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Original Article

Preliminary investigation on the tail regeneration of the juvenile marine polychaete, *Perinereis wilsoni*

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Abstract: In *Perinereis wilsoni*, gonad formation is thought to depend on germ cells supplied by the tail-end segment (pygidium). In this study, we investigated the regenerative capacity of germ cells in the pygidium following posterior amputation, using *vasa* as a putative germ cell marker. At 3 days post-amputation (dpa), *pw-vasa*-positive cells were scattered around the amputated area of the posterior segment, with some located near germ cell clusters in the distal parapodial region of the adjacent unamputated segment. By 4 dpa, *pw-vasa* expression was observed in the segment addition zone. At 7 dpa, *pw-vasa* was detected in germ cell clusters within the regenerating pygidium, and by 10 dpa, expression was evident in both the pygidium and the distal parapodia of the regenerated segment. These findings suggest that the re-emergence of germ cells in the pygidium may be supported by germ cell supply from the parapodia of the neighboring unamputated segment.

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Introduction

Polychaetes are segmented marine worms with bodies composed of serially repeated segments. Additionally, they possess non-segmental regions: the prostomium at the anterior end and the pygidium at the posterior end (Purschke, 2002). Externally, segmentation is evident as ring-like structures, whereas internally it is characterized by the serial arrangement of coelomic compartments, separated by intersegmental septa, as well as the metameric organization of organs and system components (Shimizu and Nakamoto, 2001; Purschke, 2002). New segments form at the boundary between the posterior end of the segmented region and the terminal pygidium (Purschke, 2002; Gazave et al., 2013; Niwa et al., 2013). These segments are added sequentially through a process known as posterior elongation, which depends on the presence of a segment addition zone (De Rosa et al., 2005; Gazave et al., 2013).

Some polychaetes have the remarkable ability to regenerate segments indefinitely (Niwa et al., 2013; Gazave et al., 2013). This unique trait has drawn the attention of developmental biologists seeking to understand the mechanisms underlying its seemingly limitless regenerative capacity. Niwa et al. (2013) investigated the molecular mechanisms of tail regeneration in adult *Perinereis nuntia* and found that new segments form through the coordinated recruitment of independently proliferating undifferentiated cells from the segment/pygidium boundary. This process depends on inductive signals from the pre-existing segment adjacent to the newly forming segment. Similarly, Gazave et al. (2013) studied tail regeneration in *Platynereis dumerilii* and proposed that new segments arise through multiple rounds of division from small populations of teloblast-like posterior stem cells.

While the mechanisms of segment regeneration in annelids are well-documented, little is known about the regeneration of their reproductive organs. Gonadal regeneration in annelids has been reported in *Enchytraeus japonensis*, an oligochaete capable of both asexual and sexual reproduction (Tadokoro et al., 2006). This species can regenerate gonads from any of its body fragments produced through fission during asexual reproduction. Using the *piwi* homolog (*Ej-*

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piwi) as a marker, Tadokoro et al. (2006) examined the behavior of germ cell lineages during regeneration. Their findings revealed that *Ej-piwi*-expressing cells are widely distributed throughout the body as single cells. These specialized cells are believed to function as a reservoir of germ cell precursors during asexual propagation. They migrate to regenerating tissues, where they ultimately settle into the prospective gonads and differentiate into germ cells during sexual differentiation.

Recently, a study on *Perinereis wilsoni* revealed the presence of germ cells in the tail-end segment (pygidium) during early juvenile stages. These germ cells were believed to contribute to the germ cell population of newly forming segments (Maceren-Pates et al., 2015). However, no information is available on the regenerative capacity of these germ cells in the pygidium. To investigate this, a tail amputation experiment was conducted. Therefore, this study aims to describe the regeneration of germ cells in the pygidium and parapodia of P. wilsoni following surgical removal of the posterior segments. *In situ* hybridization using *vasa* as a germline marker revealed vasa-positive cells scattered around the amputated tail region three days post-amputation. Subsequently, pygidial cells regenerated in the pygidium, followed by the reappearance of germ cells in the distal areas of the newly formed segments. Here, we present preliminary observations on the expression, localization, and potential role of *Pw-vasa*-positive cells scattered around the amputated area during germ cell regeneration.

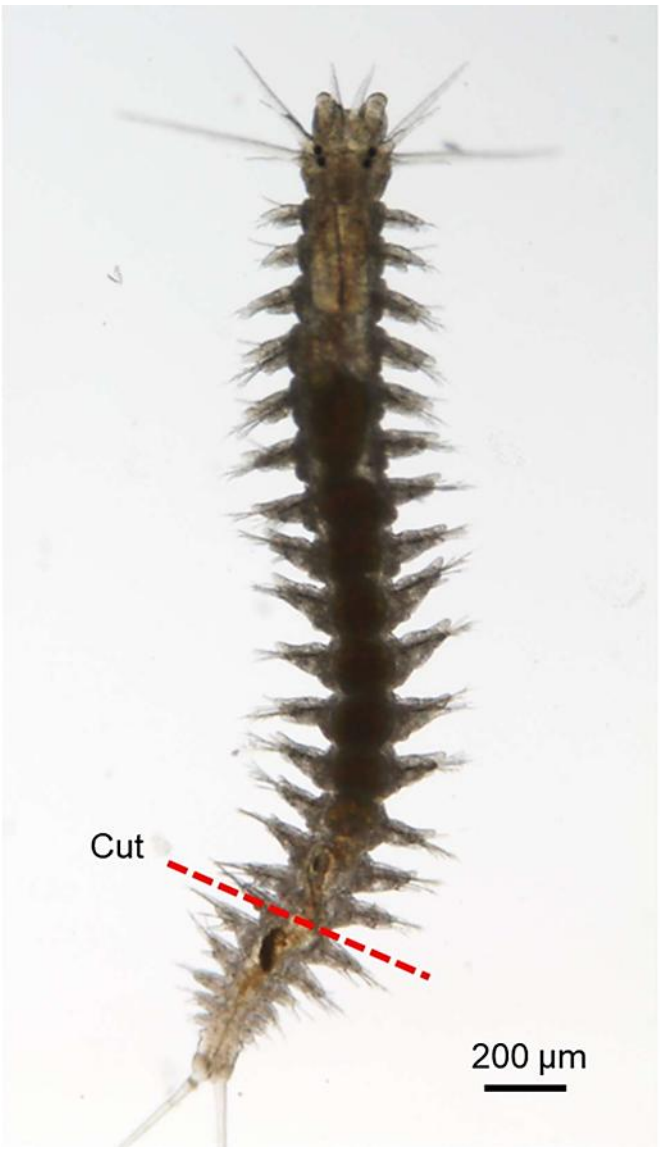

Figure 1. Surgical amputation site in a juvenile (20-30 segments) *Perinereis wilsoni*.

Materials and Methods

Mature polychaete worms, *P. wilsoni*, were obtained from a commercial hatchery in Oita Prefecture, Japan, during their spawning season (April), and maintained at the Kyushu University Fishery Research Laboratory, following the hatchery's culture methods. The animals were stocked in a culture tank with a sand bed and free-flowing seawater at a temperature of 25-27°C.

At the onset of spawning, male and female epitokes that left their burrows and swam in the water column were individually collected and placed into small plastic containers. The spawned sperm and eggs were carefully mixed and placed into 50 x 40 x 30 cm tanks with a thin sand bed and weakly flowing seawater. The embryos were grown until the early juvenile stage (20-30 segments) and then used for the experiment. Species identification was based on the recent taxonomic descriptions by Glasby and Hsieh (2006) and Tosuji et al. (2019).

cDNA cloning of *Pn-vasa* gene, phylogenetic analysis, and *in situ* hybridization: The *Pw-vasa* gene used in this study was previously cloned in our earlier research. The procedures for cDNA cloning,

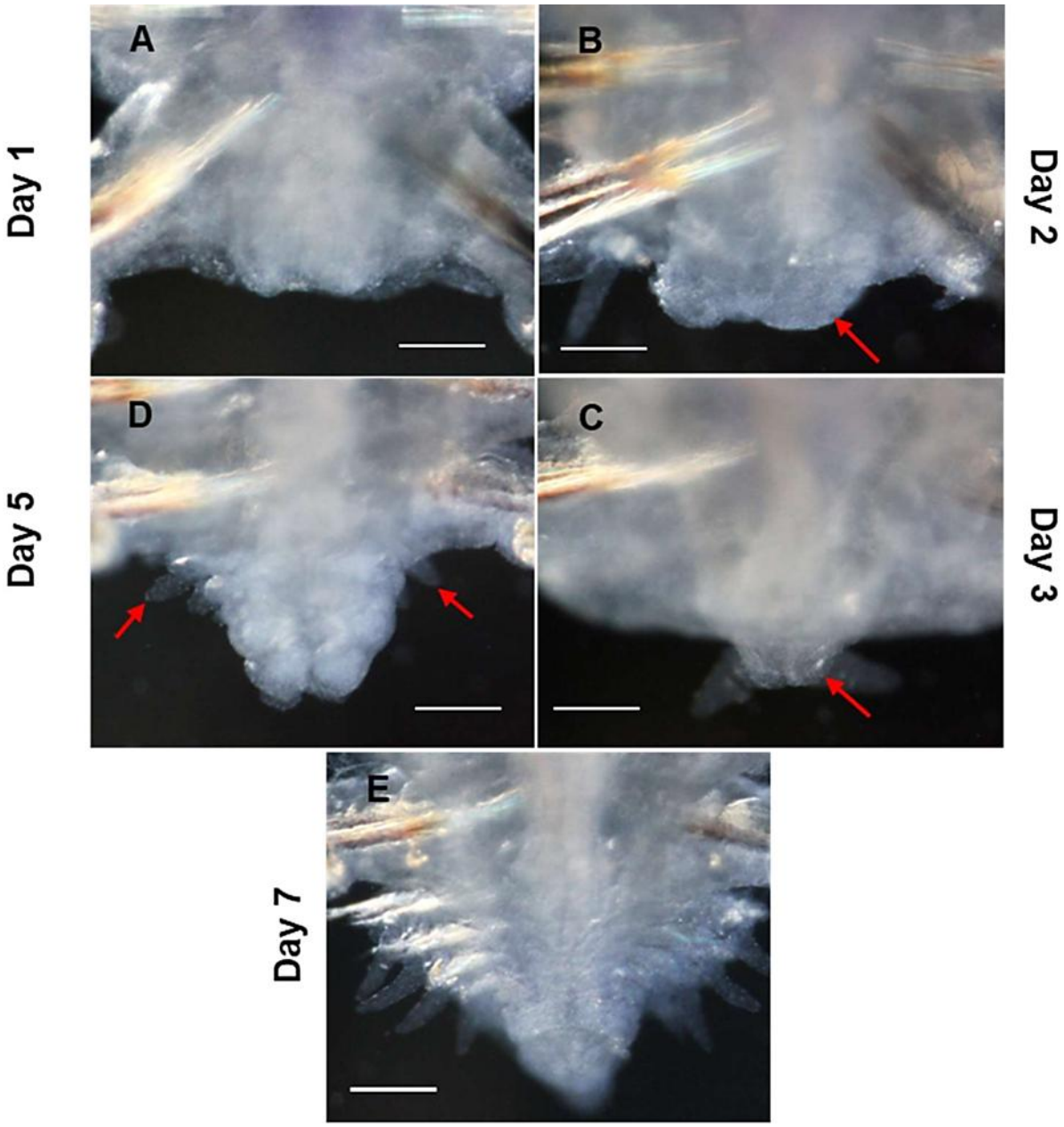

Figure 2. Morphological changes during posterior regeneration in juvenile *Perinereis wilsoni* (20-30 segments). (A) Day 1 post-surgical amputation (psa), showing a fully healed wound, (B) Day 2 psa, showing the formation of a regenerating blastema, (C) Day 3 psa, showing the development of a new pygidium, (D) Day 5 psa, showing newly regenerated segments (arrows), and (E) Day 7 psa, showing continued growth and segment regeneration (Scale bars = 100 µm).

phylogenetic analysis, and in situ hybridization are detailed in Maceren-Pates et al. (2015). Additionally, the animals used in this study are *P. wilsoni*, as identified by Tosuji et al. (2019); however, in the earlier study by Maceren-Pates et al. (2015), they were referred to as *P. nuntia*.

Surgical operation of the parapodia: Approximately 350 early juvenile marine worms (20–30 segments) were anesthetized in 0.3% ethylene glycol monohexyl ether in seawater and surgically amputated at the posterior region using a dissecting blade (Fig. 1). The amputees were then transferred to a separate culture tank. To monitor wound regeneration, 30 individuals were sampled daily. Samples were fixed overnight at 4°C in 4% formaldehyde in phosphate-buffered saline (PBS), dehydrated through a graded methanol series in PBS, and stored at -20°C until further use.

Thirty surgically amputated specimens were collected daily for *in situ* hybridization. Hole-mount in situ hybridization was performed according to the

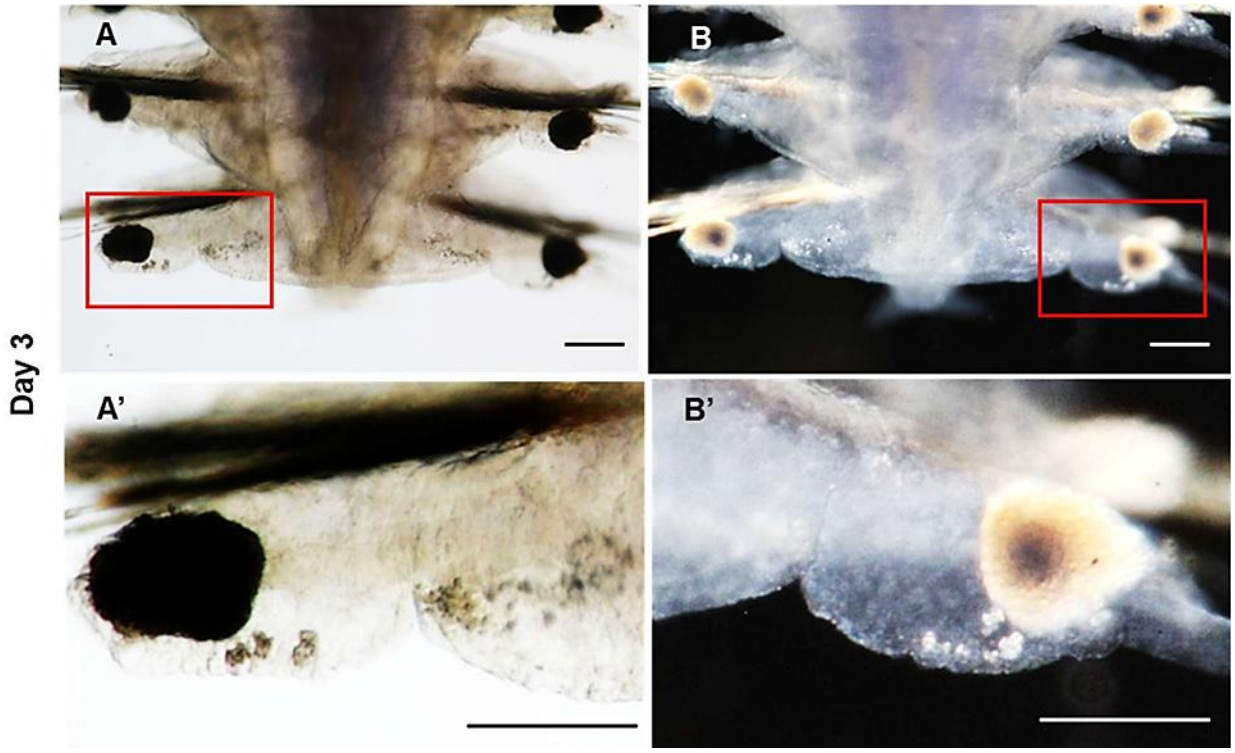

Figure 3. Expression of *Pw-vasa-positive* cells in the amputated area. (A) Hybridized worm showing Pw-vasa expression in the parapodia and in cells surrounding the amputated region, (A') Enlarged view of the boxed area in (A), highlighting smaller cells near the larger cell cluster in the parapodia, (B) Brightfield image corresponding to (A), and (B') Enlarged view of the boxed area in (B) (Scale bars = 100 µm).

protocol of Maceren-Pates et al. (2015). After hybridization, the specimens were analyzed and photographed under a microscope.

Results

***Pn-vasa* cDNA cloning and phylogenetic analysis:** A 619 bp fragment was amplified, cloned, and sequenced from the unfertilized egg of a mature worm and used as an antisense probe for the *in-situ* hybridization experiment. The deduced amino acid sequence exhibited 90% identity to the previously reported *P. dumerilii vasa* mRNA sequence (Rebscher et al., 2007) and over 60% identity to *vasa* homologs from other annelid species, including *Urechis unicinctus* (JQ665715.1), *Capitella teleta* (BK006523.1), *Enchytraeus japonensis* (AB306293.1), and *Tubifex tubifex* (AB257139.1). The molecular phylogenetic analysis was described by Maceren-Pates et al. (2015).

Time course and morphology of posterior regeneration: In *P. wilsoni*, germ cells in the pygidium are thought to contribute to newly formed segments. To investigate this, the pygidium and the last few segments were removed, and the regeneration process was analyzed by tracking the *vasa* signal. First, the number of regenerated segments post-amputation was determined. At day 1 post-amputation (psa), the wound had fully healed (Fig. 2A), and a small blastema had formed, continuing to elongate by day 2 psa (Fig. 2B, arrow). At day 3 psa, a new pygidium emerged from the blastema (Fig. 2C, arrow), and new segments became visible at day 5 psa (Fig. 2D, arrows). The rate of segment addition increased by day 7 psa (Fig. 2E) and continued through day 10 psa (Fig. 2F).

Expression of *Pw-vasa* positive cells in the regenerated pygidium: Whole-mount *in situ* hybridization was performed at daily intervals to identify the potential source of the repair process in the tail segment of *P. wilsoni*. By day 3 post-amputation (psa), *pw-vasa*-positive cells were detected around the amputation site in the tail-end segment (Fig. 3A, B). Additionally, these *pw-vasa*-positive cells were observed near the germ cell cluster in the distal region of the parapodia of the adjacent, unamputated segment (Fig. 3A', B'), along with strong

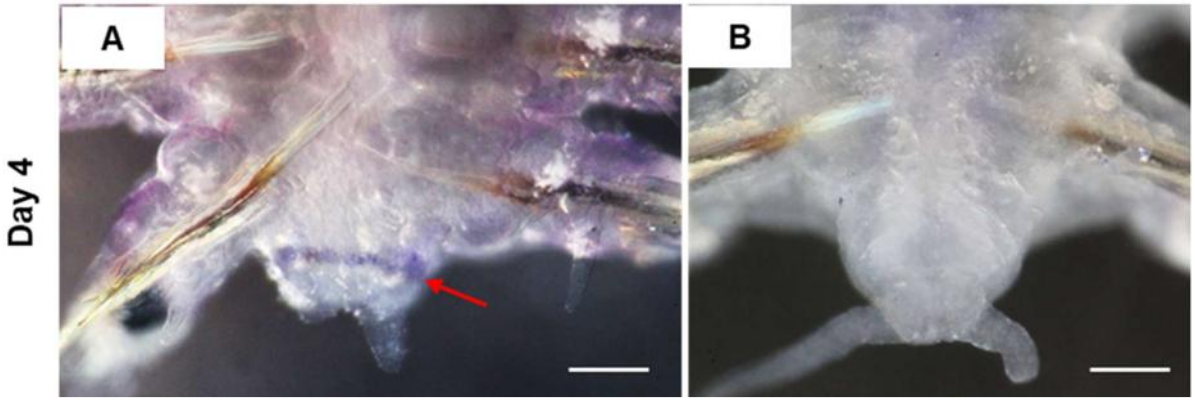

Figure 4. Expression of *Pw-vasa positive* cells in the amputated area. (A) Hybridized worm showing Pw-vasa expression in the segment addition zone, and (B) Sense control experiment at day 4 post-surgical amputation, serving as a negative control (Scale bars = 100 µm).

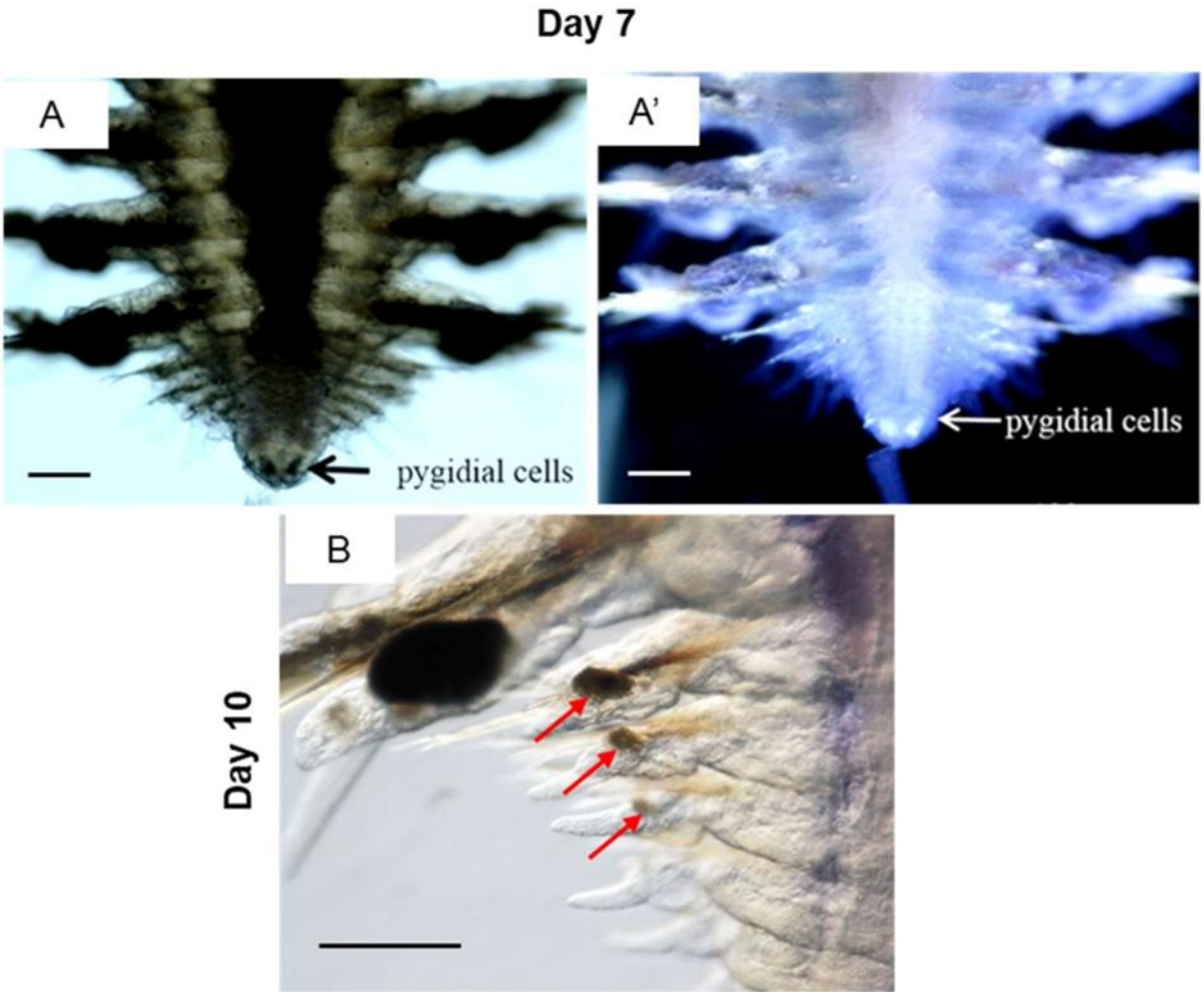

Figure 5. Germline regeneration in *Perinereis wilsoni*. (A) Hybridized worm showing regenerated germ cell clusters in the tail-end segment (pygidium), (A') Corresponding brightfield image of (A), and (B) Hybridized worm displaying Pw-vasa expression in the distal region of the parapodia within regenerated segments (arrows) (Scale bars = 100 µm).

expression in a larger cell cluster in the same distal parapodial region.

After the was pygidium regenerated, a ring-like pattern of *vasa* expression was observed at day 4 post-surgical amputation (psa) (Fig. 4A). In the sense control experiment, no such ring-like formation was detected in the pygidium (Fig. 4B). At day 7 psa, *Pw-vasa* was detected in the groups of cells in the newly regenerated pygidium (Fig. 5A, A', arrow). At day 10, psa Pw-vasa were also found to be expressed in the

distal area of the newly grown segments and in the cell clusters in the pygidium (Fig. 5B, arrows).

Discussions

In the previous study on the fully-segmented polychaete worm, *P. wilsoni* (formerly known as *Perinereis nuntia*), a mechanism of germ cell development was described (Mercedes-Pates et al., 2015), wherein germ cells specified by in situ hybridization with a vasa probe are found in the most tail-end segment (pygidium) during the early larval stage. While this species grows by the addition of a segment at the anterior border of the pygidium, two germ cells from the pygidium migrate into the newly-generated segment during its formation and settle in the parapodium on both sides of the segment. As the animals grow, germ cells in the parapodium proliferate and then divide into several clusters. Then, each cluster migrates into the deeper area of the segment and starts vitellogenesis in the coelomic cavity. Based on these observations, a model of germ cell development in fully segmented polychaetes (Mercedes-Pates et al., 2015) was proposed. In this model, the tail-end segment (pygidium) serves as a source of germ cells, which proliferate and develop into oocytes in each segment during the animals' growth.

Polychaetes also exhibit strong regenerative capacity to replace lost body segments after injury. We conducted a tail amputation experiment on a juvenile (20-30 segments) *P. wilsoni* to determine whether the germ cells in the pygidium can regenerate. After surgical amputation of the last few segments, the animal regenerated a new tail-end segment with morphological features similar to a normal pygidium, followed by the regeneration of segments. Similar regeneration processes have been reported in other polychaete species, such as *Platynereis dumerilii* (Gazave et al., 2013; Planques et al., 2019) and *Perinereis nuntia* (Niwa et al., 2013).

In most reports, *vasa* and *piwi* expression has been observed in both germ cells and somatic stem cells across various species (Rebscher et al., 2007; Juliano et al., 2010; Rebscher et al., 2012; Gazave et al., 2013; Maceren-Pates et al., 2015). In *P. wilsoni*, *vasa* expression was detected in germ cell clusters in the parapodia and pygidium, as well as in somatic cells within the segment-addition zone (Maceren-Pates et al., 2015). Similarly, previous studies on polychaete regeneration have reported *vasa* expression in the tail region (Gazave et al., 2013; Kozin and Kostyuchenko, 2015; de Jong and Seaver, 2017). In this study, we used in situ hybridization with a *vasa* probe to determine whether germ cells were present in the regenerated segments. Interestingly, *vasa*-positive cells were observed in the regenerated pygidium and in the newly formed segments, closely resembling the distribution seen in normal, unamputated animals (Maceren-Pates et al., 2015). This raises the question: how are germ cells revived following amputation? Upon closer examination, we found several cells localized near the parapodia of unamputated segments and scattered throughout the posterior-most segment at 3 days post-amputation. In situ hybridization confirmed that these cells were *vasa*-positive.

Previous studies on annelid regeneration have also investigated the cellular origin of newly formed tissues and germ cells. For example, in *Enchytraeus japonensis*, Tadokoro et al. (2006) reported that *piwi*-positive cells appearing on the dorsal surface of the ventral nerve cord migrated to the amputation site and contributed to germ cell formation. Similarly, Pates et al. (2021) suggested that *piwi*-positive cells in the skin layer of *P. wilsoni* may participate in germ cell regeneration post-amputation.

Conclusion

Based on our findings, we hypothesize that *vasa*-positive cells scattered near the tail-end segment following amputation may contribute to the regeneration of germ cells in the pygidium. These cells are likely derived from the parapodia of the nearest intact segment. However, the precise mechanisms governing germ cell regeneration in the pygidium remain unclear, given the current evidence. Further research is needed to elucidate the origin, dynamics, and fate of these regenerating germ cells. Future studies should incorporate detailed cytological

and histological analyses and investigate the mechanisms by which the pygidium reestablishes its role as a germ cell source during segment regeneration.

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