Ray–Interray Interactions during Fin Regeneration of Danio rerio

C. Murciano,* T. D. Fernández,* I. Durán,* D. Maseda,*
J. Ruiz-Sánchez,† J. Becerra,* M. A. Akimenko,† and M. Marí-Beffa*†

*Department of Cell Biology and Genetics, Faculty of Science, University of Málaga, 29071 Málaga, Spain; and †Ottawa Health Research Institute, Ottawa Civic Hospital, Ottawa K1Y 4E9, Canada

Teleost fin ray bifurcations are characteristic of each ray in each fin of the fishes. Control of the positioning of such morphological markers is not well understood. We present evidence suggesting that the interray blastema is necessary for a proper bifurcation of each ray during regeneration in Danio rerio (Hamilton-Buchanan) (Cyprinidae, Teleostei). We performed single ray ablations, heterotopical graftings of ray fragments and small holes in lateral rays which do not normally bifurcate, to generate recombinants in which the lateral rays are surrounded with ectopic interrays originating from different positions within the tail fin. These ray–interray recombinants do now bifurcate. Furthermore, we show that the interray tissue and surrounding epidermis can modulate the length of the ray. These results stress the role of the interray in inducing bifurcations of the ray blastema as well as modulating ray morphogenesis in general. In addition, gene expression analysis under these experimental conditions suggests that msxA and msxD expression in the ray and interray epidermis is controlled by the ray blastema and that bmp4 could be a candidate signal involved in these inductions. © 2002 Elsevier

Key Words: Danio rerio; fin regeneration; epidermis–blastema interactions; msxA; msxD; bmp4; graftings; ray–interray interactions; ray bifurcation.

INTRODUCTION

The exoskeleton of the fins of teleosts is composed of segmented and bifurcated rays made of dermal bone. Each ray is composed of two symmetrical concave hemirays consisting of segments joined by ligaments, and in most cases, the rays are bifurcated. The hemirays enclose a loose, vascularized connective tissue and are surrounded by a multilayered epidermis. Each ray is joined to its neighbor by an interray tissue also composed of a loose connective tissue surrounded by epidermis (Becerra et al., 1983). Following partial amputation of the fin, within the dermal skeleton, the wound is rapidly healed and a regeneration process is taking place. Regeneration of the fin involves the formation of a blastema composed of undifferentiated and proliferative mesenchymal cells in the connective tissue of each ray and interray. As distal blastema cells continue to proliferate, more proximal blastema cells differentiate and progressively lead to the restoration of the missing part of the fin.

Classical experimental analyses of partial fin ablations and grafting have been done to provide information on the regulative potential of the fin (Morgan, 1902, 1906; Nabrit, 1929; Birnie, 1947; Goss and Stagg, 1957; Marí-Beffa et al., 1996, 1999). These experiments showed that the ray has the capacity of regenerating when grafted to new positions (Birnie, 1947). However, for the ray bifurcation to occur, interactions with neighboring tissues are necessary since rays regenerating in absence of contact with other rays do not bifurcate (Marí-Beffa et al., 1996, 1999). Moreover, observations of cell morphology changes are suggestive of blastema to epidermis interactions in Carassius auratus tail fin regeneration (Marí-Beffa et al., 1996).

Results from gene expression analysis also indicate that blastema–epidermis interactions are occurring in the fin regenerate (Laforest et al., 1998). For example, shh signal emanating from basal epithelial cells has been proposed to be involved in dermal bone formation through the activa-
tion of ptc1 and bmp2b expression in the adjacent scleroblasts of the blastema (Laforest et al., 1998).

Interactions between blastema of adjacent rays have also been proposed to be involved in the normal positioning of the bifurcations of the ray. We have previously shown that the absence of neighbor rays led in C. auratus to the lack or distalization of ray bifurcations (Mari-Beffa et al., 1996, 1999). In Danio rerio, all the rays form bifurcations with the exception of the most lateral rays [the first dorsal and ventral ray in the caudal fin (R1) and the first anterior ray in the other fins], which never bifurcate. In the present study, we have investigated the influence of the adjacent interray tissues to the induction of the formation of a ray bifurcation. We made ray–interray recombinants, using a constant ray, the first unbifurcated ray, and varying the origin of the interray, and analyzed the morphological consequences of such recombinants on the formation of ray bifurcations. These studies provide evidence in favor of the hypothesis that the interray blastema provides morphogenetic information to the ray blastema for the induction and the proper positioning of the bifurcations. Our results suggest a novel mechanism of pattern formation never described in teleosts which could be similar to the mechanism involving a digit–interdigit interaction recently described in chicken foot development (Dahn and Fallon, 2000). We also show novel evidence that the onset of expression of the msx homeobox genes msxD and msxA in the distal epidermis covering the blastema in both ray and interray regions (Akimenko et al., 1995) depends on interactions with the underlying ray blastema.

The bone morphogenetic protein (BMP) signaling is involved in many inductive events during embryogenesis, and bmp4 has been shown to induce Msx gene expression in several developing systems involving epithelial–mesenchymal interactions (Vainio et al., 1993; Wang and Sassoon, 1995; Wattanabe and Le Douarin, 1996; Takahashi et al., 1996; Barlow and Francis-West, 1997; Suzuki et al., 1997; Kim et al., 1998; Chen et al., 2000). Here, we show that bmp4 is expressed in the distal blastema cells of the fin regenerate and that msxD and msxA expression in the epidermis could be activated in response to bmp4 expression in the underlying blastema cells.

MATERIALS AND METHODS

Animals

A total of 174 wild type specimens of Danio rerio have been studied following the 8 experimental protocols described below (a–h). Zebrafish were purchased at a local pet store and maintained at 28.5°C using standard methods (Westerfield, 1995). Although there is a possibility that genetic background may exert some influence on fin growth and/or regeneration (Iovine and Johnson, 2000), we have not observed such variations in the fish that were used in this study. Fish showing an unusual/abnormal regeneration process of the control rays were discarded from the experimental group. The animals were kept in aquaria of charcoal-filtered water of 30 liters capacity. Handling of fishes was under the principlesapproved by the Council of the American Physiological Society and National Laws (B.O.E. 67, 1988, Spain).

Experiments

The specimen of D. rerio were anesthetized with either tricaine (MS222; Sigma, St. Louis, MO) at a concentration of 0.2 mg/ml or 2-phenoxiethanol (Sigma-Aldrich) at a concentration of 0.25 ml/liter and then operated. After each operation, the fishes were kept for 1 day in water containing 50 mg/liter of penicillin G and then put back in the system water. We performed the following protocols:

- (a) Amputation of individual fin rays (third dorsal or ventral in the tail fin) according to Goss and Stagg (1957) (Fig. 1A). A total of 40 wild type specimens was used.
- (b) Grafting of proximal segments of the first ray (R1) of either dorsal or ventral lobe to the proximal region of the interray space (I9) of the shortest fin rays (R9) of the tail fin (Fig. 1B). To carry out the operation, the middle scale at the base of the tail fin was first removed. A fragment of R1 was then inserted through a hole in the epidermis at the level of the hypural bone. One month after grafting, the fins were cut at the level of the graft (small arrow). Morphological analysis was performed on 29 specimens. msxD expression was analyzed in 11 additional operated specimens.
- (c) A hole corresponding to the length of two or three ray segments was cut in the first lateral long rays of both lobes of the tail fin (R1) (Fig. 1C only shows operation in one lobe). After wound healing, the ray regenerate sometimes did not join the distal portion of the hole and curvedly grew outside of the fin, forming an additional ray (ectopic R1) (Fig. 1C’). One month later, the fin was amputated at the level of the base of this new ray and the morphology of the regenerate was studied in 16 specimens.
- (d) Two days after complete extirpation of R3, a fragment of the proximal part of R9 was grafted in the empty space left after R3 extirpation (Fig. 1D) following the protocol described in (b). Extirpation was carried out by opening the epidermis covering the base of the ray and excising the proximal-most part of the ray. Fifteen days postoperation, the fin was cut at the level of the graft. Sixteen specimens were studied.
- (e) Similar operation to (d), except that the grafted piece was from the proximal part of R3 (Fig. 1E). In this operation a partial extirpation of the proximal-most part of R3 was previously done. Two days later, a proximal fragment of the remaining R3 was grafted in the empty space left by the extirpated tissue. Twelve specimens were studied.
- (f) Similar operation to (b), except that the grafted piece was from the proximal part of R8 (Fig. 1F). Fifteen specimens were used.
- (g) Following single ray (R3) ablation performed as in (a) and amputation of the other lobe as an internal control for gene expression under the normal regeneration process (Fig. 1G), the fins of 22 specimens were allowed to regenerate for 4 days, fixed in 4% paraformaldehyde in phosphate-buffered saline (PFA/PBS) for 2 h at room temperature, and processed for in situ hybridization.
- (h) Same operation as in (g) except that R3 ablation was preceded by the amputation of rays 1 and 2 five days before (Fig. 1H); msxD expression was analyzed, in nine specimens, 4 days after single ray ablation.

Anatomy and Histochemistry

Anatomical inspections were performed by using a dissecting microscope (SMZ800; Nikon, Japan). Morphometrical analysis was...
done from the digital pictures with a Visilog program. Statistical analysis (X) was carried out according to Sachs (1978). Whole-mount preparations were obtained from fins fixed in 4% PFA/PBS 1 month postoperation after dehydrating the tissue in alcohol, clearing with xylene, and mounting in Eukitt. Histological sections (7 or 10 μm) of the regenerates obtained after operation (b) were stained with hematoxilin–eosin or picrosirius–hematoxilin according to Becerra et al. (1983). Sections were analyzed by using a Microphot FXA (Nikon, Japan) microscope and photographed with Nomarski optics.

**FIG. 1.** Schematic representations of the operations carried out in this paper. (A) Partial ablation of the third ray of either dorsal or ventral lobe at a proximal level. (B) Heterotopic graft of proximal fragment of the first ray (R1) into the internay (I9) between the middle shortest rays (R9). After the operation, the fin was cut at the level of the graft (small arrow). (C) Ablation of two to three segments in the proximal region of R1. The result of such “hole” is shown in (C’). An ectopic R1 (eR1) is obtained by regeneration from the proximal stump of the first ray. The horizontal line in (C) and asterisk in (C’) indicate the level of amputation following regeneration of the grafted piece. (D) Two days after complete extirpation of R3 (X) (see Materials and Methods), a fragment of the proximal part of R9 was grafted in the empty space left after extirpation of R3. Fifteen days postoperation, the fin was cut at the level indicated by the horizontal line (small arrow). (E) Similar operation to (D), except that the grafted piece originated from the proximal part of R3. (F) Similar operation to (B), except that the grafted piece originated from the proximal part of R8. (G) Following single ray (R3) ablation performed as in (A) and amputation of the other lobe as an internal control for gene expression under the normal regeneration process, the fins were allowed to regenerate for 4 days, then fixed and processed for in situ hybridization. (H) Same operation as in (G) except that R3 ablation was preceded by the amputation of rays 1 and 2 five days before. msxD expression was analyzed 4 days after single ray ablation.

**In Situ Hybridization**

In situ hybridization with msxA, msxD, bmp4, and shh antisense RNA probes was carried out according to previous protocols (Akimenko et al., 1995). The probes consisted of antisense RNA corresponding to the longest available cDNA for msxD (Ekker et al., 1992; probe size: 1145 nucleotides); to a PCR fragment of 641 bp for msxA (Akimenko et al., 1995); to a 1440-bp C-terminal fragment of shh (Krauss et al., 1993); to the full-length cDNA (1782 bp) of bmp4 (Nikaido et al., 1997).
**Dil labeling**

Dil injection in the connective tissue between the hemirays and in the epithelial tissue was carried out as described in Poleo et al. (2001). Injection was done in proximal regions of R1 in four individuals. A fragment of R1 labeled with Dil was then grafted in the I9 interray region. One day after this operation, the fin was amputated at the level of the graft. Four days later, Dil staining was analyzed in transversal cryosections of the regenerate. Observations were carried out by using light and fluorescence microscope (Zeiss Axioskop) and photographed under Nomarski optics by using a Northern Eclipse version 6.0 program.

**RESULTS**

**After Single Ray Ablation, Formation of a Bifurcation in the Regenerate Depends on the Presence of Surrounding Interray Blastemas**

In *D. rerio*, all the rays bifurcate (Fig. 2A) except for the most lateral ones in the caudal fin or the anterior-most of the rest of the fins. In addition, after the first bifurcation of the second ray (R2) of the caudal fin, the daughter branch closest to R1 does not undergo any further bifurcation in contrast to the other R2 branch and the rest of the rays (R3–R9) (Fig. 2B). To examine the relative importance of the surrounding tissue in the formation of ray bifurcations, we performed single ray ablation experiments. The third dorsal rays of the caudal fin were cut below the level of the first bifurcation. In 23 out of 40 cases, the epidermis rapidly covered the stump and healed the lateral tissues of R2 and R4, being in continuous contact with the outgrowing ray (Fig. 2C). In all these cases, several days later, the rays regenerated and formed normal bifurcations (Fig. 2D). However, in the remaining 17 out of 40 cases, following this cut, wound healing occurred in such a way that the epithelial tissue filled the entire empty space as a wedge between R2 and R4 (Fig. 2E). In this context, 7 of the regenerates grew outside of the fin. The other rays regenerated within a well-differentiated and well-developed interray tissue. In these conditions, the rays regenerated and they did not bifurcate (Fig. 2F) or they bifurcated very distally, in the last four segments of the ray, without forming an internal interray space (14 of 17 cases).

Previous results suggested that the ray needs to be surrounded by adjacent rays to bifurcate normally (Marti-Beffa et al., 1996, 1999). The present results suggest that the fin ray only requires two adjacent interray blastemas to bifurcate normally. In absence of the surrounding interray blastemas or in the presence of differentiated interray tissue, the rays lack bifurcations or these bifurcations are aberrant.

**Nonbifurcating Long Rays Can Form Bifurcations When Grafted in the Interray Tissue of Small Bifurcating Rays**

To analyze the potentiality of interray tissues to induce bifurcation in a normally nonbifurcating ray, we grafted a proximal fragment of the first nonbifurcated ray (R1) in an interray tissue (I9) between the shortest bifurcating rays (R9) (Fig. 1B), let it regenerate, and then cut the whole fin through the graft. We then examined whether the grafted piece could initiate the formation of a bifurcation in this new environment.

After heterotopic graftings, the implanted tissue perfectly healed in the host tissue (Figs. 3A and 3B). Two days after grafting, the epidermis of the grafted fragment was still covering the lepidotrichia (Fig. 3C), but it disappeared several days later (data not shown). In some cases (5 of 29 cases), no sign of regeneration was observed and the graft showed the same anatomical appearance than at the first
day postamputation (Fig. 3A). However, in 24 cases, a regenerative process occurred 3 or 4 days after grafting. In most of the cases (18 of 24), the regenerate from the grafted piece grew outside of the fin. In these cases, the ray never bifurcated and showed a shorter length than R9 (Fig. 3D). In the rest of the cases, when the grafted piece grew inside the fin, either complete regenerates, similar to control, or small and thin regenerates were obtained which did not form bifurcation. The regenerated interray epidermis was also modified, showing, on cross-sections, a diminished thickness which resembled the epidermis of a normal ray (Fig. 3E).

In order to determine the origin of the cells forming the regenerative process, we labeled the grafted material with Dil. Dil was injected 2 days before grafting in the connective tissue located between the hemirays of R1 as well as in the epithelial cells surrounding this ray. One day after grafting, the fin was cut at the level of the labeled graft. Four days after cut, a blastema was formed and Dil-labeled cells were observed by using a fluorescence microscope. A diffuse pattern of labeling was observed in the blastema but no labeled epithelial or interray cells were detected (Fig. 3F). These results indicate that the epidermis covering the grafted tissue is of host origin. They also suggest that there is not significant lateral mixing between ray and interray blastemas during blastema formation.

One month postgrafting, a second cut at the level of the graft was made across the whole fin. By this procedure, we obtained ray 1-interray 9 recombinants (R1-19) in which the first nonbifurcating ray (R1) was in contact with the interray between rays 9 (R9), which do normally bifurcate. To follow the differentiation of this R1-19 recombinant, we took advantage of the presence of a specific type of pigment cells, called white cells (Johnson et al., 1995), which are characteristic of the long rays of the dorsal and caudal fins, such as R1, and are absent from the other rays (Fig. 3G). These white cells show an orange pigmentation when mounted in the resin which is easily distinguishable from the melanocytes, displaying a characteristic black pigmentation (Fig. 3G). Although most of the grafted fragments were unpigmented, white cells were observed along the distal region of the graft regenerate after this second operation, revealing the R1 origin of the graft (Fig. 3H). The analysis of the length of the recombinants revealed another origin-feature in a herkunftsgemäss (origin-dependence, according to classical terms) regeneration of the recombinants. Although on average, the total length of the R1-19 regenerates was not significantly different from that of the neighbor R9 (Table 1), we observed in some instances a slight overgrowth of the recombinant as compared with their neighbors (Fig. 3I). However, in no case was the complete R1 length obtained.

Interestingly, R1 regenerated, in the R1-19 recombinants (8 of 24 cases), bifurcating in 4 of 8 cases (Fig. 3I). According to white cell distribution, a significant cell mixing among neighboring rays can be ruled out in these cases. These bifurcations were morphologically intermediate between those occurring in their neighbor R9 rays and the bifurcations of the long rays, in terms of the distance between the base of the ray and the bifurcation (Table 1). The differences observed between R1, R9, and R1-19 segment length, however, were not significant (Table 1). These observations suggest that R1, in R1-19 recombinants, is able to show hidden morphogenetic capabilities that can be induced by the new environment in an ortsgemäss (new position-dependent) regeneration.

Altogether, these results show that unbifurcated long rays placed in the interray tissue of small bifurcating rays can regenerate as small rays and can bifurcate with a pattern similar to that of their neighbors.

### Nonbifurcating Long Rays Can Form Transient Bifurcations When They Are Surrounded by Interray Tissue

We previously showed that when R3, which normally bifurcates, regenerates independently of the surrounding rays R2 and R4, it does not form bifurcations (Marí-Beffa et al., 1996, 1999). To further determine whether the formation of bifurcations depends on the presence of the surrounding interrays, a third operation was carried out to surround the ray1 (R1) with an ectopic interray 1 (I1). We made a hole of a few segments in both R1 below the level of the first bifurcation. It has been shown that this procedure leads to the formation of an ectopic fin fragment which regenerates from the proximal level of the hole, does not fuse with the distal part of the ray, and grows distally as a new independent tissue (Nabrit, 1929).
In 21 of 32 tail fin lobes analyzed, an ectopic R1 (eR1) grew outwards from the proximal surface of the hole and induced the regeneration of an ectopic interray I1 (eI1) which joined the original R1 to the ectopic one (Fig. 4A). In this condition, R1 with two lateral interrays, I1 and eI1, was obtained. After 20–30 days of regeneration, these fins were cut just above the origin of the eR1 that was labeled with DII prior grafting. DII labeling is restricted to the blastema cells (b). In contrast, epithelial cells (e) and interray tissue are not labeled. (G). White cells (arrows) are restricted to the distal portion of the lateral long rays (R1–R4) of each lobe of the tail fin. After mounting the fins, these cells become orange and are easily distinguished from the more abundant melanocytes, which are black (arrowhead). R1 and R2, original first and second rays. (H) Detail of a regenerate obtained following amputation at the level of the R1–I9 graft. The regenerate is surrounded by the central interray tissue (I9) and presents white cells (arrow) characteristic of R1 and melanocytes (arrowhead), which are found in most of rays. (I) Detail of the distal margin a regenerated R1–I9 heterotopical graft. The graft regenerate shows a bifurcation and segmentation pattern similar to neighbor rays but it slightly overgrows the neighboring rays and presents the characteristic white cells of R1. Arrowheads show segmentation process in the bifurcated ray. Scale bars represent 25 (F), 33 (C), 50 (E), 100 (A, G, H), and 200 μm (B, D, I).

However, we observed a number of transient bifurcations (4 in R1 and 8 in eR1 out of 21 fins) and one case of a complete bifurcation. The aborted bifurcations initiated in a similar manner both in eR1 (Fig. 4C) and R1, but in some segments (1–5) distal to the bifurcation, the new ray branches fused in a single ray (Fig. 4D). Three out of four fins showed transient bifurcation in both eR1 and R1 (Table 2). A much lower incidence of transient bifurcations was observed in control R1 (1 of 11 cases) (those operated R1 which did not regenerated an ectopic R1).

Our results suggest that R1 does not bifurcate because of the absence of one of the two neighbor interrays. They also
demonstrate the inductive potential of the interray tissue on patterning the adjacent fin ray.

The Length of the Regenerated Ray Can Be Modulated Depending on the Neighboring Epidermis and/or Interray

As described above, we observed that grafting R1 in the interray of R9 leads to a regenerate of small size resembling neighboring R9 rays, raising the possibility that interray tissue and/or epidermis may regulate the length of a ray. To further analyze the influence of the interray tissue on the length of the ray, we analyzed the effects of grafting different ray fragments into different interray positions. In the first experiment, R9 fragments were heterotopically grafted in place of R3, which was previously extirpated (Fig. 1D). When examined 1 month later, R9 poorly grew in most of the cases (12 of 16 cases). However, in 3 of 16 cases, the regenerate overgrew the normal length of R9 rays becoming 3–6 segments longer than the original rays (Fig. 4E).

In the control operation (e), a proximal fragment R3 was grafted in the empty space left following extirpation of R3 (Fig. 1E). One month later, the length of the regenerate was similar to the original R3 in 5 of 12 cases (Fig. 4F); in the other cases, the regenerate did not grow. We performed another control experiment in which proximal fragments of R8 were grafted in the interray 9 (I9) (Fig. 1F); the fin was then cut at the level of the graft and the regenerate examined 1 month after amputation. As expected, since R8 and R9 are normally of very similar size, in the 3 cases which regenerated out of 15 cases tested, the grafted R8 was either slightly smaller (Fig. 4G) or as long as R9 (data not shown). These grafted regenerates showed a pattern similar to neighboring R9 rays (Fig. 4H). In all of the above cases that properly regenerated, the rays formed bifurcations, although slightly distal than normal.

These results suggest that the rays have a morphogenetic plasticity which depends on the interray and/or epidermis environment. When grafted to ectopic positions, short rays may overgrow when they are adjacent to long rays and long rays can regenerate as shorter rays when placed in the context of short rays.

The Ray Blastema Induces msxD and msxA Expression in the Overlying Ray and Interray Epithelial Tissue

To further analyze the hypothesis of inductive interactions between the distal epidermis and the underlying mesenchymal cells of the blastema as well as interactions between ray and interray blastemas, we examined the expression, after single ray ablation, of genes known to play important role in epithelial-mesenchymal interactions during morphogenesis of many systems, such as members of the msx homeobox gene family and of the bone morphogenetic proteins (Fig. 1G). msxA and msxD are expressed in the distal epidermis covering the ray blastema as well as the

![Image](image-url)
blastema. Induction of the distal ray epidermis may be induced by distalizing ray tissue, we analyzed neighbor ray blastema. distal interray epidermis also requires the presence of a cvent to induce misx. Under these conditions, we did not observe msxD expression in the overlying epithelial cells. When the ray blastema (Figs. 5B and 5C). Although msxD expression was observed when epithelial healing of the empty R2-R4 space occurred without growth of a ray blastema (data not shown), msxD was only expressed in the epithelial tissue in contact with the ray blastema (Figs. 5B and 5C). msxA presented a similar pattern of expression as msxD under these conditions (Fig. 5D).

When R3 ablation was preceded by the amputation of the first two rays (R1 and R2) (Fig. 1H), the epithelial tissue healed the wound and filled the space between the regenerating R2 and the uncut ray R4 (Fig. 5E). In this case, R3 grew within a well-differentiated interray tissue. Under these conditions, we did not observe msxD expression in the distal epidermis before the blastema of R3 reached the level of the R2 cut. This result suggests that the mesenchymal cells in the interray may not be sufficient to induce msxD expression in the overlying epithelial cells. When the distalizing ray blastema contacted the distal epidermis, only then, msxD started to be expressed in the interray distal epidermis (Fig. 5F). The expression in this region was more or less homogeneous although gradually diminishing in the most distal part.

These results suggest that msxD and msxA expression in the distal ray epidermis may be induced by distalizing ray blastema. Induction of msxD and msxA expression in the distal interray epidermis also requires the presence of a neighbor ray blastema.

To further determine whether the blastema induction of gene expression depends on the origin of the epithelial tissue, we analyzed msxD expression during the growth of the grafted piece of R1 in the I9 interray tissue (cf. Fig. 1B). As described above, the ray growing from the grafted fragment is overlaid by the epithelial tissue of the host (Fig. 3F). Although msxD expression was very weak, enhanced staining enabled us to observe that msxD expression was restricted to the covering epidermis near the distal blastema (Fig. 5G), as during regeneration following single ray ablation. This observation indicates that the blastema of grafted rays can induce msxD expression in an ectopic fin epidermis.

The bone morphogenetic protein bmp4 has been shown to induce msx gene expression in many developing systems (Vainio et al., 1993; Wang and Sassoon, 1995; Wattanabe and Le Douarin, 1996; Takahashi et al., 1996; Barlow and Francis-West, 1997; Suzuki et al., 1997; Kim et al., 1998; Chen et al., 2000). We thus analyzed the expression of bmp4 under normal conditions of regeneration and following single ray ablation (Fig. 1G). We observed that bmp4 expression is restricted to the distal ray blastema during normal (Figs. 5H and 5I) and single ray regeneration (Figs. 5J and 5K). No expression is observed in the interray region under these conditions (Figs. 5H, 5J, and 5K). This analysis reveals a correlation between bmp4 expression in the distal blastema and the induction of msxD expression in the overlying epithelial tissue, suggesting that msxD inducing capacity of the distal ray blastema may be mediated through bmp4 signaling.

shh is normally expressed in a subset of cells of the basal layer of the epidermis in a pattern suggesting a role in the dermal bone formation and/or patterning (Laforest et al., 1998). We observed that, during single ray regeneration, following reepithelization, shh is restricted to the basal layer of the epidermis covering the proximal ray blastema (Fig. 5L). Interestingly, shh domain of expression was split in two in one of the hemirays, a characteristic that was shown to precede the formation of a bifurcation (Laforest et al., 1998).

**DISCUSSION**

The shape of the teleost fins has been proposed to be the outcome of the developing potential of each ray, their structural units, as analyzed during regeneration (Nabrit, 1929). In this paper, however, we have presented evidence in favor of the hypothesis that some particular morphological aspects of each ray, as the bifurcation and, to a lesser extent, the total length of the ray requires the presence of

<table>
<thead>
<tr>
<th>Variables</th>
<th>R1</th>
<th>Ectopic R1</th>
<th>Control R1</th>
<th>R2</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bifurcation length</td>
<td>1115.1 ± 1205.2*</td>
<td>335.3 ± 315.1*</td>
<td>—</td>
<td>328.9 ± 165.7</td>
<td>4</td>
</tr>
<tr>
<td>Total length</td>
<td>5356.4 ± 589.2</td>
<td>3719.8 ± 1294.6*</td>
<td>4957 ± 866.3</td>
<td>5367.2 ± 714.7</td>
<td>6</td>
</tr>
</tbody>
</table>

Note. (a ± b); a: mean; and b: standard deviation.

* Three cases of each bifurcated ray in four specimens.

Significance of statistical difference (α: 0.01).
FIG. 5. (A) Normal expression pattern of msxD in the epidermis distal to the blastema in control fins. Note that msxD is expressed in the epidermis covering the ray and the interray (asterisks). (B) Following single ray ablation, msxD expression is restricted to the epidermis overlying the distal blastema of the regenerating ray. (C) Cross-section of a fin similar to that shown in (B) at the level of the ray blastema, showing msxD expression in the epithelial tissue surrounding the blastema (asterisk). The lateral epidermis (e) does not express this gene at this level. (D) msxA expression is similar to that of msxD under the same experimental conditions. (E) When ablation of R3 is preceded by the amputation of R1 and R2 a few days before, the epidermis covering R3 (e) remains in contact with neighboring R2 blastema. (F) Expression of msxD under this experimental condition when the ray blastema has reached the distal epidermis (arrow). Regeneration of tissue without ray is faster than normal regeneration but stops at the level of the neighboring blastemas (arrowhead) because it needs two neighboring rays to occur. Control neighboring ray blastemas do not induce msxD expression in the interray (asterisk). (G) During growth of the R1 grafted piece to the I9 interray, msxD expression in the overlying epidermis of the host is only induced at the level of the graft blastema (arrowhead). (H) During normal regeneration, bmp4 is expressed in the distal part of the regenerate (arrow). No expression is observed in the interray region (asterisk). (I) Longitudinal sections of ray shows that bmp4 expression is restricted to the distal blastema (arrow). (J) During single ray regeneration, bmp4 expression is induced in the region occupied by the distal ray blastema (arrow). (K) Cross-section of a single ray regenerate showing that bmp4 is expressed in the distal ray blastema (arrow). There is no expression in the interray blastema and covering epidermis (e). (L) During single ray regeneration, shh is expressed in the basal layer of the epidermis covering the proximal blastema (arrow). Note that shh is expressed in two subdomains (asterisks) in one of the hemirays, suggesting that a bifurcation is about to occur. Scale bars represent 25 μm (I), 50 μm (C, K), 100 μm (A–B, D–G, J, L), 200 μm (E), and 250 μm (H). R1, R2, R3, R4, and R5, original first, second, third, fourth, and fifth ray, respectively; e, epidermis; b, ray blastema.
Ray and Intercell Interactions

the neighbor interrays during their development. This evidence come from regeneration studies in which ray blastemas are recombined with ectopic interrays from different dorsoventral positions of the tail fin of D. rerio. We previously showed that rays regenerating in isolation from their neighbors did not present any sign of bifurcation (Marí-Beffa et al., 1996, 1999). Here, we have shown that: (1) single rays do not bifurcate or only do it at a distal position depending on the presence of a distal epidermis (in this condition no neighbor ray blastema is present ruling out the necessity of its presence for a proper bifurcation to occur); (2) similarly, grafted rays regenerating outside the fin, without neighbor interrays (operation c) do not bifurcate or show distal aborted bifurcations; (3) normally nonbifurcating rays grafted into the interray of bifurcating rays (R1–I9 recombinant) may bifurcate (in this condition, new interrays (2 instead of 1 in the control) are obtained); and (4) normally nonbifurcating rays surrounded by ectopic interrays of the same ray (R1–I1 recombinant) may form transient bifurcations. This suggests that interrays of nonbifurcating rays might have a reduced potential to induce a bifurcation when compared to R1–I9 recombinants. Based on this evidence, we propose that: The interray blastema can induce the complete bifurcation of the neighbor ray blastema at a normal position. In absence of an interray blastema, the ray may bifurcate only distally to their normal position or lack any sign of bifurcation. For this induction to occur, the distal epidermis must be in contact with the distal blastema.

In our R1–I9 recombinant (operation b), R1 showed a length very similar to R9 when growing inside the fin and was reduced when growing outside the fin. These results suggest that the interray not only controls the position of bifurcations but it also modulates ray length. Further evidence comes from the grafting of R9 to R3 region in which R9 regenerate are longer than original R9. In addition, R3 homotopically grafted in its original position or R8 grafted in I9 have a similar length and pattern to neighboring rays.

Melanocytes have been proposed to derive from nonpigmented stem cells ubiquitously distributed over the fin structure (Rawls and Johnson, 2000). Regarding the white cells, our results are consistent with the hypothesis that they might be derived from unpigmented precursor cells present in the grafted fragment since no white cells have been observed in I9 unless heterotopical R1–I9 unpigmented grafts are done.

Epidermis–blastema interactions have been previously proposed to occur during fin regeneration (Marí-Beffa et al., 1996), regulating genes such as ptc-1 by shh (Laforest et al., 1998) or msc via FGFR1 activity (Poss et al., 2000), but no direct evidence was presented. In the present work, we show that the distal blastema induces mscD and mscA expression in the covering epidermis and the differentiation of a characteristic ray epidermis which is thinner than the epidermis of the interray (operation d). We also show evidence that the distal blastema can also activate mscD expression in the interray epidermis. During the normal regeneration process, mscD is expressed in the epidermis covering the ray blastema and the interray tissue. However, under our experimental conditions, expression of mscD is also restricted to the epithelial tissue covering the ray blastema either after a previously differentiated epidermis or grafting experiments. In these conditions, there is no sign of expression in the neighboring interray. Expression of mscD in the interray epidermis only appears when the ray blastema contributes to its direct or indirect induction in distal regions during regeneration. The fact that an interray tissue can regenerate in the absence of mscD expression further suggests that mscD may not be necessary for interray differentiation. We have shown that bmp4 expression is restricted to the distal blastema cells of the single ray and whole fin regenerate. This expression correlates with the induction of mscD expression in the overriding epidermal cells. This observation, combined with the demonstration that msc gene expression is upregulated in response to bmp4 signaling in many systems involving epithelial–mesenchymal interactions (Vainio et al., 1993; Wang and Sassoon, 1995; Wattanabe and Le Douarin, 1996; Takahashi et al., 1996; Barlow and Francis-West, 1997; Suzuki et al., 1997; Kim et al., 1998; Chen et al., 2000), raises the possibility that bmp4 may activate mscD gene expression in the epidermis covering the distal blastema. Finally, as a consequence of epithelial–mesenchymal interactions, expression of shh is induced in basal layer of the epidermis adjacent to the blastema during single ray regeneration.

Evidence of the control exerted by interdigit on digit morphogenesis by bone morphogenetic proteins during chick limb development has been recently shown (Dahn and Fallon, 2000). Modulation of BMP levels with Noggin in an interdigit of the chick foot causes the digit immediately anterior to the treated interdigit to take on the identity of the next more anterior digit. In this paper, a control of this region on digit identity has been claimed, although no HoxD gene misregulation was observed. Similarly, the results presented here indicate that the interray region can modulate ray morphogenesis (position of bifurcation and ray size) during regeneration. This induction capacity varies along the dorsoventral of the caudal fin, as it was demonstrated to occur along the anterior–posterior axis of the chick limb (Dahn and Fallon, 2000). However, white cell differentiation shows that the ray identity is not perturbed and that a homeotic transformation cannot be proposed to occur. The interray modulates morphogenesis of the fin rays, in our experiment, by a mechanism different to homeotic transformations.

The results presented here suggest that fin ray morphogenesis and patterning depend not only on interactions between the ray blastema and the overlying epidermis but also on interactions between the ray blastema and neighboring interray tissue during regeneration. Further analysis must be carried out in order to characterize the signals involved in this process.
ACKNOWLEDGMENTS

We thank Lynda Laforest for technical assistance and Drs. M. Tada and V. Korzh for providing bmp4 and shh cDNAs, respectively. This work has been supported by grants from the Spanish DGESC, PB98-1049 (to M.M.-B.), and the Canadian CIHR, MT-11775 (to M.-A.A.). Some of the information contained in this article has been presented at the III congress of SEBD, Málaga 2001.

REFERENCES


Received for publication January 28, 2002
Revised September 6, 2002
Accepted September 6, 2002
Published online November 7, 2002