

Regeneration and the need for simpler model organisms

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The problem of regeneration is fundamentally a problem of tissue homeostasis involving the replacement of cells lost to normal 'wear and tear' (cell turnover), and/or injury. This attribute is of particular significance to organisms possessing relatively long lifespans, as maintenance of all body parts and their functional integration is essential for their survival. Because tissue replacement is broadly distributed among multicellular life-forms, and the molecules and mechanisms controlling cellular differentiation are considered ancient evolutionary inventions, it should be possible to gain key molecular insights about regenerative processes through the study of simpler animals. We have chosen to study and develop the freshwater planarian *Schmidtea mediterranea* as a model system because it is one of the simplest metazoans possessing tissue homeostasis and regeneration, and because it has become relatively easy to molecularly manipulate this organism. The developmental plasticity and longevity of *S. mediterranea* is in marked contrast to its better-characterized invertebrate cohorts: the fruitfly *Drosophila melanogaster* and the roundworm *Caenorhabditis elegans*, both of which have short lifespans and are poor at regenerating tissues. Therefore, planarians present us with new, experimentally accessible contexts in which to study the molecular actions guiding cell fate restriction, differentiation and patterning, each of which is crucial not only for regeneration to occur, but also for the survival and perpetuation of all multicellular organisms.

Keywords: planarians; regeneration; evolution; molecular pathways

All models are wrong. Some models are useful.
(George E. P. Box, cited in Launer & Wilkinson 1979, p. 202)

1. METAZOAN MOLECULAR PATHWAYS: HOW OLD ARE THEY?

In 1962 the economist and Nobel Prize winner Herbert Simon (1916–2001) made a point about the evolution of complex systems by drawing a parable involving two watchmakers he called Hora and Tempus. Both manufactured very fine watches consisting of a thousand parts each. Demand for these timepieces was very high; however, only Hora prospered while Tempus eventually went out of business. Interestingly, the equally exceptional watches of Tempus became extinct not because of a deficiency in their function, but rather because of how they were being assembled:

The watches the men made consisted of about 1000 parts each. Tempus had so constructed his that if he had one partly assembled and had to put it down—to answer the phone say—it immediately fell to pieces and had to be reassembled from the elements. The better the customers liked his watches, the more they phoned him, the more difficult it became for him to find enough uninterrupted time to finish a watch. The watches that Hora made were no less complex than those of Tempus. But

he had designed them so that he could put together sub-assemblies of about ten elements each. Ten of these sub-assemblies, again, could be put together into a larger subassembly; and a system of ten of the latter sub-assemblies constituted the whole watch. Hence, when Hora had to put down a partly assembled watch in order to answer the phone, he lost only a small part of his work, and he assembled his watches in only a fraction of the man-hours it took Tempus.

(Simon 1962, p. 470)

Simon's parable is useful in that it illustrates two fundamental points about the evolution of complex systems: that a deduced history of an extant form must take into account environmental disruptions (an elemental force of natural selection); and second, that the extant form is possible only because stable subsystems were unperturbed by such disturbances. Modular assembly, therefore, provides an error correction strategy that is both economical in the number of its components, and lavish at accelerating the progression of structural complexity.

Current thinking on the evolution of life-forms is not too dissimilar from Simon's parable. For example, only seven major types of pathway are believed to perform cell signalling during the early development of bilaterians: the TGF- β , Wnt, Notch, Hh, nuclear receptor, JAK/STAT and receptor tyrosine kinase pathways (Gerhart 1999; Barolo & Posakony 2002). One cardinal trait of these and other molecular pathways involved in field and cell-type specification such as the Hox, Pax, Distal-less, MASH and Nkx2.5 is that they are widely distributed among the Metazoa. Their conservation suggests that they must have

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been largely present in the common ancestor to both protostomes and deuterostomes. That indicates that these pathways are at least a billion to half a billion years old (Lake 1990). In fact, many of the cell signalling and adhesion molecules used by extant metazoans, such as components of the tyrosine kinase signalling pathway and cadherins, may be significantly more ancient, as they can be found in choanoflagellates, unicellular and colonial protozoans whose origins predate the origin of multicellular animals (King *et al.* 2003). Therefore, to make it to the present time, these pathways had to survive unknown and known environmental and ecological changes, some of them colossal in magnitude such as the mass extinctions of the Proterozoic (Schopf 1991) a billion years ago and the Cambrian explosion of 550 Myr ago (Lake 1990; Carroll *et al.* 2001).

In the framework of Simon's parable, such conserved molecular pathways represent stable subsystems capable of surviving a breadth of environmental disruptions. Their stability, therefore, has generated an opportunity for these modules to extensively iterate their spatial and temporal relationships in the context of multicellular life during the past billion years or so. Under the inescapable evolutionary constraints to which all of life is subjected, novel functions must have arisen from such iterations resulting in the eventual assembly of systems (organisms) of increasing complexity. This is perhaps reflected by the pleiotropy of most if not all developmentally regulated molecular pathways, i.e. their repeated use in a wide variety of developmental contexts (Godwin & Capecchi 1998; Telford 2000; von Dassow *et al.* 2000; Fukuchi-Shimogori & Grove 2003; Hooper 2003; Pinto *et al.* 2003; Steiling *et al.* 2003). A good example of pleiotropy is provided by the *Hox* module in the cephalopod *Euprymna scolopes*. In this species of squid, the *Hox* genes are expressed in the expected co-linearity along the anteroposterior axis of the animal. However, the same *Hox* genes are also expressed in several morphological novelties found *only* in cephalopods such as the stellate ganglia, the ectodermally derived components of the brachial crown, the metabranchial vesicles, and the buccal crown, funnel tube and light organs (Lee *et al.* 2003). Interestingly, the anteroposterior expression domains of the *Hox* genes in these structures are not those predicted by *Hox* co-linearity. Instead, the *Hox* module appears to participate in new developmental programmes unrelated to anteroposterior patterning (Lee *et al.* 2003). This unexpected result suggests an association between novel temporo-spatial iterations of the *Hox* module with increased morphological complexity and function in the cephalopods.

The recent data from cephalopods as well as a wealth of experimental evidence accrued in recent years by the combined fields of genetics (McGinnis 1994), evolutionary (Chervitz *et al.* 1998; Rubin *et al.* 2000) and developmental biology (Ruvkun & Hobert 1998) seem to support the notion of modularity in metazoans (Hartwell *et al.* 1999). A modular architecture for the assembly of complex systems such as living organisms is an attractive theoretical construct with which to frame the history of the extant metazoan forms. When the frequency of environmental disturbances increases, so does the need for stable subsolutions. Such systems evolve through the gradual replacement and addition of modules (Li & Noll

1994; Force *et al.* 1999; Holland 1999) and themselves outlast ecological and environmental changes as well as the larger system itself. More importantly, modularity takes into account the assembly of individual parts into modules, their interactions and the evolutionary constraints in which they operate (Hartwell *et al.* 1999). This provides us, in principle, with a general and experimentally testable model for the emergence of novel attributes and increased functional complexity during evolution.

2. MODULAR ASSEMBLY AND REGENERATION

Regeneration is widely distributed among the different metazoan phyla (Sánchez Alvarado 2000, 2003b; Brookes *et al.* 2001), and has even been shown to occur in the simplest multicellular organism known so far, the placozoan *Trichoplax adhaerens* (Ruthmann & Terwelp 1979). The capacity shown by animals to replace their cells as a result of tissue turnover or injury and its significance to inform a wide gamut of biological processes has been discussed in detail in a previous review (Sánchez Alvarado 2000). Of interest to us is that the multiphyletic distribution of regenerative capacities is likely to be underscored by the conservation through common ancestry of a finite number of regulatory molecular pathways. Although recent efforts have begun to identify several molecules involved in various aspects of metazoan regeneration (Odelberg *et al.* 2000; da Silva *et al.* 2002; Imokawa & Brookes 2003), the minimum number of pathways and factors that are necessary and sufficient to carry out regeneration in multicellular organisms have not yet been determined. Given the remarkable similarities that exist between the orfeomes of those metazoans whose genomes have thus far been sequenced (Rubin *et al.* 2000; Lander *et al.* 2001), it is quite possible that the study of regeneration will not uncover novel molecular pathways. Instead, the most likely initial outcome will be the identification of pleiotropic functions for known genetic pathways in the milieu of regeneration. That known pathways play a role has, in fact, been shown during salamander limb regeneration for the retinoic acid nuclear receptor and FGF pathways (Boilly *et al.* 1991; Mullen *et al.* 1996; Pecorino *et al.* 1996).

However, if regeneration merely uses developmental pathways, why should we be bothered with its study? The answer is simple. Like development, regeneration involves the self-assembly of new tissues but, very much unlike development, regeneration also entails the anatomical and functional integration of the newly made parts to older, pre-existing tissues. Even though all organisms share a common set of developmental pathways, not all of them are capable of replacing structures lost to damage or amputation. Organisms such as *Caenorhabditis elegans* and *Drosophila* (in which most of the known signalling pathways have been identified and studied in exhaustive detail) are simply inept at regenerating body parts. Hence, the study of regeneration may in fact help us understand a central problem posed by modularity: how can a relatively small number of molecular pathways be responsible for the breadth of developmental fates, morphologies and cellular functions displayed by the metazoans? A probable explanation is that cellular context (presence or absence of a given signal, as well as a variety of potential local, cell-

and tissue-specific factors) may affect the mechanisms by which these pathways control the transcriptional state of their target genes. For example, in the case of signalling pathways such as Notch, Wnt, Hh and nuclear receptors, the mechanism for repression or activation of target genes is mediated not only by the same DREs in the promoter regions of the target genes, but also by the same transcription factors (Su(H)/CBF1 in the Notch pathway; Lef/Tcf proteins in the Wnt pathway; Ci/Gli in the Hh pathway, and the nuclear receptors themselves) (Neumann & Cohen 1996, 1997; Johnston & Edgar 1998; Barolo & Posakony 2002). Therefore, neither the DREs nor the transcription factors render output specificity to these pathways. Instead, specificity is established by dosage of signal, the strength of the default repression of the target genes, and by the cooperative activation of different transcription factors (Barolo & Posakony 2002). All of these conditions for pathway specificity are independent from the molecular pathways themselves, but thoroughly beholden to the particular environment (developmental, functional, etc.) in which the cell may encounter itself.

Regeneration exposes known and well-studied genetic pathways to contexts not found during embryogenesis, and as such should help us identify novel determinants of pathway specificity. An example of how systematic studies of animals endowed with robust regenerative capacity can inform the problem of modular specificity is provided by the recent identification in freshwater planarians of *nou darake*, a novel but evolutionarily conserved fibroblast-receptor-like molecule capable of modulating the activities of the FGF pathway in both planarians and vertebrates (Cebrià *et al.* 2002). Additionally, pathway specificity is known to be regulated by molecules other than proteins such as, for instance microRNAs. These small (21 or 22 nucleotide-long) RNAs have been shown to control the timing of specific cell fate switches during development (Lee *et al.* 1993; Reinhart *et al.* 2000), stem cell maintenance (Schauer *et al.* 2002), and even affect the downstream events of the Notch pathway in *Drosophila* (Brennecke *et al.* 2003). Consequently, many uncharacterized modulators may exist in metazoans whose molecular nature may be both predicted and unpredicted. Their identification and characterization will largely depend on the availability of varied and experimentally accessible biological contexts.

3. REGENERATION AND THE FRESHWATER PLANARIAN *SCHMIDTEA MEDITERRANEA*

One way to solve a complex problem is to reduce it to a simpler problem that has been previously solved. Reducing the complexities of regeneration to the study of a simple system in which the puzzle of pathway specificity was solved early on in evolution could significantly accelerate the identification of such pathways and their regulators. Two basic criteria for selecting such a model system should be met. First, the selected animal should be one of the simplest metazoans in which the trait of interest (regeneration) is patently manifested; and second, the selected animal should be relatively easy to manipulate experimentally. Planarians fulfill these criteria, as they are one of the simplest bilaterians known to display robust regenerative capacities, and are amenable to molecular

manipulations (Sánchez Alvarado *et al.* 2002; Newmark *et al.* 2003). The suitability of freshwater planarians, in particular *Schmidtea mediterranea*, to address the question of regeneration has been the focus of several recent reviews (Newmark & Sánchez Alvarado 2002; Sánchez Alvarado 2003a) and will not be discussed here. Suffice it to say, that it is now possible to use molecular tools in *S. mediterranea* to study its phenomenology (Robb & Sánchez Alvarado 2002; Oviedo *et al.* 2003), the function of its genes (Sánchez Alvarado & Newmark 1999; Newmark *et al.* 2003), and to functionally compare its developmental and regeneration processes against each other (Sánchez Alvarado 2003a). Moreover, the sequencing of the *S. mediterranea* genome should be completed in about a year (Sánchez Alvarado *et al.* 2003), opening the door to comparative genomics and bioinformatic approaches (systems analyses) aimed at informing the biology of regeneration in this metazoan.

The relative simplicity of *S. mediterranea*, combined with the molecular and genomic tools available and under development should make the problem of regeneration much more amenable to reductionistic and systems analysis approaches. Reductionistic studies will help identify the minimal components or stable subassemblies that drive regeneration in the bilaterians, whereas systems analysis will aid in the elucidation of the collective properties arising from the individual components and their interactions. In addition, the remarkable biological attributes of *S. mediterranea* (Newmark & Sánchez Alvarado 2002) provide novel contexts in which to study molecular pathways and define mechanisms for their specificity. For instance, a key component of planarian biology are the perpetuation, maintenance and regulation of a population of somatic stem cells known as neoblasts. Their division progeny are responsible for replacing cells lost to continuous tissue turnover and for the regeneration of body parts lost to injury and/or amputation (Newmark & Sánchez Alvarado 2000). This planarian attribute is a stable solution to the dauntingly complex task of interpreting local and global signals so as to maintain the form and functions of the organism. Rather than becoming malnourished, a starved planarian simply shrinks in size, a reversible phenomenon known in the literature as degrowth (Newmark & Sánchez Alvarado 2002). Such degrowth occurs by the loss of cells rather than by a reduction of cell size (Oviedo *et al.* 2003). Remarkably, the degrowing planarian never loses its form or functions and are, in fact, indistinguishable anatomically or behaviourally from their well-fed cohorts.

How can a population of cells embedded in a noisy environment detect, parse, amplify and transform the signals for growth, degrowth and regeneration into a coordinated response? This can only be accomplished through the operation and integration of multiple regulatory mechanisms (Hartwell *et al.* 1999). To distinguish signal from background, amplification strategies must exist along with means for coincidence detection, especially when the system is exposed to multiple stimuli (metabolic state and injury, for example). In addition, simultaneous activation of multiple pathways requires adaptation and insulation mechanisms as the various stimuli may differ from each other by orders of magnitude (prolonged starvation versus a small injury, for instance), and the unequal activities of the involved pathways may have undesired effects on each

other. The molecular nature of these various mechanisms in planarians is unknown. However, studying the population dynamics of neoblasts as these cells are exposed to a multiplicity of factors should help us uncover a wide variety of molecular pathways and the modulators governing their collective interactions. But more importantly, the experimental accessibility of neoblasts and their totipotentiality may provide us with a platform from which to glean and perhaps study some of the general organizing principles guiding modular assembly in multicellular organisms.

4. CONCLUSION

In the past few years, experimental evidence has been accumulating from a variety of organisms suggesting that a finite number of widely conserved genetic pathways are responsible for the determination and patterning of the vast majority of metazoan body plans (Carroll *et al.* 2001). The extensive phylogenetic distribution of these pathways or molecular modules argues for their ancient evolutionary origins and implies that they are simultaneously stable and evolvable. The multi-component interdependence of biological functions, however, makes uncovering the general principles guiding biological organization a daunting task. Hence, the dynamics of how these molecular subassemblies came together from their individual parts is not entirely clear. At the evolutionary level such organizing principles must be involved in the regulation of the processes guiding the appearance of novel attributes in the Metazoa; whereas at the organismal level, these principles are manifested in the formation of a multicellular organism from a single cell (development), and in the maintenance of the body plan after birth (tissue homeostasis and regeneration).

Consequently, even if a list of pathways involved in regenerative capacities were to become available today, those of us working on regeneration will be confronting the same problem currently being faced by many other developmental biologists. The problem, in a nutshell, is that the identification of a finite, widely distributed number of developmental pathways, along with the cataloguing and functional characterization of their pleiotropic activities, does not explain how a limited number of subassemblies can yield the fantastic array of multicellular diversity and biological functions found in the extant metazoans. This is partly due to the fact that biological function arises from the interaction of multiple mechanisms, and thus cannot be easily reduced to, nor fully explained by its individual components. Therefore, to truly understand biological function it becomes necessary to explain how the interactions of many different and independent components can establish meaningful relationships to produce the precise and reproducible biological activities required for the survival and perpetuation of living things. Although crucial, knowledge of the signalling and genetic pathways activated during any given biological activity is, unfortunately, insufficient to explain the biological activity itself.

Nevertheless, by studying the function of genetic pathways and their relationships in a variety of contexts, it should be possible to begin to identify the molecular strategies by which their pleiotropic behaviour is channelled to produce different and specific activities. Regenerating

organisms such as the planarian *S. mediterranea* provide both unexplored contexts to study widely conserved pathways, and the experimental means by which to perturb and study these molecular modules. Whether or not the study of planarians will uncover novel genetic pathways is an open question. What is clear, however, is that a diverse number of local activators, repressors and/or enhancers, and mechanisms adapting the known, widely conserved genetic pathways to specific biological functions should be uncovered. It is in these local effectors that evolutionary constraints may have had its strongest influence in yielding the uneven distribution of regenerative events in the Metazoa (Sánchez Alvarado 2000; Brockes *et al.* 2001). As such, the potential exists for the study of *S. mediterranea* to expose unsuspected properties for developmentally regulated pathways and their individual components, as well as to reveal the nature of some of the determinants responsible for the functional specificity of their output. By providing a more complete picture of the molecular activities governing biological function, the study of regeneration is bound to bring us closer to understanding the general principles guiding the organization and order of living organisms.

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GLOSSARY

- DRE: DNA response elements
 FGF: fibroblast growth factor
 Hh: Hedgehog
 Orfeome: the full complement of open reading frames found in the genome of a given organism
 Wnt: Wingless