Developmental Cell
Perspective

Keeping at Arm's Length during Regeneration

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Regeneration of a lost appendage in adult amphibians and fish is a remarkable feat of developmental patterning. Although the limb or fin may be years removed from its initial creation by an embryonic primordium, the blastema that emerges at the injury site fashions a close mimic of adult form. Central to understanding these events are revealing the cellular origins of new structures, how positional identity is maintained, and the determinants for completion. Each of these topics has been advanced recently, strengthening models for how complex tissue pattern is recalled in the adult context.

Introduction

Regeneration in the simplest terms of developmental biology means the replacement of tissue components lost by injury. Oftentimes, a regenerative response may be of little consequence in the face of a more significant repair response like scarring. For instance, the adult mammalian heart has a measurable, but severely limited, capacity to create new cardiac muscle cells after a myocardial infarction, and fibrosis is the dominant outcome (Kikuchi and Poss, 2012; Laflamme and Murry, 2011; Senyo et al., 2013). Regenerative responses can be compensatory, restoring functional mass but not necessarily the structures that were lost; for example, rodent hepatic tissue is recovered in spared lobes after hepatotectomy but is not created at the injury site (Michalopoulos, 2007). Additionally, spatiotemporal variables restrict many or most regenerative events, making the extent or type of injury, and the developmental stage or age of the injured animal, key variables (Poss, 2010). Regeneration in its most successful form restores an intricate pattern to a lost complex tissue, generating a near-perfect replica even at adult stages.

An adult newt that has had one or more limbs amputated will restore skeletal muscle, bone, nerves, connective tissue, epidermis, and vasculature to a form that can be indistinguishable from its preinjury appearance. These events occur robustly whether at digit- or shoulder-level, and have been considered by many as regeneration in its truest manifestation. The Italian scholar Spallanzani initiated questions in the mid-18th century about the memory and recovery of complex adult pattern during newt limb regeneration that have remained in many ways unanswered (Spallanzani, 1768), and later that century bony fish were shown to regenerate amputated fins (Broussonet, 1786). At the time, luminaries like Spallanzani and Bonnet debated whether regeneration is a version of preformation relying on "germs" or miniature versions of adult structures (Dinsmore, 1991). This concept faded as experimental embryology surged a century later and when Morgan studied regeneration in various creatures prior to his better-known work in *Drosophila* genetics. Morgan classified appendage regeneration as an "epimorphic" process that hinges on cell proliferation at the injury site, and some of his important investigations of regeneration involved the study of pattern renewal after a series of elaborate amputation injuries to killifish fins (Morgan, 1901).

Axolotls have become a popular model for limb regeneration, and zebrafish for fin regeneration, because of the research tools that have been developed for studying these animals. Teleost fins and urodele limbs are structurally distinct, but it is clear from years of work that they progress through similar fundamental regeneration stages. Following an amputation injury, epithelial cells migrate to cover the wound site, and a multilayered epidermis forms. Proliferation in the underlying mesenchymal compartment, which is controlled in part by influences of the wound epidermis, generates a cell mass called the blastema. Multiple structures and factors have been shown to modulate blastemal proliferation, including nerves, specialized glands, vasculature, and activators/inhibitors of classic developmental signaling pathways (Kumar and Brockes, 2012; Nacu and Tanaka, 2011). In limbs, the blastema grows to a large mass that is then patterned into the upper arm, lower arm, and hand segments. In regenerating fins, new structures grow by a process that maintains a proliferative blastemal compartment in the distal region of each individual bony fin ray, while simultaneous osteoblast patterning events occur proximal to this growth to direct bone matrix deposition. In each case, pattern is restored across multiple axes to the complex structure.

Appendage regeneration has been reviewed many times, and key aspects and classic experiments not covered here are examined in recent publications (Kumar and Brockes, 2012; Monaghan and Maden, 2013; Nacu and Tanaka, 2011; Simon and Tanaka, 2013). We focus here on features of regeneration that arguably are most germane to the lost form that is recovered: activating the cellular sources, recalling positional identities, and slowing/stopping the process. Very recent discoveries we discuss here (and others outside the scope of this review) have established pivotal concepts and mechanisms that are anticipated to direct future investigations of appendage regeneration.

The Starting Materials

Much has been learned from studies of developing embryos about how appendages first form and acquire skeletal pattern along the proximodistal (PD), anteroposterior (AP), and dorsoventral (DV) axes (Zeller et al., 2009). This information has been applied to generate molecular markers and to suggest mechanisms of various aspects of limb regeneration (Nacu and Tanaka, 2011). Yet, while a limb bud forms and is patterned concomitantly with morphogenesis of other tissues in the embryo proper, a blastema emerges from cells engaged in the homeostasis and

function of a differentiated adult structure, within an organism that may have reached its final developmental stage years prior to insult. Knowing which cells give rise to the blastema, and whether these cells maintain or switch lineages, is the terminus a quo for most questions in appendage regeneration.

The source or sources of the blastema, and the diversity and developmental potential of its cellular constituents, have been under continual investigation for several decades. For some in the field, the term ''blastema'' has implied a homogeneous population of stem cells, each with an equal ability to differentiate in one of multiple directions. Additionally, the dominant view in appendage regeneration has been that blastemal cells are primarily derived from the reversion of a differentiated state—commonly referred to as ''dedifferentiation,'' and at its extreme is analogous to reprogramming phenomena induced by defined factors. In 2009, Kragl and colleagues examined this first idea by specifically labeling most major limb cell types in the axolotl by grafting the embryonic region that produces that limb tissue from green fluorescent protein (GFP)-labeled transgenic donors into unlabeled host embryos, or by directly grafting a specified GFP^+ limb tissue to an unlabeled host (Kragl et al., 2009). Their analyses of labeled, regenerating limbs produced a theme of lineage restriction. That is, regenerated cell types largely retain their developmental identity as they transition through the blastemal stage, and do not normally demonstrate a potential to create diverse cell types. These findings support the idea of a compartmentalized, rather than homogeneous, blastema. Transgenic technologies have also matured rapidly for the zebrafish model system, and recent studies asked similar questions with respect to the different cell types in regenerating fins by genetic fate-mapping and mosaic transgene analysis (Knopf et al., 2011; Singh et al., 2012; Sousa et al., 2011; Stewart and Stankunas, 2012; Tu and Johnson, 2011). These studies indicated that fin cells largely remain restricted to give rise to like cells, whether they are epidermis, endothelium, fibroblasts, or osteoblasts. Along with lineage-tracing of various cell types during mouse digit tip regeneration and even crustacean limb regeneration, the results support an evolutionarily conserved model of a compartmentalized blastema (Konstantinides and Averof, 2014; Lehoczky et al., 2011; Rinkevich et al., 2011).

From this composite of work, several interesting questions arose, with some of these questions addressed in more recent studies. For example, to what extent are tissue origins developmentally plastic; in other words, can secondary sources be induced to replace lost cells? In fins, which contain intramembranous bone and lack skeletal muscle, osteoblasts are the primary cell type of interest, and Cre-recombinase-based fate mapping demonstrated that osteoblasts only give rise to other osteoblasts (Knopf et al., 2011). Yet, when the vast majority of fin osteoblasts were genetically ablated, Singh et al. (2012) found that osteoblasts recovered and fins regenerated with normal rate and pattern. In this scenario, newly formed osteoblasts could not be traced to preexisting osteoblasts and ostensibly regenerated de novo from a secondary source (Singh et al., 2012). Thus, there is a degree of plasticity that allows other cell types to make osteoblasts under unique conditions, although the identities of these alternative source cells remain to be uncovered by informative molecular markers and lineage-tracing. Classic experiments in salamander limbs suggest that analogous plasticity exists in amphibians (Dunis and Namenwirth, 1977; Namenwirth, 1974; Thornton, 1938).

As alluded to above, it is possible that lineage restriction involves dedifferentiation to enable a proliferative state, but activation of a restricted progenitor cell is also a plausible mechanism. During zebrafish fin regeneration, live imaging visualized the reduction in expression of *osteocalcin*, a factor secreted by differentiated osteoblasts. This change, and ultrastructural changes detectable by electron microscopy, indicated that osteoblasts undergo some degree of dedifferentiation (Knopf et al., 2011). The key limb cell type to assess in this respect is skeletal muscle, which regenerates via a satellite cell compartment in mammals but has been investigated over many decades as a potential example of dedifferentiation in salamanders. Various studies examining histology, transplanted cells, or in vitro cultured myotubes have supported the idea that muscle dedifferentiation occurs as the newt blastema forms (Kumar et al., 2000, 2004; Lo et al., 1993; McGann et al., 2001). However, salamanders are known to contain a $PAX7^+$ satellite cell population, and transplanted newt satellite cells have been shown to support new muscle regeneration (Morrison et al., 2006).

Using *Cre-loxP* genetic fate mapping during limb regeneration in newts and axolotls for the first time, Guzmán and colleagues recently reassessed the endogenous contributions by these two potential sources (Sandoval-Guzmán et al., 2014). The authors tagged differentiated muscle cell nuclei in newts via a transient transgenic genetic fate-mapping approach and then traced the labeled cells through regeneration. They found that labeled myofibers trace into the blastema after amputation, where they occasionally mark cells positive for a proliferation marker and/or negative for a contractile marker. There was no evidence that muscle satellite cells were derived from labeled myofibers (Figure 1A). At later stages of regeneration, new myofibers contained the lineage label, similarly indicating derivation from differentiated muscle cells. Surprisingly, the authors found opposing results in axolotls using a similar fate-mapping technique. In this species, whereas myofibers underwent morphological changes at the amputation plane, contributions to the regenerated limb were not detected. Instead, the authors found that $PAX7^+$ cells are abundant in the axolotl blastema, much more so than in the newt blastema, making satellite cells a clear candidate cell type as the main source of regenerated muscle in axolotl limbs (Figure 1B). Thus, there appear to be unexpected fundamental differences in the origins of blastemal cells and regenerating tissue between two salamander species. It will be critical, as the authors point out, to directly mark and trace the endogenous satellite cell populations in axolotl and newts using the most rigorous possible methodology to determine the scope of their contributions. These intriguing findings route conversation to perhaps the most common question surrounding limb regeneration—why is it limited to a group of vertebrate species? Although the capacity for limb regeneration is unique to salamanders among tetrapods, selective pressures appear to have forged distinct paths in two species to maintain high regenerative potential. Mammals had other evolutionary priorities, but these studies imply that reawakening an ancestral program for regenerating complex muscle from an appendage stump has a flexible entry point that could include manipulation of the