doi: 10.1387/ijdb.082615sb

THE INTERNATIONAL JOURNAL OF
DEVELOPMENTAL
BIOLOGY
www.intjdevbiol.com

SUPPLEMENTARY MATERIAL

corresponding to:

Zebrafish development and regeneration: new tools for biomedical research

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Results and Discussion

Here, we report some preliminary observations on the use of carrier beads to deliver retinoic acid into regenerating caudal fin blastemas of the zebrafish.

Bead loss

In 16 out of 63 fish examined 24h after surgery, the bead could not be found and had presumably fallen out.

Phenotypes and their RA dose-dependency

Most implantations did not result in phenotypically abnormal regeneration. The length of the rays appeared appropriate to their dorsoventral position. The length of the segments and the number of dichotomies was the same as in the control side of the fin (data not shown). In general, microbeads soaked with different concentrations of RA, implanted into regenerating zebrafish caudal fins resulted in phenotypes that we classify into four mutually exclusive categories:

1. no notch	No gross defects in the margin of the regenerate
2. shallow notch	1-2 d. postimplantation, a shallow indentation is visible in the distal margin of the regenerate (Figure 1A). The indentation does not approach the bead, and disappears 1-3 d. later (Figure 1B), demonstrating that the effect is reversible
3. deep notch	Differs in degree from shallow notches in that indentation approaches the bead and persists longer (Figure 2). Unlike 'permanent notches', persistent 'deep notches' do ultimately become completely closed by growth of the interray tissue. Therefore, these notches are reversible.
4. permanent notch	These notches resemble the deep notch phenotype, but differ in that they do not become closed during the duration of observation (120d). Thus the caudal fin has a persistent indentation at its distal margin.

No notch

The 'no notch' phenotype, while observed in at least some cases in all RA concentrations used, was seen mainly at lower dosages. The entire control group (RA: 0 mg/mL) produced a 'no notch' phenotype (Table 1), indicating that effects in other experiments were due to RA.

TABLE 1

SUMMARY OF RESULTS ON NOTCH INDUCTION BY RA IMPLANTED ON MICROBEADS

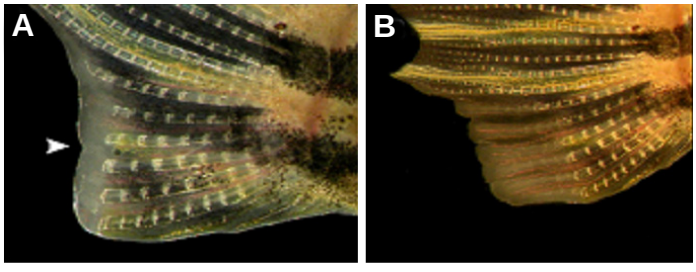
RA (mg/mL)	*N*	survivors	Normal	Shallow Notch	Deep notch	Permanent notch
0.0	8	8	8	0	0	0
0.2	13	13	7	3	2	1
1.0	17	16	4	5	7	0
5.0	15	13	3	3	4	3
10.0	10	9	1	3	0	5

Shallow notch

In most cases, for all RA concentrations used, implantation of a micro-bead soaked in RA resulted in a shallow notch in the blastema (Figure 1). Concentrations of RA of 0.2 mg/mL and higher produced in some cases a 'shallow notch' (3/13; 23.1 %; Table 1). Neither the concentration of RA used, nor the duration of the bead stay in the tissue, affected the observed reversibility of the effect. No changes in the number of elements in the rays were observed when regeneration had been completed. The regenerates have the appropriate number of bifurcations, and the length of the rays and size of the segments were unchanged (data not shown). A number of segment boundaries were irregular in shape, but this did not occur at a higher frequency than in the control fish.

Deep notch

This phenotype occurred sporadically in the 0.2 mg RA group (2/



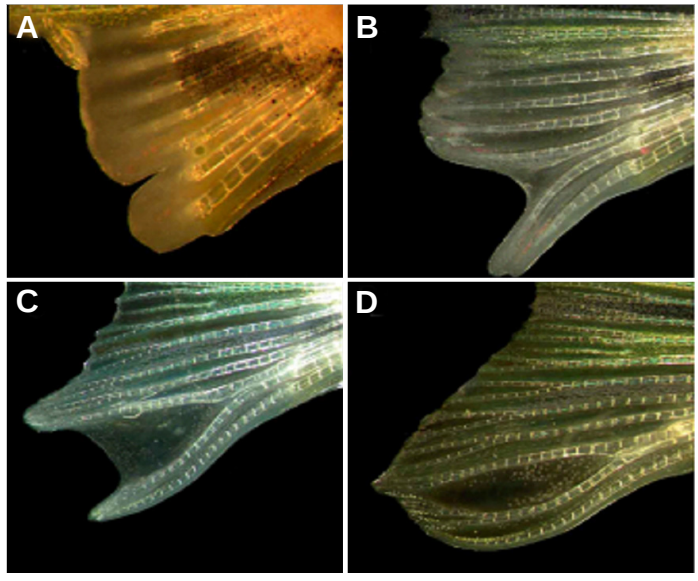


Fig. 1 (Left). A 'shallow notch' is reversible. *In this fish a 1 mg/mL RA bead was implanted.* **(A)** *2 days postimplantation, a shallow notch is visible in the regenerate (white arrowhead). The bead is still in the tissue (to the right of the arrowhead).* **(B)** *6 days postimplantation. The notch has disappeared, the regenerate looks normal and will continue to regenerate in a normal fashion.*

Fig. 2 (Right). An example of the regeneration process of the 'deep notch' phenotype in a single fish at different timepoints. *A bead with RA concentration of 1 mg/mL was used.* **(A)** *Regenerate 3 days after implantation. Note that the bead is still in the tissue. The regenerate is clearly affected by the RA in the bead.* **(B)** *10 days postimplantation. The most proximal part of the notch has shifted distally, but the notch itself has increased in size. Some interray tissue begins to grow back.* **(C)** *31 days postimplantation. Clearly the interray tissue is regenerating and filling up the notch. The first pigment cells become visible. Note that the angle between the bead rays decreases in comparison with B.* **(D)** *55 days postimplantation. Endpoint of the regeneration in this specimen. The outer shape of the fin has been restored. First bifurcation is distalised. Pigment cells are abundant in the interray tissue. Note that the bead rays proximal to the notch have slid over each other, hereby reducing the angle of the notch. This only happened in this fish; in other fish the notch was closed without sliding of the rays.*

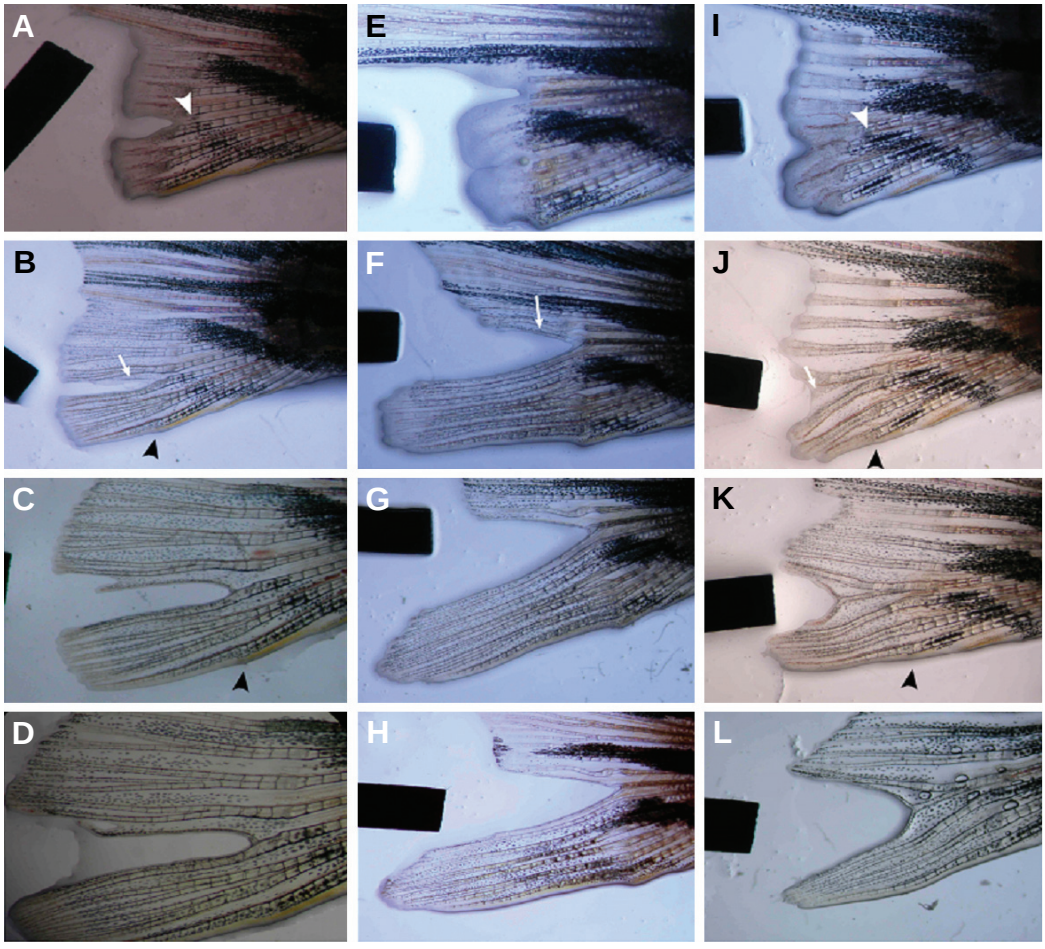


Fig. 3. Phenotypes in regenerating adult zebrafish caudal fins induced by RA-soaked bead implantation. *The ventral half of the fin was amputated; dorsal is at the top. (A-D) Bead (white arrowhead), with a RA concentration of 0.2 mg/mL was implanted. Result at 4, 11, 18 and 40 days post-implantation (dpi), respectively. In* **(B)** *the interray tissue starts to regrow and the first contours of some ectopic ray tissue appear (white arrow).* **(C)** *The bony structure has developed into an ectopic fin ray. Note that the parent ray, ventral to the notch, is bifurcating at approximately the same level as the adjacent rays.* **(E-H)** *Fish with two beads (RA concentration 5.0 mg/mL). Shown are 5, 20, 34 and 92 days postimplantation, respectively.* **(E)** *Both beads have induced a notch, of which the ventral one closes and the other develops into a 'permanent' notch (see F).* **(F)** *The dorsal bead ray has regrown along the ventral border of the notch. Dorsally from the notch other ray tissue is forming (white arrow), but this is not connected to the 'mother' ray. In the next photo of the series* **(G)**, *(34 dpi), this ectopic ray has fused with the mother ray.* **(H)** *After 92 days, the first regenerated ray and the ectopic ray did increase in length to some extend but not up to the appropriate length. Both rays now have clear well formed ray borders. (I-L) A 10 mg/mL RA bead (white arrowhead) was implanted in this fish. Photographs depicted are of 6, 9, 17, and 92 days post implantation.* **(I)** *Six dpi, the notch is only halfway the regenerate. Prior, the proximal border of the notch was closer to the amputation site, but the notch has been filled up with tissue up to the current level. In* **(J)** *one can see the outward fanning of the bead rays. The more dorsal of these two displays the first signs of a bifurcation (white arrow), which becomes perfectly clear in (K). The question is whether the induction of the bifurcation is a result of the outward fanning of the bead rays, or the fanning is a result of the induction of the bifurcation.* **(K)** *17 dpi, the daughter ray has grown and became obstructed by the proximal border of the notch. It did not stop there. Instead of termination of the regeneration, the rays branched into two daughter rays that follow dorsal and ventral border of the notch. In* **(L)**, *92 dpi, these rays have become more distinct rays, but only the most ventral one has grown to nearly the edge of the tail, whereas the dorsal one stranded after approximately 5 segments. Notice that the bifurcation of the dorsal bead ray is at the same proximity as the dorsally situated neighbour ray and that this ray bifurcates again about 7 segments later. Black arrowheads in B,C,J,K indicate amputation level. White arrowheads in A,I indicate bead implantation site. White arrows in B,F,J show initiation of ectopic rays.*

13; 15.4 %; Table 1), but at higher frequencies for higher RA concentrations.

However, with increasing concentration, the frequency of deep notches decreases since they then become 'permanent' notches. Deep notches are reversible, and even if one persists into late stages of regeneration, they ultimately become closed by growth of interray tissue. In one specimen the adjacent rays slid over each other to reduce the width of the gap (Figure 2; Figure 3), and in all of the fishes in which the notch was filled up with interray tissue, the width of the interray tissue in the notch also appeared to decrease (Figure 3) so as to approximate the notch margins. Thus, the outer shape of the caudal fin is ultimately restored.

Permanent notch

Some notched fins retain an indentation in their distal margin that does not close (Figure 3), even at the maximum time point recorded (120 dpi). This phenotype was only observed in the fish treated with the highest dosages of RA (5 & 10 mg/mL).

Lack of effects of RA bead implantation on skeletal patterning

We found no evidence of changes in skeletal patterning (ray parameters) at any concentration. In some cases, the fin is malformed and daughter rays are absent, but these phenotypes are difficult to ascribe to a specific effect of RA on positional values. In a few 'deep notch' and 'permanent notch' regenerates, ectopic ray structures were formed (Figure 3). The ectopic rays appeared to develop autonomously within the interray tissue, or were derived from a bifurcating ray (Figure 3).

(Géraudie *et al.,* 1993) have shown that RA added to the aquarium water ($2x10^{-6}$ or $2x\ 10^{-7}$ M for 4 days) and administered 24 hours postamputation can increase the number of dichotomies

in the regenerating zebrafish caudal fin. Additionally, it was observed that regenerated dorsal fins were sometimes longer than the control fins, the lepidotrichium segments were often more irregular in shape and length and their number appeared to be higher in RA than in animals treated with vehicle (DMSO, dimethylsulphoxide) alone. These results suggest that systemic RA affects the proximo-distal axis of the dorsal lobe of the caudal fin.

In our hands, local RA targeting via microbeads was not found to affect pattern formation. This conclusion was drawn from statistical analysis of the following ray patterning parameters:

Ray length

Rays adjacent to the bead implantation site appear to become shorter as the RA concentration increases, but no significant differences were found between groups (Kruskal Wallis: p= 0.073), and the Spearman correlation coefficient is low and not significant (one sided R= -0.059; p= 0.356). No significant increase of dichotomies was observed, nor a higher amount of irregular shaped segments (data not shown).

Length of ray to first bifurcation

It appeared that the distance and number of segments from base to first bifurcation is larger in amputated fins than in non-amputated controls. This was the case for all RA concentrations used and also the controls (0 mg/mL). T-tests between normal (unoperated) fish and the 0mg/mL RA bead regenerates control fish also show significant differences in distance from base to first bifurcation(p< 0.001). These results presumably reflect the proximalising effect of surgery itself as previously reported (Géraudie *et al.,* 1994; Géraudie *et al.,* 1995).

All ratios are higher than 1.0, indicating that the first bifurcations on the experimental side of the fins are at a more distal level than at the control side. This is reflected in a higher number of segments between base of the tail and first bifurcation. The segments are of comparable size to the controls (data not shown). Although there seems to be a minor relationship between this ratio and the RA concentration, it is not significant (Pearson Correlation, one sided R = 0.209, p = 0.092). In addition, no differences in variance between groups are observable for both absolute distance and the number of segments making up this first non-branched part of the rays (one-way ANOVA; p = 0.565). Thus it appears that the proximodistal level of the formation of the first bifurcation is not affected by RA concentration.

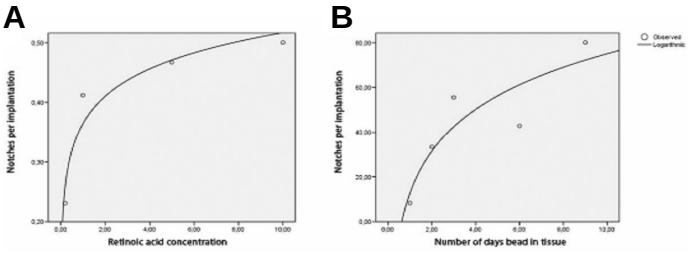


Fig. 4. Dose and time dependency of notch induction by RA. *The notches referred to in these graphs are deep or permanent notches. Logarithmic regression estimation curves are drawn with SPSS (solid lines). In both graphs, datapoints (0,0) were omitted for mathematical reasons.* **(A)** *Shows a strong logarithmic relation between the RA concentration and the number of induced notches (R^2= 0.924; p= 0.039). (Each datapoint is this graph represents the mean of respectively 8, 13, 17, 15 and 10 cases).* **(B)** *This graph shows a weaker, though considerably strong relation between the number of days the bead stayed in the tissue (bead stay) and the number of induced notches (R^2= 0.790; p= 0.044). (Each datapoint is this graph represents the mean of respectively 13, 12, 9, 9, 7 and 5 cases).*

Notch induction as a function of RA concentration and duration of exposure

Two factors likely to affect notch induction are: (i) RA concentration used; (ii) the number of days the bead stays in the tissue. As can be seen in Figure 4A, the frequency of notch induction depends on the concentration of RA in the bead. We also found a correlation between the duration of the treatment (that is how long the bead stays in the tissue) and the induction of a notch (Figure 4). The longer the bead remains in the tissue, the more likely it is that a notch will form.

To establish how the frequency of notch induction depends on RA concentration and on time the beads are present, we performed a regression analysis. Using curve estimation, it appeared that a significant logarithmic relation exists. In other words, the frequency of notch induction is related to both the RA concentration and the duration of exposure. The RA concentration gives a better prediction of notch induction (R^2= 0.924; p= 0.039) than duration of exposure (R^2= 0.790; p= 0.044).

Materials and Methods

Animals

Adult (wild-type) zebrafish (Danio rerio) were purchased from Ruinemans BV (Montfoort, the Netherlands) and were kept at 26°C ± 1°C using standard methods (12-12 light- dark cycle; pH 8.2 – 8.3; NH_3 0 mg/mL; NO_2^- 0 mg/mL; NO_3^- <100 mg/mL). Each fish was kept separately in a breeding basket. All procedures were carried out under license from the Netherlands animal experimental ethics Committee (DEC).

Fin amputation

Zebrafish were anesthetized prior to fin amputation with MS-222 (tricaine methane sulphonate) at a concentration of 300 mg/L aquarium water. Dorsal or ventral halves of the caudal fins were amputated proximal to the first bifurcation using a scalpel (morphological description of fins based on (Becerra *et al.,* 1983). In the majority of cases, the ventral half was amputated; in all cases, the unoperated (contralateral) half of the fin acted as control.

Bead preparation

The protocol is modified from (Eichele *et al.,* 1984; Scadding and Maden, 1986). Anion exchange resin beads (AG1-X2, Biotechnology Grade, 200 – 400 mesh Hydroxide Form, wet size 75 – 180 μm; Bio-Rad Laboratories, the Netherlands) were washed 4 to 5 times with PBS and soaked for 1h at room temperature in all-trans- retinoic acid (RA: Sigma) solution with concentrations of 0.2, 1.0, 5.0 or 10 mg/mL, dissolved in dimethyl sulphoxide (DMSO). Controls were DMSO only. Beads were then rinsed twice with ultra pure distilled water to remove the unbound solute and to exchange the DMSO for aqueous medium. Surplus water was then pipetted away and the beads were stained for 1 minute with aqueous methylene blue to increase the visibility of the transparent beads during implantation.

Bead implantation

Twenty-four hours after partial fin amputation, fish were re-anesthe-

tized with MS-222. A bead, prepared as described above was implanted through an incision made in the inter-ray skin with a tungsten needle. The bead was inserted so that it came to lie at the proximal margin of the blastema, between two rays. After bead implantation, fish were returned to a separate aquarium tanks and each was assigned a unique ID. Fish were maintained with normal feeding and photographed regularly (for which they were briefly re-anaesthetised).

Analyses

The experiment was terminated when the tail had regenerated sufficiently to restore its original dimensions (>40 d). The fins were not fixed before analysis. The two rays adjacent to the bead implant site were analysed. The first measurement made was the distance between the base of the tail and the first bifurcation. The second parameter recorded was the number of segments between the base of the tail and the first bifurcation. The third measurement was the length of the adjacent rays. The length of a ray was measured by starting from the base of the tail, to the tip, following the daughter rays which were closest to the implantation site. All measurements were performed with ImageTool software on digital images of the tails with a scale bar for calibration.

As it was not always possible to implant each bead between the same rays, all measurements taken were compared with the positionally equivalent ray on the control side of the tail. This is legitimate provided there is no significant difference between dorsal and ventral halves of the caudal fin in untreated fish. To determine whether this was the case, we measured all rays (8 rays per dorsal or ventral half) of untreated fish (n=6) and compared them to their unoperated counterparts using a paired T-Test. We named the rays from outer to inner, ray 1 being the most outer long ray, for both ventral and dorsal halves of the fin. The T-test showed that the only feature that differed between the dorsal and ventral halves of a normal, unoperated caudal fin, was the length rays 2 & 3: these were significantly longer on the dorsal half. We therefore did not use these rays for statistical analysis regarding ray length. For both distance and number of segments between base of the tail and the first ray bifurcation, no significant differences were found, so we were able to compare the ratios of different rays with each other.