A method to specifically ablate melanocytes in a genetically tractable organism would facilitate the analysis of melanocyte regeneration and regulation. We have demonstrated that a Q-switched neodymium:yttrium–aluminum–garnet dermatology laser kills larval melanocytes in zebrafish. Following melanocyte ablation, new melanocytes regenerate from unpigmented precursors. We show that melanocyte regeneration following laser ablation requires *kit* receptor tyrosine kinase.

Key words: *kit*/laser ablation/melanocyte/regeneration/zebrafish

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Regeneration of cells or tissues is an important process for many multicellular organisms to repair damaged tissues. This may occur by the recruitment of precursors or stem cells to reenter developmental pathways, thereby giving rise to new cells that then replenish the target cell population. Several vertebrates, especially poikilotherms, display great capacity to regenerate specific cell types or tissues (Dinsmore, 1991). Among them, zebrafish stands out for providing an opportunity to explore the genetic mechanisms underlying regeneration (Johnson and Weston, 1995; Vihtelic and Hyde, 2000; Rawls and Johnson, 2001; Poss *et al.*, 2002; Huang *et al.*, 2003). For instance, studies on the reestablishment of melanocyte stripes in the regenerating zebrafish adult caudal fin have demonstrated that *kit* receptor tyrosine kinase is required for recruiting melanocyte precursors or stem cells for regeneration of the majority of melanocytes that reconstitute fin stripes (Rawls and Johnson, 2001). Despite the elegance of the fin regeneration model, progress is hampered by the necessity to rear animals to adult stages before new mutations can be assessed. In order to gain more insight into the genetic mechanisms of melanocyte regeneration, a simple method to elicit melanocyte regeneration in embryonic or larval stages is needed.

Melanocytes in zebrafish, similar to other vertebrates, develop from neural crest cells (Raible *et al.*, 1992; Raible and Eisen, 1994; Groves and Bronner-Fraser, 1999). The appearance of the pigmented melanocytes begins at 24 h postfertilization (hpf), and the number of the pigmented melanocytes increases through approximately 60 hpf, when the embryo has approximately 400 melanocytes. This number of larval melanocytes remains nearly constant with only minimal birth and death of melanocytes occurring until the onset of pigment pattern metamorphosis at 2 wk postfertilization (Milos and Dingle, 1978a, b; Parichy *et al.*, 1999; reviewed by Rawls *et al.*, 2001). This constancy in melanocyte number from 60 hpf to 2 wk provides a window to explore melanocyte regeneration independently of normal ontogenetic mechanisms for melanocyte development.

The ability of lasers to provide intense localized energy to cells or tissues has been widely adapted for clinical procedures and biomedical research (Kimble, 1981; Montell *et al.*, 1991; reviewed by Spicer and Goldberg, 1996). Treatment of pigmentary disorders and removal of tattoos by lasers are two such applications. The notion behind this type of application is that pigments or dyes can selectively absorb the energy of the laser beam. When the laser irradiation is nearly instantaneous, the energy absorption causes localized thermal damage by rapidly superheating the target pigment-containing cells or tissues, resulting in their death and allowing the surrounding cells and tissues to remain intact (Anderson and Parrish, 1983). Although melanin has a broad absorption spectrum, several studies in mammals have shown Q-switched lasers, including Nd:YAG (neodymium: yttrium–aluminum–garnet) dermatology lasers, that emit monochromatic light in nanosecond pulses, can selectively target melanin-containing cells (Pollia *et al.*, 1987; Anderson *et al.*, 1989). Nevertheless, the fact that melanin in mammalian systems is deposited in non-melanocyte cells such as keratinocytes in the skin would prevent the specific absorption by melanocytes of the laser energy in mammals (for a review see Quevedo and Holstein, 1998). In contrast, melanocytes in teleosts, such as zebrafish, do not export melanosomes to other cell types and, in consequence, are the only cell type in the skin to contain melanin (for a review see Bagnara, 1998). We reasoned that this feature of zebrafish should allow specific laser ablation of melanocytes with minimal damage to other cell types, thus allowing investigation of whether larval melanocyte can regenerate following ablation.

Here, we investigate the ability of a Q-switched dermatology laser to ablate larval melanocytes in zebrafish. We find that the Nd:YAG Q-switched laser emitting 532 nm light selectively kills melanocytes through energy absorption by melanin. We show that larval melanocytes regenerate from melanoblasts following laser ablation. We then used a temperature-sensitive allele of the *kit* receptor tyrosine...
kinase to show that kit is required for melanocyte regeneration following laser ablation.

Results

Five hundred and thirty two nanometer Q-switched Nd:YAG laser selectively ablates melanocytes We first asked whether irradiation with a Medlite II Nd:YAG Q-switched dermatology laser can selectively kill pigmented larval melanocytes in zebrafish. The Medlite II Q-switched laser provides a 532 nm laser beam at 1–5 J per cm² density with 5–7 ns pulsewidth and has been widely applied to ablate pigment for medical and cosmetic purposes (reviewed by Halder and Nordlund, 1998). The diameter of the laser beam can be selected to cover approximately one-third of a 60 hpf larval fish. Accordingly, 60 hpf larval fish were anesthetized and exposed to one pulse of 0.2–0.5 J per cm², 532 nm laser beam (Fig 1).

Twelve hours after the laser irradiation, melanocytes in the beam path appear contracted and fragmented (Fig 1B and C). Over the ensuing 2 d, these fragmented melanocytes are then extruded through the skin (Fig 1D), a consequence of typical melanocyte cell death in teleosts (Parichy et al, 1999; Sugiarto et al, 2000). We suggest that the absorption of laser energy by melanin in melanocytes specifically heats and kills the irradiated melanocytes.

We observed that at high energy levels (5 J per cm²) larval fish were often killed by the laser irradiation. We wondered whether the laser energy was also directly absorbed by other tissues to cause damage and ultimately fish death or whether the fish death observed at higher energy was the result of energy absorption through the melanin in melanocytes that was then passed to the other cells as heat. To test between these models, we reared some embryos in phenylthiourea (PTU) solution, a chemical that prevents melanin synthesis in otherwise normally pigmented melanocytes (Milos and Dingle, 1978a; Rawls and Johnson, 2000). When the PTU-treated larvae were exposed to high laser energy (5 J per cm²), we found that most larvae (97%) survived, compared with the 3% survival rate of the pigmented larvae treated with the same laser irradiation (Table I). These results indicate that the means by which laser irradiation causes either melanocyte death at low fluence or fish lethality at high fluence is through the absorption of laser energy by melanin in melanocytes, and that the laser energy is not directly absorbed by other cell types.

Figure 1
Larval melanocytes regenerate following selective laser irradiation. Sixty hours postfertilization (hpf) larvae were irradiated with one pulse of neodymium:yttrium–aluminum–garnet laser at 0.5 J per cm². An irradiated region (marked with black arrowheads in E) with disrupted melanocyte pattern (black arrow in B) was observed at 12 h postlaser-irradiation (hpl) (B), whereas unirradiated larvae showed no such disruption in melanocyte pattern (A). Melanocytes in the irradiated region appear punctate and fragmented (black arrow in C), in contrast to dendritic melanocytes outside the irradiated region (white arrow in C). Fragmented melanocytes were typically observed being extruded from the skin within the irradiated regions (black arrow in D) on 4 d postlaser-irradiation (dpl) larvae (D). Nuclei (yellow arrowheads in E) of cells above or contiguous to laser-irradiated melanocytes (outlined with red dashed line in E) stay intact and display histology of normal tissue, revealing by staining with Hoechst 33528 (E). In contrast, the cells surrounding the punctate melanocytes (outlined with red dashed line in F) in the larvae that were poked by flamed needles have pycnotic nuclei (yellow arrowheads in F), consistent with cell death, as well as general histological disorder in the region of thermal insult (F). Simultaneously with the extrusion of contracted melanocytes (black arrow in D), dendritic and fainter melanocytes (blue arrow in D) reappear in the laser-irradiated region. At 6 dpl (G), melanocytes in the beam path were regenerated to a normal larval pigment pattern. (A) A normal 72 hpf larva that is equivalent to a 12 hpl larva (B), (B) and (G) are the same fish at 12 hpl and 6 pl, respectively. (C) and (E) are images collected from the same fish at 12 hpl. (D) A different fish at 4 dpl. (F) A fish at 12 h after thermally damaged by a flamed needle. Black arrowheads mark the path of the laser beam. Scale bars: 500 μm in (A, B, and G); 25 μm in (C); 200 μm in (D); 20 μm in (E, F).
To further confirm that the laser irradiation selectively targets on melanocytes, we examined the cells surrounding punctate melanocytes at 12 h postlaser-irradiation (hpi) by staining them with a nuclear dye, Hoechst 33258. We found that the nuclei in cells above or adjacent to punctate melanocytes stay intact and display the histology of normal tissue (Fig 1E). In contrast, when we examined the cells surrounding the melanocytes that were poked by flamed needles (see Materials and methods), we found pyknotic nuclei, consistent with cell death, as well as general histological disorder in the region of thermal insult (Fig 1F).

These results suggest that the energy transferred from laser irradiated melanocytes to the neighboring cells as heat is minimal or insufficient to damage neighboring non-melanin-containing cells. Thus, the method described here specifically and effectively ablates melanocytes in the zebrafish larvae.

**Melanocytes regenerate from unpigmented precursors following laser ablation**

We were next interested in whether melanocytes regenerate following laser ablation. We observed that simultaneously with the extrusion of contracted and dying melanocytes, potentially new melanocytes begin to appear in the laser-irradiated region at 2 d postlaser-irradiation (dpi) (Fig 1D). These melanocytes initially appear fainter than melanocytes outside the beam path, and are more dendritic (a feature of newly differentiating melanocytes) than the extruding melanocytes in the beam path, suggesting that they are newly differentiated (Fig 1D). By 6 dpi, melanocytes in the laser-irradiated region regenerate to a pigment pattern indistinguishable from that on normal larvae (Fig 1G). The number of the larval melanocytes in the laser-irradiated regions at 6 dpi is approximately 87.5% ± 6.2% of that in the same regions prior to laser irradiation (n = 5). These results indicate that the larval zebrafish can repair its pigment pattern after laser-induced melanocyte cell death.

To determine whether the melanocytes that reconstitute the laser-irradiated region are derived from unpigmented precursors (melanoblasts or melanocyte stem cells) or through the migration of pre-existing melanocytes, we incubated larval fish in PTU solution after laser irradiation (Fig 2A and B). Because PTU prevents melanin synthesis in otherwise normal melanocytes, melanocytes that differentiate in the presence of PTU can be discriminated from those that differentiate and melanize prior to laser and PTU treatment (for instance, see Milos and Dingle, 1978a; Rawls and Johnson, 2000). If melanocytes in the laser-irradiated region come from the migration of the pre-existing melanocytes outside the beam path, we expect that the laser-irradiated region should be reconstituted with pigmented melanocytes in the presence of PTU. Alternatively, if the melanocytes in the laser-irradiated region derive from unpigmented precursors, this laser-irradiated region should remain unpigmented in the presence of PTU. Following laser irradiation and PTU incubation, we found that the laser-irradiated region becomes completely clear of melanocytes and melanin by 6 dpi (Fig 2C). That melanocytes had in fact developed in the laser-irradiated region was revealed by the presence of slightly pigmented melanocytes shortly after PTU was washed out (Fig 2D). Together, these results confirm that the melanocytes in the laser-irradiated region that reconstitute the pigment pattern following melanocyte ablation by laser irradiation are derived from unpigmented precursors (melanoblasts or melanocyte stem cells) rather than through the inward migration of pre-existing melanocytes outside the beam path.

**kit is required for the melanocyte regeneration following laser ablation**

We are interested in understanding the genetic mechanisms underlying larval melanocyte regeneration in zebrafish. One candidate gene for a role in melanocyte regeneration is the kit receptor tyrosine kinase. *kit* function is essential for melanocyte development in vertebrates (for a review see Besmer et al, 1993). Mutants for *kit* in zebrafish develop melanocytes initially, but they fail to migrate to proper positions and subsequently undergo apoptosis (Parichy et al, 1999). These *kit* mutant melanocytes begin to die at 4 d postfertilization (dpf), and no new melanocytes regenerate to restore the pigment pattern until metamorphosis (approximately 14 dpf). In the light of our finding of larval melanocyte regeneration following laser ablation, the observation that *kit* mutants fail to regenerate new melanocytes after their initial melanocytes die raise the possibility that *kit* might be required for larval melanocyte regeneration following laser ablation as well. To test this, we took advantage of a temperature-sensitive *kit* allele, *kit*1e99 (Rawls and Johnson, 2003). When reared at the restrictive temperature (33 C), *kit*1e99 larvae develop a pigment pattern identical to that of the null mutants. In contrast, when *kit*1e99 animals are reared at the permissive temperature (23 C–25 C), the larval melanocyte pattern is similar to that of wild-type larvae and those melanocytes survive throughout the larval stage (Rawls and Johnson, 2003).
mutation allows us to generate embryos with normal melanocytes, then ablate them with laser irradiation and assess the role of *kit* in melanocyte regeneration by rearing animals at permissive or restrictive temperatures. Accordingly, we laser-irradiated 60 hpf-old *kit*<sup>1e99</sup> animals that were reared at the permissive temperature (25°C), and then shifted the larvae to 30°C after laser irradiation, a temperature that partially abolishes *kit* function (Fig 3, Rawls and Johnson, 2003). We found that the laser-irradiated region is clear of any melanocytes through 6 dpl, suggesting *kit* is required for melanocyte regeneration following laser ablation (Fig 3B). Surprisingly, when we irradiated *kit*<sup>1e99</sup> animals with the laser and returned the animals to 23°C or 25°C, temperatures usually permissive for *kit*<sup>1e99</sup> function, we found that melanocytes also failed to regenerate throughout the 2 wk of postlaser-irradiation development (Fig 3D; 25°C data not shown), indicating that the *kit* activity of *kit*<sup>1e99</sup> mutants at the permissive temperature (23°C and 25°C) is partially compromised and insufficient for larval melanocyte regeneration, whereas the wild-type larvae regenerate normally after all temperature shifts. Taken together, these results suggest that *kit* function is required for melanocyte regeneration following laser ablation. Moreover, a higher functional threshold of *kit* activity is required for melanocyte regeneration than for normal embryonic melanocyte development.

**Discussion**

Q-switched Nd:YAG laser-irradiation effectively ablates zebrafish melanocytes. We have developed a method to specifically ablate melanocytes in the larval zebrafish. We demonstrated that irradiation with a Q-switched Nd:YAG dermatology laser specifically kills larval melanocytes in zebrafish, and this occurs through the absorption of laser energy by melanin in melanocytes (Table I). An important feature of the Q-switched laser is that it delivers laser energy in a pulse of 5–7 ns. This rapid energy delivery is essential for killing melanocytes without also causing extensive damage to surrounding tissues or death of larvae (Fig 1E and F). We have also tried to ablate zebrafish melanocytes with a continuous Nd:YAG 532 nm laser beam that provides the same amount of energy in 0.1 s pulses. In the latter case, we could not find a fluence that specifically killed melanocytes without also severely damaging or killing the larvae (data not shown). Presumably, the faster rate of providing energy to the melanosomes with the Q-switched laser allows for the super-heating of the local cellular environment of the melanosomes, thus killing the melanocytes without transferring sufficient heat to damage neighboring cells or to kill the larvae. By this logic, we suggest that the slower rate of the laser energy delivery in the 0.1 s pulse laser cannot sufficiently superheat the melanocytes without also overheating the surrounding tissues, resulting in the damage or death to the entire larvae.

Another key feature for the success of this method in ablating melanocytes in zebrafish is that melanocytes are the only melanin-containing cells in teleosts. Melanin is synthesized and stored in melanosomes that are retained within melanocytes, rather than being transferred to other cells such as keratinocytes in the mammalian pigmentary system. We suggest that the melanosome retention in poikilotherm melanocytes together with the rapid energy delivery of Q-switched lasers account for the specificity of melanocyte ablation that we describe.
The requirement for and Johnson, 2001). The different dose requirements of melanocyte regeneration in the regenerating fin (Rawls suggested, including embryonic melanocyte development and melanocyte regeneration than that for other roles we have shown that following laser ablation, larval melanocytes regenerate from unpigmented precursors to reconstitute the pigment pattern. This ability to regenerate melanocytes suggests the existence of melanocyte precursors or stem cells at larval stages that are capable of sensing melanocyte deficits and can be recruited to generate new cells, although we are unable to formally exclude the possibility that other neural crest-derived pigment cells, such as xanthophores or iridophores, may undergo transdifferentiation to reconstitute the larval melanocyte pattern (see Ide, 1986). We and others have previously demonstrated regeneration capacity in adult tissues in fish and mouse (Rawls and Johnson, 2000, 2001; Nishimura et al, 2002). Nevertheless, the genetic mechanisms underlying how these melanocyte precursors are established in the embryos, maintained through development, and finally recruited to reenter developmental pathways at later stages remains mysterious. The ability to specifically ablate melanocytes in larval stages with the Q-switched laser and the demonstration that larval melanocytes regenerate following laser ablation now provide an opportunity to bring zebrafish genetic analysis to bear on these questions. This study with the conditional kit receptor tyrosine kinase mutation demonstrates the potential of this approach.

The kit receptor tyrosine kinase contributes to melanocyte development through multiple cellular mechanisms and at multiple stages of the zebrafish life cycle. Our analysis of the role of kit following melanocyte laser ablation now reveals that kit is also required for larval melanocyte regeneration. Furthermore, the finding that the temperature-sensitive kit allele fails to promote larval melanocyte regeneration at temperatures permissive for embryonic melanocyte development suggests that the kit1e99 allele is partially compromised at 23°C and 25°C, and that the requirement for kit function is greater during larval melanocyte regeneration than that for other roles we have suggested, including embryonic melanocyte development and melanocyte regeneration in the regenerating fin (Rawls and Johnson, 2001). The different dose requirements of kit signaling between ontogenetic development and proliferation/regeneration have been reported in other systems, for instance, hematopoiesis in mice (Tajima et al, 1998). The kit1e99 allele has a single nucleotide change resulting in a leucine-to-proline substitution in the 754 amino acid in the second tyrosine kinase domain of the kit receptor tyrosine kinase (Rawls and Johnson, 2003). Our finding here suggests that this change may cause a potential reduction of kit signaling at the permissive temperature that compromises melanocyte regeneration. By this logic, the reduction is not sufficient to compromise embryonic melanocyte development. Alternatively, melanocyte regeneration and embryonic melanocyte development may have distinct signaling pathways downstream of kit, and the 754 residue may play an essential role selective for melanocyte regeneration.

The mechanisms for which kit is required for larval melanocyte regeneration remain unclear. One possibility is that kit is required to establish melanocyte precursors or stem cells during embryogenesis. Alternatively, melanocyte precursors or stem cells might be normally established in kit mutants, but lacking kit function, larvae fail to properly recruit melanocyte precursors or stem cells back into the developmental pathway to replenish melanocytes following laser ablation. Lacking a more discriminating temperaturesensitive kit allele, we are unable to distinguish between these two models. We have previously suggested that the requirement for kit in reestablishing the melanocyte stripes in the adult regenerating fin is in recruiting melanocyte stem cells following amputation, rather than establishing the melanocyte stem cells during embryonic or larval stages (Rawls and Johnson, 2001). If the previously suggested stem cell population(s) that plays a role in fin melanocyte regeneration is also important for larval melanocyte regeneration, then kit may also be involved in recruitment of stem cells to reenter developmental pathways following larval melanocyte ablation. Identification of markers that unambiguously label melanocyte stem cells in embryonic, larval or adult stages will be necessary to firmly establish how kit is required for recruiting stem cells following larval melanocyte ablation or fin amputation.

**Materials and Methods**

**Stocks** Zebrafish were reared according to standard protocols at 28.5°C, unless otherwise noted (Westerfield, 1993). Temperature-shift experiments were conducted by using the permissive temperature of 23°C or 25°C and the restrictive temperature of 33°C (Johnson and Weston, 1995; Rawls and Johnson, 2003). All developmental staging in hours and dpf corresponds to staging at the standard temperature of 28.5°C. Staging at restrictive and permissive temperatures is translated to 28.5°C stages according to the following equation: (dpf at 23°C) (0.695) = (dpf at 25°C) (0.805) = (dpf at 28.5°C) = (dpf at 33°C) (1.245) (Kimmel et al, 1995). kit1e99 has been previously described (Rawls and Johnson, 2003). All references to this mutant allele correspond to homozygous embryos. The protocol of animal care and uses is approved by the Animals Studies Committee of Washington University (A-3381-01).

**Laser irradiation and melanocyte regeneration** For laser irradiation, larvae were anesthesia and put in a trough cut in 1% agarose plates with minimum egg water to minimize the reflection of the laser beam by water. A Medlite II Q-switched Nd:YAG derma-
tology laser (HOYA-ConBio, Fremont, California) was set to 532 nm. The laser beam was delivered by a 3 mm handpiece placed 17 cm above the animals. Each animal was exposed to at least one pulse of laser beam. After laser irradiation, larvae were immediately rescued by adding more egg water and transferred to 60 mm petri dishes for future observation. This procedure of handling and transferring larvae between irradiation plates and tubes contributes 2%–9% lethality (see Table I). Laser-irradiated melanocytes were indistinguishable from unirradiated melanocytes until 12 hpl. Then, animals with regions of irradiated melanocytes were selected, raised at appropriate temperatures, and observed at least through 10 successive days. For PTU (Sigma-P7629, St Louis, Missouri) treatment of fish, 0.1–0.2 mM PTU was added to the egg water and changed every 2 d (Millos and Dingle, 1978a).

Viability of the cells surrounding irradiated melanocytes was determined by staining with the nuclear dye Hoechst 33258 (Sigma-B1782). Thus, larvae were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) 12 h following irradiation, washed into PBS, incubated 10 min in 5 μg/ml Hoechst 33258 solution, then washed again in PBS before observations. As a positive control for thermal-induced death, we poked similar aged larvae with flame needles, waited 12 h, and processed as described above for nuclei examination.

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