The use of planarians to dissect the molecular basis of metazoan regeneration

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Freshwater planarians possess remarkable regenerative abilities that make them one of the classic model organisms for the study of regeneration. These free-living members of the phylum Platyhelminthes are representatives of the simplest triploblastic organisms possessing bilateral symmetry and cephalization. Furthermore, planarians occupy an important position in the evolution of Metazoa, which allows for the possibility of vertically integrating molecular studies of regeneration in this organism to other, more widely studied animal model systems. Because of their relative simplicity, developmental plasticity, and evolutionary position, planarians are an attractive system to dissect the molecular processes underlying regeneration. The objective of this article is to present a molecular strategy to identify and functionally manipulate genes involved in the process of blastema-derived regeneration. Ultimately, the genes identified in planarians and their interactions during regeneration will define a series of useful molecular templates that may help unravel the more complex epigenetic processes of vertebrate regeneration and may perhaps uncover the factors that make regeneration permissive in some, but not all, metazoans. **(WOUND REP REG 1998;6:413–420)**

Regeneration is one of the most fascinating and intriguing problems of biology, a fact that is clearly shown by the continuous research that it has inspired since Trembley first reported its occurrence in hydra over 250 years ago. The timeless attraction of this problem springs from the unique set of questions it poses to the experimental biologist: How are polarity and pattern determined in regenerates? What are the permissive and inhibitory factors required for regeneration? How are cell-type–specific transcription factors restricted during regeneration? How are size and proportion controlled? Obviously, the answers to such questions go further than understanding regeneration itself and impinge directly on some of today's most intensively studied aspects of biology and developmental biology (e.g., cell proliferation, morphogenesis, and organogenesis), yet the study of regeneration is still in its molecular infancy. Hence, most, if not all, of these questions remain largely unanswered. Indeed, there is currently not one established model system for the study of regeneration that would allow for a systematic elucidation of the molecular events underpinning regeneration. The experiences derived thus far from the study of regeneration in amphibians, combined with the limited regenerative abilities of genetic vertebrate models such as teleosts and mice, suggest that some of the answers to the key problems of metazoan regeneration will, in all likelihood, not come entirely from vertebrates.

Unfortunately, the same can be said of the available invertebrate genetic systems that display limited regenerative powers (e.g., imaginal disc regeneration in *Drosophila*) or no regeneration at all (*Caenorhabditis elegans*). In order to study regeneration, it would be best to identify an organism in which regeneration plays a prominent role in its life cycle and whose physiological makeup is still complex enough to integrate vertically any molecular findings into more derived animal models. One such organism with the potential of becoming a molecular-genetic model for

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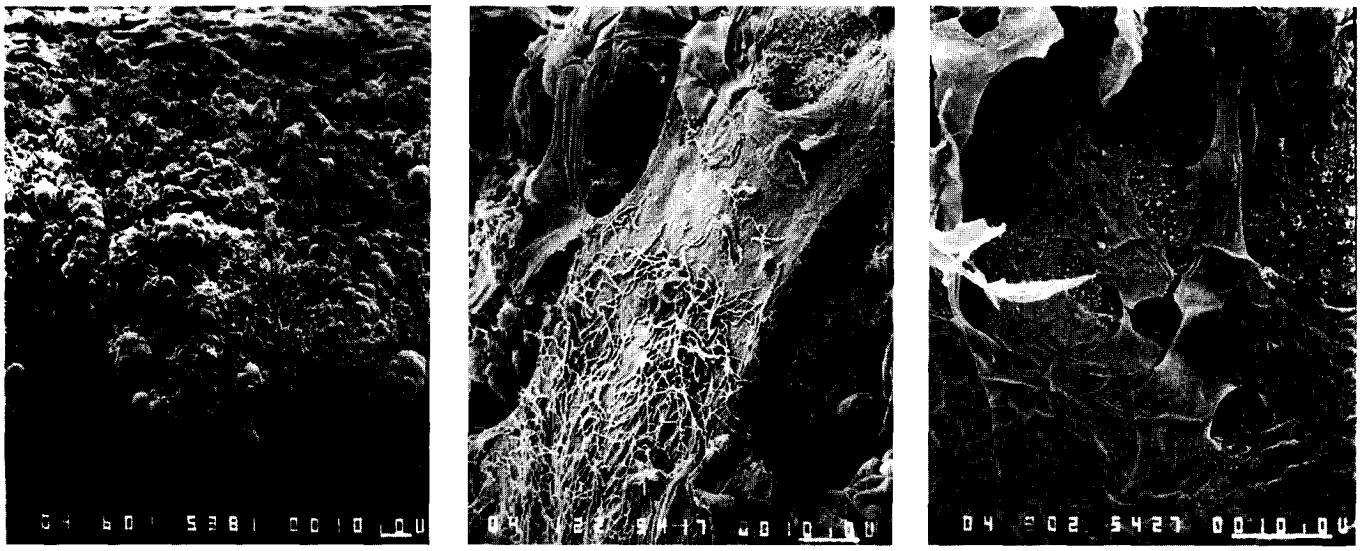

Figure 1 Scanning electron micrographs of a planarian surface wound. 0 hour: surface of the uncovered wound immediately after amputation (x601). 0.5 hour: epithelial cell with remnant ventral cilia losing its polarized morphology and beginning to migrate over the wound surface (x122). 1 hour: completely flattened out epithelial cells covering the surface of the wound (x202). No proliferation of epithelium is observed.

the study of regeneration is the planarian. The objective of this article is to discuss the merits of reintroducing the use of planarians as a regeneration system in which to dissect the molecular basis of epimorphic regeneration in metazoans.

REGENERATION IN PLANARIANS

Planarians have been studied in great detail for their regenerative abilities, and a large body of classic literature exists on this problem.[1–3] The most commonly studied order of planarians is Tricladida, so named because of the one ascending and two descending branches of their gastrovascular organ system. The Tricladida are members of the class Turbellaria of the phylum Platyhelminthes, and members of each of the three suborders (Paludicola, Maricola, and Terricola) are known to possess remarkable regenerative capacities.[3] Flatworms are the stereotypical representatives of the simplest organism in the tree of life, possessing three tissue layers (triploblastic), bilateral symmetry, cephalization, and complex organ systems. These phenotypic traits place flatworms in a key position of metazoan evolution, a fact supported by molecular analyses of their 5S and 18S ribosomal RNA[4,5] and, more recently, by the studies of *Hox* phylogeny of Dr. Adoutte at the University of Paris-Sud in France (personal communication).[6]

Epimorphic regeneration in planarians, as in vertebrates, requires the formation of a bud or blastema that subsequently grows and differentiates into the missing part(s). Similarly, regeneration in planarians begins with the formation of a wound epithelium within hours after amputation.[7] Nevertheless, the cellular dynamics of this process differ from that of vertebrates in that the wound is covered not by an active proliferation of epithelial cells, but rather through a series of drastic changes in both the morphology and the migratory properties of the planarian epithelium (Figure 1). Yet once formed, a gross morphological comparison of planarian and vertebrate blastemas reveals several common characteristics, the most prominent one being their well-defined epithelial and mesenchymal compartments (Figure 2). In vertebrates, once the wound epithelium is formed, an apical ectodermal cap is formed. One important characteristic of the apical cap is that its underlying basal lamina (adepidermal membrane) is disorganized and partially absent. Normally, the basal lamina is closely adherent to the epithelium at the interface with the underlying mesoderm, physically separating these two compartments. The absence of this barrier at the apical cap permits a direct contact between the cap's epithelial cells and the underlying mesenchyme.[8] Such contact is necessary for the normal progression of regeneration,[9,10] indicating the need for an ongoing

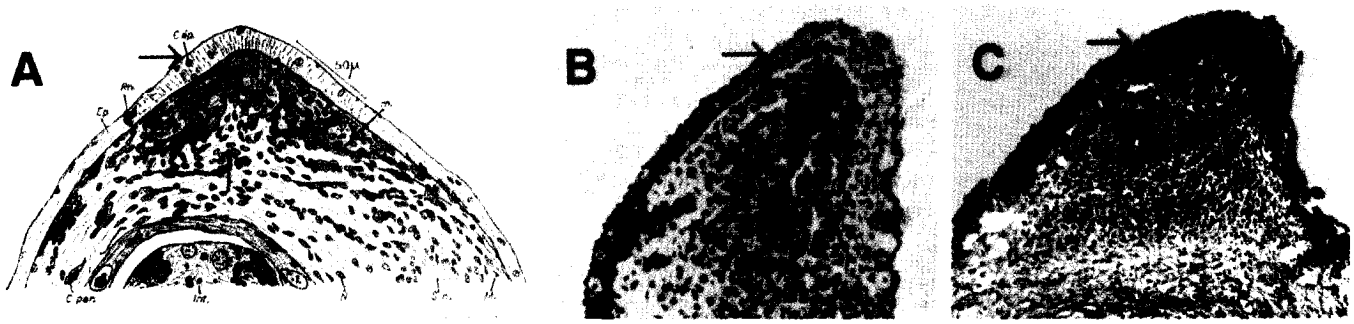

Figure 2 Histological comparison of regenerating blastemas and normal limb bud illustrate the shared epithelial (*blue arrow*)/mesenchymal (*red arrow*) interactions taking place during regeneration (**A** and **B**) and limb bud formation (**C**). **A**, Camera lucida drawing of a planarian (*Dugesia lugubris*) blastema. (Reprinted from Brøndsted[3] after Dubois.[38]) **B**, Regenerating limb blastema of a salamander (*Ambystoma opacum*). (Reprinted from Thornton.[13]) **C**, Normal limb bud of *Rana temporaria*.

molecular interaction between these two compartments. In planarians, the wound epithelium is analogous to the apical cap in vertebrates in that it also no longer adheres to a basal membrane. Thus, a direct physical contact is established between the mesenchymal and epithelial components of the blastema,[11] allowing possible molecular interactions to occur.

One important factor that contributes to the progress of regeneration in vertebrates is innervation.[12] Innervation of regenerating tissues is necessary for the maintenance of the apical cap[13] and the promotion of cell proliferation.[14] Also, injuring and deflection of brachial or sciatic nerves in salamanders result in the generation of ectopic structures such as limbs, fins, or tails,[15,16] all of which rely for their normal and induced ontogeny on the formation of an epithelial–mesenchymal bud similar in structure to that of the regeneration blastema. Whether innervation plays a role in the establishment and/or progression of regeneration in planarians is unclear. However, neuronal input effects have been described in the annelid worm *Spirographis spallanzanii*, in which cutting the nerve cord and deflecting the resulting severed ends to the body wall result in polar-specific regenerates. Thus, the nerve originating from the head and whose severed end faces caudally before deflection will produce a tail regenerate, whereas the other posterior half facing toward the head will induce a cephalic regenerate after its deflection to the body wall.[17]

Several experiments have shown that apical cap maintenance and cell proliferation in vertebrates and blastema polarity in some worms require unidentified trophic factors released by injured neurons.[18] However, such factors may not be nerve specific after all, as demonstrated by limb-regeneration studies in aneurogenic animals obtained through parabiosis. Aneurogenic limbs can be generated experimentally by the surgical posterior twinning of two tailbud-stage salamander embryos, in which the neural tube and adjacent neural crest from the anterior trunk and hindbrain level are removed from one of the parabionts.[19,20] Forelimbs of both host and parasite develop, the latter lacking innervation,[21] and both are capable of regenerating normally and completely.[19] Transplantation experiments have shown that skin grafts from aneurogenic limbs can completely replace the contribution of neurons to regenerating limbs,[22] indicating that in aneurogenic animals, the trophic factors required for regeneration are produced by the skin. These observations may explain one apparent discrepancy between vertebrate and planarian regeneration: planarians do not need a nerve cord to be present in order to regenerate, because as little as 1/300th of the organism (approximately 1×10^4 cells) devoid of this neuronal tissue is still capable of regenerating a whole planarian.[1,3] Hence, it is possible that if trophic factors are required for regeneration to occur in planarians, they may also be found in the wound epithelia. The characterization of such factors in planarians may ultimately result in the identification of the long sought-after neurotrophic factor(s) of vertebrate regeneration.

The establishment during regeneration of histologically similar epithelial and mesenchymal interac-

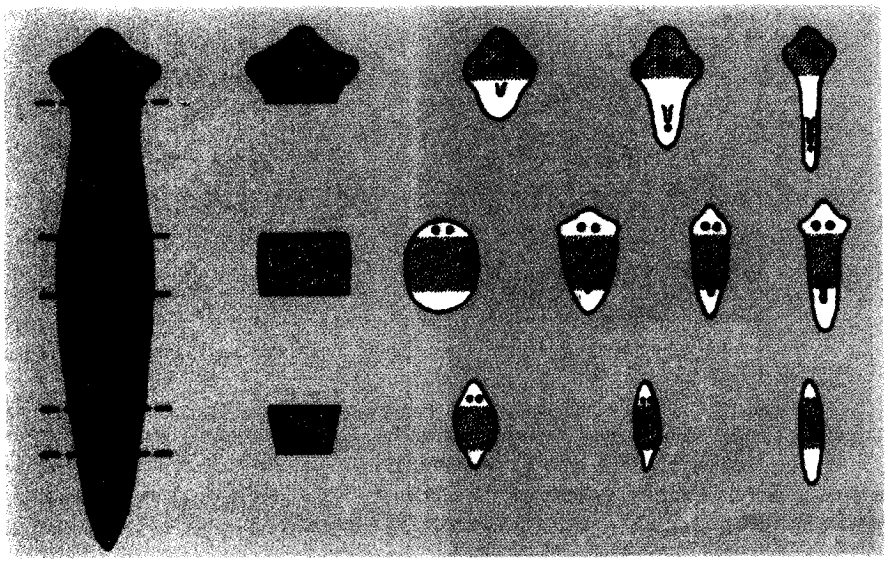

Figure 3 Polarity maintenance in regenerating blastemas of planarians (after Morgan[1] and Hay[39]).

tions in vertebrates and Platyhelminthes may reflect the evolutionary conservation of a molecular plan for the definition of cellular identities during morphogenesis. Although the role of the wound epithelium in planarians in regeneration has not been conclusively established, several lines of experimentation suggest that it plays a pivotal organizing role in the blastema. One interesting feature of planarian blastemas is their ability to maintain polarity so that a posterior blastema will give rise to a tail, whereas an anterior blastema differentiates into a head (Figure 3). Chandebois noted that in planarians, anterior amputation wounds are covered by dorsal epithelium, whereas posterior wounds are covered by ventral epithelium.[23] Also, it has been observed that the identity of the blastema may be determined by its position along the anterior/posterior axis of the organism[24] and that such position may be established by the asymmetric distribution of proteins on the epithelium.[7,25] These observations combined with the fact that blastema epithelium is known to activate *Hox* genes in the mesenchyme of both vertebrate[26] and planarian blastemas[27] suggest an active role for the epithelium in the early patterning of the regenerate.

THE PLANARIAN NEOBLAST

Unlike vertebrates, no regression of tissues, dedifferentiation, or both have been observed in planarian regeneration.[28] Regression and dedifferentiation in vertebrate regeneration are required for the formation of the pluripotential cells that make up the bulk of the blastema. In planarians, however, stem cells residing in the parenchyma (mesenchyme) undergo a short-range migration (200–300 μm) from the site of amputation to the wound epithelium and give rise to the blastema.[28] These stem cells, or neoblasts, make up 20%–30% of the cell population in adult planarians and are the only mitotically active cells in these organisms. Thus, it appears that blastema formation in planarians bypasses the dedifferentiation required in vertebrates by virtue of a pre-existing population of undifferentiated cells.

Neoblasts serve two purposes. First, they constantly replace the dying, nonproliferating differentiated cells of the adult organism.[29] Second, they make up the bulk of the mesenchymal component of the regenerating blastema.[28] Baguñà et al. elegantly demonstrated the totipotential nature of neoblasts[30] by injecting cell fractions highly enriched in neoblasts into x-ray irradiated planarians (Figure 4). A dose of 8,000 rads is sufficient to stop cell division in planarians almost immediately and causes death after 4–6 weeks. Irradiation also results in the abrogation of regeneration as early as 3 days after treatment. Injection of purified neoblasts from normal donors into irradiated hosts results in the survival of the host and in the restoration of regenerative abilities.[30] The totipotentiality of neoblasts is further demonstrated when donor neoblasts from a sexually reproducing strain are injected into irradiated asexual hosts. The neoblasts not only repopulate the host and reactivate its regenerative abilities, but also "transform" it into a sexual planarian because functional reproductive

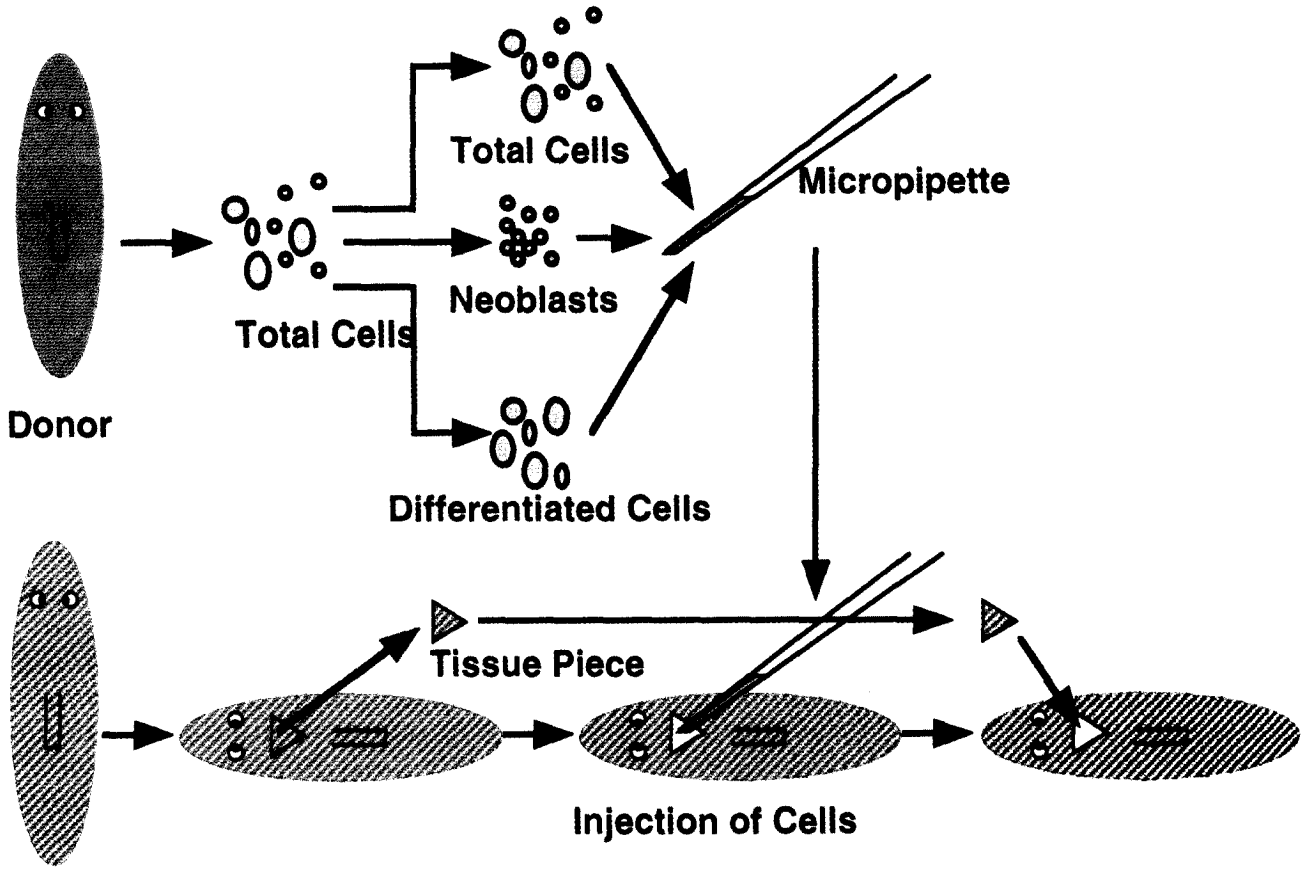

Figure 4 Schematic of the procedure employed to introduce donor cells into irradiated host planarians. Disaggregation of host cells is accomplished in a calcium/magnesium-free medium. The cells are then size-fractionated through Nytex sieves of different pore sizes. A triangular window is made in the donor, and cells are injected into the parenchyma of the host using a micropipette. The triangular tissue piece is replaced and allowed to heal. If successful, the host will survive and will in 2–3 weeks be able to regenerate its tissues again (adapted from Baguñà et al.[30]).

organs and copulatory apparatus are formed.[31] These experimental results confirm the role of neoblasts in planarian regeneration and provide evidence for the totipotent, stem-cell nature of this parenchymal cell population.

GENE ISOLATION AND TRANSGENESIS IN PLANARIANS: CURRENT AND FUTURE DIRECTIONS

Our laboratory has applied a gene expression screen devised by Wang and Brown at the Carnegie Institution[32] to regenerating and nonregenerating planarian tissues in order to identify differentially expressed genes. This method has the unique advantage of being able to estimate the number of upregulated and downregulated genes in any given screen.[32] The methodology requires small amounts of the obtained poly(A)$^+$ RNA populations; these are reverse transcribed, restricted, ligated to linkers, and amplified by polymerase chain reaction. The cDNAs are then subjected to a series of subtractive hybridizations to enrich for differentially expressed transcripts.[32] Thus far, we have identified a total of 59 unique cDNA fragments whose expression is modulated by regenerative events. Examples of such fragments are shown in Figure 5. The data indicate that it is not only possible to enrich for regeneration-modulated genes that may be required for the generation of a planarian blastema (Figure 5, *a*), but also to identify genes that are specific to either cephalic or caudal blastemas, that is, polarity-specific genes (Figure 5, *b* and *c*). Currently, our laboratory is engaged in the sequencing and characterization of the temporal and spatial expression patterns of these and other isolated transcripts.

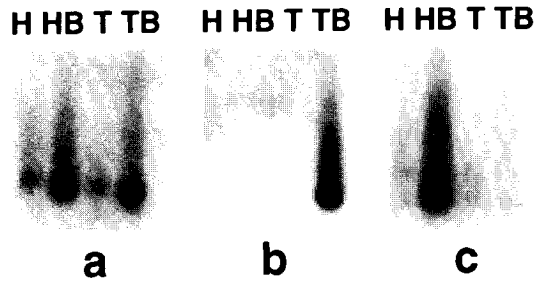

Figure 5 Regeneration modulated cDNA fragments during planarian regeneration. Individual fragment obtained from the subtractions are radioactively labeled and hybridized to filters containing 1 μg per lane of either head (*H*), head blastema (*HB*), tail (*T*), or tail blastema (*TB*) subtraction enriched cDNA fragments. **a**, Blastema upregulated fragment. **b**, Tail blastema upregulated fragment. **c**, Head blastema upregulated fragment.

The ability to study gene function in vivo is crucial and defines an organism's usefulness as an experimental model system. The study of regeneration has suffered under this tenet because organisms that are well suited to genetic manipulations (mouse, zebrafish, *Drosophila,* and *C. elegans*) display limited or no regenerative powers, and those that are widely used to study regeneration (axolotls, salamanders, and *Pleurodeles*) are quite refractory to genetic analyses. Perhaps the most attractive feature of planarians is that their biology allows for the real possibility of generating transgenic lines in which to study those genes that may be involved in the process of epimorphic regeneration. The biology of planarians and their developmental plasticity make it possible to test effectively the viability of several well-established transgenic methodologies currently being used in other animal model systems.

As previously discussed, the totipotency of planarian neoblasts, combined with their ease of reintroduction into irradiated animals (Figure 4), makes these cells prime vectors for the introduction of exogenous DNAs into naive individuals. If one were to homologize planarian neoblasts with murine embryonic stem cells, for example, it is not difficult to envision the use of several well-established methodologies such as electroporation, germ-cell injections, and even transposable-element–driven transgenesis in order to generate recombinant cells. Hence, genetically modified neoblasts may be used to repopulate irradiated animals, creating, in essence, a transgenic animal whose cells originated from the introduced recombinant neoblasts. We are attempting to take advantage of the pluripotentiality of planarian neoblasts, as well as of these well-established methodologies to introduce exogenous DNA into these organisms for the production of transgenic lines.

SUMMARY

What does one hope to learn from the study of regeneration in planarians? An indication of the exceptional biological secrets guarded so jealously by the Turbellarians is exemplified by one remarkable property of the regenerating blastema: its morphogenetic equipotency. One of the earliest discoveries of experimental embryology was the production of more than one normal larva by the physical fragmentation of early-stage echinoderm embryos, an observation that led Driesch[33] to postulate the idea of "harmonic equipotential systems." This idea was extended into the study of vertebrate embryology and eventually led to the discovery of the mosaic nature of the early vertebrate embryo.[34] The term *mosaic* was chosen because a series of heterotopic and heterochronic transplantation schemes demonstrated that the morphologically homogeneous mesodermal layer of the early embryo was already subdivided into areas fated to give rise to various organs later during development. These observations led to the idea that organogenesis has its ontogeny at developmental stages in which no overt signs of differentiation can be discerned.

The most complete studies on this matter were carried out by Harrison[35] and Detwiler[36] using the limb as a model. They noted that defined, undifferentiated mesodermal areas of early tail bud and midgastrulating stage embryos could develop into limbs. Harrison referred to these areas as morphogenetic fields. He termed them autonomous because they could assemble organs by themselves and equipotent because any part within the field could give rise to the whole organ. These "organ fields" resemble the whole organism in the premosaic stage (pregastrulation), in combining a general determination with an epigenetic mode of development.

The regenerating blastema in planarians can be conceived of as a morphogenetic field. First, it produces either a head or a tail, depending on its location. Second, it is autonomous because its transplantation gives rise to the appropriate structure.[3] Third, the blastema is equipotent because its parts are able to generate a complete rather than an incomplete structure (Figure 6). Considering the evolutionary position of planarians, the autonomy and equipotency of their blastemas point to a set of widely conserved properties

of morphogenetic fields among very distant phyla of the animal kingdom. Planarians provide us with an example of a triploblastic organism whose morphogenesis occurs in the absence of embryogenesis. In fact, regeneration in planarians is morphogenesis. Planarians that reproduce by fission do not have the luxury of gastrulation to establish their anterior/posterior and dorsal/ventral axes epigenetically. Hence, the adult organism must rely on a defined mechanism(s) to maintain polarity in the absence of any kind of embryonic development. Such a mechanism(s) may evolutionarily precede embryogenesis proper and, if conserved in sexually reproducing animals, may provide unique insights into aspects of protostome and deuterostome embryogenesis. Thus, it seems likely that a molecular study of planarian regeneration could shed light on the molecular basis of morphogenetic field establishment, as well as on the mechanisms used for its differentiation. Nevertheless, and most likely the result of a historical accident,[37] the study of planarians at the molecular level has been largely neglected. Therefore, identifying those genes that are under temporal and spatial regulation during the formation and differentiation of planarian blastemas, that is, regeneration, may ultimately provide us with the molecular skeleton at the root of the complex morphogenetic events that occur in higher organisms.

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