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Review

Stem Cell System in Asexual and Sexual Reproduction of *Enchytraeus japonensis*

(Oligochaeta, Annelida)

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Runnnng Title: Asexual and Sexual Reproduction of *E. japonensis*

Abstract

Enchytraeus japonensis is a small oligochaete species that proliferates asexually via fragmentation and regeneration. As sexual reproduction can also be induced, it is a good model system for the study of both regenerative and germline stem cells. It has been shown by histological study that putative mesodermal stem cells called neoblasts, and dedifferentiated epidermal and endodermal cells are involved in blastema formation. Recently, we isolated three region-specific marker genes expressed in the digestive tract and showed by *in situ* hybridization that morphallactic as well as epimorphic regulation of the body patterning is occurred during regeneration. We also cloned two *vasa*-related genes and analyzed their expression during development and in mature worms that undergo sexual reproduction. The results arising from these studies suggest that the origin and development of germline stem cells and neoblasts may be independent. Furthermore, we performed functional analysis using RNA interference (RNAi) and showed that a novel gene termed *grimp* is required for mesodermal cell proliferation at the initial stages of regeneration. These findings indicate that the stem cell system in *E. japonensis* is regulated by both internal and external environmental factors.

Keywords: Annelida, germline, neoblast, regeneration, stem cell

Features of *Enchytraeus japonensis*

Description and phylogeny

Enchytraeus japonensis, the only fragmenting terrestrial enchytraeid (Oligochaeta, Annelida, Lophotrochozoa, Protostomia) found in Japan, was first described in 1993 (Nakamura 1993). Most enchytraeid species (pot worms) are 2–20mm long with a body diameter of 0.05–0.5mm, usually white in color. The size and color distinguish them from their larger relatives, the earthworms. Worldwide, several hundred species of Enchytraeidae have been described (Didden et al.1997). Among them, eight have been reported to reproduce asexually by fragmentation and subsequent regeneration (Bell 1959; Christensen 1959, 1964; Bouguenec & Giani 1989; Nakamura 1993; Dózsa-Farkas 1995; Schmelz et al., 2000). Among the eight fragmenting enchytraeid species, *E. japonensis* most closely resembles *E. bigeminus* (Christensen 1964).

Structure of the body

Full body length of *E. japonensis* is 10-15mm, diameter is approximately

0.2mm, and body color is white or almost transparent. The worm has a well-developed ladder-like central nervous system, a closed blood-vascular system, a highly developed endocrine system, a coelom, an excretory system, and a segmented body. The anterior head region is composed of seven segments that contain specific organs such as the mouth, pharynx, brain, ventral nerve cord and sensory organ (prostomium), and sepal glands. In the posterior region, the worm has a growth zone adjacent to the pygidium. Though the trunk region appears to be the repeat of same segments, regional specifications exist in the digestive tract (Fig. 1).

Life cycle

The life cycle of this worm is unique in that it undergoes asexual reproduction. Under regular mass culture conditions, this worm reproduces asexually by spontaneous autotomy (fragmentation) and regeneration (whole body regeneration). When they grow to full body length (60-80 segments), the worms spontaneously autotomize into 5-10 fragments, each consisting of about 10 segments. A head (seven head segments) is regenerated from the anterior plane in 4-5 days and a tail consisting of a pygidium and growth zone is regenerated from the posterior plane in 2-3 days after fragmentation

at 24°C. After regeneration completes, the worm starts growing by the addition of new segments at the growth zone and becomes to be the original size in about 2 weeks.

On the other hand, sexual reproduction can also be induced by low-density culture, in which mature worms with fully differentiated sexual organs with gametes can be obtained approximately in 10 days at 24°C (hermaphrodite). These gametes are fertilized to produce embryos by allogamy (cross-fertilization). The entire developmental process takes approximately 7 days and the newborn worms start growing to the maximum length. Thus, the sexual reproduction cycle requires approximately 4 weeks to be completed (Fig. 2).

New experimental model for stem cell study

E. japonensis has been adapted to laboratory use. As this worm is small in size, it is easy to culture and handle. The worm were cultured on 0.6-1.0% agar plate made in 100 or 150 mm diameter disposable Petri dishes at 18-25°C, fed once or twice a week with powdered rolled oats that had been sterilized briefly by a microwave oven. Under these conditions, asexual reproduction by fragmentation occurred every 2 weeks, and sexual reproduction never occurred.

Fragmentation can be artificially induced under laboratory conditions (Myohara et al., 1999; Inomata et al., 2000) by electric shock or removal of head (decapitation). To induce sexual reproduction, usually 10 worms were cultured in a dish (low-density culture). As asexual and sexual reproduction cycles of *E. japonensis* can be controlled experimentally and progress in a relatively short period, it is an ideal model animal for studying stem cell systems in regeneration and germ cell formation (Myohara et al., 1999).

As the asexually reproduced worms are genetically identical clones, they are also suitable for molecular studies. Recently we introduced some molecular techniques such as cDNA cloning, *in situ* hybridization and RNA interference (RNAi) into the worm, in addition to the BrdU labeling study for the detection of active stem cells.

Large-scale regeneration of an entire body is widely known to occur in only a few animals such as hydras (Fujisawa, 2003; Fujisawa, 2008) and planarians (Agata et al., 2007; Umesono & Agata, 2009). Although many classical regeneration studies in Annelidae were conducted using Polychaetae and Oligochaetae (Christensen, 1964; Herlant-Meewis, 1964), for a long time thereafter, the field was barely advanced.

Recently, though, some pioneering molecular biological studies have been reported on asexual regeneration in the aquatic Oligochaeta *Pristina leidyi*, which reproduces through paratomy (Bely and Wray, 2001), and on the early development of Annelid (Seaver and Shankland, 2001; Seaver et al., 2001; Prud'homme et al., 2003). An Aquatic Oligochaete, *Lumbriculus variegatus* has also been reported to exhibit notable regeneration ability (Drewes and Fourtner, 1990). Bely (2006) reviewed the distribution of segment regeneration ability in the Annelida. According to his review, most of the Polychaeta has low ability of anterior regeneration. In contrast, some of the Oligochaeta species such as Enchytraeidae, Lumbricidae and Tubificidae show high ability of regeneration, while Hirudinida, on which many molecular and developmental studies have been done, has no ability of anterior regeneration. In order to clarify the mechanisms involved in the evolution of regeneration abilities and the nature of regenerative stem cells, comparative study on the groups of relatively close species might be very useful.

Asexual reproduction

Fragmentation

Spontaneous fragmentation (autotomy) usually occurs once every 2 weeks when the worm becomes full-grown (Myohara et al. 1999). Autotomy can also be induced experimentally by head (anterior-most seven segments) removal (decapitation) when the worm is less than the full-grown size (Inomata et al. 2000; Myohara et al. 1999). Decapitated worms placed on agar plates autotomize within 24 hr. Fragments can also be obtained artificially by cutting with a surgical blade (amputation) or by electrical stimulus (Christensen 1964; Kawamoto et al., 2005).

During spontaneous fragmentation and fragmentation induced by decapitation, circular body wall muscles contract in the middle of the segment, which causes constriction and results in fission of the body (fission zone; Yoshida-Noro et al., 2000; Kawamoto et al., 2005). Initial constriction is generally observed in the posterior region of the body before proceeding in an anterior direction. During fragmentation induced by electric shock, the plane of fission is identical to that seen during spontaneous fragmentation. Even in the fragments obtained by amputating at random locations, corrective autotomy occurs following amputation, resulting that the both ends

of the amputated fragment becomes identical to natural ends (Yoshida-Noro et al., 2000).

Regeneration processes

Regeneration of basic body components completes in 4-5 days after fragmentation at 24°C. Then the worms grow by the addition of new segments anterior to the posterior growth zone and reach their original length. Thus, the entire asexual reproduction cycle in the worm is approximately 2 weeks.

The anterior-most fragment with a head (head fragment) regenerates only a tail (the most posterior part, consisting of a pygidium and growth zone) from the posterior plane by in 2-3 days after autotomy (i.e., Day 2-3 in regeneration), while the posterior-most fragment with a tail (tail fragment) regenerates a head (seven head segments: Segments I to VII) from the anterior plane by Day 4-5. The rest of the fragments from trunk region regenerate both a head from the anterior plane by Day 4-5 and a tail from the posterior plane by Day 2-3 at 24°C.

This process is called epimorphic regeneration or epimorphosis, which means regeneration of the lost parts of the body through blastema formation and cell

proliferation. After epimorphosis completes, the worm starts growing by the addition of new segments at the growth zone and the whole body pattern is re-organized by Day 7 (Myohara et al., 1999). This process is called morphallactic regeneration or morphallaxis, which means the restoration of the proportion of the whole body, including original tissues and the regions regenerated by epimorphosis. The capacity for regeneration in this worm is basically the same throughout the body and is independent of the antero-posterior position. It has also been reported previously that regeneration does not occur from only one segment (Tochinai, 2000). We have precisely determined the regeneration stages and present these stages in Fig. 3 (Kawamoto et al., unpublished)

Stem cells in regeneration

In many species of oligochaete worms, neoblasts exhibit morphological characteristics common to undifferentiated cell types, including a high nucleocytoplasmic ratio, a large nucleus with a large nucleolus, and basophilic cytoplasm. Neoblasts in *E. japonensis* also have similar morphological features. A pair of neoblasts is usually located at the septa next to the ventral nerve cord and the

cells which possess similar morphological characteristics to neoblasts but smaller in size are located more dorsally on the septa adjacent to the body wall.

In order to investigate the precise role of neoblasts in regeneration, we used bromodeoxyuridine (BrdU) labeling for the analysis of proliferating cells during regeneration (Tadokoro et al., 2006 ; Takeo et al. in press; Sugio et al., in preparation). In the very early stages of regeneration (6-12 hrs after fragmentation), only the neoblasts at the septa were labeled (Fig. 4. A). To trace the fate of the proliferating neoblasts, we undertook a pulse-chase experiment (BrdU labeling 9-21 hrs after fragmentation and chase; Fig. 4. B). The results indicated that the labeled neoblasts migrated to the anterior and posterior regions and contributed to the mesodermal tissues in the blastema.

On the other hand, proliferation of epidermal cells is also detected at the anterior region in 12-18 hrs after fragmentation (Fig. 4. B). Transdifferentiation of epithelial cells has been suggested to form a new anterior structure including brain (Yoshida-Noro et al., 2000; Tochinai, 2000; Sugio et al., in preparation). In the current model, the major source of stem cells for anterior regeneration are neoblast lineage for mesoderm (Tadokoro et al., 2006; Sugio et al., 2008), dedifferentiation of epidermis for

ectoderm (Yoshida-Noro et al., 2000; Tochinai, 2000), and intestinal cells for endoderm (Takeo et al., 2008; Fig. 4.C), while neoblasts are thought to be the primary source for posterior regeneration (Tadokoro et al., 2006; Sugio et al., 2008).

Role of nervous system in fragmentation and regeneration

Three-dimensional confocal images with an antibody against acetylated β -tubulin (a neuronal marker; Jellies et al., 1996) showed that nerve fibers from the brain are connected to the ventral nerve cord through the subesophageal ganglion and also to the prostomium, which is an olfactory organ found in the most anterior region of the worm (Yoshida-Noro et al., 2000). There are two major circumferential structures in the body muscle of each segment that react strongly with α -bungarotoxin (an antagonist of nicotinic acetylcholine receptors; neuromuscular junction marker; Balice-Gordon and Lichtman 1990) and nerve fibers just underneath these structures in the trunk region. During the fragmentation process, the circular body wall muscles contract near one of these neuromuscular junctions in the middle of the segment (fission zone), which causes constriction and results in fission of the body (Yoshida-Noro et al., 2000; Kawamoto et al., 2005). Spontaneous fragmentation never occurs anterior to the

seventh segment where no obvious neuromuscular junctions were found. Although each of the segments posterior to the seventh segment contains a fission zone, constriction usually occurs in several segment intervals. On the other hand, decapitation or the removal of at least two anterior-most segments that contains brain and subesophageal ganglion induces fragmentation. Even a small incision made in the ventral side of the trunk causes fragmentation in the body posterior to the incision (Inomata et al., 2000). In addition, we failed to obtain autotomy in decapitated or electrically stimulated worms that were anesthetized (Kawamoto et al, 2005). Therefore, it is suggested that the nervous system controls selective fragmentation, which is important for the correct regeneration of the worm (Inomata et al., 2000; Kawamoto et al., 2005; Müller 2004).

During anterior regeneration nerve fibers begin to extend from the remaining ventral nerve cord on Day 1 in regeneration at 24°C, followed by the formation of a fine neural network that covers the entire blastema on Day 1.5. On Day 2.5, segmentation of the nerve cord and innervations of the differentiated prostomium is clearly observed, and nerve fibers begin to enter the new brain primordium at this stage. A complex neural network is seen in the brain on Day 3.

During posterior regeneration nerve fibers from the remaining ventral nerve cord begin to expand posteriorly on Day 1.5, and the network around the pygidium is formed by Day 2.5 at 24°C (original data was obtained at 18°C has been shown in Yoshida-Noro, et al., 2000). Thus, in both anterior and posterior regeneration a neural network is formed over the entire blastema. This neural network is formed prior to the formation of the brain and prior to the differentiation of the pygidium. These results are consistent with the hypothesis that the nervous system is involved in the dedifferentiation and redifferentiation of cells to form regenerated structures (Dinsmore and Mescher 1998; Thouveny and Tassava 1998; Müller 2004). Even though the neoblasts are considered to be dominant in posterior regeneration (Christensen 1964; Herlant-Meewis 1964; Kobari et al., unpublished results), the nervous system in the posterior region may also be involved in regeneration.

Molecular markers for the early step of regeneration process

As mentioned above, cells involved in the blastema formation are considered as follows; neoblast lineage for mesoderm, dedifferentiation of epidermis for ectoderm and intestinal cells for endoderm. In order to demonstrate stem cell lineages for

regeneration, identification of accurate molecular markers for stem cells are necessary. Thus, we constructed cDNA subtraction library between intact growing and early regenerating worms at 6 and 12 hr after fragmentation. We obtained approximately 400 clones for analysis. As a result, we isolated five genes whose expression levels changed during the regeneration process. Although there was not much genetic information available for Annelids in the common database, four of these genes showed some homology to known genes or EST.

One of the isolated genes is *grimp* (Takeo et al., in press). Database searches for sequence of *grimp* full-length cDNA obtained by RACE did not result in the identification of any known homologous genes. Although *grimp* contains a triplicate repeat in the 5' region with RGDS (integrin recognition) sequences and protein kinase C phosphorylation sites, it remains difficult to predict the function of *grimp* based on its structure alone.

In our result shown by *in situ* hybridization (ISH), *grimp* was expressed transiently from 3 to 12 hr post fragmentation mainly at the tip of blastema in mesodermal cells just underneath epidermis. We found that *grimp* was initially expressed in cells found not only around the wound site but also over the entire

fragment from 3 to 6 hrs after fragmentation. The expression of *grimp* weakened throughout the body by 12 hrs. Cross-section analysis showed that *grimp* was expressed only in flat mesodermal cells and neoblasts, both of them had a morphological feature of neoblast, with large nuclei and dense nucleolus. On the other hand, *grimp* expression was never observed in the epidermis, the muscle or the digestive tract. To investigate the association between *grimp* expression and cell proliferation, simultaneous ISH for *grimp* and BrdU immunohistochemistry was made. We found that *grimp* was expressed transiently only in the neoblasts and in a population of mesodermal cells that incorporated BrdU.

We succeeded in inhibiting *grimp* expression by using RNA interference (RNAi) that was established for the first time in Oligochaeta (Takeo et al., in press). Since oligochaetes have a wide coelom space, any substances injected into the coelom are diffused promptly throughout the body. The expression of *grimp* was down-regulated by *grimp* dsRNA injection. The suppression of *grimp* caused inhibition of cell proliferation in mesoderm and differentiation of anterior structures. Thus, *grimp* appears to be a good candidate molecule for the initiation of the early stages of regeneration.

Sexual reproduction

Induction of sexual reproduction

The sexual maturation was density dependent as in *E. bigeminus* (Christensen 1973). Under a low population density, *E. japonensis* occasionally regenerates hermaphroditic gonads and undergoes sexual reproduction (Myohara et al., 1999) while at a high density, sexual maturation was suppressed. To induce sexual reproduction, 10 trunk fragments without heads or tails obtained by applying an electrical stimulus to the asexual worms (Kawamoto et al., 2005) transferred to 100 mm Petri dishes and were fed after regeneration completed.

Emergence of gonads

Worms with fully differentiated sexual organs with gametes can be obtained within two weeks after the low-density culture started. Pairs of testes and seminal vesicles that are attached to the posterior surface of the anterior septum in Segment VII appear at 5 days after induction. The ovary in Segment VIII that consists of a number

of packets attached to the anterior septum at one end and freely extending into the coelom at the other (Sugio et al., 2008). The gonads develop and mature germ cells are observed at day 10 after induction (Tadokoro et al., 2006). It has been demonstrated that all fragments are able to produce functional gonads following induction, regardless of where the fragments were derived (Tadokoro et al., 2006). Mature worms lay eggs within a few days.

Although asexual worms have no identifiable gonads, immature small testis-like structures are actually observed in the presumptive gonad region (Kutsuna et al., 2001). Tadokoro et al. (2006) have demonstrated that *Ej-Piwi*-positive germ cells appeared in the anterior blastema at Day 2-3 in regeneration. *Ej-Piwi* expression is then restricted to Segments VII and VIII at Day 5 in regeneration. However, this immature structure fails to develop and mature under regular mass culture conditions.

Developmental processes

The embryonic development of *E. japonensis* was found to be similar to that of the enchytraeid *Lumbricillus lineatus* (Lasserre, 1975), *Tubifex* (Arai et al., 2001) and *Enchytraeus coronatus* (Berger et al., 2004). On average, two fertilized eggs of

approximately 150µm in diameter are laid in a cocoon. These embryos then undergo a holoblastic spiral cleavage and form mesoblasts and ectoteloblasts, followed by the formation of teloblasts and germ bands. Along with germ band elongation, the embryo gradually curves round and segmentation becomes visible. Within 4 –5 days, the embryo becomes 700 –900µm long and actively moves in the cocoon. The juvenile hatches as a miniature adult approximately 1 mm long with 13–14 segments at 5–6 days after oviposition (Myohara, 2004; Takeuchi, unpublished). Interestingly, sexual worms that appear directly from embryonic development exhibit gonads in Segments X and XI, while worms derived from regenerates contain gonads in Segments VII and VIII.

Molecular markers for germ cells

In order to define the origin of germ cells and the mechanisms underlying their differentiation in *E. japonensis*, we isolated two *vasa*-related genes (*Ej-vlg1* and *Ej-vlg2*) by RACE-PCR from RNA of mature sexual worms, and analyzed their expression by using *in situ* hybridization (Sugio et al., 2008) along with other germline marker gene *Ej-piwi* (Tadokoro et al, 2006).

Transcripts of both *Ej-vlg1* and *Ej-vlg2* were found in the testis, seminal vesicle, and ovary of mature worms. *Ej-vlg1* mRNAs were detected in spermatogonia and spermatocytes, but not in spermatids or in sperms. Strong signals of *Ej-vlg1* were detected in the cytophore (a structure that connects germinal cells through cytoplasmatic bridges during spermatogenesis) regions of cysts. In contrast, *Ej-vlg2* transcripts were observed in more restricted cells in the seminal vesicle. *Ej-vlg1* and *Ej-vlg2* transcripts were detected in oogonia, primary oocytes, and secondary oocytes in the ovary. *Ej-piwi* transcripts were present in the testis, seminal vesicle, and ovary. As in the case of *Ej-vlg1*, *Ej-piwi* mRNAs were detected in spermatogonia and spermatocytes. However, with the present method the *Ej-piwi* signals were barely detectable in oogonia and oocytes and apparently absent in secondary oocytes.

Origin of germ stem cells and regenerative stem cells

Germline separation from the somatic-line during early development is considered a universal phenomenon in both vertebrate and invertebrate species. It has been reported previously that putative mesodermal stem cells called neoblasts contribute to blastema formation in *E. japonensis* (Myohara et al., 1999) and that

Ej-piwi⁺ germline stem cells participate in gonad regeneration (Tadokoro et al, 2006).

In order to delineate the origin and formation of germline cells and neoblasts, we analyzed expression of *Ej-vlg1*, *Ej-vlg2* along with *Ej-piwi* (Fig. 5) (Sugio et al., 2008), as the *vasa*-related genes are not only expressed in germline cells but also in pluripotent stem cells in hydras (Mochizuki et al., 2001) and planarians (Shibata et al., 1999).

In the asexual worms, *Ej-vlg1* and *Ej-vlg2* were expressed in *Ej-piwi*⁺ germline stem cells situated on the ventral nerve cord, and germ cells in immature gonads, while only *Ej-vlg2* mRNAs were detected in neoblasts and in the cells located more dorsally on the septa adjacent to the body wall. *Ej-vlg1* and *Ej-vlg2* mRNAs were also detected in the growth zone cells, while *Ej-piwi* mRNAs were not detectable there.

During embryogenesis, clusters of *Ej-vlg1/Ej-vlg2* cells, located at the posterior ventral region in late embryos, became *Ej-vlg1+/Ej-vlg2+/Ej-piwi+* germline stem cells just after embryogenesis. On the other hand, *Ej-vlg2* single positive cells with morphological characteristics of neoblasts became detectable much later after embryogenesis at the ventral position on each septum where adult neoblasts exist, although these early-detected cells were much smaller in size than adult neoblasts.

These results suggest that germline stem cells specified just after embryogenesis are derived from *Ej-vlg1+/Ej-vlg2+* cells which appear at the posterior ventral region in late embryos and, and that neoblasts appear much later in development (Fig. 5) (Sugio et al., 2008).

Body plan

Restoration of the antero-posterior axis during regeneration

During normal regeneration, the antero-posterior axis in the regenerates is restored. The capacity for regeneration in this worm was found to be basically the same throughout the body, in a manner that was independent of the antero-posterior position (Fig. 6. A). However, artificially amputated fragments occasionally regenerate a head posteriorly, resulting in bipolar head (dicephalic) regeneration (Fig. 6. B; Myohara et al., 1999; Kawamoto et al., 2005). On the other hand, as head regeneration is dominant in this species, no bipolar tail worm has been observed. Amputation anterior to Segment VII frequently induces bipolar head regeneration in the head fragment (Myohara et al. 1999; Kawamoto et al., 2005). Since there is no fission

zone in Segments I to VI, the head segments are incapable of undergoing autotomy. Correct autotomy is thought to be required for posterior regeneration. Histological observations revealed that the posteriorly regenerated head exhibited almost normal structure. The intestinal tract and the ventral nerve cord were formed continuously with the original part in the right dorso-ventral polarity.

Bipolar head worms usually spontaneously autotomized at the original fragment region. The posterior-half fragments, that include the posterior head, regenerated the head anteriorly from the anterior autotomy plane. This process resulted in the formation of a bipolar head worm. The posterior head does not appear to exhibit the reversal effects on global antero-posterior axis of the worm (Fig. 6. B).

Role of the head in the regulation of body plan

How does the worm know its own length and the timing of spontaneous autotomy? It has long been known that the fission (fragmentation) of asexually reproducing planarians can be induced by removing the head (Child 1910). In *E. japonensis*, decapitation also results in fragmentation of the rest of the body, even when the body length is not enough for natural fragmentation. (Myohara et al., 1999; Inomata

et al., 2000). Of the seven head segments, the removal of the two anterior-most segments that include brain and subesophageal ganglion was sufficient for inducing fragmentation (Inomata et al, 2000).

When the decapitated full-grown sized worms kept in solution for a week, fragmentation could not take place as mechanical support by the surrounding substrata is required for autotomy. Instead, worms cultured in this manner regenerated a new head. These worms with new heads did not autotomize even after placed on the agar plate. These results suggest that inhibitory signals or factors for fragmentation are produced in the head region and that spontaneous fragmentation occurs when inhibitory signals from the brain are weakened in 2 weeks (Inomata et al, 2000), or the worm becomes long enough for the inhibitory signal to fail to reach the posterior end.

In hydras and planarians, bipolar head regeneration occurs in very short fragments made along the antero-posterior axis and in chemically treated fragments (Berrill and Karp, 1981). It has been widely accepted that hydras and planarians have a head-forming gradient that is high in the head region and decreases toward the tail, because the quality and quantity of head regeneration decrease from the head toward the tail (Rose, 1970). Though the presence of head-forming gradient in *E. japonensis*

remains to be solved, none of the newly formed posterior heads in bipolar regenerates ever reversed the original antero-posterior axis of the worm as described above.

Morphallaxis and epimorphosis

In order to express the positional information present along the anterior-posterior axis, we reported the analysis of alkaline phosphatase enzyme activity, suggesting the possibility of morphallactic regulation (Agata et al., 2007) in the trunk region (Myohara et al., 1999; Myohara, 2004). For the precise analysis of the pattern formation during regeneration, we screened the cDNA subtraction library mentioned above to isolate three region-specific genes (*EjTuba*, *mino*, and *horu*) expressed in the digestive tract from the cDNA subtraction library mentioned above (Fig. 6; Takeo et al., 2008). *EjTuba* is a tubulin alpha gene that was expressed in the head, anterior trunk, and posterior trunk region. *mino* was expressed in the trunk region just behind the head and *horu* was expressed in the middle of the trunk. We analyzed temporal and spatial changes in the expression of these genes during growth and regeneration.

In growing worms, the expression of *EjTuba* in the head (Segments III to VII), and *mino* in the trunk region just posterior to the head (Segments VIII to X,

corresponding to a crop and a gizzard form its histological feature) were observed in defined body segments, while the size of the expression regions of *EjTuba* in the trunk and *horu* were proportional to the total number of body segments. These results suggest that the entire body proportion is maintained by morphallactic regulation because new segments are continuously added posteriorly to the trunk region during growth (Takeo et al., 2008).

In normal regeneration (Fig. 6. A), though there are several differences among fragment types (original position; head, trunk or tail), the expression of all marker genes disappeared within 2 days after fragmentation; each gene was then re-expressed as regeneration proceeded, and the gene expression pattern finally became the same as that of growing worms by the 7th day in regeneration. Despite the quick disappearance of gene expression, no massive cellular death or no obvious activation of cell proliferation in the digestive tract was observed. These results suggest that regeneration and reorganization of the digestive tract is achieved by dedifferentiation and re-differentiation rather than by replacement of intestinal cells (Takeo et al., 2008).

In abnormal regeneration such as a bipolar head or secondary lateral head (Fig. 6. B), *mino* was still expressed in the region next to both the normal and the ectopic

heads (three segments after the seven head segments), suggesting that *mino* expression is induced by the head (Takeo et al., 2008). In contrast, the ectopic head was unable to invert the polarity of the external tissues (Kawamoto et al., 2005), like an event that occurs in hydras and planarians (Saito et al., 2003; Broun and Bode, 2002; Kobayashi et al., 1999a, b; Browne, 1909). Since Annelids have a more highly developed body plan, the digestive tract and epidermis–muscle system are clearly separated by the coelom; this may result in a relatively independent control of the polarity of the digestive tract and epidermis–muscle system. These results suggest that there is morphallactic as well as epimorphic or inductive regulation of the body patterning during regeneration of *E. japonensis*.

Stem cell response to environmental factors

Proliferation signals for neoblasts

A pair of neoblasts is located at each septum after Segment VII next to the ventral nerve cord, and the cells which possess similar morphological characteristics to neoblasts but smaller in size are located more dorsally on the septa adjacent to the body

wall (smaller neoblast-like cells). *Ej-vlg2* transcripts were detected in both of these cells, whereas *Ej-vlg1* and *Ej-piwi* were not expressed in these cells.

During growth, active proliferation of cells occurs at the posterior growth zone only, and new segments including neoblasts are formed (Myohara et al., 1999, Honda *et al.*, 2003), while neoblasts at the septa of other part of the body does not show active proliferation. After fragmentation, neoblasts at each septa, regardless of their distance from the position of autotomy, actively proliferate during the early stages of regeneration and participate in forming the mesodermal region of blastemas, a region where *Ej-vlg2* mRNAs was detected (Tadokoro et al., 2006, Sugio et al., unpublished data) (Fig. 4).

From these results, it appears that the stem cell system in *E. japonensis* behaves in accordance to its surrounding environmental conditions. Recently numerous studies have suggested the existence of stem cell niche that preserves quiescent stem cells. Once a signal comes from an injury site or other environmental stimulus, stem cells leave the niche and begin to proliferate and migrate (Arai et al., 2004; Claudinot et al., 2005; Martinez-Agosto et al., 2007). It is likely that the neoblasts in *E. japonensis* are also housed within a kind of niche on the ventral side,

produce the smaller neoblast-like descendants in the growing phase, and respond to some proliferation signals after fragmentation to participate in the blastema formation (Fig. 4.C). In order to confirm this model, it is necessary to trace the precise lineage of stem cells by introducing some molecular markers into the neoblasts, and to clarify the molecular nature of the stem cell niche and proliferation signals.

Wound healing and regeneration

During spontaneous fragmentation or fragmentation induced by electric shock, the plane of fission is located at the definite position in the segments and the observed wound tissue is minimal. Even when fragments are obtained by amputation, autonomous corrective fragmentation occurs and that the plane of fission is identical to the position above (Yoshida-Noro et al., 2000). In these cases, posterior regeneration occurs normally and the correct tail structure is formed.

In contrast, the posterior head is formed when the posterior end of the fragment is located at the non-autotomic position and disordered closure of the wound is observed (Yoshida-Noro et al., 2000). The incomplete short segments observed at the base of the posterior head in bipolar head worms may be vestiges of the segments that were

produced by the amputation at the non-autotomic position. Strangely, the anterior amputation plane always regenerates a normal head, no matter where the amputation occurred within a segment. These results suggest that correct autotomic plane is required for posterior regeneration. Although the mechanism of this phenomenon is under investigation, completion of wound healing might be essential for correct regeneration.

As we found a novel gene *grimp* that is expressed shortly after fragmentation around the wound site and over the entire fragment, and is important for regeneration, it is a good candidate molecule for the study of the relationships between wound healing and regeneration.

Morphallactic signal and morphogenetic gradient

From the molecular study in the digestive tract, it was revealed that morphallactic regulation occurred during growth and regeneration in *E. japonensis*. The cells in the digestive tract underwent transdifferentiation depending on their relative position along the antero-posterior axis to the whole body. On the other hand, the worms appeared to recognize their full body length and the timing for autotomy. The

fission occurs at appropriate intervals along the body to form fragments containing several segments. The fact that the removal of the head induced autotomy suggests that the existence of inhibitory signals or factors for fragmentation produced in the head.

The bipolar head analysis showed that the posterior head did not appear to exhibit the reversal effects on global antero-posterior axis of the worm. Thus, head-forming gradient along the antero-posterior axis may not exist in this worm, suggesting that the mechanisms underlying these processes are not as simple as for hydras and planarians. In order to clarify these mechanisms, it is necessary to identify and characterize the factors released from the tissues around the amputation planes, as well as the head and nervous system.

Environment for sexual maturation

Under a low population density, *E. japonensis* occasionally regenerates hermaphroditic gonads and undergoes sexual reproduction (Myohara et al., 1999), while worm reproduces asexually under regular mass culture condition. Although small clusters of germ cells are observed at the Segment VII and VIII, maturation of the

gonad is suppressed in the asexual phase.

Numerous studies have been undertaken to increase the efficiency of sexual reproduction. Culture density, original worm length, starvation prior to induction, agar pH and the presence of wet leaf mold were all tested. In order to clarify the nature of the environmental factors involved in the switch for the sexual phases, chemical biological approaches as well as molecular analysis are underway.

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Figure legends

Fig. 1. Body Structure of *E. japonensis*. **(A)** Illustration of head, trunk and tail structure (sagittal view drawn by Kawamoto, S.). Head (left) consists of 7 segments (Segment I-VII) equipped with specific organs such as brain (br), ventral nerve cord (vnc), sensory organ prostomium (pr), mouth (mo), pharynx (ph), esophagus (es), dorsal blood vessel (dbv), ventral blood vessel (vbv), peptonephridium (pe), septal gland (sg) and setae (set). Trunk region (middle) contains intestine (int) surrounded by coelom (co), nephridium (nep) and outer muscle. Neoblasts exist at the ventral region of each septum (sep) and small neoblast-like cells (sne) are located more dorsally on the septa adjacent to the body wall. Tail (right) contains growth zone (gz) just anterior to pygidium (py). **(B)** SEM images of head (right) and tail (left) region. prostomium (pr), growth zone (gz), pygidium (py).

Fig. 2. Life Cycle of *E. japonensis*. The worms undergo both asexual and sexual reproduction. In regular mass culture conditions, worms reproduce asexually by fragmentation and regeneration. Under low-density conditions, the worms occasionally mature and lay eggs. The entire cycle of asexual and sexual reproduction takes

approximately 2 weeks and 4 weeks respectively at 24°C. Scale bars indicate 1mm.

Fig. 3. Regeneration Table in *E. japonensis*. Regeneration of basic body components completes in 4-5 days after fragmentation. Standard progression stages in regeneration of trunk fragments longer than 4 segments at 24°C are shown in the table. Stage 0: After autotomy, wound healing is occurred. Stage 1: Blastemas are formed at both anterior and posterior ends. Stage 2: Blastema elongates and forms pygidium (py) at the posterior end. Stage 3: Formation of the intersegmental furrows (isf) is observed in anterior blastema, while tail regeneration completes at the posterior end. Stage4: Segmentation completes and formation of primordial of setae (st) and septal glands (sg) is occurred. Stage5: Regeneration completes and the worms start growing by the addition of new segments at the growth zone (gz).

Fig. 4. Stem Cells involved in blastema formation. (A) Bromodeoxyuridine (BrdU) labeling was used for the analysis of proliferating cells during regeneration. In the very early stages of regeneration (6-12 hrs after fragmentation), only the neoblasts (yellow arrowheads) at the septa were labeled. (B) To trace the fate of the proliferating

neoblasts, we undertook a pulse-chase experiment (BrdU labeling 9-21 hrs after fragmentation and chase). As a result, labeled neoblasts (yellow arrowheads) migrated to the anterior and posterior regions (white arrowheads), contributed to the mesodermal tissues in the blastema in 6-48 hrs. On the other hand, proliferation of epidermal cells (arrows) is also detected at the anterior blastema in 12-18 hrs after fragmentation (bottom right). Transdifferentiation of epidermal cells has been suggested to form a new anterior structure including brain (Sugio et al., in preparation). (C) In the current model, the major source of stem cells for anterior regeneration are neoblast lineage for mesoderm, dedifferentiation of epidermis for ectoderm, and intestinal cells for endoderm, while neoblasts are thought to be the primary source for posterior regeneration. Neoblasts at septa are thought to proliferate and form their descendants, followed by the migration of these descendant cells to the blastema.

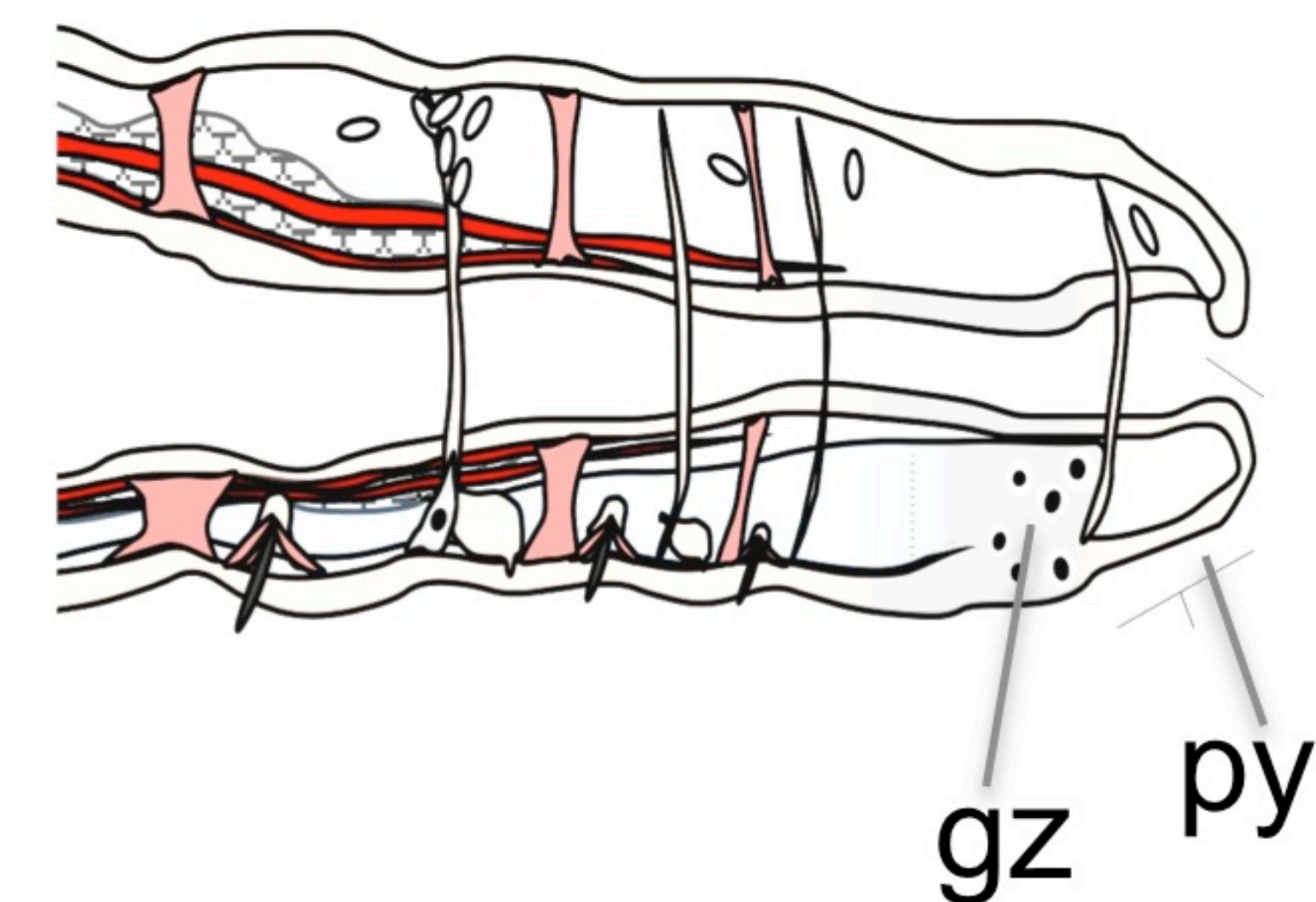
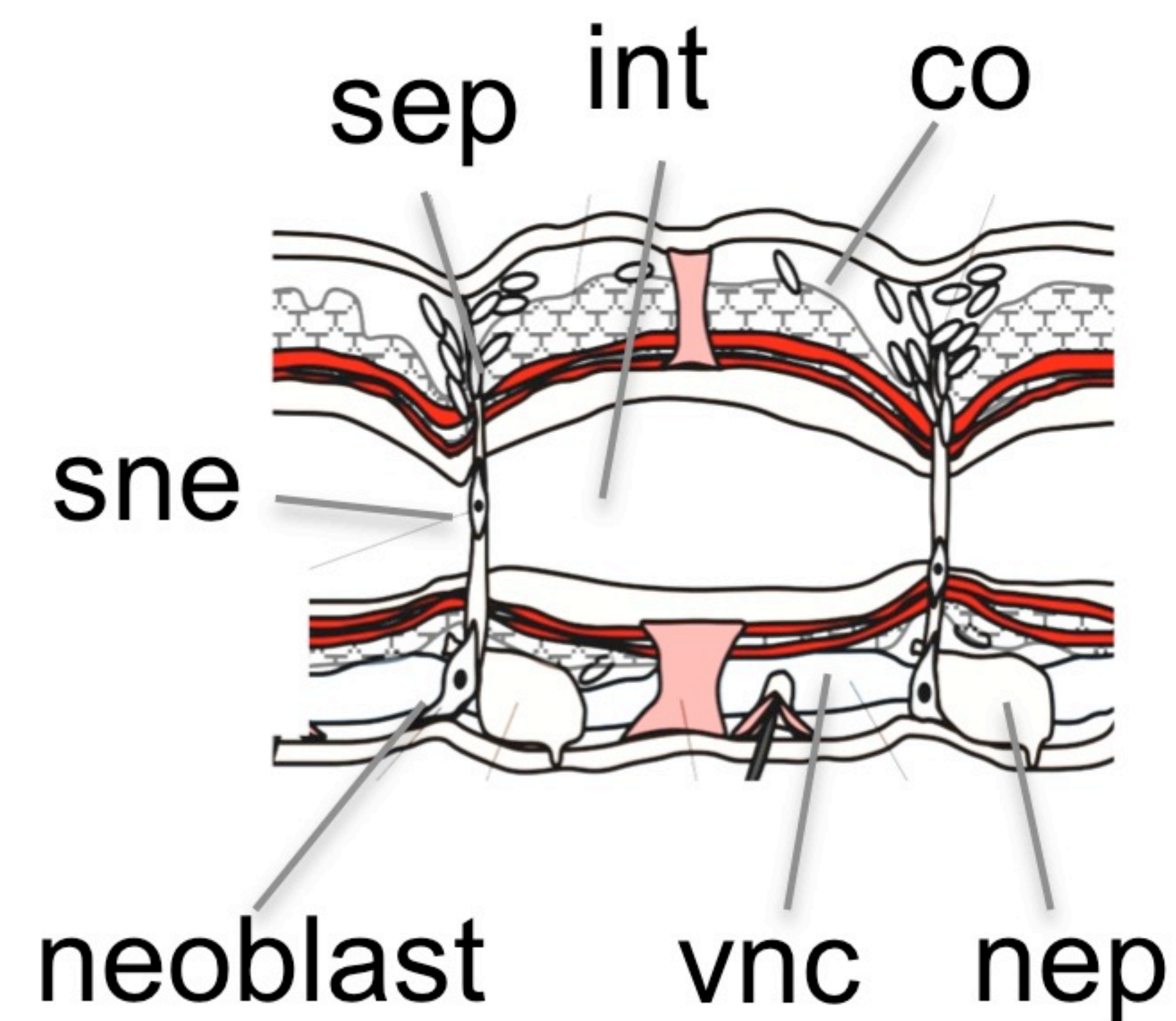
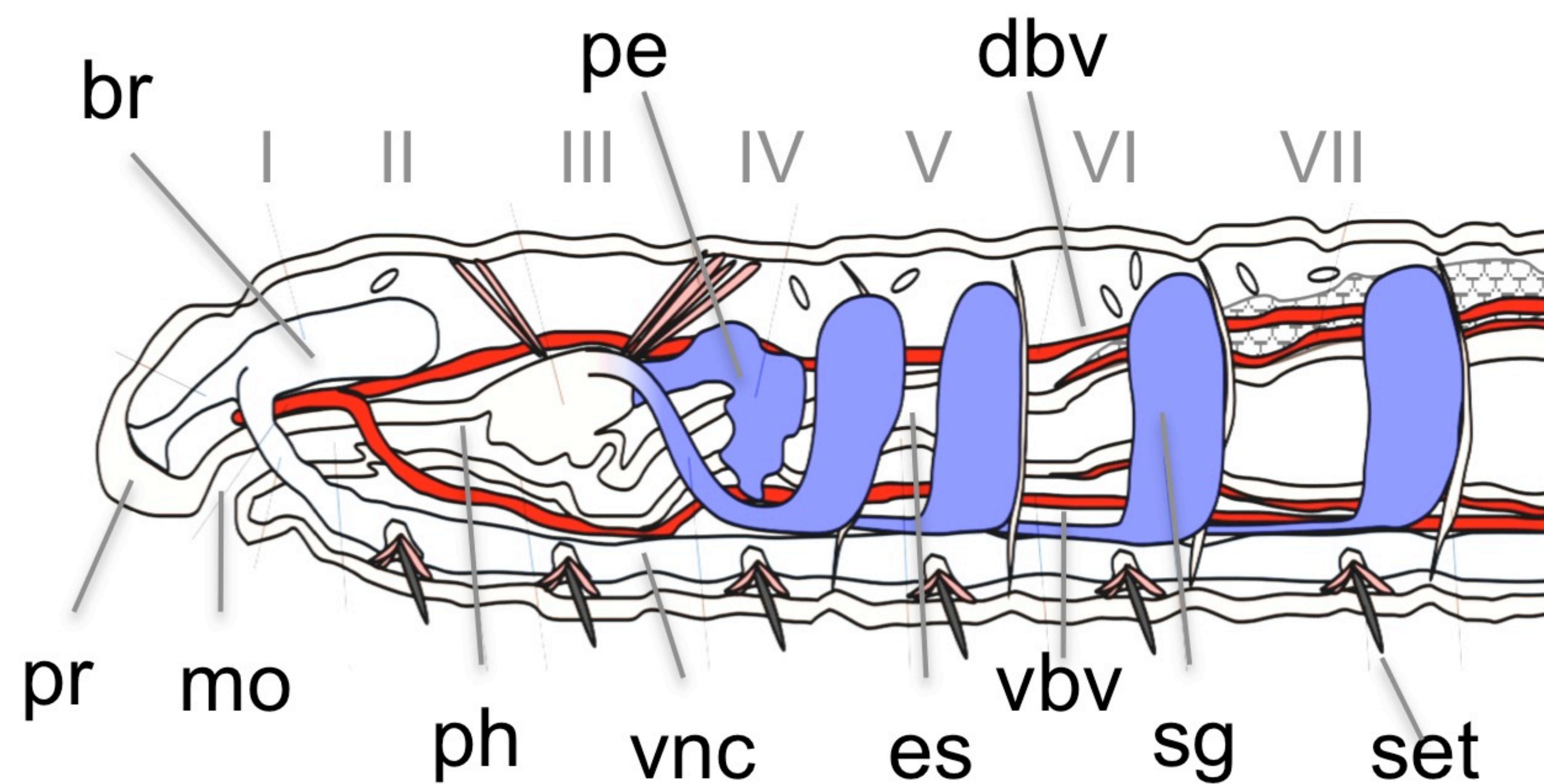
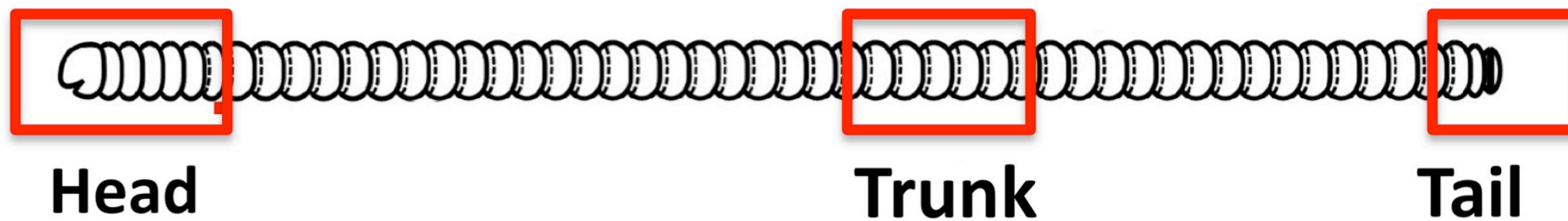
Fig. 5. Neoblasts and Germ Line Stem Cells. Schematic illustration of the expression of *Ej-vlg1*, *Ej-vlg2*, and *Ej-piwi* during growth, regeneration and development. In zygote and cleavage stage, both *Ej-vlg1* and *Ej-vlg2* are expressed in the perinuclear cytoplasm of all blastomeres. When the ventral nerve cord (gray band) is

being formed, *Ej-vlg1⁺/Ej-vlg2⁺* cells (blue) are observed at the posterior ventral region and at the posterior end of the embryo, while *Ej-vlg2⁺* cells (red) are distributed all over the whole body. After development completes, *Ej-vlg1⁺/Ej-vlg2⁺/Ej-piwi⁺* germline stem cells (green) are observed on the ventral nerve cord, and *Ej-vlg1⁺/Ej-vlg2⁺* cells (blue) are found in the mesodermal region of the growth zone. *Ej-vlg2⁺* cells (red) remain scattered throughout the body. After the 18-segment stage, *Ej-vlg2* signals (red) are detectable in neoblasts and cells in the intestinal wall. The expression pattern at this stage is almost the same as the adult pattern. These results suggest that germline stem cells specified just after embryogenesis are derived from *Ej-vlg1⁺/Ej-vlg2⁺* cells which appear at the posterior ventral region in late embryos and, and that neoblasts appear much later in development.

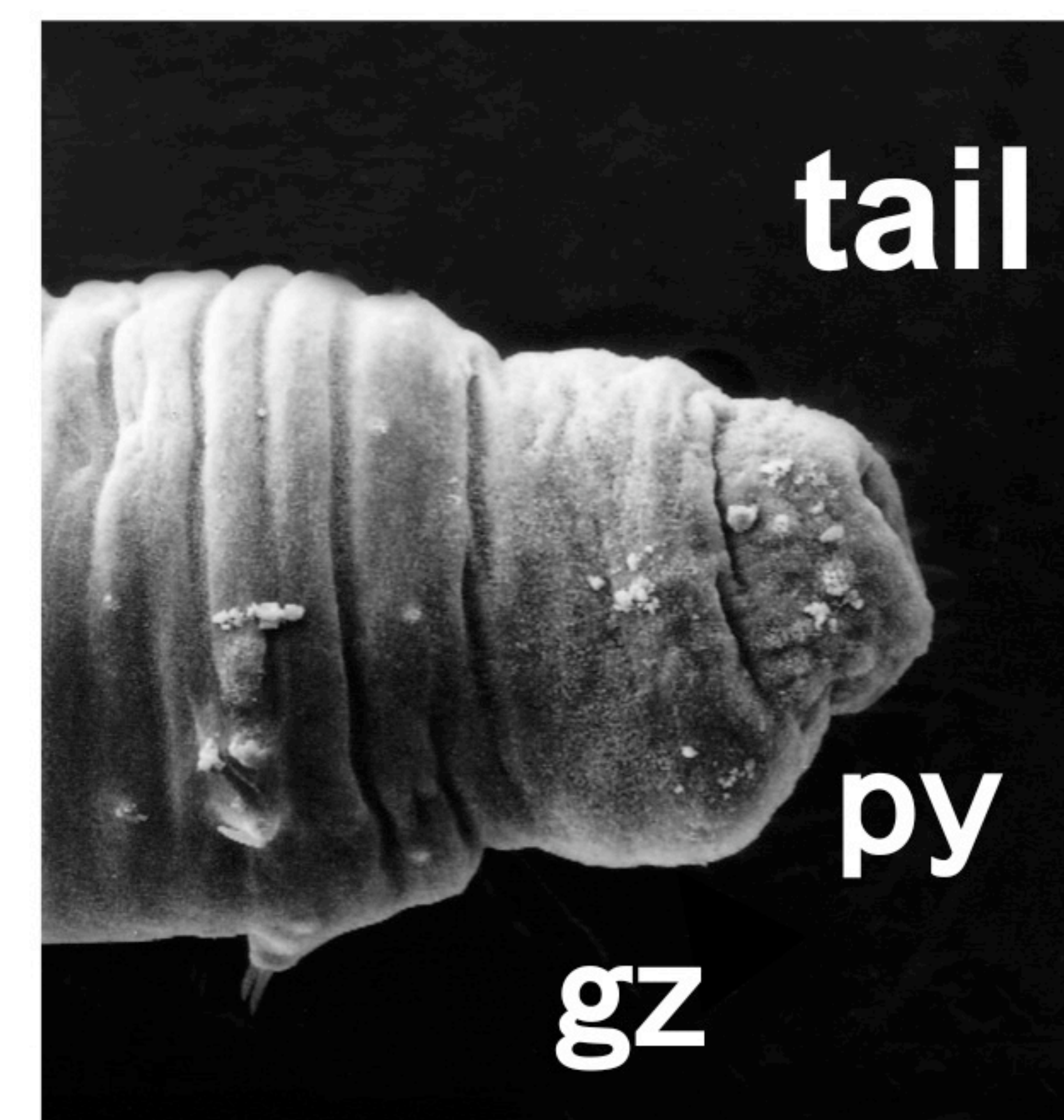
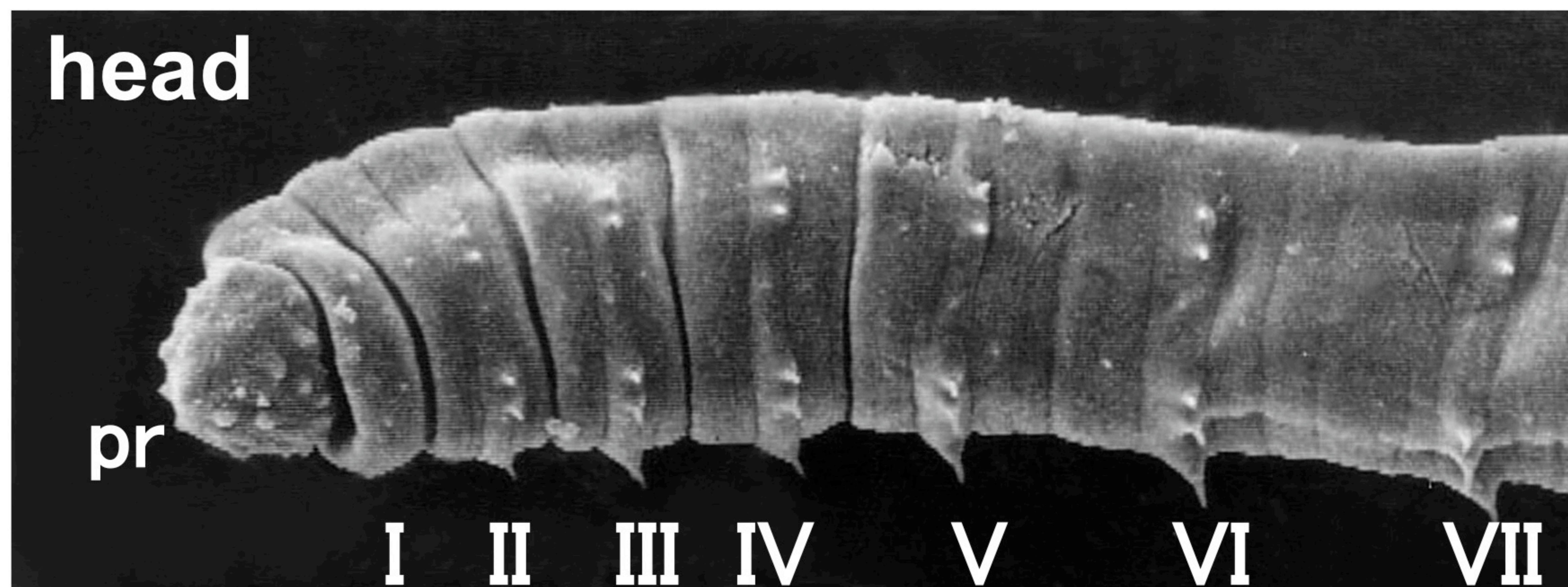
Fig. 6. Schematic figure summarizing the results of the analysis of the pattern formation during regeneration by using molecular markers (*EjTuba*, *mino* and *horu*) expressed in the digestive tract of *E. japonensis* (Takeo et al, 2004). Though there are several differences among fragment types (original position; head, trunk or tail), during normal regeneration (A), expression of *EjTuba* in the regions 1 and 2, and *horu*

disappeared at Day 0-1 in regeneration when the blastema was formed. By Day 2, expression of all marker genes including *EjTuba* in the regions 3 and *mino* disappeared once (dedifferentiation), and new expressions of *EjTuba* at region 3 and *mino* started after tail regeneration completes at Day 2-3, followed by the reappearance of the *EjTuba* expression at the posterior growth zone. After the head regeneration completed at Day4-5, each gene was re-expressed and the expression pattern finally became the same as original intact growing worms (top) by Day 7 (morphallaxis). In abnormal regeneration (B), *mino* is expressed in the region next to both the normal and the ectopic heads of bipolar-headed worms (upper), while *horu* expression is not observed. The ectopic head did not induce reversal of whole body axis polarity. In the worms with a secondary head (lower), expression of *mino* was observed only on the side of the intestine facing the ectopic head, not only in the posterior direction of the primary axis but also extended in the anterior direction.

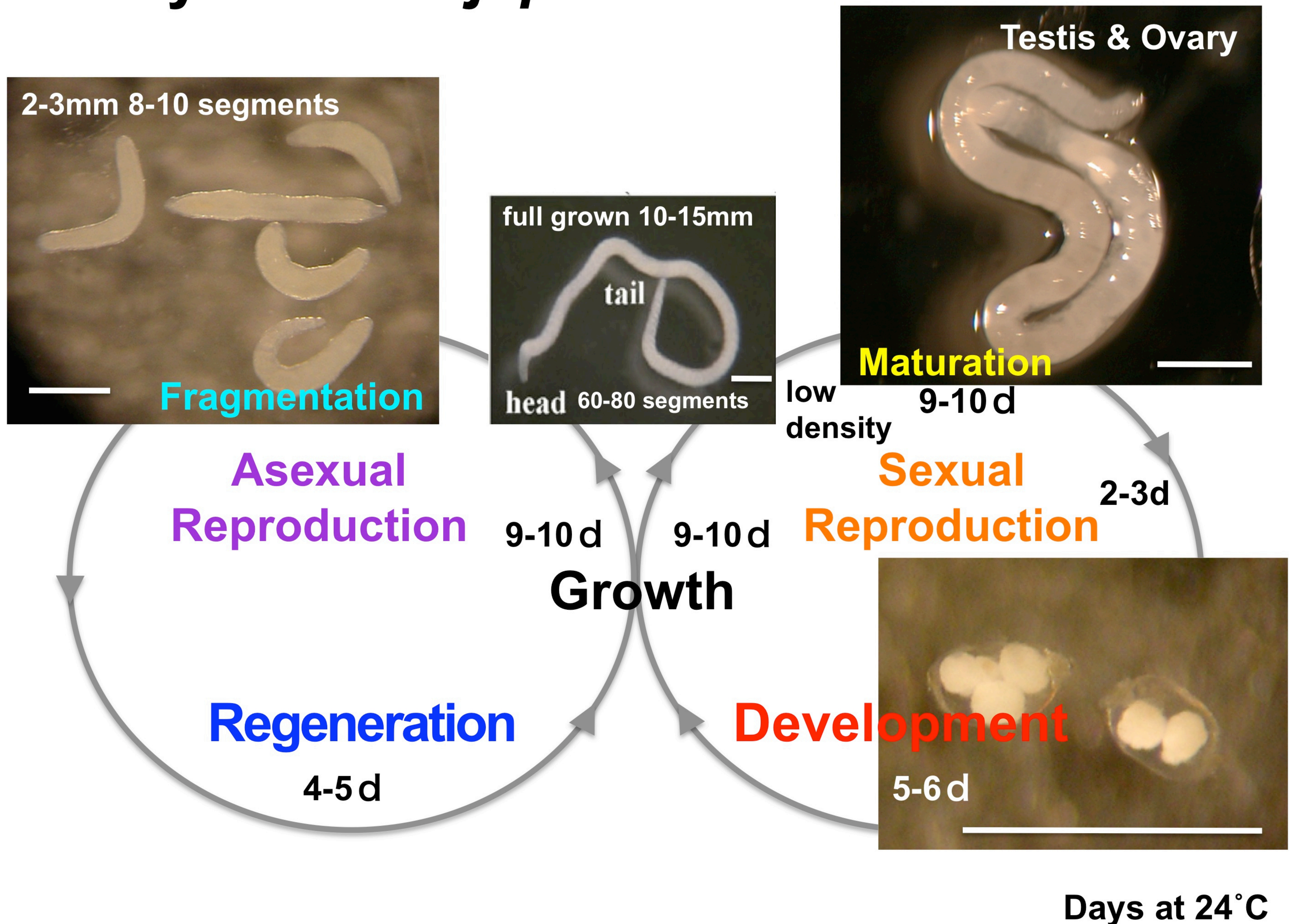
A



B

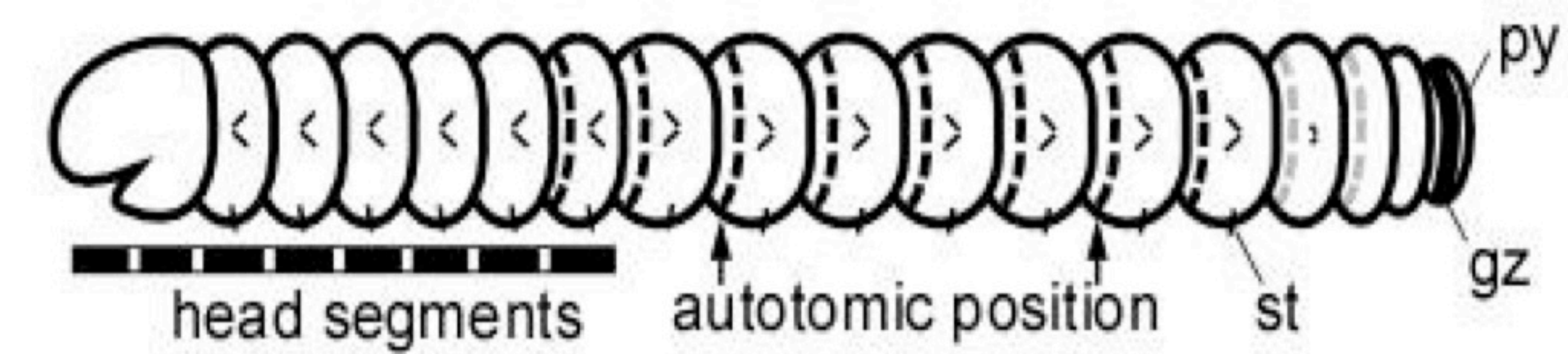


Life Cycle of *E. japonensis*



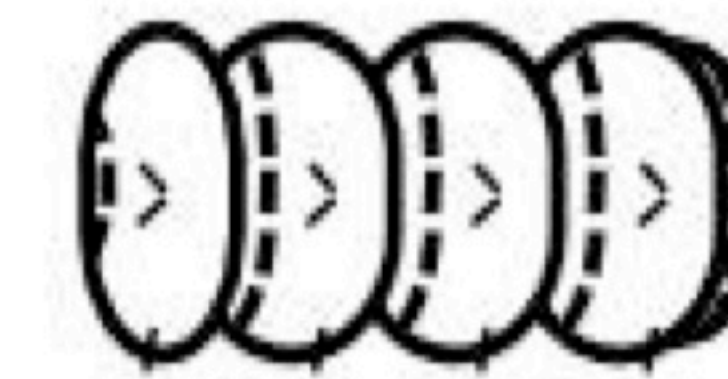
Regeneration table in *E. japonensis*

(Longer than 4 segments fatty fragment at 24 °C)



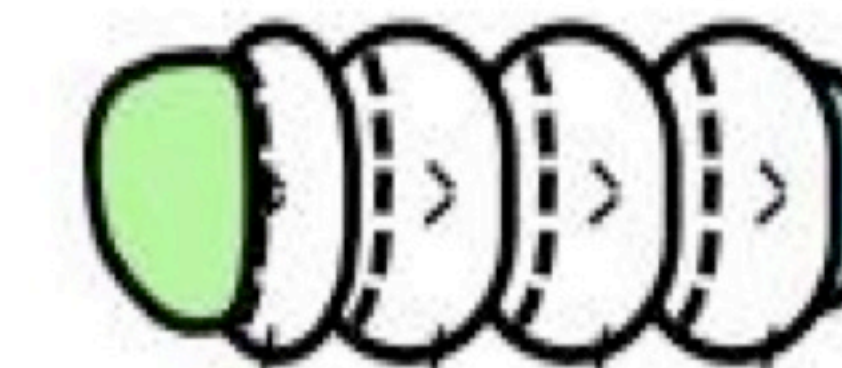
stage 0: Autotomy and Wound healing

- 0 h: Autotomy and primary wound closure by the muscles.
- 3 h: Secondary wound closure by plug.
- 6 h: Proliferation of the mesenchymal cells in wound site.
- 9 h: Tertiary wound closure by tissue healing. Formation of the anus in posterior end.



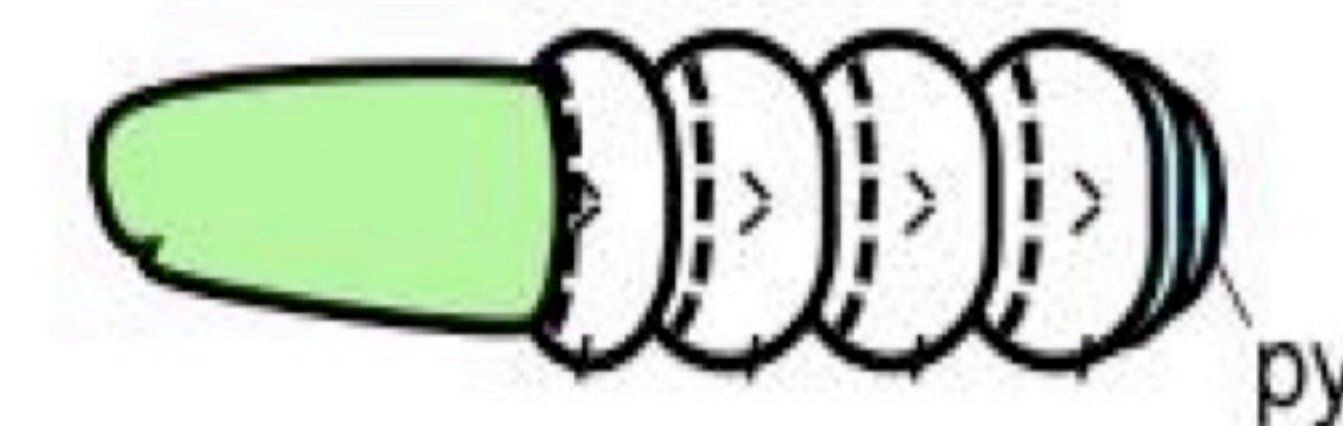
stage 1: Blastema formation

- 12 h: Proliferation of the epidermal cells in wound site.
- 12 h: Proliferation of the neoblasts in trunk segments.
- 24 h: Length of the head blastema < Width of the head blastema.



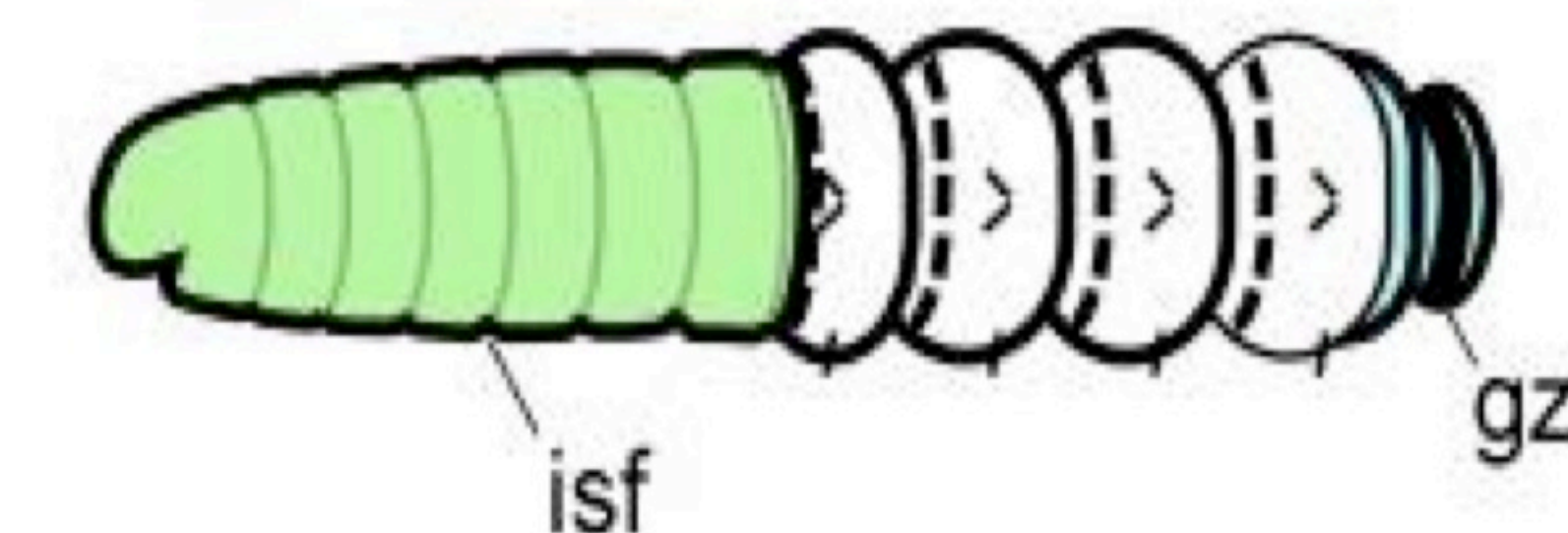
stage 2: Blastema elongation

- 12 h: Formation of the pygidium (py) in the posterior end.
- 48 h: Detectable of the head specific promordia.
- 48 h: Length of the head blastema $\geq$ Width of the head blastema.



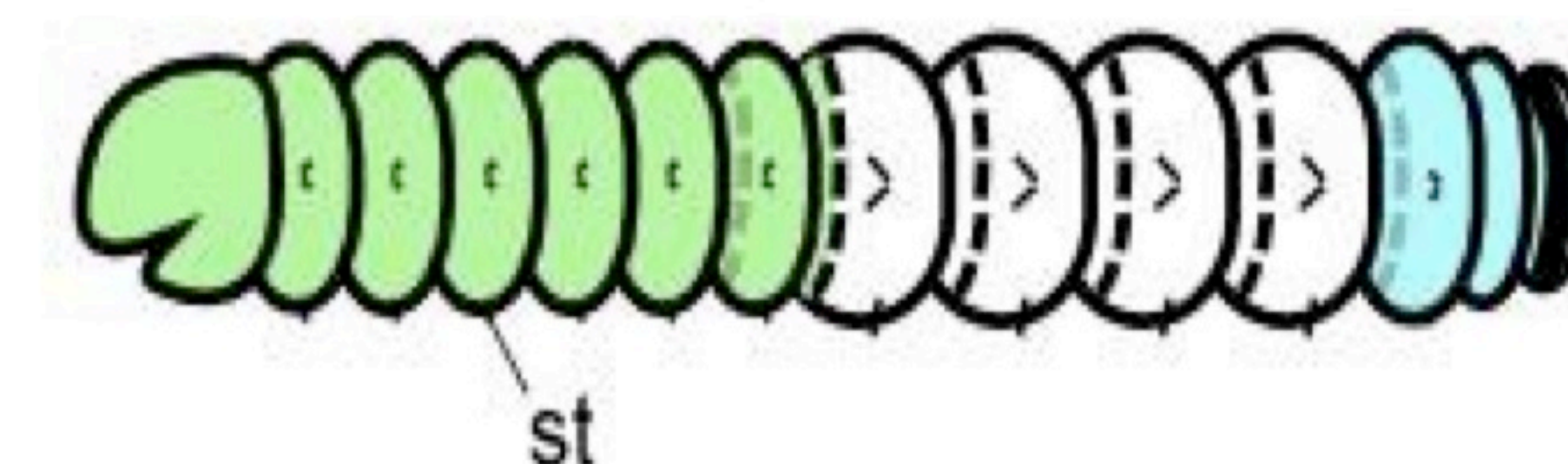
stage 3: Blastema segmentation

- 72 h: Completion of the cuticulated epithelium and the body wall muscles formation.
- 72 h: Formation of the intersegmental furrows (isf) and coelon.
- 72 h: Tail blastema regeneration completes.
- 72 h: Addition of the new trunk segments by growth zone (gz).



stage 4: Segmentation completes

- 96 h: Formation of the interannula furrows, primordia of setae (st) and septal glands.
- 96 h: Completion of the intersegmental furrows, mouth, and nervous system.
- 96 h: Worms become capable of feeding and digging.



stage 5: Regeneration completes

- 120 h: Elongation of the each head segments.
- 120 h: Width of the head blastema = Width of the original segments.
- 120 h: Completion of the septal glands (sg) formation.

