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Stem cells in animal models of regeneration

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ABSTRACT: Discovered centuries ago, regeneration is a fascinating biological phenomenon that continues to intrigue. The study of regeneration promises to inform how adult tissues heal and rebuild themselves such that this process may someday be stimulated in a clinical setting. Although mammals are limited in their ability to regenerate, closely and distantly related species alike can perform astonishing regenerative feats. Many different animals representing almost all phyla harness an innate ability to rebuild missing adult structures lost to injury. However, it is unclear which aspects of regeneration are conserved and which are unique to a given context. One aspect of regeneration that appears to be shared is the use of stem/progenitor cells to replace missing tissues. In this chapter, we review what is known about the natural role of stem cells during animal regeneration. While many animals regenerate, we limit our discussion to amphibians, zebrafish, and planarians, well-studied bilateral organisms that invoke cell proliferation in response to injury. With the exception of planarians, the cellular source of regeneration remains mysterious. Are stem cells that rebuild missing tissues present prior to injury, or are they generated during the injury response? If they are generated *de novo*, where do they come from? Can regenerative stem cells give rise to all of the missing cell types, or are multiple lineage-restricted stem cells required? Current studies in the field of animal regeneration are intensely focused on answering these central questions.

Introduction

Regeneration is arguably among the most awe inspiring biological phenomena known to exist. The history of the Western canon is populated by many examples of the indiscriminate, powerful grip regeneration has exerted on the human mind. For instance, when Lazzaro Spallanzani reported in 1768 that decapitated snails regenerate their heads, scientists, philosophers and the public alike scoured their gardens in an attempt to replicate this fascinating experiment (Odelberg, 2004). It was also discovered that salamanders can regenerate limbs and tails (including the spinal cord), while planarians can regenerate entire animals from small body fragments.

Despite the longstanding interest in this biological problem, and the knowledge that animals from all walks of life perform regenerative feats, we are still in the early stages of describing these events in cellular, molecular, and mechanistic terms. However, the genetic and molecular tools to address the problem of regeneration are rapidly improving. Aside from the curiosity it normally elicits, the study and understanding of regeneration could dramatically impact the practice of medicine.

Just as relevant is the understanding derived from the investigation of **stem cells**, undifferentiated cells that have the capacity to replace themselves indefinitely and to produce specialized cell types. While embryonic stem cells divide and ultimately give rise to all the differentiated cell types of the body, adult stem cells from specific tissues are normally lineage restricted to a specific set of cell types (Anderson *et al*., 2001; Morrison, 2001; Wagers *et al*., 2002). In order for an adult animal to replace missing structures with an exact copy of what is missing, it is clear that developmental programs must be redeployed. However, the dynamics of cell communication and proliferation are vastly different, as are the cell types involved. To accomplish regeneration, adult animals may invoke the proliferation of differentiated cells, the activation of reserve stem cells, the formation of new stem cells with limited capacity to self renew (**progenitor** cells), or a combination of these strategies.

Which cells in an adult animal divide and differentiate to replace the multiple cell types required during a regenerative response? While this is a very basic, indeed, fundamental question that has been formulated and reformulated through successive generations of biologists, its resiliency against experimental attacks has proved surprising, and in many cases quite frustrating. Nonetheless, it is apparent that different tissues (both within the same organism as well as the same tissues from different organisms) use different strategies to achieve tissue repair or regeneration. For example, the vertebrate liver invokes compensatory regeneration after the removal of two lobes, whereby the remaining lobe proliferates to reacquire the original tissue mass without replacing the missing lobes. In fact, regeneration can be compensatory (liver), tissue-specific (heart, skeletal muscle, liver, pancreas, lens, retina), or it can rebuild complex structures containing multiple tissue and organ types (e.g., limbs, fins, tails).

The goal of researchers studying model organisms of regeneration is to discover how these animals naturally accomplish the seemingly impossible task of restoring body parts lost to trauma. This chapter will focus on what we have learned about stem/progenitor cell identity and function from the most common, non-mammalian model organisms that invoke cell proliferation to drive regeneration. Although we will limit our discussion to bilaterian animals, we predict that significant overlap in regeneration mechanisms and concepts will emerge from the simultaneous study of regeneration and stem cells in pre-bilaterian animals such as hydra. These particular aspects of hydra stem cells and regeneration, have been exhausitively reviewed elsewhere (Bosch, 2007; Bosch, 2007; Galliot *et al*., 2006). Still, a set of common, fundamental questions remain unanswered for all of animal regenerartion: Where do regenerative stem/progenitor cells come from and what can they do? Given that no single animal is a model for all biological contexts, it is essential to study the multiple ways nature has solved the problem of regeneration. Therefore, the best way forward is to integrate the information derived from multiple model systems of regeneration as they are subjected to genetic, cellular, and molecular interrogation.

Regeneration in Amphibians: Urodeles (salamanders, newts, axolotls)

Background

Among the vertebrates, urodele amphibians are unmatched in their regenerative capacities. When injured, these animals regenerate an impressive array of body parts, including the upper and lower jaw, lens, retina, limb, tail, spinal cord, and intestine (Brockes and Kumar, 2005). In some cases, the restoration of complex anatomy involves the formation of a **blastema**, a mass of morphologically undifferentiated, proliferating progenitor cells that is covered by epithelium and differentiates to replace the missing structures. Upon amputation, epithelial cells migrate to cover the wound, forming a **wound epithelium (WE**). The WE thickens via distant epithelial proliferation and continued migration. The thickened structure is called an **apical ectodermal cap (AEC)**, which is thought to be similar in function to the apical ectodermal ridge (AER) that forms in the limb bud during embryonic development. While histolysis and fragmentation occurs near the wound, undifferentiated cells accumulate to form a blastema via proliferation and migration from the stump tissue. This is followed by cell specification and patterning (Chalkley, 1954; Hay and Fischman, 1961).

But, where do blastema cells come from? Are all cells of the blastema undifferentiated and equally potent or are they restricted to become only the tissue type from whence they came? Could blastema cells have multiple, intermingling developmental origins and outcomes? Early histological studies provided evidence that cells remaining after injury "dedifferentiate" and migrate to form the blastema (Brockes and Kumar, 2002; Bryant *et al*., 2002; Chalkley, 1954; Hay and Fischman, 1961; Muneoka *et al*., 1986; Straube and Tanaka, 2006; Thornton, 1938). Among these early studies, elegant cell tracking experiments using tritiated thymidine showed that muscle, neural sheath, periosteum, and loose connective tissue cells up to 1 mm away from the amputation plane proliferate and migrate to give rise to the blastema. In contrast, epithelial or blood cells do not appear to contribute to the growing mass of mesenchymal tissue (Hay and Fischman, 1961).

The term “dedifferentiation” is often employed in the regeneration literature to describe the loss of differentiated characters of cells after amputation and their concurrent acquisition of an undifferentiated morphology during blastema formation. In Hay's classic paper, she describes the dedifferentiation process as “the release of living cells from the confines of their previous organization with accompanying active mitosis of these cells (Hay and Fischman, 1961).” However, “dedifferentiation” is easily misinterpreted to imply that a cell has attained a multi-potent undifferentiated state. Because there is currently insufficient evidence to either suggest or rule out a major role for reserve stem/progenitor cells in urodele blastema formation, the term dedifferentiation as originally coined may therefore refer to a reversal of the differentiated state, an activation of reserve stem/progenitor cells, or a combination of both (see Figure 1A; Morrison *et al*., 2006).

We emphasize that the true makeup of the urodele blastema remains unknown. The extent of multi-potentiality and/or heterogeneity is presently questionable because long-term cell lineage tracing has been extremely difficult to perform in these organisms. Although blastemal cells have lost both differentiated morphology and the expression of genes associated with their tissue-specific differentiated fate (*e.g.*, muscle myosin heavy chain), it is quite possible that these cells have entered a migratory or proliferative state without changing their respective tissue identity or potentiality. In addition, recent evidence suggests that a satellite-like cell population in urodele muscle contributes to the blastema and eventually to cartilage and epidermis (see Figure 1C; Morrison *et al*., 2006). Evidence of pre-existing progenitor or stem cell populations for other tissues has not been forthcoming, and thus needs to be adequately explored with modern techniques. For example, the largest known contributor to the urodele blastema is dermal tissue, including connective tissue fibroblasts (Bryant *et al*., 2002; Muneoka *et al*., 1986). Yet, very little is known about the heterogeneity or potentiality of this cell population on a cellular or molecular level (Bryant *et al*., 2002; Muneoka *et al*., 1986). The recent identification of a potential progenitor cell population in the regenerating zebrafish heart illustrates the targeted type of approach that may be necessary to detect elusive blastemal progenitor cells (see discussion below).

It is doubtful that the cultured A1 myotubes contain the satellite-like cells that are present *in vivo*. Therefore, whether or not *in vitro* generated myotubes represent *in vivo* myotube behavior needs to be fully determined. This is important because results from these cell lines have been interpreted to represent the urodele and mammalian condition. Because the cultured cells were serum starved to trigger myotube formation in the first place, an alternative interpretation of these experiments is that newt A1 cells are more flexible or “plastic” than mouse C2C12 cells. This may turn out to be the general case for urodele cells relative to mammalian cells, or it may be a feature unique to newt A1 cells, which may not be fully differentiated. In addition, while newt A1 cells do enter S phase after serum treatment, they do not go on to divide and the myotubes do not fragment (Straube and Tanaka, 2006).
Forced expression of the *Msx1* gene (a homeodomain protein with known repressor functions) in C2C12 myotubes causes a small fraction (5%) of cells to fragment into proliferating mononuclear cells (Odelberg *et al*., 2000). Under the proper culture conditions, these cells can differentiate into adipocytes (fat), chondrocytes (cartilage), myocytes (muscle), or osteocytes (bone). Because C2C12 cells are multipotent to begin with, these results should be treated with caution. Nevertheless, this was a key demonstration that mammalian myofibers can be induced to reverse their differentiated state. Complementary studies were also carried out in newts in which primary larval limb myofibers that normally fragment upon dissociation were inhibited from doing so via *Msx1* knockdown (Kumar *et al*., 2004). Given the caveats mentioned above for cultured cells, the data argue that *Msx1* may be necessary and sufficient for differentiated muscle to fragment into proliferating mononuclear cells. To the contrary, *in vivo* morpholino-mediated knockdown of *Msx1* in individual larval axolotl tail muscle cells had no negative effect on the ability of these cells to fragment (Schnapp and Tanaka, 2005). This discrepancy has several potential explanations, including but not limited to the differences likely to exist between *in vitro* and *in vivo* conditions, and that the muscle cells of the limb and tail could exhibit a differential requirement for *Msx1* expression during fragmentation.

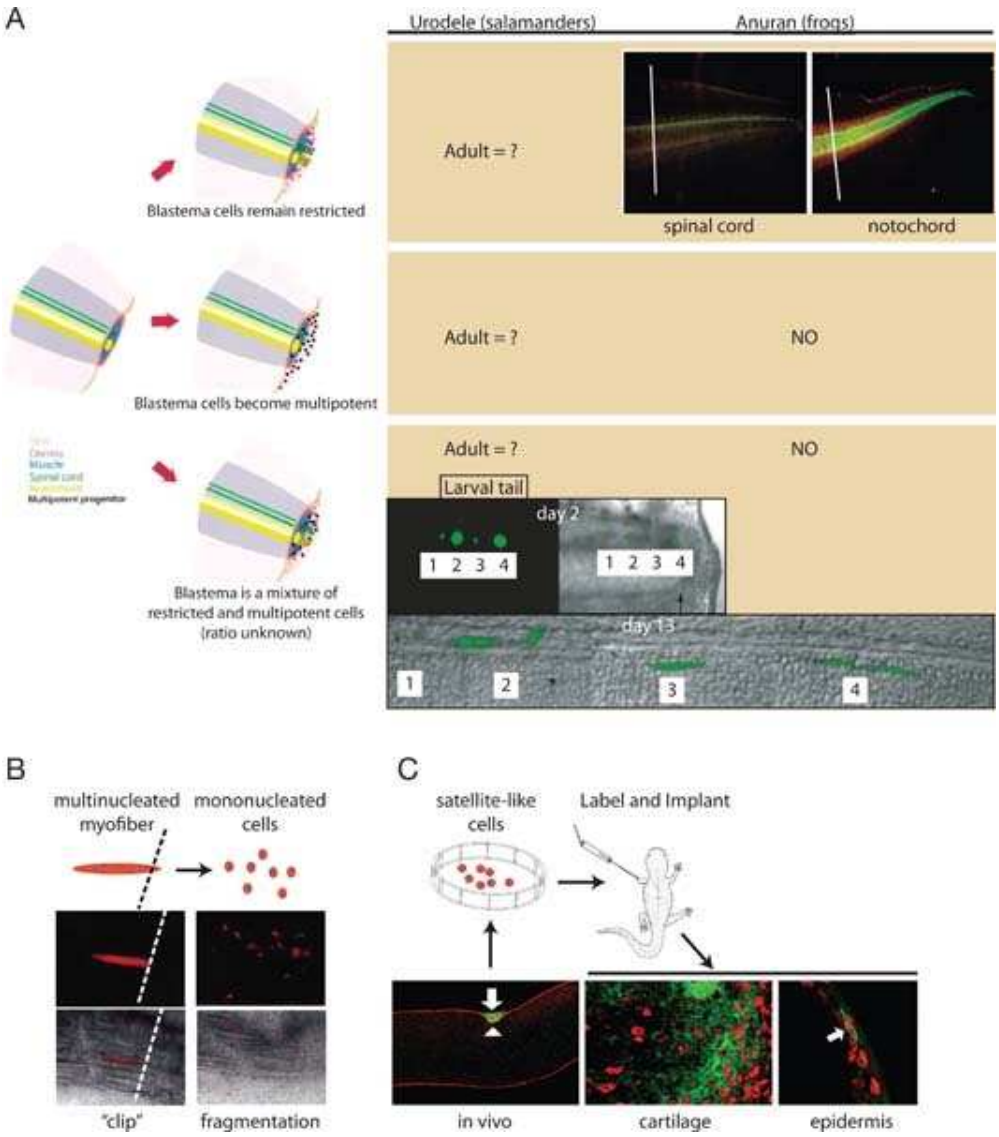

Figure 1A What is the differentiation potential of cells in the blastema?

The blastema may be composed of 1) cells that are restricted to give rise to the same tissues from which they were derived, 2) cells that are multipotent and give rise different tissue types, or 3) a complex mix of cells with a variety of origins and potentials. In the larval tail of urodeles, fluorescently tagged glial cells of the spinal cord proliferate during regeneration and can give rise to tissue outside of the spinal cord. Data currently indicates that at least in larval tissues, the urodele blastema contains a complex mix of lineage-restricted and multipotent cells. However, very little is known about the potential of blastema cells in adult urodeles. In anuran amphibians, GFP+ tissue grafts into GFP- hosts illustrate that cells of the regenerating spinal cord and notochord are derived from cells of those very same tissues. Therefore, the progenitor cells during anuran tadpole regeneration appear to be restricted in their potential. **B** Injection of dye into multinucleated muscle fibers prior to amputation illustrates that once clipped, some these fibers can fragment into proliferating mononuclear cells that contribute to the blastema. The long-term fate of these cells is still under active investigation. **C** (left) Muscle fibers in adult newts contain satellite-like cells that express Pax7 (green) and are separated from the rest of the cell by a basement membrane (red). (middle, right) When these cells are isolated, cultured, tagged (red) and implanted into regenerating newt limbs, they contribute to the blastema and give rise to unrelated tissue types including cartilage (green) and epidermis (green). Images are adapted from: (1) Echeverri, K., and Tanaka, E. M. (2002). Ectoderm to mesoderm lineage switching during axolotl tail regeneration. Science *298*, 1993–1996. Reprinted with permission from AAAS. (2) Gargioli, C., and Slack, J. M. (2004). Cell lineage tracing during Xenopus tail regeneration. Development *131*, 2669–2679. Reproduced with permission of the Company of Biologists. (3) Reprinted from Cell, 113, Tanaka, E. M., Regeneration: if they can do it, why can't we?, 559–562, 2003, with permission from Elsevier. (4) Echeverri, K., Clarke, J. D., and Tanaka, E. M. (2001). In vivo imaging indicates muscle fiber dedifferentiation is a major contributor to the regenerating tail blastema. Dev Biol *236*, 151–164. (5) Kumar *et al*., 2004, PLOS Biology, (6) © Morrison *et al*., 2006. Originally published in *The Journal of Cell Biology*. doi:10.1083/jcb.200509011.

To track dedifferentiation events, cultured cells can be labeled and implanted under the skin of regenerating limbs. *In vitro* differentiated myotubes, labeled with dye or viral insertion, remain stable in culture, but about 25% fragment upon implantation to generate proliferating mononuclear cells that contribute to the blastema (Kumar *et al*., 2000; Lo *et al*., 1993). A few cells were eventually observed to form cartilage cells, suggesting a change in cell fate, but this was an extremely rare event (Lo *et al*., 1993). On the other hand, reserve satellite-like cells also appear to contribute to the blastema (Morrison *et al*., 2006). Proliferating cells derived from satellite-like precursors were isolated in culture from adult newt myofiber explants. When tagged and implanted into regenerating adult limbs, these cells contributed to the blastema and many appeared to switch lineage into cartilage and even epidermal cells. To the contrary, another group found that implanted primary myofibers from juvenile axolotls can fragment and proliferate in the absence of satellite cells (Kumar *et al*., 2004). It is possible that these disparate results can be explained by the difference in species and life cycle stages (adult versus juvenile), or by the different criteria used to assess whether satellite cells were present. It also remains possible that both myofiber fragmentation and satellite cell proliferation contribute to the blastema *in vivo* and their relative contribution may be age and/or species dependent. A definitive explanation for these discrepancies will be important and most likely awaits *in vivo* cell tracking experiments.

In another set of implantation experiments, cells isolated from the newt heart (cardiomyocytes, CMs) were isolated, tagged, and implanted into un-amputated or amputated limbs (Laube *et al*., 2006). While CMs implanted into un-amputated limbs were stable and exhibited no special behavior, they were activated when implanted into day 5 regenerating limb blastemas and 65% gave rise to skeletal myotubes while a few expressed a cartilage cell marker. This is clear evidence of the plasticity of the differentiated state in adult urodele amphibians, but the magnitude of fate change is unclear because the transplanted CMs continued to express desmin, a marker found in many muscle cell types. While these experiments do not rule out a role for reserve stem/progenitor cells, they clearly illustrate that a large fraction of isolated newt CMs can at least switch muscle types.

***In vivo* studies**

The most convincing support for muscle fragmentation and cell fate switching during regeneration comes from *in vivo* cell tracking experiments. Unfortunately, technical constraints imposed by *in vivo* cell imaging have restricted these compelling investigations to larval axolotls that range from 2–5 cm in size from nose to tail. Axolotls are generally considered juveniles at around 5 cm in length and adults average roughly 23 cm long. This 5/10-fold difference in animal length between larvae and adults translates into a large difference of scale in both the limbs and tails for which regeneration programs must be deployed. In addition, it is currently unclear whether larval and adult animals utilize the same mechanisms, as formal evidence demonstrating the equivalence of these two biological contexts of regeneration is presently lacking. Nonetheless, the *in vivo* data provide strong evidence to support the notion that differentiated cells can in fact change their functional state. These data are represented by the following three key experiments.

Experiment (1): Multinucleated myofibers were injected *in vivo* with a cell tracking dye and monitored during regeneration (see Figure 1B; Echeverri *et al*., 2001). Amputations that removed 50% or more of the muscle cell led to degradation, while amputations that "clipped" the muscle cell caused it to fragment into proliferating mononuclear cells. Only 15/58 (~25%) clipped myofibers fragmented, which may indicate either an inefficient fragmentation process or point to a heterogeneous population of myofibers, some of which mononucleate more readily than others. On the other hand, since fragmentation was observed in a small number of animals, this may be a rare event that does not play a major role in blastema formation. The authors calculate that muscle fragmentation contributes to roughly 17% of the blastema. In contrast, earlier work using triploid/diploid transplants suggested that dermal tissues contribute to roughly 43% of the blastema (Muneoka *et al*., 1986).

Experiment (2): To track individual neural progenitor cells (glial cells) during tail regeneration, spinal cords were electroporated immediately after tail amputation to force expression of GFP under the control of a glial-specific promoter (see Figure 1A; Echeverri and Tanaka, 2002). While most of the cells gave rise to the expected neuronal and glial cell types, in 24% of the animals spinal cord cells migrated out of the regenerating spinal cord, contributed to the blastema, and gave rise to muscle. In 12% of animals glial cells gave rise to cartilage. These findings are significant because they clearly demonstrate that at least in the larval axolotl, neural progenitor cells of ectodermal origin can switch fate into mesodermally derived tissues during a regenerative response.

Experiment (3): A series of transplants and single cell electroporations were recently performed to trace the lineage of spinal cord cells during tail regeneration (McHedlishvili *et al*., 2007). Spinal cord tissue transplants from GFP(+) to GFP(-) animals showed that the cellular precursors used to regenerate the spinal cord are recruited from within 500 μm of the amputation plane. The cells close to the amputation give rise to distal spinal cord cells, while cells farther from the amputation give rise to proximal cells. Single cell GFP electroporations and embryonic GFP(+) neural plate transplants revealed that in most cases, cells retain their regional identity during regeneration such that dorsal and ventral cells each give rise to cells of the same respective position (McHedlishvili *et al*., 2007). However, in 8 of 21 electroporations and 3 of 5 transplants, cells changed dorso-ventral (DV) identities. In addition, a fraction of ventro-lateral cells near the terminal vesicle, a temporary structure that forms at the tip of the spinal cord during regeneration, migrated out of the spinal cord. These migratory cells apparently contributed to the tail blastema and gave rise to blood vessels, Schwann cells, and occasionally to muscle and cartilage cells. The results suggest that glial cells can serve as multipotential stem/progenitors during regeneration and that the terminal vesicle may represent an accumulation of de-dedifferentiated or reserve stem/progenitor cells. However, the exact nature of the transplanted cells remains in question and may include migratory neural crest cells, providing an intriguing line of questions for future investigation. These data add a layer of complexity to the regionalization and cellular make-up of the larval tail blastema and suggest that both lineage restricted and multipotent cells exist in the regenerating urodele spinal cord. Whether this is also true during adult urodele tail regeneration is unknown.

Unanswered questions

The future is promising, but a number of challenges lie ahead. The modern and classic urodele regeneration literature is derived from a mixture of regeneration paradigms (limb, tail, and spinal cord) and from a mixture of life stages (*e.g.*, adult newt, adult and larval axolotl), which vastly affects the scale on which regeneration takes place and the cell types that may be present prior to injury. While important progress has been made, the definitive source of regenerative cells in urodeles remains unknown and is most likely a complex mix of cell types and potentials. The current data suggest that the blastema may be composed of both de-differentiated and reserve stem/progenitor cells, but this has not yet been rigorously elucidated. Studies aimed at establishing fundamental differences and similarities between tail and limb regeneration need to be performed. These should include long-term cell lineage analyses during limb regeneration such that issues of cell potentiality and the relative contribution of cells to the final regenerated structures can be unambiguously resolved. In addition, studies that incorporate molecular dissections of the regionalization that occurs during proximal to distal patterning of the early limb blastema need to be expanded (Echeverri and Tanaka, 2005). Until cells are clearly marked *in vivo* and lineage traced, the nature of the limb blastema will remain enigmatic and controversial.

Likewise, much has been made of the ability of the multi-nucleated urodele myotubes to fragment and produce proliferating mono-nucleated cells that contribute to the blastema following injury (Brockes and Kumar, 2002; Brockes and Kumar, 2005; Straube and Tanaka, 2006; Tanaka, 2003). While the *in vitro* and *in vivo* data show that muscle fragmentation and proliferation occur, and that some cells switch identity during regeneration, it remains unclear as to what role this plays in the regeneration process. Because muscle only contributes to roughly 17% of the blastema, future research should focus on *in vivo* cell characterization/tracking of other cell types, including dermal cells, to determine their contribution to the blastema and to assess their differentiation potential. Moreover, a potential role (compensatory differentiation) for lineage switching can be deduced from early experiments showing that limbs devoid of bone regenerate normal limbs, including the missing bone (Weiss, 1925). This suggests that the blastema can regenerate structures absent from damaged pre-existing tissues. These experiments clearly need to be revisited with modern cell tracking techniques (Carlson, 2003).

The *in vivo* and *in vitro* data alike suggest that some differentiated cells of urodele amphibians display plasticity, as they are able to convert from one lineage to another. The question remains as to how differentiated these cells are to begin with. One potential approach to assess the extent of the differentiated state is to observe the epigenetic state of the genome. Myotubes induced from newt A1 and mouse C2C12 cells appear differentiated and no longer respond to growth factors, but the epigenetic state of the genome is completely unknown for these cells. What is the state of genome methylation and other epigenetic markers of differentiation in newt A1 and mouse C2C12 cells? This same question about epigenetics can be asked of adult *vs.* larval urodele amphibians and should be asked in all regeneration contexts. Is there a difference in the epigenetic differentiated state that can account for the flexibility of cells in their response to injury? What are the epigenetic differences between the adult and larval axolotls? In fact, it remains possible that data from larval cells and tissues may not apply to the adult context. Differences are bound to exist between the limbs of a 3cm-long larval axolotl and a 25cm-long adult. For example, de-differentiation in the adult newt limb does not begin until about day 4 or 5 while the limb blastema of a larval axolotl is already

subdivided into at least 3 proximal-distal zones by day 3 post-amputation (Echeverri and Tanaka, 2005; Odelberg, 2005). Because most if not all of the *in vivo* experiments described above were performed in larval animals, repeating these experiments in adult newt and axolotl limbs and/or tails will be extremely informative.

Regeneration in Amphibians: Anurans (frogs, toads)

Background

The frog has been a mainstay in the field of developmental biology for many years. As a research model, this animal has provided researchers with key insights into how animals coordinate the progression from a single fertilized egg into billions of organized, communicating cells that function in the complex tissues and organ systems of an entire animal. To study frog development, important tools such as transgenic overexpression, were developed to allow a detailed molecular interrogation of frog biology. These tools are now proving useful for the study of frog regeneration.

Anuran amphibians can regenerate limbs and tails as tadpoles. This regenerative ability rapidly declines during differentiation and metamorphosis at stage 52, such that by stage 56 differentiated cell types and ossified bones are present and the regenerative response has diminished. This illustrates a common theme of correlation between cell plasticity and regenerative ability. Tadpoles can only regenerate complex structures while they are going through a period of large-scale morphological change, including limb development and tail regression during metamorphosis. This suggests that regeneration in anuran amphibians may depend upon the presence of undifferentiated cells, which are no longer present once differentiation has set in. Does anuran "regeneration" represent the ability to regenerate, or instead the ability to forge on with development following damage (Slack *et al*., 2004)? Whether anuran limb or tail regeneration can be considered equivalent to adult tissue regeneration remains an open question.

Tadpole tail and limb regeneration

Besides the loss of regeneration at stage 56, there is a refractory period in which the regenerative ability of the tail is lost between stages 45 and 47 (4–6 days of development), but is regained after stage 48 (Beck *et al*., 2003). Therefore, the anuran amphibian provides a model system that can be used to study the transition between the loss and gain of regenerative abilities. For example, the blockage of the BMP or Notch signaling pathways inhibits normal tail regeneration while overexpression rescues regeneration during the refractory period (Beck *et al*., 2003). *Msx1* is a direct target of BMP signaling and forced expression of a hyperactive form of *Msx1* is sufficient to allow tail regeneration during the refractory period. However, manipulation of these pathways did not allow regeneration of late stage tadpoles, again implicating the need for responsive cell types that are likely present prior to state 56, but absent thereafter.

Regeneration of the anuran amphibian tail proceeds through the formation of an undifferentiated blastema-like structure. However, recent studies using GFP(+) tissue transplants and Cre-Lox mediated cell tracking have shown that each tissue of the frog tadpole regenerates in an independent manner, giving rise to the same tissue during regeneration (muscle to muscle, notochord to notochord, neural plate to spinal cord, and melanophores to melanophores; see Figure 1A; Gargioli and Slack, 2004; Lin *et al*., 2007; Ryffel *et al*., 2003). Unlike urodele amphibians, there is currently no evidence to suggest that anuran amphibian cells cross lineage boundaries during regeneration, and unlike urodele muscle, anuran tail muscle clearly regenerates from a satellite stem cell population (Chen *et al*., 2006). Interestingly, overexpression of a constitutively active form of Notch (NICD) during the refractory period leads to the regeneration of tails with no muscle (Beck *et al*., 2003). Notch signaling is a key regulator of satellite cell fate in mammals and its role in satellite cells during tadpole tail regeneration remains to be determined (Conboy *et al*., 2003; Conboy and Rando, 2002). What coordinates the proliferation and differentiation of multiple tissue types, and why does regenerative ability decline despite the continued presence of satellite cells?

In anurans, as in urodeles, regeneration depends on the formation of a WE and an AEC. The formation of the WE and properly specified AEC is critically important and during periods of lost regenerative ability, amputation leads to the formation of a skin-like epithelium instead of a wound epithelium. WE and AEC specification are tightly coordinated with blastema formation using at least 4 signaling pathways. Disruption of BMP, Wnt, or Notch signaling causes the loss of regenerative abilities by affecting either the formation or maintenance of a properly stratified AEC structure and the loss of *Msx1* expression in the underlying mesenchymal tissue (Beck *et al*., 2006; Beck *et al*., 2003; Kawakami *et al*., 2006; Yokoyama *et al*., 2007)). These pathways appear to control the expression of coordinated FGF signals, FGF-8 and FGF-10, which are also likely to be key players in the epidermal/mesenchymal interactions that drive early blastema formation, proliferation, and regenerative outgrowth

(Slack *et al*., 2004; Yokoyama *et al*., 2001; Yokoyama *et al*., 2000). An elegant mix of pharmaceutical and transgenic overexpression strategies recently showed that regeneration of the frog tail requires FGF and canonical Wnt signaling (Lin and Slack, 2008). These studies also suggest that Wnt signaling functions upstream of FGFs and that both pathways are inhibited when noggin, which interferes with BMP signaling, is overexpressed (Lin and Slack, 2008). While it is clear that signaling pathways play an essential early role to establish proper epidermal/mesenchymal interactions, and that each tissue type is derived from lineage-restricted cells, it remains unclear exactly which cells of which specific tissues respond to which signals.

Regeneration in Zebrafish

Background

The zebrafish has emerged as a powerful model organism for the application of genetics to study not only vertebrate development, but also regeneration (Poss, 2007; Poss *et al*., 2003). Among other tissues, zebrafish can regenerate retina, fins, and heart. It remains unknown whether zebrafish fin regeneration relies on "dedifferentiation" or stem/progenitor cell activation. Because cell implantation or tissue grafting is not yet an option in adult zebrafish, transgenesis has become the method of choice to address mechanisms of regeneration. The recent employment of transgenic lines during zebrafish heart regeneration clearly implicates a reserve progenitor cell (discussed below) and points the way for future experiments that should be designed to track down the origin of the blastema.

Appendage (fin) regeneration

Characterization

While the zebrafish response to appendage amputation is very similar to that of amphibians, the anatomy of the fish fin differs significantly. Fish appendages are composed of multiple fin rays, each of which produces their own blastema (see Figure 2A). Wound healing occurs through a process of epithelial cell migration to form a wound epidermis, which thickens to form a layered structure containing a basal layer of distinctive cuboidal cells. Like urodele amphibians, the uninjured tissues in zebrafish close to the amputation plane become "disorganized", and cells of the mesenchyme proliferate, appear to migrate and give rise to a blastema (12–48 hpa, hours post amputation). The zebrafish blastema then compartmentalizes at the onset of regenerative outgrowth (48 hpa), a period of rapid cell division, while differentiation and patterning conspire to rebuild the missing structures.

Presently, the heterogeneity of early and late blastemas is unknown, but appears to be a crucial aspect of early progenitor cell organization. Cells of the early blastema are proliferative and the G2 phase of the cell cycle at this stage is greater than 6 hrs long, indicating that cells are cycling slowly (Nechiporuk and Keating, 2002). Cells in this region express markers including *msxb*, *msxc*, *sly1*, *mps1*, *fgf20a*, and *hsp60* (see Figure 2A). Thus, the early blastema may consist of a homogeneous cell population, or it may instead consist of a heterogeneous population of cells that differentially express these markers.

By the onset of regenerative outgrowth (48 hpa) the blastema begins to resolve into two distinct domains (see Figure 2A). Cells of the proximal blastema proliferate rapidly, achieving a G2 length of roughly 1 hr by 72 hpa, and express *mps1*, *hsp60*, and PCNA (Nechiporuk and Keating, 2002). Cells of the distal blastema do not proliferate and express *msxb*, *msxc*, and *sly1*. In addition, the distal-most cells of the distal blastema region express *fgf20a* by 72 hpa, illustrating sub-regionalization within the distal zone (Whitehead *et al*., 2005). It has been suggested that the distal zone is important to direct regenerative outgrowth and that the distal zone cells may be undifferentiated progenitor cells because they express *msxb* and *msxc*, which associate with undifferentiated cell types in other model organisms (Nechiporuk and Keating, 2002). Further experimentation will be necessary to validate or refute these possibilities.

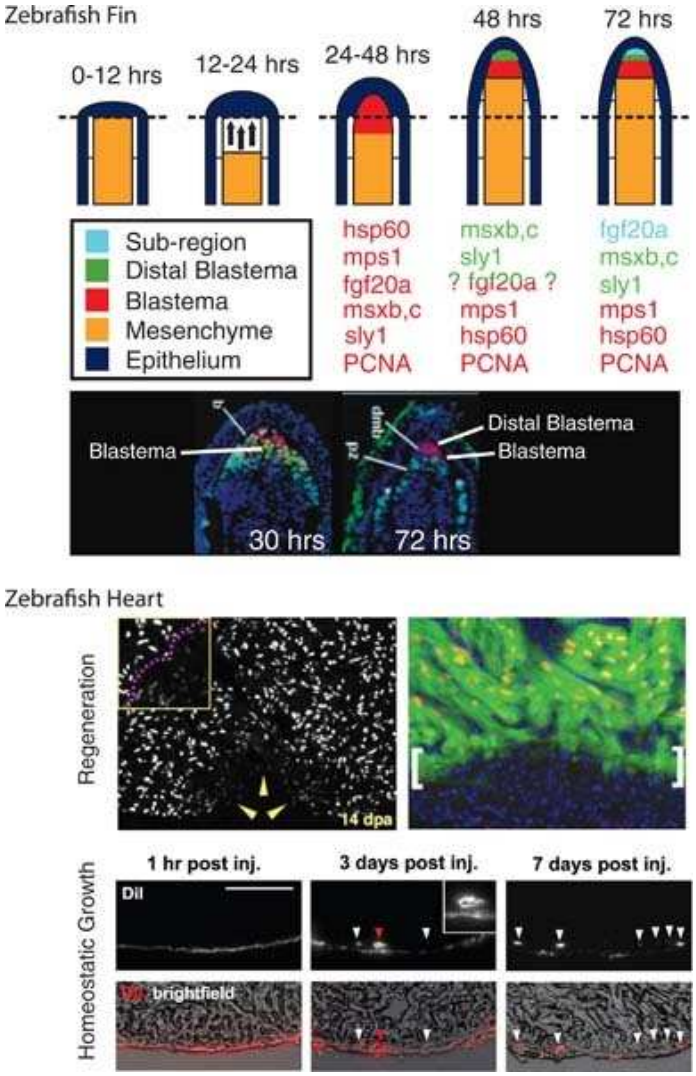

Figure 2A Stages of zebrafish caudal fin regeneration as longitudinal sections.

(top) The dotted line demarcates the amputation plane. The wound is closed by migrating epithelial cells to form a wound epidermis within the first 12 hours postamputation (hpa). Over the next 12 hours, the wound epidermis thickens as cells near the amputation disorganize and migrate toward the amputation plane. By 24–48 hpa, a blastema has formed that contains cells expressing a number of molecular markers. Near the end of this period, the blastema has compartmentalized into proximal and distal zones that can be distinguished by the level of cell proliferation as well as the expression of molecular markers. This marks the onset of regenerative outgrowth. By 72 hpa the distal zone can be further subdivided by the expression of *fgf20a*. (bottom) Cell proliferation (blue) and gene expression (red) show the compartmentalization that occurs in the blastema. **B** The identification of progenitor cells in the zebrafish heart. (Upper panels – Left) Expression of Red Fluorescent Protein (RFP) driven by a heart muscle-specific promoter. Cells along the boundary of regenerating tissue exhibit reduced "dim" expression of this marker, which could indicate either that the cells are dedifferentiating and reducing the expression of tissue specific genes, or instead that progenitor cells are just beginning to express this gene for the first time. (Upper panels – Right) To distinguish these possibilities, Lepilina et. al. engineered fish to express both GFP and RFP from the same heart muscle-specific promoter. Because GFP folds faster than RFP and RFP is more stable once it is made, GFP+/RFP- cells should represent cells that have turned this gene on for the first time (stem/progenitor progeny) while GFP-/RFP+ cells should represent cells that have turned off the gene at this promoter (dedifferentiation). As illustrated, GFP+/RFP- cells are clearly present near the edge of the amputation, indicating that these "dim" cells are in the process of activating heart muscle-specific genes for the first time and therefore represent differentiating stem/progenitor cells. (Lower panels) After injection of the fluorescent dye near the heart of unamputated fish, epithelial cells around the exterior of the heart fluoresce red. During normal tissue homeostasis, these cells migrate inward and eventually give rise to vasculature within the heart, illustrating the unexpected dynamics of cellular activity in the unamputated zebrafish heart. Images are adapted from: (1) Tales of regeneration in zebrafish., Vol. 226, 2003, 202–210; Reprinted with permission of John Wiley & Sons, Inc. (2) Reprinted from Cell, 127, Lepilina, A., Coon, A. N., Kikuchi, K., Holdway, J. E., Roberts, R. W., Burns, C. G., and Poss, K. D., A dynamic epicardial injury response supports progenitor cell activity during zebrafish heart regeneration. 607–619, 2006, with permission from Elsevier. (3) Wills, A. A., Holdway, J. E., Major, R. J., and Poss, K. D. (2008). Regulated addition of new myocardial and epicardial cells fosters homeostatic cardiac growth and maintenance in adult zebrafish. Development *135*, 183–192. Reproduced with permission of the Company of Biologists.

BrdU pulse-chase experiments suggest that cells segregate to the distal blastema at the end of blastema formation around 36–48 hpa, and that these cells are descendents of formerly proliferating cells (Nechiporuk and Keating, 2002). However, it is unclear how this compartmentalization occurs. If the early blastema is composed of a homogeneous cell population, perhaps these cells are induced to restrict their expression profile at the onset of compartmentalization. Alternatively, if the early blastema is a heterogeneous cell population in which each cell already expresses its position-specific profile, then perhaps compartmentalization occurs by a process of cell sorting. How the shift from slow cycling to rapid cycling is controlled and how it is coordinated with compartmentalization of the blastema remains unknown. New tools such as promoter driven cell ablation should help dissect the biological function of the distal and proximal domains (Curado *et al*., 2007). Additionally, gene specific targeting combined with CreLox strategies should allow cell lineage tracing to definitively dissect the origin of the blastema in zebrafish appendages (Doyon *et al*., 2008; Meng *et al*., 2008).

Functional genetics

Mutagenesis screens for temperature sensitive alleles that affect regeneration brought caudal fin regeneration to the forefront of regeneration research (Johnson and Weston, 1995; Poss *et al*., 2002). Although these screens are limited by the fact that complete coverage of the genome with temperature sensitive alleles is not possible, genes associated with regeneration have been uncovered. By combining forward genetics, pharmacology, and transgenic overexpression a potent toolbox is now available in zebrafish for a thorough investigation of its regenerative processes.

These strategies have already provided strains that are defective at various stages of regeneration. For example, fish harboring a mutation in either *fgf20a* (discussed below) or *hsp60* never form a blastema (Makino *et al*., 2005; Whitehead *et al*., 2005). Interestingly, *hsp60* is expressed in early mesenchymal blastema cells and appears to play a role in the mesenchymal cell response to nearby amputation (Makino *et al*., 2005). On the other hand, fish with a mutation in *mps1*, a mitotic checkpoint protein, exhibit normal wound healing and early blastema formation, but are defective in the regenerative outgrowth phase (Poss *et al*., 2002). Defects in *mps1* animals are observed in the proximal blastema zone at roughly 48 hpa during the transition to rapid cell proliferation. While *mps1* is clearly essential for proper regeneration, it sems to play a "house-keeping" type of role in rapidly proliferating cells. In fish with a mutation in the *sly1* gene, wound healing and mesenchymal disorganization are unafftected, but the proximal blastema zone is never established and the distal blastema zone is enlarged. Because the yeast *sly1* gene product is involved in protein trafficking and zebrafish *sly1* expressing cells segregate into the distal zone during normal blastema compartmentalization, the *sly1* mutation has been hypothesized to cause defective signaling from the distal blastema cells to the more proximal proliferating cells (Nechiporuk *et al*., 2003). However, the nature of the presumed signal(s) remains unknown. While the *mps1* and *sly1* mutant phenotypes provide resources to study the compartmentalization stage of zebrafish regeneration, the genomic regions surrounding these genes should also provide useful enhancer sequences for future investigations.

In fish, the FGF signaling pathway plays a very early role in establishing proper epidermal/mesenchymal interactions and blastema formation, just as suggested for FGF signaling during anuran amphibian regeneration (Whitehead *et al*., 2005; Yokoyama *et al*., 2001; Yokoyama *et al*., 2000). The FGF signaling pathway is essential for fin regeneration as evidenced using pharmacological (Poss *et al*., 2000), morpholino (Thummel *et al*., 2006), and dominant negative transgenic (Lee *et al*., 2005; Tawk *et al*., 2002) approaches. Fish harboring an *fgf20a* loss of function mutation display an abnormally thickened wound epidermis, improper basal layer formation, failure of uninjured tissue near the amputation to disorganize, and absence of a blastema (Whitehead *et al*., 2005). *fgf20a* is expressed within the first 6 hours after amputation in the mesenchyme directly beneath the wound epidermis while the FGF receptor, *fgfr1*, is expressed by18 –24 hpa in fibroblast-like pre-blastemal mesenchymal cells just proximal and distal to the amputation plane (Poss *et al*., 2000; Whitehead *et al*., 2005). These combined data cement FGF signaling, either directly or indirectly, as an upstream regulator of stem/progenitor cell formation, migration, proliferation, or organization. The cells expressing *fgfr1* may represent a fruitful target of future research focused on elucidating the nature of regenerative stem/progenitor cells in zebrafish.

Two other pathways, activin/TGFβ and Wnt, have also been implicated in the earliest steps of the regenerative response. Microarray experiments revealed that *activin-βA*, a member of the secreted TGFβ superfamily, is upregulated as early as 1 hpa (Jazwinska *et al*., 2007). Manipulations that inhibit activin/TGFβ signaling cause an early block in the regenerative response by reducing mesenchymal disorganization, *msxb* expression, and/or cell proliferation (Jazwinska *et al*., 2007; Kawakami *et al*., 2006). On the other hand, inhibition of canonical Wnt signaling (via overexpression of DKK or dominant negative TCF3) or activation of non-canonical Wnt signaling

(via overexpression of Wnt5a), blocks stratification of the wound epidermis and blastema formation (Kawakami *et al*., 2006; Stoick-Cooper *et al*., 2007). Wnt signaling inhibition also eliminates expression of both *fgf20a* and downstream FGF targets, suggesting that Wnt signaling is upstream of FGF signaling following amputation. Thus, regeneration of the zebrafish tail fin is akin to that of the anuran tadpole tail in that both systems utilize the same signaling pathways in apparently similar ways (Lin and Slack, 2008). These data provide a glimpse into the complex coordination of signaling pathways during the early regenerative response (Kawakami *et al*., 2006; Stoick-Cooper *et al*., 2007). Elucidation of the interactions between the basal layer of the wound epidermis and the responsive pre-blastemal stem/progenitor cells is paramount to understanding the vertebrate response to regeneration.

Unanswered questions

Naturally, great progress has extended old questions while raising a cohort of new ones:

1. Do blastema cells derive from differentiated cells or undifferentiated reserve cells? While long term pulse-chase BrdU studies have apparently ruled out label retaining (slow cycling) progenitor cells (Nechiporuk and Keating, 2002), the presence of rapidly cycling stem/progenitor cells in the fin is suspected because there is a high steady-state level of cell proliferation in the fins of intact, unamputated animals.
2. What is the source of the basal layer of the wound epithelium and how is it specified? *Lef1* appears in cells prior to the formation of a morphologically recognizable basal layer (Poss *et al*., 2000), but are they really presumptive cells of the basal layer? How are the multiple essential signaling pathways coordinated during this specification? The basal layer appears to be essential for regeneration, but why? Does it signal and induce blastema formation or is it simply a source of growth factors or extracellular matrix?
3. What purpose is served by "disorganization" of the uninjured mesenchymal tissue? Do these cells give rise to the proliferating blastemal cells, do they modify the extracellular environment to make it permissible for blastema activity, or both?
4. What is the biological purpose of blastema compartmentalization? Is the distal blastema a source of progenitor cells for the proximal blastema, or is it a source of secreted factors as implied by the *sly1* mutant phenotype?

Given the ever-expanding toolkit available for the study of zebrafish biology, it is expected that answers to these questions will be forthcoming in the foreseeable future.

Organ (heart) regeneration

Studies of zebrafish heart regeneration illustrate that careful lineage experiments are a pre-requisite for assessing the cellular source of regeneration (Lepilina *et al*., 2006). When adult fish hearts expressing red fluorescent protein (RFP) driven by a cardiomyocyte(CM)-specific promoter are transected, cells in the regenerating region express markers of cardiac embryonic progenitor cells and fluoresce red at a much reduced level compared to the surrounding differentiated CMs (see Figure 2B). This could be interpreted as the dedifferentiation of CMs, or as the differentiation of progenitor cells turning on the CM promoter for the first time. To distinguish between these possibilities, fish expressing both RFP and GFP from the same CM promoter were analyzed. Because RFP folds and fluoresces slower than GFP and it is more stable, the appearance of RFP-/GFP+ cells after amputation would represent newly differentiating progenitor cells, while RFP+/GFP- cells would represent cells undergoing dedifferentiation. When the experiment was performed, these double transgenic hearts yielded RFP-/GFP+ cells throughout the regeneration process, indicating that regeneration of the myocardium results from the differentiation of progenitor cells and not from dedifferentiation (see Figure 2B; Lepilina *et al*., 2006). While this provides important support for the progenitor hypothesis, the approach includes a key assumption: if a cell was to dedifferentiate, the cardiomyocyte promoter would turn off and proteins would undergo their normal turnover. Because the potential mechanism used to accomplish dedifferentiation is unknown, it remains possible that a dedifferentiating cell would rapidly degrade a large number of protein products and the differences between RFP and GFP stability would no longer be observable.

Where do the progenitor cells come from? Using similar approaches during tissue growth in unamputated animals, recent work revealed that CM progenitor cells are induced throughout the entire adult myocardium during rapid homeostatic tissue growth (Wills *et al*., 2008). In addition, epicardial-derived cells from the outer edge of the heart actively migrate into the myocardium during both regeneration and homeostasis to build vascular tissue (see Figure 2B; Lepilina *et al*., 2006; Wills *et al*., 2008). Additional analysis of cell proliferation with more markers and at

earlier time points will be required to fully address the origin of the progenitor cells. Nonetheless, the type of careful and creative experimental approaches used to dissect cardiac regeneration in zebrafish will be necessary in all vertebrate regenerative contexts to critically evaluate the contributions of dedifferentiation and stem/progenitor cells.

Regeneration in planarians

Background

Planarians are bilaterally symmetric animals that possess derivatives of all three germ layers (endo-, ecto-, and mesoderm) and display astonishing regenerative abilities (Reddien and Sánchez Alvarado, 2004). Relative to cells involved in the vertebrate regeneration response, the source of regenerative cells in planarians is much less controversial. Planarians recruit an experimentally accessible population of adult stem cells called neoblasts that are distributed throughout the body. Classically defined by morphology, sensitivity to γ-irradiation, and mesenchymal distribution, neoblasts are undifferentiated cells with a large nucleus and very little cytosol (Reddien and Sánchez Alvarado, 2004). With the exception of the germline, neoblasts are thought to be the only planarian cells capable of division. After animals are exposed to γ-irradiation, cell division ceases and neoblasts are lost, rendering the animals incapable of regeneration or homeostatic tissue turnover (Pellettieri and Sánchez Alvarado, 2007). The loss of neoblasts is manifested by a characteristic ventral curling and the eventual lysis of the animals (Reddien *et al*., 2005). In contrast to irradiation, neoblasts respond to amputation with a proliferative burst, which results in the formation of a regeneration blastema and the eventual restoration of the missing body parts. Because only injections of neoblast-enriched preparations can restore longevity and regeneration to irradiated animals (Baguñà *et al*., 1989), it is believed that in planarians, regeneration and tissue homeostasis are primarily driven by neoblast function.
Because small neoblast-containing fragments cut from almost any location in the adult animal can produce entire, properly proportioned planarians, it is thought that neoblasts are collectively totipotent. While the neoblasts are toti-potent as a population, the differentiation potential of any given neoblast is unkown and the molecular nature of the neoblast population remains poorly described. In recent years, the molecular dissection of planarian regeneration has been aided immensely by the discovery that RNA interference (RNAi) can be used to interrogate gene function (Newmark *et al*., 2003; Sánchez Alvarado and Newmark, 1999). In one broad stroke, it was shown that over a thousand genes from an RNAi library could be screened for phenotypes in a relatively short amount of time. This work identified 240 genes associated with regeneration defects. From this collection, 140 gene perturbations blocked, limited, or reduced regeneration, 48 of which caused the characteristic curling/lysis phenotype observed after irradiation, indicating that neoblast function was compromised (Reddien *et al*., 2005).
In addition to RNAi, sequencing of the planarian genome (http://www.genome.gov/Pages/Research/Sequencing/SeqProposals/PlanarianSEQ.pdf), generation of EST libraries (Sánchez Alvarado *et al*., 2002; Zayas *et al*., 2005), production of antibodies (Agata *et al*., 1998; Guo *et al*., 2006; Kobayashi *et al*., 2007), development of cell-specific whole mount in situ hybridization (WISH; Cebrià *et al*., 2007; Eisenhoffer *et al*., 2008; Gurley *et al*., 2008; Umesono *et al*., 1999), and fluorescence in situ hybridization (FISH) methods (Cebrià *et al*., 2007; Eisenhoffer *et al*., 2008; Gurley *et al*., 2008; Umesono *et al*., 1999), labeling of proliferative cells (Newmark and Sánchez Alvarado, 2000), development of fluorescence activated cell sorting (FACS) protocols (Hayashi *et al*., 2006; Reddien *et al*., 2005), and single cell RT-PCR techniques (Higuchi *et al*., 2008) have catapulted planarians from an academic curiosity to a viable and fascinating model for regeneration research. Because the cells involved in planarian regeneration and homeostasis are identified and these cells can be studied *in vivo*, the set of questions one can currently address using planarians are very different from those being addressed by amphibians or zebrafish experimentation. These questions are focused on the biology of planarian stem cells and the mechanisms utilized to control their self-renewal, fate choice, and differentiation:

1. Are all neoblasts the same or are there subsets of lineage-restricted cells?
2. What is the extent of their molecular heterogeneity?
3. How, when, and where do neoblasts choose their fate and decide to differentiate, especially in the context of regeneration?
4. How is this decision controlled?
5. Is differentiation immediate, or is it a slow process?
6. Does it happen in stages?
7. If it does happen in stages, what are the choices made at each step?

Heterogeneity and lineage of neoblasts

Researchers have begun to answer the above questions using the tools of modern molecular biology, but there is much more to learn. Studies using the DNA analog bromodeoxyuridine (BrdU) have shed light on the distribution and fate of neoblasts because this technique allows researchers to both visualize cells that are actively proliferating and follow those cells over time. After a single pulse of BrdU, only neoblasts are initially labeled, indicating that this population is rapidly dividing (Eisenhoffer *et al*., 2008; Guo *et al*., 2006; Newmark and Sánchez Alvarado, 2000). Labeled cells are distributed throughout the parenchyma (mesenchyme) of the animal and are conspicuously absent from the pharynx and from a region anterior to the photoreceptors, the two regions of the animal that are unable to regenerate. This distribution fits the classical criteria for neoblast distribution. An antibody that recognizes phosphorylated histone H3 (H3P), which marks dividing cells, reveals a similar neoblast distribution as do cell cycle specific probes (*mcm2* and *pcna* homologs; Newmark and Sánchez Alvarado, Orii *et al*., 2005; Salvetti *et al*., 2000).
If animals are fixed at various time points after BrdU is administered, labeling is observed in differentiated cell types roughly 35 hours after the BrdU pulse. Labeled cells can be observed in the post-mitotic epithelium several days later. When planarians are exposed to long-term continual doses of BrdU, all neoblasts, as defined by morphology, are eventually labeled. Finally, if a pulse of BrdU is administered prior to amputation, a large number of labeled cells are found in the regenerating tissue, showing that the progeny of neoblasts that were actively dividing prior to amputation make a major contribution to the regenerating tissue (Eisenhoffer *et al*., 2008; Newmark and Sánchez Alvarado, 2000). Combined, these data argue that the neoblast population constantly divides to replace cells lost to turnover. In addition, the cells of regenerating tissue are derived from the dividing neoblasts.
The neoblast population likely contains both stem cells and their committed progeny. However, the heterogeneity of the neoblast population has been difficult to address because neoblasts are defined by their morphology. Evidence of heterogeneity among neoblasts has recently been suggested by electron microscopy and FACS experiments that associate chromatoid body-containing cells with what appears to be different stages of differentiation (Higuchi *et al*., 2007). Chromatoid bodies are electron dense structures found in the cytoplasm of many cells that fit the classical definition of a neoblast. While two types of neoblast-like “stem” cells were noted, these most likely represent neoblasts in different stages of the cell cycle because they were separated based on DNA content. At the moment, general cell morphology and fluorescent dyes combined with γ-irradiation are the only available parameters to sort cells, which results in the sorting of clearly mixed cell populations (Eisenhoffer *et al*., 2008; Higuchi *et al*., 2007). The promising potential of FACS analysis awaits the identification of markers that can be used to separate cells based on molecular criteria.
The best example to date of a lineage-restricted neoblast fate is the presumptive primordial germ cells. The germline stem cells derive from somatic cells because head fragments devoid of germ cells regenerate them and can eventually make oocytes and sperm (Morgan, 1902). Germ cells represent a lineage-restricted stem cell type and are indistinguishable from neoblasts at the ultrastructural level (Higuchi *et al*., 2007). However, these cells can be identified by their distribution and their specific expression of the planarian *nanos* homolog (*Djnos*, *Smed-nanos*; Handberg-Thorsager and Salo, 2007; Sato *et al*., 2006; Wang *et al*., 2007). *nanos* encodes an RNA binding protein with a known role in germ cell differentiation and maintenance, and in planarians the undifferentiated *nanos*-expressing cells are located near the testes and ovaries. Importantly, silencing *Smed-nanos* by RNAi does not affect neoblast function or planarian regeneration, but abolishes the formation, regeneration, and maintenance of gonads in sexual planarians (Wang *et al*., 2007). In addition, the germline represents the only clear case of cycling cells besides neoblasts because these cells: 1) are sensitive to irradiation (Handberg-Thorsager and Salo, 2007; Sato *et al*., 2006; Wang *et al*., 2007; 2) incorporate BrdU (Sato *et al*., 2006); 3) express PCNA (Sato *et al*., 2006); and 4) stain positive for H3P in the spermatocyte cysts (Wang *et al*., 2007). Interestingly, expression of the *nanos* homolog in asexual animals revealed that although these animals do not form functional gonads, they still specify germ cells that do not divide (Handberg-Thorsager and Salo, 2007; Sato *et al*., 2006; Wang *et al*., 2007).
A recent study illustrates the vast potential of modern molecular tools to dissect the nature of planarian stem cells (Eisenhoffer *et al*., 2008). Comparing microarray expression profiles of non-irradiated and irradiated animals at either 24 hrs or 7 days post-irradiation generated a list of genes enriched in neoblasts and their progeny. These comparisons were crucial because neoblasts, and therefore neoblast-specific genes, disappear by 24 hrs after irradiation. However, genes that disappear soon thereafter represent post-mitotic cell types that are lost because no neoblasts remain to replenish them. Three categories of neoblast-related genes were identified based on their ordered rate of disappearance following irradiation, and on their expression pattern in intact animals (see Figure 3A). WISH, BrdU pulse-chase, and double-labeling FISH experiments showed that BrdU labeled cells, which are neoblasts at the time of labeling, exhibit a stereotyped progression of cell differentiation. At early time points, 99.3±0.5% of BrdU+ cells are positive for Category 1 markers. By 2 days after a BrdU pulse, BrdU+ cells instead express category 2

markers and by 4 days they express category 3 markers. While cells expressing category 2 and 3 markers are descendents of neoblasts, the precise relationship between these cells remains to be determined (Eisenhoffer *et al*., 2008).

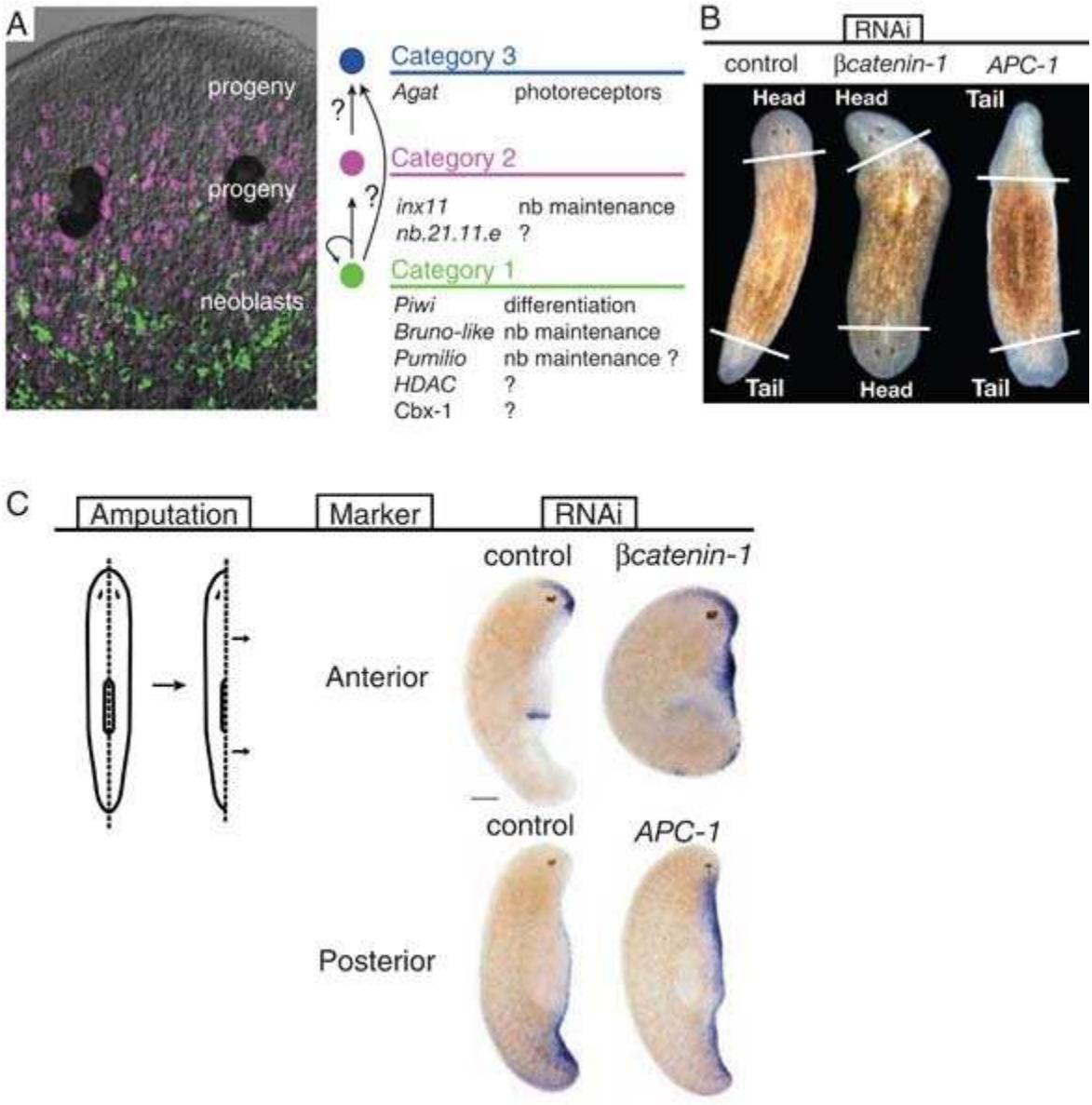

Figure 3A Planarian neoblasts (green) are present throughout the mesenchyme of the animal and express category 1 genes.

Neoblasts give rise to cells that express category 2 genes and to cells that express category 3 genes. It is not yet clear whether neoblasts can directly give rise to category 3-expressing cells, or whether those cells must first enter a temporary category 2-expressing state. Regardless, cells that express either category 2 or category 3 genes are more peripherally located than the neoblasts, indicating that as neoblasts differentiate, they turn on different gene expression programs and move toward the periphery of the animal. Many of the identified genes have been assigned particular functions based on RNAi experiments, while others await careful characterization. Not all genes are listed and descriptions of gene functions are discussed in the text. **B** RNAi of *Smed-βcatenin-1* causes stem cell progeny to acquire a head fate regardless of the location of amputation. In contrast, RNAi of *Smed-APC-1* causes stem cell progeny to adopt a tail fate. **C** Molecular markers of anterior and posterior fate on day 4 of regeneration following amputation down the center of the animal. After RNAi of *Smed-βcatenin-1* (low β-catenin activity) or *Smed-APC-1* (high β-catenin activity), stem cell progeny along the entire amputation plane adopt an anterior or posterior fate, respectively. Images provided by George T. Eisenhoffer. the authors, and adapted from (Gurley *et al*., 2008).

This study unambiguously identified markers to functionally investigate the self-renewal of neoblasts and the differentiation of their progeny (see Figure 3A). However, this is just the tip of the iceberg. While the WISH pattern of each category 1 marker is similar and marks neoblasts, careful double FISH of all category 1 markers may reveal long sought-after molecular heterogeneity among neoblasts. These studies significantly expand on earlier expression analysis of irradiated animals from a related planaria species (Rossi *et al*., 2007). However, the genes screened by microarray still represent only a small fraction of the planarian genome and additional early lineages remain to be discovered. Moreover, the experiments addressing the temporal regulation of gene expression were performed on intact animals (Eisenhoffer *et al*., 2008). During the homeostatic conditions studied, all differentiated adult cell types were present. How the animal senses which body parts to replace after amputation, how it selects the appropriate lineages, and how this corresponds to the lineage relationships observed in intact animals are fascinating questions for future investigation.

Gene function and neoblasts

Several neoblast studies have focused on planarian genes that encode homologs of RNA binding proteins associated with germ granules in other animals. While some planarian RNA binding proteins are expressed in a neoblast-like pattern (*piwi* homologs*)* (Guo *et al*., 2006; Reddien *et al*., 2005 Salvetti *et al*., 2005), some are expressed in both neoblasts and differentiated tissues (*pumilio*, *bruno*, and *musashi* homologs; Guo *et al*., 2006; Higuchi *et al*., 2008; Salvetti *et al*., 2005), and others are expressed exclusively in the germline (*vasa* and *nanos* homologs; Handberg-Thorsager and Salo, 2007; Sato *et al*., 2006; Shibata *et al*., 1999; Wang *et al*., 2007) or in differentiated tissues (*musashi* homologs; Higuchi *et al*., 2008). RNAi-mediated silencing of *pumilio, piwi, or bruno* homologs leads to regenerative failure, but through different mechanisms (Guo *et al*., 2006; Reddien *et al*., 2005 Salvetti *et al*., 2005). While neoblasts are eventually lost in all 3 cases, careful evaluation of early phenotypic stages has led to important insights. When a *bruno* homolog (*Smed-bruno-like*) is silenced, stem cell maintenance is defective while differentiation is normal (Guo *et al*., 2006). These animals can initiate regeneration and begin forming new tissue, but as neoblasts are depleted, this tissue regresses and the animals die. On the other hand, when a *piwi* homolog (*smedwi-2*) is silenced by RNAi, neoblasts proliferate, their progeny migrate, but differentiation is defective (Reddien *et al*., 2005). These studies illustrate the potential to understand various aspects of stem cell control during tissue homeostasis and regeneration. They also illustrate that it is crucial to distinguish primary from secondary regeneration phenotypes, because the loss of neoblasts is a common secondary consequence of earlier defects in distinct processes (Guo *et al*., 2006; Oviedo and Levin, 2007; Reddien *et al*., 2005 Salvetti *et al*., 2005).
Genes that do not encode RNA binding proteins can also be silenced to elicit irradiation-like stem-cell-defective phenotypes. After silencing *Smed−cdc23*, neoblasts arrest in anaphase, cannot divide to replace tissue during homeostasis, and the animals curl and lyse as if they had been irradiated. In addition, RNAi of a planarian innexin homolog (*smed-inx11*) abolishes regenerative capacity and leads to ventral curling (Oviedo and Levin, 2007). This gene is expressed in post-mitotic cells anterior to the photoreceptors (Oviedo and Levin, 2007) much like the category 2 genes discussed above (Eisenhoffer *et al*., 2008). smedinx-11 expression may reflect a transition state from neoblasts to differentiating progeny, but this supposition has not been rigorously tested.

Fate choice

In 1904, Thomas Hunt Morgan observed that anterior and posterior facing amputations both have the potential to become either a head or a tail (Morgan, 1904). Scientists had to wait over 100 years for molecular understanding of how planarians sense which structure to replace. This new insight stems from silencing the intracellular core components of the canonical Wnt signaling pathway (see Figure 3B). RNAi silencing of a planarian *β-catenin* homolog (Gurley *et al*., 2008; Iglesias *et al*., 2008; Petersen and Reddien, 2008) or *dishevelled* homologs (Gurley *et al*., 2008) induces the regeneration of a head even after tail amputation. Conversely, silencing the *adenomatous polyposis coli (APC)* homolog, which encodes an antagonist of β-catenin, causes increased β-catenin activity and consequently, a tail regenerates even if the head is removed (Gurley *et al*., 2008). These data indicate that under normal circumstances, β-catenin is a molecular switch: activity is inhibited or never initiated at anterior wounds to induce head formation and is highly activated at posterior wounds to induce tail formation. The control of β-catenin activity is also crucial for homeostasis because RNAi of *β-catenin* (*Smed-βcatenin-1*) in intact animals causes neoblast progeny throughout the animal to adopt an anterior fate, leading to the transformation of other tissue types into heads (Gurley *et al*., 2008; Iglesias *et al*., 2008; Petersen and Reddien, 2008). Thus, the silencing of specific planarian genes has uncoupled fate decisions of new neoblast progeny from their location in the animal.

The control of β-catenin activity does not actually specify head or tail fate, but instead anterior or posterior fate, which consequently leads to head or tail formation. This is supported by at least 3 lines of evidence. First, after cutting *Smed-βcatenin-1(RNAi)* animals just anterior to the photoreceptors, they do not regenerate a new head anterior to the old one, but instead specify the anterior tissue that needs to be replaced (Petersen and Reddien, 2008). Second, tail fragments of untreated animals, which must regenerate a trunk and a head, specify the anterior margin of the regenerating animal before an actual head or trunk forms (Gurley *et al*., 2008). Regeneration and tissue remodeling then replace the missing regions. Third, after lateral amputations of RNAi treated animals, nearly all of the cells along the entire amputation plane adopt an anterior (low β-catenin activity; Gurley *et al*., 2008; Petersen and Reddien, 2008) or posterior (excessive β-catenin activity; Gurley *et al*., 2008) fate (see Figure 3C). Hence, while β-catenin activity must be kept low in the anterior and high in the posterior during lateral regeneration, this activity must be maintained at intermediate levels at intermediate positions so that neoblast progeny can adopt fates other than anterior or posterior. This implies that there may be a gradient of β-catenin activity along the AP axis or perhaps a third state (head, body, tail) of the β-catenin switch. The mechanism by which this gradient is established or this switch controlled during regeneration remains the focus of current research.

The discovery that β-catenin activity is dynamically controlled during planarian homeostasis and regeneration has provided a foothold to study how planarians recognize that the anterior or posterior structures have been removed. Two main questions remain unresolved. First, which cells turn up or down β-catenin activity to determine head or tail identity? Is it the stem cells, their progeny, or both? Second, what are the upstream signals that control β-catenin activity? Secreted Wnt ligands and antagonists are the most likely candidates and their RNAs are expressed in a complex AP gradient, but silencing these genes has not yet led to head or tail misspecification defects (Gurley *et al*., 2008; Petersen and Reddien, 2008). If Wnts are used to control the β-catenin switch, then what mechanisms are in place to control Wnt expression?

Concluding remarks

Regeneration is widespread throughout the animal kingdom suggesting that it is not just a captivating subject, but that the underlying biology is fundamental. In fact, regeneration may be an ancestral feature of metazoan life that has been lost to varying degrees in multiple lineages (Sánchez Alvarado, 2000). Unfortunately, while mammals can regenerate certain organs to some extent, our regenerative powers are comparatively far less impressive.

Because mammals exhibit limited regenerative capacities, we aim to understand the many ways that other organisms use the same basic genetic toolkit to achieve regeneration. It is important to remember that embryonic, larval, and adult regeneration are very different with respect to overall scale and the ratio of differentiated to undifferentiated cells that are present. However, in any context, regeneration critically depends on a source of proliferative stem/progenitor cells. While great strides have been made in understanding vertebrate regeneration and the complex cell interactions that are involved, very little is known about the definitive source of regenerative cells or their fate. This should soon change as techniques for probing into such questions are rapidly improving. For example, transgenic GFP+ tissue transplants have already ruled out significant plasticity for blood stem cells during axolotl tail regeneration (Sobkow *et al*., 2006), while the contribution of blood stem cells during zebrafish regeneration remains unexplored. Planarians, on the other hand, provide a remarkable model system to study the regenerative response of undifferentiated cells because the stem/progenitor cells have been identified, are abundant, and are experimentally accessible *in vivo*.

Comparing how different animals achieve regeneration can now be approached from multiple fronts. The study of signaling pathways serves as an example of how the integration of data across multiple regenerative contexts has informed general concepts. In zebrafish and amphibians, Wnt signaling is required for regeneration because its inhibition leads to an improperly stratified AEC and absence of a blastema. In planaria, decreases or increases in β-catenin activity, the standard readout of canonical Wnt signaling, causes stem cell progeny to make an improper fate choice, but regeneration in general is not affected. This may indicate that the primary defect following Wnt signaling inhibition in vertebrates is caused by the improper fate specification of either AEC cells, or of would-be stem/progenitor cells. Thus, improper fate choice may lead to an improper regenerative response. Alternatively, it is also possible that the same signaling pathway is deployed in a completely different way by different animals to achieve a similar outcome, *i.e.*, the replacement of missing structures. Similarly, Hedgehog signaling appears to control the proliferation of posterior blastema cells during tail regeneration in larval axolotls (Schnapp *et al*., 2005), but has a tissue patterning function during axolotl limb and zebrafish fin regeneration (Avaron *et al*., 2006; Quint *et al*., 2002; Roy *et al*., 2000). Whether Hedgehog signaling is involved in planarian regeneration remains to be determined.

In zebrafish and planarians, steady-state cell turnover is a constant process. Zebrafish change their rate of cell proliferation in response to changes in population density and planarians constantly adjust their rate of proliferation to match nutritional status and will even shrink when starved. During specific phases of a regenerative response in zebrafish, cellular proliferation abruptly switches between slow and fast cycling modes. In planarians, proliferation increases in response to amputation, but the precise kinetics of the cell cycle during regeneration are currently under active investigation. It could be argued that at least in zebrafish and planarians, regeneration may be an exaggerated version of homeostasis. However, this is not likely to be a universal rule for regeneration, because many mammalian tissues exhibit extensive homestatic cell turnover while their regenerative response is quite limited (Pellettieri and Sánchez Alvarado, 2007). In contrast to planarians and zebrafish, urodele amphibian limbs exhibit little steady-state cell proliferation outside of epithelial tissue (Hay and Fischman, 1961) and yet, these animals provide the most dramatic examples of regeneration.

Fertilization provides a single totipotent cell that will divide many times with a concomitant decrease in potency of the resulting progeny as development ensues. This restriction correlates with changes in chromatin structure (epigenetics), which usually consists of varying degrees of DNA methylation, histone methylation and acetylation, and the presence or absence of repressive chromatin binding complexes at specific genomic locations. Recent work suggests that even in differentiated mammalian cell types, the lineage restriction process is reversible and cells can be "reprogrammed" to adopt a multi-potential state (Aoi *et al*., 2008; Meissner *et al*., 2007; Okita *et al*., 2007; Takahashi *et al*., 2007; Takahashi and Yamanaka, 2006; Wernig *et al*., 2007; Yu *et al*., 2007). If urodeles do in fact dedifferentiate their cells to a multipotent state, the natural ability to "reprogram" cells during regeneration may critically correlate with the epigenetic state of differentiated cells throughout the intact animal.

For example, the differentiated cells of the salamander may contain a different degree of epigenetic changes than similar cells in mammals, thus allowing more flexibility in their response to injury. What is the status of methylation in amphibian, fish, and mouse cells and where are the repressor complexes bound? Do changes in epigenetic state occur in specific cell types during regeneration? How does the epigenetic state of cultured newt A1 and mouse C2C12 cells compare? In planarians, one would ask if and how epigenetics contribute to the maintenance of neoblasts, and which changes occur when neoblasts differentiate. A comparison of the epigenetic state and transcriptional profiles in regenerating and non-regenerating tissues from amphibians, zebrafish, mice, and planarians could help identify commonalities and differences associated with regenerative capacities. Along these lines, recent work has focused on obtaining expression profiles of zebrafish fin and heart regeneration (Lien *et al*., 2006), as well as defining the transcriptional profile of the planarian neoblasts (Eisenhoffer *et al*., 2008; Lien *et al*., 2006).

Because each model system of regeneration has its own advantages and disadvantages, the field stands to benefit from the integration of the molecular and cellular knowledge garnered from these organisms. For example, 3 planarian genes (*piwi*, *bruno*, and *pumilio* homologs) that encode members of different RNA binding families are essential for apparently different biological functions within neoblasts. Do these genes play roles during zebrafish and/or amphibian regeneration? Can the expression of these genes or other known stem cell markers, like those used for "reprogramming," be used to locate vertebrate progenitor cells? The proteins PROD1 and AG play a role in proximal/distal fate specification and cell proliferation in urodeles, but do they have a role in anuran, zebrafish, or planarian regeneration (da Silva *et al*., 2002; Echeverri and Tanaka, 2005; Kumar *et al*., 2007)? Likewise, microRNAs (miRNA) play a key role in controlling differentiation during zebrafish regeneration (Yin *et al*., 2008). While miRNA is likely to function in all regenerative contexts, which miRNAs control which aspects of regeneration in each of the different contexts? Could any pattern or general rules be gleaned from such comparisons? As more information is gleaned from the study of pre-bilaterian animals such as hydra and nematostella, we will also need to incorporate these data with what is learned from the study of bilateral animals. Will common mechanisms emerge?

The future of regeneration research is exhilarating and full of promise. Central to the continued progress of the field will be to determine if animals use disparate mechanisms to achieve this incredible biology, or whether there are underlying principles and gene networks common to regeneration. In either case, the molecular and cellular mechanisms gleaned from these studies may help identify promising experimental strategies to either promote innate or introduce new regenerative capacities in mammalian tissues.

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