HOKKAIDO UNIVERSITY

Title	Stem cells in asexual reproduction of Enchytraeus japonensis (Oligochaeta, Annelid): Proliferation and migration of neoblasts
Author(s)	Sugio, Mutsumi; Yoshida-Noro, Chikako; Ozawa, Kaname; Tochinai, Shin
Citation	Development, Growth & Differentiation, 54(4), 439-450 https://doi.org/10.1111/j.1440-169X.2012.01328.x
Issue Date	2012-05
Doc URL	http://hdl.handle.net/2115/52713
Rights	The definitive version is available at wileyonlinelibrary.com
Type	article (author version)
File Information	DGD54-4_439-450.pdf

Instructions for use

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Develop. Growth Differ. (2012) 54, 439–450 doi: 10.1111/j.1440-169X.2012.01328.x

Original Article

Stem Cells in Asexual Reproduction of *Enchytraeus japonensis* (Oligochaeta, Annelid) : Proliferation and migration of neoblasts

Mutsumi Sugio[1], Chikako Yoshida-Noro[2, 3 4*], Kaname Ozawa[2] and Shin Tochinai[1]

1. Division of Biological Sciences, Faculty of Science, Hokkaido University, Sapporo 060-0810, Japan

2. Department of Applied Molecular Chemistry, College of Industrial Technology, Nihon University, Chiba 275-8575, Japan

3. Advanced Research Institute for the Sciences and Humanities, Nihon University, Tokyo 102-8251, Japan

4. Department of Medical Science, Division of Cell Regeneration and Transplantation, Nihon University School of Medicine, Tokyo 173-8610, Japan

* Corresponding author

Department of Applied Molecular Chemistry, College of Industrial Technology, Nihon University, 1-2-1 Izumi-cho, Narashino, Chiba 275-8575, Japan

Tel: +81-474-2584, Fax: +81-474-2584,

E-mail: noro.chikako@nihon-u.ac.jp

short running title: Stem Cells in Asexual Reproduction of *E. japonensis*

Authors' contributions:

The work presented here was carried out in collaboration between all authors. MS, CY-N and ST defined the research theme. MS and ST designed methods and experiments, carried out the BrdU labeling experiments, CY-N and KO designed and carried out Telomerase experiments. MS, CY-N and ST analyzed the data, interpreted the results and wrote the paper. All authors have contributed to, seen and approved the manuscript.

Abstract

Enchytraeus japonensis is a small oligochaete that reproduces mainly asexually by fragmentation (autotomy) and regeneration. As sexuall reproduction can also be induced, it is a good animal model for the study of both somatic and germline stem cells. To clarify the features of stem cells in regeneration, we investigated the proliferation and lineage of stem cells in *E. japonensis*. Neoblasts, which have the morphological characteristics of undifferentiated cells, were found to firmly adhere to the posterior surface of septa in each trunk segment. Also, smaller neoblast-like cells, which are designated as N-cells in this study, were located dorsal to the neoblasts on the septa. By conducting 5-bromo-2'-deoxyuridine (BrdU)-labeling–experiments, we have shown that neoblasts are slow-cycling (or quiescent) in intact growing worms, but proliferate rapidly in response to fragmentation. N-cells proliferate more actively than do neoblasts in intact worms. The results of pulse-chase experiments indicated that neoblast and N-cell lineage mesodermal cells that incorporated BrdU early in regeneration migrated toward the autotomized site to form the mesodermal region of the blastema, while the epidermal and intestinal cells also contributed to the blastema locally near the autotomized site. We have also shown that neoblasts have stem cell characteristics by

expressing *Ej-vlg2* and by the activity of telomerase during regeneration. Telomerase activity was high in the early stage of regeneration and correlated with the proliferation activity in the neoblast lineage of mesodermal stem cells. Taken together, our results indicate that neoblasts are mesodermal stem cells involved in the regeneration of *E. japonensis*.

Keywords: neoblast, proliferation, regeneration, stem cell, telomerase

Introduction

Enchytraeus japonensis is a terrestrial enchytraeid worm (Oligochaeta, Annelida) found in Japan (Nakamura 1993). *E. japonensis* has a full body length of 10–15 mm and diameter of approximately 0.2 mm, and its body color is white or almost transparent. The life cycle of this worm is unique because it can proliferate by asexual or sexual reproduction (Myohara *et al*. 1999; Yoshida-Noro & Tochinai 2010).

In regular mass culture, *E. japonensis* reproduces asexually by spontaneous autotomy (fragmentation) and regeneration. When the worm grows to full body length

(60–80 segments), it spontaneously autotomizes into 5–10 fragments, each consisting of about 10 segments. Following fragmentation, a head (7 segments that contain specific organs such as the mouth, pharynx, brain, ventral nerve cord, and sensory organ [prostomium], and septal glands) is regenerated from the anterior plane by 3–4 days, and a tail consisting of a pygidium and growth zone is regenerated from the posterior plane by 2–3 days. Regeneration is completed 5 days after fragmentation, and the worm then starts growing by the addition of new segments at the growth zone next to the pygidium. The worm becomes its full body length 10 days after the completion of regeneration. Thus, the entire asexual reproduction cycle in the worm requires approximately 2 weeks. On the other hand, sexual reproduction can be induced by low-density culture and the sexual reproduction cycle requires approximately 4 weeks (Myohara *et al*. 1999; Sugio *et al.*, 2008; Yoshida-Noro & Tochinai 2010).

E. japonensis has been adapted to laboratory use. The asexually reproduced worms are genetically identical clones, which is advantageous for molecular studies. Because autotomy can be artificially induced by electric shock, decapitation or amputation (Myohara *et al*. 1999; Inomata *et al*. 2000), simultaneous regeneration experiments are possible. Since asexual and sexual reproduction cycles of *E. japonensis*

can be controlled experimentally and progress in a relatively short period, this worm is an ideal animal model for studying stem cell systems during regeneration and germ cell formation (Myohara *et al*. 1999; Yoshida-Noro & Tochinai 2010).

The results of histological analysis suggested that the stem cells involved in regeneration are as follows: neoblast lineage for mesoderm, dedifferentiation of epidermis for ectoderm, and intestinal cells for endoderm (Myohara *et al*. 1999). The nervous system might also contribute to the formation of the new brain (Yoshida-Noro *et al*. 2000). Many species of oligochaete worms possess cells called neoblasts, which share common morphological characteristics with undifferentiated cells such as a high nucleocytoplasmic ratio, a large nucleus with a large nucleolus, and basophilic cytoplasm. Unlike neoblasts in planarians (Shibata *et al*. 2010), neoblasts in oligochaeta are not pluripotent somatic stem cells but contribute to form a new mesodermal region of the regenerating segments in some species (Randolph 1892; Krecker 1923; Turner 1934, 1935; Bilello & Potswald 1974). Neoblasts in *E. japonensis* have the morphological features of undifferentiated cells (Sugio *et al*. 2008), but their characteristics and precise roles in regeneration remain unclear.

To define the origin and lineage of germ cells and regenerative stem cells in *E. japonensis*, we previously isolated two *vasa*-related genes (*Ej-vlg1* and *Ej-vlg2*) and analyzed their expression, together with that of another germline marker gene (*Ej-piwi*; Tadokoro *et al*. 2006) by *in situ* hybridization (Sugio *et al*. 2008). *Vasa*-related genes are not only expressed in germline cells but also in pluripotent stem cells in hydras (Mochizuki *et al*. 2001; Bosch *et al*. 2010) and planarians (Shibata *et al*. 1999; Shibata *et al*. 2010; Hayashi *et al*. 2010). The result indicated that *Ej-vlg2* single positive (*Ej-vlg1*$^{-}$/*Ej-vlg2*$^{+}$/*Ej-piwi*$^{-}$) cells with morphological characteristics of neoblasts appear later than germline stem cells (*Ej-vlg1*$^{+}$/*Ej-vlg2*$^{+}$/*Ej-piwi*$^{+}$) in development, suggesting that neoblasts are somatic stem cells which develop independently of germ cells in *E. japonensis* (Sugio *et al*. 2008).

We also previously isolated a novel gene, *grimp*, which is expressed transiently from 3 to 12 h post-fragmentation in *E. japonensis*, mainly at the tip of the blastema in mesodermal cells located just underneath the epidermis (Takeo *et al*. 2010). By using *in situ* hybridization and 5-bromo-2'-deoxyuridine (BrdU)-labeling, we found that *grimp* is expressed only in the proliferating neoblasts and a population of mesodermal cells that have similar morphological features to neoblasts. Expression of *grimp* was never

observed in the epidermis, the muscle, or the digestive tract. As the suppression of *grimp* mRNA by RNA interference caused inhibition of cell proliferation in the mesoderm and differentiation of anterior structures, *grimp* appears to be a key molecule for the initiation of mesodermal regeneration in *E. japonensis* (Takeo *et al*. 2010).

Telomeres are repetitive DNA sequences located at the ends of chromosomes in most eukaryotes and provide chromosomal stability (Greider *et al*. 1985; Greider *et al*. 1996). The vertebrate telomere motif $(TTAGGG)_n$ is conserved in many animals including annelids (Traut *et al*. 2007). Telomerase is a reverse transcriptase involved in maintaining telomere length, which plays an important role in replicative life-span, and is known to present at high levels in germline and embryonic tissues (Thomson *et al*. 1998; Takahashi *et al*. 2007), and cancer cells (Kim *et al*. 1994). In most human somatic cells except for stem cells and lymphocytes, the activity of telomerase is diminished after birth and telomere length shortens with each cell division. The level of telomerase is low even in the majority of human somatic stem cells, whereas it is upregulated in cells that undergo rapid expansion (Hiyama & Hiyama, 2007). As the stem cells in *E. japonensis* show a remarkable replicative capacity, they supposed to have high telomerase activity.

In this paper, in order to clarify the features of stem cells in asexual reproduction of *E. japonensis*, we have investigated the proliferation and migration of neoblasts by BrdU pulse- and pulse-chase labeling experiments.. It is generally accepted that the characteristics of stem cells are slow-cycling when they stay in the niche and starting active self-renewal and differentiation by some stimuli (Moore *et al.*, 2006). We have shown here that neoblasts are slow-cycling in intact growing worms but starts to proliferate extensively in 12 h after autotomy. Neoblast progeny cells migrate toward the anterior and posterior stump and contribute to the mesodermal regeneration in the blastema. We have also shown stem cell characteristics of neoblasts by that they express stem cell marker gene *Ej-vlg2* and by the activity of telomerase during regeneration.

Materials and Methods

Animals and cultures

Oligochaeta worms, *E. japonensis*, were originally collected in the National Agricultural Research Center for Tohoku Region (Fukushima Prefecture, Japan) and were cultured asexually in our laboratory, as described previously (Kawamoto *et al.* 2005). Briefly, the worms were cultured in 0.6%–1.0% agar in 100- or 150-mm

diameter disposable Petri dishes at 24°C, and were fed once or twice a week with powdered rolled oats that had been sterilized briefly in a microwave oven. Under these conditions, asexual reproduction by fragmentation occurred every 2 weeks, and sexual reproduction was never observed.

Regeneration experiment

Body fragments for experiments were obtained either by autotomy, which was induced by application of an electrical stimulus (Yoshida-Noro *et al.*, 2000; Kawamoto *et al*. 2005), or by amputation with a disposable surgical blade. The anterior and posterior planes of the amputated fragments become identical to the automized ends because local autotomy occurs near the amputation site within 1h after amputation (Yoshida-Noro *et al.*, 2000). Fragments from the trunk region comprising about 10 contiguous segments with stumps at both anterior and posterior ends were collected and cultured on 0.8% agar plates (60 mm in diameter) at 24°C, and fixed at a specified time period after fragmentation.

Histological analysis and *in situ* hybridization

Intact worms and regenerating fragments were fixed in Bouin's fluid for 4 h, embedded in paraffin, and sliced into 5-mm–thick sections. The sections were stained with Delafield's hematoxylin and eosin. *In situ* hybridization of whole mounts and sections was performed according to the method of Sugio *et al*. (2008).

BrdU pulse-labeling and detection

Intact worms and regenerating fragments were labeled with BrdU (Sigma-Aldrich, St. Luis, MO, USA) by soaking them in 10 mM BrdU-containing deionized water. Specimens were fixed in Carnoy's solution for 15 min at room temperature, embedded in paraffin, and sectioned serially at 5-mm thickness. BrdU detection was performed according to the method of Yoshino & Tochinai (2004). For BrdU pulse-chase experiments, the worms were induced to undergo autotomy by application of an electrical stimulus, and then incubated on agar plates and allowed to regenerate for 9 h. The regenerating fragments were then exposed to BrdU solution for 12 h and washed repeatedly. Both anterior and posterior blastemas were then removed by amputation, and after various chase periods, the fragments were fixed for observation of labeled cells.

Telomerase assay

Fragments from the trunk region, each comprising 10 segments, were obtained by amputation and then cultured on 0.8% agar plates (60 mm in diameter) at 24°C. After an appropriate time period, the fragments were collected and frozen at −80°C. Telomerase activity was assayed by using *TeloTAGGG* Telomerase PCR ELISA (Roche Molecular Biochemicals, Manheim, Germany) according to the manufacturer's instructions, based on the Telomeric Repeat Amplification Protocol (TRAP) as described by Kim *et al*. (1994). Briefly, fragments were resuspended in lysis buffer and incubated for 30 min on ice. After centrifugation at 16,000 *g* for 20 min at 4°C, aliquots of the supernatant were rapidly frozen and stored at −80°C. The protein concentrations of extracts were determined with the Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc., Hercules, California, USA). We then performed photometric enzyme immunoassays for quantitative determination of telomerase activity by utilizing the kit above together with a SpectraMax Microplate reader as a detector (Molecular Devices, Inc., Sunnyvale, California, USA). Telomerase activity was shown as absorbance (A_{450nm}-A_{690nm}) values according to the kit protocol comparing with control cell extract in the kit as a positive

control (PC) and heat inactivated sample in lysis buffer as a negative control (NC).

Results

Neoblasts and N-cells

Neoblasts in *E. japonensis* exhibit their morphological characteristics that are a high nucleocytoplasmic ratio, a large nucleus with a large nucleolus, and basophilic cytoplasm (Sugio *et al.*, 2008). Neoblasts were found to adhere to the septa on each trunk segment (Fig. 1A, B). A pair of neoblasts was usually located laterally next to the ventral nerve cord (Fig. 1C). In addition, small cells possessing morphological features similar to those of large neoblasts were frequently observed dorsal to the neoblasts on the septa (the smaller neoblast-like cells in Sugio *et al.*, 2008) (Fig. 1C, D). For the sake of clarity, we hereafter define "the cells 1) existing on the septa dorsal to the neoblasts, 2) adhereing to the body wall, 3) having morphological features of neoblasts; i.e. a high nucleocytoplasmic ratio, a large nucleus with a large nucleolus, and basophilic cytoplasm, and 4) having smaller sizes than neoblasts" as "N-cells".

Spatial distribution of $BrdU^+$ cells in intact worms

E. japonensis grows continuously by adding new segments at the growth zone in the tail region. To identify the cells involved in growth and tissue renewal, we examined DNA-synthesizing (S-phase) cells in intact fully-grown worms (>8 mm in length) by continuously exposing the worms to BrdU for 6, 24, or 48 h.

In the head region of intact fully-grown worms, $BrdU^+$ cells were barely detected after 6 h incubation in BrdU solution (Fig. 2A), whereas many positive cells were observed in the intestine after 24- and 48-h incubation periods (Fig. 2B, C); most of the labeled cells in the head region were located in the pharynx and intestine (Fig. 2C). In the trunk region (Fig. 2D−F), $BrdU^+$ cells were observed in the intestine and the coelom after a 24-h incubation period. In the tail region, $BrdU^+$ cells were concentrated in the mesodermal and epidermal regions of the last segment (Fig. 2G–I). Extended incubation resulted in the distribution of $BrdU^+$ cells in the second last segment as well (Fig. 2I). We refer to this region as the posterior growth zone.

Incorporation of BrdU into neoblasts could not be detected even after 24- or 48-h incubation periods, which indicated the slow-cycling feature of neoblasts in the intact worms (Fig. 2E, F). We could not perform longer labeling experiments because the worms died if incubated in BrdU solution for longer than 48 h. In contrast to

neoblasts, some N-cells did incorporate BrdU after a 24-h incubation period (Fig. 2E). Although the number of septa associated with $BrdU^+$ N-cells was only 2 out of the 73 septa counted after a 6-h incubation period (worm number, n = 5), it increased up to as many as 56 out of the 97 septa after a 24-h incubation period (n = 6). Some septa had more than two $BrdU^+$ N-cells. These results suggest that that neoblasts were slow-cycling (or quiescent) and that the N-cells divided more actively than neoblasts in intact worms.

Spatial distribution of $BrdU^+$ cells in regenerating fragments

To investigate the contribution of stem cells to regeneration, fragments were incubated in BrdU solutions for 6 h at different stages after autotomy by application of an electrical stimulus (n >6 at each stage). In fragments that were continuously labeled with BrdU in the period 0–6 h after autotomy (Fig. 3A, E), only a few $BrdU^+$ cells were found in the intestine, coelom, and anterior end. In fragments incubated with BrdU in the period 6–12 h after autotomy (Fig. 3B, F), several $BrdU^+$ cells were found in the coelom around the anterior and posterior stumps, and some neoblasts and N-cells were BrdU-positive. In fragments incubated with BrdU in the period 12–18 h after autotomy

(Fig.3G), $BrdU^+$ cells were abundant in the coelom and in the epidermis around the anterior and posterior ends. In fragments labeled in the period 18–24 h (Fig.3H), $BrdU^+$ cells were observed throughout the anterior and posterior blastemas and in the intestine adjacent to the blastemas. Furthermore, most of the neoblasts and/or N-cells at the septa were BrdU-positive in the fragments labeled in the period 12-18h and 18-24h after autotomy (data not shown, see Fig. 4). In fragments incubated in the period 48–54 h and 90–96 h (Fig. 3C, D), most $BrdU^+$ cells were concentrated in the anterior blastema and in the growth zone regenerating from the posterior blastema. Some $BrdU^+$ cells were interspersed in the intestine of the trunk region. On the 5th day when regeneration was morphologically completed, the distribution pattern of $BrdU^+$ cells was similar to that in intact worms (data not shown).

$BrdU^+$ neoblasts and N-cells on the septa during regeneration

Because marked BrdU incorporation in neoblasts and N-cells on the septa was observed in the early stage of regeneration, we examined serial cross sections of regenerating fragments and counted the percentage of septa associated with BrdU-labeled neoblasts and/or N-cells at different stages of regeneration (n >3,

counted septa >12, at each stage). Positive septa with $BrdU^+$ neoblasts or $BrdU^+$ N-cells were hardly detectable for continuous incubation with BrdU in the 0–6 h period after autotomy (Fig. 4A, D). In fragments labeled in the period 6–12 h after autotomy, 40% of the septa had both $BrdU^+$ neoblasts and $BrdU^+$ N-cells, while the rest 40% had only $BrdU^+$ N-cells (Fig. 4D). In fragments labeled 12–18 h and 18–24 h after autotomy, more than 50% of septa had both $BrdU^+$ neoblasts and $BrdU^+$ N-cells (Fig. 4B, D). During the 24–30 h period, the number of septa with $BrdU^+$ neoblasts drastically decreased (Fig. 4C, D). By the 48–54 h period, no labeled neoblasts were observed, although septum with $BrdU^+$ N-cells were still detectable, albeit fewer in number than those present in the 24-30 h period (Fig. 4D). These results suggest that neoblasts and N-cells at the septa synthesize DNA most actively around 12–24 h after fragmentation. In addition, there was no correlation between the position of the septa in the fragment (distance from the position of autotomy) and the frequency of septa with $BrdU^+$ neoblasts and N-cells (data not shown).

BrdU pulse-chase experiment for tracing the migration of stem cells

To examine how neoblasts and N-cells contribute to blastema formation, we

conducted a pulse-chase tracing experiment using BrdU to label S-phase cells. Regenerating fragments were incubated in 10 mM BrdU-containing deionized water from 9 h to 21 h after fragmentation to maximize the BrdU incorporation into neoblasts and N-cells, and then the fragments were washed repeatedly with deionized water. Both the anterior and posterior blastemas of the fragments were removed by amputation to restart the regeneration process. The remaining fragments were then allowed to regenerate for appropriate time period (chase), fixed, and processed for immunohistological observation (n >4, at each stage) (Fig. 5A).

After a 1 h chase, BrdU-labeled cells were hardly observed around the anterior and posterior stumps though $BrdU^+$ neoblasts and N-cells at the septa were found (Fig. 5B). After a 6 h chase, some $BrdU^+$ cells adhering to the body wall in the coelom were found around the anterior and posterior ends (Fig. 5C). After a 12 h chase, $BrdU^+$ mesodermal cells filled the coelom adjacent to the anterior and posterior ends (Fig. 5D). After a 24 h chase, $BrdU^+$ cells were observed at a higher density in the anterior and posterior blastemas, although the BrdU signals in these cells had become granulated and weak. The signal intensity in the posterior region was stronger than that in anterior region (Fig. 5E). After a 48 h chase, the remaining BrdU signals were very finely

granulated and became broad and weak (Fig. 5F). These results indicate the migration and contribution of neoblast progeny to the mesodermal region in blastema.

Expression of a *vasa*-related gene in neoblasts and N-cells

To define the lineage of regenerative stem cells, marker genes expressed in stem cells are useful tools for investigation (Sugio *et al*. 2008; Shibata *et al*. 2010; Hayashi *et al*. 2010). A *vasa*-related gene, *Ej-vlg2* is known to be expressed in neoblasts in *E. japonensis*, smaller neoblast like cells located more dorsally on the septa adjacent to the body wall (i.e. N-cells), as well as growth zone cells, and a small number of cells in the intestine in intact worms (Sugio *et al*. 2008). All these cells are considered undifferentiated. Here, we confirmed that *Ej-vlg2* was expressed in neoblasts and N-cells on the septa in intact worms (Fig. 6A). In addition, we found that *Ej-vlg2* was expressed in the mesodermal cells adhering to the body wall in the coelom and the cells in both anterior and posterior tip of blastema in regenerating fragments (Fig. 6B). Thus, N-cells on the septa and mesodermal cells adhering to the body wall could be in the same lineage of cells derived from neoblasts. In contrast, it was not expressed to a detectable level in the cells floating in the coelom, which are probably coelomocyte or

haemocytes (Hartenstein 2006) (Fig. 6A, B). In anterior blastema within 48 h in regeneration (Fig. 6C), *Ej-vlg2* was expressed in the mesodermal and endodermal (intestinal) cells. Thus, we propose that in *E. japonensis* neoblasts are mesodermal stem cells and that N-cells are progeny of neoblasts, though some cells in the intestine might also show the stem cell features.

Telomerase activity during regeneration

To show the activity of stem cells in regeneration, we investigated the levels of telomerase activity during the regeneration process by using the Telomerase PCR ELISA kit (Roche). We found that telomerase activity was very low immediately after autotomy (0 h) and rose to 3.5 times the original level by 12 h after autotomy (Fig. 7A). By examining early time points with finer resolution, we showed that telomerase activity was low throughout the early stages of regeneration (0–6 h after fragmentation) and started increasing between 6 and 12 h after autotomy (Fig. 7B). The peak of the telomerase activity was at 72 h (day 3) and the level of activity decreased gradually until 120 h (day 5) (Fig. 7A). As we mentioned above, neoblasts started incorporating BrdU between 6 h and 12 h post-fragmentation and neoblasts and N-cells on the septa

synthesize DNA most actively around 12–24 h after fragmentation (Fig. 4D). Regeneration was finished by 72–96 h (3–4 days) and proliferation of stem cells occurred only in the posterior growth zone after 120 h (5 days). Taken together, these results suggest that stem cells in *E. japonensis* have high levels of telomerase activity, and that there is a correlation between the upregulation of telomerase activity and proliferation of neoblast lineage stem cells.

Discussion

Growth and cell renewal in intact worms

In growing *E. japonensis*, new body segments are formed at the growth zone located near the posterior end (Myohara *et al*. 1999). Here we showed that extensive BrdU incorporation occurred in the posterior growth zone (Fig. 2G, H). After extended exposure to 48 h, $BrdU^+$ cells were present not only in the last segment but also in the second last segment (Fig. 2I), indicating that a new segment had been added by this time. Similar results have been reported by Honda *et al*. (2003).

Many BrdU-labeled cells were also observed in the pharynx, intestine, and coelom, indicating that tissue renewal and/or growth occurred in these regions (Fig. 2C,

F); however, it remains to be determined whether these DNA-synthesizing cells were differentiated cells retaining the capacity to proliferate or undifferentiated stem cells.

Induction of stem cell proliferation by autotomy

Here, we showed that slow-cycling (or quiescent) neoblasts could be induced to enter S-phase soon after autotomy. Such an ability to proliferate rapidly in response to certain stimuli is a known characteristic of stem cells (Miller *et al*. 1993). In planarian, the frequency of proliferating neoblasts increases in response to amputation or feeding (Baguñà 1976a, 1976b; Nimeth *et al*. 2007). In larval *Xenopus laevis*, after partial removal of the telencephalon, neural stem cells proliferate actively and participate in regeneration (Yoshino & Tochinai 2004). In mouse corneal epithelium, large numbers of slow-cycling stem cells can be induced to proliferate by wounding or by application of phorbol myristate acetate (Lehrer *et al*. 1998).

In the present study, within several hours after autotomy, neoblasts on most of the septa, regardless of their distance from the position of autotomy, started to proliferate and produce N-cells (Fig. 3, 4). We consider that the nervous system might be involved in signal transmission from the autotomized site to the quiescent neoblast in

the niche at the ventral side. To examine the possible involvement of head or tail regions on this 'burst' of mitotic activity, we conducted similar BrdU-pulse experiments on fragments containing either head or tail regions. In the presence of head or tail regions, neoblast and/or N-cells were stimulated to incorporate BrdU to a similar level as that found in fragments without head or tail regions (data not shown).

Migration of the neoblast lineage cells to form blastema

In pulse-chase experiments, BrdU-labeling in the period from 9 h to 21 h after the initial autotomy produced a substantial number of $BrdU^+$ neoblasts and $BrdU^+$ N-cells. After the removal of both anterior and posterior blastemas, the remaining $BrdU^+$ cells in the fragments were mainly neoblasts, N-cells, mesodermal cells on the body wall in the coelom, and some intestinal cells. After chases for 6–24 h, BrdU-labeled cells were found in newly formed blastemas (Fig. 5C–F). Neoblasts in oligochaetes have been reported to possess migratory ability (Bilello & Potswald 1974). By using regional x-irradiation, O'Brien (1942) demonstrated that posterior neoblast migration occurs in the oligochaeta worm *Nais paraguayensis* to supply neoblasts to the irradiated region. Thus, we propose that the progeny of neoblasts and N-cells, and/or the

mesodermal cells adhering to the body wall in the coelom migrated to form the mesodermal part of the blastemas.

Epidermis and intestinal cells also contributed to regeneration

While BrdU incorporation in the epidermis of intact worms was observed only in the growth zone (Fig. 2I), $BrdU^+$ cells were found in the epidermis around the anterior and posterior end of the regenerating fragments (Fig. 3G) when BrdU labeling was performed 12–18 h or 18-24h after autotomy. In pulse-chase experiments, no labeled cells were found in the epidermis at any stage (Fig. 5), showing that the neoblast and N-cell lineage did not contribute to the epidermis. These results imply that epidermal cells in the blastemas were derived from epidermis adjacent to the autotomized sites. Histological analysis of blastemas stained with hematoxylin and eosin suggests that epidermal cells of blastemas become enlarged and possess the morphological characteristics of undifferentiated cells, and that they redifferentiate not only into epidermal cells but also into brain and nerve cord cells (data not shown).

$BrdU^+$ cells were also detected in the intestinal epithelium adjacent to the autotomized sites (Fig. 3H) when labeling was performed 18–24 h after amputation. In

the worm *Enchytraeus bigeminus*, cells called entodermal neoblasts have been reported to be present in the intestine and to form the oesophagus during regeneration (Christensen 1964). Because no $BrdU^+$ intestinal cells were observed to migrate a long distance from the center of the fragments toward the blastema region in our experiments (data not shown), we propose that intestinal stem cells contribute to local regeneration or renewal of intestine in *E. japonensis*.

Stem cells and telomerase activity

Telomerase activity is known to correlate with the self-renewal potential of cells (Hiyama & Hiyama, 2007). Although telomerase activity in humans has been detected only in immortal, germ, and tumor cells, Elmore *et al*. (2008) reported that telomerase activity is present in most stem cells in many animals including fish and inbred mice. They also reported the upregulation of telomerase activity during the regeneration of fin tissue in Medaka and other fish, indicating the importance of maintaining telomere length and integrity during tissue regeneration.

The results of our experiments suggest that stem cells in *E. japonensis* have telomerase activity and that there is a correlation between the period of upregulation of

telomerase activity and the period of proliferation of stem cells during regeneration. Telomerase activity in the fragments increased from 12 h after autotomy and became approximately 3.5 times higher than that at 6 h after autotomy (Fig.7B). The activity continued to increase, and reached peak at 72 h, being approximately 1.3 times higher than that at 12 h (Fig.7A). Most of the neoblasts and/or N-cells on the septa incorporated BrdU at 12 h after autotomy though they were BrdU-negative at 0-6 h after autotomy (Fig.4D). The major BrdU-positive cells at 6-12 h after autotomy were neoblasts, N-cells and the mesodermal cells in the coelom around the anterior and posterior stumps (Fig.3B, F). The results of pulse-chase experiment suggested that these mesodermal cells derived from the neoblast lineage (Fig.5). The mesodermal cells intensively proliferated to form blastema after 12 h, and the number of BrdU-positive cells had been dramatically increased, much more than 1.3 times than that at 12 h (Fig. 3C, G, H). Thus, although small number of the cells in the intestine and epidermis also proliferated (Fig.3G, H), we consider that the major population of cells that had high telomerase activity would be neoblast, N-cells and their progeny mesodermal lineage stem cells, though there might be some contribution of stem cells in the intestine and epidermis. To identify the cells that have telomerase activity, we need to introduce

immunohistochemical approaches.

Neoblasts and mesodermal stem cell lineage in *E. japonensis*

Here we showed that neoblasts are normally slow-cycling (or quiescent), but are induced to proliferate rapidly in response to autotomy stimulus. Neoblasts migrate to the automized ends and contribute to the mesodermal regions of blastems.

We demonstrated that N-cells divided more actively than neoblasts in intact worms, that they were induced to actively proliferate within 24 h after autotomy, and that they participated in blastema formation in a similar manner to neoblasts. We found that DNA synthesis in neoblasts occurred only between 6 h and 30 h after autotomy, and that N-cells continued to proliferate after neoblasts ceased to synthesize DNA. It is generally accepted that there exists an intermediate population of stem cells called transient amplifying (TA) cells that proliferate more rapidly than quiescent stem cells (Miller *et al*. 1993). Because N-cells express *Ej-vlg2* and possess similar morphological characteristics to neoblasts except for their smaller size, we propose that in *E. japonensis* neoblasts are mesodermal stem cells and that N-cells are progeny of neoblasts playing a role as TA cells. The cells expressing *Ej-vlg2* found attached to the

body wall in the coelom might be another TA population in the neoblast lineage, possibly equivalent to the N-cells. Taken together with the result of BrdU pulse-chase experiments, it is suggested that neoblast lineage stem cells migrated to the blastemas, proliferated and contributed to regeneration.

On the other hand, we previously showed that *grimp* is expressed only in neoblasts and flat mesodermal cells, both of which have the morphological features of neoblasts (i.e., large nuclei and dense nucleolus), and that *grimp* expression is never observed in the epidermis, the muscle, or the digestive tract (Takeo *et al*. 2010). The results of simultaneous *in situ* hybridization for *grimp* and BrdU immunohistochemistry indicated that *grimp* expression is associated with cell proliferation. Furthermore, suppression of *grimp* causes inhibition of cell proliferation in mesoderm and differentiation of anterior structures. These results suggest that *grimp*-expressing cells correspond to the neoblast lineage cells including N-cells and mesodermal cells on the body wall in the coelom. To confirm this, we need to perform double i*n situ* hybridization for *Ej-vlg2* and *grimp*.

In many coelomate invertebrates such as annelids, there is a population of cells called the mesothelium, which is derived from mesoderm and lines the body wall in the

coelom. Mesothelia also produce coelomocytes, or hemocytes which spread out into the coelom and blood vessels (Hartenstein, 2006). Thus there is a possibility that cells attached to the wall of the coelom, and the cells floating in the coelom, are of the same lineage as neoblasts. Because BrdU labeling becomes diluted with repeated cell divisions, we could not trace labeled cells for a longer time to confirm the fate of BrdU-labeled cell differentiation. In addition, *Ej-vlg2* was not expressed to a detectable level in coelommocytes or haemocytes. Thus, in order to study the precise fate of the neoblast cell lineage, we are attempting to develop more refined methods for tracing cell lineages, such as GFP-gene transfer.

Acknowledgements

This work was supported by KAKENHI 22570208 to C. Y-N. and the 21st Century Center of Excellence (COE) Program on the ‘Neo-Science of Natural History’ (Program Leader: Hisatake Okada) at Hokkaido University, financed by the Ministry of Education, Culture, Sports, Science and Technology, Japan to S. T.

References

Baguñà, J. 1976a. Mitosis in the intact and regenerating planarian *Dugesia mediterranea* n.sp. I. Mitotic studies during growth, feeding and starvation. *J. Exp. Zool.* **195,** 53–64.

Baguñà, J. 1976b. Mitosis in the intact and regenerating planarian *Dugesia mediterranea* n.sp. II. Mitotic studies during regeneration, and a possible mechanism of blastema formation. *J. Exp. Zool.* **195,** 65–80.

Bilello, A. A. & Potswald, H. E. 1974. A cytological and quantitative study of neoblasts in the naid *Ophidonais serpentina* (Oligochaeta). *Dev. Genes Evol.* **174**, 234-249.

Bosch, T.C.G., Anton-Erxleben, F., Hemmrich, G. & Khalturin, K. 2010. The Hydra polyp: Nothing but an active stem cell community. *Dev. Growth, Differ.* **52**, 15-25.

Christensen, B. 1964. Regeneration of a new anterior end in *Enchytraeus bigeminus* (Enchytraeidae, Oligochaeta). *Vidensk. Meddel. Dansk Naturh. Foren. Kjobenh.* **127**, 259-273.

Elmore, L. W., Norris, M. W., Sircar, S., Bright, A. T., McChesney, P. A., Winn, R. N., & Holt, S. E. 2008. Upregulation of telomerase function during tissue

regeneration. *Exp Biol Med* (Maywood). 233, 958-67.

Greider, C. W. & Blackburn, E. H. 1985. Identification of a specific telomere transferase activity in Tetrahymena extracts. *Cell* 43 405-413.

Greider, C. W., Collins, K. & Autexier, C. 1996. Telomerases. In: ML DePamphilis, Editor, DNA Replication in Eukaryotic Cells, Cold Spring Harbor Laboratory Press, New York, pp. 619–638.

Hartenstein, V. 2006 Blood cells and blood cell development in the animal kingdom. Annu Rev Cell Dev Biol. 22, 677-712.

Hayashi, T., Shibata, N., Okumura, R., Kudome, T., Nishimura, O., Tarui, H. & Agata, K. 2010. Single-cell gene profiling of planarian stem cells using fluorescent activated cell sorting and its “index sorting” function for stem cell research. *Dev. Growth, Differ.* **52**, 131-141.

Hiyama, E. & Hiyama, K. 2007. Telomere and telomerase in stem cells. *British J. Cancer*. **96**, 1020-1024.

Honda, M., Suzuki, T., Matsumoto, S. & Gamou, S., 2003. Segment formation of *Enchytraeus japonensis* (Oligochaeta: Enchytraeidae). *Pedobiologia* **47**, 522-525.

Inomata, K., Kobari, F., Yoshida-Noro, C., Myohara, M. & Tochinai, S. 2000. Possible neural control of asexually reproductive fragmentation in Enchytraeus japonensis (Oligochaeta, Enchytraeidae). J. *Invert. Reprod. Dev*. **37**, 35–42.

Kawamoto, S., Yoshida-Noro, C. & Tochinai, S., 2005. Bipolar head regeneration induced by artificial amputation in Enchytraeus japonensis (Annelida, Oligochaeta). *J. Exp. Zool. A Comp. Exp. Biol*. **303**, 615–627.

Kim, N. W., Piatyszek, M. A., Prowse, K. R. Harley, C. B., West, M. D., Ho, P. L., Coviello, G. M., Wright, W. E., Weinrich, S. L. & Shay, J. W. 1994 Specific association of human telomerase activity with immortal cells and cancer. *Science* **266**: 2011-2015.

Krecker, F. H. 1923. Origin and activities of the neoblasts in the regeneration of microdrilous annelida. *J. Exp. Zool.* **37**, 26-46.

Lehrer, M. S., Sun, T-T. & Lavker, R. M. 1998. Strategies of epithelial repair: modulation of stem cell transit amplifying cell proliferation. *J. Cell. Sci*. **111**, 2867-2875.

Miller, S. J., Lavker, R. M. & Sun, T-T. 1993. Keratinocyte stem cells of cornea, skin

and hair follicle: common and distinguishing features. *Dev. Biol.* **4**, 217-240.

Mochizuki, K., Nishimiya-Fujisawa, C. & Fujisawa, T. 2001. Universal occurrence of the *vasa*-related genes among metazoans and their germline expression in *Hydra*. *Dev. Genes Evol.* **211**, 299-308.

Moore, K. A. & Lemischka, I. R. 2006. Stem cells and their niches. *Science* **311**, 1880-1885.

Myohara, M., Yoshida-Noro, C., Kobari, F. & Tochinai, S., 1999. Fragmenting oligochaete Enchytraeus japonensis: a new material for regeneration study. *Dev. Growth Differ*. **41**, 549–555.

Nakamura, Y. 1993. A new fragmenting enchytraeid species, *Enchytraeus japonensis* from a cropped Kuroboku soil in Fukusima, northern Japan (enchytraeids in Japan 5). *Edaphologia* **50**, 37–39.

Nimeth, K., T., Egger, B., Rieger, R., Salvenmoser, W., Peter, R. & Gschwentner, R. 2007. Regeneration in *Macrostomum lignano* (Platyhelminthes): cellular dynamics in the neoblast stem cell system. *Cell Tissue Res.* **327**, 637-646.

O'Brien, J., P. 1942. Studies on the effects of x-rays on regeneration in the fragmenting

oligochaete *Nais paraguayensis*. *Growth* **6**, 203-229.

Randolph, H., 1892. The regeneration of the tail in Lumbriculus. *J. Morphology*. **7**, 17–344.

Shibata, N., Meson, Y., Orin, H., Sakurai, T., Watanabe, K. & Agata, K. 1999. Expression of vasa(vas)-related genes in germline cells and totipotent somatic stem cells of planarians. *Dev. Biol*. **206**, 73–87.

Shibata, N., Rouhana, L. & Agata, K. 2010. Cellular and molecular dissection of pluripotent adult somatic stem cells in planarians. *Dev. Growth, Differ.* **52**, 27-41.

Sugio. M., Takeuchi, K., Kutsuna, J., Tadokoro, R., Takahashi, Y., Yoshida-Noro, C. & Tochinai, S. 2008. Exploration of embryonic origins of germline stem cells and neoblasts in *Enchytraeus japonensis*. *Gene Expr. Patterns.* **8**, 227-36.

Tadokoro, R., Sugio, M., Kutsuna, J., Tochinai, S. & Takahashi, Y. 2006. Early segregation of germ and somatic lineages during gonad regeneration in the annelid Enchytraeus japonensis. *Curr. Biol.* **16**, 1012–1017.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., & Yamanaka, S. 2007. Induction of pluripotent stem cells from adult human

fibroblasts by defined factors. *Cell* **131**, 861–872

Takeo, M., Yoshida-Noro, C. & Tochinai, S. 2010. Functional analysis of *grimp*: a novel gene required for mesodermal cell proliferation at an initial stage of regeneration in *Enchytraeus japonensis* (Enchytraeidae, Oligochaete) *Int. J. Dev. Biol.* **54**, 151-160.

Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S. & Jones, J. M. 1998. Embryonic stem cell lines derived from human blastocysts. *Science* **282**, 1145–1147.

Traut, W., Szczepanowski, M., Vítková, M., Opitz, C., Marec, F., & Zrzavý, J. 2007. The telomere repeat motif of basal Metazoa. *Chromosome Res.***15**, 371-82.

Turner, C. D. 1934. The effects of x-rays on posterior regeneration in *Lumbriculus inconstans*. *J. Exp. Zool.* **68**, 95-119.

Turner, C. D., 1935. The effects of x-rays on anterior regeneration in *Lumbriculus inconstans*. *J. Exp. Zool*. **71**, 53-81.

Yoshida-Noro, C., Myohara, M., Kobari, F. & Tochinai S. 2000. Nervous system dynamics during fragmentation and regeneration in *Enchytraeus japonensis*

(Oligochaeta, Annelid). *Dev. Genes Evol*. **210**, 311-19

Yoshida-Noro, C. & Tochinai, S. 2010. Stem Cell System in Asexual and Sexual Reproduction of *Enchytraeus japonensis* (Oligochaete, Annelida). *Dev. Growth, Differ.* **52**, 43-55.

Yoshino, J. & Tochinai, S. 2004. Successful reconstitution of the non-regenerating adult telencephalon by cell transplantation in *Xenopus laevis*. *Dev. Growth Differ*. **46**, 523-534.

Figure legends

Fig. 1. Neoblasts and N-cells. (A) Diagram of the major organs in an asexual worm. A sagittal section of a worm in the trunk region (B) and a cross section of a worm around the septum in the trunk region (C) stained with hematoxylin and eosin. (D) A high-magnification view of the area enclosed with the box in (C). A pair of large neoblasts is usually located next to the ventral nerve cord (black arrows). N-cells are present more dorsally on the septa adjacent to the body wall (blue arrows). Asterisk, ventral nerve cord. b, brain; b.w., body wall; ce, coelom; g.z., growth zone; int, intestine; ph, pharynx; v.n.c.,ventral nerve cord.

Fig. 2. Spatial distribution of $BrdU^+$ (S-phase) cells in intact worms. Worms were fixed after 6 h (A, D, G), 24 h (B, E, H), and 48 h continuous incubation (C, F, I) in BrdU solution. (A-C) In head region, $BrdU^+$ cells were found mainly in pharynx (ph) and intestine (int). (D-F) In trunk region, the number of $BrdU^+$ cells increased in intestine and coelom (ce), as BrdU incubation times were longer. In a worm incubated for 24 h (E), N-cells were labeled by BrdU (yellow arrowheads) though neoblasts were BrdU-negative (white arrows) even after 48h labeling (F). (G-I) In tail region, a worm

incubated for 6 h (G) and 24 h (H), the concentration of $BrdU^+$ cells were detected in the last segment (1). (I) In a worm incubated for 48 h, $BrdU^+$ cells were present in the last and second last segments (2). py, pygidium.

Fig. 3. Spatial distribution of $BrdU^+$ (S-phase) cells in regenerating fragments. Worms were induced to undergo autotomy by application of an electrical stimulus, incubated with BrdU solution during the last 6 h before fixation. The fragments were incubated with BrdU in the period 0-6 h (A) and 6-12 h (B) after autotomy. Only a few $BrdU^+$ cells were scattered in the intestine and the coelom in (A). $BrdU^+$ signals were detected in the coelom around the anterior and posterior stumps, as well as in neoblasts (white arrows) and N-cells (yellow arrowheads) in (B). In fragments incubated with BrdU in the period 48-54 h (C) and 90-96 h (D), $BrdU^+$ cells were concentrated in the anterior blastema and the regenerated growth zone in the tail region. The regions indicated by the line with two arrows were anterior blastemas. (E-H) $BrdU^+$ cells in the anterior (left panels) and posterior (right panels) end of the fragments during early regeneration stages. The fragment were incubated with BrdU in the period 0–6 h (E) and 6-12 h (F) after autotomy. $BrdU^+$ cells were found in coelom

around the anterior and posterior stumps in (F). In the fragment incubated in the period 12-18 h (G) and 18-24 h (H), $BrdU^+$ cells filled in the coelom and are present within the wound epidermis and intestine. g. z., growth zone.

Fig. 4. BrdU-positive neoblasts and N cells on the septa during regeneration. Cross section of regenerating fragments incubated with BrdU in the period 0-6 h (A), 12-18 h (B), and 24-30 h (C) after autotomy. showing BrdU-negative neoblasts (white arrowheads), BrdU-positive neoblast (white arrow), and BrdU-positive N-cells (yellow arrows). (D) Percentage of the septa that contain BrdU-positive (+) and negative (-) neoblast (Neo) and/or BrdU-positive (+) and negative (-) N-cells showed by counting on cross sections of worms ($n > 3$, counted septa >12, at each stage). Mitotic change started from 6-12 h after amputation.

Fig. 5. BrdU pulse-chase experiment for tracing the migration of stem cells. (A) Schematic outline of the BrdU pulse-chase experiment. Fragments obtained by electrical stimulus regenerated for 9 hr and then labeled by BrdU for 12 h and washed extensively. After removal of both anterior and posterior blastemas by amputation, the

fragments were allowed to regenerate for appropriate time period (chase) and fixed to observe labeled cells by immunohistochemistry. (B-F) $BrdU^+$ cells in the anterior (left panels) and posterior (right panels) ends of the fragments. (B) After a1 h chase, BrdU signals were observed mainly in neoblasts (arrows) and N-cells at the septa. Some mesodermal cells on the body wall in the coelom, and some intestinal cells were also BrdU-positive. (C) After a 6 h chase, BrdU-labeled cells adhering to the body wall in the coelom (arrowheads) were observed around the anterior and posterior ends. $BrdU^+$ cells filled the coelom adjacent to the anterior and posterior ends after a 12h chase (D), and observed in the anterior and posterior blastemas at a high density after a 24 h chase (E). After a 48 h chase (F), BrdU signals were very finely granulated and became broad and weak. No labeled cells were found in the epidermis at any stage vnc, ventral nerve cord; int, intestine

Fig. 6. Expression of *Ej-vlg2* in neoblasts and N-cells. (A) Cross section of a intact worm. (B) Transverse section of a fragment within 24 h in regeneration. (C) Anterior blastema within 48 h in regeneration. *Ej-vlg2* was expressed in neoblasts (black arrow), N-cells (red arrow) and the mesoderaml cells adhering to the body wall in the coelum

(black arrowheads). Cells in blastema (blue arrows) and some cells in the intestine (int) also expressed *Ej-vlg2*.

Fig. 7. Telomerase activity during regeneration. Telomerase activity was analysed by using *TeloTAGGG* Telomerase PCR ELISA and the results were shown as absorbance (A_{450nm}-A_{690nm}) values according to the kit protocol comparing with positive control cell extract (PC), and heat inactivated negative control (NC). (A) Telomerase activity was very low just after fragmentation (0 h) and rose up to 3 times higher at 12 h after fragmentation. The peak of the telomerase activity was at 72 h (day 3) and decreased gradually to 120h (day 5) (n=3 at each stage). (B) Precise time course study showed that telomerase activity was low in the early stage of regeneration (0-6 h after fragmentation) and increased at 12h (n=3 at each stage).

Fig.1

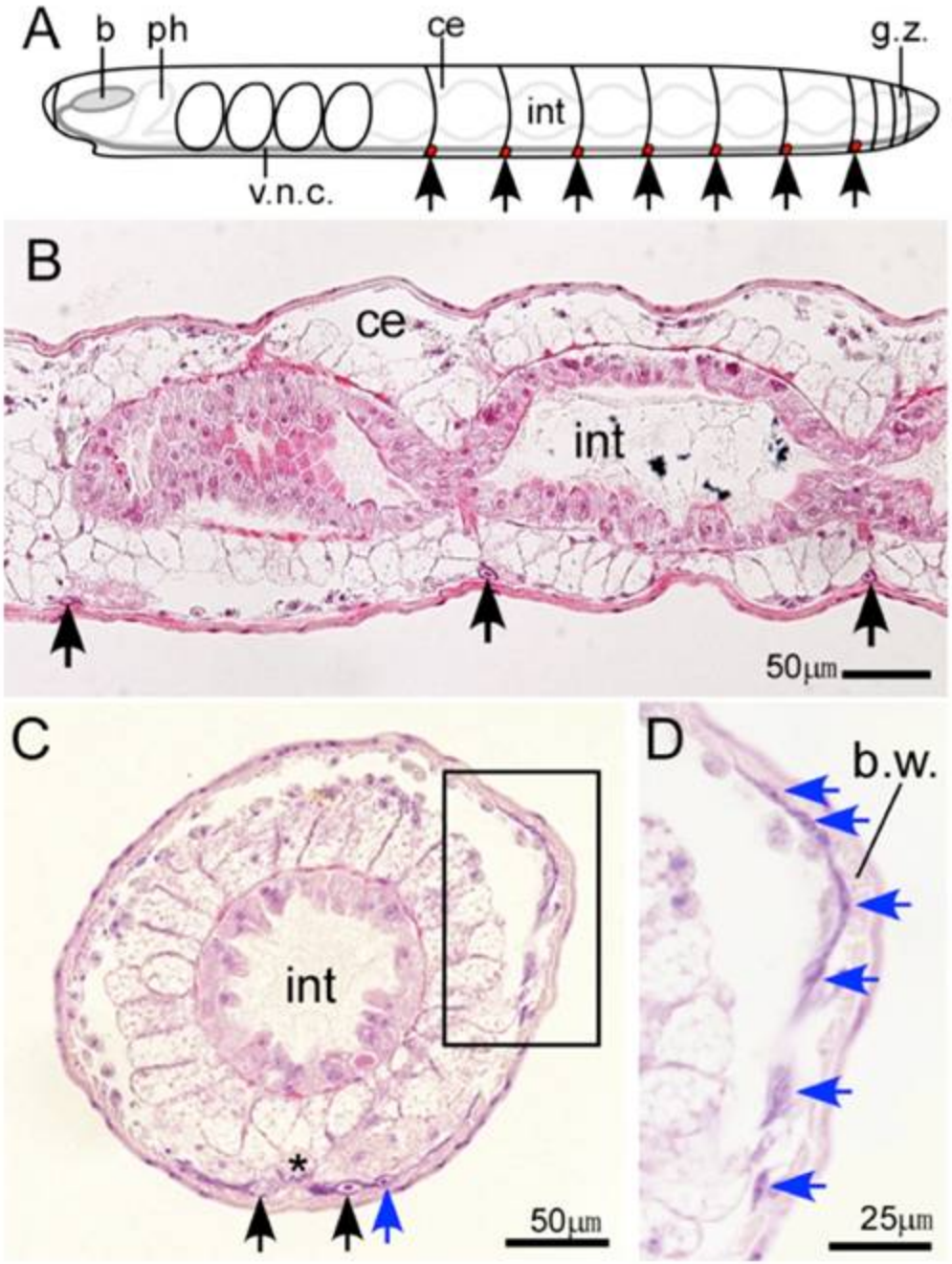


Fig.2

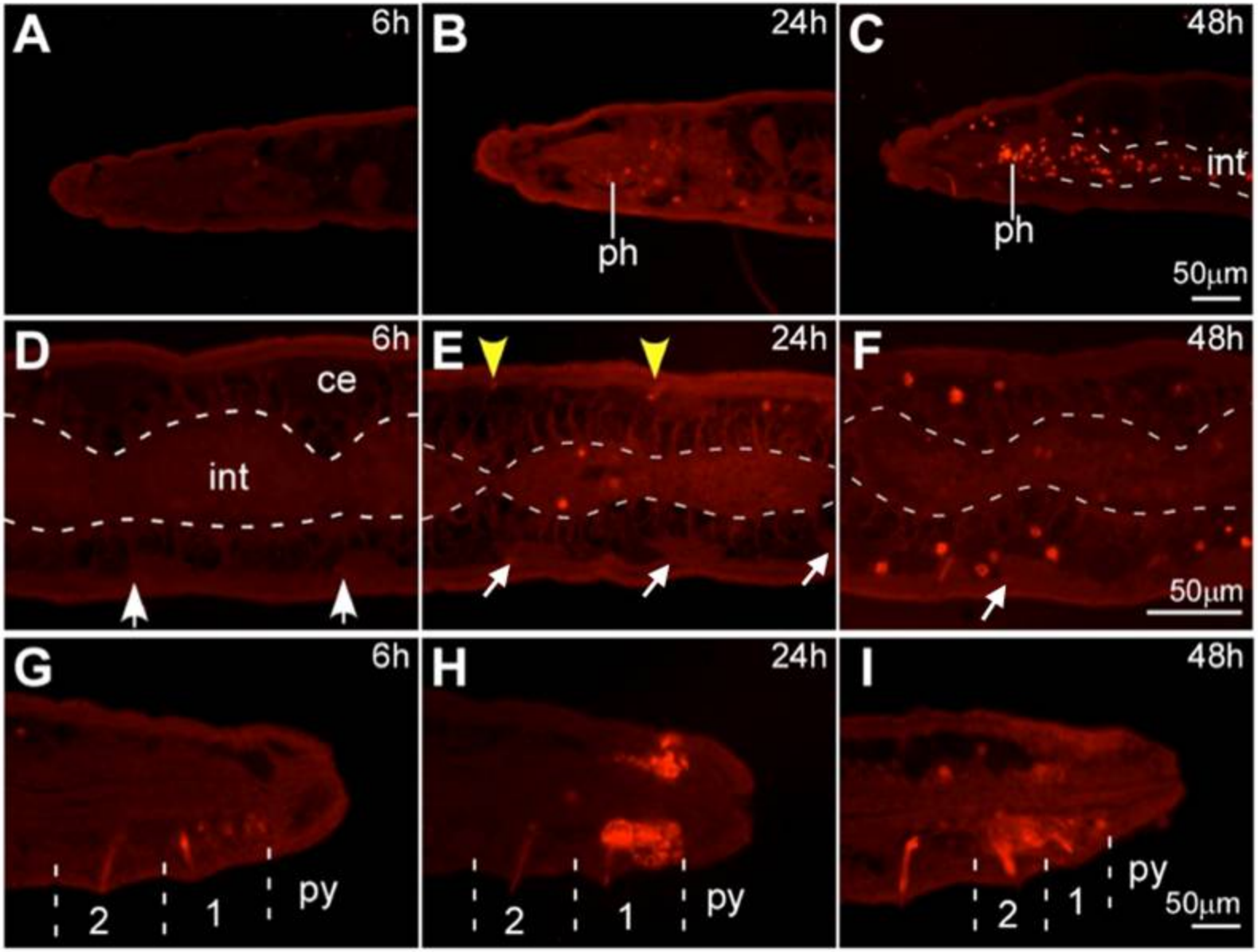

A
6h
B
24h
ph
C
48h
int
ph
50μm
D
6h
ce
int
E
24h
F
48h
50μm
G
6h
2
1
py
H
24h
2
1
py
I
48h
2
1
py
50μm

Fig.3

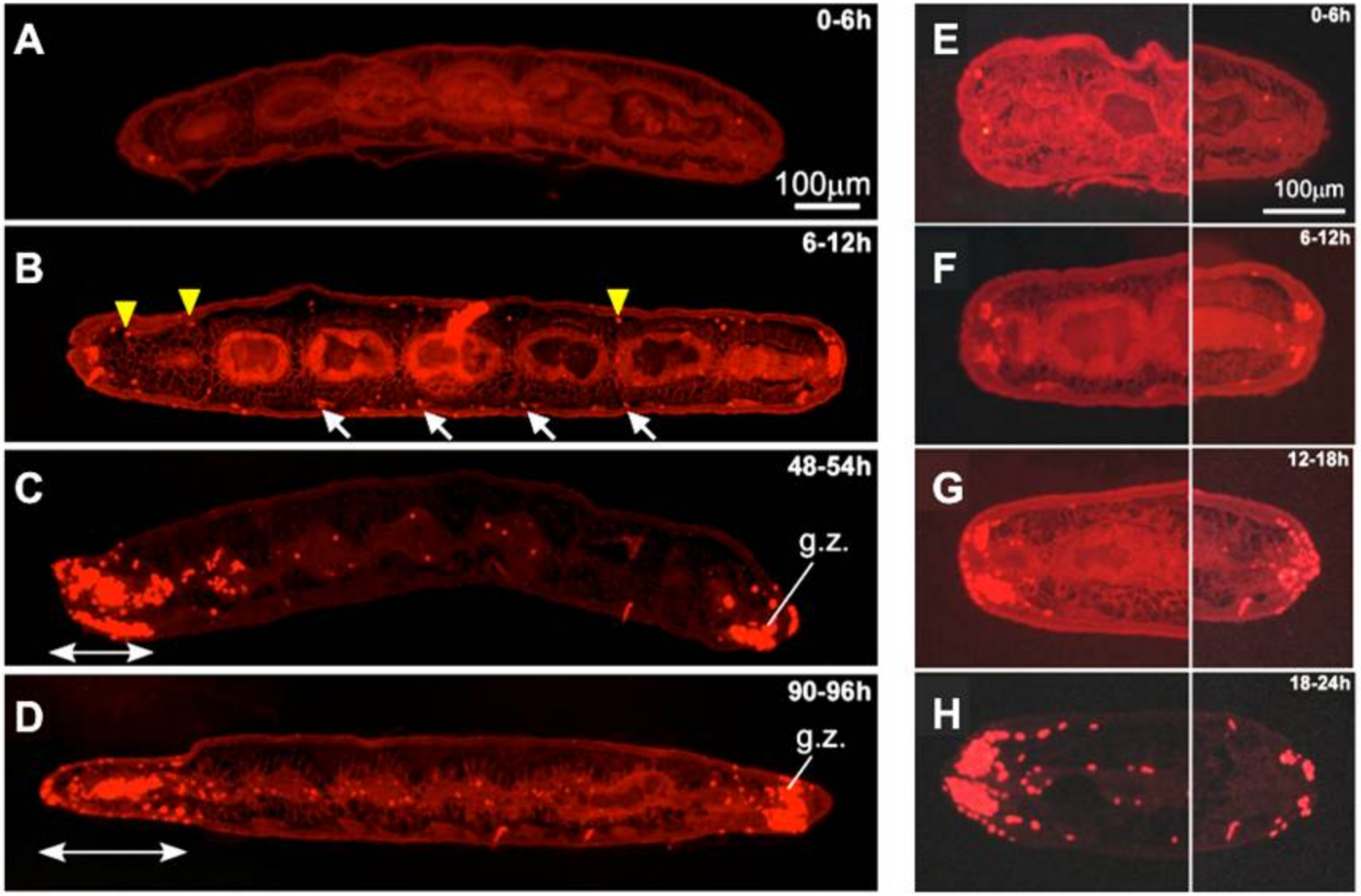

A
0-6h
100μm
B
6-12h
C
48-54h
g.z.
D
90-96h
g.z.
E
0-6h
100μm
F
6-12h
G
12-18h
H
18-24h

Fig.4

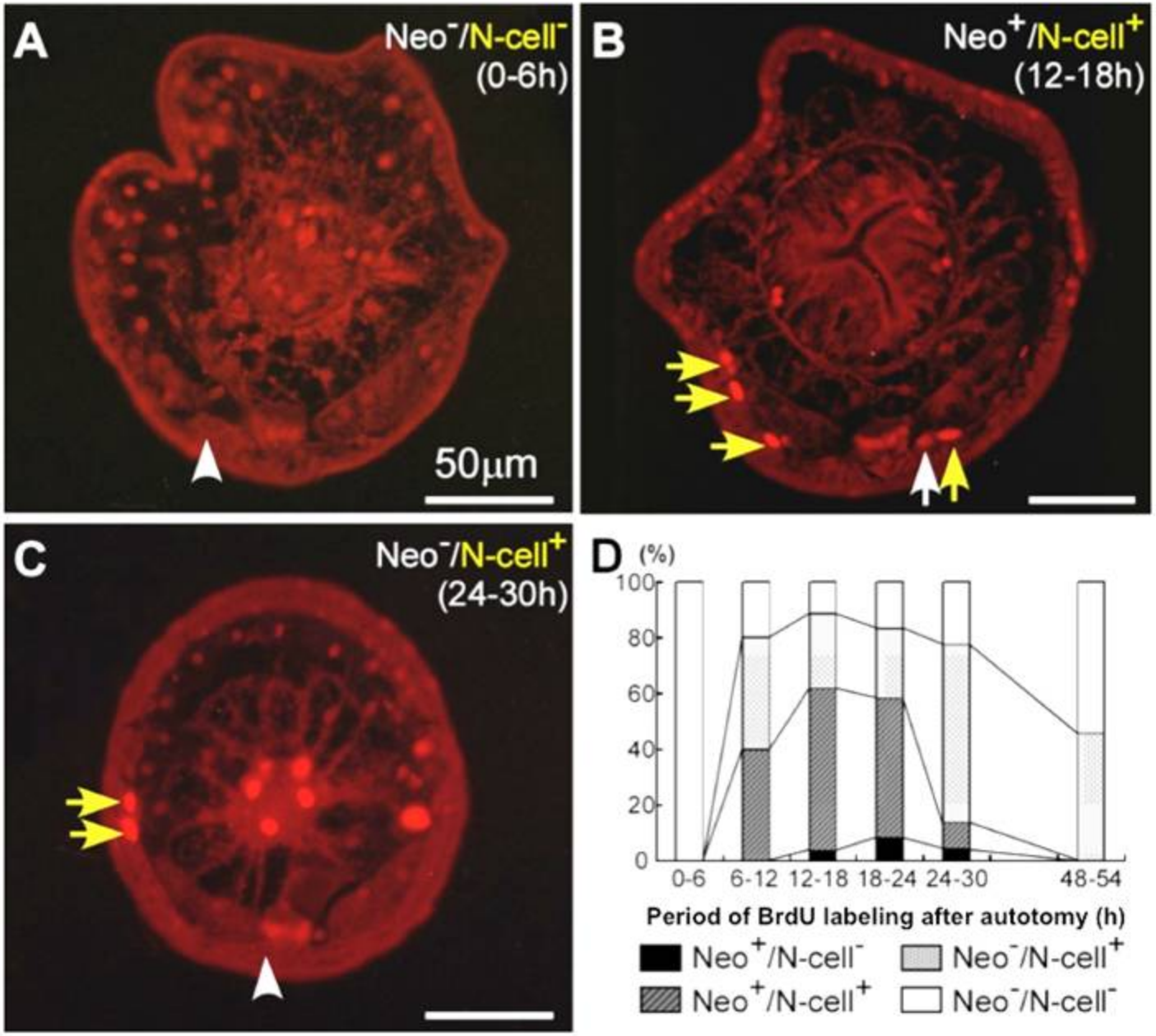

A
Neo⁻/N-cell⁻
(0-6h)
50μm
B
Neo⁺/N-cell⁺
(12-18h)
C
Neo⁻/N-cell⁺
(24-30h)
D
(%)
100
80
60
40
20
0
0-6
6-12
12-18
18-24
24-30
48-54
Period of BrdU labeling after autotomy (h)
Neo⁺/N-cell⁻
Neo⁻/N-cell⁺
Neo⁺/N-cell⁺
Neo⁻/N-cell⁻

Fig.5

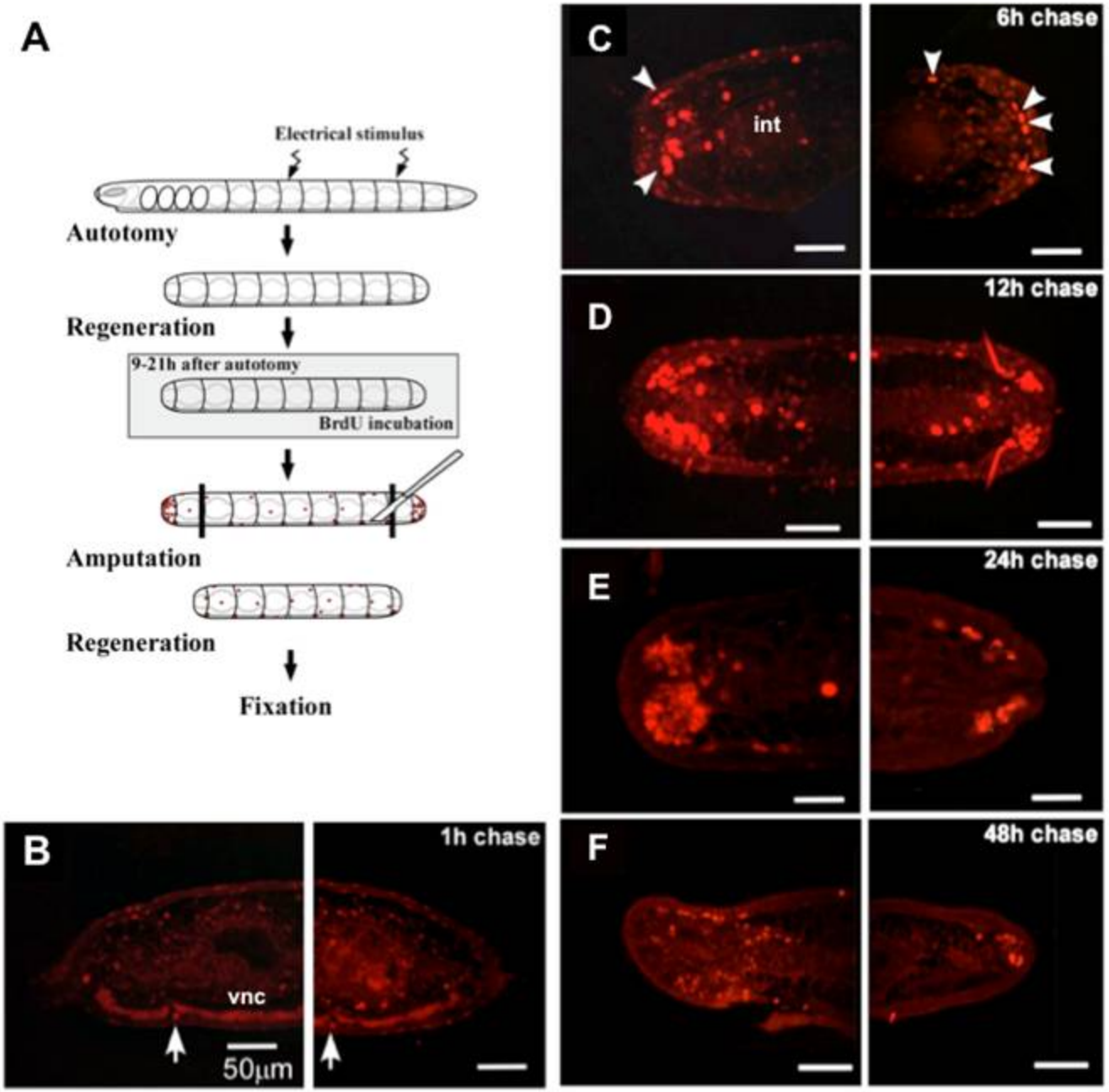

A
Electrical stimulus
Autotomy
Regeneration
9-21h after autotomy
BrdU incubation
Amputation
Regeneration
Fixation
B
vnc
50μm
1h chase
C
int
6h chase
D
12h chase
E
24h chase
F
48h chase

Fig.6

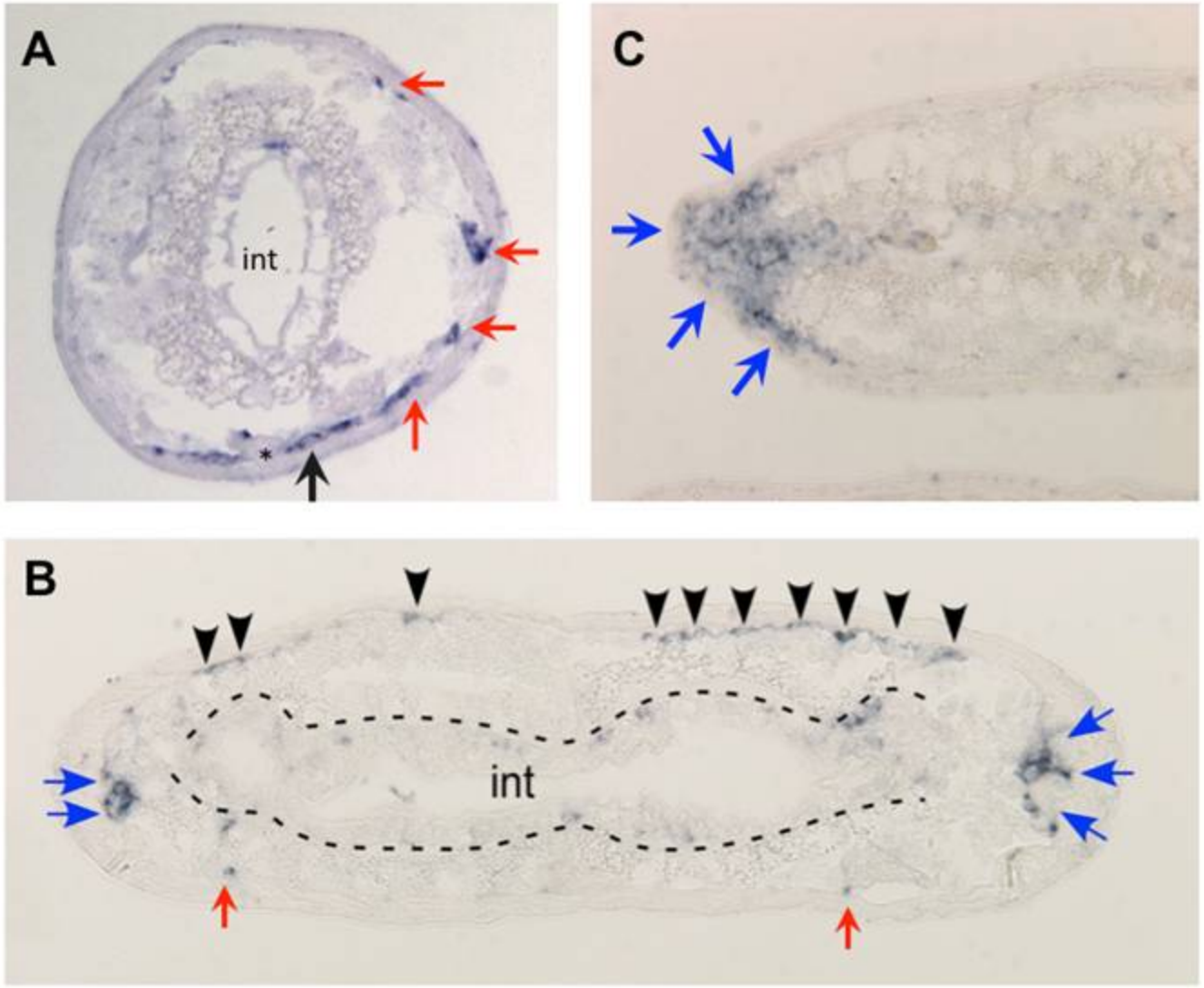
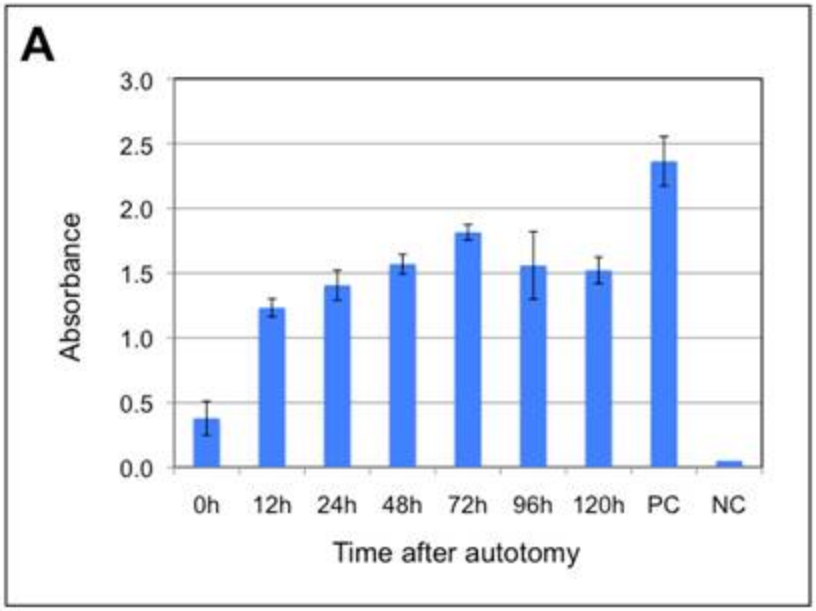
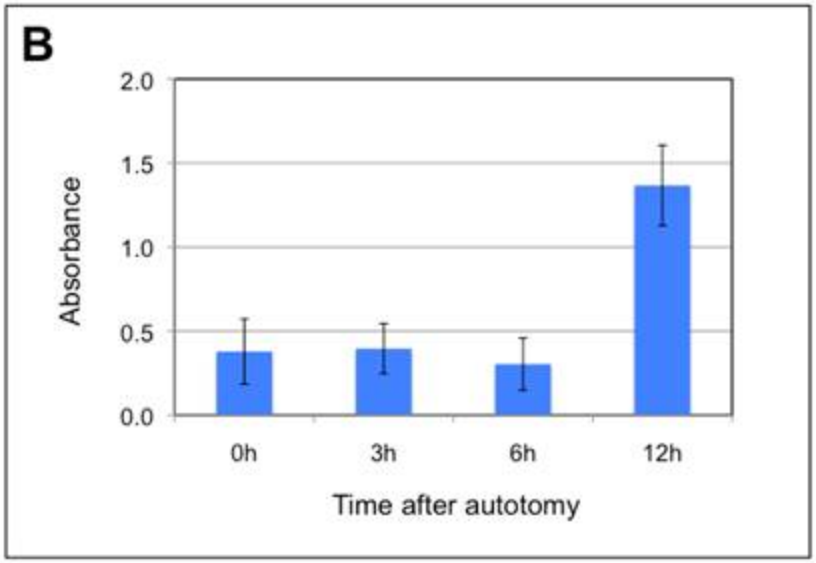


Fig.7


A
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Absorbance
0h
12h
24h
48h
72h
96h
120h
PC
NC
Time after autotomy


B
2.0
1.5
1.0
0.5
0.0
Absorbance
0h
3h
6h
12h
Time after autotomy