Doctoral Dissertation (Censored)

博士論文（要約）

Histological and molecular developmental studies on the regeneration of digestive tract in the sea cucumber *Eupentacta quinquesemita*

（ナマコ綱イシコの消化管再生における組織学的および分子発生学的研究）

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Abstract

Sea cucumbers (echinoderm) have a high regenerative capacity such that, following ejection of their almost entire inner organs, called evisceration, the lost organs regenerate. There are two ways of evisceration: from the mouth (anterior) or from the anus (posterior), depending on species. What is intriguing is that the regenerating tissues are formed at both the anterior and posterior regions and extend toward the opposite ends, and merge to form a completed digestive tract. From the posterior side, the digestive tract regenerates extending a continuous tube from the cloaca, which still remains at evisceration, in both types of species. In the posterior-eviscerating species, the esophagus also remains in the body, and a new tube regenerates continuously from it. However, in the anterior-eviscerating species, no tubular tissue remains in the anterior region, raising a question how the new digestive tract is formed in the anterior region. I addressed this question by detailed histological observation of the regenerating anterior digestive tract using a small sea cucumber, *Eupentacta quinquesemita* ("ishiko" in Japanese) after induced-evisceration. I found that an initial rudiment consisting of mesenchymal cells is formed along the edge of the anterior mesentery from the anterior end, and then, among the mesenchymal cells, multiple clusters of epithelial-like cells appear simultaneously and repetitively in the extending region by using toluidine blue staining. Subsequently, it was observed that multiple cavities were formed surrounded by these epithelial cells, coalesce with each other to form multiple lumens, and eventually became a single tube.

This anterior tube then fused to the tube regenerated from the posterior rudiment. Thus, I could decide the process of regeneration of the anterior portion of the gut in an anteriorly eviscerating species. Based on the observation that the epithelial-like cells appear among the mesenchyme, I suggest the involvement of mesenchymal-epithelial transition (MET) and fusion of cavities/lumens in the regeneration of the digestive tract.

Histological observations in Chapter 1 suggest that the regeneration process after posterior evisceration in *E. quinquesemita* involves mesenchymal-epithelial transition (MET). In Chapter 2, therefore, expression patterns of factors known to be involved in the MET pathway, i.e., transcription factor *twist* and *snail* were investigated by realtime qPCR method, after the identification of orthologs for those factor genes from the transcriptome database constructed based on RNA-sequencing result. As results, I showed that *twist* was up-regulated from relatively earlier stages of the digestive-tract regeneration, followed by the up-regulation of *twist*. Furthermore, I also focused on Hox and Parahox genes, because it was predicted that the differentiation of the digestive tract should occur according to the spatial information along the body axis. As expected, the expression patterns of Hox and Parahox genes showed an expression pattern along the anterior-posterior axis. Interestingly, the timing of Hox/Parahox expression followed the expression of MET-related factors, indicating that the detailed differentiation of digestive tract take place after the formation of gut tube by MET. This predicted differentiation process was also congruent with the results of histological observations.

In contrast to the posterior evisceration, the anterior evisceration observed in species of Dendrochirotida ejects the whole anterior structures, including the oral complex. This structure contains the nerve ring that is thought to function as a part of the central nervous system (CNS) in echinoderms. Therefore, in chapter 3, histological observations on the regeneration of the nerve ring was carried out, followed by gene expression analyses on some neural patterning genes. The result of the histological observations focusing on the nerve-tissue regeneration showed that the nerves are regenerated from the anterior ends of the remaining radial nerve chords, later connecting to each other to form the ring structure. Gene expression analyses by realtime qPCR showed that the genes responsible for the nervous-system development, in particular *otx*, *six3/6* and *pax6* genes, were up-regulated in the anterior body part during the regeneration process. These results suggest that the anterior-eviscerating species recruit the developmental genes regularly used for the normal echinoderm development.

Contents

Abbreviations

CNS: central nervous system

DIG: digoxigenin

dpe: days post evisceration

EDTA: ethylenediaminetetraacetic acid

EF1α: elongation factor 1 alpha

EMT: epithelial-mesenchymal transition

HE: hematoxylin-eosin

KCl: potassium chloride

MET: mesenchymal-epithelial transition

$MgCl_2$: magnesium chloride

MOPS: 3-(N-morpholino) propanesulfonic acid

NaCl: sodium chloride

NADH: nicotinamide adenine dinucleotide + hydrogen dehydrogenase

PBS: phosphate buffered saline

PBST: PBS containing 0.1% Tween-20

qPCR: quantitative polymerase chain reaction

RPS18: ribosomal protein S18

SSC: saline-sodium citrate

TB: toluidine blue

TUBB: tubulin beta chain

WISH: whole mount *in situ* hybridization

General Introduction

Defense and autotomy in animals

In animals, except for the top predators, almost all species perform or exhibit same types of defensive strategies to avoid predation (Rojas and Burdfield-Steel 2018). There are diverse types of defensive strategies, such as mechanical, chemical and behavioral defense (Spiteller 2008). Among those, autotomy, defined as the shedding of a body part, is a conspicuous type of defense strategy, in which preys can escape from predators while the predators are attracted to the autotomized body part (Fleming et al. 2007). It is also well known that, after autotomy, regeneration of the lost body part occurs in most of the cases, to recover the original function of the lost parts (Maginnis 2006). Therefore, regeneration after autotomy should occur in most cases, except for the case of autotomy at adult stage when the regeneration ability disappears. In echinoderms, many species in all the five extant classes perform autotomy, followed by regeneration of autotomized bodyparts (Emson and Wilkie 1980, Lawrence 1987, Dolmatov 1996, Byrne 2001, Wilkie 2001, Byrne 2020).

Regeneration

Regeneration is the re-growth of missing or damaged tissues or organs in adults or even in larvae or embryos and is often comparable to embryogenesis in that they involve morphogenesis. In embryogenesis, the whole embryo develops various tissues in a coordinated manner, whereas during regeneration, the animal that has lost a part of their body restores that part only without affecting other parts. Thus, regeneration is a self-organizing phenomenon, and its underlying mechanism may differ from that

of the normal embryogenesis. Although there are many studies, regeneration is full of unanswered questions, such as how regeneration is made possible, what molecules triggers regeneration, and why the ability to regenerate differs among animals, just to mention a few (Tanaka and Reddien 2011, Grillo et al. 2016).

Various animals are known for their high ability to regenerate and are used for the study of regeneration, for example, planarians and newts (Oviedo and Beane 2009). Echinoderms, consisting of five classes regenerate efficiently and are also studied although the less attention has been paid on them (Arnone et al. 2015, Ferrario et al. 2018, Candia Carnevali 2001). For example, sea stars, brittle stars and crinoids have high potential for regeneration, and are able to self-amputate (autotomize) their arms, but then are able to completely regrow them. Some sea stars are known to regenerate the whole body even from an arm, and some crinoids also regenerate their viscera (Arnone et al. 2015). Furthermore, in echinoderms, regeneration is a form of asexual reproduction such as fission (Dolmatov et al. 2018).

Evisceration in sea cucumbers

In general, the movements of echinoderm individuals in any species are relatively slow, so that they can easily be caught by predators. Therefore, they have defensive tactics to avoid such predation risks (Wilkie 2001). For examples, they possess hardened body walls made of sclerites (ossicles) and sometimes numerous spines (Brusca et al. 2016). As mentioned above, many species such as starfishes (Asteroidea) exhibit autotomy of their arms, followed by complete regeneration. It is also known that there are a number

of poisonous species (James 2010). Among those strategies, sea cucumbers (Holothuroidea) show the most distinctive defensive strategy, i.e., "evisceration," that is one of the types of autotomy (Smith and Greenberg 1973, Byrne 1985, Dolmatov 1996, Garciá-Arrarás et al. 1998).

Sea cucumbers are known to exhibit extensive regenerative ability and regenerate a wide spectrum of body parts, e.g., their body wall, the nervous system, and internal organs such as the digestive system, reproductive organs and respiratory trees. Ejection and regeneration of the sea cucumber digestive system have been the object of studies, and observations has been documented since more than 100 years (Mosher 1956).

Evisceration, the ejection of almost the entire internal organs, occurs under natural conditions as a defense strategy, i.e., autotomy (Mosher 1956), as well as in response to unnatural or experimental environmental cues like foul water or external mechanical or chemical stimuli. In evisceration, the digestive tract first autotomizes at the posterior or anterior ends, and is ejected from either the mouth (anterior evisceration) or the anus (posterior evisceration), respectively, depending on the species (García-Arrarás and Greenberg 2001, Mashanov and García-Arrarás 2011), and in either case, the mesentery remains, which connects between the internal lining (mesothelium) of the body cavity and the digestive tract.

In the posterior evisceration, a part of digestive tract between esophagus and cloacal stump are eviscerated, while in the anterior evisceration, not only digestive tract, but also anterior part of the body including esophagus and oral complex are eviscerated, depending on species (**Fig. GI-1**, Mashanov and García-Arrarás 2011). Species performing anterior

evisceration are seen in the order Dendrochirotida, while species showing posterior evisceration are seen in the orders Synallactida and Holothuriida (**Fig. GI-2**; the phylogenetic tree is based on Reich et al. 2015 and Miller et al. 2017). In contrast to anterior evisceration, the oral complex including the nerve ring which acts as the central nervous system, the ring canal constituting the water vascular system, and the tentacles, is eviscerated, so that the regeneration after the anterior evisceration is a more cost-consuming phenomenon.

Study purpose

Therefore, in the present thesis, I focused on the anterior-eviscerating sea cucumber, *Eupentacta quinquesemita*, to investigate the regeneration process after the anterior evisceration and the underlying developmental mechanisms that enable the sea cucumber to regenerate the whole structures of the eviscerated body parts (**Fig. GI-3**). In Chapter 1, I extensively carried out histological observations during the regeneration process after the anterior evisceration. The results showed that the mesenchymal-epithelial transition (MET) was involved in the regeneration of digestive tract. Based on this, in Chapter 2, I carried out the gene expression analyses on the MET-related genes, which were up-regulated in the relatively-early stages of regeneration process. Furthermore, Hox genes were also shown to be involved in the regeneration of digestive tract. In the anterior evisceration in *E. quinquesemita*, the central nervous system, i.e., nerve ring, is also discarded, so that there should be a mechanism for regeneration the nervous system. Therefore, in Chapter 3, I also focused on the genes that are thought

to contribute to the development of nervous system, showing the re-recruitment of genes responsible for the nervous development that are normally used during embryogenesis. Taken together, I discuss the developmental mechanisms underlying this distinctive regeneration phenomenon in *E. quinquesemita*, and the evolutionary meaning of this intriguing phenomenon which have been acquired only in sea cucumbers.

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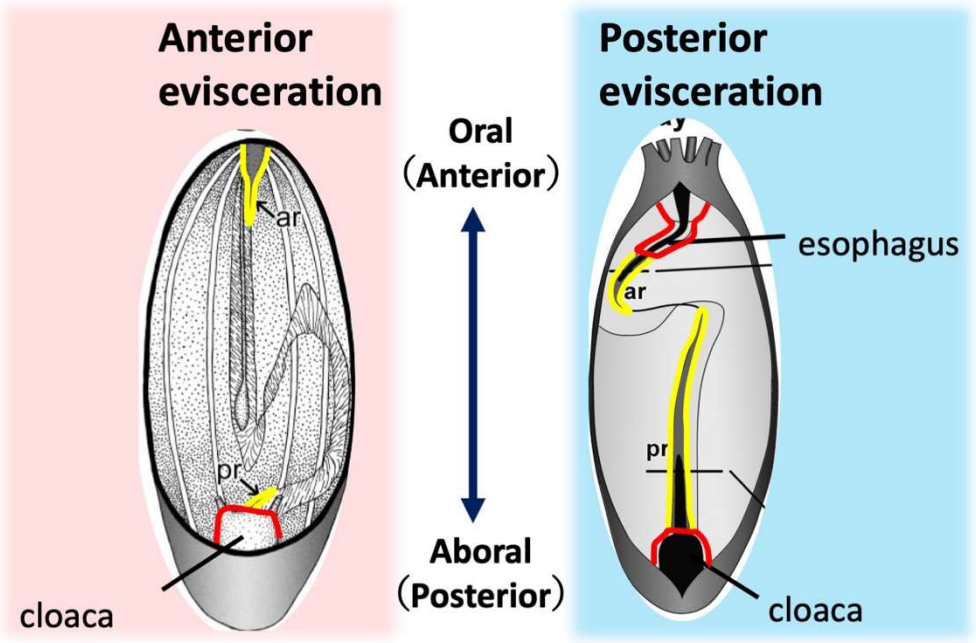

Figure GI-1.

Anterior and posterior evisceration seen in sea cucumbers (Holothuroidea). In posterior evisceration, a part of digestive tract between esophagus and cloacal stump, while in anterior evisceration, not only digestive tract, but also anterior part of the body including esophagus, oral complex, and respiratory trees, depending on species. Yellow indicates the body parts that are eviscerated and regenerated afterwards. Red indicates the body parts that are not eviscerated. Pictures were after Mashanov and García-Arrarás (2011).

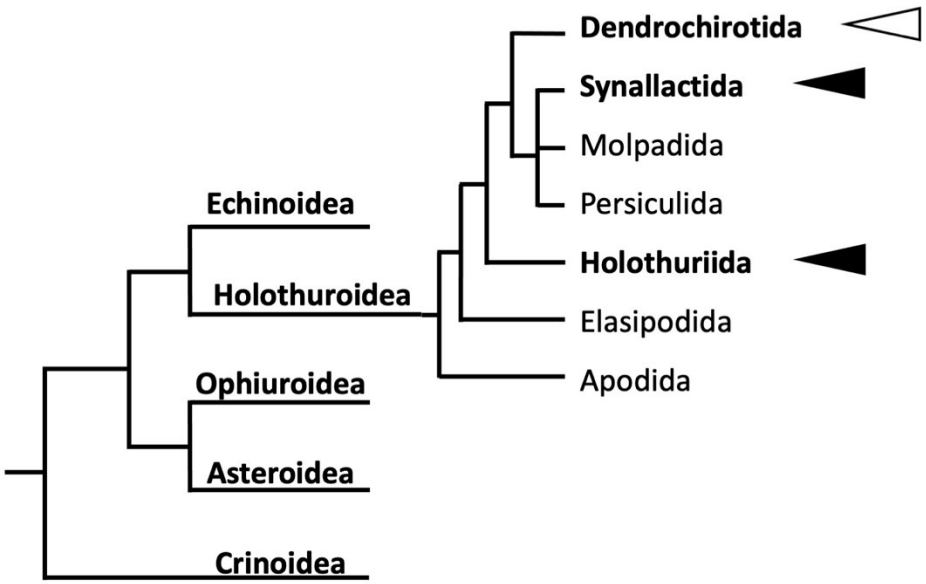

Figure GI-2.

Phylogenetic tree showing the echinoderms and the holothuroid species exhibiting anterior and posterior evisceration. Anterior evisceration is seen in species belonging to the order Dendrochirotida (white arrowhead), while posterior evisceration is seen in the orders Synallactida and Holothuriida (black arrowheads). The phylogenetic tree is based on Reich et al. (2015) and Miller et al. (2017).

Figure GI-3.

The material species focused in this study, an anterior-eviscerating sea cucumber, *Eupentacta quinquesemita*. Photo taken by Hisanori Kohtsuka.

Chapter 1

Regeneration of the digestive tract of an anterior-eviscerating sea cucumber, *Eupentacta quinquesemita*, and the involvement of mesenchymal-epithelial transition in digestive tract formation

The content of this chapter has already been published as Okada A, Kondo M (2019) Regeneration of the digestive tract of an anterior-eviscerating sea cucumber, *Eupentacta quinquesemita*, and the involvement of mesenchymal-epithelial transition in digestive tube formation. Zoological Letters 5: 21.

Introduction

Sea cucumbers are known to exhibit extensive regenerative ability and regenerate a wide spectrum of body parts, e.g. their body wall, the nervous system, and internal organs such as the digestive system, reproductive organs and respiratory trees. Ejection and regeneration of the sea cucumber digestive system has been the object of studies and observations has been documented since more than 100 years (Mosher 1956). Evisceration, the ejection of almost the entire internal organs, happens under natural conditions (Mosher 1956), as well as in response to unnatural or experimental environmental cues like foul water or external mechanical or chemical stimuli. In evisceration, the digestive tract first autotomizes at the posterior or anterior ends, and is ejected from either the mouth (anterior evisceration) or the anus (posterior evisceration), respectively, depending on the species (García-Arrarás and Greenberg 2001), and in either case, the mesentery remains, which connects between the internal lining (mesothelium) of the body cavity and the digestive tract.

Animals that eviscerate anteriorly release the entire digestive tract except for the cloaca and loses the entire anterior end structures such as the oral (aquapharyngeal) complex and the tentacles, and those that eviscerate posteriorly release the intestine between the esophagus and the cloaca. During regeneration, despite the difference in the type of evisceration, the free edge of the mesentery thickens, which contributes to the gut primordium. In posterior eviscerating sea cucumbers, the wound at the end of the remaining esophagus and cloaca due to autotomy are closed and anterior and

posterior blind gut rudiments are formed, and these tubes grow and eventually join to form a continuous digestive tract (García-Arrarás et al. 1998). By contrast, in anterior eviscerating species, a blind tube is formed continuously from the remaining cloaca, but in the anterior part, a rudiment of a mass of cells is first formed and then the tube somehow regenerates and extends posteriorly. This phenomenon has been studied using a dendrochirotid sea cucumber, *Eupentacta fraudatrix*, as an example (Mashanov et al. 2005). Based on histological data, it was described that a rudiment consisting of a solid rod of connective tissue covered by coelomic epithelium appears at the oral end of the body. According to Mashanov et al. (2005), "dedifferentiated mesothelium" covering the ventral side of the rudiment developed "folds that penetrate the underlying connective tissue," and after several days of regeneration, this "epithelial lining of the folds detaches from the surface" and forms "a number of lumina that fuse into a single blind lumen lined with a newly formed luminal epithelium," which is "derived from the external mesothelium of the rudiment," and they propose transdifferentiation of coelomic epithelium into digestive epithelium. If this is the case, the mesothelium of mesodermal origin could transdifferentiate into the digestive epithelium of endodermal origin, but this kind of transdifferentiation beyond the realm of germ layers as the epithelium structure is retained has never been reported as far as I know. Therefore, I was not totally convinced with their description, since it was not clearly shown in their data in what manner invagination occurs, how or if multiple lumens are formed, and how they fuse to form a single lumen. I thus addressed this

question in this study using another sea cucumber, *Eupentacta quinquesemita*, or "ishiko" in Japanese.

E. quinquesemita (ishiko) (**Fig. 1-1**) is a small sea cucumber that belongs to same genus as the species handled in previous studies on intestine regeneration (Leibson 1992, Mashanov et al. 2005), and they inhabit the seas around Japan. They also eviscerate anteriorly and seasonally in nature (Byrne 1985a, b). Evisceration may be induced experimentally by chemicals such as KCl (Byrne 1985c), and the presence of an endogenous "evisceration factor" has been reported (Harrington et al. 2009). Using induced evisceration, the morphology of the sites of autotomy has been studied (Byrne 2001).

The digestive tract of ishiko is made up of the oral complex, stomach, intestine and cloaca, and they are anchored to body wall by the mesentery (**Suppl. Fig. 1-1a**). The intestines can be divided into three parts: the first descending intestine, the ascending intestine, and the second descending intestine. When evisceration occurs, autotomy happens at four regions between 1) the oral body wall/the oral complex (the introvert), 2) the longitudinal muscle/the retractor muscle, 3) intestine/mesentery and 4) intestine/cloaca, and, after evisceration, the mesentery and cloaca remain in the body cavity (Mashanov et al. 2005, Byrne 2001). The general structure of the mesentery of sea cucumbers has been described by Hyman (1955) to be subdivided into three parts. I have dissected ishiko, and similarly, the three parts of the mesentery was observed, and the mesentery does not run straight along one side of the body wall, but is connected to the dorsal side near the mouth and ends on the ventral side. The first part is an anterior part

that attaches to the dorsal body wall, connected to the oral complex up to the middle part of the intestine near the gonads (1st descending intestine) (**Suppl. Fig. 1-1b**). The second middle part curves to the left and anterior side, supporting the ascending intestine, up to the ventral side of the body wall. The direction of the mesentery then changes to the posterior, and this third part of the mesentery supports the 2nd descending intestine and the cloaca, attached to the ventral side of the body (**Suppl. Fig. 1-1c**). Due to the curvature of the mesentery, when the animal is dissected open and flattened, the mesentery appears to be in a S-shape (**Suppl. Fig. 1-1a**).

In a trial to induce evisceration in ishiko, I confirmed that oral tissues were lost and a hole-like wound was made. These results were consistent with previous studies (for example, Byrne 2001). As mentioned earlier, evisceration in this species has already been reported, but to my knowledge, there are no descriptions on the process of regeneration, except for the external morphology of the regenerating digestive tract following seasonal evisceration (Byrne, personal communication). In order to provide insight into the formation of the regenerating anterior digestive tract in ishiko, I performed histological observations during the course of regeneration.

Materials and methods

Animals

The sea cucumber, *Eupentacta quinquesemita* was collected by diving at the depth of about 3 m in Sagami Bay, near the pier of Misaki Marine Biological Station in Miura, Kanagawa Prefecture, or at the depth of about 5-10 m near Aquamarine Fukushima in Onahama, Fukushima Prefecture. Animals of body lengths between 5 and 10 cm were kept in tanks until used for experiments. Referring to Byrne (1986), evisceration was induced by injection of approximately 100 mL of 0.45 M KCl into the coelom. Eviscerated animals were placed in aquaria with sea water at 13-18℃ without feeding, up to 3 weeks. The day of evisceration was designated as 0 days post evisceration (dpe). For each series of experiments, multiple individuals were induced to eviscerate simultaneously, and several animals were used for observation at various stages of regeneration.

Morphological and histological observations

Animals were anesthetized in 72 g/L $MgCl_2$ in sea water for approximately 15 minutes to 1 hour before being dissected. They were dissected along the body length at the right ventral interambulacral zone, exposing the whole body cavity, and examined under a stereomicroscope. Gonads that lie over the intestine and mesenteries were removed. The mesentery with regenerating tissues were cut out with the piece of the body wall where it was attached, and fixed in Bouin's Fixative overnight at room temperature. Subsequently, the fixative was replaced with 70% ethanol and the specimens

were stored at room temperature until embedding in paraffin (Paraplast X-TRA, SIGMA) for histological observation. Serial sections of 6-10 μm thickness were produced, and they were stained with hematoxylin-eosin (HE) or toluidine blue (TB) and observed on a light microscope (ECLIPSE TE300, Nikon). Sections were mostly made perpendicular to the anterior-posterior (oral-aboral) axis of the body. In total, 86 animals were used for morphological observations, out of which 20 were used for histological observations.

Results

Morphological observation of the regenerating digestive tract

When *Eupentacta quinquesemita* (ishiko) was induced to eviscerate by the injection of KCl, the internal organs were ejected from the mouth within about 15 minutes. A part of the gonads, the oral complex and the digestive tract were ejected anteriorly (**Suppl. Fig. 1-2**), leaving a hole at the anterior tip.

The eviscerated animals were returned to tanks and were allowed to regenerate, and some were collected every few days for examination. To record the progress of regeneration, the animals were dissected and the internal morphology was observed (**Fig. 1-2**). The degree of regeneration of the digestive tract was not the same in all animals, i.e., some individuals regenerated faster than the others, so based on morphology, I divided the process into 4 stages, stages I to IV (**Fig. 1-3**). It took 2 to 3 weeks until a continuous gut rudiment was formed after evisceration.

Stage I (just after evisceration): At 0 days post evisceration (dpe), the oral area was not healed, and an opening remained as a result of anterior evisceration. In the body cavity, only the mesenteries and cloaca remained (**Fig. 1-2**, 0dpe). The edge of the mesenteries that had been in connection with the digestive tract was free. Some gonadal tissues, the gonadal duct (embedded in the mesentery), and respiratory trees were also left in the body cavity. These features were observed 0-1 dpe.

Stage II (formation of anterior regenerating tissues): In a specimen at 4 dpe (**Fig. 1-2**, 4 dpe), the oral wound was closed. The anterior tips of the pentaradial longitudinal muscles and radial water canals were converged

at the anterior end of the body, and formed a mass of cells (depicted in yellow in **Fig. 1-3**, Stage II). This mass of cells is potentially the rudiment from which the oral complex and the digestive tract regenerate, and is termed the "anterior rudiment" in Mashanov et al. (2005). In contrast, no cell mass nor thickening of the mesentery were observed at the region where the cloaca was attached to the mesentery. Similar features were observed in animals 1-4 dpe.

Stage III (formation of posterior regenerating tissues and growth of the anterior regenerating tissues): I observed in a specimen at 10 dpe that regenerating tissues in a rod-like shape extended from the anterior rudiment (**Fig. 1-2**, 10 dpe). This tissue was connected to and appeared to elongate along the free edge of the mesentery. On the posterior side, a thickening of the edge of the mesentery was observed. At the free edge of the middle part of the mesentery, no obvious thickening was found, and the anterior and posterior gut rudiments (depicted in yellow in **Fig. 1-3**, Stage III) were separated. Similar features were observed in other specimens between 5 and 14 dpe.

Stage IV (formation of a continuous rudiment): The presence of a continuous rudiment was observed between 14 and 20 dpe, depending on the animal. In a specimen at 17 dpe (**Fig. 1-2**, 17 dpe), a thickening of the edge was observed in the entire mesentery, indicating that the anterior and posterior regenerating tissues are connected. The continuous regenerating tract appeared straight without curving, although the mesentery is attached to the body wall in a S-shaped curve. It has been reported that the mesentery grows wider during the course of regeneration (Tracey 1972), and this allows

the rudiment to take a short route between the mouth and the anus. In another specimen at 20 dpe (**Fig. 1-2**, 20 dpe), the gut rudiment had thickened and elongated to be observable without a microscope, though it was still smaller than an intact one. The middle part of the rudiment expected to become the 1st descending intestine to the ascending intestine was looped. After this stage, the connected rudiment of the digestive tract grew further, forming loops.

Histological observation of the intact digestive tract

Before looking at the histology of regenerating tissues, I observed intact tissues for comparison. Thin slices were stained with hematoxylin-eosin (HE).

The intact digestive tract is mainly composed of 4 kinds of tissues; the coelomic epithelium, muscle, connective tissues and the luminal epithelium. The tissue structure differed depending on the part of the digestive tract. In the stomach the muscle layer developed well and luminal epithelium was covered with cuticles (**Fig. 1-4a**). The muscle layers of the three parts of the intestine were thinner than the stomach and the luminal epithelia was folded (**Fig. 1-4b, c, e, f, g, i**). The luminal epithelia of the 1st descending intestine was thicker than the other two parts of the intestine. I observed additional luminal epithelia in the lumen of the thin sections, which I interpret that the luminal epithelium extended into the lumen and was folded (**Fig. 1-4b**). The cloaca is linked to body wall with tendons, and at the sites of the connection, there were protrusions on the coelomic side, which consisted of coelomic epithelium and connective tissues. A thin layer of muscles, thick

connective tissues and smooth luminal epithelium were observed as layers (**Fig. 1-4h, j**).

Histological observation of the regenerating tissues

I investigated histologically the regenerating tissues of one specimen at stage Ⅰ, three at stage Ⅱ, 13 at stage Ⅲ, and three at stage Ⅳ. Two staining methods were used, HE and toluidine blue (TB). Through the course of my experiments, I found that staining with TB is better to distinguish cell types, namely between epithelial-like cells and mesenchyme.

Stage Ⅰ: In the specimen about 1 to 1.5 hours after evisceration, only the mesentery and cloaca remained in the body cavity. The mesentery at the oral side was thin, though the free edge appeared to be wider than closer to the body wall (**Fig. 1-5a, b**), and no cell mass that was observed in later stages were present. The gonadal duct is embedded in the mesentery, between its distal end where the intact digestive tract is attached and the body wall (**Suppl. Fig. 1-1b**). The gonadal duct survives evisceration except for the segment that had been connected to the oral complex, and this was confirmed (**Fig. 1-5c, d**). The intestine detached from the posterior mesentery, anterior to the cloaca, and did not show any mass of cells at the free edge (**Fig. 1-5e, f, g)**.

Stage II: As described earlier, according to morphological observation at this stage, thickening of tissues on the free edge of the mesentery was observed on the anterior side. The thickening of the free edge of the anterior mesentery was confirmed with histological observation to be a mass of cells (**Fig. 1-6a**). The coelomic epithelium surrounding the

regenerating tissue (gut rudiment) appeared strongly stained with hematoxylin than the inside (**Fig. 1-6b**). Inside of the regenerate, no specific structures were observed, and the cells were mesenchymal. At the posterior side, the part of the mesentery where it connected with the cloaca appeared wider (**Fig. 1-6c**). I was not able to tell if this was the gut rudiment that regenerates continuously from the cloaca. In a region of the mesentery closer to the anterior side, I did not observe any regenerating tissue at the edge (**Fig. 1-6d**).

Stage Ⅲ: For the observation of this stage, specimens at 6, 7 and 12 dpe were used. To what extent regeneration progressed does not always match the period of regeneration and I did not detect clear differences between the 6 and 7 dpe specimens. In all specimens, thickened tissues were observed at both the anterior and posterior mesenteries in both specimens. The anterior regenerating tissue elongated posteriorly and became thicker in more anterior regions (**Fig. 1-7a**). In the regenerated tissues that had grown larger, multiple cavities were found (**Fig. 1-7b**). The cells in the anterior regenerate did not show uniform staining intensities with HE, and the cavities were surrounded with cells that were stained stronger than the others (**Fig. 1-7b**). TB staining of another specimen revealed that most of the mesenchyme of the regenerate was stained reddish purple and the cells surrounding the lumens were stained light blue (**Fig. 1-7c, d**). Cells stained light blue were also found scattered among mesenchymal cells (**Fig. 1-7d**). The coelomic epithelium of the regenerate was stained purple and some tissues basal to the epithelium were colored light blue (**Fig. 1-7d**). This light blue tissue was observed more on the side closer to the mesentery, and may

reflect difference in tissue differentiation. Sagittal sections of another specimen revealed that the light blue-colored tissues surrounding the cavities existed in the regenerating tissue posterior to the regenerating oral complex (**Fig. 1-7e**). The cavities were not in contact with the coelomic epithelium and the cavities extended in the anterior-posterior direction independently or possibly fused to each other (**Fig. 1-7f**). I observed that on one side of the regenerating tissue the coelomic epithelium was stained in purple whereas the on the other side a layer of cells were stained light blue (**Fig. 1-7f**). I am not able to explain why a purple layer of cells were not detected in this figure, but together with Fig. 7d, the data suggest that there may be a difference in differentiation among the cells/tissues in the periphery of the regenerate at the same distance from the anterior end of the body. On the aboral side, a hollowed tube was found, extending from the cloaca (**Fig. 1-7g**). The luminal epithelium was also continuous from the cloaca. The tissue in the regenerating tissue other than the coelomic and luminal epithelia were mesenchymal (**Fig. 1-7h**).

Stage IV: At this stage, the rudiment of the digestive tract was continuous by the elongation and fusion of the both anterior and posterior tissues (**Fig. 1-8g**). However, not all regenerating sea cucumbers formed continuous tubes. I observed in a specimen at 17 dpe that in the anterior portion of the regenerate a single lumen was formed (**Fig. 1-8a**). There was a layer of cuticle in this tissue that was expected to become the stomach (**Fig. 1-8a** and **Fig. 1-4a, d**). This suggests that differentiation of different regions of the digestive tract occurs as the lumen is formed. Posteriorly, there were regions that had multiple cavities/lumens (**Fig. 1-8b, e**), no obvious cavities

(**Fig. 1-8c**), or a clear single lumen (**Fig. 1-8d**), and close to the cloaca in the posterior portion of the regenerate, a single lumen was formed inside (**Fig. 1-8f**). In a further regenerated specimen at 20 dpe, a single lumen penetrated the digestive tract from the anterior to posterior end like in intact animal. In the middle of the digestive tract, the intestine meandered and I was able to observe a histological section of different regions in the same view (**Fig. 1-8h**), though I could not distinguish the 1st descending and ascending intestines since the loops were zigzagged. The tissues were not totally identical to the intact intestine, but some folds in the luminal side was observed, as well as a difference in tissue compositions between the 1st descending and ascending intestines versus the 2nd descending intestine, indicating that the intestines have differentiated or in the process of differentiation.

In summary, from histological observations, I detected the start of formation of the lumen at stage III and the completion at stage IV (**Fig. 1-9**). There was a difference in how the lumen is formed between the anterior and posterior gut rudiments. In the anterior region, small multiple cavities/lumens surrounded by epithelium-like tissues arose among the mesenchyme and the lumen develops by fusing with each other. On the posterior side, a new intestinal tube elongates as a hollow continuous tube from the cloaca, consistent with previous studies in other sea cucumbers (see discussion). At stage IV, the regenerated digestive tract is completed by the coalescence of new tubes generated from the anterior and posterior.

Discussion

This study is the first to report the regeneration of the digestive tract of *Eupentacta quinquesemita* (ishiko) in detail. As reported in other sea cucumber species as well as *E. fraudatrix*, the edge of the mesentery thickens and regenerating tracts expand from both the anterior and posterior rudiments toward the other end of the body. However, my histological observations of the formation of the anterior digestive tract in ishiko is different from what had been reported in the case of *E. fraudatrix*, in which the luminal epithelium is supposed to be formed by invagination of "dedifferentiated mesothelium" (Mashanov et al. 2005). I did not observe any mesothelium penetrating into the mesenchymal cells or invagination. Instead, I found that multiple small cavities are simultaneously formed inside the rudiment, over the period of regeneration, and the lumens fuse with each other, so that the number of lumens decrease and their diameters increase, which eventually results in the formation of a single lumen. The occurrence of multiple epithelia-like cells and cavities in the rudiment at different positions clearly showed that in the anterior gut rudiment, the tube is not formed by simply expanding the lumen or boring a hole inside the rod-like rudiment. In stage IV animals that appeared to have regenerated a continuous gut, nevertheless, the histology revealed that in the middle of the whole rudiment there were parts where tubes were not present or were discontinuous (**Fig. 1-8**). There has been investigations in other anterior (*Thyone okeni*) or posterior eviscerating species (*Stichopus mollis*), in which lumina were formed discontinuously and independently (Tracey 1972, Dawbin

1949). It would be interesting to examine other species whether multiple cavities are formed inside the mesenchyme.

The mechanism how epithelial tissues arise or cavities/lumens merge to each other remains a question. In the course of my study, I found that epithelium-like tissues or cells may be distinguished from mesenchyme by color by toluidine blue (TB) staining. TB stains tissues blue, but when the dye binds to acidic sugar chains the color changes to reddish purple or violet. This phenomenon is called metachromasia and is used for detection of acid polysaccharides and dyeing of cartilage (Bergeron and Singer 1958). The staining with TB allowed us to realize that there are multiple epithelium-like tissues differentiating in the anterior gut rudiment, leading to my understanding that multiple cavities are formed within them. I think that this staining method is convenient and effective in studying tube formation in the regenerate, and should be applied to *E. fraudatrix* to compare with ishiko.

I point out that tube formation in the anterior rudiment resembles secondary neurulation, a process observed in the development of the caudal portion of the spinal cord of amniotes (reviewed in Harrington et al. 2009, Lowery and Sive 2004). At the onset of secondary neurulation, mesenchymal cells condense to form a cord-like structure, and lumens are formed inside the compact cord, a process called cavitation. The transformation of mesenchymal cells into an epithelial sheet is known as mesenchymal-epithelial transition (MET) (reviewed in Thiery and Sleeman 2006, Chaffer et al. 2007). Formation of the epithelium in secondary neurulation is made possible through MET (reviewed in Shimokita and Takahashi 2011, Catala 2002, Criley 1969), and MET is also known to be involved in other

developmental programs, for example, in somitogenesis (Christ and Ordahl 1995), kidney development (Ekblom 1989), and coelom formation (Funayama et al. 1999). From my current study, the formation of the epithelial lining of the regenerating lumen of the anterior gut in anterior eviscerating sea cucumbers is added to the example of MET. MET is considered a reverse process of epithelial-mesenchymal transition (EMT), and effectors of MET and EMT are supposed to influence each other (reviewed in Thiery and Sleeman 2006). As for the molecular mechanism of MET, in some instances such as nephrogenesis, the involvement of signaling molecules are implicated, including cell adhesion molecules (e.g. R-cadherin), growth factors, and transcription factors (reviewed in Chaffer et al. 2007). It was reported that N-CAM, a cell adhesion molecule, is one of the candidate molecules implicated in secondary neurulation. This molecule is modified with sialic acid, an acidic sugar, but the intensity of cell adhesion is altered by the quantitative difference in glycosylation (Sunshine et al. 1987). It is my speculation that also during regeneration, changes in the amount of polysaccharides or sugar chains alter the properties of cells to acquire stronger binding to each other to form an epithelium-like tissue, and difference in coloring with TB reflects the differentiation of the cells. It is plausible to think that the molecules involved in MET may be also involved in the process of gut regeneration, and it is a topic that should be looked into to understand the process how the lumen may be reconstructed from a mass of mesenchymal cells.

In this chapter, I was able to elucidate the process of regeneration of the digestive tract in an anterior eviscerating sea cucumber, *Eupentacta*

quinquesemita (ishiko). The posterior digestive tract regenerated by forming a continuous lumen from the cloaca. I observed that in the anterior gut rudiment, multiple cavities are formed surrounded by epithelial-like cells, simultaneously and repeatedly, in multiple sites of the rudiment. The cavities inside the rudiment is not formed by invagination from the outer layer of the rudiment, but I confirmed clearly that mesenchymal-epithelial transition (MET) is involved. The cavities merge to form lumens, which further coalesce with each other and with the lumen from the posterior, to form a single tube. Staining histological sections with toluidine blue enables distinguishing different cell types and differentiation, and would be a convenient and effective method to further study regeneration of the sea cucumber digestive tract.

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Figure 1-1.

The animal used in the study. A small sea cucumber, *Eupentacta quinquesemita*, is about 5 cm in size. The left side in this figure is oral (anterior), and the right side is aboral (posterior).

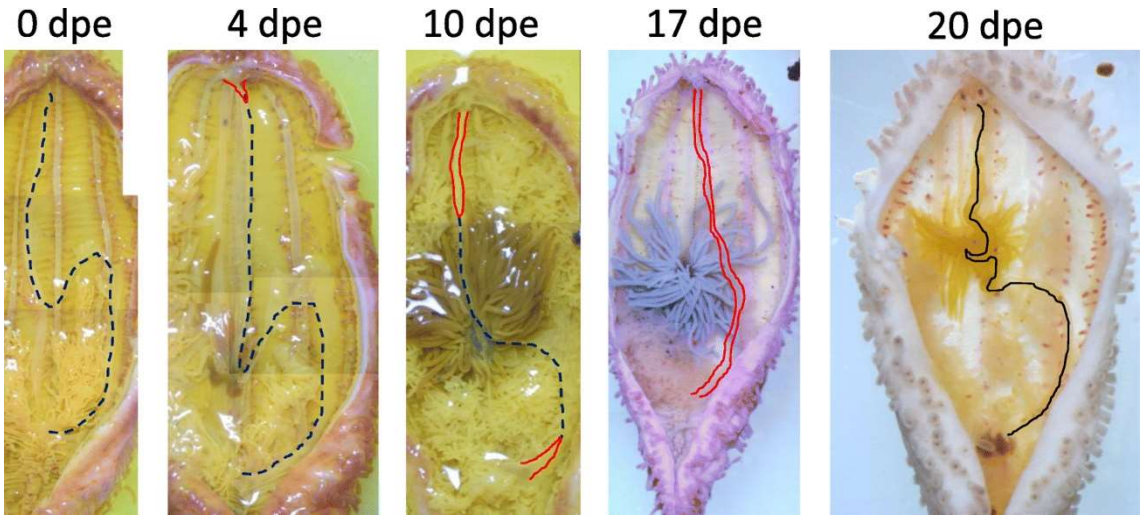


Figure 1-2.

The internal morphology of regenerating animals. Animals just after evisceration (0 dpe) to 20 dpe were dissected along the body length, at the right side of the mid-ventral ambulacrum and flattened. There are slight color variations of the animals, but specimens at 0, 4 and 10 dpe were fixed with Bouin's fixative and thus appear yellowish. The animals were about 4-5 cm in length. Black broken line: the edge of mesentery; red solid line: area along the edge of the mesentery, with thickened gut rudiments; black line: grown gut rudiment observable without the microscope.

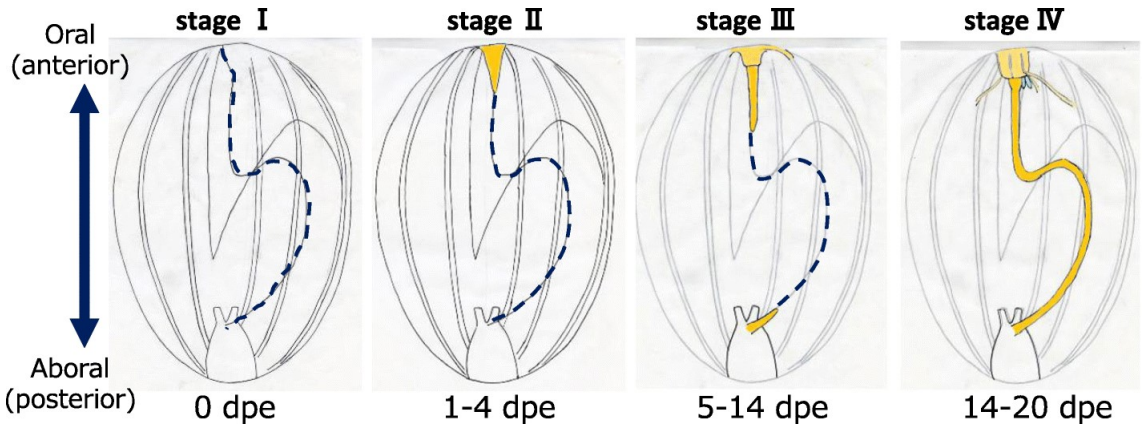


Figure 1-3.

Stages of regeneration. Based on the internal morphology of regenerating animals, I defined four stages of regeneration, stages I to IV, and schematic diagrams of each are shown. Blue broken line: the edge of mesentery without regenerating tissues; yellow colored area: regenerating tissues (gut rudiment).

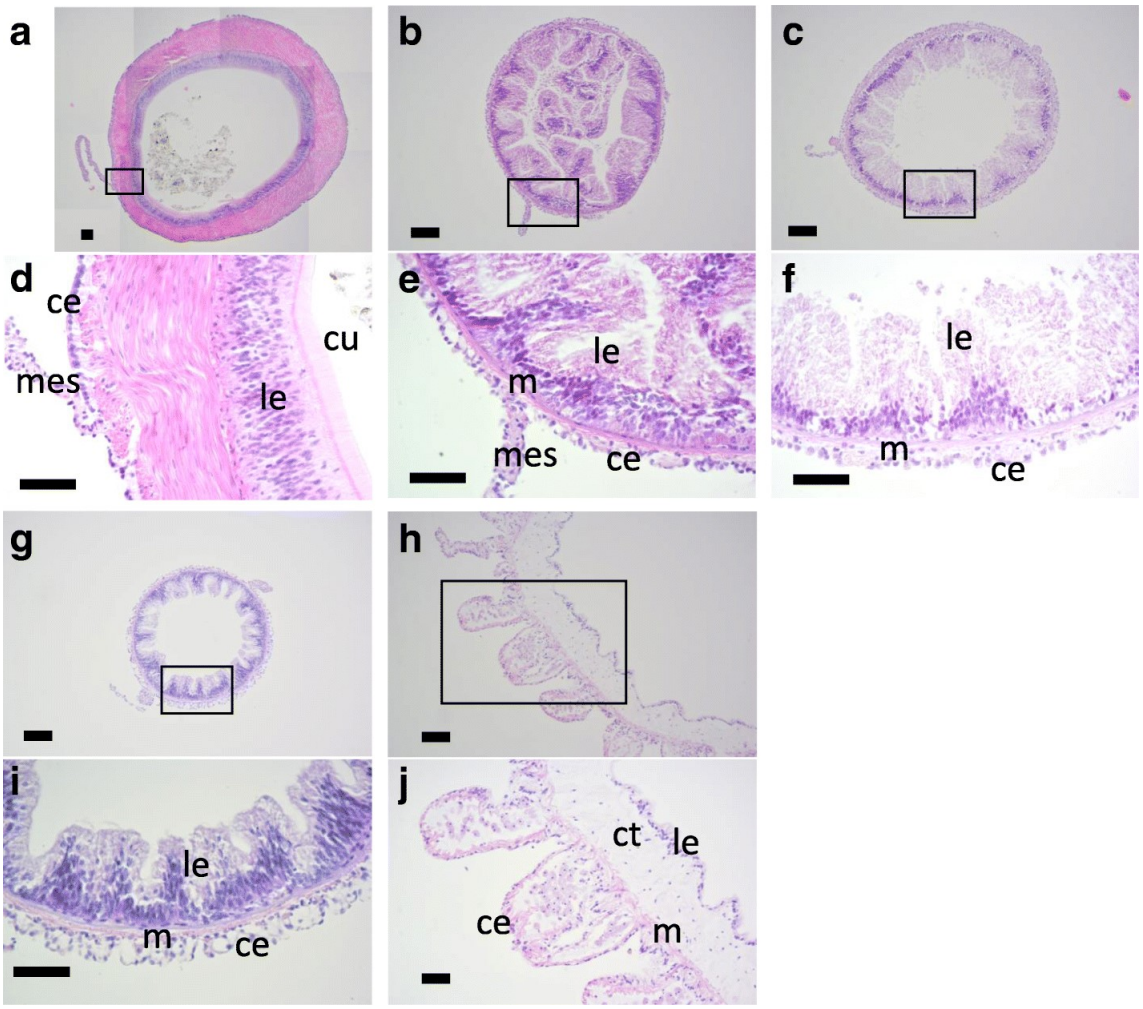


Figure 1-4.

Histology of the intact digestive tract. Cross sections of the digestive tract of *E. quinquesemita* were stained with HE. **d**, **e**, **f**, **i**, **j** are high-magnification views of the boxed areas in **a**, **b**, **c**, **g**, **h**, respectively. **a, d** Stomach. Some contents of the stomach are also observed in **a**. **b**, **e** 1st descending intestine. **c**, **f** Ascending intestine. **g**, **i** 2nd descending intestine. **h**, **j** Cloaca. ce, celomic epithelium; ct, connective tissue; cu, cuticle; gd, gonadal duct; le, luminal epithelium; m, muscle; mes, mesentery. Scale bars100 μm in **a**-**c**, **g**, **h**; 50 μm in **d**-**f**, **i**, **j**.

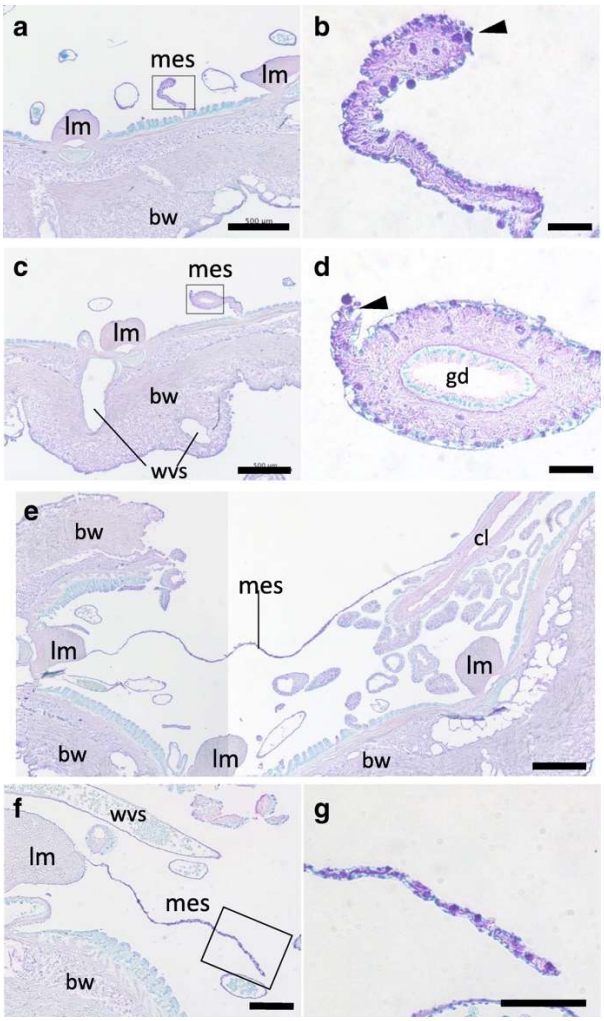


Figure 1-5.

Histology of the tissues at stage I. Cross sections of the body at stage I (0 dpe) were stained with TB, mainly focusing on the anterior (**a**-**d**) and posterior (**e**, **f**) mesenteries. **b**, **d** and **g** are high-magnification views of the boxed areas in **a**, **c** and **f**, respectively. **a**, **b** Anterior tissue, posterior to the anterior rudiment. **c**, **d** Anterior tissue posterior than **a** and **b**. The gonadal duct remains in the mesentery, but no structures are present at the free end. **e** Posterior tissue. The cloaca and the body wall are connected by the mesentery. **f**, **g** Posterior tissue, anterior than **e**. In **b**, **d** and **g**, no tubular structures nor developed cell mass are observed at the free edge of the

mesentery (arrowhead). bw, body wall; cl, cloaca; gd, gonadal duct; lm, longitudinal muscle; mes, mesentery; wvs: a part of the water vascular system (radial water canals, etc.). Arrowheads indicate the free edges of the mesentery. Scale bars 500 μm in **a**, **c**, **e**; 200 μm in **f**; 50 μm in **b**, **d**, **g**.

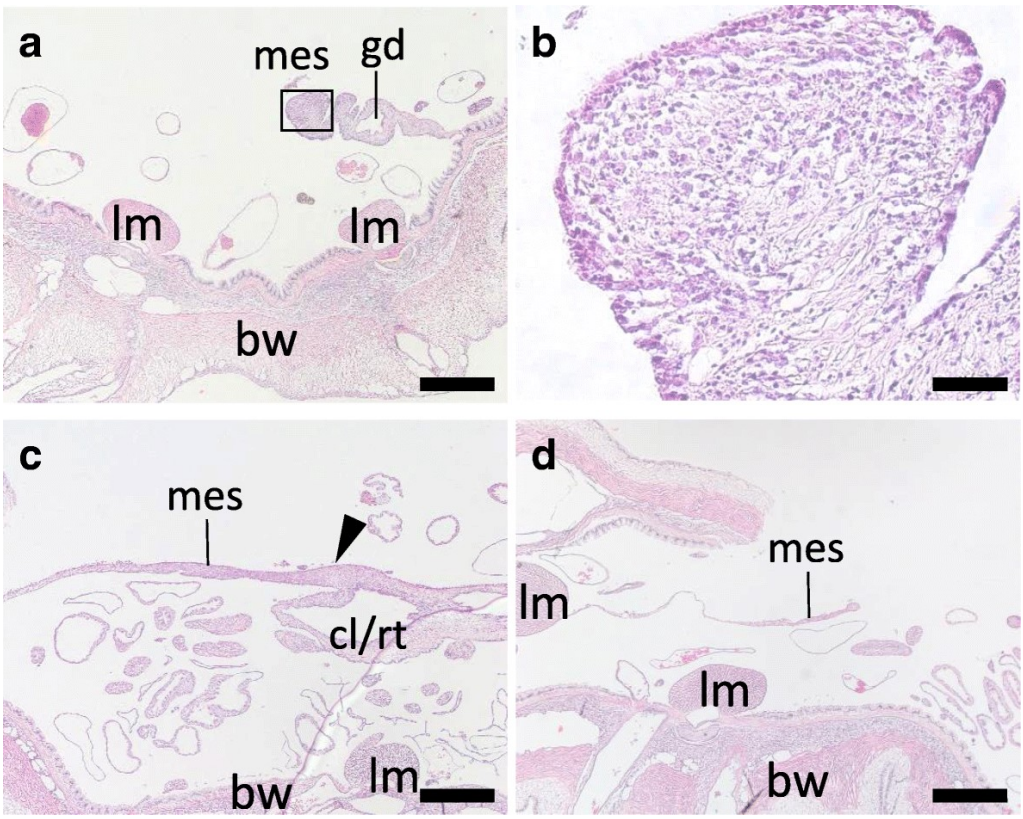


Figure 1-6.

Histology of the tissues at stage II. Cross sections of the body at stage II (4 dpe) were stained with HE, focusing on the mesentery at the anterior (**a**, **b**) and posterior (**c**, **d**) regions of the body. **a**, **b** Anterior mesentery. A mass of cells is present on the free edge of the mesentery. **b** is a high-magnification view of the boxed area in **a**. **c** Posterior tissue. A thickening of tissue (arrowhead) is observed at the connection of the mesentery and the cloaca. Respiratory trees are an organ continuous from the cloaca, and thus these two tissues could not be strictly distinguished. **d** Posterior tissue, anterior than **c**. A part of the mesentery with a free edge is shown. bw, body wall; cl/rt, cloaca or respiratory trees; gd, gonadal duct; lm, longitudinal muscle; mes, mesentery. Scale bars 500 μm in **a**, **c**, **d**; 50 μm in **b**.

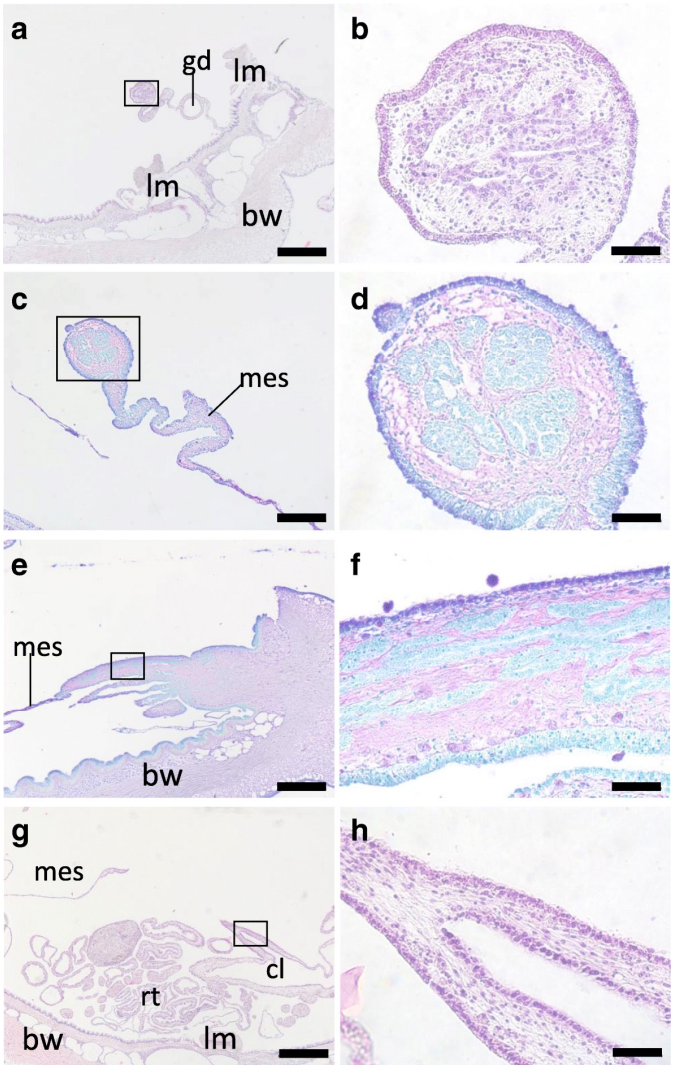


Figure 1-7.

Histology of the tissues at stage III. Histological sections of the anterior (**a**-**f**, **i**) and posterior (**g**, **h**) regions of the body at stage III. **b**, **d**, **f**, **h** are high-magnification views of the boxed areas in **a**, **c**, **e**, **g**, respectively. **a, b** Cross section, 6 dpe, stained with HE. **c, d** Cross section, 7 dpe, stained with TB. **e, f** Longitudinal section of the oral region, 7 dpe, stained with TB. The right side of this figure is anterior. **g, h** Posterior region, 6 dpe, stained with HE. The animal was sliced perpendicular to the oral-aboral axis but a longitudinal section of the regenerating tube was made. In the lumen, a mass of obscure

material is observed (arrowhead). bw, body wall; cl, cloaca; gd, gonadal duct; mes, mesentery; rt, respiratory trees. Scale bar 500 μm in **a**, **e**, **g**; 200 μm in **c**; 50 μm in **b**, **d**, **h**, **f**.

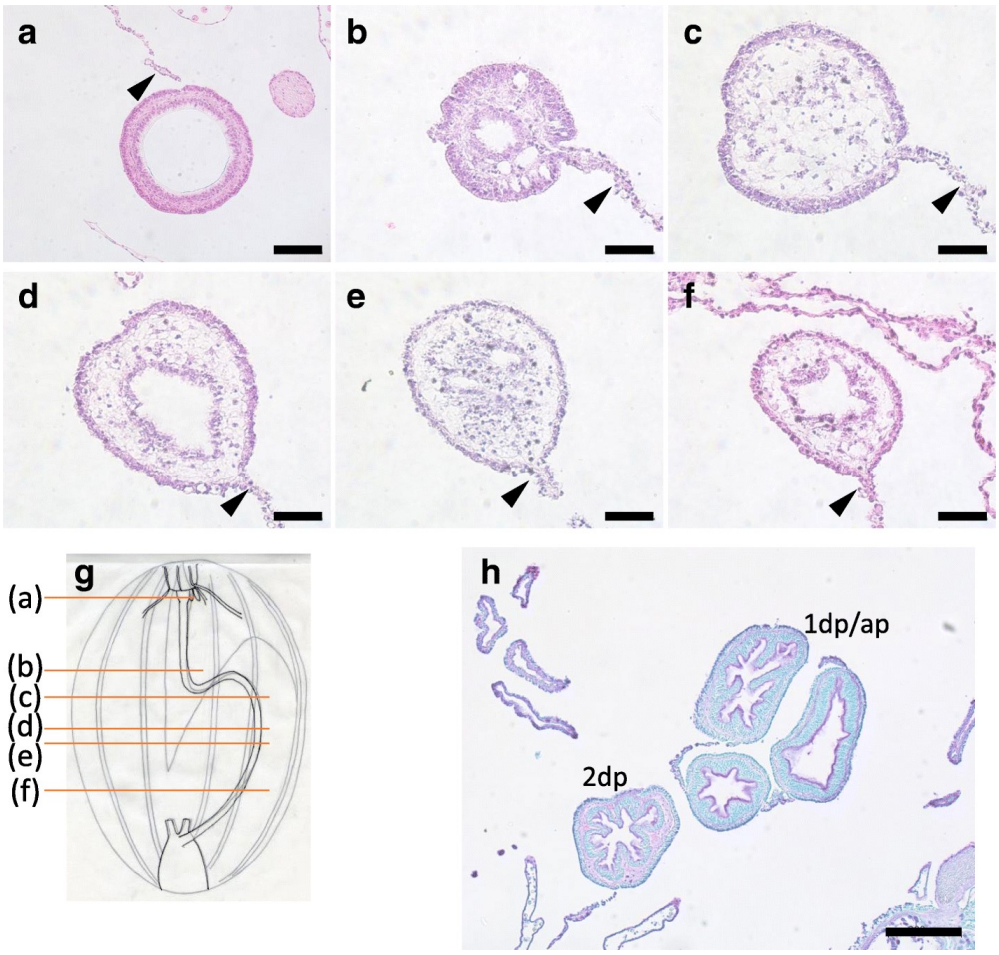


Figure 1-8.

Histology of the tissues at stage IV. Cross sections of the body at stage IV focusing on the regenerated digestive tract. Sections **a**-**f** were made at positions indicated in **g**, of a specimen at 17 dpe. **h** The middle part of the animal with 1st descending, ascending and 2nd descending intestines, at 20 dpe, stained with TB. The slice was made roughly between **b** and **c** in a further regenerated animal with a single tube throughout the digestive tract. The 1st descending and ascending intestines could not be distinguished, since the intestines meandered. 1st descending or ascending intestine; 2dp, 2nd descending intestine. Arrowheads indicate mesenteries. Scale bar 50 μm in **b**-**f**; 100μm in **a**; 200 μm in **h**.

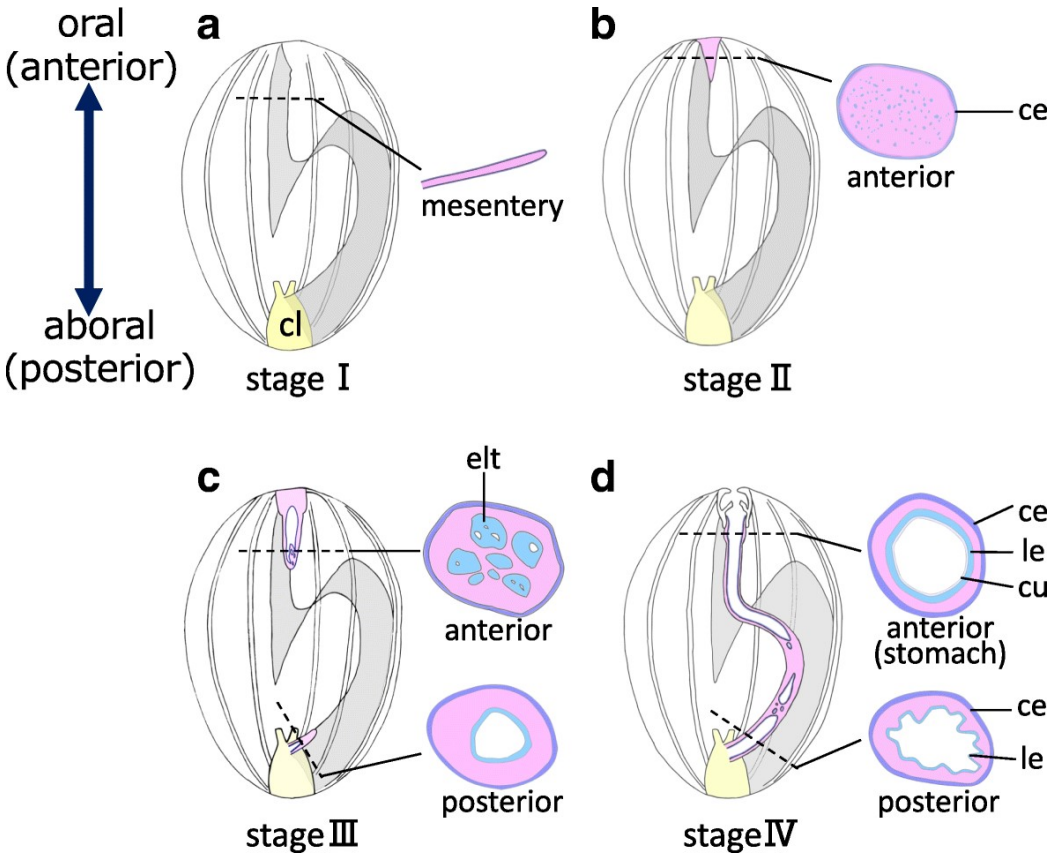
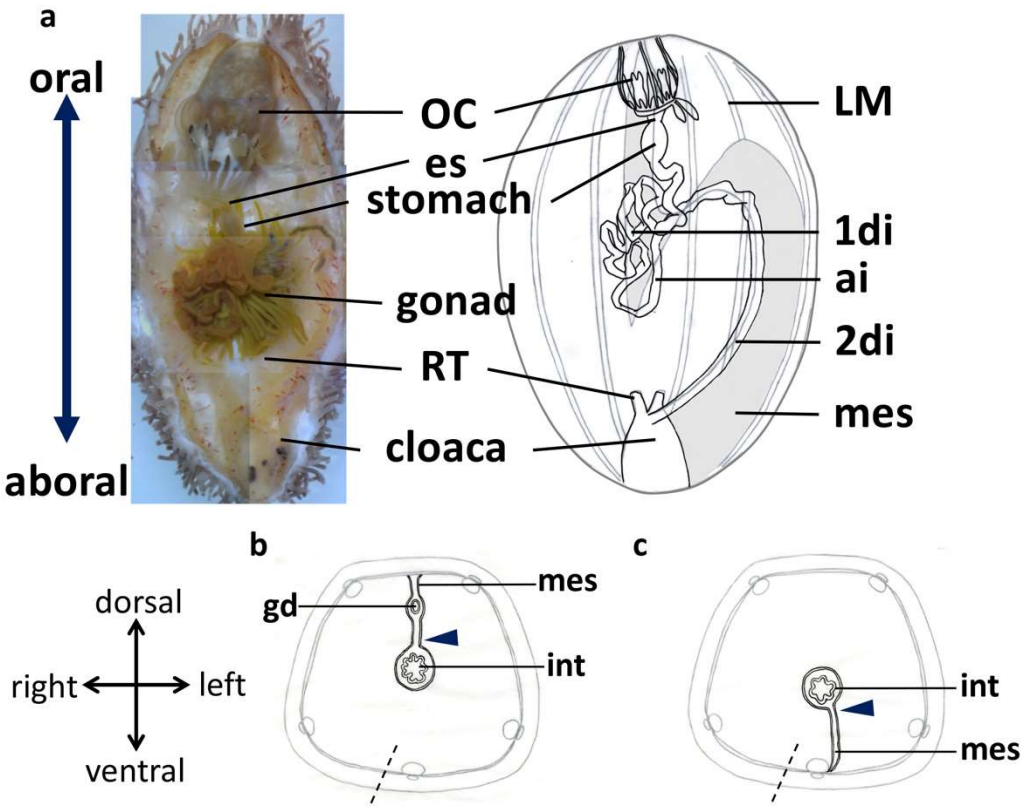


Figure 1-9.

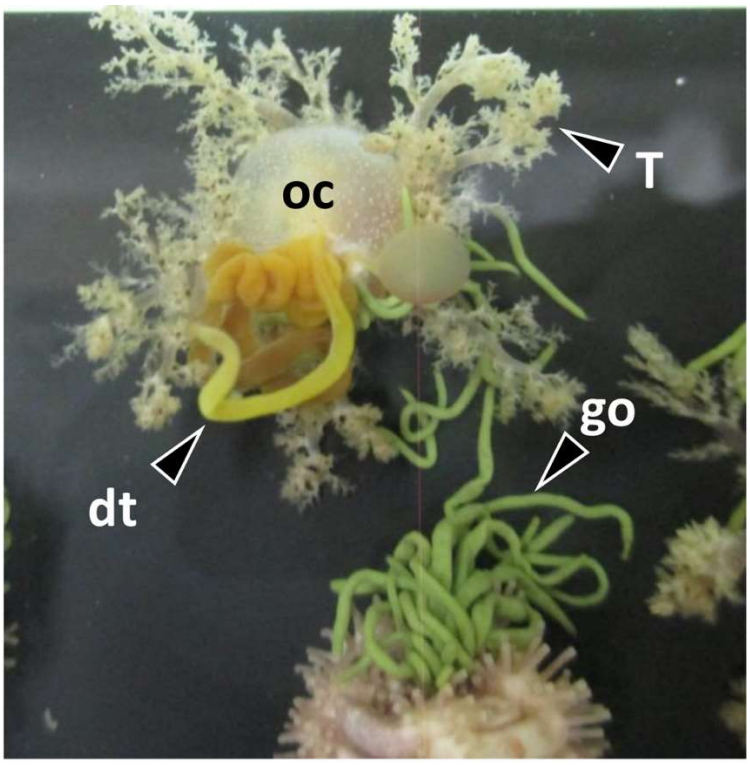
Schematic diagram of regeneration of the digestive tract during stages III and IV. During stage III multiple cavities are formed in the oral regenerating tissue and these coalesce with each other to form lumens. At stage IV when regeneration is more progressed, the gut rudiment (thickened tissue on the mesentery) becomes continuous between the anterior and posterior sides. The anterior and posterior lumens are connected and a single continuous tube is completed.



Supplementary Figure 1-1.

Internal morphology of intact *E. quinquesemita*. **a** View of a dissected and flattened *E. quinquesemita* (left) and a schematic diagram of the organs and tissues (right). The animal was dissected at the right side of the mid-ventral ambulacrum (at the dotted lines in **b** or **c**), so the dorsal midline is at the middle of the view and diagram. Five rows of longitudinal muscles (LM) run on the body wall. Gonads, respiratory trees and retractor muscles, etc. that are not relevant to regeneration of the digestive tract are omitted or simplified in the right drawing. **b**, **c** Schematic diagrams of cross sections of an animal at the level of the 1st descending intestine (**b**) and the 2nd descending intestine (**c**). Arrowheads indicate sites of autotomy at evisceration. Note that the mesentery is attached to the dorsal body wall in **b** and to the ventral body wall in **c**. 1 di, 1st descending intestine; ai, ascending intestine; 2di,

2nd descending intestine; es, esophagus; int, intestine; LM: longitudinal muscle, mes, mesentery; OC, oral complex; RT: respiratory tree.



Supplementary Figure 1-2.

Evisceration of *E. quinquesemita.* The oral complex, intestine and gonads are expelled from the hole made by rupturing the anterior end of the body. The digestive tract (dt) exhibits light yellow to orange colors and gonads (go) exhibits yellow-green to green. In this photo, tentacles (T) that are normally folded in the oral complex (OC) is extended and is visible.

Chapter 2

Expression analyses of MET-related and developmental genes during the digestive-tract regeneration in *Eupentacta quinquesemita*

Introduction

Sea cucumbers are distinctive echinoderms that perform evisceration as a type of autotomy, responding to some extrinsic stimuli like attacking by predators (Byrne 2001). Patterns of evisceration are classified into two major types, i.e., anterior and posterior evisceration (Mashanov and García-Arrarás 2011). In contrast to posterior evisceration, in which only a digestive tract between esophagus and cloacal stump is discarded, many other parts including esophagus, oral complex, and respiratory trees are also discarded in anterior evisceration (Mashanov and García-Arrarás 2011). Therefore, it is considered that the regeneration after anterior evisceration requires more time and costs.

Evisceration in sea cucumbers can also be induced by chemicals, so that it is possible to artificially induce the evisceration by injecting chemicals like KCl solution (Byrne 1986). By applying this method, extensive histological observations were carried out in Chapter 1, in an anterior-eviscerating species *Eupentacta quinquesemita*. Results strongly suggest that the distinctive developmental mechanisms should be recruited for the regeneration processes (Okada and Kondo 2019). In particular, it was revealed that the regeneration of digestive tract after the anterior evisceration involves mesenchymal-epithelial transition (MET) (Thiery and Sleeman 2006, Chaffer et al. 2007, Okada and Kondo 2019).

Mesenchymal-epithelial transition (MET) is referred to as a phenomenon in which the adhesiveness of mesenchymal cells changes and obtains the epithelial characteristics (Pei et al. 2019). MET is known to occur

in the opposite direction to epithelial-mesenchymal transition (EMT) (**Fig. 2-1**). MET and EMT are also involved in cancer metastasis and/or infiltration, so that there are numerous studies on the underlying mechanisms leading these phenomena (reviewed in Kalluri and Weinberg 2009, Foroni et al. 2012, Bhatia et al. 2020). Furthermore, it is also known that in vertebrate development, MET plays important roles in the formation of kidney, somite and coelom (Ekblom 1989, Christ and Ordahl 1995, Funayama et al. 1999). In the epithelial regeneration, it is known that wound healing process involves the process of re-epithelialization which is a case of EMT (Rousselle et al. 2019). In both EMT and MET, similar molecular mechanism is suggested to be activated, in which E-cadherin plays the major roles (Liu et al. 2016). In the process, particularly, two key transcription factors, i.e., Snail and Twist, are known to be involved (Barrallo-Gimeno and Nieto 2005, Kang and Massagué 2004, Lamouille et al. 2014). It is suggested that TGFß and Wnt signaling pathways are involved in the upstream of this EMT/MET pathway.

In the regeneration after evisceration in sea cucumbers (Holothuroidea), gene expression analyses on the MET-related factors have yet to be carried out. Therefore, in this study, by analyzing the expression patterns of MET-related factors, I expected to reveal the detailed mechanisms underlying the digestive-tract regeneration after evisceration. In addition to focusing on the MET factors, it seems also important to look at expressions of patterning genes, that provide the spatial information to the regenerating tissues to recover the functional digestive tract.

The expression patterns of Hox genes during development have so far been investigated in some species of echinoderms (reviewed in Byrne et al.

2016). In some sea urchin species (Echinoiddea), larvae were shown to express a series of Hox and Parahox genes, showing a spatially coliniear patterns of expressions in coelomic mesoderms (Arenas-Mena et al. 2000, Arnone et al. 2006, Tsuchimoto and Yamaguchi 2014). Similar expression patterns were also shown in *Metacrinus rotundus* (Crinoidea) (Hara et al. 2006). In a sea cucumber *Apostichopus japonicus* (Holothuroidea), during embryogenesis, the formation of digestive tract involves the expressions of Hox genes, that would provide the spatial information for the differentiation of digestive tract parts (Kikuchi et al. 2015). Therefore, it is curious whether the expressions of Hox genes are also involved in the regeneration after evisceration, for providing the identity of digestive-tract parts.

In this chapter, therefore, focusing on MET-related genes and Hox genes, extensive gene expression analyses were carried out in by applying realtime qPCR. Before the gene expression analyses, to obtain orthologous gene sequences in *Eupentacta quinquesemita*, RNA-seq transcriptome analysis was firstly performed and constructed a gene database. After the qPCR analyses, in situ hybridization was also carried out for some genes to identify the localization of expression sites.

Materials and methods

Animals

The sea cucumber, *Eupentacta quinquesemita* was collected by diving at the depth of about 3 m in Tokyo Bay, near the pier of Hakkeijima Sea Paradise in Yokohama, Kanagawa Prefecture, or at the depth of about 5-10 m near Aquamarine Fukushima in Onahama, Fukushima Prefecture. Animals of body lengths between 5 and 10 cm were kept in tanks until used for experiments. Evisceration was induced by injection of approximately 100 mL of 0.45 M KCl into the coelom, according to the method described in Byrne (1986). Eviscerated animals were placed in aquaria with sea water at 13-18°C without feeding, up to 3 weeks. The day of evisceration was designated as 0 days post evisceration (0 dpe). For each series of experiments, multiple individuals were induced to eviscerate simultaneously, and several animals were used for experiments at various stages of regeneration.

RNA-sequencing analysis

To identify MET-related genes, Hox and Parahox genes in the focal sea cucumber species, RNA sequencing was performed. Total RNAs were extracted from 2 embryonic stages (early and late gastrula) and juveniles of *E. quinquesemita* by QIAzol lysis reagent (Qiagen, Venlo), and samples were subjected to RNA-sequencing analysis (Eurofin Genomics, Tokyo). After constructing the contig database in *E. quinquesemita*, to obtain the target orthologous genes, local blast searches were carried out with target gene orthologs in *Strongylocentrotus purpuratus* and *Apostichopus japonicus* (Tu

et al. 2012, Zhang et al. 2017). The top-hit sequences from the *E. quinquesemita* transcriptome database were defined as putative orthologs of the target genes. To confirm the orthologs, reciprocal blast searches and phylogenetic analyses were performed (**Suppl. Figs. 2-1 to 2-6**).

RNA extraction for qPCR

To analyze gene expression patterns by realtime quantitative-PCR, total RNAs were firstly extracted from the stages during regeneration after the anterior evisceration in the focal sea-cucumber species (**Fig. 2-3**). Since it was already shown that the regeneration required about 3 weeks, the focal developmental stages were defined as 0, 4, 7, 14, 26 dpe (days post-evisceration). At each time point, regenerating digestive tracts were isolated by dissection from several individuals (the number of used individuals depended on stages, because the tissue amounts differed). Anterior and posterior digestive tracts were separately isolated, in which total RNAs were extracted. Intact digestive tracts from mature individuals were also used for comparison. For total RNA extraction, QIAzol lysis reagent (Qiagen, Venlo) was again applied, and the purification was performed with Agencourt AMPure XP (Beckman Coulter, Brea). The purified RNA samples were restored in a deep freezer (-80°C) until the following qPCR experiments.

Realtime qPCR analysis

The extracted RNA was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Relative quantifications of target transcript levels were performed using Fast SYBR

Green Master Mix and an ABI Prism 7500 instrument (Applied Biosystems). To evaluate endogenous control of constitutive expression, putative reference genes, i.e., elongation factor 1 alpha (*EF1α*), tubulin beta chain (TUBB), NADH dehydrogenase (NADH) and ribosomal protein S18 (RPS18), were evaluated using geNorm (Vandesompele et al. 2002) and Normfinder (Andersen et al. 2004). *EF1α* was the most appropriate reference gene for comparisons among stages and body parts. Quantitative PCR (qPCR) primers were designed for the target genes (**Table 2-1**) using Primer Express software (ver. 3.0.0, Applied Biosystems). Data acquisition and analyses were performed using ABI Prism 7500 software ver. 2.0.4 (Applied Biosystems) with the relative standard curve method. Quantification of gene expression involved biological triplicates, and the results were subjected to one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons tests ($p<0.05$) using R ver. 4.0.2.

in situ hybridization

To investigate the localization of target genes, *in situ* hybridization was carried out according to previous studies (Mashanov et al. 2010, Omori et al. 2011, Kikuchi et al. 2015). Digoxigenin (DIG)-labeled riboprobes were prepared using PCR-generated DNA templates. The PCR products were used as templates to transcribe riboprobes with DIG RNA Labeling Kit (Roche, Mannheim, Germany) following the manufacturer's protocol.

Whole-mount *in situ* hybridizations (WISHs) were performed as described in Mashanov et al. (2010) with minor changes. After dissection, the tissue samples were fixed with 4% paraformaldehyde in 0.5M NaCl, 0.1M 3-

(N-morpholino) propanesulfonic acid (MOPS pH7.0) overnight at 4°C. The samples were washed with phosphate buffered saline (PBS), and then decalcified with 0.5M ethylenediaminetetraacetic acid (EDTA) (pH8.0) in *PBS* for 3-4 days at room temperature. After decalcification, the samples were kept in 99.5% ethanol at -20°C until use.

The samples were washed in PBS containing 0.1% Tween-20 (PBST), treated with 1/50 volume of proteinase K (TaKaRa, Shiga) in PBST for 20 min at 37°C, acetylated sequentially in 0.25% and 0.5% acetic anhydride in 0.1 M triethanolamine, 5 min each. After washing the samples by two times PBST for 5 min, Prehybridization was performed at 58°C for 2 h or longer in hybridization buffer containing 50% formamide, 5× saline-sodium citrate (SSC), 100 μg/mL yeast RNA, 5× Denhardt's solution, 0.1% Tween 20. The riboprobes were diluted in hybridization buffer at 58°C to a final concentration of about 400 ng/ml and denatured at 80°C for 5 min. The hybridization was carried out at 58°C overnight. After hybridization, the samples were washed in 50% formamide in 5× SSC at 58°C for 20 min, 5× SSC at 58°C for 50 min, 2× SSC at 58°C for 50 min, 0.1× SSC at 58°C for 15 min and then PBST at room temperature for 15 min twice.

Subsequently, the samples were incubated in 0.1 % blocking reagent (Roche) in PBST (blocking buffer) at room temperature for 30 min, followed by an incubation in 1/2000 volume of anti-DIG-AP (Roche) in blocking buffer at 4°C overnight. Following a wash with PBST (15 min, 8 times), immunodetection was performed using BM purple (Roche) at room temperature. After detection, the samples were washed in PBST and then kept in 10% formalin/PBS.

Results

RNA-sequencing and ortholog searches

RNA-sequencing analysis on the transcriptome derived from gastrula embryos and juveniles of *Eupentacta quinquesemita* yielded 28,686,525 pairs of 100-bp reads. After filtering, 26,497,155 paired reads were retained for further analyses. *De novo* transcriptome assembly of the sequence reads generated 350,742 contigs with a total of 296,553,944 bases and N50 of 1,513 bp. Using the transcriptome data, the gene database was constructed to search target genes, i.e., MET-related and Hox genes. Successfully, the gene orthologs from the focal sea cucumber species were obtained from the database, i.e., twist and snail for the MET-related genes, Hox genes and Parahox genes. They were confirmed to be the orthologs by reciprocal blast searches and by constructing phylogenetic trees (**Suppl. Figs. 2-1, 2-2, 2-3**). Based on the obtained gene sequences from the RNA-sequencing result, primers for qPCR were designed (**Table 2-1**).

以下、Chapter 2 の詳細については、5 年以内に雑誌で刊行予定のため、非公開。

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Table 2-1 については、5 年以内に雑誌で
刊行予定のため、非公開。

Figures

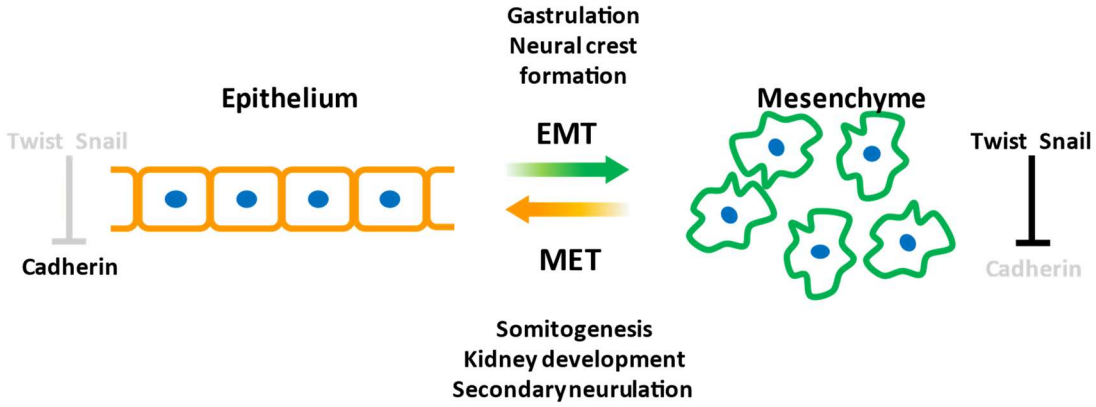

Figure 2-1.

MET (mesenchymal-epithelial transition) and EMT (epithelial-mesenchymal transition) pathway. Two important transcription factors, i.e., Twist and Snail play important roles in the regulation of Cadherin expression. At the time during MET occurs and lasts, it is considered that the *twist* and *snail* expressions are down-regulated, while the *cadherin* expression is up-regulated. This figure was drawn based on Foroni et al. (2016).

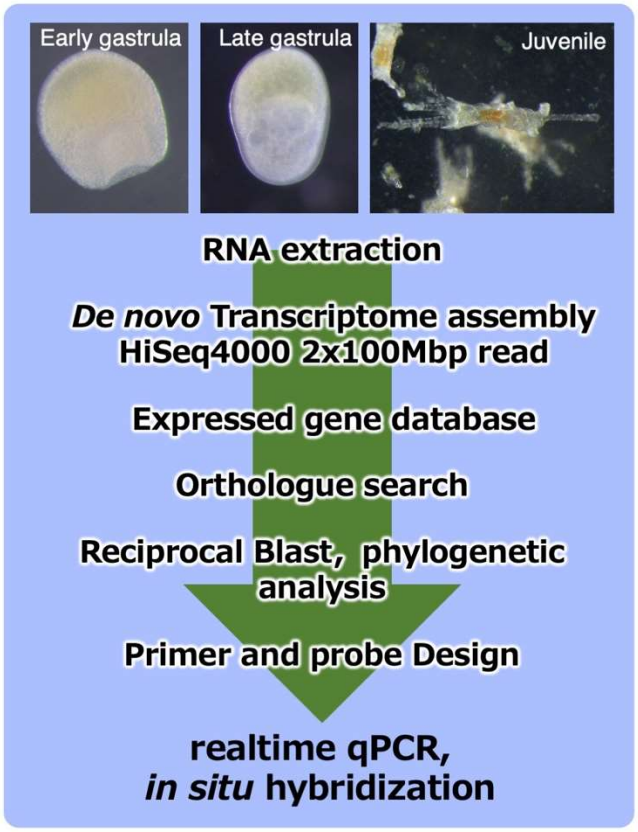

Figure 2-2.

Flow chart showing the experimental process for the gene expression analyses, focusing on the regeneration process after posterior evisceration in the focal species *Eupentacta quinquesemita*.

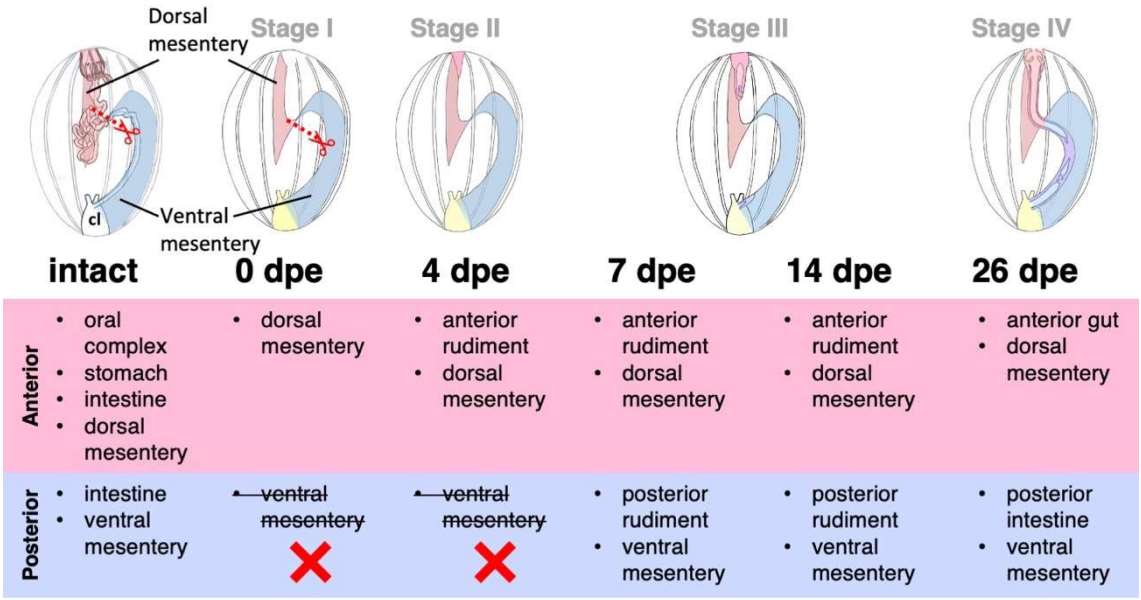

Figure 2-3.

Sampling scheme for the realtime qPCR analyses during the regeneration after posterior regeneration. The developmental stages are based on Okada and Kondo (2019). The below list shows the tissues included by the anterior or posterior body parts, in which RNAs were extracted for the analyses. RNA extraction from posterior parts at 0 dpe (days post-evisceration) and 4 dpe was failed since the tissue amounts were extremely small (indicated by X marks).

Fig. 2-4 ~ 2-8 については、5 年以内に雑誌で刊行予定のため、非公開。

Suppl. Fig. 2-1~2-3 については、5 年以内に雑誌で刊行予定のため、非公開。

Chapter 3

Histological observations and gene-expression analyses on the regeneration of circumoral nerve ring after evisceration in *Eupentacta quinquesemita*

Introduction

Sea cucumbers are distinctive echinoderms that perform evisceration as a type of autotomy, responding to some extrinsic stimuli like attacking by predators (Byrne 2001). Patterns of evisceration are classified into two major types, i.e., anterior and posterior evisceration (Mashanov and García-Arrarás 2011). In contrast to posterior evisceration, in which only a digestive tract between esophagus and cloacal stump is discarded, many other parts including esophagus, oral complex, and respiratory trees are also discarded in anterior evisceration (Mashanov and García-Arrarás 2011). Therefore, it is considered that the regeneration after anterior evisceration requires more time and costs.

In anterior eviscerating holothuroids, not only digestive tract but also oral complex and tentacles are ejected by the evisceration process (Byrne 2001, Mashanov et al. 2005). The oral complex contains nerve ring, that constitutes the central nervous system in sea cucumbers, together with radial nerves in body walls (Mashanov et al. 2009) (**Fig. 3-1**). After the anterior evisceration, the nerve ring is also regenerated, at the same time when the digestive tract is regenerated (Dolmatov 1992).

It is known that factors secreted from nerves play important roles in the regeneration of nerve tissues (Kumar and Brockes 2012). Also in the regeneration of digestive tract in holothuroids, it is predicted that such factors secreted from injured nerve tissues, although little is known about the regeneration process of nervous system during the regeneration. Furthermore, while only a histological study has been reported so far

(Dolmatov 1992), it has not yet revealed what sorts of genes are involved in the regeneration of nervous system in the regeneration process.

In this study, therefore, in order to reveal the regeneration process of nerve ring and the underlying developmental mechanism, extensive histological observations focusing on the nerve-ring regeneration and expression analyses on the neural-patterning genes were carried out in the anterior-eviscerating sea cucumber *Eupentacta quinquesemita* (Holothuroidea).

The genes focused in this study are *otx*, *six3/6*, and *pax6*, that play important roles in neural patterning and sensory-organ formation in vertebrates (Hill et al. 1991, Oliver et al. 1995, Acampora et al. 2001, Zuber et al. 2003, Manuel and Price 2005), amphioxus (Glardon et al. 1998, Tomsa and Langeland 1999, Castro et al. 2006), hemichordates (Lowe et al. 2003), fruitfly (Czerny et al. 1999, Hirth and Reichert 1999, Seo et al. 1999) and annelids (Arendt et al. 2001, 2002, Denes et al. 2007). In echinoderms, it was shown that these three genes are expressed in the oral nervous system during the early development in a stalked crinoid *Metacrinus rotundus* and a feather star *Anneissia japonica*, both of which belong to Crionoidea (Omori et al. 2011, 2020). In addition to these tree genes, other genes were also focused in this chapter, since a previous study showed that many other neural factors were involved in the neurogenesis in a sea cucumber in *Holothuria glaberrima* (Mashanov et al. 2015). Among those, three genes that are known to play critical roles in neuronal differentiation were especially focused, i.e., *Elav*, *mushasi* and *neuroD* (Robinow and White 1988, 1991, Good 1995, Okano et al. 2005, Pascale et al. 2008, Roybon et al. 2009, Bouritn et al.

2010, Horisawa and Yanagawa 2012, Colombrita et al. 2013).

Materials and methods

Animals

The sea cucumber, *Eupentacta quinquesemita* was collected by diving at the depth of about 3 m in Tokyo Bay, near the pier of Hakkeijima Sea Paradise in Yokohama, Kanagawa Prefecture, or at the depth of about 5-10 m near Aquamarine Fukushima in Onahama, Fukushima Prefecture. Animals of body lengths between 5 and 10 cm were kept in tanks until used for experiments. Evisceration was induced by injection of approximately 100 mL of 0.45 M KCl into the coelom, according to the method described in Byrne (1986). Eviscerated animals were placed in aquaria with sea water at 13-18°C without feeding, up to 3 weeks. The day of evisceration was designated as 0 days post evisceration (0 dpe). For each series of experiments, multiple individuals were induced to eviscerate simultaneously, and several animals were used for experiments at various stages of regeneration.

Histological observations on the regeneration of nerve ring

Animals were anesthetized in 72 g/L $MgCl_2$ in sea water for approximately 15 minutes to 1 hour before being dissected. They were dissected along the body length at the right ventral interambulacral zone, exposing the whole body cavity, and examined under a stereomicroscope. The dissected animals were fixed in Bouin's Fixative or 4% paraformaldehyde in 0.5M NaCl, 0.1M 3-(N-morpholino) propanesulfonic acid (MOPS pH7.0) overnight at 4°C. The specimens were washed with PBS, and then decalcified with 0.5M EDTA (pH8.0) in PBS for 3-4 days at room temperature. After decalcification, the

specimens were kept in 99.5% ethanol at -20°C until embedding in paraffin (Paraplast X-TRA, SIGMA) for histological observation. Serial sections of 6 μm thickness were produced, and they were stained with hematoxylin-eosin (HE) or toluidine blue (TB) and observed on a light microscope (ECLIPSE TE300, Nikon). Sections were mostly made perpendicular to the anterior-posterior (oral-aboral) axis of the body. In total, 4 animals were used for histological observations.

RNA-sequencing analysis

To identify orthologous genes to neuronal patterning gens from the focal sea cucumber species, RNA sequencing was performed (see Chapter 2). Total RNAs were extracted from 2 embryonic stages (early and late gastrula) and juveniles of *E. quinquesemita* by QIAzol lysis reagent (Qiagen, Venlo), and samples were subjected to RNA-sequencing analysis (Eurofin Genomics, Tokyo). After constructing the contig database in *E. quinquesemita*, reciprocal blast searches and phylogenetic analyses were performed to confirm the orthologs (**Suppl. Figs. 3-1 to 3-4**).

RNA extraction for qPCR

To analyze gene expression patterns by realtime quantitative-PCR, total RNAs were firstly extracted from the stages during regeneration after the anterior evisceration in the focal species. Since it was already shown that the regeneration required about 3 weeks, the focal developmental stages were defined as 0, 4, 7, 14, 26 dpe (days post-evisceration). At each time point, regenerating digestive tracts were isolated by dissection from several

individuals (the number of used individuals depended on stages, because the tissue amounts differed). Anterior and posterior digestive tracts were separately isolated, in which total RNAs were extracted. Intact digestive tracts from mature individuals were also used for comparison. For total RNA extraction, QIAzol lysis reagent (Qiagen, Venlo) was again applied, and the purification was performed with Agencourt AMPure XP (Beckman Coulter, Brea). The purified RNA samples were restored in a deep freezer (-80°C) until the following qPCR experiments.

Realtime qPCR analysis

The extracted RNA was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Relative quantifications of target transcript levels were performed using Fast SYBR Green Master Mix and an ABI Prism 7500 instrument (Applied Biosystems). To evaluate endogenous control of constitutive expression, putative reference genes, i.e., elongation factor 1 alpha (*EF1α*), tubulin beta chain (TUBB), NADH dehydrogenase (NADH) and ribosomal protein S18 (RPS18), were evaluated using geNorm (Vandesompele et al. 2002) and Normfinder (Andersen et al. 2004). *EF1α* was the most appropriate reference gene for comparisons among stages and body parts. Quantitative PCR (qPCR) primers were designed for the target genes (**Table 3-1**) using Primer Express software (ver. 3.0.0, Applied Biosystems). Data acquisition and analyses were performed using ABI Prism 7500 software ver. 2.0.4 (Applied Biosystems) with the relative standard curve method. Quantification of gene expression involved biological triplicates, and the results were subjected to

one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons tests (p<0.05) using R ver. 4.0.2.

Results

Histological observations on the regeneration of nerve ring

Based on the histological sections, focusing on regeneration of the nerve ring during the regeneration process after posterior evisceration, the process of nerve ring formation was clearly observed (**Fig. 3-2**). At the time when anterior evisceration occurred (0 dpe), the anterior end of sea-cucumber body was shrunk to close the wounds, so that the body wall wound containing the radial nerve tissues came close to each other. At the early stage (4 dpe), neural tissues seemed to proliferate to be extended from wounds of radial nerves in the body wall, entering the regenerating tissues at the center of body trunk and extending toward the posterior (aboral) direction (**Fig. 3-2A**). At 6 and 12 dpe, the apical ends of regenerating nerves were elongated in the perpendicular direction, forming the ring structures by connecting to each other (**Fig. 3-2B, C**). At 12 dpe, a ring-like structure was almost formed by the elongated neural tissues connecting to each other (**Fig. 3-2C**). At 21 dpe, the formation of nerve ring was completed (**Fig. 3-2D**).

以下、Chapter 3 の詳細については、5 年以内に雑誌で刊行予定のため、非公開。

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Table 3-1 については、5 年以内に雑誌で刊行予定のため、非公開。

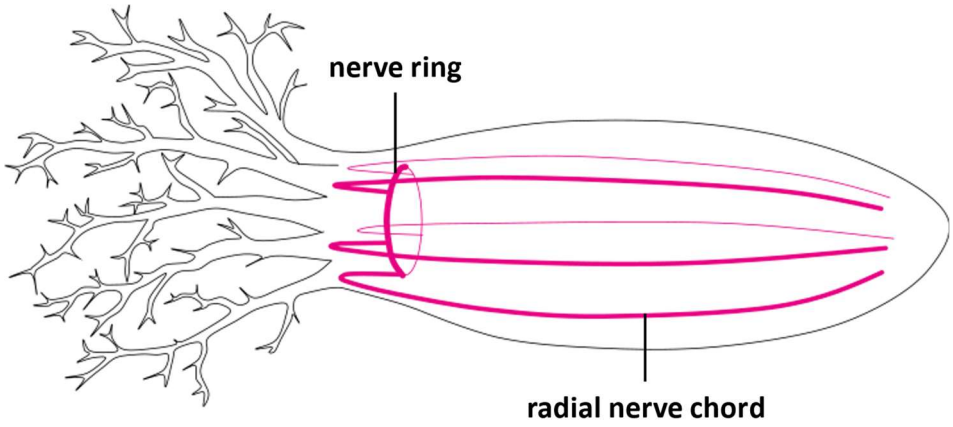

Figure 3-1.

Nervous system of a sea cucumber (Holothuroidea). It consists of nerve ring (also known as circumoral nerve ring), tentacular nerves and radial nerve chords.

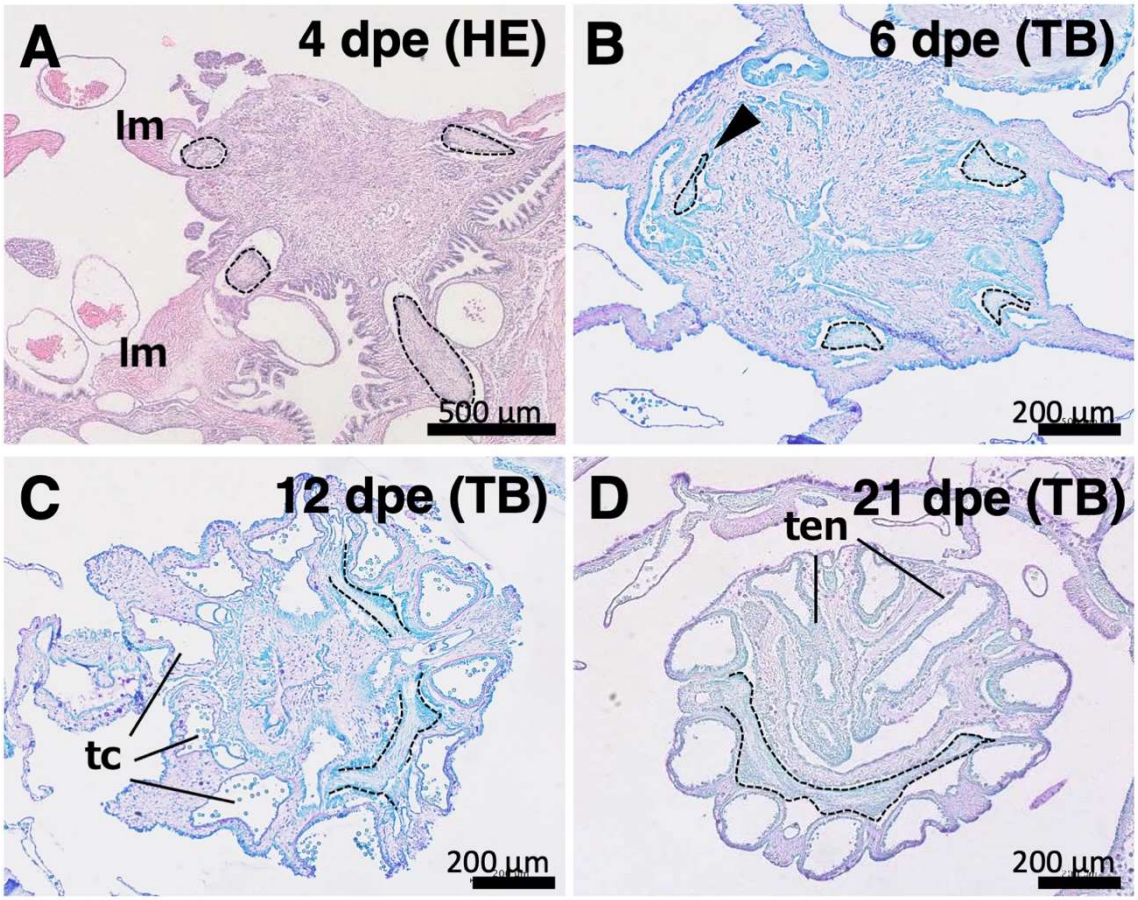

Figure 3-2.

Histological images showing the regeneration process of circumoral nerve ring.

Fig. 3-3 ~ 3-5 については、5 年以内に雑誌で刊行予定のため、非公開。

Suppl. Fig. 3-1 ~ 3-4 については、5 年以内に雑誌で刊行予定のため、非公開。

General Discussion

Regeneration after evisceration in holothuroids

"Regeneration" is a developmental phenomenon in which lost body parts are acquired to form original functional parts, and seen in variety of animals (Vogg et al. 2019; Reddien 2018; Haas and Whited 2017; Goss 1969). Species in Echinodermata, a phylum of deuterostomes, are also known to have distinctive abilities of regeneration as seen in starfishes (Astoroidea), in which arms or discs are often regenerated. Many species of sea cucumbers (Holothuroidea) also require such regeneration ability since many sea-cucumber species show "evisceration", i.e., discarding digestive tract in response to extrinsic stimuli, as a defensive strategy at the time when they are attacked by predators (García-Arrarás and Greenberg 2001, Mashanov and García-Arrarás 2011).

Patterns of evisceration are classified into two major types, i.e., anterior and posterior evisceration, that differ in the direction of gut-discarding and in the discarded parts. In anterior evisceration, which is seen in many species of the order Dendrochirotida, anterior parts of the body including esophagus, oral complex are also ejected in addition to digestive tract. In contrast, in posterior evisceration seen in species of the order Aspidochirotida, only digestive tract between esophagus and cloacal stump are discarded from anus. In this case, lost parts of digestive tract are regenerated from the remained digestive tract at the anterior and posterior body (García-Arrarás et al. 1998, Leibson 1992, Mashanov and García-Arrarás 2011). However, in the case of anterior evisceration, no tissues of digestive tract are remained at the anterior region, and the detailed process of digestive-tract regeneration has yet to be elucidated (Mashanov et al.

2005). Therefore, in my Ph.D. thesis, focusing on the regeneration after anterior evisceration in a Japanese dendrochirotid species *Eupentacta quinquesemita*, extensive histological observations and gene expression analyses were carried out to reveal the underlying mechanisms for the distinctive regeneration process.

Histological process (Chapter 1)

By the anterior evisceration in *E. quinquesemita*, the ejected digestive tract is isolated by cutting between body wall and the oral complex, between the second descending intestine and the cloaca, and at the connection between mesentery and digestive tract. Histological observations revealed that the regenerated gut was formed from both side of the body, i.e., from anterior and posterior body, and the regenerated gut were formed along the remained mesentery. The regeneration process was divided to 4 distinctive stages, i.e., Stage I (just after evisceration), Stage II (formation of anterior regenerating tissues), Stage III (formation of posterior regenerating tissues and growth of the anterior regenerating tissues), and Stage IV (formation of a continuous rudiment). During these stages, regenerating epithelial tissues were observed in hypertrophic tissues derived from mesentery or body wall, suggesting that mesenchymal-epithelial transition (MET) is involved in the regeneration process after the anterior evisceration.

Expression analyses on MET-related and Hox genes (Chapter 2)

Histological observation results in Chapter 1 suggest that the regeneration process after posterior evisceration in *E. quinquesemita* involves

mesenchymal-epithelial transition (MET). In Chapter 2, therefore, expression patterns of factors known to involved in the MET pathway, i.e., transcription factor *twist* and *snail* were investigated by realtime qPCR method, after the identification of orthologs for those factor genes from the transcriptome database constructed based on RNA-sequencing result. As the results, it was shown that *twist* was up-regulated from relatively earlier stages of the digestive-tract regeneration, followed by the up-regulation of *twist*.

Furthermore, Hox and Parahox genes were also focused in the same manner, because it was predicted that the differentiation of digestive tract should occur, according to the spatial information along the body axis. As expected, the expression patterns of Hox and Parahox genes show the colinear pattern along the anterior-posterior axis. Interestingly, the timing of Hox/Parahox expression followed the expression of MET-related factors, indicating that the detailed differentiation of digestive tract take place after the formation of gut tube by MET (**Fig. GD-1**). This predicted differentiation process was also congruent with the results of histological observations.

Regeneration of central nervous system (Chapter 3)

In contrast to posterior evisceration, anterior evisceration seen in species of Dendrochirotida ejects whole the anterior structures, including the oral complex. This structure contains, the nerve ring that is thought to function as a part of the central nervous system (CNS) in echinoderms (Mashanov et al. 2009). Histological observations focusing on the nerve-tissue regeneration showed that the nerves are regenerated form anterior ends of the remained radial nerve chords, later connecting to each other to

form the ring structure. Gene expression analyses by realtime qPCR showed that the genes responsible for the nervous-system development, in particular *otx*, *six3/6* and *pax6* genes, were up-regulated in the anterior body part during the regeneration process. These results together suggest that the anterior-eviscerating species have acquired the CNS-regeneration mechanism by recruiting the developmental genes regularly used for the normal echinoderm development.

Conclusion and perspectives

Overall, in my Ph.D. thesis, I exclusively focus on the regeneration process after anterior evisceration in *E. quinquesemita*. Although the process of evisceration has been so far investigated (Byrne 2001), and the regeneration after posterior evisceration was reported by some previous studies (García-Arrarás et al. 1998; Yuan et al 2019), the regeneration after anterior evisceration was investigated in detail by the thesis of my studies. One of the main discoveries in my study is the involvement of MET in the regeneration process. This mechanism could also be recruited in the regeneration after posterior evisceration, so that expression patterns of MET-related genes should be investigated in future studies with posterior-eviscerating species. Furthermore, it is well-known that the MET/EMT pathways are responsible for the cancer-inducing mechanisms, so some application studies could also be expected based on the results obtained in echinoderms. Furthermore, in the evolutionary point of view, the regeneration mechanism should have acquired in association with the acquisition of evisceration mechanism itself, since regeneration must occur after evisceration for the survival of sea cucumbers.

I hope my studies will help to understand such evolutionary processes that have occurred in an echinoderm lineage, i.e., Holothuroidea (sea cucumber) species.

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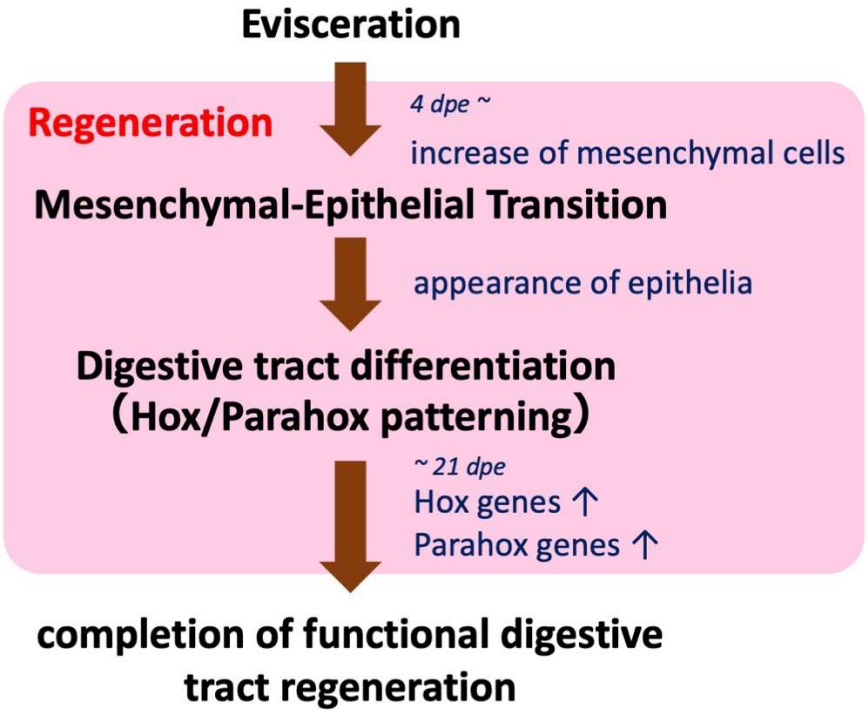

Figure GD-1.

Schematic diagram explaining the regeneration process after the anterior evisceration in *Eupentacta quinquesemita*. The mechanistic events like mesenchymal-epithelial transition (MET) and up-regulations of MET-related genes and Hox/parahox genes are also shown.

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