18 Echinoderms
Temnopleurus reevesii

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CONTENTS

18.1 INTRODUCTION

Sea urchins have been used as model organisms in biological fields for more than a century. Their usefulness as such comes from certain aspects and characteristics: sea urchin adults are easily collectable from the oceans, their gametes are easily spawned by the simple intrablastocoelar injection of KCl and embryos and larvae develop synchronously in small containers like beakers. In addition, because their early development occurs outside of the adult bodies, scientists can routinely apply embryology techniques, such as microinjection and micromanipulation (Yaguchi 2019a; George et al. 2019), leading researchers to a number of high-impact achievements in various biological fields (Davidson 2010; Evans et al. 1983). On the other hand, because the life cycle of sea urchin is generally very long and it takes almost two years to obtain the next generation, it has been impossible to apply genetics to sea urchin studies in the laboratory. However, we have found that a sea urchin species, *Temnopleurus reevesii* (Figure 18.1a), can produce the next generation in a half-year, which is much shorter than the more commonly used species of sea urchins, such as *Strongylocentrotus purpuratus* and *Hemicentrotous pulcherrimus*, and has a potential to be applied to genetics. Therefore, in this chapter, I will introduce the biological characteristics of *T. reevesii* and its high potential to contribute to genetic studies of echinoderms.

18.2 HISTORY OF THE MODEL

Although most sea urchin species are attractive to human beings as tasty food ingredients, especially in Japan, *T. reevesii* is one of the exceptions due to its bitter taste. In addition, compared with other model sea urchins, such as

S. purpuratus, *Lytechinus variegatus* in North America, *Paracentrotus lividus* in Europe and *H. pulcherrimus* in East Asia, *T. reevesii* has not been well studied in biology. Therefore, the presence of the species has been reported (Hegde et al. 2013), but there are only a handful of experimental biological data. As a comparative analysis of the developmental processes among the Temnopleurus group, the Kitazawa lab in Japan first described the development of *T. reevesii* (Kitazawa et al. 2010, 2014). Following this work, our group reported the high temperature tolerance and the neurogenesis of the embryos and larvae (Yaguchi et al. 2015). While culturing embryos/larvae/juveniles, we recognized that *T. reevesii* has a fast generation cycle, about half a year. By focusing on these characteristics, our group expected it would be possible to introduce the study of gene functions using genetics to this sea urchin and has started to prepare the genome and transcriptome resources, which will be published elsewhere soon. The genome information allowed us to use the CRISPR/Cas-9 system to knock out some genes, and in fact, we managed to obtain the first homozygous knock-out strain using this species (Yaguchi et al. 2020).

18.3 GEOGRAPHICAL LOCATION

It has been reported that *T. reevesii* is found in the western Pacific and Indian Oceans (Clark et al. 1971; Hegde et al. 2013). Since historically there have been few scientific groups using this species for research, there is a possibility that new habitats will be found elsewhere in the near future. In Japan, the Kitazawa group has reported that they used *T. reevesii* collected from the Seto Inland Sea (Kitazawa et al. 2010, 2014), whereas our group found the adults of this species in our research center's aquarium, into which seawater
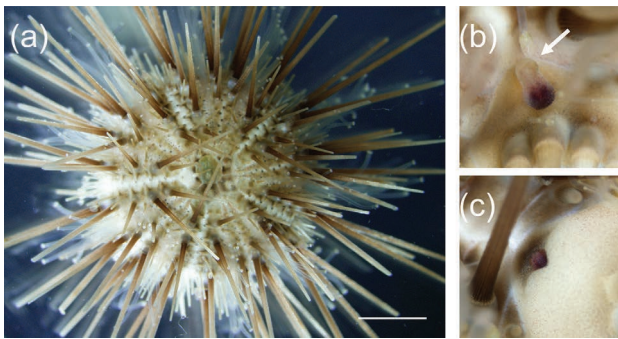
FIGURE 18.1 The adult of *T. reevesii*. (a) *T. reevesii* is a regular sea urchin whose body has pentaradial symmetry. Bar = 5 mm. (b) The genital papilla from the gonopore of adult males (arrow). This is not observed in the gonopore of females (c).

is continuously pumped. It is expected that the larvae swim in the general area around the Shimoda Marine Research Center, University of Tsukuba, including the Sagami Bay and the Pacific Ocean, and were pumped into the aquarium overflow system, in which they metamorphosed. On the other hand, although we have tried to identify the habitat of *T. reevesii* around the Shimoda Marine Research Center, we have never succeeded in finding it through scuba diving or a remotely operated underwater vehicle (ROV). Some dredge investigations picked up young individuals of *T. reevesii* but never found mature adults. Some pictures on divers' private websites show the adults of *T. reevesii* in the Izu peninsula near Shimoda, suggesting that there is a suitable habitat around Shimoda Marine Research Center, but the population of these animals is not likely to be dense.

18.4 LIFE CYCLE

Like other model sea urchins, *T. reevesii* undergoes indirect development, in which the gametes spawned from the male and female are fertilized outside the adults' bodies and the early and late development proceed as plankton in the ocean. They swim in the ocean via the movement of cilia, which are located at the surface of each ectodermal cell. Because they sink in seawater if the ciliary beating stops, the embryos/larvae essentially keep afloat using their cilia. In addition, sea urchin larvae have anti-gravitaxis, prompting them to stay at the surface of the ocean (Mogami et al. 1988). Due to their benthic lives, the adults cannot migrate over a large area, suggesting that it is likely that they spread their geographical distribution during the planktonic embryo and larval stages. Larva consume micro-algae as a food source, and in the laboratory culture, we feed them a diatom species, *Chaetoceros calcitrans*, which is commercially available (SunCulture, Marinetech, Aichi). After 1 to 1.5 months, the adult rudiment appears on the left side of the eight-armed larval body, and it grows until metamorphosis. In our laboratory, the competent larvae of *H. pulcherrimus*, the major sea urchin model in Japan, rarely metamorphose without an inducer like biofilm, which is generally localized on rocks and/or the sea floor. However, the competent larvae

of *T. reevesii* easily metamorphose in glass beakers by simply stopping the stirring of water (Yaguchi 2019b).

Juveniles eat the adhered diatoms until the shell diameter size is 1.5 mm, but their food preference changes to carnivorous when they become larger (Yaguchi 2019b). Therefore, they start to eat meat of fish, shellfish and even small sea urchins. It is surprising to note that they eat their same species but never other vegetarian species like *H. pulcherrimus*. The most prominent characteristic of *T. reevesii* as a model sea urchin in biology is that they grow very fast from juveniles to sexually mature adults. General model sea urchins like *S. purpuratus* or *H. pulcherrimus* take more than one to two years until they are stably producing gametes (Strathman 1987), but *T. reevesii* can reach the stage after a half-year by culturing above 20°C. Another advantage as a model sea urchin is the timing of producing eggs and sperm. In the general model sea urchins, they need a temperature stimulus from warm to cold (e.g. in *H. pulcherrimus*, the temperature change from 23°C to 13°C induces the maturation of gonads), but in *T. reevesii*, keeping the culturing seawater warm (above 20°C) is enough to induce the accumulation of sperm or eggs in the adult gonads. This characteristic allows scientists to repeatedly use the same individuals unless they become damaged due to spawning and to save a number of adult sea urchins for research purposes.

18.5 EMBRYOGENESIS

Because the adults that hold matured gonads were observed from May to December in the outside aquarium, it is expected that spawning and early embryogenesis occur during summer/fall in the wild, when the temperature of seawater is above 20°C. In addition, the fact that the embryos of this species have a wide range of temperature tolerance between 15 and 30°C has been described (Yaguchi et al. 2015). Therefore, in laboratory conditions, we generally culture them at room temperature (RT) (about 20°C) for long-term experiments like creating inbred strains and at 22°C for the purposes of developmental biology. The diameter of unfertilized and fertilized eggs of *T. reevesii* is about 80 μm (Figure 18.2a, b), which is smaller than that of *H. pulcherrimus*. When we culture them at RT, the first cleavage occurs between 1 and 1.5 hours, and the embryos reach the four-cell stage at about two hours. During these early cleavages, blastomeres do not attach to each other, unlike other model sea urchins. The blastomere strongly attaches to the hyaline layer (Figure 18.2c, d, arrow) (Yaguchi et al. 2015). These separated blastomeres group together around the 60-cell stage, an event called "compaction", and the development continues like other sea urchin embryos after that. At several hours after hatching (Figure 18.2e), primary mesenchyme cells (PMCs) ingress into the blastocoel from the posteriorly located vegetal plate, and gastrulation occurs from the same region. PMCs will be spiculogenic cells in prism/pluteus larval stages. As observed in other model sea urchin embryos, from the tip of the invaginating gut, the secondary mesenchyme cells (SMCs) ingress into the blastocoel (Figure
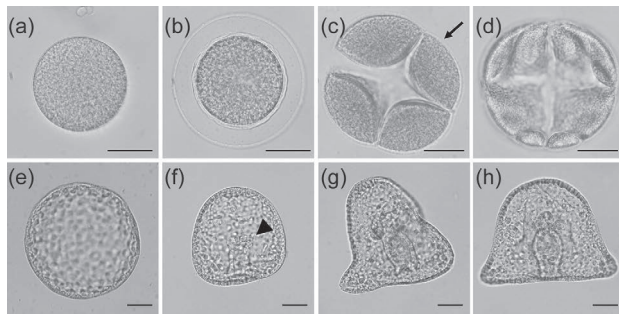
FIGURE 18.2 Development of *T. reevesii* embryos/larvae. (a) Unfertilized egg. (b) Fertilized egg with fertilization envelope and hyalin layer. (c) Four-cell stage. Arrow indicates the hyalin layer. (d) Sixteen-cell stage. (e) Hatched blastula. (f) Gastrula. Arrowhead indicates the ingress of secondary mesenchyme cells from the tip of invaginating gut. (g) Prism larva, lateral view. (h) Prism larva, ventral view. Bars = 40 μm.

18.2f, arrowhead). SMCs will be differentiated into muscles, pigment cells, the coelomic sac and blastocoel cells during the larval stages. After the tip of the gut fuses to the oral ectoderm in order to open the mouth, the endoderm starts to constrict to form the tripartite gut, which is composed of the esophagus, stomach and intestine (Figure 18.2g, h). The completion of gut differentiation allows the larvae to start food consumption (Yaguchi et al. 2015; Yaguchi 2019b). The number of larval arms increases during late pluteus stages from two to eight, as observed in other model sea urchins (Kitazawa et al. 2014). After 1 to 1.5 months after fertilization, the adult rudiment appears at the left side of the body, and it begins to metamorphose.

18.6 ANATOMY

Since *T. reevesii* is one of the regular sea urchins, the adult body has pentaradial symmetry covered with spines (Figure 18.1a). They move using tube feet, which are driven by the contractions of muscle and water force through the hydraulic system. All major anatomical characteristics are the same as those observed in the other regular sea urchins, but the genital papilla are notable in this species. The genital papilla clearly protrude from the gonopores in the male (Figure 18.1b) of *T. reevesii* but not from those of female (Figure 18.1c) (Yaguchi et al. 2015). This allows scientists to distinguish males and females when they obtain gametes, saving the time to collect eggs or sperm and saving the number of adults, because the researchers do not have to try multiple KCl injections on several individuals. The body shape and spine distribution of *T. reevesii* appear to be very similar to *Temnopleurus toreumaticus*. However, the spines of the former do not have a stripe pattern, while those of the latter do. The body color is essentially light brown, but it is variable; in fact, the strain kept in our laboratory is mutant, and its body color is highly pigmented and almost magenta. The size of the endoskeleton of adult *T. reevesii* is <5 cm in captivity in the laboratory, and the length of the spine is between about 1 to 3 cm.

18.7 GENOMIC DATA

In North America, Echinobase (Cary et al. 2018), a database for echinoderms (www.echinobase.org/entry/), publishes the genomic and transcriptomic data of several echinoderm species. In Europe, the genome and other genetic tools of the European model sea urchin, *P. lividus*, are in preparation (http://marimba.obs-vlfr.fr/organism/Paracentrotus/lividus) and will be made public soon. In Asia, we have the genome and transcriptome of *H. pulcherrimus* and have made a publicly available database for them, HpBase (Kinjo et al. 2018, 2021). The genome and transcriptome data of *T. reevesii* are in preparation, and the database is under construction and not yet publicly available but will be added to HpBase in near future. However, our laboratory used the information for gene knockout using the CRISPR/Cas-9 system (see Section 18.7), and it proved useful for these experiments. The genomic and transcriptome data will be available upon request to the author.

18.8 FUNCTIONAL APPROACHES: TOOLS FOR MOLECULAR AND CELLULAR ANALYSIS

As is the case for other model sea urchins, knockdown techniques using morpholino anti-sense oligonucleotides (MOs) and misexpression experiments using *in vitro* synthesized mRNA are available in *T. reevesii* (Suzuki and Yaguchi 2018). These reagents are introduced into unfertilized or fertilized eggs by microinjection. The microinjection techniques are common in any sea urchin species, and our laboratory uses an injection buffer that contains 22.5% glycerol for *H. pulcherrimus* eggs or blastomeres (40 mM HEPES, pH 8.0, 120 mM KCl, 22.5% glycerol). This buffer is also used for the North American *S. purpuratus*. On the other hand, glycerol-containing buffer kills the eggs of *T. reevesii*. Therefore, we use the injection buffer without glycerol. The details of the comparison and the methods of microinjection into sea urchin species are available elsewhere (Yaguchi 2019a).

To analyze the function of genes, *in situ* hybridization and immunohistochemistry are essential techniques and available to this species like other sea urchins. *T. reevesii* embryos/larvae have transparent bodies, which allow us to see the chromogenic and fluorescent signals very clearly (Figure 18.3a, b). In addition, almost all antibody reagents, which work against *H. pulcherrimus*, cross-react to *T. reevesii* embryos and larvae, but very few exceptions are present. For example, anti-phospho-Smad2/3 antibody (Abcam, Eugene, OR, USA) recognizes the phosphorylation site at the C-terminal of *H. pulcherrimus* Smad2/3 protein (. . . KQCSS*VS*; *phosphorylation site) but does not for *T. reevesii* because of its sequence difference (. . . KVCSS*MS*) (Suzuki and Yaguchi 2018).

One of the most prominent techniques in genetics is the knock-out. As mentioned, sea urchins have been considered not useful for genetics because of the length of their generation cycle. However, it takes about six months for *T. reevesii* to produce the next matured generation, which allows us
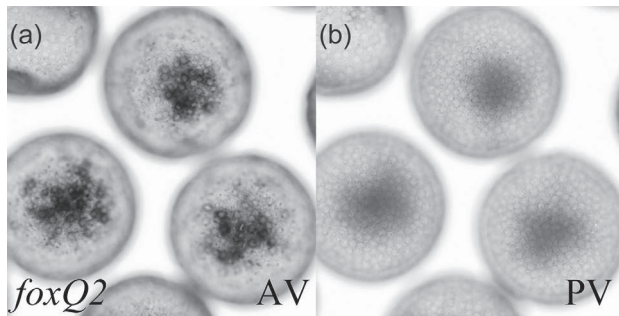
FIGURE 18.3 *In situ* hybridization using *T. reevesii*. (a) The expression of *foxQ2*, which is an essential transcription factor for the specification of anterior neuroectoderm. Anterior view (AV). (b) *foxQ2* does not express at the posterior end. Posterior view (PV).

FIGURE 18.4 Pks1 knock-out *T. reevesii*. (a) The late control (Cas-9 only injected) larva, which has an adult rudiment at the left side of the body. (b) Pks1 knock-out F0 late pluteus larva, which loses pigmentation. (c) The heterogenous F2 adult (inbred magenta mutant is used as a control strain). (d) The homogenous Pks1 knock-out F2 albino adult.

to challenge the status quo for sea urchins by introducing gene knock-out techniques to this species. In addition, the innovation of the CRISPR/Cas-9 system makes it easy for scientists to knock out genes in any organism, including sea urchins (Doudna and Charpentier 2014; Jao et al. 2013; Lin and Su 2016; Oulhen and Wessel 2016). The combination of the relatively short life cycle of *T. reevesii* and CRISPR/Cas-9 allowed us to produce the first homozygous knock-out strain of an albino sea urchin (Yaguchi et al. 2020). We focused on knocking out polyketide synthase 1 (Pks1), which plays the essential role in pigmentation (Akamatsu et al. 2010). We designed and synthesized five gRNAs against the second exon of the gene. Each gRNA was microinjected with hCas9 mRNA, which is synthesized from the plasmid (pCS2+hSpCas9; #51815 Addgene) *in vitro*. The efficiency of mutation was calculated with T7E1 assay (Vouillot et al. 2015), and #4 gRNA showed the highest efficiency. The injected embryos/larvae were cultured in 3L beakers with stirring until metamorphosis (Figure 18.4a, b), and the juveniles and young adults were cultured in a closed aquarium system (Yaguchi 2019b). Because the injected generation, that is, F0 generation, frequently contains mosaic genomic patterns even in one individual, the sperm or eggs are fertilized with wild type gametes and researchers obtain heterogeneous F1 generations. After confirming the genotype of individuals, we used the same types of sperm and eggs and then fertilized them to obtain the homozygous knock-out F2 mutant (Figure 18.4c, d). This research showed strong evidence for the availability of *T. reevesii* as a model organism in genetics, although the span of the life cycle is a little longer than those of other model organisms in this field, such as mice and fruit flies.

18.9 CHALLENGING QUESTIONS BOTH IN ACADEMIC AND APPLIED RESEARCH

Based on a number of previously published studies, gene regulatory analyses using sea urchin embryos have contributed much to biological fields and to an understanding of how gene expression is regulated. In fact, the most detailed and f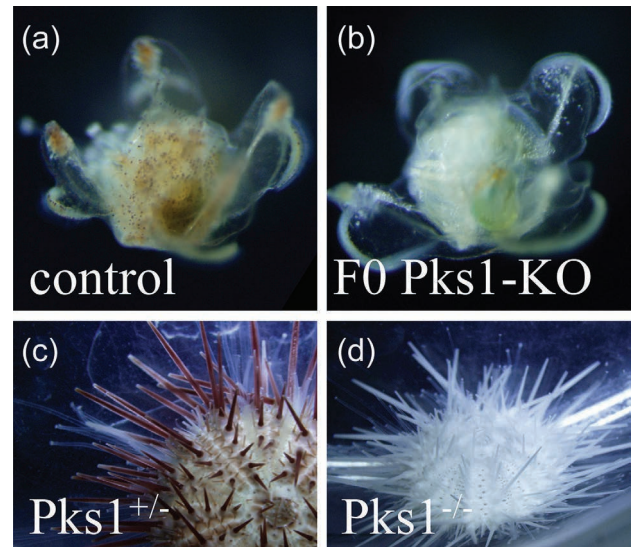amous gene regulatory network in the world is about the specification of sea urchin endomesoderm (Davidson 2010; Cui et al. 2014). To investigate cis-regulatory elements, scientists utilized the microinjection of BAC-based reporter constructs into fertilized eggs (Nam et al. 2007; Sodergren et al. 2006; Buckley et al. 2019) and analyzed the data, which came from the mosaically integrated reporter constructs and variable patterns of individuals. A large number of experiments and the efforts of statistical processing helped scientists to confirm the results. Therefore, if people can analyze the endogenous gene expression pattern in embryos in which cis-regulatory elements were homozygously deleted by the CRISPR/Cas-9 system, the results will be more reliable and we can re-build more sophisticated gene regulatory networks.

Simple gene knock-outs are also available and efficient for analyzing gene functions in sea urchins. Although gene knock-downs using MO injection techniques can target only early embryogenesis, CRISPR/Cas-9-based knock-outs can target genes that function in later developmental stages and adults. This technique will help scientists understand the biology of sea urchins more thoroughly. However, metamorphosis during the sea urchin's life might be a barrier for genetics, because it is a really drastic event, and one expects that a number of genes function to create the adult body. In fact, when we knock out Smad2/3 with CRISPR/Cas-9, the mutants were all dead at the timing of metamorphosis (data not shown). It is also true that it is still not easy to obtain the next generation of sea urchins in the laboratory, even if *T. reevesii* is easier than other model sea urchins. Taken together, however, the combination of the CRISPR/Cas-9 system and *T. reevesii* promises to reveal numerous biological insights through sea urchin knock-out strains. Knock-in techniques have not yet been successful in sea urchins.

Although many sea urchin species are famous for being a source of tasty ingredients worldwide, *T. reevesii* is not suitable for food. The Japanese name of *T. reevesii* is "hari sanshou uni", and the meanings of "hari", "sanshou" and "uni" are "spined", "bitter/hot" and "sea urchins", respectively. Therefore, it is said that *T. reevesii* is not good as food, and, in fact, people do not find this species in seafood markets. However, in genetics, *T. reevesii* can be useful to understand gene functions related to the taste and the size of gonads. At the same time, when compared with other model sea urchins which are commonly used in food, it is a mystery why *T. reevesii* can grow faster. If this question can be answered using *T. reevesii*, sea urchin farmers in the fishery industries will obtain ideas for culturing sea urchins from the basic sciences.

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