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A proliferation gradient between proximal and *msxb*-expressing distal blastema directs zebrafish fin regeneration

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SUMMARY

Previous studies of zebrafish fin regeneration led to the notion that the regeneration blastema is a homogeneous population of proliferating cells. Here, we show that the blastema consists of two components with markedly distinct proliferation properties. During early blastema formation, proliferating cells are evenly distributed. At the onset of regenerative outgrowth, however, blastemal cells are partitioned into two domains. Proximal blastemal cells proliferate at a high rate, shifting from a median G_2 of more than 6 hours to approximately 1 hour. By contrast, the most distal blastemal cells do not proliferate. There is a gradient of proliferation between these extremes. Using bromodeoxyuridine incorporation and anti-phosphohistone H3 labeling, we find a 50-fold difference in proliferation across the gradient that extends approximately 50 μm, or ten cell diameters. We show that during early regeneration, proliferating blastemal cells express *msxb*, a homeodomain transcriptional repressor. While *msxb* is widely expressed among proliferating cells during blastema formation, its expression becomes restricted to a small number of non-proliferating, distal blastemal cells during regenerative outgrowth. Bromodeoxyuridine pulse-chase experiments show that distal and proximal blastemal cells are formed from proliferating, *msxb*-positive blastemal cells, not from preexisting slow-cycling cells. These data support the idea that blastema formation results from dedifferentiation of intraray mesenchymal cells. Based on these findings, we propose a new model of zebrafish fin regeneration in which the function of non-proliferating, *msxb*-expressing, distal blastemal cells is to specify the boundary of proliferation and provide direction for regenerative outgrowth.

Key words: Zebrafish regeneration, Blastema, Proliferation, BrdU incorporation, Cell cycle, *msxb*

INTRODUCTION

For more than two centuries, the subject of regeneration has fascinated natural scientists. Mammals can regenerate some structures, including skin, skeletal muscle, bone, digit tips, liver and blood. By contrast, urodele amphibians and teleost fish have extensive regenerative capabilities. These organisms regenerate limb (fin), eye lens, optic nerve and spinal cord. Despite its importance, the cellular mechanisms of regeneration are poorly understood.

Regeneration model organisms have included invertebrates, like hydra and planarians, and vertebrates, such as axolotl. Recently, zebrafish fin regeneration has emerged as an attractive model system. The advantages of zebrafish include: (1) rapid regeneration time; (2) relatively simple architecture of the caudal fin; (3) the ability to maintain and study zebrafish in large numbers; (4) advances in zebrafish embryology and genetics; (5) the fact that standard genetic approaches, including forward genetics, can potentially be applied to dissect molecular mechanisms.

The zebrafish caudal fin is a symmetric organ composed of multiple bony fin rays or lepidotrichia. Each fin ray consists of a pair of concave hemirays formed by multiple segments joined end to end by ligaments. Blood vessels, nerves, pigment cells, and fibroblasts are in the mesenchymal compartment between rays, as well as in the intraray space. Each hemiray is surrounded by a monolayer of bone secreting cells, scleroblasts. Zebrafish fins are continuously growing throughout life by addition of ray segments to the end of the fin (Johnson and Bennet, 1999a; Iovine and Johnson, 2000).

Stages of caudal fin regeneration at various temperatures have been described (Becerra et al., 1983; Geraudie and Singer, 1992; Johnson and Weston, 1995; Poss et al., 2000b). At 33°C, the wound is closed by a thin layer of epithelium within 12 hours of amputation. In the next 12 hours, the wound epithelium thickens and mesenchymal tissue proximal to the amputation plane disorganizes. Mesenchymal cells begin to proliferate and appear to move in a distal direction. The blastema, a mass of mesenchymal cells thought to be pluripotent, is formed within the next 24 hours. The origin of the blastema remains

controversial. It is unknown whether the blastema forms through the process of dedifferentiation or through the recruitment of stem cells. The onset of the next phase, regenerative outgrowth, is marked by deposition of new bone. It has been postulated that distal blastemal cells proliferate, while proximal blastemal cells differentiate into missing structures. Although, it has been assumed that blastemal cell proliferation is critical to regeneration, blastemal cell cycle properties during regenerative outgrowth or blastema formation have not been characterized.

Previous reports investigated proliferation patterns in regenerating teleost fins using bromodeoxyuridine (BrdU) incorporation (Santamaria et al., 1996; Poleo et al., 2001) and Hoescht dye labeling (Johnson and Bennet, 1999a). However, these studies led to the idea that the fin blastema is a homogeneous population of proliferating cells. Here we show that the fin blastema is subdivided into two domains, a non-proliferating distal blastema and a highly proliferating proximal blastema, with a steep gradient of proliferation between these regions. During blastema formation, *msxb*, a homedomain transcriptional repressor, is expressed in a large number of proliferating cells. However, *msxb* expression becomes restricted to a small number of non-proliferating distal blastemal cells during regenerative outgrowth. BrdU pulse-chase experiments show that blastemal cells are formed from proliferating mesenchymal cells, not from a population of preexisting slow-cycling cells, consistent with the notion that the blastema is derived from dedifferentiated intraray mesenchyme. We have also determined that the length of the G_2 phase of the cell cycle during regenerative outgrowth is dramatically shorter than during blastema formation, which is likely a reflection of rapid cell cycle during outgrowth. Based on these findings, we propose a novel model of zebrafish fin regeneration, in which we argue that the function of the non-proliferating distal blastema is to delineate the boundary of proliferation and direct regenerative outgrowth.

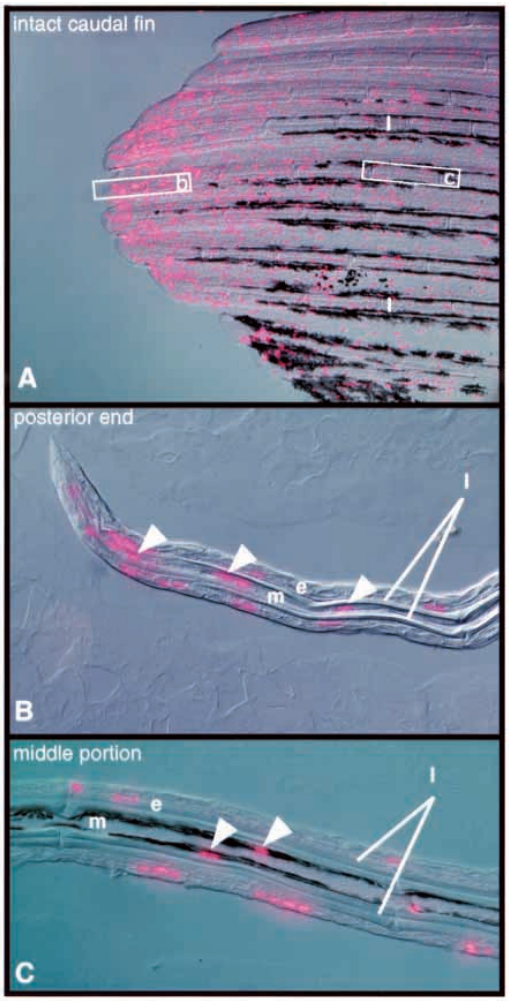

Fig. 1. Proliferation in non-regenerating caudal fin**.** Zebrafish (*n*=5) were incubated in BrdU-containing water for 18 hours and BrdU was detected using immunolabeling (red). Posterior is at left and anterior is at right. (A) Whole-mount caudal fin shows abundant labeling concentrated at the posterior end of the fin. Rectangles indicate the approximate site of longitudinal sections. (B) Longitudinal section from the posterior end of intact caudal fin. Note positive labeling of both epithelial and mesenchymal cells (arrowheads).
(C) Longitudinal section through the middle of the caudal fin. Note that both epithelial and mesenchymal cells are labeled (arrowheads). e, epithelium; l, lepidotrichia; m, mesenchyme.

MATERIALS AND METHODS

Fish care and surgery

Zebrafish 4-6 months of age were obtained from EKKWill Waterlife Resources (Gibsonton, FL) or were derivatives of WIK strain (Johnson and Zon, 1999b). Fish were maintained in our laboratory by random pair mating. Fish were anesthetized in 0.1% tricaine and caudal fin amputations were performed using razor blades. Animals were allowed to regenerate for various times at 33°C, a temperature that accelerates regeneration. Fish were anesthetized and regenerates were collected for further analysis.

BrdU incorporation assays

BrdU was dissolved in fish water at a final concentration of 50 μg/ml. Fins were amputated and fish were kept at a density of five animals per liter. For long incubations, BrdU was changed daily. Fish survived normally and demonstrated no unusual behavior while in the BrdU solution.

Simultaneous detection of *msxb* and *shh* transcripts and PCNA

msxb and *shh* RNA antisense probes were generated as previously described (Poss et al., 2000b). We used a previously published whole-mount in situ hybridization protocol with the following modifications (Poss et al., 2000b). PCNA mouse monoclonal antibody (Oncogene, 1:100 dilution) was added during sample incubation with anti-digoxigenin antibody (Roche Molecular Biochemicals) coupled to alkaline phosphatase. Fins were washed for at least 2 hours in multiple changes of phosphate-buffered saline (PBS)-0.1% Tween 20 (PBT), followed by the last wash in PBT with 2 mg/ml bovine serum albumin (PBTs). Fins were treated with anti-mouse secondary antibodies conjugated to Alexa 488 (Molecular Probes) in PBTs overnight at 4°C. On the following day, fins were washed in multiple changes of PBT for at least 2 hours and processed for the alkaline phosphatase reaction. Fins were washed three times in reaction buffer (0.1 M Tris-HCl pH 8.0, 0.1 M NaCl. 0.01 M MgCl, 0.1% Tween 20, 0.01 M levamisol) and incubated in the reaction buffer with HNPP/Fast Red TR substrate (Roche Molecular Biochemicals) for 3 hours, changing substrate solution at the end of each hour. Following the detection reaction, fins were washed several times with PBT and processed for cryosectioning. Fins were embedded in 1.5% agarose/5% sucrose blocks and treated in 30% sucrose overnight. Frozen blocks were sectioned at 14 μm, mounted on slides using Vectashield with DAPI (Vector), and digital images were

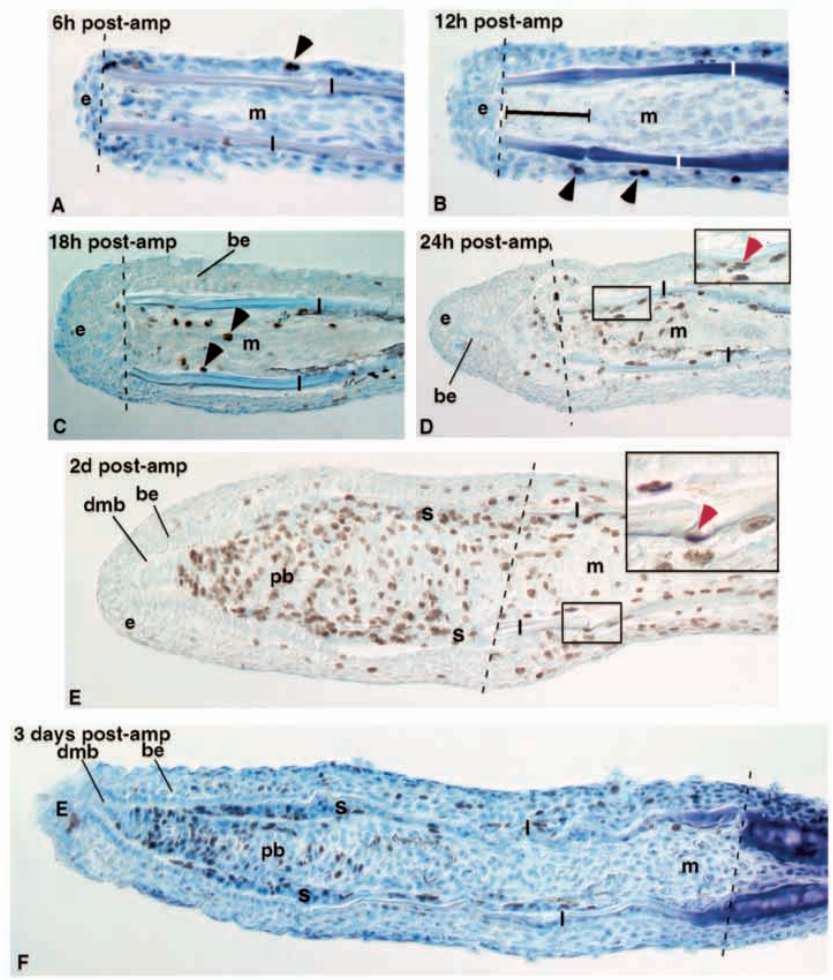

Fig. 2. Fin regeneration blastema consists of two functionally distinct regions, a non-proliferating distal and proliferating proximal blastema. Fish were incubated with BrdU for 6 hours before harvesting their fins. Longitudinal sections were stained with BrdU antibody (brown signal indicated by black arrowheads) and then counterstained with Hematoxylin. (A) 6 hours post-amputation (h.p.a.), a thin layer of wound epidermis covers the stump. The region immediately proximal to the amputation plane shows basal levels of epidermal proliferation; no BrdU-positive cells are detected in the intraray mesenchyme. (B) Early signs of mesenchymal disorganization are seen 12 h.p.a. (bracket). The number of proliferating epithelial cells is at basal levels, but there is no proliferation in the mesenchyme. (C) BrdU-positive mesenchymal cells were first detected 18 h.p.a. (arrowheads). Wound epidermis 24 h.p.a. thickens and the basal epidermal layer is initiated. (D) The number of BrdU-positive mesenchymal cells continues to increase, with some positive cells detected distal to the amputation plane. In addition to fibroblast-like cells, proliferating scleroblasts (D,E and insets) are seen (red arrowheads). (E) 2 days post-amputation, a functional blastema is formed. Note that the number of BrdU-positive cells is high in the proximal blastema, while the distal blastema remains unlabeled. Proliferating scleroblasts are observed immediately distal to bone stumps. (F) 3 days after amputation, newly formed bone is visible distal to old bone stumps. High levels of BrdU incorporation are seen in the proximal blastema, while the distal blastema remains unlabeled. Note that newly formed, BrdU-positive scleroblasts were apparent immediately behind the central zone of proliferation. The amputation plane is indicated by a dashed line. b, blastema; be, basal epidermal layer; e, wound epidermis; dmb, distal-most blastema; l, lepidotrichia; m, intraray mesenchyme; pb, proximal blastema; s, scleroblasts. Original magnifications: (A) 400×, (B) 400×, (C) 250×, (D) 200×, (E) 126×, (F) 126× and (G) 100×.

captured using an Axiocam CCD camera equipped with Axiovision software (Zeiss). Images were processed in Adobe Photoshop 5.5 (Adobe Systems Inc.) or Canvas 8 (Deneba Systema, Inc).

Immunohistochemistry

For sections, fin regenerates were fixed in 70% ethanol in 50 mM glycine (pH 2.0) overnight at 4°C. The next day, fins were rinsed twice in 100% methanol and rehydrated through a methanol/PBS series (75% methanol/25% PBS, 50% methanol/50% PBS, and 25% methanol/75% PBS) and embedded for cryosectioning. Sections (14 μm) were made from frozen blocks as described above. BrdU incorporation was visualized using a detection kit from Roche Molecular Biochemicals. After processing, sections were counterstained with Hematoxylin (Vector Laboratories), dehydrated and permanently mounted for photography. For whole mounts, fin regenerates were incubated in Carnoy's fixative (60% ethanol, 30% chloroform, and 10% acetic acid) overnight at 4°C. BrdU and H3P were detected using an adaptation of a previously published protocol (Newmark and Sanchez Alvarado, 2000). Fins were washed twice in methanol and rehydrated through a methanol/PBS+0.3% Triton X-100 (PBTx) series, they were then washed twice in 2 N HCl in PBTx and incubated in 2 N HCl in PBTx for 30 minutes. Fins were rinsed twice in PBTx and blocked for 4 hours in PBTx+0.25% BSA, followed by incubation with anti-BrdU monoclonal antibodies (Chemicon International Inc., 1:100) and anti-H3P rabbit polyclonal antibody (Upstate Biotechnology, 1:100) overnight at 4°C. On the next day, fins were washed in PBTx multiple times throughout the day (last wash PBTx+0.25% BSA) and incubated overnight at 4°C with anti-mouse antibody conjugated to Alexa 594 (Molecular Probes) and with anti-rabbit antibody conjugated to Alexa-488 (Molecular Probes), both diluted 1:200. Finally, fins were washed in PBTx and processed for cryosectioning and photography.

Quantification and statistical analysis

To determine the distribution of BrdU- or H3P-positive cells compared with the length, we overlaid each photographed section (all sections computer magnified to ×454 for analysis) with a 10 μm-square grid with zero length corresponding to the most anterior blastemal cell. At each length point a number of positive cells was normalized to the total number of squares and then to the averaged number of DAPI-stained nuclei per square. Only signals that had corresponding DAPI-stained nuclei were considered positive. Values were given as means ± standard deviation (s.d.) or standard error (s.e.m.). Because the data sets did not have a normal distribution, we used nonparametric Mann-Whitney test to compare means. Data sets were analyzed using Microsoft Excel 98 Data Analysis Tool package. Z, $Z_{critical}$, and *P* values were given for each case.

RESULTS

Proliferation in non-regenerating caudal fin

To determine the spatial distribution of proliferating cells in the intact zebrafish caudal fin, fish (n=5) were incubated in BrdU-

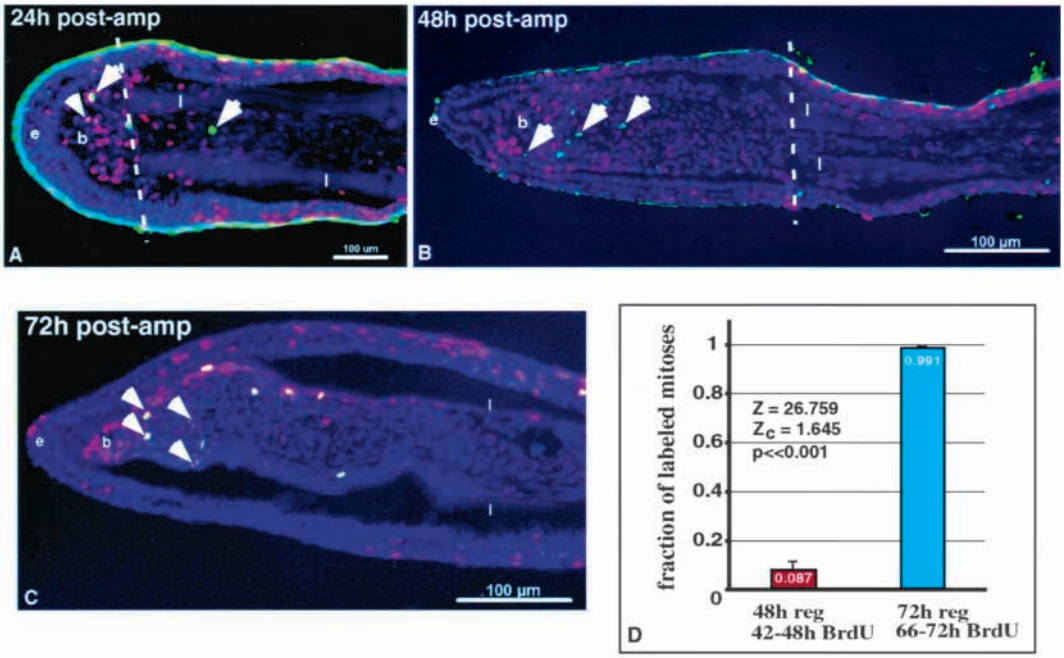

Fig. 3. G_2 shortens dramatically with the shift from blastema formation to regenerative outgrowth. (A-C) Longitudinal sections obtained from fins that were regenerating for (A) 24 hours (*n*=5), (B) 48 hours (*n*=3), and (C) 72 hours (*n*=4). Fins were treated with BrdU for 6 hours, followed by detection of BrdU (red) and anti-phosphohistone (H3P) (green), and counterstaining with DAPI (blue) to reveal nuclei. Double labeling appears yellow (arrowheads). (A) The first mesenchymal mitotic cells are seen 24 hours after amputation. (B) The number of cells undergoing mitosis continues to increase by 48 hours. Note that only a small fraction of H3P-positive cells (arrows) are also BrdU-positive 24 or 48 h.p.a., indicating that the duration of the median blastemal G_2 is greater than 6 hours. (C) By 72 hours post-amputation, almost all H3P-positive cells are also BrdU-positive, indicating that the median blastemal G_2 is less than 6 hours. (D) The fraction of labeled mitoses is shown for blastema formation (48 hours h.p.a.; 252 H3P-positive cells counted from 3 regenerates, 0.087±0.033) and during regenerative outgrowth (72 h.p.a.; 337 H3P-positive cells counted from 4 regenerates, 0.991±0.005). Note that the fractions are significantly different. Error bars indicate s.e.m. Z value for Mann-Whitney test is 26.759 ($Z_{critical}$ is 1.645), $P<<0.001$.

containing water for 18 hours. BrdU is a thymidine analog that incorporates into DNA during replication and can be detected using immunostaining. As expected, we found proliferating cells concentrated at the distal end of the fin (Fig. 1A). Sections through the posterior end of the fin showed BrdU labeling of both epidermal and mesenchymal cells (Fig. 1B). Sections through the middle of the fin revealed that epithelial cells incorporated the majority of label. We found a small number of positive mesenchymal cells, including scleroblasts, in the intraray mesenchyme (Fig. 1C and data not shown). These data indicate that proliferating epithelial cells are distributed throughout the fin, while proliferating mesenchymal cells are concentrated distally, where bone growth occurs.

Fin regeneration blastema consists of two functionally distinct regions, a non-proliferating distal and proliferating proximal blastema

To determine the distribution of proliferating cells in the regenerating caudal fin, we performed BrdU incorporation studies. To initiate these experiments, we empirically determined the length of time necessary to achieve BrdU incorporation during fin regeneration. Zebrafish that had been regenerating for 24 hours were incubated with BrdU for 2, 6, 12 and 18 hours and caudal fin regenerates were analyzed for the presence of BrdU-positive cells (data not shown). At 6 hours of BrdU treatment, incorporation was relatively high, whereas at 2 hours there was much less staining. In many subsequent experiments, therefore, we labeled for 6 hours.

We next examined levels of BrdU incorporation during wound healing (*n*=5, 20-40 sections examined for every time point described below). During the first 6 hours of regeneration, the wound was closed with a thin layer of epithelium. Closure most likely occurred by migration of epithelial cells from the lateral edges over the wound because proliferation in lateral epithelial cells was similar to basal levels (Fig. 2A). In the next 6 hours, the wound epidermis thickened, while the number of BrdU-positive cells in the lateral epidermis remained at basal levels (Fig. 2B). Mesenchymal tissue between hemirays displayed early signs of decondensation and disorganization. However, we did not detect BrdU-positive mesenchymal cells at this stage (Fig. 2B). Fig. 2C,D shows that a columnar basal epithelial layer was formed by 18-24 hours post-amputation (h.p.a.). These data indicate that wound healing results from epithelial cell migration, not proliferation.

Next, we examined BrdU incorporation during blastema formation (12-48 h.p.a.). The earliest evidence of proliferating mesenchymal cells was detected 18 h.p.a. (Fig. 2C). The extent of proliferation was one to two segments proximal to the amputation plane. The number of BrdU-positive cells continued to increase 24 h.p.a., at which point the first labeled mesenchymal cells distal to the amputation plane were detected (Fig. 2D). In addition to proliferating fibroblast-like cells, we detected proliferating scleroblasts as early as 18-24 h.p.a. (red arrows on Fig. 2D and data not shown). These cells were readily distinguished because of their characteristic elongated nuclei and positioning next to lepidotrichia. In the next 24 hours, a large number of proliferating cells accumulated in the blastema and epidermis (Fig. 2E). Scleroblasts aligned in two lateral domains, just distal to the amputation plane, and could be detected by darker Hematoxylin staining in Fig. 2E,F. These data indicate that most proliferating cells in the early stages of caudal fin regeneration (12-24 h.p.a.) are concentrated in the developing blastema, whereas epithelial proliferation remains at basal levels.

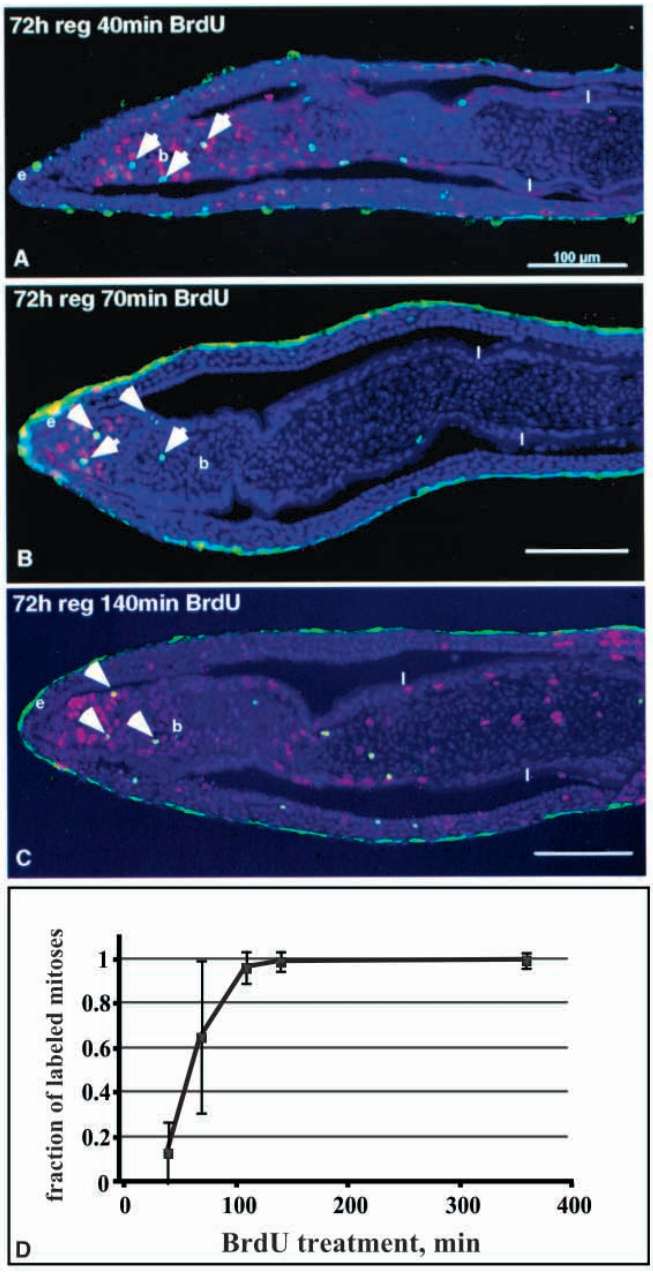

Fig. 4. Rapid G_2 during regenerative outgrowth.
(A-C) Representative sections of fins treated with BrdU for (A) 40 minutes, (B) 70 minutes, and (C) 140 minutes and stained for BrdU (red) and H3P (green). Double labeling appears yellow (arrowheads). Note the absence of double-labeled cells after 40 minutes, presence of some double-labeled cells after 70 minutes, and labeling of all H3P-positive cells with BrdU after 140 minutes. (D) Plot of the fraction of labeled mitoses over time. Each time point represents measurements ±s.d. from 40 sections obtained from three or four regenerates. Scale bars: 100 μm.

We next examined levels of BrdU incorporation in regenerating caudal fins during regenerative outgrowth, which begins approximately 48 h.p.a.. A previous study suggested that BrdU incorporation was limited in the distal portion of goldfish blastema during outgrowth (Santamaria et al., 1996). We found that the number of BrdU-labeled cells remained high in proximal blastema and adjacent aligned scleroblasts. However, the distal-most blastema (DMB) was scarcely labeled (Fig. 2E,F). This finding indicated a marked difference in the length of cell cycle between DMB and proximal blastema. Alternatively, DMB cells may have withdrawn from the cell cycle.

G_2 shortens dramatically with the shift from blastema formation to regenerative outgrowth

To define the onset and distribution of mitotic cells during caudal fin regeneration, we used anti-phosphohistone H3 (H3P) antibody. Serine (ser10) phosphorylation of histone H3 is correlated with, and required for, chromosome condensation during mitoses (Hendzel et al., 1997). Because this modification is highly conserved, H3P antibodies are a specific mitotic marker (Wei et al., 1999). Although the first BrdU-positive mesenchymal cells were detected 12-18 h.p.a., we saw no H3P-positive cells until 24 h.p.a. (Fig. 3A; n=5). By 36 and 48 h.p.a., the number of mitotic cells increased. We detected H3P-positive mesenchymal cells distal and proximal to the amputation plane (Fig. 3B and data not shown). Only 8.7% (n=3, 252 H3P-positive cells from 42 sections examined) of all H3P-labeled cells were also BrdU-positive 48 h.p.a., following a 6-hour BrdU incorporation (Fig. 3B,D), indicating that the median G_2 of the blastemal cell cycle is longer than 6 hours during this phase of blastema formation (Aherne, 1977).

We next examined the distribution of mitosis during regenerative outgrowth. Fig. 3C shows a 3-day fin regenerate stained with BrdU and H3P antibodies. Fig. 3D shows that in all fins analyzed (n=4, 337 H3P-positive cells examined from 40 sections), 99% of H3P-positive cells were also BrdU positive, indicating that the median G_2 is less than 6 hours. These data indicate that G_2, and probably the mesenchymal cell cycle, during regenerative outgrowth is much shorter than the cell cycle during blastema formation (Z=26.759, P<<0.001, Mann-Whitney test).

To calculate G_2 of the blastemal cell cycle during regenerative outgrowth, we determined the fraction of H3P-positive cells that were also labeled with BrdU during various BrdU treatments 72 h.p.a. (Aherne, 1977). We limited our analyses to the blastemal cells located in the distal third of regenerates, where most proliferative cells are concentrated (Fig. 4A-C). As early as 40 minutes after BrdU treatment, ~12% of mitotic cells were labeled with BrdU (n=4 regenerates, 356 mitoses from 40 sections) and by 70 minutes, ~65% of mitotic cells were labeled (n=3 regenerates, 217 mitoses from 40 sections). Almost 96% of mitotic cells were labeled with BrdU after 110 minutes (n=4 regenerates, 272 mitoses from 39 sections). We plotted the fraction of BrdU-labeled mitoses against time to generate a fraction of labeled mitoses (FLM) curve (Fig. 4D). From this curve, we concluded that 50% of mitoses are labeled at ~60 minutes, which represents the median length of G_2 during outgrowth, with a minimum G_2 duration of ~30 minutes (Fig. 4D).

A steep proliferation gradient is formed between distal-most and proximal blastema at the onset of regenerative outgrowth

To define the exact time when fin blastema segregates into distal and proximal portions, we closely examined BrdU incorporation in the DMB at various times: 36, 48, and 72 h.p.a. (n=3 or 4, 34-39 sections examined for each time point). We did not detect

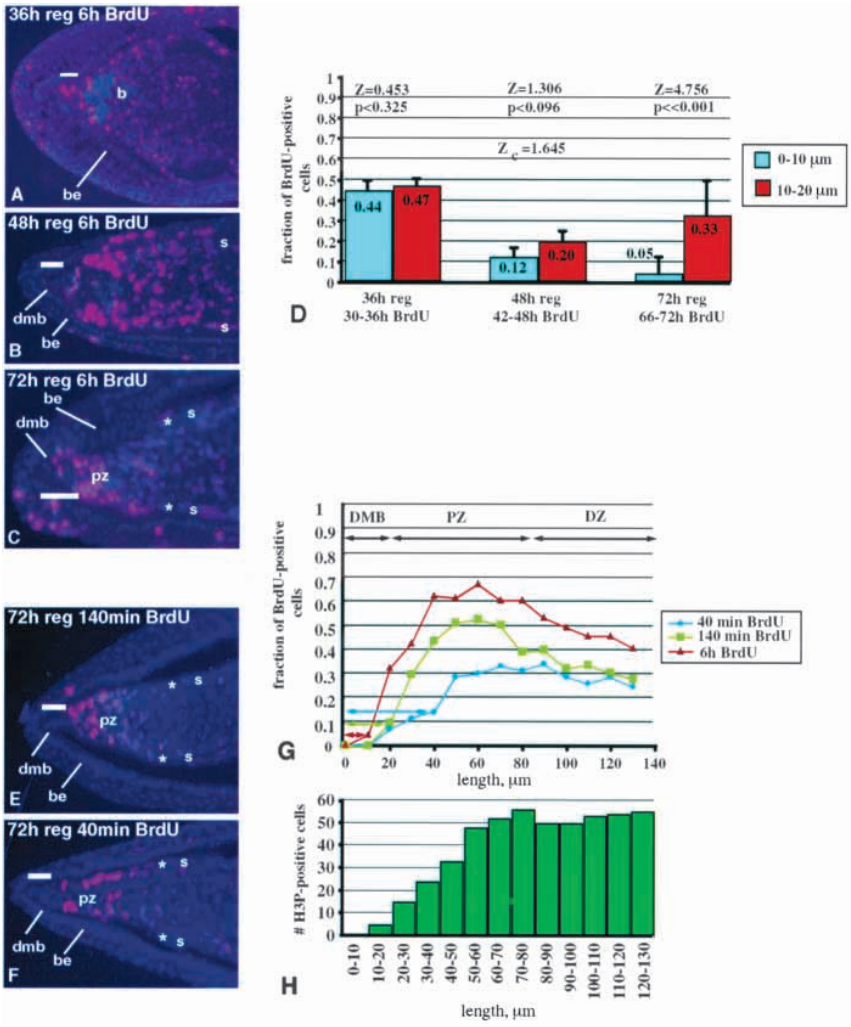

Fig. 5. Steep proliferation gradient is formed between distal-most and proximal blastema at the onset of regenerative outgrowth. (A-C) Longitudinal sections obtained from fins regenerating for (A) 36 hours, (B) 48 hours, and (C, E, F) 72 hours. BrdU incubations were for 6 hours (A-C), 140 minutes (E) and 40 minutes (F). BrdU labeling is red, DAPI is blue. A 20 μm scale bar is aligned with the most distal blastemal cells. (A) Distal portion of 36-hour regenerate showing proliferation in distal blastemal cells (*n*=4, 39 sections examined). (B) Distal-most blastema is established at the end of blastema formation and the beginning of regenerative outgrowth, 48 h.p.a. (*n*=3, 36 sections examined). Note the absence of BrdU labeling in the distal blastema. (C) During regenerative outgrowth, 72 h.p.a., the distal-most blastema is limited to 20 μm (*n*=4, 34 sections examined). Note the absence of proliferation within the distal 10 μm of DMB, and limited proliferation within the next 10 μm of DMB. (D) Differences (±s.e.m.) in the fraction of BrdU-positive cells of between 0-10 μm (left, blue bars) and 10-20 μm (red, right bar) indicate that DMB is established at the beginning of blastema formation and maintained throughout regenerative outgrowth. Z and *P* values for Mann-Whitney test are shown above each time point. (E,F) Representative fields of short BrdU treatments, (E) 140 minutes (*n*=4, 35 sections examined) and (F) 40 minutes (*n*=4, 36 sections examined) are shown. Note with progressively shorter BrdU treatment fewer distal blastema cells are labeled. (G) Fraction of BrdU-positive blastemal cells in 72-hour regenerates during 40 minutes, 140 minutes, and 6-hour treatments are plotted as a function of length from the distal tip of the blastema. Note the decrease in the zone of limited proliferation (double headed arrows of the corresponding color) with increased length of BrdU treatment. (H) Number of H3P-positive cells shown as a function of length from the distal tip of the blastema. Note that the trend closely follows that observed during BrdU incorporation experiments. Stars indicate the most distal scleroblasts. b, blastema; be, basal epidermal layer; dmb, distal-most blastema; m, intraray mesenchyme; pz, blastema proliferation zone; s, scleroblasts.

significant differences between cells distributed within 20 μm (5 μm approximately corresponds to a one cell diameter as estimated by electron microscopy) (A. N. and M. T. K., unpublished observations) from the distal end of the fin blastema at 36 h.p.a. (Fig. 5A,D). However, the difference in BrdU incorporation between distal cells and those immediately proximal to them became apparent at 48 h.p.a. and even more pronounced at 72 h.p.a. (Fig. 5B-D). These data indicate that distal blastemal cells attain their functional differences during transition from blastema formation to regenerative outgrowth, approximately 48 h.p.a.

During various lengths of BrdU treatment, we noticed that the number of proximally labeled DMB cells progressively increased with longer treatments (Fig. 5C,E,F), suggesting that proximal DMB cells cycle at a slow rate. To better characterize this finding, we plotted the fraction of BrdU-positive cells as a function of length with zero corresponding to the most distal blastemal cells (Fig. 5G; *n*=4, approximately 40 sections examined for each time point). We determined that a zone of reduced proliferation in the DMB became gradually smaller from approximately 40 μm to 20 μm, and finally to 10 μm, during 40-minute, 140-minute and 6-hour BrdU treatments, respectively. No BrdU-positive cells were detected within the first 10 μm of the DMB, even after 24-hour treatments (data not shown). To determine whether this difference is also reflected in the distribution of mitotic cells, we plotted the number of H3P-positive cells as a function of length, starting with the most distal end of the fin blastema (Fig. 5H; *n*=12, 495 mitoses from 135 sections). We never observed mitotic cells within the first 10 μm (approximately two cell diameters), while in more proximal regions the number of mitoses increased until it peaked at 50-60 μm. These findings indicate that there is no distinguishable boundary between the DMB and the proximal blastema. Instead, there is a 50-fold gradient of proliferation, as determined by H3P staining, established between the DMB and the proximal blastema. This gradient extends over 50 μm, approximately 10 cell diameters.

To further define zones of proliferation and differentiation in the regenerating fin, we analyzed levels of BrdU and H3P labeling in specific cell types. First, we defined the DMB as a domain with markedly reduced proliferation, approximately 10-40 μm deep with no clear boundary. Second, immediately proximal to the DMB is a blastemal zone with intense proliferation activity, the proliferating blastemal zone (PZ). As shown in Fig. 5F, the PZ labels first during the shortest BrdU treatment. Just proximal to the PZ is a region of moderate proliferation, the differentiation zone

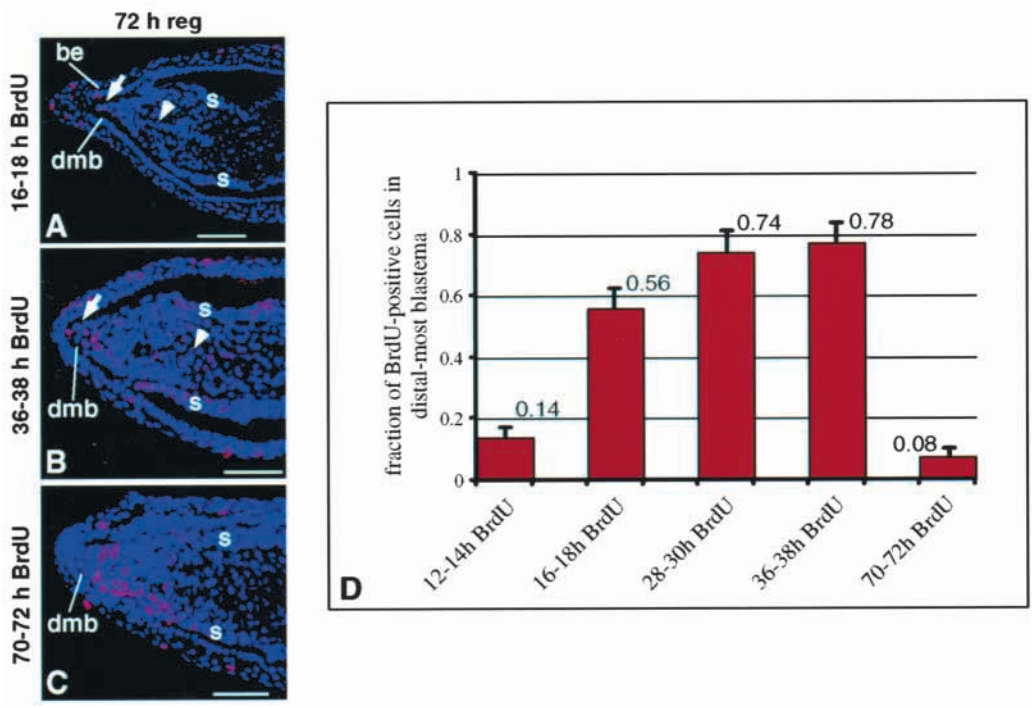

Fig. 6. Early proliferating mesenchymal cells contribute to the blastema. Fin sections from BrdU (red) pulse-chase experiments are shown. Fish were pulsed with BrdU for 2 hours at (A) 16 h.p.a., (B) 36 h.p.a. and (C) 70 h.p.a. and chased until harvesting at 72 hours. Fluorescent sections were counterstained with DAPI (blue) to reveal nuclei. (A) When a 2-hour BrdU pulse was given during early blastema formation (16 h.p.a.), staining is most apparent in the DMB and apical epidermis (arrows), including the basal epidermal layer. Note that BrdU label is diluted, indicating that blastemal cells divided before segregating into the DMB. BrdU label is even more diluted in the proximal parts of the regenerate (small dots, indicated by arrowheads) during outward growth of the regenerate. (B) When fish were pulsed at the end of blastema formation (36 h.p.a.) BrdU label in the DMB is mostly undiluted, while it is diluted in the proximal blastema. (C) By contrast, a late pulse of BrdU did not result in staining in the DMB (72 h.p.a.), indicating the absence of DNA synthesis during outgrowth. (D) A bar graph of the fraction of BrdU-positive cells in the DMB (0-20 μm) for the various 2-hour pulse times, 12 h.p.a., 16 h.p.a., 28 h.p.a., and 36 h.p.a.. Note that the fraction of BrdU-positive DMB cells is greatest when a pulse occurs late in blastema formation (36-38 h.p.a.), indicating that blastemal cells segregate into DMB late in blastema formation. For each case, the number of cells in the DMB from pulse-chase experiments was compared to 70-72 hour BrdU-treated fins using a Mann-Whitney test. *P* and Z values are: $P<0.05$, Z=1.649; $P<<0.001$, Z=7.558; $P<<0.001$, Z=9.518; $P<<0.001$, Z=11.249 for pulses administered at 12, 16, 28, and 36 hours respectively ($Z_{critical}=1.645$). Error bars indicate s.e.m. Scale bars: 50 μm.

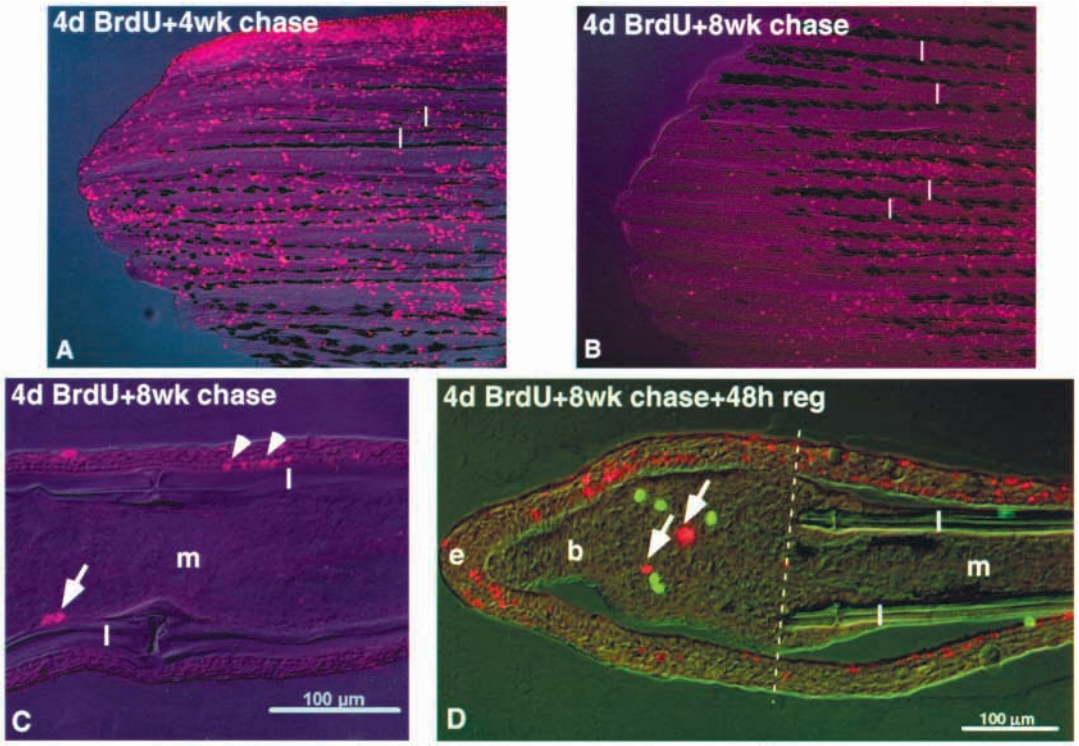

Fig. 7. Fin blastema is not formed from slow cycling stem cells. Representative fins from long-term pulse-chase experiments are shown. All the fins had continuous BrdU treatment for 4 days, followed by chases of various times. BrdU is indicated by red and H3P by green. (A) Intact caudal fin after a 4-week chase showing a high number of labeled transit-amplifying (TA) cells, mostly in the epidermis. In whole-mount (B) and longitudinal sections (C) of intact caudal fins after an 8-week chase, most of the BrdU label is lost from TA cells and concentrated in basal epidermal cells (arrowheads in C). (D) Longitudinal section through regenerating fin 2 days post-amputation. Note that BrdU-positive cells in the regenerate are rare and strongly labeled (arrows), indicating that these cells had not likely undergone division. H3P-positive cells are not BrdU-positive, suggesting that proliferating blastemal cells in the regenerate are not derived from slow-cycling cells. Original magnification (in A and B): 50×.

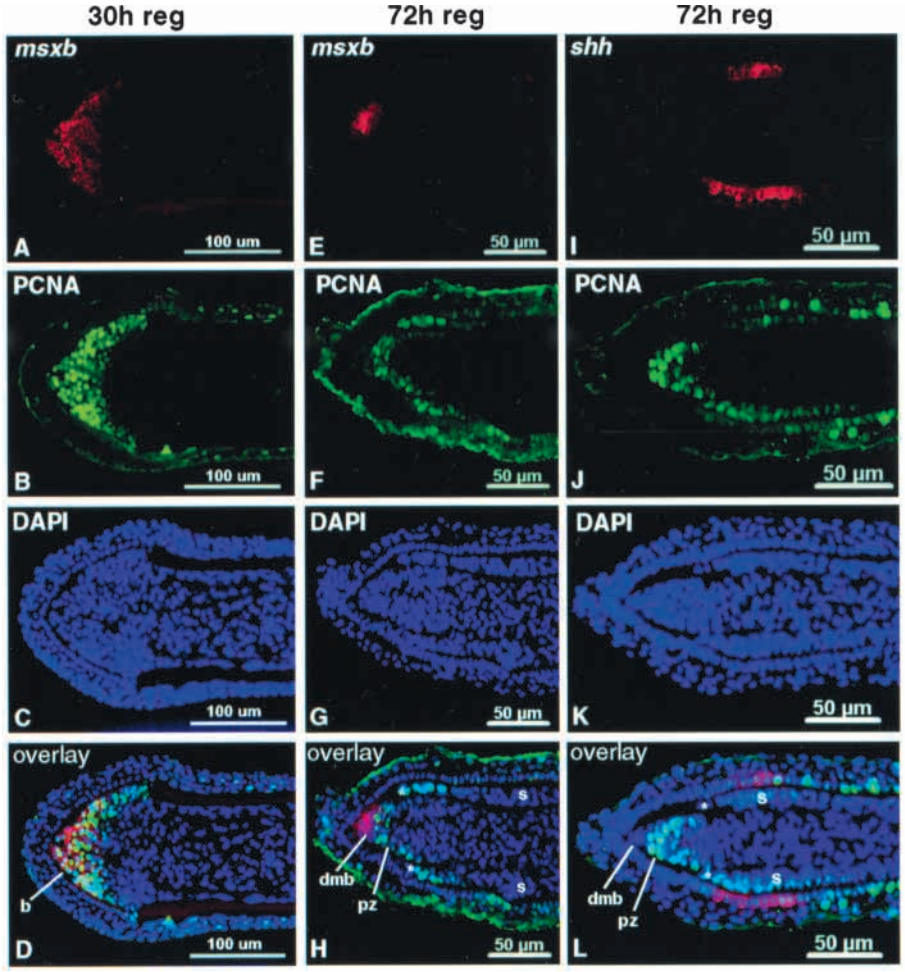

Fig. 8. *Msxb* expression is restricted to non-proliferating DMB. Representative sections from 30- and 72-hour fin regenerates (*n*=4-6) were processed for detection of *msxb* (A-H) and *shh* (I-L) mRNA (red) and PCNA protein (green). Fluorescent sections are counterstained with DAPI to reveal nuclei (blue). (A-D) Note that during blastema formation, a large number of distal blastemal proliferating cells are positive for *msxb*, while during regenerative outgrowth (E-H), *msxb* expression is restricted to a small number of non-proliferating DMB cells. Although, *msxb* and PCNA domains partially overlap, there is no PCNA staining in the DMB. (I-L) The level of *shh* staining correlates with the end of the blastema proliferation zone and the onset of differentiation (appearance of new scleroblasts, indicated by stars).

(DZ), which consists of proliferating and differentiating scleroblasts and mesenchymal cells (Fig. 5G). Thus, there is a gradient of proliferation in the blastema during regenerative outgrowth, with three major zones, DMB, PZ and DZ (Fig. 5G).

Early proliferating mesenchymal cells contribute to the blastema

To define the origin of cells in the DMB, we carried out pulse-chase experiments using BrdU. We treated animals for 2 hours beginning 12, 16, 28 and 36 h.p.a. Fish were then transferred to fresh water and regenerates were collected 72 h.p.a. (Fig. 6A,B and data not shown; *n*=4, 40 sections examined in each case). We administered BrdU pulses at early, middle and late stages of blastema formation and collected regenerates when the PZ and DMB were easy to distinguish. The intensity and pattern of BrdU staining in treated fins was compared to controls that had been regenerating for 70 hours and then treated with BrdU for 2 hours (Fig. 6C). DMB cells derived from all four pulse-chase experiments were significantly labeled (Fig. 6D). The earliest pulse resulted in labeling of DMB cells and apical epidermis, including the basal epidermal layer (Fig. 6A). Previous studies reported the absence of proliferation in the apical region of the basal epidermis (Santamaria et al., 1996). However, these cells were labeled during the earliest pulse-chase experiments, indicating that they undergo limited proliferation during early blastema formation (Fig. 6A). Proximal mesenchymal regions contained fewer BrdU signals, most of which appeared diluted. We concluded that labeling of various intensities reflected the cell's proliferation rates; that is, cells with lower intensity staining were the result of subsequent cell divisions. During late pulses, BrdU label in the DMB was undiluted, indicating that these cells had recently segregated into the DMB, before any cell division had occurred. Overall, these data indicate that the DMB originates from proliferating blastemal cells that segregate into the DMB at the end of blastema formation. Moreover, these data suggest that the DMB is not formed from a special pool of stem cells.

Fin blastema is not formed from slow cycling cells

A reliable, commonly used method to identify stem cells takes advantage of the fact that these cells are typically slow cycling (label-retaining cells) (Bickenbach, 1981; Cotsarelis et al., 1989; Cotsarelis et al., 1990; Morris and Potten, 1994; Johansson et al., 1999; Morris and Potten, 1999). To detect these cells, an extended period of labeling is followed by a long chase period. Label is retained in slow-cycling cells (stem cells), but lost from all other cycling, transit-amplifying (TA) cells. To determine the time it takes to clear label from TA cells, we treated fish for 2 or 4 days with BrdU followed by a chase of 1, 2, 4 and 8 weeks (*n*=7 fins examined in each case). As noted in Fig. 1A,C, zebrafish caudal fins contain rapidly renewing epithelial cells. Therefore, we used zebrafish caudal fin epithelium as a reference for label clearance from TA cells. After a one-month chase, some label was retained in epithelial cells (Fig. 7A). However, after two months, most of the label was lost (Fig. 7B,C). Most BrdU-positive cells were concentrated in the basal epithelial layer, but we also found a small number of BrdU-labeled mesenchymal cells (Fig. 7C). Thus, two months is a minimum time necessary to chase the label from TA cells.

To determine if slow-cycling mesenchymal cells contributed to the regeneration blastema, we carried out long-term pulse-chase experiments. Fig. 7D shows a section from a 48-hour regenerating caudal fin after 8 weeks of chase. Out of 193

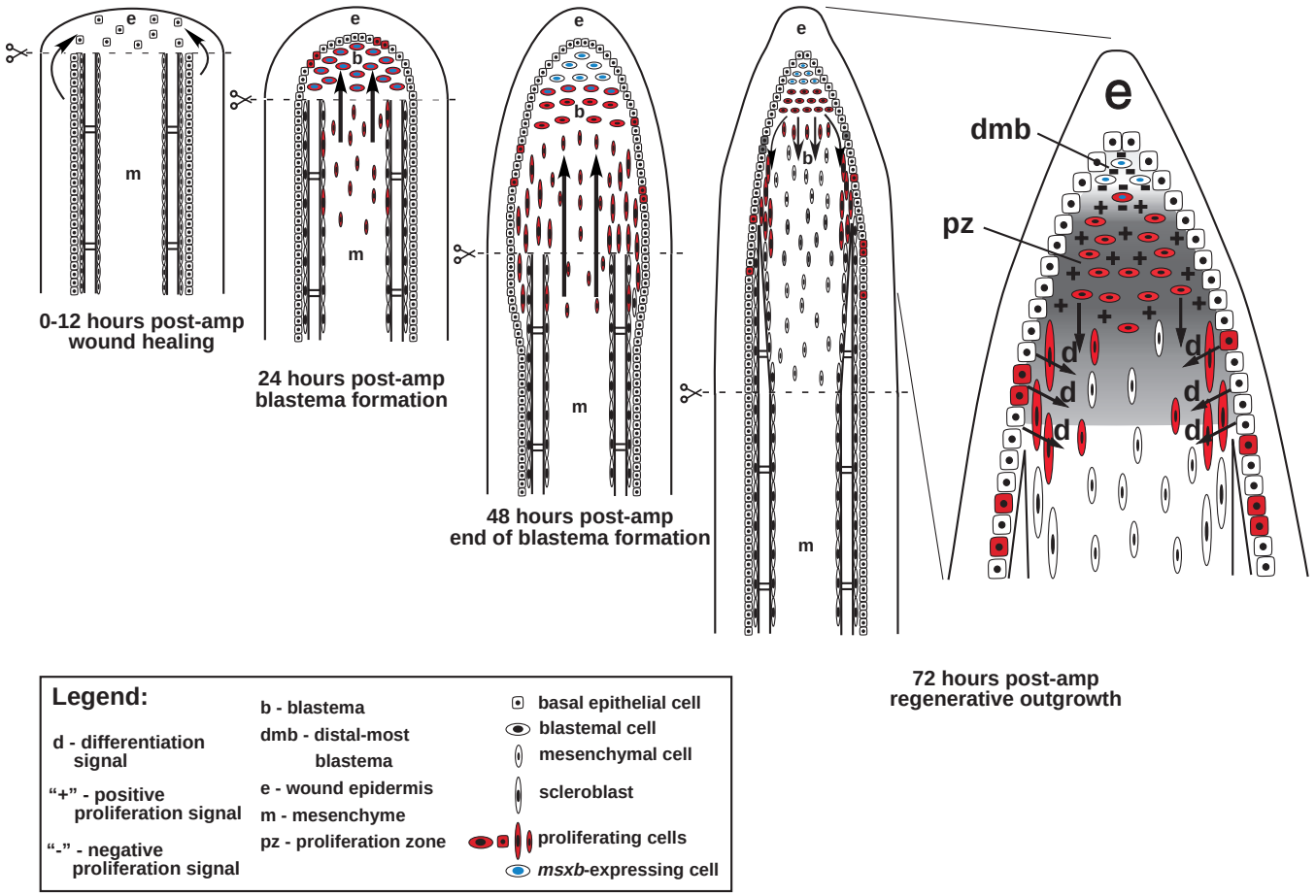

Fig. 9. A cellular model of zebrafish fin regeneration. During stage 1 (0-12 hours; wound healing), the wound epidermis is formed by migrating epithelial cells. In stage 2 (12-48 hours; blastema formation), the basal epidermis is formed, mesenchymal tissue proximal to the amputation plane begins to disorganize and intraray mesenchymal cells proliferate and move upward. A subset of early mesenchymal proliferating cells (red) expresses *msxb* (blue nuclei). A number of *msxb*-positive proliferating cells continue to increase throughout blastema formation. Just before the onset of regenerative outgrowth, blastemal cells segregate into *msxb*-positive, non-proliferating DMB and *msxb*-negative proliferating PZ, with a gradient of proliferation between the two domains. During stage 3 (48 hours ~1 week; regenerative outgrowth), the gradient of *msxb* expression and proliferation is maintained, controlling the direction of outgrowth. Cells in the PZ proliferate vigorously and move in the proximal direction to differentiate. A zone of negative proliferation in the DMB maintains the directionality of the outgrowth by inhibiting proliferation.

sections examined from four regenerates, 33 contained one or more BrdU-positive mesenchymal cells (0.47±1.32). No labeled DMB cells were identified in 191 of 193 sections. In two sections we found a single labeled DMB cell. Most of the BrdU-positive cells in the blastema were strongly labeled (Fig. 7D), suggesting that they had not undergone cell division. We simultaneously stained 2-day-old regenerates with BrdU and H3P antibodies to determine whether mesenchymal BrdU-labeled cells had undergone mitosis. Although we examined 150 sections from six regenerates, we only observed one doubly labeled cell. It is unlikely that BrdU label was diluted beyond detection levels, as we could detect faint BrdU staining in epithelial TA cells (Fig. 7C,D). These data indicate that slow-cycling cells did not significantly contribute to the blastemal cell population in regenerating caudal fin.

msxb expression is restricted to non-proliferating DMB

Previous studies have shown that blastemal cells express the homedomain transcriptional repressor *msxb* during blastema formation (Akimenko et al., 1995; Poss et al., 2000b). Subsequently, *msxb*-expressing cells are limited to a distal part of the blastema, and expression is maintained in this group of cells throughout regenerative outgrowth. To define the relationships between *msxb*-expressing cells and blastemal proliferation, we simultaneously stained fin regenerates with *msxb* antisense riboprobe and proliferating cell nuclear antigen (PCNA) antibodies (Fig. 8A-D). PCNA is expressed throughout G_1, S, and G_2/M, enabling detection of a spectrum of proliferating cells (Waseem and Lane, 1990; Woods et al., 1991). We found that a subset of proliferating mesenchymal cells expressed *msxb* as early as 18 h.p.a. (data not shown). By mid-blastema formation (30 h.p.a.) a large number of proliferating blastemal cells expressed *msxb* (Fig. 8A-D; n=5 regenerates). In contrast, during regenerative outgrowth, *msxb* expression was limited to a small number of non-proliferating DMB cells. The majority of proliferating PZ cells did not express *msxb* (Fig. 8E-H; n=5 regenerates). However, in 50%

of sections examined (18 of 36), *msxb* and PCNA domains partially overlap (Fig. 8H). These data indicate that during transition from blastema formation to regenerative outgrowth, *msxb* expression is restricted to a small number of non-proliferating or slow-cycling DMB cells.

Shh, a secreted factor that is thought to play a patterning role during regenerative outgrowth, is expressed in two lateral domains of the basal epidermis adjacent to distal scleroblasts (Laforest et al., 1998). To determine the relationship between proliferation and differentiation during regenerative outgrowth, we simultaneously stained 72 h.p.a. fin regenerates with *shh* antisense riboprobe and PCNA antibodies (Fig. 8I-L; n=5 regenerates). *shh*-expressing epithelial cells were located at the level of distal proliferating scleroblasts. The most distal *shh*-expressing cells were located just proximal to the PZ (Fig. 8I-L). Thus, the zone of differentiation begins just proximal to the zone of proliferation.

DISCUSSION

We conclude that the regeneration blastema is partitioned into two domains with distinct functional properties at the beginning (48 h.p.a.) of regenerative outgrowth. Non-proliferating, distal blastemal cells express *msxb*, while proliferating proximal cells do not. There is a 50-fold proliferation gradient between these two domains. Distal blastemal cells arise, at least in part, from previously proliferating blastemal cells, not from slow cycling mesenchymal stem cells. Finally, G_2 of blastemal cell cycle shortens dramatically during the transition from blastema formation to regenerative outgrowth. Thus, there are major changes in cell proliferation properties at the end of blastema formation, resulting in a blastema with intricate functional organization.

Regeneration blastema is divided into two domains, rapid cycling proximal blastema and slow cycling distal blastema

We conclude that proximal and distal blastema are both formed by proliferating mesenchymal cells, but that cycling in distal cells dramatically slows at the beginning of regenerative outgrowth. These conclusions are supported by the following observations. First, distal blastemal cells, unlike proximal blastemal cells, do not proliferate during regenerative outgrowth as shown by BrdU and H3P labeling. Second, during short-term pulse-chase experiments, distal and proximal blastema are both labeled, suggesting that they were derived from early cycling blastemal cells. Third, we observed the greatest labeling in the DMB when BrdU was given at the end of blastema formation, indicating that blastemal cells segregate into DMB just before the onset of regenerative outgrowth.

In this study, we found no evidence that pre-existing, slow cycling stem cells contribute to the regeneration blastema. This conclusion is based on the analysis of fin regenerates after long-term (two month) BrdU pulse-chase experiments. Although, we found a small number of labeled cells in the regenerate, we never observed evidence of mitoses in these cells. Recently, Rawls and Johnson suggested the existence of pigment stem cells that give rise to the melanocyte population in the regenerate (Rawls and Johnson, 2000). It is possible that these cells are slow cycling pigment stem cells. We suggest that regenerating blastema is unlikely to arise from slow cycling stem cells. However, our data do not exclude the possibility that fast cycling stem cells may contribute to blastema formation.

G_2 is slow during blastema formation and accelerates dramatically during regenerative outgrowth

We discovered that G_2 is slow during blastema formation, but rapidly accelerates during regenerative outgrowth. Using the fraction of labeled mitoses technique (Aherne, 1977), we determined that the median length of G_2 before 48 h.p.a. is longer than 6 hours, whereas it is approximately 1 hour during regenerative outgrowth. These data suggest that blastemal cell cycle before the transition to regenerative outgrowth is slow, reflecting the necessary reorganization and cells movements that must occur during blastema formation. Although very high doses of BrdU have been shown to slow G_2 and may result in G_2 arrest (Rabinovitch, 1983), the fact that ~95% of mitoses are labeled after 110 minutes of treatment suggests the dose used here did not cause G_2 arrest. Moreover dilution of BrdU during short-term pulse-chase experiments suggests that this dose does not have inhibitory effects on cell division and differentiation.

msxb expression correlates with slow cell cycle

In the present study, we discovered that *msxb* expression becomes restricted to the non-proliferating DMB during regenerative outgrowth. *msx* genes have been implicated in the maintenance of undifferentiated, pluripotent cells (Song et al., 1992; Odelberg et al., 2000). Initially, *msxb* is expressed in a large number of proliferating blastemal cells throughout the regenerate. At the onset of regenerative outgrowth, however, *msxb* expression becomes restricted to a small number of non-proliferating DMB cells. Interestingly, *msxb* expression appears to be strongest in the most distal cells. *Msxb* levels gradually decrease in the proximal direction (A. N. and M. T. K., unpublished observations), precisely opposite to the pattern of proliferation established between DMB and the blastema proliferation zone (PZ). Thus, *msxb* expression correlates with a slow cell cycle during blastema formation and with non- and slow-cycling DMB cells during regenerative outgrowth. We conclude that upregulation of *msxb* expression may be necessary to slow cell cycling during blastema formation and inhibit proliferation in the DMB during regenerative outgrowth.

A number of studies in other systems have correlated *msx* gene expression with reduced proliferation and suppression of the differentiated state. For example, in the progress zone of developing chick limb buds, *MSX2* is expressed in two distinct domains that are correlated with reduced proliferation (Ferrari et al., 1998). The anterior mesodermal domain of *MSX2* demarcates the anterior boundary of the limb progress zone, while another *MSX2* expression domain at the mid-proximal posterior margin of the limb bud is located at the proximal posterior boundary of the progress zone. Ectopic *MSX2* expression via a retroviral expression vector in the posterior mesoderm of the progress zone significantly reduces proliferation and impairs morphogenesis (Ferrari et al., 1998). Thus, it has been proposed that *MSX2* may be part of a regulatory network that delineates the progress zone (Coelho et al., 1992; Ferrari et al., 1998). We propose, therefore, that one function of *msxb*-expressing DMB may be to suppress differentiation by reducing cell proliferation. This, in turn, may

direct the patterning of the regenerate by controlling the direction of proliferation and differentiation.

A cellular model of zebrafish fin regeneration

Observations described here and by others suggest a possible model for cell proliferation during fin regeneration (Fig. 9). Following amputation, the wound is covered by migrating epithelial cells. Mesenchymal proliferation begins shortly after amputation in the intraray space, proximal to the amputation plane. During early blastema formation, *msxb* expression is induced in a subset of slow proliferating blastemal cells. Extensive reorganization and cell movement occurs among scleroblasts and blastemal cells in preparation for regenerative outgrowth. At the onset of regenerative outgrowth, the blastema is subdivided into the DMB and the PZ, with a gradient of proliferation established between these two domains. While *msxb* expression is maintained in the DMB, it is downregulated in the PZ. A negative proliferation field encompasses the DMB, while a positive field surrounds the PZ. We hypothesize that negative and positive proliferation fields are established by factors secreted from the apical or lateral basal epidermis.

Fgf signaling pathways may be directly involved in the induction and maintenance of *msxb* expression as well as blastemal proliferation. Recent studies demonstrated that *fgfr1* is expressed in blastemal cells during blastema formation, but its expression becomes limited to the DMB during outgrowth. Even a brief treatment with a specific *fgfr* inhibitor abolishes *msxb* expression (Poss et al., 2000b), implying that *msxb* expression is maintained via Fgf signaling. Another good candidate for regulating blastemal proliferation is a Wnt signaling pathway. Recently, expression of *wnt3a* and *lef1* has been reported in wound epidermis and blastema during regenerative outgrowth (Poss et al., 2000a). However, further studies are necessary to determine the exact role of these genes in blastemal proliferation.

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