Regeneration in Planaria

Secondary article

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Planarians possess remarkable regenerative abilities enabling them to replace parts of the body removed by amputation or naturally occurring fission. The regenerative process is mediated by the formation and eventual differentiation of a specialized structure known as the regeneration blastema.

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Introduction

In metazoans, regeneration of lost body parts requiring the formation of a blastema is known as epimorphic regeneration. A blastema is composed primarily of two cell populations: an outer cell layer or ectoderm, derived from the epidermis that covers the wound surface after amputation/fission; and mesenchymal cells that proliferate and accumulate beneath this wound epidermis, eventually differentiating into the lost body parts (**Figure 1**). Even though the architecture of both planarian and vertebrate blastemas is the same, the mechanism of blastema formation in planarians differs from that of vertebrates in two basic aspects. First, the wound epithelium forms by epidermal cell shape modifications rather than by cell proliferation. Second, the mesenchymal cells are derived from pre-existing undifferentiated cells (neoblasts), instead of the cellular dedifferentation observed in vertebrates. Such relative simplicity of blastema formation, combined with the planarian's basic body plan, developmental plasticity and evolutionary position, make these free-living members of the phylum Platyhelminthes a very attractive model system in which to study and understand the molecular principles governing metazoan regeneration.

Brief Historical Note

The regenerative abilities of planarians have been known for over 230 years. In 1766, Peter Simon Pallas first described how a small piece dissected from a planarian head was capable of regenerating a complete organism (see Brøndsted, 1969). This observation was confirmed a few years later by Shaw and then by Draparnauld, when they noted the ability of these animals to propagate asexually by fission (as cited by Randolph, 1897; see Brøndsted, 1969 for reference). However, the experimental analysis of regeneration in planarians begins with the work of Dalyell in 1814 and Johnson in 1822. These investigators inflicted partial or complete incisions, both longitudinally and meridionally, along the axes of a variety of planarian species in order to ascertain the regenerative potencies of these animals (see Brøndsted, 1969). The results of these experiments led Dalyell to conclude that planarians may 'almost be called immortal under the edge of the knife' (see Brøndsted, 1969). A rigorous description of planarian morphology, physiology and even behaviour is not found until the publication of Dugès' classic work in 1828 (as cited by Randolph, 1897; see Brøndsted, 1969 for reference). In his work, Dugès laid the foundations of modern planarian systematics. Between 1828 and the 1890s planarians attracted the attention of Michael Faraday, Charles Darwin and W.H. Harvey among others, but their work on this subject is mostly descriptive.

It was not until the early to mid-1890s that a renewed experimental interest in the process of planarian regeneration emerged, fuelled primarily by the ongoing arguments of vitalism versus reductionism engulfing the relatively new field of embryology. To Harriet Randolph, Jacques Loeb and Hans Driesch, planarian regeneration presented an intriguing dilemma in which isolated parts of an adult could recreate and regulate the characteristics of an entirely new and whole organism, a process they thought akin to embryogenesis. It was this body of work that led Thomas Hunt Morgan and Charles Manning Child to study planarian regeneration in the late 1890s and early 1900s. It is interesting to note, however, that even after more than 200 years of studies, most of the biological questions raised by planarian regeneration still remain unanswered.

Natural History

The planarians commonly used for regeneration experiments are free-living members (class Turbellaria; order Seriata) of the phylum Platyhelminthes, the flatworms. They are assigned to the suborder Tricladida based on the three main branches of their digestive system (**Figure 2a**). The Tricladida may be further subdivided based upon ecological habitat: there are freshwater, marine and terrestrial forms (the classification of these different forms is controversial and beyond the scope of this review). The

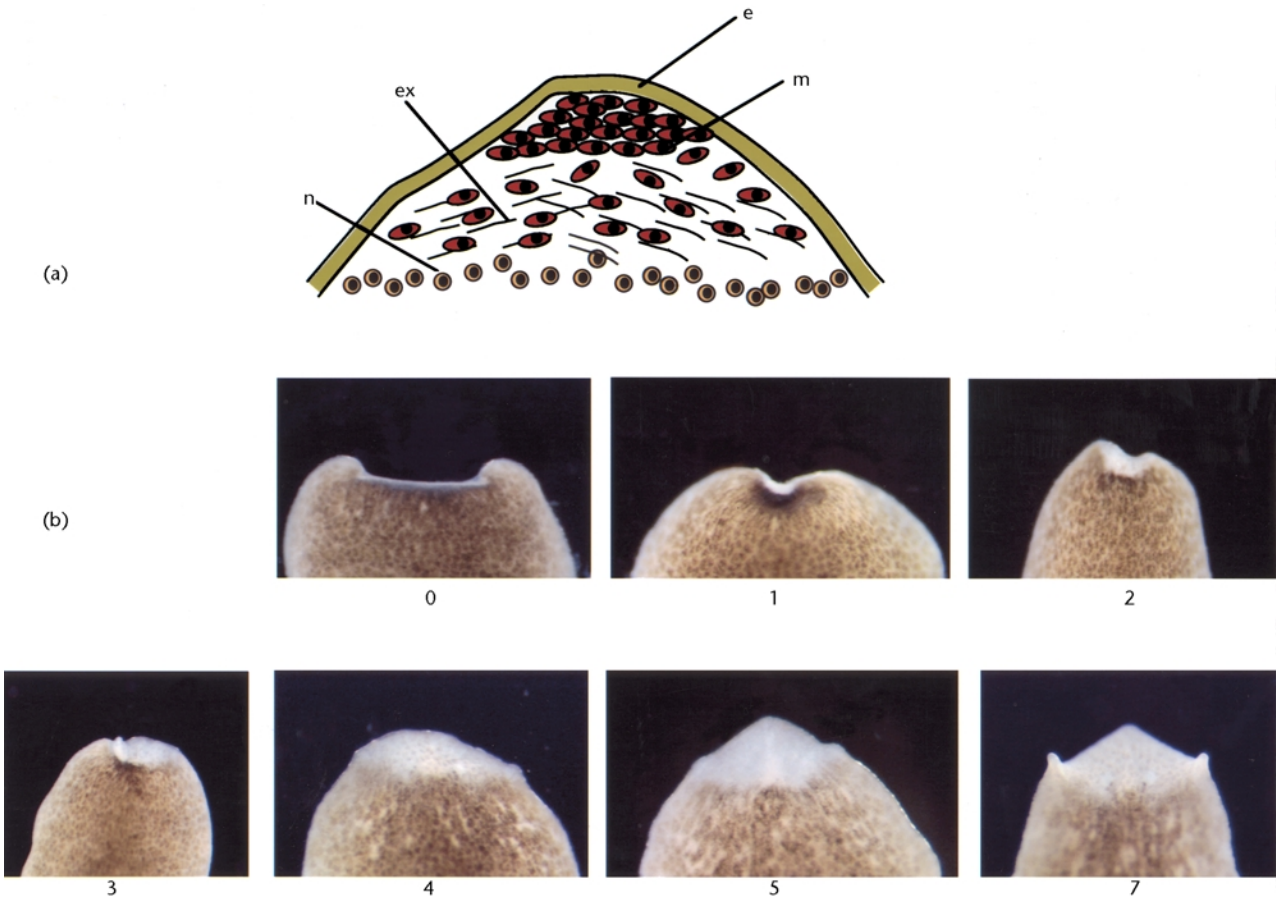

Figure 1 The process of head regeneration in *Dugesia dorotocephala*. (a) Schematic representation of blastemal components. The round cells correspond to the zone of neoblast proliferation in the vicinity of the wound (e, epithelium; m, neoblast-derived mesenchymal cells; n, neoblast; ex, extracellular matrix). (b) The same animal photographed every 24 hours after amputation. The numbers refer to days after amputation. Notice the marked cellular proliferation as evidenced by the growth of the blastema between days 2 and 3.

freshwater planarians are the most commonly studied. They are traditionally subdivided into three families: the Planariidae, Dendrocoelidae and Dugesiidae, and exhibit some differences in regenerative abilities. For example, the Dendrocoelidae can only regenerate anterior structures when transected anterior to the pharynx.

Planarians are triploblastic (possessing three tissue layers) acoelomates, with a bilaterally symmetrical body plan that is dorsoventrally flattened. They range in size from a few millimetres to 4–5 cm. The epidermal layer surrounding the flatworm is ciliated on the ventral surface; these cilia propel the animal in a gliding motion along a ventrally secreted mucous trail. Beneath the epidermal layer (and the basal lamina to which it attaches) is a complex body wall musculature composed of circular, diagonal and longitudinal muscle fibres. The planarian nervous system is composed of two ventral nerve cords that run longitudinally along the length of the organism. These nerve cords aggregate in the anterior of the flatworm to form the cerebral ganglia (**Figure 2b**), which process information received from the sensory structures (e.g. photoreceptors, rheoreceptors and chemoreceptors) that are also concentrated in the head. Planarians lack a circulatory system and must rely upon diffusion to provide the required oxygen; the highly branched digestive system, with one anterior and two posterior branches (**Figure 2a**), transports food throughout the entire body. Food enters the animal through the muscular pharynx, which is situated in the middle of the body and is extruded through the mouth opening during feeding. The digestive system lacks an anus, thus food enters and leaves the animal through the opening of the pharynx.

There are two modes of reproduction in planarians: (1) asexual reproduction by fission and (2) sexual reproduction involving pairwise mating. Asexual reproduction occurs by transverse fission, usually posterior to the pharynx; the missing pieces are then regenerated. Planarians that reproduce sexually, on the other hand, are

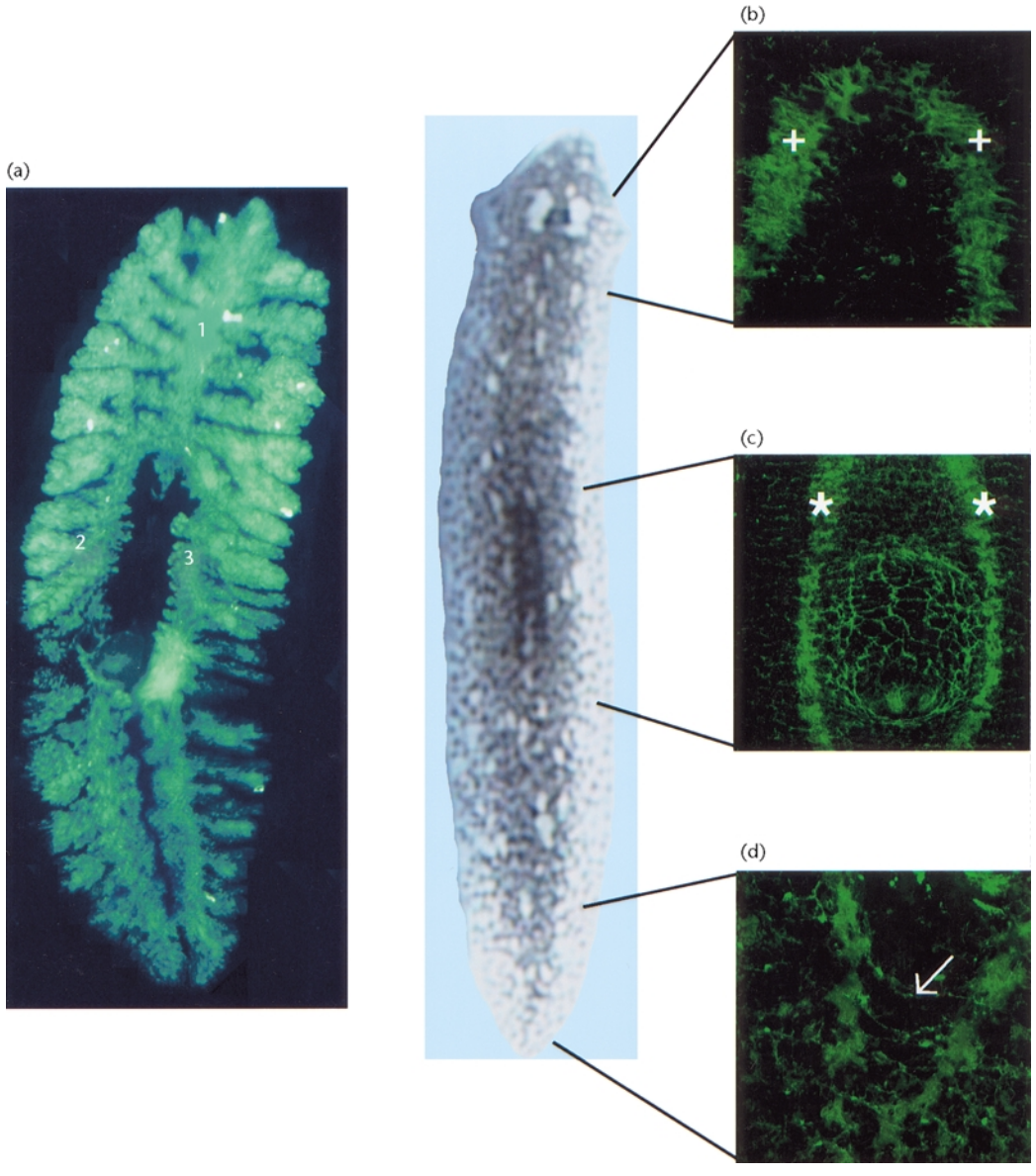

Figure 2 The planarian gastrovascular and nervous systems. (a) Visualization of the three gut branches (1, 2, 3) by feeding the animals fluorescent latex beads. Notice the intricate web of fasciculations of each of the secondary branches. This allows the transport of both oxygen and nutrients to all parts of the body. (b) The planarian brain consisting of two cerebral ganglia (+) seen at a $\times$ 40 magnification. (c) The two nerve cords, each originating in one of the two cerebral ganglia are seen here at the trunk level (*) running parallel to the pharynx ($\times$ 20 magnification). (d) The nerve cords at the tail tip of the planarian displaying commissural neurons (arrow) which connect the cords along the entire AP axis of the animal ($\times$ 40 magnification). The nervous system in panels b, c and d was visualized by labelling neurons with an anti-FMRF amide primary antibody (a neuropeptide) detected with a secondary fluorescent-conjugated antibody.We acknowledge the expert confocal microscopy assistance provided by Susanna Castel.

hermaphroditic with cross-fertilization being the norm. Some species combine these two modalities, alternating between sexual and asexual reproduction during different seasons, while others utilize only a single mode of reproduction.

Embryonic development in the sexual triclad planarians is highly modified from the spiral cleavage observed in the marine polyclad planarians, a fact reflected by the ectolecithal eggs (with yolk on the outside of the egg) observed in the triclads (Hyman, 1951). A single cocoon, or egg capsule, contains between 5 and 20 eggs (depending upon the species) surrounded by tens of thousands of yolk cells. During early cleavages, the embryonic cells show no ordered pattern of cleavage and may even separate from

each other. Eventually, some embryonic cells migrate through the yolk (away from the rest of the embryo), and flatten out, creating a membrane that surrounds the remaining embryonic cells in a yolk syncytium. These embryonic cells continue to divide. Some cells migrate to the ventral surface, where they form an embryonic pharynx, which then ingests the external stores of yolk. When the embryonic intestine is full of yolk, embryonic cells aggregate at the ventral surface, forming anterior, central and posterior masses. The embryo then begins to flatten and the embryonic pharynx, as well as the inner and outer membranes are replaced. There is no larval stage. Hence, the end of embryogenesis results in the emergence of small planarians from the cocoon.

In addition to their regenerative abilities, the morphological plasticity of planarians is demonstrated by their ability to grow and de-grow, depending upon the availability of food (Baguñà *et al*., 1990). Thus, well-fed animals will continue to grow until a maximum size is attained, whereas starved animals will shrink over a period of many months until they are the size of a newly emerged hatchling. This ability to grow and de-grow is shared by both sexual and fissiparous species and is due to different ratios of cell proliferation/cell loss under different environmental conditions, with cell proliferation prevailing when the animals are fed and cell loss prevailing when the animals are starved.

Classical Experimental Analyses of Planarian Regeneration

As described above, early investigators examined the seemingly limitless regenerative abilities of planarians. Expanding upon the work of Harriet Randolph, T.H. Morgan made numerous theoretical and experimental contributions to the study of planarian regeneration. Morgan (see Brøndsted, 1969 for references to Morgan's work) showed that a tiny fragment, corresponding to only 1/279 of the intact worm, was capable of regenerating a complete planarian. Morgan was intrigued by the regulatory shape changes that transform short, relatively wide transverse pieces into well-proportioned, long and narrow planarians. He coined the term 'morpholaxis' (later changed to morphallaxis) to reflect this remodelling of the old tissue in the absence of proliferation, and contrasted it with blastema formation, which requires cell proliferation ('epimorphosis'). Morgan introduced cell marking experiments to study remodelling in the old tissue in the terrestrial triclad, *Bipalium*. By cauterizing pigment stripes and then measuring changes in the distance between the wounded regions over time, he was able to show the migration of old tissues during morphallaxis. Morgan also used *Bipalium* to introduce grafting techniques to the study of planarian regeneration (his wife, Lilian V. Morgan, was the first to report grafts using freshwater planarians).

A fundamental question in the study of regeneration has been to understand the nature of polarity: how does the anterior end 'know' to make a head and a posterior end 'know' to make a tail? To study this problem Morgan (and others) tried to alter polarity by producing heteromorphic regenerants, in which the newly formed structures differed from the original structures removed by amputation (e.g. Janus heads in which a second head is formed at the posterior cut surface). Morgan showed that Janus heads were formed with higher frequency as transected fragments were cut shorter and shorter, leading him to suggest that longer pieces somehow had a 'stronger' polarity. Morgan initially explained this polarity as being based upon a gradation of formative materials, in which head 'stuffs' and tail 'stuffs' were distributed asymmetrically throughout the body. This suggestion is recognized as the first model of a morphogenetic gradient accounting for a developmental process (see Wolpert article in Dinsmore, 1991). Morgan ultimately abandoned both his gradient model as well as his studies of regeneration and development, believing that these problems were insoluble.

C.M. Child, a contemporary of Morgan's, became the strongest proponent of the role of gradients in developmental biology; rather than invoking the presence of formative 'stuffs', Child′s gradient model postulated differences in metabolic activity along the anteroposterior (AP) axis. Child′s model was based on two sets of results: (1) in many planarians either the ability to regenerate a head or the speed of head regeneration is different along the AP axis, often declining from head to tail (termed 'head frequency' by Child); and (2) treatments with respiratory inhibitors and anaesthetics revealed a differential susceptibility to these agents along the AP axis of the organism. Child interpreted these results to reflect a respiratory gradient throughout the animal, with a high point at the anterior region of the animal. However, subsequent investigators were unable to measure differences in respiration at different body levels (see Brøndsted, 1969).

Although respiratory gradients do not seem to exist in planarians, there is clearly, in many species, a graded ability to make heads, as reflected by the variable amounts of time required to replace missing anterior structures. Thus, the region just behind the eyes is able to regenerate more rapidly than the prepharyngeal region, which in turn can regenerate more quickly than the postpharyngeal region (reviewed in Brøndsted, 1969). H.V. Brøndsted studied this phenomenon extensively and showed that in addition to AP differences in the rate of head regeneration, there were also differences between the rates of head regeneration shown by medial and lateral tissues, with medial regions regenerating more rapidly than lateral regions. Using grafting techniques, Brøndsted showed that these differences were intrinsic to the tissue itself; thus, the rate of head regeneration of a posterior piece remains

unchanged when it is transplanted to more anterior positions. Based on these results, Brøndsted postulated the existence of a 'head-producing time-depending regeneration field'. The molecular nature of this field remains unclear to this day.

Proposed Mechanisms of Regeneration: The Role of Neoblasts

After a planarian has been transected, the wounded area is rapidly covered by a thin layer of epidermal cells. Undifferentiated cells then accumulate beneath the wound epithelium giving rise to an unpigmented structure referred to as the regeneration blastema. As regeneration proceeds, more of these undifferentiated cells continue to accumulate within the blastema, causing it to grow exponentially. Within one week of the transection, differentiation of the missing structures occurs (Baguñà *et al*., 1990).

The undifferentiated cells that constitute the regeneration blastema are called neoblasts (see Brøndsted 1969 for review) and are readily identified cytologically as relatively small cells ($\sim 10\,\mu m$ diameter) with a high nucleocytoplasmic ratio. Neoblast cytoplasm is abundant in RNA and contains numerous ribosomes as revealed by histochemical methods and electron microscopy. In uninjured planaria, neoblasts are distributed throughout the parenchyma (mesenchyme) and, as the only mitotic cells in the animal, serve as the source of replacement cells during tissue renewal. The neoblasts, thus, appear to be stem cells for the differentiated cells of the flatworm.

The origin of the neoblasts (and thus the blastema) has been a major focus of work on planarian regeneration. Two different hypotheses explaining the origin of neoblasts have predominated in discussions of this problem: (1) the cells constituting the regeneration blastema are derived by dedifferentiation of differentiated cells in the vicinity of the wound or (2) neoblasts are totipotent stem cells that proliferate in response to injury. Some evidence in favour of the former hypothesis comes from an interesting set of experiments utilizing a mosaic strain of *Schmidtea polychroa* in which somatic cells are triploid and premeiotic germline cells are either hexaploid (female germline) or diploid (male germline). Karyological and cytophotometric analyses revealed that after 3 days of regeneration $\sim 5\%$ of the nuclei within the blastemata contained a diploid chromosome complement (normally only observed in male germline cells; Gremigni *et al*., 1980). These results suggest that premeiotic germ cells may contribute to the formation of the blastema. This contribution appears to be due to a switch in the state of determination of the germ cells rather than a bona fide transdifferentiation event (Baguñà *et al*.,1990). Furthermore, since only $\sim 5\%$ of the blastema cells may be derived from premeiotic germ cells, and many species of planaria exist as asexually reproducing races that lack germ cells altogether, the origin of the remainder of the neoblasts within the blastema remains an important question.

The classic work of Dubois and Wolff provided an important indication of the role played by neoblasts in forming the regeneration blastema (reviewed in Brøndsted, 1969). This work was based upon the fact that X-irradiation abrogates the ability of planarians to regenerate and results in death several weeks after irradiation. Wolff and Dubois showed that neoblasts, the only proliferating cells in the animal, are particularly susceptible to irradiation. As the neoblasts are destroyed, the planarian loses its ability both to regenerate and to renew its tissue. By shielding various portions of the body from irradiation and then examining the regenerative abilities of irradiated animals, it was shown that the length of time required for regeneration is proportional to the length of the irradiated region. These results were interpreted to reflect the migration of neoblasts from the unirradiated tissue to the wounded area where they proliferate and give rise to the blastema. Subsequent experiments by Saló and Baguñà (1985), in which nuclear and cytoplasmic markers were used in conjunction with grafting techniques to follow the movement of neoblasts and differentiated cells, showed that this 'migration' of neoblasts is not active migration toward the blastema, but rather cell spreading due to proliferation. Faster rates of cell movement were shown to be correlated with higher mitotic indices and cells did not appear to be driven preferentially toward the wound area.

In one of the key works on planarian regeneration, Baguñà and his colleagues addressed the role of neoblasts in planarian regeneration and provided evidence that neoblasts are totipotent stem cells (Baguñà *et al*., 1989). Taking advantage of size differences between neoblasts and differentiated cells of the flatworm, serial filtration and density gradient centrifugation were used to prepare fractions highly enriched in either neoblasts or differentiated cells. These fractions were then introduced into flatworms that had previously been irradiated with a lethal dose of X-rays. Following irradiation, flatworms normally lose their ability to regenerate and they die within 3–5 weeks. However, when irradiated flatworms were injected with the fraction enriched in neoblasts, regenerative abilities and long-term survival could be restored. In contrast, injection of the differentiated cell fraction into irradiated planaria did not rescue the effects of irradiation. These results suggest that neoblasts serve as the regenerative cells of this organism. Furthermore, transplantation experiments involving sexual and asexual races of *Schmidtea mediterranea* provide compelling evidence that neoblasts serve as stem cells for all the differentiated cell types of the planarian. When neoblasts isolated from the sexual race were injected into irradiated hosts from the asexual race, formerly asexual individuals developed germ cells and the copulatory apparatus, mated and laid cocoons. Conversely, when neoblasts from the asexual

race were injected into irradiated sexual hosts, formerly sexual individuals reproduced instead by fission. To rule out the possibility that injection of neoblasts somehow revitalized host tissue, a chromosomal marker distinguishing nuclei from the asexual and sexual races was utilized to demonstrate that the mitotic cells of the rescued planaria always corresponded to the donor cells. Thus, neoblasts are capable of 'transforming' one planarian race into another, strongly suggesting that neoblasts can give rise to all of the differentiated cell types of the organism.

Projections and Summary

Given the paucity of molecular data, it would be premature to propose a molecular model of planarian regeneration. Recent developments, however, suggest that some of the necessary tools are finally available to address some of the fundamental questions: How is polarity established within the blastema and maintained within the adult? How is positional information communicated to the neoblasts? What signals the local proliferation of neoblasts in the vicinity of the wound? One approach has been to construct a hybridoma library recognizing planarian antigens (Bueno *et al*., 1997). This library has provided markers for most of the differentiated cell types of the animal, and has identified, for example, a region-specific marker that is expressed specifically in a central domain along the AP axis (Bueno *et al*., 1996). Another approach has been to use sensitive polymerase chain reaction-based subtractive hybridization screens to detect genes involved in blastema formation and maintenance, as well as genes involved in the establishment of polarity in the blastema. These types of screens, combined with the isolation of planarian homologues of *hox* (Balavoine, 1996) and other developmentally important genes (see Baguñà *et al*., 1994) should identify some of the components involved in patterning the planarian regeneration blastema. Finally, the demonstration that neoblasts can be used to 'transform' planaria provides a potentially powerful tool for studying planarian regeneration. By introducing exogenous DNA into neoblasts and reintroducing these neoblasts into lethally irradiated flatworms, it should be possible to generate planarians expressing a transgene of interest (see Baguñà *et al*., 1990).

In spite of the tremendous strides made recently in understanding the mechanisms of embryonic development, the underlying mechanisms of regeneration remain obscure. This circumstance may be attributed largely to the poor regenerative abilities of the classic genetic organisms used for studying development (*Drosophila, Caenorhabditis elegans*, mouse, zebrafish), as well as the difficulty of utilizing the commonly studied vertebrate models (the urodele amphibians) at the molecular and genetic levels. The last 30 years have seen an exodus from the study of planaria; currently, only a handful of laboratories worldwide study this fascinating organism. It is hoped that the application of molecular biological techniques to the study of planarian regeneration will reignite interest in this exciting problem and ultimately provide insight into the remarkable developmental plasticity exhibited by planarians.

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