incubated at 30 °C, a temperature that results in a female-biased sex ratio, are more sexually active and less aggressive than adult males hatched from eggs incubated at 32.5 °C, a temperature that results in a male-biased sex ratio. In contrast, females hatched from eggs incubated at 32 °C are more likely to exhibit aggressive behavior towards either sex and respond to courtship by males as if they themselves were male. In short, animals from a male-biased incubation temperature are more likely to exhibit aggression than animals from female-biased incubation temperature. Furthermore, females from a male-biased incubation temperature tend to be less attractive than females from female-biased temperatures (Flores et al. 1994). Thus, incubation temperature during embryonic development not only determines gonadal sex, but also underlying sex differences in reproductive physiology and behavior later in life (Gutzke and Crews 1988).

18.6 Disruption of Normal Sex Determination and/or Sex Differentiation by Environmental Chemicals

As described above, temperature-driven sex determination can be over-ridden by exposure of the developing embryo to sex steroid hormones. This indicates that reptiles are likely to be sensitive to environmental factors, such as endocrine disrupting chemicals (EDCs). In 1962, the book “Silent Spring” by Rachel Carson warned that dichloro-diphenyl-trichloroethane (DDT) and other pesticides were a threat to wildlife (Carson 1962). Effects of EDCs on reptilian sex determination and gonadal differentiation are improtant in terms of environmental health and risk among wildlife. As a top predator with a long lifespan and high site fidelity, the American Alligator is an excellent model for investigating the effects of chronic exposure to EDCs. To date, anti-androgenic EDCs have been implicated in reproductive abnormalities observed in juvenile male alligators. For example, environmental pollutants from a pesticide spill in lake Apopka, Florida, were associated with a decline in juvenile alligator populations (Heinz et al. 1991; Woodward et al.

1993). Alligators from this lake exhibited morphological disorders such as poorly organized testes and small phalli, and decreased plasma testosterone concentrations in male juveniles (Guillette et al. 1999b; Guillette et al. 1996; Guillette et al. 1994). This spill consisted of several organochlorine pesticides and their metabolites, including *p,p′*-dichlorodiphenyldichloroethylene (*p,p′*-DDE). These chemicals have been detected in egg yolk and plasma obtained from alligators in lake Apopka (Guillette et al. 1999a; Heinz et al. 1991). These contaminants can alter androgen receptor trans-activity (Miyagawa et al. 2015), and in ovo exposure to *p,p′*-DDE resulted in a female-biased sex ratio at the pivotal temperature in alligators and Slider Turtles (Matsumoto et al. 2014; Milnes et al. 2005; Willingham and Crews 1999). Importantly, the concentrations reported to cause sex reversal, alterations in gonadal gene expression and steroidogenesis are well within the range of concentrations measured in alligator eggs from lake Apopka (Kohno et al. 2014). Not only anti-androgen, but also estrogenic EDCs such as bisphenol A (BPA) directly affect sex ratio or gonadal differentiation in Caiman and Painted Turtles, as demonstrated by an in ovo exposure experiment (Jandegian et al. 2015; Stoker et al. 2003).

18.7 Adaptive Advantages of TSD

18.7.1 Adaptation

Segregation of sex chromosomes in heterogametic GSD systems causes an equal sex ratio between male and female offspring, resulting in enhanced parental fitness. However, species with TSD could exhibit highly skewed sex ratios depending on environmental condition. In lizards, it has been postulated that TSD is the ancestral form of a sex determination mechanism, and GSD evolved from it via the gain of sex chromosomes (Gamble et al. 2015). As TSD is universally observed in a number of species, it must have some adaptive advantage when compared with GSD. The specific advantages of TSD over GSD have been a topic of active debate for several decades. There are several general explanations for this, e.g. maternal choice of thermal qualities of nest sites to promote group fitness (Mitchell et al. 2013; Janzen and Phillips 2006; Roosenburg 1996; Woodward and Murray 1993), inbreeding avoidance (Ewert and Nelason 1991; Burke 1993), or neutral (Bull 1980), but the most theoretically robust model is the "differential fitness" hypothesis (the Charnov and Bull model). TSD would be adaptive when reproductive fitness of males and females are differentially influenced by the temperature (Charnov and Bull 1977).

The Charnov and Bull model is empirically supported and is supported by experimental manipulations using the Jacky Dragon (*Amphibolurus muricatus*), a relatively short-lived and fast maturating oviparous small lizard. This species exhibits the FMF TSD pattern: female offspring are produced from eggs incubated at low and high temperatures and both sexes are produced at intermediate incubation temperatures. Males produced from eggs incubated at an intermediate temperature

experienced higher reproductive success in their lifetime when compared to hormone-manipulated (aromatase inhibitor-treated) males incubated at either the high or low temperatures. In contrast, females from incubation at either high or low temperatures displayed higher reproductive success in their lifetime than those incubated at the intermediate temperature (Warner and Shine 2008). In this species, temperature affects metabolic rate and embryos develop faster at warm temperatures (normally resulting in female offspring). These females reach the minimum size required for sexual maturity by the time hibernation begins, implying that they would be able to reproduce a year earlier than females from incubated at lower temperatures. It is also plausible that it is better for the female to be big so that she can lay more eggs. In contrast, early-maturing males would not be advantageous, as they would have to compete for territories and partners with older and larger males. Juvenile males presumably benefit from an additional year to allow more growth prior to maturity (Warner and Shine 2005). Differential fitness by sexual body size and seasonal hatchling timing also occurs in the fish species Atlantic Silverside (*Menidia menidia*) (Conover and Kynard 1981). This theory, however, may not be applicable for larger and longer living animals with delayed sexual maturation, because the temperature-effect on offspring growth rate can be removed in such animals when they reach sexual maturity. Furthermore, temperature effects can influence not only body size and growth rate, but also physiological and behavioral traits (as aforementioned in *E. macularius*), and there may be a hidden but effective trait elicited by differential thermal conditions.

18.7.2 Transition of TSD

The distribution of sex-determination mechanisms among reptiles and the lack of an apparent heteromorphic sex chromosome homolog suggests that several independent transitions between GSD and TSD occurred in reptiles during a relatively short evolutionary period (Sarre et al. 2011; Gamble 2010; Sarre et al. 2004) [but several recent studies suggest that evolutionary transitions from GSD to TSD appear to be less frequent than estimated (Gamble et al. 2015; Pokorna and Kratochvil 2016)]. There are two scenarios regarding the two sex determination modes, (1) GSD and TSD are fundamentally different underlying mechanisms. In this case, transition from GSD to TSD would occur through the acquisition of thermosensitivity and the rapid elimination of the sex chromosomes from the population. (2) GSD and TSD are not dichotomous states, but rather are extremes in a continuum. This model may eliminate "pure" TSD systems (i.e. offspring sex is determined by temperature only, without apparent genetic effects). Rather, temperature effects are greater than genetic factor in such animals and masks any other effects in a natural environmental range. Sexual development by shared genetic signals, maternally-deposited yolk hormones or other transient influences prior to temperature effects have been suggested in the past (Mork et al. 2014).

Evidence for both of the models has been reported previously. The continuous sex determination model is substantially established in some lizard species, in which both sex determination modes (GSD and TSD) can co-occur. The Central Bearded Dragon (*Pogona vitticeps*) has a ZW/ZZ GSD system and the sex ratios do not differ significantly from 1:1 between 22 °C and 32 °C. However, temperatures above 34 °C over-ride genotypic sex and induce sex reversal to a ZZ female (Quinn et al. 2007). Sex-reversed ZZ females have been discovered in nature recently and loss of the W chromosome in the population prompted rapid transition to TSD among the test *P. vitticeps* population (Holleley et al. 2015). Likewise, the Eastern Three-lined Skink (*B. duperreyi*) has an XY/XX GSD system, but male- or female-biased offspring were frequently induced from low or high incubation temperatures, respectively (Shine et al. 2002). The Japanese Gecko, *Gekko japonicus*, also possesses heteromorphic sex chromosomes, yet at the same time exhibits TSD (Gamble 2010; Tokunaga 1985). However, a different karyotype was reported for this species with no evidence of heteromorphic sex chromosomes, indicating the presence of a cryptic species (Gamble 2010). The spotted Skink (*N. ocellatus*) is a unique viviparous lizard showing intra-species GSD-TSD transition. This skink is climatically widespread, with populations having evolved in distinct thermal environments (i.e., mountain populations experience short summers and coastal populations experience warm conditions with long summers). Intriguingly, only the coastal population is influenced by thermal effects with MF pattern. It is proposed that an earlier birth allows females a longer growing opportunity until maturity, which may be adaptive. In contrast, the mountain population does not exhibit thermal effects on sex allocation because of a shorter activity season, more synchronized birth, and slower growth until maturity. This results in relatively limited bias in birth period for reproductive output (Pen et al. 2010).

18.8 Conclusion

Reptiles, in particular the larger reptile species, are difficult to maintain and breed in the laboratory. Relatively long reproductive cycles make it difficult to apply multi-genetic studies and gene modified experiments. In addition, slow sexual maturity makes it difficult to diagnose their sex by appearance in early life. Histology of the gonads is reliable for diagnosing the sex; however, such diagnostic interference may not be permitted for endangered species. Due to these limitations, clarification is needed on thermal sensors, gene network, adaptive significance of TSD systems, and evolutional history of sex determination mode. As mentioned above, reptiles are sensitive to environmental factors including climate changes and exposure to EDCs. Understanding of environmental regulation of reptilian sex determination and differentiation system will provide important information for not only adaptive basis of wildlife but also impact on ecological and environmental research as a sentinel animal species.

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Chapter 19
Sex Determination and Differentiation in Birds

Asato Kuroiwa

Abstract The sex of birds is genetically determined. Females have a heterogametic sex chromosome constitution (ZW), whereas males are homogametic (ZZ). Genes carried on these sex chromosomes control gonadal differentiation and development during embryogenesis. There are two hypotheses for the mechanisms of sex determination. One proposes that the dosage of genes on the Z chromosome determines the sexual differentiation of undifferentiated gonads, and the other proposes that W-linked genes dominantly determine ovary differentiation or inhibit testis differentiation. Z-linked *dmrt1*, which is a strong candidate for an avian sex-determining gene, supports the former hypothesis. Although no candidate for the W-linked gene has been identified, extensive evidence for spontaneous sex reversal in birds and aneuploid chimeric chickens with an abnormal sex chromosome constitution strongly supports the latter hypothesis. Undifferentiated and bipotential gonads differentiate into either testes or ovaries via several genes. The developed gonads release sex hormones to masculinize or feminize the brain and body. However, this process cannot explain spontaneous sex-chimeric birds, i.e., gynandromorphs, in which one side of the bird appears male and the other female. This chapter introduces the sex-determining mechanism as well as the genes and sex hormones mainly involved in gonadal differentiation and development of the chicken.

Keywords Z chromosome · W chromosome · ZZW · Sex reversal · Estrogen · Gynandromorph

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19.1 The Sex Chromosomes of Birds: Z and W Chromosomes

The sex of birds is determined by the inheritance of sex chromosomes. Females have the heterogametic sex chromosomes ZW, whereas males have the homogametic ZZ. This sex-determining system is highly conserved in avians, which include nearly ten thousand species.

The typical karyotype of birds consists of several pairs of macrochromosomes and many microchromosomes. The macrochromosomes are distinguishable by their size, morphology, and banding pattern, which can be obtained by treatment with enzymes or salt solutions. By contrast, microchromosomes are too small to distinguish individually. The chromosomes of a female chicken (*Gallus gallus domesticus*), which is the most useful experimental model for birds, are shown in Fig. 19.1. The chromosome number of chickens is $2n = 78$, and ten pairs of macrochromosomes (Chromosomes 1–9, Z, and W) and 29 pairs of microchromosomes can be observed. The Z chromosome is relatively large. Draft genome sequences have been available since 2004 (International Chicken Genome Sequencing Consortium 2004). The genome size is approximately 1 billion bp, the estimated size of the Z chromosome is 82 million bp, and the number of genes reported on the Z chromosome is 1146 (NCBI, Gnome: http://www.ncbi.nlm.nih.gov/genome/111?genome_assembly_id=22848). The W chromosome has degenerated during evolution, and its estimated size is 1.25 billion bp. Only 13 genes are located on the W chromosome.

19.2 Gonadal Differentiation in Chickens

The development of the urogenital system in chickens is similar to that in other amniotes. Urogenital tissues arise from the intermediate mesoderm and the first evidence of gonadal development is observed at embryonic day (E) 3.5, characterized

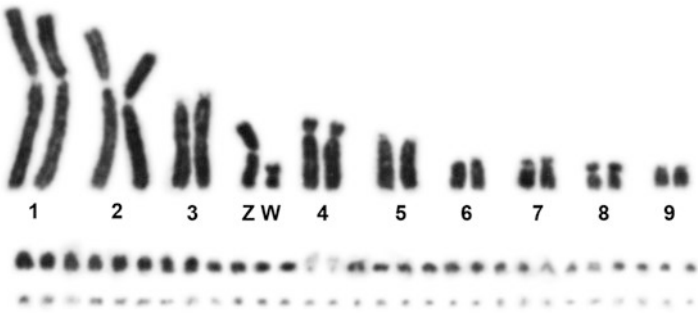

Fig. 19.1 Chromosomes of female chickens (*Gallus gallus domesticus*), $2n = 78$. The karyotype of chickens includes ten pairs of macrochromosomes and 29 pairs of microchromosomes

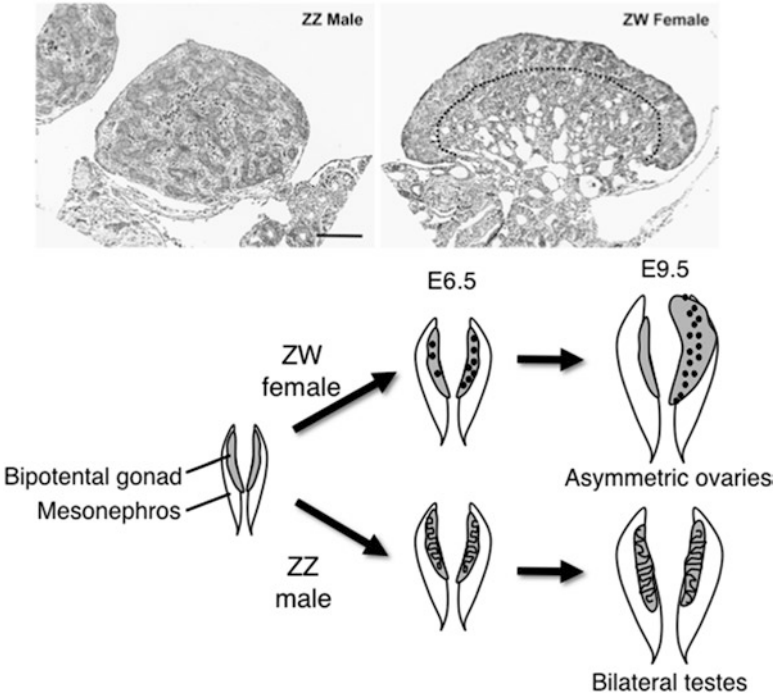

Fig. 19.2 Section of embryonic gonads with hematoxylin and eosin (HE)-staining. *Upper left*: the ZZ male gonad exhibits a developed medulla characterized by seminiferous tubules with Sertoli cells and prospermatogonia. *Upper right*: the left gonad of a ZW female exhibits a diagnostic thickened cortex and lacunae in the cortex by becoming vacuolated. *Lower*: a schematic image of gonadal development of chicken

by a thickening of the celomic epithelium ventral to the mesonephros. Until this embryonic stage, primordial germ cells (PGCs) of extragonadal origin migrate into the gonads through the blood stream.

The sex of birds is determined by genes located on the sex chromosome. In chickens, it is thought that sex determination occurs at E4.5. After sex determination, the gonads differentiate to testes or ovaries according to the sex chromosome. However, until around E6.5, the gonads are considered "bipotential," which means they are able to differentiate into either testes or ovaries.

After E6.5, histological differentiation of the gonads is observed between sexes. The gonads are differentiated to bilateral testes in ZZ male embryos. The medulla is developed and is characterized by seminiferous tubules with Sertoli cells and prospermatogonia (Fig. 19.2). The pre-Sertoli cells produce anti-Müllerian hormone (AMH), which degenerates embryonic oviducts (Müllerian ducts). Leydig cells adjacent to the seminiferous tubules in the testicle produce testosterone to differentiate round cords (Wolffian ducts) to internal genitalia. The PGCs become enclosed in developing seminiferous cords and undergo mitotic arrest in males. Meiosis only occurs after hatching.

In ZW female embryos, the right gonads are gradually depressed and fail to develop. The left gonads rapidly develop into ovaries with diagnostic thickened cortexes (Fig. 19.2). This asymmetric morphology between right and left gonads in females becomes apparent at E6.5 (Fig. 19.2). The proliferating germ cells exhibit a cortical distribution and begin to enter meiosis at E15.5 in the left gonad. PGCs in the right gonad undergo some proliferation, but do not enter meiosis (Ukeshima 1996). The Müllerian duct on the right side degenerates to form a dysfunctional vestige (Carlon and Stahl 1985). The medullary cords in the cortex of the female left gonad form lacunae by becoming vacuolated during development. The left ovary finally develops into a functional organ, in which follicles are formed.

19.3 The Two Hypotheses for Sex Determination

Two hypotheses have been proposed for the mechanism of avian sex determination (Fig. 19.3). The dosage of a Z-linked gene may mediate sex determination, whereby two copies are required for male development (ZZ). This is called the "Z dosage" model. This model is supported by the observation that birds have no dosage compensation system for the Z chromosome, like X inactivation observed in mammals (McQueen et al. 2001; Kuroda et al. 2001; Kuroiwa et al. 2002; Itoh et al. 2010). A strong candidate gene for sex determination under this hypothesis is the Z-linked *dmrt1* (doublesex and mab-3-related transcription factor 1) gene.

Alternatively, the other hypothesis is the "W dominant" model. According to this model, the W chromosome carries a dominant-acting ovary determinant or an inhibitor of testis differentiation. Two W-linked genes have been reported as candidates for sex-determining genes in chickens.

Hintw (histidine triad nucleotide-binding protein W) was reported as the best candidate for the W-linked ovary-determining gene (also known as *wpkci* and *asw*) at approximately the same time by two research groups (Hori et al. 2000; O'Neill et al. 2000). *Hintw* encodes an aberrant form of a nucleotide hydrolase enzyme (HINT). HINT proteins generally have endogenous adenosine 5′ monophosphoramidate

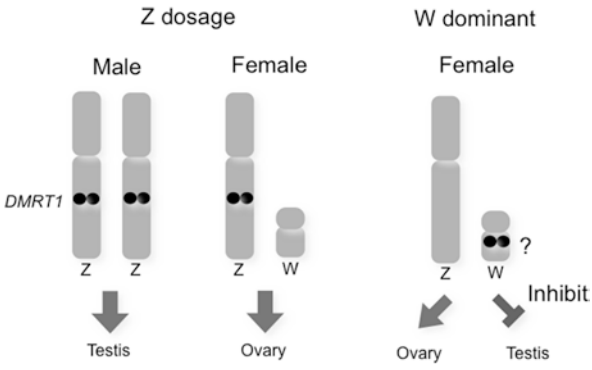

Fig. 19.3 Two hypotheses of sex-determining mechanisms in birds

enzyme activity. Its Z homolog, *hintz*, has a functional catalytic domain, the HIT motif, similar to other HINT proteins. These have been known as orthologs of *Hint1* (histidine triad nucleotide binding protein 1) on autosomes in mammals. By contrast, this motif is absent in *hintw*. Several in vitro biochemical experiments showed that *hintz* function can be inhibited via the formation of *hintz/hintw* heterodimers (Pace and Brenner 2003). However, ZZ embryos that overexpress *hintw* develop to normal males with bilateral testes (Smith et al. 2009a). This provides genetic evidence against a role for *hintw* in avian sex determination.

Fet1 (female expressed transcript 1) is another candidate for the W-linked ovary determinant (Reed and Sinclair 2002). This gene is found only in the chicken genome, i.e., there are no orthologs in another bird species. The expression was almost exclusively observed in the female urogenital system. In particular, it is strongly expressed in female left gonads leading up to sexual differentiation, at E4.5–E6.5. However, genome sequencing analyses revealed that the gene is located on chromosome 4. Therefore, there are no suitable candidates for W-linked ovary-determining genes at present.

19.4 Abnormal Sex Chromosomes

Abnormal sex chromosome constitutions are useful to understand the sex-determining mechanism of a species. For instance, mammals and fruit flies (*Drosophila melanogaster*) have XX/XY sex chromosome constitutions. However, their mechanism for sex determination differs: mammals have a male-dominant Y chromosome, whereas the sex of fruit flies is determined by X chromosome dosage. To distinguish between these, sex chromosome aneuploids could be particularly helpful. XO individuals are female in mammals, but male in fruit flies. By contrast, XXY animals are male in mammals, but female in fruit flies. Therefore, distinguishing between the mechanisms of sex determination in birds would be straightforward if sex chromosome aneuploids were available (Graves 2003).

Despite intensive studies of chickens with aberrant sex chromosome constitutions, there are no reports of ZO chickens (Graves 2003). This means that ZO chromosomes may indeed be lethal to embryos. By contrast, ZZW triploid chickens were described by Thorne and Sheldon (1991). These chickens are sterile intersex with both ovarian and testicular tissues. Furthermore, Lin et al. (1995) gave a detailed description of the gonads of 63 ZZW triploid chickens ranging in age from 1 day to 4.5 years. The right gonads of 1-day-old chickens (chicks) had testes with developed seminiferous tubules, as observed in normal diploid ZZ males. After 3 months, the development of seminiferous tubules was retarded. The slow growth in the diameter of seminiferous tubules up to 7 weeks of age was associated with a twofold increase in the number of Sertoli cells from hatching (Lin et al. 1995). In 6-month-old chickens, few spermatozoa among a large number of round, condensed spermatid nuclei in the seminiferous tubules were observed. In 9-month-old chickens, the seminiferous tubules were degenerated.

ZZW triploid chickens differed with respect to the development of the left gonads. In 1-day-old chicks, the left gonads showed ovotestis features: oocytes in the cortex and seminiferous tubules in the medulla. After 1 week, more than 50% of oocytes in cortical cords degenerated and contained no nucleus or a poorly defined nucleus. There were seminiferous tubules in the medullae of all left gonads. From 3 to 7 weeks of age, the cortex continued to degenerate and was infiltrated by leukocytes that were mainly small lymphocytes. The seminiferous tubules continued to grow in the medulla. By 6 months of age, leukocytic infiltration of the cortical region stopped, and there were no ovarian components in the gonads. By contrast, the structure of seminiferous tubules in the medulla was similar to that in the right gonads. Gonadal degeneration began at 9 months old, and all left gonads degenerated, leaving a large portion of parenchyma composed of loose connective tissue. In chickens older than 1 year, seminiferous tubules could not be observed in the left gonads.

These observations in ZZW triploid chickens, which are complete triploids with all autosomes present in triplicate, indicated that the W chromosome is associated with ovarian development to some degree; however, ovarian features are ultimately over-ridden by two Z chromosomes (reviewed in Lambeth and Smith 2012). Interestingly, chimeric chickens with a mixture of diploid and triploid cells are also informative. Although the estimated ratio of 2AZZ/3AZZW chimeras is only 5 %, the left gonads consistently developed into an ovary (Thorne and Sheldon 1993). This means that the ovary-determining gene is located on the W chromosome because the presence of a small number of cells including the W chromosome is sufficient to induce ovarian development (reviewed in Lambeth and Smith 2012). This observation supports the “W dominant” model.

19.5 *Dmrt1*: A Strong Candidate for Sex Determination in Avians

A strong candidate for the avian sex determinant under the “Z dosage” model is the conserved Z-linked gene *dmrt1*. *Dmrt1* is a transcription factor that possesses a DNA-binding domain named the DM domain. This gene is highly conserved in vertebrate and non-vertebrate species and is involved in the development of male reproductive organs (Raymond et al. 1999). In vertebrates, *dmrt1* expression is essential for testis differentiation. The overexpression of *dmrt1* in XX mouse fetal gonads drives the development of testes and represses the expression of key markers of ovarian development (Zhao et al. 2015). *Dmrt1* paralogs were identified as sex-determining genes in Medaka (*Oryzias latipes*, *dmy* in the Y chromosome) and African Clawed Frogs (*Xenopus laevis*, *dmw* in the W chromosome).

The chicken homolog of *dmrt1* is located on the Z chromosome. It is expressed more highly in male undifferentiated gonads than in females (Smith et al. 1999). *Dmrt1* knock-down via RNA interference results in the feminization of embryonic

gonads in genetically male (ZZ) embryos (Smith et al. 2009b). The feminized left gonad shows female-like histology, disorganized testis cords, and a decline in the testicular marker *sox9* (SRY-box 9). The ovarian marker aromatase is ectopically activated. The feminized right gonad shows a more variable loss of *dmrt1* and ectopic aromatase activation, suggesting differential sensitivity to *dmrt1* between the left and right gonads. Germ cells also show a female pattern of distribution in feminized male gonads. Furthermore, ectopic expression of *dmrt1* in the left and right gonads of ZW embryo induces masculinization, characterized by increased expression of male marker genes and reduced expression of female marker genes (Lambeth et al. 2014). These reports indicate that *dmrt1* is a master regulator for testis determination in the chicken and also support the "Z dosage" model for avian sex determination.

19.6 Genes Involved in Male Sexual Differentiation

Dmrt1 expression begins at ~3.5 days of incubation. The expression is observed in the medulla of gonads and is higher in ZZ males than in ZW females. After high *dmrt1* expression, *sox9* functions in testis development in ZZ chicken embryos (Fig. 19.4). In placental mammals, SRY (sex-determining region Y), directly activates *Sox9* expression by binding to the *Sox9* enhancer together with the Nr5a1 protein in the undifferentiated gonads of XY embryos (Sekido and Lovell-Badge 2008). However, in chickens, there is a time-lag between the initial expression of *dmrt1* and *sox9*, which occur at days 3.5 and 6.5, respectively. Therefore, other

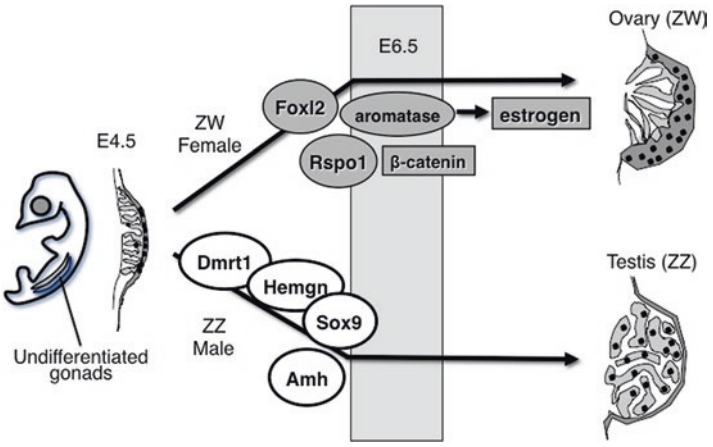

Fig. 19.4 Potential molecular mechanisms underlying gonadal differentiation in the chicken embryo

factors, which are probably chicken-specific, must be components of the molecular cascade between *dmrt1* and *sox9*.

Hemgn, located on the Z chromosome (hemogen), is a specific factor to chicken species that mediates *sox9* regulation under *dmrt1* (Nakata et al. 2013, Fig. 19.4). In mice, *Hemgn* (also known as *EDAG* in humans) is a recently characterized hematopoietic tissue-specific gene encoding a nuclear protein (Yang et al. 2001). *Hemgn* expression is restricted to the blood islands of the yolk sac and the fetal liver during embryogenesis, the adult spleen, and bone marrow. In humans, *EDAG* shows a similar expression pattern. High *EDAG* expression is observed in bone marrow cells in acute myeloid leukemia, suggesting that *EDAG* plays a regulatory role in acute myeloid leukemia (An et al. 2005). However, the gene is not expressed in the gonads during embryogenesis in mammals. In chickens, *hemgn* is expressed not only in hematopoietic tissues, but also in the early embryonic gonads of male chickens. Male-specific expression was observed in the nuclei of (pre-)Sertoli cells after the sex determination period and prior to the expression of *sox9*. The expression of *hemgn* was induced in ZW embryonic gonads that were masculinized by aromatase inhibitor treatment. ZW embryos overexpressing *hemgn*, generated by infection with a retrovirus carrying *hemgn*, had masculinized gonads. These findings suggest that *hemgn* is a transcription factor that is specifically involved in chicken sex determination.

Amh is a glycoprotein belonging to the Transforming Growth Factor-β (TGF-β) superfamily. This hormone is secreted by the gonads and plays a role in sexual differentiation of reproductive organs. *Amh* is synthesized and secreted by Sertoli cells of the embryonic testis and directly acts to regress the paired Müllerian ducts of males (Josso and Picard 1986; Josso et al. 1993; Vigier et al. 1983). In mammals, Sox9 directly regulates *Amh* transcription together with *nr5a1*, *Gata4*, and Wt1. However, in chickens, *amh* mRNA is expressed prior to *sox9* mRNA. *Amh* mRNA is also present in the female gonads of embryonic chickens, and acts to regress the right female Müllerian ducts (Hutson et al. 1981). It is thought that estrogens protect the left duct from regression by *amh* (Josso et al. 2001; Hutson et al. 1982; Tran and Josso 1977). However, the exact mechanism is not known.

Amh is widely conserved in vertebrates. Its function is primarily related to Müllerian duct regression, whereas Y-linked duplicated *amh* functions in male sex differentiation in the Patagonia Pejerrey (*Odontesthes hatcheri*). In chickens, *amh* expression precedes that of *sox9* (Fig. 19.4), indicating that *amh* plays a more central role in avian testis development, similar to fish species. Lambeth et al. (2015) suppressed *amh* expression in embryonic gonads of chickens using RNA interference, and did not observe an effect on the expression of the key testis pathway genes *dmrt1* and *sox9*, and male embryos exhibited normal testicular structure. However, the sizes of the mesonephros and gonads were reduced, with normal phenotypes in male and female embryos. These findings indicate that *amh* is required for proper cell proliferation and urogenital system growth, irrespective of sex but *amh* does not directly contribute to testicular or ovarian differentiation (Lambeth et al. 2015).

19.7 Genes Involved in Sexual Differentiation of Females

In birds, gonadal sex differentiation in females is sensitive to the sex steroid hormone estradiol. This hormone is only detected in female embryonic gonads and is required for ovarian development (Elbrecht and Smith 1992). The enzyme aromatase is responsible for converting androgens to estradiol. The aromatase protein is expressed in the medullae of female gonads from E6.5 onwards and its expression increases during ovarian development (Smith et al. 2005).

Although there are no candidates for the W-linked ovary-determining genes, several genes are involved in female sex determination. *Foxl2* (forkhead box L2) is an essential factor that is widely conserved in vertebrates, including chickens (Loffler et al. 2003; Wang et al. 2007; Pisarska et al. 2011, Fig. 19.4). The expression patterns of *foxl2* and aromatase transcripts are highly correlated in the developing ovary at 4.7–12.7 days of incubation (Govoroun et al. 2004). The proteins encoded by both genes are co-localized in the nuclei of medullar cord cells, and *foxl2* is mainly expressed in the granulosa cells of developing follicles. *Foxl2* is expressed just prior to aromatase, suggesting that it directly or indirectly regulates aromatase transcription. Aromatase inhibitor treatment reduces *foxl2* expression in vivo, suggesting that there is a feedback regulator loop between *foxl2* and aromatase (Hudson et al. 2005).

Rspo1 (R-spondin 1) and *wnt4* (wingless-type MMTV integration site family, member 4) activation of β-catenin signaling plays an important role in the developing ovary in several vertebrate species (Biason-Lauber and Konrad 2008; Liu et al. 2010; Chue and Smith 2011), including chickens (Fig. 19.4). *Rspo1* mRNA is expressed in the left and right gonads of ZW chicken embryos from E4.5, and levels increase from E8.5 onward (Smith et al. 2008). By contrast, its expression remains low in the gonads of ZZ embryos. *Wnt4* expression is observed in the bipotential gonads of ZZ and ZW embryos at E4.5. However, in the left gonads of ZW embryos, it is up-regulated during sexual differentiation, at E6.5–8.5. *Rspo1* and *wnt4* are strongly expressed in the cortex of the developing ovary (Ayers et al. 2013). They may act synergistically to activate β-catenin.

19.8 Sex Reversal

Aristotle (384–322 B.C.) seems to have been the first to record the phenomenon of abnormal sex development in poultry. He observed hens that changed into cockerels (reviewed in Taber 1964). Examples of spontaneous sex reversal in chickens and many other bird species have been reported. Forbes (1947) described the following in his review of sex reversal in birds: “Throughout centuries, a farmer has seen an old hen gradually assuming masculine behavior and plumage, or a hunter has found that the ‘cock’ pheasant he had shot was really a female. Such unnatural transformations sometimes chilled the observer with terror of evil things to come, or

sometimes, more reasonably, tired a naive or intelligent curiosity" (Forbes 1947). Interestingly, only the masculinization of female birds has been documented (i.e., female to male), and there are no reports of male-to-female sex reversal. Aristotle recorded the reciprocal event, cockerel to feminine behavior, but this has not been confirmed.

Sex-reversed chickens can be generated experimentally. ZZ embryos treated with exogenous estrogen prior to gonadal differentiation show feminization of the left gonads, resulting in ovaries or ovotestes. Additionally, the administration of the anti-estrogen tamoxifen disturbs normal female development (Scheib 1983). Aromatase inhibitors, such as fadrozole, can effectually lead to feminization in ZW embryos (Elbrecht and Smith 1992). These inhibitors induce female-to-male sex reversal in ZW females when applied between E0 and E7.5. Bilateral gonads develop and a testis-like structure with a thick cortex and dense medulla is observed, although the embryo is genetically female (ZW) (Fig. 19.5). Furthermore, genes involved in testis differentiation are unregulated; by contrast, female marker genes are down-regulated.

Evidence obtained from experimental sex-reversed chickens suggests that sex hormones and their enzymatic pathway are very important for ovary development in chickens, and potentially in all bird species. Furthermore, there are no examples of male-to-female sex reversal in birds under natural conditions, indicating that birds cannot be female without the W chromosome. This inference strongly supports the "W-dominant" hypothesis, which maintains that W-linked genes dominantly determine ovary differentiation (or inhibit testis differentiation).

19.9 Sex Chimeras and Gynandromorphs

In general, gynandromorphs have been observed in insects and crustaceans; they have the physical characteristics of both genders, usually displaying a bilateral difference. In vertebrates, gynandromorphs are found only in birds. One side of the animal appears male and the other female. These birds are rare, but researchers have focused on this interesting phenomenon as a "genetic mosaicism" (Hollander 1944). Gynandromorph birds have been observed in pigeons, zebra finches, and especially domestic fowls (Hollander 1975; Agate et al. 2003; Cock 1955), among other species. A long-standing theory on the etiology of gynandromorphs proposes that a single sex chromosome is lost at the two-cell stage on one side of the animal (Cock 1955). However, it is now understood that gynandromorph birds arise as a result of a failure in the extrusion of a polar body during meiosis and subsequent fertilization of both a Z- and W-bearing female pronucleus (Hollander 1975; Zhao et al. 2010).

Zhao et al. (2010) examined three lateral gynandromorph chickens. All chickens were ISA brown commercial hybrids with sex-linked coloration in which males show white plumage and females show brown plumage. Gynandromorph chickens show a marked bilateral asymmetry: half of the body is phenotypically female and the other side is phenotypically male. The female side with brown plumage had a

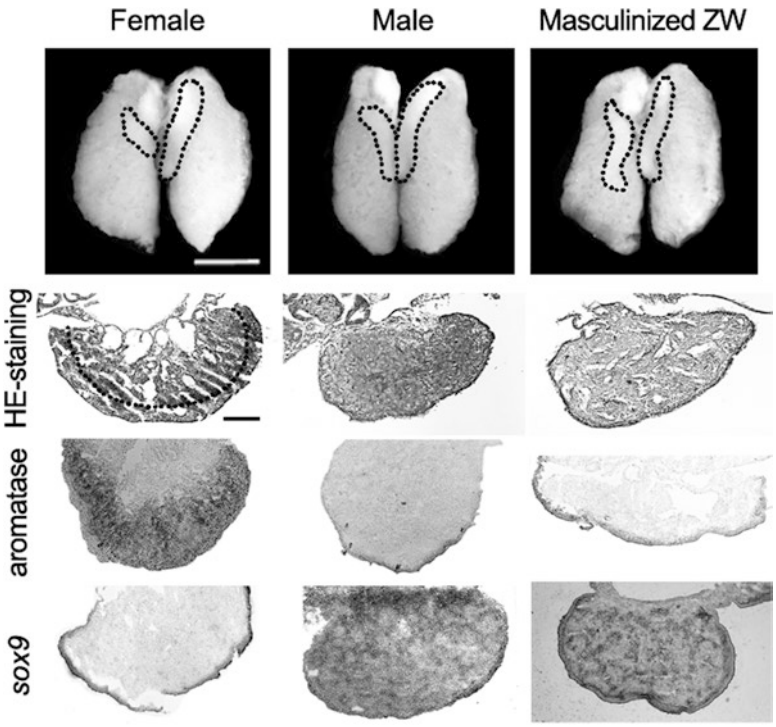

Fig. 19.5 The gonads of masculinized ZW embryos induced by aromatase inhibitor treatment. *Upper*: gonads on top of the mesonephros of female, male, and masculinized ZW embryos at day 10.5. The gonads of masculinized ZW embryos showed bilateral development, similar to male gonads. Dashed lines indicate gonads. Scale bar, 1 mm. *Middle*: HE-staining of gonad sections from female, male, and masculinized ZW embryos. The left masculinized ZW gonad has a testis-like phenotype with a dense medulla and thin cortex, although a slightly fragmented medulla was observed. The dashed line indicates the border between the cortex and medulla in the female gonad. Scale bar, 100 μm. *Lower*: aromatase and *sox9* in situ hybridization in male, female, and masculinized ZW gonad frozen sections at day 10.5. Aromatase is detected in female gonads, but no expression is observed in male or masculinized ZW gonads. By contrast, *sox9* expression is not detected in female gonads, but is present in male and masculinized ZW gonads

small wattle and small leg spur. By contrast, the male side, which was white, showed a large wattle, a large leg spur, a heavier leg structure, and an obviously greater mass of breast muscle, like a typical cockerel (Zhao et al. 2010).

To identify the sex chromosome constitutions of somatic cells, fluorescence in situ hybridization using Z and W chromosome probes was performed using chromosome preparations obtained from cells in blood and skin samples from both sides of the three gynandromorph birds. All three birds were composed of a mixture of normal diploid male and female cells. Tissues of the male side were mainly composed of ZZ (male) cells, whereas tissues of the female side mainly contained ZW (female) cells.

Understanding gonadal differentiation in gynandromorph chickens is highly complicated because gonadal structure does not correspond to external appearance (Zhao et al. 2010). Two gynandromorphy chickens (G1 and G2) appeared female on the left side and male on the right, whereas G3 showed the reverse external appearance (left: male, right: female). The left gonad differed in appearance between these three gynandromorph chickens. G1 contained a testis-like gonad composed primarily of sperm-containing seminiferous tubules. G2 contained an ovary-like gonad composed predominantly of large and small follicles. G3 contained an ovotestis comprised of a mixture of empty tubules and small follicular-like structures. The morphological appearance of the gonads reflected the cellular composition of the individual organs. Testis-like and ovary-like gonads were composed principally of ZZ- and ZW-containing cells, respectively, whereas the ovotestis comprised a mixture of ZZ- and ZW-containing cells.

19.10 Paradox in Avian Sex Development: Genes or Hormones?

The traditional view of sexual development in birds and other vertebrates is that the gonads develop into either ovaries or testes during the embryonic stage and then release sex hormones to masculinize or feminize the rest of the body. However, this process cannot explain gynandromorphy because hormones are expected to flow equally to both sides of the body. Nevertheless, the organs exhibit male or female phenotypes depending on the cellular composition, ZZ or ZW.

Gonadal chimeras generated by transplantation of presumptive mesoderm show a similar pattern. Zhao et al. (2010) transplanted sections of presumptive mesoderm from green fluorescent protein (GFP)-expressing embryos at developmental stage 12 to the equivalent tissue on non-GFP embryos at the same stage of development between sexes. The transplanted embryos were allowed to develop until stage 35, and the expression patterns of the male marker *amh* and female marker aromatase were examined. Interestingly, donor male cells expressed *amh* and donor female cells expressed aromatase in mixed-sex chimeras. Donor cells appear to be incapable of contributing to specialized compartments of the host gonad. Female donor cells in the testis of a male host cannot be recruited into the functional male Sertoli cell compartment, and male donor cells in the ovary of a female host are excluded from the functionally female compartment.

19.11 Sex Identity in Somatic Cells

Hormones must play a role in early sexual development because genetically female chicken embryos develop as males with testes when treated with an aromatase inhibitor, as mentioned in Sect. 19.8. However, studies on gynandromorphy and

gonadal chimeras provide evidence that all somatic cells recognize their sex, ZZ male or ZW female. This observation led to the idea that male and female chicken somatic cells possess a cell-autonomous sex identity (CASI) (Zhao et al. 2010; Clinton et al. 2012).

Based on gynandromorph chicken studies, Zhao et al. (2010) proposed that Z-linked genes underlie sex determination throughout the avian body. This idea is supported by the fact that birds have no chromosome-wide dosage compensation mechanism, previously mentioned in Sect. 19.3. The dosage of most Z-linked genes is twofold higher in male than in female cells, and this might determine the sex identities of each cell.

19.12 Conclusion

Our understanding of the molecular mechanisms of avian sex determination and gonadal differentiation is lagging behind that of mammals and fishes. Although genes and regulatory networks that govern the fate of gonads were recently identified, many gaps in knowledge remain. There is conflicting evidence regarding the importance of sex hormones in sex differentiation. Additional investigations of the role of genes involved in sex determination and differentiation and the relative contribution of the genetic sex of each somatic cell and hormones to sexual differentiation are expected in the future.

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Chapter 20
Sex Determination and Differentiation in Mammals

Kento Miura, Ayako Tomita, and Yoshiakira Kanai

Abstract In most mammals, sex determination is initiated by transient expression of *Sry*, *Sex-determining region Y gene*, encoding an HMG-box transcription factor in bipotential gonadal supporting cells. In XY mouse gonads, SRY activates SOX9, another SRY-related HMG-box factor, during a critical time window (i**.**e**.** embryonic days 11.0–11.5) in male supporting cells. The maintenance of high-level SOX9 expression consequently induces Sertoli cell differentiation, leading to testis formation during the early organogenic stages. In XX gonads without *Sry* expression, bipotential supporting cells express FOXL2, a forkhead transcription factor, shortly after this time window, resulting in pre-granulosa cell differentiation and its subsequent contribution to ovarian folliculogenesis after birth. At later fetal stages, after cessation of SRY expression, the balance between masclinizing FGF9 and feminizing WNT4 signals affects the maintenance of high-level SOX9 expression in the supporting cells. During the perinatal and postnatal stages, each sex of the supporting cells is maintained by the balance between the masclinizing actions of SOX9 and DMRT1 and the feminizing actions of FOXL2, estrogen, and retinoic acid. In this chapter, we review recent knowledge regarding the SRY-dependent sex determination system during the critical time window and discuss the antagonistic interaction between testicular and ovarian factors during the late fetal, perinatal, and postnatal periods in mice.

Keywords SRY · SOX9 · AMH · Bipotential supporting cells · Critical time window · FGF9 · TESCO · Retinoic acid · Sex reversal · Mouse

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20.1 Introduction

In mammals, the testis and ovary are critical organs producing gametes, which give life to the next generation by producing sperm or oocytes. Mammalian sex is determined based on sex chromosome constitution (i.e. XY or XX) at the time of fertilization. In most mammals, including humans and mice, both XY and XX embryos develop equally in a non-sexually dimorphic fashion until the early organogenic stage, leading to the formation of long and narrow gonadal primordia along the mesonephric region of the posterior trunk. Such bipotential gonads develop into either testes or ovaries in the presence or absence of *Sry, Sex-determining region Y gene*, at the critical time window during embryonic day 11.0 (E11.0)~E11.5 in mice (see reviews by Kashimada and Koopman 2010; Harikae et al. 2013a; Larney et al. 2014) (Fig. 20.1-A, B). After gonadal sex determination, the differentiating gonadal somatic cells produce various sex-dimorphic signaling factors for the maintenance of each sex of the supporting cells and simultaneously secrete testis- or ovary-specific hormones that affect the sexually dimorphic development of the intra- and extra-reproductive organs during the late fetal and peri- and postnatal stages. Such sexually dimorphic hormonal secretion from the gonads results in the sexual maturation of the adult male or female.

One important feature of mammalian sex differentiation is that the sex of gonadal supporting cell lineages controls sex differentiation in all other cell lineages, such as germ cells, steroidogenic cells, and so on. Briefly, both testes and ovaries consist of germ cells and supporting cells (**i.e.** Sertoli cells and granulosa cells) inside a tubular or follicular structure, which is surrounded by steroidogenic cells (**i.e.** Leydig cells and internal theca cells) and myoid cells in the interstitial regions (see Fig. 20.1-C). In mouse XY gonads, SRY is transiently activated in only supporting cells, leading to Sertoli cell differentiation. Differentiating Sertoli cells play a central role in the male sex determination of the other cell lineages, such as germ cells, interstitial steroidogenic cells, and vascular patterns, at later stages. In the absence of SRY, the XX supporting cells are fated to become granulosa cells, which causes female sex differentiation in the other cell lineages.

Another feature of mammals is that the sex determination/differentiation of the fetal gonads proceeds inside the mother's womb (i.e. in an estrogen-rich environment) through the placenta. Such an estrogen-dominant environment may lead to the low sensitivity to estrogens in most parts of the sex-determination process, at least during the fetal stages of mammals. This may explain the dominant regulation of male hormones, such as anti-Müllerian hormones (AMHs) and androgens, produced in embryonic testes until birth, after which newborns of both sexes will mature due to the sex hormones produced in the gonads of each individual.

In this chapter, we review recent advances in the molecular and cellular mechanisms upstream and downstream of SRY and SOX9 (SRY-related HMG-box 9) actions and discuss the antagonistic interactions between testicular and ovarian factors at the later stages, after gonadal sex determination.

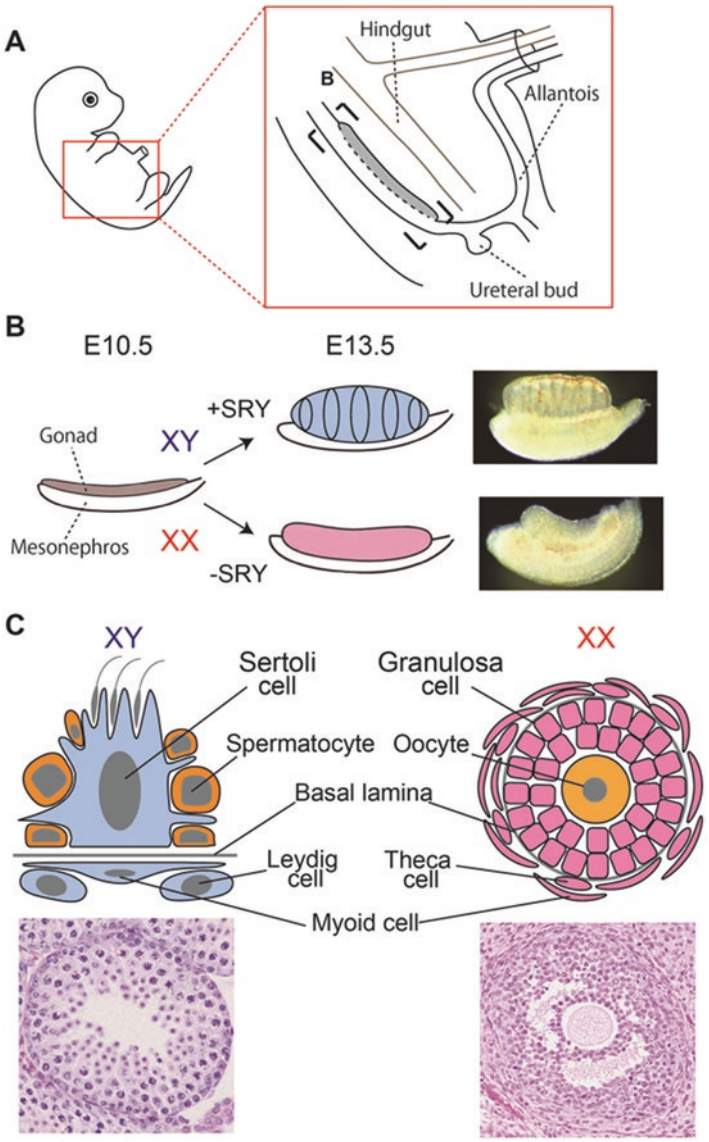

Fig. 20.1 Gonadal structure and sex differentiation in mammals. (**A**, **B**) Schematic representation showing mouse embryo with the genital ridges extended along the anterioposterior axis of the posterior trunk (**A**), and male and female gonads before and after sex determination at E10.5 and E13.5 (**B**). The dissecting microscopic images of the testis and ovary at E13.5 are also shown in the right-hand side (note testis cords [in future seminiferous tubules] in the testis). (**C**) Schematic representation and HE-stained images showing the seminiferous tubule and ovarian follicle at the postnatal and adult stages. In both testis and ovary, gonadal supporting cells (i.e. Sertoli and granulosa cells) and germ cells are tightly packed within the basal lamina layer, which forms the seminiferous epithelium or ovarian follicle. Steroidogenic cells (i.e. Leydig cells and theca cells) and myoid cells are located in the interstitium

20.2 Sex Differentiation in Supporting Cell Progenitors

20.2.1 SRY Expression in Pre-Sertoli Cells

20.2.1.1 Early Gonadogenesis and *Sry* Expression Patterns

Gonadal primordium arises from the thickening coelomic epithelium covering the mesonephric tissues of the posterior trunk and forms a pair of long and narrow structures that extend widely along the anterioposterior (AP) axis (Harikae et al. 2013a; Wainwright et al. 2014). Meanwhile, the primordial germ cells migrate into genital ridges via the morphogenic movement of hindgut (Hara et al. 2009; also see a review by Harikae et al. 2013a), leading to the establishment of gonadal primordium by E10.5. The formation of genital ridges was previously shown to require various transcription factors, including Wilms tumor 1 homolog (WT1) (-KTS isoform) (Hammes et al. 2001); nuclear receptor subfamily 5, group A, member 1 (NR5A1/SF1/Ad4Bp; Luo et al. 1994); empty spiracles homeobox 2 (EMX2; Kusaka et al. 2010); CBX2 (Katoh-Fukui et al. 2012); LIM homeobox protein-9 (LHX9; Birk et al. 2000); and GATA binding protein 4 (GATA4; Hu et al. 2013). Among these transcription factors, NR5A1 appears to play a crucial role in the proliferation and energy metabolism of gonadal somatic cells during early gonadogenesis (Baba et al. 2014; see a review by Morohashi et al. 2013). A direct reprogramming experiment demonstrated that concomitant expression using NR5A1, WT1, and GATA4 in addition to two major masclininzing transcription factors, DMRT1 and SOX9, efficiently reprogrammed mouse fibroblasts into induced embryonic Sertoli-like cells (Buganim et al. 2012). This is consistent with the crucial roles of these three factors, NR5A1, WT1, and GATA4, in the formation of gonadal somatic cells, including supporting cell progenitors, during early gonadogenesis.

Sry is first activated in the supporting progenitor cells located just beneath the coelomic epithelium at around E11.0 (Sekido et al. 2004; Kidokoro et al. 2005; see a review by Kanai et al. 2005). *Sry*-positive cells appear in the central region of the gonads along the anterioposterior (AP) axis at around E11.0, and they expand toward both the anterior and posterior poles by E11.5. Thereafter, their expression is rapidly downregulated in the anterior and central regions, becoming restricted to the posterior pole before completely disappearing at around E12.5. Such a center-to-pole wave of transient SRY expression (~8 h per cell [Sekido et al. 2004]) appears to be regulated in a tissue-autonomous manner, because a center-to-pole wave of expression is reproducible even in the segment assay using XY genital ridge cultures of three equal (anterior, middle, and posterior) segments along the AP axis (Hiramatsu et al. 2010). Because ectopic SRY expression in the whole gonad region from earlier stages does not allow for ectopic and advanced SOX9 expression within the coelomic epithelium (Kidokoro et al. 2005), it is likely that SRY-positive cells just beneath the coelomic epithelium reflect the initial recruitment of supporting cell progenitors from multi-lineage progenitor cells within the coelomic epithelium

(Karl and Capel 1998). This in turn suggests that the supporting cell progenitors appear to be recruited in a center-to-pole wave-like manner from the coelomic epithelium in developing XY gonads during E11.0–E11.5.

20.2.1.2 Regulatory Cascades to Induce *Sry* Expression in the Supporting Cell Progenitor

It is known that several transcriptional factors, such as FOG2/ZFPM2 (a co-factor of GATA4; Tevosian et al. 2002), sine oculis-related homeobox-1/-4 (SIX1/4; Fujimoto et al. 2013), chromobox 2 (CBX2/M33; Kato-Fukui et al. 2012), and WT1 (+KTS isoform) (Hammes et al. 2001), function as potential regulatory factors to promote *Sry* expression, because these null mutant gonads showed the loss of or a reduction in *Sry* expression, leading to XY sex reversal. Because ectopic SRY expression could rescue the sex-reversal phenotype in *Cbx2*- or *Six1/4*-null mice, CBX2 and SIX1/4 are likely involved in the proper SRY expression in the supporting cells of developing XY gonads (Fujimoto et al. 2013; Kato-Fukui et al. 2012).

GATA4 is required for development during early gonadogenesis (Hu et al. 2013). Recent genetic analyses using *Sf1-cre*;*Gata4*$^{flox/flox}$;*Gata6*$^{flox/flox}$ revealed that GATA4, together with GATA6, regulate proper gonadal somatic differentiation of both XY and XX gonads but does not affect initial sex differentiation (Padua et al. 2014, 2015). Although the XY gonads with *Fog2* (encoding a cofactor for GATA-mediated transcription)-null and *Gata4*$^{ki/ki}$ (lacking the interaction of GATA4 with FOG cofactors) mutations show reduced *Sry* expression and sex reversal (Tevosian et al. 2002; see a review by Tevosian 2014), GATA4 appears to have a crucial function in the differentiation and maintenance of gonadal somatic cells rather than in their sex differentiation. Two types of signaling factors, growth arrest and DNA-damage-inducible 45 gamma (GADD45γ), and mitogen-activated protein kinase kinase kinase 4 (MAP 3 K4) may transiently activate GATA4 by phosphorylation, leading to the establishment and recruitment of supporting cell progenitors at a competent state to induce *Sry* expression (Gierl et al. 2012; Warr et al. 2012; see a review by Carre and Greenfield 2014). Interestingly, insulin/insulin-like growth factor (IGF) signaling is crucial for the proper progression of the differentiation programs of supporting cell progenitors, as gonads missing these receptors, insulin receptor (INSR) and insulin-like growth factor I receptor (IGF1R), show a considerable delay in the onset of *Sry* expression (1~2 days), leading to ovarian differentiation in developing XY gonads (Pitetti et al. 2013). R-spondin homolog 1 (RSPO1) and wingless-type MMTV integration site family 4 (WNT4), which are involved in WNT signaling, are also known to function as positive regulators of cell proliferation of the coelomic epithelium during early gonadogenesis (Chassot et al. 2012). In *Rspo1/Wnt4* double-null XY gonads, the defective recruitment of supporting cell progenitor from the coelomic epithelium appear to partially lead to aberrant testis differentiation (Jeays-Ward et al. 2004; Chassot et al. 2012). Taken together, these signaling factors/pathways may be involved in the proper differentiation and/or

recruitment of the supporting cell progenitors from the coelomic epithelium rather than in defective SRY/SOX9 expression in supporting cells in developing XY gonads.

In addition to the results showing that DNA hypomethylation in the *Sry* promoter regions is stage- and gonad-dependent (Nishino et al. 2004, 2011), it is clear that *Sry* expression is regulated epigenetically by the histone demethylase enzyme, JMJD1A, through the *Sry* linear promoter (Kuroki et al. 2013). The 7.8-kb 5′-flanking regulatory sequences of *Sry* may be involved in a center-to-pole wave-like pattern of *Sry* expression (Albrecht and Eicher 2001; Sekido et al. 2004; see a review by Kanai et al. 2005), but the molecular mechanisms of the *cis*-regulatory core sequences involved in spatiotemporal *Sry* expression remain unclear (Quinn et al. 2014; Larney et al. 2015; also see review by Larney et al. 2014). Moreover, the protein stability of mouse SRY was shown to be regulated through the C-terminal polyglutamine (polyQ) domain (Zhao et al. 2014), in addition to its regulation of nuclear import and export by importin-β, calmodulin, and exportin-4, and so on (Sim et al. 2011, and references in therein).

20.2.2 SRY Actions in Initial Sertoli Cell Differentiation

20.2.2.1 SRY Directly Upregulates SOX9 in pre-Sertoli Cells

SRY directly induces SOX9 expression, and SOX9 plays a central role in Sertoli cell differentiation/maintenance in developing XY gonads. In mouse XY gonads, SRY, in cooperation with NR5A1, directly upregulates the *Sox9* gene through testis-specific enhancer core sequences (TESCO) during the sex-determining period (Sekido and Lovell-Badge 2008) (Fig. 20.2). It is likely that SOX9, together with SOX8 and SOX10 (two SOX-E subfamily members downstream of *Sox9*; Schepers et al. 2003; Polanco et al. 2010), maintains its own gene expression via these enhancer sequences, including TESCO, in a similar autoregulatory manner during the later stages after cessation of SRY expression (Sekido and Lovell-Badge 2008; Mead et al. 2013). In contrast, in developing XX gonads, several ovarian transcription and signaling factors, such as the nuclear receptor subfamily 0, group B, member 1 (NR0B1/DAX1); forkhead box L2 (FOXL2); and RSPO1/WNT4/CTNNB1 (β-catenin) pathways, appear to antagonize *Sox9* expression at least in part by inhibiting SRY/SOX-E/NR5A1 actions via these enhancer sequences (Uhlenhaut et al. 2009; Bernard et al. 2012; Ludbrook et al. 2012) (see Sect. 20.2.4).

Recently, it becomes evident that SRY is able to activate several SOX9 target genes, such as cerebellin 4 precursor protein (*Cbln4*; Bradford et al. 2009), *Sox8*, and *Cyp26b1* (Nicol and Yao 2014). A chromatin immunoprecipitation and whole-genome promoter tiling microarray also demonstrated that SRY and SOX9 recognize numerous common target genes and bind preferentially to the same regulatory sequences at the promoters of their common targets during sex differentiation (Li et al. 2014). Moreover, *Sox8*/*Sox9* double-null fetal Sertoli cells were previously

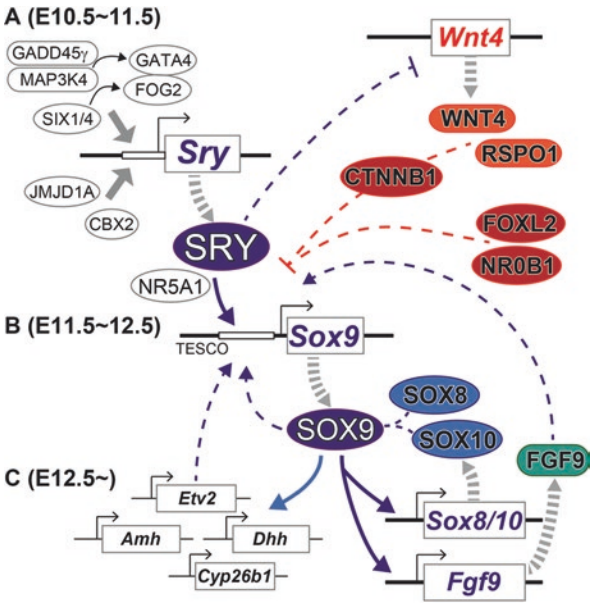

Fig. 20.2 Putative positive and negative feedback regulation of SOX9 expression in Sertoli cell differentiation. SRY expression (~8 h per each cell) is positively regulated by SIX1/4 action and GADD45γ/MAP 3 K4 signals partially through the GATA4/FOG2 action in addition to through epigenetic modification by JMJD1A and CBX2 (**A**). Together with NR5A1, SRY directly upregulates *Sox9* expression through several enhancer sequences, including TESCO (**B**). In addition to other target and downstream genes, *Etv2, Cyp26b1*, *Amh* and *Dhh*, SOX9 induces *Sox8, Sox10* and *Fgf9* in a testis-specific manner. Note that SRY and SOX9 recognize numerous common target genes and bind preferentially to the same regulatory sequences at the promoters of their common targets. After the cessation of SRY actions, SOX9, together with SOX8 and SOX10, contributes to the autoregulatory loops to maintain high-level SOX8/9/10 via the same regulatory sequences (**C**). Moreover, the SOX9-FGF9 positive feedback system appears to maintain the autoregulatory loops of SOX8/9/10 expression. In XX gonads, such regulatory loops appear to be suppressed by ovarian factors CTNNB1, FOXL2, and NR0B1, partially through the SRY-binding regulatory sequences. WNT activation of ovarian genes can be bound and repressed by only SRY, but by not SOX9, in XY gonads

shown to have severe defects in the maintenance of testicular function, in contrast that each single-null mutant displayed normal testis development (Barrionuevo et al. 2009; Georg et al. 2012). Therefore, it is likely that SRY, SOX9, and other SOX-E factors (SOX8 and SOX10) share target genes in Sertoli cells and have redundant functions in the regulation of these genes in stage- and dose-dependent manners throughout the fetal and postnatal stages (Barrionuevo et al. 2009; Polanco et al. 2010; Georg et al. 2012). This is consistent with several recent genetic interaction studies showing testis development in the complete absence of *Sox9* actions, such as *Sox9/Rspo1*- or *Sox9/Ctnnb1* double-null XY mice (Lavery et al. 2012; Nicol and Yao 2015).

20.2.2.2 A Critical Time Window for SRY Action in Developing Gonads

To examine SRY action in gonadal supporting cells, we previously developed a transgenic (Tg) mouse line bearing an inducible *Sry* transgene under control of the *Hsp70.3* promoter, which allows for testis development in XX gonads by a transient heat-shock (HS) treatment at the appropriate stages (Hiramatsu et al. 2009). Using this Tg system, it was shown that the critical time period for SRY action to induce testis development is limited to E11.0–11.25, corresponding to a time window of only 6 h after the normal onset of SRY expression in XY gonads (Fig. 20.3). After this window, SRY can induce SOX9 expression in XX gonads by E11.5–11.75, but its SOX9 expression cannot be maintained in developing XX gonads after cessation of HS-dependent SRY expression, consequently leading to ovarian formation at the later stages. These data clearly indicate the overarching importance of *Sry* action during the initial 6-h phase for the maintenance of SOX9 expression in supporting cells during early-phase gonadal sex differentiation (Hiramatsu et al. 2009). One of the overarching SRY actions is the repression of ovarian WNT4 signaling in developing XX gonads (see Sect. 20.2.4). This is because of the following: (i) the expansion of the critical period of SRY action by E11.5 in the background of *Wnt4* heterozygote mutant (Hiramatsu et al. 2009); and (ii) only SRY, not SOX9, can bind

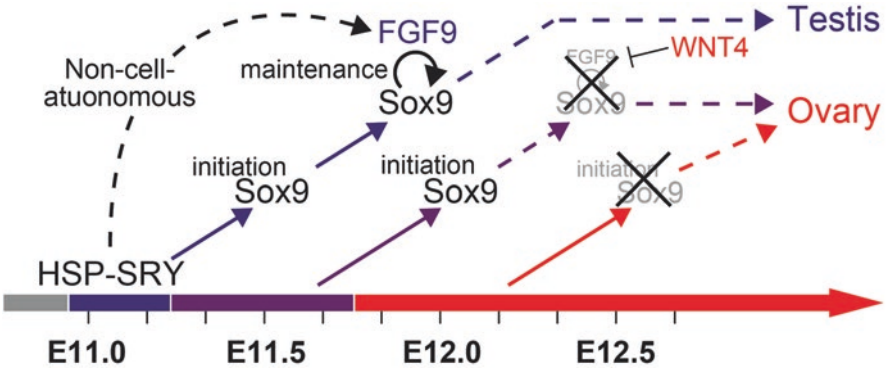

Fig. 20.3 Two distinct critical time windows for SRY actions for the initiation and maintenance of SOX9 expression in developing XX gonads. The horizontal bar represents the bipotential period (blue) and the early (purple) and late (red) ovarian differentiation phases of developing XX gonads. The initiation and maintenance expression patterns of endogenous SOX9 by ectopic SRY induction at each developmental stage are shown (note the data from the experiments using a *HSP-Sry* transgenic mouse line; Hiramatsu et al. 2009). In XX gonads at E11.0–11.25, heat shock (HS)-dependent SRY induction promotes initial SOX9 activation in pre-Sertoli cells, leading to the establishment of a SOX9-FGF9 feedback system in a non-cell autonomous manner. Such XX gonads are fated to have high-level SOX9 expression in Sertoli cells and undergo testis formation at the later stages. In XX gonads at E11.3–11.75, delayed SRY induction is still capable of promoting initial SOX9 activation in XX supporting cells. However, after cessation of HS-dependent transient SRY expression, these cells show defective maintenance of SOX9 expression, resulting in ovarian development at later stages (purple arrows). Beyond E12.0, ectopic SRY cannot induce SOX9 activation in most of the XX gonadal somatic cells (red arrows)

to various ovarian differentiation genes and repress their activation through WNT/β--catenin signaling (Li et al. 2014). Another mechanism is the fibroblast growth factor (FGF) signal-dependent feedback system immediately downstream of SOX9, as described in the next section (see Sect. 20.2.3).

20.2.3 High-Level SOX9 Expression Is Maintained by FGF9 Signals in a Positive Feedback Manner

Transient SRY action initiates *Sox9* expression through its enhancer elements, after which its expression appears to be maintained through the same enhancers, at least in part by the auto-regulatory loop of SOX9 (Sekido and Lovell-Badge 2008). SOX9 induces the expression of *Sox8* and *Sox10*, which also contributes to auto-regulation via the same enhancers (Mead et al. 2013), as described in Sect. 20.2.2.1 (also see Fig. 20.2). A similar regulatory loop with SOX9-target transcription factors was observed in one target gene, *ets variant 2* (*Etv2*; encoding an ETS-domain transcription factor), in Sertoli cells, in which ETV2 promotes *Sox9* expression via positive feedback during fetal and adult stages (DiTacchio et al. 2012).

The diffusible positive feedback signals immediately downstream of SRY/SOX9 were shown to function as another SOX9 maintenance system that is advantageous to the rapid and synchronous switch of testis determination in XY gonads (see a review by Harikae et al. 2013a). One of the positive feedback signals is FGF9, which functions as a maintenance factor for SOX9 expression in differentiating Sertoli cells (Colvin et al. 2001; Kim et al. 2006; Hiramatsu et al. 2010) (Fig. 20.2-B, C). In *Fgf9*-null XY gonads (Colvin et al. 2001) and XY gonads with mutations in *Fgfr2* (encoding main gonadal receptor for FGF9) (Kim et al. 2006; Bagheri-Fam et al. 2008; Siggers et al. 2014), SRY expression and initial SOX9 expression are properly induced in pre-Sertoli cells, but defective maintenance of SOX9 expression (especially in the pole domains) leads to the formation of ovaries or ovotestes at later stages. These results indicated that FGF9 signals promote high-level SOX9 expression via positive feedback, leading to the establishment of Sertoli cells in XY gonads.

As described in Sect. 20.2.1.1, in mouse XY gonads, *Sry* expression starts in the central region of the XY gonad at E11.0 and extends to both the anterior and posterior ends by E11.5 (Fig. 20.4-A). This causes a more than 6-h delay in the onset of SRY expression between the central and anterior/posterior pole regions due to the long and narrow structure extending to a large part of the posterior trunk at E11.0–11.5. Our previous study, conducted by Hiramatsu et al. (2010), employed a reconstruction/partition culture assay using three equal [anterior, middle, and posterior] segments of the XY genital ridge to reveal the following: (i) the central domain of the XY gonad is indispensable for the maintenance of SOX9 expression and subsequent testis cord formation in both anterior and posterior pole domains; (ii) FGF9 is one of the center-derived diffusible factors that engages the high-level SOX9

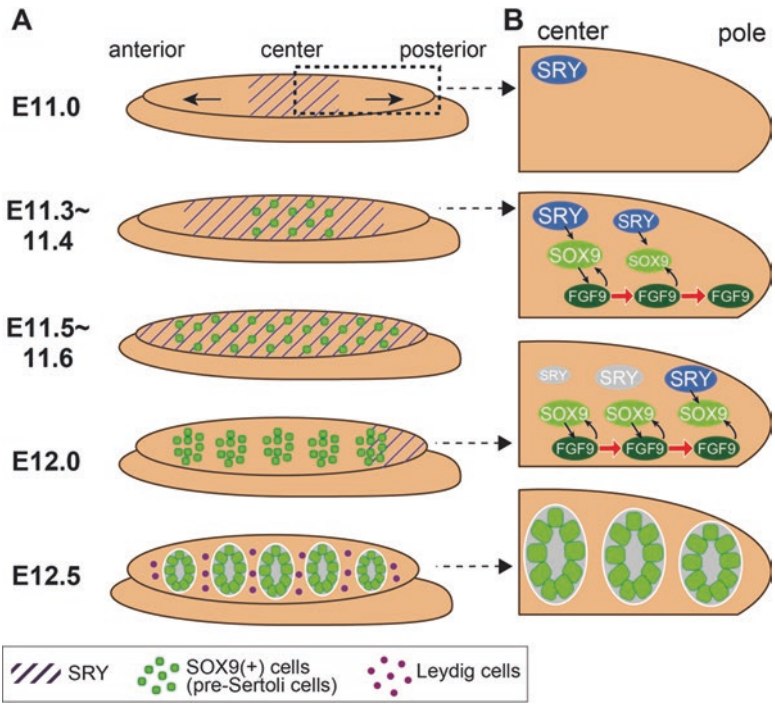

Fig. 20.4 Spatiotemporal expression dynamics of SRY, SOX9, and FGF9 along the anterioposterior (AP) axis of a developing XY gonad. (**A**) Schematic representation showing a center-to-pole wave-like pattern of SRY expression (blue oblique lines) in developing XY gonads during E11.0–12.0 (Bullejos and Koopman 2001; Kidokoro et al. 2005), which leads to the synchronous establishment of SOX9-positive Sertoli cells (green circle) and testis cord formation throughout the whole gonadal area at E12.5. (**B**) The spatiotemporal molecular dynamics of SRY, SOX9, and FGF9 along the AP axis of the developing XY gonads (Hiramatsu et al. 2010). In the central domain, SRY is initially activated at E11.0, which leads to SOX9 expression at E11.2 and FGF9 expression at E11.3–11.4. FGF9 signals maintain high-level SOX9 expression in a positive feedback manner in the center domain. At the same time, FGF9 signals rapidly diffuse toward both anterior and posterior poles (red arrows). A center-to-pole wave-like pattern of SRY expression causes its delayed expression in the pole domain, but the diffused FGF9 signals support the rapid establishment of high-level SOX9 expression, which ensures synchronized testis cord formation throughout the whole gonad

expression in pole domains; (iii) *Fgf9* is activated in the central region immediately after the onset of SOX9 expression in the center; (iv) after this, *Dual-specificity phosphatase-6* (*Dusp6*; a downstream target of FGF signaling) is rapidly upregulated throughout a whole gonadal (surface) area, including both anterior and posterior poles, immediately after the onset of center-restricted *Fgf9* expression; and (v) inhibition of FGF signaling represses the expansion of the SOX9 expression domain toward the poles, leading to typical ovotestis formation (i.e. testicular structure in the central region and ovarian structure in the anterior and posterior ends). Therefore, these findings suggest that, in the central domain, SOX9 induces FGF9 expression,

leading to the establishment of the SOX9–FGF9 feedback loop. Meanwhile, FGF9 appears to rapidly diffuse toward both the anterior and posterior poles, and these signals contribute to the rapid establishment of high-level SOX9 expression in the pole domains (Fig. 20.4-B). Such a feedback system of SOX9 and diffusible FGF9 signals may be crucial for the rapid and synchronous establishment of SOX9-positive Sertoli cells in the whole area of a long and narrow gonadal parenchyma within a very short time (E11.5–12.0). Excluding FGF9 signals, FGF10 (Hiramatsu et al. 2010), prostagrandin D2 (Malki et al. 2005; Wilhelm et al. 2005, 2007), and soluble extracellular matrix components (a niche component for various signaling factors; Matoba et al. 2008) were previously shown to be upregulated immediately downstream of SOX9 and to promote high-level SOX9 expression in a positive feedback manner, suggestive of their potential contribution to the synchronous and rapid establishment of SOX9 expression in the pole domains.

FGF9, together with Activin/Nodal, is known to induce the male-specific expression of RNA-binding protein NANOS2 (a master regulatory factor for male differentiation Suzuki and Saga 2008; Barrios et al. 2010; Bowles et al. 2010; Wu et al. 2013, 2015), leading to male germ cell differentiation/survival in XY gonads (DiNapoli et al. 2006) (see Sect. 20.3.2). Moreover, FGF9 promotes various testis-specific cellular events, such as proliferation of coelomic epithelium and meseonephric migration during E11.5–12.5 (Colvin et al. 2001; Schmahl et al. 2004).

Finally, SOX9-positive pre-Sertoli cells may be transferred into stable DMRT1/SOX9-double positive Sertoli cells, which produce various signals and hormones to induce sex determination in other cell lineages, such as AMH (see references in Shinomura et al. 2014), CYP26B1 (an enzyme that degradates retinoic acids), and DHH (desert hedgehog signals to induce steroidogenic cell differentiation) in stage-dependent and sexually dimorphic manners (Fig. 20.5-B). In addition, it was recently shown that a microRNA (mmu-miR-124) prevents *Sox9* transcription and translation in mouse ovarian cells (Real et al. 2013), and the role of miRNAs in the genetic control of mammalian sex determination requires further study (Wainwright et al. 2013; see a review by Rastetter et al. 2015).

20.2.4 Pre-granulosa Cell Differentiation by FOXL2 Transcriptional Factor and RSPO1/WNT4/CTNNB1 (β-Catenin) Signaling

Mammalian gonadal sex differentiation progresses inside an estrogen-dominant maternal uterus, and ovarian determination/differentiation are believed to thereby undergo a passive program in the absence of SRY or if SRY functioning is impaired. However, several ovarian transcription/signaling factors such as forkhead transcription factor (FOXL2) and R-spondin1 (RSPO1; a secreted WNT agonist)/WNT4/CTNNB1 (β-catenin) have active roles in the determination of pre-granulosa cells in developing XX gonads (Fig. 20.5-C) (also see a review by Nicol and Yao 2014).

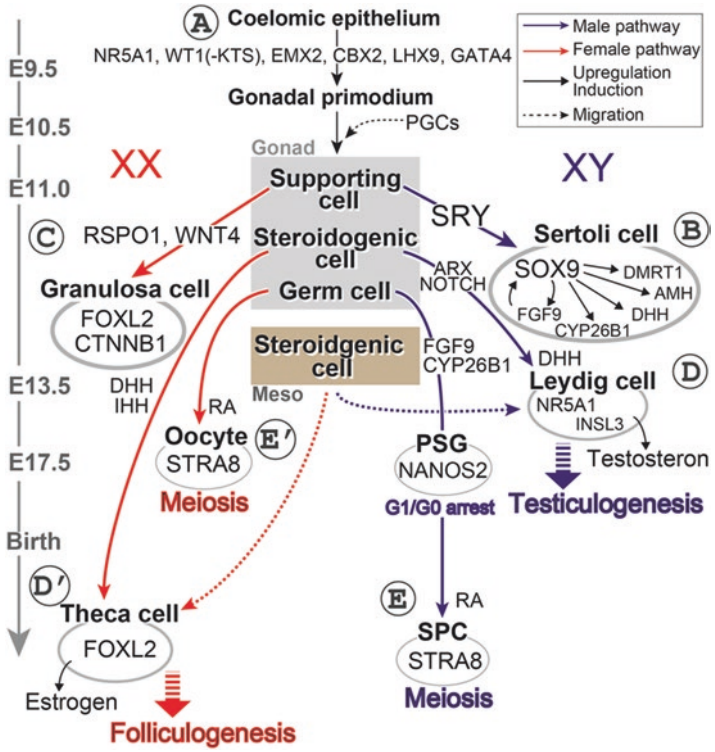

Fig. 20.5 Molecular and cellular cascades of gonadal sex differentiation in mice. Gonadal primordium arises from the thickening coelomic epithelium covering the mesonephric tissues by the actions of WT1, NR5A1, EMX2, CBX2, GATA4, and LHX9 during E9.5–10.5 (**A**). During E11.0–12.0, SRY promotes SOX9 expression in a male-specific manner, leading to pre-Sertoli cell differentiation (**B**). In pre-Sertoli cells, SOX9 induces various testis-specific factors, such as FGF9, cytochrome P450 enzyme (CYP26B1, which degradates retinoic acids), desert hedgehog (DHH), and anti-Mullerian hormone (AMH), leading to the establishment of DMRT1/SOX9-double positive Sertoli cells by E12.5–13.5. In XX supporting cells, forkhead transcription factor (FOXL2) and CTNNB1 (β-catenin), together with RSPO1/WNT4 signals, are activated during E12.0–12.5 in a female-specific manner, leading to pre-granulosa cell differentiation (**C**). Both Leydig cells and theca cells appear to originate from two distinct cell lineages of (i) the gonadal somatic cells, and (ii) the mesonephric perivascular cells; their steroidogenic cell differentiation is temporally regulated by Hedgehog (HH) signals that are secreted by Sertoli cells from E12.5 or by granulosa cells soon after birth (**D**, **D′**). The differentiation and/or maintenance of the steroidogenic cell progenitors are regulated by several other factors, such as aristaless-related homeobox (ARX), NOTCH signals, and so on. In both male and female germ cells, retinoic acid (RA) induces meiotic initiation through the upregulation of STRA8 (a meiosis inducer stimulated by RA) (**E**, **E′**). NANOS2 (a germ cell-specific RNA-binding protein) suppresses STRA8 expression and promotes male germ cell differentiation. *Meso* mesonephros, *PSG pro-spermatogonia*, *RA* retinoic acid, *SPC* spermatocyte

FOXL2 was previously shown to play a crucial central role in pre-granulosa cell differentiation as a potential direct regulator of CYP19A1, an aromatase that synthesizes estrogens (Pannetier et al. 2006 <goat>; Wang et al. 2007 <Nile tilapia>). Several loss- and gain-of-function genetic studies provided direct evidence that goat *FOXL2* functions as a female-determining gene in developing XX gonads (Boulanger et al. 2014 <goat>; and references therein). In mice, FOXL2 is activated primarily in pregranulosa cells from E12.0 in a female-specific manner. Unexpectedly, *Foxl2*-null gonads in mice show proper ovarian differentiation at the morphological level by the perinatal stages, albeit with defective differentiation/maintenance of granulsoa cells after birth (Uda et al. 2004; Ottolenghi et al. 2007; Garcia-Ortiz et al. 2009; Uhlenhaut et al. 2009). These findings suggest that FOXL2 action alone is insufficient for the initial ovarian differentiation/determination in the developing XX gonads of mice. It was also shown that FOXL2, together with estrogen receptor 1[α] (ESR1), negatively regulate *Sox9* expression directly via the TESCO enhancer sequences in mouse embryos (Uhlenhaut et al. 2009) (also see Fig. 20.2), in addition to the repression of NR5A1 expression by FOXL2 (Kashimada et al. 2011; Takasawa et al. 2014).

RSPO1/WNT4/CTNNB1 signals are involved in the proliferation and recruitment of the gonadal somatic cells from the coelomic epithelium in both XY and XX gonads until E12.5 (Chassot et al. 2012). After E12.5, RSPO1/WNT4 signals induce coelomic epithelial proliferation together with recruitment of LGR5 (a RSPO1 receptor)-positive gonadal somatic cells (Mork et al. 2012; Rastetter et al. 2014), leading to ovarian cortex formation by the perinatal stages (see a review by Suzuki et al. 2015). Ectopic expression of a stabilized form of CTNNB1 is sufficient to induce male-to-female sex-reversal in XY gonads (Maatouk et al. 2008). Therefore, it is likely that such RSPO1/WNT4 signals are able to directly repress the positive feedback loop between SOX9 and FGF9 downstream of SRY action (Kim et al. 2006).

Based on recent genetic studies, it was shown that the partial/complete XY sex-reversal phenotypes achieved by deletion of either the *Sox9, Fgf9,* or *Fgfr2* gene are rescued by the additional deletion of *Wnt4*, *Rspo1*, or *Ctnnb1*, in which testis formation occurs in the double *Wnt4/Fgf9*-, *Wnt4/Fgfr2*-, *Rspo1/Sox9*- or *Ctnnb1/Sox9*-null XY gonads (Jameson et al. 2012; Lavery et al. 2012; Nicol and Yao 2015). These data may reflect the absence of redundant factors in the canonical WNT signaling pathway (WNT4, CTNNB1, and RSPO1) in XX gonads, which is in contrast to the redundant function of the same subfamily members (i.e. FGF10 instead of FGF9 and SRY/SOX8/SOX10 instead of SOX9) in developing XY gonads. This suggests that, during the fetal stages, the redundant backup system of FGF/SOX-E may need to continually reinforce the male program under the maternal environment, including feminizing signals/hormones.

Most importantly, in developing XX gonads, the earliest sign of pre-granulsoa cell differentiation is the loss of SRY-dependent SOX9 inducibility (SDSI) during E11.5–12.0 (Harikae et al. 2013b). By monitoring the loss or reacquisition of SDSI in XX gonads of the *Hsp-SRY* mouse line, XX pre-granulosa cells maintain the

SDSI until E11.5, after which most cells rapidly lose this ability in an anterior-to-posterior manner by E12.0, indicating that the loss of SDSI is one of the earliest key events in developing ovaries. Such a loss of SDSI in pre-granulosa cells appears to be cell-autonomously regulated in an independent manner to either FOXL2, WNT, or retinoic acid (RA) actions, suggestive of an additional ovarian-determining or anti-SRY gene that has not yet been identified but is crucial for this initial step of pre-granulosa cell differentiation in mice.

20.3 Regulation of Sex Differentiation in Other Cell Lineages by Sertoli and Granulosa Cells

20.3.1 Hedgehog (HH) Signaling Regulates the Sex-Specific Timing of Steroidogenic Cell Differentiation

Differentiation of fetal Leydig cells is known to be regulated by Hedgehog (HH) (Bitgood et al. 1996), NOTCH (Tang et al. 2008), and PDGF (Brennan et al. 2003; Schmahl et al. 2008) signaling pathways, in addition to Aristaless related homeobox factor (ARX; Miyabayashi et al. 2013), by functioning during the progenitor stages (Fig. 20.5-D). Among these signaling factors, desert Hedgehog (DHH) starts to be expressed in SOX9-positive pre-Sertoli cells from E11.5 in a testis-specific manner (Bitgood et al. 1996), which promotes Leydig cell differentiation in *Patched 1* (*Ptch1*)-expressing precursor cells located outside the testis cords at E12.5~ (Yao et al. 2002). It was also shown that ectopic activation of the HH pathway in XX gonads transformed NR5A1-positive somatic ovarian cells into functional fetal Leydig cells, such as those involved in the secretion of androgens and insulin-like growth factor 3 (INLS3), which are crucial for testis decent and which cause virilization of female embryos and ovarian descent (Barsoum et al. 2009). These findings suggest that HH signals are necessary and sufficient for Leydig cell differentiation soon after testis differentiation.

In XX gonads, the recruitment and differentiation of female steroidogenic cells, internal theca cells, occurs during the initial wave of folliculogenesis soon after birth (Fig. 20.5-D′). Such theca cell differentiation is also induced by DHH and Indian HH (IHH) derived from the granulosa cells of the developing ovarian follicles (Liu et al. 2015). In granulosa cells, both *Dhh* and *Ihh* genes are upregulated by growth differentiation factor 9 (GDF9) (Liu et al. 2015), which is an oocyte-derived TGFβ molecule essential for granulosa cell growth and theca cell recruitment (Elvin et al. 1999). Taken together, these data indicate that HH signals regulate the sex-specific timing of steroidogenic cell differentiation in mouse gonads of both sexes.

In developing gonads, either male or female steroidogenic cells appear to be derived from at least two distinct progenitor lineages: the WT1-positive gonadal somatic cells derived from coelomic epithelium and specialized cells migrated from the mesonephric side (DeFalco et al. 2011; Liu et al. 2015; Miyabayashi et al. 2015).

It remains unclear whether these two distinct lineages may be partially correlated with the distinct molecular and morphological features of fetal and adult Leydig cells, which are replaced after the early postnatal stage (DeFalco et al. 2013; Shima et al. 2013; see references therein). However, the mesonephros-derived steroidogenic cell progenitors appear to be perivascular cells that migrate into the gonadal primordium from the mesonephric side in a stage-dependent and sex-dimorphic manner; such mesonephric migration occurs during E11.5~E12.5 for XY gonads and during the perinatal stages (E17.5~P7) for XX gonads (Fig. 20.5-D, D′). Because this timing of the migration of mesonephric progenitors coincides with the onset of testis cord formation and ovarian folliculogenesis, such mesonephric migration of the steroidogenic progenitors contributes to the morphogenesis of germ cells and supporting cells, leading to proper testiculogenesis and folliculogenesis in both sexes. In addition, mesonephros-derived progenitors appear to be heterogeneous, as adrenal corticotropic hormone (ACTH)-responsive adrenal-like steroidogenic cells were previously shown to be contaminated in mesonephros-derived steroidogenic progenitors (Val et al. 2006), suggestive of a mixture of multilineages of the gonadal steroidogenic cells in mammalian gonads.

20.3.2 Retinoic Acid Regulates Sex-Specific Timing of Meiotic Differentiation in Germ Cell Linages

In XY gonads, male proliferating germ cells enter G1/G0 arrest (i.e. quiescent prospermatogonia; PSG) from E13.5 until the perinatal stages. Pro-spermatogonia restarts proliferation soon after birth, after which some enter meiotic prophase, leading to preleptoten spermatocytes at the early postnatal stages. In XX gonads, the majority of female germ cells enter meiotic prophase from E13.5 in an anterior-to-posterior manner, leading to the formation of oocyte nests surrounded by the ovigerous cords by E17.5 (see review by Suzuki et al. 2015).

Retinoic acid (RA) is a crucial regulator for the meiotic initiation of both male and female germ cells (see reviews by Hogarth and Griswold 2013; Feng et al. 2014) (Fig. 20.5-E, E′). In XY gonads, fetal Sertoli cells produce CYP26B1, a degrading enzyme of RA, and inhibit meiosis by reducing the RA concentration in testes (Bowles et al. 2006; Koubova et al. 2006). RA likely induces the expression of Stimulated by retinoic acid gene 8 (STRA8) (Menke and Page 2002), which is essential for meiotic initiation of both male and female germ cells in fetal ovary and postnatal/adult testes, respectively (Anderson et al. 2008; Endo et al. 2015). Interestingly, *Stra8*-deficient ovarian germ cells do not precede with premeiotic chromosome replication and the subsequent chromosomal events of meiosis, but some surviving oocyte-like cells can synthesize zona pellucida, organize surrounding somatic cells into follicles, undergo asymmetric cell division to produce polar bodies, and cleave to form two-cell embryos upon fertilization (Dokshin et al. 2013). These findings are indicative of the independent contributions of meiosis and

oocyte differentiation to generating a functional egg. This is consistent with previous data showing events indicative of the uncoupling between the mitosis–meiosis decision from the sperm–oocyte decision in *C. elegans* (Morgan et al. 2013 <nematode>). In the teleost fish medaka, FOXL3 was shown to function as a germ cell-intrinsic cue for the egg-fate decision (Nishimura et al. 2015 <medaka>). Further studies on the identification of the *Stra8*-independent pathway that governs oocyte growth and differentiation in mouse ovaries should increase our understanding of sex determination in mammalian germ cells.

NANOS2 is a well-known, evolutionarily conserved RNA-binding protein that is specifically expressed in only male germ cells within the gonads and plays a key role in male germ cell differentiation in mice (see a review by Saga 2010). *Nanos2* is continuously expressed in spermatogonial stem cells (SSCs) after sexual maturation, which functions as an intrinsic factor to maintain the SSC population during spermatogenesis (Suzuki and Saga, 2008; Suzuki et al. 2010; Sada et al. 2009; Zhou et al. 2015). In male germ cells, NANOS2 is required to suppress *Stra8* expression and subsequent meiotic initiation at the fetal stages, and it also promotes male germ cell development independent of meiosis suppression (Saba et al. 2014). Prostaglandin D2 (PGD2) signaling, as well as FGF9 signals (see Sect. 20.2.3), contribute to the proper differentiation of male germ cells at least in part through direct effects on germ cells by upregulating *Nanos2* (Moniot et al. 2014).

It is also known that Notch signals are required for the maintenance of male germ cells in mouse testis development (Spiller et al. 2012; Garcia et al. 2013). Activin/Nodal signals, together with FGF9 (Barrios et al. 2010; Bowles et al. 2010), appear to suppress RA signaling and induce *Nanos2* expression through two intrinsic signals, SMAD2 and p38 (Wu et al. 2013, 2015). This is consistent that signaling through the TGFβ/Activin receptors ALK4/5/7 is required for promoting the differentiation of male germ cells and their entry into mitotic arrest (Miles et al. 2013).

20.4 Sex Reversal in Testes and Ovaries After Perinatal Stages

In various genetically engineered and mutant mice, a sex-reversal phenotype occurs predominantly (or becomes evident) after birth (i.e. the release of estrogen-rich mother's womb). It was previously shown that the XX gonads lacking FOXL2 display normal sex determination and differentiation throughout the fetal stages (Uda et al. 2004). In *Foxl2*-null ovaries without germ cells and *Foxl2/Wnt4* double-null ovaries, the ectopic appearance of SOX9-positive Sertoli–like cells is first detectable after the perinatal stages (Ottolenghi et al. 2007). In *Esr1/Esr2* (estrogen receptor α/β) double-null prepubertal ovaries, the initial transdifferentiation of the follicles into the seminiferous-like tubules with SOX9-positive Sertoli-like cells appears during the first wave of folliculogenesis in the early postnatal stages (Couse et al. 1999; Dupont et al. 2003). In the experimental sex reversal of E13.5 ovaries

grafted into adult male nude mice, the switch from a maternal to a paternal environment leads to transdifferentiation of the grafted ovarian medulla to the testis-like structure with SOX9-positive Sertoli-like cells only from 14 days post-transplantation (corresponding to the stages P7–10; Harikae et al. 2013b). These findings suggest that some specific mechanisms may positively support the maintenance of the femaleness of pre-granulsoa cells, even in the absence of either FOXL2, ESR1/2, or estrogen action in mammalian ovaries during fetal stages.

In *Wnt4*/*Rspo1* single- and double-null ovaries, the transdifferentiation of granulosa cells into Sertoli-like cells occurs around the perinatal stages, at the time of the transition from pre-granulosa cells into a differentiated granulosa cell state (Maatouk et al. 2013; see a review by Chassot et al. 2014). In developing fetal ovaries, FOXL2-positive pre-granulosa cells are mitotically arrested within the ovarian ovigerous cords (in future, follicles) and then start to undergo folliculogenesis at the perinatal stages (Mork et al. 2012; Maatouk et al. 2013; see a review by Suzuki et al. 2015). This implies that pre-granulosa cells may be incapable of upregulating SOX9 expression while mitotically arrested (Maatouk et al. 2013). Taken together, such a silent and dormant state of pre-granulosa cells at the fetal stages may be associated with the maintenance of their femaleness, even in the absence of FOXL2, ESR1/2, and WNT actions by the perinatal stages (Maatouk et al. 2013).

The DM domain transcription factors, such as DMRT1, function as a testis differentiation factor that is evolutionarily conserved among various vertebrate species, in addition to their roles as sex-determining genes, such as *dmrt1* on chicken Z chromosome, *dm-w* in frogs (*Xenopus laevis*), and *dmy* in medaka fish (*Oryzias latipes*). In developing mouse gonads at the early stage of sex determination, *Dmrt1* is expressed in both testes and ovaries at similar levels by E12.5 (Raymond et al. 1999, 2000). In the testes, its expression is continuously maintained at high levels until the adult stage, but it shows rapid downregulation by E14.5 in ovaries. In *Dmrt1*-null XY gonads, testis formation proceeds normally during the fetal stages, but some Sertoli cells show reduced expression of both SOX9 and SOX8 after birth, leading to a partial sex-reversal to FOXL2-positive granulosa-like cells (Matson et al. 2011). Ectopic FOXL2, as well as ectopic ESR1/2 or CTNNB1, efficiently drives male-to-female transdifferentiation in *Dmrt1*-null Sertoli cells (Minkina et al. 2014). Moreover, RA, a crucial regulator of the meiotic initiation of female germ cells (see Sect. 20.3.2), exerts feminizing effects in *Dmrt1*-mutant testes at the postnatal stages via the action of RA receptor alpha (RARα). Therefore, it is possible that at the postnatal and adult stages, DMRT1 actions maintain testis differentiation and development, partially by antagonizing the actions of *Wnt4*/RA/*Foxl2* (Minkina et al. 2014). Moreover, ectopic *Dmrt1* expression in XX ovaries is able to repress the expression of *Foxl2* and *Wnt4* in pre-granulosa cells and to induce transdifferentiation into SOX9-positive Sertoli-like cells, at least from E14.5 (Lindeman et al. 2015; Zhao et al. 2015). Overall, it is likely that, throughout the fetal to adult stages, DMRT1 action plays a crucial role in the maintenance of the maleness of Sertoli cells partially through its continuous suppression of the feminizing actions of FOXL2, ESR1/2, WNT/CTNNB1, and RA signals in addition to its upregulation of SOX9/8 expression in Sertoli cells.

20.5 Conclusions

In this chapter, we reviewed the molecular and cellular mechanisms of the SRY-dependent sex-determination system in mouse embryos and also described the antagonistic interactions between testicular and ovarian factors in late fetal, perinatal, and postnatal mice (see Fig. 20.6). Briefly, in XY gonads, SRY initiates male differentiation by its direct activation of *Sox9* at E11.0–12.0, after which the SOX9–FGF9 positive feedback system, together with the cooperative and redundant actions of their target SOX8/10 factors, leads to the establishment of maleness in the fetal Sertoli cells by E13.5. However, even after the establishment of fetal Sertoli cells,

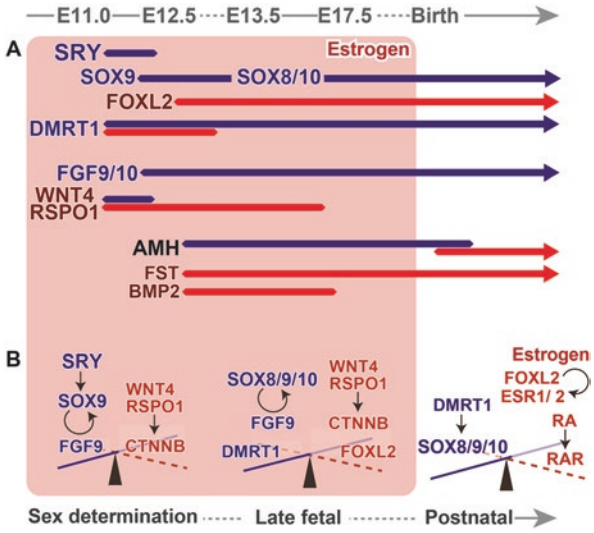

Fig. 20.6 Sex-dimorphic expression profiles and antagonistic roles of various key transcriptional and signaling factors from the early sex determination period to the late-fetal/postnatal stages. (**A**) Schematic representation showing sex-specific and stage-dependent expression profiles of masculinizng (SRY, SOX-E [SOX8, SOX9, SOX10], FGF9, etc.) and feminizing (FOXL2, WNT4, etc.) factors in addition to TGFβ family members (AMH, FST, and BMP2). (**B**) Schematic representation of the seesaw showing antagonistic interactions between testicular and ovarian factors at the sex-determining period (left), at the late fetal stages (middle), and after birth (right). Before the sex-determining period, the seesaw slightly tilted toward the ovarian pathway, in which SRY induces SOX9 expression, leading to the onset of the SOX9–FGF9 positive feedback loop. Such a SOX9–FGF9 system maintains the maleness of Sertoli cells, which is antagonized by RSPO1/WNT4 actions in XX gonads. After cessation of a transient SRY expression, DMRT1, together with SOX8/9/10 autoregulation, antagonizes FOXL2 and RSPO1/WNT4/CTNNB1 actions until late fetal stages. After birth, both male and female pups are released from their estrogen-rich mother's womb, in which their gonads are growing up under the environment of sex hormones produced by its own gonads. At these postnatal and adult stages, DMRT1 action is required for the maintenance of SOX8/9/10 expression in Sertoli cells, whereas FOXL2, ESR1/2, and RA/RARα actions are essential for the maintenance of the femaleness of granulosa cells in ovaries

DMRT1 and SOX9/8/10 continuously function as maintenance factors for the maleness of the supporting cells throughout the postnatal and adult stages, because the loss of either DMRT1 or SOX9/8 action causes a sex-reversal phenotype in postnatal XY testes. In XX gonads without SRY action, the RSPO1/WNT4/CTNNB1 and RA signals, together with FOXL2 action, appear to be activated in a passive manner, leading to differentiation into the pre-granulosa cells with a mitotically silent and static state. During the fetal stages (i.e. under the estrogen-dominant maternal environment), the femaleness of pregranulosa cells can be maintained even in the absence of these feminizing signaling and transcription factors through some specific mechanisms. After birth, pre-granulosa cells become mitotically active by the transient reduction in estrogens, leading to folliculogenesis by granulosa cells. In the postnatal and adult ovaries, ESR1/2 activation by estrogens, which appear to be synthesized through CYP19A1, a potential direct target of FOXL2 in granulosa cells, is essential for the femaleness of granulosa cells. AMH and TGFβ family members may contribute to the maintenance of the supporting cell function in both testes and ovaries in a stage-specific manner.

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Part III
Diversity in the Mechanism of Fertilization

Chapter 21
Modulation of Sperm Motility and Function Prior to Fertilization

Manabu Yoshida and Kaoru Yoshida

Abstract Spermatozoa are generated in the testis but have neither motility nor fertility. They gain these abilities while approaching eggs. During this process, spermatozoa control their motility, swimming direction, and structure to approach their final destination. Previously, these modulations of sperm motility and function in the fertilization process have been investigated mainly in marine animals with external fertilization. Recently, intensive work on mammalian fertilization has partly revealed the molecular mechanisms of sperm events. In this chapter, we first introduce features of sperm events in the process of fertilization, including initiation and activation of flagellar motility, chemotaxis, capacitation, hyperactivation, and the acrosome reaction. We then discuss the molecular mechanisms of these sperm events, specifically focusing on the main characters: Ca^{2+}, cyclic nucleotides, phosphorylation, membrane potential, pH, and lipid components.

Keywords Sperm motility · Capacitation · Acrosome reaction · Calcium · cAMP · pH · Cholesterol

21.1 Introduction

After spermatogenesis and spermiogenesis, sperm cells are still immature, and further modification of the sperm is required to accomplish fertilization. Specifically, mammalian spermatozoa undergo further maturation in the epididymis after

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spermiogenesis in the testis. Mature spermatozoa remain nonmotile in the male body and gain motility through ejaculation or spawning. In many species, spermatozoa are attracted to the egg of the same species by chemoattractants released from eggs or female organs. In the early days, these phenomena were investigated mainly in animals exhibiting external fertilization, such as marine invertebrates and fish. In recent decades, many molecular biological studies on mammalian sperm have revealed molecular mechanisms underlying the fertilization process. In this chapter, we focus on the regulation mechanisms of sperm motility and function prior to fertilization, and we discuss the generality and diversity of the molecular mechanisms.

21.2 Features of Sperm During Fertilization

During the process of fertilization, spermatozoa show initiation and/or activation of their motility, chemotactic behavior toward eggs/female organs, capacitation of fertility, hyperactivation of their motility, and the acrosome reaction (see Fig. 21.1). Some of these phenomena are controlled by female factors, and some are inhibited by male factors. These modulations of sperm function seem to lead to successful fertilization. First, we introduce features of such events in sperm behavior and function in the process of fertilization.

21.2.1 Initiation and Activation of Sperm Motility

The initial event for the sperm is the initiation and activation of their motility. Since the energy for movement contained in the sperm is limited, sperm motility is suppressed in the male body and is activated when sperm are ejaculated/spawned.

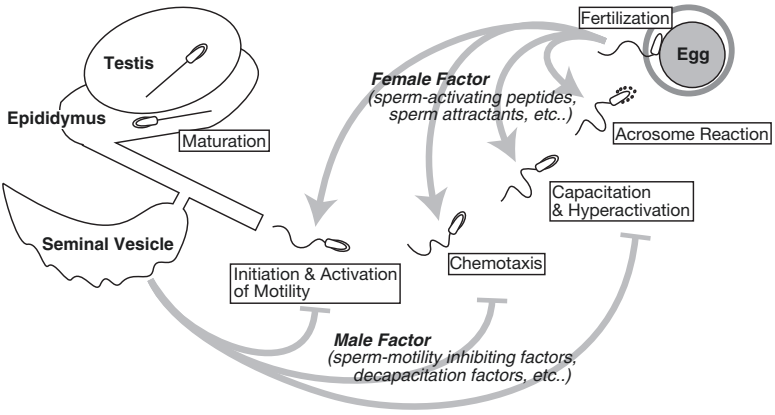

Fig. 21.1 Schema of sperm events during the process of fertilization

There are various triggers of sperm activation. In many animals exhibiting external fertilization, changes in the media surrounding the sperm trigger sperm motility (see review Morisawa 1994). One important factor in sperm activation is a change in osmolality. In general, osmolarity in the vertebrate body is roughly 250–400 mOsm/L, whereas the osmolarities of freshwater and seawater are about 0 and 1000 mOsm/L, respectively (Gllles and Delpire 2011). Thus, when the spermatozoa of many vertebrate species exhibiting external fertilization (such as teleosts and amphibians) meet such a dynamic osmotic change, they use the changes in ambient conditions as a trigger for initiating sperm motility: sperm motility of marine teleosts is induced by hypertonic shock, and that of freshwater teleosts and frogs is induced by hypotonic shock (Morisawa 1994) (Movie 21.1). The sperm of tilapia, which can reproduce in both freshwater and seawater, display interesting phenomena. The sperm of freshwater-acclimated tilapia show motility only in hypotonic conditions, but the sperm of seawater-acclimated tilapia can move in both hypertonic and hypotonic conditions; an increase in the intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ from external environments seems to mediate sperm motility in hypertonic conditions such as seawater (Morita et al. 2004). On the other hand, salmonid sperm do not use osmolality for controlling sperm motility. The seminal plasma of salmonid fish contains a high concentration of K^+, and a decrease in K^+ around the sperm initiates sperm motility (Morisawa and Suzuki 1980).

The osmolality in the invertebrate body is almost the same as that in the external conditions; thus, invertebrate sperm cannot use osmotic changes for sperm activation. Instead, the sperm of some species are activated by a female factor released from eggs or egg accessory cells. In corals and ascidians the sperm-activating factors released from the eggs have been identified as unsaturated fatty alcohols (Coll et al. 1990) and sulfate-conjugated hydroxysteroids (Yoshida et al. 2002; Matsumori et al. 2013), respectively. On the other hand, the motility of horseshoe crab sperm is initiated by a peptide released from the egg (Clapper and Brown 1980; Clapper and Epel 1982). Echinoderm spermatozoa initiate their motility in seawater; nevertheless the egg jelly of a sea urchin contains substances that activate sperm respiration and motility in acidic conditions (Ohtake 1976), and sperm-activating peptides (SAPs) have been identified in egg jelly from sea urchins and starfish (Suzuki 1990; Nishigaki et al. 1996). Even in vertebrates, sperm motility–initiating proteins have been found and identified in some species, such as newts (Watanabe et al. 2010) and herring (Pillai et al. 1993; Oda et al. 1998). Some sperm-activating factors also have sperm-attracting activity.

The triggers of motility in mammalian sperm are not obvious. Sperm collected from the cauda epididymis show flagellar beating, although it is slow. In these cases, the sperm become active after ejaculation in the female reproductive tract. Interestingly, primate semen, including human semen, is clotted by seminal vesicle secretion proteins, and sperm motility is physically suppressed. The seminal clot is liquefied by prostate-specific antigen (PSA), and the sperm then begin to move vigorously (Robert and Gagnon 1999).

21.2.2 Sperm Chemotaxis

In many animals, the eggs or female reproductive organs release sperm attractants, and sperm are attracted toward the egg. Sperm chemotaxis is obviously seen in marine invertebrates—such as jellyfish, echinoderms, and ascidians—and has been seen in internal fertilizers such as mammals (see review Yoshida and Yoshida 2011). Several sperm chemoattractants have been identified in echinoderms (Ward et al. 1985; Böhmer et al. 2005; Guerrero et al. 2010), ascidians (Yoshida et al. 2002; Matsumori et al. 2013), mollusks (Zatylny et al. 2002; Riffell et al. 2002; Hirohashi et al. 2013), corals (Coll et al. 1994), and a frog (Olson et al. 2001). Among mammals, many candidates for sperm chemoattractants have been proposed (Iqbal et al. 1980; Zamir et al. 1993; Villanueva-Diaz et al. 1995; Spehr et al. 2003; Fukuda et al. 2004), but progesterone has recently been suggested as the native chemoattractant (Guidobaldi et al. 2008; Oren-Benaroya et al. 2008; Teves et al. 2009; Lishko et al. 2011; Strünker et al. 2011). Sperm chemotaxis in many marine invertebrates is a species- or genus-specific phenomenon (Miller 1979, 1982, 1985; Yoshida et al. 2013). Thus, sperm chemotaxis may prevent crossbreeding. Little is known about the molecular basis of species specificity of sperm chemotaxis. In the case of ascidians, since the molecular structures of sperm attractants in related species are alike (Matsumori et al. 2013), the specificity of the sperm chemotaxis may be derived from the evolution of a series of enzymes involved in chemoattractant synthesis.

Sperm behavior during chemotaxis has been precisely observed in several marine invertebrate species. In the absence of sperm attractants, sperm usually show circular movement with low asymmetric flagellar movement on the surface of a glass slide (Yoshida and Yoshida 2011). In the presence of sperm attractants, sperm show quick turning movements; the swimming path curvature suddenly increases and the flagellar movement becomes highly asymmetric (see review Yoshida and Yoshida 2011) (Fig. 21.2). The changes in flagellar beating are mediated by transient Ca^{2+} increases in the sperm (see Sect. 21.3.1) and alter the swimming direction of the sperm, resulting in their approach toward the eggs.

21.2.3 Capacitation, Decapacitation, and Hyperactivation

Mammalian spermatozoa have no fertilizing capability immediately after ejaculation; they acquire this ability while in the female reproductive tract. This phenomenon is called capacitation. The capacitated spermatozoa begin to show hyperactivation and are able to undergo the acrosome reaction (see Sect. 21.2.4). Despite being initially described in 1951 (Chang 1951; Austin 1952), the capacitation process is still not fully understood. However, it is known to involve a series of changes in the sperm, including reconstitution of the plasma membrane, increases in intracellular HCO_3^- concentrations and pH, activation of second messenger

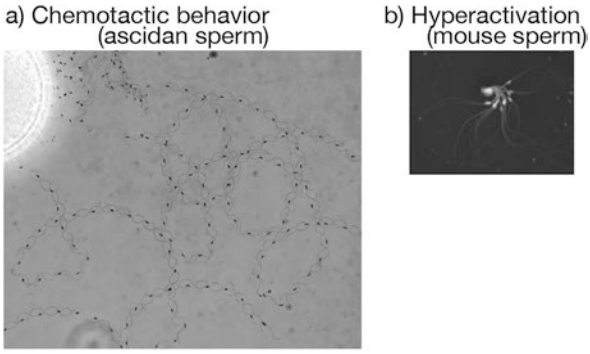

Fig. 21.2 Behavior of sperm during sperm events. (**a**) Superimposed image of the sperm of the ascidian *Ciona intestinalis* exhibiting a chemotactic response. The egg without accessory cells is placed in the *upper left area*. A movie of the sperm chemotaxis is available online on YouTube (https://youtu.be/7iqk0eQnfJQ). (**b**) Superimposed image of the hyperactivated mouse sperm

systems, and a rise in $[Ca^{2+}]_i$ (see reviews Visconti et al. 2011; Liu et al. 2012; Nishigaki et al. 2014). The activation of second messenger systems results in tyrosine phosphorylation of many sperm proteins. The necessity for cyclic adenosine 3′,5′-monophosphate (cAMP)–dependent protein tyrosine phosphorylation, especially in the sperm tail, is related to the acquisition of hyperactivated sperm motility in various species and is considered a marker of some essential elements of the capacitation process. However, the physiological inducer of this process is not clear.

While sperm capacitation is essential for mammalian fertilization, in vitro capacitated spermatozoa reversibly lose their fertilizing ability when treated with seminal plasma in several species (Chang 1957; Bedford and Chang 1962). This phenomenon is called decapacitation, and various studies have found several decapacitation factors in seminal plasma (Dukelow et al. 1966; Reyes et al. 1975; Davis 1974; Huang et al. 1999; Lu et al. 2011; Tseng et al. 2013; Kawano and Yoshida 2007). Among them, seminal vesicle secretion 2 (SVS2) is indispensable for fertilization in vivo (Kawano et al. 2014). Since SVS2 prevents cholesterol efflux from the plasma membrane of mouse sperm, it seems to maintain sperm in an incapacitated state in the uterus and unlocks sperm capacitation in the oviduct (Araki et al. 2015). The SVS2 locus is conserved in many mammals. The homologous proteins in humans are semenogelin I and II (SEMG1 and SEMG2), which block sperm capacitation triggered by various stimuli, via inhibition of superoxide anion and nitric oxide generation (de Lamirande et al. 2001; de Lamirande and Lamothe 2010).

Hyperactivation of sperm movement is observed in mammalian species, coupled with capacitation. During the hyperactivation process, the frequency of the flagella beating is markedly decreased and the amplitude of the flagellar beating is markedly increased (Fig. 21.2). The characteristic movement of the hyperactivated spermatozoa produces a slowly oscillating transverse force, perpendicular to the longitudinal sperm head axis (Ishijima 2011), which is believed to be most effective for sperm penetration through the zona pellucida (Boatman and Robbins 1991; Yanagimachi 1994).

21.2.4 Acrosome Reaction

The final sperm event prior to fertilization is the acrosome reaction: exocytosis of the acrosomal vesicle. The acrosomal vesicle, which is derived from the Golgi apparatus during spermiogenesis, is located at the anterior of the sperm head and is exocytosed when the sperm approaches the egg. The acrosomal vesicle contains factors that facilitate the penetration of the vitelline coat, and the inner membrane of the acrosomal vesicle contains the essential molecules required for sperm–egg fusion. Specific information regarding the acrosome reaction can be found in Chap. 22 of this book.

21.3 Regulating Mechanisms

As described in Sect. 21.2, sperm events—including initiation of motility, chemotactic behavior, capacitation, hyperactivation, and the acrosome reaction—are triggered by different stimulation and at different times. Furthermore, the events are triggered by highly diverse systems in animal species. However, the different events in many animals use common molecular systems: Ca^{2+} signaling, cyclic nucleotides, changes in membrane potential, protein phosphorylation, etc. In recent decades, intensive research in mammalian species, using molecular biological and genome editing techniques, has unveiled the molecular mechanisms underlying sperm events. It is now known that the regulating mechanisms of some events are almost the same in mammals and other animals, including marine invertebrates. In this section, we look at the most important systems and discuss the generality and diversity of the systems.

21.3.1 Ca^{2+}: An Important Signaling Molecule for Regulating Sperm Function

21.3.1.1 Ca^{2+} Influx and CatSper: The Sperm-Specific Ca^{2+} Channel Plays a Crucial Role in Mammalian Sperm

The most important factor for regulating sperm function is $[Ca^{2+}]_i$. Changes in $[Ca^{2+}]_i$ in sperm cells have been observed in activation of sperm motility, chemotactic behavior, hyperactivation, and the acrosome reaction (see reviews Morisawa 1994; Darszon et al. 2005; Publicover et al. 2007; Yoshida and Yoshida 2011; Lishko et al. 2012; Correia et al. 2015) (Fig. 21.3). One of the important systems for mediating $[Ca^{2+}]_i$ increase is the influx of Ca^{2+} from the outside of cells via Ca^{2+} channels on the plasma membrane. In sperm, many types of Ca^{2+} channels are found—the conventional voltage-gated Ca^{2+} channels (Ca_v), CatSper, transient receptor

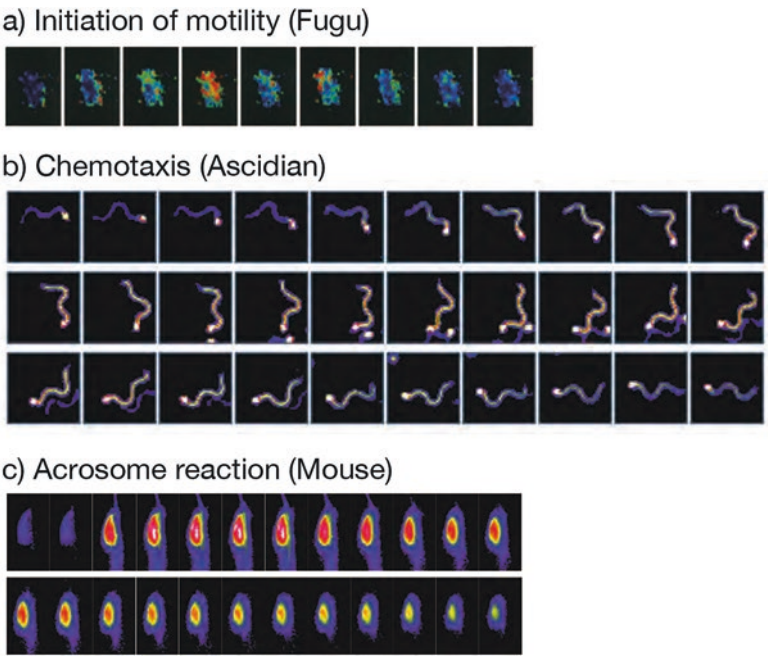

Fig. 21.3 Various Ca^{2+} responses in sperm events. Ca^{2+} changes are measured by using the fluorescent probes fura-2 (**a**) and fluo-4 (**b** and **c**), and are visualized as pseudocolor images. (**a**) Motility initiation of teleost sperm (*Takifugu niphobles*) (Image courtesy of Dr. Masaaki Morisawa). (**b**) Chemotactic response of ascidian (*Ciona intestinalis*) sperm. (**c**) Acrosome reaction of mouse sperm

potential (TRP) channels, and cyclic nucleotide–gated channels—and the channels have classically been considered to be involved in sperm function (see review Darszon et al. 2005). In these channels, a sperm-specific Ca^{2+} channel, CatSper, plays a crucial role in regulating sperm function. CatSper was initially identified as a Ca_v-like protein with six transmembrane regions (Ren et al. 2001), and it localizes four linear domains along the principal portion of the flagellum (Ren et al. 2001; Chung et al. 2014). The CatSper channel consists of four pore-forming subunits (CatSper1–4) and three auxiliary subunits (CatSperβ, γ, δ); sperm of mice lacking any of the CatSper1–4 or δ genes do not undergo hyperactivation, and this results in male infertility (Carlson et al. 2003; Qi et al. 2007; Quill et al. 2001; Chung et al. 2011). CatSper is activated by alkalization (Kirichok et al. 2006) and by ligands such as progesterone; progesterone and other low molecular weight compounds seem to activate CatSper directly in human sperm (Lishko et al. 2011; Strünker et al. 2011). Since, in mammalian sperm, progesterone is a key molecule regulating sperm chemotaxis (Ralt et al. 1991; Villanueva-Diaz et al. 1995; Teves et al. 2006) and the acrosome reaction (Wistrom and Meizel 1993; Roldan et al. 1994), CatSper is also considered to be involved in these phenomena (Strünker et al. 2011).

Additionally, since chemotaxis of sea urchin sperm also seems to be mediated by CatSper (Seifert et al. 2015), CatSper might be the universal key channel for sperm chemotaxis. However, mouse CatSper is insensitive to progesterone, although progesterone can induce the acrosome reaction in mouse sperm (Lishko et al. 2011; Strünker et al. 2011). Furthermore, CatSper seems to be missing in all protostomes and some deuterostomes, including teleosts, amphibians, and birds (Cai and Clapham 2008).

There is controversy as to whether Ca^{2+} channels other than CatSper are involved in regulation of sperm function. As described above, many types of Ca^{2+} channels found in sperm are thought to be involved in sperm function; however, the function of Ca^{2+} channels other than CatSper in mature mammalian sperm has been questioned (Lishko et al. 2012). In fact, alkalization- and voltage-activated current are not observed in the sperm of CatSper1 and Slo3 double-knockout (double-KO) mice on patch clamp recordings (Zeng et al. 2013), and mice lacking the T-type voltage-gated channels $Ca_v3.1$ and $Ca_v3.2$, which are supposed to be expressed in male germ cells, show normal fertility and sperm function, even though $Ca_v3.2$ participates in Ca^{2+} influx (Stamboulian et al. 2004; Escoffier et al. 2007). These results suggest that CatSper is the only Ca^{2+} channel functioning in mouse sperm. On the other hand, mouse sperm lacking another voltage-gated channel, $Ca_v2.3$, show abnormal Ca^{2+} responses, a reduced acrosome reaction, and a subfertile phenotype (Cohen et al. 2014). Furthermore, TRP channels are thought to be involved in the function of the sperm (Darszon et al. 2012) (see Sect. 21.3.1.3). Precise functional analyses should be carried out in order to resolve these issues.

21.3.1.2 Ca^{2+} Release from Internal Stores

Another important system for regulating $[Ca^{2+}]_i$ is Ca^{2+} release from internal Ca^{2+} stores. Generally, Ca^{2+} is stored in cells in the endoplasmic reticulum, and the release of Ca^{2+} from internal stores is mediated by the Ca^{2+} channels, inositol 1,4,5-trisphosphate (IP_3) receptor, and ryanodine receptor (Berridge 1993). However, there is no organized endoplasmic reticulum in sperm cells. Instead, sperm use the acrosomal vesicle (Herrick et al. 2005) and the redundant nuclear envelope (RNE) at the sperm neck (Ho and Suarez 2001, 2003) as the internal Ca^{2+} stores. In fact, the IP_3 receptor is located on both the acrosomal vesicle and the RNE in mammalian spermatozoa (Walensky and Snyder 1995; Ho and Suarez 2001, 2003). Of the two internal Ca^{2+} stores, the acrosomal vesicle seems to be related to the acrosome reaction (Herrick et al. 2005), and the RNE seems to be involved in hyperactivation (Ho and Suarez 2001). Interestingly, activation of Ca^{2+} release from the RNE by thimerosal partly induces hyperactivation in CatSper1-/CatSper2-KO mouse sperm (Marquez et al. 2007) and in CatSper-blocked human sperm (Alasmari et al. 2013), suggesting that Ca^{2+} release from

the RNE occurs downstream from CatSper-mediated Ca^{2+} influx (see review Correia et al. 2015 for more detailed information). Usually, the IP_3 receptor is activated by the production of IP_3 by phospholipase C (PLC), which is the enzyme responsible for catalyzing the cleavage of phosphatidylinositol 4,5-bisphosphate to IP_3 and diacylglycerol. Moreover, PLCδ4 is essential for the acrosome reaction and $[Ca^{2+}]_i$ changes in the sperm (Fukami et al. 2001, 2003), suggesting that activation of PLCδ4 generates IP_3 and induces Ca^{2+} release from internal Ca^{2+} stores during the acrosome reaction (Rossato et al. 2001; Fukami et al. 2003). On the other hand, there is no evidence for the generation of IP_3 during hyperactivation and capacitation; the sperm of PLCδ4-KO mice show normal capacitation and motility (Fukami et al. 2003). The substitution model for inducing Ca^{2+} release is that the IP_3 receptor on the RNE is activated by a CatSper-mediated Ca^{2+} increase (Alasmari et al. 2013).

Internal Ca^{2+} stores in the sperm of nonmammalian animals are obscure; there is no evidence for the existence of stored internal Ca^{2+} in any electron microscopy observations.

21.3.1.3 Capacitative Ca^{2+} Entry

Related to Ca^{2+} release from the internal store, capacitative Ca^{2+} entry (CCE) also mediates sperm function, although the Ca^{2+} channel that mediates CCE in the sperm is still unknown. Generally, CCE is observed when internal Ca^{2+} stores are depleted, i.e., CCE is activated when IP_3-induced Ca^{2+} release occurs (Putney et al. 2001). The Ca^{2+} release–activated Orai Ca^{2+} channels and the Stim calcium sensor proteins mediate CCE in somatic cells (Prakriya et al. 2006; Yeromin et al. 2006), and they are also present in mammalian sperm, though their function in the sperm is unclear (Costello et al. 2009; Lefievre et al. 2012; Darszon et al. 2012). On the other hand, CCE ultimately induces the acrosome reaction (Patrat et al. 2000; Florman 1994; O'Toole et al. 2000; Barratt and Publicover 2001), and the TRP "canonical" (TRPC) channels—other channels involved in CCE (Liao et al. 2008, 2009; Cahalan 2009)—seem to mediate the phenomenon (Jungnickel et al. 2001; Treviño et al. 2001; Castellano et al. 2003; Darszon et al. 2012). In ascidian sperm, CCE seems to mediate sperm chemotaxis, although the presence of TRP and/or Orai on the sperm is still not clear (Yoshida et al. 2003). Furthermore, TRPC channels also exist in sea urchin sperm (Darszon et al. 2012). Thus, TRPC channels may be one of the universal and important channels for mediating sperm functions. However, definitive evidence that TRP channels really mediate $[Ca^{2+}]_i$ changes and sperm function has not been reported. Precise experiments on sperm function and $[Ca^{2+}]_i$ changes, using gene-modified animals, should elucidate the role of CCE in sperm functions.

21.3.2 Cyclic Nucleotides and Protein Phosphorylation: Regulation of Sperm Motility and Capacitation

21.3.2.1 cGMP-Dependent Sperm Activation and Chemotaxis in the Sea Urchin

Cyclic nucleotides are other important signaling molecules in cells, as well as in sperm. Participation of cyclic guanosine 3′,5′-monophosphate (cGMP) in sperm motility is well known in the sea urchin. The sea urchin eggs release many SAPs from their jelly layer (Suzuki 1990), and SAPs induce the generation of cGMP in the sperm (Bentley et al. 1986; Kaupp et al. 2003). Furthermore, some SAPs also act as sperm attractants (Ward et al. 1985; Guerrero et al. 2010). The receptor of resact—the SAP chemoattractant from the sea urchin *Arbacia punctulata* (Ward et al. 1985)—is a membrane-type guanylyl cyclase, which is the enzyme catalyst in the formation of cGMP, thus interaction of resact and its receptor directly results in production of cGMP (Shimomura et al. 1986; Singh et al. 1988). On the other hand, the receptor of speract—another common SAP in sea urchins, including *Strongylocentrotus purpuratus* and *Hemicentrotus pulcherrimus* (Hansbrough and Garbers 1981; Suzuki 1990)—is not a guanylyl cyclase (Dangott and Garbers 1984; Dangott et al. 1989), though it also activates guanylyl cyclase (Bentley et al. 1986). The speract receptor may be associated with a guanylyl cyclase (Bentley et al. 1988); however, they are not colocalized in the same lipid raft fraction (Ohta et al. 2000). Activation and chemotaxis of starfish sperm is also mediated by cGMP; asterosaps SAPs and chemoattractants of the starfish *Asterias amurensis* (Nishigaki et al. 1996; Böhmer et al. 2005) also induce generation of cGMP in sperm, and the receptor of asterosaps is also a guanylyl cyclase (Nishigaki et al. 2000; Matsumoto et al. 2003). On the other hand, the involvement of cGMP in regulating sperm function is not known in species other than echinoderms.

cGMP induced by SAPs induces an increase in $[Ca^{2+}]_i$ in sea urchin sperm (Kaupp et al. 2003). cGMP opens potassium-selective cyclic nucleotide–gated (KCNG) channels, resulting in hyperpolarization (Babcock et al. 1992; Strünker et al. 2006; Galindo et al. 2007), followed by an increase in intracellular pH (pH_i; cytosol alkalization) (Nishigaki et al. 2001; Seifert et al. 2015). The increase in pH_i may trigger CatSper, resulting in a $[Ca^{2+}]_i$ increase, as described in Sect. 21.3.1.1 (Seifert et al. 2015).

21.3.2.2 cAMP and cAMP-Dependent Phosphorylation

cAMP is a key molecule for regulating sperm motility (see review Morisawa 1994; Morisawa and Yoshida 2005). The trigger for activating sperm motility immediately induces a transient increase in cAMP in the sperm of teleosts (Morisawa and Ishida 1987) and ascidians (Yoshida et al. 1994). The medium inducing capacitation also induces a progressive rise in cAMP in mammalian sperm (Okamura et al. 1985; White and Aitken 1989).

Classically, adenylyl cyclase, which catalyzes the formation of cAMP, is a protein with 12 transmembrane regions (tmAC), and its activity is regulated by trimetric G proteins. However, the presence and function of tmAC in sperm is in dispute, especially in mammals; some papers have shown the presence and effects of tmAC in sperm (Leclerc and Kopf 1999; Baxendale and Fraser 2003; Spehr et al. 2004; Livera et al. 2005; Wertheimer et al. 2013), while other studies have suggested that trimetric G proteins and tmAC are not involved in sperm function (Strünker et al. 2011; Brenker et al. 2012). The sperm of nonmammalian species seem to use tmAC (O'Brien et al. 2011; Shiba and Inaba 2014), which may partly play a role in sperm regulation.

In contrast, soluble adenylyl cyclase (sAC)—an atypical adenylyl cyclase identified in 1999 (Buck et al. 1999)—has an important role in sperm function, especially in mammals. sAC has no transmembrane region; it is activated by bicarbonate ions (HCO_3^-) and its activity is modulated by Ca^{2+} (Tresguerres et al. 2011). In mammals, HCO_3^- in the medium surrounding the sperm increases after ejaculation and is beneficial for capacitation and hyperactivation (Yanagimachi 1994; Tresguerres et al. 2011). Increases in HCO_3^- and Ca^{2+} result in production of cAMP (Okamura et al. 1985) and thus activation of sAC (Tresguerres et al. 2011).

In most cases, cAMP conveys phosphorylation of proteins catalyzed by protein kinase A (PKA). Moreover, PKA-dependent phosphorylation of sperm proteins is observed in several species (see reviews Tash and Means 1983; Morisawa 1994; Yanagimachi 1994; Morisawa and Yoshida 2005; Signorelli et al. 2012). In salmonid fish, several proteins are cAMP-dependently phosphorylated, and the 15 kDa protein localized in the basal part of the flagellum and the 22 kDa Tctex2-related dynein light chain are closely related to the initiation of sperm motility (Morisawa and Hayashi 1985; Jin et al. 1994; Inaba et al. 1999). In the sperm of the ascidian *Ciona intestinalis*, the 21 kDa dynein light chain Tctex2 and the 26 kDa axonemal protein are phosphorylated PKA dependently during the activation of sperm motility by sperm-activating and -attracting factor (SAAF) (Nomura et al. 2000). Furthermore, PKA-dependent phosphorylation of many proteins is observed during capacitation of mammalian sperm (Visconti et al. 1995). Interestingly, in many cases, PKA promotes tyrosine phosphorylation of sperm proteins such as A-kinase anchoring protein 4 (AKAP4), AKAP3, and calcium-binding tyrosine phosphorylation–regulated protein (CABYR) (Hayashi et al. 1987; Visconti et al. 1995), although PKA itself is a serine/threonine kinase. Thus, PKA must activate some tyrosine kinase in the sperm. Some studies have shown that c-Src, and the src family kinases Csk and c-Abl, are candidates for the PKA-activated tyrosine kinases in mouse and human sperm (Baker et al. 2006; Lawson et al. 2008; Mitchell et al. 2008; Baker et al. 2009). These studies also showed that Src inhibitors blocked capacitation-induced tyrosine phosphorylation. However, capacitation-induced tyrosine phosphorylation in c-Src-KO mouse sperm is the same as that in wild-type mouse sperm (Krapf et al. 2010), and c-Src does not affect capacitation-induced tyrosine phosphorylation (Stival et al. 2015). Thus, c-Src seems to be involved in capacitation of mammalian sperm but does not lie downstream from PKA activity (Visconti et al. 2011). The role of c-Src was proposed as suppression of a serine/

threonine phosphatase (Krapf et al. 2010) and/or activation of the Slo3 K^+ channel (Stival et al. 2015). At any rate, the identity of the assumed tyrosine kinase that is PKA-dependently activated during sperm capacitation is still unclear. Furthermore, the identity of the PKA-dependent tyrosine kinase in the sperm of nonmammalian animals is completely unknown.

Capacitation-associated tyrosine phosphorylation in mammalian sperm has been well investigated (Visconti et al. 2011). Recently, a proteomic study revealed 62 pY sites on 45 proteins in capacitated mouse sperm, including AKAP4 and AKAP3 (Chung et al. 2014). Interestingly, phosphorylation is potentiated in CatSper-KO sperm, suggesting that CatSper restricts the site of phosphorylation.

21.3.3 Membrane Potential and pH: Key Events Regulating Intracellular Ca^{2+} and Modulating Sperm Motility

21.3.3.1 Regulation of pH

Although they are not molecules, changes in membrane potential and pH are key events for controlling sperm functions. An increase in pH is observed in initiation and activation of motility, chemotaxis, capacitation, hyperactivation, and the acrosome reaction (Morisawa 1994; Darszon et al. 2008; Lishko et al. 2012). Intracellular pH is affected according to environmental pH. Since adenosine triphosphatase (ATPase) activity of flagellar dynein depends on pH (Gibbons and Fronk 1972), sperm motility is inhibited in lower-pH conditions. In sea urchins, pH in the testes was kept lower and resulted in sperm quiescence; spawning them into seawater induced alkalization in the sperm and triggered sperm motility (Johnson et al. 1983; Lee et al. 1983). In contrast to observations in sea urchin sperm, environmental pH has little effect on the activation of teleost sperm motility (Alavi and Cosson 2005).

Changes in intracellular pH are mainly controlled by intracellular signaling. As described in Sect. 21.3.1.1, an increase in pH (alkalization) induces gating of the CatSper channel (Kirichok et al. 2006). Furthermore, a pH increase induces the pH-sensitive K^+ current in sperm (KSper) (Navarro et al. 2007). Thus, a change in pH directly modulates Ca^{2+} concentration and membrane potential in mouse sperm. Although regulation of pH in the sperm is not completely elucidated, voltage-dependent H^+ channels (H_v) and/or Na^+/H^+ exchangers (NHE) seem to be involved in pH regulation. Gating of H_v is regulated by membrane potential and pH gradients; if the intracellular condition is acidic, H_v is gated without depolarization (Sasaki et al. 2006; Ramsey et al. 2006). Moreover, an increase in pH in human sperm seems to be mediated by H_v1 (Lishko et al. 2010). However, mouse spermatozoa do not possess H_v1, and an H_v1 current is not observed in other mammalian spermatozoa (Miller et al. 2015). On the other hand, male mice lacking sperm-specific NHE (sNHE) are completely infertile because of dysfunction of sperm motility (Wang et al. 2003). Further study has revealed that sAC is missing in sNHE-KO mice, suggesting that sNHE and sAC associate with one another to form a signaling complex

at the mouse sperm plasma membrane (Wang et al. 2007). In sea urchin sperm, sAC also seems to be associated with sNHE (Nomura and Vacquier 2006). Thus, sNHE appears to be involved in pH regulation, although there is a lack of direct evidence for this concept, since the activity of sNHE cannot be measured by traditional electrophysiological techniques (Miller et al. 2015). Furthermore, metabolism and transportation of HCO_3^- may contribute to the pH increase in the sperm (Nishigaki et al. 2014).

21.3.3.2 Hyperpolarization of the Plasma Membrane

A change in membrane potential is also observed with the initiation of sperm motility, chemotaxis, and capacitation. Generally, when cells undergo a change in membrane potential, which acts as a trigger for cell events, depolarization and action potential are observed. However, hyperpolarization is important for these sperm events. As mentioned in Sect. 21.2.1, the spermatozoa of teleosts exhibiting external fertilization are immotile in the testis and seminal fluid, and motility is triggered by osmotic shock and/or a decrease in K^+ surrounding the sperm (see review Dzyuba and Cosson 2014). Although the triggers are different, membrane hyperpolarization occurs commonly in these species. The K^+ efflux through specific ion channels induces hyperpolarization, followed by depolarization by Na^+ influx, inducing the activation of Ca_v. Since there is no contribution of Na^+ channels, NHE is responsible for the Na^+ influx. Therefore, alkalization should occur, and it is possible that CatSper contributes to the Ca^{2+} influx. However, the molecular mechanism in teleosts remains unclear.

The molecular mechanism of hyperpolarization in sperm has been well studied in mammalian species. Spermatozoa from several mammalian species exhibit capacitation-associated plasma membrane hyperpolarization, which is necessary for the acrosome reaction to occur (Zeng et al. 1995; Arnoult et al. 1999; De La Vega-Beltrán et al. 2012; López-González et al. 2014). Since alkalization in the sperm cell activates the KSper current, efflux of K^+ via some K^+ channels is assumed to be involved in hyperpolarization (Navarro et al. 2007). Further studies have shown that the sperm-specific K^+ channel Slo3 mediates the KSper current in mouse sperm. Spermatozoa of Slo3-KO mice exhibit neither capacitation-dependent nor alkalization-induced membrane hyperpolarization (Santi et al. 2010; Zeng et al. 2011). Furthermore, Slo3-KO spermatozoa do not show Ca^{2+} entry via CatSper, thus Slo3 may regulate changes in pH (Chávez et al. 2014). In contrast to mouse sperm, the KSper current mediated by Slo3 in human sperm is not induced by an increase in pH but is sensitive to $[Ca^{2+}]_i$ (Brenker et al. 2014). As K^+ channels, Slo3 and the prototypical Ca^{2+}-activated K^+ channel Slo1 could share the preponderant role in the capacitation-associated hyperpolarization of human sperm, in contrast to what has been previously reported for mouse sperm, where Slo3 channels are the main contributors to the hyperpolarization event (López-González et al. 2014).

During chemotaxis of sea urchin sperm, hyperpolarization appears to be directly induced by cGMP (Strünker et al. 2006). The cGMP-mediated hyperpolarization is

due to K^+ efflux through the cyclic nucleotide–gated K^+-selective channel (CNGK) (Galindo et al. 2007; Bönigk et al. 2009). Zn^{2+} also activates CNGK, resulting in increases in Ca^{2+} and pH (Beltrán et al. 2014). In sea urchins, hyperpolarization seems to activate sNHE (Lee and Garbers 1986), and the sea urchin sNHE (Nomura and Vacquier 2006) contains a region similar to the voltage sensor domain (Wang et al. 2003). Thus, it is possible that CNGK activates sNHE and CatSper in sea urchin sperm (Seifert et al. 2015).

21.3.4 Lipid Component in the Plasma Membrane: Regulation of Mammalian Sperm Capacitation in the Female Reproductive Tract

21.3.4.1 Lipid Component (Phospholipid, Glycolipid, Sterol)

During epididymal sperm maturation, the lipid composition of the sperm plasma membrane is modified, which is critical for sperm motility and fertility. The lipid component of the plasma membrane comprises phospholipids, glycolipids, and sterols. Among these, sterol (cholesterol) is thought to be the most important component for fertilization. Recently, there have been reports regarding KO mice, showing that an imbalanced lipid composition resulted in reduced fertility (Busso et al. 2014; Björkgren et al. 2015).

A loss of cholesterol from the sperm plasma membrane is one of the most definitive events described in capacitating mammalian spermatozoa (Davis et al. 1979). In the female genital tract, there are abundant proteinaceous acceptors for cholesterol, such as albumin, high-density lipoproteins, and apolipoproteins. Albumin is the most important component of the in vitro fertilization (IVF) medium, although it is not essential for all species (Choi et al. 2003). Apolipoproteins are the responsible acceptors for cholesterol in serum; however, there have been few reports concerning their role in sperm capacitation. Methyl-β-cyclodextrin (MβCD) is a strong acceptor for cholesterol in a mouse IVF medium (Takeo et al. 2008). Cholesterol stabilizes the plasma membrane of the spermatozoa during epididymal transit and prevents the intermolecular interactions responsible for achieving a capacitated state (Davis et al. 1980). Several factors are added to the surface of the plasma membrane of sperm during transit through the epididymal tract and vas deferens. Most of these factors concern the prevention of cholesterol efflux. These factors are called“decapacitation factors” (Yanagimachi 1994). The decapacitation factors are predicted to coordinately prevent the triggering of cholesterol efflux. Among them, SVS2 is the most important factor in mice; it is an acceptor for cholesterol and strongly incorporates cholesterol into the sperm membrane (Araki et al. 2015). The “key” mechanisms are still unknown; however, disappearance of the decapacitation factors “unlocks” the first step in capacitation.

21.3.4.2 Mechanism of Cholesterol Efflux

How can the acceptors for cholesterol access the cholesterol in an unlocked sperm membrane? Proteinaceous acceptors are thought to be able to access the cholesterol in the sperm membrane through the mediation systems of cholesterol transport. Generally, cholesterol release from the plasma membrane involves the adenosine triphosphate (ATP)–binding cassette (ABC) transporters (Tall et al. 2008). However, little evidence has been presented concerning the presence of ABC transporters in sperm (Morales et al. 2012). The other mechanism is esterification: a small percentage of sperm cholesterol (~6%) is stabilized in the membrane as cholesteryl sulfate, and the cleavage of the sulfate moiety within the female reproductive tract may trigger a cascade of events leading to sperm capacitation and fertilization (Langlais et al. 1981; Sion et al. 2001). The cholesterol efflux from the sperm plasma membrane during capacitation is also thought to be controlled by oxidative stress. Sterols can become oxidized during capacitation, and the increased hydrophilicity of the oxidation products facilitates their transfer to albumin (Brouwers et al. 2011). The involvement of reactive oxygen species (ROS) in the capacitation of mammalian spermatozoa has been appreciated since the pioneering studies of Claude Gagnon in the 1990s (de Lamirande and Gagnon 1993). Moreover, there is a great deal of evidence about the responsibility of ROS (see review Aitken and Nixon 2013). Whatever the source of the ROS that drives capacitation is, this strategy is a double-edged sword. These cells will suffer oxidative stress and will be driven into the intrinsic apoptotic cascade. ROS drive tyrosine phosphorylation, cAMP production, and cholesterol efflux from the plasma membrane, ultimately inducing a state of apoptosis (Aitken 2011).

21.3.4.3 Signaling After Cholesterol Efflux

Preferential loss of cholesterol from the nonraft pool may be the stimulus that promotes raft clustering over the anterior sperm head (Shadan et al. 2004). The distribution of monosialotetrahexosylganglioside (GM1) gangliosides is used as a marker for lipid rafts. However, the physiological role of GM1 is not yet clear. Nowadays, the acrosome reaction is considered a kind of exocytosis from the acrosome [acrosomal exocytosis (AE)]. Sterol efflux and focal enrichment of GM1 trigger the Ca^{2+} influx necessary for AE through $Ca_V2.3$ (Cohen et al. 2014). Sperm lacking $Ca_V2.3$'s pore-forming a1E subunit have shown altered Ca^{2+} responses, reduced AE, and a strong subfertility phenotype. GM1/$Ca_V2.3$ regulatory interaction requires GM1's lipid and sugar components and $Ca_V2.3$'s a1E and a2d subunits. These results provide a mechanistic understanding of membrane lipid regulation of Ca^{2+} flux and therefore Ca^{2+}-dependent cellular and developmental processes such as exocytosis and fertilization (Cohen et al. 2014).

21.4 Conclusion and Perspective

Here, we have introduced the molecular mechanisms regulating sperm function. As described above, intensive studies in mammalian species have revealed many contributing molecules in the signaling cascade in sperm. Interestingly, some signals (such as Ca^{2+}, pH, and membrane potential) and their mediators (such as CatSper, sNHE, and Slo3) are commonly used for sperm events in many animals, although the order of the signaling cascades is dependent on each event. On the other hand, participation of cGMP production and cGMP-gated channels seems to be restricted to the events in sea urchin sperm, and the change in lipid composition in the plasma membrane appears to be a mammalian sperm–specific phenomenon.

How does a spermatozoon distinguish between the same signals for different events? One explanation is the localization of signaling. For example, the $[Ca^{2+}]_i$ increase for the acrosome reaction is mainly observed in the head, whereas that for hyperactivation and chemotaxis seems to be localized in the flagellum (see Fig. 21.3). The time course of the signaling may be also important. Previously, analysis of signal localization in sperm was difficult, since the spermatozoon is too small to observe with an optical microscope. Recently, the development of super-resolution microscopy has enabled this analysis (Chung et al. 2014). Microscopy techniques for high-resolution and high-speed analysis should resolve the remaining questions.

One remaining matter is the role that PKA-dependent tyrosine kinase plays in the phosphorylation cascades. It is possible that there is some sperm-specific kinase, since key molecules such as CatSper and Slo3 are sperm-specific proteins. Identification of Ca^{2+} targets will be required, though important Ca^{2+} targets have been previously reported, including calaxin in the chemotaxis of ascidian sperm (Mizuno et al. 2009, 2012) and calcineurin in the hyperactivation of mouse sperm (Miyata et al. 2015). Calmodulin and calmodulin-dependent protein kinases may be involved in the signaling cascade (Tash et al. 1988; Nomura et al. 2004; Suarez 2008); nevertheless, targets of the kinases should be identified. Furthermore, in order to understand the generality and diversity of the sperm events in all animals, it is necessary to identify the relevant molecular mechanisms in nonmammalian sperm. Specifically, little is known about the molecular mechanisms in teleost sperm, although there is an abundance of knowledge from physiological and pharmacological studies (Morisawa 1994). Efforts to resolve these issues should expand our horizons further.

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Chapter 22
Postcopulatory Reproductive Strategies in Spermatozoa

Mei Matsuzaki, Tomohiro Sasanami, Yoko Iwata, and Noritaka Hirohashi

Abstract To reproduce sexually, males and females produce very different gametes (sperm and eggs) in many animals. This difference gives rise to very different strategies in the two sexes and in gamete cells from the two sexes. Sperm meet eggs in harmony; however, the male and female do not always have common interests in reproduction. The battle of the sexes continues even after copulation. Female promiscuity is key to the understanding of reproductive behaviors not only in male individuals but also in sperm cells, because sexual selection continues after mating through sperm competition. Here, we highlight multiple sperm traits—the sperm acrosome reaction in sea urchins, sperm storage in birds, and sperm dimorphism in squid—that are tightly associated with postcopulatory reproductive strategies.

Keywords Sperm acrosome reaction · Postcopulatory sexual selection · Sperm competition · Seminal receptacle · Sperm cooperation

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22.1 Introduction

In sexually reproducing organisms, fertilization—the integration of haploid paternal and maternal genomes—is primarily achieved by fusion between the spermatozoon and the egg. Because male gametes are generally produced and released more abundantly than female ones and yet sperm–egg fusion must occur at a ratio of 1:1, spermatozoa are always subject to strong selection or competition. This applies not only between spermatozoa from the same individual but also between those from different individuals. Thus, promiscuous mating by females can bring about the possibility of sperm competition: the process of spermatozoa from two or more males fertilizing a given set of ova (Parker 1970). Sperm competition theory has developed continuously over the last four decades, with empirical evidence from insects, birds, fish, and mammals (Birkhead et al. 2009). Currently, it is recognized as an evolutionary form of postcopulatory sexual selection. Sperm competition theory can explain why and how female promiscuity could drive the evolution of giant spermatozoa. However, recent studies have focused on more complicated situations where sperm size is well correlated with the structure of the female reproductive organs, which are known as sites for "cryptic" female choice. Thus, researchers have faced new problems in explaining the evolutionary forces that have driven the observed traits in sperm biology. In essence, the ultimate goal of a tiny swimming spermatozoon is to reach the surface of the egg at the right place, at the right time. It does not mean that faster-swimming spermatozoa are always superior in achieving fertilization; rather, adaptation to postcopulatory circumstances is mandatory for the ejaculates to maximize male fitness in terms of reproductive success. Male mating tactics also influence postcopulatory circumstances, and males often choose a favored tactic depending on the condition of rival males. In this chapter, we introduce representative processes in spermatozoa that could have evolved in complex postmating reproductive contexts.

22.2 The Sperm Acrosome Reaction As a Fertilization Strategy Differing Between Male and Female Organisms

In many species, the most relevant postcopulatory process is the sperm acrosome reaction (AR), by which spermatozoa discharge/expose the contents of the acrosomal vesicle located at the apical tip of their head upon certain stimuli derived from the egg's extracellular investments (such as the jelly coat in echinoderms and newts, the vitelline envelope in birds and frogs, and the zona pellucida/cumulus oophorus in mammals) during fertilization. As a consequence of the AR, IZUMO1 in mice and bindin in sea urchins are exposed on the surface of the sperm; thereafter these proteins play a role in sperm–egg fusion. Thus, the AR is an essential exocytotic process leading to successful fertilization (Satouh et al. 2012). Other acrosomal

contents might have fundamental roles in sperm–egg interactions or sperm penetration through the egg coat (lysin); however, the molecular identification of such participants has been extremely difficult. Not all species produce spermatozoa with an acrosome; for example, most fish do not, and those species produce eggs with a micropyle—a small opening in the egg coat, through which a spermatozoon can enter. Thus, the sperm AR is required for spermatozoa to penetrate the egg coat in general, with a few exceptions. Conversely, the presence of an egg micropyle is not always coincidental with the absence of an acrosome in the spermatozoa, as in the case of amphioxi (Morisawa et al. 2004), which show the acrosome or the AR in sperm.

In sea urchins, the AR is a species-specific process, which ensures conspecific fertilization (Vilela-Silva et al. 2008). In fact, the purple urchin, *Strongylocentrotus purpuratus*, produces sulfated fucans that differ in their pattern of sulfation from those of the sympatric sister species, *Strongylocentrotus franciscanus*, resulting in species-specific induction of the AR (Vilela-Silva et al. 1999). Hence, sperm–egg interactions play essential roles in prezygotic barriers, particularly for many broadcast spawners.

Interestingly, our (unpublished) observations suggest that spermatozoa of *S. purpuratus* are not able to penetrate—or can penetrate only with difficulty—the jelly layer of the egg from *S. franciscanus*. Spermatozoa of *S. purpuratus* can undergo the AR to some extent with sulfated fucans from *S. franciscanus* if high enough concentrations are applied (Hirohashi et al. 2002). Therefore, even if unfavorable collisions occur between heterospecific gametes, fertilization is normally strongly prohibited. Moreover, premature induction of the AR of foreign spermatozoa on the surface of the jelly layer might be beneficial for the eggs to reduce the risk of cross-fertilization, because of rapid loss in their fertilizing competence after the AR. Male organisms need to produce spermatozoa that recognize only conspecific eggs, possibly because of sperm competition, whereas females produce eggs that also can trigger the sperm AR of other species because of the presence of multiple reproductive barriers (Vilela-Silva et al. 1999; Hirohashi et al. 2002). Thus, a conflict of interest arises between the sperm and the egg. This hypothesis can be seen in the theory of sexual conflict, in which the two sexes have conflicting fitness strategies concerning reproduction. It is of particular interest to consider why only the patterns of sulfation on the largest carbohydrate polymer in the egg jelly confer this specificity of fertilization. It is possible that point mutations in genes encoding sulfotransferases could result in global and drastic changes in the sulfation pattern, thus giving a high potential for speciation.

22.3 Sperm Storage Is a Reproductive Strategy That Facilitates Fertilization Success

In marine animals that use broadcast spawning, such as sea urchins and ascidians, gametes from both male and female individuals are released in synchrony, and spermatozoa are then guided by a chemoattractant secreted from the eggs (Yoshida et al. 2008). Such chemotaxis, together with synchronized release of gametes, can yield better fertilization outcomes. However, in internal fertilizers, better fertilization success is primarily achieved by the coordinated arrival of both sets of gametes at the site of fertilization. It is unlikely that the sperm–egg encounter occurs accidentally in vivo, because only a small fraction of spermatozoa succeed in making the long journey through the female genital tract to reach the site of fertilization. Notably, the timing of ovulation does not always coincide with that of insemination except in some mammals such as rabbits and domestic cats, where copulation stimulates ovulation (Dal Bosco et al. 2011; Brown 2011). Occasionally, female animals store spermatozoa in the reproductive tract. Thereafter, sperm use is under the female's control and stored spermatozoa are released during the ovulation "window." This sperm-storing capacity in the female reproductive tract is well documented in a variety of animals—including insects, fish, amphibians, reptiles, birds, and mammals (Birkhead and Møller 1993; Holt and Lloyd 2010; Orr and Zuk 2012)—which possess specialized organs in their genital tracts, such as sperm reservoirs in mammals (Suarez 2008), sperm storage tubules in birds (Sasanami et al. 2013), spermathecae in amphibians (Watanabe and Onitake 2002), or spermathecae and seminal receptacles in insects (Heifetz and Rivlin 2010). These organs hold the spermatozoa until ovulation takes place or the oocytes become fertilizable.

Although the duration of sperm storage varies between species, it is known that spermatozoa can be stored in the genital tracts of reptiles for years, and in those of insects for decades (Birkhead and Møller 1993; Gobin et al. 2006). Nonetheless, the mechanisms by which spermatozoa can maintain their fertilization competence for such a long time at ambient or body temperatures remain elusive. Although it was believed that sperm storage is unique to animal phyla, recent findings suggest that plants can also store male gametes in the female until fertilization. In flowering plants, when the first pollen tube fails to fertilize, a second pollen tube that is quiescent in the pistil initiates migration toward the ovule and overcomes this fertilization failure (Kasahara et al. 2012). In addition, fertilization in the Fagaceae (*Fagus japonica*) is delayed by between 4 days and more than 1 year after pollination until the ovule becomes fully developed (Sogo and Tobe 2006). These phenomena appear to be similar to that of sperm storage in the female reproductive tracts of animals (Iwata et al. 2011). Sperm-storing phenomena typically occur in female individuals whose ovulatory cycles do not coincide with the timing of insemination. In some species, such as insects and birds, the mating opportunity is quite small relative to the female's reproductive period, and yet the oocytes quickly lose their fertilizability after ovulation. Thus, sperm must keep their ability to fertilize oocytes for a considerable length of time after ejaculation. Hence, the primary role of sperm

storage is to adjust the timing of sperm arrival at the site of fertilization. In some mammalian species—such as sheep, pigs, and cows—spermatozoa are trapped in the lower isthmus of the oviduct (referred to as the sperm reservoir) until the time of ovulation (Suarez 2008). In pigs and cows, this trapping is mediated via cell adhesion molecules on the sperm head and carbohydrate moieties on the epithelial surface of the caudal isthmus (Ignotz et al. 2001). Spermatozoa are released from the sperm reservoir prior to ovulation, in response to ovarian steroids, which reactivate sperm motility sufficiently to break the bond between the spermatozoon and the sperm reservoir (Hunter 2008). In the Japanese quail (*Coturnix japonica*), the spermatozoa stored in the sperm storage organ are released in response to stimulation by circulating progesterone (Ito et al. 2011). Although the duration of sperm storage in mammalian species is relatively brief (up to several days), bats can store spermatozoa in the oviduct for 6 months (Racey 1979). These bats reside in temperate zones, where hibernation interrupts the reproductive cycle (Racey 1979). Birth occurs in early summer, which is suitable for raising offspring. In this way, bats are able to maximize the fitness of their reproductive cycle in terms of the timing of copulation and fertilization. A recent study in the greater Asiatic yellow bat (*Scotophilus heathii*) indicated that sperm storage is dependent on the maintenance of high levels of circulating androgens, produced by the ovaries interacting with androgen receptors in the uterotubal junction, where sperm storage occurs (Roy and Krishna 2010). Although the details are still unclear, these phenomena are linked to the expression of B-cell lymphoma factor 2 (Bcl-2), a key regulator of apoptosis in the uterotubal junction (Roy and Krishna 2011). From these results, the authors hypothesized that androgen-dependent expression of Bcl-2 might act as an antiapoptotic factor in the uterotubal junction and play a key role in long-term sperm survival at this storage site.

Storing spermatozoa for long periods over more than one breeding season (i.e., for more than 1 year) allows female individuals to produce offspring without the need for additional mating in the next breeding season. In general, mating is costly for females because of the risks of predation, infection, and injury (Watson et al. 1998; Archie et al. 2014; Hamilton and Zuk 1982). Furthermore, copulation itself can be harmful; in *Caenorhabditis elegans*, mating reduces a female's life-span (Gems and Riddle 1996). Thus, the sperm-storing system can ensure fecundity without payment of a precopulatory price during multiple breeding cycles. In fact, in reptiles such as turtles, snakes, and alligators, sperm storage can last for up to 7 years (Birkhead and Møller 1993; Mangusson 1979). In various elasmobranch species, the duration of sperm storage ranges from days to years (Fitzpatrick et al. 2012). Although sperm storage is an excellent strategy for producing offspring without unnecessary mating, it should be noted that parthenogenesis might be an alternative way to produce embryos if female animals have long been isolated from potential male partners. Therefore, we should carefully evaluate whether the offspring generated by these sperm-storing animals might actually result from parthenogenesis rather than from conventional sexual reproduction.

As noted, the period of sperm storage in mammals is usually short. In contrast, the queens of eusocial Hymenoptera (ants, bees, and wasps) often store spermatozoa

for more than a decade (den Boer et al. 2009a; den Boer et al. 2009b). They store them in their storage organs (spermathecae) early in adult life after copulation and never mate again during the rest of their life. The spermathecae often have accompanying glands, and their secretions have been hypothesized to play a role in survival of stored spermatozoa. However, the chemical compositions of the secreted compounds—and their physiological actions—have not been elucidated. In these species, queens initially store several hundred million spermatozoa, which could reduce multiple risks such as predation and injury during copulation. Although a direct merit of sperm storage has not been established, it has been reported in *Drosophila melanogaster* that females with repeated mating survived for a much shorter time than virgin females. Furthermore, the exposure to seminal fluid products from the male accessory gland reduces the female's propensity to remate and increases the egg-laying rate (Chapman et al. 1995; Rice 1996). These findings indicate that females are discouraged from multiple mating by the first male's copulation.

22.4 Promiscuity Drives Multiple Reproductive Tactics

Paradoxically, females do not always mate with one partner (monogamy); rather, they mate with multiple male partners (polyandry) during the reproductive season or even during a single spawning event. Female promiscuity is common in various animal taxa, from insects to humans. Females can receive a sufficient number of spermatozoa from a single copulation to fertilize all of their eggs, but they still mate with multiple males. Thus, promiscuous mating does not increase overall fertilization success; rather, it increases genetic variation in the offspring and therefore offspring fitness (Mays and Hill 2004). When a female mates with more than one male partner, spermatozoa from different males may compete for fertilization. This is called sperm competition and is one of the strongest selective forces driving the evolution of reproductive strategies (Parker 1970). Where sperm competition occurs varies among different mating systems. In external fertilizers—such as marine invertebrates, frogs, and fish—sperm competition may occur in the water environment (Byrne 2004; Gage et al. 2004). Here, the rate of sperm release, the rate of sperm dilution, and the timing of gamete release from both sexes impact on fertilization success (Levitan and Petersen 1995). In internal fertilizers—such as insects, birds, and mammals—sperm competition occurs in the female's reproductive tract. The mating order, the number of transferred spermatozoa, successful arrival in the sperm storage organs, and the survival duration in these organs are the factors that may influence fertilization success. Furthermore, sperm–female interactions lead to more important and complex situations. When females store spermatozoa from more than one male, they frequently select only "favorable" ones by eliminating others either actively (via direct sperm displacement (Sato et al. 2014)) or passively (via interactions with the female seminal receptacle (Manier et al. 2010)). Such female preference after copulation is called cryptic female choice and is regarded as

the other strongest postcopulatory force in sexual selection besides direct sperm competition (Eberhard 1996). Indeed, mice lacking the seminal vesicle secretion 2 (SVS2) protein are infertile, and their spermatozoa are killed by uterus-derived cytotoxic factors. Thus, the SVS2 protein coats the surface of spermatozoa and protects them from attack by the uterus (Kawano et al. 2014). Those spermatozoa that can protect themselves from such female attack increase their chance of winning the race to the ova. This male/sperm selection by the female might maximize offspring fitness and the number of offspring by selecting among spermatozoa in the oviduct, deposited by different males at different times (Eberhard 2009; Holt and Fazeli 2010). Hence, the female reproductive tract has been recognized to serve as a site not only for sperm storage but also for sperm selection in favor of the female's cryptic preference.

22.5 Characteristics of Alternative Reproductive Tactics and Postcopulatory Sexual Selection in the Squid

It is not unusual to have more than one reproductive phenotype in a population. Such phenomena involve alternative reproductive tactics (ARTs)—one of the major current issues in evolutionary and behavioral ecology. The most commonly observed ART is a case in which large "consort" male individuals compete with each other to gain access to females, whereas small "sneaker" male individuals attempt to "steal" mating and avoid direct male–male competition. ARTs are manifested in a wide variety of behavior, morphology, physiology, and life history among individuals, and lead to male polymorphism; thus, secondary sexual characteristics that engage precopulatory sexual selection are highly developed in consorts but poorly in sneakers (Oliveira et al. 2008). Substantially more prevalent and prominent in consorts are not only a male individual's body display, such as bright colors or enlarged ornaments, but also postmating behaviors, such as mate guarding and male parental care. In contrast, sneakers usually have at least one dominant competitor at the moment of copulation and thus exhibit elaborate behaviors in order to access the female, such as sneak or parasitic mating, and female mimicking (Taborsky 2001; Parker 1990b). In addition, sneaker males would have a higher sperm competition risk than consort males, which could lead to adaptive trait evolution in sperm.

Many species have ARTs with two different male types—consorts and sneakers—and studies have focused on the differences in the modes of sperm competition between these different tactics (Oliveira et al. 2008). In contrast, some squid species in the Loliginidae exhibit unique reproductive biology: female squid have their seminal receptacle near the mouth in their array of arms and receive spermatophores from sneakers with "head-to-head" copulation. Thus, mature female squid that arrive in the spawning areas have already stored some spermatozoa in their seminal receptacle (Drew 1911). After migration to the coastal spawning areas, consort male squid fight with rivals and court females using body color display (DiMarco and

Hanlon 1997). Thereafter, they copulate with the females in a "male-parallel" position—that is, males hold females from the dorsal side and transfer their spermatophores directly into the opening of the oviduct in the mantle cavity. Sneakers rush to the paired squid and attempt to transfer their spermatophores around the seminal receptacle in a head-to-head manner (Hanlon 1996). This difference in the site of sperm deposition determines the fate of the sperm's journey. Because the site of sperm transfer by consort males is near the ovipositor, their spermatozoa acquire a positional advantage in gaining access to eggs. In contrast, although the exact site of fertilization by sneaker spermatozoa is unknown, it is thought that it occurs when the female holds the egg mass in her arm crown (Iwata et al. 2011). This positional advantage results in greater fertilization success for consorts, as revealed by DNA paternity analysis (Iwata et al. 2005). Spermatozoa from sneaker males are stored in the seminal receptacle and participate to some extent in fertilization, suggesting that both consort and sneaker male squid have a constitutive risk of sperm competition, but sneakers have a higher risk than consorts. In squid, ARTs result in two sperm placement and storage locations, with possible differences in fertilization success.

22.6 Evolution of Sperm Number Strategies Associated with Alternative Reproductive Tactics

Theoretical modeling predicts that males exposed to greater competition from other males allocate greater resources to sperm production in order to compensate for their reduced chances of fertilization success (Parker 1990a, b; Parker et al. 1997); the results of several studies of external and internal fertilizers support this prediction (Gage and Barnard 1996; Evans et al. 2003). In the bluegill fish, *Lepomis macrochirus*, Leach and Montgomerie (Leach and Montgomerie 2000) showed that the milt of sneaker males contained 50% more concentrated sperm than that of consort males. In the squid *Heterololigo bleekeri*, Iwata and Sakurai (Iwata and Sakurai 2007) analyzed morphological characteristics both inside and outside the adult body to look for adaptive traits in each ART. Although no dimorphism was observed in external body characteristics, clear dimorphism was observed in spermatophores. The length of the spermatophore is generally associated with body size, but this relationship was lost between sneakers and consorts. The long-type (consort) spermatophores contained around five times more spermatozoa than did the short-type (sneaker) spermatophores (Iwata et al. 2011). This finding was inconsistent with those of previous studies showing that males in the species with a high sperm competition risk have a higher gonadosomatic index (ratio of gonad to body size) to produce more sperm (Harcourt et al. 1981). The other model in conjunction with empirical evidence (Pilastro et al. 2002) predicted that sperm expenditure should decrease when there are more than two competing male individuals (Parker et al. 1996). In *H. bleekeri*, spermatozoa from a consort and from multiple sneakers can be involved in fertilization during a single spawning episode. Indeed, a paternity analysis identified that more than two male squid were involved in the fertilization

of eggs spawned as a single clutch (Iwata et al. 2005). The small sneaker-derived spermatophores contained fewer spermatozoa than the larger consort-derived ones, which is consistent with the prediction that sneakers would have greater exposure to sperm competition and lower sperm expenditure. Such dimorphism in spermatophore morphology could have resulted from adaptation to the structure of the seminal receptacle rather than via sperm allocation. During mating, the spermatophores of cephalopods stimulate a spermatophoric reaction, which involves extrusion of inner sperm sacs called spermatangia (Mann et al. 1966; Marian 2012). The extruded spermatangium also shows a clear dimorphism between sneakers and consorts in *H. bleekeri* (Iwata et al. 2015). The morphology of the spermatangium is "drop-like" in the short-type (sneaker) spermatophore and "rope-like" in the long-type (consort) spermatophore. The design of the drop-like spermatangia reduces mechanical tension, so the risk of detachment from the female tract is reduced. Moreover, the drop-like spermatangium has an anchor on its base, enabling it to hook onto the female body surface near the seminal receptacle (Iwata et al. 2015). Such spermatophore dimorphism is also known in *Euchaeta norvegica* (a copepod) (Hopkins and Machin 1977) and *Chionoecetes opilio* (the snow crab) (Beninger et al. 1993). Males of these species produce two different types of spermatophore at the same time: one for long-term fertilization opportunities and the other for a short-term strategy (Eberhard 1996). Spermatophore dimorphism in squid might also be an adaptation to male mating behavior associated with sperm transfer and storage, but it differs from the cases above in that individual male squid produce only one of the two types of spermatophore associated with each mating tactic.

22.7 Evolution of Sperm Size Associated with Alternative Reproductive Tactics

Sperm competition theory predicts that intense sperm competition will lead sperm morphology toward an optimum form (Varea-Sanchez et al. 2014). In addition, sperm morphology must match a particular mode and strategy of fertilization (Higginson et al. 2012; Scharer et al. 2011). Therefore, morphological diversification within a species is severely limited by both sperm competition and the mode of fertilization. Interestingly, comparisons among closely related species in some groups have demonstrated that male individuals exposed to more intense sperm competition make larger spermatozoa (Gomendio and Roldan 1991; LaMunyon and Ward 1999). Interspecific comparisons among cichlid fish have demonstrated that the spermatozoa of highly promiscuous species are larger, and a positive relationship is seen between sperm size and sperm swimming velocity (Fitzpatrick et al. 2009). In addition, being larger is better for spermatozoa to occupy a female's storage organ, possibly outcompeting those of others. Hence, males facing bad situations in mate access tend to make larger and/or faster sperm to improve their chances of fertilizing eggs. In this context, comparisons between consorts and sneakers have attracted particular attention because both types of spermatozoa share the same

fertilization environment but are exposed to different intensities of sperm competition. However, no clear dimorphism has been found in sperm size between consorts and sneakers (Pitnick et al. 2009) except in the case of the bluegill, where sneaker spermatozoa are slightly longer and swim faster than consort spermatozoa (Burness et al. 2004).

What about the case of the male squid that have two different fertilization strategies and two different sperm competition risks? In *H. bleekeri*, clear sperm dimorphism was found, in that the sneaker spermatozoa had a ~50% longer flagellum than consort spermatozoa (Iwata et al. 2011). However, there was no significant difference between sneaker and consort spermatozoa in their initial swimming speed (Iwata et al. 2011). Fertilization competency was examined in both types of spermatozoa and was much greater in sneaker spermatozoa than in consort spermatozoa when assayed in vitro. Although this difference could be attributed to the duration of sustainable motility after initial dilution, it is unlikely that a longer flagellum is associated with sperm longevity. The other possible explanation for the divergence in sperm types is that it arose in coevolution with the female seminal receptacle because only sneaker sperm can be stored in the seminal receptacle. Recent studies have demonstrated that morphological variations in male genitalia and sperm characteristics have coevolved with the morphology of the seminal receptacle (Pitnick et al. 2003; Hosken and Stockley 2004). In the squid, comparative studies with closely related species are needed to clarify this issue. Although the adaptive significance of sperm dimorphism is still elusive, manifold insemination strategies undoubtedly play essential roles in this phenomenon. In turn, basic information on postmating events is needed to estimate the intensity of competition in sperm transfer, sperm precedence in the seminal receptacle, and fertilization success in squid species.

22.8 Evolution of Sperm Cooperation Associated with Alternative Reproductive Tactics

Sperm competition can sometimes drive cooperation among sperm. Cooperation is seen in changes in sperm behavior that enable other spermatozoa from the same individual to increase their chances of successful fertilization. A representative case of sperm cooperation is in sperm aggregates, which are found in opossums (Moore and Taggart 1995), rodents (Moore et al. 2002), and insects (Hayashi 1998). Spermatozoa in a bundle can swim faster than single spermatozoa under experimental conditions (Immler et al. 2007). However, whether spermatozoa ascend the oviduct to reach the oocytes in such cooperating assemblages is purely hypothetical. In deer mice, spermatozoa tend to assemble with "brothers," suggesting that kin selection leads to cooperation (Fisher and Hoekstra 2010). To our knowledge, this phenomenon of sperm cooperation has been reported only for species with internal fertilization. In the squid *H. bleekeri*, only sneaker spermatozoa show chemotaxis toward CO_2, and respiratory CO_2 emitted from spermatozoa induces self-clustering

(Hirohashi et al. 2013). Unlike other cases of sperm assembly, the clustering of squid spermatozoa does not involve their physical contact with each other; rather, each one swims independently. In addition, the clustering of sneaker spermatozoa does not facilitate enhanced movement in any particular direction. The physiological significance of sperm clustering in squid remains to be determined.

22.9 Conclusions

In this chapter, we have reviewed the postcopulatory fertilization strategies observed in various externally and internally fertilizing species (Fig. 22.1). In general, because the sperm AR is crucial for fertilization, it has been thought that timely commitment of this reaction would increase reproductive fitness in both male and

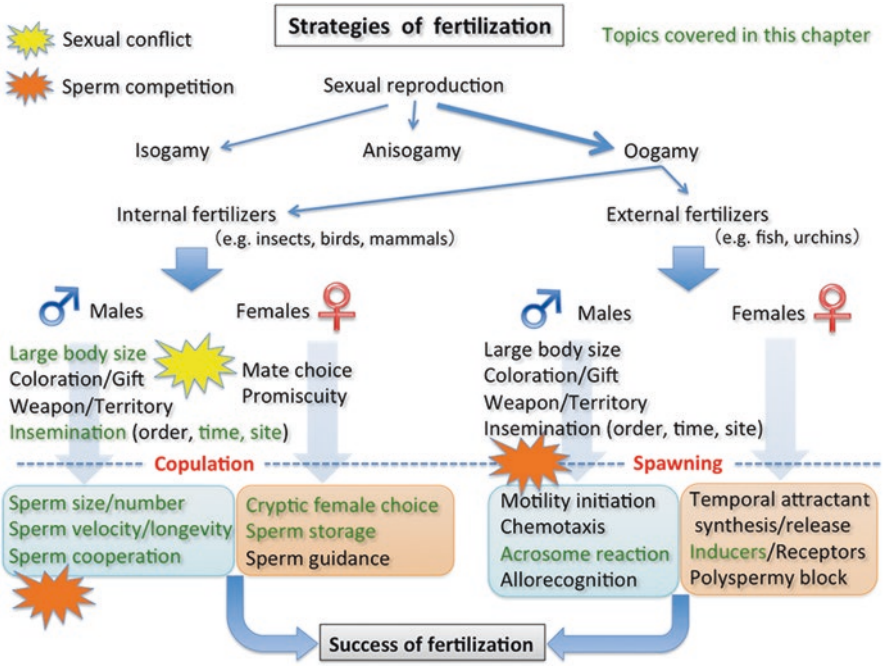

Fig. 22.1 Summary of sex-biased fertilization strategies. The most successfully widespread reproductive system is oogamy, where each sex makes either spermatozoa or eggs to pass on genes between generations. In essence, eggs are always more costly to produce than spermatozoa because of their huge difference in size. Such a difference in each gamete's cost performance drives a conflict of interest between the sexes (sexual conflict), resulting in phenomena such as sexual dimorphism, male–male competition, and female choice. These differences in morphological and behavioral characteristics are consistently observed in internal and external fertilizers. Notably, sexual conflict continues even after copulation/spawning, which is now recognized as postcopulatory sexual selection (sperm competition and cryptic female choice)

female individuals. However, as discussed in the case of sea urchins, specific induction of the AR is much more important for male individuals (spermatozoa) than for female individuals (eggs). In contrast, the threat of cross-fertilization within closely related species is more serious for females than for males because of the higher cost of egg production. Therefore, we should consider the possibility of a conflict of interest between sexes at a molecular level, which has been largely overlooked. In this regard, the limited structural variation of the AR inducer might represent the strategic regimen of oogamy. One adaptive explanation for successful sperm storage is that there are benefits gained by temporal synchronization between copulation and ovulation. These events are thought to be regulated during the ovulatory cycle, which allows sperm release from the storage site to synchronize perfectly with ovulation. Because the ability to promote sperm survival at the storage sites is astonishingly long in some taxonomic groups, elucidation of the mechanisms of sperm storage in different species must include examination of possible solutions to the problem of keeping sperm alive for long periods, which might lead to the development of new strategies for sperm preservation at ambient temperatures. We have also discussed unique sperm traits—sperm flagellar dimorphism and CO_2-mediated self-clustering—that might be associated with ARTs in the squid *H. bleekeri*. In the other species that exhibit ARTs, it is common that consort and sneaker male individuals attempt different mating tactics but release spermatozoa in the same place. In this situation, the sperm traits are still the same with the two tactics. For these squid, differences in the route to reach the ova, the storage sites, the storage period, and fertilization environments between different ARTs would lead to phenotypic variation in sperm biology. It will be important to dissect the evolutionary forces that give rise to new traits in spermatozoa.

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Chapter 23
Ascidian Sexual Reproductive Strategies: Mechanisms of Sperm-Egg Interaction and Self-Sterility

Hitoshi Sawada, Shiori Nakazawa, and Maki Shirae-Kurabayashi

Abstract Ascidians (protochordate) are hermaphrodites that release sperm and eggs nearly simultaneously. However, self-fertilization is prohibited by self-sterility mechanisms during interaction between sperm and the vitelline coat (VC) of the eggs in many ascidian species, including *Ciona robusta* (former name: *Ciona intestinalis* type A) and *Halocynthia roretzi*. A recent genetic study in *C. robusta* revealed that two tightly linked gene pairs in loci A and B, *i.e.,* the sperm *PKDREJ*-like receptor *s-Themis-A* and the VC fibrinogen-like ligand *v-Themis-A*, and *s-Themis-B* and *v-Themis-B*, which include highly variable regions among individuals, are responsible for self-recognition. Sperm recognizes an egg as a self-egg when both alleles of *s/v-Themis-A* and *s/v-Themis-B* possess the same haplotypes. When attached to the VC of self-eggs, acute and drastic Ca^{2+} influx takes place in the sperm head and flagella probably via the Ca^{2+}-conducting cation channel in the C-terminal region of s-Themis-B, which results in sperm detachment from the VC or decrease in sperm motility. We recently identified v-Themis-like, an acid-extractable VC protein with no allelic polymorphism, as a new candidate that participates in self-sterility. This self-sterility mechanism is closely related to the self-incompatibility systems in angiosperms. A different ascidian, *Halocynthia roretzi*, utilizes a different self/nonself-recognition system during fertilization, using an EGF-like repeat-containing VC protein, HrVC70. Moreover, the genome database of *H. roretzi* contains four pairs of *s/v-Themis* homologs. These gene products may also play a role in self-sterility in this species. This chapter describes the historic and current understandings of the mechanisms of gamete interaction and self/nonself-recognition in ascidian fertilization.

Keywords Sperm · Sperm-egg interaction · Self/nonself-recognition · Self-sterility · Self-incompatibility · Ascidian

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23.1 Introduction

Most terrestrial organisms reproduce by sexual reproduction rather than asexual reproduction. The former is a reproductive strategy to create genetic diversity in the next generation, which is beneficial for adaptation to possible environmental changes. To avoid inbreeding, many hermaphroditic organisms have acquired self-sterility mechanisms, the nature of which is referred to as self-incompatibility, mainly in flowering plants (Sawada et al. 2014a; Iwano and Takayama 2012; Takayama and Isogai 2005). In this chapter, the term "self-sterility" is used for ascidians to distinguish their reproductive strategy from the plant self-incompatibility system.

Ascidians (Urochordate), sessile marine invertebrates, are hermaphroditic animals that release sperm and eggs almost simultaneously during the spawning season. Ascidian eggs are covered by a glycoproteinaceous egg coat called the VC (previously called chorion) and test cells within the perivitelline space. There is a single layer of follicle cells surrounding the outer surface of the VC. Interestingly, several ascidians, including *Ciona robusta* (Phlebobranchia) (*Ciona intestinalis* type A has been renamed *Ciona robusta* (Brunetti et al. 2015)) and *Halocynthia roretzi* (Stolidobranchia), show strict self-sterility in the interaction between sperm and the VC of the eggs. *C. robusta* is a useful animal for studying the self/nonself-recognition system because genetic analyses are easy due to the short generation time (2–3 months) and because genome databases for this species have been available since 2002. (For genome database of *Ciona intestinalis (Ciona robusta)*, see two websites referred to as Ghost database (http://ghost.zool.kyoto-u.ac.jp/cgi-bin/gb2/gbrowse/kh/), and JGI database (http://genome.jgi.doe.gov/Cioin2/Cioin2.home.html); for protein database of *Ciona intestinalis (Ciona robusta)*, see the website CIPRO (http://cipro.ibio.jp/current/); for genome databases of ten ascidians (*Ciona robusta* (former name: *Ciona intestinalis* type A), *Ciona intestinalis* (former name: *Ciona intestinalis* type B), *Ciona savignyi, Molgula occidentalis, Molgula occulta, Botryllus schlosseri, Phallusia mammilata, Phallusia fumigata, Halocynthia roretzi*, and *Halocynthia aurantium*), see the website ANISEED (http://www.aniseed.cnrs.fr.)) Several techniques for gene modification and targeting experiments have been developed in this species. *H. roretzi* (type C), which is farmed for human consumption, produces large quantities of sperm and eggs from one individual and this is helpful in biochemical studies. However, this species is not useful for genetic analyses because it takes 3 years for them to reach sexual maturation. This chapter focuses on the mechanisms of gamete interaction and self/nonself-recognition during fertilization of ascidians (see also other reviews on the allo-recognition and lysin systems in ascidians: Hoshi 1985; Sawada 2002; Sawada et al. 2005, 2014a, b).

23.2 Candidate Molecules Involved in Self-Sterility and Gamete Interaction in *C. robusta*

In the early part of the twentieth century, Thomas Hunt Morgan first investigated self-sterility in ascidians using *Ciona intestinalis* (probably *Ciona intestinalis* and *Ciona robusta*) (Morgan 1910, 1923). He first reported that the sperm from *Ciona*

could not fertilize eggs from the same individuals (Morgan 1923). Then he discovered that the VC-free or VC-torn eggs could be fertilized by self-sperm (Morgan 1910, 1923). Since he had already observed that self-sperm exist in the perivitelline space, he first thought that self-sterility must be caused by the test cells or the substances produced by them, which had to be removed by tearing the VC. At that time, Morgan believed that the self/nonself-recognition site must reside on the test cells rather than the VC. However, he later found that the self-sterile eggs became self-fertile by treatment with acidic seawater (adjusted to pH 2.6 by adding HCl (or by adding lemon juice, citric acid, sulfuric acid, or nitric acid) for 2–5 min) or with proteases (e.g., 2% trypsin for 30 min or crab stomach juice) but not with ether, alcohol, or other reagents tested (Morgan 1939). Furthermore, no induction of self-fertilization was observed by the same treatment of spermatozoa (Morgan 1939). Therefore, he concluded that a certain protein responsible for self-sterility, which is susceptible to acid and trypsin, must be present in the VC.

Following Morgan's studies, efforts were made by De Santis and co-workers to clarify the self/nonself-recognition mechanisms. They showed that spermatozoa of *Ciona* (probably *Ciona robusta*) can bind to the VC of glycerinated eggs without undergoing an acrosome reaction (Rosati and De Santis 1978). They also revealed that nonself-sperm can tightly bind to the VC of glycerinated eggs, indicating that the sperm binding to the VC of glycerinated eggs is not only species-specific but also allo-specific (Rosati and De Santis 1978). Notably, sperm binding to the VC of glycerinated eggs requires external Ca^{2+} in seawater. Neither Mg^{2+} nor Mn^{2+} can replace Ca^{2+} in its ability to aid sperm binding to the VC (Casazza et al. 1984). Recently, we observed that sperm easily detach from the VC of glycerinated self-eggs but not from that of nonself-eggs (Saito et al. 2012). Our observations coincide with previous observations of Rosati and De Santis (1978). Furthermore, we showed that an acute and drastic increase in sperm intracellular Ca^{2+} concentration takes place immediately after the binding of sperm to the isolated VC of self-eggs, a phenomenon that was revealed by using the Ca^{2+} indicator Fluro-8H AM (Saito et al. 2012; see below). The intracellular Ca^{2+} increase never occurred in Ca^{2+}-deprived seawater, indicating that this increase depends on Ca^{2+} influx. This Ca^{2+} increase has not been observed in sperm binding to the VC of nonself-eggs. These results indicate that Ca^{2+} influx is a self-recognition response in *C. intestinalis* sperm, although the downstream intracellular signals are still unknown. In the flowering plant Papaveraceae, an acute increase in intracellular Ca^{2+} occurs within a self-pollen, resulting in caspase-like protease-mediated apoptosis in the self-incompatibility system (Iwano and Takayama 2012). This self-incompatibility system may be similar to the self-sterility mechanisms in *C. intestinalis.*

This allo-specific binding of sperm to the VC seems to also be prevented by several monosaccharides, most strongly by fucose (Rosati and De Santis 1980; De Santis et al. 1983). Fucose residues in the VC may participate in the sperm-egg interaction. Sperm α-L-fucosidase has been proposed to be a binding partner for the fucose residues on the VC by making an enzyme-substrate complex under seawater conditions (Hoshi 1986).

Self-sterility appears to be acquired several hours after germinal vesicle breakdown (GVBD) in *C. intestinalis* (probably *C. robusta*) (De Santis and Pinto 1991). Removal of follicle cells prevents the onset of self-sterility. Therefore, follicle cells may produce a self-sterility factor that binds to the VC during GVBD (De Santis and Pinto 1991).

Self/nonself-recognition in ascidian fertilization was once considered to involve a major histocompatibility complex (MHC)-like locus, which orchestrated the somatic self/nonself-recognition by coelomocytes and body tissues in some ascidians (Weissman et al. 1990; Saito et al. 1994). Based on this idea, searches were conducted for MHC homologs in ascidians. However, the attempts were unsuccessful (Scofield et al. 1982a, b; Marino et al. 1998), although another locus responsible for allo-recognition was identified in colonial ascidian *Botryllus schlosseri* (De Tomaso et al. 2005; Voskoboynik et al. 2013). Marino et al. (1998) focused on the MHC-related gene *hsp70*, a candidate for the ancestral MHC gene, since this gene resides in the MHC class III region and also is the oldest protein known to possess a peptide binding domain (Marino et al. 1998). Marino et al. (1998) showed that the *Cihsp70* gene is expressed during oogenesis in follicle cells of previtellogenic and vitellogenic oocytes and that two anti-Cihsp70 antibodies prevented the switch from self-fertility to self-sterility during oogenesis. In addition, since a proteasome inhibitor, clasto-lactacystin beta-lactone, inhibited the onset of self-sterility, it has been proposed that putative peptides produced by the proteasome are presented by Cihsp70 on the VC, which is responsible for self-sterility (Marino et al. 1999). However, no products of *hsp70* genes or its homologs have been detected in the VC by LC/MS analysis under our experimental conditions, suggesting that the amount of Cihsp70 protein on the VC, if any, must be very low (Yamada et al. 2009).

Since self-sterile eggs become self-fertile after treatment with acidic seawater (Morgan 1939), acidic seawater might contain an allo-recognizing sperm receptor. Based on this idea, Kawamura and co-workers explored a self/nonself-recognizing factor in the VC of *C. robusta* (Kawamura et al. 1991). They identified several factors in the acid extract of the VC that were able to inhibit the binding of nonself-sperm while not affecting the binding of self-sperm to the VC of glycerinated eggs. These factors included a non-allo-recognizing glucose-enriched inhibitor of the gamete interaction and Glu/Gln-enriched peptide-modulators, which serve as cofactors for allo-recognizing sperm receptors (Kawamura et al. 1991). These factors inhibited the binding of nonself-sperm to the VC (Kawamura et al. 1991; see also review by Harada and Sawada 2008).

To explore a putative allo-recognition-related factor in the acid extract of the VC, we carried out a proteomic analysis of the VC and the acid extract of the VC (Yamada et al. 2009). Several proteins were specifically extracted by low pH from the isolated VC, with one of the major proteins being a VC protein referred to as v-Themis-like (with a molecular architecture similar to the other allo-recognition proteins v-Themis-A and -B (described below)). v-Themis-like has no apparent polymorphism in the sequence among individuals, although it consisted of an N-terminal signal sequence, a coiled-coil domain, and a C-terminal fibrinogen-like domain that is also seen in v-Themis-A and -B. v-Themis-like is expressed in imma-

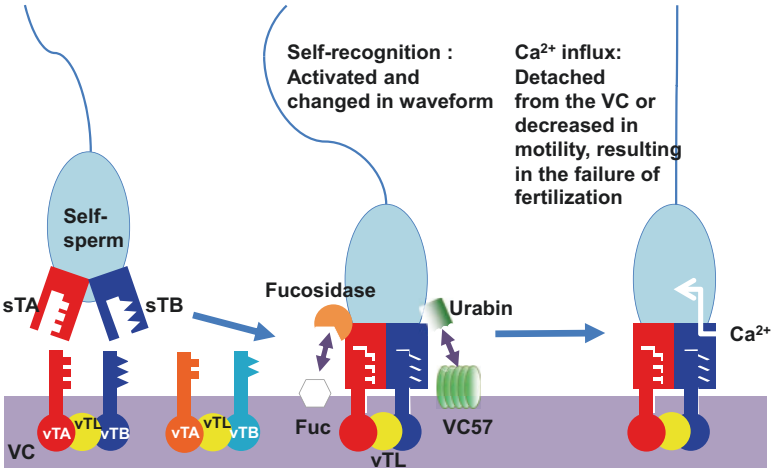

Fig. 23.1 Working hypothesis of the self-sterility mechanism in *Ciona robusta*. In *C. robusta*, fibrinogen-like ligand proteins, v-Themis-A and v-Themis-B, on the VC interact with sperm PKDREJ-like proteins, s-Themis-A and s-Themis-B, respectively. If the alleles of *s-* and *v-Themis-A* and of *s-* and *v-Themis-B* are from the same haplotypes, the sperm recognizes the VC as self-egg, which allows Ca^{2+} influx, probably via the TRP-type Ca^{2+}-conducting cation channel at the C-terminus of s-Themis-B. This results in the detachment of sperm from the VC or in sperm waveform change and decrease in motility. The s/v-Themis-mediated self/nonself-recognition process may be supported by the interaction between sperm α-L-fucosidase and fucose residue in the VC and also between sperm Urabin and VC57. v-Themis-like may be involved in the formation of a complex of v-Themis-A and -B.

ture oocytes and transferred to the VC during oocyte maturation. After this transfer, this protein is found exclusively in the VC of mature eggs. By using yeast two-hybrid screening, sperm trypsin-like protease was found to be a candidate for the binding protein of v-Themis-like (Otsuka et al. 2013). Furthermore, preliminary data indicated interaction between v-Themis-A/B and v-Themis-like. This suggests that v-Themis-like supports the self/nonself-recognition system in the sperm-VC interaction (Otsuka et al. unpublished data) (Fig. 23.1).

Other investigators carried out PCR-based subtraction and compared gonad cDNAs in genetically unrelated individuals (Khalturin et al. 2005; Kürn et al. 2007a, b). They identified several genes that are expressed in developing oocytes and/or follicle cells and are variable among individuals: *CiS7* (EGF-like repeat-containing gene), *vCRL1* (Sushi/SCR domain-containing gene), and several homologs of *HrVC70* (EGF-like repeats- and ZP-domain-containing genes; see below) (Khalturin et al. 2005; Kürn et al. 2007a, b). However, based on their genetic analyses, it was concluded that *s-* and *v-Themis* genes (Harada et al. 2008; described below) rather than *vCRL1* genes are responsible for self/nonself-recognition in *C. intestinalis* type B (Sommer et al. 2012).

We have also analyzed several other gamete proteins responsible for sperm-VC interaction in *C. robusta*. Sperm Urabin, a GPI-anchored CRISP (Cys-rich secretory protein) family protein located at the sperm head region is also involved in the bind-

ing of sperm to the VC by associating with VC57, a main VC glycoprotein with EGF-like repeats (Yamaguchi et al. 2011; Yamada et al. 2009) (Fig. 23.1). It was also reported that the 100-kDa ApoBL (apolipoprotein B-like) localized in the VC as well as egg cytoplasm may interact with a 70-kDa sperm protein (Yamada et al. 2009), which has not been identified. These interactions support the gamete interaction and self/nonself-recognition system. Genome database analysis and recent proteomic analysis have revealed that an uncharacterized protein having homology to HrTTSP-1 (Type-II transmembrane serine protease-1 of *H. roretzi*, a probable binding partner of HrVC70) (Harada and Sawada 2007; Sawada et al. 2004) is exposed to the sperm cell surface in *C. robusta* (Nakazawa et al. 2015), implying the possible participation of the HrTTSP-1-like protein in gamete interaction. Sperm-VC interactions mediated by these proteins may support self/nonself-recognition and allow the penetration of nonself-sperm through the VC, which might be mediated by the sperm extracellular ubiquitin-proteasome system functioning as a lysin in ascidians (Sawada et al. 1998; see also reviews by Sawada, 2002; Sawada et al. 2005, 2014b).

23.3 Genetic Analyses of Self-Sterility in *C. robusta*

Morgan reported results of several genetic analyses of self-sterility in *C. intestinalis* (probably *C. robusta*) (Morgan 1910, 1939, 1942, 1944). By using acid-induced self-fertilization, he raised many selfed F1 siblings and examined cross-fertility and cross-sterility among them (Morgan 1942, 1944). Cross-sterility is rarely observed in wild populations, but cross-sterile combinations are easily observed in selfed or experimentally cross-fertilized siblings. These results reinforced the theory that self-sterility is genetically controlled. The selfed F1 siblings produced sterile combinations between different individuals. Among them, Morgan noticed two types of cross-sterility, i.e., reciprocal and one-way (Morgan 1942, 1944). In reciprocal cross-sterility, sperm of one individual cannot fertilize the eggs of another individual, but this was not the case in the opposite combination in one-way cross-sterility. Morgan proposed the haploid sperm hypothesis to explain one-way cross-sterility (Morgan 1942, 1944): self-sterility is determined by haploid expression of the determinant gene in sperm and diploid expression in eggs. According to his hypothesis, a parent that is heterozygous at the self-sterility locus (A/a) produces two populations of sperm (A-expressing sperm and a-expressing sperm), either of which can fertilize homozygous eggs (a/a and A/A eggs), since a/a eggs have a-sperm receptors but not A-sperm receptors and A/A eggs have A-sperm receptors but not a-sperm receptors. In contrast, sperm (A-expressing sperm and a-expressing sperm) from two types of homozygotes (A/A and a/a individuals) cannot fertilize heterozygous eggs (A/a eggs), since the VCs of heterozygous eggs express both receptors for A-sperm and a-sperm (see Fig. 23.2).

Murabe and Hoshi (2002) and Harada et al. (2008) repeated Morgan's experiments and obtained reproducible results. When a one-way incompatible combination was observed in nonself-fertilization between selfed F1 siblings, "male"

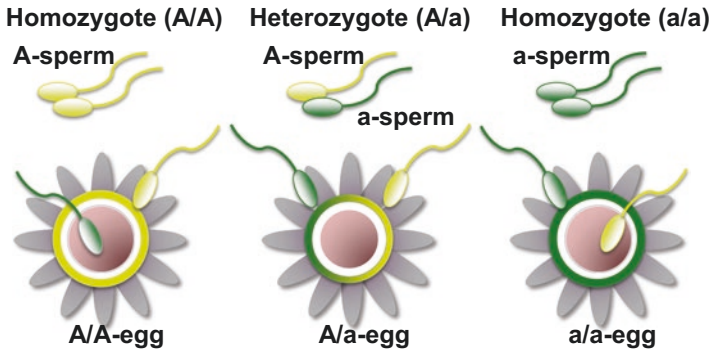

Fig. 23.2 Morgan's hypothesis on the mechanism of one-way self-sterility in *Ciona.* Cross (or nonself)-sterile combinations are observed in cross-fertilization experiments among selfed F1 siblings. There are two types of cross-sterile combinations: one is a reciprocal cross-sterility and the other is a one-way cross-sterility. In order to explain this phenomenon, Thomas Hunt Morgan proposed a "haploid sperm hypothesis," in which the sperm determinants are in haploidic expression and the egg determinants are in diploidic expression. According to his hypothesis, heterozygous individuals produce A-sperm (yellow) and a-sperm (green) and eggs express both A-sperm receptors (yellow) and a-sperm receptors (green). In contrast, homozygous (A/A or a/a) individuals produce A-sperm or a-sperm alone. Therefore, sperm of homozygous individuals cannot fertilize the eggs of heterozygous individuals, while sperm of heterozygous individuals can fertilize the eggs of both homozygous individuals. Ascidian eggs are arrested at the first meiotic metaphase, and it is reasonable to consider that both A-sperm and a-sperm receptors are expressed in heterozygous individuals

individuals should be heterozygous and "female" individuals should be homozygous in the self-sterility-determining locus in the sterile combination, according to the Morgan's hypothesis (see Fig. 23.2). About 70 marker genes in 14 chromosomes were investigated to determine whether each gene marker in the siblings is homozygous or heterozygous by PCR-based DNA sequence analyses. By this approach, two loci (locus A in chromosome 2q and locus B in chromosome 7q) were found to be responsible for self-sterility in *C. robusta.* Approximately 20 genes resided in locus A, among which only a fibrinogen-like gene product was detected in the isolated VC of the eggs by proteomic analysis (Yamada et al. 2009; Harada et al. 2008). In addition, only polycystin 1-like protein showed polymorphisms among 4 locus-A genes that were expressed in the testis. There was no overall synteny between loci A and B, but only pairs of fibrinogen-like protein and polycystin 1-like protein were commonly observed in both loci. The entire region of fibrinogen-like proteins was highly polymorphic among individuals and the N-terminal regions of polycystin 1-like proteins were also highly variable among individuals, and these regions were referred to as hyper-variable regions.

These gene pairs are promising candidates for self-sterility determinants in *C. robusta* (Harada et al. 2008), and they were designated as s(sperm)-Themis-A and s-Themis-B for polycystin-1-like (or PKDREJ-like) proteins expressed in the testis and as v(vitelline coat)-Themis-A and v-Themis-B for fibrinogen-like proteins on

the VC (Themis is a Greek Goddess who is the embodiment of divine order and prohibits incest). Interestingly, *v-Themis* genes resided in the first introns of *s-Themis* genes in both loci A and B but were transcribed in the opposite directions (Fig. 23.3a). Such a tight linkage between *s-Themis* and *v-Themis* genes will not allow segregation of *s-* and *v-Themis* pairs during meiosis. Based on genetic analysis, it was hypothesized that when sperm s-Themis-A and -B recognize v-Themis-A and -B, respectively, as the same haplotypes, spermatozoa must recognize the VC as self-egg, resulting in the establishment of a self-fertilization block.

23.4 s/v-Themis-A and -B As Key Proteins in Self-Sterility of *C. robusta*

s-Themis-A and -B contain an N-terminal hyper-variable region, REJ (receptor for egg jelly) domain, GPS (G-protein coupled receptor proteolysis) site, and LH2 (lipoxygenase homology 2) domains. In addition, s-Themis-A contains C-terminal 5 transmembrane domains, while s-Themis-B contains C-terminal 11 transmembrane domains, including a C-terminal Ca^{2+}-conducting cation channel (PKD channel) domain made up of 6 transmembrane domains (Fig. 23.3a, b). This channel shows homology to TRP (Transient Receptor Potential) channels and possesses a P-loop, which is generally involved in regulation of the cation influx, between the 10th and 11th transmembrane domains (Sutton et al. 2006, 2008; Florman et al. 2008). The molecular architecture of s-Themis-B is very similar to those of mammalian PKDREJ and sea urchin (*Strogylocentrotus purpuratus*) Sp-REJ proteins (Gunaratne et al. 2007) (Fig. 23.3c). Sea urchin sperm Sp-REJ1 contains a C-terminal single transmembrane domain and this protein is specifically expressed in the testis. Sp-REJ1 localizes on the plasma membrane covering the acrosome and functions as a receptor for egg jelly fucose sulfate polymer, an acrosome-reaction-inducing substance in sea urchins (Moy et al. 1996; Vacquier and Moy 1997), probably with the aid of two N-terminal C-type lectin domains (Gunaratne et al. 2007; Mengerink and Vacquier 2001). On the other hand, Sp-REJ3 is widely expressed in tissues including the testis (Gunaratne et al. 2007) and is localized on the plasma membrane covering the acrosome (Mengerink et al. 2002). Sp-REJ3 is cleaved at the GPS site. No antibodies against Sp-REJ3 induced the acrosome reaction, which was observed when treated with anti-Sp-REJ1 antibodies (Moy et al. 1996). These results indicate that Sp-REJ1 rather than Sp-REJ3 is involved in the acrosome reaction. In humans, there are five family members of PKD1 (hPKD1, hPKDL1, hPKDL2, hPKDL3, and hPKDREJ) (Gunaratne et al. 2007) consisting of a GPS domain, LH2/PLAT domain, and 11 transmembrane domains. PKD1 possesses no C-terminal cation channel domain but is capable of interacting with PKD2, which consists of a TRPC cation channel (Sutton et al. 2006). Human PKDREJ contains a C-terminal cation channel domain, as do Sp-REJ3 and Cr-v-Themis-B, and is specifically expressed in the testis (Sutton et al. 2006). Human PKDREJ can interact

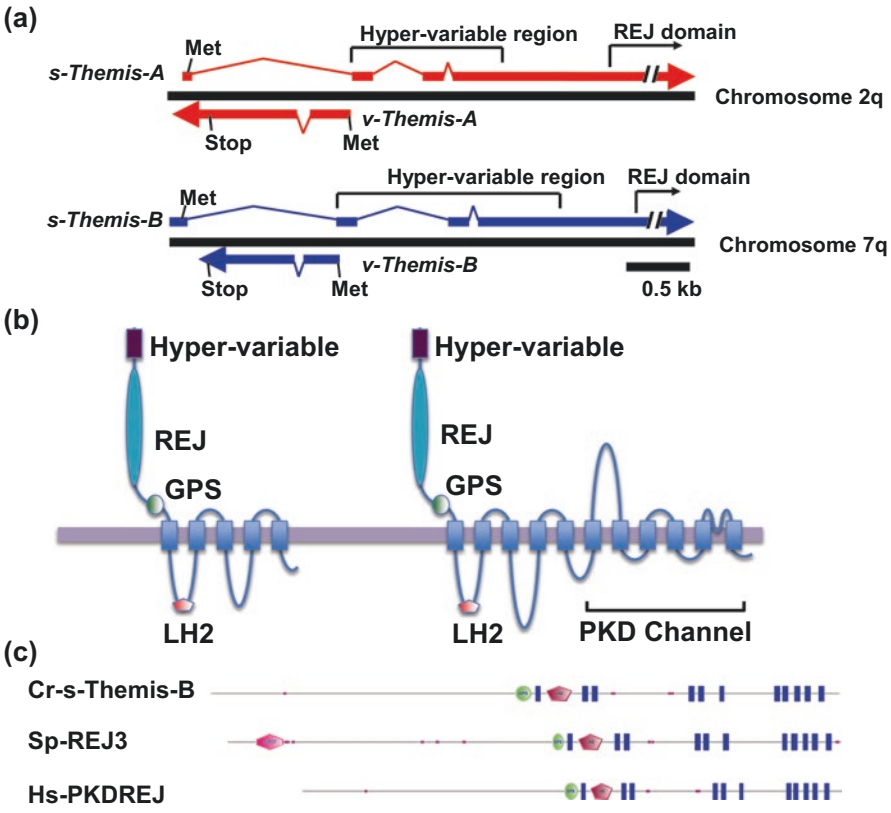

Fig. 23.3 Molecular architectures and models of *s-Themis* and *v-Themis* genes and their gene products in *Ciona robusta.* (**a**) A pair of *s-Themis-A* and *v-Themis-A* genes resides in locus A in chromosome 2q, while a pair of *s-Themis-B* and *v-Themis-B* genes resides in locus B in chromosome 7q. In both loci, *v-Themis* genes are located at the first introns of *s-Themis* genes but are transcribed in the opposite directions. (**b**) Schematic representations of s-Themis-A and s-Themis-B. Both proteins contain N-terminal hyper-variable regions, REJ (receptor for egg-jelly) domains, GPS (G-protein coupled proteolysis site) domains, and LH2 (lipoxygenase homology 2) domains. s-Themis-A possesses C-terminal 5 transmembrane domains, while s-Themis-B possesses 11 transmembrane domains including 6 transmembrane domains homologous to the TRP-type cation channel domain. (**c**) *Ciona robusta* s-Themis-B showed homology to human PKDREJ, a PKD1 family protein specifically expressed in the testis, and also to sea urchin *(Strongylocentrotus purpuratus)* Sp-REJ3, an REJ family protein specifically expressed in the testis

with PKD2 and PKD2L1, both are expressed in the testis (Sutton et al. 2006). PKDREJ is not essential for fertility, but regulation of the acrosome reaction is partially affected in *PKDREJ*-defective mouse spermatozoa (Sutton et al. 2008). Therefore, it seems likely that PKDREJ or PKDREJ-like proteins are widely involved in deuterostome fertilization.

In ascidians, a small “acrosome” (Fukumoto 1988; De Santis et al. 1980; Rosati and De Santis 1978) and “apical substance” (Fukumoto 1988) have been observed at

the tip of the sperm head. However, it is unclear whether the ascidian sperm acrosome is functionally homologous to the acrosome in mammalian and sea urchin spermatozoa and whether the "acrosome reaction" or vesicular exocytosis takes place during the binding of sperm to the VC (De Santis et al. 1980; Rosati and De Santis 1978) or prior to sperm-egg fusion after sperm penetration of the VC (Fukumoto 1988, 1990a, b, c). Instead of the acrosome reaction, the sperm reaction, which is characterized by vigorous movement and mitochondrial translocation and eventual shedding through the sperm flagella, is a well-known phenomenon in ascidian sperm induced by Ca^{2+} influx (Lambert and Koch 1988). Therefore, it is thought that ascidian spermatozoa may undergo the sperm reaction by controlled Ca^{2+} influx, in which s-Themis-B might also be involved, and that acute and drastic Ca^{2+} influx may occur upon the interaction of s/v-Themis-A and -B of the same haplotypes. During evolution, hermaphrodite ascidians might have adopted a PKDREJ-like protein, s-Themis-B, as an allo-recognition protein in addition to a gamete-interacting protein.

Within locus B, genome sequence data showed one additional pair of *s-Themis* and *v-Themis* genes, residing about 60 kbp apart from *s-* and *v-Themis-B* genes (unpublished data). We recently determined the DNA sequence in this region after cloning from a genome library derived from one individual. The data confirmed the existence of a novel pair of *s/v-Themis* genes, which were tentatively referred to as *s-* and *v-Themis-B2* (unpublished data). The sequences of this gene pair were almost identical to those of the pair of *s/v-Themis-B* except for the *v-Themis-B2* region and a hyper-variable region of *s-Themis-B2* (see Fig. 23.3b). Further genetic and biochemical studies on the expression and functions of s/v-Themis-B2 as well as s/v-Themis-A and -B are needed to clarify the roles of these gene products in *C. robusta* self-sterility.

As described in the previous section, it is proposed that a self/nonself-recognition factor is produced in follicle cells, hydrolyzed by the proteasome, and then attached to the VC during oogenesis (Marino et al. 1998, 1999; Pinto et al. 1995). In connection with this, it is intriguing to investigate the expression timing and expressing cells of *v-Themis*. Our preliminary data by RT-PCR showed that *v-Themis-A* mRNA is expressed in the ovary and ovarian eggs but not in mature eggs including follicle cells (unpublished). Furthermore, *v-Themis-A* mRNA appears to be expressed in the germinal vesicle-containing immature oocytes on the basis of *in situ* hybridization, though we cannot rule out a possibility for its expression in follicle cells (unpublished). Since follicle cells in immature oocytes appear to play a key role in the acquisition of self-sterility (Pinto et al. 1995), it is plausible that v-Themis proteins might be processed and attached to the surface of the VC with the aid of follicle cells.

23.5 Candidate Molecules Involved in Self-Sterility and Gamete Interaction in *H. roretzi*

In *H. roretzi*, in contrast to *C. robusta*, follicle cells are indispensable for fertilization. Follicle cells suspended around follicle-free eggs were not effective. Therefore, attachment to the VC was indispensable for successful fertilization. In order to

clarify whether follicle cells are responsible for self/nonself-recognition between gametes in *H. roretzi*, Fuke (1983) prepared mosaic eggs in which follicle cells had been removed by treatment with EGTA, and follicle cells from different individuals were attached by increasing the Ca^{2+} concentration. This experiment found that the self-sterility in *H. roretzi* depended on the VC and not the follicle cells.

In addition, it has been reported that follicle cells are involved in the acquisition of self-sterility during oocyte maturation, since follicle-free immature oocytes failed to acquire self-sterility after GVBD (Fuke and Numakunai 1996). Previous studies showed that a proteinaceous trypsin inhibitor such as soybean trypsin inhibitor can inhibit GVBD (Sakairi and Shirai 1991) as well as the acquisition of self-sterility during oocyte maturation (Fuke and Numakunai 1999). Furthermore, pancreatic trypsin can make the eggs self-sterile during the follicle-free oocyte maturation (Fuke and Numakunai 1999). Therefore, a certain trypsin-like protease may be released from follicle cells and play a key role in the formation of self-sterile eggs during oocyte maturation.

As in *C. robusta*, putative self/nonself-recognition factors may be detached from the VC by acidic conditions (pH 2–3, 1 min) in *H. roretzi*. To test this possibility, we isolated VCs from immature and mature oocytes and subjected them to SDS-PAGE to compare the protein compositions. The results showed that the amount of a 70-kDa main component, HrVC70, in the VC of mature oocytes was much larger than in the VC of immature oocytes (Sawada et al. 2002), suggesting that the amount of HrVC70 attached to the VC was increased during oocyte maturation. HrVC70 was extracted from the isolated VC by 1–10 mM HCl, which coincided with the fact that acid treatment of the VC allowed self-fertilization. In addition, nonself-sperm rather than self-sperm efficiently bound to HrVC70-immobilized beads, and HrVC70 isolated from nonself-eggs more strongly inhibited fertilization than did HrVC70 from self-eggs (Sawada et al. 2004). cDNA cloning of *HrVC70* was carried out and *HrVC70* was found to be expressed as a 120-kDa precursor HrVC120 exclusively in gonads. Whereas HrVC120 consists of 13 EGF (epidermal growth factor)-like repeats and a C-terminal ZP (zona pellucida) domain, HrVC70 consists of 12 EGF-like repeats, as determined by N- and C-terminal protein sequencer and LC-MS/MS analyses. *HrVC70* showed polymorphisms among individuals, having synonymous and non-synonymous substitutions. These mutations are unlikely to be random and are restricted to regions between the third and fourth Cys residues in each EGF domain and at the EGF-domain-connecting regions (Sawada et al. 2004). Fewer replacements were observed in *HrVC70* than in *s/v-Themis-A* and *-B*. However, two or three amino acid substitutions in HrVC70 might be sufficient to cause significant change in protein-protein interacting ability, since even a single amino acid replacement in Notch, another EGF-like repeat protein, can cause Notch-signaling diseases (Artavanis-Tsakonas et al. 1995). From these results, HrVC70 is considered to be a promising candidate for self/nonself-recognition protein during fertilization in *H. roretzi* (Fig. 23.4).

As sperm-borne binding partners for HrVC70, HrTTSP-1 (type-II transmembrane serine protease) and HrUrabin (*u*nique *RA*FT-derived *bin*ding partner for

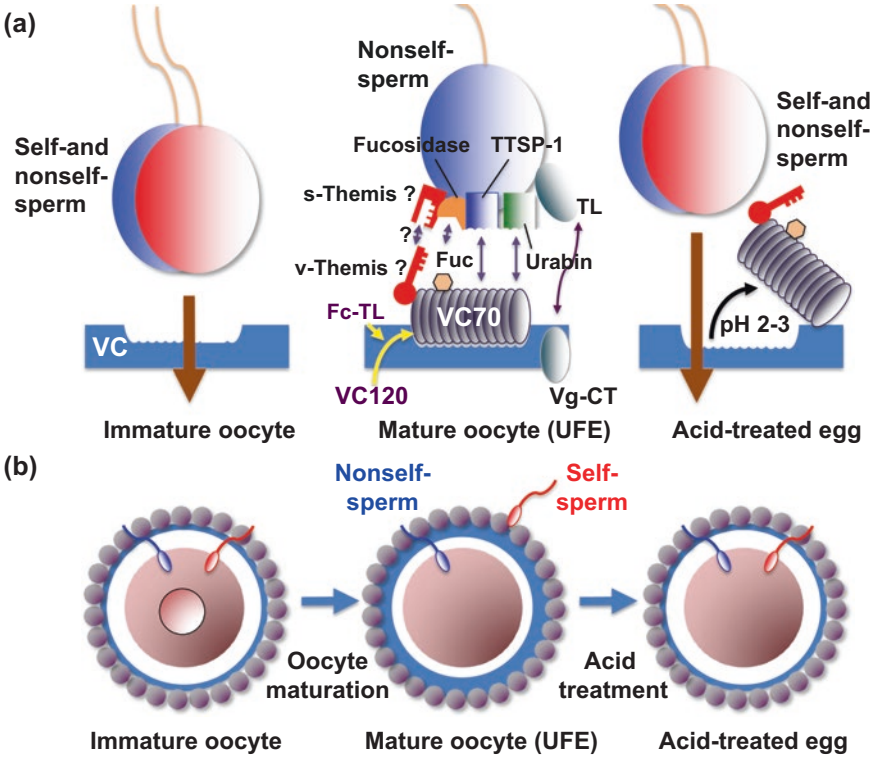

Fig. 23.4 Working hypothesis of sperm and VC molecules involved in gamete interaction and self/nonself-recognition in *Halocynthia roretzi*. Germinal vesicle-containing immature oocytes in the gonads are self-fertile (a and b, left). Spontaneously matured oocytes or released eggs are self-sterile (a and b, middle). HrVC70, consisting of 12 EGF-like repeats, which is expressed by oocytes as its precursor HrVC120 and is processed by a trypsin-like protease, appears to attach to the VC during oocyte maturation (coinciding with the acquisition of self-sterility). HrVC70 exhibits polymorphism among individuals. Acid treatment (pH 2-3) of eggs results in both elution of HrVC70 and self-fertility (a and b, right). Moreover, isolated HrVC70 is able to interact with nonself-sperm rather than self-sperm. Therefore, HtVC70 is thought to be responsible for self/nonself-recognition during fertilization in *H. roretzi*. Allo-recognizing molecular interactions may be supported by interactions between other gamete proteins, such as the interaction between sperm fucosidase and fucose residues in the VC, and between HrVC70 and sperm HrUrabin and HrTTSP-1. The L-chain of spermosin and CUB domains of trypsin-like protease (TL) appear to interact with a C-terminal fragment of vitellogenin (*Vg-CT*) attached to the VC. v-Themis might also be associated with HrVC70 on the VC. These interactions may support the gamete interaction and/or self/nonself-recognition process. The polymorphisms in *v-Themis* genes are found in the Aniseed genome database. s/v-Themis-mediated interaction might also play a role in self/nonself-recognition in *H. roretzi*

HrVC70: a GPI-anchored CRISP-family protein) have been identified by yeast two-hybrid screening (Harada and Sawada 2007) and Far-Western analysis, respectively (Urayama et al. 2008). The molecular mass of HrTTSP-1 was estimated to be 337 kDa, containing 23 CCP/SCP/Sushi-domains, three ricin B domains and one CUB

domain in its extracellular region. HrTTSP-1 is capable of binding to HrVC70 and contains several domains potentially involved in protein-protein interaction. However, the biological roles of these domains, particularly in the process of self/nonself-recognition, remain elusive.

HrUrabin must play some roles in self/nonself-recognizing gamete interaction because an anti-HrUrabin antibody potently inhibited self-sperm and nonself-sperm binding to HrVC70-agarose beads as well as fertilization (Urayama et al. 2008). Since HrUrabin has little or no polymorphism among individuals and since it showed no apparent difference between the binding ability to HrVC70 from self-eggs and that from nonself-eggs, HrUrabin may indirectly take part in discrimination between self- and nonself-HrVC70.

An orthologous gene of the *HrVC70* precursor, *HrVC120*, which is referred to as *HaVC130*, has been identified in *Halocynthia aurantium*, a close relative species to *H. roretzi*, inhabiting the northern part of Japan and the west coast of North America. The mature protein designated as HaVC80 is made up of 13 EGF-like repeats (one repeat longer than HrVC70) on the basis of sequence analogy at the C-terminal processing site of HrVC70. The precursor protein HaVC130 consists of 14 EGF-like repeats and a C-terminal ZP domain (Ban et al. 2005). HrVC120 is very similar to HaVC130 (83.4% identity in amino acids), but the 8th EGF domain of the *HrVC120* gene appears to have been duplicated in *HaVC130* during evolution. Generally, most sperm receptor proteins on the egg coat are made up of a repetitive structure of a certain domain or unit: for example, VERL (vitelline envelope receptor for lysin) in the abalone egg vitelline coat and EBR1, a binding receptor in the sea urchin egg envelope, consist of repeated structures of a certain unit or domain, and these molecular architectures are thought to be related to the rapid evolution of gamete recognition proteins (Vacquier and Sawanson 2011; Kamei and Glabe 2003). HaVC80 is also polymorphic among individuals in regions between the first and second Cys residues, between the third and fourth Cys residues, and the EGF-domain-connected regions, where similar polymorphisms are observed in HrVC70 (Ban et al. 2005). Since the mutation sites of *HrVC70* and *HaVC80* among individuals do not occur randomly, these nucleotide substitutions are unlikely to be single nucleotide polymorphisms (SNPs) introduced by random point mutations.

A genome database of *H. roretzi* is currently available (Brozovic et al. 2015). According to this database, there appear to be four homologous gene pairs of *s*- and *v-Themis* in *Halocynthia roretzi* (unpublished data), although it is still unclear how many multi-allelic gene loci exist in *H. roretzi.* Similarly, *H. aurantium* also possesses *s/v-Themis* homologs (unpublished data). *s*- and *v-Themis* genes are unlikely to be classified into A- or B-type *s/v-Themis* as they are in *C. robusta*. However, at least the sequences of *v-Themis* genes in *H. roretzi* are highly variable among individuals, and some alleles were identified in cDNAs prepared from the ovary (unpublished). Further studies are needed to elucidate the participation of s/v-Themis in *H. roretzi* fertilization, although it is plausible to consider *s/v-Themis* genes as candidates for self-sterility determinants in *H. roretzi.*

Besides allo-recognition-related proteins, several other gamete proteins are also involved in sperm-egg interaction in *H. roretzi.* A Pro-rich region in the light chain

of spermosin, a sperm trypsin-like protease with narrow substrate specificity, can interact with the C-terminal region of vitellogenin associated with the VC (Akasaka et al. 2010, 2013).

Carbohydrate moieties of gamete glycoproteins and their interacting proteins such as lectins or glycosidases are candidate molecules involved in sperm-egg interaction (Lambert and Koch, 1988). It is thought that sperm 54-kDa α-L-fucosidase is involved in the binding of sperm to the VC, since the antibody against 54-kDa α-L-fucosidase inhibited fertilization (Matsumoto et al. 2002). The optimum pH of sperm fucosidase is around pH 4–5, but this enzyme functions as a sugar-binding protein by making an enzyme-substrate complex under seawater conditions (pH ~8). On the other hand, egg 65-kDa *N*-acetylglucosaminidase appears to participate in the establishment of a polyspermy block (Lambert 1989). *N*-acetylglucosaminidase is released from the egg upon fertilization. The purified enzyme was capable of binding to the VC in a GlcNAc-specific manner, which was specifically inhibited by an antibody raised against purified *N*-acetylglucosaminidase (Matsuura et al. 1993). A 70-kDa inhibitor against the egg *N*-acetylglucosaminidase was purified from the VC (Matsuura et al. 1995). This inhibitor might be HrVC70. Sperm α-L-fucosidase must support the binding of sperm to HrVC70 on the VC via the fucose residue in the carbohydrate moiety of HrVC70 in addition to HrUrabin and HrTTSP-1. Since HrVC70 possesses fucose and *N*-acetylhexosamine residues (unpublished), *N*-acetylglucosaminidase released from the fertilized eggs could bind to HrVC70 via the GlcNAc residues, which may in turn interfere with the binding of sperm fucosidase to HrVC70, resulting in the block to polyspermy.

23.6 Conclusion

Gamete proteins involved in self/nonself-recognition and sperm-egg interaction in *C. robusta, H. roretzi*, and *H. aurantium* are summarized in Table 23.1. In *C. robusta*, s-Themis-A, B, and B2 in the sperm plasma membrane recognize v-Themis-A, B, and B2 in the VC, respectively. If these pairs are from the same haplotypes, spermatozoa will detach from the VC or decrease the sperm motility to prevent self-fertilization. Although this appears to play a central role in self-sterility of *C. robusta*, the self-sterility system in *H. roretzi* appears to be different. In fact, the self-sterility in *H. roretzi* is more strictly controlled compared to the system in *C. robusta* under natural conditions. Self-fertilization is observed in *C. robusta* by prolonged incubation with self-sperm, but self-fertilization has never been observed in *H. roretzi* even in the presence of large quantities of sperm added to the eggs and with prolonged incubation. In *H. roretzi*, we proposed that HrVC70 on the VC and sperm HrTTSP1 and HrUrabin are responsible for self-sterility. However, it is still unclear whether the polymorphisms of HrVC70 are recognized by a certain sperm protein in order to establish self-sterility. s-Themis on the sperm surface membrane and v-Themis on the VC may play a key role in *H. roretzi*. Further studies are

Table 23.1 Proposed gamete proteins involved in self/nonself-recognition and gamete interaction in ascidians

Species and events	Sperm proteins	Egg/vitelline coat (VC) proteins
Ciona robusta		
Self/nonself-recognition	s-Themis-A, -B, -B2	v-Themis-A, -B, -B2
	Trypsin-like protease	v-Themis-like
Gamete interaction	70-kDa protein	Apolipoprotein B-like
	Urabin	VC57
	α-L-Fucosidase	Fucose (VC glycoprotein)
	ND	(Gln-enriched VC peptides,
		Cihsp70, vCRL1, etc.)
Ciona savignyi		
Self/nonself-recognition	s-Themis-A and -B	v-Themis-A and -B
Halocynthia roretzi		
Self/nonself-recognition	Urabin, TTSP-1	VC70
	s-Themis	v-Themis
Gamete interaction	Proacrosin, Spermosin	Vitellogenin (VC)
	α-L-Fucosidase	Fucose (VC70)
Halocynthia aurantium		
Self/nonself-recognition	ND	VC80
	s-Themis	v-Themis

ND not determined

necessary to demonstrate whether s- and v-Themis interaction or EGF-containing protein-urabin interaction or both play a pivotal role in self-sterility.

The self/nonself-recognition mechanism in *C. robusta* seems to be very similar to the self-incompatibility system in flowering plants. In flowering plants, self-incompatibility-determinant proteins are different in respective families. In Brassicaceae, pollen *SP11/SCP* and pistil *SRK* (S-receptor kinase) genes are highly polymorphic among alleles and the loci are tightly linked (Takayama and Isogai 2005; Iwano and Takayama 2012). Taking the proteins involved in fertilization into account, sexual reproductive strategies may be more common between animals and plants than was previously thought.

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Chapter 24
Universality and Diversity of a Fast, Electrical Block to Polyspermy During Fertilization in Animals

Yasuhiro Iwao and Kenta Izaki

Abstract In the sexual reproduction of animals, fertilization is indispensable for the initiation of diploid embryonic development. Most animals exhibit monospermy, in which only one sperm enters an egg during normal fertilization. In monospermic species, a fast, electrical block on the egg membrane is one of the most important blocks to polyspermy. A fertilizing primary sperm usually causes a positive-going fertilization potential to prevent the subsequent entry of excess sperm. An increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) in the egg cytoplasm induced by the fertilizing sperm is necessary for egg activation and blocks polyspermy. The mechanism of voltage-dependent fertilization in monospermic amphibians is presented as a model system of vertebrate fertilization. The electrical polyspermy blocks in various animals are reviewed and their universality and diversity across the animal kingdom are discussed. Relationships between the fast, electrical block and $[Ca^{2+}]_i$ increases in egg cytoplasm are discussed, as well as their changes throughout the course of animal evolution.

Keywords Fertilization potential · Fast polyspermy block · $[Ca^{2+}]_i$ increase · Egg activation · Sperm-egg fusion

24.1 Introduction

During sexual reproduction, a zygote nucleus is formed by the fusion of a sperm and an egg nucleus to produce a new diploid organism. In most animals, only one sperm fuses with an egg during normal fertilization; this is known as monospermy. However, a large number of sperm usually reach an egg to ensure the fertilization of

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all spawned eggs during external fertilization. This produces the risk of polyspermy; more than one sperm entering an egg during fertilization. As polyspermic eggs typically undergo abnormal cleavage and die shortly after fertilization, animals have evolved several elaborate mechanisms to exclude excess sperm during fertilization, i.e., blocks to polyspermy. First, animals adequately regulate the number of sperm simultaneously reaching the egg. In external fertilization of monospermic frogs, for example, insemination with a relatively high concentration sperm suspension (at least 10^5 cells/ml) is necessary to achieve fertilization. In this case, more than one thousand sperm can reach the outside of the extracellular coats, jelly layers and vitelline envelope surrounding the egg membrane. However, it is estimated that approximately 20 sperm reach the egg membrane at an interval of 2–20 s over 20 min (Schlichter and Elinson 1981; Iwao 1987). The extracellular coats of amphibian eggs, therefore, play an important role in reducing the number of effective sperm and delaying the arrival of other sperm following fertilization by the primary sperm. The fast blocks to polyspermy at the egg membrane are elicited after the entry of the primary sperm, and are mediated by physical and/or biochemical changes in the properties of the egg membrane. Amongst them, the fast electrical block to polyspermy was originally demonstrated in the fertilization of sea urchins (Jaffe 1976). This block was subsequently identified in many monospermic animals including both vertebrates and invertebrates by intensive investigations for four decades (for reviews, Jaffe and Gould 1985; Gould and Stephano 2003; Iwao 2000a, 2012). Typically, the increase of the egg membrane potential to a positive level, i.e., a positive-going fertilization potential, prevents the entry of the next sperm. However, given that the fertilization potential is transient, this temporary block is followed by a permanent block through the formation of a fertilization envelope (membrane) mediated by cortical granule exocytosis, which is induced by an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in the egg cytoplasm. As sperm cannot pass through the fertilization envelope, a complete and permanent block to polyspermy is established after egg activation.

While sea urchins have been traditionally used as models in the study of fertilization in marine invertebrates, amphibians, whose fertilization occurs externally in fresh water, provide an excellent model of vertebrate fertilization. Additionally, amphibians comprise two groups with distinct systems of fertilization (Elinson 1986; Iwao 2000a, 2012). Most frogs and some primitive salamanders exhibit monospermy, whereas higher newts exhibit physiological polyspermy in which several sperm enter an egg during normal fertilization. In the latter, only one sperm nucleus is selected to fuse with the egg nucleus and form a zygote, while excess sperm nuclei degenerate without participating in embryonic development. Thus, the development of a diploid genome derived from a male and a female is ensured by recruitment of several blocks to polyspermy. As vertebrate polyspermy blocks are closely linked to egg activation induced by an increase of $[Ca^{2+}]_i$ in the egg cytoplasm, we have reviewed the different mechanisms of egg activation between monospermic and physiologically polyspermic vertebrates species (Iwao 2012). This review will first focus on the fast, electrical block to polyspermy at the level of the egg membrane in amphibians, and then compare this with other vertebrate and

invertebrate species across the animal kingdom. We refer to the $[Ca^{2+}]_i$ increase at fertilization only in reference to the fast block to polyspermy, as the important roles and molecular mechanisms of the $[Ca^{2+}]_i$ increase during egg activation have been well-reviewed elsewhere for various animal species (e.g., Stricker 1999; Runft et al. 2002; Miyazaki 2006; Whitaker 2006, 2008; Kashir et al. 2013). Finally, we consider the evolutionary processes and consequences of variations in the electrical block to polyspermy during animal fertilization.

24.2 A Model System of Vertebrate Fertilization: Egg Activation by $[Ca^{2+}]_i$ Increases and Blocks to Polyspermy in Amphibians

24.2.1 Differential Mechanisms of Egg Activation Between Monospermic and Physiologically Polyspermic Amphibians

The class Amphibia (phylum Chordata, subphylum Vertebrata) contains the orders anurans (frogs and toads) and urodela (newts and salamanders). In the majority of anurans, monospermic fertilization occurs externally with coupling between a male and a female occurring in fresh water (Elinson 1986; Iwao 2000a, 2012). In contrast, most urodeles exhibit physiological polyspermy (Iwao 2000a, 2012), with exception of a monospermic primitive salamander, *Hynobius nebulosus* (Iwao 1989). In the physiologically polyspermic urodeles, eggs are inseminated by the sperm released from female spermatheca, in which the sperm transmitted by a male are stored (Akiyama et al. 2011). In the African Clawed Frog, *Xenopus laevis*, which has been used in experiments worldwide, ovulated eggs are surrounded with a thin vitelline envelope (VE) approximately 5 μm in thickness, consisting of several glycoproteins (Fig. 24.1; for reviews, Kubo 2005; Hedrick 2008). An acrosome reaction-inducing substance in *Xenopus* (ARISX) is secreted on the VE during passage through the upper-most region of the oviduct (Ueda et al. 2002, 2003), and subsequently three layers of jelly (J_1–J_3) are secreted outside the VE (Fig. 24.1). Mature unfertilized eggs are deposited into fresh water at mating and then inseminated by the sperm. The sperm are activated by a chemotaxis protein in outer jelly layers, allurin, and guided to the VE (al-Anzi and Chandler 1998; Olson et al. 2001; Burnett et al. 2011). After undergoing the acrosome reaction in response to ARISX, the sperm pass through the VE probably with enzymatic assistance from matrix metalloproteinase 2 (MMP-2) (Kubo et al. 2008; Iwao et al. 2014), and reach the egg membrane (Fig. 24.1). Observations using scanning electron microscopy demonstrate that the penetration of *Xenopus* sperm into the egg membrane occurs at the tip of the sperm head in a perpendicular manner (Boyle et al. 2001).

In monospermic frog eggs, a single, transient increase of $[Ca^{2+}]_i$ in the egg cytoplasm is induced by the fertilizing sperm (for reviews, Iwao 2000a, 2012). In

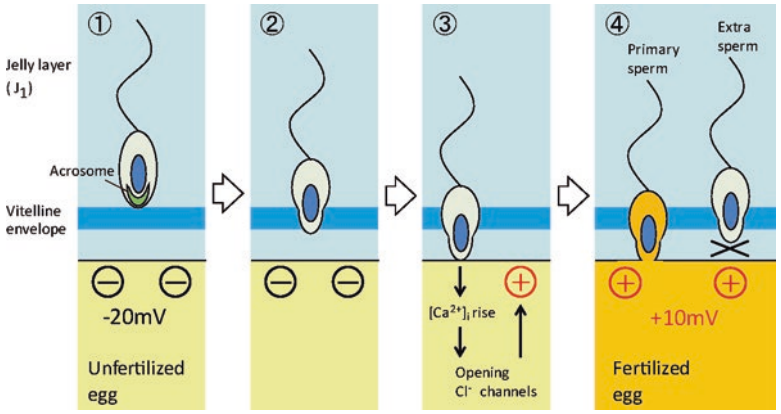

Fig. 24.1 A schematic diagram showing the process of a fast, electrical block to polyspermy during monospermic fertilization of the frog, *Xenopus laevis*

Xenopus, an increase in $[Ca^{2+}]_i$ is initiated at the sperm entry site on the animal hemisphere and spreads over the entire egg surface as a Ca^{2+} wave (Fig. 24.2, Busa and Nuccitelli 1985; Grandin and Charbonneau 1991; Nuccitelli et al. 1993; Fontanilla and Nuccitelli 1998; Iwao et al. 2014). The $[Ca^{2+}]_i$ in unfertilized eggs (0.3 μM) increases to a peak of 1.2–2.2 μM after fertilization. As the velocity of Ca^{2+} wave is 8.5–8.9 μm/s in the egg cortex (Fig. 24.2, Fontanilla and Nuccitelli 1998), it takes approximately 3 min for the Ca^{2+} wave to reach the opposite side of the egg. Relatively high $[Ca^{2+}]_i$ is maintained for approximately 10 min following fertilization. Initiation of the $[Ca^{2+}]_i$ increase is mediated by the serial activation of Src protein kinase (Src) and phospholipase Cγ (PLCγ) to produce inositol 1,4,5-trisphosphate (IP3) around the sperm entry site after the shedding of Uroplakin III (UPIII) from the egg surface in response to sperm interaction (Sakakibara et al. 2005, Mahbub Hasan et al. 2005, 2007, see also Chap. 27). The Ca^{2+} wave is caused by the propagative release of Ca^{2+} from endoplasmic reticulum (ER) through the sensitization of IP3-receptors and/or the stimulation of PLCγ by the local $[Ca^{2+}]_i$ increase.

Although it has not yet been determined whether the initiation of the increase in $[Ca^{2+}]_i$ occurs before or after sperm-egg membrane fusion, the exocytosis of cortical granules induced by the $[Ca^{2+}]_i$ increase is observed approximately 20 μm away from the site of the sperm head attachment on the egg membrane (Boyle et al. 2001), indicating that the $[Ca^{2+}]_i$ increase occurs soon after the interaction of the sperm head tip with the egg membrane. A leading hypothesis to account for egg activation during *Xenopus* fertilization is that the fertilizing sperm binds to a receptor on the egg plasma membrane, which transmits a signal to induce the $[Ca^{2+}]_i$ increase (Iwao 2000b, 2012). Egg activation accompanied by a $[Ca^{2+}]_i$ increase is caused by treatment with peptides containing Arg-Gly-Asp (RGD) residues, known as a ligand for integrins (Iwao and Fujimura 1996), or with those from the disintegrin domain of

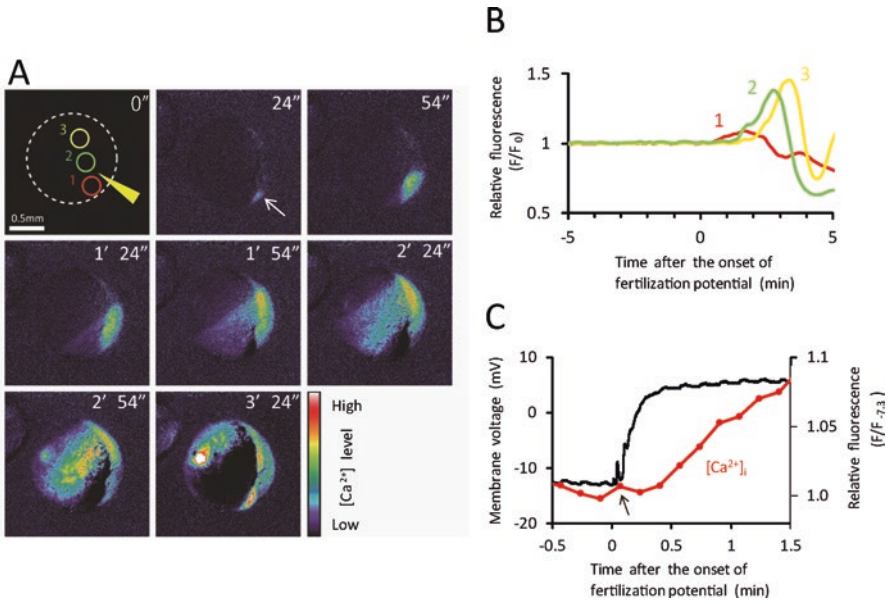

Fig. 24.2 A typical increase in $[Ca^{2+}]_i$ in the monospermic *Xenopus* egg pre-injected with Ca^{2+}-sensitive fluorescence dye of Oregon Green 488 BAPTA-1, concomitantly with the recording of egg's membrane potential, showing propagation of a Ca^{2+} wave from the sperm entry site lasting about 3 min with a rate of 8.5 μm/s (**A** and **B**). Graphs 1 – 3 in B show the changes in fluorescence intensity in ROIs 1 – 3 in A, respectively. A positive-going fertilization potential was elicited approximately at the initiation of a very small $[Ca^{2+}]_i$ increase (arrow in **C**) followed by a large and propagative Ca^{2+} wave. The changes of $[Ca^{2+}]_i$ and membrane potential were measured every 5 s and 5 ms, respectively. Arrow head, the position of microelectrode; arrow, the initiation of a Ca^{2+} wave

the metalloprotease/disintegrin/cysteine-rich family 16 (xMDC16) (Shilling et al. 1998), suggesting the involvement of integrins on the egg membrane in signal transduction during fertilization. As phosphorylation and activation of Src localized in membrane micro-domains (MD, membrane rafts) is induced by treatment with RGD peptides (Sato et al. 1999, 2003), the peptides stimulate the Src-PCLγ cascade. Recently, the peptide corresponding to a partial amino acid sequence of a hemopexin domain (HPX) of metalloproteinase-2 (MMP-2: HPX peptide, GMSQIRGETFFFK) was shown to cause egg activation accompanied by a $[Ca^{2+}]_i$ increase (Iwao et al. 2014), and the treatment of eggs with anti-HPX antibody was shown to inhibit fertilization. The arginine residue of the HPX peptide is important for the induction of egg activation via the transmission of the signal on the egg membrane, as in the activation by RGD peptides (Iwao and Fujimura 1996). The sperm also contains a tryptic protease activity against a GRR sequence (Iwao et al. 1994, Mizote et al. 1999) and the cleavage of the GRR sequence on UPIII causes egg activation through Src activation (Mahbub Hasan et al. 2005). Although the precise mechanisms need to be investigated, the interaction of the HPX domain of

MMP-2 and/or the tryptic protease on the sperm membrane with the components of MD on the egg membrane, plays the most important role in the earliest signal transduction of the $[Ca^{2+}]_i$ increase during *Xenopus* fertilization. In addition, a major involvement of phosphatidic acid (PA), produced by phospholipase D1b from phosphatidylcholine (PC), has been proposed in the activation of Src and PLCγ during *Xenopus* fertilization (Bates et al. 2014; Stith 2015). It is important to understand how the fertilizing sperm stimulates the production of PA following the interaction between the sperm-egg membranes. Given that a fertilizing sperm does not contain sufficient sperm factor in its cytoplasm for egg activation (Harada et al. 2011), a model whereby a sperm introduces factors into the egg to induce the $[Ca^{2+}]_i$ increase after sperm-egg fusion is unlikely for *Xenopus* egg activation.

During fertilization of the physiologically polyspermic newt, *Cynops pyrrhogaster*, 2–20 sperm enter an egg across the whole egg surface (Iwao et al. 1985; Iwao and Elinson 1990). A Ca^{2+} wave is initiated in the egg cytoplasm around each sperm entry site and some waves are preceded by a spike-like $[Ca^{2+}]_i$ increase (Harada et al. 2011). As each Ca^{2+} wave spreads across approximately one-quarter of the egg's surface, the increases in $[Ca^{2+}]_i$ spreads across the whole egg surface approximately 40 min after fertilization. Although the initial spike-like $[Ca^{2+}]_i$ increase is due to the sperm protease on the egg surface (Harada et al. 2011), the Ca^{2+} wave necessary for egg activation is mainly induced by a soluble sperm factor derived from sperm cytoplasm. Injection of sperm extract causes a similar $[Ca^{2+}]_i$ increase to that induced by sperm (Yamamoto et al. 2001; Harada et al. 2007; Ueno et al. 2014), and the major component of sperm factor has been identified as a sperm-specific form of citrate synthase (CS) (Harada et al. 2007, 2011). Sperm-specific CS is highly phosphorylated and binds the component containing microtubules and IP3 receptors (Ueno et al. 2014). After sperm entry, the sperm CS localized in the mid-piece region disperses in the egg cytoplasm, and subsequently PLCγ in the egg cytoplasm accumulates around the mid-piece region, in association with the sperm CS. The sperm CS forms a complex of microtubules, ER with the IP3 receptor and PLCγ around the sperm entry site (Ueno et al. 2014). As the microtubules are necessary for the propagation of Ca^{2+} waves, the whole complex is necessary for the maintenance of the Ca^{2+} waves. Although sperm CS may produce acetyl CoA and oxaloacetate, which can trigger the Ca^{2+} increase in the egg cytoplasm (Harada et al. 2011), the precise mechanism of ignition of Ca^{2+} increase remains unknown. Interestingly, the sperm of monospermic *Hynobius* not only transmit a signal for egg activation through an egg membrane receptor, but also contain a large amount of CS outside their mitochondria (Iwao 2014). The extra-mitochondrial localization of CS in the sperm may have been acquired at the transition between monospermy and physiological polyspermy in vertebrate evolution, as described below.

24.2.2 The Fast, Electrical Block to Polyspermy in Monospermic Species

Monospermy in anuran amphibians is ensured by several polyspermy blocks operating in the fertilized egg, as previously described. A fast but temporary polyspermy block occurs on the egg membrane, because the fertilization of the denuded eggs surrounded only by the egg membrane is monospermic (Elinson 1973; Katagiri 1974; Stewart-Savage and Grey 1984; Mizote et al. 1999). The fast, electrical block is dependent upon a positive fertilization potential in monospermic anurans and primitive urodeles (Cross and Elinson 1980; Grey et al. 1982; Iwao 1989). In *Xenopus*, when an unfertilized egg with negative membrane potential of approximately −18 mV is inseminated, the positive-going fertilization potential of +3 – +10 mV is rapidly elicited several minutes after insemination in a solution containing about 30 mM NaCl (Fig. 24.3A, Grey et al. 1982; Webb and Nuccitelli 1985). The positive fertilization potential is mediated by a wave-like opening of Cl^- channels on the egg membrane (Jaffe et al. 1985; Kline and Nuccitelli 1985) in response to the Ca^{2+} wave spreading from the sperm entry site, accompanied by the opening of K^+ and Na^+ channels in the frog, *Rana pipiens* (Jaffe and Schlichter 1985; Jaffe et al. 1985).

The positive fertilization potential functions as the fast block to polyspermy in anurans, as supported by the following evidence: (1) When the membrane potential of unfertilized eggs is held stable at positive levels (i.e., more than approximately 0 mV) by voltage-clamping with two microelectrodes, both egg activation and sperm penetration are prevented in *Xenopus* (Fig. 24.3B, D, Glahn and Nuccitelli 2003; Iwao et al. 2014). No significant change in $[Ca^{2+}]_i$ is observed during clamping at the positive potential (Fig. 24.4). (2) When the membrane potential of unfertilized eggs is held at −10 mV to −70 mV, a large amount of inward current (Cl^--current) is observed accompanied by $[Ca^{2+}]_i$ increases (Fig. 24.3C, D, Glahn and Nuccitelli 2003; Iwao et al. 2014). (3) When the holding potential is shifted from +10 to −20 mV 10 min after insemination when several sperm have reached the egg membrane but fertilization is blocked, $[Ca^{2+}]_i$ increases are initiated simultaneously at different sites on the animal hemisphere soon after the voltage-shift and each increase propagates as a Ca^{2+} wave (Fig. 24.4). Thus, the membrane potential regulates both the initiation of $[Ca^{2+}]_i$ increase and sperm penetration. (4) The voltages of positive fertilization potential induced by sperm are well-correlated with the voltages that prevent fertilization in various amphibian species (Fig. 24.3A, D and Table 24.1, Iwao 2000a). (5) The delay in the arrival of secondary sperm (2–20 s) is sufficient for the rising membrane potential to prevent the entry of extra sperm, as a relatively small number of sperm pass through jelly layers to reach the egg membrane in amphibians (Schlichter and Elinson 1981; Iwao 1987; Reinhart et al. 1998). (6) External halide ions cause negative-going fertilization potentials in order of periodic law ($I^- > Br^- > Cl^- > F^-$) (Cross and Elinson 1980; Grey et al. 1982; Webb and Nuccitelli 1985), which corresponds to the effectiveness in induction of polyspermy not only during fertilization in vitro (Cross and Elinson 1980; Grey et al. 1982), but

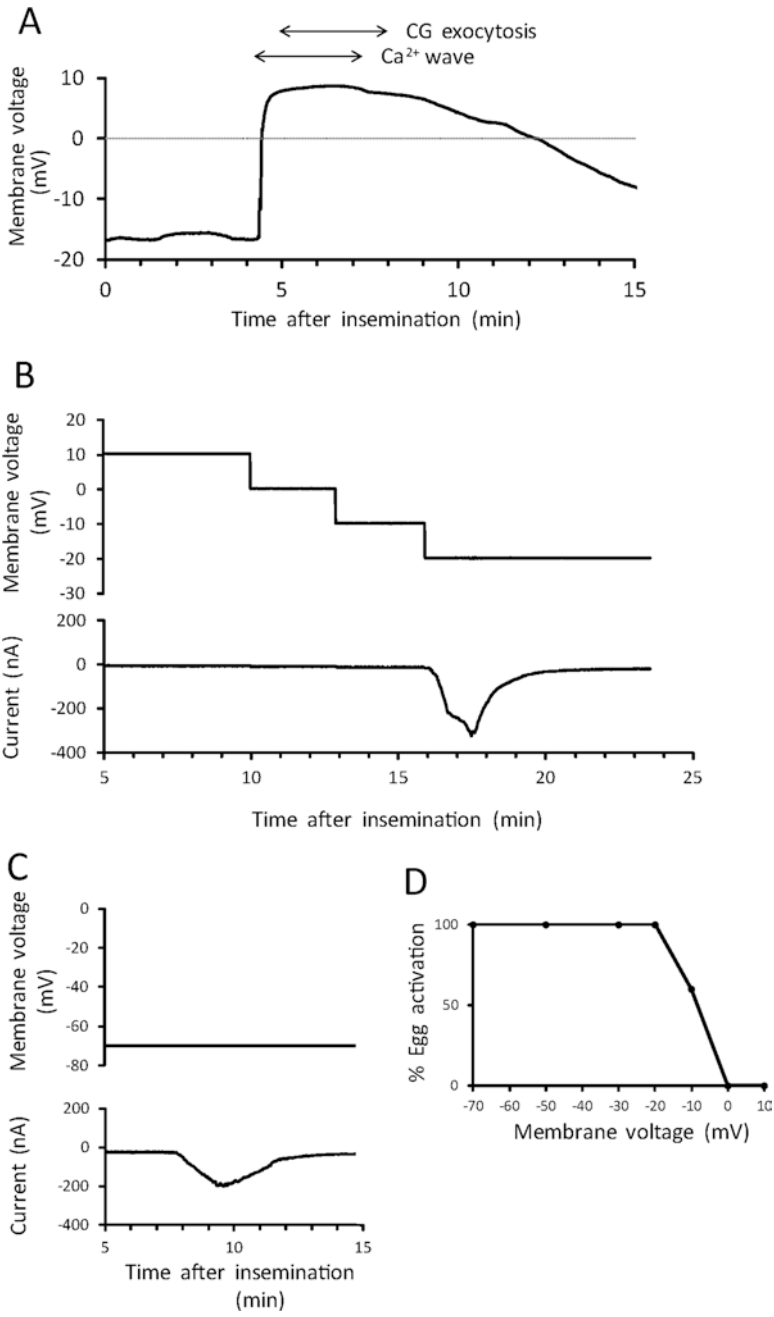

Fig. 24.3 (**A**) A typical positive-going fertilization potential of a *Xenopus* egg, showing the rise of membrane potential from −18 to +8 mV within about 2 s in 50% Steinberg's solution about 4 min after insemination. The positive potential had been maintained for about 8 min after fertilization.

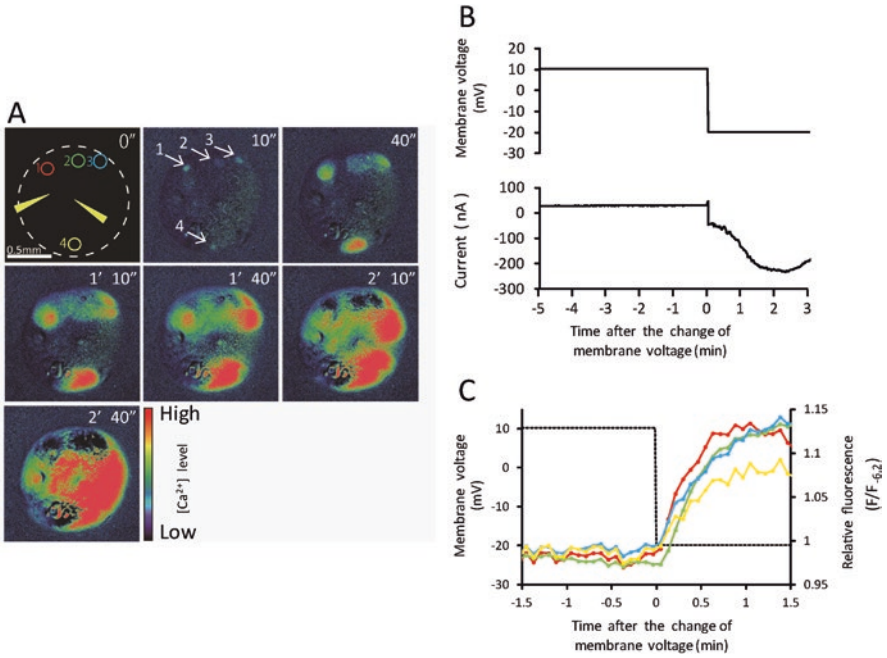

Fig. 24.4 Typical Ca^{2+} waves in a polyspermic *Xenopus* egg, concomitantly with the recording of membrane potential, showing propagation of a Ca^{2+} wave from each sperm entry site (**A**) just after the egg's membrane potential was shifted from +10 to −20 mV (**B** and **C**). Afertilization current was elicited approximately at the initiation of $[Ca^{2+}]_i$ increase (**C**). The changes of $[Ca^{2+}]_i$ and membrane potential were measured every 5 s and 5 ms, respectively. Allow heads, the position of glass microelectrodes for monitoring the membrane potential and the injection of current; arrows, the initiation of Ca^{2+} waves

also during natural mating (Grey et al. 1982). Thus, voltage-dependent fertilization ensures monospermy by a fast, electrical block to polyspermy in anurans.

Furthermore, the electrical polyspermy block in monospermic amphibians is supported by the correlation between voltage-dependent fertilization in monospermic species and the lack of voltage-sensitivity during fertilization in physiologically polyspermic species of urodeles. The eggs of monospermic *Hynobius* exhibit a

Fig. 24.3 (continued) "Ca^{2+} wave" and "CG exocytosis" show the period of spreading Ca^{2+} waves and exocytosis of cortical granules to form a fertilization envelope, respectively. (**B**) Voltage-dependent fertilization of a *Xenopus* egg whose membrane potential had been voltage-clamped at +10 mV and then inseminated. Ten min after insemination, the voltage was negatively shifted by incremental steps (10 mV-step, 3 min). A large inward current (Ca^{2+}-activated Cl^-- current, fertilization current) appeared just after the change to −20 mV. The polyspermic egg underwent multipolar cleavage. (**C**) Fertilization of the egg whose membrane potential had been clamped at −70 mV, showing a fertilization current about 7 min after insemination. (**D**) Voltage-dependent egg activation (fertilization) of *Xenopus*, no fertilization occurred at the voltages higher than 0 mV, but fertilization was not affected at the voltages from −20 to −70 mV

Table 24.1 Voltage-dependency and Ca^{2+}-increases during fertilization in various animals

Phylum	Typical genus	Mode of fertilization	Typical fertilization potential (mV)	Major ion channels of fertilization potential	Voltage preventing fertilization (mV)	Type of $[Ca^{2+}]_i$ increase	Type of egg activation
24.3.1 Radiata							
Cnidaria	*Cytaeis Hydractinia*	Mono	−64	–	None	Single	–
Ctenophora	*Beroe*	Poly	+38[a]	Na^+	–	–	–
24.3.2 Bilateria							
3.2.1 Protostomia:	Lophotrochozoan						
Nemertea	*Cerebratulus*	Mono	+44	Na^+	–	Cortical flash & repetitive	Sperm factor
Annelida	*Chaetopterus Pseudopotamilla*	Mono	+40	–	–	Single or repetitive	Sperm factor
	Urechis	Mono	+51	Na^+	> +25		Egg receptor/ acrosomal peptide
Mollusca	*Mytilus Supisula*	Mono	+60	Na^+	–	Cortical flash & repetitive	–
3.2.2 Protostomia:	Ecdysozoan						
Arthropoda	*Maia Sicyonia*	Mono	−80[b]	K^+	> −65 < −75	–	External Mg^{2+}
3.2.3 Deuterostomia							
Echinodermata							
Asteroidea	*Asterina Mediaster*	Mono	+12	Na^+	> −5	Cortical flash & single	–
Echinodea	*Lytechinus Strongylocentrotus*	Mono	+20	Ca^{2+}, Na^+	> −5 < −20	Cortical flash & single	Sperm factor or egg receptor
Chordata							
Urochordata	*Ciona Phallusia*	Mono	+28	Ca^{2+}, Na^+	> +10	Action potential & repetitive	Sperm factor

Phylum	Typical genus	Mode of fertilization	Typical fertilization potential (mV)	Major ion channels of fertilization potential	Voltage preventing fertilization (mV)	Type of $[Ca^{2+}]_i$ increase	Type of egg activation
Vertebrata							
Agnatha	*Lampetra*	Mono	+36	Cl^-	> +20	Single[c]	Egg receptor
Osteichthyes	*Oryzias*	Mono[d]	−78[b]	K^+	None	Single	–
Amphiba							
Anura	*Xenopus*	Mono	+8	Cl^-	> ± 0	Single	Egg receptor/ sperm proteases
	Discoglossus	Poly[e]	+20	Cl^-	None	Multiple[c]	–
Urodela	*Hynobius*	Mono	+47	Cl^-	> +40	Single[c]	Egg receptor (CS)[f]
	Cynops	Poly	−23[b]	–	None	Multiple	Sperm factor (CS)
Aves	*Coturnix*	Poly	–	–	–	–	Sperm factor (PLCζ, AH, CS)
Mammalia	*Mesocricetus Mus*	Mono	−80[b]	K^+	None	Repetitive	Sperm factor (PLCζ)

[a]Short-lived and multiple depolarization
[b]Hyperpolarization
[c]Based on Ca^{2+}-dependent Cl^- current
[d]Blocked by a micropyle
[e]Occasional polyspermy at an animal dimple
[f]Egg activation is caused by a signal through egg receptor, but with a large amount of CS in sperm cytoplasm
AH aconitate hydratase, *CS* citrate synthase, *PLCζ* phospholipase Cζ, – not determined

positive fertilization potential of +47 mV, mediated by the opening of Cl^- channels comparable to monospermic anurans (Iwao 1989). Fertilization is blocked at +40 mV (similar to the voltage of fertilization potential). In contrast, the eggs of polyspermic *Cynops* exhibit a very small amount of hyperpolarization, probably in response to each sperm entry (Iwao 1985). No change or a small negative-going shift in membrane potential is observed during polyspermic fertilization of the newt, *Pleurodeles waltl* and the Axolotl, *Ambystoma mexicanum* (Charbonneau et al. 1983). Given that the fertilization of *Cynops* and *Pleurodeles* is independent of the egg membrane voltage (Charbonneau et al. 1983; Iwao and Jaffe 1989), voltage-dependent fertilization is characteristic of monospermic species of amphibians.

24.2.3 Molecular Mechanisms of the Electrical Block to Polyspermy in Amphibians

To identify the component responsible for voltage-dependent fertilization, crosses between species with different voltage-sensitivities have been explored in marine animals (Jaffe et al. 1982, see *Urechis* fertilization). In amphibians, cross-fertilization between the eggs of voltage-insensitive *Cynops* and the sperm of voltage-sensitive *Hynobius* has been shown to be voltage-dependent (Table 24.2, Iwao and Jaffe 1989). In the cross between the voltage-sensitive species *Bufo japonicus* egg and *Hynobius* sperm, the voltage to prevent fertilization is comparable to that of the sperm providing species (Iwao and Jaffe 1989). Conversely, the sperm of a voltage-insensitive species (*Cynops* or *Notophthalmus* sperm) enter into an egg of a voltage-sensitive species (*Bufo* or *Xenopus* eggs) in a voltage-independent manner (Jaffe et al. 1983a; Iwao and Jaffe 1989; Iwao et al. 1994). A model of sperm voltage-sensing has been proposed in which a positively charged molecule on the sperm membrane is responsible for binding and/or fusion with the egg membrane (Iwao and Jaffe 1989). There are several candidate sperm molecules acting as the voltage-sensor during amphibian fertilization. Both *Xenopus* and *Hynobius* eggs are activated by external treatment with the RGDS peptide (pI 6.45) (Iwao and Fujimura 1996; Iwao 2014), or xMDC16 peptide (CRMPKTEC; pI 8.29) (Shilling et al. 1997). Egg activation by RGDS is insensitive to a positive potential (Iwao and Fujimura 1996), but xMDC16 peptide activates eggs in a voltage-dependent manner. The inhibition voltage (greater than +20 mV) is, however, higher than that of the sperm (±0 mV). As xMDC16 is localized only on the posterior region of the sperm head (Shilling et al. 1998), it is unlikely to be involved in the sperm-egg interaction during the earliest phases of fertilization.

In this regard, we have recently found that matrix metalloproteinase-2 (MMP-2) on the sperm surface is involved in voltage-dependent fertilization of *Xenopus* (Iwao et al. 2014). Although MMP activity seems to be involved in the sperm-egg fusion of sea urchins (Kato et al. 1998) and mammals (Correa et al. 2000), the enzy-

Table 24.2 Voltage-dependency during normal and cross-fertilization between monospermic and physiologically polyspermic amphibians

Eggs	Sperm	Voltage preventing fertilization (mV)	References
Anurans			
Bufo (mono)	*Bufo* (mono)	+20	(4)
Bufo (mono)	*Hynobius* (mono)	+40	(4)
Xenopus (mono)	*Xenopus* (mono)	±0	(2, 6)
Xenopus (mono)	*Cynops* (poly)	None	(5)
Xenopus (mono)	*Notophthalmus* (poly)	None	(7)
Urodeles			
Hynobius (mono)	*Hynobius* (mono)	+40	(3)
Cynops (poly)	*Cynops* (poly)	None	(4)
Cynops (poly)	*Hynobius* (mono)	+40	(4)
Pleurodeles (poly)	*Pleurodeles* (poly)	None	(1)

(1) Charbonneau et al. (1983), (2) Glahn and Nuccitelli (2003), (3) Iwao (1989), (4) Iwao and Jaffe (1989), (5) Iwao et al. (1994), (6) Iwao et al. (2014), (7) Jaffe et al. (1983a)

matic activity of MMP-2 is unnecessary for membrane interactions during *Xenopus* fertilization. Instead, the HPX domain of sperm MMP-2 is important for interactions with the egg membrane. The HPX peptide (GMSQIRGETFFFK; pI 10.15) causes egg activation in a voltage-dependent manner, with the inhibition voltage (+10 mV) more comparable to that during fertilization. The voltage-dependency in egg activation seems to correspond to the degree of positive charge in the peptide.

The HPX peptide binds to the egg membrane to transmit a signal into the egg cytoplasm for the initiation of $[Ca^{2+}]_i$ increase. MMP-2 localizes on the sperm membrane at the tip of the acrosome-reacted sperm, at which the sperm first binds to the egg membrane (Boyle et al. 2001). The HPX peptide binding to the membrane microdomain (MD) of unfertilized eggs is dependent on the presence of ganglioside GM1 with a sialic residue, but not asialo GM1 or GM3 with a sialic acid residue (Iwao et al. 2014). This is shown in the inhibition of *Xenopus* fertilization by a high concentration of GM1 (Mahbub Hasan et al. 2007) and restoration of the ability of sperm to fertilize anti-UPIII antibody-treated eggs by the MD fraction obtained from unfertilized eggs (Mahbub Hasan et al. 2014). The sperm membrane can bind a large amount of exogenous GM1 (Iwao et al. 2014). Furthermore, the treatment of sperm with a lower concentration of GM1 or anti-MMP-2 HPX antibody allows the sperm to fertilize an egg clamped at approximately 0 mV, whereas untreated sperm are unable to fertilize the egg (Iwao et al. 2014). The change in voltage-sensitivity is specific for GM1, but not for asialo GM1 or GM3. Thus, an interaction between the positively charged HPX domain and negatively charged GM1 plays an important role in voltage-sensing during *Xenopus* fertilization. In addition, the sperm surface glycoprotein (SGP) is involved in sperm-egg interactions (Nagai et al. 2009; Kubo et al. 2010). The secreted type of MMP-2 localizes on the sperm membrane in association with SGP (Fig. 24.5). The membrane complex containing SGP and MMP-2 binds with the egg membrane more densely on the animal hemisphere than the vegetal hemisphere (Nagai et al. 2009), corresponding to the preferential pene-

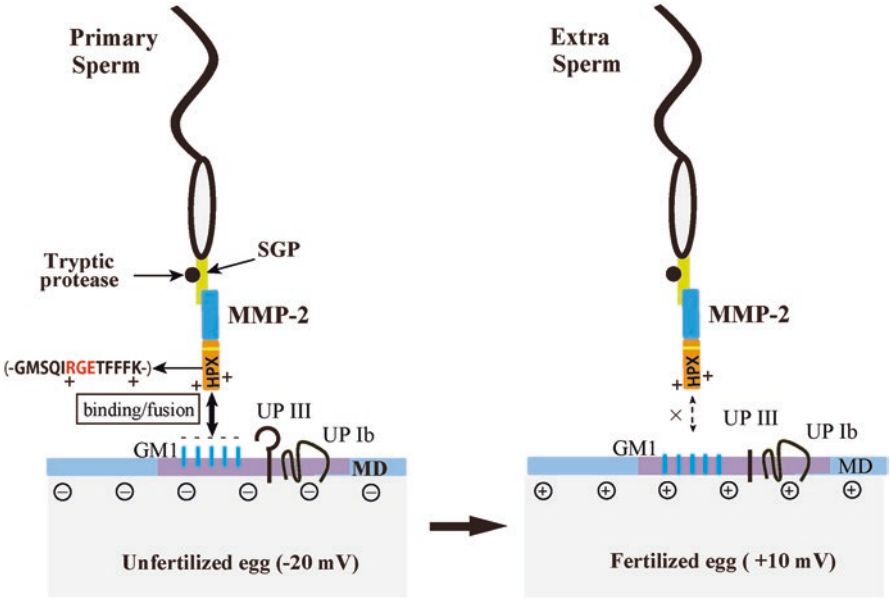

Fig. 24.5 A schematic model of voltage-dependent fertilization (egg activation) during *Xenopus* fertilization. GM1, ganglioside GM1; HPX, hemopexin domain; MD, microdomain; MMP-2, matrix matalloproteinase-2; SGP, sperm-surface glycoprotein; UP Ib, uroplakin Ib, UP III, uroplakin III

tration and adhesiveness of sperm on the animal hemisphere of *Xenopus* eggs (Grey et al. 1982; Stewart-Savage and Grey 1987; Kline 1988). Based on such evidence, we have proposed a model for voltage-sensing during *Xenopus* fertilization (Fig. 24.5, Iwao et al. 2014). An egg membrane with a negative potential (−20 mV) allows the primary sperm to bind to the egg membrane by the interaction between the positively charged HPX domain and negatively charged GM1 in the MD. After signal transduction on the MD, IP3 produced by PLCγ opens the Ca^{2+} channels (IP3-receptor) in the ER to release Ca^{2+} into the egg cytoplasm. The $[Ca^{2+}]_i$ increase generates a positive-going fertilization potential (+10 mV) through the opening of Cl^- channels. The binding and/or fusing of extra sperm is prevented in the fertilized egg.

However, additional mechanisms may be involved in the fast, electrical block to polyspermy. It has been proposed that sperm voltage-dependent phosphatase regulates sperm-egg fusion during *Xenopus* fertilization (Ratzan et al. 2011), but this is not supported by other amphibians (Mutua et al. 2014). It is also suggested that a lipovitellin/glycoproteins complex, or integrins and HSP70, are involved in the sperm-egg binding in *Discoglossus pictus* (Campanella et al. 2011) and *B. arenarum* (Coux and Cabada 2006), respectively. Further investigation is needed on the molecule responsible for the membrane fusion process in amphibian fertilization.

24.3 Comparison of Polyspermy Blocks Across the Animal Kingdom

24.3.1 Radiata (Radially Symmetric, Diploblastic Animals)

Phylum Cnidaria

In this phylum, jellyfish and hydrozoans have a radially symmetrical body plan, consisting of diploblastic layers. They live mostly in the marine environment, but some inhabit fresh water. These animals change from sexual to asexual reproduction in free-living medusa and sessile polyps, respectively. Hydrozoan sperm bind and penetrate at the animal pole of the egg. An increase in $[Ca^{2+}]_i$ is initiated at the fertilization site, which then propagates as a Ca^{2+} wave for 2–10 min (Deguchi et al. 2005; Kondoh et al. 2006). The Ca^{2+} wave is probably mediated by an intracellular Ca^{2+} release by IP3 receptors (Deguchi et al. 2005). As the earliest initiation of the Ca^{2+} wave occurs within 1 s of the attachment of sperm (Arakawa et al. 2014), the $[Ca^{2+}]_i$ increase appears to be induced directly following sperm-egg membrane binding.

Monospermy in Hydrozoa is ensured by the fast $[Ca^{2+}]_i$ increase at fertilization, as not only chelation of $[Ca^{2+}]_i$ in egg cytoplasm causes polyspermy, but also an advanced $[Ca^{2+}]_i$ increase inhibits fertilization, i.e., sperm-egg fusion (Arakawa et al. 2014). Thus, the $[Ca^{2+}]_i$ increase functions as a fast block to polyspermy at the level of the egg membrane. However, the electrical block may not be involved. It is reported that although the egg membrane slightly depolarizes from −72 to −64 mV during fertilization, fertilization is not affected by voltage-clamping at −65 – +50 mV in the hydrozoan *Hydractinia echinata* (Berg et al. 1986), and a low-Na^+ concentration, expected to inhibit the depolarization of the egg membrane, does not cause polyspermy (Arakawa et al. 2014). After the fast block by $[Ca^{2+}]_i$ increase, the dephosphorylation of mitogen-activated protein kinase (MAPK) in the egg cytoplasm irreversibly blocks sperm–egg fusion and reversibly suppresses sperm attraction as a late block to polyspermy (Arakawa et al. 2014).

Phylum Ctenophora

The bi-radially symmetric comb jellies in this phylum have eight rows of ciliated comb plates. Sperm are incorporated at any site of the egg surface (Carré and Sardet 1984). Although surface contraction and formation of a fertilization cone occurs around sperm entrance sites, the mechanism of egg activation is unknown.

Ctenophores exhibit physiological polyspermy, whereby more than 20 sperm enter an egg during fertilization in *Beroe ovata* (Carré and Sardet 1984; Carré et al. 1991). There seems to be no electrical membrane block to polyspermy, as each fertilizing sperm only induces a short-lived (approximately 60 s), Na^+-dependent depolarization from −66 to +38 mV, preceded by an action potential, and sperm-egg fusion appears independent of an egg membrane potential ranging from −78 to +16 mV (Goudeau and Goudeau 1993b). However, only one sperm pronucleus is selected to form a zygote nucleus with an egg pronucleus in the egg cytoplasm

(Carré et al. 1991; Rounvière et al. 1994). All incorporated sperm nuclei form sperm pronuclei, which are surrounded with a clear cytoplasm, the sperm pronuclear zone (SPZ). The egg pronucleus approaches the sperm pronuclei in the periphery of egg, moving towards different sperm pronuclei several times over a period of hours, finally entering the SPZ of one sperm pronucleus to form a zygote nucleus. Thus, ctenophore monospermy is ensured by a block to polyspermy in the egg cytoplasm. The mechanisms for both selection of one sperm pronucleus and exclusion of other accessory sperm pronuclei remain to be investigated.

24.3.2 Bilateria (Laterally Symmetric, Triploblastic Animals)

24.3.2.1 Lophotrochozoan (Protostomia)

Phylum Nemertea

In most ribbon worms of the phylum Nemertea, fertilization occurs externally in sea water. Sperm can enter at any area of the egg and a cortical flash increase in $[Ca^{2+}]_i$ occurs on the whole egg cortex during fertilization, followed by repetitive increases (oscillations) in $[Ca^{2+}]_i$ propagating as Ca^{2+} waves every 2–8 min for 20–60 min after fertilization (Stricker 1996; Stricker et al. 1998). The cortical flash is caused by an influx of Ca^{2+} dependent on Src-family kinases, though the Ca^{2+} waves are due to Ca^{2+} release from internal stores dependent on IP3 production (Stricker et al. 2010). The injection of a sperm extract (SE) into the egg triggers Ca^{2+} oscillations, indicating that egg activation is induced by a proteinaceous sperm factor (Stricker 1997), the detailed properties of which remain unknown.

Monospermy in *Cerebratulus* is ensured by a fast, electrical block to polyspermy on the egg membrane (Kline et al. 1985, 1986). A positive-going fertilization potential is elicited during fertilization in sea water (486 mM NaCl), consisting of a rapid increase from −65 to +44 mV, followed by a long-lasting positive potential of +24 mV for 80 min (Kline et al. 1986). The fertilization potential is mediated by the opening of Na^+ channels induced by the $[Ca^{2+}]_i$ increase. When the external Na^+ concentration is reduced to 51 mM, the amplitude of fertilization potential decreases and polyspermy occurs. The initial rapid increase and the long-lasting positive fertilization potential are probably due to the cortical Ca^{2+} flash and the following Ca^{2+} oscillations during fertilization, respectively (Kline et al. 1986). Thus, the fast increase in $[Ca^{2+}]_i$ is necessary for the fast rise of fertilization potential to prevent polyspermy at the level of the egg membrane in nemerteans.

Phylum Annelida

The eggs of marine rag worms (Polychaeta) exhibit monospermy and undergo Ca^{2+} oscillations, with an initial non-propagated $[Ca^{2+}]_i$ increase followed by propagating Ca^{2+} waves in *Chaetopterus pergamentaceus* (Eckberg and Miller 1995). The eggs of *Pseudopotamilla occelata* show a two-step $[Ca^{2+}]_i$ increase (Nakano et al. 2008), whereby the first local $[Ca^{2+}]_i$ increase around the sperm entry site is followed by a

global $[Ca^{2+}]_i$ increase. The initial increase is due to Ca^{2+} release from IP3-sensitive Ca^{2+} stores, whereas the second is caused by a Ca^{2+} influx via voltage-gated Ca^{2+} channels across the entire egg surface (Nakano et al. 2008; Nakano and Kyozuka 2014). Soluble sperm extract (SE) evokes a $[Ca^{2+}]_i$ increase by the external application of SE, but not by its microinjection (Nakano and Kyozuka 2014). As local application of SE induces a non-propagative $[Ca^{2+}]_i$ increase, the fertilizing sperm may trigger the first $[Ca^{2+}]_i$ increase before sperm entry. In the marine echiuran worm, *Urechis caupo*, a non-propagative $[Ca^{2+}]_i$ increase mediated by an influx of external Ca^{2+} occurs during fertilization (Stephano and Gould 1997). The $[Ca^{2+}]_i$ increase is probably driven by the opening of voltage-dependent Ca^{2+} channels due to the positive fertilization potential mediated by the opening of Na^+ channels (Gould-Somero et al. 1979; Jaffe et al. 1979; Gould-Somero 1981). As external application of sperm acrosomal peptide (P23, Val-Ala-Lys-Lys-Pro-Lys; pI 10.3) causes the opening of Na^+ channels independently of Ca^{2+} (Stephano and Gould 1997), *Urechis* sperm activate eggs through direct interaction with the Na^+ channels or associated receptors on the egg membrane.

A fast, electrical block to polyspermy is involved in the monospermic fertilization of annelids. The *Chaetopterus* egg elicits a positive-fertilization potential rising from −58 mV to a peak of +40 mV (Jaffe 1983a), whereas polyspermy is induced by a low external Na^+ concentration (Eckberg and Anderson 1985). For *Urechis*, there is sufficient evidence to support the electrical block to polyspermy on the egg membrane (Gould-Somero et al. 1979; Jaffe et al. 1979). A positive fertilization potential rising from −33 mV to a peak of +51 mV is elicited and can be maintained for 5–10 min. The positive potential is mediated by the opening of Na^+ channels at the site of sperm entry (Jaffe et al. 1979; Gould-Somero 1981). Maintaining an unfertilized egg's membrane potential at a positive level reduces the probability of sperm entry, whereas changing to a negative level by lowering the external Na^+ concentration induces polyspermy (Gould-Somero et al. 1979). Given that the sperm or the acrosomal peptide induces an electrical response in the positively-clamped eggs, but sperm penetration is prevented (Gould and Stephano 1987), membrane fusion-—rather than the egg activation—is dependent on the egg's membrane voltage. Thus, monospermy in *Urechis* eggs is ensured by a fast, electrical block at the egg membrane. Furthermore, cross-fertilization between sea urchin eggs and *Urechis* sperm demonstrates that the voltage-dependence of fertilization results from a component from the sperm (Jaffe et al. 1982), but the role of the sperm acrosomal peptide remains to be investigated.

Phylum Mollusca

The phylum Mollusca includes a large number of invertebrate species, and accordingly, encapsulates a wide variety of reproductive systems. Eggs are inseminated either externally or internally (see Chap. 22). External fertilization occurs in some marine bivalves, for example, the Surf Clam *Spisula solidissima* (Longo 1973). A single or repetitive increase(s) in $[Ca^{2+}]_i$ occurs during bivalve and limpet fertilization (for a review, Kashir et al. 2013). In the Purple Mussel, *Septifer virgatus*, an initial cortical flash of $[Ca^{2+}]_i$ increase is induced across the whole egg by a Ca^{2+}

influx through voltage-gated Ca^{2+} channels, followed by Ca^{2+} oscillations with 5–10 waves lasting 30 min (Deguchi et al. 1996; Deguchi 2007). A Ca^{2+} influx is responsible for the single $[Ca^{2+}]_i$ increase and the IP3 receptor-mediated Ca^{2+} release is necessary for the subsequent Ca^{2+} waves in *Mytilus edulis* (Deguchi et al. 1996), whereas the Ca^{2+} influx is the only Ca^{2+}-mobilizing system functioning during limpet fertilization (Deguchi 2007).

Monospermic bivalve eggs elicit a positive fertilization potential depolarizing from −66 to +60 mV in *Mytilus edulis* (Togo et al. 1995), from −18 to +20 mV in *Spisula solidissima* (Finkel and Wolf 1980), and from −65 to ±0 mV in *Ruditapes philippinarum* (Leclerc et al. 2000). Lowering Na^+ concentration in external media induces polyspermy in bivalves (Finkel and Wolf 1980; Dufresne-Dubé et al. 1983; Togo et al. 1995). The fast and temporary electrical block to polyspermy probably functions during monospermic molluscan fertilization. In addition, an aminopeptidase released from fertilized eggs is necessary for the complete block to polyspermy via suppression of the acrosome reaction in *Mytilus edulis* (Togo et al. 1995; Togo and Morisawa 1997).

24.3.2.2 Ecdysozoan (Protostomia)

Phylum Arthropoda

Arthropods (including crustaceans and insects) are the largest group of invertebrates and they inhabit a variety of marine, fresh water and terrestrial environments. Their reproductive systems therefore vary greatly between species. Although monospermy is a major mode of crustacean and insect fertilization (for reviews, Gould and Stephano 2003; Snook et al. 2011), physiological polyspermy occurs in some insects, such as crickets (for reviews, Ginzburg 1972; Sato and Tanaka-Sato 2002; Snook et al. 2011). Changes in $[Ca^{2+}]_i$ and the polyspermy block during fertilization have been well-investigated in marine prawns, crabs, and fruit flies. Egg activation during arthropod fertilization is usually not triggered by the fertilizing sperm. As parthenogenetic reproduction without fertilization naturally occurs in some insects, egg activation independent of sperm entry seems to be necessary for the initiation of arthropod development. The eggs of the Marine Shrimp *Sicyonia ingentis* are activated by Mg^{2+} in sea water during spawning (Lindasy et al. 1992). An initial Mg^{2+}-induced Ca^{2+} wave is caused by the Ca^{2+} released from intracellular Ca^{2+} stores through activation of tyrosine kinase and IP3 production (Lindsay and Clark 1994), followed by the second Ca^{2+} increase approximately 15 min after spawning. In the Fruit Fly *Drosophila melanogaster*, the eggs are activated during their movement through the female reproductive tracts at oviposition (Heifetz et al. 2001). The pressure on the *Drosophila* eggs induces a Ca^{2+} wave propagating from each pole of the egg, which requires an influx of Ca^{2+} through mechano-sensitive ion channels (Kaneuchi et al. 2014). The propagation of the Ca^{2+} wave is dependent on IP3 production in the egg cytoplasm.

Sicyonia eggs exhibit monospermy in normal sea water, but polyspermy in Mg^{2+}-free sea water, suggesting that the Ca^{2+} wave is involved in a polyspermy block

(Lindsay et al. 1992). During monospermic fertilization of the crab *Maia squinado*, the egg membrane hyperpolarizes from −50 to −80 mV (Goudeau and Goudeau 1989) due to the opening of K^+ channels. Eggs voltage-clamped at +20 to −65 mV elicit a fertilization current (K^+ current) in response to each sperm interaction, which is not induced at voltages more negative than −80 mV. In addition, the sperm do not penetrate eggs between +20 and −60 mV or at voltages more negative than −75 mV, indicating that membrane potentials between −65 and −75 mV are required for sperm incorporation into the egg. Although sperm-egg fusion does not appear to be simultaneous with the onset of the fertilization current, hyperpolarization (a negatively-going fertilization potential) is involved in an electrical block to polyspermy in crab eggs. It should be noted, however, that sperm entry into the *Drosophila* egg is restricted through a narrow micropyle on the hard chorion, such that only one large sperm can reach the egg membrane, indicating that a fast polyspermy block is unnecessary for *Drosophila* fertilization.

24.3.2.3 Deuterostomia

Phylum Echinodermata

Within the phylum Echinodermata, the classes Asteroidea (starfishes) and Echinodea (sea urchins), have been well-investigated as model systems for fertilization. In monospermic sea urchins, sperm can enter at any site on the egg and it causes an increase in $[Ca^{2+}]_i$ (see for reviews, Jaffe et al. 2001; Townley et al. 2006; Whitaker 2006; Kashir et al. 2013). A cortical $[Ca^{2+}]_i$ increase due to a Ca^{2+} influx through voltage-gated Ca^{2+} channels is elicited at the beginning of fertilization, and a Ca^{2+} wave subsequently propagates from the sperm entry site across the whole egg cortex with a latent period of 15–20 s (McDougall et al. 2000b). The cortical flash is mediated by external Ca^{2+} influx, but the subsequent Ca^{2+} wave is caused by Ca^{2+} release from ER dependent on IP3 production. Ryanodine receptors are also necessary for maintenance of the Ca^{2+} wave, and NAADP may contribute to these pathways (see reviews for details, Whitaker 2006, 2008; Kashir et al. 2013). The sperm probably stimulates Src-family kinase (SFK) during egg activation (see Chap. 27). During starfish fertilization, a Ca^{2+} wave, sometimes preceded by a cortical flash, also occurs (Stricker 1995; Carroll et al. 1997). However, the mechanism of the initial Ca^{2+} increase induced by the sperm still remains to be investigated in echinoderms.

The eggs of sea urchins and starfish show a positive-going fertilization potential reaching +12 to +20 mV during fertilization in sea water (Jaffe 1976; Chambers and de Armendi 1979; Miyazaki and Hirai 1979; Lansman 1983; David et al. 1988). An action potential is immediately followed by a sustained depolarization, mainly mediated by Ca^{2+} and Na^+ influxes, or Na^+ influx. A fast block to polyspermy by the positive fertilization potential was first demonstrated in sea urchins (Jaffe 1976). Holding the membrane potential of unfertilized eggs at a level more positive than approximately +5 mV, thus mimicking the fertilization potential, prevents sperm entry. Sperm attach to the egg surface, though the egg displays no electrophysiological response (Jaffe 1976; Lynn and Chambers 1984). Sperm-egg fusion is also

blocked at the positive potential (McCulloh and Chambers 1992). Conversely, polyspermy is induced if the membrane potential is transiently stepped to a negative level after the occurrence of the fertilization potential (Jaffe 1976). Interestingly, if the membrane voltage is continuously held at a negative level, sperm-egg fusion occurs, but the sperm nucleus does not enter the egg cytoplasm (Lynn et al. 1988; McCulloh and Chambers 1992). The block of sperm entry at negative potential is mediated by Ca^{2+}, which crosses the membrane and acts at an intracellular site (McCulloh et al. 2000). The molecular mechanism of block by the positive potential remains unknown. As bindin in the sperm acrosomal process may mediate membrane fusion between sperm and egg (for a review, Vacquier 2012), it will be important to investigate the role of bindin and associated molecules during voltage-dependent fertilization in sea urchins.

Phylum Chordata

Subphylum Urochordata

Ascidians living in the marine environment exhibit external fertilization, with egg activation caused by an increase in $[Ca^{2+}]_i$ due to the fertilizing sperm (for reviews, Nixon et al. 2000; Dumollard et al. 2002, 2004; Runft et al. 2002). A non-propagative Ca^{2+} action potential mediated by an influx of Ca^{2+} through voltage-gated Ca^{2+} channels is induced by depolarization of the egg membrane at the beginning of fertilization, followed by a larger propagative Ca^{2+} wave across the whole egg for about 10 min, and subsequent Ca^{2+} oscillations that continue for about 20–30 min (Speksnijder et al. 1989; Brownlee and Dale 1990; Goudeau et al. 1992; Kyozuka et al. 1998; Yoshida et al. 1998). Injection of sperm extract into an unfertilized egg triggers Ca^{2+}oscillations as observed during fertilization (Dale 1988; Kyozuka et al. 1998; McDougall et al. 2000a; Runft and Jaffe 2000). The sperm extract equivalent to that contained in one sperm can induce Ca^{2+} oscillations (Kyozuka et al. 1998), dependent upon a SFK-PLCγ signaling cascade (Runft and Jaffe 2000). Although the active component in the sperm extract appears to be a heat-labile, high molecular weight-molecule, the property of the sperm factor remains unknown.

Monospermic fertilization in ascidians is ensured by polyspermy blocks at several levels. A positive-going fertilization potential is observed during ascidian fertilization (Dale et al. 1983; Goudeau et al. 1992). The fertilization potential in *Phallusia mammillata* comprises an initial depolarizing shift from −80 to −5 mV and a second shift to +28 mV for 0.4–2 s, which is mediated by the opening of Na^+ and Ca^{2+} channels, respectively (Goudeau et al. 1992; Goudeau and Goudeau 1993a). The positive membrane potential remains at +20 mV for 1–2 min and then maintains a positive level (around ±0 mV) for 6–17 min. A series of oscillations in membrane potential begins approximately 5 min after fertilization dependent on external Ca^{2+} (Goudeau and Goudeau 1993a), probably corresponding to the oscillations in $[Ca^{2+}]_i$. The rate of fertilization is not affected by the voltage-clamping of unfertilized eggs below ±0 mV, but is reduced at positive potentials. With insemination with relatively low concentrations of sperm, fertilization rarely occurs at greater potentials than +10 mV (Goudeau et al. 1994). Thus, ascidian fertilization is

dependent on the voltage of the egg membrane and positive fertilization potential functions as a fast block to polyspermy, in addition to another block on the follicle cells (Lambert et al. 1997).

Subphylum Vertebrata

Class Agnatha

The Agnatha class, primitive and jawless vertebrates, contains hagfish and lampreys. Monospermic fertilization of lampreys occurs externally in fresh water. Although the changes in $[Ca^{2+}]_i$ during fertilization have not been investigated, lamprey eggs elicit a positive fertilization potential, mediated by the opening of Ca^{2+}-activated Cl^- channels (Kobayashi et al. 1994), indicating that a transient, single Ca^{2+} increase occurs during lamprey fertilization. The fertilization potential rises rapidly from −12 to +36 mV (Kobayashi et al. 1994), mediated by Cl^- efflux through Cl^- channels in a low ionic strength external media (e.g., 14 NaCl, 0.3 KCl, 0.2 $CaCl_2$ in mM). When eggs are clamped at various voltages, sperm-egg fusion is completely blocked at greater than +20 mV (Kobayashi et al. 1994). However, most eggs clamped even at +40 mV undergo activation. No inhibition of either sperm-egg fusion or egg activation is observed in eggs clamped at −60 mV. Thus, egg activation occurs without the sperm-egg fusion, suggesting a signal transduction for egg activation through egg-membrane receptors. As sperm-egg fusion is dependent on the voltage of the egg membrane, positive fertilization potential acts as a fast block to polyspermy in lampreys.

Class Osteichthyes

The bony fish (teleosts) in this class exhibit external and monospermic fertilization, with a chorion surrounding the egg necessary to prevent sperm penetration for monospermy, whereas chorion-free eggs become polyspermic (for reviews, Hart 1990; Iwamatsu 2000). As only one sperm can pass through a micropyle (a very small canal) at the animal pole of the chorion at a time, the number of the sperm reaching the egg is strictly limited. A Ca^{2+} wave is elicited from the sperm entry site under the micropyle and propagates in cortical cytoplasm in Medaka (*Oryzias latipes*) and Zebrafish (*Danio rerio*) (for reviews, Kashir et al. 2013; Webb and Miller 2014). The Ca^{2+} wave is mainly caused by a Ca^{2+} release from intracellular Ca^{2+} stores through IP3-receptors in response to IP3 produced by a Fyn-PLCγ cascade (see Chap. 27). Although the injection of sperm extract into unfertilized eggs induces activation in Medaka (Iwamatsu and Ohta 1974), PLCζ, which is known as a potent sperm factor for egg activation in mammals, is not involved in egg activation (Coward et al. 2011). The mechanism of sperm-induced egg activation remains to be investigated in teleosts.

The Medaka egg (with a resting membrane potential of −47 mV) generates a fertilization potential consisting of a small depolarization phase (4 mV, 20 s) followed by a longer hyperpolarization (31 mV, 155 s) under low ionic conditions (14 NaCl, 0.6 KCl, 0.6 $CaCl_2$ in mM) (Nuccitelli 1980a). The depolarization is independent of external ions and may be due to non-specific leak during sperm-egg fusion. The hyperpolarization is mediated by an increase in K^+ permeability, which may be caused by the addition of cortical vesicle membranes with K^+ channels after the

spread of a Ca^{2+} wave (Nuccitelli 1980a, 1987). As fertilization is not affected by voltages of the egg membrane between −80 mV and +48 mV (Nuccitelli 1980b), the electrical polyspermy block does not exist in teleost fertilization. Polyspermy is prevented by several non-electrical mechanisms, such as a fertilization cone extension into the micropyle to exclude extra sperm, and closure of the micropyle by shrinkage of the chorion (Iwamatsu 2000).

Class Amphibia

Most anurans (frogs and toads) exhibit external and monospermic fertilization in fresh water ponds or small streams. As previously described, monospermy in anurans is ensured by the positive fertilization potential created by the propagative opening of Cl^- channels induced by the preceding Ca^{2+} wave. There are, however, some species that exhibit an interesting state between monospermy and physiological polyspermy. In the primitive frog, *Discoglossus pictus*, sperm penetrate into a very restricted area on the animal pole, an animal dimple (Talevi and Campanella 1988). Several sperm reach the dimple and elicit a long-lasting positive fertilization potential (+20 mV) mediated by the opening of Ca^{2+}-activated Cl^- channels (Talevi et al. 1985; Nuccitelli et al. 1988; Talevi 1989). Most eggs are monospermic, but 36% are polyspermic (Talevi 1989). As the sperm penetration is independent of the egg's membrane potential, *Discoglossus* sperm have lost sensitivity to the positive fertilization potential. The eggs lack cortical granules, indicating no formation of a fertilization envelope to prevent sperm from approaching the egg membrane.

In most urodeles (newts and salamanders), eggs do not elicit a positive membrane shift during fertilization, rather physiological polyspermy is apparent, as previously described. However, primitive salamanders belonging to the genus *Hynobius* are monospermic (Iwao 1989, 2000b). Their eggs elicit a very high positive fertilization potential and exhibit voltage-dependent fertilization (Iwao 1989). In *Hynobius*, eggs lack cortical granules and the sperm contain a large amount of citrate synthase (CS) outside mitochondria (Iwao 2014), which is responsible for egg activation in higher urodeles (Harada et al. 2007, 2011) and birds (Mizushima et al. 2014). Thus, *Hynobius* exhibits intermediate characteristics that might have be acquired during the transition between monospermy and physiological polyspermy in the course of vertebrate evolution.

Class Reptilia and Aves

The amniotes (reptiles and birds) deposit large and yolky eggs on land. Their embryos develop in an amniotic cavity filled with amniotic fluid, which is an adaptation for embryonic development in dry conditions on land. Their fertilization occurs in the female reproductive tracts, such as upper oviducts, before secretion of yolk white and chorion. As fertilization in both reptiles and birds is physiologically polyspermic (for a review, Ginzburg 1972), a large number of sperm enter an egg, for example, 62 sperm into the egg of domestic fowl (Nakanishi et al. 1990). After the formation of a zygote nucleus between a sperm and an egg nucleus in the center of the germinal disc, other accessory sperm nuclei localize in the periphery. Some accessory sperm nuclei form extra bipolar spindles, but no extra cleavage furrow is induced at the first cleavage (Perry 1987; Waddington et al. 1998). All accessory

sperm nuclei degenerate after the first cleavage. The mechanisms for the selection of one sperm nucleus and for the exclusion of other accessory sperm nuclei remain unknown.

Although changes of $[Ca^{2+}]_i$ in the egg cytoplasm have not yet been investigated during bird fertilization, injection of sperm extract into an unfertilized egg evokes an initial transient $[Ca^{2+}]_i$ increase, followed by multiple spiral-like Ca^{2+} waves lasting for at least 1 h (Mizushima et al. 2014). The former $[Ca^{2+}]_i$ increase dependent on IP3 production is caused by PLCζ derived from the fertilizing sperm and is required for the resumption of meiosis (Mizushima et al. 2009). The latter long-lasting Ca^{2+} oscillations induced by aconitate hydratase (AH) and CS in the sperm cytoplasm are necessary for cell cycle progression. As cRNAs encoding PLCζ, AH and CS support the full development of zygotes by intracytoplasmic sperm injection (ICSI) (Mizushima et al. 2014), all three factors are essential for egg activation during fertilization. A block to polyspermy at the level of the egg membrane is unlikely to operate during bird fertilization.

Class Mammalia

Mammalia (mammals) consists of the subclass Prototheria, including the order Monotremata (Platypus), and the subclass Theria including infraclasses: Metatheria (marsupials) and Eutheria (mouse, human). Among them, the oviparous Platypus, *Onithorhynchus anatinus* deposits large yolky eggs (4 mm in diameter) (Hughes and Hall 1998) and their fertilization appears to be polyspermic (Gatenby and Hill 1924). It is also reported that in the small marsupial *Sminthopsis crassicaudata*, some eggs with a small yolk mass in the center are occasionally polyspermic (Breed and Leigh 1990). In contrast, monospermic fertilization of Eutheria occurs internally in the female oviducts and repetitive increases in $[Ca^{2+}]_i$ (oscillations) are observed during mammalian fertilization (for reviews, Miyazaki 2006; Swann and Lai 2013; Kashir et al. 2014). In mouse and hamster eggs, a $[Ca^{2+}]_i$ increase is induced around the sperm entry site and propagates as a Ca^{2+} wave across the whole egg, followed by a series of Ca^{2+} waves. Each Ca^{2+} wave originates from a site different from the sperm entry point and lasts 1–2 min, with an interval of approximately 30 min. The Ca^{2+} waves are mainly dependent on IP3-mediated Ca^{2+} release from ER (Miyazaki et al. 1992; Wakai et al. 2012), and a Ca^{2+} influx is involved in the maintenance of Ca^{2+} waves (Kline and Kline 1992; Mohri et al. 2001). As the initial $[Ca^{2+}]_i$ increase is initiated 1–3 min following sperm-egg fusion (Lawrence et al. 1997), the sperm appears to provide a soluble sperm factor into the egg cytoplasm for the $[Ca^{2+}]_i$ oscillations. Although integrin-mediated signaling may be involved in egg activation because RGD-containing peptides can stimulate protein kinase C and induce egg activation (Tatone and Carbone 2006; White et al. 2007), a sperm-specific PLCζ is the most predominant sperm factor in mammals (Saunders et al. 2002; Kouchi et al. 2005; for a review, Kashir et al. 2014). Another candidate sperm factor of PAWP with a WW-domain binding protein localizing in the post-acrosomal region (Aarabi et al. 2014) is not required for mouse egg activation (Satouh et al. 2015).

No positive fertilization potential is observed in mammals. During hamster fertilization, the egg undergoes a gradual hyperpolarization from −20 to −40 mV for several minutes following fertilization, in association with recurring, transient hyperpolarizations reaching −80 mV (Miyazaki and Igusa 1981; Igusa et al. 1983; Kline and Stewart-Savage 1994). The oscillations of hyperpolarization are due to the opening of Ca^{2+}-activated K^+ channels on the egg membrane in response to the $[Ca^{2+}]_i$ oscillations after fertilization (Igusa and Miyazaki 1986). As the sperm can enter into the egg at any membrane potential between −160 and +50 mV (Miyazaki and Igusa 1982), a voltage-dependent block of sperm entry does not exist in the hamster egg. In addition, a positive fertilization potential is not observed in other mammalian eggs (Jaffe et al. 1983b; Gianarol et al. 1994; Tosti et al. 2002) and the number of sperm penetrating into the egg does not increase with a reduction of Na^+ or Ca^{2+} in the external medium (Jaffe et al. 1983b). Thus, there is no fast, electrical block to polyspermy in mammalian fertilization. In the internal fertilization of mammals (e.g., mice and humans), a large number of sperm ($>10^8$ sperm) are ejaculated into the vagina, but a very small number reach the ovulated eggs after the long journey through the female reproductive tract (La Spina et al. 2016; Muro et al. 2016; for reviews, Yanagimachi 1994), such that the fast block to polyspermy on the egg membrane may not be necessary. It should be noted that the loss of an egg receptor JUNO (Folate receptor 4) against a sperm ligand IZUMO on the egg membrane is involved in a polyspermy block at the level of the egg membrane (Bianchi et al. 2014; see Chap. 26).

24.4 Conclusions

Within the animal kingdom, most animals exhibit monospermy, but physiological polyspermy is scattered across phyla, such as ctenophores (comb jellies), arthropods (insects), and vertebrates (urodele amphibians, reptiles, and birds). Physiological polyspermy appears to be acquired in relation to each reproductive condition without a close correlation with phylogeny (Table 24.1, see for details, Wong and Wessel 2006; Snook et al. 2011; Iwao 2012). Monospermy is probably an original mode of animal fertilization. Indeed, primitive metazoan sponges (Phylum Porifera), lacking true tissues and body symmetry, exhibit monospermic fertilization (for a review, Schatten and Chakbabarti 2000). It will be worthwhile to investigate the mechanism of polyspermy block during sponge fertilization to understand the origins and diversity of polyspermy blocks during animal evolution.

Monospermy is essentially ensured through several polyspermy blocks at the levels of the egg membrane and/or egg investments, such as fertilization membrane. The elicitation of positive fertilization potential is widely observed during fertilization of monospermic species from invertebrates to vertebrates (Table 24.1). The positive fertilization potential probably provides the fast polyspermy block at the level of sperm-egg binding and/or fusion in various animals. As the concentrations of Na^+ and Ca^{2+} in sea water are significantly higher than those in egg cytoplasm, the

rapidly rising and positive fertilization potential is mediated by the opening of Na^+ and/or Ca^{2+} channels on the egg in response to the primary sperm entry in marine animals. In contrast, animals whose fertilization occurs in fresh water, such as lampreys and frogs, have evolved the opening of Cl^- channels during fertilization for production of the positive fertilization potential under the lower ionic conditions. Thus, the positive potential of the egg membrane as opposed to the property of ions moving across it is important for the prevention of polyspermy. In this respect, the positively charged HPX domain of MMP-2 in *Xenopus* is a novel candidate for sensing the egg's membrane potential during voltage-dependent fertilization. To more fully understand the mechanisms involved in voltage-dependent fertilization, investigation is needed into the voltage-dependency during fertilization in MMP-2-deficient sperm. It will be important to investigate whether a similar molecule is involved in voltage-dependent fertilization in other monospermic species.

In the animal kingdom, Na^+-dependent positive fertilization potential appears in comb jellies (Radiata, Ctenophore), though the polyspermy block by positive potentials may have been acquired after the emergence of Bilateria (Protosomia, Lophotrochozoan) (Table 24.1). The electrical polyspermy block, however, also functions during plant fertilization. In the Brown Fucoid algae *Fucus* and *Pelvetia*, eggs elicit a Na^+-dependent, depolarizing fertilization potential (Brawley 1987, 1991), and a Ca^{2+} influx is necessary for egg activation (Roberts and Brownlee 1995; Bothwell et al. 2008). *Fucus* eggs have demonstrated a fast, electrical block to polyspermy via depolarizing fertilization potential not only under laboratory conditions (Brawley 1987, 1991), but also during natural mating in the field (Brawley 1992; Pearson and Brawley 1996; Serrao et al. 1999). Although the electrical block to polyspermy seems to have been acquired convergently during the evolution of plants and animals, it will be interesting to know whether there is a similar molecular mechanism involved in the electrical block to polyspermy of animals and plants. It should be noted that a sperm protein GCS1 (Generative Cell Specific 1) essential for gamete fusion in flowering plants (Mori et al. 2006) plays an important role in fertilization of a sea anemone (Cnidaria), *Nematostella vectensis* (Ebchuqin et al. 2014), suggesting a common molecular mechanism of fertilization has been retained among eukaryotes.

In this chapter, we reviewed the relationship between variations in $[Ca^{2+}]_i$ increases during egg activation and the modes of fertilization among vertebrates (Iwao 2012), suggesting that transient and large Ca^{2+} waves mediated by the egg membrane receptor are characteristic of monospermic eggs for a fast, electrical polyspermy block, whereas multiple Ca^{2+} waves caused by a sperm cytosolic factor are necessary for slower activation of physiological polyspermic eggs. However, multiple Ca^{2+} waves (oscillations) occur during monospermic fertilization of invertebrates, with a fast and electrical polyspermy block by positive fertilization potential. In these species, the positive fertilization potential appears to be elicited before the Ca^{2+} release from the intracellular Ca^{2+} stores. As the fertilizing sperm initially opens Na^+ and/or Ca^{2+} channels on the egg membrane, the initial rise of fertilization potential appears to be independent of intracellular Ca^{2+} releases in invertebrates.

The initial $[Ca^{2+}]_i$ increase is probably caused by Ca^{2+} influxes through voltage-dependent Ca^{2+} channels on the egg membrane or Ca^{2+} channels provided from the sperm membrane: a Ca^{2+} bolus/conduit model (Jaffe 1983b).

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Chapter 25
Preparing for Fertilization: Intercellular Signals for Oocyte Maturation

Leia C. Shuhaibar, David J. Carroll, and Laurinda A. Jaffe

Abstract In the hours preceding fertilization, oocytes prepare to begin development in a process known as maturation. This includes progression of the meiotic cell cycle and development of the ability to undergo the release of calcium that activates development at fertilization. In many species, the signal for oocyte maturation acts initially on somatic cells surrounding the oocyte, rather than on the oocyte itself. This chapter concerns the intercellular signaling events by which the maturation-inducing signal travels from the somatic cells to the oocyte. We first discuss how meiotic prophase arrest is maintained in mammalian oocytes, and how luteinizing hormone (LH) action on receptors in the cells of the surrounding follicle causes meiosis to resume. The LH receptors are located exclusively in the outer granulosa cells of the follicle and signal through a G_s-linked receptor to cause a decrease in cyclic guanosine monophosphate (cGMP). The LH-induced signal propagates inwards to the oocyte by way of cGMP diffusion out of the oocyte through gap junctions. We then briefly discuss the similarities and differences in mechanisms controlling oocyte maturation in animals other than mammals, focusing on hydrozoan jellyfish to emphasize the early evolutionary origin of these regulatory processes. G-protein-coupled receptors and cyclic nucleotides are common regulators in many animals. However, the assembly of these components into a regulatory system differs among species.

Keywords Oocyte · Ovarian follicle · Meiosis · Luteinizing hormone · Mammals · Hydrozoans · cAMP · cGMP · G-protein-coupled receptor · Gap junctions

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25.1 Signals for Oocyte Maturation in Mammals

Mammalian oocytes originate during embryonic development, duplicating their DNA and undergoing meiotic recombination (Gilbert 2014). They then arrest at prophase, which can last for months (in rodents) or years (in humans), until luteinizing hormone (LH), which is released from the pituitary gland beginning at puberty, restarts the meiotic cell cycle. Oocyte maturation is characterized by disintegration of the nuclear membrane (nuclear envelope breakdown, NEBD), individualization of chromosomes, and completion of the first meiotic division (Schuh and Ellenberg 2007; Clift and Schuh 2015). A second meiotic arrest occurs at metaphase of meiosis II, and fertilization provides the signal to complete the meiotic cycle.

During the process of maturation, the mammalian oocyte also prepares for fertilization by developing mechanisms for release of calcium, so that polyspermy can be prevented and development can proceed (Mehlmann and Kline 1994; Mehlmann et al. 1995, 1996; Lee et al. 2006; Mann et al. 2010; Wakai et al. 2012; Cheon et al. 2013). The development of calcium release mechanisms is a property of oocyte maturation in other animals as well, including starfish (Chiba et al. 1990) and frogs (Kume et al. 1997; Lee et al. 2006). The intercellular signaling processes that control the progression of oocyte meiosis also control this other aspect of oocyte maturation.

25.1.1 Structure of the Mammalian Ovarian Follicle

Within the mammalian ovary (Fig. 25.1a), each oocyte is surrounded by somatic cells called granulosa cells, forming the ovarian follicle. Surrounding the follicle are theca cells, and blood vessels that deliver LH from the pituitary gland to the follicle. During development, follicle-stimulating hormone (FSH) causes the follicle to grow to its full size (~400–500 μm diameter in mice, ~800–1000 μm in rats, ~2 cm in humans) and induces expression of the LH receptors that are required for resumption of meiosis (Hunzicker-Dunn and Mayo 2015). In a pre-ovulatory follicle (Fig. 25.1b), which is the stage that is responsive to LH, approximately ten layers of granulosa cells surround each oocyte. The cells immediately surrounding the oocyte are called cumulus cells, whereas the cells more distant from the oocyte are called mural granulosa cells. Between the cumulus and mural granulosa cells is a fluid-filled cavity called the antrum. Oocytes arrested at prophase are characterized by the presence of the nuclear envelope and nucleolus; these structures disappear when meiosis resumes.

Gap junctions connect the mural and cumulus cells, as well as the cumulus cells and oocyte, allowing equilibration of small molecules between the cells of the follicle (Gilula et al. 1978; Norris et al. 2008; Shuhaibar et al. 2015). Gap junction communication between the oocyte and the granulosa cells is essential to maintain meiotic arrest in oocytes in pre-ovulatory follicles (Sela-Abramovich et al. 2006;

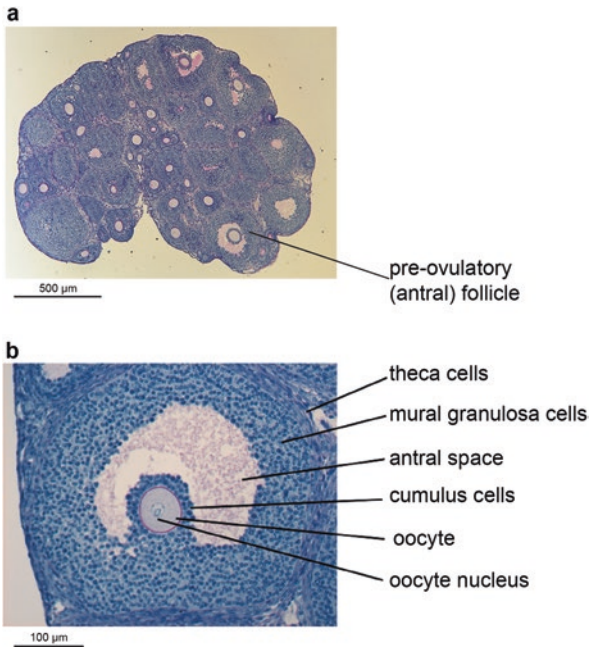

Fig. 25.1 Structure of a mouse ovary and pre-ovulatory follicle. (**a**) Histological section of a mouse ovary showing follicles at different stages of growth. (**b**) Histological section of a pre-ovulatory follicle (Part b reproduced from Robinson et al. 2012, with permission)

Norris et al. 2008; Richard and Baltz 2014). Early studies showed that rabbit oocytes are unable to maintain meiotic arrest if isolated from the follicle, indicating that an inhibitory signal from the granulosa cells controls the arrest in the oocyte (Pincus and Enzmann 1935). The requirement for cell contact to maintain arrest was established by experiments with hamster oocytes, showing that mechanically disconnecting the cumulus-oocyte complex from the surrounding mural granulosa cells, within the antral follicle, was sufficient to cause meiotic resumption (Racowsky and Baldwin 1989).

25.1.2 Inhibition of Meiotic Progression by cAMP in the Oocyte

In growing mouse oocytes, meiotic arrest is maintained because there is an insufficient amount of the protein kinase CDK1 to allow the prophase-to-metaphase transition (Chesnel and Eppig 1995). As oocytes grow to their full size, CDK1 levels increase (Firmani et al. 2017), and meiotic arrest becomes dependent instead on the activity of a complex of proteins that maintains a high level of cyclic adenosine monophosphate (cAMP) in the oocyte. The cAMP is synthesized in the oocyte by adenylyl cyclase 3 (AC3) (Horner et al. 2003). AC3 is kept active by the constitutive activity of a receptor coupled to a Gs G-protein, GPR3 (in mouse and humans) or GPR12 (in rats) (Mehlmann et al. 2002, 2004; Hinckley et al. 2005; Ledent et al.

2005; DiLuigi et al. 2008). The amount of GPR3 increases as the oocyte grows (Firmani et al. 2017). Depletion of GPR3/12, Gs, or AC3 in the oocyte causes meiosis to resume (Mehlmann et al. 2002; Horner et al. 2003; Kalinowski et al. 2004; Mehlmann et al. 2004; Hinckley et al. 2005; Mehlmann 2005; Ledent et al. 2005; DiLuigi et al. 2008). The activity of a G_s-protein is also required for maintaining meiotic prophase arrest in oocytes of fish and frogs (Kalinowski et al. 2004), but the identity of the receptor that activates G_s in these species is uncertain (see Nader et al. 2014).

Synthesis of cAMP in the oocyte leads to activation of protein kinase A (PKA) (Bornslaeger et al. 1986; Kovo et al. 2006), which in turn inhibits the activity of the phosphatase CDC25B and stimulates the activity of the kinases WEE1B and MYT1 (Conti et al. 2012; Holt et al. 2013; Mehlmann 2013). This maintains the CDK1 in an inactive form, thus inhibiting the prophase-to-metaphase transition (Jones 2004; Mehlmann 2005). Other substrates of PKA have been recently identified in the network regulating the activity of CDK1 kinase activity in *Xenopus* oocytes (Dupré et al. 2014), and the regulation in mammals may also involve additional PKA substrates.

The level of cAMP in the oocyte is determined by a balance between its synthesis, by GPR3-G_s-adenylyl cyclase signaling, and its breakdown by phosphodiesterase activity. The main cAMP phosphodiesterase in the oocyte is PDE3A, which is competitively inhibited by cyclic guanosine monophosphate (cGMP) (Norris et al. 2009; Vaccari et al. 2009).

25.1.3 Inhibition of Meiotic Progression by cGMP from the Granulosa Cells

As mentioned above, gap junction communication between the granulosa cells and the oocyte is crucial for maintenance of meiotic arrest. Gap junctions allow cGMP to diffuse into the oocyte to inhibit the phosphodiesterase activity of PDE3A and to maintain high levels of cAMP (Norris et al. 2009; Vaccari et al. 2009; Shuhaibar et al. 2015).

cGMP is generated in the mural granulosa and cumulus cells by natriuretic peptide receptor 2 (NPR2), also known as guanylyl cyclase B (Zhang et al. 2010; Kawamura et al. 2011; Robinson et al. 2012; Geister et al. 2013). NPR2 is a homodimeric transmembrane receptor activated by the C-type natriuretic peptide (CNP, also known as natriuretic peptide C, NPPC) (Potter 2011). CNP is expressed in the mural granulosa cells and activates NPR2 in both the mural and cumulus cells (Zhang et al. 2010; Tsuji et al. 2012). NPR2 is not expressed in the oocyte, and inhibition of meiosis requires diffusion of cGMP, through gap junctions, from the granulosa cell compartment into the oocyte.

25.1.4 Resumption of Meiosis in Response to Luteinizing Hormone

Luteinizing hormone is an ~30 kDa protein secreted from the anterior pituitary; it is comprised of two subunits, alpha and beta (Hunzicker-Dunn and Mayo 2015). LH is released into the bloodstream at low levels throughout the female reproductive cycle, and this release increases dramatically just preceding ovulation (Kovacic and Parlow 1972; Blake 1976). The increase in LH secretion is caused by rising levels of estrogen, which acts on receptors on cells of the hypothalamus to stimulate the release of peptides including kisspeptin that in turn stimulate the release of gonadotropin releasing hormone, which causes the synthesis and release of LH (Dungan et al. 2006; Oakley et al. 2009).

LH is delivered to the pre-ovulatory follicle by blood vessels in the theca layer surrounding the follicle, and acts on receptors in the outer layers of the granulosa cells (Bortolussi et al. 1977; Wang and Greenwald 1993). The LH receptor is a 7-transmembrane G-protein-coupled receptor, coupled to G_s as well as other G proteins. G_s stimulation of adenylyl cyclase elevates cAMP and activates PKA in the granulosa cells (Hunzicker-Dunn and Mayo 2015). LH-induced elevation of cAMP in the mural granulosa cells causes meiosis to resume by reducing the inhibitory cGMP signal to the oocyte, allowing activation of PDE3A and breakdown of cAMP in the oocyte. In mouse follicles, LH receptor activation causes a decrease in cGMP first in the mural granulosa cells, detectable within 1 min of LH application and falling to a minimum by about 10 min (Shuhaibar et al. 2015). By about 7 min, a decrease in cGMP begins in the oocyte, due to diffusion through gap junctions, and by 20 min, cGMP in the oocyte falls to a minimum. The cGMP decrease in the oocyte releases the inhibition of PDE3A, which causes a decrease in cAMP allowing activation of CDK1 kinase and the resumption of meiosis (Norris et al. 2009; Vaccari et al. 2009) (see Fig. 25.2).

This novel mechanism of signal transduction within a tissue was initially proposed 25 years ago (Törnell et al. 1991), but only recently, with the development of methods for imaging cyclic nucleotide concentrations in live tissues, was it possible to test it experimentally. As will be discussed below, another component of signaling across the follicle tissue is the release of soluble EGF-like growth factors that serve to convey the LH signal from the mural granulosa cells to reduce cGMP in other mural cells and in the cumulus cells, thus complementing intercellular diffusion of cGMP through gap junctions. A different hypothesis, that transmission of the signal from the LH receptor that reinitiates meiosis in the mammalian oocyte could occur by the release of steroid hormones, was suggested based on studies of fish and frogs (Nagahama and Yamashita 2008; Haccard et al. 2012). However, current evidence indicates that although steroids are produced in mammalian ovarian follicles in response to LH, they serve to mediate LH-induced ovulation and luteinization rather than the resumption of meiosis (Tsafriri and Motola 2007).

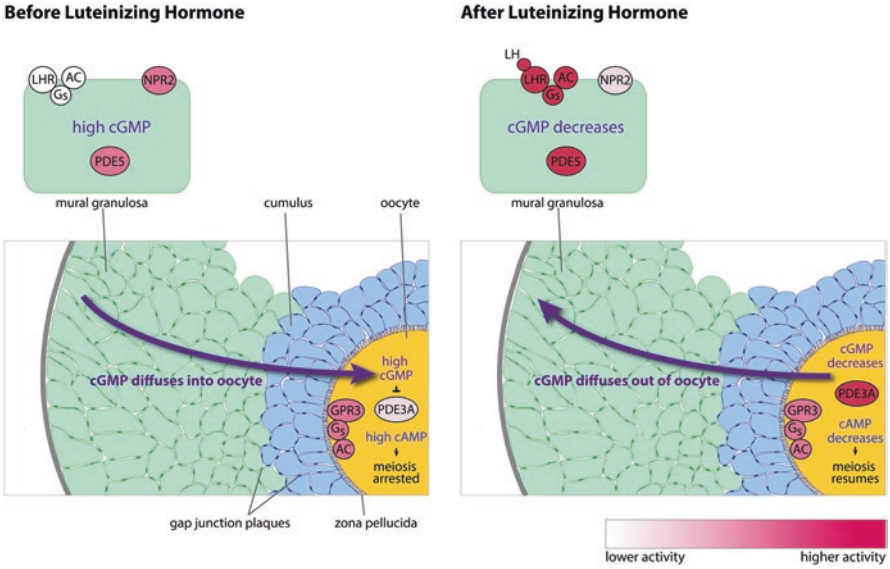

Fig. 25.2 Working model of how LH signaling rapidly decreases cGMP in the mural granulosa cells, and then via cGMP diffusion through gap junctions, decreases cGMP in the oocyte, leading to meiotic resumption in mammalian follicles. A change in enzyme activity is indicated by a change in intensity of the red color, as shown by the activity scale at the bottom. Before LH exposure (left), cGMP concentrations are elevated throughout the follicle, due to a high rate of production of cGMP by the NPR2 guanylyl cyclase in the mural granulosa and cumulus cells. cGMP phosphodiesterases, including PDE5, break down cGMP at a rate equal to its production, thus keeping the cGMP concentration at a constant level. Through gap junctions that connect all cells of the follicle, cGMP diffuses into the oocyte, where it inhibits the activity of PDE3A, maintaining cAMP at a level that inhibits meiotic resumption. The cAMP in the oocyte is produced by adenylyl cyclase 3 in the oocyte, and AC3 is kept active by the constitutive activity of the G_s-coupled receptor GPR3. After LH binds to its receptor in the mural granulosa cells (right), the activation of G_s and possibly other G-proteins results in dephosphorylation of NPR2, which decreases its rate of production of cGMP. Activation of the LH receptor also increases phosphorylation of PDE5, and increases its rate of breakdown of cGMP. Due to reduced NPR2 activity and increased cGMP phosphodiesterase activity, the concentration of cGMP in the mural granulosa cells decreases. Through the series of gap junctions that connects the oocyte to the large volume of the mural granulosa cells, cGMP in the oocyte diffuses down its concentration gradient, and the resulting decrease in oocyte cGMP relieves the inhibition of PDE3A in the oocyte, such that cAMP decreases. This model depicts only events occurring in the first 20 min after LH exposure. Subsequent events, including a decrease in gap junction permeability, an increase in EGF receptor ligands, and a decrease in C-type natriuretic peptide, also contribute to maintaining cGMP at the low level that triggers meiotic resumption as discussed in the text (Figure and legend reproduced, with permission, from Shuhaibar et al. 2015)

25.1.5 Mechanisms Leading to the cGMP Decrease in Response to Luteinizing Hormone Signaling

One mechanism by which LH signaling reduces cGMP in the follicle is that it causes dephosphorylation and inactivation of the NPR2 guanylyl cyclase, thus reducing the synthesis of cGMP. The decrease in guanylyl cyclase activity has been detected at 10 min after LH application (Robinson et al. 2012; Egbert et al. 2014; Shuhaibar et al. 2016). However, it remains to be determined exactly how activation of the LH receptor causes dephosphorylation of NPR2. LH signaling also decreases cGMP in the follicle by increasing phosphorylation of the PDE5 phosphodiesterase by protein kinase A, a modification that increases PDE5 activity (Egbert et al. 2014, 2016, 2018). Thus LH signaling reduces cGMP in the mural granulosa cells both by reducing its synthesis and by increasing its breakdown. These mechanisms have been identified using isolated follicles from mice and rats, but LH receptor activation also rapidly reduces CNP-dependent accumulation of cGMP in luteinized human granulosa cells, indicating that LH signaling could also act to decrease cGMP production and/or to increase its breakdown in the granulosa cells in human preovulatory follicles (Liu et al. 2014). Thus, although direct studies of human ovarian follicles are not feasible, it appears that this aspect of LH signaling is similar to that in follicles of mice and rats.

In addition to the rapid decrease in NPR2 activity due to dephosphorylation of the NPR2 protein, there is a slower decrease in levels of the NPR2 agonist CNP. The decrease in CNP peptide has been measured in mouse ovaries (Kawamura et al. 2011; Robinson et al. 2012; Liu et al. 2014), rat ovarian follicles (Egbert et al. 2014), and human follicular fluid (Kawamura et al. 2011), and is accompanied by a decrease in the RNA encoding the CNP precursor protein NPPC (Kawamura et al. 2011; Tsuji et al. 2012; Liu et al. 2014). The decrease in CNP presumably results in a further decrease in guanylyl cyclase activity. However, the decrease in the CNP is first detected between 1 and 2 h after LH application to mouse follicles (Robinson et al. 2012; Liu et al. 2014), too slowly to cause the initial rapid decrease in cGMP (Shuhaibar et al. 2015).

Another component of the signaling system by which LH restarts oocyte meiosis is that LH activates the EGF receptor kinase, by increasing amounts of EGFR ligands, and this contributes to the decrease in cGMP (Park et al. 2004; Panigone et al. 2008; Vaccari et al. 2009; Norris et al. 2010; Hsieh et al. 2011; Liu et al. 2014). Treatment of follicles with the EGF receptor ligands epiregulin and amphiregulin partially mimics the LH-induced decrease in cGMP in the follicle (Vaccari et al. 2009; Norris et al. 2010; Shuhaibar et al. 2015). Interestingly, EGFR signaling is not required for the rapid decrease in cGMP that occurs within the first 20 minutes after exposure of the follicle to LH (Shuhaibar et al. 2015), but prolonged inhibition of EGFR signaling attenuates the LH-induced cGMP decrease as measured at later time points, and also delays and partially inhibits resumption of meiosis (Park et al. 2004; Norris et al. 2010; Reizel et al. 2010; Hsieh et al. 2011; Wang et al. 2013; Liu et al. 2014). EGF receptor kinase activity contributes to the pathway leading to

dephosphorylation of NPR2 (Shuhaibar et al. 2016), but how LH receptor activation of G_s and other G-proteins is coupled to EGFR activation is incompletely understood. Importantly, release of EGF receptor ligands that can diffuse inwards in the follicle to act on receptors in the cumulus cells provides another way for LH signaling in the mural granulosa cells to be communicated to the oocyte (Conti et al. 2012).

In addition to regulating the production and breakdown of cGMP in the granulosa cells, LH signaling causes MAP kinase-dependent dephosphorylation of connexin 43, which decreases the permeability of the gap junctions between the granulosa cells (Sela-Abramovich et al. 2005; Norris et al. 2008). By 30 min after LH application to follicles, the decrease in permeability of the gap junctions is detectable, and it reaches a minimum by 1 h (Norris et al. 2008). The decrease is partial, and some permeability remains (Norris et al. 2008; Shuhaibar et al. 2015). Inhibition of the gap junction permeability decrease by inhibition of MAP kinase does not delay the resumption of meiosis (Norris et al. 2008; Shuhaibar et al. 2015). Thus, although application of the gap junction inhibitor carbenoxolone causes a fall in oocyte cGMP (Norris et al. 2008), and although carbenoxolone and other inhibitors of gap junctions induce meiotic resumption (Sela-Abramovich et al. 2006; Norris et al. 2008; Richard and Baltz 2014), the LH-induced decrease in gap junction permeability is not required for meiosis to resume. The permeability decrease could optimize signaling, but the primary means by which LH signaling causes meiosis to resume is by decreasing cGMP in the mural granulosa cells, resulting in a decrease in cGMP in the oocyte due to the connection of these compartments by gap junctions. For further discussion of LH signaling of meiotic resumption in mammalian ovarian follicles, see Conti et al. (2012), Mehlmann (2013), Jaffe and Egbert (2017), and Clarke (2018).

25.2 Signals for Oocyte Maturation in Hydrozoan Jellyfish and Other Animals

Oocyte maturation is a universal process among animal species (see Masui and Clarke 1979). Although a comprehensive review of this topic is beyond the scope of the present chapter, we will briefly discuss the signaling mechanisms that cause meiotic resumption in hydrozoan jellyfish, with some mention of other animals, to illustrate the evolutionary origins of the mechanisms discussed above for mammals. Like mammals, “jellyfish” are a large and diverse group of animals, of which only the hydrozoans have been studied extensively with regard to oocyte maturation, and among these only some species have been investigated. We use the term “jellyfish” to refer to this subgroup.

Like mammalian oocytes, jellyfish oocytes grow to their full size and are arrested at meiotic prophase in the ovary. They are surrounded by a layer of epithelial cells, although these cells do not form an organized follicle (Houliston et al. 2010), and

their presence is not required to maintain prophase arrest (Freeman 1987; Freeman and Ridgway 1988; Takeda et al. 2006). The signal that reinitiates meiosis in jellyfish oocytes is a change in light, usually from dark to light (Ballard 1942; Ikegami et al. 1978; Freeman 1987; Freeman and Ridgway 1988; Takeda et al. 2006; see additional references in Deguchi et al. 2011). Like LH in the mammalian ovary, the light/dark signal acts on the somatic cells of the jellyfish ovary rather than on the oocyte itself; isolated oocytes do not respond to changes in light (Freeman 1987; Takeda et al. 2006). The receptor for light belongs to the opsin family of receptors, a subgroup of G-protein-coupled receptors that is responsible for detection of light throughout the animal world (Quiroga Artigas et al. 2018). Thus, as in the mammalian ovary, the maturation-inducing signal in the jellyfish ovary is transduced by a G-protein-coupled receptor.

Exposure of the jellyfish ovary to light causes the release of a signaling molecule that then acts on the oocyte to cause meiotic resumption (Ikegami et al. 1978; Freeman 1987). Recent studies indicate that this is a small (4-amino acid) amidated peptide (Takeda et al. 2013, 2018). In this respect, signaling in the jellyfish ovary differs from that in the mammalian ovary, where the signal to the oocyte is transmitted by way of gap junctions rather than an extracellular molecule. Transmission of the signal from the somatic cells to the oocyte by way of an extracellular molecule also occurs in starfish (1-methyladenine) (Kanatani et al. 1969; Kishimoto 2011) and fish and frogs (progesterone and progesterone derivatives) (Nagahama and Yamashita 2008; Haccard et al. 2012).

As in jellyfish, the initial signal to restart meiosis in starfish, frogs, fish, and the nematode *C. elegans*, acts on the somatic cells surrounding the oocyte. In starfish, the agonist is a relaxin-like gonad-stimulating peptide (RGP) peptide released from the radial nerve (Mita et al. 2009; Mita 2013; Haraguchi et al. 2016). In fish and frogs, it is LH (see Nagahama and Yamashita 2008; Haccard et al. 2012), and in *C. elegans*, it is a protein released from sperm (major sperm protein, MSP) (Kim et al. 2013). Except for LH, the receptors for these agonists have not been identified, although evidence indicates that they are G-protein-coupled.

In jellyfish, as in mammals, the signaling pathway acts to regulate cAMP in the oocyte. Interestingly, the meiosis-inducing signal in jellyfish oocytes is an increase in cAMP, rather than a decrease (Freeman and Ridgway 1988; Takeda et al. 2006). An increase in cAMP associated with the maturation-inducing signal has also been found in oocytes of some other animals including nemertean worms and brittle stars (ophiuroids) (Deguchi et al. 2011). These findings raise the perplexing question of how cAMP can suppress meiotic progression in mammalian oocytes, as well as oocytes of fish and frogs (see Kalinowski et al. 2004) and some ascidians (Silvestre et al. 2010), but promote meiotic progression in oocytes of some other species.

As in mammals, gap junctions connect the oocyte to the surrounding somatic cells in jellyfish (Deguchi et al. 2011) and other species, including *C. elegans* (Kim et al. 2013), the fish *Fundulus* (Cerda et al. 1993), and the frog *Xenopus* (Browne et al. 1979). Since meiotic arrest is maintained in jellyfish and *Xenopus* oocytes from which the surrounding somatic cells have been removed, it appears that in

these species gap junctions do not have an essential function in maintenance of meiotic arrest as they do in mammals.

In summary, common themes, with variations, emerge in the regulation of oocyte maturation in different animals. Generally, the signal that triggers oocyte maturation acts first on the somatic cells around the oocyte. The signal then travels to the oocyte by diffusion through gap junctions and/or by diffusion through the extracellular space. G-protein coupled receptors and cyclic nucleotides are common components in the signaling pathways initiating oocyte maturation, but the specific functions of these differ. Thus, the same signaling molecules are assembled into different networks in different species.

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Chapter 26
Regulation of Sperm-Egg Fusion at the Plasma Membrane

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Abstract In fertilization, two types of gametes—sperm and egg—unite in a stepwise approach to create a single fertilized cell, which is capable of naturally developing into a new individual. Notably, membrane fusion occurring intercellularly between a sperm and an egg is essential for fertilization. In mammals, two integral membrane proteins, Izumo1 on the sperm plasma membrane and Cd9 on the egg plasma membrane, regulate sperm-egg fusion, and a new study has found a novel Izumo1 receptor, Juno, on the egg plasma membrane. Besides germ cells, Cd9 is expressed in a wide variety of cells, implying a close relationship between general fusion-related phenomena and sperm-egg fusion in particular. In invertebrate animals and in plants, Gcs1 plays an essential role in sperm-egg fusion. Considerable efforts are being devoted to understanding the molecular basis of cell-cell fusion; however, the exact mechanism(s) of the fusion process remain unclear. In this chapter we highlight the functions of some major molecules involved in the sperm-egg fusion and also discuss a possible molecular mechanism underlying this fusion.

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26.1 Introduction

During fertilization, membrane merging (hereafter referred to as "membrane fusion") occurs between the plasma membranes of the sperm and egg (Fig. 26.1). After this membrane fusion, several sperm factors enter the egg and activate cellular signaling in the egg. This restarts the cell cycle in the egg, which was arrested at the stage of meiotic metaphase II (MII). In mammals, the membrane protein families, the cell adhesion molecule "integrin" (Almeida et al. 1995; Chen and Sampson 1999), and the membrane-anchored protease "ADAM (A Disintegrin And Metalloprotease)" (Blobel et al. 1992; Evans et al. 1997; Myles et al. 1994; Waters and White 1997; Weskamp and Blobel 1994; Wolfsberg et al. 1993; Yuan et al. 1997) have been identified in the eggs and sperm, respectively. The members of the integrin family of proteins are expressed in a variety of fusion-competent and -incompetent cells, and mediate cell-cell and cell-matrix interactions (Elices et al. 1990; Gumbiner 1996; Menko and Boettiger 1987; Rosen et al. 1992; Schwander et al. 2003). Furthermore, the members of the ADAM family of proteins have a domain that is homologous to the extracellular region of the members of the integrin family of proteins (Evans 2001; Wolfsberg et al. 1993). The presence of a conserved protein domain between the integrin and ADAM families is a strong indicator of the central role of these two protein families in sperm-egg fusion (Bronson et al. 1999; Chen and Sampson 1999; Evans 2001; McLaughlin et al. 2001; Zhu et al. 2000). However, mice with manipulated integrin and ADAM genes display no overt anomalies in sperm-egg fusion or in membrane adhesion (Cho et al. 2000; He et al. 2003; McLaughlin et al. 2001; Miller et al. 2000; Vjugina et al. 2009) (Table 26.1). These findings are contradictory to the predicted functions of these proteins.

For successful fertilization in mammals, the involvement of multiple proteins with overlapping functions is believed to be necessary. The multiple proteins presumably protect against a potential malfunction during attempted fusion between sperm or eggs that lack one of the genes. Gene disruption experiments have revealed that Cd9 (Kaji et al. 2000; Le Naour et al. 2000; Miyado et al. 2000) and Izumo1 (Inoue et al. 2005) are essential for sperm-egg fusion (Fig. 26.1). Recently, Juno has been identified as the Izumo1 receptor on the egg plasma membrane (Bianchi et al. 2014). It is now time to propose a new theory for sperm-egg fusion.

26.2 Fertilization Inhibitory Antibodies

Before gene-manipulated mice were generated, an inhibition assay for in vitro fertilization (IVF) was used to identify proteins that could play a role in fertilization. At first, anti-Cd9 and anti-Izumo1 monoclonal antibodies (mAbs) were used to

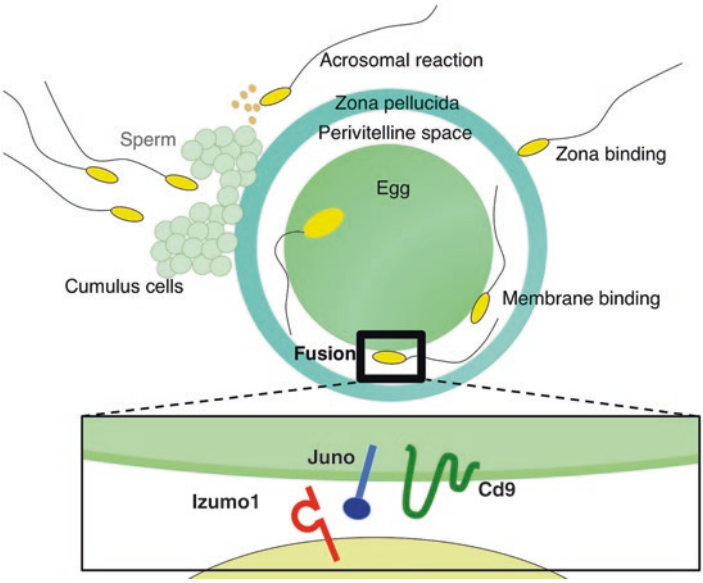

Fig. 26.1 Components identified in sperm-egg fusion. Fertilization is divided into multiple steps: interaction of sperm-somatic cells (termed cumulus cells), binding of sperm to the extracellular matrix (termed zona pellucida), and penetration of the egg. After the sperm penetrates the zona pellucida, it can bind and fuse to the egg cell membrane. Successful fertilization requires not only that a sperm and egg fuse, but also that polyspermy block occurs. In mammals, three components (Izumo1, Juno, and Cd9) are essential for the sperm-egg fusion

demonstrate an inhibition of sperm-egg fusion (Okabe et al. 1988) (Chen et al. 1999), and thereafter their roles were confirmed by the production of gene-manipulated mice (Inoue et al. 2005; Kaji et al. 2000; Le Naour et al. 2000; Miyado et al. 2000).

Two mAbs, mn7 and mc41, recognize two intra-acrosomal proteins, Acrin1/Mn7 and Acrin2/Mc41, respectively, and significantly inhibit fertilization of zona pellucida-intact (zona-intact) eggs, but do not inhibit the fertilization of zona pellucida-free (zona-free) eggs (Saxena et al. 1999).

Equatorin is a sperm-specific type I transmembrane protein that is widely distributed in the acrosomal region of mammalian sperm (Toshimori et al. 1998; Yamatoya et al. 2009). During the acrosome reaction, this protein becomes exposed at the surface of the equatorial segment. The mn9 mAb against this protein inhibits both in vitro and in vivo fertilization. In addition, the domain recognized by the mn9 mAb was identified to be near threonine 138, which was *O*-glycosylated, by using amino acid substitution and *O*-glycosylation inhibitor assays. Furthermore, immunoelectron-microscopic analysis showed that Equatorin was localized to the hybrid vesicles surrounded by amorphous substances at the advanced stage of the acrosome reaction.

Table 26.1 Predicted components in sperm-egg interaction in mammals

Gene name	Category of coding protein	Expression	Phenotypes of gametes in KO mice	References
Ace, testis-specific	Secreted protein, angiotensin-converting enzyme	Sperm	Failure of sperm transport into the oviduct and zona-binding	Kondoh et al. (2005)
Ace3, testis-specific	Secreted protein, angiotensin-converting enzyme	Sperm	No fertilizing defect	Inoue et al. (2010)
Acrin1, Mm7	Intra-acrosomal protein	Sperm	Unknown	Saxena et al. (1999)
Acrin2, Mc41	Intra-acrosomal protein	Sperm	Unknown	Saxena et al. (1999)
Adam1	Membrane protein	Sperm	Failure of sperm transport into the oviduct and zona-binding	Cho et al. (1998)
Adam2	Membrane protein	Sperm	Impaired binding of oviduct and zona with sperm	Kim et al. (2006)
Adam3	Membrane protein	Sperm	Impaired binding of oviduct and zona with sperm	Nishimura et al. (2001)
Basigin, Mc31, Ce9, Cd147	Membrane protein	Sperm	Unknown	Saxena and Toshimori (2004)
E-cadherin	Membrane protein	Egg and sperm	Impaired membrane adhesion but normal fusion	Takezawa et al. (2011)
N-cadherin	Membrane protein	Egg and sperm	Unknown	Marin-Briggiler et al. (2010)
Calmegin	ER chaperone	Sperm	Failure of sperm transport into the oviduct and zona-binding	Ikawa et al. (1997) andYamagata et al. (2002)
Calreticulin3	ER chaperone	Sperm	Failure of sperm transport into the oviduct and zona-binding	Ikawa et al. (2011)
β-catenin	Cytoplasmic and nuclear protein	Egg and sperm	Impaired membrane adhesion but normal fusion	Takezawa et al. (2011)
Cd81	Membrane protein, tetraspanin	Egg	Impaired fusion with sperm	Rubinstein et al. (2006) and Tanigawa et al. (2008)
Cd9	Membrane protein, tetraspanin	Egg and sperm	Defective fusion with sperm	Miyado et al. (2000) and Le Naour et al. (2000)

(continued)

Table 26.1 (continued)

Gene name	Category of coding protein	Expression	Phenotypes of gametes in KO mice	References
Cd98	Membrane protein	Egg	Unknown	Takahashi et al. (2001)
Crisp1	Secreted protein	Sperm	Normal fertility, but impaired fertilization in vitro	Da Ros et al. (2008)
Crisp2	Secreted protein	Sperm	Unknown	Busso et al. (2007)
Equatorin, Mn9	Membrane-anchored protein	Sperm	Unknown	Yamatoya et al. (2009) and Toshimori et al. (1998)
gcs1	Membrane fusion	Sperm cells	Defective gamete fusion in animals and plants	Mori et al. (2006)
Igsf8	Membrane protein, IgSF	Egg	No fertilizing defect	Inoue et al. (2012)
Integrin α3	Membrane protein	Egg	Normal membrane adhesion and fusion	He et al. (2003)
Integrin α6	Membrane protein	Egg	Normal membrane adhesion and fusion	Miller et al. (2000)
Integrin α9	Membrane protein	Egg	Reduced fertilizing ability	Vjugina et al. (2009)
Integrin αV	Membrane protein	Egg	Unknown	He et al. (2003)
Integrin β1	Membrane protein	Egg	Normal membrane adhesion and fusion	Miller et al. (2000)
Integrin β3	Membrane protein	Egg	Unknown	He et al. (2003)
Izumo1	Membrane protein, IgSF	Sperm	Defective fusion with egg	Inoue et al. (2005)
Izumo2	Membrane protein, IgSF	Sperm	Unknown	Grayson and Civetta (2012)
Izumo3	Membrane protein, IgSF	Sperm	Unknown	Grayson and Civetta (2012)
Izumo4	Membrane protein, IgSF	Sperm	Unknown	Grayson and Civetta (2012)
Juno	Membrane fusion	Egg	Defective fusion with sperm	Bianchi et al. (2014)
Pdilt	Protein disulfide isomerase	Sperm	Failure of sperm transport into the oviduct and zona-binding	Tokuhiro et al. (2012)
Pmis2	Unidentified	Sperm	Failure of sperm transport into the oviduct and zona-binding	Yamaguchi et al. (2012)
Spesp1	Equatorial segment protein	Sperm	Morphological defects of sperm	Fujihara et al. (2010)

(continued)

Table 26.1 (continued)

Gene name	Category of coding protein	Expression	Phenotypes of gametes in KO mice	References
Tex101	GPI-anchored protein	Sperm	Impaired binding of oviduct and zona with sperm	Fujihara et al. (2014)
Tmem190	Membrane protein	Sperm	No fertilizing defect	Nishimura et al. (2011)
Tpst2	Tyrosylprotein sulfotransferase	Sperm	Failure of sperm transport into the oviduct and zona-binding	Marcello et al. (2011)
Tssk6	Serine kinase	Sperm	Morphological defects of sperm	Spiridonov et al. (2005) and Sosnik et al. (2009)

In the field of fertilization research, antibodies have been extensively evaluated and the continuous identification and analysis of these antigens has contributed significantly to this field.

26.3 Adam3 and Its Regulatory Factors

During fertilization in mammals, sperm migrate to the oviduct, where they meet eggs ovulated from the ovary. Sperm-egg fusion occurs within the oviduct. The oviduct is the duct leading from the ovary to the horn of the uterus. Therefore, the sperm interact not only with eggs, but also with the female reproductive tract (Ikawa et al. 2010; Okabe and Cummins 2007).

Gene disruption has been used to produce at least ten mice lines for three members of the ADAM family (Adam1a, Adam2, and Adam3) (Cho et al. 1998; Kim et al. 2006; Nishimura et al. 2001), two testis-specific chaperone proteins expressed in the endoplasmic reticulum (ER) (Calmegin and Calreticulin3) (Ikawa et al. 1997, 2011; Yamagata et al. 2002), a protein disulfide isomerase (Pdilt) (Tokuhiro et al. 2012), a tyrosylprotein sulfotransferase (*Tpst2*) (Marcello et al. 2011), and a testis-specific angiotensin-converting enzyme (Ace) (Kondoh et al. 2005).

Testis-expressed gene 101 (Tex101), a glycosylphosphatidylinositol (GPI)-anchored protein, is essential for the expression of Adam3 on the sperm plasma membrane (Fujihara et al. 2014). Two proteins, Pmis1 and Pmis2, were missing in spermatozoa from *Calmegin*-deficient mice. Pmis1-deficient mice were fertile, but *Pmis2*-deficient male mice were sterile because of a failure in sperm transport into the oviducts (Yamaguchi et al. 2012). These mice showed an impaired migration of sperm into the oviduct in vivo and an impaired zona-binding ability in vitro. When cumulus-free IVF was performed, these mutant sperm were able to fuse with the

zona-free eggs, but not zona-intact eggs, due to the loss of the surface expression of Adam3. These results suggest that sperm proteins serve important functions in the interaction between sperm and the female reproductive tract via the expression of Adam3.

26.4 Other Sperm-Specific Proteins

It is widely accepted that the equatorial segment of the acrosome-reacted sperm plays a role in initiating fusion with the egg plasma membrane. When a mouse line lacking *Spesp1* (sperm equatorial segment protein 1) was generated, the number of offspring that were fathered by *Spesp1*+/− and *Spesp1*−/− male mice was significantly lower than that of wild-type male mice (Fujihara et al. 2010). Fewer sperm were found to migrate into the oviduct and fewer eggs were fertilized. The sperm produced in *Spesp1*+/− and *Spesp1*−/− male mice also showed a lower fusing ability compared with the wild-type sperm. Moreover, scanning electron microscopy revealed that the sperm membrane in the equatorial segment area, which usually forms an acrosomal sheath, was damaged after the acrosome reaction in *Spesp1*-deficient mice, suggesting that Spesp1 is necessary to produce a fully fusion-competent sperm.

Tmem190, a small transmembrane protein containing the trefoil domain, was identified by proteomic analysis of the mouse sperm (Nishimura et al. 2011). Tmem190 is an inner-acrosomal membrane protein expressed in the cauda epididymal sperm and has two distinct structural features—a trefoil domain and a small transmembrane protein—which are predicted to form a protein-protein complex that is required for sperm-egg fusion. During the acrosome reaction, Tmem190 is partly relocated onto the surface of the equatorial segment, where the sperm-egg fusion occurs. Moreover, Tmem190 and Izumo1 were co-localized to the sperm plasma membrane, both before and after the acrosome reaction. However, Tmem190 is dispensable for fertilization because *Tmem190*-deficient male mice were fertile.

26.5 Sperm-Binding Secretory Proteins

Epididymal proteins Crisp1s are a cysteine-rich secretory protein family (Da Ros et al. 2015). The *Crisp1*-deficient sperm presented a decreased level of protein tyrosine phosphorylation during capacitation, and the impaired ability to fertilize both zona-intact and zona-free eggs in vitro; but they exhibited normal fertility in vivo (Da Ros et al. 2008). Testicular Crisp2 is also expected to be involved in sperm-egg fusion (Busso et al. 2007); but the gene-manipulated mice for this protein have not yet been reported.

26.6 Cell Adhesion Proteins

Before membrane adhesion, both sperm and egg retain the cell-cell adhesion complex composed of β-catenin and E-cadherin (De Vries et al. 2004; Huelsken et al. 2001; Nagafuchi and Takeichi 1988; Nagafuchi et al. 1991; Takezawa et al. 2011; Valenta et al. 2012). Once the membrane adhesion occurs, β-catenin is immediately degraded by ubiquitination in both the sperm and egg. The absence of β-catenin results in a reduction in the ability of the sperm to adhere to an egg, but sperm-egg fusion occurs normally (Takezawa et al. 2011). N-cadherin is also expressed in the human sperm and eggs (Marin-Briggiler et al. 2010), but neither N-cadherin-deficient sperm nor eggs have yet been reported.

Basigin/Mc3/Ce9/Cd147 is a transmembrane glycoprotein that acts as a receptor for erythrocyte invasion by *Plasmodium falciparum* (Crosnier et al. 2011). The mAb against this protein significantly inhibits fertilization in cumulus-intact, zona-intact, and zona-free rat eggs (Saxena and Toshimori 2004); *Basigin*-deficient male mice are infertile due to spermatogenesis arrest (Igakura et al. 1998).

26.7 Fusion-Related Proteins

Cd98 is a glycoprotein composed of two subunits, Slc3a2 and Slc7a5, forming the large neutral amino acid transporter (Ohgimoto et al. 1995), and is expressed on mouse eggs (Takahashi et al. 2001). mAbs against Cd9 and Cd98 cooperatively inhibit fertilization in vitro (Takahashi et al. 2001), but the gene-manipulated eggs and sperm for this protein have not been analyzed yet.

26.8 GPI-Anchored Proteins

As described above (Fujihara et al. 2014), Tex101 is a glycosylphosphatidylinositol (GPI)-anchored protein involved in the expression of Adam3 on the sperm plasma membrane. Female mice deficient in egg-specific GPI-anchored proteins showed severely reduced fertility due to the failure of sperm-egg fusion (Alfieri et al. 2003).

26.9 Izumo1, Izumo1 Receptor, and Izumo1-Related Proteins

Izumo1, named after the Japanese shrine dedicated to marriage, is a sperm-specific member of the immunoglobulin superfamily and is essential for sperm-egg fusion (Inoue et al. 2005). Izumo1 is post-translationally modified by N-linked glycosylation on its extracellular domain and phosphorylation of the cytoplasmic C-terminus

(Inoue et al. 2008) (Baker et al. 2012). Remarkably, Izumo1 moves predominantly to the equatorial segment once the acrosome has reactioned (Satouh et al. 2012).

Folate receptor 4 (Folr4) has been identified as a receptor for Izumo1 on the mouse egg, and renamed Juno (Bianchi et al. 2014). The Izumo1-Juno interaction is conserved within members of several mammalian species. Although this interaction is required for efficient sperm–egg adhesion, it is insufficient for membrane fusion (Inoue et al. 2015).

Three family member genes (*Izumo2*, *3*, and *4*) are mostly expressed in the sperm with potential roles in sperm-egg fusion (Grayson and Civetta 2012), but the relevant gene-manipulated mice have not been reported yet.

Ace3, a testis-specific Ace homolog, has been identified as one of the Izumo1-interacting proteins on the sperm plasma membrane (Inoue et al. 2010). In Ace3-deficient mice, the localization of Izumo1 was found to be dispersed more widely on the sperm plasma membrane, but Ace3 is functionally dispensable for fertilization (Inoue et al. 2010).

Tssk6 is a member of the testis-specific serine kinase family of proteins and is expressed in male post-meiotic germ cells (Sosnik et al. 2009; Spiridonov et al. 2005). In *Tssk6*-deficient sperm, Izumo1 fails to relocate during the acrosome reaction. Tssk6 is involved in Izumo1 relocation via actin polymerization.

26.10 Generative Cell-Specific 1 (Gcs1)

In plants, sperm cells are delivered by the pollen tube, which extends towards an embryo sac enclosing an egg and a central cell. Gcs1 is a sperm transmembrane protein that is essential for gamete fusion in flowering plants (Mori et al. 2006). Putative *gcs1* genes are widely conserved across organisms, both in animals as well as plants (Hirai et al. 2008; Mori et al. 2010; Kawai-Toyooka et al. 2014; Ebchuqin et al. 2014). *Arabidopsis thaliana gcs1* mutant gametes fail to fuse, resulting in male sterility, suggesting that Gcs1 plays a critical role in sperm-egg fusion, and has a widely conserved function in plants and animals.

26.11 Cd9 and Cd9-Related Proteins

The Cd9gene (encoding a 24-kDa protein) is expressed in a variety of mammalian cells (Hemler 2008). This protein is localized mainly on the plasma membranes and is suspected to be involved in cell-cell adhesion, because Cd9 associates with integrin family members (Hemler 2008). Cd9 has two extracellular loops, and four transmembrane domains. Cd9 is a cell surface marker and is useful for isolating undifferentiated cells from mixed cell populations in mice and humans because of its significantly higher amount in mesenchymal and embryonic stem cells compared to fibroblastic cells (Akutsu et al. 2009).

Three laboratories independently generated *Cd9−/−* mice (Kaji et al. 2000; Le Naour et al. 2000; Miyado et al. 2000). All strains of the *Cd9−/−* mice showed severe female subfertility, whereas the *Cd9−/−* male mice were fertile. Moreover, the *Cd9*-deficient eggs exhibited severely reduced sperm fusion ability. Cd9 continues to be studied as one of the crucial factors in sperm-egg fusion in mammals.

An immunoglobulin superfamily member, Igsf8, associates with Cd9 on the egg surface and is undetectable on the surface of *Cd9*-deficient eggs (Inoue et al. 2012). However, the *Igsf8*-deficient female mice showed no fertilization defects in vitro or in vivo (Inoue et al. 2012).

26.12 Tetraspanin

Cd9 belongs to a membrane protein family termed "tetraspanin," which encompasses 35 members in mammals, such as Cd9, Cd63, Cd81, Cd82, and Cd151 (Hemler 2008), 30 in nematodes (Moribe et al. 2004, 2012), and 30 in flies (Kopczynski et al. 1996; Todres et al. 2000). The members of the tetraspanin family (hereafter referred to as "tetraspanins") act as scaffolding proteins, anchoring multiple proteins including membrane proteins and cytoplasmic proteins to a single area of the plasma membrane. Tetraspanins are involved in tissue formation (Hemler 2008) and transmission of infectious diseases (Hassuna et al. 2009). Generally, pathogenic microorganisms such as bacteria, viruses, parasites, or fungi are transmitted directly or indirectly from one organism to another (Shiino 2012). Tetraspanins are involved in the onset of infectious and parasitic diseases (e.g., cell-cell transmission of Human Immunodeficiency Virus (HIV-1) (Garcia et al. 2005; Wiley and Gummuluru 2006). After the primary infection of HIV into host cells, Cd9, Cd63, Cd81, and Cd82 are enriched at budding sites of HIV-1 virions. In mice, Cd81 is required for malarial parasites to infect the hepatocyte (Silvie et al. 2003). Malarial sporozoites, the cell form that infects new hosts, are transmitted into livers of the mammalian hosts through bites from infected mosquitoes. However, sporozoites are unable to infect hepatocytes of *Cd81−/−* mice. Cd81 is also involved in the sperm-egg fusion process (Rubinstein et al. 2006; Tanigawa et al. 2008). Female *Cd81−/−* mice are subfertile because *Cd81*-deficient eggs exhibit impaired sperm fusion ability. In addition, Cd81 is expressed on *Cd9*-deficient eggs, and Cd9 is also expressed on *Cd81*-deficient eggs, at levels comparable with that of wild-type eggs, indicating that Cd9 and Cd81 work independently in sperm-egg fusion (Ohnami et al. 2012). On the other hand, plants have more than 60 tetraspanins (Chiu et al. 2007; Huang et al. 2005), but their roles in plant cell membrane fusion are unclear.

Tetraspanin-like proteins have been identified in fungi, and their molecular masses (more than 200 kDa) are greater than those of tetraspanin proteins identified in animals and plants (20–30 kDa) (Lambou et al. 2008). An appressorium is a specialized cell typical of a fungal plant pathogen, which is used by the fungus to infect host plants. By analyzing a non-pathogenic fungal mutant of the rice blast fungus, tetraspanin-like Pls1 (Mgpls1) protein has been shown to control the appressorial

function (Clergeot et al. 2001). Similarly, *Colletotrichum lindemuthianum* Pls1 (Clpls1) is a functional homolog of Mgpls1, and the non-pathogenic *Clpls1*-deficient mutant in bean leaves exhibits a defect in the formation and positioning of the penetration pore (Veneault-Fourrey et al. 2005).

Taken together, these results suggest that the tetraspanin family is closely related to membrane fusion-related events in various organisms.

26.13 Exosomal Tetraspanins

Membrane vesicles, termed exosomes, are derived from living cells (Denzer et al. 2000). Furthermore, they have been proven to play a role in the mediation of adaptive immune reactions to pathogens and tumors through the enhancement of antigen-specific T cell responses (Couzin 2005; Simons and Raposo 2009).

Besides immune cells, exosomes are released from a wide range of normal and malignant mammalian cell types, and their diameter is estimated to range from 50 to 90 nm (Simons and Raposo 2009). The protein composition of these exosomes varies with the origin of cells; yet the exosomes commonly contain a ganglioside GM3, two kinds of heat shock proteins (Hsp70 and Hsp90), and tetraspanins (Simons and Raposo 2009). They also contain transcripts, mRNA, and microRNA (miRNA), which are thought to be shuttled from one cell to another, thereby influencing protein synthesis in recipient cells (Valadi et al. 2007).

Exosomes have potential for use as therapeutic tools due to their capacity for efficient transfer of proteins, mRNA, and miRNA to selective targets (Kosaka et al. 2010). They are rich in tetraspanins that associate with other proteins (Raposo and Stoorvogel 2013; Robbins and Morelli 2014). Since each of the tetraspanins preferentially interacts with other membrane proteins as targets, differences in tetraspanin complexes in exosomes can be used to effectively select target cells by assembling a combination of tetraspanin-associated proteins, including adhesion molecules such as integrins, MHC class I and II molecules, and PDZ domain-containing proteins (Hemler 2003).

26.14 Cd9 and Egg Microvilli

In mammals, the sperm binds to microvilli on the egg plasma membrane, and sperm-egg fusion first occurs around the equatorial region of the sperm head. Two reports suggest that Cd9 contributes to the organization of the cell membrane in eggs. First, Cd9 is transferred from the egg to the fertilizing sperm present in the perivitelline space, an interspace that completely surrounds the egg and is present between the egg cell membrane and the zona pellucida. This implies the involvement of a process similar to trogocytosis, which is a mechanism for the cell-to-cell contact-dependent transfer of membrane fragments from antigen-presenting cells to lymphocytes for the generation of immune responses against pathogens

(Barraud-Lange et al. 2007). Secondly, *Cd9* deficiency alters the length and density of the microvilli on the egg cell membrane (Runge et al. 2007). These results suggest that Cd9 is involved in membrane organization, especially in the formation of microvilli on the egg plasma membrane.

26.15 Which Came First, Microvilli or Membrane Vesicles?

Treatment with fixatives often disturbs membrane organization and modifies the localization of membrane proteins. The potential of enhanced green fluorescent protein-tagged Cd9 (Cd9-eGFP) as a reporter protein was exploited for studying sperm-egg fusion in live mouse eggs (Miyado et al. 2008). As depicted in Fig. 26.2,

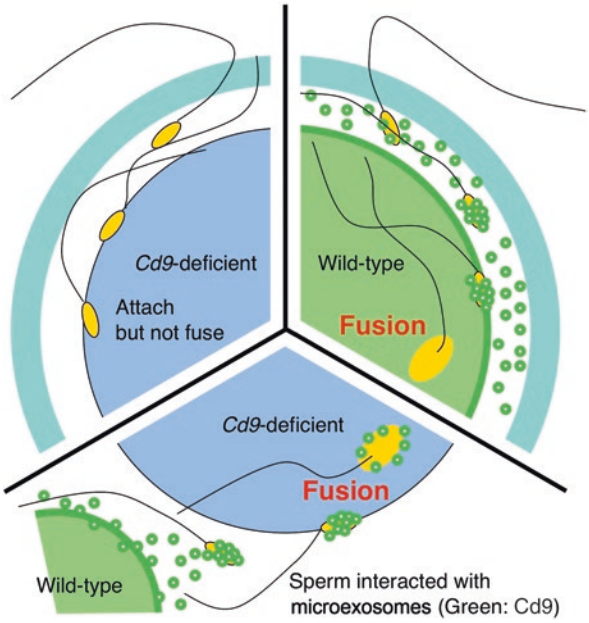

Fig. 26.2 Overview of the studies of *Cd9*-deficient eggs. In wild-type eggs, Cd9-containing microexosomes are released from wild-type eggs before any interaction with the sperm (upper right diagram). Shortly after the sperm penetrates the zona pellucida into the perivitelline space, the microexosomes are transferred on the acrosome-reacted sperm head. Then, a sperm fuses with the egg cell membrane. Interaction between the sperm and the microexosomes is an essential step for sperm-fusing ability. In contrast, *Cd9*-deficient eggs cannot release the microexosomes, which are correlated with the formation of microvilli on the egg cell membrane (upper left diagram). The sperm cannot fuse to the cell membrane of the *Cd9*-deficient egg. On the other hand, when the zona pellucida is removed from the eggs, the sperm is able to interact with the microexosomes released from wild-type eggs and can fuse with the *Cd9*-deficient egg (lower diagram). By co-incubation with wild-type eggs, the sperm can fuse with a similar number of *Cd9*-deficient and wild-type eggs. This procedure is most commonly used to overcome male infertility and fusion defects in *Cd9*-deficient eggs

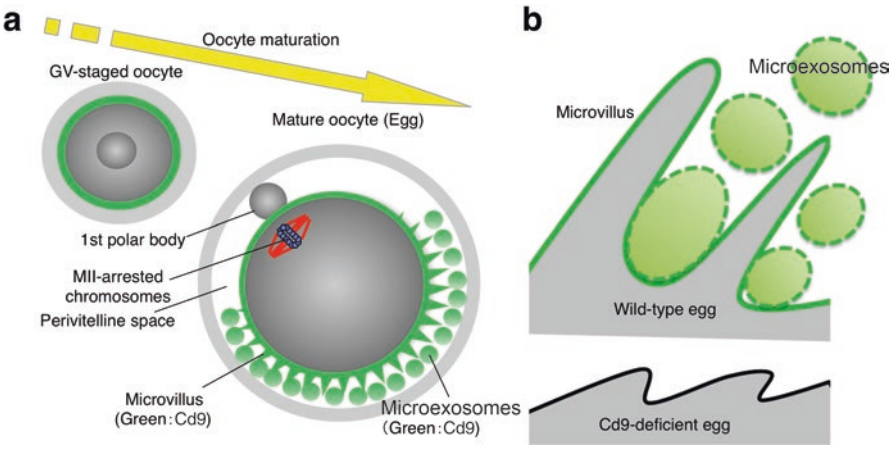

Fig. 26.3 Requirement of microexosomes for sperm-egg fusion. (**a**) Release of microexosomes from a MII-arrested egg. (**b**) Microexosome release and microvilli formation. Upper image: wild-type egg; lower image: *Cd9*-deficient egg. Green circles: Cd9-containing microexosomes. Green lines: Cd9 localized on the egg plasma membrane

Cd9-eGFP was significantly accumulated within the perivitelline space in eggs just before fertilization. Additionally, upon careful treatment of the eggs with fixatives, immunoelectron-microscopic analysis of the wild-type eggs revealed that Cd9 was not only present in the perivitelline space, but was also incorporated into vesicles of varying sizes (5–10 nm in diameter) (Miyado et al. 2008). Moreover, the membrane vesicles share Cd9, GM3, and Hsp90 with exosomes, and these components are absent in eggs lacking *Cd9* and are reproduced by Cd9-eGFP expression restricted to the eggs (Miyado et al. 2008). Membrane vesicles were also previously detected by electron microscopy within the perivitelline space of eggs in opossums (Talbot and DiCarlantonio 1984) and humans (Dandekar et al. 1992). These results reveal two features of the nature of Cd9 in mouse eggs (Fig. 26.3). First, Cd9 is essential for the formation of the Cd9-incorporated exosome-like vesicles (hereafter referred to as microexosomes) (Miyado et al. 2017) in mouse eggs. Second, the microexosomes are produced in mouse eggs and are released outside the egg cell membrane just before fertilization.

The above-mentioned findings about microexosomes hint towards the possibility that they facilitate sperm-egg fusion. As expected, Cd9-containing microexosomes rendered sperm fusion competent with *Cd9*-deficient eggs (Miyado et al. 2008) (Fig. 26.2). Furthermore, *Cd9*-deficient sperms could not fuse with wild-type eggs, but the co-existence of wild-type eggs resulted in the fusion of 60–70% of the *Cd9*-deficient eggs with at least one sperm (Miyado et al. 2008). Thus, sperm can fuse with *Cd9*-deficient eggs with impaired microvilli with the aid of the microexosomes released from wild-type eggs, which means that microexosomes, not the egg microvilli, are essential for sperm-egg fusion.

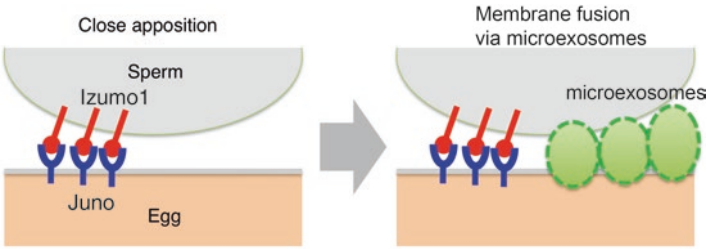

Fig. 26.4 The possible roles of Izumo1, Juno, and Cd9 in sperm-egg fusion

26.16 A Potential Mechanism of Sperm-Egg Fusion

Intercellular membrane fusion can be divided into four steps: (1) close apposition, (2) stalk formation, (3) hemifusion, and (4) formation of a fusion pore (Hui et al. 1981) (Kozlovsky et al. 2002). Hemifusion precedes the formation of a connection between membrane components, leading to the formation of a fusion pore. Although hemifusion is considered a key step in membrane fusion, this step remains unproved at the biological and even biophysical and biochemical levels. Since hemifusion is thought to start with close apposition and subsequent formation of a stalk (Kozlovsky et al. 2002), it is critical to understand the two steps prior to hemifusion in order to understand the mechanism of membrane fusion. Two membrane proteins, Izumo1 and Juno, play crucial roles in close apposition (Bianchi et al. 2014) (a left image in Fig. 26.4). Inoue et al. (2013) have determined the domains of Izumo1 that were required for the function of sperm-egg fusion. They suggest that the formation of a helical dimer at the N-terminal region of Izumo1 is required for its function. Since there are no direct interactions between Izumo1 and Cd9, the microexosomes may be involved in the steps after close apposition (right image in Fig. 26.4).

This knowledge has great potential for helping us to understand the molecular mechanisms of membrane fusion phenomena and for clinical applications, such as the induction of sperm-egg fusion.

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Chapter 27
Fertilization and Protein Tyrosine Kinase Signaling: Are They Merging or Emerging?

Ken-ichi Sato

Abstract Fertilization is a unique form of cell–cell interaction allowing two gametes of the same species—the egg and sperm, which have distinctly different genetic compositions—to unite and give rise to the birth of the next generation of the species. Protein tyrosine phosphorylation, catalyzed by enzymes of a large family collectively called protein tyrosine kinases (PTKs), was initially discovered as a protein posttranslational modification system contributing to the onset, maintenance, and progression of malignant cell transformation, and it is now known to serve as a system that regulates a variety of cell–cell interaction events and multicellularity in living animals. As summarized here, a number of research projects using several model animals have been designed to examine how the egg and sperm interact and how the egg activates to initiate development, by highlighting the roles played by PTK in the egg. This chapter aims to provide the history of the study of fertilization and PTK signaling—most extensively studied using the African clawed frog, *Xenopus laevis*—and to discuss current problems.

Keywords Egg activation · Fertilization · Protein tyrosine phosphorylation · Sexual reproduction · Signal transduction · Src family protein tyrosine kinase

27.1 Introduction

Protein tyrosine kinase (PTK) initially was identified as the oncogene product of Rous sarcoma virus (viral Src protein (v-Src)) and its proto-oncogene product in chicken cells (cellular Src protein (c-Src)) (Hunter 2009). v-Src and c-Src represent the prototypes of cytoplasmic membrane-bound or nonreceptor PTKs, other examples of which include Abelson kinase (Abl), focal adhesion kinase (FAK), and Janus

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tyrosine kinase (Jak). There is another group of PTKs whose partial structure acts as a receptor for certain extracellular ligands or growth factors; they are called receptor PTKs. Examples of receptor PTKs include epidermal growth factor receptor (EGFR), fibroblast growth factor receptor (FGFR), and nerve growth factor receptor (NGFR). Ninety PTK genes have been identified in the human genome: nonreceptor PTKs of 32 species and receptor PTKs of 58 species (Robinson et al. 2000; Watari et al. 2010). Thus, PTKs are a large multigene family with relevance to a number of cellular functions such as proliferation, differentiation, motility, and cell–cell communication. Fine tuning of intracellular PTK activity contributes to a variety of physiological phenomena; therefore, its deregulation corroborates its pathological impact. Since the discovery of PTK activity in v-Src and c-Src, much effort has been devoted to understanding when, where, and how the activity of PTKs—in other words, protein tyrosine phosphorylation—contributes to both physiological and pathological cell functions (Brown and Cooper 1996; Thomas and Brugge 1997). While the occurrence of cellular protein tyrosine phosphorylation is widely demonstrated in all metazoan multicellular animals, from sponges to humans (Srivastava et al. 2010), the same phenomenon is rarely detectable or is not seen in plants or in unicellular organisms such as bacteria, yeast and protozoa (de la Fuente van Bentem and Hirt 2009; Lee and Jia 2009); these organisms possess noncanonical PTKs. Noncanonical PTKs include protein kinases of the BY type in bacteria, those structurally related to P-loop nucleotide triphosphatase, and dual-specificity (specific to serine/threonine and tyrosine) TKL kinases. Wee1 kinase and mitogen-activated protein kinase kinase (MEK) are the other examples of dual-specificity protein kinases that are present in both yeast and metazoan cells. On the other hand, histidine-specific phosphorylation—a relatively unstable form of protein phosphorylation—is limited to prokaryote species.

Serine/threonine-specific protein kinases are present in all unicellular and multicellular organisms (Hanks and Hunter 1995). Thus, the appearance of conventional PTK activity correlates with the emergence of multicellularity in living animals. A recent breakthrough in this topic is the finding that the choanoflagellate *Monosiga brevicollis*, a unicellular protozoan species, possesses not only canonical PTK but also tyrosine-specific protein phosphatase and PTK substrates, which serve as a binding partner for other signaling proteins containing phosphotyrosine-dependent binding domains (e.g., Src homology 2 (SH2) domain) (King et al. 2008; Liu and Nash 2012; Manning et al. 2008; Segawa et al. 2006). These findings imply that the PTK system has been experimentally invented in this unicellular organism and is subjected to playing a specific role in multicellular functions (Lim and Pawson 2010; Liu and Nash 2012; Pincus et al. 2008; Suga et al. 2001, 2012) such as cell–cell communication, which includes fertilization.

Fertilization is the culmination of the specific recognition and interaction of two gamete cells—egg and sperm—followed by a series of processes called egg activation and initiation of zygotic development (Ducibella and Fissore 2008; Okabe 2013; Runft et al. 2002) (Fig. 27.1). From the early 1980s, soon after the discovery of PTKs, a number of experiments were designed to ask whether fertilization and initiation of development involve “on–off” regulation of protein tyrosine

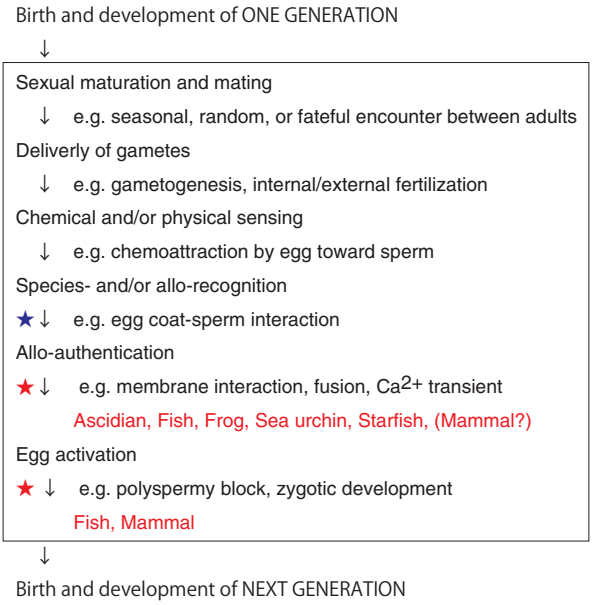

Fig. 27.1 Roles played by protein tyrosine kinase (PTK) in the fertilization system, as highlighted in this chapter. Fertilization is a biological system of fundamental importance in animal sexual reproduction, in which a sequence of events from the mating of parental adults in one generation to the activation of embryonic development in the zygote (i.e., a fertilized egg that has completed mixing of the parental genetic material) occurs to give rise to a newborn of the next generation. This chapter aims to describe the history—as well as discussing problems awaiting further investigation—of research on cellular events at fertilization in several model animals (e.g., the sea urchin, frog, and mouse), gamete membrane interaction, and activation of development (as shown by red letters)—but not egg coat–sperm interaction (as shown by blue letters)—and PTK-mediated signal transduction

phosphorylation in eggs. The physiological contribution of PTKs usually occurs in the early phase of cellular functions; irrespective of receptor-type or cytoplasmic-type PTKs, their signal-dependent activation usually occurs within minutes of the signal cue. Therefore, research was designed to determine whether oocyte/egg PTK activity is activated in response to insemination, i.e., right after interaction between the egg and the sperm at the plasma membrane level. This approach could also be a challenge to answer a long-standing question in the fertilization field: how does the egg activate; in other words, how does the egg initiate a transient elevation in the intracellular calcium concentration (Ca^{2+} transient), which is necessary and sufficient for activation of all egg activation processes (Ciapa and Chiri 2000; Kinsey 1997, 2014; McGinnis et al. 2011; Nuccitelli 1991; Runft et al. 2002; Santella et al. 2004; Sato et al. 2000a, b, 2006a)? This chapter presents experimental findings on PTK signaling and fertilization obtained so far in several animal species and discusses their physiological significance, focusing on the egg side. For a discussion of similar topics on the sperm side, see Ijiri et al. (2012) and Visconti et al. (2002).

27.2 Protein Tyrosine Kinase Signaling at Fertilization in Animal Models

27.2.1 Sea Urchins

Garber and colleagues (Dasgupta and Garbers 1983; Satoh and Garbers 1985) first reported the presence of PTK activity in sea urchin eggs and early embryos. Subsequently, Kinsey and colleagues demonstrated, for the first time, fertilization-dependent protein tyrosine phosphorylation in eggs of the same species (Jiang et al. 1991; Kamel et al. 1986; Kinsey 1984; Peaucellier et al. 1988; Ribot et al. 1984). Since then, sea urchins and some other sea invertebrate species have served as model animals for study of PTK signaling and fertilization. In the sea urchin, sperm-induced tyrosine phosphorylation of egg proteins occurs as early as several seconds to minutes after insemination (Abassi and Foltz 1994; Ciapa and Epel 1991; Wright and Schatten 1995). Consistently, SFK (Fyn) or SFK-like PTK (p57/58 kinase) activity has been reported to start within a period of seconds or up to 5 min after insemination (Abassi et al. 2000; Giusti et al. 2003; Kinsey 1995; O'Neill et al. 2004). Activation of PTK of other types (i.e., Abl) at fertilization has also been demonstrated (Moore and Kinsey 1994). Specific inhibition of SFK activity (e.g., PP1) or general inhibition of egg PTK (e.g., genistein) results in a delay or complete blockade of sperm-induced Ca^{2+} transient (Moore and Kinsey 1995; Shen et al. 1999). An alternative approach to inhibition of SFK signaling, by introducing a recombinant protein containing SH2 domain, has also been shown to inhibit normal fertilization (Giusti et al. 2003; Kinsey and Shen 2000; O'Neill et al. 2004; Townley et al. 2009). Sperm-induced activation of egg SFKs seems to contribute to the activation of PLCγ, by which IP_3-dependent Ca^{2+} transient and egg activation events are triggered (Carroll et al. 1999; De Nadai et al. 1998; Rongish et al. 1999; Shearer et al. 1999). Giusti et al. (2003) found that an SFK closely related to mammalian c-Src—named SpSFK (after *Strongylocentrotus purpuratus* SFK) or *Lytechinus variegatus* homologue AcSFK—is expressed in sea urchin egg membranes. Kinsey et al. showed the presence of a 57 kDa kinase (Kinsey 1995) and Fyn (Kinsey 1996; Kinsey and Shen 2000) in sea urchin eggs, both of which show fertilization-dependent activation and/or phosphorylation, although molecular cloning of them has not been done. Proteome analysis of phosphotyrosine-containing egg proteins before and after fertilization was also first explored in this species: the results so far obtained suggest the presence of hitherto unknown proteins as the sperm-induced tyrosine kinase substrate (Roux et al. 2006, 2008). In addition, the presence of a specific membrane domain (which we name the membrane microdomain in *Xenopus laevis* eggs) in the egg that may act as a scaffold for sperm-induced tyrosine kinase signaling has been suggested (Belton et al. 2001).

27.2.2 Ascidians, Starfish, and Other Sea Animals

The role played by egg SFKs and/or other types of PTKs in fertilization has also been implicated in some other sea animals. In ascidians—a model of hermaphroditic chordate species—preincubation of eggs in the presence of erbstatin (a PTK-specific inhibitor) attenuates sperm-induced *Ciona savignyi* egg activation (Ueki and Yokosawa 1997), suggesting that egg PTK is required for this process. In *Ciona intestinalis*, it has been shown that both egg fertilization and injection of sperm extract into the egg cytoplasm result in the occurrence of Ca^{2+} transient, which depends upon the involvement of SH2 domain–dependent signaling events (Runft and Jaffe 2000). While molecular insights into sperm–egg interaction at the vitelline envelope (for allorecognition and self/nonself recognition, see Harada et al. 2008; Saito et al. 2012; for proteasome-dependent interaction, see Sawada et al. 2002) have been well characterized in *C. intestinalis*, their relationships to egg PTK activity is not known. The molecular identity of the egg PTK that is possibly involved in egg activation is also unknown.

In the starfish *Asterina miniata*, Kinsey and colleagues demonstrated, for the first time, oocyte protein tyrosine phosphorylation in the course of hormone-induced maturation (Peaucellier et al. 1990). A subsequent study examined a possible functional relationship between PTK and sperm-induced egg activation via ectopic expression of a receptor-type PTK (i.e., platelet-derived growth factor receptor) (Shilling et al. 1994). Carroll et al. presented further evidence for the possible involvement of egg PTK in starfish fertilization in a study where a requirement of PLCγ for Ca^{2+} transient and egg activation was demonstrated (Carroll et al. 1997; Runft et al. 2004). Subsequently, Giusti et al. reported that starfish fertilization involves SH2-dependent interaction between SFK and PLCγ, and their enzymatic and sequential activation (firstly SFK, secondary PLCγ) for Ca^{2+} transient and egg activation (Giusti et al. 1999a, b, 2000, 2003). In this species, however, the molecular identity of egg SFKs to be activated upon fertilization has not been demonstrated. In addition, some other researchers have shown that starfish fertilization involves the actions of a sperm-derived protease on the egg surface (Carroll and Jaffe 1995; Santella et al. 1998), actin reorganization at the sperm–egg interaction site (Puppo et al. 2008), and egg NAADP+ as a trigger of sperm-induced Ca^{2+} transient (Lim et al. 2001); however, their relationship to egg PTK is not yet known.

Other sea animals—*Chaetopterus* (Yin and Eckberg 2009) and the protostome worm *Cerebratulus* (Stricker et al. 2010)—have also been examined; so far, however, the significance and function of egg PTK remains unclear.

27.2.3 *Amphibians (the African Clawed Frog,* Xenopus laevis*)*

For more than 50 years, the oocyte of *X. laevis* has been an excellent model system to study early embryogenesis (Nieuwkoop and Faber 1994). Hormone-induced oocyte maturation and fertilization have also been subjects of extensive study since the early 1970s (Ferrell 1999a, b; Masui 2000; Runft et al. 2002) (Fig. 27.2). The involvement of PTK activity in fertilization was explored in studies by Nuccitelli and colleagues, where ectopic expression of receptor-type tyrosine kinase (i.e., the EGF receptor) in mature oocytes and its subsequent activation with EGF resulted in egg activation accompanied by IP_3-dependent Ca^{2+} transient (Nuccitelli et al. 1993; Yim et al. 1994). The results demonstrated that activation of egg PTK is sufficient to promote egg activation. On the other hand, Runft et al. showed that SH2 domain–mediated signaling mechanisms, which are thought to be important for EGF receptor–mediated artificial egg activation, are not required for sperm-induced egg activation (Runft et al. 1999). Concurrently, however, our study demonstrated that an egg Src-related PTK is activated within 10 min of insemination (Sato et al. 1996, 1999). Later, molecular cloning demonstrated that it is *Xenopus* Src 1/2 (Iwasaki et al. 2006). Pharmacological studies showed that activity of egg PTK is required for sperm-induced Ca^{2+} transient and other Ca^{2+}-dependent cellular processes (Glahn et al. 1999; Sato et al. 1998, 2000a, 2001; Tokmakov et al. 2002; for a review, see Sato et al. 2004). The activated Src has been shown to phosphorylate and activate phospholipase Cγ, which contributes to inositol 1,4,5-trisphosphate-dependent intracellular Ca^{2+} release within the fertilized eggs (Sato et al. 2000a, b,

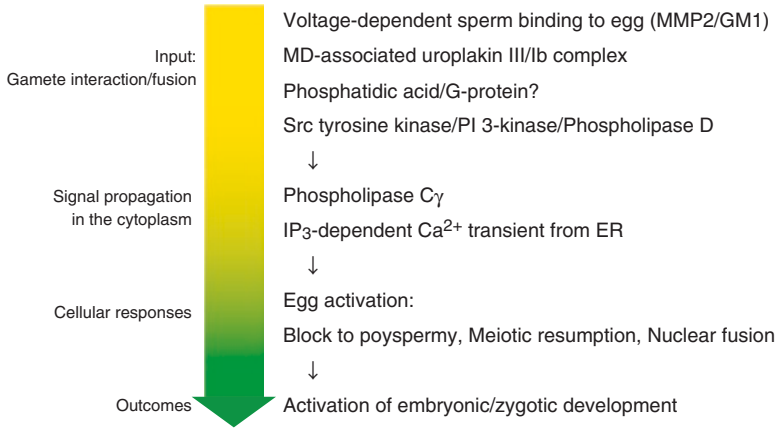

Fig. 27.2 Sperm-induced egg activation involving the egg microdomain-associated uroplakin III–Src protein tyrosine kinase (UPIII–Src PTK) signaling mechanism in the African clawed frog, *Xenopus laevis*. The egg plasma membrane–associated and PTK-dependent signaling mechanism has been extensively studied by using *X. laevis* as a model animal. This schematic diagram depicts a series of events from the egg–sperm membrane interaction to the cytoplasmic propagation of the signal of fertilizing sperm and the onset of embryonic/zygotic development in this organism

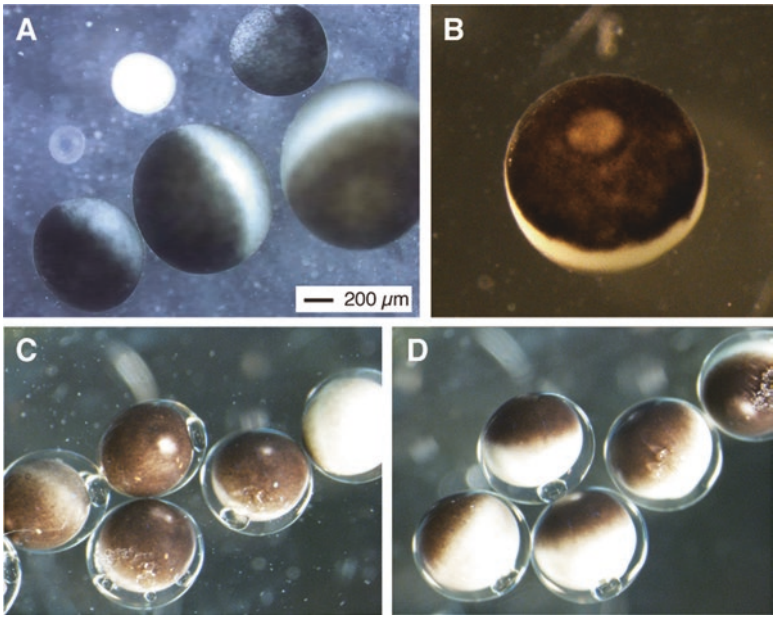

Fig. 27.3 *Xenopus laevis*: (**a**) immature oocytes, (**b**) progesterone-treated mature oocytes, (**c**) unfertilized eggs, and (**d**) fertilized eggs 5 min postinsemination

2001, 2003). Other cytoplasmic substrates of the activated Src so far identified include RNA-binding protein heterogeneous nuclear ribonucleoprotein K (Iwasaki et al. 2008), the SH2-containing adaptor protein Shc (Aoto et al. 1999), phosphatidylinositol 3-kinase (Mammadova et al. 2009), lipovitellin 2 (Kushima et al. 2011), and the intracellular region of uroplakin III (UPIII) (for details, see below) (Sakakibara et al. 2005).

In *X. laevis*, oocyte/egg membrane surface receptors and the intracellular membrane–associated interaction partner for Src have also been investigated, some of which are thought to be sperm-interacting and/or enzymatic regulators for the tyrosine kinase Src (Fig. 27.3). The first to be examined was a molecular interaction between sperm disintegrin and egg integrin. A synthetic peptide containing an Arg–Gly–Asp sequence—a disintegrin motif with high affinity to certain kind of integrin proteins—was shown to activate parthenogenetically *Xenopus* eggs (Iwao and Fujimura 1996). Later studies demonstrated the presence of sperm-borne disintegrin xMDC16 (Shilling et al. 1997, 1998) and MMP-2 (Iwao et al. 2014); the former contains Lys–Thr–Cys and the latter contains a hemopexin domain for binding to egg integrin and/or other negatively charged microdomain-associated molecules (e.g., ganglioside GM1). In fact, egg GM1—but not asialo-GM1, which does not possess a positive charge—has been shown to directly interact with sperm and to contribute to sperm-dependent egg activation; this interaction could provide a

means for a voltage-dependent *Xenopus* gamete interaction at the plasma membrane level (Iwao and Jaffe 1989; Iwao et al. 2014).

As a second candidate for the sperm–egg interacting machinery, the egg microdomain-associated single-transmembrane protein UPIII has been well characterized. This protein was initially identified as a predominantly tyrosine-phosphorylated protein that localizes to low-density detergent-insoluble membrane (LD-DIM) fractions of fertilized *Xenopus* eggs (Sakakibara et al. 2005). LD-DIM is also called a detergent-resistant membrane (DRM) or a microdomain (MD; hereafter, we use this terminology). These biochemically isolated membrane compartments are constituted by nonsaturated phospholipids, cholesterol, and sphingolipids, as well as a specific subset of receptor and/or signaling proteins (Brown and London 1998; Simons and Ikonen 1997; Mahbub Hasan et al. 2011). Several lines of evidence have demonstrated that the MD of an unfertilized *Xenopus* egg is a structural and functional platform for sperm-induced Src tyrosine kinase signaling at fertilization (Luria et al. 2002; Mahbub Hasan et al. 2005, 2007, 2011, 2014; Sato et al. 2002, 2003, 2006a, b). *Xenopus* fertilization requires tryptic protease activity of sperm at the level of gamete–plasma membrane interaction (Mizote et al. 1999). Its pharmacological inhibition results in a failure of sperm-induced activation of Src and embryonic development (Mahbub Hasan et al. 2005). UPIII is thought to be a sperm-interacting protein as a target of sperm protease (Mahbub Hasan et al. 2005, 2014). The sperm protease has been purified to near homogeneity by biochemical and chromatographic fractionation (Mizote et al. 1999); however, its molecular identity remains unclear. The mechanism by which sperm-induced partial proteolysis of UPIII promotes activation of Src and egg activation remains unknown. In mammalian cells, there are some examples showing that proteolysis of the cell surface receptor (e.g., CD44, Notch) (Fortini 2002; Nagano and Saya 2004; Vu et al. 1991) acts as a trigger for intracellular signal transduction. A possible mediator for Src and egg activation in response to UPIII proteolysis is phosphatidylinositol 3,4,5-triphosphate, a product of phosphatidylinositol 3-kinase (Mammadova et al. 2009). More recently, possible involvement of phosphatidic acid (PA)—a product of phospholipase D—in sperm-induced activation of Src has been documented (Bates et al. 2014; Stith 2015).

UPIII has a binding partner, uroplakin Ib—a tetraspanin transmembrane uroplakin family protein, which contributes to the exit of UPIII from the endoplasmic reticulum and membrane localization (Jenkins and Woolf 2007; Tu et al. 2002; Wu et al. 2009). The UPIII–UPIb complex on the egg surface physically associates with the ganglioside GM1. Therefore, it is attractive to hypothesize that the egg surface MD-associated GM1–UPIII/UPIb–Src system—in short, the UPIII–Src system—constitutes a machinery to directly interact with sperm by the positively charged moiety of GM1 (see above) and to transmit the binding signal to the egg cytoplasm by proteolysis of the extracellular domain of UPIII and intracellular phosphorylation and/or activation of UPIII–Src. More recent study has demonstrated that the UPIII–Src system in the egg MD acts not only in receiving and transmitting egg activation signals from fertilizing sperm but also in "activating" the fertilizing sperm through their membrane interaction (Mahbub Hasan et al. 2014). Isolated MDs of

unfertilized eggs are capable of interacting with fertilizing sperm, by which sperm become able to fertilize eggs blocked with an antibody that binds to the extracellular domain of UPIII. These findings suggest that interactions between the sperm and isolated MDs bypass those between the sperm and the egg surface; otherwise, fertilization of antibody-blocked eggs is not possible.

The UPIII–Src system acquires its functional competence for receiving the sperm signal and for transmitting it to the egg cytoplasm during hormone-induced oocyte maturation (Mahbub Hasan et al. 2014; Sato 2014). Biochemical experiments have shown that UPIII is expressed in ovarian immature oocytes from the beginning of oogenesis. On the other hand, the UPIII extracellular domain becomes more accessible to the anti-UPIII extracellular domain antibody after hormonal treatment of oocytes for maturation, as determined by indirect immunofluorescence studies. In vitro sperm-induced activation of the isolated MD fractions, with phosphorylation of UPIII and Src serving as one of the readouts, have also shown that the responsiveness of the MD of fully grown immature oocytes to sperm is much weaker than that of in vitro maturing oocytes or of ovulated unfertilized eggs (Mahbub Hasan et al. 2014; Sato 2014). The aforementioned ability of the MD to bypass the requirement of sperm to interact with egg surface UPIII is also weak in an MD prepared from immature oocytes. Thus, the UPIII–Src system in *X. laevis* represents the first example of the egg membrane machinery undergoing maturation.

27.2.4 Fish (the Zebrafish, Danio rerio*)*

Considerable progress has also been made in understanding the role of SFKs in fertilization and egg activation of vertebrate species other than *Xenopus*. In the zebrafish, *D. rerio*, an SFK immunochemically related to mammalian Fyn is activated within 30 s of insemination (Kinsey et al. 2003; Sharma and Kinsey 2006, 2008; Wu and Kinsey 2000, 2002). Possible involvement of a tyrosine-specific protein tyrosine phosphatase, RPTPα, in the sperm-induced activation of the egg SFK has been suggested (Wu and Kinsey 2002): RPTPα constitutively associates with the egg SFK, thereby it activates upon fertilization and dephosphorylates a C-terminal negative regulatory phosphotyrosine of the egg SFK. Application of tyrosine phosphatase inhibitors has been shown to inhibit normal zebrafish fertilization. The interaction between RPTPα and egg SFK seems to be mediated by the SFK SH2 domains, implying how the SFK SH2 domain–dependent SFK function works for sea urchin and starfish egg fertilization (see Sects. 27.2.1 and 27.2.2). Another SFK—Yes—and PYK2 (a FAK-related Ca^{2+}-dependent tyrosine kinase) have also been implicated in normal fertilization and early embryogenesis (Sharma and Kinsey 2013; Tsai et al. 2005), although their molecular functions are unknown. Phylogenetic analyses based on partial sequencing of genomic Src and Src-related genes have been challenged in the freshwater killifish species (Cyprinodontiformes)

(Meyer and Lydeard 1993) and cichlid fish (Sides and Lydeard 2000); however, functional analysis of them in relation to egg fertilization has not yet been done.

27.2.5 Mammals (Mice, Rats, and Others)

In the mouse and rat, the SFKs (Fyn, Lck, Yes, and Src) (Levi et al. 2012; Mori et al. 1991; Kurokawa et al. 2004; Talmor et al. 1998) and FAK family kinases (FAK, PYK2) (Luo et al. 2014; McGinnis et al. 2013) are expressed in eggs, and maturing oocytes and fertilized eggs contain tyrosine-phosphorylated proteins (Ben-Yosef et al. 1998; Kurokawa et al. 2004; McGinnis et al. 2013). In particular, localized accumulation of phosphotyrosine-containing proteins in the cortex, the sperm–egg interaction site, and the MII spindle of fertilized mouse eggs has been demonstrated (McGinnis et al. 2007, 2013). However, pharmacological and biochemical studies demonstrate that the activity of neither SFK nor more generally PTK is sufficient or required for fertilization-induced Ca^{2+} oscillation (Kurokawa et al. 2004; Mehlmann and Jaffe 2005). Rather, functions of PTKs such as Fer- and SH2-mediated molecular interaction seem to be important for meiotic maturation and zygotic development, respectively, in mouse oocytes, as determined by pharmacological approaches and gene knockdown approaches (Meng et al. 2006; McGinnis et al. 2009, 2011; Talmor-Cohen et al. 2004a). Moreover, Fyn is implicated in cleavage furrow ingression during meiosis and mitosis, and in the release of the oocyte from the metaphase (Levi et al. 2010a, b; Levi and Shalgi 2010). Evidence from another line demonstrates that Fyn kinase activity is required for normal organization and functional polarity of the mouse oocyte cortex (Luo et al. 2009). Interaction between Fyn and tubulin during meiosis has also been demonstrated in rat eggs (Talmor-Cohen et al. 2004b). A possible link between PTK activity and egg activation in mammals has been demonstrated in studies using pig oocytes, showing that sodium orthovanadate (a wide-specificity inhibitor for protein tyrosine phosphatase) causes parthenogenetic activation (Kim et al. 1999). In bovines and mice, sperm disintegrin (Arg–Gly–Asp sequence) and egg integrin–mediated gamete interaction have been suggested to act for egg activation (Gonçalves et al. 2007; Tatone and Carbone 2006; White et al. 2007); however, their relationship to egg PTK signaling has not yet been examined.

Involvement of PLCγ and other PLC isoforms in fertilization signaling has also been extensively studied in mouse eggs. Mehlmann et al. reported that neither SH2 domain–dependent PLCγ action nor even egg PLC activity is required for fertilization-induced Ca^{2+} oscillation (Mehlmann et al. 1998, 2001). On the other hand, Sette and colleagues reported that a truncated form of c-Kit tyrosine kinase, which is specifically expressed in sperm (Sette et al. 1997, 1998), promotes PLCγ-dependent egg activation (Sette et al. 1997, 1998, 2002) and that the mechanism involves Fyn–PLCγ–Sam68 interaction and activation of Fyn (Paronetto et al. 2003). Such a "sperm factor"–dependent mechanism of egg activation has been postulated since the late 1980s in some species, including sea urchins and mice

(Swann 1996). In this connection, two hypothesized additional candidates for the mouse sperm factor are PLCζ (Saunders et al. 2002) and PAWP (Aarabi et al. 2014; Wu et al. 2007): which of these proteins is the main sperm factor is still in dispute (Aarabi et al. 2015; Nomikos et al. 2014). PLCζ is a sperm-specific PLC that can be activated in a range of intracellular Ca^{2+} concentrations in which other isoforms of PLC are not activated, which could be a reason why this PLC isoform is solely responsible for promoting Ca^{2+} oscillation in mouse eggs, where the intracellular Ca^{2+} concentration is low (Saunders et al. 2002; Swann et al. 2004). A synthetic peptide inhibitor of SFK, peptide A7, has been shown to inhibit Ca^{2+} oscillation promoted by insemination or injection of sperm extract (Kurokawa et al. 2004). This might be due to direct binding of the peptide to PLCζ, but not due to the inhibition of SFK activity in eggs (Kurokawa et al. 2004). The WW-binding protein PAWP is also a sperm-specific, but not cytosolic, protein; it is a constituent of the postacrosomal and perinuclear theca, while other lines of evidence suggest the existence of an "insoluble sperm factor" (Kurokawa et al. 2005; Perry et al. 2000; Sutovsky et al. 2003). Although molecular interaction involving WW domain–containing protein and SFK has been demonstrated in human somatic cells (Macias et al. 1996), the molecular target of sperm PAWP remains unknown.

27.3 Perspectives

As described above, several model organisms have been actively employed to study involvement of PTK activity in fertilization. Fundamental, old, and still exciting questions in the field of fertilization study remain to be answered. How do the egg and sperm interact and fuse with each other? How does the egg–sperm interaction/fusion ignite the developmental cue? Identification and characterization of the molecular machinery for gamete interaction/fusion is the most important criterion to answer the aforementioned problems (Fig. 27.4). In the sea urchins *Strongylocentrotus franciscanus* and *S. purpuratus*, egg-associated EBR1 protein has been identified as a sperm-interacting molecule (Kamei and Glabe 2003). In *X. laevis*, UPIII/UPIb complex has been suggested as a sperm protease target on the egg surface (see above). In the mouse, sperm IZUMO1 (Inoue et al. 2005, 2013) and egg Juno proteins (Bianchi et al. 2014) have been identified as the mutual interacting partners in gamete–plasma membrane interaction: their gene knockout results in failure of gamete binding. Egg surface CD9 has also been postulated as the gamete interaction/fusion component in the mouse: its gene knockout results in failure of gamete fusion (Kaji et al. 2000; Le Naour et al. 2000; Miyado et al. 2000; 2008). The molecular identity of the candidate gamete interaction/fusion molecule has not yet been demonstrated in the other animal species highlighted in this article. Future study should clarify the functional relationship between the gamete interaction/fusion machinery and egg PTK, and its physiological importance for successful fertilization and activation of development.

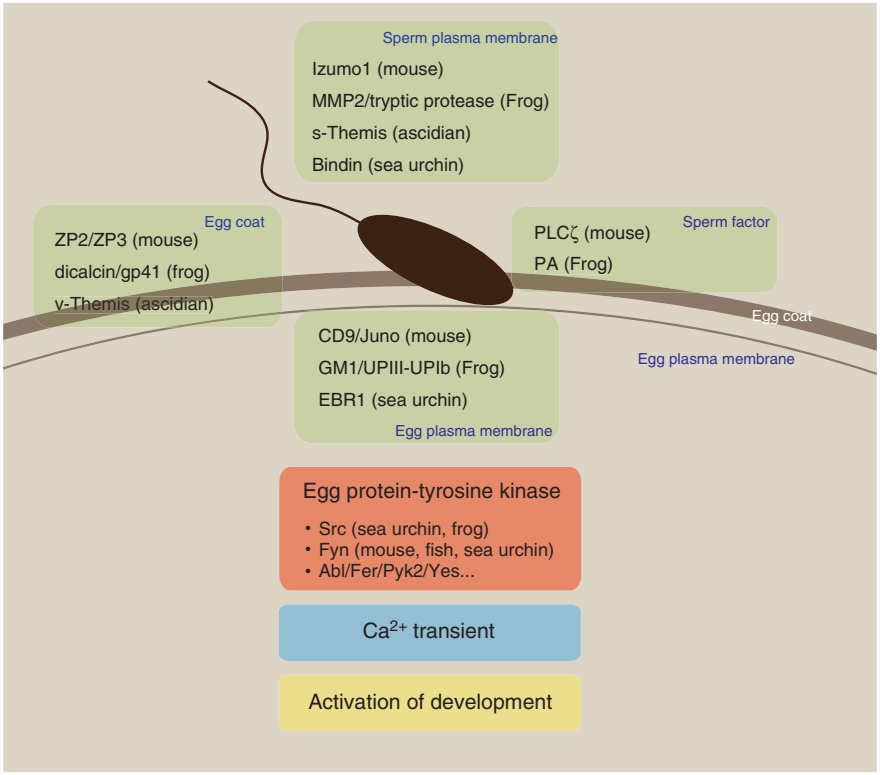

Fig. 27.4 Spatiotemporal sequence of fertilization. Fertilization involves gamete interaction at the level of the sperm–egg coat (e.g., the vitelline envelope, zona pellucida), gamete interaction at the plasma membrane level, stimulation of egg protein tyrosine kinase (PTK) and/or introduction of sperm-derived factor, transient release of intracellular calcium ions, and activation of development

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Part IV
Diversity in Axis Formation

Chapter 28
Early Embryonic Axis Formation in a Simple Chordate Ascidian

Gaku Kumano

Abstract Embryonic axes are established during early embryogenesis in animal development and serve as frameworks upon which embryonic body structures are built. A variety of tissue precursors are arranged along embryonic axes. Therefore, elucidation of the mechanisms by which these axes form in early embryos is fundamental in understanding how animal development proceeds, and this has become one of the most important questions in the field of developmental biology. In this chapter, axis formation mechanisms in the eggs and early embryos of marine invertebrates and simple chordates—ascidians—are presented. This information provides an opportunity to better understand the comprehensive and unique axis formation story of this particular animal species. However, generally important concepts of embryonic axis formation are interspersed throughout the axis-forming processes in ascidians. Therefore, readers should appreciate the complex and fascinating strategy underlying the establishment of early embryonic axes for the later development of ascidians and find the comparison of these mechanisms with those utilized in other organisms interesting.

Keywords Animal-vegetal · Ascidian · Ascidian · Maternal determinant · Dorsal-ventral · Embryonic axis formation · Left–right asymmetry · Ooplasmic movements · *Postplasmic/PEM* RNA

28.1 Introduction

Animal bodies are equipped with various tissues and organs in properly specified positions along their body axes so they can perform life activities, including feeding, protection from enemies, and reproduction. Bilaterians have three definitive

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axes: the rostral–caudal, dorsal–ventral, and left–right axes. The positioning of tissues and organs along these three axes reflects the rearrangement of embryonic tissue precursors through gastrulation in bilaterian larvae and adults. These tissue precursor cells are specified in terms of developmental fates and arranged according to pregastrula positions along early embryonic axes. Accordingly, the axes established during early embryogenesis serve as key references for appropriate tissue arrangement and, together with subsequent controlled cell movements during gastrulation, these positions contribute to the eventual body organization in larvae and adults. Thus, it is important to examine how these early embryonic axes are established, particularly because these specified regions appear to arise from a simple-looking single cell—that is, the egg.

When examining axis formation in early embryos, it is essential to note that the names of the embryonic axes do not necessarily reflect the body orientation of the larvae and adults. For example, the anterior–posterior axis is established in ascidian embryos immediately after fertilization (Fig. 28.1). Notochord and mesenchyme precursor cells are specified and present in the anterior and posterior halves of pregastrula embryos, respectively (Fig. 28.1p) (Nishida 1987). However, through gastrulation, these notochord and mesenchyme precursors end up in the posterior/tail and anterior/trunk regions of larvae, respectively (Fig. 28.1q–s) (Nishida 1987). Similarly, the so-called dorsal–ventral axis in *Xenopus* pregastrula and early gastrula embryos extends from the organizer to the contra-organizer sides, both of which comprise precursor cells that give rise to ventral tissues, referred to as blood islands, in larvae (Lane and Smith 1999; Lane and Sheets 2002; Kumano and Smith 2002).

Importantly, axis formation comprises two distinct, but not always separable, developmental processes. One process determines the positions of the axes—that is, where the axes run within the egg and/or embryo. This process, in many cases, relies on localization mechanisms through which the presence of certain molecular complexes and/or organelles is biased to one side of the egg and/or embryo. The position of such localized materials should determine the position of one end of an axis, thereby defining the entire axis. Localization depends on the cis-elements or localization signals of the localized materials and the environmental factors that localize these molecules. In addition, as a second process, these molecular complexes, or those attached to the organelles, often characterize one end of an axis on the basis of their functional domains, independently of their localization signals. For example, some localized molecular complexes create anterior structures through anteriorizing activities or generation of tissues residing in the anterior part of the body, thereby orienting the axis as an anterior–posterior axis. The distinction between these two processes is important because the latter process depends on the function of the localized materials, but not localization mechanisms per se. For example, the mislocalization of *nanos* messenger RNA (mRNA) to the anterior according to the *bicoid* RNA localization signal in *Drosophila* embryos compromised the characterization, but not the position, of what should have been the anterior–posterior axis of the embryo, forming two posterior structures with mirror image symmetry along this axis (Gavis and Lehmann 1992). The establishment of the left–right axis in

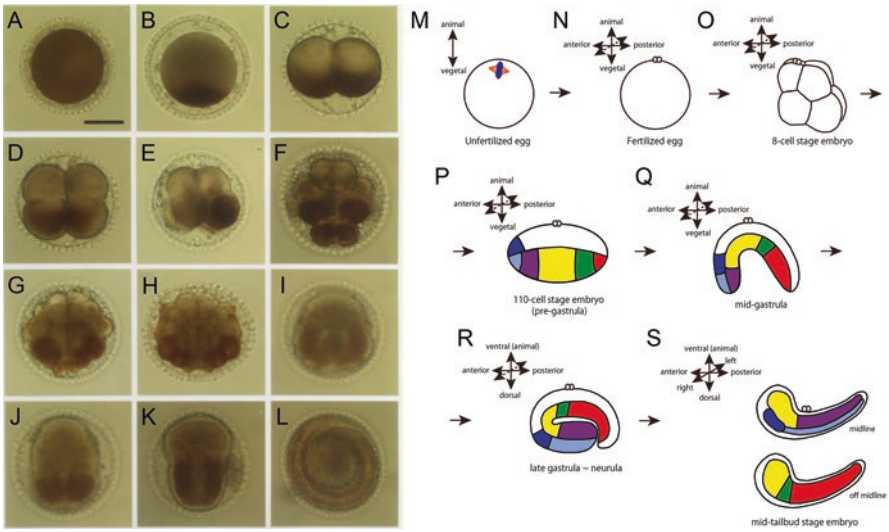

Fig. 28.1 Ascidian embryogenesis. (**a**–**l**) Embryogenesis of the ascidian species *Boltenia villosa*. The specific cytoplasm, naturally colored in brown and known as a myoplasm, is inherited by the muscle lineage cells. (**a**) Unfertilized egg. The myoplasm is distributed ubiquitously. (**b**) Fertilized egg. The myoplasm is localized to the vegetal region as a result of ooplasmic segregation. (**c**) Two-cell-, (**d**) four-cell-, (**e**) eight-cell-, (**f**) 16-cell-, (**g**) 32-cell-, and (**h**) 64-cell-stage embryos. The myoplasm is inherited by the muscle lineage cells. (**i**) Gastrula and (**j**) neurula. (**k**) Early and (**l**) late tailbud embryos. The myoplasm is observed in the muscle cells of the tail. (**b** and **e**) Animal is shown at the top. (**e**) Lateral view. (**f**–**h**) Vegetal views. (**d** and **f**–**k**) Anterior is shown at the top. (**m**–**s**) Schematic diagrams of ascidian embryogenesis. All of the embryos are oriented in a manner in which the site of the polar body extrusion (animal pole) is fixed at the top. Axes that have been established at the stage indicated are shown as *solid arrows* above on the left side of each diagram. The *dotted arrows* indicate that corresponding axes are placed but not determined in terms of orientation. (**m**) Unfertilized and (**n**) fertilized eggs. Only the case where the original and new animal–vegetal axes overlap is indicated here, for simplicity (see text). (**o**) Eight-cell-stage embryo, (**p**) 110-cell- and pregastrula-stage embryo, (**q**) gastrula, (**r**) late gastrula to neurula, and (**s**) midtailbud-stage embryo. (**o**–**s**) Lateral views. (**p**–**r**) Midsagittal sections of the embryos. (**s**) Midsagittal section (top) and sagittal section off the midline (bottom). (**p**–**s**) Colored areas indicate groups of cells fated to become certain tissues, showing how the arrangement of tissue precursors changes during gastrulation: brain in dark blue; nerve cord in light blue; notochord in purple; endoderm in yellow; mesenchyme in green; muscle in red

ascidian embryos might be another example in which the determination of the axis position and axis characterization are separable. The position is automatically determined once the other two perpendicular axes (animal–vegetal and anterior–posterior) are established in the eggs. Later in development, this axis is characterized and the left and right sides are subsequently determined (Nishide et al. 2012).

This chapter describes early embryonic axis formation during pregastrula development in ascidian species as model organisms (Fig. 28.1). In ascidians, the primary axis is established during oogenesis, defining the animal–vegetal axis (Fig. 28.1m). The second axis forms perpendicularly to the primary axis soon after fertilization,

defining the anterior–posterior axis (Fig. 28.1n). When the second axis is obtained, the left–right axis is positioned automatically (Fig. 28.1n). The orientation of the third axis is not characterized until the neurula stage (Fig. 28.1s).

Ascidians are marine invertebrates comprising a sister group of vertebrates in the phylum or superphylum (Satoh et al. 2014) Chordata. Because these organisms comprise a relatively small number of cells in the embryonic stage but still retain the common body plan for Chordata, ascidian embryos have attracted a great deal of research attention as they can be used to understand the fundamental mechanisms by which the chordate body plan is generated during development. Indeed, the distribution of tissue precursors in the fate maps of pregastrula embryos is similar in vertebrates and ascidians (Nishida 1987; Lane and Smith 1999; Lemaire et al. 2008; Nishida and Stach 2014), suggesting that the use of axes to specify tissue precursors might be conserved. This notion might be true to some extent when considering the animal–vegetal axis. Along this axis, the three germ layers are established: the endoderm forms in the vegetal region, the ectoderm forms in the animal region, and the mesoderm forms in the region between the two. In both vertebrates and ascidians, the germ layers are established by localized germ layer determinants to the vegetal poles (Lemaire et al. 2008; Kumano 2012; Houston 2013), which are localized during oogenesis and immediately after fertilization. The patterned specification of tissue precursors along the axis perpendicular to the animal–vegetal axis is also governed by localized determinants in these animals (Lemaire 2009; Langdon and Mullins 2011; Kumano 2012; Houston 2013).

Despite the many features shared by ascidians and vertebrates, mechanisms that are unique to ascidians in relation to axis formation have been discovered. One such prominent example is the left–right determination mechanism, as detailed in Sect. 28.4. Accordingly, ascidian axis formation represents both common and unique mechanisms, which are introduced in detail in Sects. 28.2, 28.3 and 28.4 to describe the basic principles of this developmentally important event.

28.2 The Primary Axis Established Prior to Fertilization: The Animal–Vegetal Axis

In most animals, the primary axis is established during oogenesis and oocyte maturation, and defines the animal–vegetal axis. This axis depends on the location of the meiotic apparatus (MA). Polar bodies are produced on the side of the oocyte where the MA is localized and the animal pole is literally defined (Figs. 28.1m and 28.2a). Thus, the vegetal pole is the opposite of this pole. Through axis-forming processes, via the eccentric positioning of the MA or the germinal vesicle (GV) from which the MA originates, the mature oocytes eventually become polarized, with molecular complexes and/or organelles differentially distributed along the axis, awaiting fertilization. Thus, unraveling the process in which the MA or GV becomes situated on one side of the oocyte is key to understanding the mechanisms underlying the establishment of the primary axis, particularly the occurrence of the earliest symmetry

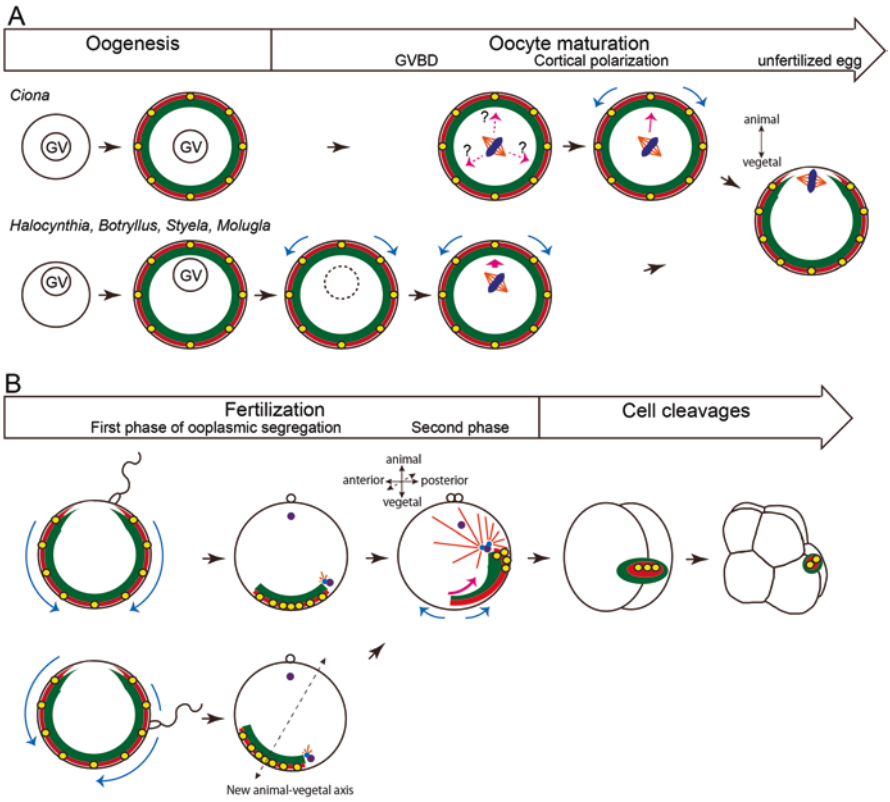

Fig. 28.2 Schematic diagram of the establishment of the primary and anterior–posterior axes. Two examples are shown for the processes of the primary axis formation during oogenesis and oocyte maturation (**a**). In *Ciona* (top), germinal vesicle (GV) breakdown (GVBD) occurs in the centric position and the meiotic apparatus (MA) migrates in a random direction to the cortex of the future animal pole. In *Halocynthia*, *Botryllus*, *Styela*, and *Molgula* (bottom), however, the GV is eccentrically positioned, and after GVBD, the MA migrates to the closest cortex and defines the animal pole. After fertilization (**b**), the first phase of ooplasmic segregation (blue arrow in the left-most embryo) concentrates molecules, including *postplasmic/PEM* RNAs (yellow circles) and organelles, such as the cortical endoplasmic reticulum (ER; red) and subcortical mitochondria (green), to the vegetal pole. Two cases where the original and new animal–vegetal axes overlap (top) and do not overlap (bottom) are indicated here. The second phase of ooplasmic segregation (pink arrow) further concentrates molecules and organelles at the future posterior poles. These molecules/organelles are retained at the future posterior pole during cleavage stages. Blue arrow: cortical movements; pink arrows: cytoplasmic movements; purple circle: nuclei; blue circle: centrosomes; orange lines: microtubules

breaking event, although the process remains largely unknown in ascidians, as detailed below in this section.

Looking across the metazoan species, the processes used to achieve the eccentric positioning of the MA in mature oocytes fall into three categories [discussed in Prodon et al. (2006)]. In some mollusk and mammal species, GV breakdown

(GVBD) and subsequent mitotic spindle formation occur at the center of oocytes and, subsequently, the MA migrates to the cortex. However, in starfish and amphibian species, the GV position is already polarized, as it is situated near the cortex on one side of growing oocytes. Following GVBD, MA formation occurs in the same eccentric position. Moreover, in certain sea urchin, sea cucumber, and fish species, the GV migrates from the center to the cortex, and GVBD and MA formation occur near the cortex. Among ascidian species, *Ciona* has GVBD at the center and the MA migrates from this point to a near-cortex position (top in Fig. 28.2a) (Prodon et al. 2006). In contrast, the eccentric position of the GV has been reported in the growing oocytes of *Halocynthia*, *Botryllus*, *Styela*, and *Molgula* (bottom in Fig. 28.2a) (Mukai and Watanabe 1976; Sawada and Schatten 1988; Sakari and Shirai 1991).

In mature oocytes of solitary ascidian species—such as *Halocynthia roretzi* (Prodon et al. 2008), *Ciona intestinalis/robusta* (Prodon et al. 2006) (formerly *Ciona intestinalis* type B/type A; Distinction at the species level is difficult at this point since *Ciona* from Roscoff where a mixture of *intestinalis* and *robusta* inhabit was used in Prodon et al. before separation of these species), and *Phallusia mammillata* (Sardet et al. 1992)—animal–vegetal differences in the distribution of molecular complexes and/or organelles have been observed. These differences include the mitotic spindle and microtubules in the animal regions, and the cortical rough endoplasmic reticulum (cER), maternal mRNAs, known as *postplasmic/PEM* RNAs, which are associated with cER, subcortical mitochondria-rich myoplasm, and microfilaments, in the vegetal regions (Fig. 28.2a).

In *Ciona*, the MA, generated at the center of the oocyte after GVBD, migrates in a random direction toward the cortex where the animal pole is established (dotted pink arrows in Fig. 28.2a) (Prodon et al. 2006). Upon arrival at the cortex, the MA excludes cortical and subcortical materials, such as *postplasmic/PEM* RNAs and organelles, away from the future animal pole, generating a biased distribution toward the vegetal hemisphere (right-most embryo in Fig. 28.2a) (Prodon et al. 2006). These events are actin dependent (Prodon et al. 2006), although the detailed molecular mechanisms remain unknown. In mouse oocytes, a similar *Ciona*-like process of eccentric MA positioning involving MA migration occurs, and molecules such as microfilament-binding protein Formin 2 and asymmetric forces exerted on the chromosome-spindle complex are essential for this process (Yi et al. 2013; Li and Albertini 2013). This cortical and subcortical movement toward the vegetal hemisphere is reminiscent of the process that occurs in anterior–posterior axis formation in *Caenorhabditis elegans* zygotes (Prodon et al. 2006). There, an actin-dependent cortical flow triggered by sperm entry draws materials at the cortex toward the anterior region, whereas a cytoplasmic flow, known as the fountain flow, pushes other materials back to the posterior region (Hird and White 1993; Goldstein and Hird 1996; Munro et al. 2004).

The involvement of cytoplasmic flow in MA positioning has been suggested in *H. roretzi* (Prodon et al. 2008). In *Halocynthia* oocytes, the GV is eccentrically positioned, and after GVBD, the MA migrates to the closest cortex (bottom in Fig. 28.2a) (Prodon et al. 2008). It has been proposed that the essential factors diffuse out from the GV upon GVBD, triggering the relaxation of actin networks at the

nearest cortex (the future animal pole) and generating cortical and cytoplasmic flows, thereby polarizing mRNAs/organelles in the cortical and subcortical regions, which accumulate in future vegetal regions (blue arrows in Fig. 28.2a), and pushing the MA toward the future animal pole (thick pink arrows in Fig. 28.2a). GVBD at the centric position, as observed in *Ciona* oocytes, would not trigger animal–vegetal differences through diffusible factors from the GV. Interestingly, because the exclusion of the mRNAs/organelles from the future animal pole is initiated before the arrival of the MA at the cortex in *Halocynthia*, the chromosomes/MA might not trigger cortical polarization along the animal–vegetal axis in this species. This contrasts with the situation in mouse oocytes, where chromosomes trigger animal pole specification upon arrival at the cortex (Deng et al. 2007). However, the chromosomes/MA could facilitate the complete exclusion of the mitochondria-rich myoplasm from the future animal pole as a later event (Prodon et al. 2008). The polarization of mRNAs/organelles and cortical and cytoplasmic flows in *Halocynthia* all depend on the actin cytoskeleton (Prodon et al. 2008). At earlier stages, the GV in *Halocynthia* young oocytes is eccentrically positioned farthest away from the follicle stalk, associated with the gonadal epithelium (Prodon et al. 2008), and this early polarization has also been observed in the colonial ascidian *Botryllus* (Mukai and Watanabe 1976). It has been recently shown in *Halocynthia* that experimental translocation of the GV of full-grown oocytes to the opposite pole completely reversed the animal-vegetal axis (Tokuhisa et al. 2017).

Accordingly, ascidian oocytes with an established primary axis are matured and await fertilization in meiotic metaphase I. Upon fertilization, another cortical contraction—more prominent than the initial exclusion from the animal pole described above—occurs, and molecular complexes/organelles accumulate further, becoming highly enriched at the future gastrulation site (the first phase of ooplasmic segregation; see blue arrows in left-most embryo in Fig. 28.2b) (Sardet et al. 2007).

The primary axis, established as described above, might not correspond to an axis along which the three germ layers are specified during pregastrula embryogenesis and at one end of which the gastrulation site is defined. Rather, the primary axis might simply guide the direction of the new axis. As described above, cortical contraction after fertilization concentrates molecular complexes/organelles at the future gastrulation site. Interestingly, the direction of the contraction could more or less deviate from the original animal–vegetal axis in *C. intestinalis/robusta* and *P. mammillata* (Fig. 28.2b bottom) (Roegiers et al. 1995; Prodon et al. 2005). Thus, the site that is richest in mRNAs/organelles as a result of ooplasmic segregation might not coincide with the original vegetal pole and could be localized 45–60° away from this pole, depending on the position of the sperm entry site (Roegiers et al. 1995; Prodon et al. 2005). The cortical contraction is also actin dependent and is triggered by sperm entry (Sardet et al. 2007). Accordingly, the sperm entry point might define a new axis in ascidians that is located either on or off the original animal–vegetal axis, which specifies the three germ layers along this axis and defines the gastrulation site during early embryogenesis. This axis might be called the dorsal–ventral axis in ascidian embryos because future dorsal tissue structures, such as the nerve cord, become situated opposite the site of polar body extrusion after gastrulation

when the embryos are viewed with the polar body site as a fixed reference point through different developmental stages (Fig. 28.1p–s) (Nishida 2005). However, as it is difficult to imagine a dorsal–ventral axis without any differentiated dorsal or ventral tissues in the pregastrula embryo, and this axis has not conventionally been described in previous studies, this axis will hereafter be referred to as the (new) animal–vegetal axis.

The concentrated cortical and subcortical domains at the future gastrulation site after the first phase of ooplasmic segregation should contain materials that specify the animal–vegetal axis. These materials could determine the developmental fate of the endoderm/mesendoderm and/or exclude that of the ectoderm from the vegetal region and specify the site of gastrulation. However, thus far, none of the molecules localized to vegetal cytoplasmic domains at this stage play such roles. Instead, these identified materials are further translocated to the future posterior site by the second phase of ooplasmic segregation (middle embryo in Fig. 28.2b), and these molecules specify the anterior–posterior axis, as described in Sect. 28.3. The existence of materials that specify the gastrulation site and the mesendoderm fate has been predicted in several species: in *Styela plicata* (Bates and Jeffery 1987) and *H. roretzi* (Nishida 1996) for those that specify gastrulation sites, and in *H. roretzi* (Nishida 1993, 1996), *C. robusta* (formerly *Ciona intestinalis* type A; assumed to be *robusta* based on current knowledge of geographical distribution, that is, *Ciona* from Japan), and *Ciona savignyi* (Imai et al. 2000) for those that specify mesendoderm fates. These materials are concentrated in a small region opposite the site of polar bodies [5–8% of the total egg volume in the case of *Halocynthia* (Nishida 1996)], between the first and second phases (see below) of ooplasmic segregation; however, these materials do not remain in this region; rather, they become dispersed into the entire vegetal hemisphere after the second segregation (left-most embryo in Fig. 28.3a) (Bates and Jeffery 1987; Nishida 1993, 1994, 1996).

A cytoplasmic component of the canonical Wnt pathway that is upstream of β-catenin has been predicted to be a maternal factor (or factors) specifying the mesendoderm fate in the vegetal hemisphere of ascidian embryos (Nishida 2005; Kumano and Nishida 2007). β-Catenin protein is ubiquitously present throughout the egg/embryo (Imai et al. 2000; Kawai et al. 2007), and nuclear or active β-catenin is only observed in blastomeres in the vegetal hemisphere starting at the 16-cell stage in *C. intestinalis robusta* (Roscoff, Hudson et al. 2013) and the 32-cell stage in *Halocynthia* and *C. savignyi* (red circles in Fig. 28.3a) (Imai et al. 2000; Kawai et al. 2007; Hudson et al. 2013). During ascidian embryogenesis, the third (four- to eight-cell) cell division—the first division along the animal–vegetal axis—after fertilization produces ectoderm cells in the animal half and mesendoderm (+ nerve cord-fated) cells in the vegetal half (Fig. 28.3a). The segregation of the first germ layer depends on the β-catenin activity in both *Ciona* and *Halocynthia*, as the inhibition of β-catenin activity results in the conversion of the mesendoderm to the ectoderm fate (Imai et al. 2000; Kawai et al. 2007; Rothbächer et al. 2007). β-Catenin specifies the endoderm and mesendoderm by activating the gene expression of transcription factors that are essential for the formation of these germ layers in *Halocynthia* and *Ciona* (Satou et al. 2001; Imai et al. 2002a, b; Kumano et al. 2006).

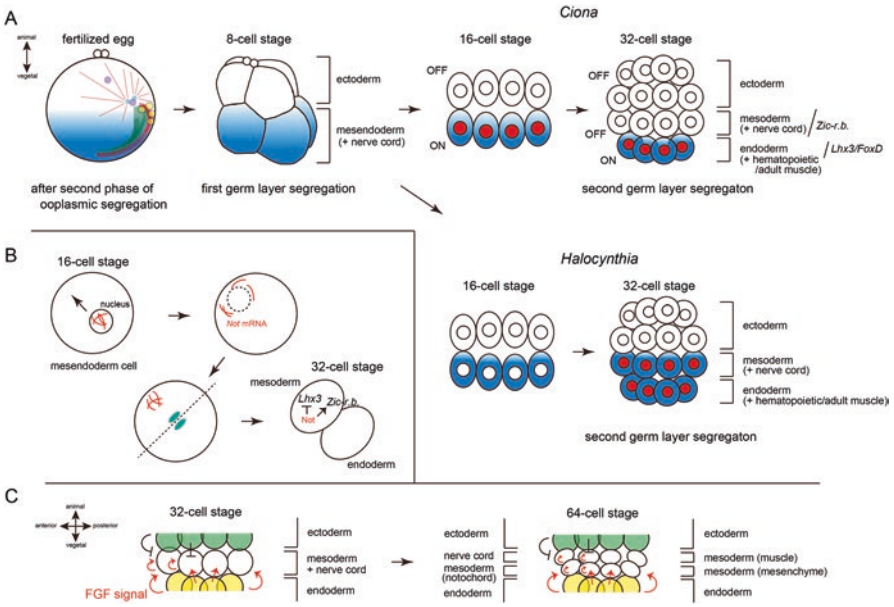

Fig. 28.3 Schematic diagram of the specification mechanisms of the animal–vegetal (ventral–dorsal) axis. (**a**) The β-catenin activities during germ layer segregation are shown in two ascidian species: *Ciona* (top) and *Halocynthia* (bottom). The distribution of an unidentified determinant of mesendoderm specification, which presumably facilitates the nuclear localization (red circles) and activation of β-catenin in the egg and the early embryo, is shown in blue. Differential β-catenin activities distinguish the endoderm, mesoderm, and ectoderm in *Ciona* and the mesendoderm and ectoderm in *Halocynthia*. All lateral views. (**b**) A β-catenin-independent mechanism of the segregation of the mesoderm from the endoderm in *Halocynthia* mesendoderm cells is shown. *Not* messenger RNA (mRNA; red lines) is asymmetrically segregated by the migrating nucleus and is inherited only by the mesoderm cells. The Not transcription factor promotes and inhibits mesoderm and endoderm differentiation, respectively, through the regulation of its target gene expression. Dotted line: future cleavage plane. (**c**) Mechanisms of further fate segregation along the animal–vegetal axis at the division to the 64-cell stage, with an emphasis on the tier comprising mesoderm and nerve cord precursor cells, are shown. Lateral views. Mesoderm (+ nerve cord) cells are exposed to the fibroblast growth factor (FGF) signal (red arrows) emanating from the endoderm (yellow) and mesoderm (+ nerve cord) cells. The daughter cells that remain in contact with endoderm cells, even after cell division, are induced to become notochord and mesenchyme precursor cells. The other daughter cells in contact with ectoderm cells (green) are exposed to less FGF signal and assume nerve cord and muscle fates as uninduced default fates. The reduction in the level of the FGF signal in the latter cells is facilitated in the anterior region by an inhibitory signal (black inhibitory arrows) emanating from the adjacent ectoderm cells and in the anterior and posterior regions by loss of FGF signal competence via transcription factor expression (not shown here) and their position far away from endoderm cells

The endoderm and mesendoderm are also specified by the restriction of the activity of an initially ubiquitous transcription factor for ectoderm differentiation to the animal region in *Ciona* (Rothbächer et al. 2007). Ascidian embryos undergo a second cell division along the animal–vegetal axis as the fifth (16- to 32-cell) division after fertilization. This division within the vegetal half of the embryo produces mesoderm

(+ nerve cord precursor) and endoderm (+ hematopoietic/adult muscle precursor) cells in the animal and vegetal localized regions of the vegetal hemisphere, respectively (Fig. 28.3a). While the second germ layer segregation continues to depend on β-catenin activity in *Ciona* (Hudson et al. 2013), this segregation is regulated by a different mechanism that does not rely on differential β-catenin activities in *Halocynthia* (Takatori et al. 2010), at least in the anterior half of the embryos of both species. Nuclear β-catenin is confined to endodermal blastomeres at the 32-cell stage in *Ciona* (right-most embryo at the top in Fig. 28.3a) (Hudson et al. 2013); the nuclear expression of this protein is observed in both mesodermal and endodermal blastomeres at the same stage in *Halocynthia* (right-most embryo at the bottom in Fig. 28.3a) (Kawai et al. 2007). Accordingly, in *Ciona*, the OFF–OFF stage (i.e., off at the 16-cell stage and off at the 32-cell stage) of β-catenin activity defines the ectoderm, while the ON–OFF stage defines the mesoderm (+ nerve cord precursor) and the expression of the transcription factor Zic-r.b, which is activated by β-catenin and specifies mesoderm (+ nerve cord precursor). Moreover, the ON–ON stage defines the endoderm and the expression of transcription factors Lhx3 and FoxD, which are essential for endoderm differentiation along the animal–vegetal axis (top in Fig. 28.3a) (Hudson et al. 2013). However, in *Halocynthia*, the nuclear migration–driven asymmetric segregation of the mRNA encoding the transcription factor Not segregates the mesoderm from the endoderm (Fig. 28.3b) (Takatori et al. 2010). Not, inherited from mesodermal blastomeres, activates Zic-r.b, which specifies mesodermal and nerve cord tissues and represses *Lhx3* expression and endoderm differentiation (Fig. 28.3b). The direction of the nuclear migration to the future mesoderm region of the mesendoderm cell is determined by localization of Phosphatydylinositol 3-kinase activity in that region generated by the first phase of ooplasmic segregation (Takatori et al. 2015).

Upon division to the 64-cell stage, the mesoderm and nerve cord precursor cells in the anterior and the mesoderm cells in the posterior halves of the region between the ectoderm and endoderm further divide along the animal–vegetal axis to produce distinct tissue precursors (Fig. 28.3c). The daughter cells located vegetally and adjacent to the endoderm cells receive more fibroblast growth factor (FGF) signal than other daughter cells (Nishida 2003; Picco et al. 2007; Shi and Levine 2008) and are induced to become notochord and mesenchyme precursor cells in the anterior and posterior halves, respectively (Figs. 28.3c and 28.4b) (Nakatani et al. 1996; Kim et al. 2000; Imai et al. 2002a). In contrast, the cells located animally and adjacent to the ectoderm cells receive less FGF signaling, likely below the threshold level needed for induction (Nishida 2003; Picco et al. 2007; Shi and Levine 2008), and take on nerve cord and muscle fates as uninduced default fates in the anterior and posterior halves, respectively (Fig. 28.3c) (Kim and Nishida 1999; Minokawa et al. 2001). The different levels of FGF signaling between these daughter cells reflect the combination of these three mechanisms. The first mechanism is observed in *Halocynthia*, representing FGF signaling emanating from the endoderm (red arrows in Fig. 28.3c) (Kim et al. 2007). The second mechanism is observed in the anterior region of both *Halocynthia* and *Ciona* embryos, representing an inhibitory effect from the adjacent ectoderm cells (black inhibitory arrows in Fig. 28.3c) (Kim et al.

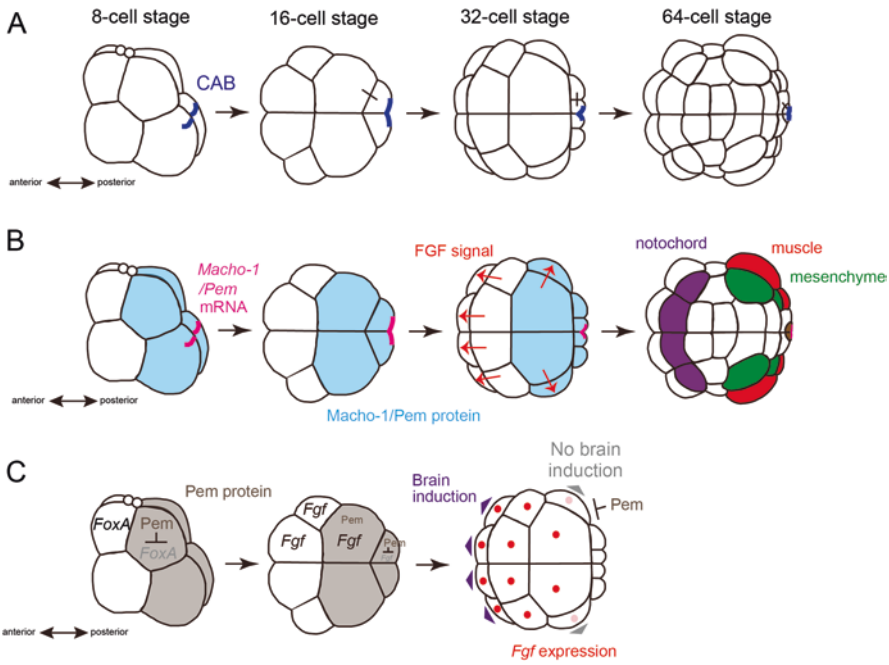

Fig. 28.4 Schematic diagram of the specification of the anterior–posterior axis. (**a**) Cell cleavage patterns and the posterior-end location of the centrosome-attracting body (CAB; dark blue) are shown. Lateral view of the eight-cell-stage embryo and vegetal views of the rest. Note that three successive unequal cell divisions occur in the blastomeres of the posterior end of the embryo at each cleavage stage (the divisions to 16, 32, and 64), producing the smallest cells in the posterior end, which inherit CAB, and accordingly generating the specific cleavage pattern for the posterior region. These unequal cell divisions are mediated by Pem and CAB, which attract the mitotic spindle toward the posterior by shortening microtubules connecting the centrosome and CAB, thereby posteriorly shifting the cleavage plane. Bars connect daughter cells after unequal cell divisions. (**b**) Developmental fate determination processes are shown. Although *Macho-1* and *Pem* messenger RNAs (mRNAs; pink) are localized to the CAB, their proteins are likely distributed throughout the posterior region (light blue). Thus, cells in the posterior region that inherit the muscle determinant Macho-1 and are not exposed to the fibroblast growth factor (FGF) signal (red arrows), which inhibits muscle differentiation, cell-autonomously become muscle cells (red). In addition, cells inheriting these mRNAs in the posterior end of the embryo are the richest in the level of Pem protein and become segregated as germline cells from somatic cells via the transcriptional repression activity of Pem (brown). Moreover, Macho-1 and Pem proteins in the posterior region differentiate anterior from posterior in response to the FGF signal (red arrows), resulting in notochord (purple) and mesenchyme (green) induction in the anterior and posterior regions, respectively. The FGF signal emanating from mesoderm (+ nerve cord) cells are not indicated here, for simplicity. (**c**) Anterior–posterior patterning in the ectoderm is shown. Pem protein is present in the posterior animal blastomeres in addition to the posterior vegetal blastomeres at the eight-cell stage, likely reflecting translation at the four-cell stage and the inheritance of this protein by both daughter cells (brown). The transcriptional repression of Pem activity restricts *FoxA* expression to the anterior region (left). Pem protein also represses *Fgf* expression in a subset of cells of the posterior vegetal region, leading to the secretion of Fgf as an anterior-specific brain inducer from peripheral anterior vegetal blastomeres in contact with competent ectoderm cells (purple arrowheads), but not from peripheral posterior vegetal blastomeres (gray arrowheads). Red circles: *Fgf*-expressing cells at the 32-cell stage

2007; Picco et al. 2007). The third mechanism is observed in *Halocynthia*, showing a loss of FGF signal competence in the anterior and posterior daughter cells located animally, exerted by the expression of transcription factors (Hashimoto et al. 2011; Kumano et al. 2014). The Fgf ligand is the downstream target of the β-catenin produced in endoderm and mesoderm (+ nerve cord) cells (red arrows in Fig. 28.3c) (Imai et al. 2002a; Kumano et al. 2006).

A similar FGF-dependent germ layer segregation mechanism is also utilized when endoderm and hematopoietic/adult muscle precursor cells in the anterior half of the vegetal region of the embryo are separated at the division to the 64-cell stage. Vegetally positioned daughter cells receive FGF signaling and become endoderm cells, whereas the other daughter cells located animally are exposed to less FGF and give rise to hematopoietic/adult muscle cells (Imai et al. 2002a, 2003; Shi and Levine 2008).

28.3 Cytoplasmic Segregation–Driven Localization of Posterior Determinants: Establishment of the Anterior–Posterior Axis

The anterior–posterior axis is established perpendicularly to the (new) animal–vegetal axis after fertilization and runs parallel to the first cleavage plane during ascidian embryogenesis. This axis reflects the relocalization of a subset of vegetally concentrated molecules/organelles to one side of the equatorial level of the fertilized eggs (the second phase of ooplasmic segregation; middle embryo in Fig. 28.2b) and the execution of posteriorizing activities according to these relocalized molecules (Kumano and Nishida 2007; Prodon et al. 2007; Sardet et al. 2007; Lemaire 2009; Makabe and Nishida 2012). Accordingly, this side is specified as the posterior region.

During the first phase of ooplasmic segregation, the sperm centrosome moves toward the vegetal pole in a microfilament-dependent manner but does not reach the pole. Thus, the side on which the centrosome resides, with respect to the animal–vegetal axis, can be predicted to be the future posterior side (blue circle in the second embryo from the left-most in Fig. 28.2b). The dynamic translocation and movement of astral microtubules that extend from the duplicated paternal centrosomes on the side of the egg drive the extensive displacement of the cER, *postplasmic*/*PEM* RNAs, and mitochondria-rich myoplasm to the equatorial region (middle embryo in Fig. 28.2b) (Chiba et al. 1999; Roegiers et al. 1999; Sardet et al. 2003; Prodon et al. 2005). In addition, cortical relaxation, called vegetal relaxation—spreading toward the equatorial region—pushes molecules/organelles back animally and further concentrates these materials at future posterior poles in *Phallusia* species (blue arrows in middle embryo in Fig. 28.2b) (Roegiers et al. 1999). Molecules/organelles with posteriorizing activities are translocated during multiple segregation events that constitute the second phase of ooplasmic segregation, and gastrulation and endoderm determinants are left behind in the vegetal

region. Retranslocated molecules, such as *Pem* and *Macho-1* (*Zic-r.a*) mRNAs, are associated with the cER through the entire period of the maturing and matured oocytes prior to fertilization, in fertilized eggs, and in early cleavage–stage embryos in *H. roretzi*, *C. intestinalis/robusta*, and *P. mammillata* (Sardet et al. 2003; Prodon et al. 2005, 2006, 2007). Importantly, however, cER–mRNA complexes and the mitochondria-rich myoplasm change the tracks by which these materials are translocated from microfilaments during the first phase of segregation to microtubules in the second phase, and how this change occurs is unknown.

Removal and transfer experiments on a small portion (8–15%) of the egg cytoplasm on the future posterior side containing the cortex and subcortex layers after the second phase of ooplasmic segregation in *H. roretzi* have shown that this region contains all necessary and sufficient materials to establish the anterior–posterior axis, and this axis is specified by posteriorization imposed on the default anterior state via these materials (Nishida 1994). Posteriorization includes posterior-specific tissue fate determination, such as the primary muscle and mesenchyme, the generation of the posterior-to-anterior difference in FGF signal competence, and the control of posterior-specific unequal cell cleavage patterns. The factors that are necessary for these posteriorization events have been identified as members of *postplasmic/PEM* RNAs in several ascidian species, including *Halocynthia*, *Ciona*, and *Phallusia*. Examples of these factors include *Macho-1* (Nishida and Sawada 2001), *Pem* (or *Pem-1*) (Yoshida et al. 1996), and *Popk-1* (Sasakura et al. 1998). Interestingly, each of these factors confers multiple posteriorizing functions, as detailed below.

The distribution of *postplasmic/PEM* RNAs in fertilized eggs has been observed by in situ hybridization in a small but relatively dispersed cytoplasmic region on the future posterior side; however, these molecules become more concentrated onto a subcellular structure at the posterior end of the embryo—known as the centrosome-attracting body (CAB) (Hibino et al. 1998)—as cell division and embryogenesis proceed (Fig. 28.4a). The CAB and *postplasmic/PEM* RNAs are only inherited by the posterior-most blastomeres of the embryo (Fig. 28.4a, b). Therefore, these mRNAs are the most concentrated in these cells. However, all other cells in the posterior half of the embryo (the descendants of the posterior two cells at the four-cell stage produced by the second cell division after fertilization, separating anterior and posterior) are also under the influence of *postplasmic/PEM* RNAs (light blue and brown cells in Fig. 28.4b, c, respectively) (Kobayashi et al. 2003; Kondoh et al. 2003; Kumano and Nishida 2009). This effect likely reflects translation from the mRNAs in the previous cell cycle, resulting in the inheritance of translated proteins from both daughter cells, including the cell that does not possess a CAB after cell division. Accordingly, this mechanism generates at least three distinct regions along the anterior–posterior axis: the anterior half, the posterior half, and the posterior-most blastomeres.

The fate determination of posterior-specific tissues depends on the inheritance of particular types of *postplasmic/PEM* RNAs and their translated products. *Macho-1*, which encodes a zinc finger–containing transcription factor, has been identified as a muscle determinant (red cells in Fig. 28.4b) in *Halocynthia* (Nishida and Sawada

2001) and later in *Ciona* (Satou et al. 2002). Molecular identification of a muscle determinant has long been sought since the discovery by Conklin (1905) of a specific egg cytoplasm inherited by muscle lineage cells. *Macho-1* is also essential for mesenchyme induction by conferring responsiveness to the FGF signal (Kobayashi et al. 2003; Imai et al. 2003). The presence of this protein in the posterior half of the embryo differentiates cells in the anterior and posterior halves in response to the same FGF signal (red arrows in Fig. 28.4b), thereby inducing mesenchyme and notochord by FGF in the posterior and anterior regions, respectively (purple and green cells in Fig. 28.4b). *Pem*, another *postplasmic/PEM* RNA, plays a similar role in *Halocynthia*. Pem confers posterior-specific FGF signal competence in cells of the posterior half (Fig. 28.4b) by suppressing the expression of *FoxA* and *Zic-r.b.* (Kumano and Nishida 2009), which are intrinsic competence factors required for notochord and anterior induction (Kumano et al. 2006). Furthermore, the germline is specified in posterior-most blastomeres, where Pem proteins are the most concentrated (brown cells in Fig. 28.4b). Pem contributes to this specification by repressing somatic-type transcription in the germline in *H. roretzi* and *C. robusta* (Japan) (see Chap. 1) (Kumano et al. 2011; Shirae-Kurabayashi et al. 2011).

Pem also patterns the ectoderm along the anterior–posterior axis in both direct and indirect manners. Pem restricts *FoxA* expression to the anterior region in the animal hemisphere by repressing zygotic transcription in the posterior region as early as the eight-cell stage in *H. roretzi* (Fig. 28.4c) (Kumano et al. 2011). Translated Pem products at the four-cell stage are inherited by posterior animal blastomeres (brown cells in Fig. 28.4c) and repress gene expression. FoxA activates anterior-specific ectodermal gene expression later in embryogenesis in *Ciona* embryos (Roscoff) (Lamy et al. 2006). In addition, Pem suppresses brain induction in the posterior region of the ectoderm, possibly by restricting cells with the inducing ability to the anterior region in the vegetal hemisphere in *H. roretzi*. Pem might attenuate the expression of Fgf (red circles in Fig. 28.4c), a brain inducer, to a level below the threshold needed for induction in posterior vegetal cells adjacent to the ectoderm cells at the 32-cell stage (gray arrowheads in Fig. 28.4c), but not in anterior vegetal cells that are in contact with the ectoderm (purple arrowheads in Fig. 28.4c) (Kumano and Nishida 2009).

Posterior specification through Pem occurs via the transcriptional repression activity of this protein, and this activity is most likely exerted in the nucleus (Kumano et al. 2011). However, Pem also posteriorizes embryos independently of this activity and regulates posterior-specific cleavage patterns (Negishi et al. 2007; Kumano et al. 2011), as detailed below.

The posterior half of ascidian embryos experiences a distinct cell cleavage pattern illuminated by three successive rounds of unequal cell divisions in cell size, starting at the division to the 16-cell stage (Fig. 28.4a). Each of the unequal cell divisions occurs in a pair of the posterior-most blastomeres. In *Halocynthia*, *Ciona*, and *Phallusia*, these divisions are controlled by a shift of the mitotic spindle toward the posterior end by shortening of one of the astral microtubules assembled and focused on the CAB located at the cortex of the posterior end of the embryo (Hibino et al. 1998). Thus, this process generates the smallest blastomeres at the posterior

end. This microtubule shortening is regulated by cytoplasmic Pem, which is localized to the CAB in *Halocynthia* and *Phallusia* (Negishi et al. 2007; Prodon et al. 2010).

Moreover, Popk-1, encoded by another *postplasmic*/*PEM* RNA, regulates most of the posteriorizing events described above by ensuring that sufficient amounts of proteins, such as Macho-1 and Pem, are supplied for the events in the posterior half. It has been suggested that *postplasmic*/*PEM* RNAs are translated on the CAB (Tanaka et al. 2004; Paix et al. 2011). Popk-1 regulates the size of the CAB as a platform for mRNA localization and therefore the amount of the localized mRNAs and their translation in *H. roretzi* (Nakamura et al. 2005).

28.4 Rotation of the Entire Embryonic Body: Left–Right Determination

As described in Sect. 28.1, the position of the third and left–right axis is automatically fixed when the second axis (the anterior–posterior axis) is established; however, this axis is not characterized and oriented until the neurula stage in ascidian embryos. In general, left–right axis determination requires two independent mechanisms. One mechanism differentiates the two opposite ends of the axis, while the other mechanism determines which side is left or right. These mechanisms were identified by mutant analyses, identifying mutants in which the orientation of the left–right axis was randomized with two different ends (Fujinaga 1997), while other mutants lost their left and right differences (Kawakami et al. 2008).

Ascidian embryos show two morphological differences between left and right: the brain vesicle is positioned off the midline, deviated toward the right in the trunk region of the larva, while the tail bends and elongates toward the left side. In *H. roretzi*, the anterior neural tube, comprising the brain vesicle, rotates approximately 45° clockwise when viewed from the posterior at the late tailbud stage, morphologically generating the first left–right asymmetry in the trunk region (Taniguchi and Nishida 2004). In *Halocynthia* and *C. robusta* (Japan), the asymmetric positioning of the brain vesicles is determined by Nodal signaling, a conserved signaling pathway that determines left and right in other animals, such as vertebrates and snails (Namigai et al. 2014; Grande and Patel 2009). *Nodal* is asymmetrically expressed in the left side of the epidermis of neurula and early tailbud embryos in *Halocynthia* (Morokuma et al. 2002; Nishide et al. 2012) and tailbud embryos in *Ciona* (Yoshida and Saiga 2008, 2011). The loss of Nodal signaling leads to different results in these two species: the positioning of the brain vesicle on the midline in *Halocynthia* (loss of left–right difference) (Nishide et al. 2012), and the formation of ocellus photoreceptor cells on both the left and right sides in *Ciona* (loss of left–right difference) (Yoshida and Saiga 2011). Nodal also determines the direction of tail bending in *H. roretzi* (Nishide et al. 2012). Although the loss of Nodal signaling results in the randomization of the direction in which the tail bends, it is not clear whether the left–right orientation is indeed randomized or lost, because the limited space of the

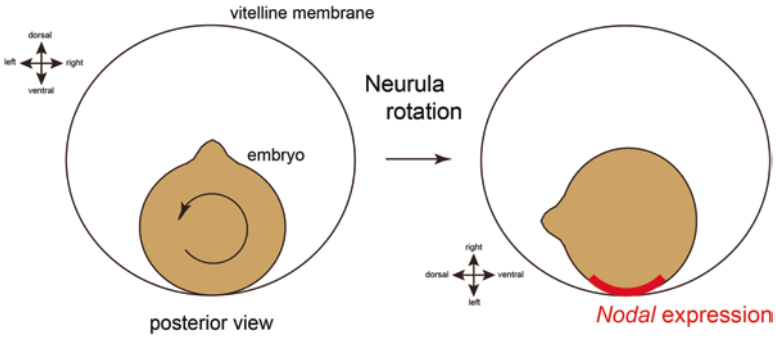

Fig. 28.5 Schematic diagram of left–right determination in *Halocynthia* embryos. Neurula rotation (arrow) brings the left side of the neurula embryo in contact with the vitelline membrane, and the contact induces *Nodal* expression in the ectoderm layer (red). Posterior views with dorsal up (left) and to the left (right)

vitelline envelope might force the tail to bend in either direction with a probability of 50/50, even when Nodal is lost (Nishide et al. 2012).

The factors that determine left-sided *Nodal* expression and orient the left–right axis in ascidian embryos have been extensively studied in *H. roretzi*. It has been proposed that the expression of *Nodal* is regulated by a unique use of cilia that has not been observed in other organisms, such as mice and zebrafish, for the determination of left and right (Hamada and Tam 2014; Blum et al. 2014a; Bakkers et al. 2009). At the early neurula stage, *Halocynthia* embryos rotate 90° in a counterclockwise direction when viewed from the posterior, known as neural rotation. This movement orients the left side of the epidermis downward and in contact with the vitelline envelope (left embryo in Fig. 28.5) (Nishide et al. 2012), and this contact activates *Nodal* expression in the contacted epidermis (right embryo in Fig. 28.5) (Nishide et al. 2012). Ciliated epidermal cells are observed at this stage, and it has been suggested that these cilia drive the rotation (Nishide et al. 2012). Interestingly, in *Ciona* embryos, rotation also occurs in the same counterclockwise direction at the neurula stage; however, this organism does not stop rotating after 90°; rather, it continues rotating more than 360° and does not end up with the left side oriented downward (Nishide et al. 2012). Therefore, although the end result of left-sided *Nodal* expression is conserved, this process might differ between these two species.

28.5 Concluding Remarks

Ascidian early embryonic axis formation has been extensively studied in three solitary ascidian species: *Halocynthia*, *Ciona*, and *Phallusia*. With the possible exception of left–right determination, the molecular and subcellular mechanisms through which the three developmental axes are established are well conserved among these

species, which are separated by more than 400 million years of evolution (*Halocynthia* and *Ciona*). This conservation is in contrast to that of nematode species, such as *C. elegans* versus *Acrobeloides*, both of which have nearly identical cell lineages and cell positions throughout the beginning of gastrulation but show different mechanisms for anterior–posterior axis specification in zygotes (Goldstein et al. 1998). Therefore, these mechanisms must be indispensable for early development in ascidians, and any changes in these mechanisms could have caused significant deterioration in these organisms compared with nematode species. In contrast, the mechanisms underlying left–right determination leading to asymmetric *Nodal* expression can be diversified, as is evident in other organisms (Blum et al. 2014b; Schlueter and Brand 2007), and might have changed, even within ascidian species, to facilitate other changes that could provide selective advantages.

Axis formation during early embryogenesis among metazoan species, such as those mentioned in this chapter, relies on polarized determinant segregation within eggs and early embryos. Particularly, ascidians use microfilament-dependent and cytoplasmic flow–driving mechanisms to establish the primary axis, and these mechanisms might be similar to those of the anterior–posterior axis formation in *C. elegans* zygotes, as described above. In addition, the mechanism of the second phase of ooplasmic segregation is also similar to that of the second axis formation (running from the future organizer to the contra-organizer) in the eggs of vertebrates, such as frogs and fish (see Chaps. 31 and 32), in that both mechanisms are microtubule dependent and generate axes perpendicular to the animal–vegetal axes. However, despite the conserved significance of the determinant segregation in axis formation and the apparent similarities in the manner in which the determinants are segregated, complex and unique mechanisms of the establishment of embryonic axes are used in each species. For example, the sperm-donated centrosome and nucleated microtubules also polarize the *C. elegans* zygote along the anterior–posterior axis independently of the abovementioned actin-dependent mechanism (Motegi et al. 2011). In addition, ascidians rely on the dynamic translocation and movement of the astral microtubules that extend from the sperm-derived centrosomes to relocalize determinants to one side of the equatorial region of the egg. However, frogs and fish use subcortical and self-organized parallel microtubule arrays on the future organizer side of the eggs (Rowning et al. 1997; Tran et al. 2012; Olson et al. 2015). Moreover, although several molecules that are used for axis specification are conserved among a variety of animal species (e.g., β-catenin, Nodal), the identities of other determinants are quite unique to each species, such as Bicoid and Gurken in *Drosophila* (Riechmann and Ephrussi 2001; Becalska and Gavis 2009), Vg1 and VegT in *Xenopus* (Kloc et al. 2001; White and Heasman 2008), and Macho-1 and Pem in ascidians.

In conclusion, early embryonic axes established through complex and unique processes in a simple chordate ascidian provide a framework for the three-dimensional embryonic body structure according to the proper positioning of axis and tissue determinants and the arrangement of tissue precursors within the embryo. These mechanisms are well conserved among ascidian species and contribute to the characteristic features of these species.

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Chapter 29
Recent Advances in Hagfish Developmental Biology in a Historical Context: Implications for Understanding the Evolution of the Vertebral Elements

Kinya G. Ota

Abstract Hagfish have been recognized as important for investigating the evolution of vertebral elements, because of their crucial phylogenetic position; however, the deep-sea habitat of most hagfish species limits the number of available embryos, thus impeding studies of their embryology in general and of their axial skeletogenesis in particular. This paucity of hagfish embryos has long impeded attempts to determine whether the absence of vertebral elements in this animal represents the ancestral morphological state. However, embryonic materials recently obtained from the Japanese inshore hagfish (*Eptatretus burgeri*) have provided an opportunity to investigate the fine histology of the embryonic somite and gene expression patterns of somite derivatives. These approaches identified segmentally arranged mesenchyme-derived nodules of cartilage at the ventral aspect of the notochord. On the basis of the clear gene expression patterns of *Twist* and *Pax1/9* (known as sclerotomal markers in gnathostomes), it is presumed that hagfish and gnathostomes share similar molecular developmental mechanisms for the vertebral elements. In sum, the common ancestor of all vertebrates likely possessed the developmental mechanisms that control expression of *Twist* and *Pax1/9* and the formation of segmentally arranged vertebral elements. Thus, it is reasonable to assume that the hagfish vertebral elements, like the rest of the skeleton, represent a secondary degenerated condition.

Keywords Vertebrates · Vertebra · Axial skeleton · Pax1/9 · Twist · Cyclostomes · Extracellular matrix

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29.1 Introduction

Vertebral elements—namely, cartilaginous or bony segmental nodules attached to the notochord—represent one of the crucial morphological characteristics that define vertebrates (Janvier 1996; Liem et al. 2001; Kardong 2011; Fleming et al. 2015). In order to investigate the evolutionary origin and process of the skeleton, early researchers conducted a number of classic comparative studies using various vertebrate species (Gadow 1895, 1933; Parker 1883; Goodrich 1930). Gadow (1895, 1933) proposed a schema in which the gnathostome vertebra consists of two dorsal and two ventral elements (Gadow 1933) (Fig. 29.1). This schema can also be applied

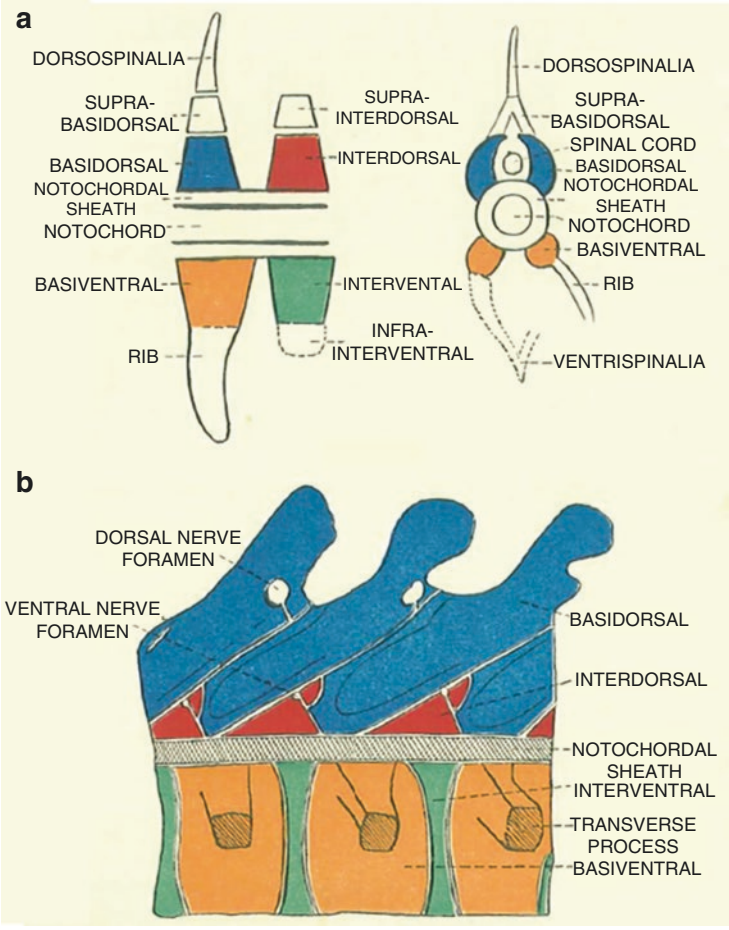

Fig. 29.1 Gadow's (1933) schema of the complete vertebra. Upper left: left side view of the vertebral elements. Upper right: transverse view of the basal elements. Bottom: left side view of the three vertebrae of the sturgeon; this schema was drawn from a plaster model. Basidorsal, basiventral, interdorsal, and interventral regions are shown in blue, orange, red, and green, respectively

to the extant agnathans—lampreys and hagfish; however, among the extant agnathans, lampreys possess only dorsal elements (Tretjakoff 1926; Janvier 1996) and hagfish lack any trace of vertebrae (Janvier 1996).

The skeletal anatomy of the hagfish was well investigated from the mid-nineteenth to early twentieth centuries by a few researchers (Müller 1835; Parker 1883; Schaffer 1897; Ayers and Jackson 1900; Cole 1905, 1906) (Fig. 29.2a–c′). Müller (1835) described the detailed musculoskeletal anatomy of the Atlantic hagfish (*Myxine glutinosa*) (Fig. 29.2a). Almost all of the musculoskeletal elements can be identified in his sketch (Fig. 29.2a), but vertebral elements were not found in this animal. Moreover, clear descriptions of the vertebral elements were absent from other anatomic studies of *M. glutinosa* (Parker 1883; Studnicka 1896; Schaffer 1897; Cole 1905) and adult *Bdellostoma* (Parker 1883). On the basis of these anatomic descriptions (Müller 1835; Parker 1883; Studnicka 1896; Schaffer 1897; Cole 1905, 1906), some textbooks indicate that the axial skeletal system of the hagfish consists of only cartilaginous fin rays and a notochord (Janvier 1996; Kardong 2011; Liem et al. 2001).

On the other hand, Ayers and Jackson (1900) described segmentally arranged cartilaginous nodules on the ventral aspect of the notochord at the caudal level in one Pacific hagfish species (*Eptatretus* species) (Fig. 29.2b, b′). Presumably because the authors did not explicitly indicate that these cartilaginous nodules are homologous to the vertebral elements of other vertebrate species, this report of axial skeletal systems in *Eptatretus* species was overlooked and was not re-examined by subsequent researchers for many decades. It is assumed that brief descriptions of the presence of unusual morphological features of the vertebrae in hagfish in later studies (Goodrich 1930; Wake 1992) may have originated from Ayers and Jackson (1900); however, these subsequent studies were also overlooked, presumably because of the vagueness of the descriptions; in fact, these brief descriptions provided no citations and no figures.

Recently, this neglected hagfish report by Ayers and Jackson (1900) was re-examined using molecular developmental techniques, resulting in a novel interpretation of the evolutionary origin and process of the axial skeletal systems of early vertebrates (Ota et al. 2011). This chapter first explains the phylogenetic significance of the morphological status of hagfish axial skeletons and summarizes the progress of hagfish embryological studies with detailed reference to the historical background, to indicate why the axial skeletal morphology of this animal has been a continuing matter of debate in the context of vertebrate evolution. Moreover, the evolutionary scenario of the axial skeleton in vertebrates on the basis of recent molecular developmental studies of the Japanese inshore hagfish is discussed (Ota et al. 2011, 2013, 2014).

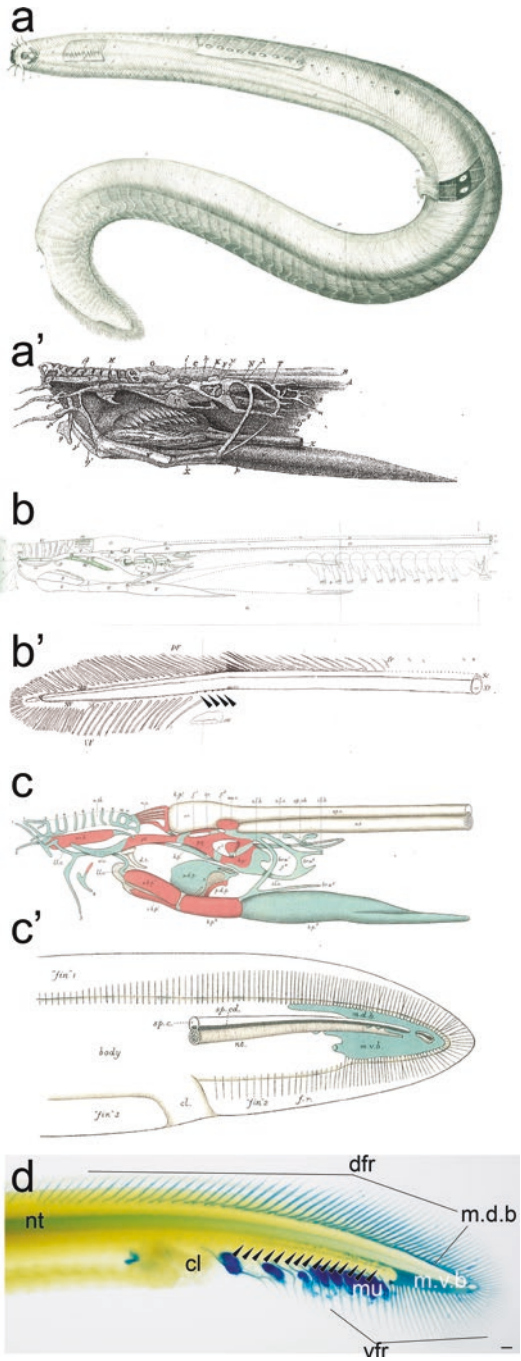

Fig. 29.2 Hagfish adult anatomy. (**a**–**c**′) Illustrations by researchers from the mid-nineteenth to early twentieth centuries. Detailed illustrations of the musculoskeletal morphology were made by Müller (1835) (**a**, **a**′). Ayers and Jackson (1900) described the cranial and axial skeletal morphology in a *Bdellostoma* species (**b**, **b**′). Cartilaginous nodules were identified at the ventral aspect of the notochord at the caudal level (**b**′) (arrowhead). (**c**, **c**′) Cole (1906) also described the skeletal anatomy at the caudal region in detail, but no segmental cartilaginous nodules are present in his illustration (**c**′). (**d**) Lateral view of a whole-mount Alcian Blue–stained adult hagfish. Segmentally arranged cartilaginous nodules (black arrowheads) are present, as in the sketch by Ayers and Jackson (1900) (Modified from Ota et al. 2011)

29.2 Phylogenetic Significance

To understand why the morphological status of the axial skeleton in hagfish was a matter of debate, one must be aware of the phylogenetic relationship of the hagfish to other vertebrates. Although detailed historical descriptions of the phylogenetic studies of this animal are beyond the scope of this chapter (for details, see Janvier 2008), the controversial relationship between morphology and molecular data relating to the axial skeleton in hagfish is described here.

There were two exclusive theories regarding the phylogenetic relationship: the cyclostome theory and the craniate theory (or vertebrate theory). The former proposed a monophyletic relationship for lampreys and hagfish, while the latter proposed paraphyly of the cyclostomes (see Janvier 2008) (Fig. 29.3). Although the cyclostome theory was initially supported in the field of paleontology (see Janvier 2008), the monophyletic relationship between hagfish and lampreys was reconsidered in the late 1970s when cladistic conceptual frameworks and methods were applied to investigation of the phylogenetic relationship of the early vertebrates (Løvtrup 1977; see Janvier 2008). In 2008, Janvier favored the craniate theory by explicitly defining the taxon Vertebrata as comprising gnathostomes and lampreys, but excluding hagfish, on the basis of the presence of homologous dorsal vertebral elements (neural arch elements or basidorsals and interdorsals) in the former two groups (Fig. 29.3); consequently, hagfish were automatically excluded from the taxa of the Vertebrata because of their absence of vertebral elements. Once the phylogenetic relationships between extant and extinct vertebrate species became testable under the parsimony-based cladistic conceptual framework, paleontologists were able to examine the phylogenetic position of their own fossil agnathan species of

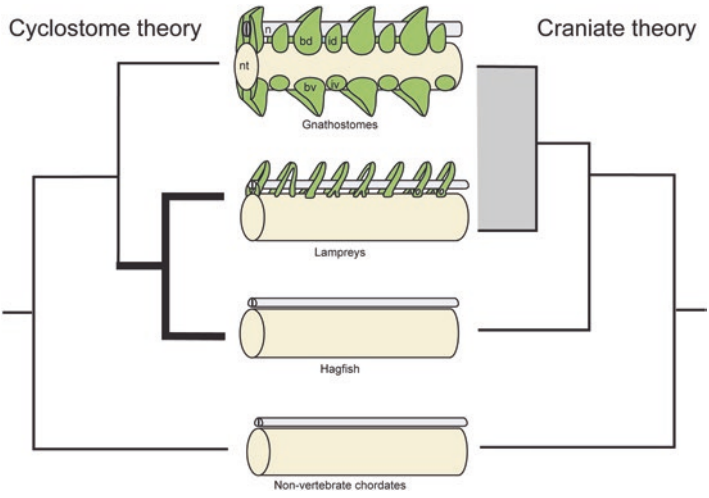

Fig. 29.3 Phylogenetic relationship of the early vertebrates. The cyclostome theory (left) supports a monophyletic relationship between lampreys and hagfish (bold line), while the craniate theory (right) excludes hagfish from the vertebrate clade (gray-colored clade)

interest; this led to the reconstruction of phylogenetic trees of early vertebrates from the 1970s to 2000s (Løvtrup 1977; Forey and Janvier 1993; Shu et al. 1999; Donoghue et al. 2000; Gess et al. 2006). In these phylogenetic trees, the hagfish were always excluded from the clade consisting of gnathostomes and lampreys (Løvtrup 1977; Forey and Janvier 1993; Shu et al. 1999; Donoghue et al. 2000; Gess et al. 2006). Although anatomic similarities between the oral apparatuses of hagfish and lampreys demonstrated by Yalden (1985) are considered to be strong morphological evidence in support of the cyclostome theory, the craniate theory became widely accepted in the field of the paleontology and in some vertebrate textbooks (Karldong 2008; Liem et al. 2001; Janvier 1996). In fact, when new fossil records of hagfish and lampreys were found, their evolution was discussed on the basis of the craniate theory (Bardack 1991; Shu et al. 1999; Gess et al. 2006). Consistent phylogenetic relationships among extant vertebrate species (gnathostomes, lamprey, and agnathans) may be insufficient motivation to re-examine character states in data matrices used by paleontologists (Forey and Janvier 1993; Gess et al. 2006; Shu et al. 1999; Donoghue et al. 2000).

Since the 1990s, the craniate theory has been criticized by researchers familiar with molecular phylogenetic analyses (Stock and Whitt 1992; Kuraku et al. 1999; Mallatt and Sullivan 1998; Delarbre et al. 2002; Takezaki et al. 2003). The application of molecular sequencing techniques to nonmodel organisms has enabled the reconstruction of molecular phylogenetic trees for early vertebrates. These molecular phylogenetic analyses tend to support the cyclostome theory (Fig. 29.3). As paleontologists excluded monophyly of cyclostomes on the basis of a cladistic conceptual framework (Løvtrup 1977; Forey and Janvier 1993; Shu et al. 1999; Donoghue et al. 2000; Gess et al. 2006), researchers using molecular evolutionary methods rejected paraphyly of cyclostomes on the basis of a solid methodological framework and large volumes of molecular data (Kuraku et al. 1999; Takezaki et al. 2003), fueling controversy between the paleontological and molecular approaches. This controversy centered on whether hagfish morphological characteristics are degenerated or ancestral (Ota and Kuratani 2006; Ota et al. 2007, 2011).

Many morphological characteristics that are interpreted as ancestral by paleontologists should be reinterpreted as secondary degenerated characteristics on the basis of the cyclostome theory (Fig. 29.3). However, there are almost no constructive arguments between paleontologists and molecular evolutionary biologists regarding the interpretation of hagfish morphological data. The axial skeletal morphology of hagfish species is not exceptional. This controversy motivated us to apply evo–devo approaches to hagfish (Ota and Kuratani 2006).

29.3 Hagfish Developmental Biology

Investigations into the embryogenesis of morphological characteristics are quite useful for determining whether the absence of a morphological characteristic in an animal represents an ancestral or derived condition. For example, if several residual

embryological traits of the morphological characteristic are recognized during development, the absence of the morphological traits may be inferred to be a derived condition. Moreover, the ancestral condition of molecular developmental mechanisms of morphological traits can be investigated through application of molecular techniques to embryos. In fact, a number of molecular developmental studies using lampreys have revealed common ancestral embryonic and morphological features of vertebrates (Kuratani et al. 2001, 2002, 2004; Kusakabe and Kuratani 2005; Murakami et al. 2001, 2004; Neidert et al. 2001; Ohtani et al. 2008; McCauley and Bronner-Fraser 2004, 2006; Zhang et al. 2006). The large number of molecular developmental studies on lampreys is mainly due to the ease of accessing embryonic materials from this animal; the freshwater habitat and large number of spawning eggs of lamprey are advantageous for evo–devo studies. On the other hand, there has been little progress in hagfish evo–devo on account of the difficulty in obtaining hagfish embryos from their deep-sea habitat (Holland 2007). Indeed, while several researchers have attempted to collect hagfish embryos (see Ota and Kuratani 2006), the rate of success has been very low (Ota and Kuratani 2006; Ota et al. 2007; Doflein 1899; Dean 1898, 1899; Price 1896a, b, 1897, 1904; Gilchrist 1918; Holmgren 1946; Fernholm 1969). The following sections discuss some successful accounts of obtaining hagfish embryos at different times and locations, with a view to explaining why axial skeletal development has not been extensively examined in hagfish.

29.3.1 Eastern Pacific

At Monterey Bay (CA, USA), several researchers succeeded in obtaining embryos of the West Pacific hagfish (*Eptatretus stoutii*, then known as *Bdellostoma stoutii*) and reported embryonic morphological and histological features of this animal (Price 1896a, b, 1897; Dean et al. 1896; Dean 1898, 1899; Worthington 1905; Doflein 1899). Of these researchers, George C. Price (an assistant professor of zoology at Stanford University) is considered to have been the first to describe the development of embryos and their histological sections (Price 1896a, b, 1897). Using these embryos, Price examined the developmental process of the trunk regions, with a particular focus on the excretory organs, with technical assistance from Carl von Kupffer (a professor at the Histological Laboratory of the University of Munich) (Price 1897, 1904).

Following the success of Price (1896a, b), Bashford Dean (an adjunct professor in zoology at Columbia University) also achieved great success. He obtained numerous *E. stoutii* embryos and was the first to publish detailed sketches of a series of different stages of these embryos (Dean et al. 1896; Dean 1898, 1899) (Fig. 29.4). In his report (1896), he introduced a remarkably skillful Chinese fisherman by the name of Ah Tack Lee; it is supposed that Lee may have provided sea products to the local Chinese village at Monterey Bay, on the basis of the description of the Chinese community at this location at the time (Lydon 1985). Lee may have been quite familiar with the distribution of the marine organisms in this sea area; in fact, he

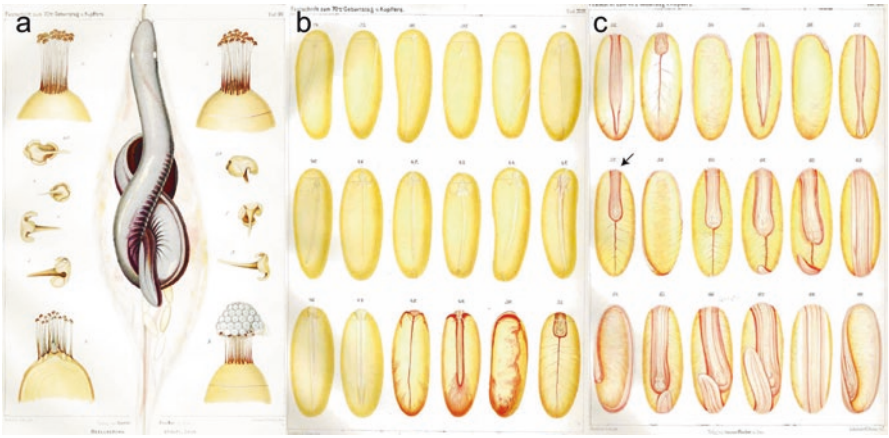

Fig. 29.4 Dean's sketches (1899). (**a**) Hagfish adult and eggs. The adult individual was caught on a longline (visible in the figure). (**b** and **c**) Series of hagfish embryos (Dean 1899). (**b**) Early to late pharyngular–stage embryos. (**c**) Late pharyngular to prehatching–stage embryos. The embryo used in Ota et al. (2013) is equivalent to the embryo in panel **c** indicated by the black arrow (see text for details) (Images courtesy of M. K. Richardson)

provided embryonic samples of the chimaeroid fish to Dean (Gregory 1930; Dean 1897). Thus, through this Chinese fisherman, Dean obtained hagfish embryos. However, the eggs appear to have been obtained by good fortune, according to the following description:

> The eggs, it was found, could only be taken by accident in the following way: The mature Myxinoid, having taken a hook of the trawl line, secretes an enormous quantity of thick and viscid slime, and if by chance during this process its writhing body comes in contact with a string of eggs these may became enslimed, and thus, together with the fish, be brought to the surface.

Through such serendipity, Dean succeeded in obtaining more than 700 eggs. Of these, 130 were fertilized and were at various stages of development, including first division–, neurula-, and pharyngular-stage embryos, and hatched larvae (Dean 1899; Fig. 29.4). He described the morphological features of the fresh embryos through the egg shells (Fig. 29.4). The clarity of his sketches and descriptions led to his reports being frequently cited by later researchers (Holmgren 1946; Fernfolm 1969). Moreover, Franz Doflein (from the Zoologische Staatssammlung München) also succeeded in collecting several hagfish embryos in the same season, period, and location as Dean (Doflein 1899). The hagfish embryos obtained by Dean (1898, 1899) and Doflein (1899) at Monterey Bay were preserved and subjected to histological analyses by subsequent researchers (Stockard 1906a, b; Conel 1929, 1931a, b, 1942; Neumayer 1938; von Kupffer 1899).

Although it is noted that some embryos of Japanese hagfish species were obtained by Saburo Hatta (from the Sapporo Agricultural College) prior to the publication of the studies mentioned above (Dean 1904), no report on the embryos obtained by Hatta was published. Thus, in effect, only studies by three authors (Price, Dean, and

Doflien) can be recognized as hagfish embryological studies of the nineteenth century.

Following the successes of the aforementioned researchers (Price 1896a, b, 1897; Dean et al. 1896; Dean 1898, 1899; Doflein 1899), dredging of Monterey Bay was performed on several occasions in failed attempts to collect developing embryos; for example, the US Fish Commission steamer *Albatross* dredged the bottom of Monterey Bay for 3 months in the spring of 1904, to no avail (Worthington 1905). In this period, Julia Worthington received developing embryos and hatched larvae of a hagfish species with five gill slits (then designated as *Bdellostoma dombeyi*) from a fisherman. Moreover, Worthington reported that developing embryos were observed under artificial conditions, although development of the obtained embryos ceased because of excessively high water temperature (Worthington 1905). Since this report did not contain any images of the hagfish embryo, its contribution to hagfish embryology was not significant. However, her study provided an idea as to how hagfish behave in the sea, and was the first account of successful incubation and observation of hagfish embryos under aquarium conditions. In addition, this report described the final published study of the collection of the hagfish embryos from Monterey Bay. An attempt to use a submersible to study the same sea area was undertaken by John Wourms (from the Department of Zoology at Clemson University), but this did not advance our knowledge of hagfish developmental biology [personal communication from John Pearse (Long Marine Laboratory of Marine Sciences, University of California) at the 2008 meeting of the Society of Integrative and Comparative Biology]. The inability to gain further information may be due to environmental changes in this sea area (see Holland 2007).

29.3.2 South Africa

Only a single report has been published on South African hagfish species (Gilchrist 1918). J. D. F. Gilchrist found five eggs of the South African hagfish (*Bdellostoma hexatrema*) on August 23, 1916, in a small bay (Fiddle Bay) on the west coast of South Africa, in which rocks were interspersed with mud and sand (Gilchrist 1918). These eggs contained embryos of 60 mm in total length. The author described strings of embryos and attached species in detail with photographs. Indeed, on the basis of circumstantial evidence that a mollusk species (*Mytilus cenatus*) exhibits a shell length of 18 mm, it was assumed that the string was attached to a substance at the bottom of the sea for a long while. To my knowledge, this report is the first to contain photographs of hagfish embryos.

29.3.3 North Sea

From the mid-nineteenth century, scientists at bases in Scotland, Norway, and Demark performed surveys for embryos of *M. glutinosa* (Steenstrup 1863; Nansen 1887; Cunningham 1886). However, none of them succeeded in obtaining hagfish embryos. In fact, no hagfish embryos of any stage were reported until 1946, when Nils Holmgren (from the University of Stockholm) published a paper describing this organism (Holmgren 1946). From 1924, he made attempts to secure hagfish of different developmental stages, making numerous dredgings with a specially constructed dredger and maintaining contact with fishermen. Although he did not obtain any eggs through dredging, he received one egg from a fisherman who was trawling with a shrimp net. Moreover, eight ripe eggs collected in Danish waters by fishermen were sent from Orvar Nybelin (director of the Gothenburg Natural Museum) (Holmgren 1946). As a result of these events, Holmgren changed his strategy. From 1939, he distributed bottles of Bouin's fixative to fishermen, promising a generous reward for each egg, in an attempt to obtain fertilized eggs. Although such attempts to collect hagfish eggs were interrupted by the Second World War, by 1944 more than 113 deposited eggs had been obtained at a depth of 100–200 m off Hållö Laesö, Hirtshals, and Stora Pölsan near Marstrand. All of these embryos were examined to determine whether fertilized eggs were present or not; well-developed embryonic bodies were observed in two eggs. Using these embryos, Holmgren described the detailed structure of the brain and cranial cartilage, as well as connections of the nerves and blood vessels (Holmgren 1946). Although only two embryos were obtained, this was the first report of the embryology of *Myxine* species.

The second report of *M. glutinosa* embryos was also conducted at the University of Stockholm, by Bo Fernholm (1969). His collaborator, G. Gustafson (from the Kristineberg Marine Zoological Station in Sweden) was able to collect a single embryo at a developmental stage close to that of the adult in the Gullmarfjord at Hällebäcksgrund on the Swedish west coast, at a depth of 70 m. Most of the contents of this report related to histology of the adenohypophysis, because questions regarding the evolutionary origin of that structure had arisen during this period (Gorbman 1958; see also Oisi et al. 2013a, b). It seems that Fernholm and some other zoologists also hoped to continue the investigation into developmental biology, but no extra embryos were obtained in this period (Fernholm 1974) despite extensive efforts to obtain hagfish embryos around the world (Foss 1962, 1968; Kobayashi et al. 1972; see Ota and Kuratani 2006, 2008). In the end, the embryo described by Fernholm became the last embryo to be studied in the twentieth century.

29.3.4 A Series of Hagfish Embryos in Japan

The hagfish evo–devo project was launched in 2006 by a group supervised by Shigeru Kuratani (from the Center for Developmental Biology, Riken, Japan) (Ota and Kuratani 2006, 2008; Ota et al. 2007, 2011, 2013; Oisi et al. 2013a, b). The group focused on the Japanese inshore hagfish (*Eptatretus burgeri*) because of the relatively easy accessibility of this species, which can be found in relatively shallow waters of the northwestern Pacific and has been caught for commercial purposes in Japan, Korea, and Taiwan by local fishermen (Ota and Kuratani 2006, 2008; Oisi et al. 2015). This species can be purchased from local fishermen and maintained relatively easily in aquarium tanks (Ota and Kuratani 2006). The evo–devo group contacted the Japan Fisheries Cooperative in Shimane and found a collaborative fisherman, Osamu Kakitani (Ota et al. 2014; Oisi et al. 2015). Thanks largely to his contribution, the group succeeded in obtaining a series of hagfish embryos (Ota et al. 2007, 2011, 2014; Oisi et al. 2015). The series of hagfish embryos included embryos at the neural, pharyngular, and prehatching stages (Ota et al. 2011; Oisi et al. 2015). The variation of stages enabled observation of axial skeletal development in this hagfish species. Moreover, fixing of these embryos was suitable for analysis of gene expression patterns, thus allowing axial skeletal development to be investigated at the level of molecular biology (Ota et al. 2011, 2013). Such analyses of these materials provided an opportunity to reconsider the evolutionary origin and process of axial skeletal systems.

29.4 Re-examination of Axial Skeletal Development

According to studies of model vertebrate animals, the vertebral elements and their related tissue are derived from the sclerotomes (Christ et al. 1998, 2000, 2004; Huang and Christ 2000; Morin-Kensicki et al. 2002). The sclerotomes are derived from the ventral part of the segmentally arranged somite (Christ et al. 1997, 2004; Huang and Christ 2000; Morin-Kensicki et al. 2002), which exhibits a mesenchyme form and expresses sclerotome-related marker genes (for example, the *Pax1/9* and *Twist* genes) (Christ et al. 2000). Thus, given that hagfish also have vertebral elements, it is highly possible that sclerotomal cells can be detected in hagfish embryos. In fact, in the histological description in Price (1904), there is a report of mesenchymal cells located at the ventral aspect of the somite. Moreover, myotomes and dermomyotomes were also observed through histological analysis. These descriptions in the Price paper suggest that hagfish have three representative somite derivatives, which can be recognized in gnathostomes (Goulding et al. 1991; Christ et al. 2000; Relaix et al. 2005; Buckingham et al. 2006). From these embryonic histological descriptions, it is assumed that the cartilaginous nodules in the *Eptatretus* species recorded by Ayers and Jackson (1900) might be derived from sclerotomal cells that

share the same molecular developmental mechanisms as their gnathostome equivalents (Ayers and Jackson 1900; Fig. 29.2).

To establish whether the sclerotomal cells described by Price (1900) are similar to the sclerotomes of gnathostomes (Christ et al. 2000), gene expression patterns of sclerotomal markers (*Pax1/9* and *Twist*) were analyzed at three different developmental stages (the early, mid-, and late pharyngular stages) (Christ et al. 2000; Ota et al. 2011). Moreover, the expression patterns of *Pax3/7* and *MyoD* genes (dermomyotome and myotome markers, respectively) were also examined (Ota et al. 2011). These hagfish somite derivative–related genes showed clear expression indicative of similarities in the molecular developmental mechanisms of gnathostomes and hagfish (Ota et al. 2011; Fig. 29.5).

Anatomic observations of *E. burgeri* suggested that the cartilaginous nodules are located at the 80th to 100th somite levels; furthermore, *Twist*- and *Pax1/9*-positive cells were consistently detected at the same levels of the somite (Ota et al. 2011). These results suggested that the *Pax1/9*- and *Twist*-positive mesenchymal somite derivatives from the 80th to 100th somite levels might differentiate into the cartilaginous nodules (Ota et al. 2011). From these results, the *Pax1/9*-positive mesenchymal cells at the ventromedial aspect of the somite epithelia at the early pharyngular stages were found to be developmentally homologous to the sclerotome in gnathostomes. In other words, hagfish and gnathostomes share homologous molecular developmental mechanisms for sclerotomal derivatives (Ota et al. 2011) (Fig. 29.5).

It is worth mentioning the exceptional gene expression patterns of the lamprey sclerotome in this context. It has been reported that the somite of the lamprey embryo (*Lethenteron camtschaticum*) lacks expression of *Pax1/9* homologues (Ogasawara et al. 2000). Reliable reports are scarce; thus, further detailed investigation will be required to decide whether this animal utilizes totally different molecular developmental mechanisms for sclerotome formation, or whether the expression level of the *Pax1/9* gene is simply lower than those in other vertebrates. However, in any case, the exceptional expression pattern of the *Pax1/9* gene in the lamprey can be considered to be a lineage-specific event on the basis of the evidence that molecular mechanisms underlying sclerotome formation are conserved in hagfish and gnathostomes.

Anatomic and gene expression pattern analyses also helped in interpreting the absence of cartilaginous nodules in the middle trunk region of hagfish. On the basis of the above results, it was suggested that *Pax1/9*- and *Twist*-positive mesenchymal cells are able to react to signals from the notochord, which induce chondrogenesis. This is consistent with the evidence that hagfish adults and embryos have a cartilaginous chordal cranium, known as the parachordal (Ota et al. 2011; Neumayner 1938). On the other hand, *Pax1/9*- and *Twist*-positive mesenchymal cells were also detected at more anterior somite levels, at which none of the cartilaginous nodules are present at the adult stages (Ota et al. 2011). These findings suggested that (1) the molecular developmental mechanisms in the hagfish somite derivatives enable the development of early sclerotome primordial cells, and (2) sclerotomal cells at the midtrunk region cannot undergo chondrogenesis (Ota et al. 2011, 2013) (Fig. 29.5).

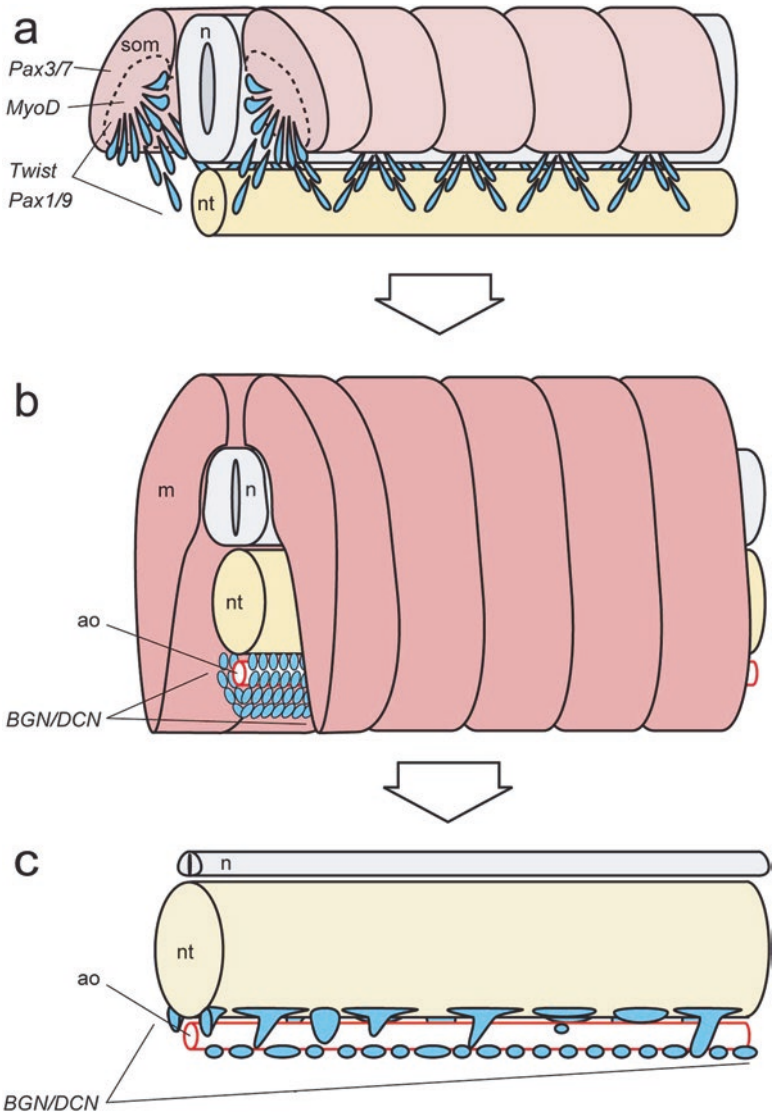

Fig. 29.5 Schematic representation of the developmental process of cartilaginous nodules in hagfish. (**a**) In the early pharyngular–stage embryo, medial ventral somite cells differentiate into *Twist*- and *Pax1/9*-positive mesenchymal cells. (**b**) The mesenchymal cells migrate and surround the dorsal aorta in late pharyngular to prehatching–stage embryos. (**c**) The mesenchymal cells express extracellular matrix proteins (*BGN/DCN*) and form the cartilaginous tissue at the adult stages (Reproduced from Ota et al. 2011)

The developmental process of cartilaginous nodules was examined not only at the early stages but also in late-stage embryos (Ota et al. 2014). The expressed sequence tag project used hagfish embryonic material (Takechi et al. 2011) to isolate the gene encoding the hagfish cartilaginous extracellular matrix (ECM) protein, biglycan/decorin (*BGN/DCN*) (Ota et al. 2013). The *BGN* and *DCN* genes are categorized into the class I small leucine-rich proteoglycan (SLRP) gene family and are expressed in the cartilaginous tissues of gnathostomes (Schaefer and Iozzo 2008; Kalamajski and Olderg 2010). The expression patterns of the *BGN/DCN* gene in the late hagfish embryo (242 days postdeposition) and the cartilaginous nodules in the adult specimen suggest that *Pax1/9*- and *Twist*-positive mesenchymal cells express *BGN/DCN* protein and ultimately form cartilaginous nodules at the ventral aspect of the notochord (Ota et al. 2013). This suggests that hagfish and gnathostomes possess common molecular developmental mechanisms from early to late development, but several parts of these mechanisms have been modified in each lineage (Ota et al. 2013) (Fig. 29.5).

29.5 Updated Evolutionary Scenario for the Vertebral Elements

The above molecular developmental analyses allowed us to devise a comprehensive evolutionary scenario based on the premise that the common ancestor of hagfish and other vertebrates possessed the same molecular developmental mechanism for the vertebral elements (Ota et al. 2011). More specifically, it is possible to hypothesize the evolutionary scenario of the vertebral elements on the basis of the cyclostome theory and molecular developmental evidence, as follows: (1) the common ancestor of all vertebrates had molecular developmental mechanisms to form cartilaginous vertebral elements at all trunk levels; (2) some fossil agnathans were equipped with vertebral elements at all trunk levels; and (3) in the hagfish lineage, the vertebral elements were lost because of regressive evolution in the middle trunk region (Ota et al. 2014) (Fig. 29.6).

Although phylogenetic trees in recent paleontological studies have been reconstructed on the basis of the cyclostome theory (Gai et al. 2011; Mallat and Holland 2013; Conway Morris and Caron 2014; Janvier 2015) (Fig. 29.6), the evolutionary history of the vertebral elements is still not completely clear. In fact, although anatomic data have led us to assume that the common ancestor of all vertebrate species had segmentally arranged dorsal and ventral vertebral elements (Fig. 29.1), with ventral vertebral elements being degenerated in the lamprey and hagfish lineages (the dorsal elements at all levels and ventral element of the midtrunk level were reduced during evolution (Fig. 29.6)), we still do not have sufficient information to examine this hypothetical evolutionary scenario. To determine whether the above evolutionary scenario is correct, more detailed analysis will be required using several fossil agnathans, including *Haikouichthys*, *Metaspriggina*, and *Euphanerops*

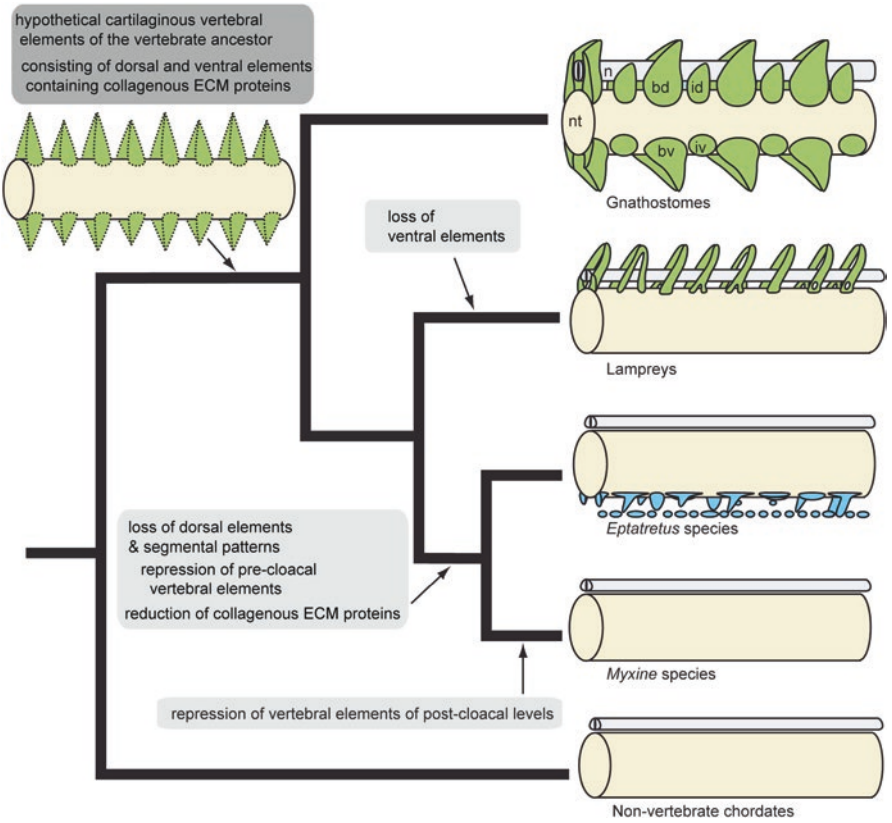

Fig. 29.6 Hypothetical evolutionary process of the vertebral elements. On the basis of the cyclostome theory, the hagfish clade (consisting of the *Myxine* and *Eptatretus* species) is clustered with the lamprey clade. On the basis of this phylogenetic tree and the skeletal anatomy of the vertebrates, it is hypothesized that the common ancestor might have had segmentally arranged vertebral elements at both the dorsal and ventral sides of the notochords (Reproduced from Ota et al. 2013, with permission)

(Shu 2003; Janvier 2011; Conway Morris and Caron 2014). It is hoped that further detailed analyses in a wider range of fossil agnathan species will provide greater understanding of the evolution and development of vertebral elements.

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Chapter 30
Left–Right Specification in the Embryonic and Larval Development of Amphibians

Ryuji Toyoizumi and Kazue Mogi

Abstract Biomolecules in living organisms, such as amino acids and double-stranded DNA, show left–right asymmetry. Even unicellular organisms, such as ciliates, have species-specific left- or right-handedness. Such molecular chiral asymmetry and cellular left–right asymmetry have likely provided a basis for the evolution of genetically determined left–right asymmetry of organ situs and the morphology of the heart, visceral organs, and central nervous system in eumetazoans. To study left–right asymmetry of the body plan, we believe that *Xenopus laevis* is a valuable model organism. The early *Xenopus* larva forms a transparent epidermis in the ventral abdominal region; thus organ development and morphology can be easily observed without dissection.

Genetic cascades involved in left–right specification during the phylotypic somite stage are conserved among vertebrates. Key left-handed genes such as *nodal*, *lefty*, and *pitx2* have been cloned and functionally characterized in *Xenopus* embryos. Here, we review advances over the last two decades of molecular embryology research on left–right specification in visceral organs. Despite this extensive research, *Xenopus* brain laterality is still elusive. This is partially explained by a lack of useful molecular markers showing left- or right-handed expression in the embryonic or larval brain in *Xenopus*, although in teleosts such as zebrafish, medaka, and flounder, several genes—including *nodal*, *lefty*, *pitx2, otx5*, and *leftover*—show temporary left- or right-handed expression in the epithalamic region. Prominent morphological left–right differences in the dorsal diencephalic habenular nuclei have been described in both anuran species (except for *Xenopus*) and urodelan species since the early twentieth century. Accordingly, it is necessary to establish a new model organism to shed light on brain laterality in amphibians.

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Keywords Laterality · *nodal* · *otx5* · *leftover* · Anura · Urodela · Tadpole · Habenula · Spiracle · Brain asymmetry

30.1 Introduction

Why do various bilaterians have a left–right asymmetric body plan? In the early phase of vertebrate embryogenesis, morphologically bilateral embryonic tissue develops into prominently left–right asymmetric organs, such as the heart, lungs, and digestive organs, via species-specific genetic information. Even the neural circuits of lower vertebrates and our brain hemispheres show anatomic and functional left–right asymmetry, called laterality. The invertebrate body plan is also left–right asymmetric, as determined by the laterality of one-sided gene expression (Grande and Patel 2009). It is not clear whether the common ancestor of protostomes and deuterostomes had a left–right asymmetric body plan, but the genetically determined left–right asymmetric body plan of most extant bilaterians suggests that some cellular properties, such as circular clockwise rotational ciliary movement, are essential components of the left–right determination process (Nonaka et al. 2002; Nonaka et al. 2005; Hirokawa et al. 2006).

The human body is made of left–right asymmetric chiral molecules: amino acids. Most amino acids in our body are L-type, while R-type amino acids are very rare; the chirality of amino acids is described by the CORN rule. Some have proposed that astrochemical mechanisms can explain this bias in the amino acid chirality (homochirality) of primordial ancestral organisms (Cronin and Pizzarello 1997; Cronin and Pizzarello 1999; Pizzarello et al. 2003). Double-stranded DNA molecules also show molecular chirality. In physiological conditions, double-stranded DNA takes a B-form or a Z-form. In the more common B-form double helix, the DNA strands are right-handed spirals. Living organisms are made of such asymmetric molecules, and the linkage between biases in molecular chirality and the mechanism determining the left–right asymmetry of internal organs—such as the heart, digestive organs, spleen, and diencephalic nuclei—is largely unknown.

Unicellular organisms such as ciliates show species-specific left–right asymmetry. Most *Paramecium* species (such as *P. caudatum*, *P. aurelia*, and *P. bursaria*), when viewed from the rear, rotate in a counterclockwise (sinistral) direction (Párducz 1967; Wichterman 1986). Interestingly, *P. calinski* exhibits clockwise rotation when viewed from the rear during migration. Unknown genetic factors may regulate the cooperative metachronal waves of ciliary beating in paramecia.

Recently, Dr. Kenji Matsuno and colleagues discovered that the left–right asymmetric rotation of the *Drosophila* hindgut reflects the chiral nature and chiral morphology of the embryonic epithelial tube. They called this left–right asymmetric driving force “planar cell chirality” (Taniguchi et al. 2011). Furthermore, they discovered that individual cells have intrinsic chiral properties in left–right asymmetric epithelial morphogenesis in the *Drosophila* hindgut (Hatori et al. 2014). We do not

yet know whether this model is applicable to vertebrate left–right asymmetric morphogenesis in general, but this novel insight is exciting because it suggests that a unified mechanism may determine both protistan chirality and our left–right asymmetric body formation based on the chiral properties of individual cells composed of chiral biomolecules. Irrespective of this, left–right specification mechanisms converge on the *nodal–pitx2* axis in vertebrate embryogenesis during the phylotypic stage, which corresponds to the somite stage. Therefore, we review this point with special reference to *Xenopus* left–right specification. In early *Xenopus* development, until the tailbud stage (the late somite stage), yolk platelets interfere with direct observation of the visceral morphology. However, early *Xenopus* larvae become transparent, especially on the ventral side, where no skin melanophores migrate and differentiate (Fig. 30.1). In early *Xenopus* larvae, we can noninvasively observe the state of the living heart and visceral organs. In addition, we can predict the future left–right orientation from early cleavage–stage embryos on the basis of the dorsoventral pigmentation gradation caused by the cortical rotation of eggs just after fertilization. Using *Xenopus laevis* embryos, a unilateral microinjection technique has been applied to cleavage-stage embryos in combination with *in situ* hybridization to detect marker genes with left-handed expression. This approach has made significant contributions to our understanding of the visceral left–right specification mechanism. Thus, we believe that *Xenopus* is a good model organism for the study of embryonic and larval left–right specification mechanisms.

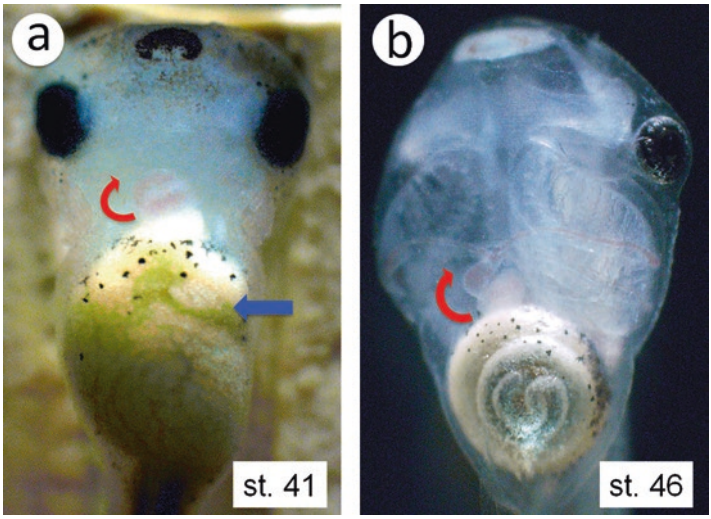

Fig. 30.1 Ventral view of early *Xenopus* larvae. Through the transparent epidermis, the left–right orientation of the heart and visceral organs is easily observed. The red arrows depict the direction of blood flux. The blue arrow depicts the ventral pancreas. In the initial phase of left–right asymmetric curvature, the gut forms a C-shape when viewed from the ventral side (**a**; stage 41). Subsequently, counterclockwise coiling of the gut starts from the upper left portion of the abdomen, and the coiled gut fills a large proportion of the abdominal cavity (**b**; stage 46)

In Part I of this chapter, we review the brief history of molecular research on left–right asymmetry in the heart and visceral organs, focusing on the discovery and comprehensive analysis of mouse *iv* and *inv* mutants and asymmetrically expressed genes in *Xenopus* and other vertebrate species. In Part II, we compare recent advances in the study of teleostean epithalamic left–right asymmetry in classical studies of anuran and urodelan left–right asymmetry of the habenulae (in Latin, *nuclei habenulae*) and introduce our recent approach to analyzing *Xenopus* brain laterality.

30.2 Part I: The *nodal–pitx2* Axis Is Pivotal for Left–Right Specification in *Xenopus* and Other Vertebrate Model Organisms

30.2.1 Discovery of Left- and Right-Handed Genes

We have observed that studies on embryonic left–right determination can be divided into two types: those that identify left–right asymmetrically expressed genes and those that examine mutants with phenotypes that show *situs inversus viscerum* (left–right asymmetry of the heart and/or visceral organs).

In the latter half of the 1990s, several left–right asymmetrically expressed genes were identified after early chick embryos at the primitive streak–somite stage were established as research organisms for molecular embryology of left–right specification in 1995 (Levin et al. 1995). Almost all of these genes are involved in the left–right specification of internal organs. In the first decade of the twenty-first century, dorsal epithalamic regions, including the pineal complex and habenular nuclei, were identified as "left-handed"—in other words, left-dominant neural circuit formation in the diencephalon is under the control of left-handed genes.

30.2.2 Two Mouse Mutants Show Situs Inversus *of the Heart and Visceral Organs*

In the mid-twentieth century, Hummel and Chapman (1959) reported a mutant strain that shows a left–right–reversed heart and visceral organs, and they named the strain *iv* (*inversus viscerum*). This mouse strain has an autosomal recessive mutation affecting left–right orientation in 50% of offspring. This finding demonstrates the existence of specific genes responsible for left–right determination. It took more than 30 years to identify the causative gene for the *iv* mutant as *left–right dynein* (*lrd*), a member of the dynein ATPases, which encode the motor proteins of the cilia (Supp et al. 1997). Before body hair formation, the semitranslucent epidermis reveals that half of the neonates of this strain show a white stomach on the right side

of the abdomen after lactation. A left–right inverted autosomal recessive mutant was isolated by Dr. Takahiko Yokoyama and named *inv* (inversion of embryonic turning). Importantly, the homozygous *inv* mutant shows 100% left–right reversal. Two research groups independently and simultaneously identified the causal gene of the *inv* mutant (Mochizuki et al. 1998; Morgan et al. 1998). The *inv* gene encodes a protein that has an ankyrin motif and a calmodulin-binding motif (Morgan et al. 2002). Inv may be a cytoplasmic protein undersealing the cell membrane. It is ubiquitously expressed in both mouse and *Xenopus* early embryos (from the cleavage stage to the tailbud stage) (Yasuhiko et al. 2001; Yasuhiko et al. 2006). The mechanism underlying left–right reversal in all homozygous *inv* mutants is unclear.

These two mouse mutants have had significant impacts on research in the field; they suggest that specific genetic cascades completely drive left–right asymmetric morphogenesis. Except for the *situs inversus* and polycystic kidney, the mutants do not exhibit severe or fatal abnormal morphologies of the external and internal organs, indicating that the grand plan of left–right specification is separable from organ-specific morphogenesis. Coupled with analyses of left- or right-handed gene expression, these mutants have facilitated developmental studies of left–right asymmetry.

30.2.3 Nodal Is a Key Regulator of Left–Right Specification

Dr. Elizabeth J. Robertson and colleagues discovered that mouse *nodal*, an organizer-expressed gene, is expressed in the left lateral plate mesoderm (LPM) of the somite-stage embryo (Collignon et al. 1996). They also observed left-dominant *nodal* expression in the node. In 1996, left-sided *Xenopus nodal* expression in the LPM was also reported (Lowe et al. 1996).

Nodal is an important member of the TGF-β superfamily; it mediates cell-to-cell communication via transmembrane hetero-tetrameric receptors (type I receptors called activin-receptor-like kinases (ALKs) and type II receptors). In general, the expression of pivotal phylotypic genes during the somite stage is conserved among vertebrate embryos of different classes (Irie and Kuratani 2011; Irie and Kuratani 2014). At the pivotal stage for left–right specification, *nodal* is expressed unilaterally in the left LPM in various vertebrate embryos, including mice, rats (Fujinaga et al. 2000), rabbits (Fischer et al. 2002), chicks (Levin et al. 1995), *Xenopus*, zebrafish (Rebagliati et al. 1998a; Long et al. 2003), and medaka (Soroldoni et al. 2007). In tunicate embryos, *nodal* is also expressed on the left side, but the expression is restricted to the epidermis and does not occur in mesodermal tissues (Morokuma et al. 2002). In lancelets, *nodal* is expressed in the left mesoderm and endoderm of their remarkably handed internal structures (Yu et al. 2002; Onai et al. 2010; Soukup et al. 2015). In hemichordates, *nodal* is first expressed in vegetal pole cells and later in the ventral ectoderm, with a slight enrichment in the right ventral ectoderm at the late gastrula stage; however, no significant left–right asymmetric expression of hemichordate *nodal* has been reported (Röttinger et al. 2015).

Strangely, *nodal* is expressed on the *right* side of sea urchin embryos, and this expression is crucial for asymmetric adult rudiment development, which originates from the left coelomic pouch (Duboc et al. 2005; Luo and Su 2012).

The first study of *nodal* expression in protostomes was performed in snails. In dextral *Lottia gigantea, nodal* is expressed on the right side, whereas in sinistral *Biomphalaria glabrata, nodal* is expressed on the left side (Grande and Patel 2009). These results indicate that the laterality of *nodal* expression corresponds to the winding morphogenesis of the spiral shell in snails.

The importance of Nodal signaling during embryogenesis has been demonstrated, using various techniques. The mouse embryo has a unique single *nodal* gene, which is indispensable for anteroposterior specification and mesoderm induction during the primitive streak stage. Thus, *nodal* knockout is embryonically lethal in mice, and it is difficult to analyze later left–right–determining events (Zhou et al. 1993). Fortunately for researchers, the left–right–determining enhancer of *nodal* ASE is independent of the other functional enhancers of *nodal*. Accordingly, conditional knockouts targeting the asymmetric enhancer have revealed essential functions of left-handed *nodal* expression (Saijoh et al. 2003; Kawasumi et al. 2011). The conditional knockout of *nodal* in the LPM specifically elicits laterality defects (Kumar et al. 2008). Mice with the hypomorph of the *nodal* allele survive until the somite stage, and the direction of embryonic turning and the situs of handed organs show left–right reversal (Lowe et al. 2001; Brennan et al. 2002).

Uterus morphology and the process of placentation are diverse among mammalian species. These phenotypes may be related to the left–right specification mechanisms. In the rabbit, the regulatory mechanism of *nodal* expression by FGF-8 differs from that of mouse *nodal* (Fischer et al. 2002; Blum et al. 2007; Feistel and Blum 2008). Midline *FGF-8* expression suppresses *nodal* expression in the right LPM in rabbit embryos, whereas in mouse embryos, midline *FGF-8* expression is a prerequisite for left-handed *nodal* expression in the left LPM.

Because of their transparent embryos, rapid development, and ease of breeding, zebrafish in the family Cyprinidae have been established as a standard lower vertebrate model organism. In zebrafish, three *nodal* homologues (so-called *nodal*-related genes)—*squint*, *cyclops*, and *southpaw*—have been identified. Among them, *cyclops* and *southpaw* exhibit left-handed expression patterns (Rebagliati et al. 1998a; Long et al. 2003). Their embryonic left-handed expression is temporal. The well-known *cyclops* is a causal gene of the cyclopia (one-eyed) mutant; *cyclops* is also involved in mesoderm induction, dorsoventral pattern formation, and anteroposterior specification. The expression of *cyclops* in the embryonic epithalamic region is left-handed (Rebagliati et al. 1998b). Additionally, *southpaw* shows left-handed expression at the somite stage of zebrafish embryos, as suggested by its name (Long et al. 2003). Expression of *southpaw* is not observed in the brain, and its earliest expression in Kupffer's vesicle is seemingly not biased. However, during the somite stage, its expression expands from the rear to the front of the left LPM (Long et al. 2003).

In the weak allelic *cyclops* mutant, frequent left–right reversal is observed; thus *cyclops* is thought to be involved in left–right specification. Dr. Michael Rebagliati

and colleagues reported that morpholino oligonucleotide injections targeting *southpaw* induce a high incidence of left–right reversal, without other defects (Long et al. 2003). In this morphant, left-sided *cyclops* expression in the diencephalon is frequently shifted to the right side; thus, left-handed *southpaw* expression in the LPM might affect brain laterality. There is a long lag between left LPM *southpaw* or *cyclops* expression and left-handed diencephalic *cyclops* expression. However, the mechanism by which left–right asymmetric signaling in the trunk triggers one-sided gene expression in the brain is unclear.

In *X. laevis* embryos, six *nodal*-related genes have been identified: *Xnr-1 to Xnr-6* (*Xenopus nodal related-1* to *-6*). Among the *Xnr* genes, only *Xnr-1* is expressed unilaterally in the left LPM (Lowe et al. 1996; Lohr et al. 1997) (Fig. 30.2). *Xnr-2* to *Xnr-6* do not show left–right asymmetric expression. *Xnr-1* expression is first detected at the late blastula stage, when it is expressed in several cells in the vegetal region (Jones et al. 1995). Subsequently, *Xnr-1* is expressed in the dorsal lip of the blastopore (organizer) at the early gastrula stage. At the early to midneurula stage, *Xnr-1* expression is not detected by reverse-transcription polymerase chain reaction (RT-PCR) or *in situ* hybridization. At the late neurula stage (stage 17), *Xnr-1* is

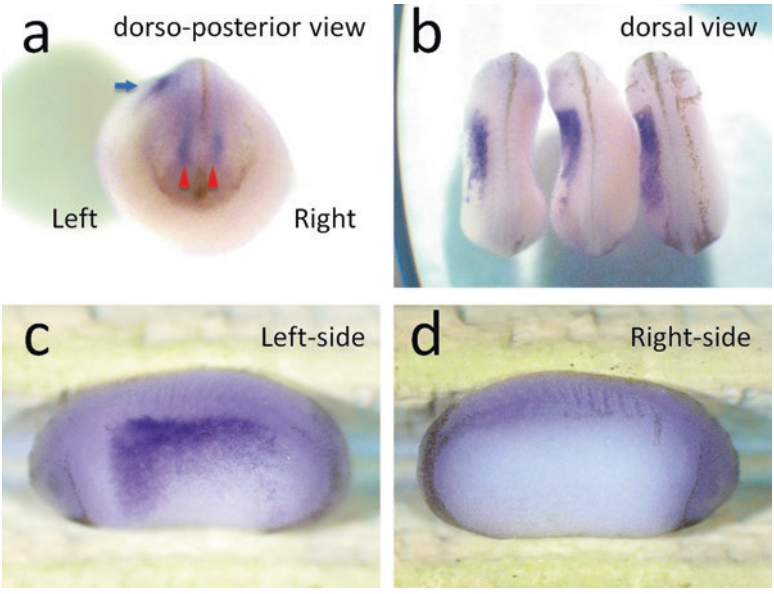

Fig. 30.2 Expression pattern of left-handed *Xnr-1* (*Xenopus nodal related-1*). Among six *Xnrs*, only *Xnr-1* is expressed in a left–right asymmetric manner during the late neurula–early tailbud stages. (**a**) Dorsoposterior view of a neural tube–stage (stage 20) embryo. In the left lateral plate mesoderm (LPM), left-handed expression is observed (blue arrow). At the rear of the dorsal arch-enteron roof, a bilateral expression domain is also recognized (red arrowheads). This embryo was permeabilized with 50% glycerol. (**b**) Dorsal view of three early tailbud–stage embryos expressing *Xnr-1* in the entire left LPM. (**c** and **d**) Lateral views of an early tailbud–stage (stage 23) embryo showing left-handed *Xnr-1* expression in the left LPM (**c**) and not in the right LPM (**d**)

expressed in the dorsoposterior region of the archenteron roof (Fig. 30.2a). At this stage, *Xnr-1* expression is bilateral in the region adjacent to the posterior notochord. This bilateral expression is regulated by the extracellular antagonist Coco, a member of the Cerberus/DAN family, identified by Dr. Ali H. Brivanlou and colleagues (Vonica and Brivanlou 2007). Coco acts as an inhibitor of Activin/Nodal/BMP signaling but, interestingly, enhances TGF-β1 signaling (Deglincerti et al. 2015).

At the late neurula stage, left-handed *Xnr-1* expression starts in the posterior portion of the LPM, near the archenteron roof, and exhibits simultaneous bilateral expression at this stage. This left-sided expression expands toward the anterior LPM, and most of the left LPM transiently expresses *Xnr-1* (Fig. 30.2b). Soon after, posterior expression ceases and left-sided expression is limited to the frontal left LPM, which might be part of the prospective heart mesoderm (Lowe et al. 1996).

To study mesoderm induction in the *Xenopus* embryo, an *Xnr-1* expression vector is frequently used to identify candidate components involved in mesoderm induction. *Xnr-1* itself has the potential to induce mesoderm and, on the basis of its expression pattern, it is suitable for induction of the prospective mesoderm; thus *Xnr-1* is regarded as an intrinsic mesoderm inducer. However, our morpholino knockdown experiments have revealed new properties of *Xnr-1*. Like the zebrafish *southpaw* gene, the phenotype of *Xnr-1* knockdown morphants was normal, except for the heart and visceral left–right asymmetry. Abrogated left-handed *Xnr-1* expression via morpholino injection into the left blastomere resulted in a randomized left–right orientation of the internal organs (Toyoizumi et al. 2005). We can predict the future dorsoventral and left–right axes in early cleavage–stage embryos by referring to the dorsoventral pigment gradation caused by the cortical rotation of the eggs just after fertilization. In contrast, posterior bilateral archenteron roof expression was not affected by left- or right-sided morpholino injection (Toyoizumi et al. 2005). Morpholino injections targeting *Xnr-1* into right-sided blastomeres did not cause the left–right reversal of internal organs, and *Xnr-1* expression in the left LPM was normal. Our results strongly suggest that *Xnr-1* is the only essential *Xenopus nodal*-related gene for the determination of left–right orientation (Fig. 30.3). We noticed that the asymmetric morphology of the heart and visceral organs was not severe; rather, most morphants exhibited nearly normal phenotypes. Similar results using *Xnr-1* morpholinos have since been obtained by Dr. Brivanlou and colleagues (Vonica and Brivanlou, 2007). We observed that bilateral expression of TGF-β5 is needed for left-handed *Xnr-1* expression (Mogi et al. 2003), as is Subtilisin-like proprotein convertase (SPC) activity (Toyoizumi et al. 2006).

Using right-sided hypodermic Activin/TGF-β/Nodal injections into *Xenopus* embryos at the early to midneurula stage, we observed that the right LPM has the potential to express *Xnr-1* and, subsequently, *pitx2* (Toyoizumi et al. 2000; Mogi et al. 2003; Toyoizumi et al. 2005). However, the absence of an initial trigger of *Xnr-1* expression might nullify this potential in the right LPM.

Components of Nodal signaling have been analyzed in detail. FAST (Forkhead Activin Signal Transducer) is an important transcription factor downstream from Nodal signaling in mesoderm induction. In the left–right specification cascade, FAST is also an essential factor (Norris et al. 2002, Saijoh et al. 2000). Smad is an

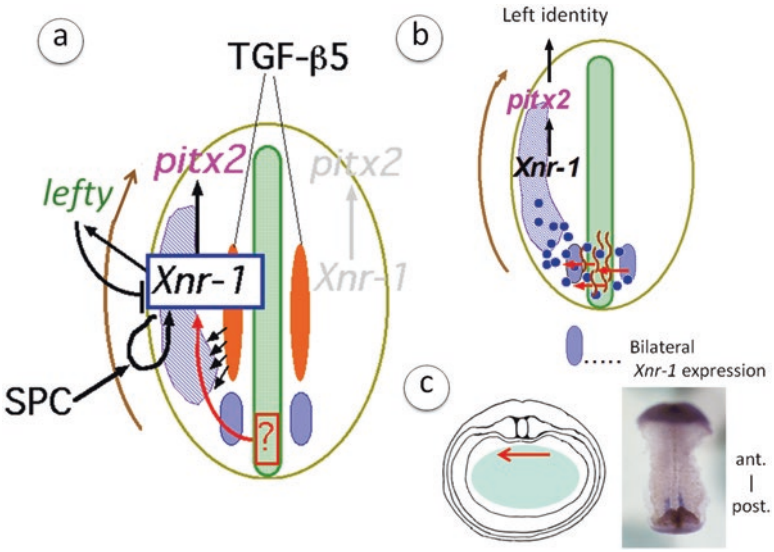

Fig. 30.3 Schematic representation of the genetic cascade controlling left–right orientation in the *Xenopus* embryo at the neurula–early tailbud stage. (**a**) Until the midneurula stage, *Xnr-1* expression ceases temporally after expression in the organizer region at the gastrula stage. An as-yet-unidentified inducer triggers *Xnr-1* expression in the rear left lateral plate mesoderm (LPM) at the late neurula stage. Using an autoregulatory positive feedback loop, the expression domain of left-handed *Xnr-1* expands toward the entire region of the left LPM. At the same stage, *Xnr-1* induces *Xenopus lefty* (*antivin*) in the left LPM, and later feedback inhibition of *Xnr-1* expression by *lefty* restricts the *Xnr-1* expression domain within the anterior left LPM. *Xnr-1* also induces *Xenopus pitx2* in the anterior left LPM, and *pitx2* is continuously expressed on the left side until the stage of actual morphological curvature of the left-right asymmetric organs. We observed that bilaterally expressed TGF-β5 in the somites and Subtilisin-like proprotein convertase (SPC) activity are prerequisites for left-handed *Xnr-1* expression (Mogi et al. 2003; Toyoizumi et al. 2006). (**b** and **c**) Nodal flow model of the *Xenopus* archenteron roof region. Dr. Martin Blum and colleagues have reported that ciliary clockwise rotational movement generates a leftward fluid flow in the dorsal roof of the *Xenopus* archenteron (gastrocoel roof plate (GRP), *leftward arrows* in (**b**) and (**c**)), and this is pivotal for *Xnr-1* expression in the left LPM (Essner et al. 2002; Schweickert et al. 2007). In the mouse early somite–stage embryo, *nodal* is expressed in the crown region (peripheral margin of the organizer region) of the node, and *nodal* expression is dominant on the left side. In contrast, expression of *Xenopus nodal related-1* (*Xnr-1*) in the late organizer of *Xenopus* embryos (GRP) at the late neurula stage is strongly bilateral and is not biased on the left side (right photograph in (**c**), *Xnr-1* expression in the piece of archenteron roof viewed from the ventral side). Nodal flow generated in the *Xenopus* late organizer region adjacent to the posterior archenteron roof (GRP) carries unidentified determinants (possibly including Xnr-1) toward the left rear portion of the LPM and might induce posterior left-handed expression of *Xnr-1*. Whether *Xenopus nodal* and/or *pitx2* expression in the brain region of the larvae or adults is left-right asymmetric or symmetric is not yet known (See Figs. 30.5 and 30.10)

intracellular mediator of Nodal signaling and the signaling of other TGF-β superfamily proteins. Because TGF-β signaling is reciprocally involved in many developmental events, it is difficult to analyze the essentiality of Smad using knockout and knockdown strategies. However, knockout mouse strategies, resulting in

severe abnormalities, have demonstrated that Smad2 (R (receptor regulated)-Smad for TGF-β/Activin/Nodal signaling) and Smad5 (R-Smad for BMP/GDF signaling) are involved in left–right specification in the mouse embryo (Nomura and Li 1998, Chang et al. 2000).

30.2.4 Lefty Is a Feedback Inhibitor of the Nodal Autoregulatory Loop

Lefty is a secretory peptide that modulates cell-to-cell communication and belongs to the TGF-β superfamily. The *lefty* gene was first identified by Dr. Hiroshi Hamada and colleagues (Meno et al. 1996). In the early phase of studies of *lefty*, it was a candidate for the left–right determinant of internal organs. It has since been precisely identified as a feedback inhibitor of Nodal signaling; it likely modulates Nodal signal transduction by extracellularly inhibiting Nodal–receptor interactions (Meno et al. 1997, 1998, 1999, 2001). Unlike most members of the TGF-β superfamily, which are dimers connected by a single disulfide bond, Lefty is a monomer. Thus, within embryonic tissues, Lefty diffuses faster than Nodal. This property facilitates its role as an intrinsic extracellular antagonist (Juan and Hamada 2001; Müller et al. 2012).

In mouse embryos, *lefty-1* and *lefty-2* have been identified. The former is mainly expressed on the left side of the axial organs, notochord, and floor plate of the neural tube, while the latter is mainly expressed in the left LPM at the somite stage. Knockout mice targeting *lefty-1* and *-2* have been systematically generated by Dr. Hamada and colleagues. Unexpectedly, they showed left isomerism (i.e., a left–left phenotype). This result was a turning point in the study of *lefty*. Consequently, a combination of experimental and theoretical studies have revealed that differences in diffusion efficiency between Nodal and Lefty result in a specific spatiotemporal pattern of Nodal activity that leads to a moderate intensity of Nodal signaling in both organizer formation and left-side specification (Branford and Yost 2002, 2004; Hamada et al. 2002).

Xenopus lefty (*antivin*) is also expressed in the left LPM and is involved in left–right specification at the somite stage (Cheng et al. 2000; Fig. 30.4). The injection of Lefty protein into the left LPM inhibits both *Xnr-1* and *pitx2* expression in the left LPM, and causes a high incidence of left–right reversal in *Xenopus* embryos (Toyoizumi et al. 2005) (Fig. 30.3). Coinjection of Nodal coupled with Lefty into the left flank of *Xenopus* neurulae restores the normal left–right orientation in a Nodal dose-dependent manner (Toyoizumi et al. 2005) (Fig. 30.3). Midline *Xenopus lefty* expression does not depend on left-handed *Xnr-1* expression (Toyoizumi et al. 2005); therefore, we speculate that axial *lefty* expression is induced and maintained by axial *Xnr-4* expression at the late neurula–neural tube stage (Joseph and Melton 1997).

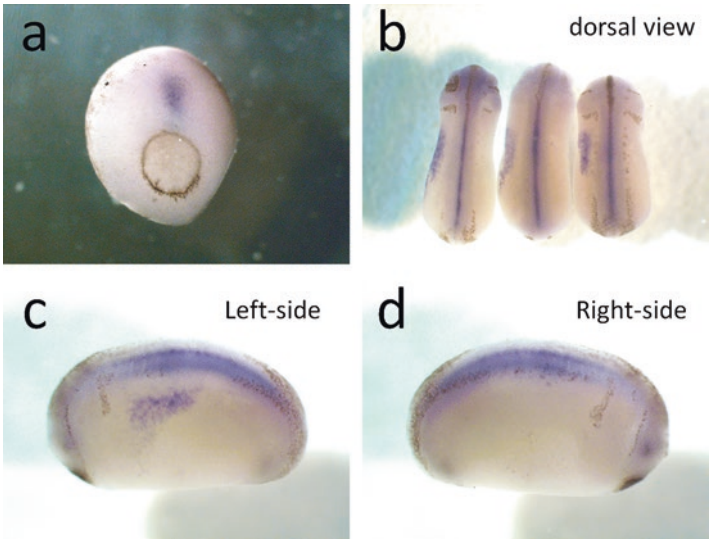

Fig. 30.4 Expression patterns of axial and left-handed *Xenopus lefty* (synonym in lower vertebrates: *antivin*), which plays a significant role in feedback inhibition of Xnr-1 signaling. (**a**) Midgastrula embryo (stage 11.5) showing *Xenopus lefty* expression in the dorsal lip portion. (**b**) Dorsal view of three embryos (stage 24) showing left-handed *lefty* expression in the left lateral plate mesoderm (LPM) and axial expression along the midline. (**c** and **d**) Early tailbud–stage embryo (stage 23) showing handed *lefty* expression in the left LPM but not in the right LPM, and axial *lefty* expression in the floor plate and hypochord, as was reported by Cheng et al. (2000)

30.2.5 Extracellular Regulation of Nodal Signaling Directs Left–Right Orientation

TGF-β superfamily members are extracellular signaling polypeptides; thus the activity of ligands in this family is—like that of secretory Wnt ligands—regulated by extracellular modulators. The intrinsic mechanism of left–right specification requires such extracellular modulators at several steps. For example, Cerberus and DAN (members of the Cerberus/DAN family) are expressed left–right asymmetrically in the chick embryo, and right-side implantation of cerberus-expressing cells in the *area pellucida* (the embryo-forming region of the blastoderm) induces left–right reversal of the heart situs and torsion of the telolecithal chick embryonic body (Zhu et al. 1999). Caronte also belongs to the Cerberus/DAN family, and its mode of action is very complicated. Caronte might directly interact with both Nodal and BMP. Although its precise role is controversial, *caronte* is thought to regulate the handed expression of *nodal* and its activity in the left LPM (Rodríguez-Esteban et al. 1999; Piedra and Ross 2002).

In lower vertebrates, members of the Cerberus/DAN family are essential for left–right specification. *Xenopus* Coco is an inhibitor of Nodal/Activin/BMP signaling and is involved in left–right axis specification. Recently, an RNA interference (RNAi) screening approach has revealed that Coco is an enhancer of TGF-β1 signaling (Deglincerti et al. 2015).

Charon has been detected in medaka, zebrafish, and flounder; it is another member of the Cerberus/DAN family. The expression pattern of *charon* is bilateral around the Kupffer's vesicle in zebrafish embryos. By regulating *southpaw* activity, *charon* is also involved in left–right specification (Hashimoto et al. 2004). Kupffer's vesicle is a ciliated organ and is regarded as a left–right organizing center, which transiently forms in the bottom of the tail region of somite-stage fish embryos. The role of Kupffer's vesicle was a mystery for more than 100 years since its discovery by Kupffer in a herring embryo, but molecular embryology has finally revealed its function in left–right specification. Interestingly, in contrast to the bilateral *charon* expression in zebrafish, medaka *charon* is dominant on the right side of the Kupffer's vesicle, and this might generate left-dominant Southpaw activity in medaka (Hojo et al. 2007). *Charon* in the flounder is bilaterally expressed around Kupffer's vesicle, although flounder fry eventually metamorphose into a severely left–right asymmetric external morphology (Hashimoto et al. 2007).

30.2.6 Role of FGF Signaling in Left-Handed nodal *Expression*

The roles of FGF signaling in left–right specification upstream from left-sided *nodal* expression are quite different among vertebrate species. In the mouse embryo, *fgf-8* expression along the primitive streak shows no left–right bias, and *fgf-8* induces *nodal* expression in the left LPM, whereas in rabbit and chick embryos, *fgf-8* inhibits *nodal* expression in the right LPM (Boettger et al. 1999; Meyers and Martin 1999; Schneider et al. 1999; Fischer et al. 2002; Tanaka et al. 2005; Schlueter and Brand 2009). Rabbit *fgf-8* expression along the streak is not biased, as observed in mouse embryos; however, in chick embryos, *fgf-8* expression around the Hensen's node (organizer region) is right biased. In zebrafish embryos, *fgf-8* is needed for ciliogenesis in the Kupffer's vesicle and *charon* expression around the vesicle (Hong and Dawid 2009). However, the role of FGF signaling in *Xenopus* embryonic left–right specification is not known.

30.2.7 Pitx2 Is a Final Determinant of Left–Right Orientation

In various vertebrate species, *nodal* induces left-specific expression of *pitx2*, a bicoid-type homeobox gene. In humans, *PITX2* is a causative gene of Axenfeld–Rieger syndrome (Footz et al. 2009). *Pitx2* is continuously expressed on the left side

of the internal organs until the actual morphological manifestation of asymmetric morphogenesis (Logan et al. 1998; Piedra et al. 1998; Tsukui et al. 1998; Yoshioka et al. 1998). In zebrafish larvae and fry, *pitx2* is expressed in the left diencephalon. In addition, *pitx2* is expressed in various tissues, such as the pituitary gland, tooth bud, pouch of Rathke, and mesenchyme around the eye primordium. As described above, *pitx2* is continuously expressed on the left side of organs, such as the heart and gut, until the onset of left–right asymmetric morphogenesis. Thus, *pitx2* is regarded as the final determinant in the complex process of left–right specification. Knockouts targeting *pitx2* in mice result in left isomerism of pulmonary lobulation. In mouse, chick, *Xenopus*, and zebrafish embryos, *pitx2* is induced by *nodal* (Logan et al. 1998; Piedra et al. 1998; Tsukui et al. 1998; Yoshioka et al. 1998) (Figs. 30.3 and 30.5). On the basis of these results, in collaboration with other various transcription factors, *pitx2* orchestrates the left–right asymmetric differentiation of internal organs and neural circuits. Downstream cellular targets of Pitx2 have long been unknown. Recently, Welsh et al. (2013) used a laser microdissection technique and site-directed overexpression in the chick embryo to identify *daam2* as a downstream target of *pitx2*. Daam2 is an effector of noncanonical Wnt signaling. They found that Daam2 directly binds to α-Catenin and N-cadherin to promote cell-to-cell adhesion of the *left* dorsal mesentery for future gut looping morphogenesis. They also found that both *gpc3* and *frizzled* are downstream targets of *pitx2* and facilitate Wnt5a signaling in the mesenchyme of the left dorsal mesentery (Welsh et al. 2013). Interestingly, *pitx2* expression on the right side is suppressed by

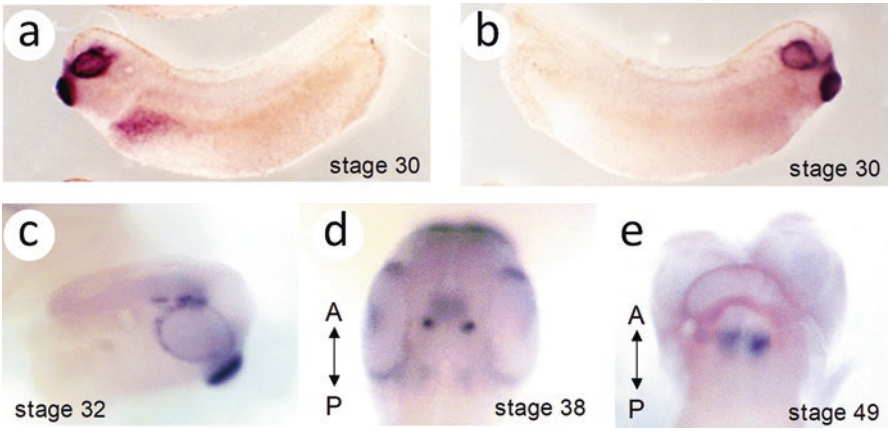

Fig. 30.5 Expression profile of *Xenopus pitx2*. (**a** and **b**) Left (**a**) and right (**b**) sides of a midtailbud-stage embryo. The left lateral plate mesoderm (LPM) shows significant expression, whereas the right LPM shows no expression. Mesenchymal expression around the eyes is bilateral, and expression in the cement gland is symmetric. (**c**–**e**) *Xenopus pitx2* expression in the central nervous system (CNS). When the samples are viewed from the lateral (**c**), dorsal (**d**), or ventral (**e**) sides, only bilateral expression of *pitx2* is recognized in the brain (our unpublished data). The embryos in (**c**) and (**d**) are permeabilized. The whole-mount head in (**e**) is viewed from the posterior ventral side

right-side expression of the long noncoding RNA *Playrr*, which is adjacent to the *pitx2* locus. On the left side, *Playrr* is suppressed by *pitx2* expression (Welsh et al. 2015). The pivotal cytoskeletal protein Shroom3 is essential for Pitx2-dependent left–right asymmetric gut coiling in the mouse embryo (Plageman et al. 2011). Cooperation among Shroom3, left-handed Pitx2, and N-cadherin in the dorsal mesentery is needed for the initial change in epithelial cell shape for gut coiling.

Another homeobox transcription factor downstream from Nodal signaling, *nkx3.2/bapx1*, is involved in left–right specification in the chick embryo. Interestingly, the laterality of its expression differs between chick and mouse embryos. In the chick embryo, left-handed *nkx3.2* is induced by Nodal in the left LPM. Potential *nkx3.2* expression on the right side is inhibited by FGF-8, whose expression is biased on the right side around the Hensen's node at the primitive streak stage. In contrast, in the mouse embryo, *nkx3.2* is expressed in the right LPM, and the upstream inducer of *nkx3.2* in the right LPM is elusive in the mouse (Schneider et al. 1999). In the mouse embryo, the right-sided expression of *nkx3.2* eventually shifts to the left side after 9.5 days postcoitum (Hecksher-Sørensen et al. 2004). The role of *nkx3.2* in *Xenopus* embryonic left–right specification is still unclear.

30.3 Part II: Amphibian Habenular Nuclei Show Left–Right Asymmetry in Their Morphology and Neural Circuits

30.3.1 Amphibian Diversity and Left–Right Morphological Asymmetry of Habenulae

In several teleost species, especially zebrafish and medaka (*Oryzias latipes*), early reports indicated that diencephalic habenular nuclei show left–right asymmetry (Signore et al. 2009; Villalón et al. 2012). Detailed studies of zebrafish embryos have revealed that the *nodal–pitx2* cascade specifies diencephalic left–right asymmetry (Concha et al. 2000, 2003). Amphibian habenular left–right asymmetry was first reported in newts in the early twentieth century (Mangold 1921; Wöellwarth 1950; Frontera 1952). In anurans, classical histological studies performed in the mid-twentieth century revealed that the left habenular nucleus is larger than the right one (Morgan et al. 1973; Morgan 1977, 1991). These studies used European and North American species (Concha and Wilson 2001). Accordingly, we investigated whether Asian and Japanese amphibian species show left–right asymmetric habenular morphogenesis (Figs. 30.6, 30.7 and 30.8). We obtained similar results to those obtained for European and North American amphibian species in both anuran (Fig. 30.6) and newt species (Figs. 30.7 and 30.8).

From a phylogenetic viewpoint, we are fascinated by the working hypothesis that diverse amphibian species share a common molecular mechanism for the control of left–right asymmetric habenular morphogenesis. Morgan (1977) reported that *Xenopus* habenulae show left–right *symmetric* morphology (Fig. 30.6c). Other

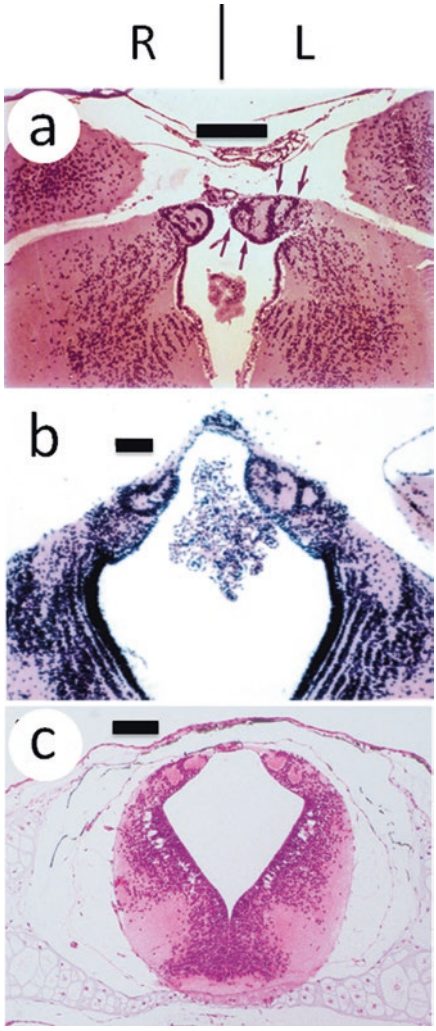

Fig. 30.6 Anuran habenular left–right asymmetry and symmetry. (**a**) Cross-section of an adult *Occidozyga lima* (floating frog, rice paddy frog) of the family Dicroglossidae. (**b**) Larval *Rana catesbeiana* (North American bullfrog) of the family Ranidae. These higher anuran species show substantial habenular left–right asymmetry. The left habenula nucleus has three subnuclei divided by septa, and the left is larger than the right. (**c**) Larval *Xenopus laevis* of the family Pipidae. Most primitive anuran *X. laevis* have left–right symmetric habenulae (Photograph courtesy of Ms. Kotomi Imura)

species in the family Pipidae should be investigated. Left–right differentiation has not been demonstrated in most plesiomorphic *Xenopus* habenulae, but recently diverged frogs and toads exhibit neural circuit differentiation related to habenular left–right asymmetry.

As far as we have established, amphibian habenulae show species-specific stable left–right asymmetry, which is consistent with previous findings. The morphology and left–right patterning of urodelan habenulae are quite different from those of anuran habenulae. In the Japanese red-bellied newt (*Cynops pyrrhogaster*), adult left–right asymmetry of habenulae is more rigid than that of larval habenulae (Figs. 30.7a and 30.8a). Therefore, we propose two possible explanations. First, before reaching the adult stage, left–right–reversed individuals selectively die out

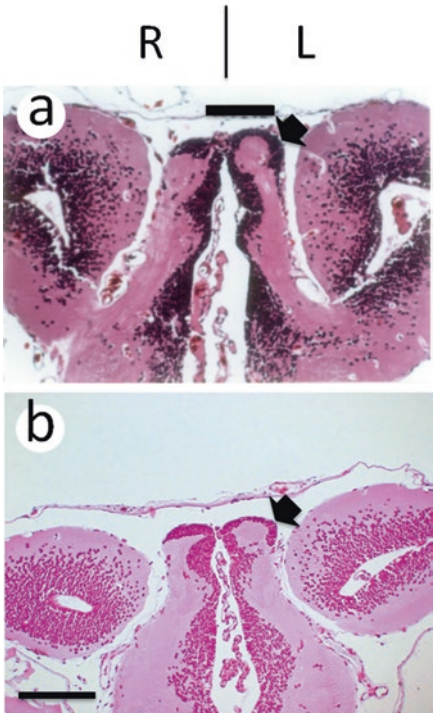

Fig. 30.7 Left–right asymmetry of the dorsal epithalamic region of the adult newt. (**a**) Adult newt of *Cynops pyrrhogaster*. (**b**) Adult newt of *Cynops orientalis*. In both species, the left habenula has many neural cells in the dorsolateral region of the nucleus (arrow), whereas in the right habenula, neuronal cell bodies are absent in the corresponding area. Such remarkable morphological left–right asymmetry has been known since the first half of the twentieth century, but the detailed mechanisms are not clear

because of a low survival rate. Second, and perhaps more likely, habenular left–right asymmetry is gradually established from the larval stage to adulthood in amphibians.

30.3.2 Possible Left–Right Asymmetry of Amphibian Neural Circuits in Association with the Habenulae and Pineal Complex

In zebrafish larvae and fry, and even in adults, the pineal complex shows absolute left–right asymmetry (Halpern et al. 2003). *In situ* hybridization using an *otx5* probe has revealed that both the pineal organ and parapineal organ are *otx5* positive and the parapineal organs are continuously positioned on the left side of the midline-located pineal organ (Gamse et al. 2002, 2003). The left-biased parapineal position is conserved throughout the life cycle of the zebrafish. Finally, in adulthood, the parapineal organ is positioned at the left bottom of the pineal stalk. It is interesting that *otx5* shows a circadian oscillatory expression pattern, similar to that of the *AANAT* gene (serotonin *N*-acetyltransferase/arylalkylamine *N*-acetyltransferase) (Gamse et al. 2002). *Xenopus otx5a/b* have been cloned and characterized (Kablar et al. 1996; Kuroda et al. 2000; Vignali et al. 2000). However, it is not clear whether

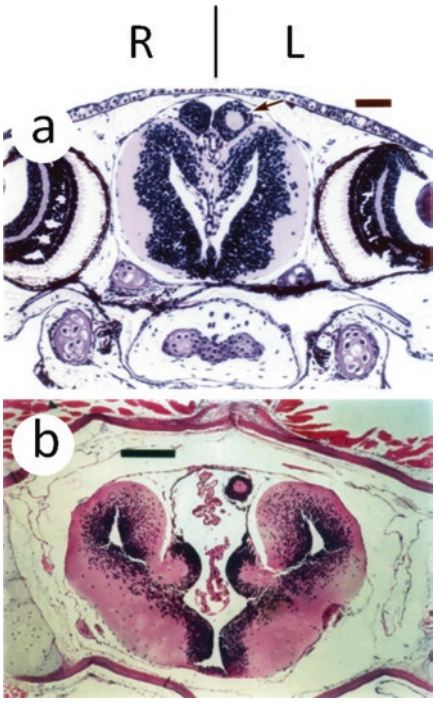

Fig. 30.8 Cross-section of the diencephalic region of the juvenile (stage 48) *Cynops pyrrhogaster* (Japanese fire belly newt) (**a**) and adult *Ambystoma mexicanum* (axolotl) (**b**), stained by the hematoxylin and eosin (H-E) double-staining method. In both species, on the basis of sectioning from anterior to posterior, the left habenular nucleus (arrow) shows a more frontal neuropil than the right habenular nucleus, indicating that the left habenula protrudes more extensively toward the frontal region. In *Cynops* larvae, 21 out of 31 individuals (68%) show strong left dominance of this "neuropil protrusion toward the anterior," but this is not reflected in the absolute index of left-handedness. Bars, 0.2 mm in (**a**) and 0.4 mm in (**b**)

Xenopus otx5 expression shows a left–right asymmetric pattern in the pineal complex. Accordingly, we re-examined the expression pattern in the brain. In contrast to zebrafish embryonic and larval *otx5* expression, no left–right asymmetric expression was detected in the epithalamic region in *Xenopus* (Fig. 30.9). *foxD3* is another marker of the pineal complex in the zebrafish embryo, but the function of this gene in pineal formation is not clear (Dadda et al. 2010; Facchin et al. 2015). Left–right asymmetric pineal formation is ensured by FGF signaling, and a loss of FGF signaling causes a decrease in the number of pineal cells (Clanton et al. 2013). In zebrafish, the left habenula is dominant, and the neuropil is denser in the left than the right habenula. Furthermore, efferent neural projections from the parapineal organ are confined to the left habenula (Concha et al. 2003).

Lipid-soluble DiI/DiO double-staining experiments and later transgenic analyses have elucidated that projections from both habenula show a characteristic "laterotopic" pattern (named by Dr. Hitoshi Okamoto's group) (Aizawa et al. 2005). The

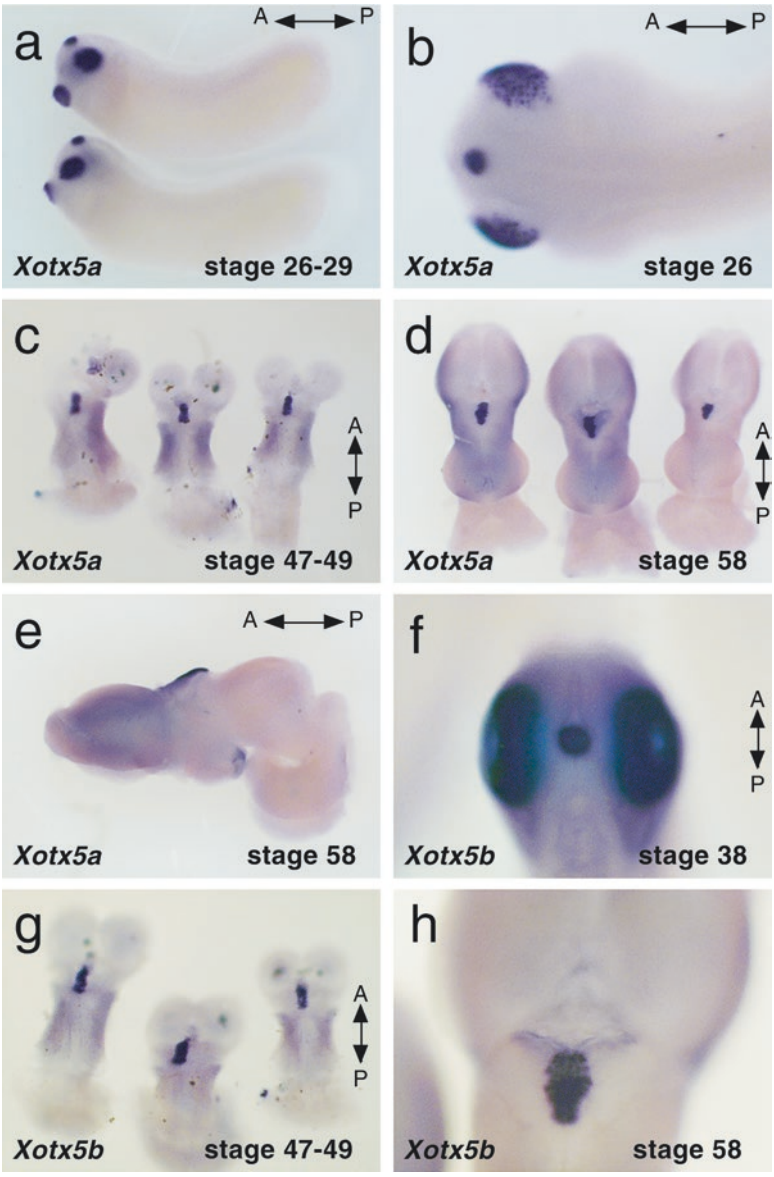

Fig. 30.9 Expression patterns of *Xotx5a* and *Xotx5b* in the brains of *Xenopus* larvae. (**a**–**e**) Expression pattern of *Xotx5a*. Expression of *Xotx5a* was clearly observed in the eyes, most frontal cement glands, and pineal glands in tailbud embryos (**a** and **b**) as well as the larval brain (**c**–**e**). Note that the *Xotx5a* expression domain is left–right symmetric in the pineal gland of the dorsal diencephalon. (**f**–**h**) Expression pattern of *Xotx5b*. Expression of *Xotx5b* is also intense in the eyes and left–right symmetric in the pineal gland of a tailbud embryo (**f**) and larvae (**g** and **h**). In all samples examined, the expression patterns of *Xotx5a* and *Xotx5b* in the brain region were thoroughly left–right symmetric. (**a**) and (**e**) are lateral views, and the others are dorsal views ($n = 49$ for *Xotx5a*, $n = 49$ for *Xotx5b*)

neural circuit connecting the habenulae and the interpeduncular nucleus (IPN) is highly conserved across vertebrate species (Concha and Wilson 2001). However, until the early twenty-first century, it was not known that projections from the left habenula innervate mainly to the dorsal IPN, while projections from the right habenula innervate mainly to the ventral IPN. This difference is regulated by Notch signaling, and the population balance of the medial nucleus and the lateral nucleus within each of the habenulae under the influence of Wnt signaling results in the "laterotopic pattern" (Aizawa et al. 2007; Hüsken et al. 2014).

Unfortunately, whether the *Xenopus* epithalamic neural circuit generates left–right asymmetry remains unknown. Kuan et al. (2007) did not detect left–right asymmetry of the habenular–IPN projections in the anuran *Rana clamitans* after DiI/DiO double-labeling injections into the habenulae. They further reported that mouse habenular–IPN projections were also left–right symmetric (*not* "laterotopic"). Moreover, no effective left–right asymmetric marker for either the habenulae or the pineal complex is known for *Xenopus*. A lack of information on left- or right-handed gene expression in neural tissue has limited molecular studies of *Xenopus* brain left–right asymmetry.

We cloned the partial sequence of the *Cynops* (Japanese newt) *nodal* gene (Toyoizumi 2005). Using our sequence information (GenBank: AB114684), Dr. Makoto Asashima's group cloned the full-length complementary DNA (cDNA) and reported the expression pattern of the *Cynops nodal* gene, which they named *Cynodal* (Ito et al. 2006). *Cynodal* is the orthologue of *Xnr-1* and mouse *nodal*. Predictably, *Cynodal* shows left-handed expression in the left LPM during the phylotypic tailbud stage (somite stage). Interestingly, *Cynodal* shows serial (at least two times) rear-to-frontal expression waves in the left LPM (Ito et al. 2006), although *Xenopus Xnr-1* shows a single rear-to-frontal expression wave along the anteroposteriorly expanded LPM. At the late tailbud stage, *Cynodal* shows left-handed dorsal expression in the diencephalon, probably in the primordia of the habenulae and/or pineal complex (Ito et al. 2006). We examined *Xnr-1* expression in the *Xenopus* larval brain, but its expression levels were equivalent in the left and right halves of the brain (Fig. 30.10).

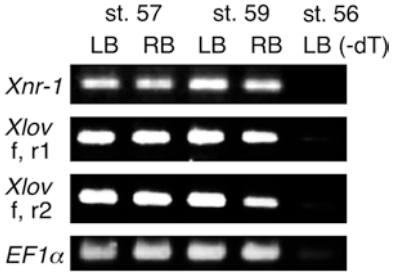

Fig. 30.10 Reverse-transcription polymerase chain reaction (RT-PCR) analysis of *Xenopus leftover* homologue (*kctd 12.1*) and *Xnr-1*. *Xenopus* larval brains were dissected and cut into halves along the midline, and RT-PCR was performed. Expression levels of both *Xenopus leftover* (*kctd 12.1*) and *Xnr-1* were equal in all larvae at stages 56–59 ($n = 4$ for each gene). Each pair of lanes corresponds to the amplified products from one individual. Ubiquitous *EF1*α was used as the loading control. *f* forward primer, *LB* left brain, *r* reverse primer, *RB* right brain

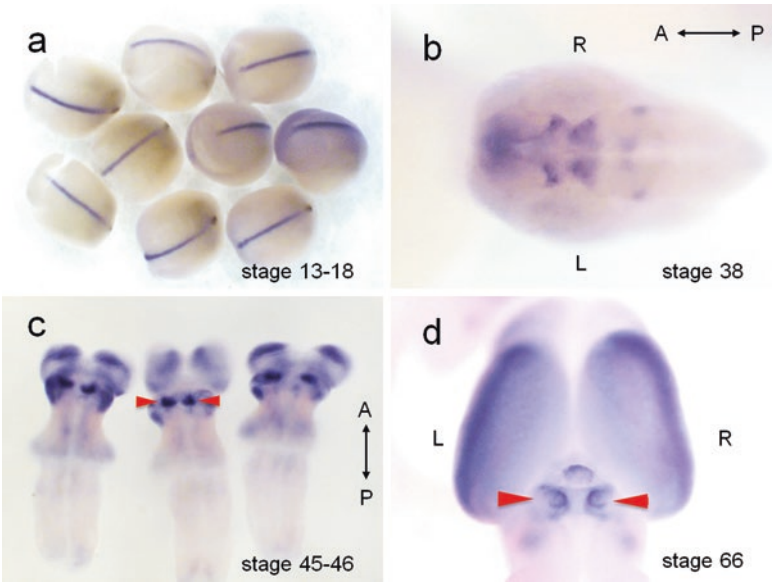

Fig. 30.11 Expression profiles of *Xenopus leftover* homologue (*kctd 12.1*) (n = 83). (**a**) At the neurula stages, expression is observed throughout the dorsal midline. (**b**) At the late tailbud stage, several brain regions are positive. (**c**) At the early larval stage, the dorsal telencephalic and diencephalic regions are bilaterally positive. Three brains were isolated and viewed from the dorsal side. (**d**) At the postmetamorphic stage, bilateral habenular expression is recognized (red arrowheads). Expression in the frontal organ (the forward extension of the amphibian epiphysis) and the dorsal pallium of the telencephalon was also recognized

The axolotl (*Ambystoma mexicanum*, Mexican salamander) has long been a powerful model organism for the study of vertebrate regeneration, and two axolotl *nodal* genes (*AxNodal-1* and *-2*) have been cloned and characterized (Swiers et al. 2010). In early gastrula embryos, both *AxNodal-1* and *-2* are expressed in the dorsal lip (organizer region), and at the later tailbud stage, only *AxNodal-1* is expressed in the left LPM. *AxNodal-2* does not show left–right asymmetric expression; instead, *AxNodal-2* shows *Xnr-4*-like dorsal axial expression at a later stage. Diencephalic *AxNodal-1* expression was not mentioned in this study.

In zebrafish embryos, the expression of *leftover* coding potassium channel tetramerization domain containing 12.1 (*kctd12.1*) is dominant in the left habenula, while *right-on* (*kctd12.2*) and *dexter* (coding *kctd8*) are dominant in the right habenula (Gamse et al. 2003, 2005). However, our RT-PCR analysis revealed that the expression levels of the *Xenopus leftover* homologue are also equivalent in the left brain and the right brain (Fig. 30.10). An *in situ* hybridization analysis did not reveal a left–right difference in habenular *leftover* expression (Fig. 30.11), although zebrafish and medaka *leftover* genes are dominant in the left habenula (Signore et al. 2009). Interestingly, Ishikawa et al. (2015) reported that the medaka parapineal organ is mainly incorporated into the left habenula, on the basis of observations of

transgenic medaka, whose pineal complexes are fluorescently visible in live organisms. Dr. Miguel L. Concha and colleagues reported the overall similarity of left-sided brain *nodal* expression in zebrafish and medaka, and heterochronic shifts in the timing of the epithalamic left–right asymmetric formation of the neural circuits, especially parapineal efferent connectivity toward the left habenula (Signore et al. 2009).

In zebrafish fry, Dr. Yoshihiro Yoshihara and colleagues revealed that the projections of olfactory neurons toward the habenulae show remarkable left–right asymmetry, and this may be functionally related to zebrafish exploratory behavior using olfaction (Miyasaka et al. 2009). Dr. Shin Tochinai and colleagues revealed the high regenerative activity of *Xenopus* olfactory neurons, olfactory bulbs and, surprisingly, the telencephalic regions (Yoshino and Tochinai 2004; Yoshino and Tochinai 2006; Endo et al. 2007). Future studies are necessary to determine whether the olfactory bulb–habenular connection is symmetric or asymmetric in *Xenopus.* In the latter case, additional studies of the plasticity and stability of the olfactory bulb–habenular connection may be useful.

30.3.3 The Spiracle Is an Eminent Left–Right Asymmetric Ectodermal Organ in Some Anurans

In higher anuran species, the spiracle is a left–right asymmetric larval organ of ectodermal origin for respiration (Fig. 30.12). Amphibian larvae are aquatic; to eliminate the fresh water within the gill chamber that is inhaled by swallowing, the spiracle is pierced through the gill chamber. The spiracle morphology differs remarkably among anurans, and these differences reflect phylogenetic relationships (Matsui 1996; McDiarmid and Altig 1999). In the anuran species of the family Ranidae, the spiracle is formed only on the left side of the posterior part of the lateral flank (Malashichev 2002). Using *Rana catesbeiana* in the family Ranidae, we investigated the position of the spiracle. All 412 larvae showed left-handed spiracle formation, without exception (Fig. 30.12). These results indicate that handed spiracle formation is precisely controlled in *R. catesbeiana. X. laevis* is a very primitive amphibian species of the family Pipidae, and *Xenopus* spiracles form bilaterally on the posterior part of the gill chamber (Fig. 30.13). Hence, when using the embryos of higher anurans, experiments are needed to examine the laterality of spiracle formation. Though no molecular analysis of left-handed spiracle formation has been performed in higher anurans, the role of the *nodal–pitx2* cascade in the control of ectodermal asymmetric morphogenesis is a fascinating issue. A linkage analysis of the laterality of the internal organs, the epithalamic organs, and the one-handed spiracle may also be useful to elucidate the grand plan and/or orchestration of left–right determination in the whole body.

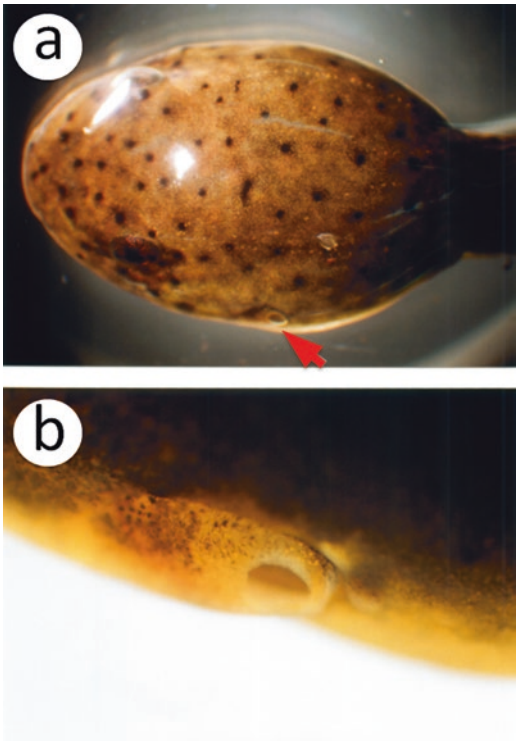

Fig. 30.12 Left-handed spiracle of a *Rana catesbeiana* larva. (**a**) One spiracle is formed solely on the left flank of the larva, and this left-handedness is highly conserved. (**b**) Magnified view of the spiracle shown in (**a**). Fresh water that is swallowed and passed through the gill chamber for respiration is exhausted asymmetrically through the spiracle

30.4 Concluding Remarks

In this chapter, we have introduced and discussed the current state of research on amphibian visceral organ and brain asymmetry, with special reference to *X. laevis* and newts. *Xenopus* brain laterality is still a mystery, and only a few pioneer studies by Dr. Michael Levin and colleagues have hinted at its existence (Pai et al. 2012; Blackiston and Levin 2013). To advance beyond morphological descriptions of amphibian brain asymmetry, it is necessary to clone *nodal* and *pitx2* in various amphibian species. In addition, left-handed and right-handed brain molecular markers are needed in amphibians. A neuroanatomic approach with labeling of the pineal and habenular projections at high resolution in various amphibian species may also be useful. A simple nursing technique for adult frogs and toads—most of which often feed only on moving prey—and methods for season-less acquisition of early embryos of higher amphibians should be developed. Newts have high regenerative activity (Okamoto et al. 2007; Berg et al. 2010), and the epithalamic region might undergo regeneration after depletion of a substantial part of the habenulae. This suggests various questions that can be examined by the dissection and regeneration of the habenular nuclei. Which half (left or right) of the habenulae is dominant? Is the speed of regeneration different between sides of the asymmetric habenulae? Are some subnuclear compartments maintained after regeneration? Such a regenerative

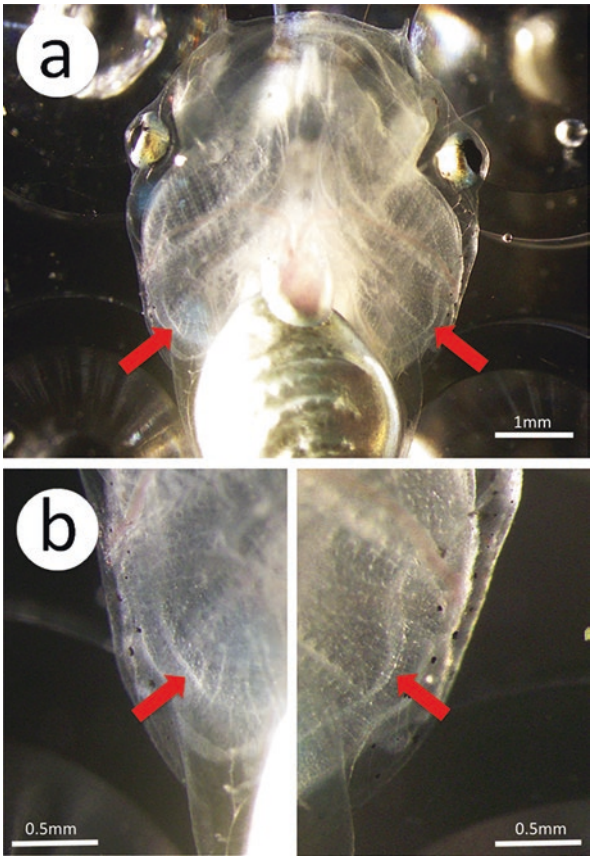

Fig. 30.13 Spiracles of a *Xenopus laevis* larva. Bilateral and left–right symmetric spiracles are formed in the case of a primitive anuran, i.e., a *Xenopus* tadpole. (**a**) Ventral view of a stage 50 larva. Red arrows depict the spiracles. (**b**) Magnified views of the spiracles of the same larva in (**a**) (Photograph courtesy of Ms. Kotomi Imura)

approach may unveil the role of habenular commissure neurons during left–right asymmetric habenular morphogenesis. Habenular left–right asymmetry is most prominent during the metamorphic stage (Kemali and Guglielmotti 1977), and seasonal differences in the extent of habenular left–right asymmetry have been reported (Kemali et al. 1990). Accordingly, the hormonal control of left–right asymmetric habenular growth is an interesting issue.

30.5 Future Prospects: Toward an Understanding of Brain Asymmetry in Humans

At first glance, the human brain appears to be morphologically bilateral. However, several brain regions show left–right asymmetry. The Sylvian fissure (lateral fissure) is longer in the left brain hemisphere than in the right, and the angles of the left and right fissures against the cerebral base differ (Toga and Thompson 2003). In

both humans and chimpanzees, the left temporal gyrus is larger than the right one. Synapse formation toward pyramidal neurons differs between the left and right dentate gyrus of the hippocampus, which controls memory and emotion (Kawakami et al. 2003). We have not yet obtained evidence that connects the *nodal–pitx2* cascade with such fine-scale left–right differential refinement of the neural circuit. Using mainly zebrafish, medaka, and flounder embryos, studies of the left–right asymmetric formation of the diencephalic region have advanced substantially over the last two decades. These findings suggest that the epithalamic region, including the habenular nuclei, is the center of left–right differential brain organization.

Roger W. Sperry and Michael S. Gazzaniga examined patients who underwent a split-brain procedure, and found that after surgical cutting of the corpus callosum, the human left and right hemispheres had different preferences and cognition (Zaidel et al. 1981; Benowitz et al. 1983; Kosslyn et al. 1985; Turk et al. 2002; Funnell et al. 2007). It is now well known that higher brain function differs significantly between the two brain hemispheres. However, there is a large gap in knowledge between left–right differential brain formation from a developmental perspective and cognitive psychology of the brain hemispheres. It may be possible to shorten the distance between the two research fields, using the amphibian brain as a model. Such an approach will connect our knowledge of cognitive science and developmental biology to clarify how our brain evolved left–right differences. Recent advances in molecular embryology have indicated that our brain is a left–right differently developed and, at the same time, unified nervous system.

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Chapter 31
The Molecular Basis of the Gastrula Organizer in Amphibians and Cnidarians

Yuuri Yasuoka and Masanori Taira

Abstract The gastrula organizer, an embryonic tissue, has a central role in early development of all eumetazoans, from cnidarians to vertebrates. In amphibians, the organizer—also known as the Spemann–Mangold organizer—is located in the dorsal blastopore lip of the gastrula embryo. This organizer is capable of inducing a secondary body axis when transplanted into the ventral region of a blastula embryo. In sea anemone embryos, the organizer has recently been recognized in the blastoporal lip, implying its ancient origin among eumetazoans. Here we review the molecular basis of vertebrate and cnidarian organizers, which have been widely studied using *Xenopus*, zebrafish, and mice for more than 20 years, and have relatively recently been studied using *Nematostella*. Recent genome-wide investigations have provided a comprehensive overview of transcription factor (TF) binding sites and regulatory principles of the gene regulatory network (GRN) in the *Xenopus* organizer. These analyses provide a platform for genome-wide evolutionary study of organizer-equivalent tissues in other organisms, including cnidarians. Here, we discuss an evolutionary prospective for the organizer, focusing on (1) the formation of the organizer by combinatorial signaling pathways such as Wnt, Nodal, and Bmp; and (2) the GRN regulating organizer formation and activity by TFs such as Lim1/Lhx1, Otx, Goosecoid, Brachyury, and FoxA.

Keywords Evolution · Development · Genome · Spemann-Mangold organizer · Gene regulation · Transcription factor · Gene regulatory network · *Xenopus* · *Nematostella*

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Abbreviations

AZ	1-azakenpaullone
BCNE	blastula *chordin*- and *noggin*-expressing
ChIP-seq	chromatin immunoprecipitation sequencing
CRM	*cis*-regulatory module
DE	distal element
GRN	gene regulatory network
PCR	polymerase chain reaction
PE	proximal element
qPCR	quantitative polymerase chain reaction
RNAPII	RNA polymerase II
RNA-seq	RNA sequencing
RPKM	reads per kilobase of exon per million mapped reads
TF	transcription factor
ZGA	zygotic gene activation

31.1 Introduction

In 1924, Spemann and Mangold discovered the gastrula organizer during transplantation experiments using the dorsal blastopore lip of newt embryos ((Spemann and Mangold (1924); republished in 2001 (Spemann and Mangold 2001)). Since then, extensive classical experimental embryology studies have revealed (1) neural and muscle induction by the organizer; (2) functional division of the organizer into head and trunk organizers; (3) posteriorization of the embryo by the trunk organizer; (4) mesoderm induction by the presumptive endoderm; (v) localization of the dorsal determinant at the vegetal pole; and (6) formation of the organizer (the dorsal mesoderm) by both the mesoderm inducer and the dorsal determinant (reviewed by De Robertis et al. 2000; Harland 2000; Kimelman and Griffin 2000; De Robertis and Kuroda 2004; Niehrs 2004; Heasman 2006). Nearly seven decades after discovery of the organizer, molecular studies of the organizer were initiated using the African clawed frog, *Xenopus laevis*; subsequently these were extended to other model organisms, such as zebrafish and mice. Consequently, various organizer-specific transcription factors (TFs) and secreted molecules for neural and mesoderm induction have been identified, which include Wnt, Bmp, Nodal, and FGF signaling components.

In the 1990s, considerable effort was expended to identify organizer genes and to determine their functions. After 2000, individual gene analyses were supplanted by comprehensive analyses intended to understand interactions at the level of gene regulatory networks (GRNs), involving individual TFs and signaling pathways. In about 2010, a dramatic turning point occurred with the development of next-generation sequencing technologies and new genomic methods, such as chromatin

immunoprecipitation (ChIP) sequencing (ChIP-seq) and RNA sequencing (RNA-seq). These advances allowed the application of genome-wide approaches to studies of embryonic development. These new techniques were initially applied to cultured cells (homogenous materials) (Barski et al. 2007; Johnson et al. 2007; Robertson et al. 2007; Mikkelsen et al. 2007) and subsequently adapted for embryos of mice (Visel et al. 2009; Blow et al. 2010; McKenna et al. 2011; Donaldson et al. 2012), zebrafish (Aday et al. 2011; Bogdanovic et al. 2012), and *Drosophila* (Harrison et al. 2011; Ozdemir et al. 2011; Bonn et al. 2012), as well as the two organisms that are subject of this chapter: the western clawed frog, *Xenopus tropicalis* (Akkers et al. 2009; Gentsch et al. 2013; Chiu et al. 2014; Gupta et al. 2014; van Heeringen et al. 2014; Yasuoka et al. 2014; Hontelez et al. 2015; Wills and Baker 2015; Nakamura et al. 2016; Charney et al. 2017a); and the starlet sea anemone, *Nematostella vectensis* (Schwaiger et al. 2014).

Because of its key phylogenetic position as a sister group to bilaterians, the phylum Cnidaria is important for understanding of the evolution of developmental mechanisms of animal body plans (Ball et al. 2004; Technau and Steele 2011). Since the first gene expression study in embryos of *N. vectensis* in 2003 (Scholz and Technau 2003), extensive developmental expression studies and functional analyses have been performed. During the initial period of about 5 years after the first report, most studies concentrated on describing expression patterns of developmental regulatory genes that had been identified in bilaterians. Then researchers began to analyze more detailed functions of genes and signaling pathways. As in *Xenopus*, *Nematostella* studies then shifted to more comprehensive genomic analyses using the whole genome sequence (Putnam et al. 2007).

This chapter first provides an overview of the molecular basis of the gastrula organizer. Then it describes recent progress in genome-wide analyses of organizer-specific TFs. Finally it considers the evolutionary origin of the organizer on the basis of recent studies using cnidarian embryos.

31.2 Overview of Molecular Mechanisms of Organizer Formation and Function

The organizer is formed on the dorsal side of the blastula embryo as a result of combined Wnt/β-catenin and Nodal signaling (De Robertis et al. 2000). Sudou et al. (2012) reported that at the blastula stage, Wnt/β-catenin- and Nodal-responsive organizer genes are separately expressed in the dorsal–animal region (corresponding to the blastula *chordin*- and *noggin*-expressing (BCNE) region; see Sect. 31.4.4 and Fig. 31.3) and the dorsal–vegetal region, respectively. However, by the gastrula stage these two expression domains overlap in the dorsal marginal zone to form the organizer. The organizer secretes Bmp signaling inhibitors such as Chordin (Chrd), Noggin, and Follistatin to form and maintain the dorsoventral axis (Smith and Harland 1992; Hemmati-Brivanlou et al. 1994; Sasai et al. 1994). During

gastrulation, the anterior and posterior parts of the organizer segregate to become the head and trunk organizers (Niehrs 2004; Heasman 2006). In the head organizer, Wnt/β-catenin and Nodal signaling pathways are also inhibited by secreted signaling antagonists such as Cerberus (Cer), Dkk1, Frzb, and Crescent (Sasai et al. 1994; Bouwmeester et al. 1996; Glinka et al. 1998; Leyns et al. 1997; Wang et al. 1997; Pera and De Robertis 2000; Shibata et al. 2000). Expression of these genes is regulated by the head organizer–specific TFs Otx2 and Goosecoid (Gsc), and the organizer-specific TF Lim1/Lhx1 (Yasuoka et al. 2014). In contrast, the trunk organizer secretes posteriorizing factors, such as Wnt, FGF, Nodal, and retinoic acid (Niehrs 2004; Heasman 2006). Thus, head and trunk organizer activities function in opposition to regulate anteroposterior patterning in the embryo.

Reporter gene analysis is generally performed to validate direct relationships between TFs and their target genes in *Xenopus* (summarized in Koide et al. 2005). In addition, genome-wide studies in *X. tropicalis*, using ChIP-seq and RNA-seq, have provided a global overview of occupancies of TFs on *cis*-regulatory modules (CRMs) near their target genes and their effects on gene expression. In Sect. 31.3, we summarize genome-wide studies using *X. tropicalis* embryos, revealing the epigenetic basis of early development, mesoderm formation by Nodal signaling, and the organizer GRN.

31.3 Genome-Wide Studies Using *Xenopus* Gastrula Embryos

To investigate genome-wide functions of TFs in early embryos, the *X. tropicalis* system is advantageous. First, thousands of synchronously developing embryos can be obtained from one female, using in vitro fertilization. This is very important for ChIP-seq analysis of TFs because approximately 10^6 cells are required for one ChIP-seq assay, which is technically difficult using mouse or chick embryos. Second, *X. tropicalis* has a diploid genome (Hellsten et al. 2010), unlike the allotetraploid frog *X. laevis* (Session et al. 2016), and this makes bioinformatic analysis more straightforward. Additionally, *X. tropicalis* genome organization is more similar to that of many mammals than to that of teleost fish such as zebrafish and medaka, which underwent an extra round of whole-genome duplication ~310 million years ago, followed by genome rearrangement and subfunctionalization of duplicated genes (Inoue et al. 2015; Jaillon et al. 2004; Howe et al. 2013). Third, embryonic manipulations such as microinjection and tissue fragment dissection are easily performed. For this purpose, *X. laevis* embryos, which are larger in size and are able to develop in a wider temperature range than *X. tropicalis* embryos, may be more convenient. Taking advantage of the *Xenopus* system, many molecular components of GRNs undergoing gastrulation have been determined (see Table 31.1). However, determination of GRNs on the basis of ChIP-seq and RNA-seq studies using whole embryos requires care, because developing embryos comprise heterogeneous populations of cells (see Fig. 31.1a).

Table 31.1 Genome-wide studies using *Xenopus tropicalis* embryos

Type of analysis		Target	Stage (Nieuwkoop and Faber stages[a])	References
ChIP-seq	Histone modifications	H3K4me3	Blastula (8, 9), gastrula (10.5, 11, 12), α-amanitin-treated gastrula (11), neurula (16), tailbud (30)	Akkers et al. (2009), Gupta et al. (2014), van Heeringen et al. (2014) and Hontelez et al. (2015)
		H3K27me3	Blastula (8, 9), gastrula (10.5, 11, 12), α-amanitin-treated gastrula (11), neurula (16), tailbud (30)	Akkers et al. (2009), Gupta et al. (2014), van Heeringen et al. (2014) and Hontelez et al. (2015)
		H3K4me1	Blastula (8, 9), SB431542-treated blastula (9), gastrula (10.5, 12), neurula (16), tailbud (30)	Gupta et al. (2014), van Heeringen et al. (2014), Yasuoka et al. (2014) and Hontelez et al. (2015)
		H3K27ac	Blastula (8, 9), SB431542-treated blastula (9), gastrula (10.5)	Gupta et al. (2014), Yasuoka et al. (2014) and Hontelez et al. (2015)
		H3K9ac	Blastula (8, 9), gastrula (10.5, 12), neurula (16), tailbud (30)	Hontelez et al. (2015)
		H3K9me2	Blastula (9), gastrula (10.5, 12), neurula (16), tailbud (30)	Hontelez et al. (2015)
		H3K9me3	Blastula (9), gastrula (10.5, 12), neurula (16), tailbud (30)	Hontelez et al. (2015)
		H3K36me3	Blastula (9), gastrula (10.5, 12), neurula (16), tailbud (30)	Hontelez et al. (2015)
		H4K20me3	Blastula (9), gastrula (10.5, 12), neurula (16), tailbud (30)	Hontelez et al. (2015)
	General transcriptional regulators	RNAPII	Blastula (7, 8, 9), gastrula (10.5, 11, 12), α-amanitin-treated gastrula (11), neurula (16), tailbud (30)	Akkers et al. (2009), van Heeringen et al. (2014), Hontelez et al. (2015) and Charney et al. (2017a)
		Ezh2	Blastula (9)	van Heeringen et al. (2014)
		Jarid2	Blastula (9)	van Heeringen et al. (2014)
		p300	Blastula (9), gastrula (10.5, 11, 12), α-amanitin-treated gastrula (11), neurula (16), tailbud (30)	Yasuoka et al. (2014) and Hontelez et al. (2015)

(continued)

Table 31.1 (continued)

Type of analysis		Target	Stage (Nieuwkoop and Faber stages[a])	References
		TLE	Blastula (8), gastrula (10.5)	Yasuoka et al. (2014) and Charney et al. (2017a)
	TFs for Nodal signaling	Smad2/3	Blastula (8, 9), gastrula (10.5), E2a-depleted gastrula (10.5)	Chiu et al. (2014) andGupta et al. (2014)
		FoxH1	Blastula (8, 9), gastrula (10.5)	Chiu et al. (2014) and Charney et al. (2017a)
	TFs for Wnt signaling	β-catenin	Gastrula (10.25)	Nakamura et al. (2016)
	Tissue-specific TFs	Bra	Gastrula (11–12.5), neurula (19–20)	Gentsch et al. (2013)
		Eomesodermin	Gastrula (11–12.5)	Gentsch et al. (2013)
		VegT	Gastrula (11–12.5)	Gentsch et al. (2013)
		Otx2	Gastrula (10.5)	Yasuoka et al. (2014)
		Lim1	Gastrula (10.5)	Yasuoka et al. (2014)
		Gsc	Gastrula (10.5)	Yasuoka et al. (2014)
		FoxA	Gastrula (10.5)	Charney et al. (2017a)
RNA-seq	Whole embryos	Time course	Every 30 min after fertilization (egg ~27), every 1 h after 24 hpf (28–42)	Owens et al. (2016)
		Time course	Every 30 min after fertilization (egg ~12)	Collart et al. (2014)
		Stages	Cleavage (2–6), blastula (8, 9), gastrula (10, 11–12), neurula (13–14, 15, 16–18, 19, 20–21), tailbud (22–23, 24–26, 28, 31–32, 33–34, 38–39), tadpole (40, 41–42, 44–45)	Tan et al. (2013)
	Dissected tissues	Head organizer region	Gastrula (10.5)	Yasuoka et al. (2014)
		Equatorial region	Gastrula (10.5)	Yasuoka et al. (2014)
		Dorsal region	Gastrula (10.5)	Yasuoka et al. (2014)
		Ventral region	Gastrula (10.5)	Yasuoka et al. (2014)

		Animal cap	Gastrula (10–10.25)	Blitz et al. (2017)
		Ventral marginal zone	Gastrula (10–10.25)	Blitz et al. (2017)
		Lateral marginal zone	Gastrula (10–10.25)	Blitz et al. (2017)
		Dorsal marginal zone	Gastrula (10–10.25)	Blitz et al. (2017)
		Vegetal mass	Gastrula (10–10.25)	Blitz et al. (2017)
	Gene/signaling-depleted embryos	*Xbra/Xbra3* morphants	Tailbud (32)	Gentsch et al. (2013)
		SB431542-treated embryos	Gastrula (10.5)	Chiu et al. (2014)
		foxh1 morphants	Gastrula (10.5)	Chiu et al. (2014)
		otx2/otx5/lim1 morphants	Gastrula (11.5)	Yasuoka et al. (2014)
		gsc morphants	Gastrula (11.5)	Yasuoka et al. (2014)
		E2a morphants	Gastrula (10.5)	Wills and Baker (2015)
		α-Amanitin-treated embryos	Gastrula (11)	Hontelez et al. (2015)
		wnt8a morphants	Gastrula (10.25)	Nakamura et al. (2016)
		wnt8a morphants + CSKAwnt8a	Gastrula (10.25)	Nakamura et al. (2016)
MethylCap-seq		DNA methylation	Blastula (9), gastrula (10.5, 12.5)	Bogdanovic et al. (2011) and Hontelez et al. (2015)

ChIP-seq chromatin immunoprecipitation sequencing, *hpf* hours postfertilization, *MethylCap-seq* MethylCap sequencing, *RNAPII* RNA polymerase II, *RNA-seq* RNA sequencing, *TF* transcription factor

[a]Stages according to Nieuwkoop and Faber (1967)

31.3.1 Gene Expression Catalogs

Transcriptomic data from normal embryonic development serve as a useful platform to understand gene regulation in the embryo. To date, several groups have reported such data from *X. tropicalis* at different developmental stages (Tan et al. 2013) or at different times after fertilization (Collart et al. 2014; Owens et al. 2016). Including a mixture of synthetic ERCC RNA Spike-In (Baker et al. 2005) RNAs of various lengths, as external controls, Owens et al. (2016) calculated the absolute numbers of individual transcripts in developing embryos and produced the most comprehensive, high-temporal-resolution data covering the first 66 h of early *X. tropicalis* development. Careful transcriptome kinetic analysis also uncovered synexpression group genes that share similar expression domains and cellular/developmental functions.

Transcriptomic data from dissected embryonic tissue fragments are important resources to identify spatially regulated gene expression patterns. In 2014, we first reported transcriptomic data from dissected tissues of *X. tropicalis* gastrulae (Yasuoka et al. 2014). More recently, another group also reported an RNA-seq analysis of dissected gastrula tissues (Blitz et al. 2017). These data allow us to more easily define spatial gene expression patterns of individual genes in *Xenopus* gastrula embryos.

31.3.2 Epigenetic Marks

The first *X. tropicalis* ChIP-seq data were reported on H3K4me3 and H3K27me3 histone marks and RNA polymerase II (RNAPII) binding in gastrula-stage embryos (Akkers et al. 2009). In general, H3K4me3 modification is associated with transcriptionally active promoters, while H3K27me3 modification is associated with repression of nearby genes or CRMs. In human and mouse ES cells, however, promoters of developmental regulatory genes are often marked with both H3K4me3 and H3K27me3, a so-called bivalent state (Azuara et al. 2006; Bernstein et al. 2006; Mikkelsen et al. 2007), but this was not the case in early *Xenopus* embryos assayed by sequential ChIP experiments for reported bivalent state genes in ES cells (Akkers et al. 2009). In addition, by analyzing H3K27me3 ChIP-seq using animal and vegetal halves of gastrula embryos, it was shown that developmental regulatory genes are enriched with H3K27me3 in animal or vegetal halves where genes are not expressed, suggesting that H3K27me3 is deposited near genes for localized expression in *Xenopus* (Akkers et al. 2009) (see Fig. 31.1a).

Dynamics of epigenetic states after the blastula stage onward have also been reported (van Heeringen et al. 2014; Hontelez et al. 2015). During the blastula stage, enrichment levels of H3K27me3 are very low throughout the embryo generally, but they greatly increase by the gastrula stage. At the blastula to gastrula stages, H3K27me3 enrichment is of two types: narrow domains (~10 kb, centered on the

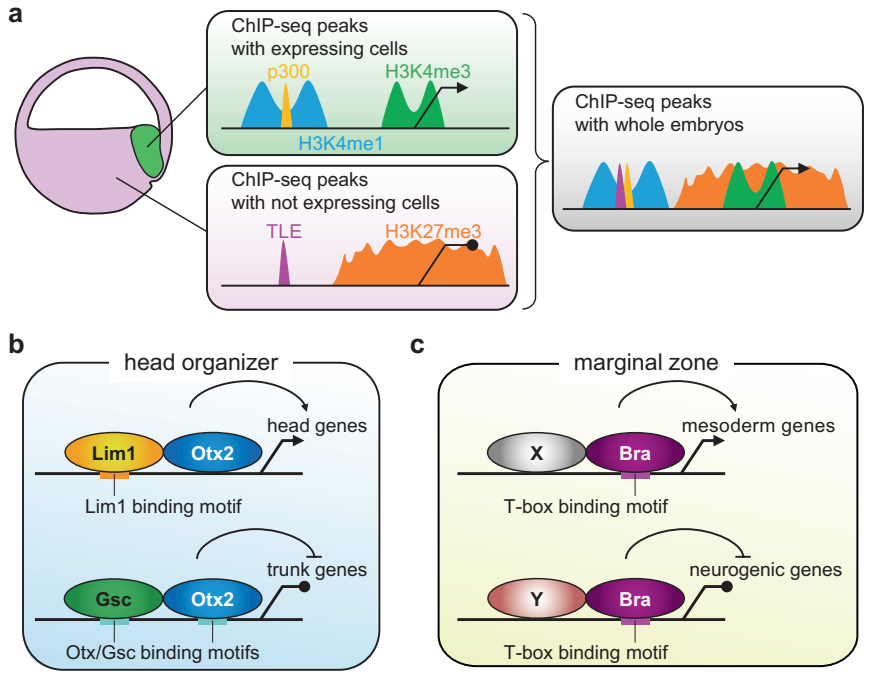

Fig. 31.1 Genome-wide studies using *Xenopus* gastrula embryos reveal regulatory principles of gene expression during development. (**a**) Current understanding of chromatin immunoprecipitation sequencing (ChIP-seq) results using whole embryos. To activate a gene in a specific area of an embryo, p300 binds to *cis*-regulatory modules (CRMs), enhancer histone marks are enriched around CRMs, and promoter histone marks are enriched around the promoter. Meanwhile, in non-expressing cells, TLE binds to CRMs and repressive histone marks are enriched around the promoter and gene body to repress the gene. Therefore, when we performed ChIP-seq assays using whole *Xenopus* embryos, p300, TLE, and enhancer histone marks were coenriched in many tissue-specific CRMs and promoter histone marks and repressive histone marks were coenriched around many gene promoters (Akkers et al. 2009; van Heeringen et al. 2014; Yasuoka et al. 2014). (**b**) A regulatory principle in the head organizer. Otx2 activates head genes with Lim1 via CRMs with Lim1-binding motif(s), whereas Otx2 represses trunk genes with Gsc via CRMs that possess Otx/Gsc-binding motifs (Yasuoka et al. 2014). **c** A regulatory principle in the marginal zone. Bra activates mesodermal genes but represses neurogenic genes. In both cases, Bra may bind to T-box-binding motifs in CRMs, but partner transcription factors (TFs) in each case have not yet been identified (Gentsch et al. 2013)

gene promoter) and broad domains (~100 kb, covering one or more genes) (van Heeringen et al. 2014). In many cases, a broad H3K27me3 domain appears to form by spreading from a local H3K27me3 site initiated during the blastula stage. Notably, this H3K27me3 nucleation correlates with pre-existing DNA methylation–free regions (Bogdanovic et al. 2011). This correlation between broad H3K27me3 domains and DNA methylation–free regions is also found in human ES cells, zebrafish embryos (van Heeringen et al. 2014), and medaka embryos (Nakamura et al.

2014). Curiously, these H3K27me3 enrichment regions are not associated with the polycomb group complex PRC2, which catalyzes trimethylation of H3K27, as assayed by PRC2 components Ezh2 and Jarid2 ChIP-seq (van Heeringen et al. 2014). Instead, PRC2 binds widely to many enhancers marked with H3K4me1 (van Heeringen et al. 2014). Because colocalization of PRC2 and H3K4me1 in the same cell has never been shown by sequential ChIP experiments, this possibility seems unlikely. Our interpretation of the data is that PRC2 and H3K4me1 separately mark CRMs in different cells, leading to the prediction of tissue-specific gene expression (Fig. 31.1a).

The question of whether epigenetic marks (H3K4me3, H3K27me3, and so on) and occupancy of transcription-related proteins (p300 and RNAPII) for embryonic transcription are controlled maternally or zygotically was examined using an RNAPII elongation inhibitor, α-amanitin, which blocks zygotic transcription (Hontelez et al. 2015, see Table 31.1). This experiment revealed that regulatory regions epigenetically marked by maternal factors contain hypomethylated promoters, whereas regulatory regions epigenetically marked by zygotic factors contain methylated promoters and distal enhancers. Most p300-bound enhancers are zygotically controlled, but the enhancers can regulate promoters in either maternally or zygotically marked (defined) regulatory regions (Hontelez et al. 2015). Maternally controlled regulatory regions are maintained well into the neurula and tailbud stages and contribute to activation of many embryonically expressed genes. Zygotically controlled regulatory regions are established from the midblastula stage onward and gradually increase in their relative contribution to gene activation later in development. Notably, maternal and zygotic regulatory regions correspond well to early (dorsalization) and late (posteriorization) Wnt/β-catenin target genes, respectively, during development (Hontelez et al. 2015). This finding is consistent with that of a previous report, in which ChIP–polymerase chain reaction (ChIP–PCR) analysis using *X. laevis* embryos suggested that β-catenin binds to promoters of early, but not late, targets before zygotic gene activation (ZGA) (Blythe et al. 2010).

Seeming colocalization of both repressive and active enhancer marks, like those of PRC2 and H3K4me1, was also observed at TLE corepressor binding sites in *Xenopus* gastrula embryos (Yasuoka et al. 2014). A significant number of TLE ChIP-seq peaks overlap with those of p300 and enhancer histone marks (H3K4me1 and H3K27ac). ChIP–quantitative PCR (ChIP–qPCR) assays using dorsoventrally bisected embryonic fragments further suggest that both p300 and TLE recognize the same CRM, but which protein binds to the CRM is dependent on specific tissue types (Fig. 31.1a). Because p300 binds to enhancers of both tissue-specific and ubiquitous genes, p300 binding alone is not a good predictor of tissue-specific enhancers. On the other hand, TLE binding seems to selectively mark tissue-specific CRMs (Yasuoka et al. 2014). To determine the temporal order of events, we examined occupancy of p300 and TLE on CRMs using early to mid- (stage 8) and late (stage 9) blastula–stage embryos, which are before and after ZGA, respectively (Yasuoka, Taira et al. unpublished data). We found that TLE, but not p300, occupies CRMs before ZGA. This result is consistent with the observation that significant p300 binding to enhancers occurs after ZGA (Hontelez et al. 2015; Charney et al. 2017a).

In summary, we postulate that spatially and temporally localized gene expression is accomplished perhaps because CRMs of these genes are actively repressed by TLE or PRC2 in the region where gene expression is not required (Fig. 31.1a). In the future, it will be important to determine whether this repressive action mediated by TLE and PRC2 is a general principle governing the spatial activity of CRMs during development.

31.3.3 ChIP-seq Analyses for Transcription Factors Involved in Mesoderm Induction by Nodal Signaling

Nodal is necessary for mesendoderm formation in vertebrates and activates one of the major signaling pathways required for organizer formation (De Robertis et al. 2000; Kimelman and Griffin 2000; Niehrs 2004; Heasman 2006). Nodal signaling is transduced by phosphorylation of Smad2/3 TFs. Phospho-Smad2/3 directly upregulates expression of a set of target genes in concert with partner TFs such as forkhead box factor FoxH1 and bHLH factor Wbscr11, both of which bind to a CRM to activate the *gsc* gene in the organizer (Watabe et al. 1995; Ring et al. 2002). Utilizing ChIP-seq data, binding properties of Smad2/3 and FoxH1, and their partner proteins, were further analyzed, as described below.

ChIP-seq analyses for Smad2/3 using *X. tropicalis* embryos provided an overview of Nodal signaling activity during mesendoderm formation (Chiu et al. 2014; Gupta et al. 2014; Wills and Baker 2015). Comparisons of Smad2/3 binding and histone marks during the blastula and gastrula stages suggest that the enhancer histone marks H3K4me1 and H3K27ac are detected by the late blastula stage, which coincides with the onset of Nodal signaling (Gupta et al. 2014). Interestingly, these enhancer histone marks at Nodal-responsive CRMs are mostly unaffected by Nodal signaling inhibition (Gupta et al. 2014), indicating that enhancer marking is regulated by a Nodal signaling–independent mechanism. These enhancers may already be poised before the onset of Nodal signaling.

FoxH1 ChIP-seq using *Xenopus* gastrula embryos revealed that PouV TFs function as cobinding partners for FoxH1 to modulate Nodal signaling (Chiu et al. 2014). Comparison of RNA-seq data between FoxH1 morphants (embryos injected with FoxH1 antisense morpholino) and embryos treated with SB431542, a chemical inhibitor of Nodal receptors, revealed a partial overlap between FoxH1 and Nodal target genes. This finding implies that FoxH1 has a Nodal signaling–independent role, and that FoxH1 is not the sole TF mediating Nodal signaling. A more recent FoxH1 ChIP-seq study using early and late blastula–stage embryos (Charney et al. 2017a) demonstrated that FoxH1 binds to Nodal-response elements in the absence of Nodal signaling during cleavage stages. In early blastulae, FoxH1 recruits TLE to CRMs, which become marked by H3K4me1 and p300 by the gastrula stage, suggesting that FoxH1 together with TLE has a role in silencing genes until ZGA. However, by the gastrula stage, a selected group of FoxH1-bound CRMs are

free of TLE, cobind with Smad2/3, and recruit FoxA. This interaction between FoxH1 and FoxA, also known as the molecular "hand-off" model, may be important to maintain the enhancer activity during mesendodermal specification (Charney et al. 2017a).

Smad2/3 ChIP-seq revealed that a bHLH TF, E2a, functions as a Smad2/3 cofactor in human ES cells (Yoon et al. 2011). In *Xenopus* embryos, E2a and Smad2/3 ChIP-seq analyses, together with RNA-seq of E2a-depleted embryos, demonstrated that co-occupancy of E2a and Smad2/3 is involved in gene activation by Nodal signaling, whereas E2a binding alone without Smad2/3 is linked to repression of the Nodal inhibitor gene *lefty*, another Nodal target (Wills and Baker 2015). This finding hints at the role of E2a in Nodal target gene regulation.

31.3.4 Organizer Gene Regulatory Network

The organizer is formed under the influence of both Wnt/β-catenin and Nodal signaling (De Robertis et al. 2000). In *Xenopus*, *siamois* (*sia1*), *twin* (*sia2*), and *Xnr3* (*nodal3*) were characterized as direct targets of Wnt/β-catenin signaling (Laurent et al. 1997; McKendry et al. 1997; Fan et al. 1998). Activation of these genes depends on promoter poising by recruitment of a histone H3-arginine-8-methyltransferase, Prmt2 (Blythe et al. 2010). Unfortunately, in comparison with Nodal signaling, comprehensive analysis of target genes downstream from Wnt/β-catenin signaling has not been as well studied. Recently, ChIP-seq analysis for β-catenin using *X. tropicalis* gastrulae was reported (Nakamura et al. 2016), but it remains to be seen how many organizer genes are directly regulated by maternal Wnt/β-catenin signaling and how Wnt/β-catenin-induced organizer genes contribute to the formation of the organizer. It will be important to perform temporal analyses and examine transcriptomes of β-catenin-depleted embryos and ChIP-seq for TCF and β-catenin together with epigenetic marks. This will reveal the fundamental mechanism that controls switching from early (maternal) to late (zygotic) Wnt/β-catenin signaling.

Organizer-forming signaling pathways mediated by Wnt and Nodal synergistically impinge upon CRMs present on selected organizer genes. For example, *gsc* is upregulated by both Nodal signaling (directly with FoxH1 and Wbscr11) and Wnt/β-catenin signaling (via Siamois and Twin) through an upstream CRM that includes the distal and proximal elements (DE and PE) (Watabe et al. 1995; Laurent et al. 1997; Ring et al. 2002; Koide et al. 2005). Subsequently, Lim1/Lhx1 (organizer gene), Otx2 (head organizer gene), and Mix1 (endomesodermal gene)—all of which are downstream targets of Nodal signaling—and VegT (vegetally localized maternal gene) coordinate *gsc* expression using the same CRM (Mochizuki et al. 2000; Sudou et al. 2012).

Expression of the Bmp/Nodal/Wnt inhibitor *cer* is directly induced by Siamois, Lim1, Otx2, Mix1, and VegT, indicating that CRMs of *cer* integrate information

necessary for dorsalization by Wnt (Siamois), mesendoderm formation by Nodal (Lim1, Otx2, and Mix1), and animal–vegetal polarity by a maternal factor (VegT) (Yamamoto et al. 2003; Sudou et al. 2012). Furthermore, ChIP-qPCR analysis using blastula, gastrula, and neurula embryos showed that the binding combination of Siamois, Lim1, Otx2, Mix1, and VegT TFs to CRMs of *gsc* and *cer* coordinates the expression dynamics of *gsc* and *cer* (Sudou et al. 2012). Among the five TFs, only Lim1 and Otx2 bind to the *gsc* CRM continuously from the gastrula to neurula stages (see Fig. 31.3a). In contrast, *cer* expression disappears during the neurula stage and none of five TFs bind to the *cer* CRM at the neurula stages. These ChIP-qPCR analyses of in vivo TF binding to specific CRMs prompted us to carry out genome-wide analyses of critical TFs by ChIP-seq.

Using *X. tropicalis* gastrula embryos, ChIP-seq analyses were performed to reveal genome-wide occupancies of the organizer-specific TFs Gsc, Lim1, and Otx2 (Yasuoka et al. 2014). RNA-seq assays were also performed using morphants for these TFs and dissected embryonic tissue fragments to gain comprehensive expression profiles of the head organizer region, the marginal zone (the trunk and posterior region), the dorsal region, and the ventral region of gastrula embryos. ChIP-seq data showed thousands of overlapping peaks among the three TFs and coregulators (p300 and TLE), leading to the identification of potential CRMs and nearby target gene candidates. RNA-seq data from *gsc*, *lim1*, and *otx2* morphants were used to assess whether these TF target gene candidates were up- or downregulated, while RNA-seq data with dissected tissues were used to assess whether these TFs and target gene candidates are coexpressed. We identified 87 head organizer genes by RNA-seq, and the list included 30 well-known head organizer genes, most of which are top ranked on the basis of their expression levels (Table 31.2). In other words, the head organizer genes were fairly thoroughly identified by these methods. After our identification, two studies analyzed the uncharacterized genes *pkdcc2* and *cnrip1*, listed among the 87 head organizer genes (Table 31.2): *pkdcc2* is expressed in the head organizer and functions in gastrulation movement (Vitorino et al. 2015); and *cnrip1* is involved in eye and neural development (Zheng et al. 2015). Analysis of the remaining uncharacterized head organizer genes is awaited to gain a whole perspective on the head organizer function (see Table 31.2).

Bioinformatic analyses showed that Otx2 and Lim1 co-occupy a specific type of CRM (type I), whereas Otx2 and Gsc co-occupy another type (type II). Type I CRMs have Lim1-binding consensus motifs and reporter genes harboring the CRM that are positively regulated upon Otx2 and Lim1 binding. Type II CRMs have Otx2- and Gsc-binding consensus motifs and reporter genes harboring the CRMs that are negatively regulated by Otx2 and Gsc binding. These data suggest that the interaction of Otx2, Lim1, and Gsc is central to establishing the proper head organizer GRN. We propose that Otx2 functions as a "molecular landmark" of the head organizer to initiate head formation (Fig. 31.1b). ChIP-seq analyses for Siamois, Mix1, and VegT have shown an additional layer of complexity in coregulating the CRMs (Kirigaya, Yasuoka, Taira et al. unpublished data; Honda, Yasuoka, Taira et al. unpublished data).

Table 31.2 Head organizer genes thoroughly identified by RNA sequencing (RNA-seq) with dissected tissue fragments of *Xenopus tropicalis* gastrula embryos

Gene name	Expression levels in the head organizer (RPKM)	Expression ratio (head organizer/ remaining regions)	References for expression in the head organizer
frzb	1120.69	14.2	Leyns et al. (1997) and Wang et al. (1997)
goosecoid	922.38	14.4	Blumberg et al. (1991) and Cho et al. (1991)
otx2	674.21	10.5	Blitz and Cho (1995) and Pannese et al. (1995)
cerberus	545.01	28.1	Bouwmeester et al. (1996)
hhex	388.71	15.9	Newman et al. (1997), Jones et al. (1999), Schneider and Mercola (1999) and Zorn et al. (1999)
dkk1	362.75	44.7	Glinka et al. (1998)
otx5/crx	343.24	5.2	Kuroda et al. (2000) and Vignali et al. (2000)
admp	327.87	16.1	Moos et al. (1995)
frizzled8	304.19	15.6	Deardorff et al. (1998) and Itoh et al. (1998)
crescent/frzb2	281.43	80.9	Pera and De Robertis (2000) and Shibata et al. (2000)
gata4	256.13	6.4	Afouda et al. (2005) and Fletcher et al. (2006)
gata6	251.75	5.6	Afouda et al. (2005) and Fletcher et al. (2006)
chordin	227.72	9.8	Sasai et al. (1994)
cndp2/darmin-r	226.19	5.7	
pkdcc2	205.49	31.1	Vitorino et al. (2015)
blimp1/prdm1	177.75	5.2	de Souza et al. (1999)
LOC100494211	167.86	6.9	
mig30	155.1	14.5	Hayata et al. (2002)
ephrinb2	140.66	5.3	
dkkx	139.8	40.8	
lim1/lhx1	136.48	6.1	Taira et al. (1992) and Taira et al. (1994a)
rihA	134.88	43.7	
slc38a3	114.75	6.1	
mmp1	112.82	7.6	
noggin	90.37	15.8	Smith and Harland (1992)
cfd/ami	82.96	15.7	
tmem150b	78.84	6.7	
prr5l	63.62	5.7	
tolloid-like1	52.5	7.7	
siamois/sia1	50.59	17.8	Lemaire et al. (1995)
slc13a3	49.39	7.3	

(continued)

Table 31.2 (continued)

Gene name	Expression levels in the head organizer (RPKM)	Expression ratio (head organizer/ remaining regions)	References for expression in the head organizer
pitx2	47.7	6.5	Faucourt et al. (2001)
gjb1	47.36	47.8	
Unknown (Xtevm_898.1.1_VX[a]*)*	42.98	159.2	
cnrip1	42.55	5.8	[b]
ets1	37.41	15.0	
nkx6–2	35.91	7.2	
slc6a8	35.41	25.3	
Xnr3/nodal3	33.52	7.9	Smith et al. (1995) and Ecochard et al. (1995)
hnf1b	33.04	7.4	Demartis et al. (1994)
neuropilin2	30.11	8.0	
vcan	28.3	6.4	
foxa1	28.24	6.3	Sinner et al. (2004)
tiki1/trabd2a	28.09	5.1	Zhang et al. (2012)
nkx2–3	26.26	12.3	
anpep	22.01	18.2	
prdm12	21.99	14.0	
hps4	20.17	5.4	
follistatin	19.72	8.4	Hemmati-Brivanlou et al. (1994)
dmbx1	19.45	29.5	
hao1	18.7	20.5	
Unknown (Xtevm_89.18.1_VV[a]*)*	18.4	5.6	
gjb2	18.22	16.3	de Boer et al. (2006)
Unknown (Xtevm_83.10.1_VV[a]*)*	18.1	21.3	
ras-dva	16.84	5.7	
LOC495835 (LOC100158459)	14.67	5.3	
st6galnac4	13.98	5.6	
fam228b (LOC100145040)	13.95	6.3	
nkx2–2	13.9	55.6	
six1	13.64	9.3	
six6	13.55	10.1	
zcchc14	13.27	5.7	
mertk	12.83	8.9	
phox2b	12.76	6.9	

(continued)

Table 31.2 (continued)

Gene name	Expression levels in the head organizer (RPKM)	Expression ratio (head organizer/ remaining regions)	References for expression in the head organizer
twin/sia2	12.49	9.2	Laurent et al. (1997)
spns2	10.45	5.6	
ripply2.1	10.03	125.4	
LOC100486741	9.48	6.5	
fgfrl1/ndk	8.92	9.7	Hayashi et al. (2004)
cadherin11	8.78	8.1	
Unknown (Xtevm_68.14.1_VV[a]*)*	8.17	6.9	
LOC100496476	7.83	31.3	
palm	7.6	7.0	
follistatin-like3	7.37	12.7	
LOC100495150	7.26	5.3	
LOC101735240	6.92	19.8	
igsf3	6.71	22.4	
cmtm5	6.64	7.8	
bhlhb5/bhlhe22	6.47	5.7	
Unknown (Xtevm_493.3.1_VV[a]*)*	6.27	7.2	
cebpa	6.17	5.4	
protocadherin9	5.77	10.9	
lypd6	5.68	6.4	
slug/snail2	5.49	8.9	Essex et al. (1993)
hoxd9	5.36	6.2	
ndnf	5.19	6.0	
kcng3	5.04	168.0	

Modified from Yasuoka et al. (2014)
RPKM reads per kilobase of exon per million mapped reads
[a]Gene models annotated by Akkers et al. (2009)
[b]Zheng et al. (2015) described a developmental expression pattern of *cnrip1* by whole-mount in situ hybridization, but unfortunately they did not examine the head organizer region, using hemi-sections

ChIP-seq analysis of the trunk (tail) organizer gene Brachyury/T (Bra), as well as Eomesodermin and VegT, has also been reported in *X. tropicalis* gastrulae (Gentsch et al. 2013). These three T-box TFs positively regulate genes for posteriorization and for mesoderm formation and differentiation (somitogenesis and myogenesis), and negatively regulate genes for neuroectoderm specification (Fig. 31.1c), thus mediating trunk organizer formation and functions and defining the mesodermal cell fate in the tail organizer at the tailbud stage. Although the exact mechanism and partner TFs are still unknown, this type of dual regulatory function (positive and

negative) of T-box factors resembles that of Otx2 in the head organizer. Because the expression patterns of both *otx* in the head region and *bra* in the blastoporal region (posterior region) are highly conserved among bilaterians (Reichert and Simeone 2001; Technau 2001), we speculate that the regulatory principle governing Otx and Bra provides evolutionary variability, plasticity, and flexibility (so-called "evolvability") of target genes and regulatory modes (positive or negative regulation) by changing CRM sequences and partner TFs. Thus, CRMs mediated by Otx2 and Bra determine gene expression output and development. In the organizer, thousands of CRMs integrate information from signaling pathways and organizer-specific TFs to temporally and spatially regulate gene expression. While a handful of organizer gene interactions have been carefully examined (Koide et al. 2005; Charney et al. 2017b), the current organizer GRN is insufficient to predict the outcome. In the future, it will be important to integrate both computational and mathematical approaches to build the organizer GRN.

31.4 Comparisons Between Cnidarians and Vertebrates

In the field of evolutionary developmental biology (evo–devo), one of the biggest unsolved issues is how the gastrula organizer evolved from its ancient prototype in animals. To assess evolutionary origins of the vertebrate gastrula organizer, its counterpart has been investigated in invertebrate embryos, including cnidarians. In evo–devo studies of cnidarian embryos, the most commonly used model animal is the sea anemone, *N. vectensis*. There are several reasons for its popularity. First, it is easy to obtain synchronously developing *Nematostella* embryos because spawning can be induced by regulating light, temperature, and nutritional conditions (Fritzenwanker and Technau 2002; Genikhovich and Technau 2009a). Second, microinjection methods are well established for molecular studies of gene function and regulation (Genikhovich and Technau 2009b; Layden et al. 2013). Third, the genome sequence is available (Putnam et al. 2007). Here we review recent progress in *Nematostella* studies in genomics, axis specification processes, and organizer gene expression.

31.4.1 Organizer Transplantation Experiments with Cnidarian Embryos

The first evidence for metazoan organizer-like activity came from transplantation experiments in *Hydra* (Browne (1909), reviewed by Bode (2012)), even before those of Spemann and Mangold in newts. In adult *Hydra*, the organizer is located in the mouth region, called the hypostome. This organizer is now known as the head organizer in *Hydra*, but in this chapter, we do not use that term, in order to avoid confusion between the *Hydra* organizer and the vertebrate head organizer. Like the

Spemann–Mangold organizer, the *Hydra* organizer can induce a secondary body axis when transplanted into the body column. Furthermore, formation of the *Hydra* organizer in the budding zone or the regenerating tip involves Wnt/β-catenin signaling (Broun et al. 2005; Lengfeld et al. 2009), superficially like that of the vertebrate organizer, even though the *Hydra* organizer is generated in the adult.

Another model organism in cnidarians is *N. vectensis*, which is useful for embryological studies. Transplantation experiments using *Nematostella* embryos demonstrated that the blastoporal lip of an early gastrula embryo induced ectopic tentacles and a pharynx when transplanted into the aboral ectoderm (Kraus et al. 2007; Kraus et al. 2016). The region named the blastoporal organizer appears to be equivalent to the gastrula organizer in vertebrates, suggesting that a prototype of the gastrula organizer existed in a common ancestor of eumetazoans.

31.4.2 Genome-Wide Studies Using Nematostella *Embryos*

To date, three cnidarian genomes have been decoded: *N. vectensis* (Putnam et al. 2007), *Hydra magnipapillata* (Chapman et al. 2010), and the coral *Acropora digitifera* (Shinzato et al. 2011). Cnidarian genomes contain an estimated 20–30,000 protein-coding genes—a number comparable to those in vertebrates—and gene repertoires of developmental regulatory proteins are well conserved among eumetazoans. This implies that neofunctionalization of existing GRNs, rather than creation of new genes, has been the primary driving force behind eumetazoan developmental systems, including the gastrula/blastoporal organizer.

The first ChIP-seq study of *Nematostella* embryos was reported in 2014 (Schwaiger et al. 2014). ChIP-seq data for histone modifications, RNAPII, and p300 coactivator, and RNA-seq data have been used to identify more than 5,000 enhancers in the *Nematostella* genome (Schwaiger et al. 2014). Transgenic reporter assays validated enhancer activity in 75% of the predicted enhancers that were examined. Remarkably, TF genes or developmental regulatory genes are associated with multiple enhancers more often than housekeeping genes, indicating a complex landscape of gene regulatory elements in *Nematostella*, which is equivalent to those in *Drosophila* and zebrafish (Schwaiger et al. 2014). *Xenopus* also regulates tissue-specific gene expression with multiple enhancers (Yasuoka et al. 2014). These findings are reminiscent of "superenhancers" occupied by master TFs to regulate cell identity genes (Whyte et al. 2013). In addition, the genomic distribution of predicted enhancers (that is, intergenic regions, introns, or near promoters) in *Nematostella* is similar to that in bilaterians (Schwaiger et al. 2014). These results suggest that the eumetazoan ancestor had already developed complex gene regulatory strategies for developmental processes, despite having simple anatomy, such as that observed in cnidarians. Further genome-wide studies of GRNs in cnidarian development should provide clues to the process of GRN evolution from the cnidarian-type ancestor to vertebrates.

31.4.3 Formation of the Blastoporal Organizer by Signaling Pathways

In *Xenopus* and zebrafish, Wnt/β-catenin signaling is responsible for organizer formation. In vertebrates, Nodal signaling induces mesoderm genes, including organizer genes, while Bmp signaling restricts the organizer region to the dorsum (De Robertis et al. 2000). So what kind of signaling molecules are involved in blastoporal organizer formation in *Nematostella*? In this section, we review studies of developmental signaling pathways in cnidarians.

31.4.3.1 Wnt/β-Catenin Signaling

The fly and nematode have lost 6–11 out of 13 Wnt subfamilies, but 12 of them are found in the *Nematostella* genome (Kusserow et al. 2005; Lee et al. 2006; Guder et al. 2006). Most *Nematostella wnt* genes are expressed in the blastoporal region from the gastrula to the planula in both the endoderm and ectoderm, whereas a Wnt antagonist *dkk* is expressed in the aboral ectoderm (Kusserow et al. 2005; Lee et al. 2006; Guder et al. 2006). In *Nematostella* eggs, Dishevelled is maternally localized near the animal pole, where the blastopore will be formed. The animal pole of cnidarian eggs is equivalent to the vegetal pole of *Xenopus* eggs, in terms of the relative position of the blastopore. By the blastula stage, nuclear localization of β-catenin is detected in the animal half of the embryo (Lee et al. 2007). These data are consistent with the observation that animal, but not vegetal, halves bisected at the eight-cell and gastrula stages can produce a complete body plan because the animal half will form the organizer (Fritzenwanker et al. 2007).

To investigate Wnt-downstream genes during early *Nematostella* development, genome-wide microarray analysis was performed using blastula embryos treated with the GSK3β inhibitors lithium chloride or 1-azakenpaullone (AZ) to activate Wnt/β-catenin signaling (Rottinger et al. 2012). The results showed that Wnt/β--catenin signaling mainly activated blastopore-associated genes such as *bra*, *foxa*, *lim1/lhx1*, and *chrd*, which are expressed in the ectoderm as a ring around the animal pole at the blastula stage (Fig. 31.3a). In contrast, expression levels of endodermal genes such as *snail*, *otx*, and *gsc* in the central region of the animal pole at the blastula stage were not greatly affected by perturbation of Wnt/β-catenin signaling.

Involvement of Wnt/β-catenin signaling in blastoporal organizer formation in *Nematostella* was further examined in combination with transplantation and genetic manipulation (Kraus et al. 2016). Detailed transplantation experiments showed that organizer activity is limited to ectodermal tissue around the blastopore, which expresses *wnt1* and *wnt3*, corresponding to the central ring, but not to the external ring or the central domain (see Fig. 31.3a). Microinjection experiments showed that ectopic expression of Wnt1 or Wnt3 induces a secondary axis when expressed in a single blastomere at the eight- to 16-cell stage, whereas knockdown of Tcf abol-

ished organizer activity, suggesting that Wnt/β-catenin signaling is necessary and sufficient for formation of the blastoporal organizer. Thus, Wnt/β-catenin signaling plays a central role in organizer formation in cnidarians, including the organizer in adult *Hydra*.

31.4.3.2 Nodal Signaling

Though *nodal-related* (*ndr*) genes have not yet been found in the *Nematostella* genome (Putnam et al. 2007), they are present in other cnidarian genomes, including those of corals and *Hydra* (Watanabe et al. 2014a). In *Hydra*, Nodal signaling by asymmetric expression of *ndr* is required for lateral bud formation (Watanabe et al. 2014a). Interestingly, β-catenin is involved in this *ndr* expression, and Ndr in turn upregulates *pitx*. The autoregulatory feedback loop of Nodal signaling and the regulatory axis from Nodal signaling to *pitx* are similar to those in left–right patterning in vertebrates (Fig. 31.2b). Thus, the asymmetric branching pattern in cnidarians is thought to share the same system as that in left–right asymmetric patterning of vertebrates (Watanabe et al. 2014a). In *Nematostella*, the loss of *ndr* may have been related to the loss of branching in its body plan.

The regulatory axis from β-catenin to Nodal is also similar to that in formation of the gastrula organizer in vertebrates (De Robertis et al. 2000). Furthermore, it should be noted that *pitx2* is listed among the head organizer genes of *Xenopus* identified by RNA-seq analysis with dissected head organizer regions (Table 31.2) (Yasuoka et al. 2014). To elucidate the common mechanism of the organizer formation, the question of whether Nodal (or ALK4/5/7-Smad2/3) signaling is involved in organizer formation and function in *Hydra*, *Nematostella*, and other cnidarians should be addressed.

31.4.3.3 Bmp Signaling

Cnidarians are often considered radially symmetric, but they actually have two orthogonal axes: the oral–aboral and the directive axes (Figs. 31.2c and 31.3b). Two *bmp* genes, *dpp* and *bmp5–8*, and their antagonist, *chrd*, are expressed asymmetrically along the directive axis in *Nematostella, Acropora*, and *Podocoryne* gastrulae (Matus et al. 2006a; Rentzsch et al. 2006; Reber-Muller et al. 2006; Hayward et al. 2015). Functional analyses of Bmp signaling components have shown that Bmp signaling is required to break symmetric expression of *dpp* and *chrd* along the directive axis during gastrulation (Saina et al. 2009). Recently, more systematic and mathematical approaches have demonstrated that the domain of restricted *chrd* expression is most important for patterning along the body axis, resulting in activation of Bmp signaling on the opposite side of the embryo (Genikhovich et al. 2015; Meinhardt 2015).

In the Bmp–Chrd signaling network, *bmp* genes can be expressed anywhere, e.g., *dpp* on the same side as *chrd* in *Nematostella*, or *bmp4* on the opposite side in

Xenopus (Fig. 31.2c). Thus, core components of the Bmp signaling network system (Bmp, Chrd, and Tolloid) are evolutionarily constrained to determine the directive axis in *Nematostella* and the dorsoventral axis in *Xenopus*, whereas *bmp* genes and other Bmp signaling modulators, such as Bambi and Smad6/7, vary significantly between *Nematostella* and *Xenopus*, reflecting strong evolutionary divergence of Bmp signaling in eumetazoans (Genikhovich et al. 2015).

It is well known that secreted Bmp antagonists, such as Chrd and Noggin, induce neural tissue on the dorsal side in *Xenopus* (Harland 2000; De Robertis and Kuroda 2004) and that in *Drosophila*, *short gastrulation* (*sog*)—the *Drosophila* orthologue of *chrd*—specifies neuroectoderm in the ventral region (De Robertis et al. 2000; De Robertis 2006). These data may create the impression that Bmp inhibition is intimately related to neural differentiation, but as exemplified by experiments with chicks (Streit and Stern 1999; De Robertis and Kuroda 2004), as well as with *Nematostella* (Watanabe et al. 2014b), this is not always true. To reconcile these observations, it should be noted that the evolutionarily conserved role of the Bmp–Chrd system is to pattern the embryo along the axis but not to differentiate types of tissue. Therefore, it is conceivable that in *Xenopus*, dorsoventral patterning by the Bmp–Chrd system is interlocked to neural induction by the organizer.

In *Nematostella*, initial blastoporal expression of *dpp* and *chrd* depends on Wnt/β-catenin signaling, suggesting that organizer formation by Wnt/β-catenin signaling is upstream from blastopore expression of *dpp* and *chrd* (Rottinger et al. 2012; Kraus et al. 2016). However, it remains to be seen whether the symmetry disruption of *bmp/chrd* expression is initiated by an as-yet-unidentified trigger or is determined by biased Bmp signaling levels in combination with a positive feedback loop and inhibitory effects on the opposite side (Fig. 31.2c).

Notably, among the *Nematostella* orthologues of Bmp antagonists that are expressed in the vertebrate gastrula organizer (De Robertis and Kuroda 2004), *chrd*, but not *noggin* or *follistatin*, is expressed in the blastoporal ectoderm (Matus et al. 2006a). *cer* is not found in the *Nematostella* genome. This suggests that *chrd* is one of the most ancient effectors of organizer functions in eumetazoans. However, ectopic expression of Chrd by mRNA injection into a single blastomere does not induce a secondary axis in *Nematostella*, in contrast to strong axis induction by Wnt1 and Wnt3 (Kraus et al. 2016). In addition, the central ring (*dpp*- but not *chrd*-expressing blastoporal ectoderm; see Fig. 31.3a) has been shown to have axis-inducing activity in transplantation experiments (Kraus et al. 2016). Finally, activation of Wnt signaling by AZ after the late gastrula stage did not affect unilateral expression of *dpp* and *chrd*, although treated embryos resulted in double-headed primary polyps (Kraus et al. 2016). These data suggest that Bmp signaling is not involved in organizer formation but is involved in patterning along the directive axis in *Nematostella*. Thus, it seems that Bmp antagonists became integrated into gastrula organizer GRNs after the divergence of cnidarians and bilaterians.

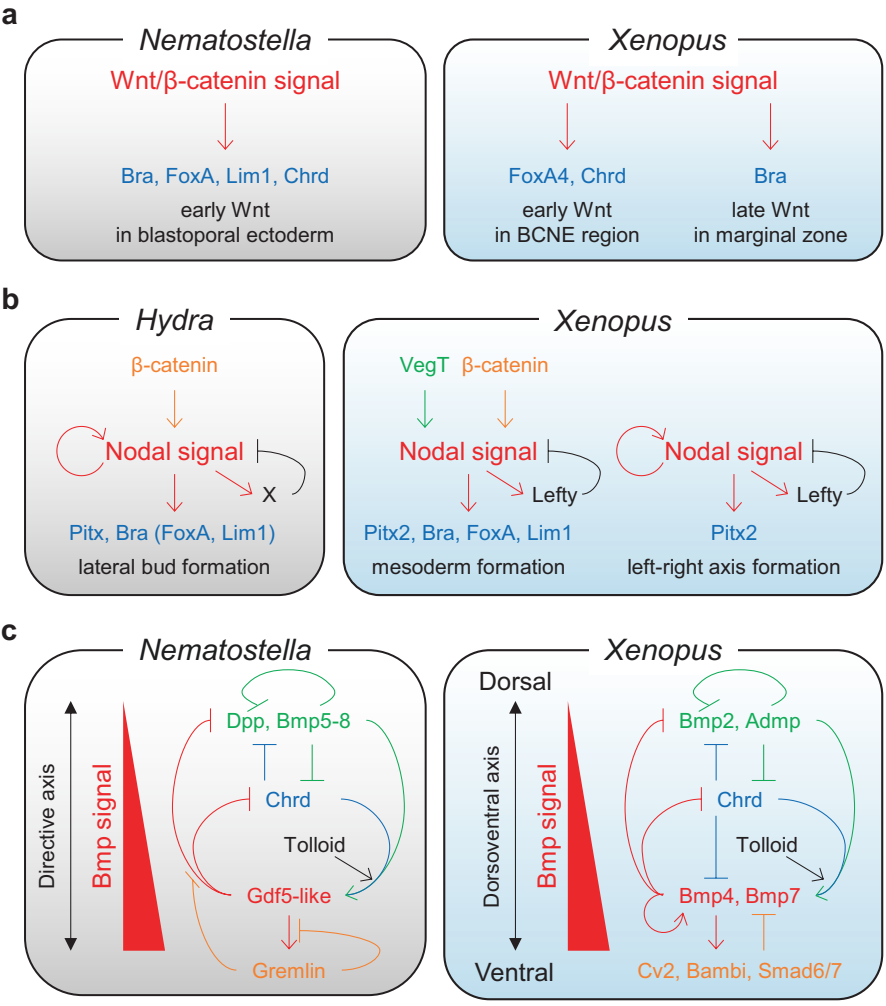

Fig. 31.2 Wnt, Nodal, and Bmp signaling pathways share conserved roles in eumetazoans. (**a**) Comparison of Wnt/β-catenin signaling target genes in *Nematostella* and *Xenopus*. In *Nematostella*, early Wnt/β-catenin signaling activates blastoporal ectoderm genes (*bra*, *foxA*, *lim1*, and *chrd*) (see Fig. 31.3a) (Rottinger et al. 2012). Among these, *foxA* (*foxa4*) and *chrd* are activated by early Wnt/β-catenin signaling in the blastula *chordin*- and *noggin*-expressing (BCNE) region of the *Xenopus* blastula (Kuroda et al. 2004; Reversade et al. 2005; Murgan et al. 2014). On the other hand, late Wnt/β-catenin signaling activates *bra* in the marginal zone of the gastrula (Vonica and Gumbiner 2002). These facts indicate conservation of regulatory axes from Wnt/β-catenin signaling to organizer genes. (**b**) Comparison of Nodal signaling mechanisms in *Hydra* and *Xenopus*. In *Hydra*, Nodal signaling has both positive and negative feedback loops (Watanabe et al. 2014a), like left–right patterning in vertebrates. In addition, the Nodal target gene, *pitx*, is also conserved. In *Xenopus*, the vegetally localized maternal transcription factor (TF), VegT, and maternal β-catenin are required for zygotic activation of Nodal signaling (Agius et al. 2000; Xanthos et al. 2002). Then Nodal signaling activates *bra*, *foxA*, and *lim1* during mesoderm formation processes (Taira et al. 1992, 1994a; Latinkic et al. 1997; Pohl and Knochel 2005; Sudou et al. 2012).

31.4.4 Establishment and Maintenance of the Gastrula Organizer by Transcription Factors

As mentioned in Sect. 31.3.4, the gastrula organizer is established and maintained through GRNs composed of organizer-specific TFs and their binding CRMs for target genes. To date, there have been a few functional studies of TFs expressed in the blastoporal organizer of cnidarians. In this section, we summarize expression patterns of so-called organizer genes in cnidarians and bilaterians, and compare them with those of *Xenopus* and vertebrates (Fig. 31.3).

31.4.4.1 Brachyury/T (Bra)

The T-box TF Brachyury/T (Bra) has a crucial role in mesoderm formation in vertebrate early development (Showell et al. 2004). Evolutionarily, its blastoporal expression in blastula to gastrula embryos is highly conserved in eumetazoans (Technau 2001). Among bilaterians other than chordates, expression of *bra* in the mouth and anus of larval stage embryos, either one or both of which are derived from the blastopore, is widely conserved (Hejnol and Martindale 2008). In chordates, *bra* is expressed in the notochord and posterior mesoderm, suggesting the loss of mouth expression and acquisition of notochord expression in chordates (Satoh et al. 2012). In *Nematostella*, it is expressed in the blastoporal organizer region and is activated by Wnt/β-catenin signaling (Figs. 31.2a and 31.3a) (Scholz and Technau 2003; Rottinger et al. 2012; Kraus et al. 2016). In *Hydra*, two copies of *bra* genes are expressed in the *Hydra* organizer and are upregulated by Wnt/β--catenin and Nodal signaling (Bielen et al. 2007; Watanabe et al. 2014a). In *Acropora*, *bra* is also expressed around the blastopore (Hayward et al. 2015; Yasuoka et al. 2016). Loss-of-function analyses for *bra*, using *Acropora* embryos, have demonstrated that *bra* is necessary for pharynx formation (Yasuoka et al. 2016). Furthermore, *Acropora bra* is activated by Wnt/β-catenin signaling directly via a 5′ *cis*-regulatory sequence of *bra*, again showing the evolutionary conservation of the regulatory axis from Wnt/β-catenin signaling to *bra*.

Fig. 31.2 (continued) These genes are also expressed in the budding zone of *Hydra*, although whether Nodal signaling activates *foxA* and *lim1* was not tested. Lefty is a negative feedback regulator in vertebrates, but its counterpart has not been identified in *Hydra*. Therefore, Nodal signaling shares similar regulatory feedback loops and target genes in *Hydra* and *Xenopus*. (**c**) Comparison of the Bmp signaling network in *Nematostella* and *Xenopus* (Modified from Genikhovich et al. 2015). In both systems, *chrd* is expressed in the Bmp signaling–negative side together with Bmp agonists, expression of which is negatively regulated by Bmp signaling. In the Bmp signaling–positive side, Bmp signaling activates other types of Bmp signaling agonists and antagonists, forming positive and negative feedback loops. Chrd is considered to act as a Bmp shuttle, defusing away from the Chrd source and promoting signaling at a distance (Plouhinec et al. 2011). This comparison suggests that Bmp signaling shares similar regulatory networks in directive axis patterning in *Nematostella* and dorsoventral patterning in *Xenopus*

Bra functions as a trunk/tail organizer gene (Taira et al. 1997), and loss of *bra* function has been shown to lead to axis truncation in vertebrates (Showell et al. 2004; Gentsch et al. 2013). In *Xenopus*, *bra* expression starts at the blastula stage in the marginal zone, induced by Nodal and Fgf signaling. Then expression is excluded from the head organizer region at the gastrula stage (Fig. 31.3a) (Latinkic et al. 1997; Evren et al. 2014). Zygotic Wnt/β-catenin signaling also contributes to maintenance of *bra* expression in the posterior mesoderm in *Xenopus*, as well as in mice (Yamaguchi et al. 1999; Vonica and Gumbiner 2002). These data suggest that Bra has functioned as an organizer gene in organizer GRNs since the common eumetazoan ancestor arose, and that regulation of *bra* expression by Wnt/β-catenin signaling has been conserved.

31.4.4.2 FoxA

The forkhead box gene, *foxa*, is known as a foregut marker gene in bilaterians (Fritzenwanker et al. 2004; Martindale et al. 2004; Martindale and Hejnol 2009). In *Nematostella, foxa* expression is initiated in the blastoporal organizer region by Wnt/β-catenin signaling and is maintained in the pharyngeal ectoderm (Figs. 31.2a and 31.3a) (Fritzenwanker et al. 2004; Martindale et al. 2004; Rottinger et al. 2012; Kraus et al. 2016). In a study of *Acropora, foxa* expression was not detected before gastrulation. Instead, it first appeared in the pharyngeal ectoderm of the late gastrula (Hayward et al. 2015). If these expression patterns imply a conserved function of FoxA for gut development in eumetazoans, the cnidarian pharynx may be comparable to the bilaterian foregut.

In *Xenopus*, there are three *foxa* ohnologs: *foxa1/HNF3α*, *foxa2/HNF3β*, and *foxa4/XFKH1/pintallavis* (Pohl and Knochel 2005, Watanabe et al. 2017). *foxa4* is expressed in the BCNE region (Fig. 31.3a). This region is induced by Wnt/β-catenin signaling and contributes to the forebrain and the midline structures, including the notochord, floor plate, and prechordal plate (Kuroda et al. 2004; Sudou et al. 2012) (Fig. 31.3a) (Pohl and Knochel 2005; Reversade et al. 2005; Murgan et al. 2014). Then *foxa4* expression is maintained in the notochord and floorplate from the neurula to the tailbud embryo (Fig. 31.3b) (Pohl and Knochel 2005). *foxa1* and *foxa2* are expressed throughout the endoderm and the dorsal organizer region in gastrula embryos (Sinner et al. 2004; Suri et al. 2004). At later stages (neurula to tailbud), both genes are expressed in the notochord, floorplate, and foregut (Fig. 31.3b) (Pohl

Fig. 31.3 (continued) in the planula (Mazza et al. 2007) but are omitted from this figure. In the *Xenopus* neurula, head organizer genes (*lim1*, *otx2*, *gsc*, and *chrd*) are coexpressed in the prechordal plate (blue), whereas trunk organizer genes (*bra*, *foxa*, *lim1*, and *chrd*) are coexpressed in the notochord (magenta). In addition, *otx* genes (*otx1*, *otx2*, and *otx5*) are expressed in the anterior neuroectoderm (orange), and *foxa* genes (*foxa1*, *foxa2*, and *foxa4*) are expressed in the floor plate (green) and the endoderm (yellow). This comparison further highlights the centralized expression domains of vertebrate organizer genes throughout embryonic development

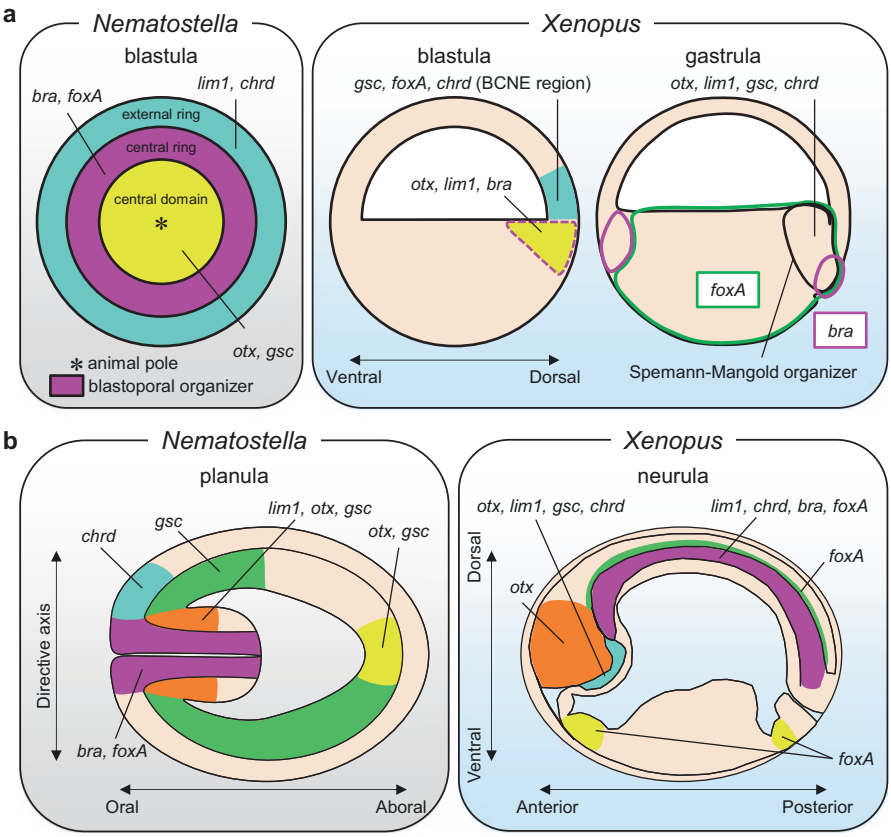

Fig. 31.3 Expression of vertebrate organizer genes is not integrated in cnidarians. (**a**) Comparison of expression patterns of *bra*, *foxA*, *lim1*, *otx*, *gsc*, and *chrd* from blastula to gastrula between *Nematostella* and *Xenopus*. In the *Nematostella* blastula, the external ring (blue), central ring (magenta), and central domain (yellow) are indicated, as described by Rottinger et al. (2012). The blastoporal organizer corresponds to the central ring (Kraus et al. 2016). In the *Xenopus* blastula, the blastula *chordin*- and *noggin*-expressing (BCNE) region (blue) expresses *gsc*, *foxA4*, and *chrd*. In the dorsal–vegetal region (yellow) of the blastula, *lim1*, *otx2*, and *bra* are expressed (Sudou et al. 2012; Fletcher and Harland 2008). In the *Xenopus* gastrula, the dorsal endomesoderm forms the Spemann–Mangold organizer, which is derived from the BCNE region and the dorsal–vegetal region in the blastula (Sudou et al. 2012). This comparison indicates that expression of organizer genes in the blastula to gastrula stages is more integrated in *Xenopus* than in *Nematostella*. (**b**) Comparison between the *Nematostella* planula and the *Xenopus* neurula. In the *Nematostella* planula, *bra* and *foxA* are coexpressed in the pharyngeal ectoderm (magenta) (Scholz and Technau 2003; Fritzenwanker et al. 2004; Martindale et al. 2004). Expression of *chrd* in the oral ectoderm is restricted to the side of weak Bmp signaling (blue) (Matus et al. 2006a) (see Fig. 31.2c). Expression of *gsc* is maintained in the endoderm but asymmetric along the directive axis. In the early planula, *gsc* is expressed in diametrically opposed domains of the body wall endoderm and the pharyngeal endoderm (green and orange) (Matus et al. 2006b). *lim1* and *otx* genes (*otxA*, *otxB*, and *otxC*) are presumably coexpressed in the pharyngeal endoderm (orange) (Mazza et al. 2007; Srivastava et al. 2010). *gsc* and *otx* genes (*otxA*, *otxB*, and *otxC*) are also expressed in the aboral endoderm (yellow). *otx* genes (*otxA*, *otxB*, and *otxC*) are also expressed in tentacle-forming regions

and Knochel 2005). Coexpression of *foxa* and *bra* in both cnidarians and vertebrates (Fig. 31.3) suggests the possibility that FoxA and Bra have functioned together throughout metazoan history to build organizer GRNs.

31.4.4.3 Lim1/Lhx1

The LIM homeobox gene *lim1/lhx1* was proposed as an ancient eumetazoan organizer gene because of the deep origin of its blastoporal expression. In *Nematostella*, *lim1* is expressed in the external ring region of the blastoporal ectoderm at the blastula stage (Fig. 31.3a) (Yasuoka et al. 2009; Rottinger et al. 2012). This expression domain overlaps with that of *chrd* (Rottinger et al. 2012), and Lim1 positively regulates *chrd* expression, as determined by knockdown experiments using antisense morpholino (Yasuoka et al. 2009). Because *chrd* is a target gene of Lim1 in *Xenopus* (Yasuoka et al. 2014; Agulnick et al. 1996; Taira et al. 1994b, 1997), the regulatory axis from Lim1 to *chrd* is conserved from cnidarians to vertebrates. However, the *lim1/chrd* coexpressing region did not show axis-inducing activity in *Nematostella* (Fig. 31.3a) (Kraus et al. 2016). Furthermore, in nonchordate deuterostomes (ambulacrarians), *chrd* is not expressed around the blastopore but is expressed in the oral/ventral ectoderm (Lowe et al. 2006; Saudemont et al. 2010; Rottinger et al. 2015), in contrast to *lim1* expression in both the blastoporal and the oral ectoderm in sea urchin gastrula embryos (Yasuoka et al. 2009). Therefore, it is more likely that the regulatory axis from Lim1 to *chrd* was incorporated into organizer GRNs in the chordate lineage.

Nematostella lim1 expression is also detected in the pharyngeal endoderm of planula larvae and primary polyps, overlapping with the pharyngeal nerve ring, which contains GABA-ergic neurons (Srivastava et al. 2010). Together with expression patterns of other LIM homeobox genes in *Nematostella*, it is proposed that fundamental roles of the LIM homeobox gene family in neuronal differentiation are conserved from cnidarians to bilaterians (Hobert and Westphal 2000; Shirasaki and Pfaff 2002; Srivastava et al. 2010).

In *Xenopus*, *lim1* expression is directly induced by Nodal signaling in dorsal endomesodermal cells from the late blastula to early gastrula stages (Fig. 31.3a) (Taira et al. 1992, 1994a; Sudou et al. 2012). At the blastula stage, initially *lim1* expression does not overlap with that of *chrd*. This is different in *Nematostella*, where *Xenopus lim1* is initially expressed in the dorsal mesodermal and endodermal regions, whereas *Xenopus chrd* begins to be expressed in the BCNE region (Fig. 31.3a). However, during later stages, the expression domains of *lim1* and *chrd* in the head and trunk organizers overlap significantly (Sudou et al. 2012). In late gastrula to neurula embryos, *lim1* expression is maintained in both head and trunk organizers, and later Lim1 protein is maintained in the prechordal plate and notochord (Sudou et al. 2012) (Fig. 31.3b). At the neurula stage, *lim1* expression starts in the pronephros, controlled by retinoic acid signaling (Taira et al. 1994a), and in the central nervous system (Karavanov et al. 1996). Because of the core regulatory axis from Lim1 to *chrd*, other downstream genes such as *gsc* and *otx* may have been recruited into organizer GRNs in the vertebrate lineage.

31.4.4.4 Otx

The paired-type homeobox gene *otx* (*orthodenticle homeobox*) functions as the head selector gene for brain formation in bilaterians (Arendt and Nubler-Jung 1996; Reichert and Simeone 2001). In deuterostomes, Otx also participates in gastrulation and endomesoderm specification (Ang et al. 1996; Harada et al. 2000; Satou et al. 2001; Davidson et al. 2002). In *Nematostella*, there are three tandemly duplicated *otx* genes (*otxA*, *otxB*, and *otxC*). All three are expressed in the central domain of the animal pole of blastula embryos, which will become the endoderm, but not in the organizer (Fig. 31.3a) (Mazza et al. 2007; Rottinger et al. 2012; Kraus et al. 2016). After gastrulation, expression of *otx* genes is maintained in the aboral endoderm and appears in an endodermal pharyngeal ring surrounding the presumptive mouth and tentacles in planula larvae and primary polyps (Fig. 31.3b) (Mazza et al. 2007). Because the *otx*-expressing pharyngeal ring corresponds to the oral nervous system of *Nematostella* (Marlow et al. 2009; Watanabe et al. 2014b), *otx* expression patterns in *Nematostella* embryos imply an evolutionary correlation between the nerve ring of cnidarians and the central nervous system in bilaterians.

In *Xenopus*, there are also three *otx* ohnologs (*otx1*, *otx2* and *otx5/crx*, derived from two rounds of whole-genome duplication, Watanabe et al. 2017). All three are expressed in anterior neural tissue as head selector genes (Andreazzoli et al. 1997; Blitz and Cho 1995; Pannese et al. 1995; Kuroda et al. 2000; Vignali et al. 2000). In addition, *otx2* and *otx5* are coexpressed in the gastrula organizer, induced by Nodal signaling in endomesodermal cells from the late blastula to gastrula stages (Fig. 31.3a) (Sudou et al. 2012). In cephalochordates (amphioxi), *otx* is expressed in the anterior ectoderm and endoderm, but not in the mesoderm, from the gastrula to larval stages (Onai et al. 2010; Onai et al. 2009). Thus, it appears that *otx* genes have acquired dorsal mesoderm expression in the vertebrate lineage to contribute to head organizer GRNs.

31.4.4.5 Goosecoid

The paired-type homeobox gene *gsc* has a common expression pattern in the foregut/mouth region (the oral ectoderm) in bilaterians (Hejnol and Martindale 2008; Martindale and Hejnol 2009). In addition, in some bilaterians, including chordates, *gsc* is expressed in endomesodermal cells in gastrula embryos (Hejnol and Martindale 2008; Neidert et al. 2000). In *Nematostella*, *gsc* is first expressed in the central domain of the animal pole at the blastula stage, which is not regulated by Wnt/β-catenin signaling (Fig. 31.3a) (Rottinger et al. 2012), but the expression level is quite low and this early expression was not detected in the first report of *Nematostella gsc* (Matus et al. 2006b). This *gsc* expression domain does not correspond to the blastoporal organizer (Kraus et al. 2007, 2016). Consistent with other genes having early expression in the endodermal lineage, *gsc* is expressed in the body wall endoderm and pharyngeal endoderm of planula larvae, but its expression is asymmetric along the directive axis (Matus et al. 2006b). Similar expression

patterns of *gsc* were also observed in *Acropora*, although it is unclear whether *gsc* is expressed along the directive axis (Hayward et al. 2015).

In contrast, *Xenopus gsc* is first expressed in the BCNE region and is induced by Wnt/β-catenin signaling (Kuroda et al. 2004; Sudou et al. 2012) (Fig. 31.3a). Subsequently, *gsc* expression is also induced by Nodal signaling in the dorsal endomesodermal region and is finally expressed in the gastrula organizer (Sudou et al. 2012) (Fig. 31.3a). During and after gastrulation, *gsc* expression is maintained in the prechordal plate by Lim1 and Otx2 (Taira et al. 1994b; Mochizuki et al. 2000; Sudou et al. 2012) and plays a crucial role in midline formation of the head (Sander et al. 2007; Yasuoka et al. 2014). In cephalochordates, *gsc* is expressed in the dorsal mesoderm during gastrulation, whereas *gsc* expression is not restricted to the anterior mesoderm but is maintained in the entire mesoderm from the late gastrula to neurula stages (Onai et al. 2015). Then from the late neurula to larval stages, *gsc* expression is restricted to the posterior mesoderm (Neidert et al. 2000). It has also been reported that *gsc* expression was activated by both Wnt/β-catenin and Nodal signaling in amphioxi, as in vertebrates (Onai et al. 2010; Yasui et al. 2002). Therefore, it is likely that *gsc* was not one of the ancestral organizer genes when eumetazoans arose. Later, *gsc* was co-opted into organizer GRNs in chordates. Then its function became more restricted to the head organizer in vertebrates.

31.5 Conclusions

In this chapter, we have described both the molecular basis of the Spemann–Mangold organizer, as revealed using *Xenopus* embryos, and also evolutionary comparisons of the gastrula organizer between cnidarians and vertebrates. Genomic studies of TFs and epigenetic marks illustrate regulatory principles of genes and CRMs in the *Xenopus* organizer GRNs (Fig. 31.1): (1) tissue-specific gene expression is highly associated with both active and repressive epigenetic marks on promoters and/or CRMs in ChIP-seq data when whole embryos are used (Fig. 31.1a); (2) Otx2 functions as a molecular landmark of the head organizer, together with Lim1 and Gsc (Fig. 31.1b); and (3) Bra determines the posterior mesodermal cell fate in the trunk organizer (Fig. 31.1c). Compared with reporter assays for determining one-by-one regulatory axes of TFs and their target genes (summarized in Koide et al. (2005)), genome-wide data offer an abundance of information on TF functions, so as to reveal target genes, binding sequences, TF–TF interactions on CRMs, and positive/negative regulation in GRNs.

Comparison of the gastrula organizer between cnidarians and vertebrates offers insight into organizer evolution (Figs. 31.2 and 31.3): (1) initiation of organizer formation by Wnt/β-catenin signaling is an ancestral feature of eumetazoans (Fig. 31.2a); (2) disruption of symmetry by Nodal signaling has an ancient origin in eumetazoans, but axis-inducing activity of Nodal signaling is not evident in cnidarians (Fig. 31.2b); (3) patterning by the Bmp–Chrd signaling network shares highly constrained core components (Bmp, Chrd, and Tolloid) among eumetazoans

(Fig. 31.2c); (4) under the control of Wnt/β-catenin signaling, Bra and FoxA may have functioned as core TFs in the organizer since eumetazoans arose (Figs. 31.2a and 31.3a); (5) the regulatory axis from Lim1 to *chrd* already existed in the eumetazoan ancestor (Fig. 31.3a) and was incorporated into the organizer GRN in the chordate ancestor; and (6) Otx and Gsc were not related to the gastrula organizer in basal eumetazoans (Fig. 31.3a) but were later incorporated into the head organizer GRN in the vertebrate ancestor.

Evolution of the vertebrate gastrula organizer required integration of more organizer genes into blastoporal organizer GRNs in comparison with the cnidarian blastoporal organizer. For example, *otx2* expression in the vertebrate head organizer is regulated by Lim1 and FoxA (Taira et al. 1997; Kimura-Yoshida et al. 2007; Kurokawa et al. 2010; Yasuoka et al. 2014) and *gsc* expression in the head organizer is regulated by Lim1 and Otx2 (Mochizuki et al. 2000; Sudou et al. 2012; Yasuoka et al. 2014). On the basis of cnidarian-like ancestral organizer GRNs, vertebrates evolved more intricate, elaborate, and centralized organizer GRNs.

31.6 Perspective

X. laevis has an allotetraploid genome, in contrast to the diploid genome of *X. tropicalis*. Recently, the *X. laevis* genome was decoded, and ChIP-seq and RNA-seq assays were performed with *X. laevis* embryos (Session et al. 2016). We are now able to perform comprehensive studies of the molecular basis of the Spemann–Mangold organizer with the most commonly used frog model system, *X. laevis*. In terms of genome biology, it would be interesting to examine whether the two *X. laevis* subgenomes (named L and S) are differentiated in organizer GRNs. As a clue, it was found that *otx2*, *gsc*, and *gsc2* genes in the S subgenome are maternally expressed, unlike those in the L subgenome and in *X. tropicalis* (Watanabe et al. 2017). TF gene expression profiles are relatively conservative between the two subgenomes (Watanabe et al. 2017), whereas expression profiles of signaling molecules, including *chrd* and *cer*, differ between subgenomes (Suzuki et al. 2017; Michiue et al. 2017). These data indicate that CRMs for signaling molecule genes have diversified more during the evolution of subgenomes than those for TF genes (Michiue et al. 2017).

Conservation of organizer formation by Wnt/β-catenin signaling is manifest in cnidarians and vertebrates, but evolution of inducers secreted from the organizer is still unclear. In vertebrates, Wnt/β-catenin signaling is involved only in organizer formation and later, Wnt ligands are expressed in the trunk organizer as posteriorizing factors. The inducers secreted from the vertebrate organizer are Bmp antagonists, which induce neural tissue and muscle, and specify the dorsal side of the embryo. Contrarily, in cnidarians, Wnt ligands induce the organizer and are also expressed in the organizer region, but the inducers secreted from the organizer are as yet unidentified. The fundamental question of the evolutionary origin of inducers of the organizer remains to be answered by deeper analyses using cnidarian embryos.

Bifunctional activities (activator and repressor) of TFs are likely to be essential to GRN evolution in conserved expression domains, as exemplified by Otx and Bra (Fig. 31.1b, c). In the *otx2*-expressing head/anterior region or the *bra*-expressing blastopore/posterior region, GRNs can evolve to modulate their morphology without changing target genes, but by changing their response between activation to repression. In fact, Bra negatively regulates SoxB genes in vertebrates but positively regulates them in *Acropora* (Yasuoka et al. 2016). Notably, *lim1* expression overlaps with both *otx* and *bra* in the head and trunk organizers of vertebrates. As mentioned in Sect. 31.3.4, Lim1 functions as a partner TF for Otx2 to activate head organizer genes in *Xenopus* (Yasuoka et al. 2014), and Lim1 interacts directly with Otx2 (Nakano et al. 2000; Yasuoka et al. 2014). On the other hand, Lim1 contributes to AP patterning of neural tissue together with Bra as a trunk organizer gene (Taira et al. 1997). Molecular interactions between Lim1 and Bra have not been reported, but homeodomain-binding motifs (Lim1 type) are enriched in *Xenopus* Bra ChIP-seq data (Gentsch et al. 2013), suggesting the possibility that Lim1 can also function as a partner TF for Bra. In *Nematostella*, *otx* and *bra* are expressed during development in a mutually exclusive manner, which is similar to bilaterian AP patterning. In addition, *Nematostella lim1* is coexpressed with *otx* in the pharyngeal endoderm of planula larvae (Fig. 31.3b), whereas *Nematostella lim1* is not coexpressed with *bra* in the blastoporal/pharyngeal ectoderm of the blastula/planula (Fig. 31.3a, b). Thus, Lim1 seems to be an ancient partner TF of Otx, but not of Bra, to activate target genes. Cooperative functions of Lim1 and Otx might be the basis for head organizer GRNs, whereas those of Lim1 and Bra need to be analyzed more thoroughly for understanding of the evolution of trunk organizer GRNs.

Molecular studies of organizer gene expression in amphioxus (a cephalochordate) have shown that many organizer genes are expressed in the dorsal mesoderm, as in vertebrates (Terazawa and Satoh 1997; Langeland et al. 2006; Yu et al. 2007), suggesting that a prototype of the vertebrate organizer was already established in the chordate ancestor. In other deuterostomes such as echinoderms and hemichordates, expression of organizer genes is not centralized in the blastoporal mesoderm, whereas some organizer genes such as *chrd*, *lim1*, *gsc*, *bra*, and *foxa* are coexpressed in the oral/ventral ectoderm, possibly controlled by Nodal signaling (Yasuoka et al. 2009; Lapraz et al. 2009; Saudemont et al. 2010; Lowe et al. 2015; Rottinger et al. 2015). In addition, Wnt antagonists such as *dkk* and *sfrp* genes, which are vertebrate head organizer genes, are not expressed in the blastoporal region but are expressed in the anterior ectoderm (the opposite side of the blastopore) of sea urchin embryos (Range et al. 2013; Cui et al. 2014). These data allow us to speculate about several steps in the evolution of the chordate organizer in deuterostomes. First, Nodal signaling was co-opted to initiate early mesoderm specification so as to induce organizer genes in the dorsal mesoderm. Second, Wnt antagonists gained expression in the dorsal mesoderm, possibly under the control of Nodal signaling and/or organizer TFs. Finally, the dorsal mesoderm region evolved self-organizing GRNs to maintain centralized organizer gene expression. To explore these hypotheses, more functional genomic data for the gastrula organizer need to be accumulated, using evolutionarily important animals.

In the near future, we will be able to develop genomic, epigenomic, transcriptomic, and proteomic resources of organs, tissues, and embryos, using many organisms from different phyla. These resources will hopefully open the next door for evo–devo genomic studies. If we uncover binding profiles and regulatory principles of organizer TFs in invertebrates, we will be able to outline an evolutionary scenario of GRNs involved in establishing the vertebrate organizer.

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Chapter 32
Axis Formation and Its Evolution in Ray-Finned Fish

Masahiko Hibi, Masaki Takeuchi, Hisashi Hashimoto, and Takashi Shimizu

Abstract In teleost embryos, the formation of the body axes is controlled by both maternal and zygotic factors. During oogenesis, the formation of the oocyte's animal–vegetal polarity is maternally controlled. A mature oocyte contains a set of factors (dorsal determinants) involved in dorsal determination at the vegetal pole. After fertilization, a parallel array of microtubules forms briefly at the yolk's vegetal pole to transport the dorsal determinants to the prospective dorsal side. The dorsal determinants activate Wnt/β-catenin signaling and induce expression of dorsal-specific genes required for forming the dorsal organizer. The molecules expressed in the dorsal organizer antagonize the signaling of ventralizing or posteriorizing factors such as Bmps and Wnts, thereby establishing the signaling gradients that are subsequently required to properly form the dorsoventral (DV) and anteroposterior (AP) axes. Genetic analyses of zebrafish mutants have identified the maternal and zygotic genes that control formation of the body axes. Comparative studies of zebrafish, the primitive ray-finned fish bichir, the basal vertebrate lamprey, and the amphibian *Xenopus* indicate that bichir embryogenesis is a good model for understanding the evolution of DV axis formation. This chapter focuses on the genetic control of DV and AP axis formation, and its evolution in ray-finned fish.

Keywords Axis formation · Wnt · Bmp · Dorsal organizer · Zebrafish · Bichir

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32.1 Introduction

In vertebrate embryogenesis, the dorsoventral (DV), anteroposterior (AP), and other body axes are formed through an intricate developmental process involving signaling, transcriptional regulation, organelle transport, and cell differentiation and movement. The molecular mechanisms that form these axes have been relatively well studied in zebrafish and other model animals. A zebrafish oocyte has clear animal–vegetal (AV) polarity in which the blastodisc and the yolk are located at the animal (A) and vegetal (V) poles, respectively, but there is no apparent DV axis until the early gastrula stage, at around 6 hours postfertilization (hpf), when the embryo's dorsal side is marked by the embryonic shield (the dorsal organizer structure). However, the program that forms the DV axis is thought to be initiated soon after fertilization. Recent studies have revealed that oocyte AV polarity is linked to the formation of the embryonic DV axis. Dorsal determinants, which are deposited at the vegetal pole of the egg during oogenesis, are thought to be transferred after fertilization to the dorsal blastomeres, where they induce the dorsal-specific genes that establish the dorsal organizer. The organizer factors interact with ventralizing and posteriorizing signals to establish the embryonic DV and AP axes. In this chapter, we first describe the molecular mechanisms in zebrafish by which (1) oocyte AV polarity is established and linked to the embryo's DV axis; (2) the dorsal organizer is formed; and (3) interactions between the organizer factors and ventralizing factors determine the DV and AP body axes. Next, we compare the developmental processes of zebrafish with those of other vertebrate species—including bichir, lamprey, and *Xenopus*—and discuss the evolution of the germ layer and DV axis formation in ray-finned fish.

32.2 Embryonic Development of Zebrafish

Zebrafish sperm enters the oocyte at the animal pole. After fertilization, the chorion is detached from the egg surface, and cytoplasmic materials in the yolk are transported toward the animal pole to form the blastodisc. After the first blastodisc cleavage at 45 minutes postfertilization (mpf), synchronous cleavages occur every 15 min until the midblastula transition (MBT) at the 512-cell stage, at around 3 hpf (Kimmel et al. 1995). Zygotic gene expression is initiated at the MBT. Cell differentiation and morphogenetic processes start concomitantly, including (1) formation of the enveloping layer (EVL), which is an epithelial cell sheet covering the blastoderm; (2) formation of the yolk syncytial layer (YSL), which occurs when marginal blastomeres (connected to the yolk) collapse and release their nuclei into the yolk; and (3) epiboly, in which the blastomeres and EVL move to the vegetal pole to cover the yolk; this event starts slightly later than the MBT (Kimmel et al. 1995). Although the YSL is an extraembryonic structure, it is important in forming the germ layer. Transplanting the YSL to the animal pole of the blastoderm ectopically induces the

endoderm and mesoderm (Mizuno et al. 1996; Ober and Schulte-Merker 1999; Rodaway et al. 1999), and depletion of RNAs from the yolk inhibits mesoderm and endoderm formation (Chen and Kimelman 2000), indicating that the YSL functions to induce the endoderm and mesoderm (Sakaguchi et al. 2002). The YSL attaches to the EVL, possibly through E-cadherin, and takes the EVL and the blastoderm to the vegetal pole during epiboly (Shimizu et al. 2005b; Solnica-Krezel and Driever 1994).

During gastrulation, the embryonic structure is further established with a series of cell movements: involution/ingression of the mesendoderm, convergent extension, and migration of the axial mesoderm, in addition to epiboly (Solnica-Krezel and Sepich 2012). Mesoderm convergence and extension bring more cells to the dorsal side to form the dorsal thick (axial) mesendoderm. The axial mesendoderm elongates in the AP axis to become the prechordal plate and notochord. Various signaling pathways, including the Wnt/PCP (planar cell polarity) pathway, are involved in convergent extension [see review by Solnica-Krezel and Sepich (2012)]. Signaling molecules generated from the embryo's dorsal and ventral regions act in coordination with gastrulation movements to establish the DV and AP axes.

32.3 Establishment of Oocyte Animal–Vegetal Polarity During Oogenesis in Zebrafish

Mature zebrafish oocytes contain dorsal determinants at the vegetal pole (see Sect. 32.4.2). Thus, the formation of AV polarity in oocytes is important for forming the embryonic DV and AP axes. A zebrafish oocyte develops through four stages until it is mature and competent for fertilization (Lessman 2009; Marlow 2010; Nagahama and Yamashita 2008). Oocyte AV polarity begins to form during stage I with the appearance of the Balbiani body (Fig. 32.1a), which is composed of a variety of messenger RNAs (mRNAs), proteins, and organelles, including mitochondria. The Balbiani body initially localizes adjacent to the nucleus on the future vegetal pole side and then moves to the vegetal pole and releases its contents (e.g., mRNAs) to the vegetal cortex (Bontems et al. 2009; Marlow and Mullins 2008). In *Xenopus* and zebrafish oocytes, some germline-specific transcripts are transported via the Balbiani body to the vegetal pole (Wilk et al. 2005; Kloc et al. 1996, 2001; Kloc and Etkin 1995; Kosaka et al. 2007).

Genetic studies with a maternal mutant have revealed that formation of the Balbiani body is controlled by Bucky ball, a protein that lacks obvious sequence similarities to other proteins (Bontems et al. 2009; Marlow and Mullins 2008). Oocytes with a *bucky ball* mutation fail to form the Balbiani body, to localize germline mRNAs to the vegetal pole, and to establish AV polarity (Bontems et al. 2009; Marlow and Mullins 2008). In these *bucky ball*–mutant oocytes, mRNAs that normally localize to the vegetal pole localize radially instead (Bontems et al. 2009; Dosch et al. 2004; Nojima et al. 2010). The vegetal pole localization of the transcripts

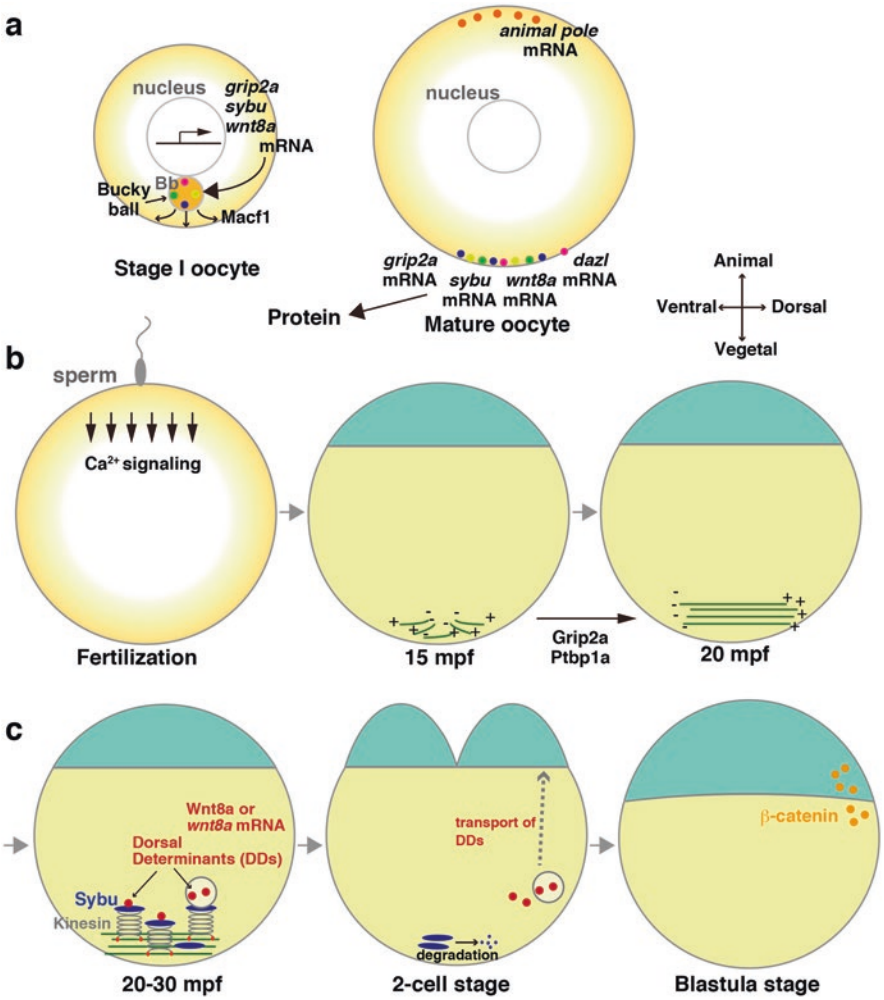

Fig. 32.1 Maternally controlled formation of the oocyte animal–vegetal (AV) axis and embryonic dorsoventral (DV) axis in zebrafish. (**a**) Formation of the oocyte AV axis. In the stage I oocyte, messenger RNAs (mRNAs) involved in dorsal determination and germ cell development are transcribed in the nucleus and deposited in the Balbiani body (Bb); these include glutamate receptor interacting protein 2a (*grip2a*), *syntabulin* (*sybu*), *wnt8a*, and *daz-like* (*dazl)* mRNAs. During oocyte maturation, mRNAs are transferred from the Balbiani body to the vegetal cortex. (**b**) Microtubule-dependent dorsal determination. Sperm entry activates Ca^{2+} signaling, which travels from the animal pole to the vegetal pole, where it induces microtubule formation. The microtubules are bundled to form an array at around 20 minutes postfertilization. (**c**) Dorsal determinants (DDs) or vesicles containing DDs are transported through the vegetal microtubule array. At the early cleavage stage, an unknown mechanism transports DDs (or vesicular DDs) to prospective dorsal blastomeres, where they activate canonical Wnt signaling, which causes β-catenin to accumulate at the blastula stage (Figure modified from Hibi et al. 2002; Langdon and Mullins 2011; Nojima et al. 2010)

for glutamate receptor interacting protein 2a (Grip2a) and Syntabulin, which are reported to be involved in dorsal determination, is dependent on Bucky ball (Nojima et al. 2010; Ge et al. 2014); *wnt8a* transcripts, which are probably involved in dorsal determination (Sect. 3.2), are also transferred from the Balbiani body to the vegetal pole (Lu et al. 2011). These data suggest that the localization of mRNAs to the vegetal pole, which depends on the Balbiani body, may be indispensable in dorsal determination. Study of another maternal mutant, *magellan*, has revealed that microtubule–actin crosslinking factor 1a (Macf1a) regulates oocyte AV polarity, possibly through microtubule-dependent transport of Balbiani body components (Gupta et al. 2010).

32.4 Microtubule-Dependent Dorsal Determination in Zebrafish

32.4.1 Microtubule Array Formation

An array of parallel microtubules forms at the vegetal pole of the zebrafish embryo at around 20 mpf (Jesuthasan and Stahle 1997) (Fig. 32.1b). Disruption of the microtubules by nocodazole treatment, cold temperatures, or ultraviolet irradiation at this point causes a loss of the dorsal organizer and ventralization of the embryo (Jesuthasan and Stahle 1997). Removal of the vegetal yolk mass at the early one-cell stage also severely ventralizes the embryo (Mizuno et al. 1999; Ober and Schulte-Merker 1999). These data have established the hypothesis that dorsal determinants initially localize to the vegetal pole and are then transported along the vegetal microtubules to the prospective dorsal side, where they activate the genetic program(s) that induce dorsal tissue.

The relevance of the vegetal microtubule array in dorsal determination was initially proposed for *Xenopus* embryogenesis. After fertilization, the *Xenopus* egg cortex rotates relative to the sperm entry point during the first cell cycle (cortical rotation). Microtubules initially appear to be randomly oriented at the vegetal cortex. However, during cortical rotation, the cortical microtubules become aligned with the plus ends toward the prospective dorsal side (Olson et al. 2015). During this process, organelles are transported along the microtubule array. Inhibition of microtubule formation disrupts dorsal tissue formation (Elinson and Rowning 1988; Houliston and Elinson 1991; Rowning et al. 1997), supporting the microtubule array's role in transporting dorsal determinants (or organelles containing the dorsal determinants) in *Xenopus* embryos. In *Xenopus*, sperm entry is proposed to control the orientation of vegetal microtubules by providing nascent microtubules originating from the sperm-derived centrosome (aster) (Houliston and Elinson 1991; Schroeder and Gard 1992).

In zebrafish, the micropyle is located at the animal pole region of the chorion, and the sperm can enter only into the animal pole of the egg. No microtubules are

seen in the area between the pronucleus (its associated centrosome) and the vegetal cortex. Therefore, the sperm entry provides little, if any, information regarding the bias of vegetal microtubule orientation. Nevertheless, the plus ends of the vegetal microtubules that form at around 20–30 mpf are oriented to the prospective dorsal side (within 30° of the embryonic shield) (Tran et al. 2012) (Fig. 32.1b). Vegetal microtubule formation depends on Ca^{2+} signaling, which is potentially activated by sperm entry (Tran et al. 2012) (Fig. 32.1b). Vesicular structures (called cortical granules) are transported along the dorsal-oriented microtubules to the prospective dorsal side (Tran et al. 2012). Some mRNAs that localize to the vegetal pole, such as *wnt8a* mRNA, are translocated slightly to the prospective dorsal side. Although it is not clear how the vegetal microtubules are oriented to the prospective dorsal side, these data suggest that microtubule-dependent transport and a cortical rotation–like movement take place in zebrafish. These events are likely to be involved in dorsal determination in zebrafish, as in *Xenopus* (Fig. 32.1c). However, the vegetal microtubules do not reach the blastodisc (Tran et al. 2012), implying that the vegetal microtubule-dependent mechanism only provides a bias in positioning the dorsal determinants at the vegetal pole, and that other mechanism(s) translocate the dorsal determinants to the dorsal blastomeres.

32.4.2 Mechanisms That Control Microtubule Array Formation and Transport of Dorsal Determinants

Studies of maternal effect mutations in zebrafish that affect the initial dorsal determination have identified molecules that function in microtubule-dependent dorsal determination (Table 32.1, Fig. 32.1b). The *hecate* mutants are deficient in genes that encode Grip2a, an adaptor protein that contains multiple PDZ domains (Ge et al. 2014), and whose mRNA localizes to the vegetal pole. The *hecate* mutant embryos fail to bundle microtubules at the vegetal pole and do not form a parallel microtubule array, implying that Grip2a is involved in bundling the vegetal microtubules (Ge et al. 2014). Embryos with the maternal effect mutant *brom bones*, which are deficient in the gene encoding polypyrimidine tract binding protein 1a (Ptbp1a, also known as hnRNPI), are also severely ventralized. Embryos with a *brom bones* mutation do not activate inositol 1,4,5-triphosphate (IP_3)-mediated Ca^{2+} release, do not undergo exocytosis of the cortical granules, and do not form a vegetal microtubule array (Mei et al. 2009), suggesting that Ptbp1a is involved in processing the pre-RNA for IP_3–Ca^{2+} signaling components. It has been suggested that the persistent cortical granules in the vegetal cortex of *brom bones* mutants secondarily affect the formation of the vegetal microtubule array (Mei et al. 2009). However, it is also possible that the IP_3-dependent activation of Ca^{2+} signaling plays a role in formation of the vegetal microtubule array. In any case, these data provide genetic evidence that the parallel microtubule array plays a pivotal role in dorsal determination. Embryos of the maternal effect mutant *tokkaebi* also show

Table 32.1 Zebrafish maternal effect mutants and the role of the mutant loci in axis formation

Gene	Mutant	Gene product	Role in axis formation	Other function
(A) Regulation of oocyte animal–vegetal axis				
bucky ball	*bucky ball*	Cytoplasmic, no known homologues	Balbiani body formation	Germ cell development
macf1a	*magellan*	Microtubule–actin crosslinking factor 1a	Microtubule-dependent transport of Balbiani body	
(B) Regulation of embryonic dorsoventral axis				
ptbp1a	*brom bones*	Polypyrimidine tract binding protein 1a	Formation of vegetal microtubule array	IP_3-dependent Ca^{2+} release
grip2a	*hecate*	Glutamate receptor interacting protein 2a, PDZ-containing adaptor	Bundling of vegetal microtubules	Germ cell development in *Xenopus*[a]
syntabulin[b]	*tokkaebi*	Syntaxin-interacting, linker for kinesin-1 motor protein	Microtubule-dependent transport	Axonal transport, germ cell development
β-catenin 2	*ichabod*	One of the two β-catenin genes in fish, downstream from the canonical Wnt pathway	Regulation of dorsal-specific genes	
kif5Ba	*kif5Ba*	Heavy chain of kinesin-1	Bundling of vegetal microtubules	Microtubule-dependent transport

IP_3 inositol 1,4,5-triphosphate

[a]Knocking down *Xenopus* Grip2.1 (XGRIP2.1) reduces the number of primordial germ cells (Tarbashevich et al. 2007)

[b]Syntabulin is involved in microtubule-dependent axonal transport of synaptic vesicles and mitochondria in cultured rat neurons (Cai et al. 2005; Su et al. 2004). Syntabulin is also involved in dorsal axis formation; *syntabulin* messenger RNA localizes to the germ plasm and is expressed later in primordial germ cells in *Xenopus* (Colozza and De Robertis 2014)

ventralization but do not display abnormalities in formation of the vegetal microtubule array (Nojima et al. 2004). The *tokkaebi* locus encodes Syntabulin, which is a linker for the kinesin motor protein and is involved in microtubule-dependent transport of organelles in neurons (Cai et al. 2005; Su et al. 2004). Since *syntabulin* mRNA is localized to the vegetal pole, Syntabulin protein is also localized to the vegetal pole until the vegetal microtubules form, after which Syntabulin is translocated from the vegetal pole in a microtubule-dependent manner and is degraded at the two-cell stage (Nojima et al. 2010). These data suggest that Syntabulin assists in transporting the dorsal determinants—or the organelles (e.g., vesicles) containing the dorsal determinants—along the vegetal microtubules and in releasing them from the vegetal microtubules after the two-cell stage. It was recently reported that maternal effect mutants of *kif5Ba*, which encodes a heavy chain of Kinesin-1, showed abnormal formation of the vegetal microtubules (random orientation or nonbundled), aberrant localization of Syntabulin and *wnt8a* mRNA, and ventralized

phenotypes (Campbell et al. 2015), suggesting that Kinesin-1 plays an important role in the vegetal microtubule formation and the subsequent microtubule-dependent dorsal determination.

32.4.3 Dorsal Determinants

The canonical Wnt pathway, which results in β-catenin accumulation, is believed to play an essential role in dorsal determination in *Xenopus* and zebrafish embryos. In these animals, activating the canonical Wnt pathway elicits ectopic formation or expansion of the dorsal organizer, whereas its inhibition impairs dorsal axis formation (Hibi et al. 2002). Furthermore, the maternal effect mutant *ichabod*, which lacks the expression of maternal β-catenin 2, fails to establish dorsal tissues (Kelly et al. 2000). In zebrafish, β-catenin accumulation is detected in the nuclei of dorsal blastomeres by the 128-cell stage, and in the dorsal blastoderm and dorsal YSL of midblastula-stage embryos (Dougan et al. 2003; Schneider et al. 1996). These data suggest that dorsal determinants activate the canonical Wnt pathway to induce dorsal-specific genes. It was initially thought that Wnt molecules may not be directly involved in dorsal determination, since inhibition of Wnt molecules by either dominant negative Wnt1/8 or a secreted Frizzled-related protein, Frzb1, did not suppress dorsal axis formation in *Xenopus* embryos (Tao et al. 2005; Hoppler et al. 1996; Leyns et al. 1997; Wang et al. 1997). However, maternal Wnt11 was shown to activate the canonical Wnt pathway, and the Wnt11/5a complex has been suggested to be a dorsal determinant in *Xenopus* (Cha et al. 2008; Cha et al. 2009). Neither *wnt11* nor *wnt5a* can activate the canonical Wnt pathway and induce dorsal-specific genes in zebrafish (Lu et al. 2011; Nojima et al. 2010). In zebrafish, *wnt8a* mRNA localizes to the egg's vegetal pole (Lu et al. 2011) and then is translocated from the vegetal pole in a microtubule-dependent manner. Expression of a dominant negative Wnt8a abolishes the expression of the dorsal-specific gene *chordin* (Lu et al. 2011; Ge et al. 2014; Tran et al. 2012), suggesting that *wnt8a* mRNA is a dorsal determinant. Many vegetal mRNAs and proteins are translocated by the movement accompanying cortical rotation. It is not clear whether it is the *wnt8a* mRNA or Wnt8a protein that acts as a dorsal determinant. It also remains to be elucidated how Wnt8a is transported to the dorsal blastoderm. Before the 128-cell stage, *wnt8a* mRNA is not detected in dorsal blastomeres, yet Wnt8a protein may be translated and transported to the dorsal blastomeres before that stage. Genetic analyses of maternal *wnt8a* mutants and detailed localization analyses of *wnt8a* mRNA or Wnt8a protein should provide compelling evidence for the role of Wnt8a in dorsal determination. In any case, Wnt8a may play a major role in activating the canonical Wnt pathway (Fig. 32.1c).

32.5 Program for Forming the Dorsal Organizer in Zebrafish

32.5.1 Wnt Signaling and Dharma

The canonical Wnt pathway induces dorsal-specific genes by binding a complex of Tcf/Lef family protein and β-catenin to their promoter/enhancer regions at the MBT; this process is controlled by both positive and negative regulators. Caveolin-1 is reported to inhibit the nuclear translocation of β-catenin, and Tob1a inhibits the formation of the Tcf/Lef and β-catenin complex (Mo et al. 2010; Xiong et al. 2006).

The dorsal-specific gene *dharma* (also known as *nieuwkoid*) encodes a homeodomain-containing transcriptional repressor, which harbors an Engrailed homology 1 (Eh1) repressor motif (Koos and Ho 1998; Yamanaka et al. 1998), The *dharma*-defective mutant *bozozok* exhibits various degrees of defects in the dorso-anterior tissues, including defects in organizer formation (Fekany et al. 1999; Koos and Ho 1999). At the MBT, *dharma* is expressed in the dorsal blastoderm; thereafter, its expression is confined to the dorsal YSL at the early gastrula stage (Koos and Ho 1998; Yamanaka et al. 1998). The canonical Wnt pathway induces *dharma* expression. The *dharma* promoter/enhancer contains many Tcf/Lef-binding sites involved in dorsal-specific *dharma* expression (Leung et al. 2003b; Ryu et al. 2001; Shimizu et al. 2000), indicating that *dharma* is a direct target of the maternal canonical Wnt pathway (Fig. 32.2a). Dharma represses the ventral expression of the homeobox genes *vox*, *vent*, and *ved* (Kawahara et al. 2000a, b; Imai et al. 2001; Shimizu et al. 2002). The expression of *vox*, *vent*, and *ved* is positively regulated by the maternal factor Runx2b type2 (Runx2bt2) (Flores et al. 2008). Vox, Vent, and Ved are also transcriptional repressors, which repress dorsal organizer genes such as *goosecoid* and *chordin* (Shimizu et al. 2002; Imai et al. 2001; Melby et al. 2000). Hence, the Dharma-mediated repression of *vox*, *vent*, and *ved* releases the expression of dorsal organizer genes in the dorsal blastomeres. Vox, Vend, and Ved also repress *dharma* expression. Thus, the mutual repression of *vox/vent/ved* and *dharma* refines the dorsal organizer domain (Kawahara et al. 2000a, b; Imai et al. 2001; Shimizu et al. 2002). The SoxB1 transcription factors Sox3 and Sox19b also restrict *dharma* expression (Shih et al. 2010). Dharma not only represses *vox/vent/ved* expression but also directly represses the gene expression of the ventralizing factors Bmp2b and Wnt8a (Erter et al. 2001; Leung et al. 2003a). The Dharma-mediated inhibition of Bmp and Wnt signals may contribute to Dharma's non-cell-autonomous role in forming dorsal and anterior tissues (Koos and Ho 1998; Yamanaka et al. 1998). The stability of the Dharma protein is regulated by protein degradation mediated by the E3 ubiquitin ligase Lnx2b (Ro and Dawid 2009). The regulation of *dharma* expression and its role in DV axis formation are summarized in Fig. 32.2a.

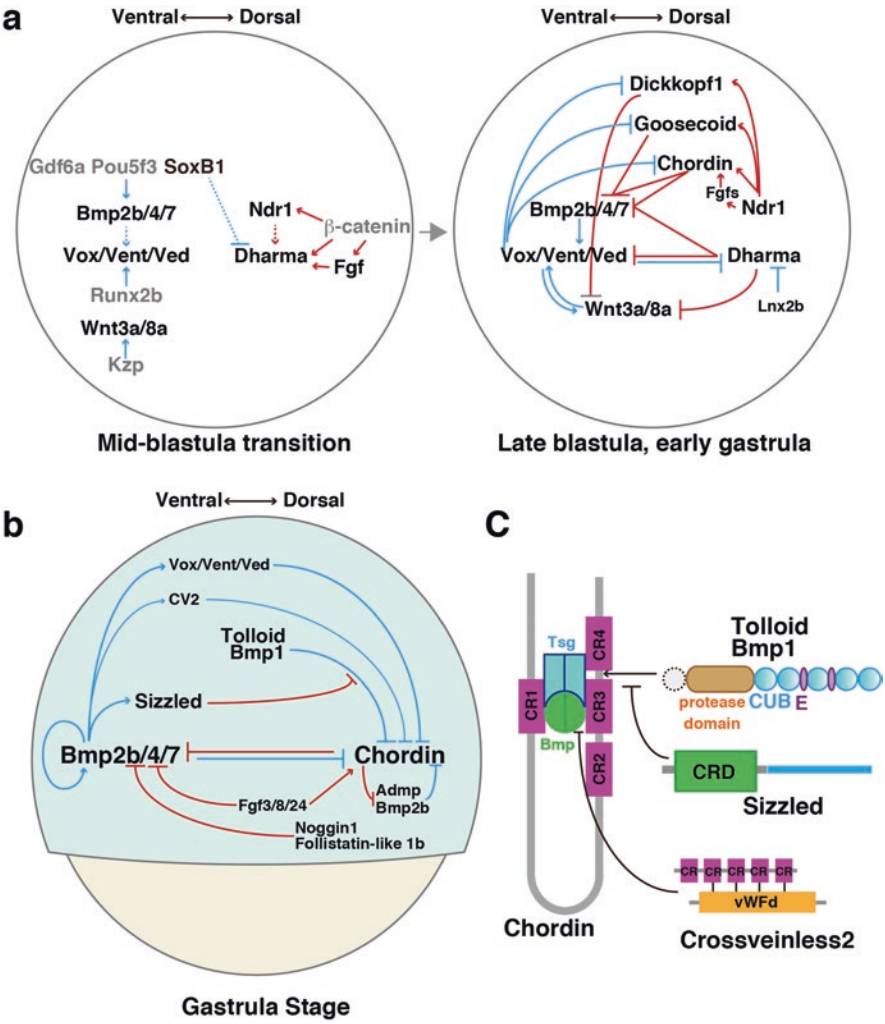

Fig. 32.2 Molecular mechanisms controlling dorsoventral (DV) axis formation in zebrafish. (**a**) Control of DV axis formation at the midblastula transition (*left panel*) and at the late blastula to early gastrula stages (*right panel*). Expression of the ventralizing factors Bmp2b/4/7, Vox/Vent/Ved, and Wnt3a/8a is regulated by the maternal factors Gdf6a/Pou5f3, Runx2b, and Kzp, respectively. Expression of the dorsalizing factors Dharma and Ndr1 is regulated by maternal Wnt signaling. The mutual repressive interaction between Dharma and Vox/Vent/Ved defines the DV axis. Dharma releases the expression of dorsal organizer genes by suppressing Vox/Vent/Ved-mediated repression of these genes. (**b**) Regulation of DV axis formation at the gastrula stage. Dorsalizing and ventralizing signals are marked by *blue lines* and *red lines*, respectively; maternal factors are indicated by *gray letters*. (**c**) Regulation of Chordin and Bmps. Chordin has four cysteine-rich (CR) domains. Tolloid and Bmp1—which have proteinase, CUB, and epidermal growth factor–like domains (E)—cleave Chordin protein. Sizzled, which has a CR domain similar to that of Frizzled, binds Bmp1 and inhibits Bmp1-dependent (and possibly Tolloid-dependent) Chordin degradation. Crossveinless 2 (CV2) contains CR domains and a von Willebrand factor domain (vWFd). The cleaved form of CV2 suppresses Chordin-mediated Bmp inhibition and thus displays pro-Bmp activity. Twisted gastrulation (Tsg) binds Chordin and Bmps, and promotes Bmp signaling. Tsg enhances Tolloid-dependent Chordin degradation (Langdon and Mullins 2011; Muraoka et al. 2006; Shimizu et al. 2000)

32.5.2 Nodal-Related Genes

The Nodal-related gene *ndr1* (also known as *squint*) is also thought to be a target of the maternal canonical Wnt pathway (Shimizu et al. 2000; Kelly et al. 2000). The dorsal expression of *dharma* and *ndr1* is lost in *ichabod* mutants, which are defective in β-catenin 2; β-catenin rescues the expression of these two genes (Kelly et al. 2000), supporting the idea that *dharma* and *ndr1* function downstream from the maternal Wnt pathway. Ndr1 functions with another Nodal-related gene, *ndr2* (known as *cyclops*), to form the endoderm and the dorsal mesoderm (Dougan et al. 2003; Erter et al. 1998; Rebagliati et al. 1998a, b; Sampath et al. 1998; Feldman et al. 1998; Schier and Talbot 2005). A combined deficiency of *dharma* and *ndr1* (zygotic combined mutations) severely reduces the dorsoanterior tissues (Shimizu et al. 2000; Sirotkin et al. 2000), revealing that *dharma* and *ndr1* play major roles in forming the dorsal organizer and the dorsoanterior tissues (Fig. 32.2a). In addition to this zygotic Ndr1 function, a role has also been proposed for maternal *ndr1* transcripts in dorsal determination. Maternally deposited *ndr1* mRNA is distributed to the two prospective dorsal blastomeres at the four-cell stage; morpholino-mediated *ndr1* knockdown causes severe ventralization (Gore et al. 2005), suggesting that maternal Ndr1 functions in dorsal determination. However, arguing against this role of Ndr1, dorsal axis formation is not severely defective in maternal effect *ndr1* mutants that cannot generate the Ndr1 protein (Erter et al. 2001; Feldman et al. 1998; Heisenberg and Nusslein-Volhard 1997; Amsterdam et al. 2004). It was recently suggested that a noncoding function of *ndr1* RNA might activate the maternal canonical Wnt pathway, although the mechanism remains elusive (Lim et al. 2012).

32.5.3 Fgf Signaling

The fibroblast growth factors (Fgf) *fgf3*, *fgf8*, and *fgf24*—which are expressed in the dorsal marginal blastomeres in the blastula period—control dorsal axis formation by repressing *bmp* gene expression (Furthauer et al. 1997, 2004). In *ichabod* mutants, β-catenin-dependent expression of the organizer genes depends on Fgf signaling. Fgf signaling is also involved in Ndr1-dependent *chordin* expression and in maintaining *dharma* expression (Maegawa et al. 2006) (Fig. 32.2a). Therefore, the *fgf* genes function in dorsal axis formation downstream from the maternal canonical Wnt pathway. The Fgf signaling in dorsal axis formation is negatively regulated by Sef, Sprouty2, and Dusp6 (Mkp3, a mitogen-activated protein kinase [MAPK] phosphatase), which function as feedback regulators (Furthauer et al. 2002, 2004; Tsang et al. 2002, 2004). Precise control of the Fgf signaling gradient should contribute to the regulation of Bmp signaling along the DV axis. Fgf signaling, together with Wnt and retinoic acid signaling, also controls the posteriorization of embryos (Koshida et al. 1998, 2002; Shimizu et al. 2005a, 2006; Kudoh et al. 2002, 2004) (Fig. 32.3c, d).

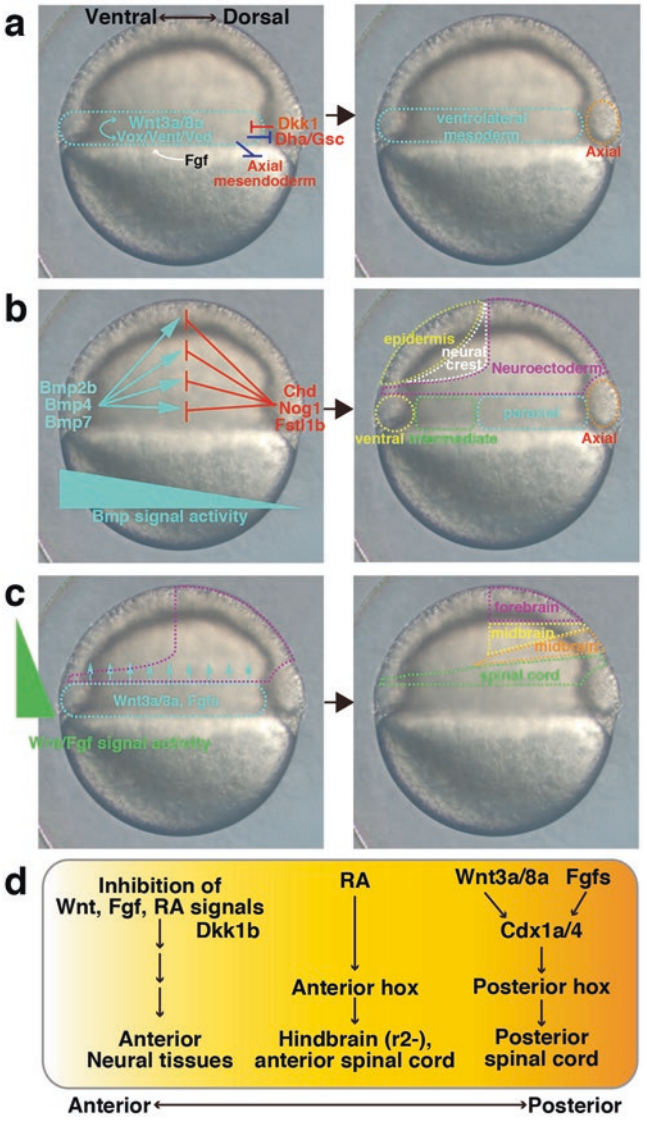

Fig. 32.3 Fate determination according to dorsoventral (DV) and anteroposterior (AP) positional information in zebrafish. (**a**) Wnt signaling controls the fates of the ventrolateral versus axial mesendoderm. Wnts and Vox/Vent/Ved are involved in forming the ventrolateral mesoderm. Dkk1, Dharma, and Goosecoid control the formation of axial mesendoderm tissue by inhibiting Wnts and Vox/Vent/Ved. (**b**) A Bmp signal gradient controls cell fates on the DV axis. Strong Bmp activity is required for formation of the ventral mesoderm and epidermis. Bmp inhibition is required for neuroectoderm formation. (**c**) Control of AP axis formation in the neuroectoderm. The posterior region of the neuroectoderm, which receives Wnt and fibroblast growth factor (Fgf) signaling from the nonaxial mesoderm, is fated to become posterior neural tissue such as the spinal cord or hindbrain. The anterior (animal pole) region of the neuroectoderm, which does not receive posterior-

32.6 Interactions Between the Dorsal Organizer and Ventralizing/Caudalizing Factors in Zebrafish

32.6.1 Goosecoid

The Wnt target genes (e.g., *dharma*, *ndr1*, and *fgf*s) cooperatively regulate the expression of dorsal organizer genes such as *goosecoid*, *chordin*, and *dickkopf1* (*dkk1*) (Hashimoto et al. 2000; Maegawa et al. 2006; Shimizu et al. 2000; Sirotkin et al. 2000) (Fig. 32.2a). The expression of *goosecoid* and *dkk1* depends on both Dharma and Nodal signaling (Hashimoto et al. 2000; Shimizu et al. 2000). However, *chordin* expression depends more on Dharma than on Nodal signaling, as *chordin* expression is relatively well maintained in Nodal signal–deficient embryos, such as the maternal zygotic *one-eyed pinhead* (*oep*) mutant (Gritsman et al. 1999; Shimizu et al. 2000). Goosecoid is a homeodomain-containing transcription factor, which harbors an Eh1 repressor motif and exhibits sequence similarity to Dharma (Cho et al. 1991; Stachel et al. 1993). Goosecoid can inhibit Bmp signaling even in the absence of the secreted Bmp inhibitors Chordin, Noggin1, and Follistatin-like 1b (Dixon Fox and Bruce 2009) (Fig. 32.2a). Thus, it is possible that Goosecoid directly binds *bmp* gene promoters/enhancers to negatively regulate their expression in dorsal blastomeres, like Dharma. Although the *goosecoid* gene exists in most, if not all, vertebrate genomes, *dharma* is found only in teleost genomes. It is conceivable that *goosecoid* was duplicated during teleost-specific whole-genome duplication (WGD), and that the expression and function of the two genes were diversified during teleost evolution: *dharma* came to be expressed earlier, *goosecoid* came to function at a later stage, and the expression of *goosecoid* came to depend on *dharma*. This hypothesis could explain the variable expressivity and penetrance of the *bozozok* mutants (Fekany et al. 1999) because *dharma* may partly function redundantly with *goosecoid*.

32.6.2 Chordin and Other Bmp Antagonists Regulate Bmp Signaling

Chordin, which is a secreted Bmp inhibitor containing four cysteine-rich domains, binds Bmp dimers, thereby inhibiting Bmp's binding to its receptors (Piccolo et al. 1996; Sasai et al. 1994; De Robertis 2009). Among the known Bmp inhibitors,

Fig. 32.3 (continued) izing signals, becomes anterior neural tissues such as the forebrain and midbrain. (**d**) Molecular mechanisms controlling AP axis formation during early neural development. Wnts and Fgfs from the nonaxial mesoderm regulate the expression of the caudal-related homeodomain proteins Cdx1a and Cdx4, which induce the posterior *hox* genes that determine the fate of the posterior spinal cord. Retinoic acid (RA), generated by the anterior paraxial mesoderm, controls expression of the anterior *hox* genes required for fate determination of the hindbrain (posterior from rhombomere 2) and the anterior spinal cord. Posteriorizing signals (Wnt, Fgf, and RA) must be inhibited to induce anterior neural tissues

Chordin functions nonredundantly in DV axis formation (Dal-Pra et al. 2006; Hammerschmidt et al. 1996; Schulte-Merker et al. 1997). The genes encoding the Bmp inhibitors Noggin1 and Follistatin-like 1b are also expressed in the dorsal organizer region at the blastula and early gastrula stages (Dal-Pra et al. 2006; Furthauer et al. 1999). These genes function redundantly with Chordin: knocking the genes down separately does not cause ventralization, but knockdown of the two genes along with *chordin* results in strong ventralization (Dal-Pra et al. 2006; Furthauer et al. 1999). Compared with Noggin1 and Follistatin-like 1b, Chordin has unique features: (1) *chordin* is expressed more broadly than other Bmp inhibitor genes and is negatively controlled by Bmp signaling (Miller-Bertoglio et al. 1997; Schulte-Merker et al. 1997); and (2) the stability of the Chordin protein is precisely regulated by a mechanism involving Tolloid/Bmp1 family proteinase, Sizzled, Twisted gastrulation, and other proteins (Blader et al. 1997; Connors et al. 1999, 2006; Jasuja et al. 2006; Little and Mullins 2004; Muraoka et al. 2006; Xie and Fisher 2005; Yabe et al. 2003) (Fig. 32.2b, c). These features make it likely that Chordin is a nonredundant factor that regulates Bmp signaling and DV axis formation.

In addition to Chordin, Crossveinless 2 (CV2)—a Chordin family protein that has von Willebrand factor C domains and is expressed ventrally—also modulates Bmp activity. Noncleaved CV2 functions as a Bmp inhibitor, but the cleaved form binds both Bmp and Chordin and suppresses Chordin-mediated Bmp inhibition (pro-Bmp activity) (Rentzsch et al. 2006; Zhang et al. 2010). The expression of the *bmp* genes *bmp2b*, *bmp4*, and *bmp7a* is regulated by the maternal factors Gdf6a (also known as Radar, a Bmp-related cytokine), Pou5f3 (also known as Pou2 or Spiel ohne grenzen, an Oct3/4 orthologue or paralogue) (Reim and Brand 2006; Sidi et al. 2003), and the zygotic SoxB1 transcription factors Sox2/3/19a/19b (Okuda et al. 2010) (Fig. 32.2a). *bmp* gene expression is also self-regulated by Bmp signaling (De Robertis 2009). Interactions between Chordin, the Chordin regulators, ventrally expressed Bmps (Bmp2b, Bmp4, and Bmp7a), and dorsally expressed Bmps (Bmp2b and Admp) define a clear Bmp signaling gradient that is required for cell differentiation along the DV axis (Fig. 32.3b). The control of Bmp signaling is beyond the scope of this chapter; for excellent reviews on the regulation of Bmp signaling in zebrafish axis formation, see Langdon and Mullins (2011) and Bier and De Robertis (2015).

32.6.3 Role of Wnt Inhibition in Forming the DV and AP Axes

Although maternal canonical Wnt signaling is involved in initiating dorsal axis formation, zygotic canonical Wnt signaling negatively regulates dorsal axis formation and positively regulates formation of posterior tissues. The mechanism by which maternal and zygotic Wnt signals regulate a different set of genes and exhibit opposite functions in axis formation has not been defined. During gastrulation, *wnt8a*

and *wnt3a* are expressed throughout the blastoderm margin except in the most dorsal region, which corresponds to the embryonic shield; this expression persists in the tailbud at the end of gastrulation (Kelly et al. 1995; Lekven et al. 2001; Shimizu et al. 2005a). The expression of *wnt8a* is at least partly regulated by the maternal transcription factor Kzp (Kaiso zinc finger–containing protein) (Yao et al. 2010) (Fig. 32.2a). The zebrafish *wnt8a* gene has two open reading frames (ORFs), which are located tandemly in the zebrafish genome and can function as a bicistronic transcript (Lekven et al. 2001). Inhibition of both *wnt8a* ORFs reduces the ventrolateral mesoderm and tail structure, and enlarges the head structure (Lekven et al. 2001). Wnt3a functions redundantly with Wnt8a. Inhibition of both Wnt3a and the Wnt8a ORFs causes more severe phenotypes than inhibition of Wnt8a alone: the ventrolateral mesoderm is reduced, while the axial mesoderm expands, and the tail structure is lost, while the head structure expands (Shimcizu et al. 2005a; Thorpe et al. 2005). Inhibition of β-catenins 1 and 2 expands *chordin* expression and causes severe dorsalization (Varga et al. 2007). These data suggest that zygotic (gastrula) canonical Wnt signaling plays two roles in axis formation: (1) it controls the fate of the axial versus ventrolateral mesoderm for the DV axis, since Wnt induces the ventrolateral mesoderm; and (2) it functions as a posteriorizing signal for the AP axis (Fig. 32.3a, c). The ventral expression of *vox*, *vent*, and *ved* is regulated by Wnt8a/Wnt3a signaling, and *wnt8a* and *wnt3a* expression is regulated by Vox/Vent/Ved (Ramel and Lekven 2004; Shimizu et al. 2005a). The cross-regulation between Wnt signaling and Vox/Vent/Ved may refine the fate determination of axial versus ventrolateral mesoderm tissue; Dharma restricts the axial mesoderm by repressing these ventralizing signals (Figs. 32.2a and 32.3a). For posteriorization, the caudal-related genes *cdx1a* and *cdx4* function downstream from Wnt signaling; inhibition of Cdx1a and Cdx4 severely truncates the posterior, as with inhibition of Wnt8a/Wnt3a (Shimizu et al. 2005a) (Fig. 32.3d). The loss of Cdx1a/Cdx4 also leads to ectopic formation of hindbrain tissue (Shimizu et al. 2006; Skromne et al. 2007), indicating that Cdx1a and Cdx4 function downstream from Wnt8a/3a to control posterior tissue formation and repress anterior tissues (Fig. 32.3d). The Sp1 family transcription factors Sp5a and Sp5l also function downstream from Wnt signaling, for both the DV and AP axes (Thorpe et al. 2005; Weidinger et al. 2005). Further studies are necessary to reveal the relationships between Sp5/5l and Vox/Vent/Ved, or Sp5/Sp5l and Cdx1a/Cdx4, for DV and AP axis formation (Table 32.2).

The dorsal organizer expresses the Wnt inhibitor Dkk1 (Glinka et al. 1998), which binds the Wnt lipoprotein receptor proteins 5 and 6 (LRP5/6) and Kremen, and downregulates Wnt signaling (Davidson et al. 2002; Mao et al. 2002). Zebrafish have two *dkk1* genes (Untergasser et al. 2011). Initially, *dkk1b* is expressed in the dorsal marginal blastoderm and the dorsal YSL at the blastula stage, and then in the prechordal plate at the gastrula stage (Hashimoto et al. 2000; Shinya et al. 2000). Canonical Wnt signaling also controls *dkk1b*, thus functioning as a negative feedback regulator (Shinya et al. 2000). In the blastoderm margin, *dkk1b* expression may be regulated by Wnt8a and Wnt3a (Hashimoto et al. 2000; Shinya et al. 2000). Thus, the Wnt8a/Wnt3–Dkk1 system is part of a reaction–diffusion mechanism that forms a Wnt signal gradient; a similar role has been proposed for Dkk in hair follicle

Table 32.2 Molecular players in dorsoventral and anteroposterior axis formation in zebrafish

Molecule	Nature of molecule	Role in axis formation
Admp	Secreted protein	Ventralization, a Bmp family member
Bmp2b/4/7	Secreted protein	Ventralizing factor
Caveolin-1	Transmembrane protein	Inhibition of nuclear translocation of β-catenin
Chordin	Secreted protein	Inhibition of Bmp signaling
Cdx1a/4	Transcription factor	Posteriorization
CV2	Secreted protein	Inhibition and promotion of Bmp signaling
Dharma	Transcriptional repressor	Repression of ventral genes
Dickkopf1	Secreted protein	Inhibition of Wnt signaling
Dusp6	Cytoplasmic protein	Inhibition of Fgf signaling, a MAPK phosphatase
Eomesa	Transcription factor	Endoderm formation
Fgf3/Fgf8/Fgf24	Secreted protein	Dorsalization, posteriorization, and mesoderm formation
Follistatin-like 1b	Secreted protein	Inhibition of Bmp signaling
Gdf6a	Secreted protein	Ventralization (maternal factor for *bmp* expression)
Goosecoid	Transcriptional repressor	Repression of ventral genes
Kremen1	Transmembrane protein	Dkk-mediated inhibition of Wnt signaling
Kzp	Transcription factor	Regulation of *wnt3a/8a* expression
Lnx2b	Ubiquitin ligase	Degradation of Dharma
LRP5/6	Transmembrane protein	Coreceptor for Wnt signaling
Ndr1/2	Secreted protein	Nodal-related molecules, organizer and mesendoderm induction
Noggin1	Secreted protein	Inhibition of Bmp signaling
One-eyed pinhead	Membrane-attached protein (GPI anchored)	Coreceptor for Nodal-related molecules
Pou5f3	Transcription factor	Ventralization (maternal factor for *bmp* expression)
Retinoic acid	Signaling molecule	Posteriorization
Runx2bt2	Transcription factor	Maternal regulator for *vox/vent/ved* expression
Sef	Transmembrane protein	Inhibition of Fgf signaling
Sizzled	Secreted protein	Inhibition of Tolloid/Bmp1 family protein (stabilization of Chordin)
Smad1/5	Signaling and transcription factor	Mediator of Bmp signaling
Sox2/3/19a/19b	Transcription factor	Ventralization (promotes *bmp* expression and represses *dharma*)
Sp5a/5l	Transcription factor	Mediator of Wnt signaling
Sprouty2	Cytoplasmic protein	Inhibition of Fgf signaling
Tbx16	Transcription factor	Paraxial mesoderm formation

(continued)

Table 32.2 (continued)

Molecule	Nature of molecule	Role in axis formation
Tcf/Lef family	Transcription factor	Involved in the canonical Wnt pathway, interacting with β-catenin
Tob1a	Transcription factor	Inhibition of formation of Tcf/Lef and β-catenin complex
Tolloid/Bmp1 family	Secreted protein, metalloprotease	Degradation of Chordin (promotion of Bmp signaling)
Vox/Vent/Ved	Transcriptional repressor	Repression of dorsal genes and mediator of Bmp signaling
Wnt3a	Secreted protein	Posteriorization and axial mesoderm formation
Wnt8a	Secreted protein	Maternal: dorsal determination Zygotic: posteriorization and axial mesoderm formation

Molecules involved in dorsoventral and anteroposterior axis formation are listed alphabetically. Molecules listed in Table 32.1 are not described in this table
Fgf fibroblast growth factor, *GPI* glycophosphatidylinositol, *MAPK* mitogen-activated protein kinase

formation (Sick et al. 2006). This mechanism may be similar to that in the Nodal–Lefty (Nodal inhibitor) system for mesoderm and endoderm differentiation (Muller et al. 2012; Schier 2009). As nonaxial mesendoderm expressing Wnt8a and Wnt3a is suggested to provide posteriorizing signals (Koshida et al. 1998; Woo and Fraser 1997), Dkk1b may generate a high Wnt signal area near the margin and a low Wnt signal area in the animal pole (Fig. 32.3c, d). This Wnt signaling gradient is required to establish AP embryonic polarity. At the late gastrula stage, Dkk1b from the prechordal plate may ensure the anterior neural fate. Since Dkk1b can rescue the formation of not only the anterior neuroectoderm but also the axial mesoderm in *dharma*-deficient *bozozok* embryos, Dkk1 may also be involved in axial mesendoderm formation (Hashimoto et al. 2000) (Fig. 32.3a). Although genetic analysis has revealed that Dkk1 is essential for head development in the mouse (Mukhopadhyay et al. 2001), the role of Dkk1 in formation of the AP axis in zebrafish has not been genetically proven. Combination knockouts of *dkk* family members expressed in zebrafish gastrula embryos (*dkk1a*, *dkk1b*, and *dkk3*) (Lu et al. 2011) should reveal the role of Dkk family proteins in body axis formation.

32.6.4 Nodal/Bmp/Fgf/Wnt Signaling Interactions for DV and AP Axis Formation

Zygotic Nodal, Bmp, Fgf, and Wnt signaling cooperatively regulate axis formation. DV patterning along the AP axis is reported to be temporally coordinated: inhibition of Bmp signaling at the onset of gastrulation controls the formation of the anterior neuroectoderm, whereas at a later stage it regulates the formation of the posterior

neuroectoderm (Tucker et al. 2008). In this process, Bmp-mediated DV patterning is temporally coordinated with the posteriorizing signals Fgf, Wnt, and retinoic acid (Hashiguchi and Mullins 2013). Fgf negatively regulates Bmp signaling by phosphorylating Smad1/5, which are Bmp signal transducers; this mechanism is involved in coordination of Bmp and Fgf signaling, at least in part (Hashiguchi and Mullins 2013).

The ventral blastoderm margin is proposed to function as the tail organizer independently of the dorsal organizer. The activation of Nodal/Bmp/Wnt signaling mimics tail organizer activity (Agathon et al. 2003). The dorsal organizer, the tail organizer, and the entire blastoderm margin are proposed to function as an organizing center, which depends on the ratio of Nodal/Bmp activity: Nodal is high on the dorsal side, and Bmp is high on the ventral side (Fauny et al. 2009). Moreover, Nodal and Bmp alone are sufficient to organize uncommitted naïve cells of the blastula animal pole into a well-organized embryo, both in vivo and in vitro (Xu et al. 2014). These data suggest that Nodal and Bmp signaling are minimal requirements for providing embryonic cells with DV and AP axis information, and that Nodal/Bmp activity gradients play a pivotal role in axis formation. Although genetic evidence may be required to prove that endogenous Nodal and Bmp levels are sufficient to generate the embryonic axis, the data imply that Nodal and Bmp signaling function as hubs in the program for DV and AP axis formation. Other signaling pathways and transcription factors that control the expression of Nodal/Bmp molecules and inhibitors function downstream from (and possibly in parallel with) Nodal/Bmp signaling in axis formation, as discussed earlier. Mathematical modeling will help to explain the intricate programs that shape the DV and AP axes.

32.7 Evolution of Axis Formation in Ray-Finned Fish (Actinopterygii)

32.7.1 Bichir Provides a Good Model for Evolutionary Developmental Biology (Evo–Devo) Studies

Although the mechanisms controlling axis formation in zebrafish are relatively well understood, the degree to which these mechanisms are conserved among fish—and whether these mechanisms are also shared by other vertebrate species—is not clear. Ancient jawless fish (Agnatha) that existed about 600 million years ago were the ancestors of all vertebrates (Blair and Hedges 2005). The extant jawless fish are in the class Cyclostomata, which includes lampreys and hagfish; other vertebrates (Gnathostomata) are derived from an ancient lineage of jawed fish that diverged from the Agnatha. The extant jawed fish are categorized into two groups: cartilaginous fish (Chondrichthyes) and bony fish (Osteichthyes); the bony fish are further classified into two subgroups: lobe-finned fish (Sarcopterygii—e.g., coelacanths, lungfish, and tetrapods) and ray-finned fish (Actinopterygii). The ray-finned fish

include several taxa: Polypteriformes (bichirs and reedfish), Chondrostei (sturgeons and paddlefishes), Lepisosteiformes (gars), Amiiformes (*Amia calva*), and Teleostei (teleosts, Fig. 32.4). Most extant fish, including zebrafish and medaka model fish, are teleosts. Early embryogenesis is similar in various teleosts, but teleost embryogenesis is quite different from that of nonteleost fish (Bolker 1993a, b; Cooper and Virta 2007). This difference may be rooted in the WGD that is proposed to have taken place in the teleosts (Amores et al. 1998; Jaillon et al. 2004). The teleost-specific WGD may have generated gene variations that contribute to teleost-specific developmental processes (Kuraku et al. 2009).

Thus, to understand the evolution of fish embryogenesis, it is important to study the development of nonteleost fish. Bichirs (order: Polypteriformes, family; Polypteridae), which live in African rivers and lakes, have been used in recent studies of developmental biology (Takeuchi et al. 2009a). The bichir lineage diverged from ray-finned fish about 400 million years ago, soon after bony fish diverged into lobe-finned and ray-finned fish (Inoue et al. 2003). Thus, bichirs are considered to be one of the most primitive ray-finned fish. Furthermore, bichirs did not undergo teleost-specific WGD. Therefore, comparative analysis of zebrafish and bichir development should provide insights into the evolution of mechanisms for embryonic axis formation.

32.7.2 Morphogenetic Processes of Bichir Embryos

A teleost embryo undergoes meroblastic cleavages, whereas a bichir embryo undergoes holoblastic cleavages, like the *Xenopus* embryo, cleaving from the animal to the vegetal pole. In *Xenopus*, the first cleavage furrow demarcates the DV axis. When one of the two blastomeres is labeled at the two-cell stage, the interface between the labeled and unlabeled halves coincides with the midsagittal plane (Klein 1987). In contrast, the first cleavage plane in zebrafish is not related to either the DV axis or the left–right (LR) axis, and the blastomeres are intermingled during the blastula and gastrula stages (Kimmel and Law 1985; Kimmel and Warga 1987), indicating that *Xenopus* and zebrafish use distinct developmental strategies for cleaving and mixing the blastomeres. In bichir embryos, injection of rhodamine dextran into one of the two blastomeres at the two-cell stage marks cells in either the left or right half of the embryo (Fig. 32.5a) (Takeuchi et al. 2009a). Therefore, the first cleavage plane in bichir embryos demarcates the DV axis, and the blastomeres are not intermingled as they are in *Xenopus* embryos. Holoblastic cleavage is associated with formation of embryonic cavities (the blastocoel and archenteron) and absence of the EVL and YSL. Bichir embryos have a blastocoel and an archenteron, and no EVL or YSL (Fig. 32.5), suggesting they have inherited an amphibian-type morphogenetic process. The embryonic structure of bichirs is also similar to that of the agnathan lamprey (Takeuchi et al. 2009b). It is tempting to speculate that the morphogenetic process of the bichir embryo may be similar to that of ancestral fish; the EVL and YSL might have evolved in the ray-finned fish lineage after the bichir had diverged from the ray-finned fish.

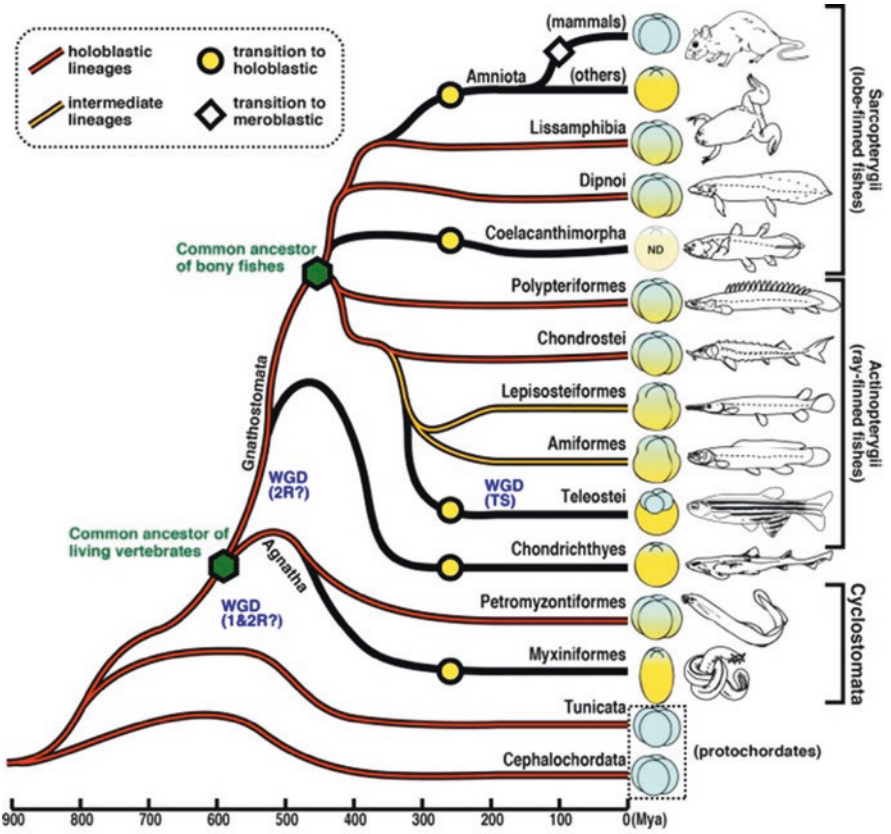

Fig. 32.4 Vertebrate phylogenic tree and cleavage patterns. Basal chordate embryos undergo holoblastic cleavages. As the common ancestor of vertebrates evolutionarily acquired a massive yolk (indicated by the yellow color in the embryos), their cleavage patterns were highly diversified. The holoblastic cleavage pattern is retained in various vertebrate lineages (indicated by red lines). The transition from a holoblastic to a meroblastic cleavage pattern occurred independently several times in the vertebrate lineages (yellow circles). Bichirs (Polypteriformes)—the most primitive ray-finned fish (Actinopterygii)—undergo holoblastic cleavages, as do amphibians. Bichirs diverged from all other ray-finned fish about 400 million years ago (Mya) during the Devonian period, before the teleost-specific (TS) whole-genome duplication (WGD) and soon after an ancestral bony fish diverged into ray-finned and lobe-finned fish (Sarcopterygii)

Fig. 32.5 (continued) midline region, fluorescent image). Dotted lines indicate the midline. (**b**) Germ layer patterning in the bichir and lamprey during gastrulation. Bichir and lamprey embryos have a blastocoel and an archenteron but no enveloping layer (EVL) or yolk syncytial layer (YSL). Both the mesoderm (red) and endoderm (green) are formed in the marginal zone of the bichir embryo and in the conical eminence of the lamprey embryo during early gastrulation. In the bichir embryo, the vegetal cell mass (VCM) is an extraembryonic structure, which does not contribute to the endoderm. The archenterons are marked by *asterisks*. (**c**) Evolution of endoderm and mesoderm induction in vertebrates. In the vertebrate ancestor, vegetal cells might have induced the endoderm (green arrow) and mesoderm (pink arrow) in the embryo; the bichir VCM retains this activity. With the holoblastic-to-meroblastic transition, this activity was retained in the YSL of teleost embryos. The activity was incorporated into the endoderm cells of amphibian embryos as the vegetal cells became the endoderm. In amniote embryos, the activity was retained in the visceral endoderm or the hypoblast, which are extraembryonic structures

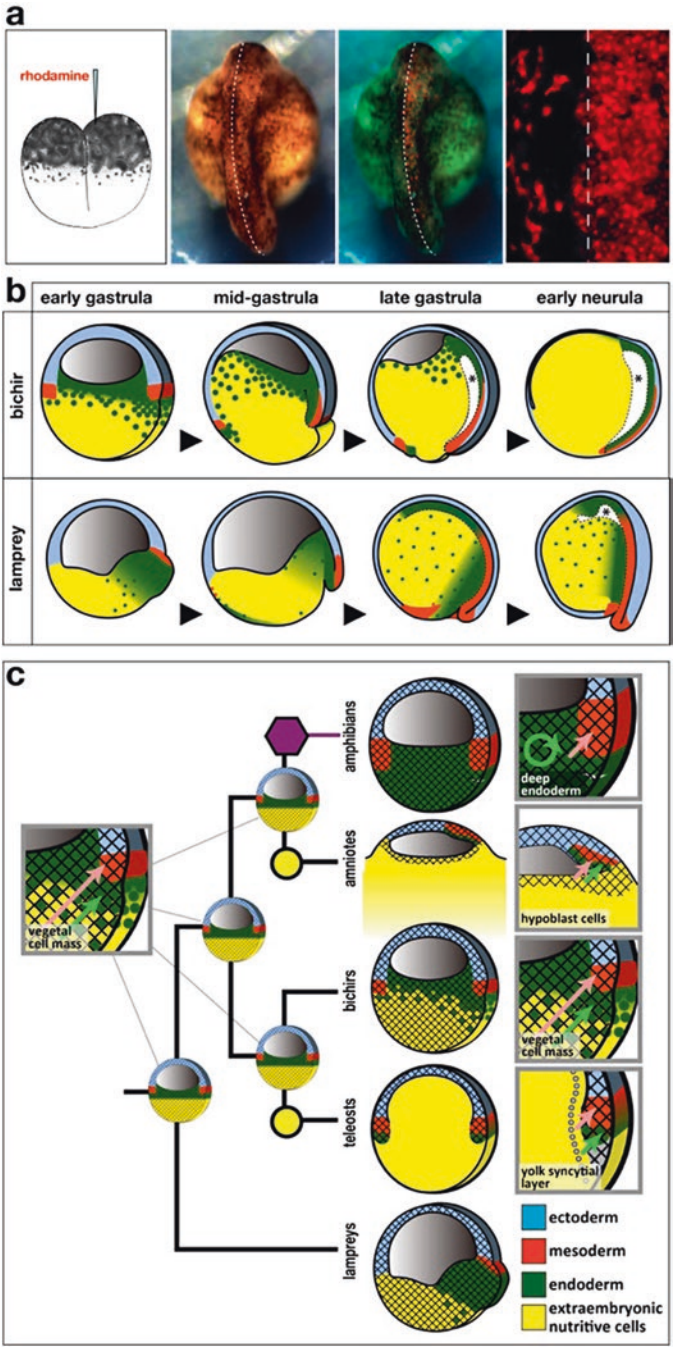

Fig. 32.5 Early bichir and lamprey embryogenesis. (**a**) In bichirs, as in amphibians, the first cleavage plane of the embryo demarcates the dorsoventral (DV) axis. However, there is no correlation between the first cleavage and the body axes in teleost embryos. In bichirs, rhodamine injection into one of the blastomeres of a two-cell-stage embryo (left panel) results in fluorescence mostly in either the left or right half of the embryo at the tailbud stage (middle left panel: bright-field image; *middle right panel*: merged images from bright-field and fluorescent images; right panel: high-magnification view of the

32.7.3 Molecular Mechanisms Controlling Bichir Embryonic Development

It is thought that a cytoplasmic fusion of vegetal blastomeres during the evolution of ray-finned fish brought about the teleost YSL (Figs. 32.4 and 32.5). The zebrafish YSL and the *Xenopus* vegetal endoderm are strikingly similar in their ability to induce the mesoderm and endoderm. However, the cellular identity of the teleost YSL is distinct from that of the *Xenopus* vegetal endoderm. The *Xenopus* vegetal endoderm expresses endodermal markers and gives rise to endoderm tissues such as the pancreas, liver, and gut, whereas the teleost YSL is an extraembryonic tissue that does not express endoderm markers. Bichir embryos, like *Xenopus* embryos, have vegetal blastomeres, which raises the question of whether the vegetal blastomeres in bichir embryos are endoderm cells.

However, recent histological analyses of bichir and lamprey embryos, using molecular markers, has revealed that the vegetal blastomeres in bichirs and lampreys are extraembryonic nutritive cells that do not express endoderm markers (Takeuchi et al. 2009b). The endoderm and mesoderm form in the equatorial (marginal) zone (the conical eminence in lampreys). The Nodal-related gene *ndr1*, which is expressed in the zebrafish YSL, is also expressed in the equatorial zone of bichir embryos (Takeuchi et al. 2009b). Although bichir and lamprey embryos are morphologically similar to *Xenopus* embryos, the localization of the mesoderm and endoderm in bichir and lamprey embryos is similar to that in zebrafish (teleost) embryos (Fig. 32.5).

The T-box transcription factor Tbx16/VegT is required for endoderm formation and for vegetal cell induction of the mesoderm in *Xenopus*. Tbx16/VegT mRNA is maternally deposited at the vegetal pole of the *Xenopus* egg (Zhang et al. 1998). In contrast to *Xenopus*, bichirs do not express *tbx16* maternally or in vegetal blastomeres (Takeuchi et al. 2009b). There is no *tbx16* homologue in the lamprey genome. The mRNA of Eomesodermin homologue a (Eomesa, Eomes, Tbr2)—another T-box transcription factor involved in endoderm formation—is maternally deposited and zygotically expressed in the prospective endoderm in zebrafish (Bjornson et al. 2005). As with zebrafish, *eomes* transcripts are maternally deposited and zygotically expressed in the endoderm region in bichir and lamprey embryos (Takeuchi et al. 2009b). These data suggest that although bichirs and lampreys follow the amphibian-type morphogenetic process, they use the teleost-type mechanism for germ layer formation. The lamprey and bichir lineages are phylogenetically distant but use similar mechanisms for embryonic morphogenesis and germ layer formation. This developmental strategy—amphibian-type (holoblastic) morphogenesis and teleost-type germ layer formation—might have been used by the common ancestors of the vertebrates (the stem lineage). As maternal *tbx16/VegT* expression is common to at least some amphibians, Tbx16/VegT-dependent differentiation of the endoderm of the vegetal cells might have evolved in the amphibian lineage (Takeuchi et al. 2009b).

32.7.4 Bichir DV Axis Formation

Injection of *Xenopus β-catenin* mRNA into both blastomeres of a two-cell-stage bichir embryo can induce a secondary axis, as in *Xenopus* (Takeuchi et al. 2009a) (Fig. 32.6). This observation indicates that the maternal canonical Wnt pathway also plays a key role in dorsal determination in the bichir embryo. What molecule(s) function downstream from the Wnt pathway to control the expression of dorsal-specific genes in bichirs? Nodal-related genes are regulated by the Wnt pathway in both *Xenopus* and zebrafish (Sect. 32.5.2). In bichir embryos, *ndr1* is expressed in the dorsal equatorial zone (Takeuchi et al. 2009b), suggesting that *ndr1* may also be involved in inducing the dorsal organizer, as proposed for zebrafish. The transcriptional repressor Dharma functions downstream from the Wnt pathway to induce dorsal tissues in zebrafish (Leung et al. 2003b; Ryu et al. 2001; Shimizu et al. 2000), whereas the transcriptional activator Siamois and its paralog Twin mediate this process in *Xenopus* (Ishibashi et al. 2008; Kessler 1997; Laurent et al. 1997; Lemaire et al. 1995). Injection of the mRNA of the bichir Siamois-related transcription activator into *Xenopus* embryos can induce a secondary axis (Masaki Takeuchi, unpublished data). These data suggest that bichirs use the amphibian-type mechanism—at least in part—for Wnt signal–mediated induction of dorsal tissues. Although *siamois*-related genes have not been identified in lampreys or other nonbichir fish, the presence of the *siamois*-related gene in bichirs suggests that common ancestors of the bony fish might have used Siamois-related transcription factor(s) for dorsal axis formation (Fig. 32.6). During teleost evolution, the *siamois*-related gene was lost, and *dharma* might have evolved (or diverged from *goosecoid*) in the teleost lineage (Leung et al. 2003b; Ryu et al. 2001; Shimizu et al. 2000).

32.8 Perspectives

Although we understand many aspects of the molecular mechanisms that control axis formation in zebrafish, there are many unanswered questions, including how the oocyte AV polarity is initially established during oogenesis, how the vegetal pole mRNAs are transferred through the Balbiani body to the vegetal pole, what initiates the formation of the vegetal microtubules, how the vegetal microtubules are oriented to the prospective dorsal side, and how patterning signals (Wnt, Bmp, Nodal, Fgf, etc.) are coordinated to control axis formation.

Many loss-of-function studies of zygotic genes have used antisense morpholinos. Recent genome-editing techniques, such as the CRISPR/Cas9 and TALEN systems, can generate mutants of genes of interest, which will allow us to determine the function of genes whose mutants were not isolated by forward genetic screening. Germline replacement with the genetic mutants will enable us to understand the role of maternal factors (Ciruna et al. 2002). It has been reported that antisense morpholino-mediated knockdown and genetic knockout (mutation) often lead to

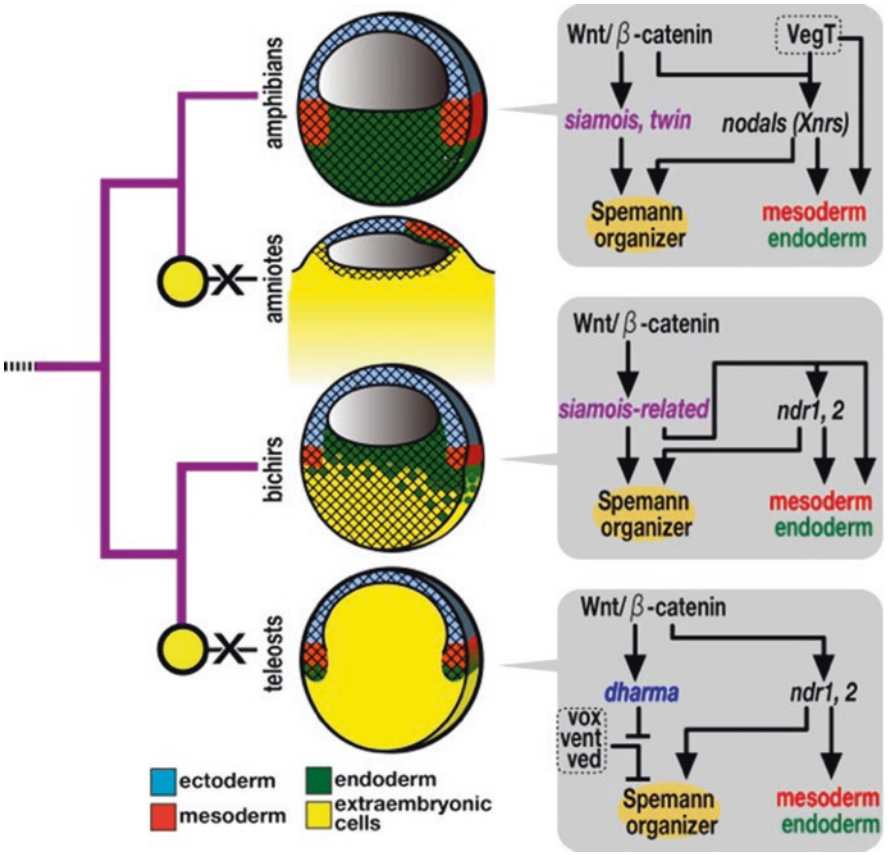

Fig. 32.6 Axis formation in bichirs and its evolution in vertebrates. A *siamois*-related gene might have mediated the canonical Wnt pathway to induce the dorsal axis in the common ancestor of the bony fish. During evolution, the *siamois*-related gene was retained in the amphibian and bichir genomes, but it was lost in the amniote and teleost genomes. In contrast, *dharma* evolved to function as a Wnt target for dorsal axis formation in teleosts

different phenotypes (Kok et al. 2015; Stainier et al. 2015). Although genetic compensation (upregulation of genes that compensate for loss of target genes) may explain such discrepancies (Rossi et al. 2015), we may need to reevaluate data obtained from antisense morpholino experiments.

In dorsal determination, regulation of microtubule formation and microtubule-dependent transport play essential roles. Visualization of the components involved in dorsal determination, along with time-lapse analysis, will reveal the molecular dynamics associated with DV axis formation. Mathematical modeling with precise transcriptome (single-cell transcriptome) data should help us understand the intricate processes that coordinate multiple signals.

There are also questions with respect to evolution, including to what degree the axis formation mechanisms are conserved among fish; whether the bichir-type developmental mechanism (amphibian-type morphogenetic process, teleost-type germ layer formation, and amphibian-type dorsal axis formation) is used by Cyclostomata (lamprey and hagfish), cartilaginous fish, lobe-finned fish, and nonteleost ray-finned fish; and how stem lineage axis formation is adapted for the amniote lineage. Sequencing with next- and third-generation sequencers enables us to reveal the genome sequences and transcriptomes of nonmodel animals, and whole-genome sequencing of bichir is in progress. Genome-editing techniques also allow us to study gene function in these animals. In the future, we will be able to discuss the details of the molecular mechanisms that form the body axes in many different vertebrate species, and find the blueprint for the evolution of axis formation in vertebrates.

Note We have recently reported that maternal *wnt8a* is dispensable for the initial dorsal determination but cooperates with zygotic *wnt8a* for ventrolateral and posterior tissue formation. Maternal *wnt6a* is an alternative dorsal determinant candidate (Hino et al. Dev Biol 434(1), 96–107, 2018). The data suggest that Wnt8a, Wnt6a, and possibly other Wnts that are expressed maternally may cooperate to activate the canonical Wnt pathway for the dorsal axis formation.

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Chapter 33
Postembryonic Axis Formation in Planarians

Yoshihiko Umesono

Abstract Planarians have a long history of attracting many biologists for the study of regeneration, one of the most intriguing phenomena in postembryonic development. Planarian regeneration absolutely depends on somatic pluripotent stem cells, termed neoblasts, which are distributed throughout the body. Recent progress in applying molecular and genetic approaches, including RNA interference, has provided increasing knowledge about cellular and molecular dynamics in planarian regeneration. Using the freshwater planarian *Dugesia japonica* as a model, we revealed that interplay between the anterior extracellular signal–regulated kinase (ERK) and posterior ß-catenin signaling pathways can account for the reconstruction of a complete head-to-tail axis via differentiation of neoblasts during regeneration. Notably, our data suggest that these two signals form opposing activity gradients along the anteroposterior axis. Surprisingly, Thomas Hunt Morgan, one of the great early investigators of planarian regeneration, predicted the existence of these two opposing morphogenetic gradients more than a century ago. Thus, our study provides, for the first time, a basic molecular framework for Morgan's hypothesis in planarian regeneration. Furthermore, our data suggest that the balance between the anterior ERK signaling and posterior ß-catenin signaling varies among planarian species, resulting in drastic differences in the head-regenerative capacity of their tail fragments.

Keywords Planarian · Regeneration · Pluripotent stem cells · ERK · ß-catenin · Polarity · Gradient · RNAi

33.1 Introduction

Regeneration is the complex process by which animals reconstitute missing body parts after disease or injury; thus it is one of the most intriguing phenomena in postembryonic development in animals (Agata et al. 2007; Stoick-Cooper et al. 2007).

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Key events in regeneration include successive phases of wound healing, formation of a blastema at the wound, and pattern formation along the body axis via activation of regenerative cells. How can regenerative animals reconstitute original body axes after injury? Planarians have a long history of attracting many biologists to answer this difficult question.

Planarians belong to an evolutionarily early group of organisms with defined bilateral symmetry, dorsoventral polarity, a central nervous system (CNS) with a simple brain structure, and high regenerative ability (Agata and Umesono 2008; Umesono and Agata 2009; Umesono et al. 2011). After amputation, an anterior-facing wound regenerates a head and a posterior-facing wound regenerates a tail—a phenomenon known as regeneration polarity. More than a century ago, Thomas Hunt Morgan, one of the most famous early investigators of planarian regeneration, found that very thin fragments of *Planaria maculata*—made by sectioning perpendicular to the anteroposterior (AP) axis—regenerated bipolar two-headed planarians, termed "Janus heads" (an allusion to the Roman god Janus) (Morgan 1904). On the basis of this observation, he attempted to explain the nature of polarity by positing two opposing morphogenetic gradients of formative substances—"head stuff" and "tail stuff"—along the AP axis (Morgan 1905; Lawrence 1988). However, until the past decade, studies on the molecular mechanisms underlying planarian regeneration made very slow progress because of many difficulties in applying molecular approaches. In 1997, we reported a whole-mount in situ hybridization method with high sensitivity, using the freshwater planarian *Dugesia japonica* as a model, which was a great contribution to studies of gene expression in planarians (Umesono et al. 1997, 1999). In 1999, Sánchez Alvarado and Newmark reported RNA interference (RNAi) in the freshwater planarian *Schmidtea mediterranea*, which for the first time provided an opportunity to perform knockdown of the function of genes in planarians (Sánchez Alvarado and Newmark 1999). At present, these two species act as a worldwide standard planarian model and are providing increasing knowledge about cellular and molecular dynamics of planarian regeneration. In this chapter, I describe how the head-to-tail body axis is properly reconstructed during planarian regeneration at the cellular and molecular levels.

33.2 *Dugesia japonica* Model

We used a clonal strain of the freshwater planarian *D. japonica* as a model planarian (5–10 mm in body length along the AP axis) that possesses extraordinarily high regeneration ability. For example, when an animal is dissected into seven body fragments along the AP axis, all of the fragments can completely regenerate a whole individual within a week, with a 100% success rate. This ability enables *D. japonica* not only to propagate asexually through fission under laboratory conditions but also to provide rapid and reliable data for regeneration studies.

D. japonica has at least four distinct body regions along the AP axis: the head, prepharyngeal, pharyngeal, and tail regions (Fig. 33.1). The separations between

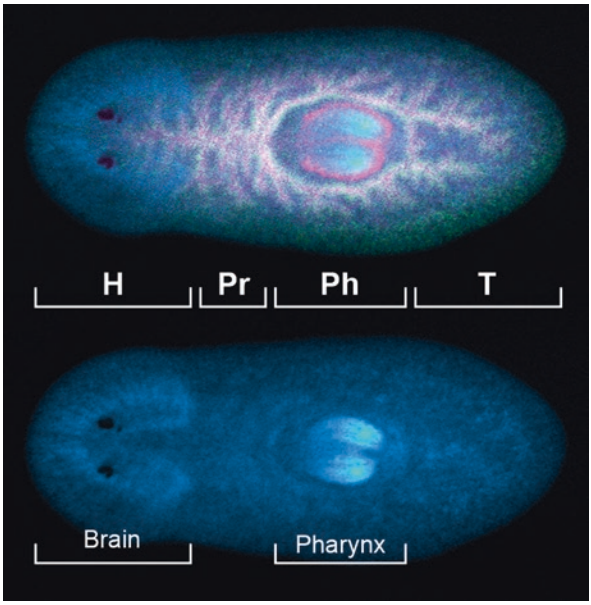

Fig. 33.1 Body structure of the freshwater planarian *Dugesia japonica*. The planarian body is subdivided into at least four regions in an anterior-to-posterior sequence: a head region containing a brain and eyes (H), a prepharyngeal region (Pr), a pharyngeal region containing a pharynx (Ph), and a tail region (T). The upper panel shows triple staining with anti–aromatic L-amino acid decarboxylase (anti-AADC) antibody (green), anti-ß-cateninA antibody (magenta), and Hoechst 33342 (cyan). The lower panel shows Hoechst 33342 staining alone in the same animal. The digestive system is composed of a three-branched gut—with one anterior branch in the head and prepharyngeal regions, which is connected to the anterior end of the pharynx, and two posterior branches in the pharyngeal and tail regions—which is well visualized by double staining with anti-AADC (Nishimura et al. 2007a, b) and anti-ß-cateninA (since ß-cateninA protein is highly accumulated in the epithelial cell membrane of the gut and pharynx). It is known that the *D. japonica* genome possesses at least two *ß-catenin* homologue genes (termed *Djß-cateninA* and *B*) (Kobayashi et al. 2007; Yazawa et al. 2009). Hoechst 33342 nuclear staining visualizes the brain and pharynx in cyan, since they are masses of numerous cells. Anterior is to the left (Reprinted from Umesono (2014), with permission from Springer)

these regions are indicated by the presence of position-specific tissues and organs associated with specific gene expression (Umesono et al. 2013). A brain and pair of eyes represent typical morphological structures specific to the head region, and the brain occupies a major field of the head region in *D. japonica*. In the middle portion of the body, there is a pharynx, which acts as an organ for feeding and egestion (Fig. 33.1). The digestive system is composed of a three-branched gut, with a single branch in the head and prepharyngeal regions, and two gut branches in the pharyngeal and tail regions (Fig. 33.1). The single gut branch is connected to the anterior end of the pharynx. Therefore, the gut patterns can subdivide the whole body along the AP axis into two subdivisions, the anterior (head and prepharyngeal) region and the posterior (pharyngeal and tail) region. The prepharyngeal region is specified as

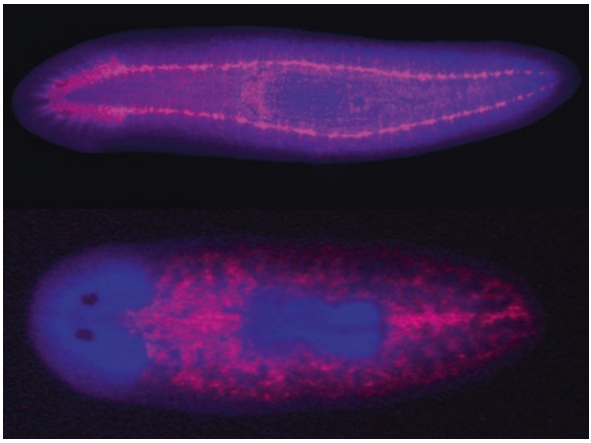

Fig. 33.2 Central nervous system (CNS) and neoblast distribution in *Dugesia japonica*. The upper panel shows anti-synaptotagmin (SYT) antibody staining in magenta (Tazaki et al. 1999), seen from the ventral view, visualizing the axonal networks of the CNS. The planarian CNS is composed of an inverted U-shaped brain in the head region and a pair of ventral nerve cords in the trunk region. The lower panel shows staining in magenta with a probe for the neoblast-specific *DjpiwiA* gene (Shibata et al. 2010). Hoechst 33342 staining visualizes the nuclei of all cells in blue. Anterior is to the left

a region with a single gut branch, no brain, and no eyes. The tail region is specified as a region with two gut branches and no pharynx.

Another unique aspect of planarians is that they occupy an interesting phylogenetic position with respect to the CNS, which in planarians is composed of two morphologically and functionally distinct structures: the anterior brain and the ventral nerve cords (Fig. 33.2; Agata et al. 1998). The brain is a bilobed structure consisting of many types of neuronal cells, including dopaminergic, GABAergic, and serotonergic neurons (Nishimura et al. 2007a, b, 2008a, b, 2010). A pair of eyes, acting as a light-sensing organ, are composed of only two cell types: pigment cells and photoreceptor cells, whose axons form an optic chiasma and project caudally onto the dorsomedial region of the brain, where the photosensory signals are integrated (Agata et al. 1998; Sakai et al. 2000; Okamoto et al. 2005; Umesono and Agata 2009). These observations strongly suggest a functional property of the brain, acting as an information processing center in planarians. In fact, planarians can sense a variety of signals coming from outside, such as light and chemicals, and perform distinct behaviors depending on the type of external signals. One of the typical examples is negative phototaxis (light avoidance behavior), in which the eyes sense light signals and transmit the signals to the brain, which interprets the direction from which the light signals come (Inoue et al. 2004; Takano et al. 2007). In this neuronal circuit, GABAergic neurons in the brain are indispensable for eliciting negative phototaxis (Nishimura et al. 2008b). Another example is negative thermotaxis (cryophilic behavior); serotonergic neurons in the brain are indispensable

for this behavioral trait (Inoue et al. 2014). An important question is how *D. japonica* can regenerate the head region, which is morphologically and functionally distinct from the rest of the body, even from tail fragments.

33.3 Neoblast Dynamics in Blastema Formation

The robust regenerative ability of planarians absolutely depends on a unique population of somatic pluripotent stem cells called neoblasts, which are distributed throughout the body (Baguñà et al. 1989; Shibata et al. 2010; Wagner et al. 2011). Neoblasts express a cohort of neoblast-specific genes, such as *piwi* genes of the argonaute family (Fig. 33.2), and are the only proliferative somatic cells in adult planarians (Newmark and Sánchez Alvarado 2000; Salvetti et al. 2000; Orii et al. 2005; Reddien et al. 2005; Shibata et al. 2010). Neoblasts are highly sensitive to X-ray irradiation, and X-ray-irradiated planarians specifically lose their neoblasts and regenerative ability (Shibata et al. 1999). Implantation of isolated neoblasts into X-ray-irradiated planarians can restore their regenerative ability (Wagner et al. 2011). This clearly indicates that the neoblasts act as the only source of regenerative cells in planarians.

Head regeneration starts with wound healing, which is followed by formation of a head blastema via activation of neoblasts after amputation. In *D. japonica*, from around 12 h after amputation, a cluster of neoblasts expressing the gene *D. japonica piwiA* appear, surrounding the presumptive blastema-forming region at the cut edge, and then start to form a blastema (Tasaki et al. 2011a). At around 24 h after amputation, a blastema can be clearly recognized as a mass of cells that are positive for staining with anti-DjPiwiA antibody (Fig. 33.3a–c). Simultaneously or a few hours later, however, almost all of the blastema-forming neoblasts have apparently decreased the expression level of *DjpiwiA* messenger RNA (mRNA) (Fig. 33.3d). Such transcriptional regulation has also been observed for other neoblast-specific genes, *proliferating cell nuclear antigen* (*Djpcna*) and *histone H2B* (*Djhistone H2B*), in *D. japonica* (Orii et al. 2005; Tasaki et al. 2011a). These blastema-forming cells already lack proliferative activity (Tasaki et al. 2011a). In contrast, there are a lot of proliferating neoblasts in a region posterior to the blastema (called the post-blastema region). At this time, head blastema–forming cells start to differentiate (Cebrià et al. 2002b). Moreover, these blastema-forming cells completely lack expression of DjPiwiA protein, in accordance with their terminal differentiation (Shibata et al. 2016). This series of neoblast dynamics has been commonly observed in both head and tail regeneration. These observations suggest that blastema formation involves a dynamic change in the cellular state of neoblasts or their progeny as the source of the blastema; that is, it involves an exit from the proliferative and pluripotent state—and an immediate entry into the differentiating state—in planarians.

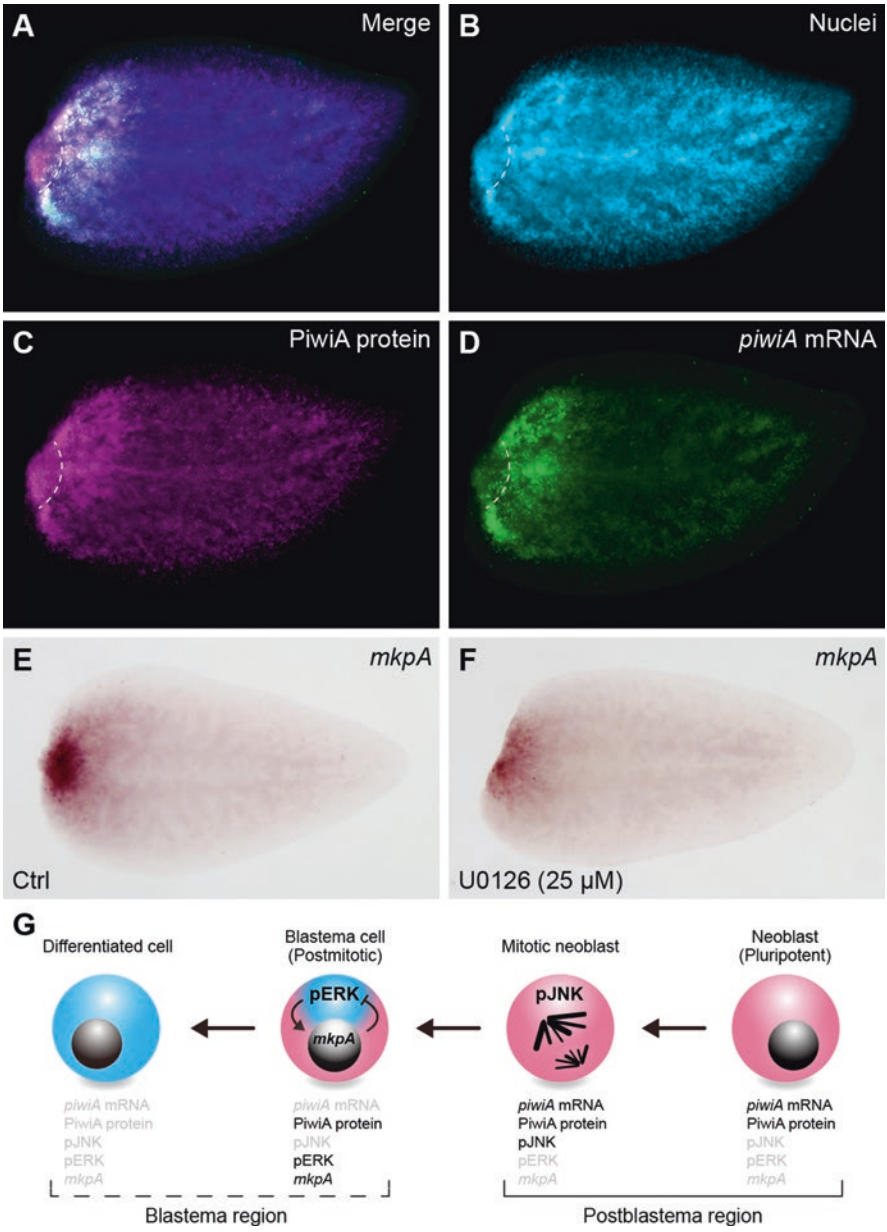

Fig. 33.3 Neoblast dynamics involved in head blastema formation. (**a**–**d**) Triple staining with a *DjpiwiA* gene probe (green), anti-DjPiwiA antibody (magenta), and Hoechst 33342 nuclear staining (blue) at 24 h of head regeneration from a tail fragment. The dashed lines indicate the gross border between the head blastema, which is recognized as a DjPiwiA(+) and *DjpiwiA*(−) region at a wound, and the rest of the body. DjPiwiA(+) and *DjpiwiA*(+) neoblasts are highly accumulated at a position posterior to the blastema (i.e., a collar of strong *DjpiwiA* signal in D)—the so-called postblastema region, where many mitotic neoblasts are present under the control of c-Jun NH2-

33.4 Mitogen-Activated Protein Kinase Signaling Pathways in Planarians

The mitogen-activated protein kinase (MAPK) signaling pathways are evolutionarily highly conserved and play crucial roles in a variety of cellular functions, including cell proliferation, differentiation, and stress response. There are four subgroups of kinases in the MAPK family—extracellular signal–regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), p38, and ERK5—which are activated by different stimuli via phosphorylation (Nishida and Gotoh 1993; Imajo et al. 2006). The active form of MAPKs is inactivated by mitogen-activated kinase phosphatases (MKPs), which dephosphorylate activated MAPKs (Kondoh and Nishida 2007). Therefore, the magnitude and duration of MAPK signaling is likely to reflect a balance between the activities of upstream activators such as MAPK kinase (MEK) and the activities of MKPs. It is well known that MKPs act as feedback-induced negative regulators of MAPK signaling pathways (Kondoh and Nishida 2007).

We found that coordinated action of JNK and ERK signaling pathways is required for the neoblast dynamics involved in blastema formation in *D. japonica* (Tasaki et al. 2011a, b). JNK is highly activated in mitotic neoblasts and is required for accelerating the entry of neoblasts into the M-phase in the cell cycle in the postblastema region, resulting in the generation of neoblast progeny to form a blastema (Tasaki et al. 2011b). Subsequently, ERK is highly activated in blastema cells and is required for exit from the proliferative and pluripotent state of blastema cells and entry into the differentiating state (Tasaki et al. 2011a). These ERK-activated blastema cells start to express *DjmkpA*, the *D. japonica* gene encoding an MKP that acts as a negative feedback regulator of activated ERK in blastema cells (Tasaki et al. 2011a; Fig. 33.3e, f). This feedback circuitry converts differential ERK activity levels into binary cell fate decisions of blastema cells regarding whether to proliferate or to differentiate. Neoblast dynamics involved in blastema formation and differentiation are summarized in Fig. 33.3g (Umesono 2014); the JNK-activated mitotic neoblasts act as a definitive source of the blastema cells, in which an ERK/*DjmkpA* feedback circuit makes the final decision about whether blastema cells undergo differentiation or not. When animals were treated with the chemical JNK inhibitor SP600125, neoblasts failed to enter the M-phase in the cell cycle, resulting in a lack of blastema cells as neoblast progeny (Tasaki et al. 2011b). In contrast, when animals were treated with the chemical MEK inhibitor U0126, which inactivates ERK, blastema cells failed to differentiate and underwent several rounds of cell cycle progression (Tasaki et al. 2011a).

Fig. 33.3 (continued) terminal kinase (JNK) activation (Tasaki et al. 2011b). (**e** and **f**) *DjmkpA* expression at 24 h of head regeneration from tail fragments. (**e**) *DjmkpA* is strongly expressed in the head blastema. (**f**) Treatment with the chemical mitogen-activated protein (MAP) kinase kinase (MEK) inhibitor U0126, which inactivates ERK signaling, causes a drastic decrease in *DjmkpA* expression at a wound. (**g**) Summary of the spatial profiles of activated JNK (pJNK), activated ERK (pERK), and *DjmkpA* expression involved in blastema formation. Anterior is to the left (Reprinted from Umesono (2014), with permission from Springer)

We also found that ERK activation is involved in the neoblast differentiation necessary for the regeneration of not only the head and tail but also the prepharyngeal and pharyngeal regions during regeneration, demonstrating the crucial role of ERK signaling in triggering dynamic reconstruction of the whole-body AP patterns in *D. japonica* (Umesono et al. 2013). Finally, we revealed that two distinct posteriorizers (Wnt/ß-catenin signaling and the *nou-darake* (*ndk*) gene) can account for the reconstruction of the whole-body AP patterns with the assistance of ERK activation as a cell differentiator (Umesono et al. 2013).

33.5 Wnt/ß-Catenin Signaling in Planarians

The Wnt signaling pathway employs components that are evolutionarily well conserved among metazoans, and it plays crucial roles in the regulation of body patterning, cell proliferation, and cell fate specification during animal development and regeneration. In the absence of secreted Wnt ligand molecules, cytoplasmic ß-catenin undergoes degradation through ubiquitination by a multiple-protein complex called the destruction complex. When Wnt ligands bind their cell surface receptor protein, Frizzled (Fz), ß-catenin can escape from degradation via dissociation of the destruction complex and, as a result, accumulate in the cytoplasm. Finally, ß-catenin translocates into the nucleus and acts as a transcriptional coactivator of the DNA-binding transcription factors T-cell factor/lymphoid enhancer factor (TCF/LEF) to promote target gene expression. This sequence in the signaling pathway is called the canonical Wnt signaling pathway or the Wnt/ß-catenin signaling pathway (Fagotto 2013).

The first great breakthrough in the study of regeneration polarity in planarians was identification of the *ß-catenin* gene (*Smed-ßcatenin-1*) in *S. mediterranea*. Knockdown of the function of *Smed-ßcatenin-1* by RNAi resulted in Janus heads at the expense of tail regeneration (Gurley et al. 2008; Iglesias et al. 2008; Petersen and Reddien 2008). Therefore, manipulation of a single gene could reproduce the phenotype observed in Morgan's classical experiments (Fig. 33.4). Indeed, a cohort set of planarian Wnt genes (e.g., *Smed-wnt1/P-1*, *Smed-wnt11-1*, *Smed-wnt11-2*, and *Smed-wnt11-5* in *S. mediterranea*) are expressed with a spatial gradient in the posterior region, with the highest level at the tail end and progressively lower levels toward the pharynx (Petersen and Reddien 2008; Gurley et al. 2010; Stückemann et al. 2017). *Smed-wnt1/P-1* RNAi also resulted in Janus heads (Adell et al. 2009; Petersen and Reddien 2009), suggesting that Smed-wnt1/P-1-mediated ß-catenin activity plays an instructive role in the specification of the tail identity. In contrast, RNAi with genes that encode a component of the ß-catenin destruction complex, such as adenomatous polyposis coli (APC) and Axin, increased ß-catenin activity independently of the Wnt ligand activities and resulted in "Janus tails" at the expense of head regeneration (Gurley et al. 2008; Iglesias et al. 2011, Stückemann et al. 2017). Furthermore, we demonstrated that the Hedgehog (Hh) signaling pathway plays a crucial role in regulating the transcription of the posterior Wnt family genes

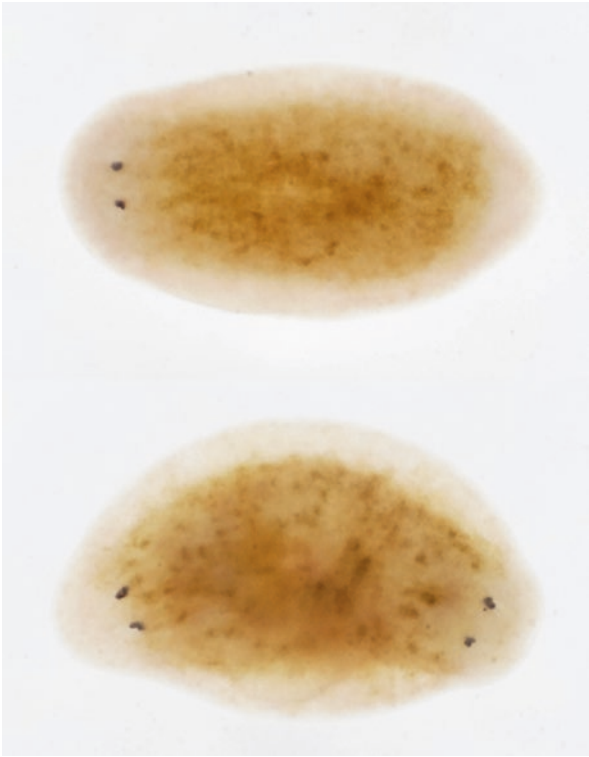

Fig. 33.4 Bipolar two-headed planarian at the expense of tail regeneration, caused by RNA interference (RNAi) with the gene *Dugesia japonica ß-cateninB* (*Djß-catB*). The upper panel shows a control regenerate from a trunk fragment containing a pharynx. The lower panel shows a *Djß-catB*(*RNAi*) regenerate. Anterior is to the left

in *D. japonica* (Yazawa et al. 2009). RNAi with the planarian gene *patched*, which encodes a putative receptor of Hh ligand, caused ectopic activation of the posterior Wnt genes in the anterior-facing blastema and resulted in Janus tails at the expense of head regeneration (Rink et al. 2009; Yazawa et al. 2009). These observations suggest that Wnt-mediated posteriorly biased ß-catenin activity fits with the instructive role of the classical concept of the tail stuff (Adell et al. 2010; Almuedo-Castillo et al. 2012 Sureda-Gómez et al. 2016; Stückemann et al. 2017). Notably, recent studies using specific antibodies have provided evidence of a tail-to-head gradient of nuclear ß-catenin along the AP axis in planarians (Sureda-Gómez et al. 2016; Stückemann et al. 2017). However, to date, no molecules have been reported to function as the classical head stuff in planarians. Interestingly, our data suggested that ERK signaling forms a deceasing activity gradient in a head-to-tail direction during regeneration (Umesono et al. 2013). On the basis of our findings, we assessed whether or not ERK signaling fits with the instructive role of Morgan's head stuff.

First, we examined the relationship between ERK and the posterior Wnt/ß-catenin signaling during regeneration. The results showed that RNAi with the gene *D. japonica ß-cateninB* (*Djß-catB*) caused transformation of the tail region into a head-like region via activation of ERK (Umesono et al. 2013). *Djß-catB* RNAi also increased the ERK activity level in the regenerating head region (Umesono et al. 2013). Conversely, the increase in ß-catB activity induced by RNAi with the gene *D. japon-*

ica APC (*DjAPC*) strongly decreased the ERK activity level in the regenerating head and interfered with head regeneration (Umesono et al. 2013). These observations suggest that posterior ß-catenin signaling plays a role in the negative regulation of ERK signaling to decide between head regeneration and tail regeneration at wounds.

We also found that interplay between the anterior ERK and posterior ß-catenin signals is required for specification of the pharyngeal region but not the prepharyngeal region. During head regeneration from tail fragments, *Djß-catB*(*RNAi*) animals showed roughly normal regeneration of the head and prepharyngeal regions but failed to regenerate a pharynx (Umesono et al. 2013). These knockdown animals had ectopic fusion of the two posterior branches of the gut in the presumptive pharyngeal region at the expense of the pharynx structure, resulting in transformation of the pharyngeal region into a prepharyngeal-like region (Umesono et al. 2013). We detected an increase in the ERK activity level in *Djß-catB*(*RNAi*) animals during this process (Umesono et al. 2013). These observations suggest that the posterior ß-catenin signaling plays a role in the negative regulation of ERK signaling to decide between prepharyngeal and pharyngeal regeneration.

33.6 *nou-darake* Gene in Planarians

Our findings, presented here, provide a molecular explanation for the specification of the head, pharyngeal, and tail regions by interplay between the anterior ERK and posterior ß-catenin signals during planarian regeneration. We also identified the *D. japonica* gene *nou-darake* (*ndk* means "brains everywhere" in Japanese), which plays a crucial role in the specification of the prepharyngeal region.

ndk encodes a putative transmembrane protein with two extracellular immunoglobulin-like domains related to those of fibroblast growth factor (FGF) receptors but with no cytoplasmic kinase domains characteristic of this receptor family (Cebrià et al. 2002a). During regeneration, *ndk* is specifically expressed in the head blastema, and its expression is promoted by anterior ERK signaling (Umesono et al. 2013). *ndk* RNAi caused gradual brain expansion to more posterior regions beyond the head region during regeneration (Cebrià et al. 2002a). Interestingly, this effect was observed predominantly in the prepharyngeal region (Umesono and Agata 2009), where ectopic eyes were often observed (Cebrià et al. 2002a), at the expense of the prepharyngeal identity via an increased ERK activity level (Umesono et al. 2013). These observations suggest that *ndk* RNAi caused transformation of the prepharyngeal region into a head-like region via activation of ERK in a non-cell-autonomous manner (Fig. 33.5).

In summary, ERK activation at a high level with low ß-catenin activity promotes the specification of the head region, where *ndk* is expressed as an output of ERK activation. In turn, *ndk* properly specifies the prepharyngeal region in a non-cell-autonomous manner. We wonder about the mechanism underlying the non-cell-autonomous effects in *ndk*(*RNAi*) animals. Interestingly, the ectopic brain

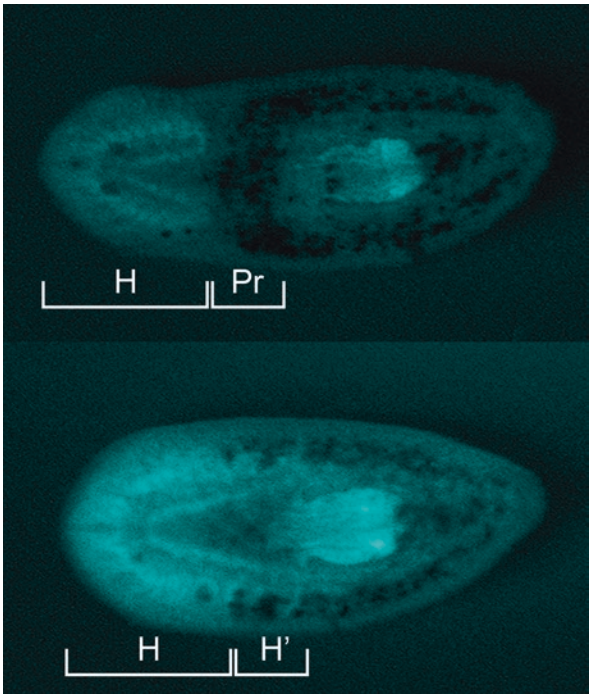

Fig. 33.5 *ndk* RNA interference (RNAi) causes transformation of the prepharyngeal region into a head-like region. The upper panel shows a ventral view of a control regenerate from a tail fragment at 7 days of regeneration. The belt of the gene *PN8* expression (dotted black signals) visualizes the prepharyngeal (Pr) region. The lower panel shows a ventral view of an *ndk*(*RNAi*) regenerate from a tail fragment at 7 days of regeneration. *ndk* RNAi results in a decrease in the number of *PN8*-positive cells in the presumptive prepharyngeal region, where ectopic brain tissues are detected. Hoechst 33342 nuclear staining visualizes the brain and pharynx in cyan, since they are masses of numerous cells. Anterior is to the left. H head region, H' head-like region, Pr Prprepharyngeal region

formation in *ndk*(*RNAi*) animals is suppressed by combinatorial inhibition of the two planarian FGF receptor genes (*DjFGFR1* and *DjFGFR2*) (Cebrià et al. 2002a) expressed in neoblasts and also in brain cells (Ogawa et al. 2002). When *ndk* mRNA was injected into *Xenopus* embryos, it inhibited FGF signaling, as did the dominant negative form of FGFR1 (XFD) (Cebrià et al. 2002a). The expression of the extracellular domain of NDK still exerted this inhibitory effect (Cebrià et al. 2002a), indicating that the extracellular domain of NDK is responsible for its ability to inhibit FGF signaling. These observations suggest that NDK functions as a dominant negative form of FGF receptor via the extracellular domain of NDK. A simple model is that NDK traps diffusible factors that induce differentiation of the brain cells from neoblasts through a direct interaction with these factors, and thus inhibits brain cell differentiation outside the head region (Cebrià et al. 2002a); that is, NDK forms a clear boundary between the head and prepharyngeal regions by establishing a proper extrinsic environment for brain cell differentiation from neoblasts in

planarians. Recent comprehensive transcriptome profiling should be useful for identifying a good candidate gene that encodes a diffusible brain-producing factor in *D. japonica*.

33.7 Head-Regenerative Versus Nonhead-Regenerative Planarians

It is widely accepted that regenerative ability varies among animal phyla and even within a given phylum (Agata and Inoue 2012). Notably, the ability to regenerate a head is known to vary even among planarian species. In the planarian *Phagocata kawakatsui*, the tail region completely lacks head-regenerative ability (Fig. 33.6), while tail regeneration occurs normally (Umesono et al. 2013). Why are tail fragments of *P. kawakatsui* completely unable to regenerate a head? We observed many mitotic cells, comparable in number to those in a planarian species with a head-regenerative trunk region (Umesono et al. 2013), suggesting that the loss of head-regenerative ability in the tail region is not due to the absence of active neoblasts. Thus, we speculated that unknown factor(s) might interfere with the ERK activation in neoblasts necessary for head regeneration from tail fragments of *P. kawakatsui*. On the basis of our findings described above in *D. japonica*, the gene *P. kawakatsui mkpA* (*PkmkpA*) and the gene *P. kawakatsui ß-cateninB* (*Pkß-catB*) were the best candidates for such unknown factors; thus, we assessed our hypothesis by silencing these genes in tail fragments of *P. kawakatsui*.

First, we isolated two distinct *P. kawakatsui* complementary DNA (cDNA) clones related to *DjmkpA* and performed RNAi experiments. Double RNAi with these two genes never induced head regeneration from tail fragments of *P. kawakatsui* (Umesono et al., 2012, unpublished data). In contrast, surprisingly, *Pkß-catB* RNAi resulted in regeneration of a head-like structure at anterior-facing wounds from tail fragments (Fig. 33.6b). This structure included a pair of eyes (Fig. 33.6b) and also brain tissues (Umesono et al. 2013). Furthermore, these regenerates sensed light stimuli and showed negative phototactic behavior, in contrast to regenerates from control tail fragments, which showed less locomotive activity (Umesono et al. 2013). Thus, we demonstrated for the first time that a single gene is responsible for the lack of head-regenerative ability within a given species (Umesono et al. 2013). In two other freshwater planarian species, *Dendrocoelum lacteum* and *Procotyla fluviatilis*, the *ß-catenin* gene is also responsible for inhibiting head-regenerative ability in the tail region (Liu et al. 2013; Sikes and Newmark 2013).

We speculate that head regeneration results from the balance between anterior ERK signaling and posterior Wnt/ß-catenin signaling, which might vary among planarian species, resulting in the diversity of head-regenerative ability among planarian species. This diversity may be associated with the way of reproduction: sexual versus asexual reproduction in planarians. Interestingly, *P. kawakatsui*, *D. lacteum* and *P. fluviatilis* all undergo sexual reproduction only, and show no asexual repro-

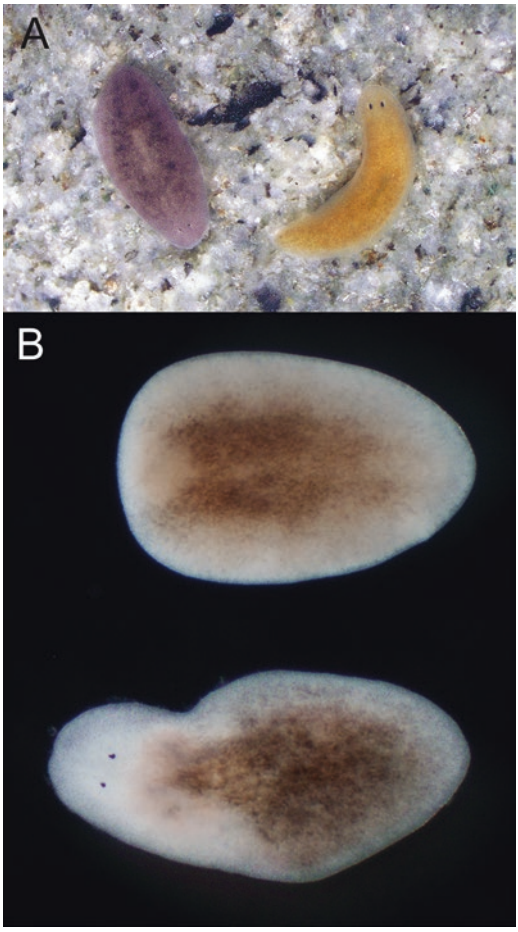

Fig. 33.6 The *Phagocata kawakatsui ß-cateninB* (*Pkß-catB*) gene encodes an anti-regenerative factor for head tissues in the tail fragments. (**a**) The freshwater planarians *P. kawakatsui* (left) and *Dugesia japonica* (right) live in the same stream (often under rocks) but show drastic differences of head-regenerative ability in the tail region. (**b**) The upper panel shows a dorsal view of a control tail fragment of *P. kawakatsui* at 20 days after amputation. The lower panel shows a dorsal view of a *Pkß-catB*(*RNAi*) tail fragment at 20 days after amputation

duction. In contrast, *D. japonica* actively undergoes asexual reproduction as well as sexual reproduction in nature. In the mode of asexual reproduction, binary fission usually takes place at a position posterior to the pharynx; thus, a tail fragment regenerates an intact individual with a head for clonal propagation of this species. In this scenario, sexual planarians do not need to have head-regenerative ability in the tail region. We speculate that planarians have evolved to gain the ability to regenerate their heads in the tail region for undergoing asexual reproduction via modulation of posterior ß-catenin signaling. Further discussion and experiments will be needed to investigate this matter.

Which *wnt* genes are involved in this process is also an intriguing question. In our preliminary experiments, RNAi with the gene *P. kawakatsui wnt11-5* (*Pkwnt11-5*) failed to induce head regeneration from tail fragments in *P. kawakatsui* (Nishimura et al., 2013, unpublished data). It was also reported that triple gene knockdown of three distinct *wnt* genes (homologues of *wnt1/P-1*, *wnt11-1*, and *wnt11-2* in *P. fluviatilis*) did not result in head regeneration from tail fragments

(Sikes and Newmark 2013). Thus, unidentified *wnt* gene(s) or other mechanism(s) might cause strong β-catenin activation in the tail region, depending on the specific planarian species.

33.8 Conclusions

Our central goal is to understand how the head-to-tail axis is re-established via activation of neoblasts during planarian regeneration. We found that ERK and posterior Wnt/ß-catenin signaling pathways establish a solid framework for de novo AP axis formation in *D. japonica* as follows (Fig. 33.7). After amputation, the interplay between ERK activity as a cell differentiator and ß-catenin activity as a posteriorizer results in a binary decision on regeneration of a head or a tail at wounds (a step of distalization) (Agata et al. 2007). Subsequent interplay between the anterior ERK and posterior ß-catenin signaling results in the establishment of the whole-body AP pattern by filling in the prepharyngeal and pharyngeal regions in the correct order between the head and tail (a step of intercalation) (Agata et al. 2003; Agata et al. 2007). In this process, interestingly, our data suggest that activated ERK may form a spatial gradient in the anterior region (Fig. 33.7b)—an opposite pattern to that of Wnt/ß-catenin signaling activity, as assayed by the expression of *DjAbd-Ba*, a downstream target gene of Wnt/ß-catenin signaling (Nogi and Watanabe 2001; Umesono et al. 2013). *ndk* is expressed under the control of the anterior ERK activation in the head blastema and is required for the specification of the prepharyngeal region, and ß-catenin activity is required for the specification of the pharyngeal region, via negative regulation of the anterior ERK activity level in both cases. This suggests that regenerative cells outside the head region are prone to undergoing differentiation into head tissues in the absence of the activities of these two posteriorizing factors, *ndk* and *Djß-catB* (Fig. 33.7b). Thus, we propose a novel concept that neoblasts might follow a default program of differentiation into head tissues such as brain cells, which is triggered by the activation of ERK, suggesting that activated ERK triggering of the default program might correspond to the classical head stuff referred to by Morgan. Interestingly, this possibility is in accordance with the neural default model for embryonic stem (ES) cells (Eiraku and Sasai 2012), a typical example of pluripotent stem cells in mammals (Evans and Kaufman 1981; Martin 1981). Furthermore, we have shown that proliferation of neoblasts is highly sensitive to ERK activation as a differentiation signal, in a way similar to the regulation of ES cells in mice (Kunath et al. 2007; Ying et al. 2008). These observations encourage us to speculate that ERK signaling was integrated into the pluripotent stem cell system early in the course of evolution and plays a crucial role in balancing proliferation and differentiation of pluripotent stem cells. Notably, we found that ß-catenin exerts an anti-differentiation activity in planarian stem cells. Thus, our findings not only have confirmed the broad outline of Morgan's hypothesis but also highlight the possible value of planarian studies for establishing a fundamental principle regarding the regulation of pluripotent stem cells.

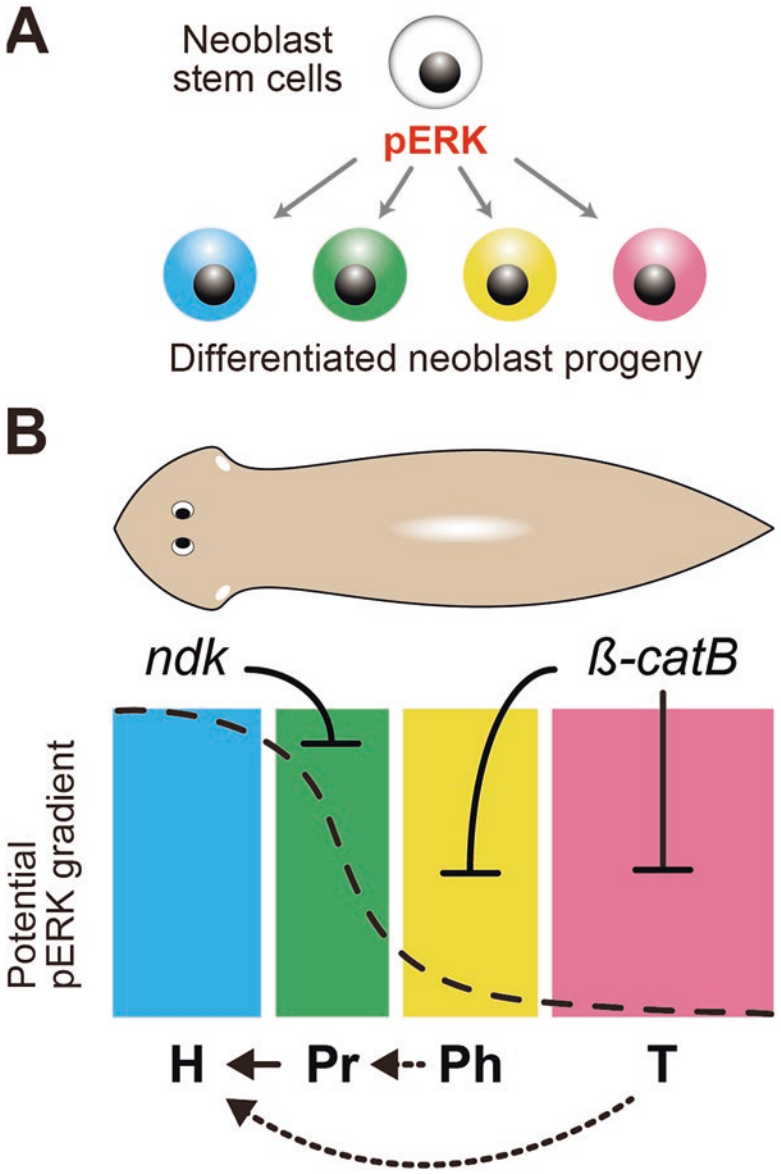

Fig. 33.7 Cell fate determination of neoblasts along the anteroposterior (AP) axis during planarian regeneration. (**a**) Activated ERK (pERK) is required for differentiation of neoblasts to establish the whole-body patterning along the AP axis. (**b**) *ndk* (which functions as an output of ERK signaling in the differentiating head) and *Dugesia japonica ß-cateninB* (*Djß-catB*) genes are involved in negative regulation of ERK signaling to shape the anterior pERK gradient in distinct manners. *ndk* RNA interference (RNAi) causes transformation of the prepharyngeal region into a head-like region via activation of ERK (arrow). *Djß-catB* RNAi causes transformation of the pharyngeal region into a prepharyngeal-like region and the tail region into a head-like region via activation of ERK (dashed arrows). H head cell, Ph pharyngeal cell, Pr prepharyngeal cell, T tail cell (Modified from Umesono (2014), with permission from Springer)

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Chapter 34
Larval and Adult Body Axes in Echinoderms

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Abstract The echinoderm body plan is amazing and unique among metazoans, with pentameral (fivefold) symmetry as adults. Fossil records indicate that most of the extinct species also had non-bilateral shapes. Five classes of extant echinoderm species show diverse morphologies incorporating the pentameral symmetry. The anterior-posterior axis of most living echinoderms is not obvious, and it appears to be radially symmetrical as well. Instead, an oral-aboral axis and a proximal-distal axis have been assigned. Moreover, the body axes are different in an embryo/larva when compared to an adult. Embryos and larvae are bilateral with animal-vegetal, anterior-posterior, left-right, dorsal-ventral (oral-aboral) axes present. The larvae metamorphose into pentameral adults, and together with the change in structure, the body axes change. This remarkable transition in morphology makes their study unique for understanding how body axes are formed and how they change in evolution. Molecular studies of the development of axes in embryos and larvae have shown that the factors and genes involved in axis formation in echinoderms are shared with other bilaterians and these mechanisms appear to be conserved. However, the development into a pentaradial adult remains a mystery. Recent findings based on analyses of *hox* genes suggest that the oral-aboral axis of the adult body is equivalent to the anterior-posterior axis of other bilaterians. In this chapter,

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we will discuss the correlation between the *hox* gene expression and transitions in this unique change in body axes.

Keywords Echinoderm · Body axis · Development · *hox* genes · Adult rudiment · Metamorphosis · Pentameral symmetry · Bilateral

34.1 Introduction

Echinoderms are one of the most eye-catching animals in the sea. These members of the phylum Echinodermata, with their non-bilateral shapes and generally visible with the naked eye, are macroscopic benthic and sessile marine animals. They are generally characterized by a calcitic skeleton composed of many ossicles, a water vascular system, mutable collagenous tissue, and pentaradial (fivefold and radially symmetrical) body organization in adults (Wray 1999).

The skeleton of echinoderms has many pores and looks like 3D mesh, or a sponge of calcite. This structure, which is called a "stereom," is considered one of the most ancient synapomorphies of the echinoderms (Smith 2005; Bottjer et al. 2006). These skeletal elements are largely divided into two groups by their origins, which is detailed in Sect. 34.2.3.

Another feature of echinoderms is the coelomic water vascular system. It is composed of intricate water canals that have one or several congested pores on the body wall to incorporate sea water. The water-filled canal is connected to the pouched podia, which are the main organs for feeding and/or locomotion of adult echinoderms. These structures originate from the larval hydrocoel, which is formed in the left side of bilateral larvae and thus integral to the significant alterations of the axes between the bilateral larvae and the pentameral (fivefold symmetrical; interchangeably used with "pentaradial" in this review) adults.

Mutable collagenous tissue, or the catch apparatus, is made of collagenous ligaments, which have special structures to adjust their thickness under nervous control. The thickness changes rapidly, so this tissue is used to control spine movements of sea urchins, body tone of sea cucumbers, autotomy of arms in crinoids, brittle stars, etc. (Motokawa 1984).

The most recognizable feature of echinoderms is their pentaradial body. Many sea stars and brittle stars have apparent pentameral star-shaped bodies and regular sea urchins (a group that has a globular skeleton) have beautiful geometric pentameral patterns on their skeletons. Crinoids and basket stars usually have highly branched arms, but they converge to five at the most proximal points. Sea cucumbers have cigar-like bodies and do not appear to be pentameral; however, they have tissues such as tentacles or podia that are arranged pentaradially in transverse sections. While the pentaradiality is usually incomplete in animals of this phylum with asymmetrical visceral organs derived from bilateral larvae, some members are non-pentameral, such as six- or more-armed sea stars and brittle stars, and bilateral-looking irregular sea urchins (sand dollars and heart urchins). However, fossil

records and phylogenetic analyses suggest these non-pentameral aspects are derived forms (Kroh and Smith 2010; Mah and Blake 2012; O'Hara et al. 2014).

In this review, we will focus on understanding the amazing body plan of echinoderms, discuss their evolutionary history, and highlight the current molecular investigations on how the body plan is established during development. The formation of the unique body plan in echinoderms is complicated, but is an impactful and evolutionarily revealing topic.

34.2 Echinoderm Phylogeny and Body Plan

34.2.1 Paleontological Studies – The Origin of the Pentameral Body Plan

Fossil records of echinoderms are particularly rich, yet their origin is still unclear. The Precambrian *Arkarua* is attractive as an origin with its pentameral body but it lacks a stereom and water vascular system. Instead, the first echinoderm probably emerged in the early Cambrian period (~510 mya), and diversified rapidly (Smith et al. 2013; Brusca et al. 2016).

Two groups of echinoderms appear to have emerged from the Cambrian period. One such group is the non-radial homalozoa, which is now regarded as a polyphyletic group including four extinct classes: *Homoiostelea* (Solutes), *Homostelea* (Cinctans), *Ctenocystoidea*, and *Stylophora* (Fig. 34.1a, David et al. 2000). These unusual echinoderms have asymmetric (*Homoiostelea*, *Homostelea*, *Stylophora*) or bilateral (*Ctenocystoidea*) flat bodies, which are usually divided into broad theca and thin stele so that the body shape resembles crooked lollipops. All viscera were in the theca and the openings on the surface, which are probable mouth, anus, and hydropore, are always positioned on the theca. One of the openings is usually positioned on the top of the theca (the point of "top of lollipop"), and is thus regarded as anterior and the tip of the stele as the posterior. With their bilateral or asymmetrical body and clear anterior-posterior (AP) axis in their body plan, they are usually regarded as the pre-pentameral echinoderms (Paul and Smith 1984; Shu et al. 2004). Recent studies place bilateral *Ctenocystoidea* and *Ctenoimbricata* species as the most basal group of echinoderms (Smith and Zamora 2013; Zamora and Rahman 2015).

The second group of uniquely-shaped echinoderms is helicoplacoids (Fig. 34.1b). They have a cigar-like body and a mouth in the middle of the body with three spiral grooves. Their bodies are thus considered as triradiate. The evolutionary position of this unusual echinoderm is still under discussion. Some scientists believe that they are derived from pentameral ancestors (Sprinkle and Wilbur 2005; Bottjer et al. 2006) and others suggest that they are the ancestors of pentameral echinoderms (David et al. 2000; Rozhnov 2012, 2014). Smith and Zamora (2013) reported on another Cambrian spiral-bodied echinoderm, *Helicocystis*, which bears five spiral grooves. The body plan of *Helicocystis* is similar to other pentameral echinoderms, thus this species could be the "missing link" between crown-group echinoderms and "more basal" triradiate helicoplacoids.

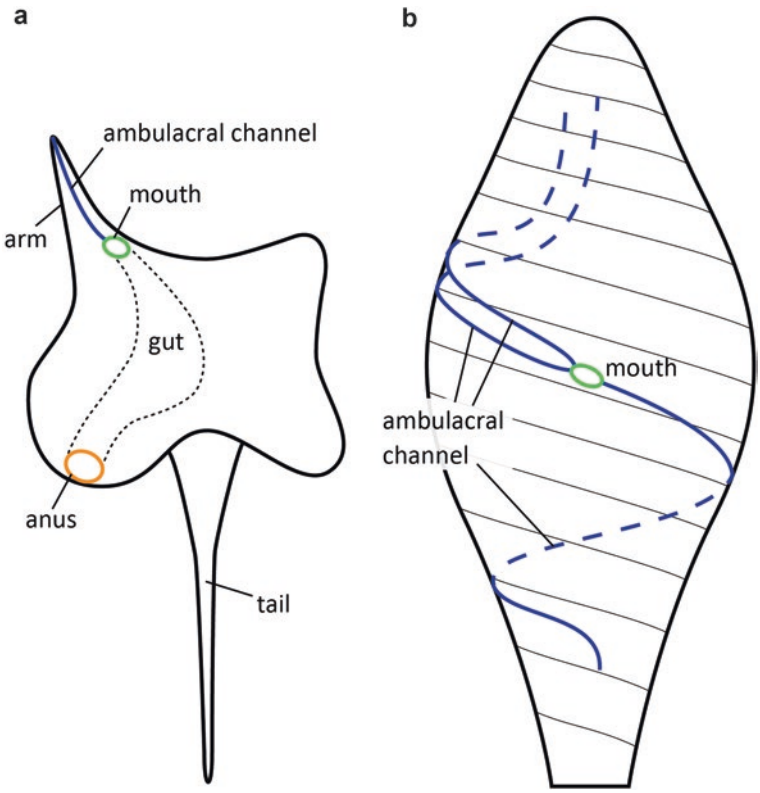

Fig. 34.1 Schematic illustrations of the basal Paleozoic echinoderms. Blue lines indicate ambulacral channels. Green and orange rings indicate the positions of the mouth and the anus, respectively. (**a**) A solutan homalozoa. This group shows an asymmetric body plan with long posterior tail, and an arm with the ambulacral channel, which is projected from the left anterior part of the body. (**b**) A helicoplacoid. This group shows a triradiate body plan with spirally plated body and three twisted ambulacral channels

In addition to these non-pentaradial groups, many pentaradial echinoderms are known from the Paleozoic period. However, there are also many "pseudo-pentaradial" species, e.g., species in which only three rays radiate from the mouth and two of them split into two rays immediately off the mouth. This 2–1–2 symmetry is observed in many fossil groups including Paleozoic crinoids (Sumrall and Wray 2007). This is regarded as the basal form of "true fivefold symmetry" in which five rays radiate directly from the mouth (Hotchkiss 1998; Sumrall and Wray 2007).

34.2.2 Phylogeny of Extant Echinoderms

While phylogenetic relationship among the classes of echinoderms still has some room for discussion, there is no doubt about the phylogenetic position of the phylum Echinodermata; one of the largest groups of deuterostomes and a sister group

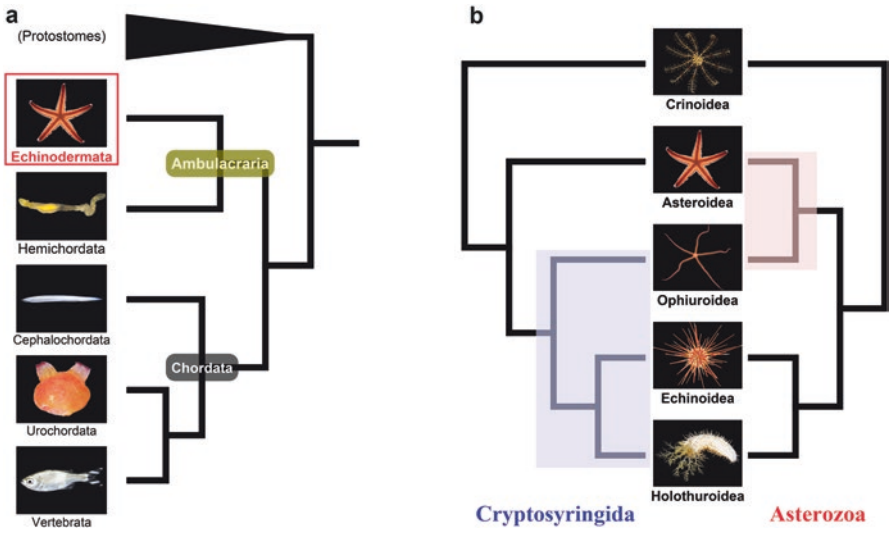

Fig. 34.2 (**a**) Phylogenetic position of the phylum Echinodermata within the deuterostome clade. Echinodermata is the sister group of bilateral Hemichordata. (**b**) Two hypotheses of the phylogenetic relationship among five classes of Echinodermata. Both hypotheses support the monophyly of basal Crinoidea, and regard Echinoidea and Holothuroidea as a sister group. In the Cryptosyringida hypothesis, Ophiuroidea constitutes a monophyletic clade with Echinoidea and Holothuroidea. In the Asterozoa hypothesis, Asteroidea and Ophiuroidea constitute a monophyletic clade

to Hemichordata (Swalla and Smith 2008; Cannon et al. 2016). These two phyla (Echinodermata and Hemichordata) are collectively grouped as the clade of Ambulacraria. In turn, the sister clade to Ambulacraria is the Chordata. The phylogenetic relationships of the clades are shown in Fig. 34.2a.

Extant echinoderms are separated into five classes: Crinoidea (sea lilies and feather stars), Asteroidea (sea stars and sea daisies), Ophiuroidea (basket stars and brittle stars), Echinoidea (sea urchins, sand dollars, and heart urchins), and Holothuroidea (sea cucumbers). Sea daisies were once classified as a sixth class, Concentricycloidea (Baker et al. 1986), but detailed phylogenetic analyses revealed that they are one of the groups of Asteroidea (Janies and Mooi 1998; Mah 2006; Janies et al. 2011). In addition to these five extant classes, many fossil classes are known, the oldest of which dates back to the Cambrian period (Brusca et al. 2016).

Echinoderms are classically divided into two groups: stalked or sedentary Pelmatozoa (which includes the class Crinoidea and many fossil classes) and the free-living Eleutherozoa (which includes the other four extant classes and a few fossil species). The Pelmatozoan clade is also supported by the homology of some plates and ossicles, components that form the skeleton (Ausich et al. 2015). However, Pelmatozoa is paraphyletic when synapomorphies are considered (Brusca et al. 2016). Whether Pelmatozoa is monophyletic or not is inconclusive, since Pelmatozoa includes numerous extinct species and thus a comprehensive molecular phylogenetic analysis is almost impossible. Unlike Pelmatozoa, the monophyly of the Eleutherozoan clade is almost definite. Yet ambiguity exists in the phylogenetic

relationship inside the Eleutherozoan clade. There are two major hypotheses about the phylogeny among the four classes of extant Eleutherozoa. The first hypothesis regards Ophiuroidea, Echinoidea, and Holothuroidea as the monophyletic clade Cryptosyringida, and Echinoidea and Holothuroidea as a sister group (Mac Bride 1906; Smith 1984) (Fig. 34.2b). This hypothesis is consistent with the resemblance of the larval form of Ophiuroids and Echinoids (explained below), and also supported by some histological and biochemical observations (Hyman 1955). In this hypothesis, the similar shapes of adult Asteroids and Ophiuroids are plesiomorphies. Two sister groups are formed in the second hypothesis, which are "armed" Asterozoa, which includes Asteroidea and Ophiuroidea, and "rounded" Echinozoa, which includes Echinoidea and Holothuroidea (Bather 1900) (Fig. 34.2b). According to this hypothesis, the similar larval form between Ophiuroids and Echinoids might just be coincidental. Some morphological and DNA sequence analyses advocate the former Cryptosyringida hypothesis (Littlewood et al. 1997; Smith 2005; Pisani et al. 2012; Telford 2013), but other morphological, molecular phylogenetic and phylogenomic analyses support the latter Asterozoa hypothesis (Janies 2001; Mallatt and Winchell 2007; Cannon et al. 2014; Telford et al. 2014; Reich et al. 2015). With strong support by genomic datasets (Telford et al. 2014; Reich et al. 2015), the Asterozoa hypothesis seems more probable. However, additional studies (such as detailed phylogenomic analysis with more clades, and evo-devo studies among all classes of echinoderms) are still needed to confirm the interrelationship of Eleutherozoa classes.

The phylogeny of the fossil and the extant echinoderms suggests dramatic changes in their body plans and axes through evolution. The following section describes details of their body plans and axes to examine the morphological relationship among the classes of echinoderms and their ancestors.

34.2.3 Adult Body Plans and Axes of Echinoderm

As introduced in Sect. 34.1, adult echinoderms are characterized by their pentameral bodies, quite distinct from other animals. They usually do not have an obvious head-like structure with an integrated nerve center. Thus, the head-tail axis, which usually corresponds to the anterior-posterior (AP) axis in bilateral animals, is difficult to define. Major axes of echinoderms are summarized in Fig. 34.3.

The oral-aboral (OA) axis is defined with the direction of mouth opening as oral, and the opposite direction as aboral. The OA axis is equivalent to the axis connecting the mouth and the anus in adult sea stars, regular sea urchins, and sea cucumbers. In crinoids and irregular sea urchins, the anus is not in the aboral direction. Instead, the anus opens in the same or in the perpendicular direction to the mouth opening. Some brittle stars lack the anus but maintain an oral-aboral axis. The OA axis has been considered to match the head-tail axis or the AP axis in many free-living animals, however, in echinoderms, only sea cucumbers show this correspondence between the OA and AP axes. Other free-living echinoderms usually move in all

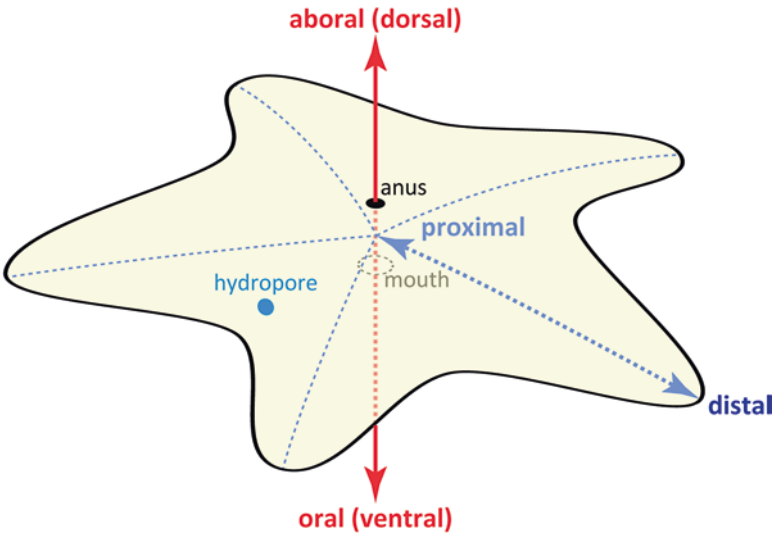

Fig. 34.3 A schematic illustration showing the major axes of echinoderms. Note that the oral-aboral (OA) axis does not correspond to the dorsal-ventral (DV) axis in some groups of echinoderms like crinoids and holothurians. The OA axis is curved in crinoids, and the mouth and anus are on the same side of the body. In holothuroids, the OA axis is equal to the anterior-posterior (AP) axis, and the DV axis is perpendicular to the OA (AP) axis

directions, perpendicularly to the OA axis, and the "anterior" direction varies and is different from the "oral" direction. Sea cucumbers show one of the most derived body plans among living echinoderms (Brusca et al. 2016), thus the correspondence between OA and AP axes in sea cucumber is regarded as secondarily acquired characteristics.

With the divergence from an AP axis, the proximal-distal (PD) axis becomes more important. This axis generally is defined by the point near the trunk being proximal, and the point away from the trunk being distal. The PD axis is strongly related to the radial axes in echinoderms, where the center of rays (including the position of mouth) is regarded as the "trunk" (Fig. 34.3), and the PD axes are perpendicular to the OA axis.

Pentaradial symmetry of the echinoderms is not only observable in their whole-body shape, but also in their internal tissues such as nerve, muscle, gonad, and water vascular system. The five rays of pentamery radiate from the center, where the mouth is positioned. These five rays correspond to the "ambulacra" which are where the rows of tube feet are arranged. However, the pentamery is usually incomplete (Sect. 34.1), and the left-right (LR) axis and the dorsal-ventral (DV) axis of some echinoderms are evident. Such an example is the sea cucumber, which has an AP

axis (which equals the OA axis), an LR axis, and a DV axis (the animal lies with its ventral side down), and these axes are perpendicular to each other. When they are cut on the transverse plane, the pentamery—though incomplete—may be observed. Other echinoderms also lie on one side of the body and keep the other side up, e.g., sea urchins keep their mouth on the bottom. However, in these cases the terms "dorsal" or "ventral" are not used and dorsal-ventral is defined as being different from oral-aboral.

Pentameral echinoderms appear to have evolved from the bilateral ancestor through an intermediate triradiate form (Sect. 34.2.1). This implies a radical change of their body plan and their axes through evolution, such that the comparison of morphological structures among fossil and extant echinoderms is necessary for understanding the evolution of their axes. Mooi et al. (1994) argued that there are two types of skeletons in each ray: axial and extraxial. This "extraxial-axial theory" (EAT) defines all skeletal structures that are generated at the terminal growth point of each ray as "axial" elements and the other skeletal structures as "extraxial" elements. The axial elements include ambulacral plates and their associated plates (e.g., cover plates and spines) and the extraxial elements are the remaining body wall skeletons. The axial elements are generated at the point just proximal to the terminal (or ocular) plate, thus the patterning rule of the generation of axial components is called the "ocular plate rule" (OPR). In contrast, extraxial skeletons are generated stochastically at the point where they are required. The axial and extraxial skeletons are associated with larval hydrocoel and somatocoel, respectively (Mooi et al. 2005). Studies based on the EAT revealed the interrelationships of the body region among extant and extinct echinoderms (e.g. Hotchkiss 1998; Mooi and David 1998; David et al. 2000), and supported the 2–1–2 symmetry as the basal form of pentaradial symmetry (Sumrall and Wray 2007).

Another approach to clarify echinoderm morphological evolution is "universal elemental homology" (UEH, Sumrall 2008, 2010). This model focuses on more detailed homology of each skeletal element compared to EAT. Therefore UEH is useful to resolve phylogenetic relationships between taxa that show highly derived morphology, or to discuss higher level phylogeny of fossil echinoderms (Kammer et al. 2013; Zamora and Rahman 2015). While comprehensive studies based on UEH among Paleozoic echinoderms are still ongoing, UEH has revised phylogenetic relationships of some Paleozoic pentaradial echinoderms with changes to some traditional clades (Kammer et al. 2013). Further UEH-based studies using non-pentaradial echinoderms will reveal the evolution of the pentaradial body pattern of echinoderms.

These theories and approaches to understanding morphological homologies seek to clarify the phylogenetic relationships and evolution of echinoderms (mostly of the extinct taxa). As for the extant taxa, a combination of morphology and molecular data is the key for studies of phylogeny. Further, molecular analyses of echinoderm development may contribute insights into how the unique morphologies of echinoderms have developed through evolution.

34.3 Axis Formation During Development of Echinoderms

34.3.1 Development of Echinoderms

In general, echinoderms show a bilateral body plan during their larval stages, but after metamorphosis, they show a pentameral symmetric body plan. Echinoderm embryos cleave radially and become symmetrical blastulae. After this stage, development of echinoderms differs between taxa, depending on whether they develop directly (develop to an adult without feeding) or indirectly (develop to an adult via a feeding larval stage), and cannot be generalized. In most of the taxa studied, the gastrula stage is highlighted by development of the archenteron (primitive gut). Its tip reaches the overlying ectoderm and merges with it, forming the mouth opening. The opposite opening is the blastopore, where invagination began, and this opening eventually becomes the anus, which is a defining character of deuterostomes. The embryos develop into bilateral larvae, sometimes called "dipleurula," which is originally a name given to a hypothetical bilateral echinoderm larva, but the forms of larvae sufficiently differ between echinoderm classes, and so are usually named differently. Crinoids develop into planktonic auricularia and/or doliolaria larvae which settle and become cystidean larvae, and then metamorphose into pentacrinoids with stalks. In other echinoderm classes, larvae are generally free-swimming until metamorphosis. Asteroids go through bipinnaria and/or brachiolaria larvae stages. Ophiuroids and echinoids have ophiopluteus and echinopluteus larvae, respectively. Holothuroids develop into auricularia larvae and then doliolaria larvae. Then all echinoderm larvae undergo metamorphosis and this step involves significant changes in morphology. These significant changes include a shift in body axis accompanied by reorientation and torsion of the coeloms. The hydrocoel, which differentiates into the water vascular system, grows and encircles the esophagus and eventually fuses to create a complete ring canal. Around the ring canal, most taxa develop an adult rudiment, which grows and forms the adult body. Sea cucumbers do not have adult rudiments but the ring canal is formed. Instead, sea cucumbers reorganize their whole body by developing adult tissues such as the adult nervous system and tentacles inside the body during metamorphosis, which starts at the auricularia stage, and the bilateral body metamorphoses into a cylindrical shape, similar to the adult. It is during this stage that the pentameral body plan is established, either in the adult rudiment or in the whole body (in sea cucumbers).

In the following sections, we review studies of the molecular basis of axis determination and patterning during echinoderm development. Molecules that are mentioned in this review are listed in Table 34.1. Molecular investigations of genes involved in development have generally used sea urchins (e.g., *Strongylocentrotus purpuratus*, *Paracentrotus lividus*, and *Lytechinus variegatus*). Recently, development of other classes of echinoderms have also been studied, and comparative analyses are reported (Lowe and Wray 1997; Shoguchi et al. 2000; Harada et al. 2002; Nakano et al. 2003; Hara et al. 2006; Cisternas and Byrne 2009; Omori et al. 2011; Kikuchi et al. 2015).

Table 34.1 Molecules Involved in the Formation of Body Axes during Development

Gene name (gene symbol)	Protein function	Axis
sine oculis homeobox 3 (*six3*)	TF (sine oculis homeodomain)	AV
frizzled 1/2/7 (*fzd1/2/7*)	Wnt receptor	AV
frizzled 5/8 (*fzd5/8*)	Wnt receptor	AV
forkhead box q2 (*foxq2*)	TF (forkhead)	AV, OA
homeobrain (*hbn*)	TF (paired-like homeodomain)	AV
dickkopf 1 (*dkk1*)	Wnt antagonist	AV
Wnt family member 1 (*wnt1*)	Ligand	AV
Wnt family member 8 (*wnt8*)	Ligand	AV
protein kinase C (*pkc*)	Protein kinase	AV
c-Jun N-terminal kinase (*jnk*)	Mitogen-activated protein kinase (MAPK)	AV
nodal	Ligand	OA, LR
univin	Ligand (TGF-beta family, Gdf1/3)	OA, LR
lefty	Nodal antagonist	OA, LR
goosecoid	TF (paired-like homeodomain)	OA
brachyury	TF (T box)	OA
forkhead box a (*foxa*)	TF (forkhead)	OA
octamer transcription factor 1/2 (*oct1/2*)	TF (POU homeodomain)	OA
T-cell factor (*Tcf*)	TF (HMG box)	OA
p38mapk	MAPK	OA
bone morphogenetic protein 2/4 (*bmp2/4*)	Ligand	OA, LR
chordin	BMP inhibitor	OA
anti-dorsalizing morphogenetic protein 1 (*admp1*)	Ligand	OA
anti-dorsalizing morphogenetic protein 2 (*admp2*)	Ligand	OA
hbox12 (common name: *pmar1*)	TF (paired-like homeodomain)	OA
not	TF (NKL homeodomain)	LR
pituitary homeobox 2 (*pitx2*)	TF (paired-like homeodomain)	LR
SRY-related HMG box E (*soxe*)	TF (HMG box)	LR
paired box 6 (*pax6*)	TF (paired homeodomain)	LR
sine oculis homeobox 1/2 (*six1/2*)	TF (sine oculis homeodomain)	LR
eyes absent (*eya*)	Transcriptional co-activator	LR
dachshund (*dach*)	TF (SKI/SNO/DAC)	LR
forkhead box c (*foxc*)	TF (forkhead)	LR
forkhead box f (*foxf*)	TF (forkhead)	LR
forkhead box y (*foxy*)	TF (forkhead)	LR
snail	TF (zinc finger, C2H2)	LR

See Figs. 34.4, 34.5, and 34.6. *AV* animal-vegetal, *OA* oral-aboral, *LR* left-right, *TF* transcription factor

34.3.2 *Animal-Vegetal Axis*

In echinoderm embryos, the first visible axis is the animal-vegetal (AV) axis. The AV axis is determined by maternal information before fertilization in sea urchin (Croce et al. 2011). The molecular mechanisms establishing the AV axis have been studied mainly using sea urchin as the model system.

Wnt/β-catenin signaling is critical in establishing posterior identity in most metazoan embryos, and is also suggested for sea urchin embryos (Petersen and Reddien 2009). β-catenin is involved in establishment of the AV axis in sea urchins. After the 5th cleavage, β-catenin accumulates in the nuclei of vegetal cells and a gradient of nuclear β-catenin is observed with the highest concentration at the vegetal pole (Fig. 34.4). This suggests that accumulation *of* β-catenin in the nuclei is necessary for vegetal specification and vegetal blastomeres play a role as the Wnt/β--catenin signaling center (Logan et al. 1998).

Another signaling center involved in patterning of the AV axis in sea urchin embryos is the animal pole domain (APD). The APD is located at the animal pole and produces the animal plate (consisting of serotonergic and non-serotonergic neurons) and cells with long immotile cilia. The APD contains regulatory genes such as *foxq2* and *hbn*, expressed at the animal pole in the early blastula. Expression of most of these genes requires *six3* function (Fig. 34.4). This function was discovered when an embryo was injected with *six3*-translation blocking morpholino and this resulted

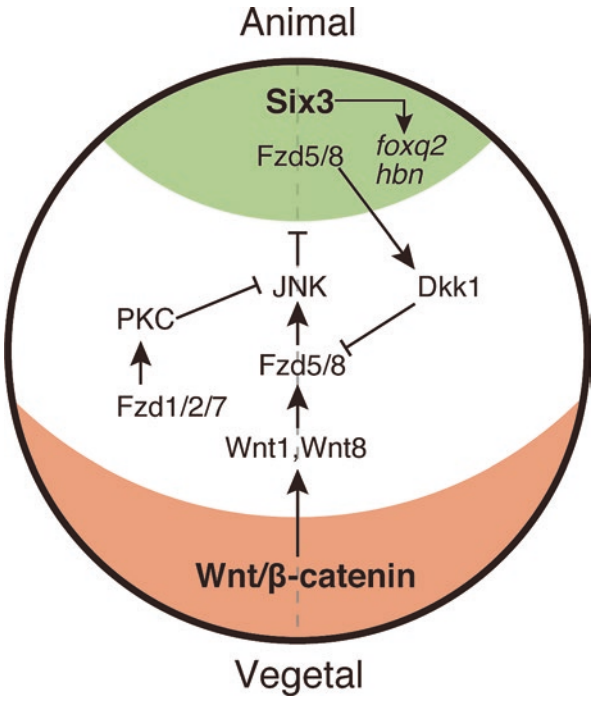

Fig. 34.4 Gene regulatory network for the formation of the AV axis in sea urchin embryos. A gradient of nuclear β-catenin is observed with the highest concentration at the vegetal pole (red area). Wnt/JNK pathway, Fzd1/2/7 signaling and secreted Wnt antagonist Dkk1 coordinate to determine the area of APD (green area). See Table 34.1 for the functions of molecules involved in axis determination

in significantly reduced levels of both *foxq2* and *hbn* transcripts (Wei et al. 2009). *six3* is expressed at the anterior end of many bilateral embryos and is essential for specification of the anterior neuroectoderm. After the 6th cleavage, APD regulatory genes are progressively restricted to cells at the animal-most region of the embryo. This process requires posterior Wnt/β-catenin signaling (Yaguchi et al. 2008).

In sea urchins, this Wnt-dependent restriction of APD regulatory genes to the animal pole is mediated by Wnt/JNK signaling, which includes Wnt1, Wnt8, and its receptor Fzd5/8 (synonym Fzl5/8), and requires c-Jun N-terminal kinase (JNK) activity (Range et al. 2013). Another Wnt receptor, Fzd1/2/7 (synonym Fzl1/2/7) is also involved in this progressive APD restriction. Fzd1/2/7 and its activation of Protein Kinase C (PKC) prevent Wnt/β-catenin and Wnt/JNK signaling activities. Finally, the secreted Wnt antagonist Dkk1 is expressed at the animal pole of the embryo, reaching the highest levels by the mesenchyme blastula stage and this antagonist prevents Wnt/JNK signaling by restriction of the APD. These Wnt pathways and Dkk activity coordinate to define the correct APD territory at the anterior end of the embryo (Fig. 34.4).

As stated above, the Wnt/β-catenin signaling center at the vegetal pole of the embryo and the APD at the animal pole are believed to coordinate to establish the AV axis by providing positional information in the sea urchin.

34.3.3 Oral-Aboral Axis (Dorsal-Ventral Axis)

The oral-aboral (OA) axis of the echinoderm larva becomes evident at the gastrula stage when the presumptive oral side of the embryo becomes flat, transitioning a radially symmetric larva to one of bilateral symmetry. The oral side is equivalent to the ventral side, and aboral side to the dorsal side, thus this axis is also defined as the dorsal-ventral (DV) axis. A number of studies on the molecular basis of the establishment of the OA axis in echinoderms have been reported using sea urchin. The factors involved in this process are illustrated in Fig. 34.5.

The earliest polarization event along the OA axis in the sea urchin is the expression of *nodal*, which belongs to the TGF-β superfamily on the future oral side of the embryo. Expression of *nodal* is zygotic and it begins around the 6th cleavage stage in the ectodermal cells. Its expression is restricted to the presumptive oral ectoderm and is required for expression of oral ectoderm regulatory genes such as *gooscoid*, *brachyury* and *foxa* (Duboc et al. 2004; Flowers et al. 2004; Molina et al. 2013).

Initiation of *nodal* expression requires several maternal factors (Fig. 34.5). One of these factors is Univin, a TGF-β homolog related to Vg1. Another maternal factor necessary for expression of *nodal* is Tcf, which is the effector of Wnt/β-catenin signaling. *nodal* expression is abolished if translation of *univin* mRNA or Tcf function is blocked (Duboc et al. 2004; Range et al. 2007). Moreover, a maternal factor Oct1/2 was reported to be crucial for initiation of *nodal* expression and the zygotic expression of *univin* (Range and Lepage 2011).

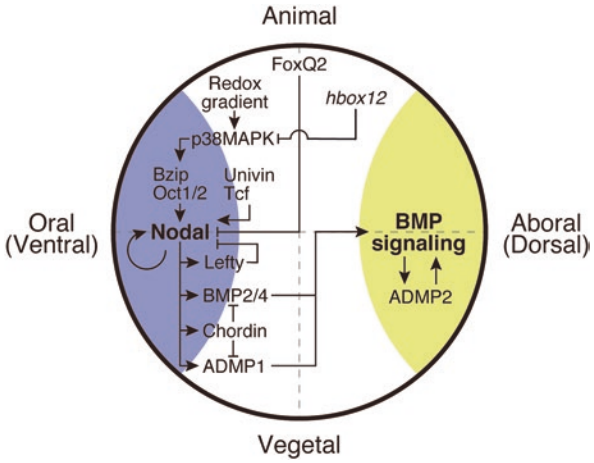

Fig. 34.5 Representation of the establishment of the OA (DV) axis in sea urchin embryos. On the oral side of the embryo, redox signaling and the maternal factors activate the expression of *nodal*, which subsequently activates *bmp2/4* and *chordin* expression (blue area). BMP2/4 is prevented from signaling by Chordin on the oral side but is translocated to the aboral side where it activates BMP signaling (yellow area). The identity of the bZIP transcription factor (Bzip) is unknown. See Table 34.1 for the functions of molecules involved in axis determination

The mechanisms establishing asymmetric expression of *nodal* on the presumptive oral ectoderm is not clear. Many years ago, the earliest physiological gradient along the OA axis was reported as the activity of cytochrome oxidase, which shows a higher respiration rate at the presumptive oral side than the aboral side as early as the 3rd cleavage stage (Child 1941). Recent studies show that this respiratory gradient is generated by maternal asymmetric distribution of mitochondria and the spatial expression pattern of *nodal* correlates with the mitochondria gradient (Coffman et al. 2009). The cis-regulatory analyses of *nodal* revealed that the 5′ cis-regulatory module contains target sites for the bZIP transcription factor (Bzip) and Oct1/2, both of which are redox sensitive (Nam et al. 2007; Range and Lepage 2011). Furthermore, it was reported that the activity of p38 MAP kinase, which is known to transfer signals responding to redox changes, is required for *nodal* expression on the oral side (Bradham and McClay 2006). Taken together, the current model suggests that at the early blastula stage, the redox gradient generated by maternally distributed mitochondria activates p38 MAP kinase on the oral side leading to the activation of Bzip and Oct1/2. These transcription factors initiate and maintain the expression of *nodal* on the future oral ectoderm. A recent study has showed that the zygotic expression of the homeobox transcription factor *hbox12* (now known as *pmar1*) also regulates *nodal* expression. *hbox12* expression begins before the onset of *nodal* expression and is restricted to the aboral side, repressing p38 MAP kinase activity, which is essential for *nodal* expression (Di Bernardo et al. 1995; Cavalieri et al. 2008; Cavalieri and Spinelli 2014) (Fig. 34.5).

Although *nodal* expression is restricted to the oral side, when its translation is blocked, specification of the aboral side does not occur, as well as the oral side. Injection of *nodal* mRNA into one blastomere at the 8-cell stage rescues complete axis formation in *nodal* morphants. Therefore, it was suggested that the oral territory expressing *nodal* has a long range organizing activity (Duboc et al. 2004). *nodal* expression on the oral side induces the expression of extracellular diffusible molecules such as Lefty, BMP2/4, and Chordin (Duboc et al. 2004, 2008; Lapraz et al. 2009). It is suggested that Lefty is more diffusible than Nodal and acts as a long-range inhibitor of Nodal, maintaining the restriction of *nodal* expression to the oral side (Duboc et al. 2008). Similarly, FoxQ2 (expressed at the APD) represses *nodal* expression at the APD, also contributing to the restriction of *nodal* expression to the oral side (Yaguchi et al. 2008). Whereas Lefty acts as a long-range inhibitor, BMP2/4 acts as a relay molecule. Although its transcripts are detected exclusively on the oral side, BMP2/4 protein diffuses over a long distance to the aboral side, activating aboral marker genes. Furthermore, *chordin* (expressed on the oral side and is downstream of *nodal*) was reported to prevent BMP signaling on the oral side (Lapraz et al. 2009). Therefore, the oral territory expressing *nodal* acts as a signaling center that is essential in establishing the OA axis patterning (Fig. 34.5).

Recently, it has been shown that both a secondary OA axis and ectopic neural tissue were induced when *nodal* signaling was ectopically activated in sea urchins (Lapraz et al. 2015). Moreover, *admp1* and *admp2* (the admp-related genes in sea urchins) cooperate with *bmp2/4* to specify the aboral region of the embryo (Fig. 34.5). The induced oral territory of sea urchin embryos expresses *admp* and *nodal*, possesses the ability to induce a secondary axis and ectopic neural tissue, and can adjust BMP-signaling by *admp* regulation. This territory also has fundamental features of the Spemann-Mangold organizer in amphibians, and is thought to be analogous to the Spemann organizer (Lapraz et al. 2015). If this is the case, the OA axis in sea urchins might be homologous to the dorsal-ventral axis of chordates.

34.3.4 Left-Right Axis

We refer to the left or right side of larvae using the common definition that refers to the oral side of the embryo as ventral. The earliest morphological asymmetry shared among echinoderms, except crinoids, is the hydropore and the hydroporic canal on the left side of the larva. This asymmetric structure consists of the opening (the hydropore in an adult as shown in Fig. 34.2) and the canal connecting it to the left somatocoel. After metamorphosis, the hydroporic canal is integrated into the adult body and it becomes part of the water vascular system. Many studies on this left-right (LR) asymmetry have been reported in both experimental embryology and molecular biological studies using sea urchins.

In sea urchins, the hydroporic canal is integrated at the late pluteus stage into the adult rudiment, which is normally formed on the left side, manifesting the most obvious LR asymmetry. Classical bisection experiments have shown that the sepa-

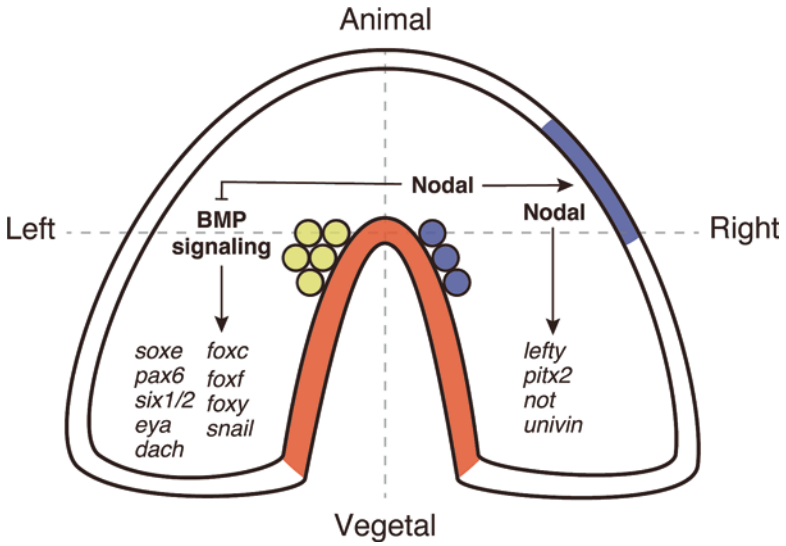

Fig. 34.6 Schematic illustration of LR asymmetry of Nodal and BMP signaling in sea urchin embryos viewed from the aboral side. First, asymmetric expression of *nodal* appears on the right side of the tip of the archenteron (blue, in the middle). Several hours later, *nodal* begins to be expressed on the right side of the ectoderm (blue, on the right). Nodal signaling blocks BMP signaling on the right side and activates right-sided genes downstream of *nodal*, whereas BMP signaling (yellow region) activates left-sided genes on the left side of the embryo. See Table 34.1 for the functions of molecules involved in axis determination

ration into the left and right halves of the embryo can affect the position of where the rudiment is formed. All embryos that are manually bisected using glass needles before hatched blastula stage can form a normal left-sided rudiment (McCain and McClay 1994). However, when separated after gastrulation, the left halves fail to form the rudiment in the normal place, whereas the right halves develop normally (Aihara and Amemiya 2001). These bisection experiments suggest that some induction from the right side of the embryo is necessary to establish normal LR asymmetry after gastrulation.

At the molecular level, the earliest reported LR asymmetrical event during sea urchin development is the expression of *nodal* in the right endomesoderm at the mid-gastrula stage (Fig. 34.6). This is consistent with reports from experimental embryology that suggest that the determination of LR polarity takes place after gastrulation (Duboc et al. 2005). This *nodal* expression in the right endomesoderm is required for subsequent *nodal* expression in the right lateral ectoderm, and in turn, this ectodermal expression of *nodal* activates Nodal target genes, such as *nodal*, *lefty*, *pitx2*, *univin*, and *not* on the right side of the embryo (Duboc et al. 2005; Hibino et al. 2006; Bessodes et al. 2012; Luo and Su 2012) (Fig. 34.6). It has also been shown that the establishment of the right-sided expression of *nodal* involves signaling pathways including Notch signaling, in which H^+/K^+-ATPase has been shown to function, suggesting that the ionic flux generated by the ATP-driven

proton pump is a factor in the early LR determination (Bessodes et al. 2012). BMP genes are initially expressed bilaterally, and BMP signaling is also required for right-sided expression of *nodal* in the endomesoderm. However, immunohistochemistry for phosphorylated Smad1/5/8 revealed that BMP signaling is stronger on the left side of the endomesoderm opposite to the nodal expressing region (Fig. 34.6). This restriction of BMP signaling to the left side requires right-sided *nodal* signaling and activates transcription factors and their cofactors, which show left-sided expression in the endomesoderm, such as *soxe*, *pax6*, *six1/2*, *eya*, *dach*, *foxc*, *foxf*, *foxy*, and *snail* (Ransick et al. 2002; Duboc et al. 2005; Hardin and Illingworth 2006; Hibino et al. 2006; Tu et al. 2006; Luo and Su 2012, Materna et al. 2013) (Fig. 34.6).

These asymmetric Nodal and BMP signaling pathways regulate left- or right-sided gene expression and are related to LR asymmetric morphogenesis. Small micromeres are precursors of part of the coelomic pouches (Pehrson and Cohen 1986). These assemble on both the right and left sides at the tip of the archenteron and receive the asymmetric signals: Nodal on the right and BMP on the left side (Fig. 34.6). When Nodal signaling is blocked using inhibitor SB-505124, the micromere distribution (normally being three and five on the right and left coelomic pouches, respectively) becomes symmetric with four on each side (Luo and Su 2012). Moreover, TUNEL assays revealed that Nodal signaling induces apoptosis of small micromeres in the right coelomic pouch. On the other hand, when BMP signaling is blocked, the hydroporic canal, which extends from the left coelomic pouch, is not formed (Luo and Su 2012).

The endomesodermal cells located at the tip of the archenteron distribute LR asymmetry to distant tissues and therefore may be considered analogous to the function of an LR organizer such as the node in mice, the Küpffer vesicle in zebrafish, the gastrocoel roof plate (GRP) in *Xenopus*, or Hensen's node in chickens. Furthermore, the opposite effects of Nodal and BMP signaling are suggested to be conserved in the establishment of LR asymmetry in deuterostomes, though in sea urchins the *nodal* expression on the right side is opposite to chordates (Nakamura and Hamada 2012).

34.3.5 Anterior-Posterior Axis and **hox** *Genes in Echinoderms*

In echinoderms, the animal-vegetal, oral-aboral, and left-right axis determinations are almost completed within the larval stages, and the mechanisms mentioned above are involved in the axes formation of larvae that show bilateral symmetry. However, echinoderms significantly rearrange their body plan during metamorphosis and show pentaradial symmetry at the adult stage. The recognizable axes in their adult forms are the oral-aboral (OA) axis and pentaradial proximal-distal (PD) axis (Fig. 34.3). This OA axis in the adult does not always correspond to that in larva since the adult OA axis is newly formed, shifted 90° in echinoids and asteroids, and 180° in crinoids from the larval OA axis, in the process of transition between larva

and adult. However, it is not shifted in holothurians and ophiuroids (Peterson et al. 2000). Therefore, the formation of the adult axes is independent of the mechanisms of larval axis determination. How this unusual body plan in echinoderm adults relates to the bilateral body plan of other phyla has been a long-standing and critical question. Current studies may enable us to resolve this question through analyses of *hox* genes.

hox genes play an important role in patterning the embryo along the anterior-posterior (AP) axis in many phyla (Lewis 1978; Krumlauf 1994; Holland and Garcia-Fernàndez 1996; Wada et al. 1999; Ikuta and Saiga 2005; Garcia-Fernàndez 2005). These genes are classified into three groups: anterior (*hox1* to *hox3*), medial (*hox4* to *hox8*), and posterior (*hox9* to *hox13*), and are organized into a cluster on the chromosome. The arrangement of the genes in the cluster are believed to correspond to their spatial expression patterns. In other words, the genes closer to the 3′ end in the cluster are expressed in more anterior regions of the body, and the genes closer to the 5′ end are expressed posteriorly. This remarkable coincidence between the arrangement in the cluster and the expression pattern of *hox* genes is referred to as spatial collinearity.

In echinoderms, the analyses of *hox* genes and their cluster organization on the chromosomes has been reported in echinoids and asteroids. The other three classes (crinoid, ophiuroid, and holothuroid) have less detailed information on the *hox* orthologs, but are critical for future studies. The four posterior *hox* genes in echinoderms, as well as in hemichordates, are named *9/10*, based on the similarity to the chordate paralogous group (PG) 9 and PG10 genes, or *11/13a*, *11/13b*, and *11/13c*, with similarities to PG11-13 genes without specific orthology relationships (Peterson 2004). The organization of the Hox cluster has been revealed in an indirect developing echinoid, *Strongylocentrotus purpuratus* (Cameron et al. 2006). This cluster contained 11 *hox* genes and its organization is quite unusual as *hox4* was not identified and *hox1* to *hox3* were translocated to the 5′ end in a reverse order. Moreover, *hox5* and *hox11/13b* were inverted in situ. In the direct developing sea urchin *Peronella japonica*, the same set of genes was identified but the organization of *hox* genes in this species was not analyzed (Hano et al. 2001). Nine partial *hox* sequences were identified by polymerase chain reaction (PCR) from the sea cucumber, *Holothuria glaberrima*, but the medial *hox* genes *hox4*, *hox6*, *hox7* and *hox8* were not reported (Méndez et al. 2000). Recently, Kikuchi et al. (2015) isolated 11 *hox* gene sequences including eight full-coding sequences (CDSs) in another sea cucumber, *Apostichopus japonicus*. Among the five medial *hox* sequences identified, three were annotated as *hox5*, *hox7*, and *hox8*, and the remaining two *hox* genes are considered to be *hox4* and *hox6* (Kikuchi et al. 2015). In an ophiuroid (*Stegophiura sladeni)* partial sequences for one anterior *hox* gene, three medial *hox* genes, and five posterior *hox* genes were isolated (Mito and Endo 2000). In asteroids, early studies identified seven *hox* sequences from each of the two species *Asterina minor* and *Patiriella exigua* (Mito and Endo 1997; Long et al. 2006). The complete organization of the Hox cluster was reported in *Acanthaster planci* (Crown-of-thorns Starfish) and 11 *hox* genes were identified (Baughman et al. 2014). Unlike the sea urchin Hox cluster, the *A. planci* Hox cluster showed no trans-

location of the genes and contained *hox4*, thus resembling chordate or hemichordate clusters. In crinoids, Mito and Endo (2000) cloned the homeobox sequences of *hox* genes from a comatulid (feather star) *Oxycomanthus japonicus* and showed that this species has at least 11 *hox* genes, but *hox4* and *hox5* were not identified. In the stalked crinoid (sea lily) *Metacrinus rotundus*, which is considered a more basal crinoid, eight full CDSs were cloned including *hox4* and *hox5* (Hara et al. 2006).

Expression patterns of *hox* genes have been reported in the sea urchin, sea lily, sea cucumber (Fig. 34.7), and sea star. In the sea urchin *S. purpuratus*, *hox7* and *hox11/13b* are expressed in the aboral ectoderm and the blastopore region before the pluteus stage (Angerer et al. 1989; Arenas-Mena et al. 2006) (Fig. 34.7a). In the late pluteus stage, slightly before metamorphosis, five *hox* genes (*hox7*, *hox8*, *hox9/10*, *hox11/13a*, and *hox11/13b*) are expressed sequentially in the left and right somatocoel along the U-shaped digestive tract (Arenas-Mena et al. 2000) (Fig. 34.7b). Later in the adult rudiment, *hox3* is expressed in the dental sacs in a pentaradial pattern (Arenas-Mena et al. 1998). In the direct developing sea urchin, *Holopneustes purpurascens*, three *hox* genes (*hox3*, *hox5*, *hox11/13b*) are expressed in numerical order along the OA axis of the adult rudiment with the anterior-most expression of *otx* (known to have an expressing region anterior to that of *hox1* in other bilaterians) (Morris et al. 2004; Morris and Byrne 2005, 2014). A recent study showed that four *hox* genes showed a radial expression pattern in the adult rudiment in *P. japonica* (*hox1*, *hox5*, and *hox11/13b* in the vestibule, and *hox3* in the interambulacra and dental sac rudiments), suggesting that a subset of *hox* genes is recruited for a new patterning role (Tsuchimoto and Yamaguchi 2014) (Fig. 34.7c).

In the sea lily *M. rotundus*, four *hox* genes (*hox5*, *hox7*, *hox8*, *hox9/10*) are expressed in the mesodermal somatocoel in numerical order along the AP axis of the larva at preauricularia and middle auricularia stages (Hara et al. 2006) (Fig. 34.7f). Moreover, Omori et al. (2011) reported that *otx* is expressed in the region just anterior to that of *hox7* in the archenteral sac. In the sea star *Parvulastra exigua*, *hox4* expression is observed in the epithelium of the anterior coelom, left and right coeloms, developing hydrocoel, and the gut at the larval stage. After metamorphosis, *hox4* is expressed in the epithelium of the somatocoel of the juvenile in a pentaradial pattern (Cisternas and Byrne 2009). In a sea cucumber *A. japonicus*, the expression patterns of eight *hox* genes (*hox1*, *hox5*, *hox7*, *hox8*, *hox9/10*, *hox11/13a*, *hox11/13b*, and *hox11/13c*) were analyzed from early development to juvenile stages (Kikuchi et al. 2015). Five *hox* genes (*hox1*, *hox7*, *hox8*, *hox11/13a*, *hox11/13b*) are expressed in the gut in numerical order along the AP axis at the early auricularia stage (Fig. 34.7d). Then at late auricularia stage, the expression of only three *hox* genes (*hox7*, *hox11/13a*, *hox11/13b*) are detectable by in situ hybridization, but they do not show a sequential expression pattern in the gut, and are expressed separately in the stomach, anal region, and the left somatocoel, respectively. This suggests that the development of the larval body is almost completed and *hox* genes are no longer involved in patterning the larva just before metamorphosis. Metamorphosis occurs after the late auricularia stage, and after metamorphosis, all eight *hox* genes studied begin to be expressed in the epithelium of the gut and/or somatocoel in a numerical order along the digestive tract. This suggests that

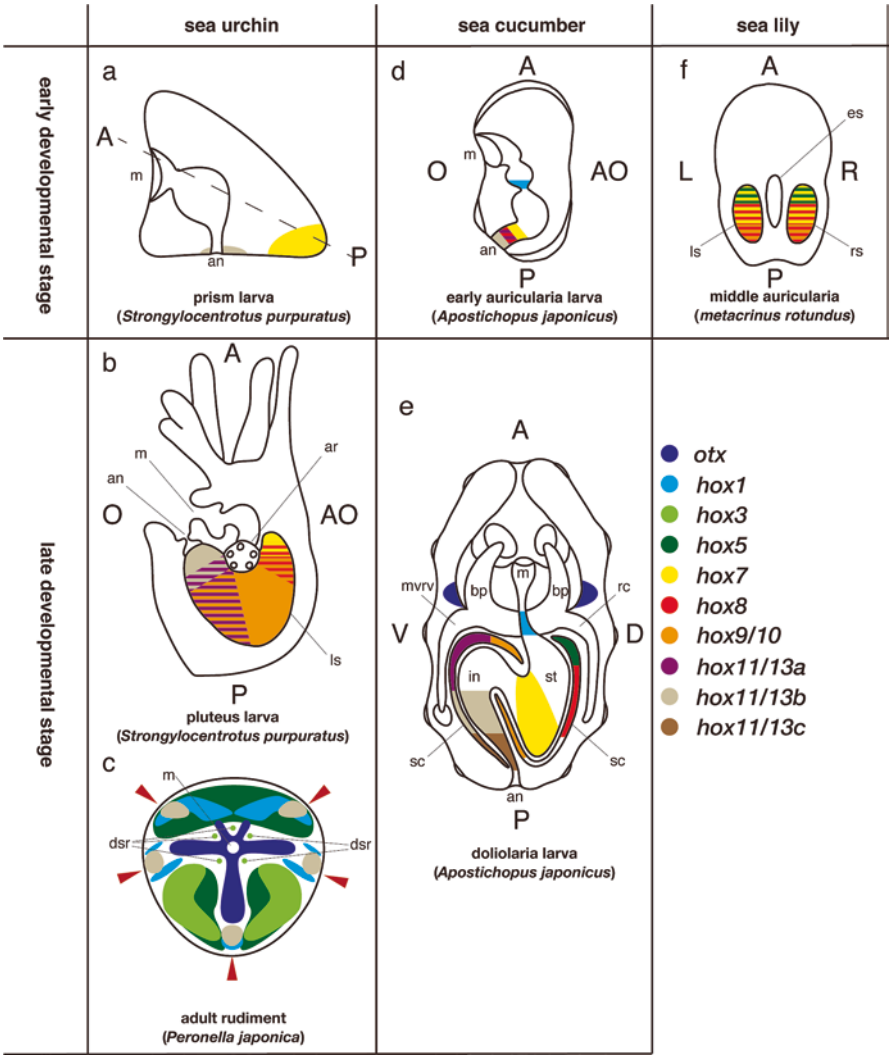

Fig. 34.7 Expression patterns of *hox* genes in the sea urchin (*S. purpuratus* and *P. japonica*), sea cucumber (*A. japonicus*) and sea lily (*M. rotundus*). Left column shows expression patterns in sea urchin: (**a**) prism larva of sea urchin viewed from the left lateral side, (**b**) pluteus larva viewed from the left lateral side and (**c**) adult rudiment of sea urchin viewed from the oral side. Middle column shows expression patterns in sea cucumber: (**d**) early auricularia larva of sea cucumber viewed from the left lateral side and (**e**) doliolaria larva of sea cucumber viewed from the left lateral side. Right column shows expression patterns in sea lily: (**f**) middle auricularia larva of sea lily viewed from the aboral side. Directions of the body axes that may be defined are also indicated. Note that the adult dorsal-ventral axis is present at the doliolaria stage larva of sea cucumber. The abbreviations are as follows: *A* anterior, *P* posterior, *O* oral, *AO* aboral, *V* ventral, *D* dorsal, *L* left, *R* right, *an* anus, *ar* adult rudiment, *bp* buccal podium, *dsr* dental sac rudiment, *es* enteric sac, *in* intestine, *ls* left somatocoel, *m* mouth, *mvrv* mid-ventral radial vessel, *rc* radial canal, *rs* right somatocoel, *sc* somatocoel, *st* stomach. Red arrowheads indicate the position of ambulacra (radial canals) in (**c**). The regions between the ambulacra are interambulacra

they play a role in patterning the digestive tract along the AP axis. Moreover, Shoguchi et al. (2000) reported that *otx* expression was observed in the buccal cavity just anterior to the region where *hox1* is expressed (Fig. 34.7e).

Since the sea urchin, which has been the most studied among echinoderms, is highly derived and the expression patterns obtained from sea lilies mainly focused on early developmental stages, comparing these data had limitations (David and Mooi 2014). However, by using the data from blastula (early developmental stage) to juveniles in sea cucumbers (Kikuchi et al. 2015), we are able to conclude that there are two phases of patterning by *hox* during development. The first is the patterning of the AP axis, seen in the patterning of the larval gut at early auricularia stage in the sea cucumber, and the second is the patterning of the adult digestive tract after metamorphosis (Kikuchi et al. 2015). It is reasonable to compare *hox* expression in the sea lily and sea urchin with that of the presumptive same stage in the sea cucumber. In the sea lily, four *hox* genes are expressed sequentially in the somatocoel at the middle auricularia stage (Fig. 34.7f). Similarly, in the sea cucumber, five *hox* genes are expressed sequentially in the gut (Fig. 34.7d). Although the regions where *hox* genes are expressed are different (the somatocoel in sea lily and the gut in sea cucumber) and the types of *hox* genes expressed are different, it could be hypothesized that *hox* genes have a conserved role in patterning the AP axis of echinoderm larvae. In later stages, five *hox* genes are expressed sequentially in the somatocoel in sea urchins (Fig. 34.7b). Since the adult digestive tract of the sea urchin elongates along the somatocoel, it may be that the expression of *hox* genes in the somatocoel gives spatial information necessary to transform the larval gut to an adult digestive tract (Arenas-Mena et al. 2000; Tsuchimoto and Yamaguchi 2014). This idea is further confirmed by the finding that eight *hox* genes are expressed in the epithelium of the gut or somatocoel sequentially at the doliolaria stage when the larval gut is being rebuilt as the adult digestive tract in the sea cucumber (Kikuchi et al. 2015) (Fig. 34.7e). It is known that *hox* genes are expressed in the visceral mesoderm providing the positional information to pattern the digestive tract in other bilaterians (Beck et al. 2000; Bienz 1994; Kawazoe et al. 2002; Roberts et al. 1995; Sakiyama et al. 2001; Yokouchi et al. 1995). Therefore, patterning the digestive tract may be the fundamental function of *hox* genes in bilaterians, including echinoderms. In later stages when pentameral structures are formed, four *hox* genes in the adult rudiment of sea urchins and *hox4* in the somatocoel of juvenile sea stars are expressed in pentameral patterns (Morris et al. 2004; Cisternas and Byrne 2009; Morris and Byrne 2005, 2014; Tsuchimoto and Yamaguchi 2014). The expression of *hox* genes could be interpreted as being involved in patterning the echinoderm pentaradial body plan, but in contrast to sea urchins and sea stars, a pentameral expression of *hox* genes is not observed in the sea cucumber, though they do have a pentameral body plan. The pentaradial expression domains of a subset of *hox* genes in the sea urchin *P. japonica* are correlated with the pentameral structures that are to develop, such as the ambulacrum, lantern, and spines (Tsuchimoto and Yamaguchi 2014). This could be considered a result of co-option of the *hox* genes to patterning these structures (Tsuchimoto and Yamaguchi 2014). Sea cucumbers do not have

these structures, which may be the reason why pentameral expression is not present.

Altogether, taking into consideration the expression of *otx*, which is expressed anterior to the *hox* genes along the OA axis, molecular data suggest that the OA axis of echinoderms is equivalent to the AP axis of other bilaterians, and that the AP axis of adult echinoderms is arranged along the digestive tract.

34.4 Conclusion and Perspectives

Studies on the body plan of echinoderms have contributed to our overall understanding of how body plans are established, how they vary in evolution, and some of the potential molecular mechanisms that might be involved in such morphogenesis. Most of this work has been carried out in the highly derived sea urchin taxon. The accumulation of molecular data on body plan determination has shown that the molecules involved in the process are orthologs of those involved in body plan determination in other model species, especially in vertebrates. This conclusion is not surprising, considering that bilaterians utilize a set of highly conserved genes. Instead, this conclusion indicates that the general body plan of echinoderms is determined using similar tools. How they use these tools may be different. It must be noted, however, that early swimming larvae are the targets of most molecular analyses, not the pentameral adult body that is unique to this phylum.

The most intriguing feature of echinoderms is their pentaradial structure, and the development mechanism for this feature is largely unknown. Minsuk et al. (2009) defined another axis to describe this adult body plan, the "circumoral" axis. This axis circles the mouth perpendicular to the oral-aboral axis, and along this circumoral axis, the five rays of pentameral symmetry is divided. Although no molecular basis is presented, treatment with $NiCl_2$ disturbed the circumoral pattern, resulting in abnormal distribution of appendages (Minsuk et al. 2009). Further studies on adult rudiment formation and metamorphosis are needed to solve the mystery of the echinoderm body plan.

To elucidate morphogenesis during metamorphosis and in the adult rudiment, modern microscopy techniques are powerful tools. What was previously only observable as a series of histological sections is now reconstructed into 3D and laser scanning confocal microscopy, which allows us to grasp the image of developing echinoderm embryos and larvae. The suitable methods for observation in combination with the diversity of species for investigation must be selected wisely. For example, during metamorphosis of the sea cucumber *A. japonicus*, the larvae shrink and condense to a much smaller size than at pre-metamorphosis, become pigmented, and therefore, the inner structure is difficult to observe, but with tissue clearing, the vision is improved, and the development of the hydrocoel may be observed (Tajika and Kondo, unpublished).

Studies based on classic experimental embryogenesis, in which embryos or larvae are manipulated to test the development of individual tissues or to see interac-

tions between tissues, may also be useful to find the mechanisms underlying metamorphosis and late stages of development. It may not be easy since embryos and larvae of echinoderms are generally smaller than other major model organisms in which manipulation experiments have been performed, and again, the species to use may have to be carefully reasoned. We have tried laser ablation of larval tissue in the developing sea cucumber, and we were able to produce fourfold symmetrical (not pentameral) juveniles (Nagai and Kondo, unpublished). These kinds of approaches may reveal new developmental phenomena.

Ever since the whole genome of the sea urchin *S. purpuratus* was sequenced in 2006, many echinoderm genomes have been and are being sequenced. Transcriptomes are produced and we will be able to access many genes previously unknown that are unique to echinoderms, and that may play crucial roles for echinoderm development. These assets are key to expanding our understanding of the echinoderm body plan development. In turn, understanding how this unique echinoderm body plan is established will likely impact our general understanding of *hox* genes, axes formation, and evolutionary selection in body plans.

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In the original version of Chapter 9, the electronic supplementary materials were inadvertently omitted. The electronic supplementary materials videos have been included in the chapter.

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