Fin Regeneration From Tail Segment With Musculature, Endoskeleton, and Scales

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ABSTRACT

It is well known that fish caudal fins can be completely regenerated after fin amputation. Although much research on fin regeneration has been carried out, there have been very few reports regarding fin regeneration after tail amputation. In this study, we used grass carp, common carp, koi carp, and zebrafish as experimental organisms. Some caudal fins could be distinctly regenerated in 2 weeks after tail amputation. After all-trans-retinoic acid treatment and tail amputation, zebrafish were unable to regenerate caudal fins that could be seen with the naked eye. However, after tail amputation, more than half of the zebrafish tested were able to regenerate caudal fins. Caudal fin regeneration depended on the presence of musculature and endoskeleton at the site of amputation. These caudal fins arose from segments of the endoskeleton, which contrast with currently accepted knowledge.


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All organisms mount biological responses to damage, but they vary widely in the degree to which they can recover from damage. These types of homeostatic renewals are thought to be mediated by resident stem cells of specific lineages. In this context, teleost fish are of particular interest, as they have a remarkable capacity to regenerate damaged organs, including heart, spinal cord, retina, and limbs/fins (Akimenko et al., 2003; Poss et al., 2003; Stoick-Cooper et al., 2007). Regeneration of organs such as amphibian limbs and fish fins are dramatic examples, where intricate structures consisting of multiple cell types are patterned into complex tissues and faithfully restored after amputation. Repair of many organs, such as the chicken retina or mouse liver, is thought to be mediated through activation of resident stem cells or through proliferation of normally quiescent differentiated cells, respectively (Fischer and Reh, 2001; Fausto et al., 2006).

Amphibian and fish appendages regenerate through a process termed “epimorphic regeneration,” sometimes referred to as “true” regeneration (Akimenko et al., 2003; Poss et al., 2003). The relative simplicity and the easy access of the caudal fin and its skeletal elements make it an excellent system for dissecting the molecular pathways involved in bone regeneration. While resection of the fin at the level of the endoskeleton is not followed by regeneration, amputation of the dermal fin rays results in complete replacement of the lost structures within approximately 3 weeks (Akimenko et al., 2003; Smith et al., 2006).

Fin regeneration occurs in three steps: (1) wound healing and formation of the wound epidermis; (2) formation of a regeneration blastema, a population of mesenchymal progenitor cells that are necessary for proliferation and patterning of the regenerating limb/fin; and (3) regenerative outgrowth and pattern reformation (Akimenko et al., 2003; Poss et al., 2003; Stoick-Cooper et al., 2007). Many functional genes in fin regeneration have been researched, including a sonic hedgehog homolog (shh), a fibroblast growth factor (fgf), and a bone morphogenic protein (bmp) (Laforest et al., ’98; Poss et al., 2000; Smith et al., 2006). Heat shock...
protein 60 (hsp60) expression is increased during formation of blastema cells, and its dysfunction leads to mitochondrial defects and apoptosis in these cells. Experimental data indicate that the nbl mutation causes dilated mitochondria and apoptosis selectively in the distal blastema cells (Makino et al., 2005).

When amputations are performed distally to the branch points or dichotomies, where a single ray bifurcates to extend two individual “daughter” rays, all-trans-retinoic acid (RA) treatment causes a dichotomy reduction, such that the two “daughter” rays fuse to once again form a single ray. It is thought that exogenous RA respecifies the pattern in the regenerating caudal fin and the blastema has been identified as a possible RA target tissue (White et al., ’94). The regenerating caudal fin is sensitive to RA treatment and shows clear teratogenic responses on the dorso-ventral axis under many of the experimental conditions investigated, both in wild type and long-fin mutants (Géraudie et al., ’95). Eve1 and evx2 products are part of the molecular signals involved in pattern formation of the fin and fin rays in connection with outgrowth. Therefore, RA might alter growth and morphogenesis of the regenerating fins by a fine regulation of these genes, among others (Brulfert et al., ’98).

Fin regeneration from anchovy tail segments with musculature and scales was reported in Freshwater Fisheries (a Chinese journal) in 1980 by Chuanmi Yuan et al. They found that some anchovies caught in the Tai lake could regenerate irregular caudal fins after part of their tail was bitten out by other animals in the water. Anchovies can seldom be caught alive, because they struggle excessively when they are in fishing nets; consequently, it is difficult to use anchovies as experimental animals. Four other kinds of fish, including grass carp, common carp, koi carp, and zebrafish (Danio rerio), were used in this study to research further into fin regeneration from fish tail segments with musculature and scales.

MATERIALS AND METHODS

Maintenance of animals

Grass carp, common carp, koi carp, and zebrafish were all purchased at a local pet store. Grass carp and common carp were approximately 3.5–5 cm in length, koi carp about 5–6 cm, and zebrafish about 2.5–4 cm. The daily photoperiod was 14 hr of light and 10 hr of darkness, and the temperature was kept in the range of 24–29°C. Animals were fed once a day with a commercial food (White et al., ’94; Santos-Ruiz et al., 2002).

Tail and fin amputation

Fish used for experiments were anesthetized by immersion in water containing 0.2 mg/mL tricaine (3-aminobenzoic acid ethyl ester). Tail amputation was carried out at the proximal 1–2 mm of the far margins of the tail that had musculature, endoskeleton, and scales. Proximal caudal fin amputations were done at the distal 1–2 mm of the far margin of the tail that had musculature, endoskeleton, and scales. Distal caudal fin amputations were carried out in the region just before the first bifurcation of the fin rays. The fish were then returned to their tanks (Akimenko et al., ’95; Padhi et al., 2004).

Histologic processing for light microscopy

Fins were fixed for 12 hr in Bouin’s fluid, decalcified for 3 days in 10% ethylenediamine tetraacetic acid, embedded in paraffin, and cut into 6-μm-thick sections. These were mounted on sylanized glass slides, deparaffinized, and rehydrated for use in immunohistochemical staining. Stained sections were observed under a microscope (Microphot FXA, Nikon, Melville, NY), and photographed with Nomarski optics (Santos-Ruiz et al., 2002). Sections (thickness, 6 μm) were cut from paraffin blocks and collected on poly-L-lysine-coated slides (Sigma, St. Louis, MO). Sections were dewaxed in xylene, hydrated in a descending alcohol series, and stained by a routine hematoxylin–eosin (H–E) staining technique, as described previously (Shidham et al., 2001; Tertemiz et al., 2005).

Alcian blue and Alizarin staining

Portions of tail and caudal fins were fixed in 4% PFA and washed in phosphate buffer solution (PBS), then stained for 6 hr at room temperature with a filtered 0.1% Alcian blue (in 30% acetic acid, 70% ethanol), and dehydrated in a graded water: ethanol series. The tissues were then stored in 100% ethanol overnight at 4°C to fix the Alcian blue in the fin rays and to de-stain the surrounding soft tissues. Fins were rehydrated in a water: ethanol series, macerated in a fresh solution of 0.5% trypsin in 2% sodium borate for 10 min at 37°C, and extensively rinsed with frequent water changes. Bones were stained by placing the fins in a fresh solution of 0.1% Alizarin red S in 0.5% KOH for 4–5 hr at room temperature. Fins were
cleared by transfer to glycerol through a graded series of 0.5% KOH–glycerol solutions and kept in 100% glycerol at 4°C (Ferretti and Géraudie, '95; Laforest et al., '98).

**Bromodeoxyuridine incorporation**

For studies involving bromodeoxyuridine (BrdU), a stock concentration of 50 mg/mL BrdU was prepared in sterile Hanks solution. The fish were anesthetized with tricaine and injected in the abdominal cavity with 250 μg/g weight BrdU. Six hours postinjection, the fish were euthanized and their caudal fins or part of the tail with musculature were amputated and fixed in Bouin’s fluid. Slides of tissue sections were prepared and treated with primary mouse anti-BrdU antibody (Sigma) at a dilution of 1:500 and secondary goat anti-mouse antibody (ZSGB-BIO) at a dilution of 1:500. 3,3’-Diaminobenzidine (DAB) was used to visualize and photograph BrdU incorporation (Poleo et al., 2001; Zodrow and Tanguay, 2003).

**Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling in situ apoptosis detection assay**

Apoptosis in fish caudal fins on tissue slides was detected by enzymatic labeling of DNA strand breaks using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL). TUNEL was carried out using a cell death detection kit (Roche, Mannheim, Germany) according to the manufacturer’s instructions. Briefly, tissue sections were dewaxed in xylene and rehydrated in a descending alcohol series (at room temperature). Samples were washed in PBS for 5 min, then pretreated with 3% hydrogen peroxide for inactivation of endogenous peroxidase for 10 min and 20 μg/mL proteinase K for 30 min. The labeling reaction was performed by adding 50 μL TUNEL reagent to each sample. A negative control was prepared with the reagent without enzyme. Incubation was carried out for 1 hr at 37°C, and then the slides were washed with PBS and incubated again with converter reagent for 30 min at 37°C. After washing, color development for localization of cells containing labeled DNA strand breaks was performed by incubating the slides with DAB substrate solution for 30 sec. Slides were lightly counterstained with hematoxylin before permanent mounting (Selam et al., 2002; Sasso-Cerri et al., 2006).

**RA treatment**

A 5 mg/mL stock solution of RA (Sigma Aldrich, St. Louis, MO) was prepared in dimethyl sulfoxide. An appropriate amount of RA at a final concentration of 10⁻⁶ M was added directly into the tank water after amputation for 4 continuous days, and the water and RA were refreshed every day. Both control and RA-treated fish were kept in the dark during these experiments (White et al., '94; Ferretti and Géraudie, '95).

**RESULTS**

**Interesting phenomena in caudal fin regeneration**

The caudal fins of experimental fish are shown in Figure 1. Amputation levels are indicated by the black horizontal lines (Fig. 1A–D). Compared with the normal caudal fin of a grass carp, the regenerated caudal fin rays do not branch as normal caudal fins do, when distal caudal fin amputations were carried out just before the first bifurcation of the fin ray (Fig. 1E–F⁰). Regenerated koi carp caudal fins bifurcate earlier than common carp after caudal fin amputation at distal 1–2 mm to the rear margin of the tail with musculature (Fig. 1G–J). It is interesting that sometimes red common carp have a fair amount of melanin pigment in their regenerated caudal fins (Fig. 1K–M).

**Caudal fin regeneration after tail amputation**

There are some differences in caudal fin regeneration when caudal fins or tails are anesthetized for amputation, which was carried out at the proximal 1–2 mm of the rear margin of the tail with musculature, endoskeleton, and scales. The regeneration rate of the caudal fin after tail amputation is about 50%, while the rate after caudal fin amputation is 100%. Only some of the tail amputations regenerated completely, while all of the caudal fin amputations completely regenerated. Caudal fins can be seen with the naked eye about 2–4 weeks after tail amputation and about 3 days after caudal fin amputation. The former regeneration comes from the segment of the tail with musculature, endoskeleton, and scales, whereas the latter regeneration comes from the caudal fins with fin rays.

Some grass carp whose tails had been cut two months before regenerated irregularly only part of...
the caudal fin. Others had not regenerated caudal fins after five months. The stem length of the fish is about 8 cm (Fig. 2A–D, part D). No caudal fins completely regenerated with their original shape after tail amputation in this experiment. Fin rays could be seen early with the naked eye in regenerated caudal fins of grass carp, which were treated with tail amputation (Fig. 2A–D). Tails of common carp had been cut about one month before, and the regenerated caudal fins were about 1–2 mm long and could be seen easily in water but not out of water. After 42 days of tail amputation, distinct fin rays still could not be seen with the naked eye and the existing fin ray was more similar to a blood vessel. Some fish had no regeneration after similar amputations even after 50 days (Fig. 2E–G).

When the common carp tail was cut about three months previously (Fig. 2H–I part H), it regenerated a complete caudal fin according to its shape after one month. However, the regenerated caudal fins had no fin rays that could be seen with the naked eye. When
the common carp caudal fin was cut distally 1–2 mm behind the margin of tail with musculature about two months before, it regenerated a complete caudal fin according to its shape after one month. These regenerated caudal fins had fin rays that could be seen with the naked eye (Fig. 2H–I).

Regenerated caudal fins of koi carp had abundant blood vessels but no distinct fin rays. For this reason, these may be useful in medical experiments regarding the development of blood vessels. The probability ratio of caudal fin regeneration after tail amputation was 100%, and most of these regenerated caudal fins were complete according to their shape. The stem length of koi carp and grass carp used in this experiment was similar, although the stem length of koi carp was longer than that of common carp or of zebrafish. Therefore, stem length did not appear to be a critical factor for fin regeneration after part of the tail was amputated (Fig. 2J–L').

The probability ratio of zebrafish caudal fin regeneration after tail amputation was about 50%. Only part of the regenerated caudal fins was regular and complete. When the regenerated caudal fin after tail amputation reached 2 mm, fin rays could be seen with the naked eye, although blood vessels could not be seen. Most of the regenerated zebrafish caudal fins after tail amputation were seen between 15 and 30 days (Fig. 2M–P).

Common carp or koi carp did not regenerate caudal fins and their caudal endoskeleton was exposed after they were partly cut. Because of muscular contractions around the broken ends of the tails that had been operated on, the caudal endoskeleton was exposed and most of these fish died. In a few cases among common carp, the broken ends of the tails that had been operated on were covered with a scale-like substance (Fig. 2Q–T).

**Alcian blue and Alizarin staining, H–E staining, BrdU incorporation, TUNEL assay, and RA treatment**

After Alcian blue and Alizarin staining, tails of koi carp and zebrafish with regenerated caudal fins after tail amputations showed dissection evidence that was comparable to that of normal koi carp and zebrafish. This clearly indicates that...
tail amputation greatly differed from caudal fin amputation (Fig. 3A–E). At a 20 × magnification, H–E staining of regenerated common carp caudal fin after tail amputation indicated that the area between the tail with musculature and the regenerated caudal fin is not smooth. In contrast, H–E staining of untouched common carp caudal fin at the same 20 × magnification shows a smooth area between the tail with musculature and a caudal fin with fin rays (Fig. 3F and G). We can see that proliferated cells stained with DAB are located at the margin of the regenerated caudal fin treated with BrdU after tail amputation, while no cell proliferation is seen in the absence of BrdU (Fig. 3H–K). Apoptotic cells occurred primarily at the margin of the caudal fin. When magnified 400 ×, TUNEL test results showed that there were no DAB-stained cells in the control group (Fig. 3L–O).

When RA was used, caudal fins of zebrafish that were cut before the first bifurcation were regenerated. Caudal fins regenerated for 10 days without RA showed no deformities (Fig. 3P–Q, part P). On the contrary, caudal fins regenerated for 1 month after RA treatment had distinct deformities (Fig. 3P–Q, part Q). This indicates that the treatment of zebrafish with RA in the experiment had an effect (Fig. 3P–Q). Zebrafish were treated with RA and with tail amputation. After 2 months, only one small caudal fin was regenerated, which could not easily be seen with the naked eye and which showed no further growth after another month (Fig. 3R and S). RA can cause anepithymia in zebrafish and common carp and was seen the day after RA treatment. Common carp are sensitive to RA and all the treated fish died within 10 days of RA treatment, whereas common carp without RA treatment remained alive and healthy. No zebrafish died from RA treatment, and all zebrafish with caudal fin amputations regenerated deformed fins after RA treatment, although they also showed anepithymia. The regeneration ratio of zebrafish after tail amputation was about 50%, whereas no zebrafish regenerated caudal fins that could be seen easily with the naked eye after identical tail amputation and RA treatment.

DISCUSSION

Caudal fin regeneration after fin amputation has been researched repeatedly, in contrast to experiments on caudal fin regeneration after tail amputation (Akimenko et al., 2003; Poss et al., 2003; Stoick-Cooper et al., 2007; Thatcher et al., 2008; Kizil et al., 2009). Fin regeneration after tail amputation was demonstrated in four species of fish, including grass carp, common carp, koi carp, and zebrafish. Anchovies have also been observed to have this ability. Normal fish and fish with general caudal fin amputation have an intact rear margin of the tail (Fig. 1A–M). It can clearly be seen that fish with regenerated caudal fins after tail amputation have no general rear tail margin (Fig. 2A–T). In the experiment using Alcian blue and Alizarin staining, tail amputation was shown to be clearly different from caudal fin amputation (Fig. 3A–E). Fin regeneration after tail amputation comes from tail segment with musculature, endoskeleton, and scales. This is different from currently held views that resection of the fin at the level of the endoskeleton is not followed by regeneration (Akimenko et al., 2003; Smith et al., 2006). Proliferated cells were found using BrdU and DAB in the regenerated caudal fins after tail amputation (Fig. 3H–K). This finding was similar to that reported for regenerated caudal fins after fin amputation (Poleo et al., 2001; Zodrow and Tanguay, 2003). Cell apoptosis occurs during regeneration of newt limbs, deer antlers, and mouse liver (Vlaskalin et al., 2004; Mount et al., 2006; Tannuri et al., 2008). In caudal fin regeneration after tail amputation, cell apoptosis was also found by TUNEL (Fig. 3L–O). RA treatment increased apoptosis in the regenerated fin after fin amputation (Ferretti et al., ’95; Géraudie and Ferretti et al., ’97).

Activation of resident muscle stem cells occurs in regenerating salamander limbs found by processes involving antibodies and labeling. Thus, it is likely that de-differentiation and stem cell activation both contribute to formation of the blastema (Morrison et al., 2006). Although de-differentiation of cells has not yet been demonstrated in regenerating structures other than amphibian limbs and tails, the morphology, ontology, and gene expression profile of the zebrafish blastema in the regenerating caudal fin suggests that zebrafish fin regeneration occurs by similar mechanisms (Stoick-Cooper et al., 2007). Tail tissue that formed after tail amputation in the region around the cross section had homologous structure to that seen in tetrapods. Therefore, fin regeneration after tail amputation has the potential to reveal general principles that are common to regeneration from tissue with endoskeleton in all vertebrate appendages. Other “fin regeneration” studies performed on actinopterygians have been limited to the lepidotrichia, which do not have an endoskeleton or musculature.

There are some issues with these experiments that still require answers: First, the morphology
Fig. 3. Alcian blue and Alizarin staining, H–E staining, BrdU incorporation, TUNEL assay, and RA treatment. (A–E) Alcian blue and Alizarin staining. Tail of koi carp (A, B) and zebrafish (D) with regenerated caudal fins after tail amputation have dissection evidence (arrow) compared with normal tails of koi carp (C) and zebrafish (E). (F, G) H–E staining. Regenerated caudal fin (F) of common carp after tail amputation is different from normal caudal fin (G) of common carp. (H–K) BrdU incorporation. Common carp tail treated with tail amputation and BrdU (H) is positive, while normal caudal fin of koi carp treated with BrdU (I) regenerated caudal fin of zebrafish after tail amputation (J) and normal caudal fin of koi carp (K) as control. Magnified parts of H, I, J, and K are shown in H', I', J', and K' respectively. (L–O') TUNEL assay. Normal koi carp caudal fin (L) is the control. Regenerated caudal fin of common carp (M) after tail amputation is positive. Regenerated caudal fins of common carp after tail amputation (N, O) are also as control. Magnified part of L, M, N, and O are shown in L', M', N', and O' respectively. (P–Q') Regenerated caudal fin of zebrafish after distal fin amputation. Some are treated without RA (P), while others are treated with RA (Q). Magnified parts of P and Q are shown in P' and Q', respectively. (R, S) Zebrafish treated with RA and tail amputation.
and molecular changes of fin regeneration between the time of tail amputation to the time that the regenerated fin has reached a length of 2 mm should be further researched. Second, for reasons that are not clear, the regenerated caudal fin of common carp and koi carp, after tail amputation, regenerate fin rays quite late, and some have no fin rays even after a complete caudal fin, according to shape, has been regenerated. Third, some caudal fins regenerate only very slowly after tail amputation and only parts of them regenerate, while amputation of the dermal fin rays results in complete replacement of the lost structures within approximately 3 weeks (Akimenko et al., 2003; Smith et al., 2006). This also requires further investigation. Finally, RA was shown to have a very powerful inhibitory effect on caudal fin regeneration after tail amputation, again for reasons that are unclear. Zebrafish with caudal fin amputation after RA treatment always regenerated deformed fins. Further experiments are needed to understand these unusual regeneration phenomena.

LITERATURE CITED


