

Stem Cell Dynamics During Growth, Feeding, and Starvation in the Basal Flatworm *Macrostomum* sp. (Platyhelminthes)

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Development, growth, and regeneration in *Macrostomum* are based—as in all Platyhelminthes—on likely totipotent stem cells (neoblasts), basic for all Bilaterians. We demonstrate dynamics and migration of neoblasts during postembryonic development, starvation, and feeding of *Macrostomum* sp. Double labeling of S-phase and mitotic cells revealed a fast cell turnover. Conflicting with recent results from planarians, we have some indication of slow cycling neoblasts. As in planarians, starvation dramatically reduced mitotic activity and a very basic level was maintained after 30 days of starvation. Afterward, feeding induced a dramatic immediate proliferative response probably caused by G2-arrested neoblasts. The following 12 hr showed a significant mitotic decline, caused by the depletion of the G2 neoblast pool. Neoblasts that pass through S-phase led to a maximum of mitoses after 48 hr. Our results allow deeper insight into cellular dynamics of an ancestral bilaterian stem cell system of a basal Platyhelminth. *Developmental Dynamics* 230:91–99, 2004. © 2004 Wiley-Liss, Inc.

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INTRODUCTION

Platyhelminthes possess a unique stem cell system within the animal kingdom (Baguñà, 1981; Ehlers, 1985; Ladurner et al., 2000). In adult organisms, neoblasts are the only cells that divide and differentiate (Hori, 1997; Hori and Kishida, 1998; Ladurner et al., 2000; Newmark and Sánchez Alvarado, 2000). Studies on free-living platyhelminths such as acoels (Drobysheva, 1986; Ladurner and Rieger, 2000; Gschwentner et al., 2001), macrostomids (Palmberg, 1990; Ladurner et al., 2000), and planarians (Baguñà, 1974, 1976a,b,

1981; Baguñà and Romero, 1981; Saló and Baguñà, 1984, 1985; Baguñà et al., 1989; Newmark and Sánchez Alvarado, 2000) have proved the presence and differentiation potential of stem cells in these groups. Planarians are in a continuous state of cell turnover (Baguñà and Romero, 1981). Their neoblasts are distributed throughout the mesenchyme but not within the plicate pharynx and the region in front of the photoreceptors. They migrate and differentiate into all cell types in intact and regenerating organisms (Newmark and Sánchez Alvarado,

2000; Saló and Baguñà, 2002). Introducing purified neoblast fractions into X-irradiated hosts has demonstrated the high differentiation potential of stem cells (Baguñà et al., 1989).

A similar stem cell system was shown more recently also in *Macrostomum* sp. (Rieger et al., 1994; Ladurner et al., 2000; Peter et al., 2001, 2004). Investigations of development (Rieger et al., 1994; Ladurner et al., 2000) and regeneration (Salvenmoser et al., 2001) have underlined the importance of stem cells in this organism.

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BrdU incorporation has been used to monitor cells in S-phase (Dolbeare, 1995) in various organisms such as yeast (Lengronne et al., 2001), *Hydra* (Holstein et al., 1991), *Drosophila* (Milan et al., 1996), *Caenorhabditis elegans* (Perez-Canellas et al., 1997; Chittka and Chao, 1999), zebrafish (Poleo et al., 2001), and mouse (Berton et al., 2001; Wang et al., 2001). Also in platyhelminths, the distribution of S-phase neoblasts was documented for several species. In planarians and the acoel *Convolutriloba longifissura*, S-phase cells and mitoses were found throughout the parenchyma (Newmark and Sánchez Alvarado, 2000; Gschwentner et al., 2001), and the total cell number and density of neoblasts turned out to be a function of the body length (Baguña, 1976a; Baguña and Romero, 1981). In contrast, in the rhabdocoel *Macrostomum* sp. mitotic cells and S-phase cells were seen along the lateral side of the animal in close proximity to the main nerve cords (Ladurner et al., 2000).

Newmark and Sánchez Alvarado (2000) have demonstrated for *Schmidtea* (formerly *Dugesia*) *mediterranea* that, after 12-hr chase, 96% of the mitotic cells are labeled with 5'-bromo-2-deoxyuridine (BrdU) and that populations of slow cycling cells or cells arrested in G2 were lacking. These data contradicted earlier results for *Schmidtea mediterranea*, showing the presence of a population of neoblasts arrested in G2 ready for mitosis (Baguña, 1976b). Saló and Baguña (1984) also identified fast and slow cycling neoblasts in *Girardia tigrina* by using hydroxyurea (HU). Only preliminary data on the cell cycle of neoblasts are available for *Macrostomum* sp. (Ladurner et al., 2000) and the acoel *Convolutriloba longifissura* (Gschwentner et al., 2001): two stages of neoblast differentiation were suggested for *C. longifissura* on the basis of light microscopical and ultrastructural morphology. In *Macrostomum* sp., Rieger et al. (1999) identified three subsequent stages by ultrastructural features of the cytoplasm and the nucleus.

Compared with planarian species, the microturbellarian *Macrostomum*

has several advantages for studying the neoblast system: (1) anatomic organization of the small Macrostromids is comparable to that of the much larger planarians, and approximately the same number of cell types occur in both groups; yet macrostromids have a total number of approximately 25,000 cells (Ladurner et al., 2000) while planarians may consist of millions of cells (Baguña, 1976a). (2) Planarians are highly derived in their embryonic development (Tyler and Tyler, 1997) while macrostromids have spiral cleavage. The ontogenetic origin of their stem cells, thus, is comparable to that of other spiralian taxa. (3) Macrostromid flatworms represent the most basal branch of all rhabdiphoran platyhelminths (Ehlers, 1985; Littlewood and Olson, 2001). Knowledge about the stem cell system in such organisms could provide cues about the evolutionary origin of a stem cell system in the Bilateria.

In this study, we show the influence of body size, feeding, and starvation on cell number, cell cycle, and cell turnover. Incorporation of BrdU was performed to identify cells in S-phase as reported first by Gratzner (1982) and mitotic cells were labeled by staining with anti-phospho histone H3 (anti-phos-H3; Hendzel et al., 1997). Specimens were treated with different protocols to study characteristics of the cell cycle: (1) incubation in colchicine to figure out the rate of mitosis, (2) incubation in HU to arrest neoblasts in early S-phase, (3) continuous labeling and pulse-chase experiments with BrdU to determine the duration of G2, and (4) starvation and feeding to specify induction and reduction of neoblast proliferation. In summary, to further the understanding of this unique postembryonic stem cell system that apparently is totipotent and might be basal to all Bilateria, we are providing new information (1) on the number and distribution of mitoses during postembryonic development, (2) on the cell cycle of neoblasts in control animals and (3) in animals after 1 to 30 days of starvation, and (4) on the induction of neoblast proliferation by feeding after a 30-day starvation period. (5) We find evidence for populations of stem

cells differing in their cell cycle: this follows from BrdU/anti-phos-H3 double labeling in combination with colchicine treatment or HU incubation.

RESULTS

Number and Spatial Distribution of Mitotic Cells During Postembryonic Development

To determine a correlation between body length and the number of anti-phos-H3-labeled cells in *Macrostomum* sp., cells were counted in 277 animals, ranging from hatchlings to adults (Fig. 1A-E; for a schematic drawing of *Macrostomum* sp., see Ladurner et al., 2000). Hatchlings (Fig. 1A,B) were 225- to 400- μm long ($n = 72$) and lacked gonads. They showed a positive correlation of size and number of mitoses, from 225 μm with 8.00 ± 0.82 mitoses to 350 μm with 14.3 ± 2.9 mitoses. Juveniles (450 to 800 μm , $n = 163$) represented transition stages from hatchling to adult, some of them featuring developing gonads. On average, 7.9 ± 5.7 somatic mitoses (mesodermal and gastrodermal) and 9.5 ± 5.1 gonadal mitoses were found. Adults (Fig. 1C,D) had a length of 850 μm to 1,150 μm , and all animals possessed gonads ($n = 42$). The number of their mitotic cells was highly variable (Fig. 1E): 30.6 ± 23.0 somatic mitoses and 35.5 ± 22.4 gonadal mitotic cells were found.

Independent of body size, almost all mitotic cells were located along the lateral sides of the animals. Only few mitotic and S-phase cells occurred along the median axis (Fig. 1C). Those cells belonged to the proliferating subpopulation of gastrodermal neoblasts (see also Rieger et al., 1999). Immunocytochemical and ultrastructural analyses indicated that approximately 75% of mitosis and S-phase cells were in close proximity (i.e., $< 5 \mu\text{m}$) to the main nerve cords (data not shown; for the pattern of the nervous system, see Ladurner et al., 1997). No S-phase or mitotic cells were found in the region anterior to the photoreceptors (the rostrum; Fig. 1C). Daughter cells migrated toward the median axis (Fig. 1D) and into the rostrum (Fig. 1F-I).

After 4-hr chase, 1.64 ± 2.73 BrdU-labeled cells had migrated anterior to the level of the eyes ($n = 11$), 7.00 ± 3.07 after 16 hr ($n = 9$), and 15.60 ± 6.95 after 24 hr ($n = 6$; Fig. 1I).

Cell Cycle

Colchicine was used to arrest cells in metaphase. All mitotic cells of adult specimens, including somatic mitoses and gonadal mitoses, were counted to determine the proliferating pool of neoblasts (Fig. 2A). As in the control animals, mitoses varied over a wide range, in a few cases standard deviations even exceeded the mean values. A number of 10.4 ± 7.15 ($n = 110$) mitoses were present in control animals (2-day starvation, see Experimental Procedures section) not exposed to colchicine. After 4 hr of colchicine treatment, the number of mitotic cells accumulated to 46.80 ± 62.01 ($n = 41$); the respective numbers were 125.96 ± 123.87 ($n = 28$) after 8 hr, 139.17 ± 119.76 ($n = 18$) after 12 hr, 204.77 ± 170.90 ($n = 22$) after 24 hr, 16.89 ± 9.69 ($n = 18$) after 48 hr, and 18.86 ± 5.76 ($n = 7$) after 66 hr, respectively (Fig. 2A). The comparatively low levels of mitotic cells after 48 hr and 66 hr of colchicine treatment indicated a toxic effect of colchicine (see Discussion section). Up to 24 hr, mitotic cells were accumulated with an average rate of 8.10 cells per hour. The accumulation rate shows a steep increase at 4–8 hr followed by a lower but steady increase for the next 16 hr (Fig. 2A).

In another series of experiments, BrdU pulse labeling was combined with varying chase times in colchicine solution and double staining for BrdU and mitosis. Control animals possessed 435 ± 79 ($n = 21$) S-phase cells and 13.5 ± 6.7 mitoses. After 30 min of BrdU incubation, no double-labeled cells (anti-BrdU/anti-phos-H3) were present (Fig. 2C). After 2 hr of BrdU pulse-chase with colchicine treatment, 13.31% ($n = 8$) of anti-phos-H3-labeled cells showed double labeling. The fraction of double-labeled cells increased to 52.61% ($n = 11$) after 4 hr, 61.15% ($n = 9$) after 16 hr, and to 88.61% ($n = 6$) after 24 hr chase (Fig. 2B; cf. also Fig. 3A,B). To test whether a toxic effect of colchicine influenced

the proliferation rate of neoblasts, we performed continuous BrdU labeling and anti-phos-H3 staining without colchicine treatment. The ratio of double-labeled mitotic cells (labeled with anti-phos-H3 and BrdU) after 2 hr was 12.33% ($n = 5$) of all cells in mitosis, after 4 hr 66.04% ($n = 7$), after 8 hr 57.70% ($n = 16$), after 24 hr 95.49% ($n = 7$), and after 48 hr 97.90% ($n = 8$; Fig. 2C). These numbers suggest that colchicine had no dramatic toxic effect at exposure times up to 24 hr. The percentage of double-labeled germ line cells in the male gonads at 4 hr and 24 hr were only 25.85% ($n = 7$) and 74.38% ($n = 7$), respectively.

To test for subpopulations of neoblasts, possibly differing in their response to releasing from a block of their cell cycle, animals were incubated in HU to arrest cells in an early stage of S-phase. After 16 hr of HU incubation followed by a 30-min BrdU pulse, we found a concentration of labeled cells around the testes (Fig. 3C). The total number of somatic S-phase cells was not reduced compared with control animals but the labeling was weaker as expected. After 24 hr and 48 hr of HU treatment, incorporation of BrdU was very weak and the number of cells could not be determined. After 7 days of incubation in HU, no S-phase cells could be found (data not shown).

The recovery of proliferation after 7 days HU incubation was studied after transfer of animals to artificial sea water (ASW) before BrdU labeling. After 12 hr of recovery, no BrdU-labeled cells were found. After 24 hr, weakly BrdU and anti-phos-H3-labeled cells were present in a small anterior region of the male gonads (Fig. 3D). In some individuals, BrdU-labeled cells appeared along the lateral margins. At 48 hr, the number of cells in the gonads had increased and BrdU-labeled cells were present at the lateral sides of the animal. A normal pattern of BrdU and mitotic cells was regained after 12 days of recovery.

Starvation and Feeding Experiments to Study Neoblast Cell Dynamics

Starvation of *Macrostomum* sp. resulted in a decrease in size, a reduction of gonads, and a decline of the

number of mitoses (Fig. 4). Well-fed animals showed high background fluorescence in the gut because of the autofluorescence of the diatoms. Animals were 954.55 ± 147.93 μm in size ($n = 22$); they featured 26.77 ± 8.47 somatic mitoses and 28.77 ± 9.90 mitoses in the gonads (Fig. 4A). After 2 days of starvation (i.e., control animals) diatoms were digested and the autofluorescence in the gut had disappeared (Fig. 1C). Animals were 894.44 ± 94.93 μm in size; the number of somatic mitoses was reduced by 49.50% to 13.52 ± 6.69 ($n = 27$), and the number of mitoses in the gonads by 46.50% to 15.41 ± 4.91 . After 9 days of starvation, the average size was 743.10 ± 84.22 μm ($n = 29$) and 9.24 ± 4.80 somatic mitoses were observed. In the gonads, a reduction by 56.70% was detected (12.48 ± 7.22). Starvation for 16 days resulted in a decrease to 704.69 ± 97.02 μm ($n = 32$), with 5.97 ± 4.84 somatic mitoses. In the gonads, the number of mitoses dropped by 90.70% (2.69 ± 3.13). After a starvation period of 30 days, the size of the animals declined to 571.74 ± 61.84 μm ($n = 23$) and the number of somatic mitoses to 0.74 ± 0.92 (Fig. 4B,C). The gonads and the copulatory organs had disintegrated.

After 30 days of starvation, animals were transferred to Petri dishes with diatoms. The recovery of proliferating cells (Fig. 4) and the rebuilding of reproductive organs were very fast. Animals started immediately to ingest diatoms. After 30 min of feeding, the gut was completely filled with diatoms, resulting in a dense uniform autofluorescence (Fig. 3E,F) of the gut. The number of mitoses increased 5.5-fold after 30 min of feeding (Fig. 4), 24-fold after 2 hr, and 55-fold after 6 hr of feeding. The number of labeled cells was 4.20 ± 2.9 ($n = 10$) after 30 min of feeding (Fig. 4), 6.00 ± 3.0 ($n = 8$) after 60 min, 17.75 ± 5.9 ($n = 12$) after 2 hr (see also Fig. 3G), and 41.00 ± 8.6 ($n = 9$) after 6 hr of feeding. After 12 hr, the number of mitoses had declined to 26.30 ± 7.5 ($n = 10$), it rose again to a maximum of 57.09 ± 19.5 ($n = 11$) mitoses after 48 hr. At day 5, a decline to 35.38 ± 11.5 was observed,

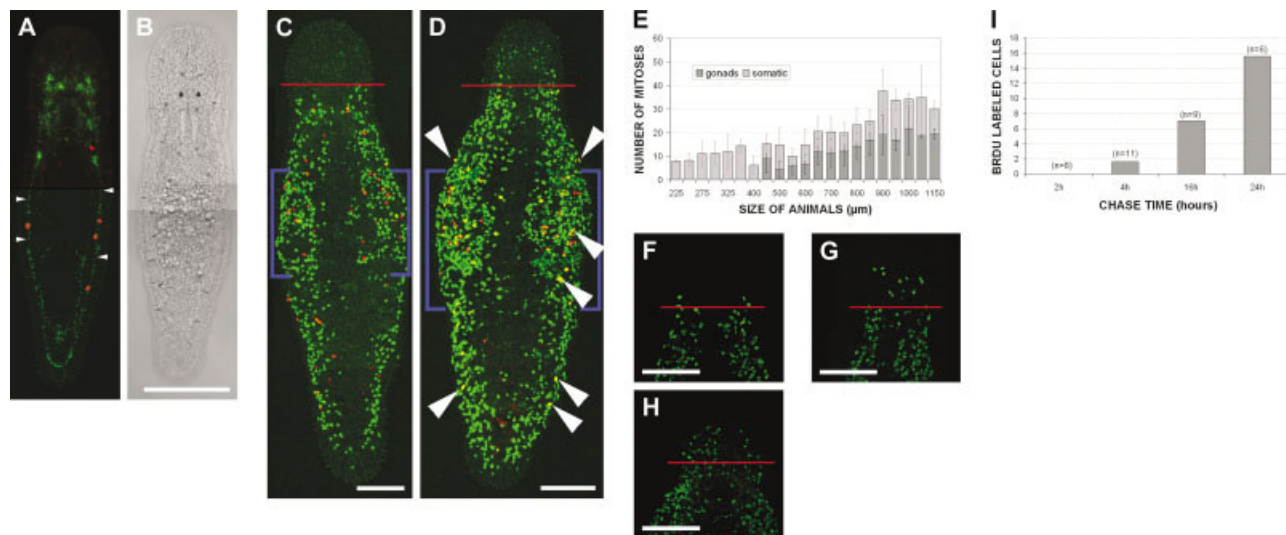


Fig. 1.

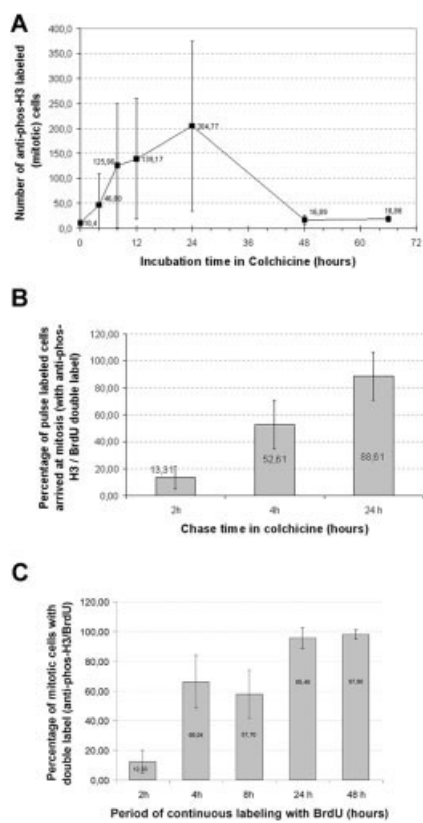


Fig. 2.

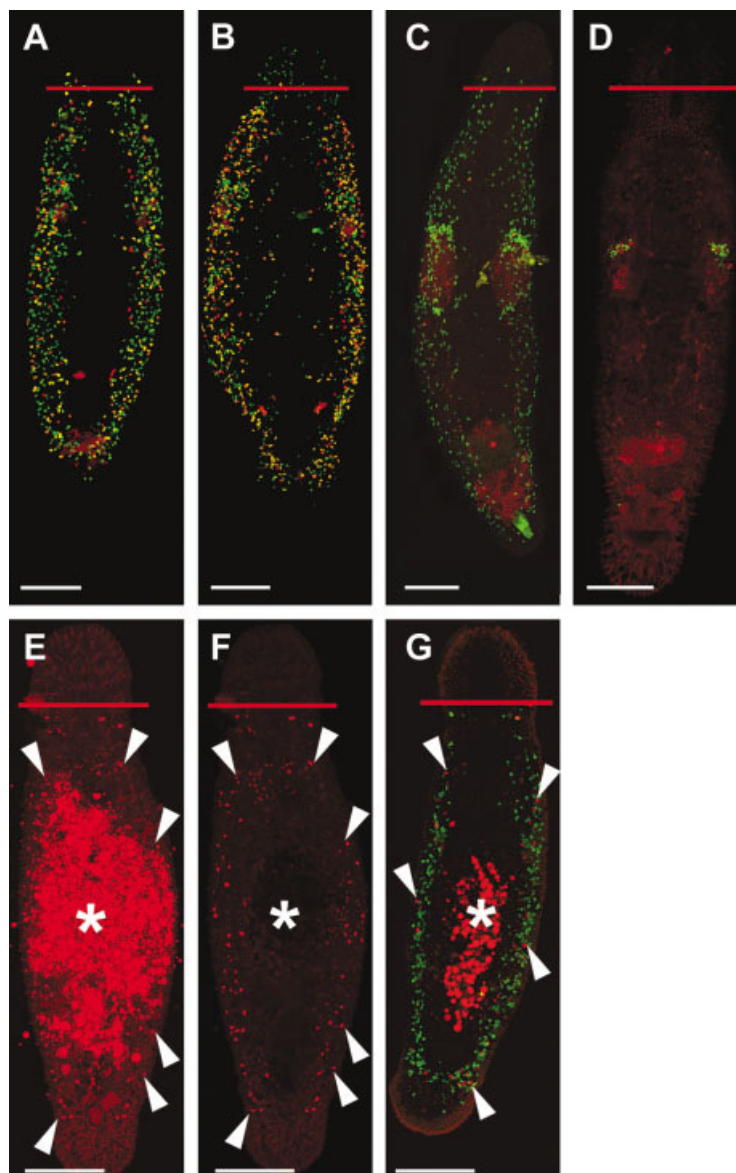


Fig. 3.

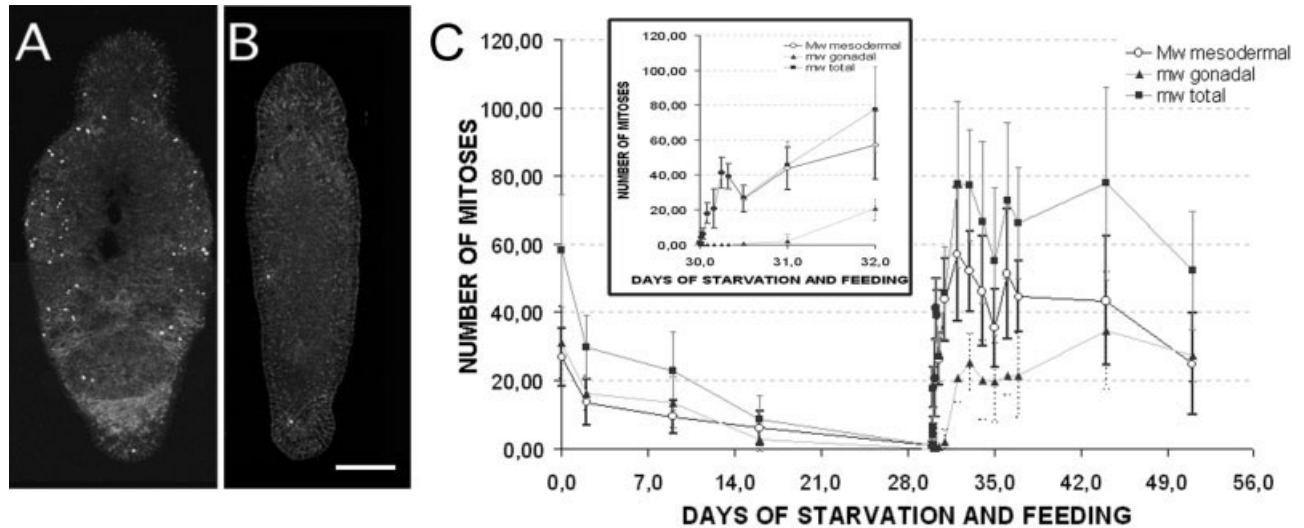


Fig. 4. Influence of starvation on mitoses in *Macrostomum* sp. A,B: Projections of confocal laser scanning images showing mitoses (light spots) in animals starved for 0 days (A) and 30 days (B). C: Graph of the number of mitoses during starvation and after feeding after a 30-day starvation period. Inset shows enlargement of curve between days 30 and 32. Note the initial increase (until 6 hr) after feeding probably based on neoblasts arrested in G2. After the depletion of the G2 pool, a minimum in the number of mitoses was observed after a 12-hr feeding time followed by another maximum at 48 hr, backed by neoblasts that passed S-phase and G2. Scale bar = 100 μ m in B (applies to A,B).

followed by another peak at day 6 (51.33 ± 19.0 ; $n = 9$). Finally, the number of mitoses decreased to 24.86 ± 14.9 ($n = 14$) at day 21, a similar value as observed for animals that were not starved (26.77 ± 8.47 ; $n = 22$).

DISCUSSION

Stem Cell System and Distribution of Mitotic Cells

The data presented here together with results from Ladurner et al.

(2000) and Rieger et al. (1999) lead to the following general conclusion about the distribution of neoblasts in the genus *Macrostomum*: S-phase and mitotic cells were located in two bands along the lateral side of

Fig. 1. Distribution and migration of mitoses and S-phase cells in *Macrostomum* sp.: Anterior is to the top, red lines indicate the level of the eyes, blue brackets indicate gonads. A: Confocal projection of mitoses in a hatchling of *Macrostomum* sp. Mitoses are in red, FMRF-amid nervous system in green (arrowheads). Note the close relation of mitotic cells to the major longitudinal nerve cords. B: Interference contrast of the same specimen as in A. C,D,F-H: Confocal projections of adult *Macrostomum* sp. Mitoses (red) and S-phase cells (green). C: Confocal projection of a 30-min bromodeoxyuridine (BrdU) pulse of adult *Macrostomum* sp. D: At 24 hr of continuous BrdU exposure of adult *Macrostomum* sp. Several cells are double labeled (arrowheads). BrdU-labeled cells have migrated into the region anterior to the eyes and toward the median axis. E: Stacking bar chart representing the number of somatic mitoses (light gray) and number of mitoses in the gonads (dark gray) from hatchlings to adults. F-H: Projections of confocal laser scanning images after 30-min BrdU pulse and 4-hr (F), 16-hr (G), and 24-hr (H) chase in colchicine. I: Graph of the number of BrdU-labeled cell that migrated into the region anterior to the eyes after specified chase times. Scale bars = 100 μ m.

Fig. 2. Temporal relationship between S-phase and mitosis. A: Graph of the number of mitoses of cells that proceeded through S-phase (anti-bromodeoxyuridine/anti-phospho histone H3 (anti-BrdU/anti-phos-H3) double-labeled cells) after different chase times (cumulative graph). We incubated animals in colchicine to accumulate mitoses. Note the dramatic decrease of mitoses after 24 hr because of a toxic effect of colchicine after longer incubation times. B: Graph of the percentage of double-labeled cells (anti-BrdU/anti-phos-H3) that accumulated during different chase times in colchicine. Numbers indicate the percentage of double-labeled cells among the total of all cells labeled with anti-phos-H3. The total numbers of accumulated mitoses per animal were 108.0 ± 43.22 ($n = 8$) after 2 hr, 204.5 ± 55.83 ($n = 11$) after 4 hr, and 256.0 ± 131.34 ($n = 5$) after 24 hr. C: Graph of the percentage of double-labeled mitoses (anti-BrdU/anti-phos-H3) in continuous BrdU-labeling experiments without colchicine incubation. Numbers indicate the percentage of double-labeled mitotic cells, based on the total number of mitoses at a given time. The total numbers of mitoses per animal were 8.8 after 2 hr ($n = 8$), 18.1 after 4 hr ($n = 11$), 12.81 after 8 hr ($n = 16$), and 24.1 after 24 hr ($n = 6$).

Fig. 3. Distribution of proliferating cells in *Macrostomum* sp. Mitoses (red) and S-phase cells (green). Anterior is to the top, red lines indicate the level of the eyes. A,B: Confocal projections of anti-bromodeoxyuridine/anti-phospho histone H3 (anti-BrdU/anti-phos-H3) double labeling of adult *Macrostomum* sp. after 4-hr (A) and 24-hr pulse-chase (B) with colchicine treatment. C: Confocal projection after 16 hr of treatment with hydroxyurea (HU) followed by 30-min BrdU exposure. D: Confocal projection after 7 days of HU and 24 hr of recovery in artificial sea water followed by a 30-min BrdU pulse. E,F: Confocal projections after 30 days of starvation and feeding for 30 min. E: Confocal projection using a long-pass filter (see Experimental Procedures section) to demonstrate mitoses (arrowheads) and autofluorescence of algae in the gut (asterisk). F: Confocal projection of the same specimen as in E but obtained with a band-pass filter to blind out autofluorescence of algae (asterisk). G: Confocal projection of anti-BrdU/anti-phos-H3 (arrowheads) double labeling after 30 days starvation and feeding for 2 hr; diatoms in the gut (asterisk). Scale bars = 100 μ m in A-G.

the animal. Proliferating cells were very scarce along the median axes and were completely lacking anterior to the eyes. Somatic neoblasts were found often close to the two main ventrolateral nerve cords, but always in the area between dorsal and ventral nerve cords while gastrodermal neoblasts occurred all over the dorsal and lateral sides of the gut. No cell proliferation was found near the neuropile, the brain, in the epidermis, or anterior to the eyes.

Studies on other representatives of the Platyhelminthes reveal a high complexity of neoblast distribution. Baguñà and Romero (1981) analyzed macerated preparations of certain regions of the two planarians *Schmidtea* (formerly *Dugesia*) *mediterranea* and *Girardia tigrina* and found a high density of nerve cells and neoblasts in the head and tail but a uniform distribution of these cell types in the middle region. In *S. mediterranea* (Baguñà, 1976a), lactic orcein revealed that peripheral regions had a higher mitotic density than the central areas, except for the region in front of the eyes that was free from mitoses. No mitotic cells were found in the pharynx. Palmberg (1986, 1990) reported that, in the turbellarian *Macrostomum lineare*, the only cells incorporating (³H)thymidine were mesenchymal and gastrodermal neoblasts. In the acoels *Convoluta convoluta* and *Oxyposthia praedator*, autoradiography after (³H)thymidine labeling furnished data on the distribution of proliferating cells within the parenchyma (Drobysheva, 1986). Proliferating cells occurred in the peripheral parenchyma and the marginal zone of the central parenchyma. No labeled nuclei were found in the middle zone of the central parenchyma that consists only of digestive elements. In the acoel *Convolutriloba longifissura*, Gschwentner et al. (2001) observed a homogenous distribution of S-phase cells and no correlation to the nerve cords. Thus, as mentioned in the Introduction section, in *Macrostomum* sp., the postembryonic neoblast cell system is uniquely associated in its position to main parts of the central nervous system and, as such, may be espe-

cially useful for analyzing the regulating role of the nervous system on cell proliferation in this likely to be totipotent stem cell system.

Effects of Starvation and Feeding

After a starvation period of 30 days, *Macrostomum* sp. were again fed with diatoms. The reaction within the first 48 hr was characterized by two steep increases of mitotic activity separated by a decrease. These changes in the number of mitoses can be ascribed to different phases of mitotic activity: (1) Initially, feeding after 30 days of starvation induces cells arrested in G2 to enter mitosis, resulting in a rapid increase of the number of mitotic cells observed from 0.5 to 8 hr of feeding. (2) After depletion of the G2 cell pool, the number of mitoses decreases. (3) The following maximum is based on cells that passed S-phase and entered mitosis. In the following 2 weeks, mitotic activity was normalized. Baguñà (1976a) has observed a comparable rapid increase of proliferation in planarians 1 hr after feeding. A maximum of mitoses was reached between 3 and 8 hr after feeding. Another possible explanation may be that a yet unknown chemical signal (from the nervous system) may cause the two peaks in the data set.

During starvation, *Macrostomum* sp. showed a steep decline of the number of mitoses compared with control animals combined with degeneration of the gonads and reduction of the body length. Bode et al. (1973) reported similarly strong effects of starvation on cell number and size of *Hydra attenuata*. Cell numbers increased after feeding and were still elevated during the first 3 days of starvation, but then a sharp decrease in size and total cell number was observed. Baguñà and Romero (1981) found a similar response to starvation in the planarian *G. tigrina*: within the first 3 days of starvation, the cell number still increased due to proliferation after feeding. Subsequently, a steady decrease in size from originally 7-mm body length was observed, resulting in animals of 2-mm body length.

Characteristics of Cell Cycle and Slow Cycling Neoblast Population

The accumulation of cells in mitosis by mitotic arrest with colchicine (Fig. 2A) revealed different cell cycle periods. As the transition of mitosis to G1 is blocked by this method, the fraction of cycling neoblasts is progressively depleted. This depletion does not proceed with a uniform rate, but varies during different time intervals: fast cycling fractions are depleted more rapidly than cells with a slow cycle. Whereas period lengths cannot be deduced from the data obtained, the fast reaction within in the first 8 hr is clearly visible.

In addition, we screened for different periods of G2 in neoblasts by anti-BrdU/anti-phos-H3 double labeling, based on two different protocols. The results after BrdU/anti-phos-H3 double labeling with colchicine incubation up to 24 hr suggest the presence of neoblasts with a very short cell cycle. After 24 hr of chase, 89% of all mitotic cells were double stained with BrdU and anti-phos-H3, representing the fraction of the main cycling neoblasts. A small percentage of 11% were not double labeled and represent an even slower cycling subpopulation.

Because of the toxicity of colchicine, continuous treatment for more than 24 hr resulted in a dramatic loss of mitotic cells. However, within the time frame of 24 hr, we found comparable fractions of cells that had passed from S-phase through G2 to mitosis in continuous BrdU-labeling experiments without colchicine and in pulse-chase experiments with colchicine (see discussion below). Therefore, a strong toxic effect of colchicine is not likely for incubation periods up to 24 hr. It is known that cytotoxic side effects of colchicine that might influence the cell cycle are negligible at concentrations below 1 mM (Sluder, 1991); a concentration of 0.125 mM was applied in this study. A 0.2 mM colchicine solution did not have detrimental effects on planarian cells or slow down mitosis in planarians with incubation periods of 12 hr and 24 hr but induced pyknosis after 6 days (Mc-

Whinnie and Gleason, 1957). These observations confirm our results.

Continuous BrdU-labeling experiments without colchicine showed that, after 24 hr, 95% of the mitotic cells were double labeled with BrdU. Continuous BrdU labeling without colchicine for 48 hr still revealed cells only labeled with anti-phos-H3. These cells could not have passed S-phase during the past 48 hr and represent, therefore, slow cycling neoblasts.

To detect possible subpopulations of neoblasts, animals were treated with HU to arrest cells at the transition of G1 and S-phase. It is known that HU inhibits DNA synthesis (Wright and Tollon, 1988; Yarbro, 1992) and arrests cells in early S-phase (Yarbro, 1992). Incubation in HU does not inhibit initiation of DNA replication but stops elongation after several kilobases (Lengronne et al., 2001). Experiments with a 30-min BrdU pulse after HU incubation should reveal if neoblasts differed in their recovery behavior when reentering the cell cycle. This appeared indeed to be the case: After 16 hr of incubation in HU and a BrdU pulse of 30 min, we observed labeled cells concentrated around the gonads. These cells were related with the reproduction system, which corresponds to observations in *Hydra* (Holstein and David, 1990). The arrest of S-phase cells with HU was also used in regeneration experiments in planarians and led to the distinction of fast (up to 8 hr) and slow (2–3 days) cycling neoblasts (Saló and Bagaña, 1984).

Mutomba and Wang (1996) observed recovery of cells infected with *Trypanosoma brucei* after incubation for 12 hr in 7.5 $\mu\text{g/ml}$ (= 0.1 mM) HU within 24 hr. Incubation at higher concentrations than 0.1 mM caused irreversible damage (see also Yeo et al., 2000). After treatment of 12 hr with 0.2 mM HU, cells did not recover within 48 hr after removal of the drug. These reports confirm that the concentration range chosen in this study was high enough to expect complete inhibition of DNA synthesis, but at the same time in a range where toxic effects could not be excluded. The latter has been shown by the results. We found weakly BrdU-labeled cells

after 24 to 48 hr of incubation in 2.5 mM HU.

After a prolonged treatment with HU for 7 days, animals were transferred to ASW for various time periods before BrdU pulse labeling. After 24 hr in ASW, we found BrdU-labeled cells within the gonads. We interpret these results, which fit well to the observations after 16 hr of HU-incubation discussed above, in the following way: Gonadal S-phase cells represent a separate subpopulation different from somatic neoblasts, which recover faster than these. This finding is corroborated by irradiation experiments: Gonadal S-phase cells showed a much higher tolerance (unpublished results, P. Ladurner). After 12 days in ASW, the recovery of the proliferation pattern of somatic neoblasts was also complete.

In *Macrostomum* sp., continuous BrdU labeling and anti-phos-H3 staining of proliferating cells in the gonads suggested that gonadal proliferating cells have slower cell cycles than somatic neoblasts: Mitoses accumulated at a lower rate in gonadal neoblasts.

A similar situation was found in the pluripotent stem cell system of *Hydra*. The interstitial cells of these organisms are multipotent stem cells and can differentiate into several classes of cell types such as nerves, nematocysts, and gland cells (Heimfeld and Bode, 1984; Holstein and David, 1990). Functional investigations on interstitial cells of *Hydra oligoactis* indicated the existence of three subpopulations of stem cells (Holstein and David, 1990). They were classified based on the length of their G2, and short cycle cells seem to be true stem cells. Long cycle cells were identified as sex cell precursors, medium cycle cells are supposed to be nematocyte precursors.

CONCLUSION

For *Macrostomum* sp., we can summarize the following characteristics concerning distribution, proliferation, and migration of neoblasts in developing and adult animals. (1) S-phase and mitotic cells could be found almost exclusively in the parenchyma along the lateral margins

of the animal. (2) Cell proliferation was spatially correlated with the course of the main nerve cords. (3) Differentiating cells migrated from the lateral margins toward the median axis and from the level behind the eyes into the rostrum. (4) Neoblasts with considerably different length of cell cycles could be identified in these animals with double-labeling experiments, representing different subpopulations. In addition, we found different tolerance of neoblast subpopulations against HU. (5) Furthermore, a high flexibility of the neoblast system concerning feeding conditions was found. The value of this study is twofold: one being the experimental advantages of *Macrostomum* sp. with only 25,000 cells, and the other being that *Macrostomum* sp. represents one of the lower bilateria with a one-way gut.

EXPERIMENTAL PROCEDURES

Cultures

Cultures of *Macrostomum* sp. were reared in Petri dishes with the diatom *Nitzschia curvilineata* according to Rieger et al. (1988). A first characterization of this new species was given by Ladurner et al. (2000), and its final description is presently in preparation (Ladurner et al., manuscript in preparation). They were maintained in a temperature-controlled chamber at 20°C, 50% humidity and a photoperiod of 14 hr of light and 10 hr of dark. For examination, animals were transferred to Petri dishes without diatoms and starved for 2 days ("control animals") in ASW (32%).

Starved and Fed Animals

We reared animals for starvation experiments in Petri dishes with *Nitzschia curvilineata*, transferred them to ASW, and kept them in darkness for various periods of time: (A) no starvation, (B) 2 days, (C) 9 days, (D) 16 days, and (E) 30 days of starvation. For feeding experiments, we transferred animals to Petri dishes with *Nitzschia curvilineata* after 30 days of starvation. After 0 hr, 0.5 hr, 1 hr, 2 hr, 4 hr, 6 hr, 8 hr, and 12 hr, daily up to 7 days, and after 2 and 3 weeks, we performed a 30-min BrdU pulse and anti-phos-H3 labeling.

Labeling Mitoses With the Anti-Phos-H3 Mitosis Marker

Specimens were rinsed in ASW (3×10 min), relaxed in ASW-MgCl₂ (20 min), fixed in 4% paraformaldehyde (60 min, room temperature), rinsed in phosphate-buffered saline (PBS, 3×10 min), and incubated in PBS-T (0.1% Triton X100 in PBS, 60 min). Animals were then incubated in BSA-T (1% bovine serum albumin in PBS-T, 30 min, room temperature). Animals were incubated in primary anti-phos-H3 (Upstate Biotechnology, 1:300 in BSA-T) at 4°C overnight. After rinsing in PBS (3×10 min), specimens were incubated (60 min, room temperature) in secondary TRITC-conjugated swine anti-rabbit (DAKO, 1:150 in BSA-T). Animals were mounted using Vectashield and observed with a Reichert POLYVAR epifluorescence microscope or a confocal ZEISS LSM 510.

Double Labeling of S-Phase Cells (Anti-BrdU) and Mitoses With the Anti-Phos-H3 Mitosis Marker

We labeled animals with 5'-bromo-2-deoxyuridine (BrdU) by incubation in 50 μ M BrdU in ASW (continuous labeling) for 14 days, or by a 30-min pulse of 5 mM BrdU in ASW. Specimens were relaxed, fixed, and incubated in PBS-T as described above. We then treated animals with 0.15 μ g/ml Protease XIV (SIGMA) at 37°C (30 min) or with 0.015 μ g/ml Protease XIV (2 hr, room temperature), followed by incubation in 0.1 N HCl (10 min, on ice). Whole-mounts were transferred to 2 N HCl (60 min, 37°C) to denature DNA, rinsed in PBS (3×10 min), and incubated in BSA-T (30 min, room temperature). Animals were incubated in a mixture of primary mouse anti-BrdU (Sigma, 1:1,000 in BSA-T) and primary anti-phos-H3 (Upstate Biotechnology, 1:300 in BSA-T) at 4°C overnight. After rinsing in PBS (3×10 min), specimens were incubated (60 min, room temperature) in a cocktail of secondary fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse (DAKO, 1:150 in BSA-T) and tetra-rhodamine isothiocyanate (TRITC)-conjugated swine anti-rabbit (DAKO, 1:150 in

BSA-T). Animals were mounted using Vectashield and observed with a Reichert POLYVAR epifluorescence microscope or a confocal ZEISS LSM 510.

Examination of cell cycle.

We used colchicine (0.005%, Sigma) to arrest neoblasts in mitosis and to accumulate neoblasts that had passed through the cell cycle within different periods of time.

Double labeling of mitoses (anti-phos-H3) and S-phase cells (anti-BrdU). To analyze the length of G₂, anti BrdU/anti-phos-H3 double labeling was applied to *Macrostomum* sp. in combination with or without colchicine treatment for different chase times. A pulse of BrdU lasting 30 min was set before colchicine treatment. Continuous labeling with BrdU was used in the experimental series without colchicine. 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 500 μ M BrdU for 0.5 hr, 1 hr, 2 hr, 4 hr, 8 hr, 24 hr, and 48 hr of continuous labeling to demonstrate BrdU-labeled cells that entered mitosis; (3) with 5 mM BrdU for 30-min pulse and 2-hr, 4-hr, 16-hr, 24-hr, and 48-hr chase times in colchicine (Sigma, 0.005% in ASW), to accumulate BrdU-labeled cells that entered mitosis and appeared double labeled after the proposed chase times. In all cases, the specimens were subjected to a treatment identical to that described above for BrdU labeling and anti-phos-H3 labeling, respectively, to visualize the labels. Instead of single antibodies, a mixture of both primary antibodies was applied in the concentrations given above.

S-phase arrest with HU. To discriminate neoblasts by their recovery behavior after blocking the cell cycle, specimens were incubated in freshly prepared 2.5 mM HU (Sigma, 2.5 mM) for 16 hr, 24 hr, and 48 hr and then labeled with a 30-min pulse of BrdU. To monitor recovery of S-phase neoblasts after the release of the S-phase arrest after 7 days of exposure to HU, the animals were transferred

to ASW and labeled by a 30-min BrdU pulse 4 hr, 12 hr, 24 hr, 48 hr, and 12 days later. Subsequently, the same protocol was applied as described above for anti-BrdU/anti-phos-H3 labeling, beginning with washing in ASW.

Microscopy.

The Reichert POLYVAR epifluorescence microscope was equipped with a TRITC (BP 546; LP560) and FITC (BP455-490; LP 515) filter set. A ZEISS LSM 510 was used to obtain confocal images. For Figure 3E,F, a long-pass filter (LP 560) and narrow-band filter (BP 560-615) were applied to discriminate between the auto fluorescence of the algae and the TRITC-labeled mitoses.

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