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FUNDAMENTALS OF PLANARIAN REGENERATION

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■ **Abstract** The principles underlying regeneration in planarians have been explored for over 100 years through surgical manipulations and cellular observations. Planarian regeneration involves the generation of new tissue at the wound site via cell proliferation (blastema formation), and the remodeling of pre-existing tissues to restore symmetry and proportion (morphallaxis). Because blastemas do not replace all tissues following most types of injuries, both blastema formation and morphallaxis are needed for complete regeneration. Here we discuss a proliferative cell population, the neoblasts, that is central to the regenerative capacities of planarians. Neoblasts may be a totipotent stem-cell population capable of generating essentially every cell type in the adult animal, including themselves. The population properties of the neoblasts and their descendants still await careful elucidation. We identify the types of structures produced by blastemas on a variety of wound surfaces, the principles guiding the reorganization of pre-existing tissues, and the manner in which scale and cell number proportions between body regions are restored during regeneration.

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INTRODUCTION

Planarians (Figure 1) are bilaterally symmetric metazoans of the phylum Platyhelminthes commonly found in freshwater streams and ponds where they prey predominantly upon insects, insect larvae, and other invertebrates. Planarians have the capacity to replace large regions of missing structures through regeneration. An extensive and disparate body of literature exists regarding both the cell biology of regeneration and the restoration of animal form that dates back to the initial observations in 1774 (Pallas 1774) and to systematic studies in the 1890s. Although many of the older studies of planarians contain outdated hypotheses about the cellular and/or molecular events related to specific experiments (Brøndsted 1969), many of the observations are still of fundamental importance to our understanding of regeneration in planarians. Techniques for studying gene function in planarians, such as RNAi (Sánchez Alvarado & Newmark 1999) (double-stranded RNA-mediated genetic interference; Fire et al. 1998), and in situ hybridizations (Umesono et al. 1997), combined with the characterization of a large number of cDNAs from the species *Schmidtea mediterranea* (Sánchez Alvarado et al. 2002), have allowed the initiation of molecular genetic studies of planarian biology. Discussions of the usage of molecular techniques to study gene and cell function in planarians have been extensively described elsewhere and are not described here (Baguñà 1998, Agata & Watanabe 1999, Cebrià et al. 2002, Newmark & Sánchez Alvarado 2002, Newmark et al. 2003). What is lacking, however, is a synthesis of more than 100 years of study of planarian regenerative phenomena that identifies key regeneration principles and unresolved issues related to these principles. Understanding what is already known and needs to be learned about these principles is essential as we enter into a period of molecular perturbation of planarian biology. Here, we aim to generate such a synthesis. In order to avoid confusion and to facilitate reading of this review, anatomical areas and types of amputations are illustrated in Figure 1.

Anatomy

Planarians lack a coelom, i.e., an organ-containing internal cavity, and possess derivatives of all three germ layers (ectoderm, mesoderm, and endoderm). All space between the various organ systems is filled with a mesenchyme, generally referred to as the parenchyma (Hyman 1951). The nervous system is organized into bi-lobed cephalic ganglia connected to two ventral longitudinal nerve cords (Cebrià et al. 2002a), which are interconnected by commissural neurons (Robb & Sánchez Alvarado 2002). Sensory structures, such as photoreceptors (Carpenter et al. 1974), chemoreceptors (MacRae 1967), and rheoreceptors (Hyman 1951),

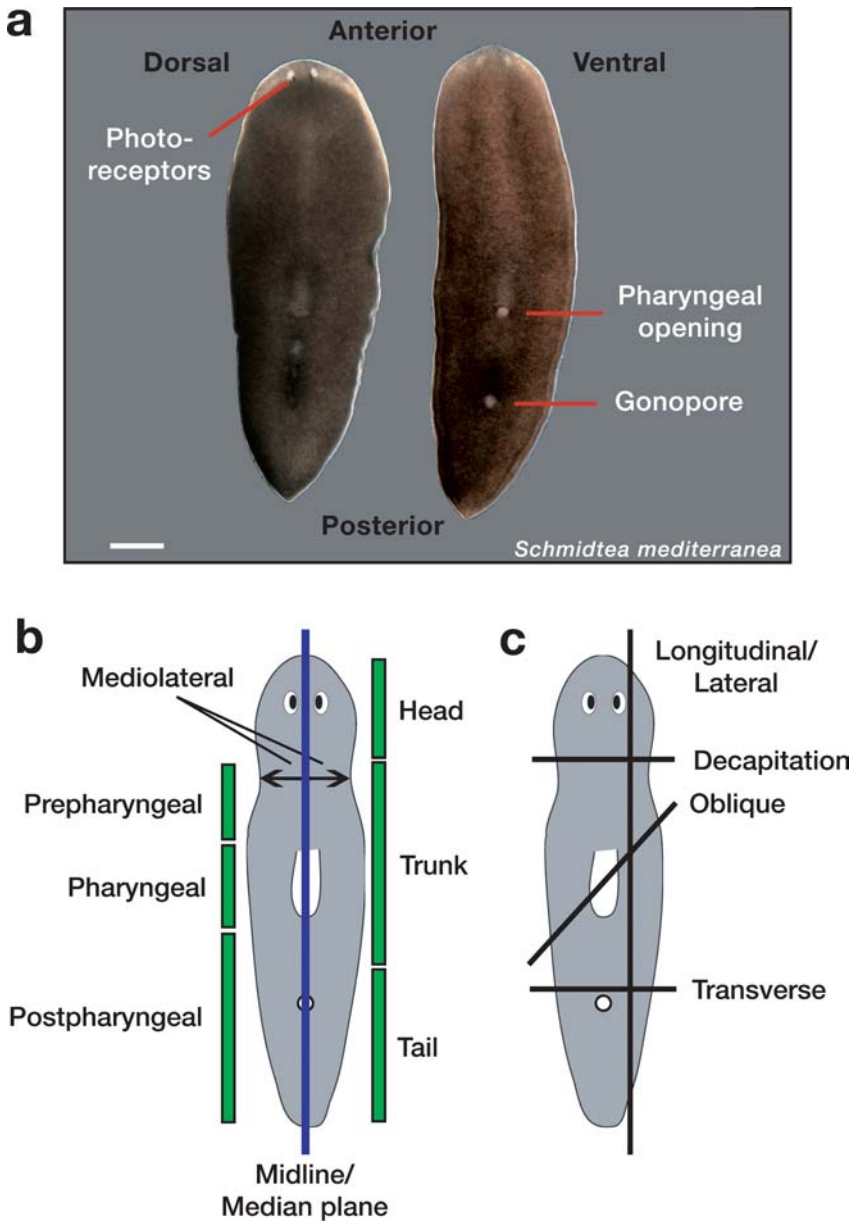


Figure 1 The freshwater planarian. (*a*) External characteristics and the anterior, posterior, dorsal, and ventral regions of the planarian body plan are shown using a sexual strain of *Schmidtea mediterranea*. Scale bar: 1 mm. (*b*) Nomenclature used to describe different body parts of the planarian anatomy. (*c*) Types of amputations used to study the regenerative properties of planarians.

are found at the anterior end of the animal and send projections to the cephalic ganglia. A submuscular nervous plexus runs beneath the body wall musculature and connects to the main nerve cords (Hyman 1951).

The body wall musculature contains longitudinal, diagonal, and circular muscle fibers (Cebrià & Vispo 1997, Orii et al. 2002) not used for locomotion, but rather for negotiating obstacles. Ventral ciliated epithelial cells accomplish locomotion. Food is ingested through a muscular, extensible pharynx that serves as both the mouth and the anus of the animal; the pharynx connects to the three-branched (triclad) digestive system, consisting of one anterior and two posterior branches (Newmark & Sánchez Alvarado 2002). Freshwater planarians reproduce either asexually by transverse fission or sexually as cross-fertilizing hermaphrodites (Hyman 1951). The reproductive system consists of paired ovaries situated behind the cephalic ganglia, with numerous testes located dorsolaterally. Posterior to the ventral pharyngeal opening is the gonopore, an aperture in the ventral surface leading to the muscular copulatory apparatus (Figure 1*a*) (Hyman 1951).

Terminology

There are many types of regenerative phenomena found throughout the Metazoa (Sánchez Alvarado 2003). These range from the replacement of limbs, to the replacement of individual cells, and to the reorganization of cells without cell proliferation (Sánchez Alvarado 2000). Such a wide variety of regenerative events can lead to a confusing set of definitions for the term regeneration. Throughout this manuscript, the phrase planarian regeneration refers to the old, and rather broad definition of regeneration: the replacement of missing structures following injury (Morgan 1901).

Historically, planarian regeneration has been subdivided into two types of processes defined by the terms "epimorphosis" and "morphallaxis" (Morgan 1901), terms that, unfortunately, have created some confusion and controversy (Saló & Baguñà 1984, Galliot 1997, Ito et al. 2001, Agata et al. 2003). Morgan defined them as follows: ". . .there are known two general ways in which regeneration may take place, although the two processes are not sharply separated, and may even appear combined in the same form. . .I propose to call those cases of regeneration in which a proliferation of material precedes the development of the new part, 'epimorphosis'. The other mode, in which a part is transformed directly into a new organism, or part of an organism without proliferation at the cut surfaces, 'morphallaxis'." (Morgan 1901). Whereas it is implied that epimorphosis involves cell proliferation at the wound site, morphallaxis defines events occurring away from the wound. Morgan does not specify the cell biology involved in morphallaxis, which was unknown at the time and remains unknown to date. He coined the word morphallaxis simply to describe that after amputation ". . .the relative proportions of the planarian are attained by a remodelling of the old tissue." Thus the term morphallaxis is useful in discussing the remodeling of pre-existing tissues, and not in discussing the cell biology of such remodeling. Here we use morphallaxis

to refer to tissue remodeling with no intended restrictions on the cell biology that might be involved, and epimorphosis to refer to the formation of blastemas at wound surfaces via cell proliferation.

THE PLANARIAN BLASTEMA

Responses to Wounding

The stimulus for regeneration is injury (Needham 1952). In planarians, amputation elicits a series of responses that ultimately result in a minimization of tissue loss. First, the animal pulls away from the wounding agent, possibly reflecting a predator avoidance reflex. A strong muscular contraction at the site of wounding occurs within seconds and minimizes the surface area of the wound (Chandebois 1980, Newmark & Sánchez Alvarado 2002). Specialized planarian cells, referred to as rhabdites, release their contents at the wound site producing a protective mucosal covering, with possible immunological functions (Reisinger & Kelbetz 1964). A head fragment containing the brain will continue to locomote, possibly to escape the fate its body might have befallen to a hungry predator. Although trunk fragments can move and typically keep their ventral sides down, they remain relatively stationary during regeneration.

A thin layer of epithelium covers the wound within 30 min (Baguñà et al. 1994, Sánchez Alvarado & Newmark 1998), a process that occurs by cell spreading rather than proliferation (Chandebois 1980). The spreading involves both dorsal and ventral epithelial cells, which lose their characteristic morphologies as they cover the wound (Sánchez Alvarado & Newmark 1998). In contrast to wounds produced in humans, scarring (i.e., deposition of dense collagenous fibers) does not seem to occur in planarians (Dubois 1949). As a result, the epithelium is in direct contact with tissues at the site of amputation. In the case of a transverse amputation, such tissues typically will involve muscle cells, nerve tracts, intestine, and mesenchymal cells.

Possible Cues for the Initiation of Regeneration

The molecular nature of the stimulus or stimuli responsible for initiating regeneration after injury in planarians is unknown. In light of the instructive morphogenetic role played by vertebrate epithelia in limb blastemas (Gardiner et al. 1995) and limb buds (Niswander et al. 1993), the planarian epithelium has, accordingly, received attention as a possible source of stimulatory signals (Chandebois 1980, Baguña et al. 1988, Kato et al. 2001). Although it is known that continuous contact between the epithelial cap and the underlying mesenchyme is required for regeneration to occur in vertebrates (Goss 1956), little evidence exists regarding the nature of this putative interaction in planarians (Baguña et al. 1988).

Because amputation and wound healing provide a context in which dorsal and ventral epidermis come into direct contact with each other, it has been suggested that this dorsal/ventral (D/V) interaction may trigger the regenerative response

(Kato et al. 2001, Ogawa et al. 2002). For example, when a fragment is transplanted with reverse D/V polarity from one planarian to another (see below), a regenerative response takes place (Kato et al. 2001). However, not all regenerative events in planarians require D/V interactions to occur. Amputation of the pharyngeal tip results in the proliferation of cells at the base of the pharynx rather than at the site of amputation, and before there is healing of the pharynx (Ito et al. 2001). Furthermore, when two different anterior/posterior (A/P) regions are placed together via transplantation, regenerated tissue appears between the two fragments (Okada & Sugino 1937, Brøndsted 1942, Saló & Baguñà 1985b, Kobayashi et al. 1999a). In these cases, when tissues from different positions contact one another, regeneration occurs without cues from an epithelial covering. In summary, the epithelium, D/V interaction-induced signals, and confrontation of tissues with different positional information might all instruct planarian tissues to regenerate. Whether these three different situations share similar molecular mechanisms is unknown.

Cell Proliferation, Cell Migration, and the Regeneration Blastema

In intact adult planarians cell proliferation is constantly occurring as part of a homeostatic mechanism by which cells lost to normal physiological turnover are replaced. Cell division has been assayed in planarians by at least three methods: direct observation of mitotic figures in paraffin sections (Dubois 1949, Saló & Baguñà 1984), electron microscopy (Le Moigne 1965, 1966; Morita et al. 1969; Pedersen 1972), and with BrdU labeling (Newmark & Sánchez Alvarado 2000). The use of a single pulse of BrdU in intact animals, for example, followed by fixation and inspection 24 h later, shows cell proliferation throughout the entire animal, with the notable exception of the areas in front of the photoreceptors and the pharynx. This result is confirmed by using antibodies against the phosphorylated form of Histone H3 (Newmark & Sánchez Alvarado 2000), a marker of the G2/M transition in the cell cycle (Hendzel et al. 1997). Because there is an absence of proliferation in both the pharynx and the distal anterior tip of the animal, and because the cells within essentially all planarian tissues appear to be replaced during normal homeostasis, these two tissues must depend on cell migration to maintain their structural integrity (Newmark & Sánchez Alvarado 2000, Ito et al. 2001). This suggests that both cell proliferation and migration are processes normally occurring in the maintenance of tissues in intact planarians.

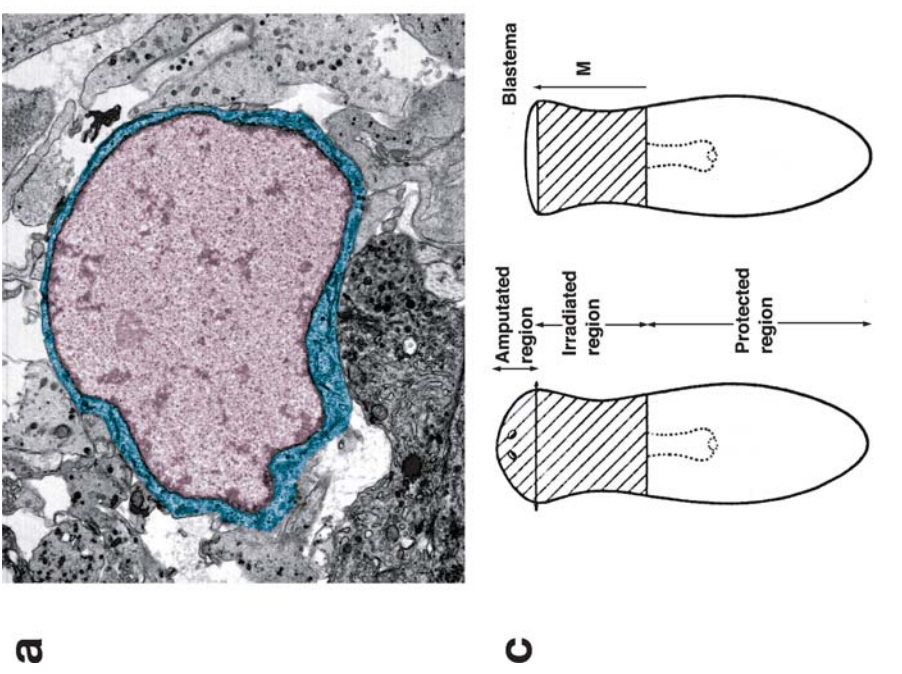
When mitotic activity is assayed in amputated animals, a local burst of proliferation is observed near the site of injury (Dubois 1949, Saló & Baguñà 1986, Newmark & Sánchez Alvarado 2000) leading to the production of an unpigmented epithelial/mesenchymal bud known as a regeneration blastema (Sánchez Alvarado & Newmark 1998). In the regeneration of a head, most cell proliferation is restricted to the region at the boundary of the old tissue and the blastema, and little to no proliferation is observed within the head blastema proper (Figure 2*b*)

(Pedersen 1972, Saló & Baguñà 1984). Because cell proliferation in planarians can be abrogated by gamma-irradiation (Dubois 1949), a number of experiments have been carried out to identify the contribution of cell proliferation to blastema formation. When animals are subjected to high doses of irradiation (e.g., 8–10,000 rads) and amputated immediately after treatment, some blastema formation is observed (Bardeen & Baetjer 1904, Chandebois 1976). However, as time proceeds, the blastema is resorbed and the animal eventually dies (Bardeen & Baetjer 1904). On the other hand, if animals are irradiated and amputation performed 7 days after irradiation, blastemas do not form and the animals also die a few days later (Dubois 1949). These experiments are interpreted as follows. First, cell division in the intact, unirradiated animal produces nonmitotic cells whose viability is unaffected by irradiation. If the nonmitotic division progeny have not differentiated to replace cells in the body normally lost to tissue turnover, they can be recruited to mount a regenerative response (blastema formation observed after amputation within 2 days after irradiation). If, on the other hand, the nonmitotic cells have differentiated, then undifferentiated cells are not available to form a blastema (amputation 7 days after irradiation). Thus elimination of proliferation by irradiation creates a situation in which the nonmitotic cells responsible for differentiating into tissues eventually turnover and are no longer renewed, resulting in the eventual demise of the organism. These data indicate that formation and maintenance of the regeneration blastema require both cell division and migration: cell division to produce nonmitotic cells, and migratory mechanisms to target these nonmitotic cells to active areas of regeneration (e.g., the regeneration blastema) (Figure 2*b*).

THE CELL BIOLOGY OF PLANARIAN REGENERATION

What Are Neoblasts?

Interest in the large numbers of embryonic-like cells distributed throughout the body of adult planarians and other platyhelminthes is long-standing (Wagner 1890, Lehnert 1891, Keller 1894), especially because the cell proliferation observed in both intact and amputated planarians is restricted to this population of small (5–8 μm in diameter), highly undifferentiated cells with large nuclei and very little cytoplasm (Figure 2*a*). These cells are referred to as neoblasts (Dubois 1949, Wolff 1962), a term first used by Harriet Randolph to describe a particular cell type in the annelid *Lumbriculus* (Randolph 1892), and later adopted to describe similar cells in planarians (Buchanan 1933, Wolff 1962). By morphology, neoblasts represent ~25–30% of all planarian cells (Baguñà et al. 1989). The progeny of neoblasts have been shown to produce epidermis (Skaer 1965, Hori 1983a, Ehlers 1992, Newmark & Sánchez Alvarado 2000), rhabdite cells (Lentz 1967, Hori 1978), muscle (Sauzin 1967, Hori 1983b, Morita & Best 1984), and germ cells (Gremigni 1974), among others. Considering that neoblasts are the only dividing cell type in planarians, they may well be totipotent stem cells. However, the paucity of specific markers



and the relatively uniform morphology of neoblasts make it difficult to determine the extent of heterogeneity that may exist in this cell population.

BrdU labeling has begun to provide key information on the population dynamics of neoblasts. For example, we now know that a large subpopulation of G2-arrested cells do not exist in the intact organism (Newmark & Sánchez Alvarado 2000). Such a subpopulation was invoked in the past to explain the early mitotic peak that occurs in the initial 5–12 h of regeneration (Baguñà 1976, Saló & Baguñà 1984). Still not clear, however, is how amputation can trigger the proliferative response of neoblasts. Moreover, even in intact animals, an average of 6% of all neoblasts are labeled soon after a single injection of BrdU, suggesting that neoblasts are entering S phase at a relatively rapid rate (Newmark & Sánchez Alvarado 2000). Although BrdU experiments have begun to shed light on the cell cycle parameters of neoblasts, these alone cannot elucidate if the proliferating compartment of planarians is made up of one or multiple types of stem cells. It is still possible that the apparent totipotency of the neoblasts may reflect the compound activities of multiple types of stem cells.

Because current methods lack the necessary resolution to distinguish between totipotent neoblasts and neoblast descendants committed to particular lineages, we find it necessary to make the following distinction of terms. We refer to those cells that proliferate, have a large nucleus and small cytoplasm, are 5–8 μm in diameter, and are presumed to be capable of producing both multiple tissue types and themselves as neoblasts. Some progeny of neoblasts might be morphologically indistinguishable from neoblasts proper, e.g., lineage-committed cells. When it is possible that neoblasts and/or these other cells were observed in a particular experiment, for clarity, we state "neoblast and/or neoblast progeny."

What Is the Source of Neoblasts?

For the most part, the planarian literature invokes dedifferentiation of somatic cells, self-renewal of a stem cell population, or a combination of the two to explain the origin of neoblasts. For reasons described below, most in the field of planarian

Figure 2 The planarian neoblast. (*a*) Electron micrograph of a neoblast near a site of amputation in the planarian *Schmidtea mediterranea*. The nucleus and cytosol are pseudocolored in light red and blue, respectively. Absence of differentiation in the cytoplasm and decondensed chromatin are prominent. Original magnification, 7,000×. (*b*) Model illustrating known and presumed neoblast activity in the formation of a regeneration blastema by both symmetric and asymmetric cell divisions. (*c*) The partial irradiation experiment of Wolff & Dubois (modified from the original, 1948). See text for details. M indicates the direction of migration. (*d*) Measurements of mitotic indices during the recovery of irradiated tissue (fragments 2, 3, 4) after amputation. Red asterisk designates the number of mitoses measured in unirradiated animals after decapitation. Note the significant difference in magnitude between both peaks (~50 versus ~200) (modified from original, Dubois 1949).

biology favor the hypothesis of self-renewal. Nevertheless, because formal demonstration of self-renewal requires the development of new techniques for lineage tracing, the elimination of the dedifferentiation hypothesis is not yet possible. As such, both hypotheses need consideration.

DEDIFFERENTIATION The evidence for dedifferentiation is based on histological and electron microscopy studies in which differentiated cells are seen to lose their morphology and participate in regeneration (Flickinger 1964, Woodruff & Burnett 1965, Hay 1966). However, other studies using similar methods failed to corroborate these findings (see below). Furthermore, dedifferentiation studies in planarians have been complicated by methodological limitations that cause difficulties in the interpretation of the results. For example, work that suggests gastrovascular cells produce neoblasts by dedifferentiating were carried out with vital dyes that can diffuse from cell to cell (Rose & Shostak 1968). Because neoblasts and/or neoblast progeny are in close proximity to the gastrovascular system (Newmark & Sánchez Alvarado 2000), the transfer of dye from gastric cells to neoblasts is a distinct possibility.

Perhaps the strongest argument for the occurrence of dedifferentiation is provided by the work of Gremigni and coworkers (Gremigni & Miceli 1980; Gremigni et al. 1980, 1982). They used a strain of *Dugesia lugubris* in which the somatic cells are triploid, the female germ cells hexaploid, and the male germ cells triploid. By taking advantage of this mixoploidy, they were able to follow the fate of these cells in amputated animals. For instance, amputation through the gonadal region produced blastemas primarily derived from somatic cells (triploid) but also from germ cells (diploid and/or hexaploid) that produced somatic tissue, e.g., pharyngeal muscle. However, because the germ cells are themselves stem cells, they could possibly be sufficiently pluripotent to produce various somatic tissues. In addition, amputation of sexually reproducing planarians leads to the degeneration of germ cells, which should prevent these cells from contributing to the formation of the regeneration blastema (Fedecka-Bruner 1967). These caveats aside, the work of Gremigni and coworkers illustrates the types of experiments that are required to resolve issues of cell origin and fate in planarians, i.e., the stable and specific labeling of cells to follow their developmental outcomes (Echeverri & Tanaka 2003).

SELF-RENEWAL Self-renewal hypotheses for the origin of neoblasts are primarily based on ultrastructural, irradiation, cell transplantation, and BrdU experiments. First, ultrastructural studies find no evidence for dedifferentiation (Le Moigne 1966, Morita et al. 1969, Pedersen 1972). Secondly, gamma-irradiation of planarians results in a loss of regenerative abilities and ultimately their demise (Bardeen & Baetjer 1904). Such loss of viability and regenerative capacity is correlated with the elimination of only neoblasts and/or their proliferating progeny, i.e., the only mitotically active cells in adult planarians (Dubois 1949, Baguñà et al. 1989, Ladurner et al. 2000, Newmark & Sánchez Alvarado 2000). Nevertheless, the possibility exists that DNA damage inflicted upon differentiated cells by irradiation

could also result in a blockade of dedifferentiation. Third, if neoblasts and/or neoblast progeny are partially purified away from other cell types by size fractionation and then injected into irradiated animals, regeneration capabilities are restored (Baguñà et al. 1989). If the same experiment is repeated using cell fractions enriched in differentiated cells, no rescue is observed. These results suggest that neoblasts are totipotent and that differentiated cells alone are not enough to restore viability. Nevertheless, there are limitations to these experiments. For example, because specific cell fates could not be effectively followed in these assays, the inability of differentiated cells to restore viability to irradiated animals does not necessarily preclude some occurrence of dedifferentiation that went undetected.

Labeling proliferating cells with BrdU provides the further evidence supporting the self-renewal of a stem cell population in planarians. After exposure to BrdU, the first and only cells that label are cells with the morphology of neoblasts (Newmark & Sánchez Alvarado 2000). Since dedifferentiation can be associated with cell cycle re-entry (Tanaka et al. 1997, Velloso et al. 2001), cells with morphologies other than those of neoblasts might be detected among the first cells labeled with BrdU if dedifferentiation occurs. This, however, is not observed. Cells with non-neoblast morphologies can be detected only 35 h after BrdU labeling, and these are presumably the differentiating progeny of labeled neoblasts. The division progeny of BrdU-labeled cells contribute to the tissues of the blastema and to differentiated structures during physiological cell turnover in intact animals, indicating pluripotentiality of this labeled cell population (Newmark & Sánchez Alvarado 2000). Formal proof of the neoblast concept and the unambiguous resolution of cell fate issues necessitate lineage-tracing methodologies, which in planarians are at their earliest stages of development (Gonzalez-Estevez et al. 2003).

Neoblast Migration

Neoblasts have very little cytoplasm (Figure 2*a*), and it is difficult to think of such cells actively moving in response to wound signals. In fact, it has been proposed that neoblast movements are the result of the slow and nondirected spreading caused by random movements linked to cell proliferation (Saló & Baguñà 1985a). However, cells with a morphology similar to neoblasts, their immediate progeny, have been unambiguously observed to migrate into the area in front of the photoreceptors (Newmark & Sánchez Alvarado 2000) and the pharynx (Ito et al. 2001), suggesting that neoblasts themselves could be capable of migrating despite their undifferentiated morphology. Furthermore, migration of stem cells with a morphology similar to neoblasts has been observed in other organisms, such as the movement of hematopoietic stem cells from the bone marrow into the blood stream of mice (Wright et al. 2001). Dubois & Wolff performed a series of ingenious experiments in the late 1940s involving partial irradiation of planarians (Figure 2*c*) that provide the most compelling evidence to date that neoblasts can migrate (Wolff & Dubois 1948, Dubois 1949). Posterior halves of animals were shielded, while the anterior halves were exposed to gamma-irradiation. If the animals were left

intact, the irradiated halves degenerated after a few weeks. If, on the other hand, the partially irradiated animals were decapitated, the anterior region did not become necrotic, and head regeneration was observed at the site of decapitation (Figure 2*c*) (Wolff & Dubois 1948). Whereas regeneration in control animals was completed in 1 week, partially irradiated animals formed blastemas 3–4 weeks after amputation. Dubois & Wolff observed that the delay in blastema formation was directly proportional to the length of the piece irradiated. They concluded that the cells forming the blastema in the irradiated tissue migrated from the unirradiated half and that such migration can be triggered only by wounding, as the neoblasts or their division progeny did not rescue the uncut irradiated half of the animal (Wolff & Dubois 1948).

The hypothesis that signals from the wound are driving cell migration and proliferation is supported by a second experiment (Figure 2*d*) in which the number and spatial distribution of mitotic figures along the A/P axis during cephalic regeneration were determined (Dubois 1949). The peak of mitotic events is localized in both normal and partially irradiated animals to the decapitation site. Moreover, in partially irradiated worms, the peak is significantly higher in magnitude and occurs 4 weeks after decapitation (Figure 2*d*), suggesting that the signals from the wound are stable over time and that a cell type with the capacity to divide can migrate long distances in planarians (Dubois 1949).

It is important to consider that even if migration to sites of amputation does normally occur, it is not necessary for regeneration because short transverse fragments of planarians in which migration is restricted still regenerate (Figure 3*b*). Nevertheless, Dubois' experiments raise a number of issues. For example, in order for an irradiated region to regain regenerative capacity following amputation, a normal distribution of neoblasts must presumably be restored throughout the animal, requiring an increase in neoblast numbers. This increase can be accomplished by symmetric cell divisions (producing two neoblasts from one neoblast). Activation of symmetric divisions of neoblasts could also explain how mitotic activity, and regenerative capacity, is regained by the regeneration blastema (see above and Figure 2*b*). These experiments stand to teach us much about the nature of neoblasts. For example, experiments in which neoblasts are labeled with BrdU and mitotic activity detected with anti-phosphoHistone H3 antibodies should help illuminate the dynamics of repopulation of the irradiated tissues and help identify normal mechanisms of neoblast regulation.

PROPERTIES OF BLASTEMA FORMATION

Above we describe the cellular events that produce the regeneration blastema and the possible cues for inducing such events. Exactly what is a planarian capable of making in these blastemas? Below we describe the blastemas produced on a variety of wound surfaces to identify the capabilities and principles underlying the replacement of missing parts by epimorphosis.

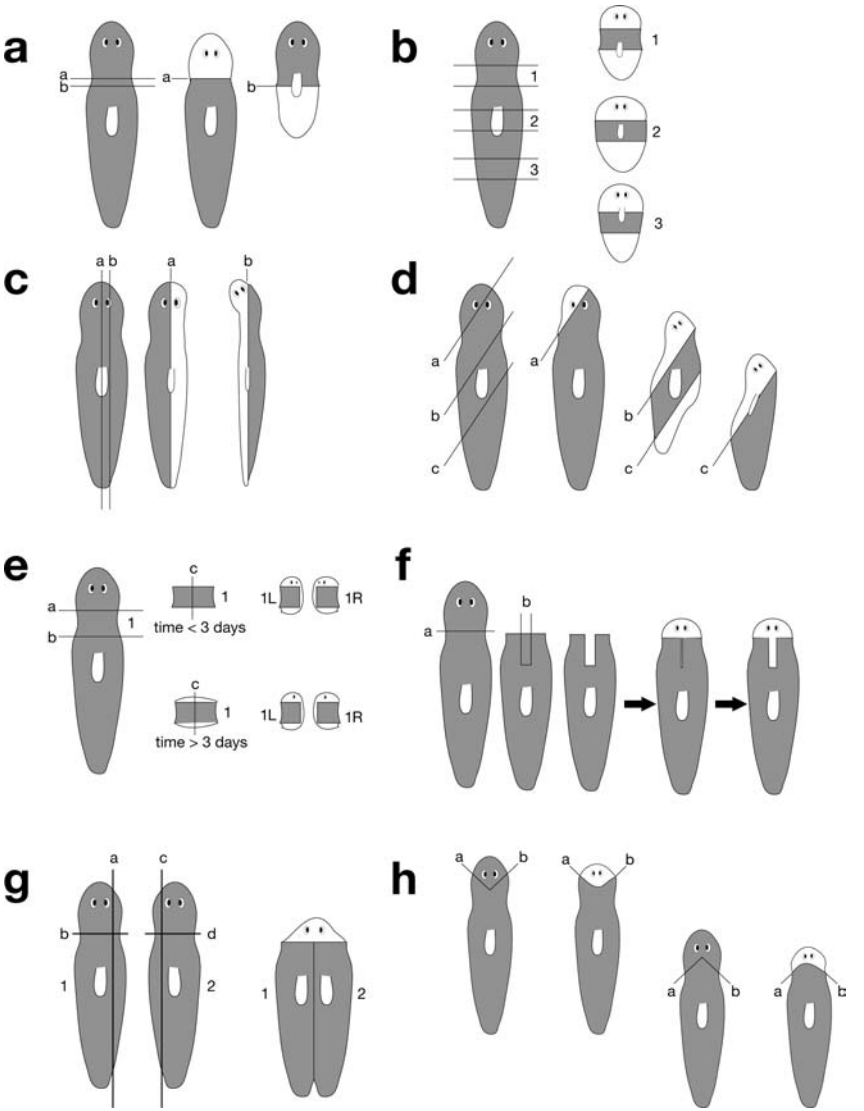


Figure 3 (*a–h*) Schematics of planarian amputations. Dark regions are pre-existing tissues and white regions are newly produced tissues during regeneration. Solid lines indicate amputations. Lower case letters label particular amputations. Numbers label body regions between amputation planes that will become fragments. Descriptions of experiments and relevant references can be found within the text.

Tissue Polarity

When a planarian is amputated transversely, two fragments are generated and are capable of regenerating (Pallas 1774; Johnson 1822, 1825). The term polarity has been used to describe the fact that an anterior-facing wound will regenerate a head and a posterior-facing wound will regenerate a tail (Morgan 1901). "Something in the piece itself determines that a head shall develop at the anterior cut surface and a tail at the posterior cut surface. This "something" is what we call polarity" (Morgan 1904c).

SMALL REGIONS OF TISSUE ARE CAPABLE OF PRODUCING EITHER HEAD OR TAIL BLASTEMAS Consider the cells within two closely spaced parallel lines, a and b, with a more anterior than b, and both perpendicular to the A/P axis (Figure 3*a*). Amputation at position b produces a head fragment, in which the cells between position a and b will become involved in the production of a tail blastema. In contrast, amputation at position a will produce a headless animal, in which cells between positions a and b will now produce a head (Figure 3*a*) (Brøndsted 1955). Short transverse fragments of planarians can be generated that have open wounds on both the anterior and posterior sides. These fragments almost invariably generate head blastemas on the anterior-facing end and a tail on the posterior-facing end, indicating that A/P polarity can be determined by even small regions of tissue (Morgan 1898) (Figure 3*b*). Two exceptions are notable. First, small fragments of tissue from in front of the photoreceptors of multiple planarian species are incapable of regenerating (Morgan 1898), probably reflecting the absence of mitotic activity in this region (Newmark & Sánchez Alvarado 2000). Second, some small fragments, for example, from *Dugesia tigrina* animals, occasionally regenerate two-headed animals (Janus-heads) (Morgan 1898, 1904a) or, for example from *D. lugubris* animals, two-tailed animals (Morgan 1904d), indicating some minimal A/P distance in a region of tissue is needed to robustly specify polarity.

THE WOUND SURFACE AND POLARITY A lateral fragment that contains no anterior-facing wound surface can, nonetheless, regenerate a head at the anterior end (Morgan 1900) (Figure 3*c*). At lower frequency, these short lateral fragments regenerate a head perpendicular to the original A/P axis rather than at the anterior end. Other types of fragments, with only an anterior point and no anterior-facing wound surface also regenerate a head at the anterior end of pre-existing tissue (Morgan 1898). These and extensive related observations in multiple species indicate that the A/P axis of new tissue is determined not by the orientation of the amputation plane but by the A/P axis of pre-existing tissue.

Gradients of Regenerative Capacity

HEAD-FREQUENCY CURVES AND REGENERATIVE GRADIENTS In a variety of species, the rate of regeneration of photoreceptors in a head blastema declines the more posterior the amputation is made (Sivickis 1931, Brøndsted 1939, Child 1941).

Such data sets are referred to as head-frequency curves. For example, the rate of regeneration declines from anterior to posterior in *Phagocata gracilis* (Buchanan 1933), *Dugesia dorotocephala* (Child 1911), or *D. lugubris* (Dubois 1949). In some species capable of asexual reproduction, regeneration speed increases in a postpharyngeal region related to the location at which fission will occur and then declines again toward the tail, e.g., *D. dorotocephala* (Child 1911). In some species, such as *Dendrocoelem lacteum* (Morgan 1904b) and *Bdellocephela punctata* (Brøndsted 1939), regeneration of a head occurs only in anterior regions, but regeneration of a tail is possible in both anterior and posterior regions. The inability to regenerate a head from posterior regions in *D. lacteum* likely resides in the differentiated tissues rather than in the regenerative cells (Stephan-Dubois & Gilgenkrantz 1961). Furthermore, lateral pieces of *D. lacteum* tissue do not regenerate photoreceptors as quickly as do similarly sized pieces from the midbody (Brøndsted 1946). Therefore, the rate of regeneration of photoreceptors can be slower in both more posterior and more lateral regions. The differences in the rate of regeneration along the A/P axis may reflect some aspect of the polarity of planarian tissue that normally allows for a head to develop on anterior-facing surfaces or a tail on posterior-facing surfaces. Studies performed by Child and colleagues in the early 1900s led to hypotheses involving metabolic gradients as determinants of developmental polarity (Child 1941). These hypotheses have not yet proved generally applicable. The polarity of planarian tissues has also been utilized to support hypotheses of developmental patterning involving morphogenetic gradients (Slack 1987). However, the molecular properties of planarian tissues reflected by these head-frequency curves have not been uncovered and, furthermore, no satisfying explanations exist for how a planarian wound surface can be specified to make a head or a tail.

What a Regeneration Blastema Makes

The blastema of a decapitated planarian will regenerate a new head; yet, the blastema of an animal with head and midbody, or more, cut off will regenerate only a new head as well. Thus the planarian blastema does not always restore all of the structures lost by injury. Below, we illustrate what is made by blastemas on a variety of wound surfaces and indicate that a blastema can be regulated by pre-existing tissues to either repair partially damaged or to replace completely missing structures. Using anterior-facing wounds as case studies, we demonstrate the propensity of anterior blastemas to produce a single and complete head, despite their capacity to form two or more heads and ability to produce a head from small body regions.

REGENERATION FROM TRANSVERSE AND LATERAL FRAGMENTS Transverse fragments made from the prepharyngeal region, the pharyngeal region, and the postpharyngeal region of planarians such as *D. tigrina* all make head and tail blastemas that are similar in extent, despite the fact that some tissues lack more anterior structures than others (Morgan 1898) (Figure 3*b*). Similarly, epimorphic regeneration

from lateral fragments can fail to replace all missing structures (Randolph 1897). Lateral fragments that contain more than half of the original animal produce, in a regeneration blastema, slightly less tissue than what is missing (Morgan 1900) (Figure 3*c*). Lateral fragments that contain less than half of the original animal, and thus missing more than half of lateral structures, produce in a regeneration blastema only slightly less tissue than the remaining pre-existing tissue (Morgan 1900) (Figure 3*c*). Therefore, the blastemas of both lateral and transverse wound surfaces are typically insufficient to replace all missing structures. In order to replace the remaining missing body regions not formed by the regeneration blastema, morphallaxis must occur (see below).

THERE IS NO PREDETERMINED PROGRAM FOR THE EXTENT OF ANATOMY PRODUCED BY ANTERIOR-FACING BLASTEMAS Although head blastemas on anterior-facing, transverse edges from regions posterior to the photoreceptors essentially always produce the same anterior structures (Morgan 1898) (Figure 3*b*), there is not an all or nothing, preset program for regeneration from an anterior-facing wound. For example, an oblique amputation through the head that leaves one photoreceptor in place triggers replacement of only the missing part of the head rather than regeneration of an entire head on the anterior-facing oblique surface (Morgan 1900) (Figure 3*d*). Oblique amputations can be made such that the wound surface is longer than the worm is wide; the anterior-facing regeneration blastema in these cases produce not only a head but also side tissue (Morgan 1900). Furthermore, lateral fragments that retain part of the head regenerate only the region of the head that is missing rather than starting an entirely new head (Randolph 1897) (Figure 3*c*). Therefore, in cases of limited injury, a regeneration blastema can regenerate only what is missing and not more, but in cases of extensive injury, a regeneration blastema can replace some structures entirely, but not all missing structures. How the tissues at the wound surface specify the extent of the regeneration blastema is unknown.

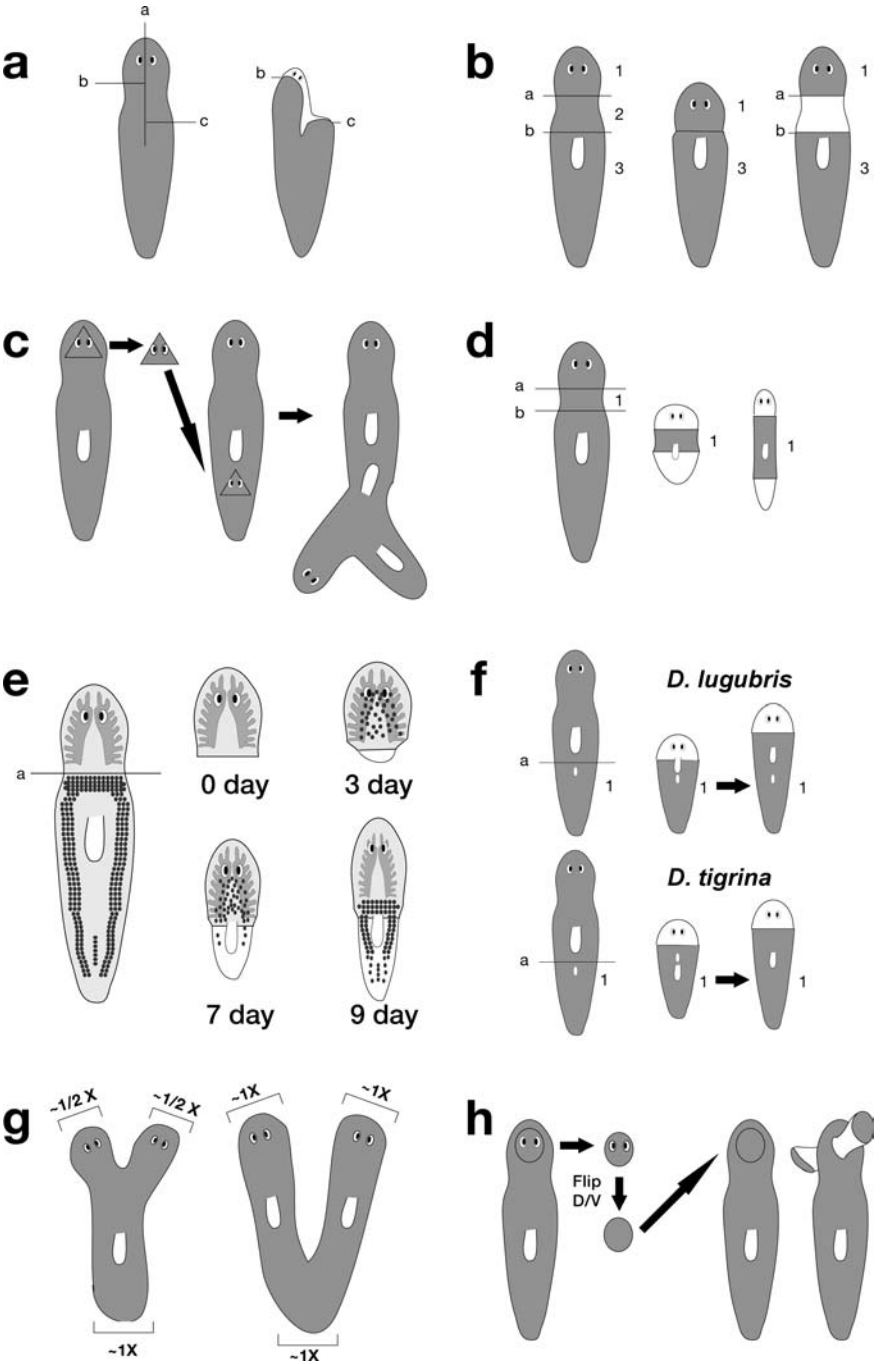
ANTERIOR-FACING BLASTEMAS HAVE THE CAPACITY TO PRODUCE MULTIPLE HEADS BUT ARE SPECIFIED TO PRODUCE ONE COMPLETE ONE In embryonic development, some regions of cells that are specified to make a particular structure can be divided, and each of the resulting regions can make the entire structure (Harrison 1918), a property defined as equipotentiality for isolated blastomeres (Driesch 1900). The early planarian head blastema displays equipotentiality (Morgan 1902). If a head blastema is split in two, within 2 days after an initial transverse cut each resulting half blastema will regenerate a complete head (Morgan 1902) (Figure 3*e*). Therefore, an early anterior-facing blastema is capable of producing one or more heads.

A complete, essentially symmetrical head is produced in the anterior-facing blastema from small pieces of many different shapes, e.g., a tiny fragment from the side of the animal (Randolph 1897; Morgan 1898, 1900; Montgomery & Coward 1974). Therefore, equipotential cells within an early anterior-facing blastema are specified to produce one complete head, even if the underlying tissues do not have

left, middle, and right regions within them. For instance, a region of a decapitated animal can be removed from the median plane and the two sides brought together (Figure 3*f*). On this anterior surface, a symmetric head is formed. By decapitating and then removing the lateral side of two planarians, it is possible to join two planarian bodies along their longitudinal wounds and produce an anterior-facing wound with duplicated pre-existing tissues (Figure 3*g*). Although these experiments, using *Planaria torva*, involve a very small sample size, they indicate that a single head can be regenerated on this anterior wound surface (Brøndsted 1956). Furthermore, wounds that reveal multiple anterior surfaces, if covered by a single blastema, can make a single head. Anterior wound surfaces in *D. tigrina* animals that come to an apex at the midline, or to a low-point at the midline, and that thus have two anterior oblique edges, still regenerate a single head (Morgan 1900) (Figure 3*h*). In a more extreme example, two anterior edges can be generated along an anterior wound surface at different A/P positions. In *D. lugubris* individuals, one large blastema can be formed that covers the most anterior surface as well as the more posterior of the anterior-facing edges (Figure 4*a*). A single head forms from this blastema but is off-set to the more anterior-edge (Morgan 1902). How off-set cephalic regeneration may occur is discussed in the section on oblique wounds below.

These and other similar results demonstrate that the cells that accumulate in the early blastema can organize themselves to produce a single structure regardless of the nature of the pre-existing tissue from which they come. In a normal planarian blastema, there may be a region of equipotential cells that are specified to make a single head structure. A similar principle has been encountered in other developmental paradigms. For example, in *Caenorhabditis elegans* vulval formation, a group of equipotential cells defines the vulval equivalence group. The fates of the cells in this equivalence group are regulated, in part, by lateral inhibitory signaling between them (Horvitz & Sternberg 1991, Wang & Sternberg 2001). Other examples exist in *Drosophila* in which numbers of structures made by equipotential cells are restricted by lateral inhibition (Jennings et al. 1994, 1995).

REGENERATION MAY OCCUR IN A MANNER THAT INITIALLY DOES NOT GENERATE SYMMETRY Some property of the pre-existing tissue specifies the median plane of the blastema around which the symmetry of, for example, a new head will be formed. For instance, consider regeneration of oblique fragments surgically generated by two transverse amputations at angles in which one or more of the amputations are not at right angles to the median plane of the animal (Figure 3*d*). The pre-existing midline can be visualized by the location of the pharynx in an oblique fragment from the pharyngeal region. Along the anterior wound of such a pharyngeal oblique piece in multiple planarian species, a head blastema will be generated initially off-set toward the anterior edge of the tissue, and somewhat perpendicular to the cut surface (Morgan 1900) (Figure 3*d*). Tail blastemas initially develop off-set toward the more posterior edge of an oblique wound surface (Morgan 1900). Therefore, the symmetry of the regeneration blastema on an



oblique fragment does not correspond to the midline of old tissue. A pharynx in oblique tail fragments of *D. lugubris* animals is regenerated at the boundary of the old and new tissue, approximately at the middle of the pre-existing tissue and initially nearly parallel to the wound surface with its highest point toward the newly regenerated head (Morgan 1902) (Figure 3*d*). This observation also demonstrates that the newly regenerated midline, presumed to be a line along which the pharynx is centered, is not in line with the old midline. In these oblique fragments bilateral symmetry around a midline is eventually restored by the process of morphallaxis combined with asymmetric epimorphic growth (Morgan 1902). The initial development of a head off-set toward the more anterior region of an oblique surface could be related to the graded capacity for regeneration rate along the A/P axis discussed above.

TRANSPLANTATIONS TRIGGER THE PRODUCTION OF NEW TISSUES A large variety of cut-and-paste experiments have put planarian tissues with different A/P, mediolateral, and D/V positional identities or polarities together. Regeneration of new unpigmented tissues between transplanted fragments is produced under such circumstances in multiple planarian species, probably the result of cell proliferation (Brøndsted 1942, Kobayashi et al. 1999a). Are these observations relevant to what is normally made by a blastema? Despite the fact that these tissues do not meet the strict definition of a blastema, they appear to be the result of new tissue production similar to that seen in other cases of epimorphosis. Understanding the extent of structures made in these cases might explain how regenerating tissues in general are specified to make particular missing body regions. These studies also add to our understanding of the events that can trigger regeneration (see above). For example, when a median section of tissue is removed from the anterior end of a decapitated planarian and the two left and right halves are brought together, new tissue appears to replace this missing median region (Morgan 1900) (Figure 3*f*). Furthermore, when anterior and posterior transverse fragments are brought together, new midbody tissue is produced between the two regions (Okada & Sugino 1937, Brøndsted 1942, Chandebois 1976, Saló & Baguñà 1985b) (Figure 4*b*). In contrast, when two regions of the same A/P location are brought together, no regenerative response is seen (Okada & Sugino 1937, Chandebois 1976, Kato et al. 1999). Therefore, it is likely that the types of tissues made in these cases are determined by what would normally be present between the two disparate regions.

←

Figure 4 (*a–h*) Schematics of planarian amputations and transplantations. See Figure 3 legend for labeling conventions. Descriptions of experiments and relevant references can be found within the text. (*e*) In situ hybridizations allow visualization of the reorganization of the brain and mucous-producing cells during regeneration (Agata et al. 2003). Light shaded and bilobed structure, brain. Dark circles, mucous-producing cells. (*g*) X indicates a unit of width. (*h*) Flip D/V indicates that the fragment is flipped such that ventral is up and dorsal is down.

The concept of intercalary regeneration has been invoked to explain the positional identities of tissues produced in a variety of transplantation experiments (Chandebois 1976, French 1976, Agata et al. 2003). However, the molecular and cellular explanations for these regenerative phenomena that occur are still lacking. In cases where incomplete injuries occur in the wild, wound healing undoubtedly brings separate body regions together; regenerative replacement of missing body regions in these situations is thus an important attribute of normal planarian regeneration.

Differentiation in the Blastema

COMMITMENT TO FORM PARTICULAR STRUCTURES WITHIN BLASTEMAS When are the cells in a blastema specified to make a head or a tail? If a head blastema is split into two after 3 days of regeneration following a transverse cut, it will form a single photoreceptor at a position that would have been appropriate for regeneration of one half of a head (Morgan 1900, 1902) (Figure 3*e*). The second photoreceptor will eventually appear as new tissue is made laterally and as symmetry is eventually obtained. This finding supports the notion that the fate of the cells within a head blastema has been specified within 2 to 3 days following amputation. Consistent with this idea, when blastemas are amputated after 2 days of growth, they can show signs of appropriate head or tail differentiation in isolation (Sengel 1960). Moreover, signs of differentiation of head-like cells have been detected by examining the expression of neuronal genes with in situ hybridizations as early as within one to 2 days following regeneration (Cebrià et al. 2002b). These experiments indicate that neoblast descendants do not differentiate into specific cell types or structures prior to entering the blastema from the parenchyma (Umesono et al. 1997, 1999; Agata & Watanabe 1999; Cebrià et al. 2002b). However, it remains unknown whether neoblasts become determined to adopt a particular fate prior to entry into the blastema and subsequently differentiate within the blastema.

INDUCTIVE AND INHIBITORY INTERACTIONS A series of surgical and biochemical experiments using lysates from particular planarian regions has led to the development of a model for tissue specification and differentiation in the blastema and neighboring tissues that involves a series of inductive and inhibitory interactions (Lender 1962, Wolff 1962). For example, if the cephalic ganglia of *Polycelis nigra* are prevented from regenerating by frequent cutting, photoreceptors fail to regenerate (Wolff & Lender 1950a,b). When this procedure is carried out and a lysate from a normal head is added, the photoreceptors regenerate (Lender 1955). Tail lysates, on the other hand, have no effect suggesting the activity within the head lysate is specific (Lender 1956). Other related phenomena, such as a possible requirement of the pre-pharyngeal region for induction of the pharyngeal region, have also been described (Lender 1962). Because some of these inductive and inhibitory effects could be nonspecific and because none of the presumed factors involved has ever been biochemically purified, the validity of these models remains in question.

REMODELING AND PROPORTION

In the prior section, we examined blastema formation on a large variety of wound surfaces and defined key principles related to the production and patterning of new tissues. Now, we turn to the changes that occur in the pre-existing tissue to identify principles by which a differentiated fragment of a whole planarian can be reorganized to produce an animal with all organ systems restored in the proper proportions. The regions of pre-existing tissue described below are identified as pigmented and the blastema as unpigmented.

Resetting Positional Information

An animal can be amputated in a variety of ways such that the underlying tissues are not symmetrically distributed. Previously, we discussed the fact that some property of the anterior blastema tends to produce a single and complete head. The pre-existing tissue posterior to this head blastema will ultimately be remodeled to produce additional structures and to restore form (morphallaxis, see below). Initially, however, as the polarity and nature of the anterior blastema is being specified, the positional values within the pre-existing tissue are apparently not entirely reset. Therefore, a regeneration blastema is produced utilizing the positional information relating to, in part, the origin of the fragment. Furthermore, as new structures are produced in pre-existing tissues, which indicate that positional values have been reset, influence upon the patterning by the prior positional information of the body can be seen.

TRANSVERSE HALF FRAGMENTS In normal regeneration from transverse wounds, the midline of the blastema corresponds to the midline of the pre-existing tissue (Morgan 1898). In contrast, when a transverse fragment of *D. tigrina* or *D. lugubris* is isolated and cut into two, the regenerated heads are shifted such that their median planes (the midline between the photoreceptors) are off-set toward the midline of the old tissue rather than being found in the middle of the fragment (Morgan 1901, 1902) (Figure 3*e*). Furthermore, the left half-piece first regenerates a left eye followed by the right, whereas the right half-piece first regenerates a right eye followed by a left (Morgan 1902). These data indicate left or right lateral regions of tissue are most proficient at regenerating left or right lateral structures, respectively. Together, these observations indicate that the positional information in pre-existing tissues influences blastema patterning and is incompletely reset before specification of a new head.

THE POSITIONAL IDENTITY OF PRE-EXISTING TISSUE CAN BE ASSAYED BY USING TWO CUTS AT DIFFERENT TIMES In regenerating thin transverse fragments, A/P polarity is occasionally lost and animals regenerate with two opposing (Janus) heads (see above). If there is a time delay between the first and the second cuts, for example 48 hours, the likelihood of producing Janus heads is decreased,

indicating that A/P polarity in these small fragments is re-established with time (Morgan 1904a, Child & Watanabe 1935). The A/P positional identity can be assessed by the rate of photoreceptor regeneration (see above). If two successive amputations are made at a similar A/P location, the rate of head formation is greater with increasing time intervals between the amputations (Brøndsted 1956), possibly identifying the rate at which A/P positional information is reset. One caveat with this experiment is the possibility that amputation near an actively regenerating region may allow more rapid regeneration than normal.

PHARYNX REGENERATION IN TRANSVERSE FRAGMENTS When events of morphallaxis begin, i.e., when remodeling and production of new structures within pre-existing tissue begins to occur, influences on the pattern of development by the prior position of the pre-existing tissues is apparent. For example, when three transverse fragments from different A/P positions are generated (1, prepharyngeal; 2, pharyngeal but lacking a pharynx; and 3, postpharyngeal; Figure 3*b*), each of these pieces will regenerate a similar head and tail (see above). The positional information of these pieces is re-established such that within the pre-existing tissue of each a pharynx will form. The prepharyngeal fragment regenerates a pharynx at the posterior end of the old tissue near the boundary of the tail blastema; the pharyngeal region regenerates a pharynx in the middle of the old tissues; and the postpharyngeal region regenerates a pharynx at the anterior end of the old tissue, near the boundary of the old tissue and the anterior blastema (Figure 3*b*). Therefore, despite the fact that pre-existing tissues in these regions are specified to make midbody structures, differences in their region of origin influence their patterning (Morgan 1898, 1900, 1902).

TRANSPLANTATIONS TRIGGER CHANGES IN THE POSITIONAL IDENTITIES OF PRE-EXISTING TISSUES Because transplanted regions can trigger lasting changes in the host tissues, these experiments might illuminate the instructive interactions between tissues that establish positional identities. We consider a case study below. When head or prepharyngeal regions (Okada & Sugino 1937) are transplanted into the postpharyngeal region of a host, outgrowths are produced (Santos 1929, 1931); this is frequently, for example, in 113/125 transplantations (Santos 1931), associated with the development of an ectopic pharynx with reversed polarity in the host (Figure 4*c*). Markers that can distinguish the host and donor cells indicate that the host tissue itself can be reorganized by the presence of the graft (Kobayashi et al. 1999a). If the graft outgrowth and the induced pharynx are removed, the ectopic pharynx will regenerate in the absence of graft tissue (Sengel 1953), suggesting a stable change of positional identity of host tissue was triggered by the graft. These observations indicate that during normal regeneration, instructive interactions between pre-existing and newly produced tissues might specify positional information.

Morphallaxis and the Restoration of Form

As described above, blastema formation does not always result in the generation of all missing structures and can be asymmetric. Therefore, in most cases of planarian regeneration, epimorphic regeneration produces animals that lack proper proportion, symmetry, and/or structures. These problems are in large part resolved by the process of morphallaxis.

EXAMPLES OF MORPHALLAXIS When a tail region is isolated by amputation, a new head is produced by epimorphosis in the anterior blastema, and in pre-existing tissue, a pharynx is produced (Morgan 1898). This pharynx arises in a region that was once postpharyngeal and contains the two main posterior branches of the gastrovascular system (see above), indicating the tail fragment entirely reorganizes its anatomy. In regenerated animals from transverse fragments, the width is initially too great for the length because the full extent of the length lost is not replaced by epimorphosis (see above), and the pharynx is often too close to the head (Morgan 1898). The new blastema tissue does not change much in length once formed, and animal proportion is created by the lengthening and thinning of the pre-existing tissues (Morgan 1898) (Figure 4*d*). This lengthening and thinning can be extensive. For example, epimorphic regeneration of a transverse fragment produces a new animal of a certain length that contains a pigmented pre-existing region and unpigmented, newly formed head and tail regions; during morphallaxis and without feeding of the animal, the pigmented pre-existing region can extend to a length greater than the length of the newly formed small animal (Morgan 1898).

REORGANIZATION OF BODY REGIONS In tail fragments, in which a pharynx will form in the pre-existing tissue, the muscle cell gene, *DjMHC-A*, is detected in mesenchymal cells in the middle of that fragment, a region where the pharynx will ultimately form. Furthermore, irradiation blocks pharynx regeneration (Ito et al. 2001). These observations indicate neoblasts may be involved in pharynx formation and specified before actual formation of the ultimate structure (Agata & Watanabe 1999, Kobayashi et al. 1999b). In situ hybridizations with probes that recognize differentiated tissues normally residing in different locations (e.g., in *D. japonica*, *DjPC2* for the CNS and *PN8* for mucous-producing cells in the prepharyngeal and lateral regions) (Agata et al. 2003) permit visualization of the way in which different tissues are repatterned during regeneration and morphallaxis (Figure 4*e*). For example, in a head fragment produced by decapitation, the brain is much too large for the size of the piece, and prepharyngeal, pharyngeal, postpharyngeal, and tail regions must all be generated. Cells that will become prepharyngeal (*PN8*-positive) appear on top of the pre-existing cephalic ganglia 3 days following amputation (Agata et al. 2003); later, these two body regions separate (Figure 4*e*). Similar positional changes in pre-existing tissues have been observed using an antibody, TCEN49, that recognizes cells in the pharyngeal

region (Bueno et al. 1996). These observations allow for visualization of the process of morphallaxis and illustrate several differences between regeneration and normal embryonic development: (*a*) Structures become present in abnormal configuration or proportion following amputation (e.g., a large brain in a decapitated head regenerating a body). (*b*) Structures can be reformed in abnormal configurations during regeneration (e.g., appearance of prepharyngeal mucous-producing cells on top of the brain in a decapitated head regenerating a body). (*c*) Order is restored from abnormal morphology and body organization through reshaping and positioning of the tissues (e.g., redistribution of mucous-producing cells to the normal region, posterior to the brain).

THE METABOLIC STATE CAN ALTER MORPHALLAXIS Depending upon available resources, two animals cut in the same way can utilize variant morphallactic remodeling strategies to restore form. For example, in laterally regenerating animals from a fragment that does not contain the original midline, a new pharynx is produced at the boundary between the old and new tissues. Because the old tissue is greater in extent than the new tissue, the pharynx is produced off-center (Figure 3*c*). In starved animals, following initial regeneration, symmetry is largely restored from remodeling from the pre-existing tissue and minimal growth of new tissue (Morgan 1900). In animals fed after the regeneration of a pharynx, symmetry is restored with growth of new tissues and minimal loss of old tissues (Morgan 1900). Implicit in the above example and others described in this section is that existing tissue can shrink. Animals that are starving can "degrow" through a process that involves loss of cell number; the form and function of existing organs can be maintained while they change in size, shape, and cell number (Abeloos 1930, Baguñà et al. 1990, Oviedo et al. 2003). During morphallaxis, starved animals lose more pre-existing material during reshaping than do well-fed animals.

SPECIES SPECIFICITY WITH MORPHALLAXIS Because not all species of planarians display the same morphallactic strategies to restore symmetry and proportion, there may not be set rules that all of the triclad planarians follow to restore form following amputation. For example, consider tail fragments produced by an amputation posterior to the pharynx but anterior to the gonopore of sexual *D. lugubris* and *D. tigrina* individuals. In *D. lugubris*, the new pharynx appears near the boundary of the pre-existing tissue and the blastema, and anterior to the gonopore, placing it much too close to the newly regenerated head compared with the normal adult proportions (Figure 4*f*). The tissue anterior to the gonopore, which contains the new pharynx, extends in length to restore form (Morgan 1900). In similar tail pieces of *D. tigrina*, the new pharynx is formed posterior to the remaining gonopore well within the pre-existing tissue (Figure 4*f*). The gonopore then disappears as part of the restoration of proper animal anatomy (Morgan 1900). Nonetheless, many of the differences are variations on a larger theme: New structures (e.g., a pharynx) can be formed, and old structures (e.g., the gastrovascular system) can be remodeled within pre-existing tissue, and pre-existing tissue can change in length and width.

THE RELATIONSHIP BETWEEN EPIMORPHOSIS AND MORPHALLAXIS It is unresolved if blastema formation through cell proliferation at the wound surface and the remodeling of pre-existing structures are separable events. For instance, a tail region deprived, by amputation, of a head blastema can regenerate a pharynx (Ziller 1973), but when the left and right sides of an anterior wound meet and seal at the midline no morphallaxis occurs (Morgan 1898). Regardless of the necessity of specific regenerative events upon each other, there may be differences in the capabilities of epimorphosis and morphallaxis. For example, the photoreceptors and brain always form in a blastema and apparently never in pre-existing tissues, indicating that the cellular events capable of producing certain cell types or organs in an adult may require events specialized to epimorphosis.

Regulation of Proportions

In the initial phases of regeneration, a complete set of organ systems is produced. However, because some systems may be pre-existing from a much larger animal, the proportions between the components of the newly reconstituted anatomy may be strikingly abnormal. Below, we illustrate evidence for the ability of planarians to tightly regulate the size and number proportions between organ systems during regeneration.

REGULATION OF PROPORTION AND FORM The gene *cintillo*, from the planarian *S. mediterranea*, is expressed in sensory neurons that vary in number proportionally to the size of the animal (Oviedo et al. 2003). When a large animal is amputated, the head blastema produces the number of *cintillo*-positive cells that correlates with the size of the newly regenerated animal, rather than with the original size of the animal (Oviedo et al. 2003). Furthermore, in decapitated heads the brain is much too large for the size of the animal to be generated. As the head piece slowly reshapes itself to obtain the proper form, the number of *cintillo*-positive cells decreases toward that appropriate for a planarian with smaller dimensions (Oviedo et al. 2003). Observations of planarians produced to have duplicate structures hint at the complexities of regulation that occur to establish the proper numbers of organ systems and cell types in the proper proportions. For instance, the planarian head can be surgically split into two or more and regenerate two or more heads (Randolph 1897, Lus 1926). The body of a two-headed animal, if it contains a single midbody region, does not double in size. A combination of new growth and tissue loss results in two heads that are each about half the width of the single body (Morgan 1902) (Figure 4*g*). In contrast, a two-headed planarian with partially duplicated midbody regions can regenerate two heads, each of the same width of the single body (Morgan 1902) (Figure 4*g*) and each with the number of *cintillo*-positive cells appropriate for an animal of that length (Oviedo et al. 2003). Although duplicated structures can regenerate, e.g., following decapitation of a two-headed animal, various amputation experiments that remove one duplicated structure near or within a single body illustrate the propensity of the animal to maintain only one set of organ systems (Morgan 1902, Rand & Browne 1926,

Rand & Ellis 1926, Li 1928, Santos 1931). These observations demonstrate that the proportion of specific tissues and cell numbers to one another and animal size is tightly regulated.

SUMMARY AND PROSPECTS

Regeneration in planarians involves the production of new tissue at the wound surface through cell proliferation. The cells involved are small, contain a large nucleus, are undifferentiated, are capable of dividing, and are called neoblasts. For decades, experiments have been performed with the aim of deciphering exactly what these cells are, using electron microscopy, histology, visualization of mitotic figures, irradiation, cell transplantation, and, more recently, BrdU labeling. The data suggest that neoblasts are stem cells; i.e., capable of producing multiple tissue types and themselves. Because the only apparent dividing cells in an asexually reproducing animal capable of regenerating any region of its body have the appearance of neoblasts, these cells may very well be totipotent. Truth be told, however, the defining characteristics of neoblasts are their morphology and capacity to divide. The population could be heterogeneous. Furthermore, it is not known, using current methods, whether some neoblast descendants committed to particular lineages are indistinguishable from self-maintaining neoblasts. In addition, although dedifferentiation has not been convincingly observed, it has not been proven that these small dividing cells are exclusively produced by themselves and not by differentiated cells. Lineage analyses will be required to establish the population characteristics and dynamics of neoblasts. Regardless, neoblasts and/or neoblast-progeny are capable of arriving at a wound from long distances, possibly as the result of directed migration. At the wound, neoblasts are signaled to proliferate. The division progeny march into the blastema, differentiate, and organize themselves to form new anatomy. In addition, neoblast numbers must increase, probably through symmetric cell division, to populate the new tissue and thus endow it with regenerative capacity.

An amputated piece of a planarian faces many challenges in the restoration of its form through regeneration. Illustrative examples of such changes following many types of amputations and the general ways in which these events occur have been reviewed here. First, the tissue has to specify what type of structures should be formed upon various wound surfaces. These decisions are determined by origin of the piece within the original animal. Even if the piece contains an irregular wound surface, anterior-facing wounds tend to make one head, and even if the piece comes from only a small region of the animal, the anterior-facing wounds tend to make one complete head. The extent of the anatomy produced in a blastema is specified by what is missing; i.e., a blastema has the ability to replace only the missing regions of a head or to completely replace a head. Injuries can occur that do not result in complete amputation but in missing body regions; wound healing tends to bring remaining tissues together creating a situation in which body regions between two juxtaposed tissues are missing. New tissue production can occur to

produce what is missing between the two. Even with these abilities of producing new structures through cell proliferation at wound surfaces, the animal faces major challenges. First, the regeneration blastema, for example a head blastema, does not have the ability to replace all missing body regions in the case of extensive injury. In fact, it does not even come close. A small piece from the tail or the side will, just like a decapitated and otherwise nearly complete animal, produce only a head. In both cases, the animals reorganize and reshape the other pre-existing tissues to produce the remaining missing anatomy and to restore proportion and symmetry. First, animals must reassign positional information to the remaining tissue; e.g., the tissue can no longer remain a small piece of the tail or side, it must establish a midline about which symmetry can be generated, produce new tissues, and/or alter existing tissues to produce the appropriate anatomy that corresponds to the new A/P, L/R coordinates. Next, once a complete complement of anatomical structures has been generated, animals must be able to correct for the fact that (*a*) many organs or organ systems are out of proportion with the body size, (*b*) the animal may be asymmetric, and (*c*) the physical distribution of organ systems may be inappropriate.

The molecular genetics of planarians can now be studied. The principles and issues described in this review should facilitate the interpretation and design of experiments to understand how the planarian genome controls the fundamentals of planarian regeneration.

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