Cell

Planarian Regeneration: Its End Is Its Beginning

Alejandro Sánchez Alvarado[1,*]
[1]Howard Hughes Medical Institute, Department of Neurobiology & Anatomy, University of Utah School of Medicine, Salt Lake City, UT 84103, USA
*Contact: sanchez@neuro.utah.edu
DOI 10.1016/j.cell.2006.01.012

Why does regeneration take place in some animals but not others? Increased understanding of gene function is required to dissect the genetics, cell biology, and physiological aspects that make regeneration possible. An unlikely model animal, the planarian *Schmidtea mediterranea*, is proving valuable in this endeavor.

In 1901, Thomas Hunt Morgan wrote: "The fact that the process of regeneration is useful to the organism cannot be made to account for its existence in the organism" (Morgan, 1901). Although such words may strike us today as being counter to the general concepts of natural selection, Morgan reached this conclusion after an exhaustive study of regeneration in both plants and animals (Morgan, 1901). Morgan did, in fact, have a point. Take for example the nemertine worm *Lineus ruber* and its close relative *Lineus viridis*, species that are almost identical in morphological attributes and share similar if not identical environmental niches. Few would disagree that natural selection has played a key role in producing both extant forms. Harder to explain, however, is the fact that these two species, often found living in the same estuary, respond very differently to amputation. Amputate a part of *L. ruber* and the missing part regenerates; amputate a part of *L. viridis* and no regeneration is observed (Brockes et al., 2001). In fact, multiple examples exist describing such intraphyletic variability (Needham, 1952), as well as the most common and equally puzzling distribution of regenerative properties among the different animal phyla (Sánchez Alvarado, 2000). Thus, looming large on the horizon is the following question: Why can some animals regenerate missing body parts and others cannot? To date, no satisfactory answer to this question exists. We are at odds in explaining regeneration as an evolutionary variable (Brockes et al., 2001) and, moreover, lack sufficient molecular evidence to either support or debunk the notion that regeneration may be a primordial metazoan attribute lost to some species for reasons that are unknown. In an effort to mechanistically address these issues, we and others have begun a systematic genetic and cellular exploration of the problem of regeneration in animals. In this essay, I aim to provide an account of these ongoing efforts and to provide an argument for the underappreciated advantage of regeneration studies to inform our understanding of fundamental aspects of animal biology, including our own. A largely ignored animal model of regeneration that is making a comeback is the planarian, a simple platyhelminth worm (see Figure 1).

"...wide was the wound,
But suddenly with flesh filled up and healed."

—John Milton, *Paradise Lost, VIII 467*

Why Study Regeneration?

Besides the obvious, important practical ramifications of improving human health, the study of regeneration also provides fertile and largely unexplored grounds for boosting our understanding of the basic molecular and cellular processes governing biological function. Unfortunately, the numerous superficial similarities that exist between regeneration and embryogenesis have engendered the misleading view that regeneration is merely a recapitulation of embryonic events, that is, a redundant problem of development that will eventually be resolved by studying the embryo. Like embryogenesis, regeneration does involve the self-assembly of new tissues. Yet, very much unlike embryogenesis, regeneration also entails the anatomical and functional integration of newly made parts into older pre-existing tissues. Consequently, many regenerated organs and organ systems are out of proportion with the body size of the animal, resulting in asymmetries that must be corrected in order for the organism to regain its proper proportion and function. Moreover, not all animals can replace structures lost to damage or amputation, even though all organisms share a finite pleiotropic set of developmental pathways (Carroll et al., 2001). If regeneration merely recycles such pathways in the adult form, why can some animals regenerate whereas others cannot? Mechanisms that sense perturbations of homeostasis must exist that are capa-

ble of reactivating and regulating the pleiotropic activities of developmental pathways in order to achieve the specificity required to restore only the missing body parts and to re-establish homeostatic balance. Therefore, multiple uncharacterized (and perhaps unpredicted) postembryonic regulators of development must exist, and their identification and characterization will largely depend on the availability of varied and experimentally accessible biological contexts. Because regeneration exposes developmental pathways to conditions not found during embryogenesis, its study may uncover mechanisms by which pathway specificity is regulated. Irrespective of what light the future may throw on deciphering the molecular nature of regeneration, it is clear that its study will also provide mechanistic insights into many fundamental and unresolved aspects of metazoan biology.

Why Planarians?

Almost nine years ago, I followed two basic criteria for selecting a suitable model system with which to study regeneration. First, the animal should be one of the simplest metazoans in which regeneration is patently manifested. Second, the organism should be relatively easy to manipulate experimentally. Of the several animals considered, planarians fulfilled these criteria best, as they are one of the simplest bilaterians known to display robust regenerative capacities. Indeed, there exists more than 100 years of scientific literature reporting experimentation with planarians (Reddien and Sánchez Alvarado, 2004). Planarians are best known for their capacity to regenerate complete individuals from minuscule body parts. Such extraordinary tissue plasticity finds its source in a population of adult somatic stem cells called neoblasts that are distributed throughout the planarian body. Neoblasts are the only mitotically active cells in planarians (Newmark and Sánchez Alvarado, 2000), and their division progeny generate the roughly 40 different cell types found in the adult organism. In intact planarians, neoblasts replace cells lost to normal physiological turnover while giving rise in amputated animals to the regeneration blastema, the structure in which missing tissues are regenerated. The pronounced limitations of somatic tissue turnover and regenerative properties in current invertebrate models, coupled with the difficulty of studying vertebrate somatic stem cells in vivo, are compelling reasons to examine and test the suitability of planarians to inform both regeneration and stem cell biology.

Why *Schmidtea mediterranea*?

There are thousands of different planarian species, but only several dozen have been characterized in some detail. Of these, the free-living freshwater hermaphrodite *Schmidtea mediterranea* has emerged as a suitable model system because it displays robust regenerative properties and, unlike most other planarians, it is a stable diploid (2n = 8) with a genome size of $\sim 4.8 \times 10^8$ basepairs (nearly half that of other common planarians). Moreover, a Robertsonian translocation (that is, the fusion of a whole arm of chromosome 1 to chromosome 3) has produced an exclusively asexual biotype (Newmark and Sánchez Alvarado, 2002). Both sexual and asexual forms have proven easy to rear in the laboratory. By serially amputating individual worms and allowing the fragments to regenerate, single animals have been expanded into clonal colonies of thousands of individuals (see Figure 1). We have succeeded in breeding clonal lines of the sexual strain that produce fertile progeny, effectively overcoming previous limitations in the sexual propagation of planarians in captivity. Thus, clonal inbred lines now can be generated for genomic and genetic analyses, and for a detailed molecular and morphological characterization of the embryogenesis of freshwater planarians, neglected since 1916 (Sánchez Alvarado, 2003). As alluded to earlier, the belief that embryogenesis and regeneration can be likened to each other has persisted in the absence of direct testing. Access to thousands of *S. mediterranea* embryos, coupled to the regenerative capacities of this species, will enable systematic comparative and functional studies of embryogenesis and regeneration, a task essential for understanding their true relationship.

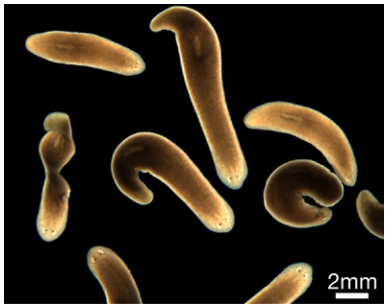

Figure 1. A Raid of Planarians
Depicted are individuals of the clonal line CIW4 of the planarian *Schmidtea mediterranea*, established in 1999 at the Carnegie Institution of Washington, Department of Embryology by Dr. Philip A. Newmark during his postdoctoral training. This line has allowed the standardization of regeneration studies in planarians. Scale bar is 2 mm.

Tools for Studying Regeneration in *S. mediterranea*

To study *S. mediterranea*, there needs to be a way to measure regenerative processes, catalog genes, visualize their expression, and interfere with their function. We have developed methods to label planarian neoblasts in order to assay cellular activities during regeneration (Newmark and Sánchez Alvarado, 2000) and fluorescent immunohistochemistry protocols (Sánchez Alvarado and Newmark, 1999). In addition, we have generated large cDNA collections and adapted whole-mount in situ hybridization methods (Sánchez Alvarado et al., 2002), and we have introduced RNA interference (RNAi) methods to disrupt gene function in planarians (Sánchez Alvarado and Newmark, 1999). Our group has characterized over 5000 nonredundant cDNA sequences (Sánchez Alvarado et al., 2002), all of which have been printed on microarrays. We have also created a publicly accessible database (SmedDb) where sequence, GenBank, PubMed, in situ, and RNAi data can be consulted (Sánchez Alvarado et al., 2002). Our analyses of the sequence data have already begun to complement current efforts

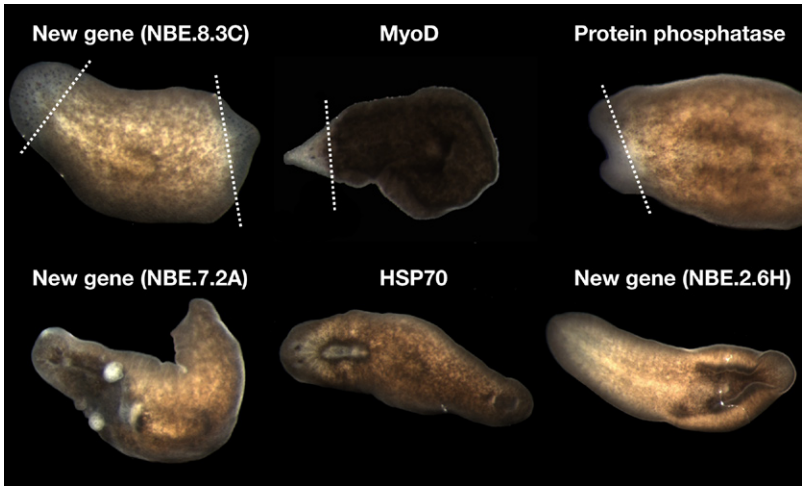

Figure 2. RNAi Phenotypes
Regeneration (top row) and homeostasis (bottom row) defects can be robustly and reproducibly obtained by RNA-mediated genetic interference in *S. mediterranea*. The first genetic screen for regeneration in planarians was spearheaded by Dr. Peter Reddien while a postdoctoral fellow and was carried out by feeding these organisms with bacteria engineered to produce dsRNA against *S. mediterranea* cDNAs (Reddien et al., 2005a). Such studies have uncovered genes with functions associated with the multiple morphogenetic events underpinning regeneration and the maintenance of differentiated tissues. (In all panels, anterior is to the left; gene names are shown). More information can be found at http://planaria.neuro.utah.edu. White dashed lines indicate approximate location of amputation planes.

to understand the evolution of genes, gene pathways, and biological processes (Cebria et al., 2002; Sánchez Alvarado et al., 2002). For example, the identification of genes present in the human genome, but absent from the genomes of the fruit fly *Drosophila* and the nematode *Caenorhabditis elegans*, has led to the proposal that such genes arose as a result of direct horizontal gene transfer between bacteria and vertebrates (Lander et al., 2001). However, our identification of *S. mediterranea* homologs of several of these genes (Sánchez Alvarado et al., 2002) suggests that these loci are not shared by bacteria and vertebrates through horizontal gene transfer but rather by descent through common ancestry (Stanhope et al., 2001). This illustrates how even a limited number of *S. mediterranea* DNA sequences can deepen our understanding of the evolution of human genes.

By introducing dsRNA technology into planarians (Sánchez Alvarado and Newmark, 1999), we have been able to transform *S. mediterranea* into a regeneration model system in which gene function can be analyzed. In contrast to *C. elegans*, the adult planarian nervous system is not refractory to RNAi, allowing the functions of neuron-specific genes to be tested. In collaboration with Kiyokazu Agata, we have studied the function of the *nou-darake* (*ndk*) gene (Cebria et al., 2002), the planarian homolog of an uncharacterized human FGF receptor-like gene not found in the genomes of *C. elegans* or *Drosophila*. By silencing *ndk* with RNAi, the product of this gene was shown to block the FGF signaling pathway of both planarians and vertebrates (Cebria et al., 2002). These findings provide proof-of-principle that planarians are a valuable model for yielding insights into aspects of human biology that are not readily accessible for study in current invertebrate genetic model systems.

An RNAi Genetic Screen for Regeneration

More recently, we have carried out an RNAi-based loss-of-function screen to begin a systematic exploration of the molecular mechanisms underpinning regeneration (Reddien et al., 2005a). One thousand and sixty five different genes intended to be a representative sampling of the *S. mediterranea* genome were selected for this screen. The appearance of defects was followed in both amputated and intact animals (see Figure 2). Of the genes studied, 240 were found to be required for diverse aspects of planarian biology, including regeneration, tissue homeostasis, and stem cell regulation. As defects in regeneration could arise indirectly from the perturbation of general cellular functions, the resolution of these phenotypic analyses was increased to identify genes specifically involved in regeneration. First, there was selection of all the genes that when perturbed blocked, limited, or reduced regeneration. Second, the mitotic activity of neoblasts in each of these gene perturbations was quantified to determine if the regeneration defects observed were caused by the misregulation of neoblast proliferation. Third, tissue homeostasis was analyzed in unamputated animals in which the genes important for regeneration were perturbed by RNAi to distinguish between gene functions shared by both regeneration and tissue homeostasis, and those specific to either of these two biological processes. This strategy identified genes involved in stem cell maintenance and proliferation, genes that affected only tissue homeostasis but had no effect on regeneration, and most importantly genes that affected only regeneration but not neoblast proliferation or tissue homeostasis (Reddien et al., 2005a). Among the collection of genes identified that affect regeneration specifically, one encodes a candidate wound-healing factor (RNAi of this gene causes cell lysis shortly after amputation). Other regeneration candidate genes are predicted to encode proteins similar to FKBP-like immunophilin, chondrosarcoma-associated protein 2, nucleostemin (a neurogenic stem cell marker in mammals), two DEAD box RNA binding proteins, SMAD4, Baf53a (a topoisomerase), and a WW-domain protein. This set of genes may represent previously unrecognized signaling mechanisms that specifically activate stem cells to mount a regenerative response following wounding. As such, the identified molecules may provide key entry points for unraveling

molecular events that may make preexisting tissues competent to restore missing body parts after wounding or amputation. Screens of this type in *S. mediterranea* clearly delineate a strategy for gaining detailed mechanistic insight into how the genome controls planarian physiology, including regeneration and the regulation of stem cells in vivo.

A recent example of how planarians can inform our understanding of regeneration and stem cell biology is provided by a study of the *piwi*-like gene *smedwi-2* in *S. mediterranea* (Reddien et al., 2005b). PIWI-like proteins are known to be functionally associated with stem cell activities, yet little is known about how PIWI proteins regulate stem cells. RNAi of *smedwi-2* blocks regeneration, even though neoblasts are present and capable of proliferating in response to wounding. Moreover, in unamputated animals, the division progeny of neoblasts in which *smedwi-2* is blocked by RNAi migrate to sites of cell turnover but, unlike wild-type cells, fail to replace aged tissue (Reddien et al., 2005b). This study suggests a new role for PIWI proteins in the generation of stem cell progeny that are able to promote both regeneration and tissue homeostasis.

Solving the Problem of Regeneration

Why does regeneration happen? What are the factors that determine the extent and varied manifestations of this metazoan attribute? Answering these questions necessitates introducing studies of gene function to begin the process of dissecting the genetics, cell biology, and physiological aspects that make regeneration possible. Using *S. mediterranea* as a model system overcomes a number of important experimental limitations in the study of regeneration. For example, an effort has been made to genetically dissect regeneration in the adult zebrafish (Nechiporuk et al., 2003). However, to avoid potential embryonic lethality that could mask the identification of postembryonic gene function, it was necessary to devise a temperature-sensitive screen in this organism. Like all conditional screens, such an approach cannot saturate the zebrafish genome and thus is limited in its scope (Nechiporuk et al., 2003). In contrast, RNAi screens can be carried out and analyzed in the adult *S. mediterranea*, bypassing the need for recovering conditional mutations. By identifying genes and genetic activities associated with regeneration and tissue homeostasis, the recently completed RNAi-based screen of *S. mediterranea* proved the practicality and effectiveness of this approach. *S. mediterranea* is a model system of regeneration in which it is possible to saturate the genome with loss-of-function phenotypes enabling the elucidation of genomic activities associated with regenerative properties. Larger and more cell/tissue-specific RNAi screens to cover a wider representation of the planarian genome are likely to yield further insights into regeneration and new ways to investigate metazoan gene function.

How will we then test the universality of discoveries made in *S. mediterranea*? First, it will be important to determine how widely the components and regulators of *S. mediterranea* regeneration pathways are distributed among the Metazoa. Conversely, it will also be important to determine how well the key developmental pathways characterized in *Drosophila*, *C. elegans*, and vertebrates (Carroll et al., 2001) are represented in *S. mediterranea*. The ongoing *S. mediterranea* genome sequencing project has begun to resolve some of these issues. So far, we have identified, cloned, and begun studies on the roles that orthologs of *hedgehog*, *patched*, *delta*, *serrate*, *notch*, six different *wnt*s, and three TGF-βs may play in regulating regeneration in *S. mediterranea*. Interestingly, we have also found an ortholog of *bambi*, a pseudo-receptor absent in *Drosophila* and *C. elegans* that is known to regulate the TGF-β pathway of vertebrates (Onichtchouk et al., 1999). The fact that modulators of the FGF (*nou-darake*) and TGF-β pathways (*bambi*) are shared by planarians and vertebrates, but seemingly missing from flies and nematodes, indicates that insights gained in *S. mediterranea* will not only translate to other organisms but also will inform human biology in ways that *Drosophila* and *C. elegans* cannot.

Finally, not all tools are yet in place to fully exploit the remarkable biology of planarians. The repertoire of genetic tools for *S. mediterranea* needs to be broadened to include the introduction of permanent modifications to the genome. For example, gain-of-function assays that take advantage of homologous and nonhomologous end-joining recombination will become important tools to study the transcriptional regulation of genes and to tag genomic output with reporter molecules to aid in the in vivo visualization of cellular activities. These and other methods will greatly facilitate studies of cell biology, a mostly uncharted aspect of planarians that is key to understanding regenerative processes. By studying gene function in a context that allows the in vivo visualization of cellular activities (such as cell migration, cell division), the power of RNAi-based screens in *S. mediterranea* will continue to expand. Not only will it expedite the discovery of genetic activities required for the formation and patterning of planarian cells, tissues, and organs, but it will also help in detailing the complexities and population dynamics of neoblasts in vivo. The planarian *S. mediterranea*, therefore, is poised to help fill voids in our understanding of how stem cells originate and are maintained and regulated during embryogenesis, tissue homeostasis, and regeneration. By extension, such knowledge should, in due course, provide the necessary mechanistic insight to help solve the problem of regeneration in animals and perhaps reveal its evolutionary and organismal significance.

ACKNOWLEDGMENTS

I thank Carl Thummel, David Grunwald, Janet Lindsley, Thomas N. Parks, and Tatjana Piotrowski for their thoughtful comments.

REFERENCES

Brockes, J.P., Kumar, A., and Velloso, C.P. (2001). J. Anat. *199*, 3–11.

Carroll, S.B., Grenier, J.K., and Weatherbee, S.D. (2001). From DNA to Diversity: Molecular Genetics and the Evolution of Animal

Design (Malden, Massachusetts: Blackwell Science).

Cebria, F., Kobayashi, C., Umesono, Y., Nakazawa, M., Mineta, K., Ikeo, K., Gojobori, T., Itoh, M., Taira, M., Sanchez Alvarado, A., and Agata, K. (2002). Nature *419*, 620–624.

Lander, E.S., Linton, L.M., Birren, B., Nusbaum, C., Zody, M.C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W., et al. (2001). Nature *409*, 860–921.

Morgan, T.H. (1901). Regeneration (New York: The Macmillan Company).

Nechiporuk, A., Poss, K.D., Johnson, S.L., and Keating, M.T. (2003). Dev. Biol. *258*, 291–306.

Needham, A.E. (1952). Regeneration and Wound-Healing (New York: John Wiley & Sons, Inc.).

Newmark, P.A., and Sánchez Alvarado, A. (2000). Dev. Biol. *220*, 142–153.

Newmark, P.A., and Sánchez Alvarado, A. (2002). Nat. Rev. Genet. *3*, 210–219.

Onichtchouk, D., Chen, Y.G., Dosch, R., Gawantka, V., Delius, H., Massague, J., and Niehrs, C. (1999). Nature *401*, 480–485.

Reddien, P.W., and Sánchez Alvarado, A. (2004). Annu. Rev. Cell Dev. Biol. *20*, 725–757.

Reddien, P.W., Bermange, A.L., Murfitt, K.J., Jennings, J.R., and Sánchez Alvarado, A. (2005a). Dev. Cell *8*, 635–649.

Reddien, P.W., Oviedo, N.J., Jennings, J.R., Jenkin, J.C., and Sánchez Alvarado, A. (2005b). Science *310*, 1327–1330.

Sánchez Alvarado, A. (2000). Bioessays *22*, 578–590.

Sánchez Alvarado, A. (2003). Curr. Opin. Genet. Dev. *13*, 438–444.

Sánchez Alvarado, A., and Newmark, P.A. (1999). Proc. Natl. Acad. Sci. USA *96*, 5049–5054.

Sánchez Alvarado, A., Newmark, P.A., Robb, S.M., and Juste, R. (2002). Development *129*, 5659–5665.

Stanhope, M.J., Lupas, A., Italia, M.J., Koretke, K.K., Volker, C., and Brown, J.R. (2001). Nature *411*, 940–944.