

Planarian pharynx regeneration revealed by the expression of myosin heavy chain-A

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ABSTRACT The pharynx is a distinctive organ in the center of the body of planarians. Although the process of pharynx regeneration has been studied previously, the details and mechanism of the process remain controversial. We examined the process of regeneration of the pharynx in the planarian *Dugesia japonica* in detail by *in situ* hybridization and immunohistochemistry for myosin heavy chain-A (DjMHC-A), which is mainly expressed in the pharynx muscles and pharynx-anchoring muscles. We also monitored the behavior of the neoblasts in this process. In the regenerating posterior body fragment, the pharyngeal rudiment was formed by accumulation of cells that were probably undifferentiated cells derived from the neoblasts. The pharynx muscles appeared to differentiate in the rudiment in a manner that was coordinated with the differentiation of the pharynx-anchoring muscles in the region surrounding the rudiment. During this process, all cells containing mRNA for *DjMHC-A* also contained the DjMHC-A protein. These results argue against a previously proposed hypothesis that in the mesenchyme, 'pharynx-forming cells', which are committed to differentiate into the pharyngeal cells but have not yet differentiated, gather in the rudiment to form the pharynx (Agata and Watanabe, 1999). Rather, the present observations suggest that regeneration of the planarian pharynx proceeds by accumulation of cells that are probably undifferentiated cells derived from neoblasts in the rudiment, followed by their differentiation into the pharyngeal cells there.

KEY WORDS: *Platyhelminthes, flatworm, neoblast, stem cell, muscle*

Freshwater planarians have remarkable regeneration ability. When a planarian is cut, the multi- or totipotent stem cells, called 'neoblasts', distributed throughout the remaining body parts seem to differentiate into all of the lost types of cells, and ultimately the planarian regenerates completely (for reviews, see Brønsted, 1969; Baguña *et al.*, 1994). The pharynx is a distinctive organ which is present in the center of the planarian body (Fig. 1). In the regenerating posterior body fragment, the pharynx forms in the central region (Kobayashi *et al.*, 1999). Regarding regeneration of the planarian pharynx, two hypotheses have been proposed: (1) undifferentiated cells from the mesenchyme accumulate to form the pharyngeal rudiment. The pharyngeal rudiment then grows by incorporation of new undifferentiated cells. The pharyngeal cells are generated *de novo* from undifferentiated cells in the rudiment (Bueno *et al.*, 1997). Alternatively, (2) 'pharynx-forming cells' that are undifferentiated, but committed to differentiate into the pharyngeal cells, appear in the mesenchyme and become concentrated in the rudiment. The pharyngeal rudiment then grows by continuous incorporation of pharynx-forming cells, (Kobayashi *et al.*, 1999;

reviewed in Agata and Watanabe, 1999). The former was based on observation using pharynx specific antibodies (Bueno *et al.*, 1997). The latter hypothesis was based on the results of *in situ* hybridization using as the probe myosin heavy chain-A gene (*DjMHC-A*), which is mainly expressed in the pharyngeal muscles. The 'pharynx-forming cells' were defined as 'committed cells', in which genes required for the differentiation of the pharynx were expressed, but their mRNAs were not translated yet (Kobayashi *et al.*, 1999; Agata and Watanabe, 1999). The difference between the two hypotheses lies in whether 'pharynx-forming cells' are present in the mesenchyme.

Recently, we were successful in the production of an anti-serum against DjMHC-A protein (Ito *et al.*, 2001; Orii *et al.*, submitted for publication). In the present study, we examined the process of regeneration of the pharynx in detail by simultaneous detection of

Abbreviations used in this paper: BrdU, bromodeoxyuridine; DjMHC-A, *Dugesia japonica* myosin heavy chain-A; PCNA, proliferating cell nuclear antigen.

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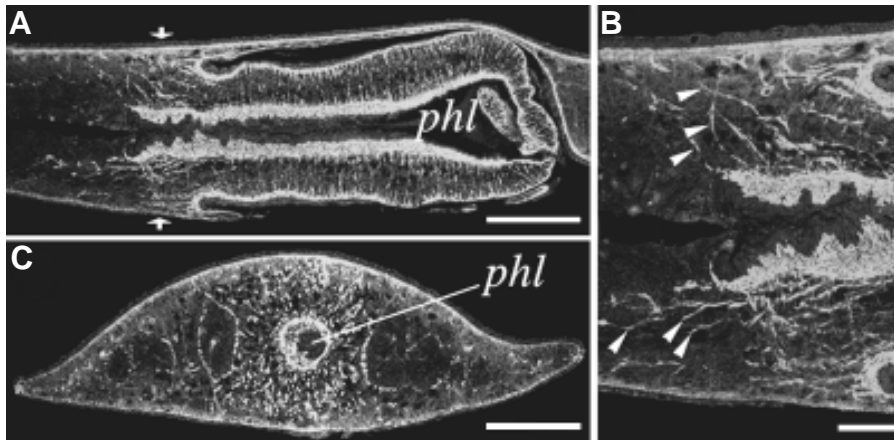


Fig. 1. Distribution of DjMHC-A muscles visualized by immunostaining with an antiserum against DjMHC-A. (A) Sagittal section. Scale bar, 200 μm . (B) Higher magnification of the base of the pharynx in (A). Arrowheads indicate pharynx-anchoring muscles. Scale bar, 50 μm . Anterior is on the left. Dorsal is on the top. (C) Cross section at the position indicated by arrows in (A). Scale bar, 200 μm . phl, pharynx lumen.

the mRNA and the protein for DjMHC-A. In addition, the behavior of neoblasts during pharynx regeneration was monitored by labeling with bromodeoxyuridine (BrdU) and by immunostaining with an antiserum against proliferating cell nuclear antigen (PCNA).

Immunohistochemical staining revealed that in the pharynx, myosin heavy chain-A (DjMHC-A) was present in the inner and outer circular muscles and the radial muscle fibers between them. In addition, DjMHC-A was present in the circular body-wall muscles, muscle fibers surrounding the intestine, and the pharynx-anchoring muscles connecting the body wall and the pharynx (Fig. 1; Orii *et al.*, submitted for publication). The pharynx-anchoring muscles extend radially from the base of the pharynx to the body wall (Fig. 1C).

To investigate the early processes of pharynx regeneration, we focused on DjMHC-A as a pharyngeal marker and examined the distribution of muscle cells containing the mRNA and the protein by *in situ* hybridization and immunohistochemistry, respectively. Previously, Kobayashi *et al.* (1999) studied the regeneration of the pharynx by *in situ* hybridization for transcripts of the *DjMHC-A* gene. According to their observations and our present observations (Fig. 2 and data not shown), at 1-1.5 days of regeneration, *DjMHC-A*-mRNA-positive cells appear in precisely the area where the pharyngeal rudiment will form. At 1.5-2 days of regeneration, DjMHC-A-mRNA-positive cells accumulate to form the pharyngeal rudiment. A slit is opened in the region posterior to the cluster of *DjMHC-A*-mRNA-positive cells. At 2-3.5 days, *DjMHC-A*-mRNA-positive cells continuously appear in the mesenchyme surrounding the rudiment. The rudiment grows to

form the pharyngeal cavity. It looks as if *DjMHC-A*-mRNA-positive cells appear in the mesenchyme, migrate to the rudiment, and form the pharynx muscles. Based on these observations, Kobayashi *et al.* (1999) proposed the existence of 'pharynx-muscle-forming cells', in which the *DjMHC-A* gene was transcribed but the mRNA was not yet translated. They suspected that 'pharynx-muscle-forming cells' were committed, but had not yet differentiated into pharynx muscle cells, and were moving into the rudiment to form a pharynx (Agata and Watanabe, 1999). Our findings did not support this proposal, as we found that all cells containing *DjMHC-A* mRNA also contained DjMHC-A protein, as indicated by double staining with an RNA probe

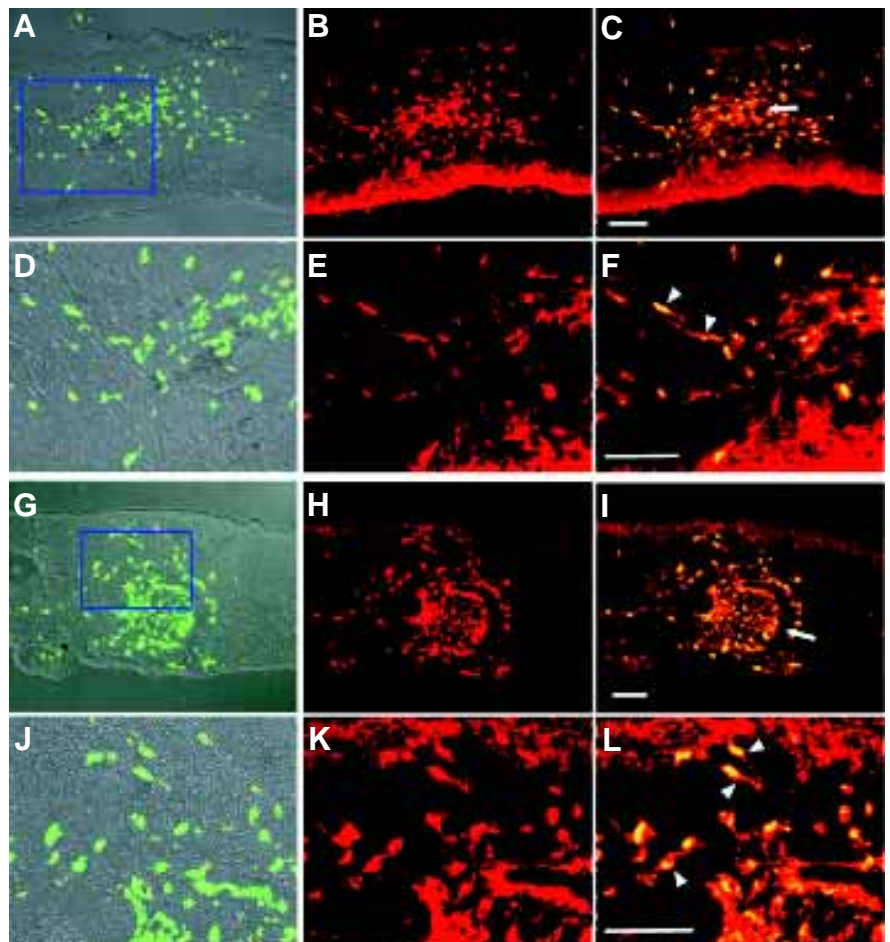


Fig. 2. Simultaneous detection of the mRNA and the protein for DjMHC-A during regeneration of the posterior fragment. (A-F) At 2 days of regeneration. (G-L) At 3.5 days of regeneration. The left column is merged images of *in situ* hybridization for mRNA derived from the *DjMHC-A* gene (green) and Nomarski images. The middle column is images of immunostaining with the anti-DjMHC-A antibody (red). The right column is merged images of the left (*in situ* hybridization) and the middle images. The areas indicated in (A) and (G) were magnified in (D-F) and (J-L), respectively. All specimens were sagittal sections. Scale bars, 50 μm .

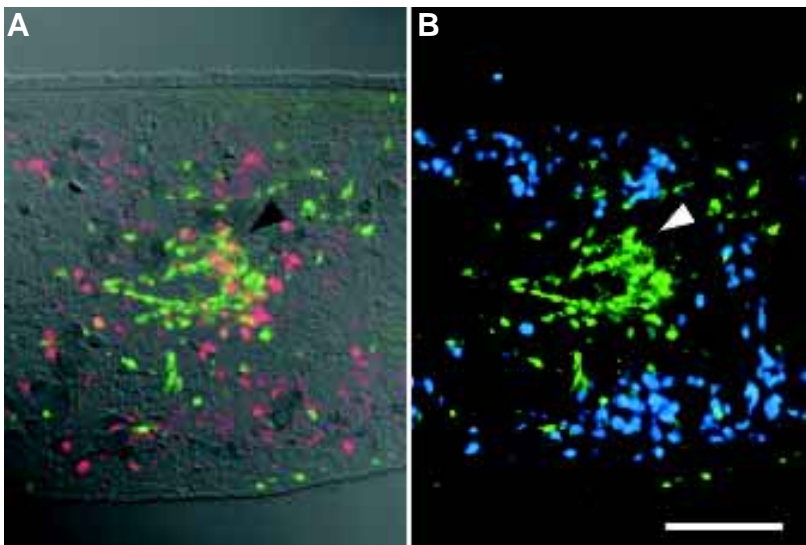


Fig. 3. Behavior of the neoblasts in the pharyngeal rudiment on day 2 of regeneration. (A) Nomarski images, and images of *in situ* hybridization for *DjMHC-A* (green) and immunostaining for BrdU (red) were superimposed. Note the presence of signals of BrdU and *DjMHC-A* in the rudiment. (B) Images of *in situ* hybridization for *DjMHC-A* (green) and immunostaining for PCNA (blue) were superimposed. Note the absence of PCNA signals in the rudiment. The frames of (A) and (B) are the same. Arrowheads indicate the pharynx rudiment. Scale bar, about 0.5 mm.

for *DjMHC-A* mRNA and an antiserum against *DjMHC-A* protein (Fig. 2). This finding indicates that the translation occurs just after the transcription of the *DjMHC-A* gene. Furthermore, these cells containing both the mRNA and protein of *DjMHC-A* already appeared to begin to elongate at 2 days of regeneration (arrowheads in Fig. 2F). These elongated cells are present in the region surrounding the pharyngeal rudiment (arrowheads in Fig. 2L). They are very similar to pharynx-anchoring muscles in their distribution and their direction of extension as indicated, in comparison with *DjMHC-A* muscle fibers in the intact organism (Fig. 1). Thus, the pharynx-anchoring muscles must correspond to 'pharynx-muscle-forming cells', which were referred to by Kobayashi *et al.* (1999). At 3.5 days of regeneration, the morphology of pharynx-anchoring muscles appeared to be even more distinct. This suggests that the pharynx regenerates in coordination with regeneration of the surrounding tissue, including pharynx-anchoring muscles.

To investigate the behavior of the neoblasts in pharynx regeneration, regenerating fragments labeled with BrdU (Newmark and Sánchez Alvarado, 2000; Kato *et al.*, submitted for publication). After cutting the worms in the post-pharyngeal region, the posterior fragment of the planarian, was pulse-labeled with BrdU for 3 hours, and then allowed to regenerate for 2 days. Sagittal sections were subjected to *in situ* hybridization for expression of the *DjMHC-A* gene, and immunostaining with anti-PCNA and anti-BrdU antibodies (Fig. 3). As the neoblasts are the only type of cells with proliferating activity, PCNA is a useful marker for identifying them. The level of PCNA decreases gradually as the neoblasts differentiate (Ito *et al.*, 2001). In contrast to the many PCNA-positive cells in the mesenchyme, we detected few PCNA-positive cells in the rudiment during regeneration of the pharynx (blue in Fig. 3B), suggesting that few neoblasts proliferate there. This is in good agreement with the previous reports which showed that there are no proliferating cells in the regenerative tissues such as the pharyngeal rudiment (Baguña, 1976) and the blastema (Morita and Best, 1984; Saló and Baguña, 1984; Orii *et al.*,

unpublished). In contrast, several cells labeled with BrdU (red in Fig. 3A) were present in the pharyngeal rudiment, and some of them also contained *DjMHC-A* mRNA (green in Fig. 3A,B). These results suggest that the cells in the rudiment are derived from the neoblasts in the mesenchyme, and have begun to differentiate somewhat to a limited extent. These processes are summarized schematically in Fig. 4. The neoblasts proliferate in the mesenchyme (red circles). During pharynx regeneration, after these cells proliferate (white circles, probably still undifferentiated), they move, accumulate, and then differentiate into the pharyngeal cells in the rudiment (green circles). Simultaneously, the tissue surrounding the pharynx must also be coordinately differentiated from the neoblasts (Fig. 4). These observations basically support a hypothesis proposed by Bueno *et al.* (1997). In addition, the coordinated differentiation of the pharynx-anchoring muscles indicates that pharynx regeneration is a re-organization process occurring in the whole fragment rather than just in the rudiment.

In this study, we have shown that pharynx regeneration proceeds by continuous accumulation in the pharyngeal rudiment of cells that are derived from the neoblasts and probably undifferentiated. The differentiation of the pharyngeal cells seems to occur in the rudiment. Although we do not know when the neoblasts moving into the pharyngeal rudiment become committed to differentiation into pharyngeal cells, at least, we do not have any data supporting the idea that committed cells such as 'pharynx-forming-muscle cells' appear in the mesenchyme and move into the pharynx rudiment.

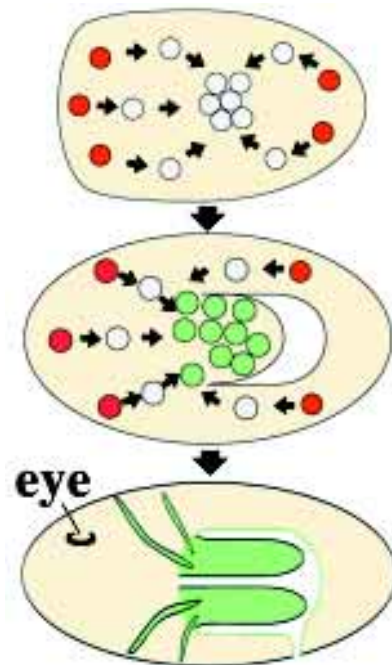


Fig. 4. Schematic diagram of pharynx regeneration. Red, white and green circles indicate PCNA-positive cells (undifferentiated cells with proliferating ability), PCNA- and *DjMHC-A*-negative cells (probably undifferentiated cells after cell proliferation) and *DjMHC-A*-positive cells (differentiated cells), respectively. The neoblasts proliferate in the mesenchyme, move, accumulate in the area where the pharynx will form, and then differentiate into the pharyngeal cells.

Recently, Koinuma *et al.* (2000) showed that a putative transcription factor gene, *DjFoxA*, was expressed in the region surrounding the pharynx in the central part of the body. It is possible that the neoblasts differentiate into the pharyngeal cells, followed by regionalization of the central part of the body under the control of some genes such as *DjFoxA*. It will also be necessary to examine when and how other types of pharyngeal cells differentiate.

Experimental Procedures

Animals

An isogenic strain, G1, of the planarian *Dugesia japonica* was used (Orii *et al.*, 1999). The worms were maintained asexually in autoclaved tap water at 22–24°C and fed chicken liver twice a week. After starvation for at least 1 week, worms about 1 cm in length were cut transversally at a position posterior to the pharynx. The posterior fragments were then allowed to regenerate.

Histochemical Analyses

Fixation and preparation of samples were described previously (Ito *et al.*, 2001). Paraffin-embedded sections were subjected to *in situ* hybridization for *DjMHC-A* mRNA using a TSA kit with streptavidin-FITC (NEN Life Science Products) (Kobayashi *et al.*, 1998; Kato *et al.*, 1999) and then to immunohistochemical staining for DjMHC-A protein using anti-rabbit IgG conjugated with Cy3 as the secondary antibody (Jackson Immuno Research Laboratories) (Ito *et al.*, 2001). The antibody against DjMHC-A protein was produced using the specific carboxyl terminal region of DjMHC-A expressed in *E. coli*, as an antigen. The antibody has no cross-reactivity with the other myosin heavy chain-B in the planarian. Specimens were observed under a confocal laser scanning microscope LSM510 (Zeiss) or a fluorescent microscope.

Labeling of the neoblasts with bromodeoxyuridine (BrdU) was performed basically according to Ladurner *et al.* (2000) (Kato *et al.*, submitted for publication). Immunohistochemistry with anti-PCNA antibody was described previously (Ito *et al.*, 2001). Simultaneous multicolor detection of *DjMHC-A* mRNA, *DjPCNA* protein and BrdU was performed using a TSA kit with streptavidin-FITC, streptavidin-coumarin and streptavidin-Texas Red (NEN Life Science Products), respectively.

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