Tales of Regeneration in Zebrafish

Kenneth D. Poss, Mark T. Keating, and Alex Nechiporuk

Complex tissue regeneration involves exquisitely coordinated proliferation and patterning of adult cells after severe injury or amputation. Certain lower vertebrates such as urodele amphibians and teleost fish have a greater capacity for regeneration than mammals. However, little is known about molecular mechanisms of regeneration, and cellular mechanisms are incompletely defined. To address this deficiency, we and others have focused on the zebrafish model system. Several helpful tools and reagents are available for use with zebrafish, including the potential for genetic approaches to regeneration. Recent studies have shed light on the remarkable ability of zebrafish to regenerate fins. Developmental Dynamics 226:202–210, 2003. © 2003 Wiley-Liss, Inc.

Key words: zebrafish; wound healing; regeneration; fin; limb; blastema; fgf; Shh

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INTRODUCTION

Damaged vertebrate organs heal through one of two mechanisms, repair or regeneration. Repair is characterized by heavy inflammation, fibrosis, and formation of a collagen-rich connective tissue scar believed to be permanent. Regeneration is the process by which damaged or lost structures are perfectly or near-perfectly replaced. Mammals contain several organ systems capable of regeneration, such as blood and liver, but the majority of organs heal by scarring. In contrast, many invertebrate and lower vertebrate species have mastered the art of regeneration (Dinsmore, 1991). Over the past few centuries, regeneration has been studied as a means to understand multiple concepts, from the existence of a soul to the development of an embryo. Today, investigation of regeneration in lower vertebrate model systems complements the modern field of stem cell research. That is, if we understand how regeneration occurs naturally in these organisms, we can learn how to optimize regenerative medicine in humans.

Perhaps the most spectacular regenerative events in vertebrates represent epimorphic regeneration. Thomas Hunt Morgan used the term “epimorphosis” to define regenerative processes involving proliferation (Morgan, 1901a). A more contemporary definition of epimorphic regeneration reflects regeneration with the presence of a specialized structure called the blastema, and the reconstitution of complex tissue with multiple cell types. The blastema is typically defined as a mass of proliferative, pluripotent progenitor cells and is a key intermediate during regeneration of the limb in salamanders and the fin in teleosts. Despite the historical significance of the field and the clear implications for biomedical progress, surprisingly little is understood regarding how the blastema forms and functions in any model system. In fact, the molecular dissection of regeneration has lagged considerably behind that of vertebrate embryology.

With the advent of new molecular tools, genome sequencing, and new attention to the field, the study of regeneration in model organisms is experiencing a rebirth. Here, we will focus on epimorphic regeneration of fins in the zebrafish, a model organism intensely used by developmental geneticists. Our review will highlight advantages of the zebrafish fin for studying regeneration, describe what is known about events that facilitate regenerative processes in the fin, and outline promising approaches with the potential to yield novel information.

ZEBRAFISH CAUDAL FIN AS A MODEL SYSTEM FOR STUDYING REGENERATION

For many years amphibians have been the organisms of choice for studying vertebrate regeneration. They are true champions of regener-
ation, able to regrow limbs, tail, eye structures, jaw optic nerve, and spinal cord, with a partial response in the heart (for recent reviews, see Brockes et al., 2001; Brockes and Kumar, 2002). However, a lack of well-developed molecular and genetic approaches in these organisms precludes systematic screening for genes required during regeneration. On the other hand, the zebrafish (*Danio rerio*), a teleost fish that also regenerates multiple structures (fins, optic nerve, scales, heart, and spinal cord; Fig. 1A; Broussonet, 1786; Morgan, 1901b; Bereiter-Hahn and Zylberberg, 1993; Bernhardt et al., 1996; Becker et al., 1997; Poss et al., 2000b, 2000; Poss, personal communication).

**Fig. 1.** Fin regeneration in zebrafish. A: The adult zebrafish (3–4 cm long) contains five fin types capable of regeneration: an, anal; ca, caudal; do, dorsal; pec, pectoral; pel, pelvic. B: Fins are composed of several, segmented bony fin rays. Each ray is comprised of concave, facing hemirays (lepidotrichia) that surround connective tissue, including fibroblast-like cells, as well as nerves and blood vessels. The anterior of the fish in this image is to the left. C: Most caudal fin structures are replaced within 1–2 weeks of amputation (dpa, days postamputation). However, as regenerative growth slows to nearly imperceptible rates after this period, it is difficult to assess precisely when the regeneration program terminates. Interestingly, distal and proximal amputations require the same amount of time to replace the amputated portion (S. Johnson, personal communication).

**Fig. 2.** Fin regeneration stages depicted as longitudinal sections through a caudal fin (adapted from Poss et al., 2000b). The distal, regenerating end is at the top. Amputation plane is designated by a dashed line. A: Wound healing. Wound is closed within 12 hr postamputation (hpa) by migrating epithelial cells. B1: Blastema formation, disorganization of mesenchymal tissue. In the next 12 hr, the wound epidermis thickens, mesenchymal tissue between hemirays disorganizes, and cells migrate distally. B2: Blastema formation. The blastema, a mass of proliferative mesenchymal cells, is formed distal to the amputation plane. C: Regenerative outgrowth. During this stage, blastemal cells proliferate and differentiate to replace missing structures.
(2002b), is amenable to standard molecular and genetic manipulations. Available to zebrafish researchers are (1) the ability to perform mutagenesis screens (Driever et al., 1996; Haffter et al., 1996); (2) detailed genetic, radiation hybrid, and expressed sequence tag (EST) maps; (3) the nearly complete sequence of the zebrafish genome; and (4) well-developed transgenesis and knockdown techniques (Hi-gashijima et al., 1997; Nasevicius and Ekker, 2000). Other practical advantages of zebrafish include a short generation time, the ability to raise and maintain a large number of animals, and the availability of useful reagents and technologies generated by a rapidly expanding cadre of zebrafish embryologists.

Although each of five zebrafish fin types regenerates, the caudal fin is primarily used to assay regeneration. This organ is easily accessed for surgery, its injury does not compromise survival, and it possesses a relatively simple, symmetric structure with limited cell types. For instance, the caudal fin does not contain skeletal muscle, or endochondral bone formed by mineralization of cartilage. The fin skeleton instead has a dermal origin and mineralizes directly as bone. The zebrafish caudal fin is composed of multiple bony fin rays or lepidotrichia, most of which are bifurcated at the ends (Montes et al., 1982; Becerra et al., 1983; Santamaria and Becerra, 1991; Geraudie and Singer, 1992) (Fig. 1B,C). Each fin ray is composed of a pair of concave hemirays that consist of multiple segments joined end-to-end by ligaments. Blood vessels, nerves, pigment cells, and fibroblast-like cells are in mesenchymal compartments between rays, as well as in intraray spaces. Each hemiray is surrounded by a monolayer of bone-secreting cells called scleroblasts. Zebrafish fins grow continuously by the sequential addition of constant length segments to the end of the fin ray (Johnson and Bennett, 1999; Lovine and Johnson, 2000).

Figure 2 illustrates the stages of caudal fin regeneration (Becerra et al., 1983; Geraudie and Singer, 1992; Johnson and Weston, 1995; Johnson and Bennett, 1999; Poss et al., 2000b). Regeneration is highly dependent on temperature; regeneration at 33°C occurs nearly twice as quickly than at 25°C (Johnson and Weston, 1995). At 33°C, the wound is closed within 12 hr postamputation (hpa) by a thin layer of epithelium. The blastema is formed within the next 24 hr. During the outgrowth phase (beyond 48 hpa), cells of the proximal regenerate appear to differentiate into missing structures, whereas outgrowth is sustained by cell division in the distal parts of the blastema. We explain these stages in more detail below and discuss expression of some factors that are thought to play important roles during each stage. We will not discuss here processes of vascularization, innervation, and pigmentation during regeneration. We also do not have space to discuss each of the growing number of genes up-regulated during fin regeneration. These genes are summarized in Table 1.

## WOUND HEALING

Remarkably, there is little bleeding or inflammation after caudal fin amputation. Within 1 to 3 hr of injury, a thin, epidermal layer migrates to cover the wound. This response does not involve cell proliferation, as determined by bromodeoxyuridine (BrdU) incorporation assays (Santamaria et al., 1996; Polo et al., 2001; Nechiporuk and Keating, 2002; Santos-Ruiz et al., 2002), and can even occur in dissected and explanted fin regenerates (our unpublished observations).

Over the next 12 to 18 hr, the epidermis accumulates additional layers. This maturation process also appears to be dominated by migration events, not by proliferation. One of the earliest molecular markers for fin regeneration is β-catenin expression in wound epidermal cells, induced in the first few hours after amputation and maintained through regeneration (Poss et al., 2000a). Here, the augmented β-catenin is presumed to function in maintaining cell–cell interactions that facilitate migration.

By 18–24 hpa, during blastema formation, a basal epidermal layer composed of cuboidal cells has manifested adjacent to blastema

<table>
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<th>Domain</th>
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<td>Blastema</td>
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<td>Scleroblasts</td>
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References: 1, Monnot et al., 1999; 2, Poss et al., 2000a; 3, Akimenko et al., 1995; 4, Laforest et al., 1998; 5, Geraudie et al., 1998; 6, Brulfert et al., 1998; 7, Borday et al., 2001; 8, Poss et al., 2000b; 9, White et al., 1994.
tissue. Because of its proximity to the blastema and the expression of developmentally important genes in this layer during later stages, it is suspected to play a primary role in communicating growth and patterning signals throughout regeneration. Little is known about signals that govern the formation of the wound epidermis and its different layers, often referred to as the apical epidermal cap (AEC). The gene lef1 is essential for epithelial-mesenchymal interactions throughout vertebrate development, such as in the tooth bud, hair follicle, and mammary gland. lef1 mRNA is detectable in epidermal cells before manifestation of the basal layer, and afterward, clearly marks basal layer cells (Pospisil et al., 2000a). Although one can only speculate on the roles for Lef1 during regeneration, its expression suggests a role in identity, proliferation, and/or signaling from basal layer epidermal cells.

BLASTEMA FORMATION

A hallmark of epimorphic limb or fin regeneration is formation of the blastema, a developmental event that distinguishes regeneration from embryogenesis. The newly formed fin blastema is a proliferative mass of mesenchymal cells that sits atop each severed fin ray and ultimately gives rise to the new structures of the fin. During maturation of the epidermal cap, fibroblast-like cells, located as far proximal as one to two segments, disorganize and orient longitudinally. Experiments using a traceable fluorescent dye or BrdU pulse-chase labeling indicate that these cells migrate distally toward the amputation plane (Poleo et al., 2001; Nechiporuk and Keating, 2002; Santos-Ruiz et al., 2002). Concurrently with migration, proliferation is stimulated in these cells, and they reorganize to form the blastema.

Blastemal cells are thought to be less differentiated than mature fin cells. This belief has been inferred from their enhanced proliferation and migratory properties, but lineage tracing studies that might demonstrate pluripotency have not been reported. It is unclear whether blastema formation involves cellular dedifferentiation, the activation of quiescent stem cells, or both processes. Cellular dedifferentiation does appear to occur during newt limb blastema formation. For instance, labeled myotubes implanted into the blastema can yield labeled mononucleated blastemal muscle, and cartilage cells (Lo et al., 1993). By contrast, the fin does not contain structural cells with clearly differentiated phenotypes like myotubes, and such cellular manipulation is considerably more difficult in the adult zebrafish fin.

Suggestive evidence that cellular dedifferentiation might occur in the regenerating fin stems from the fact that the transcription factors msx2 and msx2a genes are induced in mesenchymal cells during blastema formation (Akimenko et al., 1995; Poss et al., 2000b). These data are intriguing, as msx1, a mammalian orthologue of msx2/c, is a candidate for inducing dedifferentiation in mammalian cells and/or maintaining cells in undifferentiated state. Msx1 is expressed in the progress zone of the developing vertebrate limb (Davidson et al., 1991; Wang and Sassoon, 1995). During muscle development, Msx1 down-regulates expression of the myogenic regulatory gene MyoD (Woloshin et al., 1995; Bendall et al., 1999; Thompson-Jaeger and Raghow, 2000), and has been shown to cause fragmenting and proliferation in a low percentage of cultured murine myotubes (Odelberg et al., 2000). Slow cycling mesenchymal cells that might represent structural stem cells have been sought after but not revealed during zebrafish fin regeneration (Nechiporuk and Keating, 2002). However, elegant genetic studies indicate that a pigment stem cell population is activated by amputation to replace melanocytes (Rawls and Johnson, 2000, 2001).

The mature wound epidermis is suspected to be a source of growth factors that stimulate formation of the regeneration blastema and maintain its function. In newts, animals that are conducive to grafting and fine surgical manipulations, it has long been known that the wound epidermis is required for limb regeneration. Removal of the newt wound epidermis halts regeneration until a new epidermis forms (Thornton, 1957). Moreover, attaching the limb stump to the body cavity, thus preventing reformation of the epidermis, blocks regeneration (Goss, 1956). It is presumed that the wound epidermis has a similar role in the zebrafish fin.

One factor that might stimulate blastema formation is Wnt5, a gene product detectable 12 hpa in the wound epidermis and at later stages in the basal epidermal layer (Poss et al., 2000a). Wnt5 expression and function is essential in the tail bud during embryogenesis, as the pipetail mutant strain displays reduced posterior proliferation and extension due to a disruption of the wnt5 gene (Rauch et al., 1997). Thus, it is possible that Wnt5 has a similar role in stimulating proliferation during blastema formation. However, fin regeneration in surviving adult pipetail (wnt5) mutants is grossly normal (our unpublished observations). To explain this finding, it is possible that (1) the pipetail allele (ppf1<sup>278</sup>) examined is not strong enough to disrupt fin regeneration, (2) Wnt5 does not supply an obvious essential function during regeneration, or (3) other Wnt signals compensate for Wnt5 deficiency.

It is appropriate to consider factors like Wnt5, with embryonic roles in limb, fin, or trunk outgrowth, as potential stimulators of fin blastema formation. Many studies have documented the roles of fibroblast growth factors during organogenesis (Ohuchi et al., 1997; Martin, 1998; Reifers et al., 1998; Peters and Balling, 1999). We recently showed that SU5402, a pharmacologic inhibitor of Fgfr1, potently inhibits blastema formation (Fig. 3A–D; Poss et al., 2000b). Incubation with SU5402 also blocks induction of msx2 and msx2a (Fig. 3E,F), as well as mesenchymal cell proliferation (Fig. 3G,H). These data indicated that Fgf signaling is required for blastema formation, consistent with the observation of fgf1 transcription in mesenchymal cells during this process. There are many vertebrate Fgfs, however, and it is unclear which factors might be functioning during regeneration. Wfgf, also known as germing Fgf, is detected...
by reverse transcriptase-polymerase chain reaction (RT-PCR) in the regenerate during blastema formation and by in situ hybridization in the epidermis during outgrowth, and is a candidate for this factor (our unpublished data, Poss et al., 2000b).

Finally, we should consider the role of nerves in promoting blastema formation. Experiments performed in the middle of the past century demonstrated that formation of a functional newt blastema was dependent on innervation (reviewed in Tsonis, 1996). This dependency has been investigated to some extent in larger teleosts, where data have supported the idea that nerves provide some factor that might stimulate blastemal proliferation (Gerardie and Singer, 1977, 1985). However, similar studies have not been reported in zebrafish.

**REGENERATIVE OUTGROWTH**

**Transition From Blastema Formation to Regenerative Outgrowth**

The switch from blastema formation to regenerative outgrowth is characterized by several important morphologic and molecular changes.

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**Fig. 3.** Fgf signaling is required for blastema formation, including cell proliferation and expression of the blastemal marker msxb. A: Fin from untreated fish at 4 days postamputation (dpa), showing normal regrowth and new segmentation. Arrows demarcate the amputation plane in each photograph. The distal, regenerating end is positioned toward the top in each image. B: Fin from fish treated with SU5402 (R, 1) for 4 days immediately after amputation. These fins showed no new growth. Here, the amputated edge appears saw-toothed due to the retraction of tissue between rays. C: Hematoxylin stain of 1 dpa fin regenerate section from untreated fish (asterisk denotes new blastema). D: Fin regenerate section from fish treated with R, for 24 hr. Note the lack of blastema. However, R-treated fin regenerates showed mesenchymal disorganization (arrowheads mark boundary between organized and disorganized tissue), as well as longitudinal arrangement suggestive of migration. E: Whole-mount in situ hybridization of msxb expression at 1 dpa, indicating strong blastemal expression. F: Expression of msxb is reduced or absent in fins treated with R, for 24 hr after amputation. G: An untreated bromodeoxyuridine (BrdU) incorporation control. Animals were incubated with BrdU from 42–48 hpa. H: Animals were treated with SU4022 from 40–48 hpa, and BrdU from 42–48 hpa. BrdU incorporation (brown) in proximal blastemal cells was dramatically reduced by drug treatment. Adapted with permission from *Developmental Biology* (Poss et al., 2000b).

**Fig. 4.** During regenerative outgrowth fin regenerate is subdivided into three zones: distal and proximal blastema and patterning zone. Anterior is at the left. Shown are longitudinal sections from 2 days postamputation (dpa, A) and 3 dpa (B–E) regenerates that were (A) stained with hematoxylin; (B) colabeled with antisense msxb RNA (red) and proliferating cell nuclear antigen (PCNA) antibody (green) and counterstained with DAPI (blue); (C) colabeled with antisense shh RNA (red) and PCNA antibody (green) and counterstained with DAPI (blue); (D) labeled with wfgf antisense RNA (violet) and photographed by using Nomarski optics; E: colabeled with antisense wnt5 RNA (violet) and PCNA antibody (red) and photographed by using Nomarski optics. Note that the proximal extension of wnt5 signal in the basal epidermis (arrowheads) corresponds to the extent of proliferation (red PCNA signal) in the underlying mesenchyme. wnt5 expression is occasionally detected in the distal blastema, as indicated in this image. b, blastema; db, distal blastema; le, lepidotrichia; pz, patterning zone; pb, proximal blastema; s, scleroblasts. Scale bars = 50 μm in A–E.)
This transition occurs at approximately 4 days postamputation (dpa) at 25°C and 2 dpa at 33°C. At this point, the mesenchymal part of the regenerate consists of a mature blastema and proximally positioned differentiating tissue, consisting of at least two cell types, scleroblasts and fibroblast-like cells (Fig. 4A). Scleroblasts can be visualized by staining with the monoclonal antibody ZNS-5 (Johnson and Weston, 1995).

Analyses of the blastemal cell cycle demonstrate that its kinetics dramatically change during the transition from blastema formation to outgrowth (Nechiporuk and Keating, 2002). The median G2 length during blastema formation is greater than 6 hr, whereas it is only 1 hr during outgrowth. This difference suggests that overall length of the blastemal cell cycle during regenerative outgrowth is much shorter than during blastema formation. The extremely fast rate of the blastemal cell cycle is not unexpected, if one considers that it takes as little as 7 to 14 days for the amputated fin to regain most of its original size. Somewhat surprising is the relatively slow cell cycle during blastema formation. However, this finding may reflect the timing necessary for reorganization of the blastema and other parts of the regenerate in preparation for outgrowth.

At a molecular level, the transition to outgrowth is highlighted by changes in expression levels and/or patterns of existing genes as well as up-regulation of new genes. For instance, the level of wfgf expression is markedly higher during outgrowth than during blastema formation, when assayed by semiquantitative RT-PCR (our unpublished observations). In addition, the pattern and expression level of the blastemal marker msxb changes during the transition to regenerative outgrowth. msxb has a diffuse mesenchymal expression pattern during blastema formation. By contrast, during regenerative outgrowth, the msxb expression domain becomes limited to the distal blastema and is more highly expressed (Fig. 4B; Akimenko et al., 1995; our unpublished data). On the other hand, wnt3a is not detectable during blastema formation, but it is up-regulated in the distal portion of the epithelium during regenerative outgrowth (Poss et al., 2000a).

### Blasticom Partmentalization During Outgrowth

Recently, we discovered that the fin blastema is subdivided into two domains at the onset of regenerative outgrowth, the distal (or distal-most) and proximal blastema (DB and PB, respectively; Nechiporuk and Keating, 2002). These blastemal zones have different cellular and molecular characteristics. The distal blastema consists of non- or slow-proliferative cells, which are msxb-positive (Fig. 4B). Santamaria et al. (1996) also suggested that BrdU incorporation is limited in the distal portion of goldfish blastema during outgrowth. The intensely proliferative PB is located immediately proximal to the DB and is positive for the proliferation marker proliferating cell nuclear antigen (Fig. 4B,C). There is no defined boundary between these two zones, but instead there exists a steep proliferation gradient that extends ~50 μm, or 10 cell diameters. Finally, immediately proximal to the PB is a zone of moderate proliferation activity, called the patterning zone (PZ, or differentiation zone), which consists of scleroblasts and presumably differentiating mesenchymal cells. The distal edge of the epidermal sonic hedgehog (shh) expression domains aligns with the border between the PB and PZ (Fig. 4C). Shh is a secreted molecule that is involved in the patterning of various structures during vertebrate development (reviewed in Ingham and McMahon, 2001). At least two interesting questions remain regarding this subdivision of the mesenchymal portion of the regenerate. First, how are these zones set up and maintained? Second, what is the functional significance of blastemal subdivision?

It is likely that the DB, PB, and PZ are set up and maintained through epithelial-mesenchymal interactions. The regulation of mesenchymal msxb/c expression by epidermally released Wfgf might comprise one of these interactions. It has been suggested that msx genes are responsible for maintaining cells in an undifferentiated, pluripotent state (Muneoka and Sassoon, 1992; Song et al., 1992; Odelberg et al., 2000). Furthermore, in the developing chick limb, MSX2 is expressed in nonproliferating limb mesenchyme (Ferrari et al., 1998). Thus, it is possible that msx genes expression regulates the balance between pluripotency and differentiation in the fin blastema. Low levels of msxb/c expression during blastema formation correspond to the slow cell cycle, whereas high msxb/c expression might maintain DB cells in a non- or very slow-proliferating state. Factors secreted from the AEC, including Wfgf, might be involved in the induction and maintenance of msxb/c expression in the blastema (Fig. 4D). When Fgf signaling is blocked with SU5402 during various stages of fin regeneration, it down-regulates blastemal msxb/c expression (Poss et al., 2000b). Also, levels of epidermal wfgf expression appear to correlate with the levels of mesenchymal msxb/c during blastema formation and regenerative outgrowth (our unpublished observations).

Treatment of regenerating fin with SU5402 rapidly blocks proliferation. However, mesenchymal fgfr1 expression is restricted to the DB. Therefore, it is unclear whether Fgf signaling has a direct or indirect role in the induction and maintenance of blastemal proliferation in the PB. Fgf signals yet to be determined may directly stimulate proliferation in the PB through Fgf receptor subtypes other than Fgfr1. Or, blocking Fgf signaling during outgrowth might disrupt the supply of the blastemal progenitors (see below), and indirectly block proliferation. In addition to Fgfs, other molecular factors may promote and/or maintain blastemal proliferation. As mentioned earlier, wnt5 is expressed throughout outgrowth in the basal epidermal domain adjacent to the proliferating PB cells.

The proximal limit of the PB is delineated by epidermal expression of shh (Fig. 4C). Recent studies suggest that Shh is responsible for scleroblast alignment and proliferation. Quint et al. (2002) demonstrated that ectopic expression of shh or bone morphogenetic protein 2b (bmp2b,
member of the transforming growth factor-β family) by direct plasmid injection leads to ray fusion. On the other hand, cyclopamine, a specific Shh inhibitor (Cooper et al., 1998), blocks fin regeneration and alters expression patterns of shh and its receptor, patched 1 (ptc1). Of interest, ectopic matrix deposition caused by shh injection was inhibited by coinjections with chordin, a direct bone morphogenetic protein antagonist (Piccolo et al., 1996), indicating that Bmp signaling pathway is required for bone formation. Overall, the significance of the observed ectopic bone deposition is unclear, as it remains to be determined whether Shh is responsible for patterning (i.e., aligning scleroblast) or promoting scleroblast differentiation. We should also note that it is unknown whether new scleroblasts are derived from differentiated blastemal cells, or division of preexisting scleroblasts, or both processes.

What is the functional significance of these blastemal zones? We propose that the DB cells represent a pool of blastemal progenitors. Under this model, the size of this progenitor pool might be determined by the expression levels of growth factors in the AEC, potentially by Wgf and/or other Fgfs. During regenerative outgrowth, DB cells slowly divide and their progeny enter the PB proliferative zone. In contrast, proliferation in PB drives regeneration (Poss et al., 2002a; our unpublished results).

As with all genetic screens, attempts to detect TS regeneration mutants also have limitations. Only 10% or less of all N-ethyl-N-nitrosourea-induced mutations are thought to be TS (Suzuki et al., 1967; Bartel and Varshavsky, 1988), and it is unlikely that this type of screen could approach saturation. Certainly, some genes might not yield any TS alleles. Furthermore, it is difficult to target specific processes like formation of the AEC or blastema. Thus, forward genetic approaches must be complemented by methods that test candidate gene function during fin regeneration.

For candidate gene testing, the heat-inducible, hsp70 promoter system might represent a tool to ectopically express candidate genes in the regenerating fin (Halloran et al., 2000). However, generation and testing of such inducible lines is a time-consuming process, and normal expression from the promoter or leakiness in transgenic lines during embryogenesis could have lethal effects. Therefore, it will be beneficial to further explore the recently described direct plasmid injection system using other genes expressed during fin regeneration (Quint et al., 2002) or to test viral approaches.

Removing candidate gene function in the adult fin is also crucial for understanding regeneration. Pharmacologic studies allowed testing the roles of Fgf and Shh signaling during regeneration (Poss et al., 2000b; Quint et al., 2002). However, there is a limited number of chemical with specific effects on signaling pathways, and it is often difficult to obtain these in quantities sufficient for use with adult zebrafish. Morpholino technology, widely used by zebrafish embryologists, is not immediately applicable to adult zebrafish. It is possible that new RNA interference technology might be effective in zebrafish, as it disrupts gene expression in mammalian cells (Jacque et al., 2002; Lee et al., 2002; Leirdal and Sioud, 2002; Lewis et al., 2002; Paddison et al., 2002a,b; Paul et al., 2002; Sui et al., 2002; Yu et al., 2002).

Many recent studies have reported successful generation of transgenic zebrafish lines containing green fluorescent protein driven by promoters of zebrafish origin (e.g., Motoike et al., 2000; Park et al., 2000; Koster and Fraser, 2001). Transgenic lines that mark specific tissues or cell types during regeneration will be extremely useful for lineage analyses and isolation of various cell populations.

**FUTURE APPROACHES AND PERSPECTIVES**

Zebrafish genetics facilitates the molecular dissection of biological processes. Most large-scale mutagenesis screens have focused on embryonic development, an approach that has proved successful in dissecting vertebrate developmental pathways (Driever et al., 1996; Haffter et al., 1996). Genetics can also be applied to regeneration. However, an important prediction of a genetic screen for regeneration defects is that many, if not all, of the genes necessary for adult regeneration also will be required during embryonic development. As zebrafish survive well at a wide range of temperatures, selecting for conditional, temperature-sensitive (TS) mutants addresses this issue. In 1995, Johnson and Weston carried out a TS screen to find adult zebrafish mutants with defects in fin regeneration. They isolated several mutants, two of which (reg5 and reg6) were described in detail (Johnson and Weston, 1995). A similar strategy has been adopted in our laboratory to isolate TS mutants defective in fin regeneration (Fig. 5). From this screen, we have cloned two genes essential for fin regeneration (Poss et al., 2002a; our unpublished results).

As with all genetic screens, attempts to detect TS regeneration mutants also have limitations. Only 10% or less of all N-ethyl-N-nitrosourea-induced mutations are thought to be TS (Suzuki et al., 1967; Bartel and Varshavsky, 1988), and it is unlikely that this type of screen could approach saturation. Certainly, some genes might not yield any TS alleles. Furthermore, it is difficult to target specific processes like formation of the AEC or blastema. Thus, forward genetic approaches must be complemented by methods that test candidate gene function during fin regeneration.

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Removing candidate gene function in the adult fin is also crucial for understanding regeneration. Pharmacologic studies allowed testing the roles of Fgf and Shh signaling during regeneration (Poss et al., 2000b; Quint et al., 2002). However, there is a limited number of chemicals with specific effects on signaling pathways, and it is often difficult to obtain these in quantities sufficient for use with adult zebrafish. Morpholino technology, widely used by zebrafish embryologists, is not immediately applicable to adult zebrafish. It is possible that new RNA interference technology might be effective in zebrafish, as it disrupts gene expression in mammalian cells (Jacque et al., 2002; Lee et al., 2002; Leirdal and Sioud, 2002; Lewis et al., 2002; Paddison et al., 2002a,b; Paul et al., 2002; Sui et al., 2002; Yu et al., 2002).

Many recent studies have reported successful generation of transgenic zebrafish lines containing green fluorescent protein driven by promoters of zebrafish origin (e.g., Motoike et al., 2000; Park et al., 2000; Koster and Fraser, 2001). Transgenic lines that mark specific tissues or cell types during regeneration will be extremely useful for lineage analyses and isolation of various cell populations.
tions. For example, generation of GFP lines that mark DB cells (e.g., mxb/c promoter-GFP) may provide a useful tool for studying different blastemal zones and determining cell fates. Potentially, expression profiles of these cell populations can be studied by using zebrafish cDNA microarrays. In addition, such transgenic lines might be used during mutagenesis to increase the specificity of the genetic screen.

A combination of forward and reverse genetic approaches should garner a new understanding of zebrafish fin regeneration. As information about mechanisms of fin regeneration accrues, it will be important to compare mechanisms of fin regeneration and other types of regeneration in zebrafish and urodèles. Pursuit of these mechanisms should also have relevance as scientists in related fields try to enhance regeneration in mammalian organs.

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