Regeneration in the metazoans: why does it happen?

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Summary

Why does regeneration occur? And why, when it manifests itself, does it do so in some but not all metazoan species? Hence, what are the permissive or inhibitory factors operating behind this phenomenon? When it comes to regeneration, many questions, such as these, remain unanswered. In fact, the problem of animal regeneration has withstood the probing of scientific inquiry for over 250 years and still awaits a satisfactory mechanistic explanation. In this essay, I will review the distribution and the modes of regeneration that are found in the different metazoan phyla. Also, I will re-examine ideas on its evolutionary origins, and discuss its possible relationship to both asexual reproduction and embryogenesis. This endeavor has two objectives. First, to bring forward an interpretation of regeneration which integrates evolutionary and developmental considerations into its discussion. And second, to suggest a comparative experimental approach to this problem that may bring us closer to understanding the molecular basis of this long-standing biological problem. ***BioEssays*** **22:578–590, 2000.**

Introduction

The idea of regenerating lost body parts has captured human imagination since the beginning of history. Testament to our collective interest in this subject is found in the surviving mythologies of many ancient peoples. For instance, well-known are the Greek myths of the Hydra's ability to regenerate its many heads, and of chained Prometheus condemned to watch his own liver regenerate every time it was devoured by an eagle. In the subject of regeneration, we find that rare occasion when scientific inquiry uncovers natural events resembling those of mythology. In 1740, Abraham Trembley (1710–1784) discovered that an almost microscopic animal decorated at its cephalic end with a beautiful array of tentacles was able to completely regenerate its head after amputation. These animals' unexpected ability, and their singular morphological appearance, prompted Trembley to name them after the mythical Hydra.[1]

Trembley's published work with *Hydra* elicited a flurry of regeneration experiments in Europe. The reports matched and sometimes surpassed mythology itself. Peter Simon Pallas (1741–1811) reported in 1766 the singular regenerative properties of a then new and "obscure" species of animals, known today as the planarians.[2] And in 1768 Lazzaro Spallanzani (1729–1799) published his discovery that amphibian tadpoles were capable of regenerating their tails, and that salamanders could regenerate their jaws, limbs, tails and eyes.[3] Examples of two of these old observations are shown in Fig. 1. As for Prometheus, almost two centuries after Trembley's original discovery had to elapse before scientific research would show that the human liver is also capable of regenerating itself.[4]

Considering that the phenomenon of regeneration has been known to scientists for over 250 years,[5] and that its understanding would shed light on issues such as tissue polarity, patterning, and the control of size and proportion in animals, what do we biologists in the age of genetic engineering and genome sequencing projects know about it? Surprisingly, we know little about the biological significance and molecular mechanisms underpinning this remarkable phenomenon. In fact, of all long-standing biological problems, regeneration is one of the least understood. This state of affairs could be ascribed to a historical accident but is most likely the direct consequence of a search that began early in this century for laboratory animals with which, first, one could do genetics, and later, molecular biology. Using *Drosophila*, *Caenorhabditis elegans,* zebrafish, *Xenopus*, chickens and mice, biologists have brought about the molecular detailing of many developmental processes across varied metazoan phyla. While the study of embryology has benefited tremendously from these carefully chosen model systems, the same cannot be said for regeneration, since these animals display limited powers of regeneration or no regenerative abilities at all. Most unfortunate is the fact that the model organisms most extensively used to study regeneration (axolotls, pleurodeles and other salamanders, for example) are extremely refractory to genetics and molecular manipulations. Therefore, these animals have

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Funding agencies: Carnegie Institution of Washington; National Institute of General Medical Sciences, NIH; Grant number: RO1 GM57260-01.

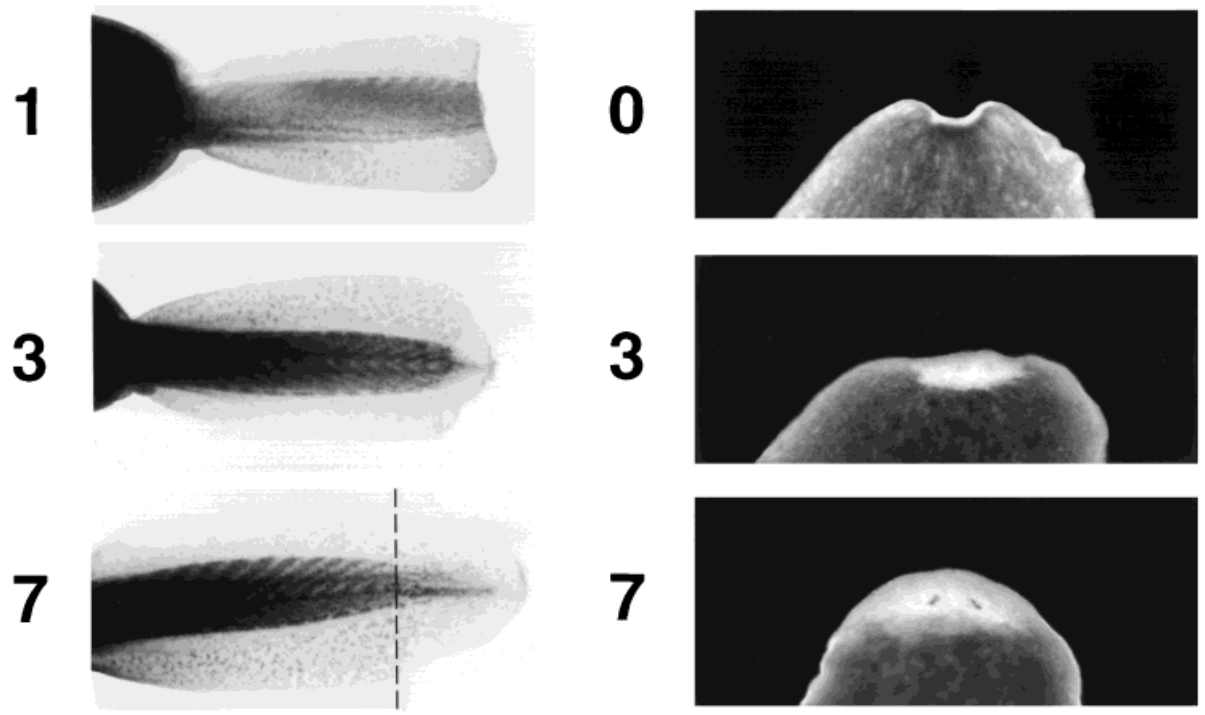

Figure 1. Vertebrate and invertebrate regeneration. Numbers to the left of each set of panels are days of regeneration after amputation. Photographs of the same amphibian tadpole from the anuran species *Rana temporaria* is shown regenerating the distal tip of its tail (left panels). Only days 1, 3 and 7 are shown. The wound in this species heals within 30 minutes post-amputation and a blastema (see text) is formed within 24 hours. In this and other anuran species such as *Xenopus laevis*, regeneration of the tail progresses at a fairly brisk pace such that the entire structure is fully regenerated by 7 days post-amputation [dashed line in 7 demarcates the limit between the old (15 mm) and the newly regenerated tail (5 mm)]. The panels to the right display cephalic regeneration of the invertebrate triclad planarian *Schmidtea mediterranea*. These organisms also heal their wound within 30 minutes post-amputation and regenerate by forming a blastema (unpigmented tissue seen at day 3, approximately 0.5 mm from bottom to top). A head, complete with cephalic ganglia (brain), photoreceptors and all other sensory components is fully regenerated by seven days. Pigmentation of the new tissue will occur within two to three weeks post-amputation.

received a lesser share of modern scientific attention and, as a consequence, so has the study of regeneration itself.

The concentration of effort that biologists have invested in understanding the molecular nature of a handful of organisms has proven successful and insightful in elucidating the molecular nature of various biological phenomena, ranging the gamut from cell division and body axes determination to the evolutionary origins of the metazoan phyla. Yet, judging by the large amount and wide-ranging scope of the literature generated by such effort, outside observers and specialists alike are often led to conclude that regeneration is uncommon, since it is rarely addressed by such work and it is described only in a few, molecularly intractable organisms. This superficial view has misled many modern developmental biologists to regard regeneration as a mere curiosity, an evolutionary footnote emerging from some kind of incomplete recapitulation of embryonic events by various tissues of a few kinds of adult organisms. This is, as we shall see, an inaccurate perception engendered by the molecular study of a handful of species initially chosen to thrive in laboratory incubators and never chosen to portray a balanced representation of the complex, organismal diversity of life.

Regeneration among the metazoans

So the question arises: is regeneration the exclusive domain of a few animal species, or is it a more widely distributed phenomenon in the metazoans? A casual inspection of any of the currently proposed phylogenetic trees of the Metazoa[6,7] clearly reveals that regeneration is a widely distributed and far more common event than is generally realized. Almost every phylum possesses one or several species capable of regenerating missing body parts; in some cases, even entire organisms may regenerate from parts of their bodies (Fig. 2). Examples are provided, on the one hand, by the urodele amphibians, which are capable of

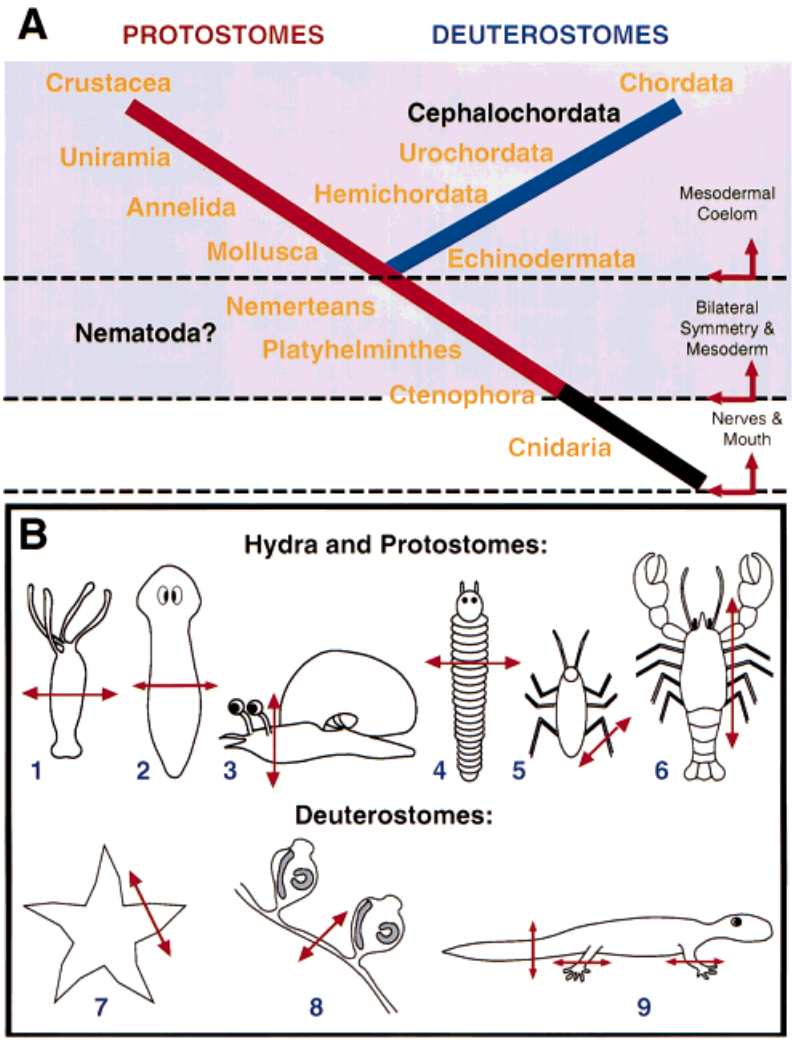

Figure 2. Distribution of regeneration in the Metazoa. **A:** Phylogenetic tree of the Metazoa. The organization and relationships of the metazoan phyla are far from settled issues. All of the phylogenetic trees available today are controversial, either in some of their regions or in their entirety, depending on the source consulted.[6,57–59,71] Regardless of the tree used to describe the phyletic relationships of the Metazoa, the conclusions made in this essay about animal regeneration remain unchanged. I have chosen to illustrate the distribution of regenerative powers in the Metazoa using the tree presented in this panel because it lends itself to a clear demonstration of how widely distributed this phenomenon is among animals. I have refrained from utilizing the newly proposed tripartite division of bilaterians[7,72] since its preliminary allocation of hox gene paralogues of key phyla needs to be corroborated by the corresponding and actual gene linkage maps. This is especially true for the newly proposed clade of Lophotrochozoans which include Brachiopods, molluscs, annelids, nemertines, and platyhelminths for which genomic maps of Hox genes are not available.[7] Also, this new clade remains controversial since it is not supported by recent triploblastic relationship analyses combining 18S rDNA of 145 terminal taxa and 276 morphological characters of 36 supraspecific taxa.[59] The tree presented here is based on several sources,[6,59,71] and like earlier ones,[73] it divides arthropods into crustaceans and uniramians based on the conclusions of Anderson,[74] Manton[75] and Valentine.[76] Such a division is also suggested by recent 18S ribosomal RNA systematics.[72,77] The area in gray encloses phyla containing all three germ layers, i.e., endoderm, mesoderm and ectoderm (triploblasts). Diploblasts (only endoderm and ectoderm) are in the white area with Ctenophora placed in between these two groups since their musculature is always derived from true mesodermal cells.[6] The branch in red denotes those phyla falling under the developmental subcategory of protostomes, while the one in blue denotes the deuterostomes. The dashed lines indicate the appearance of key morphological features in the metazoa. All phyla colored in orange contain species capable of regenerating missing body parts. For those phyla in black (Cephalochordata, and Nematoda) regeneration abilities are either unknown or conflicting evidence exists in the literature. In the cephalochordate *Amphioxus* some regeneration of the tail has been observed,[78] but infection of these animals after amputation has been blamed for lack of a rigorous test of regenerative abilities.[17] Of the seven phyla usually regarded as the pseudocoelomates (Rotifera, Gastrotricha, Kinorhynca, Nematoda, Nematomorpha, Acanthocephala, and Entoprocta), Nematoda was chosen to represent this group because of its large number of species and the vast literature it has generated. Because of the diversity in species and niches occupied by the members of these phyla, it would not be too surprising to find in this group species capable of regeneration. A report on the regeneration of rotifers exists but to my knowledge it has not been confirmed.[79] The question mark next to this phylum indicates a current controversy on the appropriate location of pseudocoelomates in the metazoan tree of life, which most recently has been grouped with the arthropods to create a new clade of metazoans referred to as the 'Ecdysozoa'.[7,59,72] **B:** Examples of regeneration found in the different metazoan phyla. Red lines with arrowheads denote planes of amputation. 1: Hydra (Cnidaria); 2. Planarian (Platyhelminthes); 3. Mollusk (Mollusca); 4. Polychaete[80] (Annelida); 5. Insect limb (Uniramia); 6. Crayfish limb (Crustacea); 7. Starfish (Echinodermata); 8. Ascidian (Urochordata); and 9. Salamander (Chordata) regeneration. Each of the two fragments resulting from the amputations in 1, 2, 4 and 7 is capable of regenerating an entire organism (bi-directional regeneration). Not included in this panel are the ctenophores, which are also capable of bi-directional regeneration.[81] See main text for further explanations and references (figure B was adapted and expanded from Brockes, Ref. 21).

regenerating complete appendages, and on the other, by the planarians which can regenerate entire organisms from tissue fragments a mere 1/279th of their body size.[8] The wide variety and evolutionary distances that exist among the animals capable of undergoing regeneration are quite astonishing (Fig. 2B). Just as embryologists wondered how conserved the developmental processes were between different phyla, so we should wonder about the underpinnings of regeneration in the Metazoa. Are there different modes of regeneration, i.e., did every phylum "invent" its own way to regenerate, or did all phyla inherit from one common ancestral organism a program, or parts thereof, with which to execute their own molecular routines of regeneration? In other words: is regeneration an analogous trait in animals, i.e., the result of convergent evolution (multiple, independent evolutionary origins), or is it a homologous trait (a single evolutionary origin) which arose very early in the evolution of metazoans (symplesiomorphy)? The answer to these questions requires that we take a look at the known strategies used by animals to regenerate.

As first noted by T. H. Morgan (1866–1945), regeneration in the Metazoa can be classified into two general groups according to the following criteria: 1) regeneration which occurs in the absence of active cell proliferation, and 2) regeneration which requires cell proliferation. The first, is referred to as *morphallaxis* (a term coined by Morgan in 1898[8] and later clarified by the same author in 1901[9]). Morphallaxis involves the re-creation of missing body parts solely by the remodeling of pre-existing cells. An example of morphallactic regeneration is provided by *Hydra*. As first shown by Trembley at the gross morphological level, pieces of hydra regenerate without creating new material,[1] a fact corroborated during the last three decades of this century by the demonstration that the differentiation of new structures in the regenerating *Hydra* occurs in the absence of cell proliferation.[10,11]

The second mode of regeneration requires cell proliferation and was originally termed *epimorphosis* by Morgan.[9] Currently, epimorphic regeneration is subdivided into two broad categories: non-blastemal and blastemal based regeneration. Non-blastemal regeneration occurs as a result of: a) transdifferentiation of the remaining tissue into the missing structure; b) limited dedifferentiation and proliferation of the surviving cells in the organ after injury or amputation; and c) by the proliferation and differentiation of stem cells already present in the damaged tissue. Examples of these are provided, respectively, by lens regeneration in urodele amphibians,[12] and by liver[13] and bone[14] regeneration in humans.

Blastemal regeneration, on the other hand, involves the formation of a specialized structure known as the regeneration blastema (Fig. 3). This structure is similar in form and organization to the early embryonic limb buds produced during vertebrate embryogenesis. As in a limb bud, a regeneration blastema is made of two, well-defined compartments: a superficial sheet of cells of epithelial origin covering the full extent of the bud, and an underlying mass of cells of mesenchymal origin (Fig. 3, C–F). Depending on the organism, the regeneration blastema may form either within hours or days after amputation or injury. The missing parts are regenerated by the eventual differentiation of the blastema. This mode of regeneration is common to planarians,[15] mollusks (gastropods,[16,17] and cephalopods[18]), echinoderms,[19] urochordates,[20] and limb[21] and tail regeneration[22] in vertebrates (Figs. 1, 2 and 3).

As if it were not remarkable enough that such diverse phyla require blastema formation to regenerate missing parts, the structural similarities that blastemas share are extraordinary.[23] One can envision a variety of ways by which a missing body part could be regenerated, yet the fact remains that in metazoans blastemas reign supreme. In 1927, Korschelt, noting the high degree of morphological resemblance that exists between the epimorphic regenerates of many different animals suggested that regeneration must have been a primordial attribute of all metazoans.[24] This idea, however, failed to gain wide acceptance because it was hard to reconcile the apparent paradox of different phyla having an evolutionarily conserved feature such as the blastema, with the obvious inability to regenerate exhibited by many related species within those same phyla. If regeneration were truly a primordial attribute, why is it then that not all animals have the ability to epimorphically regenerate lost body parts? Later, Goss would propose a simple and elegant solution to this problem: for Korschelt's hypothesis to be true, and to explain the present, erratic distribution of regeneration among the different species of any given phylum, regeneration must have been selected against, rather than for, during the evolution of the Metazoa.[25] Because all blastemas would have shared a common evolutionary origin in Goss's scenario, the remarkable conservation of blastemal structures between distant phyla would be thus explained. At the same time, negative selection would explain the non-uniform distribution of epimorphic regeneration between different taxonomic groups, since different species would have either eliminated or been indifferent to regeneration, according to their respective selective pressures.

Solid molecular evidence suggesting a common ancestral origin for regeneration is presently lacking, however. The evidence available thus far is correlational and comes primarily from the study and comparison of vertebrate regenerative events with their better understood developmental counterparts. For instance, when molecular analyses are extended to limb ontogeny and regeneration, it becomes clear that both of these events deploy similar sets of regulatory genes to accomplish their respective morphoge-

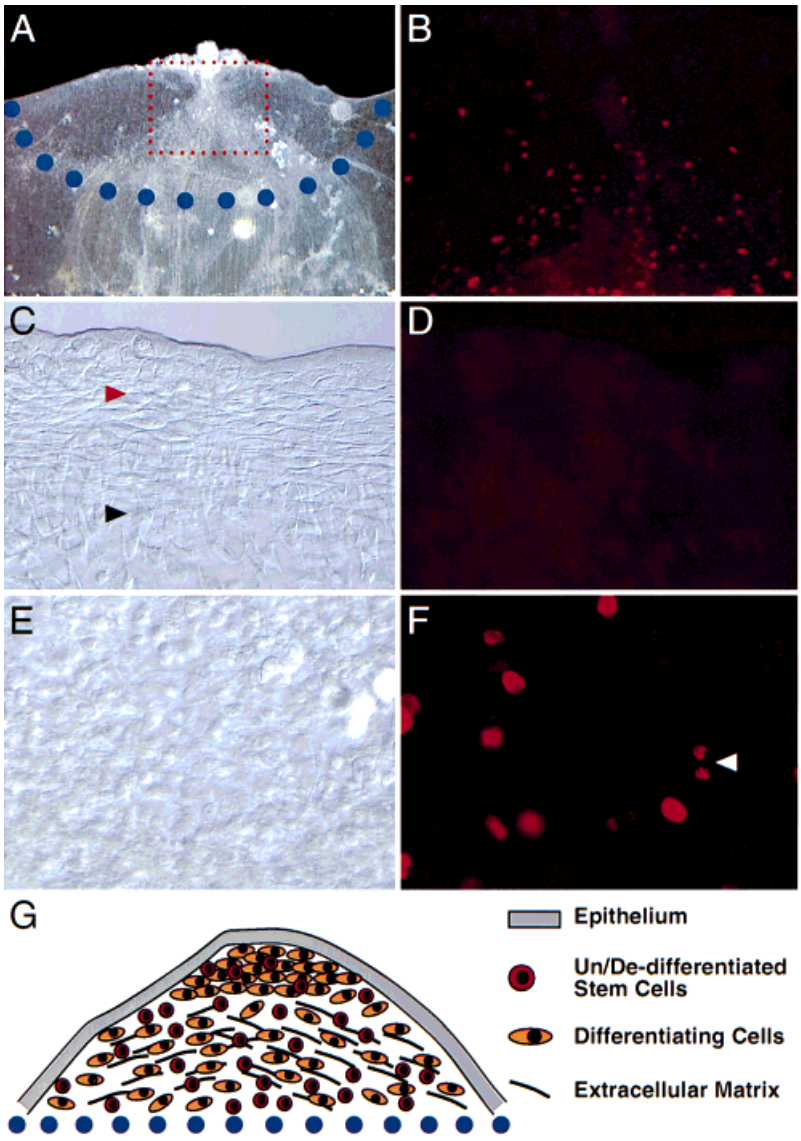

Figure 3. The regeneration blastema. The 3 day-old *Rana temporaria* tail regeneration blastema is used to illustrate this complex structure. In all panels, distal is to the top, and proximal to the bottom. **A:** A tadpole tail comparable to stage 49 of *Xenopus laevis* development displaying a 3 day-old blastema (5 ×). Blue dots indicate amputation site. The blastema lies above this demarcation. **B:** Detection of mitotic activity in the 3 day-old blastema. The dotted red rectangle indicated in **A** is shown in this panel at 20 ×. Mitotic activity is detected with an anti-phospho-histone H3 antibody[82] and is confined primarily to the region proximal to the site of amputation. **C** and **D:** Epithelial (red arrowhead) and subepithelial (black arrowhead) cells at the distal end of the blastema. At this stage, cells with well-defined cellular processes appear to be migrating and undergoing differentiation (**C**, black arrowhead). This region also appears devoid of cellular proliferation (**D**) as assayed by the mitotic marker anti-phospho-histone H3 antibody. **E** and **F:** The regeneration blastema's mesenchymal compartment. Most cells at this stage and in this blastemal region possess few cellular processes and are relatively rounded in appearance (**E**). Most of the mitoses observed in a blastema at this stage are observed in this compartment (**F**). The white arrowhead in **F** indicates a cell in telophase. **A**, **C**, and **E** Nomarski optics. **E**, **D**, and **F** epifluorescence. Magnification for **C-F** is 20x. **G**. Schematic representation of a canonical blastema based in part on **A-F**. As in **A**, blue dots represent amputation site. The epithelial compartment is shown enveloping the differentiating and proliferating mesenchyme. The mesenchymal compartment is shown composed of differentiating (brown) and proliferating (red) cells in an extracellular matrix. Among the proliferating cells, the regenerative stem cells can be found either as a reserve population (as is the case for planarians), or as the result of cellular dedifferentiation (as known to occur during salamander limb regeneration).

netic goals.[26–30] All such developmental mechanisms, in turn, appear to have evolved only once, as demonstrated by the functional conservation of key molecular events regulating the embryogenesis of both related and unrelated taxa across wide phylogenetic distances.[31,32]

Needham noted in 1952[17] that the characteristics shared by development and regeneration are underscored by the final products of each event: little if any is the difference that exists between the embryonically derived limb in a salamander, for example, and the regenerated limb in an adult organism. The implication is that both of these events must share evolutionarily conserved mechanisms. An obvious extension of this assumption is that the high degree of morphological conservation across different phyla between regeneration blastemas and their related embryonic anlagen intimates the existence of shared molecular mechanisms controlling the epithelial-mesenchymal interactions that are required for their respective ontogeneses.[23] The evolutionary relationship between regeneration and development is not entirely clear, however, and is far from resolved. Ultimately, only molecular evidence obtained from diverse organisms such as hydra, planarians and salamanders will

be able to settle the evolutionary origin of regeneration. Still, and in view of the available morphological evidence,[23] the limited molecular data,[26,27,29,30] and the high degree of conservation displayed by developmental mechanisms,[32–34] a homologous rather than an analogous origin of regeneration provides us with the most satisfactory explanation for the emergence and distribution of this phenomenon in the Metazoa.

Asexual reproduction and regeneration

A common ancestral origin of regeneration may be sufficient to explain the similarities found in blastemas across the extant metazoan phyla, yet it fails to satisfactorily explain the existence and biological significance of regeneration itself. Why did regeneration originate in evolution? Did it first emerge as such, i.e., as an independent property? Or could it be instead that regeneration has always been there as a 'pristine' (Ursprung)[24] property of life, preceding even developmental processes? Stripped of specialized functions, and reduced to its most fundamental property, i.e., the restoration of bodily components, regeneration becomes the equivalent of physiological maintenance. This is an obligatory task performed by all organisms in order to survive and thrive against the constant and generally disruptive forces of Nature. When viewed in this way, it is easy to see why Morgan[9] and Korschelt[24] considered regeneration a primordial attribute of all living beings. Needham would take this idea further a few years later and regard regeneration as being "identical to life itself",[17] since in those organisms whose parts are capable of regenerating entire individuals, their asexual mode of reproduction (budding in hydra, and fission in planarians, for instance) is indistinguishable from their morphallactic or epimorphic modes of regeneration.

Needham's concept is better illustrated by the reproductive and regenerative properties of the protozoan *Bursaria* (Fig. 4). The asexual mode of reproduction of this and other protozoans[35] generally involves a fissioning event which literally splits the organisms in half[36] (Fig. 4c,d), with the resulting halves differentiating back into complete individuals (Fig. 4d,a). Curiously, the events leading to this mode of reproduction can be triggered by cutting the animal in half and yield the same end result, i.e., reproduction (Fig. 4b′,c′). Here, the resulting fragments heal their wounds by closing the exposed surfaces (Fig. 4c′), and then differentiate in a manner that is indistinguishable from asexual reproduction (Fig. 4c′,d). Therefore, the only difference between asexual reproduction and regeneration in *Bursaria* would appear to be the stimulus triggering these two events. In the first case, the stimulus is provided by favorable environmental conditions, such as abundance of food; while in the second case the stimulus is provided by a deleterious incident such as injury. Hence asexual reproduction and regeneration in protozoans appear to be different manifestations of the same event, i.e., an ability of these organisms to reverse

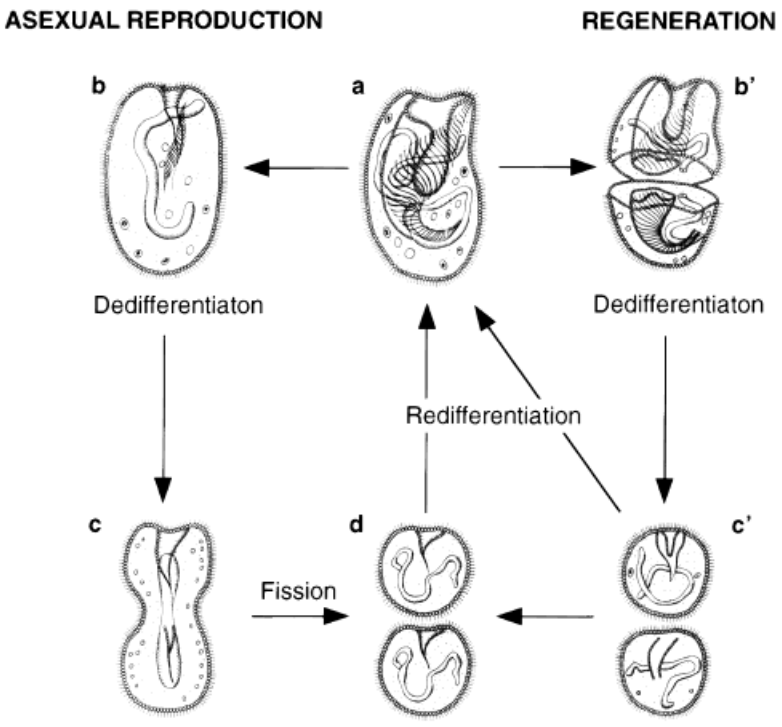

Figure 4. Asexual reproduction and regeneration. The life cycle of *Bursaria* is compared to the process of regeneration after injury in this protozoan. **a:** Adult organism with aboral zone on top. **b** and **b′:** Both the initiation of asexual reproduction and regeneration after amputation result in a dedifferentiation of the organism. **c** and **c′:** After dedifferentiation, transverse fission during normal asexual reproduction (**c**), and wound healing of the resulting fragments after amputation (**c′**) produce two individual cells. **d:** A process of redifferentiation begins to produce a new adult organism. Not pictured here, for simplicity, is the process of sporulation which the two cells in **d** are capable of undergoing, regardless of whether they are the result of normal reproduction or regeneration (After Lund[36] and Hay[39]).

their own morphogenetic processes. Even though an evolutionary relationship between regenerative phenomena in the single cells of protozoa and that of metazoans has not been uncovered, it is nevertheless clear that morphogenetic reversibility or the association of vegetative reproduction and regeneration also holds true for the way other, more complex organisms such as hydra,[6] planarians[15,37,38] and ascidians[39] reproduce and regenerate.

Could the phenomenon we call regeneration have its origins, then, in asexual reproduction? The only obvious difference between vegetative reproduction and regeneration appears to lie in their stimuli. This indicates that the molecular cascades we generally associate with regeneration may have appeared first and foremost as a way to propagate the species (asexual reproduction), and that such cascades may have been co-opted by many organisms to cope with injury or any other catastrophic event. The ability to trigger asexual reproduction after amputation has obvious adaptive advantages and it is not difficult to see how this property could have been selected for. Moreover, an explanation as to how a pre-existing feature such as asexual reproduction could have been co-opted into a new feature like regeneration need not be esoteric, or too contrived. It may have only required, for instance, that the stimulus needed for regeneration (injury) activate the same messenger molecules used to mediate asexual reproduction. In fact, strong molecular evidence supporting this idea is provided by hydra.

In hydra, both the initial stages of asexual reproduction (budding), and regeneration are mediated by secreted signaling peptides. During asexual reproduction a localized increase of the secreted peptide known as head activator (HA) is known to occur.[40–43] After binding to receptors found on the surface of pluripotential interstitial cells, HA induces an increase of intracellular cAMP which then activates protein kinase A, resulting in the phosphorylation of the transcription factor CREB.[44,45] Once modified, CREB activates the genes responsible for the terminal differentiation of nerve cells, which in turn are responsible for the budding process in hydra.[46] Finally, the emerging bud will eventually differentiate and produce a new and complete organism.[6] Likewise, amputation of hydra is known to result in the release of high levels of the peptide HA, which then triggers the formation of a bud, in this case a regeneration bud which will differentiate into the missing body parts. In other words, the inflicted injury (amputation) is capable of activating the same genetic cascade normally used to initiate asexual reproduction.[47]

Yet, there are animals adept at regenerating entire organisms from their parts in which asexual reproduction and regenerative abilities are dissociated from each other. Examples are provided by the triploblastic planarians. Asexual reproduction in these animals occurs by transverse fission along the posterior two thirds of the animal, i.e., at or below the level of the pharynx. After fissioning, the resulting anterior piece regenerates a tail, and the corresponding posterior piece regenerates a head and a pharynx. In most planarian species, amputation yields the same end results. Yet, in *Dendrocoelum lacteum* and *Bdelocephalla punctata,* amputation along the length of the pharyngeal and post-pharyngeal region fails to activate regeneration of either a head or a pharynx in the resulting posterior pieces. If these animals, however, are amputated above the pharyngeal level, both pieces will regenerate complete organisms. Interestingly, the inability to regenerate at or below the level of the pharynx in *D. lacteum* and *B. punctata* is also accompanied by a lack of asexual fission. Both of these species reproduce exclusively by sexual means even though their potential for vegetative reproduction is evidenced by the ability of prepharyngeal fragments to regenerate complete animals.[15] Cases such as these merit molecular study, since exceptions in which regeneration and asexual reproduction appear to be dissociated from each other may help us identify those factors which either allow or prevent an organism from reversing its morphogenetic processes.

Nevertheless, the high correlational incidence of asexual reproduction and regeneration in most animals may be a reflection of why regeneration emerged to begin with, i.e., as a co-option of asexual reproductive mechanisms for regenerative events; its perpetuation fueled by the adaptive advantages such a trait would have conferred upon most organisms during natural selection. Molecular analyses of other asexually reproducing animals such as the diploblastic *Hydra*, as well as the triploblastic planarians during naturally occurring fission and injury-induced regeneration should shed more light on this contention. In doing so, they should corroborate or dismiss a primordial evolutionary co-option of vegetative reproductive mechanisms for the purposes of morphallactic and epimorphic regeneration.

Cellular pluripotentiality and regeneration

There are, however, obvious and important differences between the regeneration potencies of simple organisms and those of more histologically complex animals. This is evident even in species possessing extensive blastemal-based regenerative abilities such as planarians and salamanders. While the bisection of a planarian results in two fragments, each of which is capable of regenerating an entire individual (Fig. 2B, 2), the amputation of a salamander limb, for example, results in two pieces of very different regenerative abilities (Fig. 2B, 9). One is the salamander itself, which goes on to regenerate the missing body part, and the other, the detached limb which is obviously unable to regenerate the 'missing' salamander and dies. As facetious as this comparison may appear, it raises a very important point. The ability of individual organisms or their parts to

reverse their morphogenetic processes, i.e., to go from a differentiated to an undifferentiated state in order to recreate themselves, depends entirely on that organism's capacity to regulate cellular pluripotentiality. Therefore, accounting for the presence of regeneration in complex animals such as the vertebrates, based on the interrelatedness that exists between asexual reproduction and regeneration, becomes, to say the least, quite an engaging paradox. Nevertheless, as dissimilar in regenerative properties as an amputated salamander hand may be from an amputated planarian tail, the cells that make up these two fragments have in common the remarkable biological property of being pluripotential. The difference lies in how this potential is controlled and rationed in the adult organism. In the metazoans, whether we are concerned with asexual or sexually reproducing animals, morphallactic or epimorphic regeneration, the ability to reproduce missing body parts always depends on the availability of a source of pluripotential cells.

In animals, cellular pluripotentiality is a highly regulated property. The more complex the organism becomes, i.e., the more differentiated its cells become, the harder it is to induce dedifferentiation and extricate from its cells their inherent pluripotentiality. In sexually reproducing animals, this is most apparent during embryogenesis. Beginning their life as a single, pluripotential cell, almost all embryos possess the power to regenerate missing parts at some stage of their embryogenesis, but lose this ability as their development proceeds. This was discovered almost 100 years ago by Hans Driesch (1867–1941) while experimenting with echinoderm (sea-urchin) embryos.[48] After physically separating the blastomeres of a 4-cell stage embryo, Driesch observed that each of the cells developed, not into incomplete embryos as he had expected, but into four normal, albeit smaller, individuals. In other words, during the first and second cleavages, the single-celled embryo merely regenerated or copied itself to produce 4, equipotent cells capable of producing an entire organism on their own. At the same time, Driesch also noted that this regulatory ability was lost in later developmental stages. This observation was subsequently extended to more complex organisms such as the cephalochordates (*Amphioxus*)[49] and the chordates (amphibians),[50] where again the ability of individual blastomeres to regenerate and eventually produce a complete embryo is highest very early during development and much reduced, if not completely absent, at later embryonic stages.

As in embryogenesis, the dynamics of regeneration are not exempt from the forces that drive the regulation of cellular pluripotentiality. This is clearly evidenced by the way regeneration is deployed in metazoans of increasing evolutionary complexity (Fig. 5), and by the concomitant decrease in regenerative abilities that is observed as this complexity increases. In the simple diploblastic hydra (Fig. 2), one finds that the normal condition for the adult form is a constant state of regeneration. The body column of this animal is made up mostly of undetermined, undifferentiated cells which are constantly undergoing mitosis.[51,52] The undifferentiated, pluripotential cells in the gastric column[53] are permanently changing their position in an apical or basal direction. At the same time, these cells are continually changing their morphology as they move to these poles in order to physiologically maintain the functional/structural integrity of this organism.[54] As noted previously by Galliot,[47] this property of the gastric column, i.e., to serve as a source of pluripotential cells, is not dissimilar from that of a regeneration blastema. In hydra, the gastric column can be functionally likened to a regeneration blastema, and regeneration in this animal goes directly to the post-blastemal regenerative stages observed in higher organisms, i.e., determination and differentiation (Fig. 5). Therefore, cellular proliferation and the creation of a specialized structure like a blastema during hydra regeneration is pre-empted by the existence of a normal and constant source of pluripotential cells in the body column. This explains, albeit teleologically, the morphallactic nature of regeneration in these organisms.

Triploblasts, on the other hand, have placed more regulatory checkpoints on the pluripotentiality of their cells, and pluripotentiality is severely restricted, even in those animals in which regeneration powers are still extensive. In the planarians, one of the simplest triploblasts known, cellular pluripotentiality is confined to the neoblasts, the only undifferentiated and mitotically active cell population in these organisms.[15] Unlike hydra, where cell proliferation is not required for its regeneration, planarian neoblasts have to proliferate first, such that a specialized structure (the blastema) can be formed to direct localised cellular proliferation, determination and differentiation which results in the regeneration of the missing structures. In more complex triploblasts such as the salamanders, for instance, cellular pluripotentiality is restricted even further. In these organisms, which are capable of regenerating missing body parts such as their limbs and tail (Fig. 2B, 9), no reserve of pluripotential cells is to be found in the normal, uninjured appendages. Instead, the pluripotential cells are produced de novo from pre-existing, terminally differentiated cells. After injury or amputation, the cells of this animal first have to undergo a process of dedifferentiation, then re-enter mitosis and proliferate before a source of pluripotential cells is available to regenerate the missing appendage.[21] Hence, as evolutionary complexity increases, more and more regulatory checkpoints appear to have been introduced to control the inherent pluripotentiality of all animal cells (Fig. 5). Such incremental specialization of pluripotentiality for regenerative events may, in turn, be a reflection of the selective forces that may have been acting on the evolutionary origins

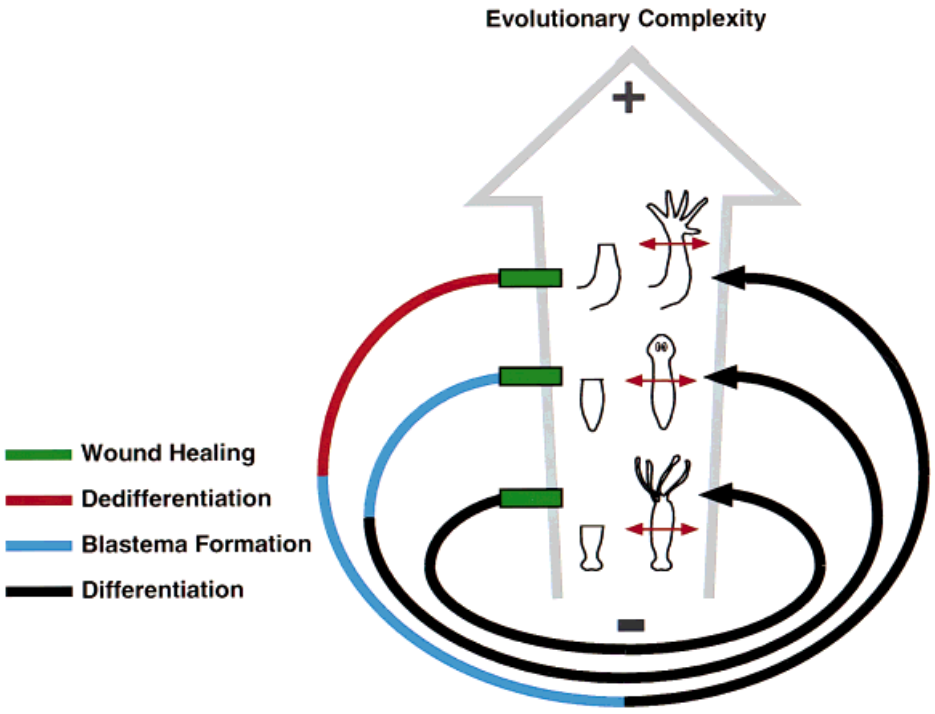

Figure 5. Increasing regulation of cellular pluripotentiality with increasing morphological complexity. The apparent addition of several regulatory steps (wound healing, blastema formation, dedifferentiation and differentiation) as evolutionary complexity increases is illusrated. Three representative organisms, all with great powers of regeneration, are included. From bottom to top: hydra (Cnidaria), planarian (Platyhelminthes) and the salamander (Chordata), represented by one of its limbs. The gray arrow points in the direction of lower (minus sign) to higher (plus sign) evolutionary complexity (refer to figure 2 A). The cycles of regeneration for these organisms are illustrated by the three ellipses, all of which find their origin in wound healing after amputation, and culminate in the fully regenerated structures of the respective organisms.

of the regeneration blastema itself. Only when we have accrued sufficient molecular data on processes such as determination and differentiation, blastema formation and dedifferentiation for hydra,[55] planarians[56] and salamanders[21] will we be able to substantiate the tentative relationships postulated in Fig. 5.

Regeneration and embryogenesis

One of the most troublesome issues of evolutionary theory involves the accounting for new structures that emerge during evolution. The appearance of appendages such as the limbs in the body plans of chordates, uniramians and crustaceans is an example of such a structure. The evolutionary conservation of the molecular events leading to limb ontogenesis among different phyla is well known, and it is used regularly as an example of a homologous metazoan trait with a functional conservation of key molecular events.[57] Nevertheless, the phylogenetic relationships that exist, for example, between insects and vertebrates indicate that none of their common ancestors possessed appendages, and thus it is difficult to explain how these appendages may have arisen homologously in the first place. The obvious implication would be that the molecular cascades utilized in the formation of limbs must have been present in the most recent, common ancestor of these organisms. A look at the phylogenetic tree in Fig. 2A or any of the newly proposed trees[7,58,59] clearly shows that such an organism is likely to have been a triploblast and thus a protostome whose phylogenetic position is either at or before the branching out of the tree into Deuterostomes. Neither Nemerteans nor Platyhelminthes nor Ctenophora nor even Cnidaria possess structures in their body plans which can be considered homologous to limbs but they almost certainly share the basic signalling cascades known to exist in more complex metazoans.

One explanation that has been put forward to account for the appearance of novel structures during the course of evolution is that of co-opting a pre-existing structure performing a particular task for a new use that may have been selectively advantageous.[57,60] Earlier, I mentioned the

similarities that exist between the regeneration blastema and the embryonic limb bud, not only among similar species but also between phylogenetically distant phyla. Both of these specialized structures are composed of well-defined epithelial-mesenchymal boundaries and are very similar at the histological level.[23] In light of what has been discussed, I would like to suggest the possibility that the molecular processes underlying blastema formation and regeneration have been co-opted by sexually reproducing animals for the production of new structures such as limbs during the evolution of their developmental processes.

In a regeneration blastema one can find the fundamental genetic interactions that are required for the patterning of a structure polarized along dorsoventral, anteroposterior and proximodistal axes. Thus, it can be said that in the case of triploblasts such as the planarians the blastema that forms after either binary fission or amputation is essentially playing the role of embryogenesis in these animals. The possibility exists then that the genetic cascades required to produce complex body plans may have appeared for the first time as a developmental module in the blastema of simple organisms such as the planarians. Developmental modules have been defined by Raff[57] and others[61] as autonomous structures with clear morphological identities, discrete genetic organization and hierarchical units, all of which are capable of undergoing temporal transformations. Standard examples of such modules are given by the domains of the *Drosophila* embryo which are fated to become segments of the body axis at later developmental stages.[62]

Interestingly, the definition of a developmental module also holds true for both limb buds and regeneration blastemas. How different then are the developmental events occurring in a limb bud from those that give rise to a body plan in the regeneration blastema of planarians? In fact, it is easy to show the modular similarities that exist between a limb bud and the embryogenesis of the vertebrate body axis within any given Chordate. Both require mesenchymal-epithelial interactions to integrate their activities; both are directed by discrete genetic cascades in which specific hierarchies of genes such as those of the Hox cluster(s) are deployed under spatial and temporal constraints. And finally, as development proceeds, both the embryonic body and the limb bud show domains of differentiation which are eventually refined into concrete elements (branchial arches into cephalic and trunk structures; regions of ossification in the limb bud to form the bones of the appendage, for example). Hence, it is likely that during evolution the discrete genetic organization that must be operating in the blastemas of simpler organisms during regeneration/asexual reproduction may have been co-opted in sexually reproducing animals to produce novel embryonic structures such as the limb bud. This co-option is likely to have occurred only after the duplication of many of the genes, and gene clusters which are involved in setting these axes and epithelial mesenchymal interactions (Hox clusters,[63,64] Msx[65] and T-box genes,[29] etc.). Finally, this idea is underscored by the fact that the morphogenetic fields that give rise to the limbs in insects and to limb/fin buds in chordates share important features with the regeneration blastemas of simple organisms such as the planarians,[23] the most striking one being the autonomy and equipotency all these structures have in common. They are autonomous because their transplantation gives rise to the appropriate structures[66,67] and equipotent because their parts are able to generate complete, rather than incomplete structures, as illustrated in Fig. 6.

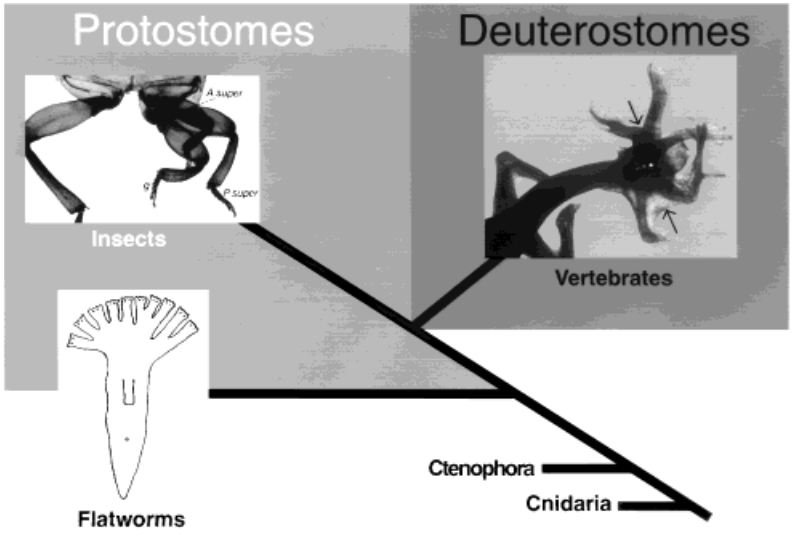

Figure 6. The conservation of morphogenetic properties between regeneration blastemas and limb fields. The figure illustrates the ability of the morphogenetic field to give rise to multiple and complete structures from its parts. Representatives of the protostomes are the uniramian *Tenebrio*[83] and the planarians.[84] The deuterostome is a *Rana temporaria* tadpole obtained by the author after treatment of the tail blastema with retinol palmitate to induce a homeotic transformation of tail-into-limbs. Some of the emerging limb buds were bisected with a scalpel resulting in the formation of two fairly complete limbs per surgically manipulated bud (arrows).

Concluding remarks

The ideas presented in this essay arise from the combined observations and the many interpretations that regeneration has elicited from experimental biologists throughout the years. Obviously, these ideas have to be tempered with molecular experimentation. The diversity of experimental tools available today demands it. As more molecular data are accumulated on the phenomenon of regeneration and life in general, we should not be surprised to find some of these ideas eliminated, others transformed, and still others that are altogether new to emerge on this subject. Subscribing to Morgan's, Korschelt's and Needham's notion that regeneration is a primitive or primordial (Ursprung) character of life, i.e., a symplesiomorphy, may provide us with a very productive intellectual framework with which to approach this problem. If regeneration, as the ideas of these authors suggest, is truly primordial, one would have to take into account the role that the molecular circuitries regulating this phenomenon may play in other, later traits that have emerged in the different phyla during evolution. This basic notion is what has prompted me to re-examine certain cardinal traits of metazoan life, such as asexual and sexual reproduction and their developmental consequences from the standpoint of regeneration.

The question of why an apparently advantageous attribute such as regeneration would be selected against during evolution is intriguing. It may be that the price of maintaining or producing the pluripotential cells required to form a blastema may have been incompatible with the long-term survival of any given species. Uncontrolled production and proliferation of such a pool of pluripotential cells, for example, may have resulted in the formation of tumors and in the eventual demise of the afflicted organism. An altogether different possibility, however, is that neither positive nor negative selection for regeneration operated in the evolutionary history of those extant species unable to carry out epimorphic regeneration, allowing this trait to disappear or become more restricted. Hence, in those organisms where regeneration either does not happen, or is severely restricted, an uncoupling of the molecular cascades involved in the creation (embryogenesis), and the repair and maintenance of the adult structure (regeneration) could have occurred. This would be likely if during evolution no selective pressures (either positive or negative) were exerted to either maintain or eliminate regenerative properties in these animals. Factors such as the size of the adult structure to be regenerated, and/or the longevity of any given species may lie at the root of this hypothetical uncoupling. Unfortunately, our knowledge of the genetic and epigenetic mechanisms regulating scale, proportion, and longevity in the Metazoa is rudimentary at best, and it is very likely that these seemingly unrelated properties will play key roles in regulating regeneration.

Considering the association that exists between regeneration and cellular pluripotentiality, and given the different strategies used by the Metazoa to regulate these properties, future research on regeneration must become multiphyletic and integrative in nature. Such an approach, as logical as it seems, would require the identification and isolation not only of those sets of genes activated during regeneration, but also during injury, wound repair, dedifferentiation and asexual reproduction. This is further complicated by the fact that there may be other unsuspected or even unknown biological processes acting on regeneration whose effects would have to be defined, and then characterized at the molecular level. Hence a comprehensive, multiphyletic and integrative approach to the problem of regeneration seems, at first glance, rather difficult to deploy. Yet, as overambitious as such a strategy may appear, the availability of new technologies such as automated DNA sequencing and DNA microarrays make this seemingly daunting task quite tangible. Conceivably, a relatively small number (approximately 10,000 per species) of expressed sequenced tags (ESTs) from organisms capable and incapable of regenerating missing body parts, and ideally representing several phyla, could be generated relatively rapidly. Species-specific DNA microarrays could then be made from such ESTs and then hybridized with cDNAs obtained from tissues of the appropriate species which have been subjected to either injury, or are undergoing wound repair, dedifferentiation, regeneration or asexual reproduction. Such an approach using hydra, ctenophores, planarians, ascidians, salamanders, and the ESTs already available for *Drosophila*, zebrafish, and mice, for example, will help delineate the common and not-so-common genetic ground shared by regenerative events among the Metazoa.

One key advantage of large-scale gene expression profiling is that it permits the study of not only the single process one initially intended to observe (regeneration, for example), but also of all the other processes normally taking place in the tissues being analyzed. By detecting the expression of unsearched-for genes in the cDNA population hybridized against the DNA microarray, genetic cascades not suspected to play a role in regeneration could be easily identified and with them, any unknown biological processes acting on regeneration. This added benefit of DNA microarrays is clearly illustrated by the recently defined transcriptional response of human fibroblasts to serum, which identified not only the genes controling the transition from G_0 to a proliferating state in these cells, but also a large and unexpected number of genes known to play key roles in wound healing.[68] This observation is remarkable in that it indicates that the proliferative response of fibroblasts to serum may simply form part of the wound healing process of vertebrates.

Since in the Metazoa, injury is the stimulus for regeneration,[17,69] it is an intriguing observation that the serum of

many vertebrates that are unable to epimorphically regenerate (chickens, sheeps, and cows) contain a factor or factors that can trigger the dedifferentiation and concomitant reentry into the cell cycle of newt myotubes in vitro.[70] Reentry into the cell cycle of differentiated cells is an obligatory step in the formation of regeneration blastemas in newts and other amphibians (Fig. 5). Such a biochemical link between dedifferentiation and wound healing provides a functional assay in which DNA microarrays could help identify the genes activated by serum in newt but not in mammalian, or chicken cells, for example. Such molecular evidence should begin to help explain the differences in regenerative abilities that exist between urodeles and other vertebrates. Furthermore, whether homologs of such serum factors will be present in simpler organisms after injury should be of interest in trying to define the extent of molecular homology that may exist between regenerative events in the Metazoa. The role of such factors in invertebrate regeneration could also be screened using DNA microarrays. Would such factor(s) be present and play a role in the regenerative abilities of ascidians, for example, which possess a well defined circulatory system? Would they be present, for that matter, in animals lacking circulatory systems and in which dedifferentiation does not occur? And if so, what would their function be and what role if any will they play during the regenerative events of these organisms? Questions such as these and the others posed throughout this essay reveal how rich and untapped the field of regeneration remains for the experimental biologist. It is my belief that a comparative molecular analysis of the regenerative processes found in animals will not only shed light on the intrinsic dynamics of this phenomenon, but also help us to understand some of the most basic and fundamental aspects of metazoan biology that continue to puzzle us today.

Acknowledgments

I thank Dr. Malcolm Steinberg for his encouragement and insightful suggestions, my colleagues at the Department of Embryology and Drs. Phillip A. Newmark and Tatjana Piotrowski for helpful comments.

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