

Regeneration in *Macrostomum lignano* (Platyhelminthes): cellular dynamics in the neoblast stem cell system

Katharina Theresia Nimeth · Bernhard Egger ·
Reinhard Rieger · Willi Salvenmoser · Roland Peter ·
Robert Gschwentner

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Abstract Neoblasts are potentially totipotent stem cells and the only proliferating cells in adult Platyhelminthes. We have examined the cellular dynamics of neoblasts during the posterior regeneration of *Macrostomum lignano*. Double-labeling of neoblasts with bromodeoxyuridine and the anti-phospho histone H3 mitosis marker has revealed a complex cellular response in the first 48 h after amputation; this response is different from that known to occur during regeneration in triclad platyhelminths and in starvation/feeding experiments in *M. lignano*. Mitotic activity is reduced during the first 8 h of regeneration but, at 48 h after amputation, reaches almost twice the value of control animals. The total number of S-phase cells significantly increases after 1 day of regeneration. A subpopulation of fast-cycling neoblasts surprisingly shows the same dynamics during regeneration as those in control animals. Wound healing and regeneration are accompanied by the formation of a distinct blastema. These results present new insights, at the cellular level, into the early regeneration of rhabditophoran Platyhelminthes.

Keywords Regeneration · S-phase · Cell cycle · Planarian · *Macrostomum lignano* (Platyhelminthes)

Introduction

Platyhelminthes have often been regarded as resembling the ancestor of all Bilateria (e.g., Moore and Willmer 1997). Although morphological data most often place them at the basis of all Bilateria (Tyler 2001), molecular data are controversial (Carranza et al. 1997; Telford et al. 2003). At present, most molecular analyses portray a phylogenetic split, with the Acoelomorpha as the basal-most taxon in the Bilateria and the remaining flatworms (Catenulida, Rhabditophora) as basal taxa in the lophotrochozoan protostomes (Pasquinelli et al. 2003; Cook et al. 2004; Baguña and Riutort 2004a,b). The conflicting views regarding the morphological and molecular phylogenies, i.e., whether the Platyhelminthes (including Acoelomorpha, Catenulida and Rhabditophora) are monophyletic, still cannot be resolved. However, a proposed synapomorphy for flatworms is the possibly totipotent stem cell system (Ehlers 1985).

Platyhelminthes apparently possess a unique stem cell system in the animal kingdom: the neoblast system. This system is responsible for postembryonic proliferation, growth, tissue homeostasis, and regeneration (Baguña 1981; Ehlers 1985; Ladurner et al. 2000; Reuter and Kreshchenko 2004; Peter et al. 2004). Neoblasts are the only cells that proliferate in adult platyhelminths (Hori 1997; Hori and Kishida 1998; Ladurner et al. 2000; Newmark and Sánchez Alvarado 2002). Studies on free-living flatworms such as acoels (Drobysheva 1986; Ladurner and Rieger 2000; Gschwentner et al. 2001), macrostomids (Palmberg 1990; Ladurner et al. 2000;

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K. T. Nimeth · B. Egger (✉) · R. Rieger · W. Salvenmoser ·
R. Gschwentner
Institute of Zoology,
University of Innsbruck,
Technikerstrasse 25,
Innsbruck 6020, Austria
e-mail: bernhard.egger@uibk.ac.at

R. Peter
Department of Genetics and General Biology,
University of Salzburg,
Salzburg, Austria

Nimeth et al. 2004), and triclads (Baguña 1981, 1998; Saló and Baguña 1984, 2002; Baguña et al. 1989; Newmark and Sánchez Alvarado 2000; Sánchez Alvarado et al. 2002; Peter et al. 2004) have shown the crucial role played by stem cells in these groups. The stem cell system of triclads and macrostomids, and also of acoels, seems to be a prerequisite for their regenerative capacity.

High regenerative potential is characteristic for many Platyhelminthes, although it varies considerably between different taxa. Asexual reproduction is often linked with a good regeneration capacity (Reuter and Kreshchenko 2004). *Macrostomum lignano*, which lacks an asexual reproduction mode, can regenerate all organ systems, with the exception of brain and pharynx (Egger et al. 2006a,b). The regeneration potential is extreme in the most potent representatives of the triclads (Brøndsted 1969; Baguña 1998), which have been most thoroughly investigated for their regenerative capacity among Platyhelminthes (see Brøndsted 1969; Gremigni and Miceli 1980; Baguña et al. 1990, 1994; Peter 2001; Saló and Baguña 2002). However, specific labeling of S-phase neoblasts by using tritiated thymidine or bromodeoxyuridine (BrdU) has not been successful until recently and is possible only via BrdU-contaminated food or micro-injections (Newmark and Sánchez Alvarado 2000; Reddien et al. 2005). Because of such technical restrictions, attempts have been made to analyze the cellular dynamics of stem cells in regenerating triclads neither with BrdU, nor with molecular markers (Salveti et al. 1998, 2000; Agata and Watanabe 1999; Agata 2003).

In this study, the cellular dynamics in regenerating *M. lignano*, a macrostomorph representative of the rhabditophoran Platyhelminthes, have been examined for the first time. Because of their developmental and morphological characteristics, macrostomorphans are considered to be the basal-most taxon within rhabditophoran flatworms, whereas triclads are believed to have a number of derived features among rhabditophorans (Ehlers 1985, Rieger 1996). Cell-cycle analyses in intact specimens of *M. lignano* have revealed a fast and flexible stem cell system in this organism (Rieger et al. 1994; Ladurner et al. 2000; Nimeth et al. 2004; for reviews, see Peter et al. 2001; Reuter and Kreshchenko 2004). Mitotic cells and S-phase cells are distributed in two bands along the lateral sides of the animal and are absent in front of the eyes and along the median axis (Ladurner et al. 2000; Nimeth et al. 2004; Egger et al. 2006a,b). Neoblasts are found in close proximity to the main nerve cords, suggesting a guiding function of the central nervous system (Rieger et al. 1994; Bode et al. 2006). Starvation and feeding experiments have revealed the high adaptability of proliferative activity and fast responses of the neoblast system in intact non-regenerating animals (Nimeth et al. 2004). These features

of the stem cell system suggest the existence of neoblast subpopulations. At the ultrastructural level, various types of neoblasts (somatic neoblasts in the mesenchymal space and the gastrodermis, and gonadal S-phase cells) have been identified and distinguished according to their location (Rieger et al. 1999; Bode et al. 2006). Within the “mesodermal” neoblasts, three different stages can be discerned because of their cytoplasmic and nuclear organization (Rieger et al. 1999).

This study has focused on the cell-cycle activity of the neoblast system in *M. lignano* at specific time points after amputation of the tail plate and during the formation of the posterior blastema. The simple application of BrdU merely by soaking the animals in a BrdU-containing medium allows the detailed analysis of cellular dynamics and makes this unpigmented hermaphrodite an excellent model organism within the Platyhelminthes, in addition to triclads.

Materials and methods

Cultures

Cultures of *M. lignano* were reared in Petri dishes with the diatom *Nitzschia curvilineata* according to Rieger et al. (1988). They were maintained in a temperature-controlled chamber at 20°C, 50% humidity, and a photoperiod of 14 h light and 10 h dark. For examination, animals were washed in Petri dishes with artificial sea water (ASW, 32‰). Amputation of the tail plate was performed in a small drop of ASW on a slide. Subsequently, specimens were transferred to Petri dishes filled with ASW. After amputation, animals could not adhere and had lost parts of their reproductive structures, but otherwise behaved normally.

Freshly hatched juveniles were collected from culture dishes containing only eggs to ensure a similar and known age of the experimental animals. These standardized animals were fed ad libitum for 4–5 weeks after hatching. For culturing and technical reasons, 11 different batches of standard animals were used for all experiments.

Double-labeling of S-phase cells (anti-BrdU) and mitoses (anti-phospho histone H3 mitosis marker)

Control animals and regenerating animals were labeled with 5-bromo-2'-deoxyuridine (BrdU; Sigma) by a 30-min pulse of 5 mM BrdU in ASW. Specimens were rinsed in ASW (3×10 min), relaxed in 1:1 MgCl₂·6H₂O (7.14%):ASW (20 min), fixed in 4% formaldehyde (60 min, at room temperature), rinsed in PBS (phosphate-buffered saline, 3×10 min), and incubated in PBS-T (0.1% Triton X-100 in

PBS, 60 min). Treatment with 0.15 $\mu\text{g/ml}$ Protease XIV (Sigma) at 37°C (30 min) was followed by incubation in 0.1 N HCl (10 min, on ice). The animals were then transferred to 2 N HCl (60 min, 37°C) to denature DNA, rinsed in PBS (3 \times 10 min), held in BSA-T (bovine serum albumin in PBS-T, 30 min, room temperature), and incubated in a mixture of primary mouse-anti-BrdU (Roche, 1:600 in BSA-T) and primary anti-phospho histone H3 mitosis marker (anti-phos-H3; Upstate Biotechnology, 1:150 in BSA-T) at 4°C overnight. After being rinsed in PBS (3 \times 10 min), specimens were incubated in a cocktail of secondary fluorescein isothiocyanate (FITC)-conjugated goat-anti-mouse (Dako, 1:150 in BSA-T) and tetramethylrhodamine isothiocyanate (TRITC)-conjugated swine-anti-rabbit (Dako, 1:150 in BSA-T) antibodies at room temperature for 60 min. Rinses in PBS (3 \times 10 min) were followed by mounting in Vectashield (Vector Laboratories) and observation with a Reichert POLYVAR epifluorescence microscope (Leica Austria), a Leica DM5000 B digital microscope equipped with a Penguin 600 CL (PIXERA) digital camera and the program Viewfinder for image processing, or a confocal ZEISS LSM 510.

To analyze cellular dynamics during regeneration, *M. lignano* were double-labeled with anti-BrdU/ anti-phos-H3 according to various protocols (1–6). Control animals were labeled (1) with 5 mM BrdU for 30 min and fixed immediately to demonstrate the number of S-phase and mitotic cells in control animals, (2) with 5 mM BrdU for 30 min (pulse) followed by 2 h and 4 h of regeneration (chase), or (3) by continuous incubation in 5 mM BrdU for 2 h and 4 h. Regenerating animals were labeled (4) after 2 h, 4 h, 8 h, 24 h, and 48 h of regeneration with 5 mM BrdU for 30 min (pulse), (5) immediately after amputation followed by a

2-h and 4-h chase (pulse-chase), or (6) by continuous incubation in 5 mM BrdU for 2 h and 4 h after amputation.

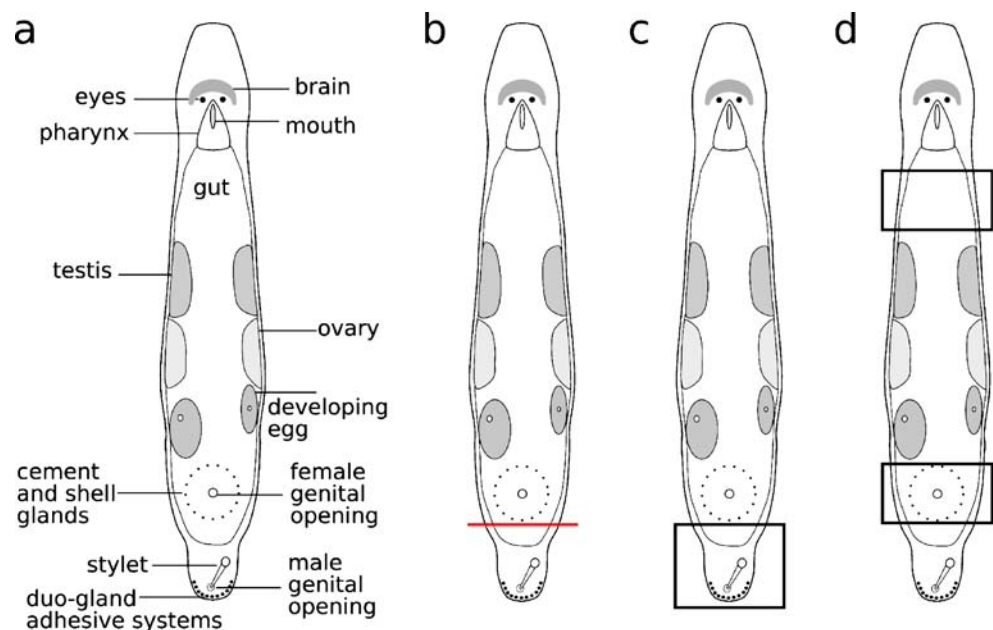
Analysis of cellular dynamics

The number of mitoses, the number of S-phase cells, and the number of anti-BrdU labeled mitoses (double-labeled cells) were determined. Amputation was performed behind the female genital opening near the caudal border of the shell and cement glands (Fig. 1b). In control animals, cell numbers in the area behind the cutting level (Fig. 1c) were counted to quantify the loss of stem cells caused by amputation. In control animals and regenerating specimens, cell numbers in the whole animal and two defined segments were counted to distinguish general and local responses to amputation (Fig. 1d). The diameter of the shell and cement glands around the female genital opening was defined as the length of the segments. The back (rear) segment lay at the female genital opening and the front segment of the same length was defined as being behind the pharynx but in front of the testes.

Staining of semithin sections according to Heidenhain

After amputation and regeneration, animals were relaxed in $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (7.14%), fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) containing 10% sucrose (1 h), postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer (1 h), dehydrated in a standard acetone series, and embedded in Epon/Araldite after Mollenhauer (1964). Semi-thin sections were cut with a Reichert Ultracut UCT and mounted on slides. Labeling was performed according to Heidenhain (1885). Sections were treated with saturated

Fig. 1 Representation of *Macrostomum lignano*. **a** Overview of the highlighted organ systems. **b** Cutting level (red line) immediately behind the shell and cement glands of the female genital opening. **c** Area examined in control animals to estimate the loss of stem cells after amputation of the tail plate. **d** The back segment and front segment were of equal lengths in order to distinguish local and general responses of the stem cell system



sodium methylate (2×2 min) to remove Epoxy resin, rehydrated through a decreasing ethanol series (100%, 95%, 70%, 50%, 30%, for 2 min each), and transferred to distilled water before removal of OsO₄ in 5% H₂O₂ for 10 min. Iron hematoxylin staining (30 min in 4% iron alum at 60°C) was followed by washes (2×3 min) in distilled water at 60°C, 30 min in Heidenhain's iron hematoxylin at 60°C, and washes (2×3 min) in distilled water at 60°C. Destaining was performed with saturated picric acid until nuclei stood out clearly against a colorless background. The sections were then rinsed in distilled water, washed in running tap water for 10 min, dehydrated (ethanol series: 90%, 90%, 100%, 100%), incubated in butyl acetate (2×10 min), and coverslipped.

Microscopy

A Reichert POLYVAR epifluorescence microscope equipped with a TRITC (BP 546; LP560) and FITC (BP455–490; LP 515) filter set was employed to examine fluorescently stained specimens. A ZEISS LSM 510 was used to obtain confocal images. For imaging, autofluorescence of the algae was eliminated by means of a narrow band filter (BP 560–615).

Results

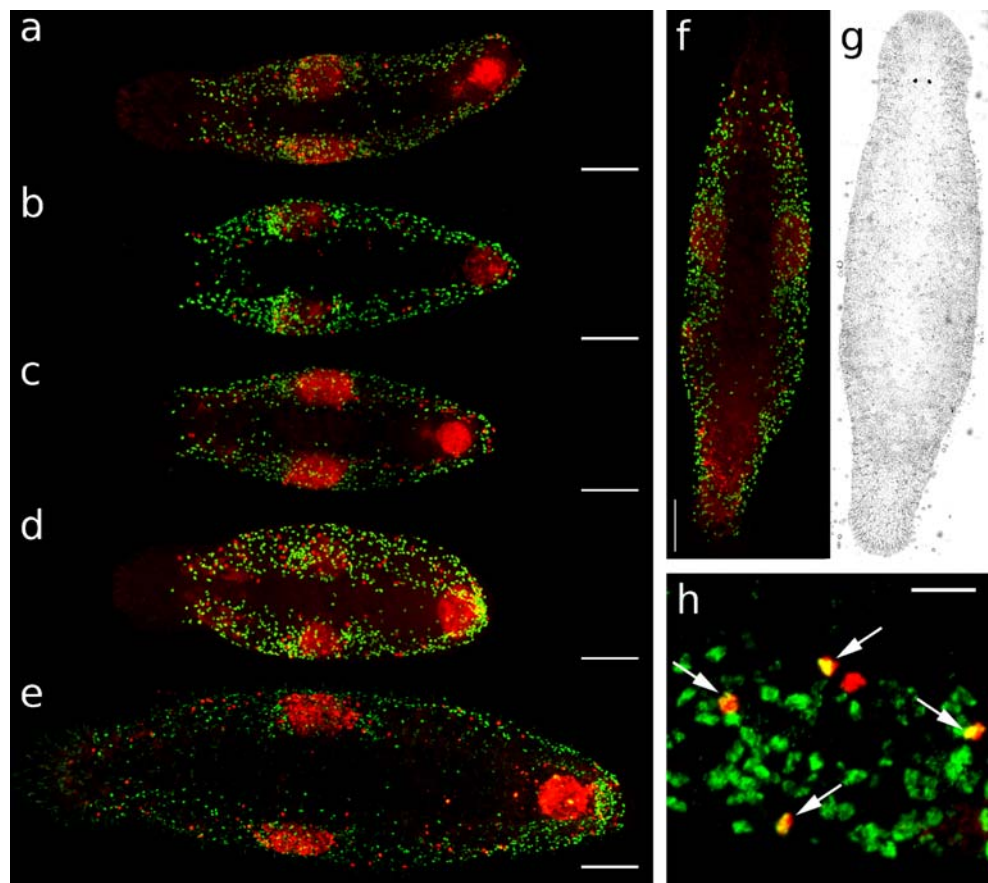
Numbers of S-phase cells and mitoses in whole-mounts

Animals were amputated behind the cement and shell gland ring of the female genital opening (Fig. 1) and left to regenerate for 2 h, 4 h, 8 h, 24 h, and 48 h (Fig. 2a–e). In controls and in amputated specimens, the total numbers of S-phase cells (green) and mitoses (red) were counted, and the total length of the specimens was measured. Additionally, the number of S-phase cells and mitoses behind the designated cutting level in control animals was determined in order to estimate the loss of proliferative cells after amputation.

Control animals had 494.33 ± 111.69 ($n=24$) S-phase cells, about 15% (76.31 ± 14.69) of which were counted behind the cutting level. Even if the total number of S-phase cells decreased soon after amputation (Fig. 3a), this decrease was not significant compared with control animals during the first 2–8 h of regeneration. After 48 h of regeneration, the number of S-phase cells (640.13 ± 51.58 , $n=5$) significantly exceeded normal values (one-way ANOVA, $F_{5,70}=9.39$, $P<0.001$).

The total number of mitoses in control animals was 38.25 ± 9.89 , about 7% (2.77 ± 2.20) of which were located

Fig. 2 Regeneration in *M. lignano*. **a–e** Confocal projections of anti-BrdU/anti-phos-H3 double-labeling (green S-phase cells, red mitoses). Regenerating animal 2 h (**a**), 4 h (**b**), 8 h (**c**), 24 h (**d**) and 48 h (**e**) after amputation. Bars 100 μ m. **f, g** Standard animals after a 30-min BrdU pulse. **f** Mitoses and S-phase cells are located in two lateral bands but not in the median axis and the rostrum. Bar 100 μ m. **g** Interference contrast image of **f**. **h** Enlargement of a labeled standard animal after a 30-min BrdU pulse and 4-h chase. Several cells are double-labeled (arrows). Bar 20 μ m



behind the cutting level. Mitotic activity showed a more significant response to amputation (Fig. 3b) than S-phase cells. During the first 2–4 h of regeneration, the number of mitoses significantly declined, started rising 8 h after amputation and significantly exceeded controls after 24 and 48 h (one-way ANOVA, $F_{5,70}=29.69$, $P<0.001$).

Numbers of S-phase cells and mitoses in segments

Neoblasts showed a distinct pattern of distribution in *M. lignano* with variable density in the different body regions. In this study, two segments of interest were defined: one located immediately at the area of amputation (back segment), and one in front of the testes (front segment, Fig. 1d), thereby excluding proliferating gonadal cells from the counts. The number of S-phase and mitotic cells in these sections was quantified at 2 h, 4 h, 8 h, 24 h, and 48 h after amputation (Fig. 3c,d).

A comparable response of S-phase and mitotic cell dynamics was observed. Although the trends observed in both segments were similar (Fig. 3c,d), not all changes were found to be equally significant.

S-phase cells

In control animals, the number of S-phase cells in the front segment (90.74 ± 27.30) was slightly higher than that in the back segment (71.58 ± 18.42 , Fig. 3c). After amputation, the number of S-phase cells in the front segment showed no significant decrease or increase during the observed time of regeneration, whereas S-phase cells in the back segment were significantly diminished at 2 h and 4 h after amputation but rose again after 8 h and significantly exceeded control values after 24 h and 48 h of regeneration (Fig. 3c, front segment: one-way ANOVA, $F_{5,64}=2.30$,

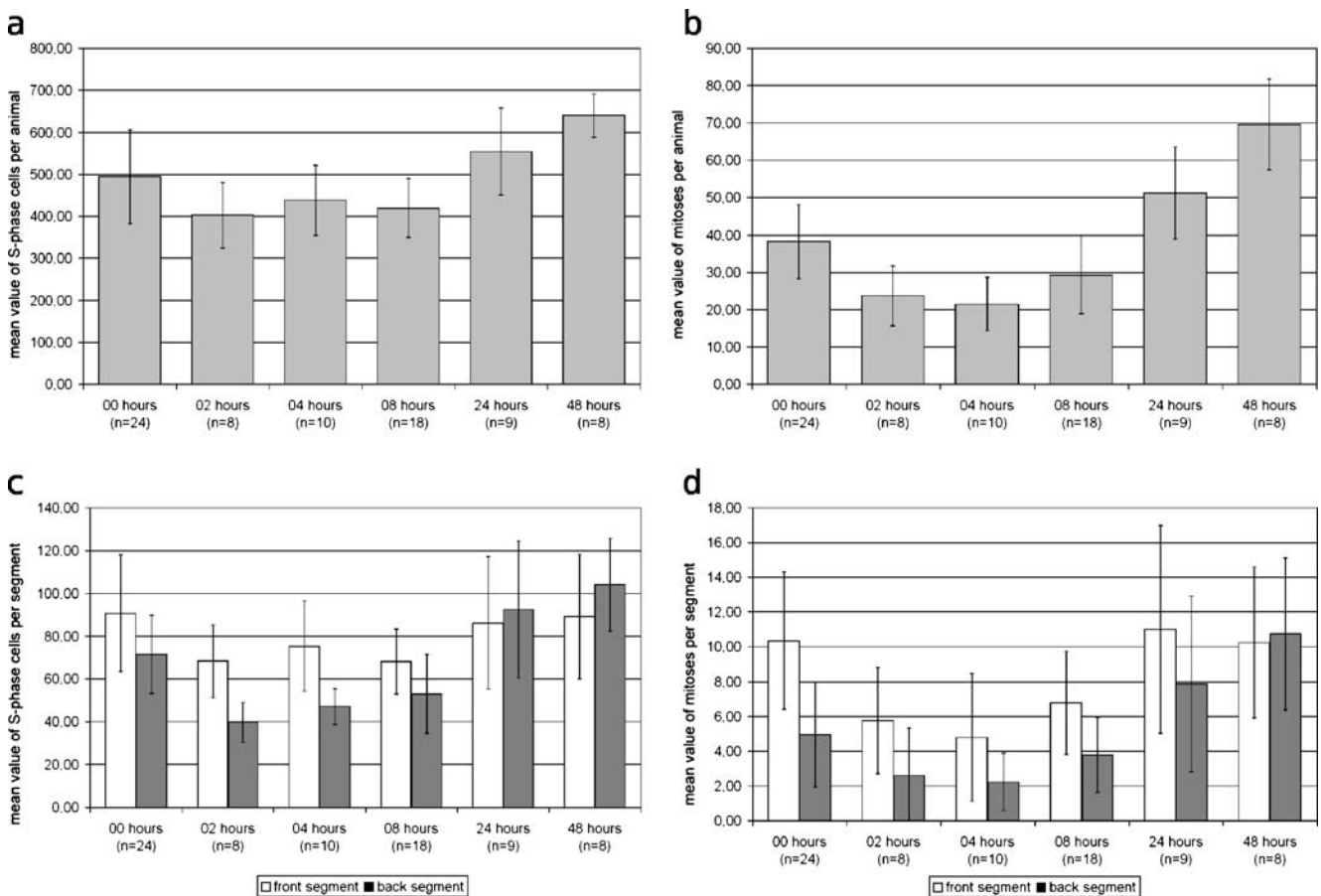


Fig. 3 Dynamics of S-phase cells and mitoses in *M. lignano*. **a, b** Cell counts at 2–48 h after amputation. **a** The number of S-phase cells decreased at 2–8 h after amputation, increased after 24 h, and significantly increased after 48 h (00 hours control animals). **b** Mitotic activity significantly decreased in the first 2–4 h of regeneration, was about the same as the control level after 8 h, and significantly increased after 24 and 48 h. **c, d** Dynamics in front and back segments. **c** No significant changes were observed in the front

segment, whereas S-phase activity in the back segment significantly decreased at 2 h and 4 h of regeneration and significantly increased after 24 h and 48 h. **d** In the front segment, mitotic activity significantly decreased at 4 h after amputation and reached control levels after 24 h. In the back segment, mitoses significantly diminished 2–8 h after amputation and showed a significant boost after 24 h and 48 h

$P=0.055$; back segment: one-way ANOVA, $F_{5,64}=15.54$, $P<0.001$).

Mitotic cells

In controls, the number of mitoses was more than two-fold higher in the front segment (10.35 ± 3.95) than in the back segment (4.96 ± 3.02 , Fig. 3d). During early regeneration, the number of mitoses in the front segment decreased and, at 4 h after amputation, was significantly lower than that in control animals (4.78 ± 3.67). After 24 h and 48 h of regeneration, mitotic values in the front region rose to standard values. A different pattern was observed for mitoses in the back region. Similar to S-phase cell dynamics, the number of mitoses diminished 2–8 h after amputation, but mitotic activity significantly exceeded normal values at 24 h (7.88 ± 5.06) and 48 h (10.75 ± 4.37) after amputation (Fig. 3d, front segment: one-way ANOVA, $F_{5,64}=4.80$, $P=0.0009$; back segment: one-way ANOVA, $F_{5,64}=9.20$, $P<0.001$).

Double-labeled BrdU mitoses

The total number of mitoses that were additionally labeled with BrdU was counted in control and regenerating animals, both in BrdU pulse-chase and in continuous BrdU experiments. In BrdU pulse-chase experiments, regenerating animals were incubated for 30 min in a BrdU solution and subsequently left to regenerate for a 2-h or 4-h chase. Animals were then fixed to reveal cells that were mitotic at the time of fixation, and that had also incorporated BrdU during the 30-min BrdU exposure. Double-labeled mitoses signified cells that went from S-phase into mitoses within 2 h or 4 h, respectively. Continuous BrdU exposure with subsequent labeling should yield more double-labeled mitoses. The difference from pulse-chase experiments would then lie in those cells that had entered S-phase after the initial 30 min and that were also in mitosis after the designated regeneration time of 2 h or 4 h. These were the so-called fast-cycling cells.

After 2 h, significantly different ratios of double-labeled mitoses were found for continuous BrdU exposure and pulse-chase experiments (Fig. 4). In regenerating animals, only $22.2\pm 7.3\%$ of mitoses were BrdU-labeled in pulse-chase experiments, whereas $40.2\pm 21.6\%$ of mitoses were double-labeled after continuous BrdU exposure. However, no difference was found between regenerating and untreated control animals (two-way ANOVA for effect of factor treatment, $F=0.28$, $P=0.60$, for effect of factor time, $F=1.41$, $P=0.24$, interaction $F=1.21$, $P=0.27$; test for normality: Shapiro-Wilk W -test: $W=0.98$, $P<W=0.39$). In the latter, $18.7\pm 9.8\%$ double-labeled mitoses were found in

pulse-chase experiments and $37.9\pm 10.8\%$ after continuous BrdU exposure.

After 4 h, the values in continuous experiments were similar to those in pulse-chase experiments, for both control and amputated animals: by 4 h after amputation, $71.3\pm 15.5\%$ double-labeled mitoses were found in pulse-chase experiments, and $70.4\pm 38.5\%$ double-labeled mitoses after continuous BrdU exposure. In control animals, the ratios were $68.3\pm 11.8\%$ double-labeled mitoses in BrdU pulse-chase experiments, and $73.8\pm 13.8\%$ double-labeled mitoses in continuous BrdU treatments.

Blastema formation

Regenerating animals were examined up to 72 h after amputation in the tail region (Fig. 1b) by using light microscopy in vivo (differential interference contrast) and semi-thin sections of fixed material. After 24 and 48 h of regeneration, a caudally located blastema distinctly contrasted the surrounding tissue (Fig. 5, a 48-h blastema). Analyses of semi-thin sections revealed the blastema to consist mainly of undifferentiated cells (neoblasts) and a small number of differentiated duo-gland adhesive systems (for a description of the duo-gland adhesive system, see Tyler 1976). The blastema was terminally bordered by partially newly built epidermis (Fig. 5b).

By 72 h after amputation, the onset of differentiation of the male copulatory organ was observed, the predominant organ of the tail plate. This indicated that a true blastema of mostly undifferentiated cells only existed up to 48 h of regeneration. Hence, the present study of cell dynamics in regenerating animals was limited to 48 h after amputation.

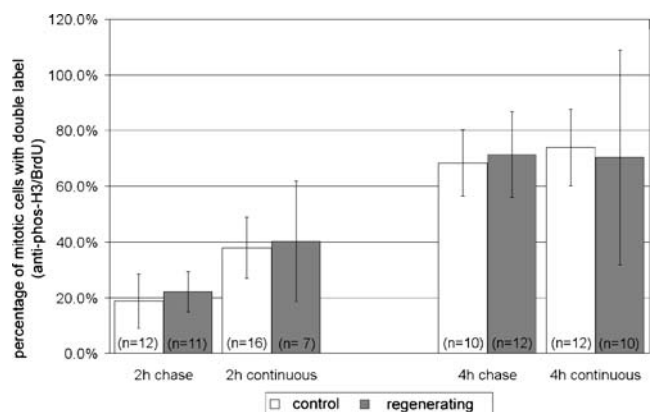


Fig. 4 Graph of ratio of double-labeled mitoses of *M. lignano*, both in control and amputated animals. After 2 h, the difference between the pulse-chase and continuous BrdU treatments is significant. After 4 h, no differences can be found, either in control animals or in amputated specimens

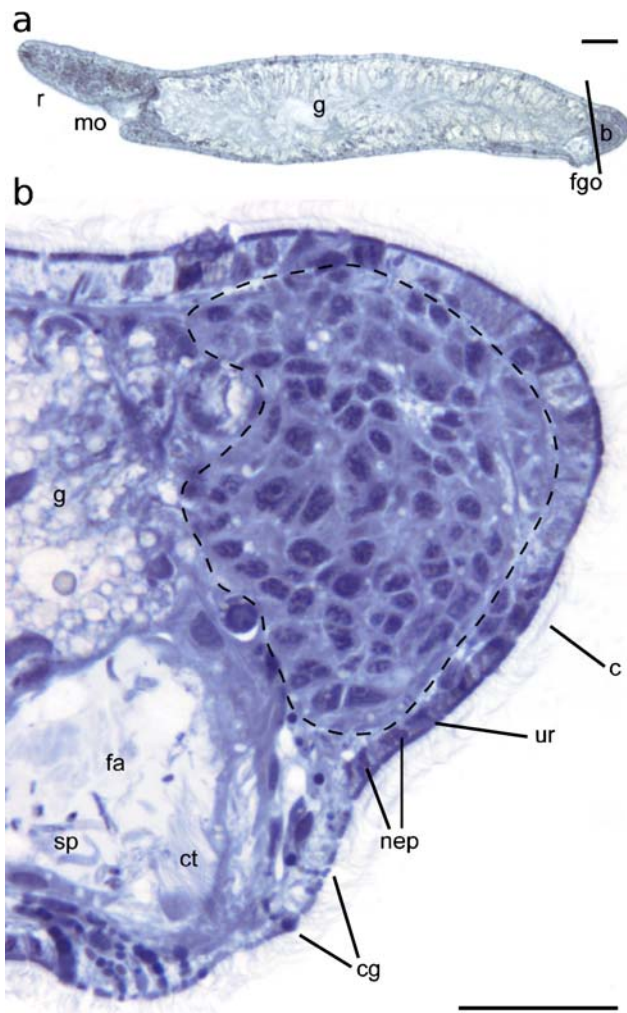


Fig. 5 Images of semi-thin sagittal sections of regenerating *M. lignano*. **a** Overview of a regenerating animal 48 h after amputation (black line cutting level, **b** blastema, **fgo** female genital opening, **g** gut, **mo** mouth opening, **r** rostrum). Bar 50 μ m. **b** Posterior end of specimen after 48 h of regeneration (dotted lines blastema region, **cg** cement glands, **c** cilia, **ct** ciliary tuft, **fa** female antrum, **g** gut, **nep** newly built epidermal cells, **sp** spermatozoa, **ur** ultrarhabdites). Bar 20 μ m

Discussion

Regeneration in Platyhelminthes

In this study, the early aspects of cell-cycle activity in the neoblast stem cell system have been shown during regeneration in the platyhelminth *M. lignano*. Platyhelminths are well-known for their unique stem cell system responsible for postembryonic proliferation, growth, tissue homeostasis, and regeneration (Baguña 1981, 1998; Ehlers 1985; Newmark and Sánchez Alvarado 2000; Ladurner et al. 2000; Agata 2003; Reuter and Kreshchenko 2004; Peter et al. 2004). Triclad and macrostomids, the two best-studied taxa in this respect among free-living flatworms, are

in a state of permanent cell turnover (Baguña and Romero 1981; Ladurner et al. 2000). The only cycling and differentiating cells in adult organisms are the neoblasts, which are located within the mesodermal space but are absent in the pharynx and in front of the photoreceptors. Labeling experiments with BrdU and proliferating cell nuclear antigen have shown that neoblasts migrate in Macrostomida (Nimeth et al. 2004) and in Tricladida (Newmark and Sánchez Alvarado 2000; Ito et al. 2001). Unlike in mammalian cell renewal, a single population of totipotent stem cells is competent for all cell renewal in Platyhelminthes (Newmark and Sánchez Alvarado 2000; Saló and Baguña 2002; Peter et al. 2004). The rescue of lethally X-irradiated hosts after transplanting neoblasts from healthy donors is one of the most convincing experiments that suggest totipotency of triclad neoblasts (Baguña et al. 1989), although it has not been replicated in another laboratory to date. This apparent totipotency may play a crucial role in regenerative capacity, enabling flatworms to regenerate entirely from small body fragments (Montgomery and Coward 1974; Egger et al. 2006a,b). After amputation, the wound is rapidly closed by muscle contraction, whereby the dorsal part adheres to the ventral side (Salvenmoser et al. 2001). During regeneration, an unpigmented tissue (the blastema), which is densely packed with neoblasts, develops at the wound site. In triclad, mitotic activity is observed in a narrow region adjacent to the blastema, the so-called postblastema, but not in the blastema proper (Baguña 1976; Saló and Baguña 1984). This applies to anterior and posterior blastemas. Mitoses during regeneration show a bimodal response with a first maximum at 4–8 h and a second maximum at 2–3 days after amputation (Baguña 1976; Saló and Baguña 1984). The highest maxima occur in the postblastema. This general mitotic response throughout the body indicates a key role for intercellular communication mechanisms in the regeneration of triclad (Saló and Baguña 1984).

In the acoel *Convoluta naikaiensis*, mitoses have been found in the stump (postblastema) and in the blastema itself (Hori et al. 1999). Observations in *Microstomum lineare* (also a macrostomid) in which no blastema has been described have revealed a more complicated situation. In this case, regeneration starts from organ primordia within the stump (Palmberg 1986, 1990).

Mitotic response to amputation

In regenerating *M. lignano*, a significant decrease of mitoses occurs in the first few hours of regeneration, succeeded by a boost of mitoses at 1–2 days after amputation. However, in feeding experiments after 30 days of starvation, a rapid increase of mitoses as soon as 30 min has been observed after feeding in *M. lignano*. This mitotic

boost lasts for 6 h and is followed by a relative minimum at 12 h and a subsequent increase and then a decrease over the next couple of days (Nimeth et al. 2004). Thus, in *M. lignano*, feeding and regeneration do not have the same effect on cell-cycle activity.

An initial decrease of mitotic activity after amputation, as occurs in *M. lignano*, is not known in triclad flatworms in which an increase first occurs after about 4 h, later declining and subsequently rising to a second maximum after 2–4 days (Saló and Bagaña 1984 and the literature therein). In several other studies of triclads, no increase during the first 24 h of regeneration has been observed, although no experimental data have been obtained for the first 12 h in these investigations; the authors report a general rise of mitotic indices after several days (e.g., Dubois 1949; Best et al. 1968; Gabriel 1970). With regard to this temporal point of view, a similar pattern (number of mitoses increases, then decreases, and finally increases, instead of a decrease-increase-decrease) to the observations of Lindh (1957) and Bagaña (1974, 1976) is found in *M. lignano* after starvation and subsequent feeding (Nimeth et al. 2004).

The data on the spatial effects of regeneration in *Macrostomum* are in accordance with observations in triclads by Lindh (1957), who has found a local boost of mitotic activity and no general activation. In other triclads, Saló and Bagaña (1984) have recorded an increase in mitotic activity also in body regions far from the amputation level, but on a lower scale than in regions near the wound. Although an overall rise of mitoses during regeneration has been observed in *M. lignano*, this rise can be attributed to a significant increase of mitoses near the wound site.

The initial decrease in mitotic activity after the loss of a body part coincides with wound closure in *M. lignano* (Salvenmoser et al. 2001) and may prevent additional loss of regenerative cells through the wound opening.

S-phase cells in regenerating *M. lignano*

The incorporation of BrdU into S-phase cells is an important tool for cell-cycle analyses. BrdU as a thymidine analog is incorporated into DNA during replication and thereby specifically labels cells in S-phase. BrdU labeling was not possible in triclads until a new application method was introduced (Newmark and Sánchez Alvarado 2000). Although the application of BrdU is now also available for triclads (application through feeding or injection), these methods do not allow the full advantage to be taken of all the experimental procedures carried out here with *M. lignano*. BrdU application in *M. lignano* can be performed simply by “soaking”, viz., BrdU is added to the culture media for an appropriate time and in an appropriate

concentration. This permits pulse, pulse-chase, and continuous BrdU labeling experiments with a high accuracy in timing in this flatworm (Ladurner et al. 2000; Nimeth et al. 2002, 2004; Peter et al. 2004; Egger et al. 2006a,b). Even the percentage of S-phase cells among the neoblast population (all proliferative cells comprising true stem cells and differentiating cells) can be determined (Bode et al. 2006).

In pulse experiments, a decrease has been observed in the number of S-phase cells in the whole animal during early regeneration but which is not significant compared with values in control animals. However, at 48 h after amputation, the number of S-phase cells is significantly increased. Again, as with mitoses, labeled cells in the whole specimen and in two segments have been counted. Even after 48 h of regeneration, no significant changes occur in the front segment, but in the back segment, the number of S-phase cells is significantly diminished 2 h and 4 h after amputation. After 1 and 2 days of regeneration, the S-phase levels significantly exceed control levels in this rear segment.

The later upregulation of S-phase activity in the wound region is in accordance with data of regenerating triclads (Newmark and Sánchez Alvarado 2000). However, as these worms were labeled with BrdU 18 h prior to amputation, the staining also shows cycling and differentiating cells.

General aspects of the cell cycle during regeneration

To obtain insights into the cell-cycle characteristics, the number of mitotic cells double-labeled with BrdU was surveyed. Nimeth et al. (2004) previously reported a pool of fast-cycling cells passing G2 within 1 h, whereas the majority of cells passed G2 within 4 h; in their experiments, specimens of *M. lignano* were fed ad libitum and starved for 2 days prior to BrdU application. In our present regeneration study, the experiments were designed without a starvation period to avoid a possibly diminished capability of regeneration attributable to a starvation effect. Pulse-chase and continuous BrdU treatment were performed on regenerating and control animals to determine the number of cells that passed from S-phase through G2 to mitosis within 2 and 4 h. At both 2 h and 4 h of regeneration, specimens were not significantly different compared with controls. This means that, during early regeneration of *M. lignano*, no extra activation of cells in G2 occurs compared with control animals. This is different from triclads in which Saló and Bagaña (1984) have found that the first mitotic response to amputation can be largely attributed to pre-existing G2 cells. In *M. lignano*, at 2 h, a marked difference is seen in the ratio of double-labeled mitoses between pulse-chase and continuous BrdU treatment, both

in regenerating and in control animals. This difference is attributable to the additional labeling of fast-cycling neoblasts in the continuous experiments, going through the whole S-phase and G2 into mitosis in only 2 h. After 4 h, the effect of the fast-cycling cells is less predominant. In starved *M. lignano*, the ratio of double-labeled mitoses is even lower at 2 h, but at 4 h, the values are almost the same as for fed specimens (Nimeth et al. 2004). Thus, starvation seems to affect only the fast-cycling cells, causing an increase in G2 length. However, amputation causes no change in cell-cycle characteristics during the first 4 h compared with controls. This is an indication that the duration of the cell cycle (at least the steps from S-phase to mitoses) cannot be reduced in *M. lignano*, even in emergency situations. On the other hand, as previously pointed out, mitotic activity significantly decreases during the first few hours of regeneration, and so an accelerated cell cycle might be observed after 24 h of regeneration.

Amputation of *M. lignano* at first delays and then stimulates proliferation; this indicates that changes occur in cell-cycle activity. As mentioned above, specimens of *M. lignano* grow during regeneration by a general rise of proliferation, although local effects are more striking. Similar observations have been reported for triclads (Dubois 1949; Gabriel 1970; Newmark and Sánchez Alvarado 2000).

Further investigations should reveal more details of the stem cell system and blastema formation in *M. lignano* compared with those of other Platyhelminthes.

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