CHAPTER 16

Growth Control in the Ontogenetic and Regenerating Zebrafish Fin

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The growth and regeneration of the zebrafish fin provide yet another opportunity to exploit genetics to study important vertebrate problems. Mutants have been identified in zebrafish that affect the development of the embryonic fin, disrupt the normal growth relationship of fin and body, or disrupt the regeneration of the fin. Analysis of a regeneration mutation suggests that the developmental checkpoints that ensure developmental integrity in normal growth are absent in the early stages of regeneration. These stages correspond to the only time in fish development when differentiated bone cells divide.

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I. Introduction

"Most of them grow as long as they live, and apparently live until they fall victim of some stronger species."

—David Starr Jordan, 1905

With this simple observation, the great ichthyologist Jordan sums up for us one intriguing difference between the fishes and mammals—the potential for unlimited growth. Despite this potential for unlimited growth, fish show remarkable properties of growth control. Different species grow at different rates to attain different typical sizes—contrast the tiny zebrafish with the mighty sturgeons or the great sharks. Another sign of exquisite growth control is the great variety of shapes attained by different fishes—consider the simple tube shape of the eels, the disk shape of angel fish, and the specialized heads of cod or hammerhead sharks. Differences in the shapes and sizes of fins also speak to the problem of growth control. How, for instance, do the different rays of caudal fins achieve the length relative to one another in order to achieve the bilobed caudal fin of the zebrafish, the paddle-shaped fin of the American minnow (*Fundulus*), or the elaborate swords of the swordtail (*Xiphophorus*)?

The fins of many fish have an even more remarkable property of growth control. When amputated, most fins will regenerate to restore the original size and shape of the missing fin. The property of fin regeneration was first observed by Broussonet (1786), following up the remarkable observations of animal regeneration first by Trembley (1744) in hydra, repeated by Reamur (1742), and extended to worms and other forms by Bonnet (1745) and Spallanzini (1768). For a thorough description of the early history of regeneration research in fish and other animals and its role in the foundations of developmental biology, the reader is urged to read Morgan (1901) on regeneration. Questions of growth control, patterning, and the underlying evolutionary changes that led to differences in fin morphology or are responsible for the precise regeneration of the fin have captured the imagination of early biologists, such as Morgan (1900) and Goss and his colleagues (see Goss and Stagg, 1957; Tassava and Goss, 1966), and later workers employing the tools of molecular biology (Akimenko *et al.*, 1995; Sordino *et al.*, 1995) and genetics (Johnson and Weston, 1996; van Eeden *et al.*, 1996).

Before we can delve into the specific properties of regeneration, we must first acquaint ourselves with the basic anatomy of the fin. In Fig. 1A (see color plate) we used Alizarin Red and Alcian Blue staining of a whole fish skeleton to reveal the organization of the caudal fin rays. This preparation rather simply shows that the caudal fin is composed of multiple branched rays (stained red) that articulate at the base of the fin to bony plates that are themselves articulated to the vertebra. Each fin ray is in turn composed of multiple segments that are separated by joints (stained blue in this preparation). The growth of the fin on

its long axis is achieved by adding new segments to the distal tip of the growing fin ray. Thus, once established, fin-ray segments do not increase in length as the fin grows. Each ray is actually a pair of hemirays, as revealed in the longitudinal section (Fig. 1C) and in the transverse section (Fig. 1B) of regenerating fins. The mature hemiray, or lepidotrichium, is surrounded by a monolayer of osteoblasts that synthesize the bone matrix. In Fig. 1C, differentiated osteoblasts are labeled specifically with the monoclonal antibody ZNS5 (Johnson and Weston, 1996), allowing for the unambiguous distinction between osteoblasts and fibroblasts in regeneration studies. Protected between the hemisegments are the blood vessels, nerves, pigment cells, and fibroblast-like cells. Surrounding the dermal compartment is the epidermis, separated from the osteoblasts and fibroblasts by a typical basement membrane. For our discussion of growth control, we will only consider the $ZNS5^+$ differentiated osteoblasts and the $ZNS5^-$ unlabeled fibroblasts or undifferentiated mesenchyme.

The regeneration of the fin occurs in several discrete stages. These regeneration stages have been described for a variety of fishes (Goss and Stagg, 1957; Becerra *et al.,* 1996) and vary depending on the fish and other conditions, such as the temperature. Here we will consider the stages of regeneration attained for a typical zebrafish caudal fin challenged to regenerate at 25°C (Johnson and Weston, 1996). In the first day following amputation, wound-healing acts to cover the stump with a thin layer of epidermis. The overlaying epidermis thickens over the stump, and by the second day after amputation fibroblasts and osteoblasts near the amputation plane in the stump appear generally looser and begin to migrate distally to form a regeneration blastema distal to the amputation plane by the third day. New bone matrix is first apparent between regeneration osteoblasts between three and four days of regeneration, and new joints are typically visible by dissection microscope by the fifth day of regeneration. At 33°C, zebrafish regeneration typically achieves each of these landmarks in about one-half the time as at 25°C. By convention, regeneration in the zebrafish caudal fin at these different temperatures is referred to in terms of regeneration at 25°C.

Several lines of evidence point to the supremacy of the fin ray in the growth control of the regenerating fin. Broussonet (1786) first concluded from observations of regenerating fins that some part of the fin ray (or "osselets") is required for the regeneration. Nabrit (1929) came to the same conclusion, finding that when he first removed fin rays from regions of the caudal fins in *Fundulus,* allowed the fin to heal, then amputated portions of the fin, the fin failed to regenerate in regions where the fin ray had been completely removed. Birnie (1947) found that transplanted goldfish fin rays regenerate to an extent commensurate with the original location of the fin ray rather than to the extent of neighboring fin rays, further speaking to the autonomous role of each fin ray in controlling its own regeneration. Critical roles for the nerve supply have also been indicated for fin regeneration (Goss and Stagg, 1957), similar to the requirement for nerves in salamander limb regeneration (Singer, 1952).

II. Mutations Affect the Growth, Development, and Regeneration of Fins

A. Mutations Affecting the Development of the Embryonic Pectoral Fin and Fin Fold

Large-scale mutant screens from several labs have identified upwards of 30 loci affecting the development of embryonic fins. A preliminary description of one of these mutant collections is available (van Eeden *et al.,* 1996). The initial characterization of these mutants has suggested that they can be divided into several categories. These include mutations that apparently result in the degeneration of pectoral fins and fin folds (8 loci). Most (six of eight) of the mutations in this class are viable. In the initial study, adult phenotypes for this class of mutation are described as subtle and may vary from individual to individual. For instance, mutants of the *pinfin* locus have a variably reduced number of lepidotrichia in the caudal fin of the adult animal. A second major class of mutations includes those that affect the development of the ventral fin fold, but not of the pectoral fin (6 loci). Notably, these include mutations that result in ventralization or dorsalization along the dorsal–ventral axis of embryo, such as *dino, mercedes,* and *swirl.* A third major class (15 loci) includes mutations that affect the pectoral fin with little or no affect on the fin fold. Among these are mutants that cause small pectoral fins (11 loci). Interestingly, 5 of the 11 loci that cause smaller pectoral fins also cause reduced pharyngeal arches, suggesting common roles for the genes that affect pectoral fin development and arch development. Mutants at two other loci, *dakel* and *boxer,* have smaller pectoral fins and abnormal jaws, but not reduced gill arches. *In situ* hybridizations of *dakel* mutants shows that the signaling molecule *shh* is initially expressed at low levels in *dakel* pectoral fins, and by 32 hours development *shh* expression has disappeared from *dakel* fins. Presumably the *dakel* mutation causes a defect in the *shh* signaling pathway that is sufficient to result in defects in limb growth similar to that shown for *shh* mutants in mouse (Chiang *et al.,* 1996). Thus, it seems likely that the genetic analysis of embryonic fin development in the zebrafish will continue to enhance our understanding of the signaling pathways that control the patterning and growth of the mammalian limb.

B. Mutations Affecting Growth of the Adult Fin

Relatively few mutations have been identified that affect the development of the adult fin. This can be attributed to several causes. For instance, we might expect some fraction of the genes that affect the embryonic fins to also affect the development of the adult fins. Because many embryonic fin mutants are lethal, the affect of these genes on the growth and development of the adult fin cannot yet be ascertained. Additionally, it is important to remember that the embryonic fins and the adult fins are dramatically different tissues. Most of what we see externally as fin in the adult is bony fin, a tissue that is not present in

the embryo. Thus, we might also expect that phenotypes recognizable in the embryo are a poor prognostic for growth phenotypes of the adult bony fin rays. Most large-scale mutant screens have focused on embryonic phenotypes. F3 animals in those mutant screens were not uniformly and systematically reared to adult stages to look for defects in the bony fin rays. Mutant screens employing parthenogenetic reproduction to render new mutations homozygous are more useful for screens targeting adult phenotypes (Johnson and Weston, 1996). Nevertheless, a handful of interesting mutations have been identified with profound affects on development of the adult fin. In the large-scale screens, two notable mutations were found; including *finless* (*fls*^{ts370f}) whose mutants show no embryonic fin defect but completely lack adult fins, and *another long fin* (*alf*^{dty86d}), which causes an overgrowth of the fin similar to the previously described *long fin* (*lof*) mutation (Tresnake *et al.,* 1981; Johnson and Weston, 1996). Presumably *lof* and *alf* cause defects in growth control regulation that couples fin growth to body growth (Johnson and Weston, 1996; S. Johnson, unpublished). The dominant overgrowth mutants *lof* and *alf* are joined by a recessive mutation, *short fin* (*sof*^{b123}), a spontaneous mutation isolated by Charline Walker at the University of Oregon using heat-shock parthenogenesis to generate homozygous zebrafish. The *sof* mutants have normal-shaped fins and fin rays that are approximately half the size of normal fins (S. Johnson, unpublished). It remains to be demonstrated that the dominant *lof* and *alf* mutations are distinct loci from the recessive *sof* locus, a problem best resolved by placing each mutation on the zebrafish genetic map (Johnson *et al.,* 1996). Clearly, the study of overgrowth and undergrowth mutations, such as *lof, alf,* and *sof,* and of newer, less characterized mutations arising in adult parthenogenesis screens may provide new insights into the problem of proportionate growth and allometry.

C. Mutations Affecting Regeneration of the Fin

Traditional studies of fin or limb regeneration in fishes and salamanders have been held back by the scarcity of mutations that affect or prevent regeneration. We might expect that many or most of the genes involved in the regeneration of the fin or limb might also be involved in the initial development or growth of fin or limb. For instance, axolotl *short toes* mutants fail to regenerate amputated limbs (Rio-Tsonis *et al.,* 1992) or show impaired regeneration (Gassner and Tassava, 1997). Mutations in other genes required for the regeneration of the fin might be lethal, resulting in embryos that die prior to development of the adult fin or limb. The problem of studying the late roles for genes with requirements in the early stages of development is traditionally approached by generating conditional mutations. Because zebrafish are cold-blooded and develop normally over a wide range of temperatures (23–34°C), one might reasonably expect to identify temperature-sensitive mutations that have no effect on embryonic development and fin growth at low temperatures but that disrupt the regeneration of the fin at high temperatures. Such screens have been successful employed to

identify seven regeneration mutations (Johnson and Weston, 1996), including *reg5* and *reg6* described here.

The screen for regeneration mutants, outlined in Fig. 2, is simple. Parthenogenetic techniques of reproduction, such as early pressure (EP) (Streisinger *et al.*, 1981) are used to render homozygous the newly induced mutations from mutagenized stocks. Parthenogenesis techniques are essential for screening for relatively rare phenotypes such as temperature sensitivity because they allow large numbers of independent mutations to be rendered homozygous in a single generation. Briefly, EP parthenogenetic progeny are reared at permissive temperature—typically 25°C, for around 6 to 8 weeks of development. Ideally, in well-mutagenized stocks ~50% of the progeny that would normally survive EP parthenogenesis are homozygous for embryonic lethal mutations and fail to live to adult stages. The remaining EP progeny are potential conditionally expressing mutants and can be screened for temperature-sensitive defects in fin regeneration by amputating approximately one-half of the caudal fin and shifting the fish to 33–34°C. By the end of 1 week, most fish will have regenerated 1–2 millimeters of fin, typical of wild-type stocks. Mutants that have failed to regenerate can then be easily detected in swimming populations, or all the fish can be anesthesized and screened by dissection microscope for abnormal regeneration, including disorganized growth or tumors. Potential founder mutants identified in this manner can then be reamputated and shifted to the permissive temperatures to test for temperature sensitivity, and again reamputated and shifted back to the restrictive temperature to test for repeatability. Our ability to test the same individual repeatedly for regeneration at different conditions is analogous to replica plating of bacteria and yeast colonies.

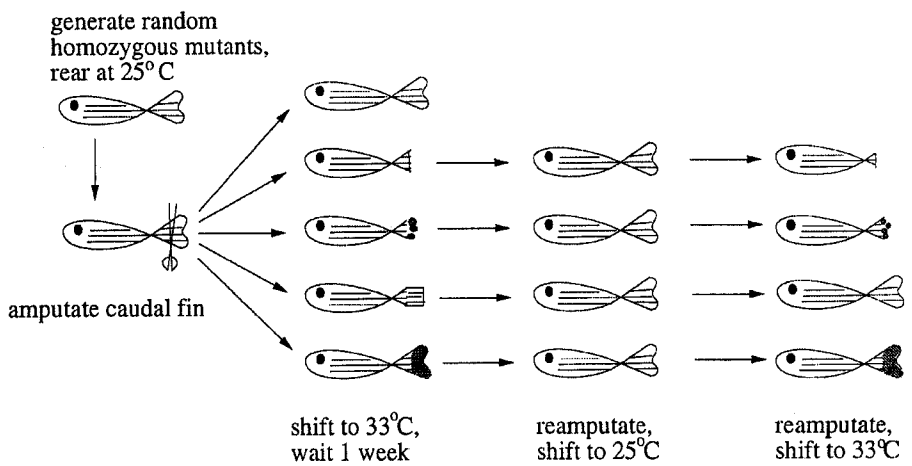

Fig. 2 Screen for temperature-sensitive mutations that affect regeneration of the zebrafish caudal fin.

Two regeneration mutants were described from the initial screen. Mutants for *reg5* are described as regeneration-defective, accumulating a regeneration blastema that subsequently fails to differentiate new bone in the regenerating fin, resulting in regeneration arrest. In contrast, mutants for *reg6* are able to regenerate fins at the restrictive temperature, but the regenerated portion of the fins show dysmorphic growth, including disorganized lepidotricia and an average of four to five blood blisters per fin, apparent by stage 8 of regeneration at the restrictive temperature. Reciprocal shift experiments indicate that the critical period for *reg6* function during regeneration falls between stage 4 and stage 8 of regeneration. Anatomically, stage 8 corresponds to a morphogenetic transition from regenerative growth to normal growth. This transition includes the shift from a stage where ZNS5$^+$ differentiated osteoblasts are observed in mitosis to a stage when differentiated osteoblasts are rarely observed to divide.

III. Developmental Checkpoints in Fin Growth

Studies of the differences in the effect of the *reg6* mutation on ontogenetic growth and regeneration have led Johnson and Weston (1996) to propose that developmental checkpoints that act to ensure the integrity of normal growth are not active during early (before stage 8) stages of regeneration. When *reg6* mutants are challenged to grow their fins at the restrictive temperature (by being shifted to 33°C at two weeks development, after the embryonic lethal period for *reg6* and prior to the development of lepidotrichia), they typically show few or no blood blisters that characterize the mutant fin during regeneration. One possible explanation for this result is that the *reg6* gene is not expressed during normal growth, and therefore is not required in ontogeny. An alternative possibility is that the *reg6* mutation also causes growth defects during the normal development of the fin, but that growth control mechanisms or developmental checkpoints act to survey the integrity of the growing fin; that is, when mistakes such as that caused by *reg6* during regeneration are found, they can be fixed prior to the onset of catastrophes (e.g., the blood blisters that characterize the regeneration defect). The prediction of the model that developmental checkpoints allow for the repair of *reg6*-induced damage during normal growth is that removing the developmental checkpoints in normal growth should result in blood blisters similar to those caused by the *reg6* mutation during regeneration.

One candidate for a mutation that disrupts the developmental checkpoints is the *long fin* mutation. In *lof* mutants, fin growth continues into adult stages at the same initial rate as in juvenile fish, rather than the growth slowing as the growth of the body slows with maturity (S. Johnson, unpublished). Thus, *lof* relieves an apparent dependent relationship of fin growth on body growth. The relief of an apparent dependent relationship is one criterion indicating that a developmental checkpoint may act to ensure developmental integrity (Hartwell and Weinhart, 1989), or in this case, that the *lof* mutation disrupts a develop-

mental checkpoint. The finding that double mutants for *reg6* and *lof* show blood blisters similar to that caused by *reg6* in regeneration (Johnson and Weston, 1996) tends to confirm the notion that developmental checkpoints act during normal growth and development to prevent catastrophes caused by mutations such as *reg6*. The effect of *reg6* during the early stages of regeneration raises the possibility that early stages of regeneration have dispensed with the developmental checkpoints, perhaps to accomodate the rapid growth typical of the early stages of regeneration.

IV. Cellular Basis of Growth in the Fin

An understanding of growth-control mechanisms that regulate the size and shape of the fin and the regenerate requires some knowledge of where cell division responsible for growth occurs. To date, the analysis of cell division in the growing fin has not been reported, and a single report describing BrDU incorporation in the late-stage regeneration of goldfish caudal fin has been reported (Santamaria *et al.,* 1996). To better understand the role of cell division in fin growth and regeneration in the zebrafish caudal fin, we examined sections through fin rays for mitotic figures in normally growing or regenerating caudal fins. Fixed fins or regenerates were immunostained with antibody ZNS5 to reveal differentiated osteoblasts; then cryosectioned and stained with the fluorescent dye Hoescht to reveal the state of chromosome condensation. The cells in the mesodermal layers of longitudinal sections through bony rays were counted, taking into account the number of $ZNS5^+$ differentiated osteoblasts and $ZNS5^-$ fibroblasts or otherwise unlabelled cells, as well as the position of cells with condensed chromosomes or mitotic figures, indicative of cells in late stages of the cell division cycle. Examples of mitotic figures in $ZNS5^+$ and $ZNS5^-$ cells are shown in Fig. 3 (see color plate).

A. Differentiated Bone Cells Typically Never Divide in Normal Growth of the Fin

To determine the contribution of cell division of the differentiated bone cells to the growth of the fin, we prepared eight caudal fins from young adult zebrafish for mitotic figure analysis. We counted a total of 214 sections with 24,200 $ZNS5^+$ bone cells and 32,300 $ZNS5^-$ fibroblast-like mesenchymal cells. Among these, we found 26 mitotic figures in the $ZNS5^-$ compartment (0.08% of $ZNS5^-$ cells) and a single mitotic figure among $ZNS5^+$ labelled cells. Inconsistent with notion that the growing bone grows by division of distal bone cells, the single mitotic bone cell that we observed was 3.4 mM from the distal tip of the fin, or 10–15 ray segments distant from the site of new ray segment formation. Similarly, the 26 mitotic figures observed in $ZNS5^-$ cells were distributed throughout the length of the fin ray, rather than being concentrated in the distal end of the fine. The 19-fold difference between the mitotic figures seen for differentiated bone and

fibroblast compartments makes it unlikely that the division of bone cells contributes significantly to the formation of new bone cells. These results are consistent with previous models that fin rays grow and new bone forms by condensation from the fibroblast compartment (Hass, 1962; Becerra *et al.,* 1996) and tend to demonstrate that osteoblasts that condense into the bone compartment are postmitotic. Presumably, the site of condensation of newly born osteoblasts is in the distal-most region of the growing bone, although we cannot rule out the notion that bone cell number increases by recruiting new osteoblasts up and down the length of the fin ray.

B. Differentiated Bone Cells Divide Following Amputation

In contrast to our observation that differentiated osteoblasts typically do not divide in growing fin rays, we found that ZNS5$^+$ osteoblasts near the amputation plane enter the cell cycle beginning between 1.5 and 2 days after amputation (see Fig. 4, see color plate). These findings are consistent with the previous observations of Goss and Stagg (1957), who noted that osteoblasts surrounding the bone in the stump of amputated tilapia fins typically loosen from one another in the first two days following amputation and then enter the regeneration blastema. Our findings that stump osteoblasts enter the cell cycle suggests that the blastema arises, in part, due to cell division in the stump. Similarly, we observe high rates of cell division in the fibroblast layer, tending to confirm earlier models that the regeneration blastema is derived from both fibroblasts and osteoblasts.

The division of ZNS5$^+$ osteoblasts continues in the stump through 3 days after amputation. By the 4th and 5th day following amputation, cell division of osteoblasts persists, but is only observed at the distal end of the regenerating fin ray. BrDU incorporation of distal osteoblasts in similar-stage goldfish regenerates has also been described (Santamaria *et al.,* 1996). The observation that ZNS5$^+$ osteoblasts in the growing ray of early stage regenerates continue to divide raises the possibility that osteoblasts in the early stage regenerate (prior to day 8) are derived exclusively from the division of pre-existing osteoblasts, rather than by condensation from the regeneration blastema, as suggested by a number of workers (Goss and Stagg, 1957; Johnson and Weston, 1996). Clearly, lineage experiments will be required to address the contribution of the regeneration blastema to fin ray and fibroblast compartments of the early stage regenerates.

By 8 days after regeneration, no cell division is observed in the ZNS5$^+$ osteoblasts of the regenerating fin ray. Presumably, new osteoblasts in the growing fin arise now exclusively by condensation from the dividing cells in the ZNS5$^-$ blastema, which persists in the distal part of the growing regenerate. Previously, we suggested that regeneration undergoes a transition to a more normal growth pattern at 8 days. The finding that cell division is still concentrated in the regeneration blastema at stage 8 and is uniformly distributed among fibroblast cells indicates that the transition from regeneration to normal growth has not yet

occurred by stage 8. We have not yet carried these studies out far enough in time in order to determine when the regeneration program ceases and normal growth ensues.

V. Conclusion

In summary, we have found differences in the cell-division patterns of the early and late regenerate and the normal growth of the fin. Typically, differentiated osteoblasts never or rarely divide in the normally growing caudal fin. When a portion of the fin is amputated, differentiated osteoblasts near the amputation plane are recruited to enter the cell cycle. These cells or their daughters stream distally into the regenerate. Whether they dedifferentiate and become multipotent, or immediately recondense into the growing fin ray, is not clear. The cell division of differentiated osteoblasts persists through 5 days after amputation, but has ceased by 8 days after amputation. Thus, around 8 days after amputation, the regenerating zebrafish caudal fin transits to a cell-division pattern similar, but not identical, to the cell-division pattern of normally growing fins. This regeneration stage transit corresponds to the end of the critical period for *reg6*, suggesting that another aspect of this transit is the acquisition of developmental checkpoints that are absent in early stage regeneration.

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