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Functional analysis of *grimp*, a novel gene required for mesodermal cell proliferation at an initial stage of regeneration in *Enchytraeus japonensis* (Enchytraeidae, Oligochaete)

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ABSTRACT ***Enchytraeus japonensis* is a small oligochaete species, which has a remarkable regeneration capacity. It has been proposed as a new model animal for the study of regeneration, and some histological studies of this species have been carried out. On the other hand, the molecular biological mechanism of regeneration is almost unknown in this species. To clarify the molecular biological mechanism operating at an initial stage of regeneration in *E. japonensis*, we isolated by the cDNA subtraction method five genes whose expression levels changed in the regeneration process occurring between growing and early regenerating worms. One of the isolated genes (a novel gene named *grimp*) was expressed transiently from 3 to 12 h post amputation only in neoblasts and a population of mesodermal cells (the non-neoblast *grimp*-expressing cells) incorporating BrdU simultaneously showed mitotic activity. We succeeded in inhibiting *grimp* expression by RNA interference (RNAi), thus applying this technique for the first time in Oligochaeta. In knock-down worms, the number of BrdU-positive neoblasts and the non-neoblast *grimp*-expressing cells in the coelom drastically decreased. Moreover, the elongation and the segmentation of blastemas were inhibited, while no statistically significant inhibitory effect was observed in epidermal and intestinal cells. These results suggest that *grimp* is required for initial proliferation of neoblasts and some mesodermal cells for regeneration.**

KEY WORDS: *RNAi, mesodermal cell proliferation, regeneration, Oligochaeta Annelida*

Introduction

Although the regeneration phenomenon is widely distributed in the animal kingdom, the degree and manner of regeneration greatly vary depending on the species. For example, in amphibian limb regeneration, the lost part is regenerated through the formation of a blastema formed by aggregation of undifferentiated cells in the amputated stump (epimorphosis). In contrast, in planarians or hydras, large-scale regeneration of an entire body and the reorganization of remaining tissues are observed (morphallaxis). Moreover, the type of cells contributing to regeneration varies among animals; multipotent stem cells in the regeneration of hydras or planarians, and dedifferentiated cells in amphibian limb or lens regeneration (Sanchez and Tsonis, 2006; Gardiner *et al*., 2002; Baguna *et al*., 1989; Bode, 1996).

An annelid oligochaete, *Enchytraeus japonensis*, has been proposed as a new model animal for the study of regeneration because of its remarkable regeneration capacity and ease of breeding. This worm regenerates by the combination of epimorphosis and morphallaxis (Takeo *et al*., 2008; Myohara *et al*., 1999). Fragments either obtained by autotomy or amputation regenerate a tail from the posterior plane in 2-3 days, a head from the anterior plane in 4-5 days, and the whole body pattern is reorganized in 7 days. The worms grow to the original size in about 2 weeks. It has been suggested that both multipotent stem cells and dedifferentiated cells are involved in whole body regeneration of the worm (Yoshida-Noro *et al*., 2000). The multipotent meso-

Abbreviations used in this paper: dsRNA, double stranded RNA; ISH, in situ hybridization; ORF, open reading frame; RNAi, RNA interference; WISH, whole-mount in situ hybridization.

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dermal stem cells, the neoblasts, participate in blastema formation (Myohara 2004; Myohara *et al*., 1999).

Recently, molecular biological techniques such as cDNA subtractive hybridization and RNA interference (RNAi) have been applied to regeneration research in many species. Especially, RNA interference is a very useful technique to know directly the gene function. Thus, some genes needed for regeneration have been identified in hydras and planarians by utilizing this technique (Mannini *et al*., 2008; Miljkovic-Licina *et al*., 2007; Sanchez-Alvarado, 2006; Agata, 2003; Lohmann *et al*., 1999). Although molecular biological research on regeneration has been conducted in oligochaetes (Niva *et al*., 2008; Takeo *et al*., 2008; Myohara *et al*., 2006; Tadokoro *et al*., 2006; Martinez *et al*., 2005; Bely and Wray, 2001; Dupin *et al*., 1991), gene knock-downs have not yet been succeeded. So far, only four studies utilizing RNAi in other orders of annelids (leech, Hirudinea) have been reported (Baker *et al*., 2008; Shefi *et al*., 2006; Biswas *et al*., 2002; Baker and Macagno, 2000).

In this paper, in order to clarify the molecular mechanisms operating in the early stages of regeneration, we have isolated genes that show changing levels of expression during the regeneration process of *E*. *japonensis*. One of them, named *grimp* (gene required for initial mesodermal cell proliferation), is expressed only at the initial stages of regeneration. This molecule has 3 repeat sequences in the N-terminal half and shows no obvious homology to any known genes. We performed a functional knock-down approach to *grimp* by utilizing RNAi, which was established for the first time in oligochaetes. From our results, it was evident that *grimp* is necessary for the initiation of mesodermal cell proliferation including neoblasts at a very early stage of regeneration in *E*. *japonensis*.

Results

Isolation of genes showing different expression levels between regenerating and growing worms

By cDNA subtraction made between growing and early regenerating worms, five genes with differential expression levels were obtained. RT-PCR showed that three of them (*EjPsmd*, *EjTuba* and *grimp*) were up-regulated while two others (*horu* and *mino*) were down-regulated in an early stage of regeneration than in the intact growing worms (Fig. 1). Although the complete sequences of *EjTuba* (*Enchytraeus japonensis alpha tubulin*-like gene), *horu* (derived from the Japanese word “horumon”, which means “intestine”) and *mino* (which means “stomach” in Japanese) have yet to be obtained, *EjPsmd* (*Enchytraeus japonensis Psmd*-like gene) and *EjTuba* showed high homology in NCBI Blastx search to the 26S proteasome non-ATPase regulatory subunit gene and the alpha tubulin gene, respectively (Table 1). We analyzed the expression patterns of these five genes by whole mount and section *in situ* hybridization (WISH and ISH). *EjTuba*, *horu* and *mino* were shown to be expressed in different intestinal parts (Takeo *et al*., 2008), while no signals of *EjPsmd* was detected so far examined (data not shown). The strong expression of *grimp* was observed in the wound site as described later. Since *grimp* expression seemed to be associated with regeneration, it was further analyzed.

By making 5' and 3' RACE, a 1093 bp-long cDNA fragment

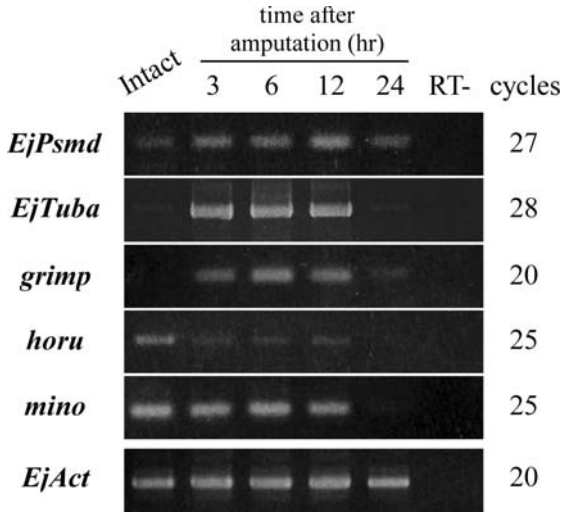

Fig. 1. Gene expression levels in intact and regenerating worms (3, 6, 12 and 24 h post amputation) shown by RT-PCR. *The expression levels of* EjPsmd, EjTuba *and* grimp *were transiently up-regulated at an early stage, and those of* mino *and* horu *decreased as regeneration proceeded. The actin gene (*EjAct*) was used as an internal control. ‘RT-’ and ‘cycles’ indicate a control reaction without reverse transcriptase and PCR cycle numbers, respectively.*

of *grimp* containing a putative open reading frame (ORF) and a poly (A) sequence was obtained (Fig. 2A). By Northern blot analysis of the total RNA isolated from 6 hr-regenerated worms; a single band about 1.2 kbp long was observed (Fig. 2B). These results indicate that the entire sequence of *grimp* was successfully isolated. Database searches for nucleotide and deduced amino acid sequence of *grimp* did not identify any known homologous gene. From the result of ORF analysis in NCBI ORF finder, it was predicted that the region from the 175th to 717th bp has high probability of containing an ORF (*grimp* mid region in Fig. 2A). Although no conserved large domains were found in domain analysis for deduced amino acid sequence, it was suspected that the possible polypeptide product of this gene seems neither a transmembrane nor a secretory protein deduced from secondary structure prediction (SOSUI, http://bp.nuap.nagoya-u.ac.jp/sosui/). On the other hand, from the result of motif search (GENETYX, GENETYX CORPORATION;

TABLE 1

CLONES ISOLATED BY cDNA SUBTRACTIVE HYBRIDIZATION BETWEEN INTACT AND REGENERATING WORMS

obtained genes		most similar sequence found in NCBI Blastx search			
name	**length(bp)**	**definition**	**accession #**	**bit score**	**E-value**
EjPsmd	2070	similar to LOC561267 protein isoform 1 [*Danio rerio*]	XP_686470	469	3e-130
EjTuba	1259	alpha-1 tubulin [*Hirudo medicinalis*]	AAB07727	833	0.0
grimp	1102	none			
horu	778	hypothetical protein [*Monodelphis domestica*]	XP_001375856	36.2	3.8
mino	1026	hypothetical protein NCU06948 [*Neurospora crassa* OR74A]	XP_958218	72.8	4e-11

In the five clones of which expression level was different between growing and regenerating worms, only *EjPsmd* and *EjTuba* showed high homology to known genes in NCBI Blastx search.

PPSearch, EMBL-EBI, http://www.ebi.ac.uk/ppsearch/), it was revealed that the deduced polypeptide contains three integrin-binding sequences RGDS (red bars in Fig. 2A and C), five protein kinase C (PKC) phosphorylation sites (black bars in Fig. 2A and C), one cAMP- and cGMP-dependent protein kinase phosphorylation site (blue bar in Fig. 2A), two N-myristoylation sites (green bars in Fig. 2A) and one casein kinase II phosphorylation site (yellow bar in Fig. 2A). The polypeptide sequence contains 11 chymotrypsin cleavage sites (slashes in Fig. 2A and C) and one possible signal peptidase cleavage site (arrowhead in Fig. 2A). More interestingly, the deduced amino acid sequence revealed that there is a triplicate repeat of a very similar sequence containing RGDS sequences and protein kinase C phosphorylation sites (shaded box in Fig. 2A, and Fig. 2C). Further analyses thus far failed to obtain any helpful information to presume the function of *grimp*.

In the present study, the predicted ORF was amplified in RT-PCR, and the entire sequence of *grimp* excepting the poly (A) sequence was used to synthesize a probe for Northern blot analysis and ISH. For RNA interference (RNAi) experiment, double-stranded RNAs (dsRNAs) were synthesized using three different regions as follows; the mid region that was expected to contain an ORF (*grimp* mid region in Fig. 2A), the 5' and 3' regions of a mid region (*grimp* 5' and 3' region in Fig. 2A).

Spatial and temporal expression patterns of grimp *during early stages of regeneration*

In regenerating *E. japonensis*, wound closure occurs by 3 h post fragmentation (hpf), and mesodermal cell proliferation is observed in the wound site from 6 hpf. The mesodermal and epidermal cells there actively proliferate at 12-24 hpf resulting in blastema formation. RT-PCR analysis revealed that *grimp*, which was expressed strongly from 3 hpa, peaked from 6 to 12 hpa, and then only very weak expression was observed at 24 hpa when the formation of blastema began (Fig. 1).

The spatiotemporal analysis by WISH (Fig. 3 A-F) showed that *grimp* was expressed in cells found not only around the wound site (arrowheads in Fig. 3 A and B) but also over the entire fragment from 3 to 6 hpa (Fig. 3 A, B and E). The expression of *grimp* weakened throughout the body by 12 hpa (arrowheads in Fig. 3C). At 24 hpa, the expression of *grimp* was no longer detectable (Fig. 3D). Detailed observation on sagittally sectioned ISH (Fig. 3 G-I') showed that *grimp* was expressed only in flat cells (the non-neoblast *grimp*-expressing cells, arrowheads in Fig. 3G, Fig.3I and I') and neoblasts (Fig. 3H and H'), both of which were found in the coelom between the body-wall musculature and the digestive tract. On the other hand, *grimp* expression was never observed in the epidermis, the muscle or the digestive tract (Fig. 3G). Both types of *grimp*-expressing cells had a large nucleo-cytoplasmic ratio and a large nucleolus (Fig. 3 H-I'). In addition, neoblasts were several folds larger than the other mesodermal cells, and they were always localized in the particular position close against to the septum, the body wall and the ventral nerve cord. On the other hand, the non-neoblast *grimp*-expressing cells were scattered in the coelom, and they assumed a flat shape because they were always tightly apposed to the body wall.

Simultaneous grimp *ISH and BrdU immunohistochemistry*

To investigate the association between *grimp* expression and cell proliferation, simultaneous ISH for *grimp* and BrdU (5'-bromo-

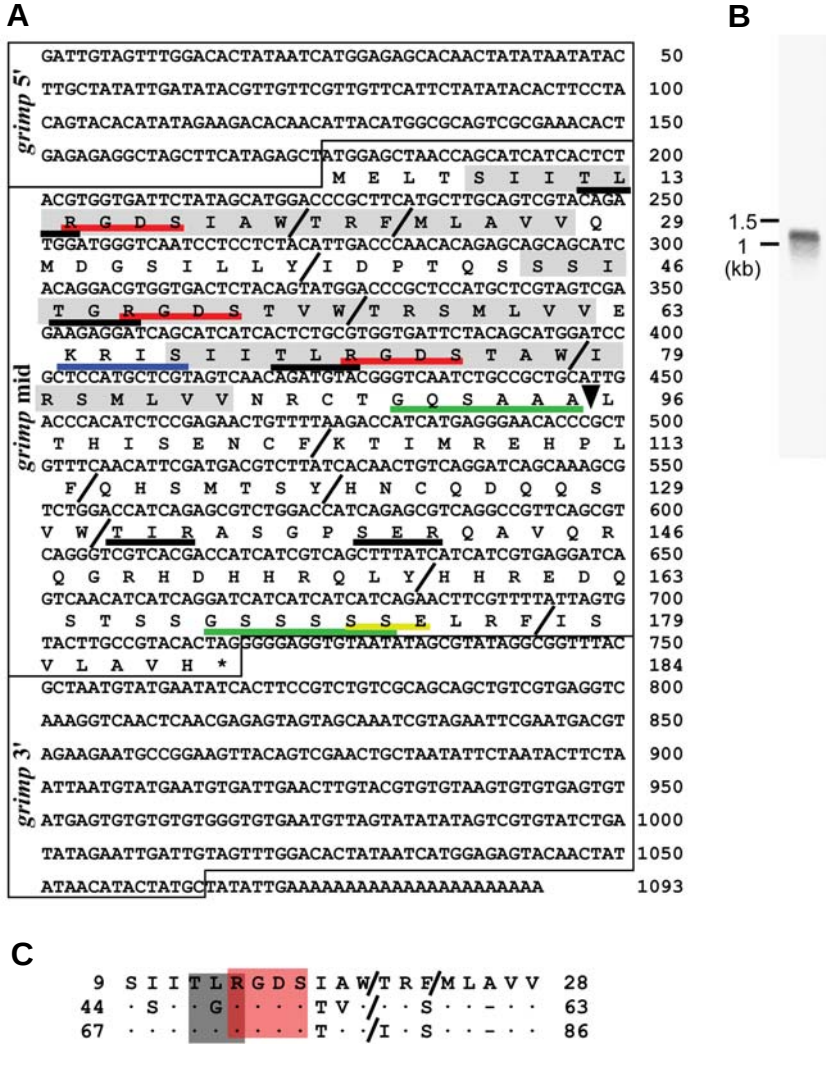

Fig. 2. Sequence and Northern Blot analysis. (A) *Nucleotide and deduced amino acid sequences of* grimp. *Deduced amino sequence contains cell attachment integrin-binding sequences (RGDS, red bars), protein kinase C phosphorylation sites (black bars), cAMP- and cGMP-dependent protein kinase phosphorylation site (blue bar), N-myristoylation sites (green bars) and casein kinase II phosphorylation site (yellow bar), chymotrypsin cleavage site (slashes), possible signal peptidase cleavage site (arrowhead), and a triplicate repeat of a very similar sequence (shaded box). In RNA interference examination, dsRNA were synthesized using ORF, and its 5' and 3' regions, respectively.* **(B)** *Northern blot analysis. A single band of about 1.2 kbp long was observed. Numbers to the left show the position of the RNA base marker.* **(C)** *Amino acid sequence alignment among thee repeated sequences. Red and black boxes indicate RGDS sequences, protein kinase C phosphorylation and chymotrypsin cleavage sites, respectively.*

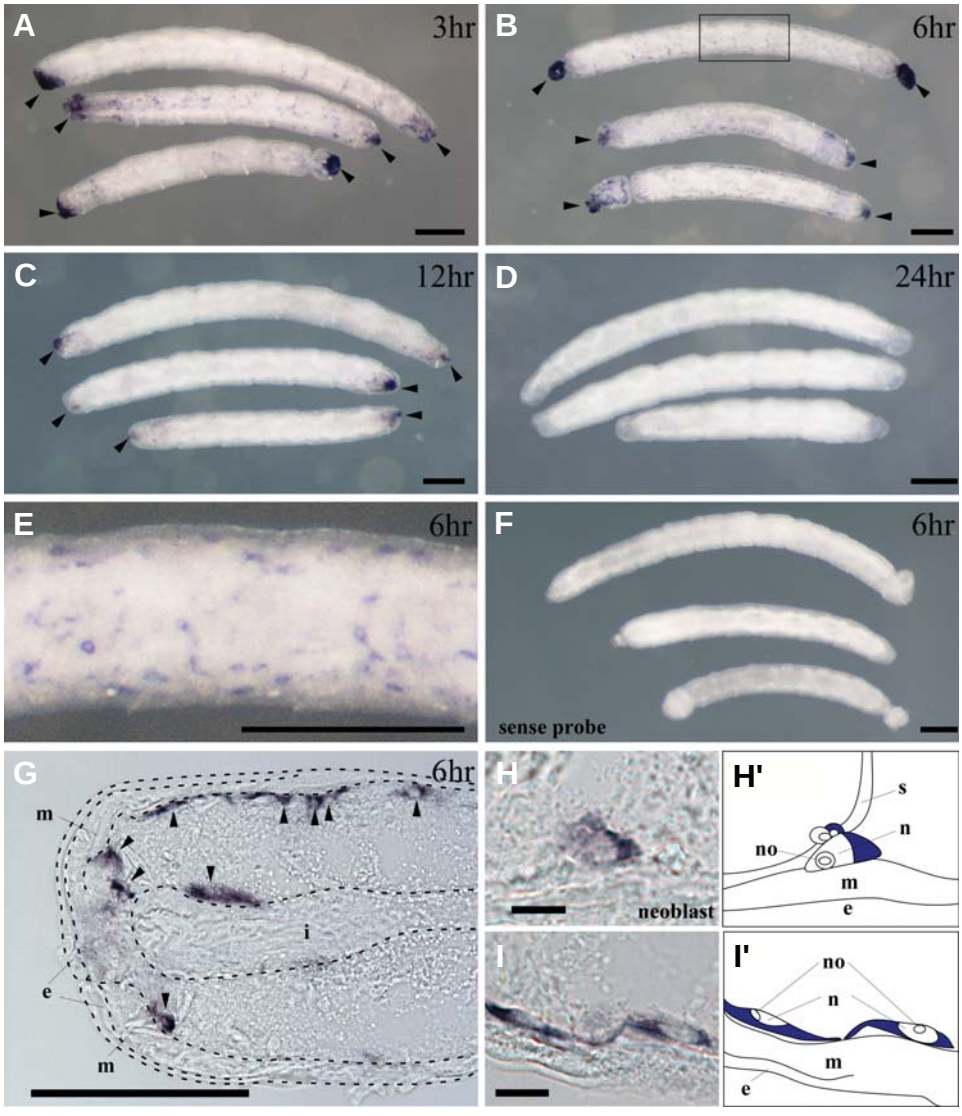

Fig. 3. Spatiotemporal changes of *grimp* expression in regenerating worms. (A–F) *Whole mount* in situ *hybridization. From 3 to 6 hpa, strong expression of* grimp *was observed in the wound sites, and numerous* grimp*-expressing cells were also found over the whole body* **(A,B,E)**. *Then* grimp*-expressing cells decreased according to regeneration, and were no longer detectable by 24 hpa* **(C,D)**. *No signal was observed by using sense probe (F). Panel E is a magnified image of the box in B. Arrowheads indicate the* grimp *expression near the wound site.* **(G–I)** *Sagittally sectioned* in situ *hybridization at 6 hpa.* grimp *was expressed in mesodermal cells including neoblasts; see arrowheads in* **(H)**. **(H' ,I')** *Trace drawings of* **(H,I)**, *respectively. e, epidermis; i, intestine; m, musculature; n, nucleus, no; nucleolus; s, septum. In all photographs, the left side is the anterior. Scale bars, 200 μm in (A-G); 10 μm in (H,I).*

2'-deoxyuridine) immunohistochemistry was made (Fig. 4). BrdU was incorporated for 3 h in intact worms prior to fixation. In regenerating worms, successive BrdU incorporation was made (Fig. 4A). The number of *grimp* and/or BrdU-positive cells per segment was counted since the total number of segments varied in intact (32 to 61 segments) and regenerating (8 to 15 segments) worms.

While no *grimp*-expressing cell was observed in intact worms (n = 4, 48.5 ± 12.4 segments/worm; Fig. 4B and F), 6.7 ± 4.8, 6.5 ± 5.2 and 0.92 ± 0.72 *grimp*-expressing mesodermal cells/segment were found at 3hpa (n = 6, 12.5 ± 3.0 segments/fragment; Fig. 4C and F), 6hpa (n = 10, 12.1 ± 1.3 segments/fragment; Fig. 4D and F) and 12hpa (n = 10, 11.7 ± 2.6 segments/fragment; Fig. 4E and F), respectively. In addition, at 3 and 6hpa, 0.24 ± 0.26 and 0.24 ± 0.22 neoblasts also expressed *grimp* (insets in Fig. 4C and D, Fig. G). The number of *grimp*-expressing cells peaked at 3 to 6hpa, and *grimp*-expressing cell was no longer observed after 12hpa.

In contrast, the number of BrdU-positive mesodermal cells increased according to regeneration as follows; 19.8 ± 11.5 mesodermal cells and 0.22 ± 0.13 neoblasts/segment in intact worm, 30.0 ± 15.9 mesodermal cells 0.76 ± 0.88 neoblasts at 3hpa, 45.1 ± 23.9 mesodermal cells and 0.49 ± 0.25 neoblasts at 6hpa, and 38.8 ± 16.2 cells and 0.12 ± 0.09 neoblasts at 12hpa (white arrowheads and insets in Fig. 4 B' – E', Fig. F and G). In addition, at 12hpa, active cell proliferation of epithelial cells was also observed in the wound site (white arrows in Fig. 4E'). BrdU-positive cells were also observed in the epidermis and the digestive tract (epidermis, arrows; digestive tract, unmarked BrdU-positive cells in Fig. 4B') in intact worms

Moreover, 16.6 ± 22.1% of non-neoblast *grimp*-expressing cells and 37.5 ± 49.0% of *grimp*-expressing neoblasts at 6hpa, and 8.9 ± 14.9% of non-neoblast *grimp*-expressing cells at 12hpa were double-positive for both *grimp* and BrdU (arrowhead with an asterisk and inset in Fig.4 D - D'', Fig. 4F and G).

During regeneration, there is no axial variation in *grimp* expression/BrdU incorporation related to the distance from the wound except for the wounded segments at the amputation sites.

RNA interference using dsRNA

To clarify whether *grimp* expression is linked to the following cell proliferation, an attempt was made to interfere with gene function (RNAi) using dsRNA. Since oligochaetes have a wide coelom space that is separated by net-like septal tissues, any substances injected into a coelom diffuse promptly throughout the body. In fact, in *E. japonensis*, an injected 25 nt-long rhodamine-conjugated test siRNA in the coelom diffused immediately after injection (Fig. 5A and B). Moreover, siRNA was mostly observed only in nephridia but detected very few coelomic cells at 1 h after injection (Fig. 5C). It was suspected that the injected siRNA were promptly expelled through nephridia. By repeated injections made once a day for 3 days, it was found that

siRNA could be incorporated into mesodermal cells, while a major amount of siRNA was still detected in the nephridium (arrowheads in Fig. 5 D and E).

Thus, *grimp* (*grimp* mid region in Fig. 2) or control *EGFP* dsRNA was injected once a day for 3 days, worms were amputated 3 h after the last injection, and allowed to regenerate for 6, 12 and 96 hr. ISH made at 6hpa revealed that the aggregation of *grimp*-expressing cells was observed in *EGFP* dsRNA-injected worms similar to non-treated regenerating worms (yellow dashed area in Fig. 6A, see also Fig. 4C). On the other hand, in *grimp* dsRNA-injected worms, it was found that only a few mesodermal cells expressed *grimp* (arrowheads in Fig. 6B). In RT-PCR analysis, it was also confirmed that the expression level of *grimp* in *grimp* dsRNA-injected worms was much lower than that in *EGFP* dsRNA-injected worms (Fig. 6C). These results clearly indicate that the expression of *grimp* was repressed by *grimp* dsRNA injection. More interestingly, at 12 hpa, the number of BrdU-positive mesodermal cells per segment was drastically decreased in *grimp* dsRNA-injected worms (5.6 ± 4.3 cells/segment, n = 9, 7.1 ± 1.1 segments/fragment) compared to that in *EGFP* dsRNA-injected worms (32.7 ± 11.2 cells/segment, n = 9, 9.7 ± 0.9 segments/fragment; $t = 6.94$, $p < 0.001$; *t*-test; Fig. 6 D, E and F). The difference in the number of BrdU-positive neoblasts was statistically significant between *EGFP* and *grimp* dsRNA-injected

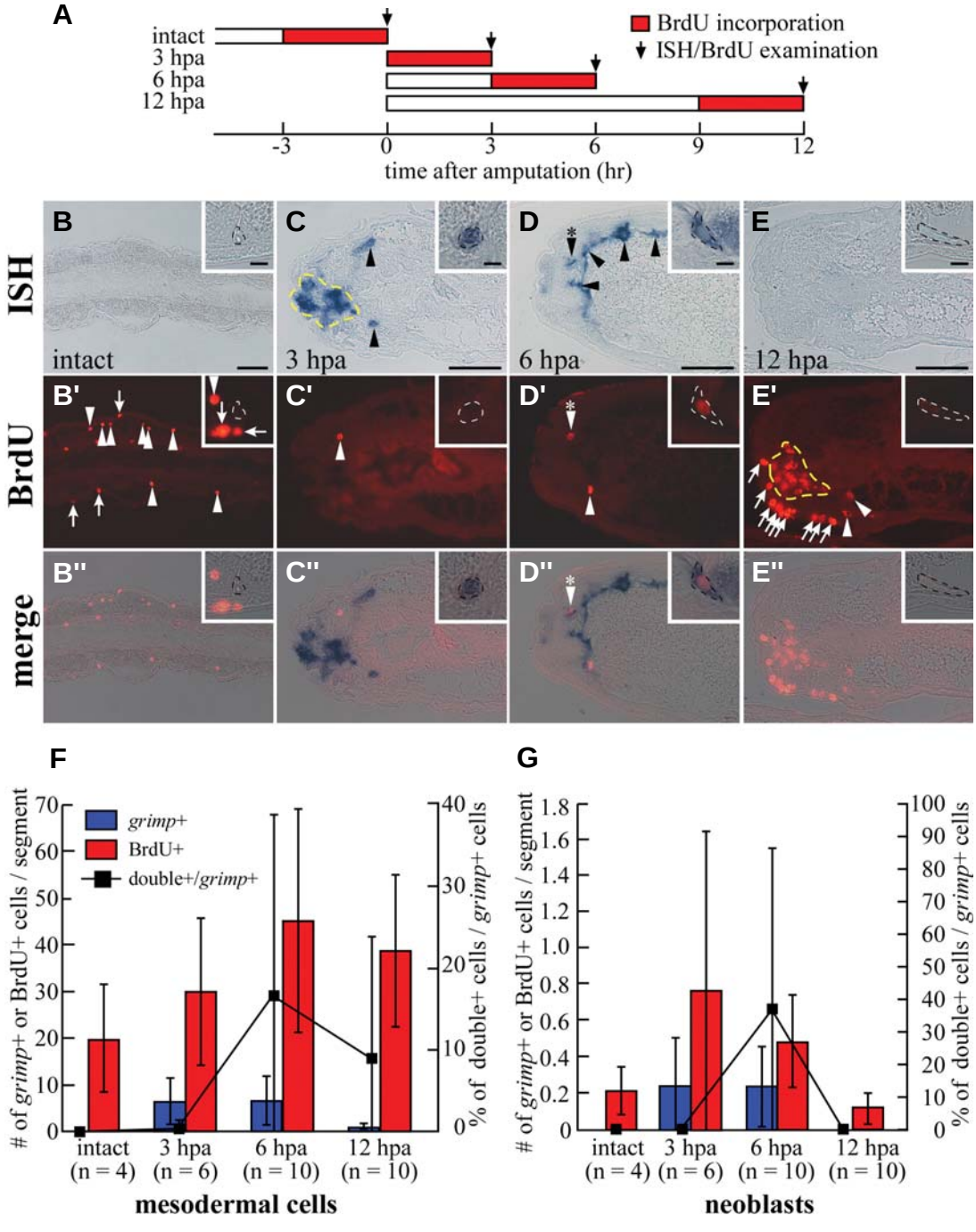

Fig. 4. Simultaneous ISH and BrdU immunohistochemistry. (A) *Experimental scheme. BrdU was incorporated for 3 h prior to fixation.* **(B-E'')** *ISH* **(B-E)**, *BrdU immunohistochemistry* **(B'-E')** *and merged images* **(B''-E'')** *on sagittal sections. In intact worms, only BrdU single-positive cells were found* **(B,B')**. *At 3 and 6 hpa, many mesodermal cells near the wound site and neoblasts were positive for* grimp, *see arrowheads and insets in* **(C,D)**, *and BrdU-positive mesodermal cells were observed in the coelom* **(C', D')**. *A few double-positive cells were also observed at 6 hpa (arrowhead with asterisk and insets in D-D''). At 12 hpa,* grimp *expression was no longer observable* **(E)**, *while the incorporation of BrdU continued in the wound site* **(E')**. *White arrowheads and white arrows in* **(B'-E')** *indicate BrdU-positive cells in the coelom and the epidermal layer, respectively. Dashed area in insets indicates neoblast. Dashed yellow areas indicate aggregation of* grimp-*expression or BrdU-positive cells. Scale bars: 50 μm and 10 μm in insets.* **(F,G)** *Numbers of* grimp-*expressing (blue) and BrdU-positive (red) cells/segment are shown by columns for mesodermal cells* **(F)** *and for neoblasts* **(G)**, *respectively. The rate of double-positive cells to the total number of* grimp-*expressing mesodermal cells and neoblasts are also plotted in each graph by black squares. Vertical bars show standard deviations.*

worms (*EGFP* dsRNA-injected worms, 0.6 ± 0.35 cell/segment; *grimp* dsRNA-injected worms, 0.27 ± 0.21cell/ segment; $t = 3.04$, $p < 0.01$; *t*-test; Fig. 6F). In contrast, the number of BrdU-positive epidermal cells showed no significant difference between them (*EGFP* dsRNA-injected worms, 17.0 ± 8.0 cells/segment; *grimp* dsRNA-injected worms, 15.0 ± 5.5 cells/segment; $t = 0.41$, $p > 0.5$; *t*-test; Fig. 6F). The number of BrdU-positive intestinal cells was 36.0 ± 8.6 and 22.8 ± 11.2 cells/segment in *EGFP* and *grimp* dsRNA-injected worms, respectively (not significantly different, $t = 2.921$, $p > 0.1$; *t*-test; Fig. 6F).

In normal regeneration of *E. japonensis*, the segmentation of an anterior blastema is observed and head regeneration is completed by 96 hpa (Myohara, 2004). When regenerating worms was repeatedly injected with dsRNA once a day for 4 days (96 hr), segmentation of the anterior blastemas was observed in 94.1% of *EGFP* dsRNA-injected worms (n = 34, segments inscribed with Roman numerals in Fig. 6G, Fig. 6I), while the segmentation and the elongation was observed only in 4.2% of *grimp* dsRNA-injected worms (n = 24, Fig. 6 H and I).

To assess the specificity of the inhibitory effect of *grimp* RNAi, RNAi experiments were made using *grimp* 5' and 3' dsRNA (synthesized using *grimp* 5' and 3' regions in Fig. 2). In both cases, the elongation and the segmentation of anterior blastema were also inhibited (*grimp* 5' dsRNA, 50.0% of segmentation, n = 20; *grimp* 3' dsRNA, 53.9% of segmentation, n = 26), although the apparent inhibitory effects decreased in comparison with the experiment using *grimp* dsRNA (*grimp* mid region in Fig.2). In intact *grimp* RNAi-treated animals without amputation, they did not show any defect of proliferation of mesodermal cells in the intact state (data not shown).

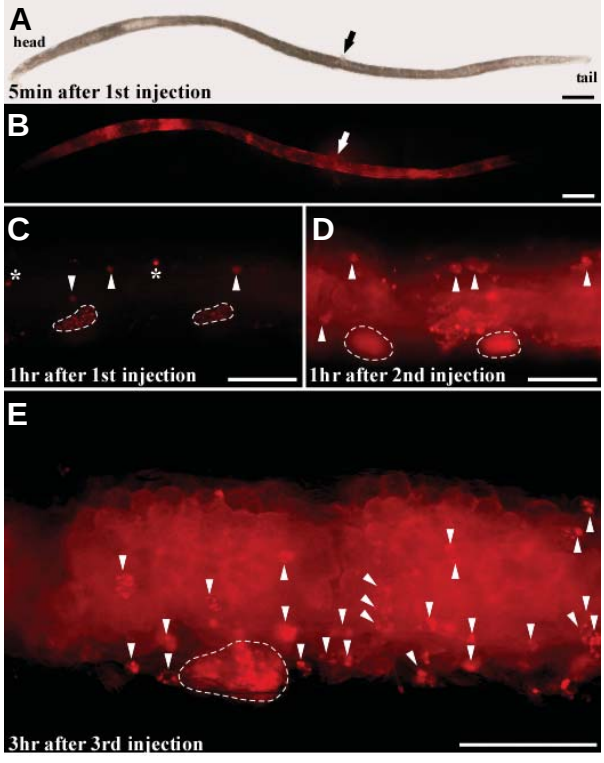

Fig. 5. Repetitive injection of 25 nt-long rhodamine-conjugated test siRNA in the coelom. *Injected 25 nt-long rhodamine-conjugated test siRNA was promptly diffused in the coelom within 5 min after injection* **(A,B)**. *However, siRNA was mostly observed in nephridia even at 1 h after injection* **(C)**. *By repeated injections made once a day for 3 days, siRNA-incorporated mesodermal cells were easily found, while a major amount of siRNA was still detected in the nephridium* **(D,E)**. *Asterisks indicate autofluorescence of setae. Arrow, dashed areas and arrowheads indicate the injection point, nephridia and siRNA-incorporated mesodermal cells, respectively. Scale bars: 500 μm in (A,B); 20 μm in (C-E).*

Discussion

Genes isolated by subtraction

The expression levels of isolated five genes by cDNA subtraction technique changed during the initial stages of regeneration. Among them, *EjPsmd* and *EjTuba* showed high homology to the 26S proteasome non-ATPase regulatory subunit and the alpha tubulin genes, respectively by NCBI Blastx search (Table 1). In *E. japonensis*, a proteasome subunit-like gene has already been reported, and that expression level was shown to be up-regulated during regeneration (Myohara *et al*., 2006). In many species, the proteasome involves multiple biological processes in the ubiquitin-proteasome pathway, operating during such processes as remodeling of the muscle and progression of the cell cycle by regulating the amount of the cyclin-dependent kinase inhibitor p27 (Taillandier *et al*., 2004; Montagnoli *et al*., 1999; Pagano *et al*., 1995; Pal *et al*., 1994). Although the spatial expression pattern of *EjPsmd* was not clarified in the present study, it could be assumed that this gene is associated with regeneration-related processes such as progression of the cell cycle or wound closure, on the grounds that transient upregulation of *EjPsmd* expression occurs at an early stage of regeneration (Fig. 1). Generally, it has been known that alpha tubulin is a component of cytoskeletal microtubules that are essential for cell division. In fact, in *E. japonensis*, it has been indicated that *EjTuba* is expressed in growth zone and blastema where cells actively proliferate (Takeo *et al*., 2008). The transient upregulation of *EjTuba* in an early stage of regeneration might be caused in association with the cell proliferation.

There are several reasons why homologs of the remaining three genes (*grimp*, *horu*, *mino*) could not be identified from existing databases. First, it is highly probable that the many genes expressed in Enchytraeidae may not be common with animals examined so far including even oligochaete species such as *Lumbricus* and *Eisenia*. Moreover, it is apparent that part or entire coding regions are missing from *horu* and *mino* sequences because the 5' sequence has yet to be obtained. In the case of *grimp,* whose entire sequence has been obtained, the expression is very much limited to the early stage of regeneration, resulting in the possible failure of registration even in the earthworm EST databases (LumbriBASE and EandreiBASE).

Success of RNA interference

RNA interference is a very useful technique to know the function of given genes, and it has been utilized in regeneration studies of hydras and planarians (Mannini *et al*., 2008; Miljkovic-Licina *et al*., 2007; Sanchez-Alvarado, 2006; Agata, 2003; Lohmann *et al*., 1999). However, the same technique has not

yet been established in any oligochaete species, while four experiments have so far been reported on another order of annelids (leech, Hirudinea) (Baker *et al*., 2008; Shefi *et al*., 2006; Biswas *et al*., 2002; Baker and Macagno., 2000).

In the analysis utilizing RNAi, a variety of animals are soaked in, injected with, or fed with RNA solutions to make their cells incorporate dsRNA or siRNA. Almost all test siRNA injected into the coelom of *E. japonensis* was promptly taken into nephridia in the present study (Fig. 5). Either the use of lipofection reagent or keeping worms at low temperature failed to make mesodermal cells in the coelom incorporate siRNA. However, it was found that siRNA was incorporated into mesodermal cells by simply injecting solutions once a day up to 3 days, while much siRNA was still detected in the nephridia. This might be because the total amount of injected siRNA exceeded the capacity of the nephridia.

By injecting *grimp* dsRNA once a day for 1 to 2 days, worms regenerated as control *EGFP* dsRNA-injected worms (data not shown). On the other hand, when the injection of *grimp* dsRNA was extended to 3 days, *grimp* expression, BrdU incorporation in coelomic mesodermal cells, and the elongation and the segmentation of the anterior blastema were all inhibited (Fig. 6). The same phenotype was also observed in similar experiments using *grimp* 5' or 3' dsRNA, although the inhibition was less severe than the experiment using *grimp* dsRNA. It has been indicated that the inhibitory efficiency of dsRNA injection increases with increasing dsRNA length (Yang *et al*., 2000). The length of *grimp*, *grimp* 5' and 3' dsRNA was 543, 174 and 347 bp, respectively and this difference in length may be the cause of the inhibitory efficiency among experiments. Because H_2O- and *EGFP* dsRNA-treated worms who were injected with technically possible highest concentration of dsRNA (5μg/μl) regenerated as non-treated worms (data not shown), it is highly improbable that there was non-specific effect of RNA injection. These results suggest that the function of the *grimp* gene was specifically down-regulated by the injection of *grimp* dsRNA

Apart from the findings described above, the establishment of the RNAi technique in this species will increase the usefulness of *E. japonensis* as a model animal for regeneration studies.

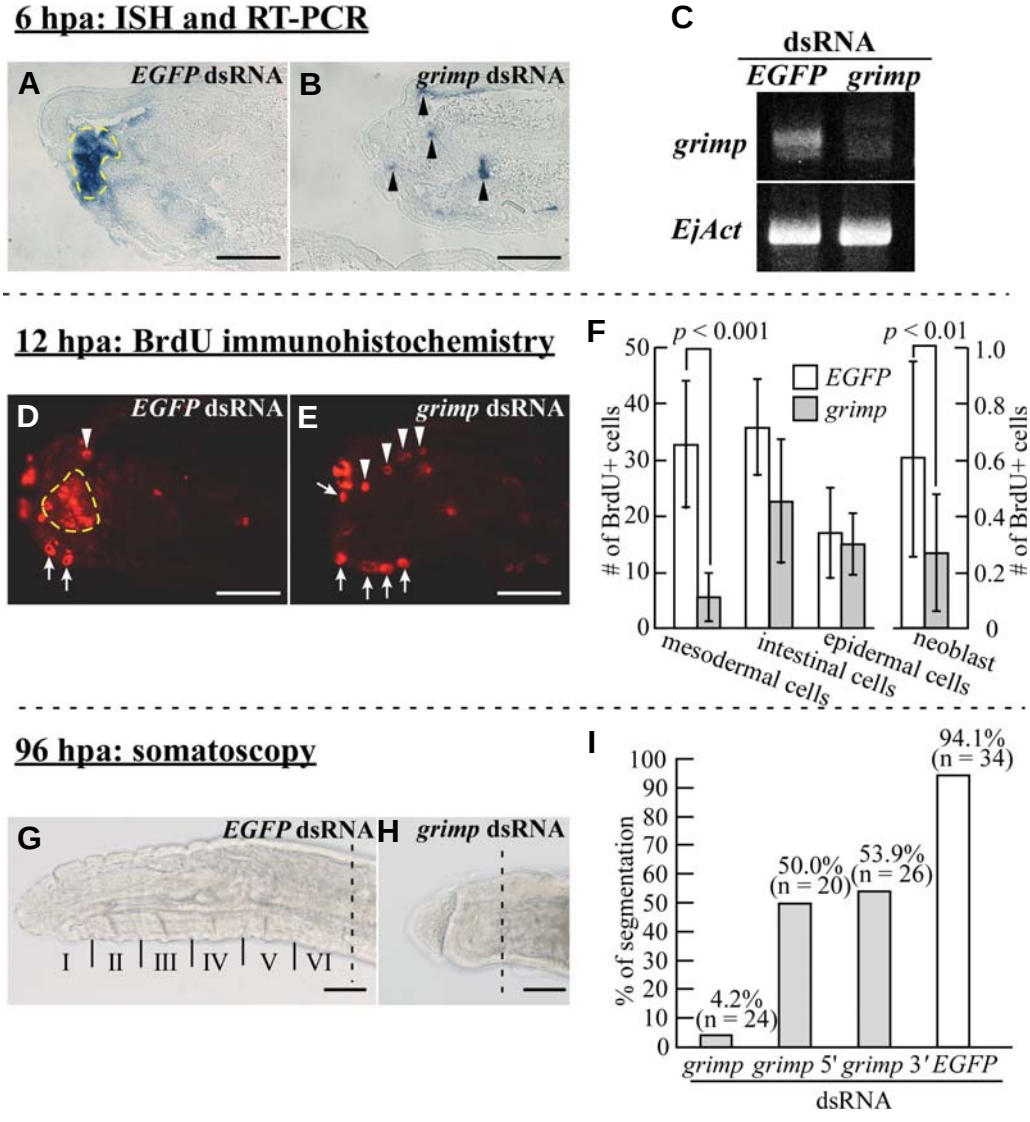

Fig. 6. RNA interference using *grimp* dsRNA. (A-C) *Sagittal section ISH at 6hpa. Although aggregation of* grimp*-expressing cells was observed in* EGFP *RNA-injected worms (yellow dashed area in A), only a few positive cells were found in* grimp *RNA-injected worms (arrowheads in B). Likewise, the* grimp *expression level of* grimp *RNA-injected worms greatly decreased, as shown by RT-PCR* **(C)**. *Scale bars, 100 μm.* **(D-F)** *While numerous BrdU-positive cells were observed in* EGFP *RNA-injected worms (yellow dashed area in D) at 12 hpa, only a few positive mesodermal cells were found in* grimp *RNA-injected worms (white arrowheads in E). The significant difference of BrdU-positive mesodermal cells and neoblasts were confirmed between* EGFP *and* grimp *RNA-injected worms* **(F)**. *White arrows indicate BrdU-positive epidermal cells. Vertical bars show standard deviation (SD).* **(G-I)** *External appearance at 96 hpa after 5 successive injections. Although the segmentation of the anterior blastema was observed in* EGFP *RNA-injected worms (inscribed with Roman numerals in G), it was strongly inhibited in* grimp *RNA-injected worms* **(H)**. *dsRNA of 5' - and 3' -regions of* grimp *were less effective than* grimp *ORF* **(I)**. *Scale bars, 50 μm.*

Expression of grimp promotes mesodermal stem cells to proliferate in initial stage of regeneration

In zebrafish and axolotls, it has been indicated that the proliferation and migration of mesenchymal cells are inhibited and the blastema is not formed by obstructing the TGF-beta or FGF signaling pathway (Jazwinska *et al*., 2007; Levesque *et al*., 2007; Thummel *et al*., 2006; O'Kane and Ferguson, 1997). In addition, it has also been reported that Smad4 involved in the TGF-beta pathway is necessary for the proliferation of stem cells in planarians (Reddien *et al*., 2005). In Oligochaeta, including *E. japonensis*, although some molecular biological research has been made, no genes required for the initiation and the progression of regeneration have yet been identified (Myohara *et al*., 2006; Martinez *et al*., 2005; Bely and Wray, 2001; Dupin *et al*., 1991). In the present study, we found one of the key genes required for the initiation of regeneration, *grimp* is strongly expressed in neoblasts and in a subset of mesodermal cells in the coelom (Figs. 3 and 4). In addition, *grimp* was expressed only transiently from 3 hpa (when wound closure occurred) to 12 hpa (when active cell proliferation occurred at wound site), while occasional cell proliferations were observed even in intact worms (Figs. 1 and 3). Some *grimp*-expressing cells also incorporated BrdU at the same time (Fig. 4). In *grimp* dsRNA-injected worms, both the expression level of *grimp* and the number of *grimp*-expressing cells, and also the number of proliferating neoblasts and mesodermal cells scattered in the coelom decreased. Moreover, at 96 hpa, the elongation and segmentation of the anterior blastema was also inhibited in these worms (Fig. 6). The influence of RNAi, in contrast, was not significantly affected the proliferation of intestinal cells and epidermal cells in which *grimp* was not expressed at all (Fig. 6). Non *grimp*-expressing intestinal proliferation was reduced by about one third, although that reduction was not statistically significant. It could be that the RNAi effect is simply less penetrant in intestinal cells but that *grimp* is nevertheless important for that proliferation, or that *grimp*-expressing cells affect intestinal proliferation indirectly.

These results suggest that *grimp* is not related to cell proliferation in general, but is closely connected to a key process for the initiation of proliferation only in mesodermal cells, including the neoblasts, at initial stages of regeneration. Thus, we named this gene *grimp* (gene required for initial mesodermal cell proliferation). It seems that mesodermal cells may have two proliferation systems; 1) *grimp*-independent the physiological proliferation for body maintenance and 2) *grimp*-dependent emergent proliferation for regeneration triggered by the amputation signal.

The deduced amino acid sequence of *grimp* has a triplicate repeat of a very similar sequence containing RGDS sequences and protein kinase C (PKC) phosphorylation sites and a chymotrypsin cleavage site. It has been previously indicated that the integrin-binding sequence (RGDS) was also found in fibronectin (Ruoslahti and Pierschbacher, 1986), that RGD-mediated cell adhesion was necessary for cell migration in gastrulation and wound closure in axolotls and, quite interestingly, intestinal regeneration in sea cucumbers (Cabrera-Serrano and Garcia-Arraras, 2004; Donaldson *et al*., 1987). Although it is not certain whether the translated product of *grimp* is a distinct signal peptide carried outside cells because a distinct signal peptide was not identified, and RGD-mediated cell adhesion might play an important role in initiation of mesodermal cell proliferation in *E. japonensis*. Connected to this, it should be mentioned that the peptide contains a possible site which is cleaved by the signal peptidase. In addition, it has been known that PKC takes part in cellular responses to various signals such as hormones and growth factors and that its phosphorylating activities are essential for numerous processes, including cell proliferation and differentiation (Moraczewski *et al*., 2008; Nishizuka, 1992). Although any helpful information for presumption of interaction between *grimp* and other genes was not obtained in the present study, the presence of many PKC phosphorylation sites may indicate that *grimp* may be located downstream of growth factors such as TGF-beta and FGF. The function of *grimp* is no more than a speculation, as depicted above, at this time. It is still unclear whether this gene is actually translated into a polypeptide or not. Attempts to raise antibodies against partial peptide sequence or recombinant protein of *grimp* are in progress for further analysis. Also, screening of genes affected by *grimp* dsRNA is under way.

Earthworms have ectodermal, mesodermal and endodermal stem cells (Jamieson, 1981), and neoblasts are thought as mesodermal stem cells in *E. japonensis* (Tadokoro *et al*., 2006). Although the size is quite different between the non-neoblast *grimp*-expressing cells and neoblasts, they have common histological features such as a large nucleo-cytoplasmic ratio, and enhanced proliferation during early stages of regeneration. In addition, it has been reported that a *vasa*-like gene (*Ejvlg2*) is expressed in both neoblasts and that smaller cells are similar to neoblasts in morphology (Sugio *et al*., 2008). From these and the present RNAi experiment, it is highly probable that neoblasts and the non-neoblast *grimp*-expressing cells have a common property, suggesting that the cells in blastemas were provided from not only neoblast but also the non-neoblast *grimp*-expressing cells. That means, in turn, that there are two types of mesodermal stem cells over the entire body in *E. japonensis*.

Materials and Methods

Animals and regenerating fragments

An enchytraeid worm *Enchytraeus japonensis* has been cultured asexually in our laboratory as described previously (Kawamoto *et al*., 2005). All fragments were cut artificially using a disposable surgical blade to contain about 10 segments. The fragments from the trunk region having amputated

TABLE 2

GENE-SPECIFIC PRIMERS FOR RT–PCR

Gene name	primer sequences
EjPsmd	5' – ACTTGAGAGCTAGCTTACCAC – 3' 5' – ACATGCTGCTCTGTAAGATCA – 3'
EjTuba	5' – CAATGCCTGCTGGGAGTTGTA – 3' 5' – ACTCCGACCTCCTCGTAATCT– 3'
grimp	5' – ATGGAGCTAACCAGCATCATC – 3' 5' – CTAATAAAAACGAAGTTCTGA – 3'
horu	5' – TTGCTCTGTTTGCAACCAAGA – 3' 5' – TGTCACGGTCAGGGTTACGAC – 3'
mino	5' – ACGCGGGGTTCAGTATTATCT – 3' 5' – ACTCAAAGAACTTCCTCAACC – 3'
EjAct	5' – AATTGGGATGATATGGAGAAG – 3' 5' – ATCCACATTTGTTGGAAGGTG – 3'

stump at both ends were cultured on a 0.8% agar plate (60-mm in diameter) at 24°C, and fixed at 3, 6, 12 and 96 hours after amputation (hpa).

Subtractive hybridization

To isolate differentially expressed genes, we performed cDNA subtractive hybridization between growing worms and regenerating worms 6 or 12 h after amputation according to a previously described protocol (Takeo *et al*., 2008). Through cDNA subtractive hybridization and RT-PCR analysis, five genes were isolated and expanded by 3' and 5' direction RACE. Then, sequences were compared with known and predicted nucleotide sequences using the National Center of Biotechnology Information (NCBI) and the earthworm EST databases (LumbriBASE and EandreiBASE), and also searched for conserved domains on each predicted amino acid sequence using NCBI Conserved Domain Search server. The open reading frame was found by the NCBI ORF Finder. *EjPsmd* (Gene accession number AB430779) and *EjTuba* (Gene accession number EU118298) were shown to have high homology to the 26S proteasome non-ATPase regulatory subunit and the alpha tubulin gene, respectively. Any homologous genes for the remaining three were not identified (*grimp*, Gene accession number AB430780). Gene accession numbers of *mino* and *horu* are EU118299 and EU118300, respectively.

Semi-quantitative RT-PCR

Total RNA was extracted from intact worms and regenerating fragments at 3, 6, 12 and 24 h after amputation using ISOGEN (NIPPON GENE), then cDNA was synthesized using SMART™ PCR cDNA Synthesis kit (CLONTECH). The *E. japonensis* actin gene *EjAct* was amplified as an internal control. The list of gene-specific primers is presented in Table 2.

Northern blot analysis

By using an entire sequence of *grimp* other than poly (A) tail, the digoxigenin-labeled RNA probes were synthesized using the DIG RNA labeling kit (SP6/T7, Roche), and were degraded by alkaline hydrolysis to fragments of approximately 500 nt in length. Total RNA (10 μg per lane) was fractioned by 1% formaldehyde–agarose denaturing gel electrophoresis. Following electrophoresis, the gel was incubated in 50 mM NaOH for 25 min, 200 mM NaOAc for 20 min twice. Then total RNA was transferred to a Hybond-N+ membrane using upward capillary transfer with 20x SSC. Gel electrophoresis and transfer of total RNA to the membrane were carried out in accordance with standard protocols (Sambrook *et al.,* 2001). The transferred membrane was incubated in 0.05 N NaOH for 5 min, 2x SSC for 30 min, then baked at 80°C for 2 hrs to fix total RNA. Following prehybridization that was carried out at 50°C for 1 h in hybridization buffer (50% deionized formamide, 5x SSC, 2x Denhardt's solution, 1% SDS), the membrane was incubated in fresh hybridization buffer containing 100 ng/ml of the probe at 50°C for 16 hr. The hybridized membrane was washed twice in 2x SSC for 5 min each, washed twice in 0.2x SSC/0.1% SDS at 60°C for 30 min each. Washed membrane was incubated at room temperature for 1 h in 1% blocking reagents (Roche) in maleic acid solution (0.1 M maleic acid, 0.15 M NaCl, 0.1% Triton-X 100, pH 7.5), then incubated at room temperature for 1 h with 1/2000 Anti-Dig/AP antibody (Roche) in 1% blocking reagents. After incubation, the membrane was washed three times in PBS for 20 min each, and a color reaction was performed with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) solution (Sigma).

Whole-mount and section* in situ *hybridization (WISH and ISH)

We synthesized a probe in a manner similar to that in Northern blot analysis, and performed whole-mount and section *in situ* hybridization essentially as described previously (Takeo *et al*., 2008). The major modifications were that the prehybridization and hybridization were carried out at 50°C.

Simultaneous ISH and BrdU immunohistochemistry

The intact and regenerating worms were soaked into 20 mM BrdU (5'-bromo-2'-deoxyuridine, SIGMA) for 3 hrs. After soaking, the worms were fixed with 4% paraformaldehyde at room temperature overnight. Following usual ISH as described above, the specimens were treated with 2 N HCl for 30 min, neutralized with 0.1 M sodium borate buffer (pH 8.5) twice for 15 min each, washed in PBS, incubated with a 10% Fetal Calf Serum (FCS) blocking solution for 10 min followed by the rat monoclonal anti-BrdU antibody (50 μg/ml in PBS with 10% FCS and 0.1% NaN_3, abcam) for 2 hrs. The sections were then visualized with a rhodamine-conjugated goat anti-rat IgG antibody (30 μg/ml in PBS, ROCKLAND) for 2 hrs, rinsed with PBS, mounted with 50% glycerol and observed with a fluorescence microscope.

RNA interference experiments

Double stranded RNAs (dsRNAs) were synthesized according to the protocol for *Drosophila* (Kennerdell and Carthew, 1998). The template for three different types of *grimp* and *EGFP* dsRNA synthesis were amplified using gene-specific primers containing 18 or 19 gene-specific nucleotides and a T7 promoter sequence with an extra "gggcg", as presented in Table 3. Single stranded RNA was synthesized using T7 RNA polymerase (Roche), purified by RNeasy Mini kit® (Qiagen), annealed at 65°C for 15 min, at 37°C for 60 min, and then at 4°C overnight. The quality and quantity of dsRNA were analyzed by agarose gel electrophoresis. RNA interference (RNAi) was performed by injection of *grimp* dsRNA or *EGFP* dsRNA (1 μg/μl, 100 nl per worm) as a negative control. Under anesthetization in a saturated L-menthol solution, intact worms were injected with dsRNA into the coelom once a day for three days. Thee hours after the last injection the worms were transected without anesthesia, and allowed to regenerate for 6, 12 and 96 h with additional dsRNA booster injection 24 h after amputation (1 μg/μl, 20 nl per fragment).

TABLE 3

GENE-SPECIFIC PRIMERS FOR dsRNA SYNTHESIS

dsRNA	primer sequences
grimp	5' – TAATACGACTCACTATAgggcgTAGGAGCTAACCAGCATCA – 3' 5' – TAATACGACTCACTATAgggcgCTAATAAAAACGAAGTTCT – 3'
grimp5'	5' – TAATACGACTCACTATAgggcgGATTGTAGTTTGGACACT – 3' 5' – TAATACGACTCACTATAgggcgAGCTCTATGAAGCTAGCC – 3'
grimp3'	5' – TAATACGACTCACTATAgggcgTGTACTTGCCGTACACTA – 3' 5' – TAATACGACTCACTATAgggcgGCAAGTATATTATACAGT – 3'
EGFP	5' – TAATACGACTCACTATAgggcgATGGTGAGCAAGGGCGAG – 3' 5' – TAATACGACTCACTATAgggcgTTGAAGTTCACCTTGATG – 3'

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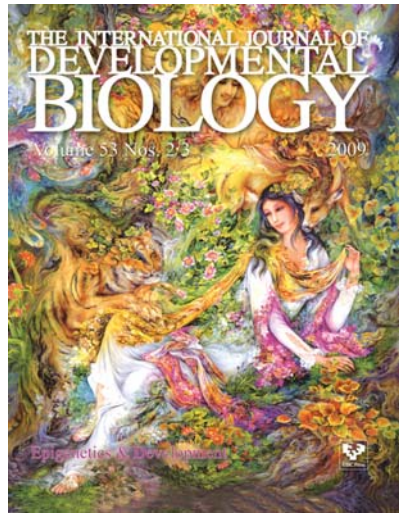

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