Regeneration of the acorn worm pygochord with the implication for its convergent evolution with the notochord

Running title: Regeneration of the pygochord

Authors

Koji Yoshimura, Yoshiaki Morino and Hiroshi Wada*

Graduate School of Life and Environmental Sciences

University of Tsukuba

1-1-1 Tennodai, Tsukuba 305-8572 JAPAN

*Author for correspondence: H. Wada

hwada@biol.tsukuba.ac.jp

tel: +81-29-853-4671

Abstract

The origin of the notochord is a central issue in chordate evolution. This study examined the development of the acorn worm pygochord, a putative homologue of the notochord. Because the pygochord differentiates only after metamorphosis, we followed the developmental process by inducing regeneration after artificial amputation in *Ptychodera flava*. We found that although the regeneration of the posterior part of the body did not proceed via formation of an obvious regeneration bud, pygochord regeneration was observed within a few weeks, possibly via trans-differentiation of endoderm cells. We detected the expression of the fibrillary collagen gene (*Fcol*) and *elav* in the pygochord during regeneration. This indicates that pygochord cells are not part of gut epithelial cells, but that they differentiate as a distinct cell type. Our gene expression analyses do not provide supporting evidence for the homology between the pygochord and notochord, rather favoured the convergent evolution between them.

Keywords: pygochord, notochord, hemichordate, regeneration, *elav,* collagen

Introduction

The innovation of the notochord was essential for establishing the chordate body plan. The notochord functions as a physical support during the typical swimming mode of chordates and also serves as a signalling centre for patterning other body parts, such as the nervous system and paraxial mesoderm, which also characterise the chordate body plan. Because of these critical contributions to the establishment of the chordate body plan, the origin of the notochord has been studied in non-chordate invertebrates (Annona et al., 2015; Lauri et al., 2014; Miyamoto & Wada, 2013; Peterson et al, 1999).

The acorn worms belong to the phylum Hemichordata, which constitutes the sister group of chordates together with echinoderms (Castresana et al., 1998; Philippe et al., 2011). Compared with the highly specialised pentaradial form of most echinoderms, the acorn worm retained a longitudinal body plan, which likely reflects the condition of its deuterostome ancestors.

Consequently, early studies of notochord homologues focused on acorn worms. Bateson (1884, 1886) postulated that the stomochord, which is the anterior protrusion of the acorn worm endoderm tissue, was a putative homologue of the notochord. Histologically, the stomochord is similar to the notochord, with prominent vacuoles, and is supported by a well-developed extracellular sheath, the proboscis skeleton (Miyamoto & Wada, 2013). Recently, Miyamoto & Wada (2013) showed that *hedgehog* is expressed in the stomochord and this tissue might serve as a signalling centre for patterning the dorsal nerve cord along the dorsal–ventral (DV) axis. However, the homology between the stomochord and the notochord is doubtful because DV body axis

was proposed to be reversed between acorn worms and chordates (Ferguson, 1996; Lowe et al., 2006). BMP signalling has a conserved role in DV patterning of the body axis within Bilateria, although BMP signalling has doralizing activity in protostomes and ventralising activity in chordates (reviewed in Ferguson, 1996). The reversal of BMP activity in DV axis patterning seems to correlate with the organ system, i.e., the nervous system is ventral and the circulatory system dorsal to the digestive gut in protostomes, while the nervous system is dorsal and the circulatory system ventral to the digestive gut in chordates. This evidence supports inversion of the DV body axis between protostomes and chordates. BMP signalling in the acorn worm indicated that the inversion occurred in deuterostomes in the lineage leading to chordates after the divergence of hemichordates and echinoderms (Lowe et al., 2006). Therefore, the acorn worm stomochord, which is the dorsal protrusion of the digestive tube, is unlikely to be homologous to the chordate notochord, which differentiates from the dorsal wall of the archenteron. Miyamoto & Wada (2013) suggested that hedgehog signalling in the stomochord may have been co-opted during notochord evolution. Satoh et al. (2014) rather suggested homology between the stomochord and the endostyles of chordates.

Another putative homologue of the notochord that is more consistent with the DV inversion hypothesis is the pygochord, which is located on the ventral wall of the posterior gut. In addition, pygochord cells often contain vacuoles (Hyman, 1959; Willey, 1899). However, two issues undermine likelihood of homology between the pygochord and the notochord. First, the pygochord is not universally observed in acorn worm species; rather it has been found only in Ptychoderidae (Hyman, 1959; Willey, 1899).

Therefore, the pygochord was sometimes completely ignored and regarded just as extension of ventral mesentery or gut epithelium (Benito & Pardos, 1997). Second, the position of the pygochord relative to the blood vessel along DV axis is not consistent with the DV inversion hypothesis (Brunet et al., 2015). In the ventral part of the acorn worm, the gut, pygochord, blood vessel, and nerve cord are located in a dorsal to ventral direction, while in chordates the gut, blood vessel, notochord, and nerve cord are located in a ventral to dorsal direction. In the acorn worm, blood flows anteriorly in the vessels dorsal to the gut and posteriorly in the ventral vessels (Benito & Pardos, 1997). In chordates, the anterior flow occurs in vessels ventral to the gut and the posterior flow in the dorsal vessels (Ruppert, 1997). These patterns are consistent with the DV inversion hypothesis and suggest the homology of blood vessels. In that case, pygochord cannot be homologous with the notochord.

Recently, Lauri et al. (2014) proposed another candidate as the notochord homologue in annelids. The ventral longitudinal muscle of annelids differentiates from bilateral precursor cells that converge toward the midline. Because the precursor cells are specifically marked by transcription factors, whose homologues also mark notochord cells, such as *brachyury*, *foxA*, *foxD*, and *twist*, Lauri et al. (2014) proposed homology between the longitudinal ventral muscle, the axochord, and the chordate notochord. This hypothesis was criticised from a phylogenetic perspective because deuterostomes seem to lack structures similar to the axochord (Hejnol & Lowe, 2014). The axochord hypothesis assumes multiple loss of the axochord–notochord because the axochord–notochord is claimed to be present in the common ancestors of bilaterians. To

counter this criticism, (Brunet et al., 2015) reported axochord-like ventral longitudinal muscles in several invertebrates. However, gene expression profiles of these organs remain to be elucidated.

Although recent molecular developmental analyses have stimulated the debate on the origin of the notochord, its origin is still an open question (Annona et al., 2015). Among the candidates, we know very little about the acorn worm pygochord. This is partly because the pygochord cells differentiate during metamorphosis after weeks in the swimming larval stage. Consequently, differentiation of the pygochord is observable only after rearing swimming larvae for a long time. To overcome the difficulty obtaining metamorphosing larvae of Ptychoderidae acorn worms, we decided to analyze the development of the pygochord by inducing regeneration by amputating the posterior part of the trunk. Regeneration is astonishing in the acorn worm and can occur in nature after amputation or spontaneously (Miyamoto & Saito, 2010). Several authors have reported details of the regeneration process, although these studies mostly describe regeneration of the anterior portion (Humphreys et al., 2010; Luttrell et al., 2016; Miyamoto & Saito, 2010; Rychel & Swalla, 2008), partly because anterior regeneration is easily observed. After anterior amputation of the trunk, a regeneration bud is clearly observed, and this is followed by differentiation of the proboscis and collar region. In comparison, we did not observe an obvious regeneration bud during posterior-ward regeneration. We found that, under appropriate culture conditions, regeneration of the pygochord occurred within a few weeks. Using this regeneration process, we analysed the mechanisms involved in differentiation of the pygochord. Here we addressed two

issues. First, we asked whether the pygochord cells differentiate as distinct cell types expressing cell specific effector genes, or they just behave as extension of gut cells or others. Second, we seek for any molecular marker expression supporting the homology between the notochord and pygochord.

Materials and Methods

Induction of regeneration

Adult *Ptychodera flava* were collected on Bise Beach, Okinawa. Live specimens were kept in artificial sea water (Marine Art BR, Tomita Seiyaku) at 23°C, with sand and bubbling air. Adult specimens of 100-150 mm body length were used in this study.

To induce regeneration, the animals were cut with a scalpel at the level of the hepatic region. The anterior fragment of the cut body was kept in artificial sea water at 23°C with sand in the bottom of the dish. The animals were transferred to a dish without sand to clean their gut contents 2 days before fixation, because sand in the gut interferes with making transverse sections.

Histology

Specimens of either complete adults or regenerating parts of the body were fixed for 2 days in Bouin's fixative at 4°C, and stored in 80% ethanol at –30°C. The fixed specimens were dehydrated and embedded in Paraplast (Sigma-Aldrich, St. Louis, MO, USA). The specimens were sectioned at 7 μm, stained with Delafield's haematoxylin and eosin Y, and observed under a light microscope (Nikon Eclipse Ni-U).

Gene expression analysis

The nucleotide sequences of fibrillar collagen (*FColA*), myosin heavy chain (*MHC*), *elav*, *brachyury*, *foxa*, *soxd* and *soxe* were retrieved from databases of the transcriptome and genome sequences of *P. flava* (http://marinegenomics.oist.jp/acorn worm/viewer/infor?project_id=33; Simakov et al., 2015). Then, cDNA was isolated by PCR using RNA from the regenerating portion of the body and the primers listed in Supplementary Table 1. The alignment of Clade A fibrillar collagen genes from several deuterostome species were conducted by ClustalX (Larkin et al., 2007), and edited manually using SeaView (Gouy et al., 2009). Amino acids of C-terminal region were used for tree construction. The maximum likelihood tree was constructed using RAxML (version 8.2.0; Stamatakis 2014) with ModelGenerator (Keane et al., 2006) to estimate the appropriate evolutionary model.

Specimens used for *in situ* hybridisation were fixed in 4% paraformaldehyde with 0.1 M MOPS (pH 7.5) and 0.05 M NaCl for 2 days at 4°C. *In situ* hybridisation of frozen sectioned materials was done as described in Miyamoto and Wada (2013).

Results

Histology of the pygochord

From transverse sections of the trunk region of complete adult specimens of *P. flava*, we confirmed the original description of the pygochord as a cellular extrusion of the ventral wall of the gut (Willey, 1899), ventral to a blood vessel (Fig. 1). The cellular extrusion is restricted to a small area of the posterior part of the trunk. In most specimens, the anterior limit of the pygochord was approximately 10 mm from the posterior end of the fixed specimens and the posterior end was approximately 0.4 mm from the posterior end (Fig. 1C-E). In the more anterior part of the body, a single layer of columnar cells constitutes the intestine, and the ventral blood vessel is observed as a space surrounded by extracellular matrix (Fig. 1B, C).

Induction of posterior regeneration

To observe regeneration of the pygochord, we amputated the trunk of *P. flava* at the level of the hepatic region (Fig. 2). By doing so, we are certain that no pygochord was left in the amputated body (Fig. 3A, B). In contrast to the regeneration of the anterior portion in which an obvious regeneration bud was observed, we did not observe a regeneration bud with posterior regeneration. Furthermore, because the acorn worm body shrinks on physical stimulation, body length is not stable. Consequently, body length is a poor indicator of the regeneration process. It was difficult to distinguish

whether regeneration involved growth toward the posterior end. Instead, we observed that the hepatic processes became smaller at the posterior end (Fig. 2).

To determine whether the pygochord differentiates during posterior regeneration, we followed the time course of regeneration by making transverse sections. We did not observe the pygochord in specimens 3 or 7 days post amputation (dpa) (n = 0/3 for 3 dpa and 0/5 for 7 dpa: pygochord observed/specimens observed; Fig. 3C). In these specimens, a blood vessel was observed ventral to the gut wall (Fig. 3C). The earliest sign of pygochord regeneration was observed 14 dpa, as a cellular protrusion arising ventrally from the gut wall (Fig. 3D). These pygochord cells were distinguishable from gut cells because the pygochord cells do not form a single cell layer, but form a cell aggregate as a ventral protrusion from the gut wall. The pygochord was observed approximately 400 μm from the posterior end, which is comparable with the location of the adult pygochord. The distance from the posterior end seems to be maintained during regeneration, although pygochord differentiation continues for more than three weeks, and the longitudinal length of the regenerated pygochord became longer than 600 μm (Fig. 3F-H).

Pygochord gene expression profile

Because we observed the process of pygochord differentiation during regeneration, we examined the gene expression profile during pygochord regeneration. First, we examined expression of the fibrillar collagen gene (*Fcol*), because it is a conserved effector gene enabling the notochord to function as a hydrostatic skeleton (Wada et al.

2006). Molecular Phylogenetic analysis indicated that the *P. flava Fcol* form a monophyletic clade with the clade A fibrillar collagen genes including that for notochord sheath (*col2a1*; Wada et al., 2006). The expression of *Fcol* was detected in pygochord cells, but not in gut epithelium (Fig. 4A), indicating that pygochord cells possess a cellular identity distinct from gut epithelium. Weak *Fcol* expression was observed in muscle cells, which specifically expressed *MHC* (Fig. 4A, B), that are not expressed in the pygochord.

We examined several genes whose homologues have critical functions in chordate notochord development, *Brachyury*, *foxa*, *soxd*, and *soxe*. But we could not detect reproducible signal by *in situ* hybridization to sectioned specimens. In comparison, we detected strong *elav* expression in pygochord cells (Fig. 4C). *Elav* is also expressed in cells in the ventral nerve cord and in some cells in the gut epithelium (Fig. 4C). Interestingly, this endoderm expression was detected in regenerating buds in which pygochord cells are not observed histologically (Fig. 4D).

Discussion

Histology of the pygochord

After the original description by Willey (1899), there have been few reports of the acorn worm pygochord. In this study, we observed essentially the same features in the pygochord of *P. flava*. The pygochord was observed as a cell mass of a ventral protrusion from the ventral midline of the gut in the trunk region. No pygochord was observed in the anterior part of the trunk; it was restricted to the posterior end and extended approximately 10 mm.

Pygochord cells are relatively rich in vacuoles and express a fibrillary collagen gene (Fig. 1C, 4A). These cellular features of the pygochord are similar to those of the chordate notochord, and supporting the idea that the pygochord function as a skeleton support for the tail (Willey, 1899).

The expression of *elav* in the pygochord was surprising, because *elav* is specifically expressed in nerve cells in most animals. The *elav* gene encodes an RNA-binding protein, and in many taxa, *elav* is involved in the differentiation and maintenance of nerve cells (Simone & Keene, 2013). Indeed, nerve cells also expressed *elav* in the acorn worms (Nomaksteinsky et al., 2009; Fig. 4C, D). In some species, however, such as in starfish larvae, *elav* expression was reported in non-nerve cells (Yankura et al., 2013). We do not know the functional significance of *elav* in the pygochord. It is noteworthy that the specific expression of *elav* and *Fcol* indicate that pygochord cells possess specific identity as a cell type, and are not just a ventral protrusion of gut cells.

Regeneration of the pygochord

Details of anterior-ward regeneration have been reported for some species of acorn worm (Humphreys et al., 2010; Luttrell et al., 2016; Miyamoto & Saito, 2010; Rychel & Swalla, 2008). Regeneration commences as a protruding regeneration bud, which subsequently develops into the proboscis. The collar region appears at a slightly later stage, and regeneration of the more posterior region follows. In comparison, posterior regeneration has not been documented. We did not observe a regeneration bud. In addition, because the acorn worm body is quite elastic and expands and contracts easily, body length is not a good indicate of regeneration. One clear sign of regeneration is degradation of the hepatic sacs, which suggests that regeneration is driven by trans-differentiation. Therefore, we could not judge which part of the posterior end of the regenerating worm is actually the regenerating portion.

To evaluate whether the pygochord regenerates after amputation, we need to ensure that the pygochord cells observed in the posterior part of the regenerating worm are cells that appeared during regeneration, rather than cells present before amputation. Therefore, we decided to amputate worms at the level of hepatic region, because the pygochord does not exist anterior to this point. We found that pygochord regeneration was reproducibly observed in worms at 14 dpa. The differentiation process of pygochord cells during regeneration is likely to be regulated by *elav*. Furthermore, the presence of *elav* expression in gut epithelial cells suggests that pygochord cells differentiate via the de-differentiation of gut cells.

Evolutionary origin of the notochord

By amputating the acorn worm body, we succeeded in observing differentiation of the pygochord. We examined gene expression profiles during this differentiation process to test whether the acorn worm pygochord is a homologue of the chordate notochord. Although *Fcol* expression was observed in both cell types, we did not detect the expression of developmental genes involved in chordate notochord development, such as *brachyury*, *soxd*, *soxe*, or *foxa*. However, we cannot exclude the possibnility that the differentiation of the pygochord during regeneration may proceed in a different manner from that during ontogenic process. Indeed the anterior regeneration process does not follow exactly the ontogenic process (Luttrell et al., 2016). Difference between the regeneration process and the ontogenic process was also described in other systems (e.g., Burton and Finnerty, 2009). Therefore, the homology between the pygochord and notochord need to be tested by further studies on the ontogenic process, which is not impossible but quite laborious (Miyamoto and Wada, 2013). Our study may rather suggest that the pygochord is a convergent structure providing physical support for the tail of Ptychoderidae acornworms by fibrillary collagen, and their differentiation is possibly under control of *elav*.

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Figure legends

Figure 1. Histology of the pygochord. (A) Dorsal view of *Ptychodera flava*. (B) Transverse section at the level shown by the broken line B in (A). (C) Magnified view of the ventral part shown by the square of (B). (D) Transverse section at the level of shown by the broken line D in (A). At this level, no pigochord observed, and ventral wall of the gut connected directlt to the ventral blood vessel. (E) Transverse section at the level of shown by the broken line E in (A). At this level, pygochord is observed, but much smaller than that at the level of B. bv: blood vessel, ge: gut epithelium, p: pygochord, vnc: ventral nerve cord. Scale bars in (A) = 10 mm, (B) = 1 mm, and (C-E) = 100 μm.

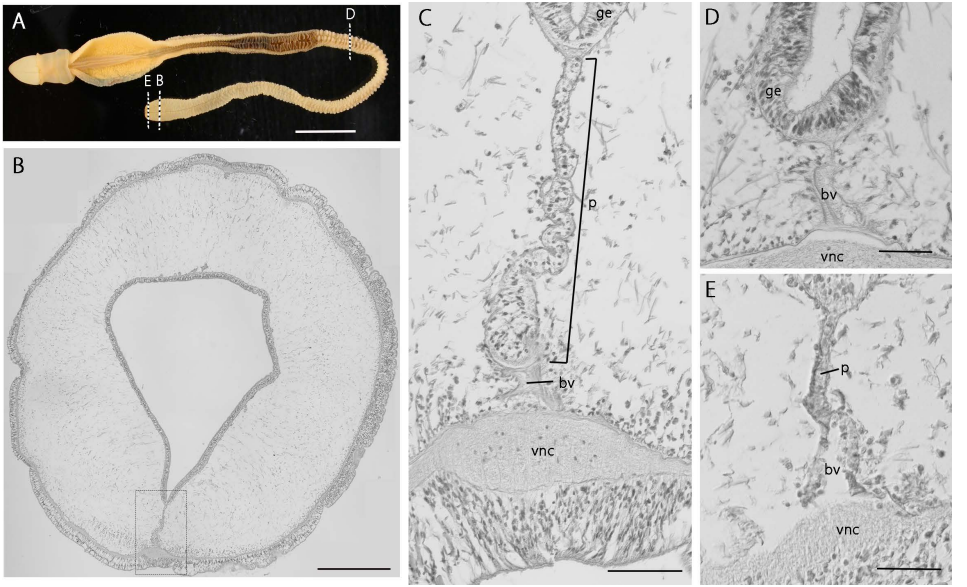
Figure 2. Time course of the posterior regeneration. Dorsal views (A, C, E, G. I) and lateral views (B, D, F, H, J) of the trunk region. (A, B) immediately after amputation. (C, D) 9 days post amputation (dpa). (E, F) 17 dpa. (G, H) 21 dpa. (I, J) 23 dpa. Some of the hepatic processes are marked by arrows.

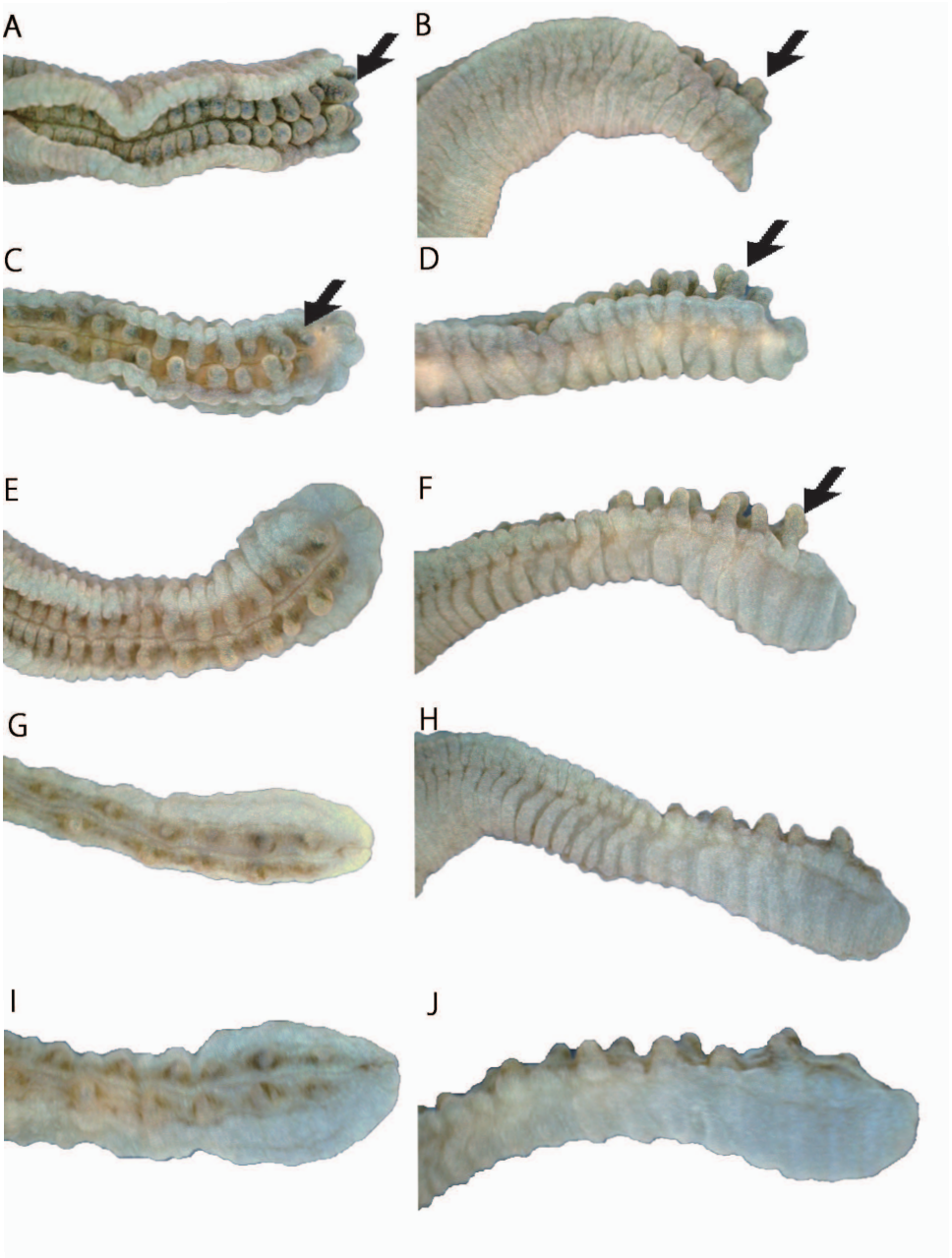
Figure 3. Regeneration of the pygochord. (A) dorsal view of the trunk immediately after amputation at the level of hepatic region. (B) Transverse section of the specimen just after amputation at the level close to where amputation was done. No pygochord observed at this level in 0 dpa. This figure was reconstructed from two images. (C) Transverse section of 7 dpa specimen. (D) At 14 dpa, pygochord was observed as ventral protrusion from

the single cell layer of gut wall. (E) Pygochord cells are observed at 16 dpa. (F-G) Pygochord is more obvious at 21 dpa. Transverse sections at the level about 1,000 μm from the posterior end (F), about 600 μm from the posterior end (G), and about 400 μm from the posterior end (H). bv: blood vessel, ge: gut epithelium, p: pygochord, vnc: ventral nerve cord. Scale bars = 50 μm.

Figure 4. Gene expression pattern during pygochord regeneration. (A) Expression of *Fcol* in the specimen of 21 dpa at the level of about 650 μm from the posterior end. Expression is observed in pygochord cells and weakly in muscle cells. (B) *MHC* expression marks muscle cells but not pygochord cells in 21 dpa specimen at the level of about 950 μm from the posterior end. (C) Expression of *elav* is detected in pygochord cells and some cells in gut wall (arrows), as well as in ventral nerve cord cells in 21 dpa specimen at the level of about 950 μm from the posterior end. (D) At the level of about 2,000 μm from the posterior end (about 1,000 μm anteriorly from the anterior end of the pygochord), *elav* expression is observed in some cells in gut wall (arrows). Pygochord was surrounded by thin broken lines. Basal line of the gut epithelium was shown by thick broken lines. Figures A and D were reconstructed from two images. bv: blood vessel, ge: gut epithelium, p: pygochord, vnc: ventral nerve cord. Scale bars = 50 μm.

Fig. 1

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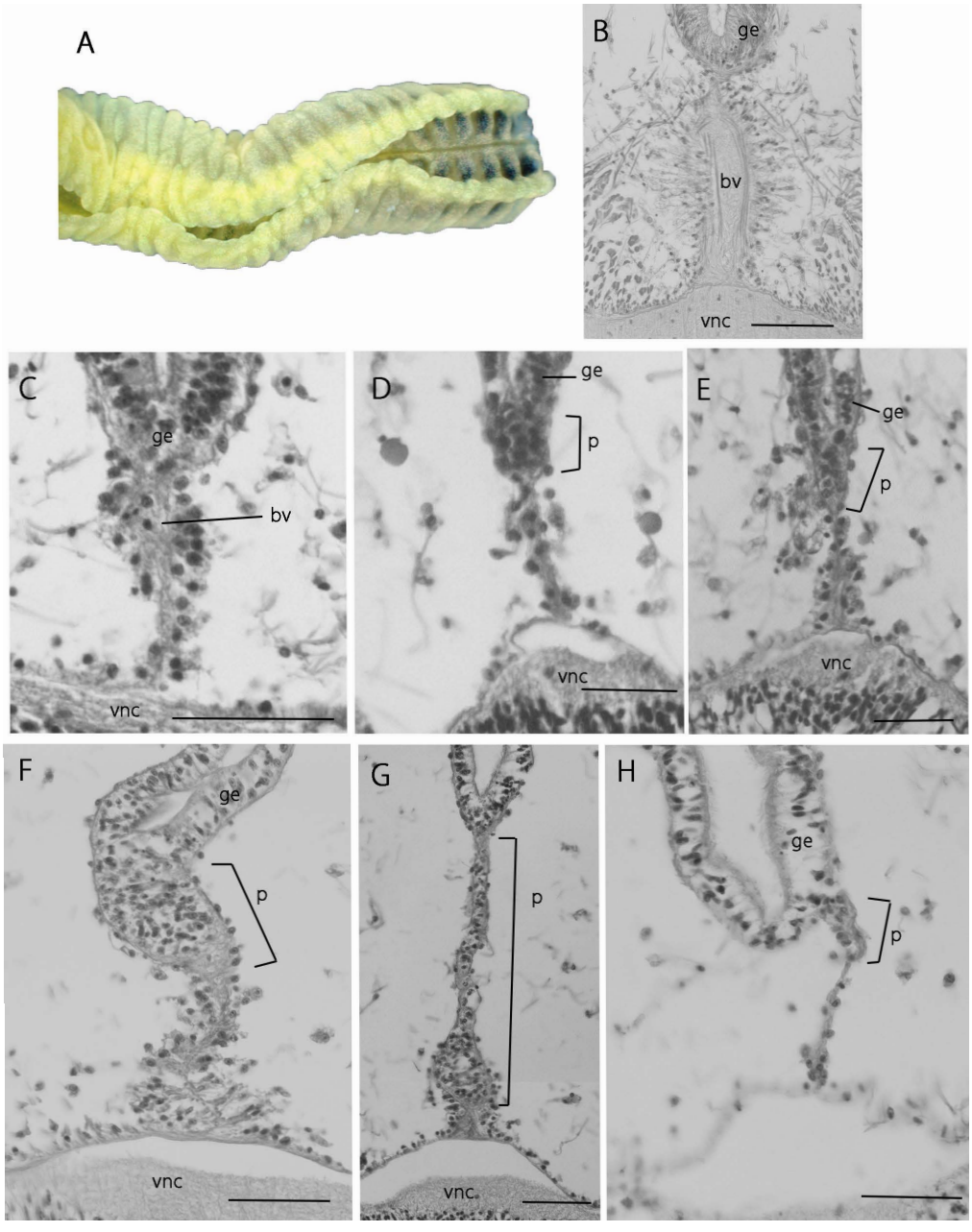
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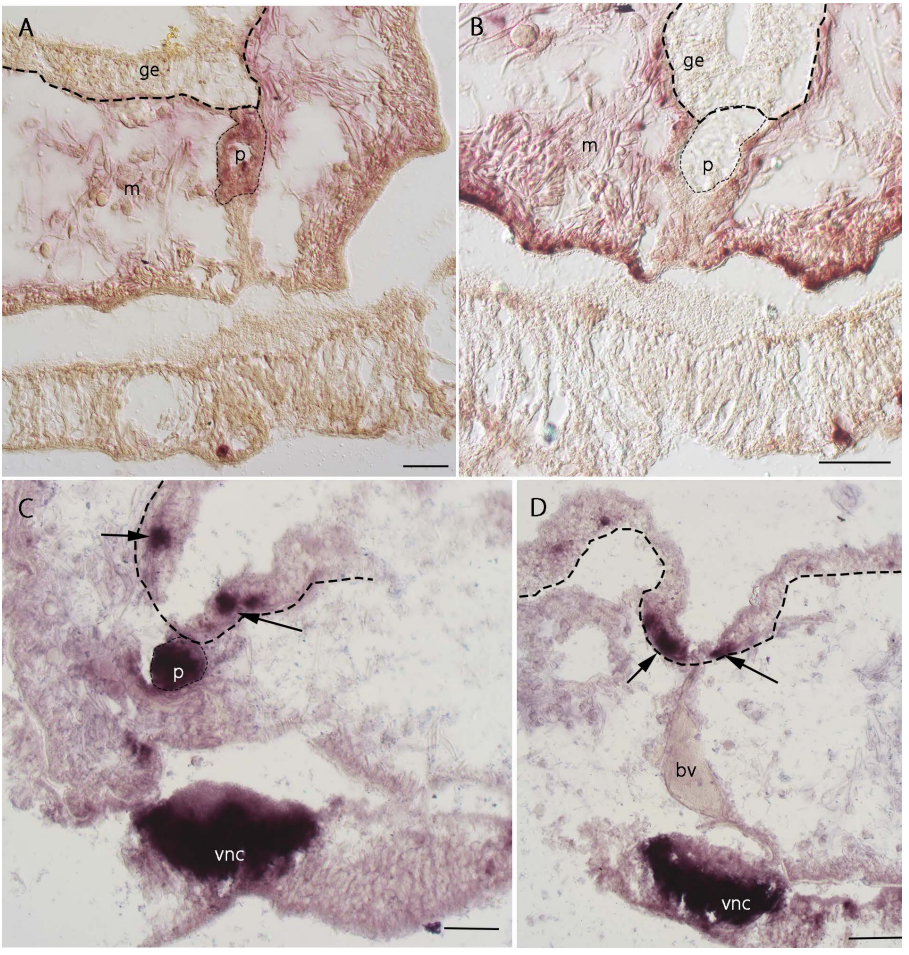
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D
bv
vnc