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Research Article

Analysis of cell proliferation and migration during regeneration in *Lumbriculus variegatus* (Clitellata: Lumbriculidae)

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Abstract. Regeneration of anterior and posterior structures following fragmentation is a well-known characteristic of *Lumbriculus variegatus*, a freshwater oligochaete. Elucidation of cellular activities occurring during regeneration in oligochaetes has relied on classic histological methods. In this project, molecular and chemical approaches were used to analyze the role of cell proliferation and cell migration in *L. variegatus* regeneration. Mitosis was blocked through incubation of worm fragments in 2.5 mM colchicine or 25 μg/ml vinblastine sulfate. Worm fragments incubated in these drugs no longer regenerated heads or tails. Because colchicine and vinblastine may affect cellular processes other than mitosis, more direct evidence of cell proliferation was obtained by soaking regenerating worms in 5 mM bromodeoxyuridine (BrdU). At various times following BrdU treatment, worms were fixed and macerates of regenerating tissues were prepared. The dispersed cells were stained with antibodies against BrdU followed by incubation with fluorescein-conjugated secondary antibodies. Proliferation of cells, based on BrdU labeling, was observed in both regenerating heads and tails. Incorporation of BrdU into cell nuclei was observed, with extensive labeling detected in cells harvested at 120 hours into regeneration compared to tissues harvested early in the regenerative process (24 to 72 hours). To investigate the potential role of cell migration in the regenerative process, worm fragments were incubated in migration blockers, locostatin (7.5 μM) or latrunculin B (0.15 μM). Both drugs inhibited regeneration of heads and tails. The studies suggested that regeneration in *L. variegatus* involves both cell migration and cell proliferation and that BrdU labeling can be used to monitor these processes.

Introduction

Following fragmentation, *Lumbriculus variegatus*, a freshwater oligochaete, exhibits regeneration. Fragments regenerate complete worms with anterior fragments forming blastemas that differentiate into posterior structures (and vice versa) and interior fragments forming both anterior and posterior structures. Over several days, the original segments in the oligochaete fragments morphallactically acquire phenotypes typical of their new position in the worms (Berrill, 1952; Drewes and Fourtner, 1990). Another interesting feature in *L. variegatus* is that the ability of the worms to regenerate head structures progressively decreases along the anterior-posterior axis (Hyman, 1916). Research on regeneration in *L. variegatus* and other oligochaetes has relied on histological ap-

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proaches. Turner (1935) obtained indirect evidence for mitotic activity during regeneration by blocking formation of new heads and tails through exposure of tissues to x-rays. Anterior regeneration in *Nais paraguayensis* was determined by O'Brien (1946) to involve rapid cell proliferation based on observation of mitotic figures. The cell division appeared to occur in already differentiated epithelial cells. Posterior regeneration was described by O'Brien (1946) to involve proliferation of epidermal cells and extensive cell migration and proliferation of cells with morphologies characteristic of neoblasts. Stephan-Dubois (1956) suggested that migrating and dividing neoblast cells might be involved in both anterior and posterior regeneration in *L. variegatus*. Consistent with this view are the histological observations by Myohara et al. (1999) indicating that epimorphic mechanisms, of which cell division is a component, were responsible for head and tail regeneration in *Enchytraeus japonensis*, a terrestrial oligochaete. Studies over several decades support roles for both cell migration and cell proliferation in tissue regeneration in oligochaetes. However, the definitive nature and origin of the cells involved in these processes remain unknown. We initiated studies on *L. variegatus* to explore its potential as a model system for elucidation of the cellular and molecular events of regeneration. The project was designed to determine if the cell proliferation occurring during regeneration could be detected by soaking worm fragments in 5-bromo-2-deoxyuridine (BrdU), a drug that is incorporated into DNA during its replication (Newmark and Sánchez Alvarado, 2000). If so, this molecular technique could be used to visualize the location and movement of proliferating cells in regenerating tissues. Another objective was to reaccess the requirement for cell migration during regeneration through use of cell migration inhibitors such as locostatin.

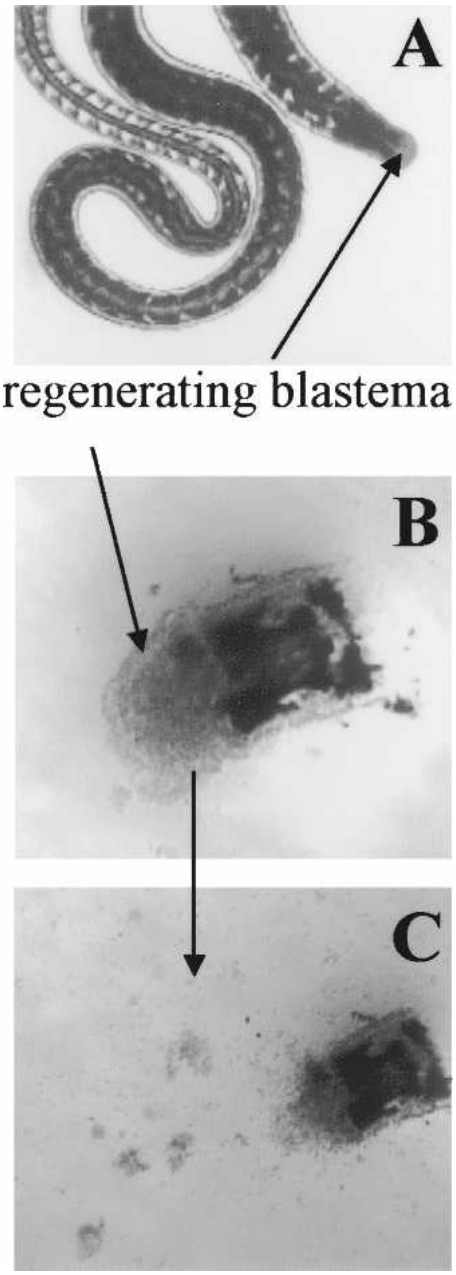

Figure 1. Preparation of regenerating tissue for analysis of BrdU incorporation. Regenerating blastemas (B) were removed from worms (A), fixed in acetic acid:glycerol:water [1:1:13], and dissociated into cells (C) which were then stained with antibodies against BrdU.

Materials and Methods

Maintenance of worm cultures

Lumbriculus variegatus were obtained from Carolina Biological Supply Company and maintained in spring water (Culligan) in four-liter plastic containers in an incubator held at 24°C. Worms were fed crushed *Lumbriculus* food pellets (Carolina Biological Supply) once per week

and the spring water was changed twice per week. Prior to cutting the worms to initiate regeneration, worms were placed in fresh spring water without food for seven days.

Treatment of worms with mitosis- or cell migration-blocking drugs

To evaluate the use of chemical methods for analysis of cell proliferation during regeneration in *L. variegatus*, worms were cut midpoint along their longitudinal axes with a razor blade into anterior halves that regenerated tails and posterior halves that regenerated heads. These fragments were then incubated in 0.5 to 25 mM colchicine or 5 to 50 μg/ml vinblastine sulfate in spring water. To investigate the role of cell migration in the regenerative process, worm fragments were incubated in 1 to 10 μM locostatin (Zhu et al., 2005) or 0.1 to 10 μM latrunculin B (Spector et al., 1982) in spring water.

Regeneration of anterior and posterior structures was observed over ten days and compared to that of control worms that were cut and incubated in spring water. At least 25 worms were exposed to each drug concentration. Worms were examined with an Olympus BH-2 microscope and photographed with a Sony color video camera (Model CUC8995). Using an Advanced

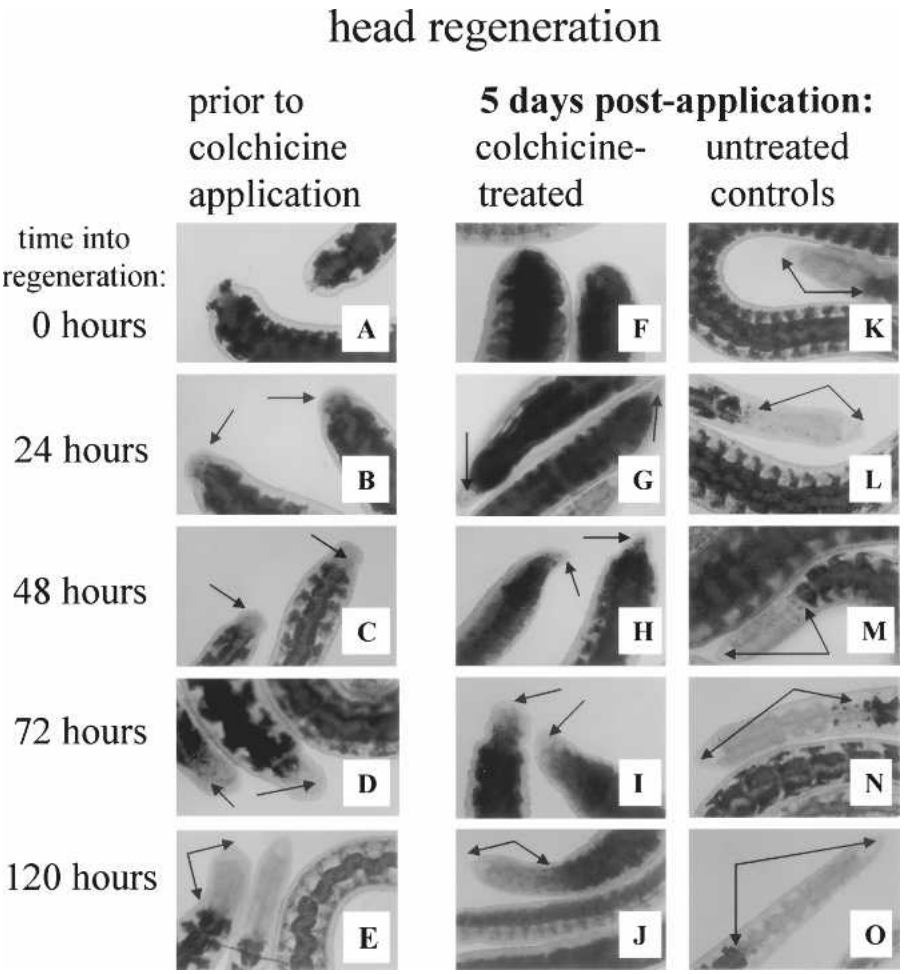

Figure 2. Effect of colchicine on regeneration of heads. Posterior fragments of worms were allowed to regenerate heads in spring water for 0 (A), 24 (B), 48 (C), 72 (D) or 120 hours (E). These fragments were then incubated in 2.5 μM colchicine (F-J) or spring water (K-O) for 5 days. Arrows bracket regions of new tissue formation.

DV Converter (Model ADVC-100), images were captured into iMovie on a Macintosh PowerMac G4 computer.

BrdU treatments

At 0, 24, 48, 72, or 112 hours into the regenerative process, worm fragments were incubated for 24 hours in 5 mM BrdU in spring water. Blastemas were then excised with scalpels from the regenerating worms (Figure 1A and B), fixed, and dissociated (Figure 1C) in acetic acid:glycerol:water (1:1:13). Using the protocol of Newmark and Sánchez Alvarado (2000), dissociated cells were air-dried on SuperFrost Plus slides, washed with phosphate-buffered saline + 0.5% Triton X-100, incubated in 2N HCl to denature the DNA, and neutralized with 0.1 M borax. The cells were then stained with anti-BrdU (Sigma monoclonal B2531, 1:500 dilution) followed by fluorescein-conjugated anti-mouse IgG (Sigma F5387, 1:100 dilution). Controls consisted of harvested tissues that were stained with the secondary antibody. Slides were analyzed using an Olympus BH-2 microscope and digitally photographed using a Spot Insight camera (Model 11.1) and Spot (Version 4.0.6) software (Diagnostic Instruments, Inc.).

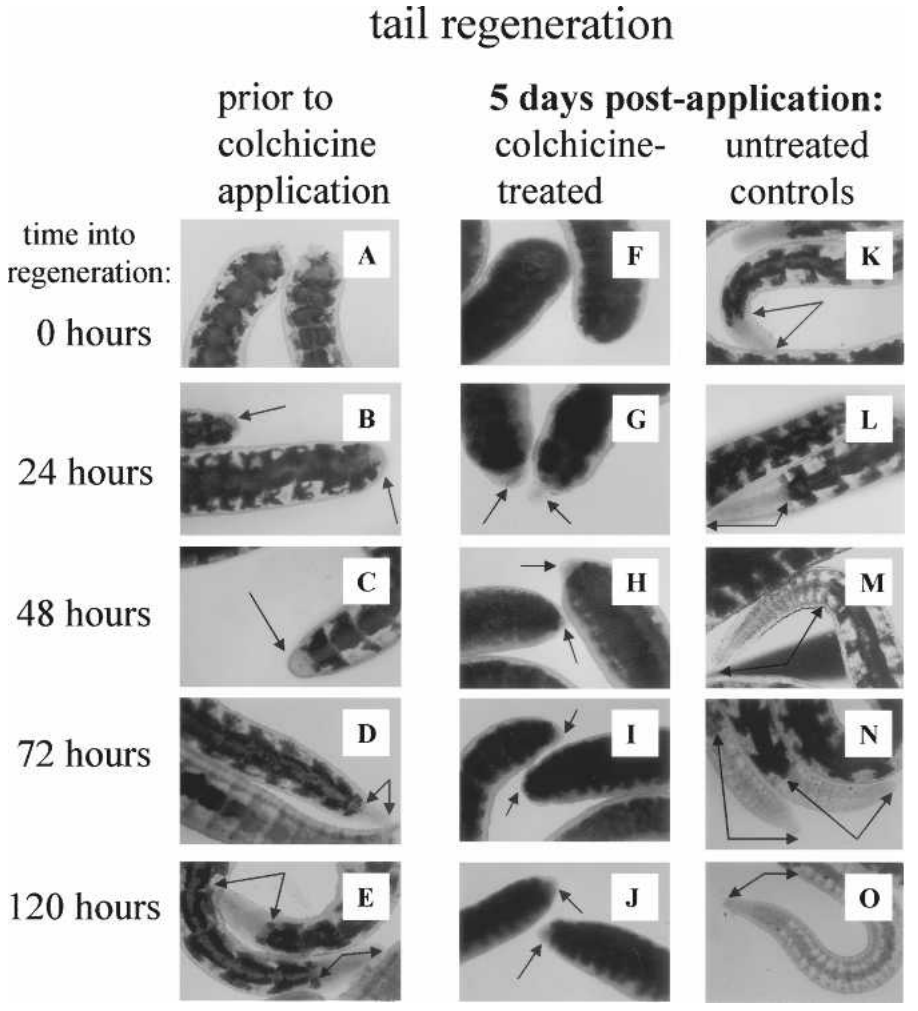

Figure 3. Effect of colchicine on regeneration of tails. Anterior fragments of worms were allowed to regenerate tails in spring water for 0 (A), 24 (B), 48 (C), 72 (D) or 120 hours (E). These fragments were then incubated in 2.5 μM colchicine (F-J) or spring water (K-O) for 5 days. Arrows bracket regions of new tissue formation.

Results

Effect of mitosis-blocking drugs on regeneration

Worm fragments treated with 2.5 mM colchicine (Figures 2 and 3) or 25 μg/ml vinblastine sulfate (data not shown) did not regenerate heads or tails. To further explore this inhibitory effect, worm fragments were allowed to regenerate for 0, 6, 12, 24, 48, 72, 96, or 120 hours before being placed in 2.5 mM colchicine. Compared to controls not exposed to colchicine (Figure 2K-O),

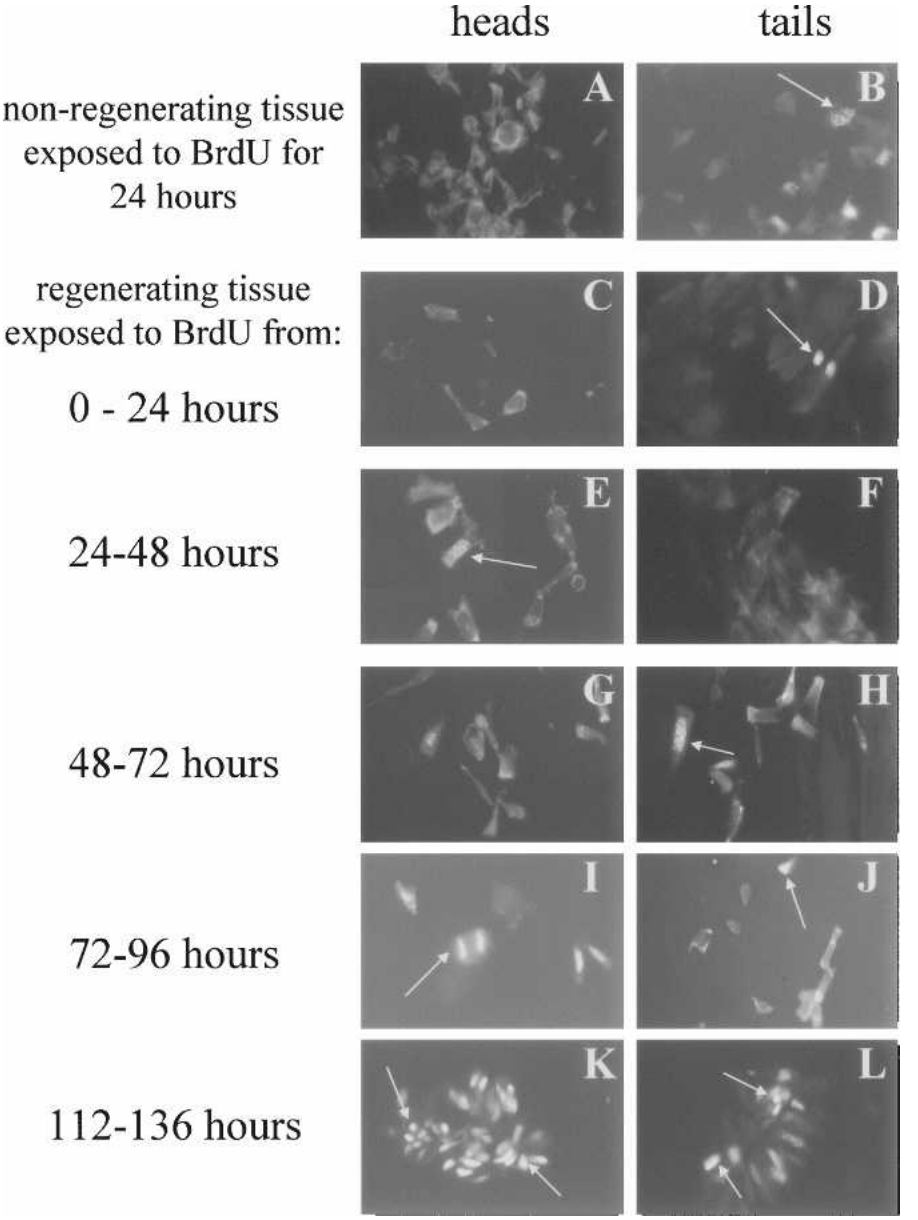

Figure 4. Fluorescence microscopy showing BrdU incorporation into cells from regenerating heads or tails. Worm fragments were incubated for 24 hours in 5 mM BrdU in spring water at 0, 24, 48, 72, or 112 hours into the regenerative process. Blastemas were removed and dissociated into cells as described in the caption to Figure 1. After denaturation of the DNA, the cells were stained with antibodies against BrdU followed by fluorescein-conjugated secondary antibody. Arrows indicate BrdU-labeled nuclei.

worm fragments that regenerated for 0, 24, 48, 72, or 120 hours and were then incubated in 2.5 mM colchicine (Figure 2F-J) did not exhibit head regeneration beyond that already present at the time the colchicine was added (Figure 2A-E). Compared to untreated control fragments (Figure 3K-O), any regeneration that had occurred in tails prior to incubation in the drug (Figure 3A-E) was lost upon exposure to the colchicine (Figure 3F-J). Fragments exposed to colchicine at 6, 12, or 96 hours into regeneration showed similar patterns of response to the drug (data not shown).

Incorporation of BrdU into regenerating worm tissues

Because colchicine and vinblastine may affect cellular processes other than mitosis such as cell motility or cell adhesion (Gordon and Staley, 1990; Hastie, 1991), direct evidence of cell proliferation was obtained by soaking regenerating worms in 5 mM bromodeoxyuridine (BrdU). Incorporation of BrdU was not observed in cells isolated from non-regenerating head (Figure 4A) tissue. Since segment formation in tails is a process that continues after regeneration is completed, BrdU incorporation into nuclei of some cells from non-regenerating tail tissue was expected and was observed (Figure 4B). About 11–15% of the cells harvested from anterior and posterior regenerating tissue at 24 hours (Figure 4C and D) and 48 hours (Figure 4E and F) into regeneration showed incorporation of BrdU into nuclei. In the time period from 48 to about 96 hours into regeneration of heads and tails, BrdU labeling was observed in about 13–15% of the nuclei (Figure 4G-J). The most extensive labeling was detected at 112 to 136 hours into regeneration where about 29–32% of the cells from both regenerating head (Figure 4K) and tail (Figure 4L) tissues showed BrdU incorporation.

Effect of cell migration-blocking drugs on regeneration

The results of the BrdU studies suggest that blastema formation during the first few days of regeneration consisted of cellular processes besides cell proliferation. To explore the potential role of cell migration in regeneration in *L. variegatus*, worm fragments were incubated with locosatin (Zhu et al., 2005) or latrunculin B (Spector et al., 1982), drugs known to inhibit cell migration. Compared to control worms (Figure 5A

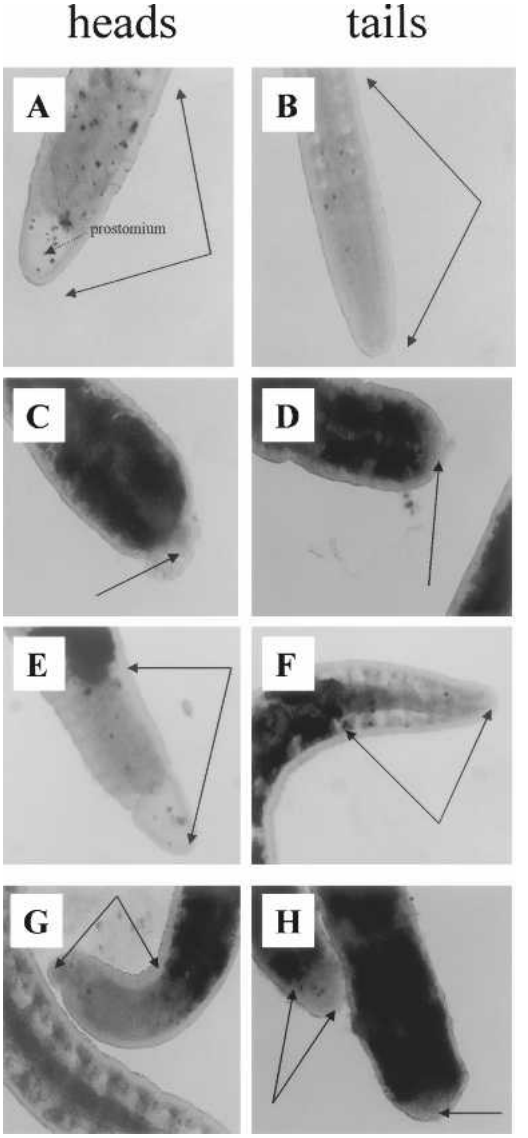

Figure 5. Effect of cell migration inhibitors locostatin and latrunculin B on regeneration. Worm fragments were incubated in spring water (A and B) or 7.5 μM locostatin (C and D) for 10 days. Following treatment with locostatin, fragments (E and F) were then incubated in spring water for an additional 10 days. Other fragments (G and H) were incubated in 0.15 μM latrunculin B for ten days. Arrows bracket regions of new tissue formation.

and B), locostatin inhibited regeneration of both heads and tails with a minimum inhibitory concentration of 7.5 μM. Small outgrowths of tissue were observed in regenerating heads incubated in this drug (Figure 5C) but no tail blastema formation was observed (Figure 5D). This effect was reversible with full regeneration of heads and tails occurring when treated worms were removed from the drug and incubated in spring water (Figure 5E and F).

When worm fragments were treated with 0.15 μM latrunculin B, no outgrowth was observed in the posterior regions of the fragments indicating that regeneration of tails was inhibited by this drug (Figure 5H). The inhibitory effect of latrunculin B on head regeneration was less dramatic (Figure 5G). Head structures were formed; however, compared to untreated controls (Figure 5A) they lacked a prostomium (Hyman, 1916) and were not segmented.

Discussion

The results demonstrate that BrdU was incorporated into nuclei of cells from regenerating tissues of *L. variegatus* following soaking of worm fragments in this thymidine analog. Similar patterns of BrdU incorporation were seen in both anterior and posterior regeneration (Figure 4C-L), with uptake of BrdU detected throughout regeneration. Supportive of these results is the inhibition of head and tail regeneration by mitosis blocking drugs (Figures 2 and 3). These observations confirm that the cellular and molecular mechanisms of regeneration of heads and tails in *L. variegatus* share some epimorphic features in common, with cell proliferation necessary for formation of new tissues. Stephan-Dubois' (1956) analysis of *L. variegatus* first provided histological evidence that this is the case and Myohara et al. (1999) observed similar histological events in a terrestrial oligochaete. The successful labeling of cells in *L. variegatus* with BrdU showed that this molecular approach can be used in further analysis of cell proliferation in these organisms.

Because extensive cell proliferation, based on BrdU labeling of cells, was not observed until approximately 120 hours into regeneration and because regeneration was blocked by exposure of worm fragments to locostatin and latrunculin B, our studies suggest that regeneration in *L. variegatus* also involves cell migration. These results are consistent with the histological observations by Turner (1935) of *L. inconstans* where development of new blood vessels and muscle tissue appeared to arise from differentiation of cells that accumulated and then proliferated in regenerating outgrowths.

It remains to be determined if the BrdU-labeled cells are neoblasts that migrate into the cut site or are cells derived from differentiated tissues already in the region of the wound. Treatment of regenerating head tissues with latrunculin B and locostatin provided some insight on this question. Assuming that latrunculin B and locostatin inhibited cell migration without affecting cell proliferation, the observation of partial anterior regeneration in cut worms treated with these drugs (Figure 5C and G) suggest that some anterior tissues are formed without cell migration. We conclude that, unlike planaria in which regeneration relies initially on migration and then proliferation of neoblasts (Newmark and Sánchez Alvarado, 2000), head regeneration in *L. variegatus* may more closely resemble regeneration in vertebrates such as amphibians. In these animals, dedifferentiation, proliferation, and redifferentiation of cells at the wound site comprise much of the regenerative process (Sánchez Alvarado and Tsonis, 2006). This reveals the potential usefulness of *L. variegatus* as a model system in which to study the regulation and molecular processes of regeneration. On the other hand, blastema development in tails was completely blocked by both locostatin and latrunculin B (Figure 5 D and H), suggesting that regeneration of tails is dependent on migration of cells to the cut site.

Our interpretations are presented tentatively since locostatin, by inhibiting MAP kinase signaling (Trakul and Rosner, 2005), and latrunculin B, by disruption of microfilaments (Spector et al., 1983), can also affect cell proliferation. The low levels of cell proliferation observed during the early stages of regeneration could have been blocked by these drugs with resultant inhibition

of regeneration. To further examine the nature, origin, and position of cells that are involved in formation of new tissues during regeneration in *L. variegatus*, BrdU uptake is being analyzed in regenerating tissues that have been fixed, embedded in paraffin, and sectioned. Regenerating tissues are also being treated with aphidicolin, a specific inhibitor of DNA synthesis (Spadari et al., 1985), to determine when during regeneration cell proliferation is essential. These studies will further clarify the role and time course of cell migration and proliferation in the outgrowth of new heads and tails in *L. variegatus*.

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