Zebrafish kit mutation reveals primary and secondary regulation of melanocyte development during fin stripe regeneration

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SUMMARY

Fin regeneration in adult zebrafish is accompanied by re-estabishment of the pigment stripes. To understand the mechanisms underlying fin stripe regeneration and regulation of normal melanocyte stripe morphology, we investigated the origins of melanocytes in the regenerating fin and their requirement for the kit receptor tyrosine kinase. Using pre-existing melanin as a lineage tracer, we show that most fin regeneration melanocytes develop from undifferentiated precursors, rather than from differentiated melanocytes. Mutational analysis reveals two distinct classes of regeneration melanocytes. First, an early regeneration class develops dependent on kit function. In the absence of kit function and kit-dependent melanocytes, a second class of melanocytes develops at later stages of regeneration. This late kit-independent class of regeneration melanocytes has little or no role in wild-type fin stripe development, thus revealing a secondary mode for regulation of fin stripes. Expression of melanocyte markers in regenerating kit mutant fins suggests that kit normally acts after mitf and before dct to promote development of the primary kit-dependent melanocytes. kit-dependent and kit-independent melanocytes are also present during fin stripe ontogeny in patterns similar to those observed during regeneration.

Key words: Melanocyte, Fin, Regeneration, Regulation, sparse, Danio rerio, kit, mitf, dct

INTRODUCTION

Organisms maintain their form throughout life by replacing lost cells with new ones. In some cases, cells are replaced by proliferation of well-defined undifferentiated precursors. A variety of vertebrate tissues are maintained in this way, including the circulating blood (Morrison et al., 1995), the intestinal epithelium (Potten et al., 1997) and the hair follicle (Lavker et al., 1993). In other developmental scenarios such as the regeneration of the amphibian limb, cells are replaced by precursors that are not as well characterized (Brockes, 1997). The mechanisms that regulate the balance between precursor cells and their daughters, and the patterning of these cells to facilitate the maintenance of complex form are not well understood.

The zebrafish pigment pattern provides several opportunities to study the development of melanocytes from their undifferentiated precursors. Genetic dissection of the adult pigment pattern reveals multiple populations of melanocytes that develop during the transition from larval to adult form. The first of these populations begins to appear after 2 weeks of development and is absent in mutants for the zebrafish homologue of the kit receptor tyrosine kinase. The zebrafish kit locus was originally identified as sparse and subsequently renamed kit, to preserve a common nomenclature with amniotes (Parichy et al., 1999). A second population begins to appear during the fourth week of development (at 28°C) and is absent in rose (ros) or leopard (leo) mutants. Fish doubly mutant for kit and ros, or kit and leo, lack virtually all melanocytes in the body. Persistence of melanocyte stripes in the caudal and anal fins of kit;ros double mutants suggests the existence of yet a third distinct population of melanocytes, independent of the function of kit and rose (Johnson et al., 1995). The inference drawn from these studies is that precursors for adult pigment cells remain quiescent through embryonic and larval development and are recruited to re-enter the developmental pathway upon the onset of larval-adult metamorphosis. These precursors might also generate new cells during growth as new stripes are added or during regeneration of fin stripes following fin amputation.

Re-establishment of the pigment pattern in the regenerating zebrafish fin may provide insights into how quiescent melanocyte precursors are recruited to differentiate. Concomitant with regeneration of the fin, melanocytes (black pigment cells) and xanthophores (yellow pigment cells) re-establish stripes in the regenerated tissue. Goodrich and colleagues described two stages of melanocyte appearance during regeneration of the caudal fin, in which the melanocyte and xanthophore stripes are parallel to the fin rays, or parallel to the vector of growth (Goodrich and Nichols, 1931; Goodrich et al., 1954). During the first day following amputation, the wound epithelium forms and no melanocytes are present in the regenerate. Beginning approximately 3 days postamputation, a few melanocytes appear in regions immediately distal to the
preexisting melanocyte stripes (Fig. 1A). This phase is followed by the appearance of many melanocytes in a uniform band along the proximal regenerate parallel to the amputation plane (distal to both melanocyte and xanthophore stripes) beginning at approximately 4 days (Fig. 1B). The stripe pattern is then re-established by disappearance of melanocytes from regions distal to the original xanthophore stripes (Fig. 1D).

The origins of melanocytes in the regenerating zebrafish fin are not clear. Lacking methods to label melanocytes, Goodrich and his colleagues failed to distinguish between potential roles for pre-existing melanocytes migrating into the regenerating fin and melanocytes arising via de novo differentiation from unpigmented precursors (Goodrich and Nichols, 1931; Goodrich et al., 1954). The model that, in intact fins, unpigmented melanocyte precursors exist that could be recruited to re-establish the melanocyte stripe in regenerating fins was suggested by the de novo appearance of melanocytes in xanthophore regions following localized destruction of xanthophores (Goodrich et al., 1954). Unpigmented melanocyte precursors have also been shown to exist in adult mice, where they could contribute to the maintenance of the adult pigment pattern (Kunisada et al., 1998).

The processes by which melanocyte precursor cells are recruited to re-enter developmental pathways are poorly understood. Indeed, the existence of melanocyte precursors in zebrafish is only inferred, as molecular markers for these cells are not yet identified. To understand the role of melanocyte precursors in zebrafish fin stripe regeneration, we studied the regenerative development of wild-type caudal fins and identified mutants defective in those processes. We show that the fin stripe is re-established almost entirely by melanocytes that differentiate de novo from unpigmented precursors. Mutants for the zebrafish kit receptor are defective for this de novo melanocyte population, and analysis of early markers of melanocyte differentiation suggests kit is required for these primary regeneration melanocytes to progress beyond a mitf-positive stage (Lister et al., 1999). Study of kit mutants reveals a secondary class of de novo regeneration melanocytes that develops independently of kit function, and regulates its activity to reconstitute the stripes as fin regeneration passes to a late outgrowth stage (stage 8; see Johnson and Bennett, 1999). Furthermore, we demonstrate that these two de novo populations are similarly utilized during wild-type or kit mutant fin stripe ontogeny. This suggests that fin stripe growth and regeneration use common pathways to recruit melanocyte precursors and regulate stripe pattern.

MATERIALS AND METHODS

Stocks
Wild-type and mutant stocks were maintained on a 14L:10D light cycle at a constant temperature of 25°C. lofD2, nacn2, jagb250, leol1, rosb40, kitb5 and kitb141 have been previously described (Tresnake, 1991; Johnson and Weston, 1995; Lister et al., 1999; Johnson et al., 1995, 1996; Streisinger et al., 1986; Parichy et al., 1999). kitb14 is a spontaneous allele isolated and kindly provided by Charlene Walker. All references to kit mutants correspond to the kitb5 null allele, except where noted. kitb5 encodes a deletion at bp 846 of the coding sequence, resulting in a frame shift and premature stop codon (Parichy et al., 1999).

Fin regeneration and ontogeny experiments
For regeneration studies, caudal fins were amputated as described (Johnson and Weston, 1995). Briefly, mature fish (3-12 months of age) were anesthetized, the distal two-thirds of the caudal fin amputated with scissors or a scalpel, and fish returned to fresh water at 25°C on a regular feeding schedule for the duration of the experiment. Regenerating fins were staged by days postamputation according to Johnson and Weston (1995). Juveniles were staged according to body length and age. For phenylthiourea (PTU) treatment of fish, 0.1-0.2 mM PTU was added to the water and changed every 2 days (Milos and Dingle, 1978). Efficacy of PTU solutions was confirmed in parallel by ability to block pigmentation in 1 day embryos. Eight or more fish were analyzed at each time point and treatment.

Fin stripe melanocyte counts
To visualize individual melanocytes in adult fin stripes, fish were immersed in 1 mg/ml epinephrine for 5 minutes to contract melanosomes. Ontogenetic fin stripe melanocyte densities were assessed by observing intact caudal fins in wild-type and kitb5 adult siblings. Regenerated fin stripe melanocyte densities were determined by observing regenerated adult caudal fins in wild-type and kitb5 siblings 8 weeks following amputation. Melanocyte density was assessed in the three stripes of the caudal fin. Observation of late regeneration de novo melanocytes was accomplished by treating wild-type and kitb5 sibling regenerates with PTU during stages 7-13. Images were taken of each regenerate at stage 13 and several hours after PTU washout, and de novo melanocytes were identified as those unpigmented at stage 13 and present after PTU washout. Images of regenerating central fin stripes were then divided into thirds (proximal, medial and distal regenerate), and de novo melanocytes in each third of the central stripe were counted. Statistical analysis was performed with aid of JMP statistical package.

In situ hybridization
Whole-mount in situ hybridization of fins was performed according to Thissee et al. (1993), with the following modifications. Proteinase K digestion was carried out at 10 mg/ml for 30-45 minutes, and hybridization and stringency washes occurred at 68°C. Full-length antisense digoxigenin-labeled riboprobes for zebrafish tyrosinase-related protein 2dopachrome tautomerase (dct) (Kelsh and Eisen, 2000), microphthalmia-associated transcription factor (mitf) (Lister et al., 1999), and kit (3545 bp; Parichy et al., 1999) were synthesized from linearized templates with T7 and T3 RNA polymerases.

RESULTS

Most regeneration melanocytes arise from unpigmented precursors
Melanocytes may appear in the regenerate through migration of pre-existing pigmented melanocytes from the old stripe, or through de novo differentiation from unpigmented precursors (de novo melanocytes). To distinguish between these models, we sought to identify previously differentiated melanocytes in the regenerating fin. We took advantage of the property of phenylthiourea (PTU) to inhibit the synthesis of new melanin, thereby preventing visualization of any newly differentiated melanocytes (Milos and Dingle, 1978). Thus, for regeneration in the presence of PTU, any pigmented melanocytes must have differentiated prior to amputation and drug treatment. At 3 days postamputation (stage 3), we observe that PTU-treated regenerates (not shown) are indistinguishable from untreated controls (Fig. 1A), each with a few melanocytes (approx. 2-10 per stripe) within 0.2 mm of the amputation plane and
immediately distal of the old stripe. This pattern persists through stage 7 in PTU-treated fins (Fig. 2B), suggesting that previously differentiated melanocytes are a minor component of the total regeneration melanocyte pool.

In contrast, the vast majority of regeneration melanocytes develop by de novo differentiation from unpigmented precursors. When PTU-treated fish are returned to fresh water at stage 7 to remove the drug, lightly pigmented melanocytes become visible within a few hours in the normal pattern of untreated fish (Fig. 2C), indicating that these PTU-treated melanocytes had developed normally albeit without melanin. Since no melanin was apparent in these cells prior to drug washout, we conclude that the melanocytes that regulate fin stripe regeneration are derived from previously unpigmented precursors. Interestingly, wild-type fins are able to regenerate their stripes normally through at least ten rounds of amputation and regeneration (not shown), suggesting that these precursors have an apparently inexhaustible capacity for self-renewal. Therefore, although a few previously pigmented melanocytes migrate into the proximal regenerating stripe, fin stripes are reconstituted almost entirely by de novo melanocytes that differentiate beginning around stage 4.

**Melanocyte precursors are uniformly distributed in the fin**

We were interested to know whether melanocyte precursors are uniformly distributed throughout the fin, or instead reside in proximity to the differentiated melanocyte stripes in the fin. To begin to locate melanocyte precursors in the fin, we examined the expression of genes involved in early stages of melanocyte differentiation. In situ hybridization analysis reveals that *mitf*, the earliest known melanoblast marker in zebrafish (Lister et al., 1999), is expressed by mesenchymal cells in the stump beginning at stage 1 (Fig. 3A). The number of mesenchymal *mitf*-positive cells increases through stage 3, evenly distributed along a region approximately 1 mm proximal to the amputation plane (not shown; see Discussion). Later melanoblast markers, the *kit* receptor (Parichy et al., 1999) and the melanin synthesis enzyme *dct* (Steel et al., 1992; Kelsh and Eisen, 2000), are expressed in mesenchymal cells distributed throughout the

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**Fig. 1. Stages of melanocyte development during caudal fin regeneration.** (A) Whole mount of stage 3 wild-type regenerate showing a few melanocytes (white arrowheads) just distal of the amputation plane (black arrowheads in all images) and old stripe stump, and melanocyte detritus in the distal regenerate (black arrow). (B) Stage 4 wild-type regenerate displaying melanocytes (white arrowheads) in the regenerate along the amputation plane, distal to both old melanocyte and xanthophore stripes. (C) Stage 5 wild-type regenerate showing increasing numbers of melanocytes in the regenerate (white arrowhead). (D) Stage 7 wild-type regenerate displaying more melanocytes in the regenerate, and the initial clearance of melanocytes from the presumptive xanthophore stripe (white arrowheads). Different fins are shown at each stage. Scale bars: A,B, 200 μm; C,D, same scale as B.

**Fig. 2. Regeneration melanocytes arise by de novo differentiation from unpigmented precursors and are absent in *kit* mutants.** (A) Stage 7 wild-type regenerate displays the normal pattern of regeneration melanocytes (black arrows). (B) Stage 7 wild-type after regeneration in the presence of PTU during stages 0-7 renders most regeneration melanocytes invisible. A few previously pigmented melanocytes migrate out from the stripe stump (white arrowheads). (C) The same PTU-treated fin several hours after PTU washout reveals many pigmenting melanocytes in the normal pattern (black arrows). (D) Stage 7 *kit* mutant regenerate lacks the normal pattern of regeneration melanocytes. Similar to wild type, a few pigmented cells migrate into the proximal regenerate (white arrowheads). A blood vessel is indicated by black arrow. Amputation planes are indicated by black arrowheads. Scale bars: A-D, 200 μm.
distal stump and the proximal regenerate beginning at stage 1.5 (Fig. 3C) and stage 2 (Fig. 3E) respectively. By stage 3, cells expressing mitf (Fig. 3G), kit (Fig. 3H) or dct (not shown) are also found to express melanin, verifying their melanoblast identity. Since cell division and gross morphological reorganization do not begin until around stage 1.5-2 of regeneration (Johnson and Bennett, 1999), we suggest that these observed patterns of differentiating mitf-kit/dct-positive cells early in regeneration reflect the position of de novo melanocyte precursors prior to amputation.

Although cell division and gross morphological reorganization do not begin until around stage 1.5-2 of regeneration (Johnson and Bennett, 1999), it remained possible that a few melanocyte precursors initially associated with the differentiated melanocyte stripes disperse laterally (approximately 1 mm) from positions in the stripe stump prior to stage 1 expression of mitf. To challenge this model, we examined fin melanocyte regeneration in jaguar (jag); long fin (lof) double mutants, where the stripes are more widely spaced than in wild-type fins (Fig. 3I), but otherwise regenerate melanocyte pattern through stage 7 identical to wild type (not shown). This allowed us to resect the dorsal and ventral regions of jag; lof caudal fins, which contained melanocyte stripes, and then amputate the distal one-third of the remaining medial fin section. Following such compound amputation, fins have no differentiated melanocytes along the distal amputation plane. Moreover, the differentiated melanocytes closest to the distal amputation plane are at least 7 mm away in the proximal fin stumps. Melanocytes appeared dispersed throughout the distal regenerate as early as stage 3.5 after compound amputation (Fig. 3J), identical to the time when melanocytes regenerate in jag; lof mutants following simple distal amputation (not shown). Distal regeneration melanocytes in jag; lof compound amputees do not appear when regeneration occurs in the presence of PTU (not shown), consistent with our earlier finding (see above) that regeneration melanocytes do not arise from previously pigmented melanocytes migrating from the proximal fin stumps or the body stripes. Melanocyte regeneration along the proximal amputation planes occurs with similar timing (Fig. 3J), and the original fin pigment pattern is

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**Fig. 3.** Melanocyte precursors are distributed evenly throughout the fin and are unable to progress beyond a mitf-positive stage in regenerating kit mutants. Whole-mount in situ of wild-type (A) and kit mutant (B) regenerating fins reveals mitf-positive cells at stage 1 in the fin stump. Kit transcript is found in cells proximal of the amputation plane as early as stage 1.5 in wild-type (C; white arrowhead), but is never observed in similarly staged kit mutants (D). Similarly, dct transcript is observed as early as stage 2 in wild-type regenerates (E; white arrowhead), but not in kit mutant regenerates (F). Cryosections along the proximodistal axis of stage 3 wild-type regenerates reveals mitf-positive (G) and kit-positive (H) mesenchymal cells along the amputation plane beginning to express melanin. In situ images were all taken from presumptive xanthophore stripes, which are devoid of previously pigmented melanocytes, although similar patterns are observed in presumptive melanocyte stripes (not shown). (I) Intact jag; lof caudal fins contain melanocytes only in the most dorsal, ventral and distal regions (black arrows). The portion of the fin to be resected is shown by a black line. (J) The same fin at stage 3.5 after amputation reveals several melanocytes in the distal and proximal regenerates (black arrows). The inset shows a melanocyte in a higher magnification of the area outlined in the black box. Distal is to the right in all images. Amputation planes are indicated by black arrowheads. Scale bars: A-F, 20 μm; G,H, 10 μm; I, 2 mm; J, 1 mm.
eventually reconstituted (not shown). Although we cannot formally exclude unusually rapid melanoblast migration from pre-existing stripes at the proximal amputation planes in this experiment, our finding of normal pattern and timing of melanocyte development in the distal region of these compound regenerates supports the notion suggested by in situ analysis of melanoblast markers (above, Fig. 3A,C,E) that melanocyte precursors are distributed evenly throughout the intact fin and are locally recruited by the amputation plane.

The normal development of new melanocytes is dependent on kit

To identify genes involved in fin stripe regeneration, we surveyed a panel of adult pigment pattern mutants (including jagb120, nacw2, leoy1, rosb140 and kitb5) for defects in fin stripe regeneration. After amputating and observing the regeneration of these different mutants, two mutations caused deficits in regeneration melanocytes at stage 7: kitb5, a null-allele of the zebrafish kit gene (Fig. 2D; Parichy et al., 1999), and nacw2, a loss-of-function allele of zebrafish mitf (not shown; Lister et al., 1999). Since kit mutants form melanocyte stripes during fin ontogeny (Johnson et al., 1995) yet show dramatic deficits in early stages of regeneration (see below), we further investigated the melanocyte regeneration defect in kit mutants.

kit mutant fin regeneration initially appears similar to wild type, with several melanocytes appearing distal of the old stripe stump by stage 3 (not shown). The migration of these cells into the fin regenerate is therefore not dependent on the kit gene. However, no additional melanocytes appear in the regenerate through stage 7, suggesting that kit mutant animals lack de novo melanocytes during fin stripe regeneration (Fig. 2D). To determine if this defect was due to mutations other than kitb5 fixed in the background, other kit mutant alleles were tested (kitb1e1, kitb134, not shown). These other kit alleles displayed regeneration defects identical to kitb5, confirming that the observed de novo melanocyte deficit in kit mutants is a consequence of loss of kit function. Therefore kit is required for development of de novo regeneration melanocytes that pigment beginning at stage 4 in wild-type animals.

kit is required for melanoblast differentiation prior to dct expression

The kit mutant fin stripe regeneration phenotype suggests that kit promotes the development of melanocyte precursor cells. To help determine when kit might be required for melanocyte precursor development, we observed kit expression patterns using in situ hybridization. As previously described, kit is expressed during embryonic and larval development in the melanocyte lineage (Parichy et al., 1999), suggesting that the kit mutant regeneration defect could result from a requirement for kit in establishing melanocyte precursors early in ontogeny. Alternatively, kit may be required during regeneration to recruit melanocyte precursors to form melanocytes. As discussed above and shown in Fig. 3C, kit is expressed in melanoblasts as early as stage 1.5, consistent with a role for kit during regeneration. Almost all melanocytes in the regenerate express kit by stage 5, but this expression wanes and becomes undetectable in most melanocytes by stage 10 (not shown).

If kit is required during regeneration rather than for establishing precursor populations, then differentiating melanoblasts might develop up to the stage of kit requirement in kit mutant regenerates. To test this possibility, we observed melanoblast marker expression in kit mutant fins. Similar expression patterns of mitf in kit mutant and wild-type regenerates at stage 1 (Fig. 3A,B) and continuing through stage 3 (not shown) suggests that kit is not required for generating

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**Fig. 4.** Late regeneration melanocytes arise by de novo differentiation, and contribute differently to kit mutant and wild-type regeneration. (A) Stage 13 kit mutant regenerate after regenerating in the presence of PTU from stages 7-13 reveal few pigmented melanocytes in the regenerate (black arrows). (B) The same kit mutant fin several hours after PTU washout reveals many new melanocytes in the proximal regenerate (white arrowheads). (C) Stage 13 wild-type regenerate after regenerating in the presence of PTU during stages 7-13 of regeneration displays holes in the regenerated stripe (outlined in red). (D) The same wild-type fin several hours after PTU removal reveals new melanocytes filling the holes in the stripe, and in the distal regions of the fin (white arrowheads). The proximal one-third of the regenerated fins are indicated by blue brackets in B and D. Fish were treated with epinephrine prior to imaging to contract melanosomes. Amputation planes are labeled by black arrowheads. Scale bars: A-D, 200 μm.
mitf-positive presumptive melanoblasts (see Discussion). However, no mesenchymal kit-positive (Fig. 3D) or dct-positive (Fig. 3F) cells are detected through stage 7 in kit mutants, suggesting that kit is required for melanoblasts to progress to a kit/dct-positive stage during regeneration. In wild-type fins, low levels of kit transcript were detected in an unidentified population of cells scattered throughout the epidermis (not shown). The identity of these cells remains unclear. Interestingly, kit mutant fins retain the unidentified kit-positive epidermal cells (not shown), suggesting that their development is not dependent upon kit function.

**kit-independent de novo melanocytes re-establish melanocyte stripes in kit mutant regenerates**

Although regenerating kit mutant fins lack a wild-type pattern of de novo melanocytes, the fin stripes are eventually re-established. From stage 0-7, only a few melanocytes appear in the kit mutant regenerate immediately distal to the old stripe stump (Fig. 2D). These cells result from migration of pre-existing melanocytes into the regenerate (see below). Subsequent to stage 8, kit mutant melanocyte stripes are slowly re-established as melanocytes appear in the presumptive stripe until the regenerate stripe is filled to the distal edge of the fin (not shown). While a regenerating wild-type caudal fin stripe extends to the distal edge of the regenerating fin by stage 5, regenerating kit mutant fin stripes typically do not complete extension to the distal edge until around stage 20 (not shown).

To distinguish whether kit-independent melanocytes arise from unpigmented precursors or from previously pigmented melanocytes, kit mutant caudal fins were amputated and challenged to regenerate in the presence of PTU. Since regenerating kit mutant fin stripes begin to extend distally around stage 8 and the efficacy of PTU treatment is temporally limited to about 7 days, kit mutant fish were treated with PTU from stage 7 to stage 13. Before PTU was removed at stage 13, melanocytes in the regenerating stripe were minimal in number and distal extension (Fig. 4A). At least some of these pigmented cells represent pre-existing melanocytes similar to those observed in wild-type and do not require kit, because kit mutant regenerates treated between stages 0 and 7 with PTU retain several melanocytes immediately distal of the stripe stump (data not shown). It is also possible that some of these pigmented cells represent kit-independent de novo melanocytes that pigment prior to stage 7. Following the removal of PTU at stage 13, melanocytes appeared in the presumptive stripe immediately distal to previously pigmented cells (Fig. 4B). Therefore, while regenerating kit mutant fins do not display a wild-type pattern of de novo melanocytes early in regeneration (primary regulation melanocytes), their stripes are eventually re-established by kit-independent de novo melanocytes beginning around stage 8 (secondary regulation melanocytes). Interestingly, wild-type regenerates also form de novo melanocytes during late regeneration; however, their pattern is distinct from that observed in kit mutants (Fig. 4C,D; see below).

**Ontogeny of melanocyte fin stripes is similar to regeneration**

Fin regeneration serves to investigate how cells are stimulated to replace missing tissue and regulate form, and may also provide an accelerated reflection of the processes employed during ontogenetic development. To explore the relationship of regeneration and ontogeny, we sought to determine whether ontogenetic development of caudal fin stripes displayed patterns of de novo melanocytes similar to those observed in regenerative development. Observation of wild-type and kit mutant juveniles suggested at least some similarity. Melanocytes initially appear in growing wild-type caudal fins around the 4.5 mm stage (approx. 21 days postfertilization at 25°C in these experiments, dpf) and cover the entire fin by the
mutant regeneration. We amputated wild-type and kit mutant siblings lack all melanocytes in their caudal fins (Fig. 5D). To determine if these kit-dependent melanocytes develop from unpigmented precursors, 4.5 mm wild-type juveniles were treated with PTU and allowed to continue development. After 6 days in PTU (approx. 5 mm stage), no new melanocytes were visible (Fig. 5A), while untreated siblings had developed more melanocytes in their fins (not shown). New fin melanocytes became visible after removal of PTU (Fig. 5B), confirming that fin stripe melanocytes develop from undifferentiated precursors rather than division of previously differentiated melanocytes in the body.

In contrast, melanocytes do not appear in developing kit mutant caudal fins until the 8-9 mm stage (approx. 40 dpf), when they fill in the presumptive stripe, progressing proximal to distal (Fig. 5F). Since kit mutant juveniles are devoid of both fin and body melanocytes at stages immediately prior to development of fin melanocytes (Fig. 5D; Parichy et al., 1999), these kit-independent melanocytes must also be formed by de novo differentiation. We conclude that both kit-dependent and kit-independent fin melanocytes develop with similar temporal and spatial relationships during ontogeny and regeneration of the fin.

**kit-independent melanocytes regulate during kit mutant fin stripe development**

Is there a role for kit-independent melanocytes during wild-type fin development? Previous studies of kit mutants showed that kit-dependent melanocytes form approximately 50% of the wild-type complement of body stripe melanocytes, and kit-independent melanocytes form the remaining 50% (Johnson et al., 1995). The inference drawn from these studies is that kit-independent melanocytes in the body stripes develop regardless of kit-dependent melanocytes. We were interested in whether kit-independent melanocytes contribute similarly to fin stripe development. Counts of wild-type and kit mutant fin stripe melanocytes revealed that these genotypes have similar melanocyte densities in both ontogenetic and regenerated fin stripes (Fig. 6A). This demonstrates that kit-independent melanocytes are able to regulate to wild-type melanocyte density during kit mutant fin development.

The above finding of similar melanocyte density in wild-type and kit mutant regenerates (Fig. 6A), together with the observation that regeneration melanocytes in kit mutants arise late at proximal positions (Fig. 4A,B), raises the possibility that the pattern of late proximally developing (kit-independent) melanocytes observed in kit mutants may be a specific response to lack of kit function. Alternatively, kit-independent melanocytes may develop during wild-type regeneration in a pattern similar to kit mutant regeneration. To distinguish between these two models, we compared the positions of late de novo melanocytes during wild-type and kit mutant regeneration. We amputated wild-type and kit mutant siblings and treated them with PTU during regeneration stages 7-13. We imaged the regenerating fins at stage 13 and after PTU washout, and assessed the number of de novo melanocytes in the proximal, medial and distal thirds of each regenerating central stripe. Comparison of wild-type and kit mutant fish revealed approximately five-fold greater numbers of de novo melanocytes in the proximal third of kit mutant regenerates than in wild type. In contrast, most late regeneration melanocytes in wild type appeared in the distal growth zone of the regenerate (Fig. 6B). This finding suggests that kit-independent de novo melanocytes do not develop in a similar pattern in wild-type regeneration compared to kit mutants. Rather, it suggests that development of these cells is a regulatory response to the lack of kit function or kit-dependent melanocytes during the late stages of regeneration.

**DISCUSSION**

**Regeneration melanocytes arise from uniformly dispersed stem cells**

PTU experiments described above show that most fin regeneration melanocytes arise from undifferentiated precursors. Although a few melanocytes migrate a short distance from the stump into the proximal regenerate and make no additional contribution to the regenerating stripe, the vast majority of regeneration melanocytes are PTU-sensitive (lack melanin during regeneration in PTU) and therefore arise from undifferentiated precursors. We can generally exclude the possibility that regeneration melanocytes arise from previously differentiated melanocytes that are partitioning their melanosomes to progeny, as we see no evidence of partitioned melanosomes at early stages of regeneration (S. L. J.,
Fig. 7. Primary and secondary regulation of melanocyte development during fin stripe regeneration. De novo differentiation of primary kit-dependent melanocytes begins at stage 4 (st4) in wild-type (WT; kit-dependent melanocytes are represented in blue, with mature cells in dark blue and differentiating cells in light blue). These new cells appear in a band along the proximal regenerate. By stage 8 (st8), de novo differentiation of primary melanocytes occurs mainly in the distal regenerate. The secondary kit-independent class of melanocytes begin to differentiate around stage 8 in response to the absence of kit function or kit-dependent melanocytes (kit-kit-independent melanocytes are represented in red, with mature cells in dark red and differentiating cells in light red). These cells appear in the proximal presumptive melanocyte stripe. Both primary and secondary melanocyte classes re-establish the fin stripe by stage 30 (st30) in their respective genotypes.

unpublished data). Further, early melanoblast markers (mitf, kit, dct) identify cells that develop uniformly distributed in the distal stump at early stages (stages 1-2; Fig. 3A,C,E), or in the proximal regenerate at later stages (stages 3-4; Fig. 3G,H). This pattern of differentiating melanocytes persists in jaguar;long fin mutants where the amputation plane is selected at great distance from differentiated melanocytes (Fig. 3I,J), supporting our hypothesis that previously differentiated melanocytes do not give rise to undifferentiated precursors. We suggest in arguments below that these undifferentiated precursors are stem cells.

Several lines of evidence suggest that melanocyte stem cells exist in adult animals that provide new melanocytes in the regenerating fin. Here, we minimally define a precursor cell as one that can generate differentiated progeny, and a stem cell as a cell with the capacity for both producing differentiated progeny and for self-renewal. Goodrich first suggested the existence of such melanocyte stem cells by showing that new melanocytes could be recruited to develop in non-melanocyte regions in the intact fin (Goodrich et al., 1954). This model is also supported by the finding that unpigmented melanocyte stem cells are present in adult mice, where they might be responsible for maintaining the adult pigment pattern through multiple hair follicle cycles (Kunisada et al., 1998). Our previous findings that melanocytes develop in larval kit mutants that lack all differentiated melanocytes shows that unpigmented melanocyte precursors are responsible for generating at least part of the adult zebrafish pigment pattern (Johnson et al., 1995). An alternative to the model that regeneration melanocyte stem cells exist in the intact fin is that stem cells are generated in the regeneration blastema. Our finding that fin stripe regeneration and ontogeny share developmental and genetic pathways tends to suggest that the stem cells contribute both to ontogenetic growth of the fin and to regeneration, rather than being a property solely of the regenerating fin (Fig. 5). Furthermore, their capacity for self-renewal appears to be significant, since wild-type fin stripes are able to form new melanocytes along a normal time course through multiple rounds of regeneration. Taken together, these data suggest that melanocyte stem cells scattered evenly throughout the intact fin are recruited following amputation to divide in a self-renewing manner to produce new melanocytes.

**kit-dependent and kit-independent mechanisms of melanocyte regeneration in the fin**

A surprising finding that prompted this study was that, while kit mutants have apparently normal fin stripes prior to amputation, these mutants show profound defects in melanocyte regeneration. Our finding that two different temporal classes of regeneration melanocytes contribute to the regeneration and development of the stripe tends to explain this result. The first class develops dependent on the kit gene early in regeneration (absent in kit mutants), and a second kit-independent class develops in late stage regeneration of kit mutants (see Fig. 7). Like kit-dependent melanocytes, we suggest that kit-independent regeneration melanocytes arise from unpigmented stem cells rather than by division of previously pigmented cells. This is supported by the findings that their pigmentation is inhibited by PTU (Fig. 4A,B), and that kit mutant juveniles develop fin stripe melanocytes prior to the formation of any other adult melanocytes (Fig. 5D,F). Although the regeneration stage when kit-independent melanocytes begin to appear is somewhat variable (Fig. 4A), these cells generally first appear around stage 8. Interestingly, stage 8 marks a transition from regenerative developmental anatomy to normal growth anatomy (Johnson and Weston, 1995; Johnson and Bennett, 1999), suggesting that kit-independent melanocyte differentiation may be a response to this transition. By analogy, the finding of two independent populations of melanocytes in the zebrafish body stripes, one dependent on kit and the other dependent on rose (Johnson et al., 1995), supports the model of two distinct populations of regeneration melanocytes. Furthermore, the kit-dependent melanocytes develop before the second melanocyte population in both the body stripe and regenerating fin. There are, however, important distinctions between body stripe development and fin stripe regeneration. For instance, fin stripe development and regeneration is largely independent of rose, as kit;rose double mutants develop or regenerate fin stripes indistinguishable from the kit single mutant (not shown). Additionally, kit-independent regeneration melanocytes are able to regulate their number to recover normal melanocyte density in the absence of kit function (see Fig. 6A). In contrast, kit-independent (rose-dependent) body stripe melanocytes fail to compensate for the missing kit-dependent melanocytes in kit mutants (Johnson et al., 1995).

The finding of distinct classes of differentiated melanocytes in the regenerating fin (as assessed by their different timing of development and different genetic control) does not distinguish between models for distinct or shared stem cells for the different types of melanocytes. Characterization of melanocyte stem cell potency and proliferative ability awaits the generation of molecular markers for these cells.
Several models could account for the defect in melanocyte development during kit mutant fin stripe regeneration. For example, kit could be required for proper establishment of kit-dependent melanocyte stem cells during the embryonic and larval stages. Alternatively, kit might be required for recruiting melanocyte stem cells to re-enter the developmental pathway during regeneration. Consistent with this second model, kit is expressed in developing melanocytes during fin regeneration (Fig. 3C,H). In situ hybridization in kit mutant regenerates reveals that normal mitf expression persists through stage 3 in the absence of kit function; however, dct-positive or kit-positive melanoblasts are not detected (Fig. 3A-F). Inability to detect kit transcript in kit mutant regenerates may be due to mutant transcript instability, rather than failure of kit mutant melanoblasts to proceed to a kit-positive stage of development. However, the normal development of mitf-positive cells and the absence of dct-positive cells in kit mutant regenerates suggests that kit acts downstream of mitf to promote progression to a dct-positive stage of melanoblast development in kit-dependent melanocytes. This inference is made cautiously, however, since mitf may be expressed in other cell types during regeneration that appear unaffected in kit mutants (Parichy et al., 2000). In the absence of molecular markers for melanocyte stem cells, these models could be further tested with a conditional allele of kit. Conditional mutations could be generated by screening for temperature-sensitive alleles (Johnson and Weston, 1995).

**kit-independent melanocytes represent a secondary regulatory mode and are largely absent in wild-type regeneration**

Is kit-independent de novo differentiation of fin melanocytes specific to kit mutant regeneration, or does this phenomenon also occur in wild-type regeneration? Considering the fact that new melanocytes develop in the proximal presumptive stripe after stage 8 in kit mutants (Fig. 4A,B), it was possible that the mutant revealed a role for these kit-independent melanocytes in the same pattern during late wild-type regeneration. This model tends to be refuted by the low number of late de novo melanocytes appearing in the proximal regenerating wild-type stripe compared to kit mutants (Fig. 6B). This suggests that kit-independent melanocytes or their precursors regulate their development in response to the absence of kit function and/or early kit-dependent melanocytes. The ability of the kit-independent melanocytes to regulate to fully populate the fin stripes is further demonstrated by the finding of similar melanocyte densities in wild-type and kit mutant fin stripes (Fig. 6A). This is in contrast to kit-independent (rose-dependent) melanocytes in the body stripe, which only form about half the wild-type complement of melanocytes (Johnson et al., 1995). We describe two redundant modes of regulation involved in reconstituting the melanocyte stripes of the zebrafish fin: a primary mode that develops melanocytes early in regeneration dependent on the kit gene, and a secondary mode that develops melanocytes late in regeneration in the absence of kit function (see Fig. 7).

Several models could account for the pattern of secondary regulation during kit mutant fin regeneration. By one model, there may be two distinct melanocyte stem cell populations, one for primary (kit dependent) regeneration melanocytes, the other for secondary (kit-independent) regeneration melanocytes. The absence of primary regeneration melanocytes (for instance, in kit mutants) may provide the opportunity for secondary stem cells to be recruited to develop after a lag of approximately one week. A second model posits a single stem cell population, which is first recruited to produce melanocytes in a kit-dependent fashion. Lacking kit, these differentiating melanoblasts presumably die, similar to kit-defective melanoblasts in fish or mouse embryos (Parichy et al., 1999; Wehrle-Haller and Weston, 1995). The subsequent absence of melanocytes in the regenerate may provide the conditions by which the same stem cell population is again recruited to produce melanocytes, this time by a kit-independent mechanism. A third model is that a single stem cell population is recruited only once to produce regeneration melanocytes. These differentiating melanoblasts may require kit to promote specific morphogenetic processes, such as the migration of developing melanoblasts into the regenerate, or their differentiation in the regenerate. Lacking kit function, these melanoblasts may arrest or delay their development at one of these steps. After a delay, secondary (kit-independent) mechanisms may be recruited to rescue the development of these cells, accounting for the apparent delay described for appearance of secondary regeneration melanocytes. We feel this last model is less likely, as other melanoblasts and melanocytes that utilize kit for their development die when kit function is ablated (Parichy et al., 1999; Wehrle-Haller and Weston, 1995), rather than simply delay development until other means can be found to promote their development. Rather, we support a model that kit-independent melanocytes are a distinct developmental lineage from kit-dependent melanocytes, arising either from a common stem cell population or alternatively from discrete stem cell populations.

These results show that kit-independent melanocytes arise through a secondary regulatory mode during kit mutant fin regeneration. Do they also play a minor role in wild-type fin development? One possibility is that kit-independent melanocytes function during wild-type development to help regulate the final form of the stripe. For instance, perhaps the mechanism of early kit-dependent stripe development leads to holes and gaps in the stripe that are later filled by kit-independent melanocytes (one possible example is shown in Fig. 4C,D). While regulation among melanocyte precursors and other neural crest derivatives during embryonic vertebrate development is well described (Huszar et al., 1991; Raible and Eisen, 1996; for review see Vaglia and Hall, 1999), our findings imply that at least partial regulatory potential is retained in adult neural crest derivatives. Although redundant secondary mechanisms of regulation are probably common in adult vertebrate development, they are only beginning to be appreciated (for instance, see Alison and Sarraf, 1998). The zebrafish genetic system raises the possibility of identifying mutants defective for the secondary kit-independent melanocyte class, allowing for understanding of the molecular mechanisms underlying the regulation of these cells.

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