

***In Vivo* Imaging Indicates Muscle Fiber Dedifferentiation Is a Major Contributor to the Regenerating Tail Blastema**

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During tail regeneration in urodele amphibians such as axolotls, all of the tissue types, including muscle, dermis, spinal cord, and cartilage, are regenerated. It is not known how this diversity of cell types is reformed with such precision. In particular, the number and variety of mature cell types in the remaining stump that contribute to the blastema is unclear. Using Nomarski imaging, we followed the process of regeneration in the larval axolotl tail. Combining this with *in vivo* fluorescent labeling of single muscle fibers, we show that mature muscle dedifferentiates. Muscle dedifferentiation occurs by the synchronous fragmentation of the multinucleate muscle fiber into mononucleate cells followed by rapid cell proliferation and the extension of cell processes. We further show that direct clipping of the muscle fiber and severe tissue damage around the fiber are both required to initiate dedifferentiation. Our observations also make it possible to estimate for the first time how many of the blastema cells arise specifically from muscle dedifferentiation. Calculations based on our data suggest that up to 29% of nondermal-derived cells in the blastema come from dedifferentiation of mature muscle fibers. Overall, these results show that endogenous multinucleate muscle fibers can dedifferentiate into mononucleate cells and contribute significantly to the blastema. © 2001 Academic Press

Key Words: blastema; dedifferentiation; muscle fibers; regeneration; Axolotl; *Ambystoma mexicanum*.

INTRODUCTION

Urodele amphibians have the impressive ability to perfectly regenerate their tails throughout adulthood. Amputation initially results in wound healing, followed by the formation of a blastema of proliferating cells that go on to form the complete array of tissue types. It is still unknown how this diversity of cell types is reformed with such precision during regeneration. Two major questions need to be addressed: first, the number and type of mature cells in the remaining stump that contribute to the blastema. Though tissue-grafting experiments have shown that nerve, dermis, bone, and muscle all contribute cells to the blastema, the grafted tissue represented a complex mixture of cell types so such experiments have not completely resolved whether cells arise from the reactivation of a resident, interstitial stem cell, or through dedifferentiation (Stocum, 1995). Second, it is difficult to distinguish be-

tween different populations of cells within the blastema to determine whether it contains segregated progenitor cells specific for muscle, bone, and nerve, as opposed to a pluripotent progenitor.

Studies on limb regeneration suggest that there is considerable dedifferentiation and cell plasticity during the formation of the blastema. Detailed histological studies of the fate of muscle fibers at the plane of amputation suggested that muscle fibers lose their myofibrillar structure, their nuclei enlarge, and then bud off as mononucleate cells into the blastema (Thornton, 1942; Hay, 1959). In these studies, however, the process of dedifferentiation was inferred from static images, and, as a result, the true relationship between the muscle cells and blastema cells remained unproven. It has been argued that the observations actually represented the fusion of myoblasts into newly forming muscle fibers instead of budding off of mononucleate cells. The first experimental evidence to support this unusual “budding off” mechanism of producing progenitor cells was provided by Lo *et al.* (1993). Multinucleate myotubes formed in culture were purified by size-selective sieving, injected

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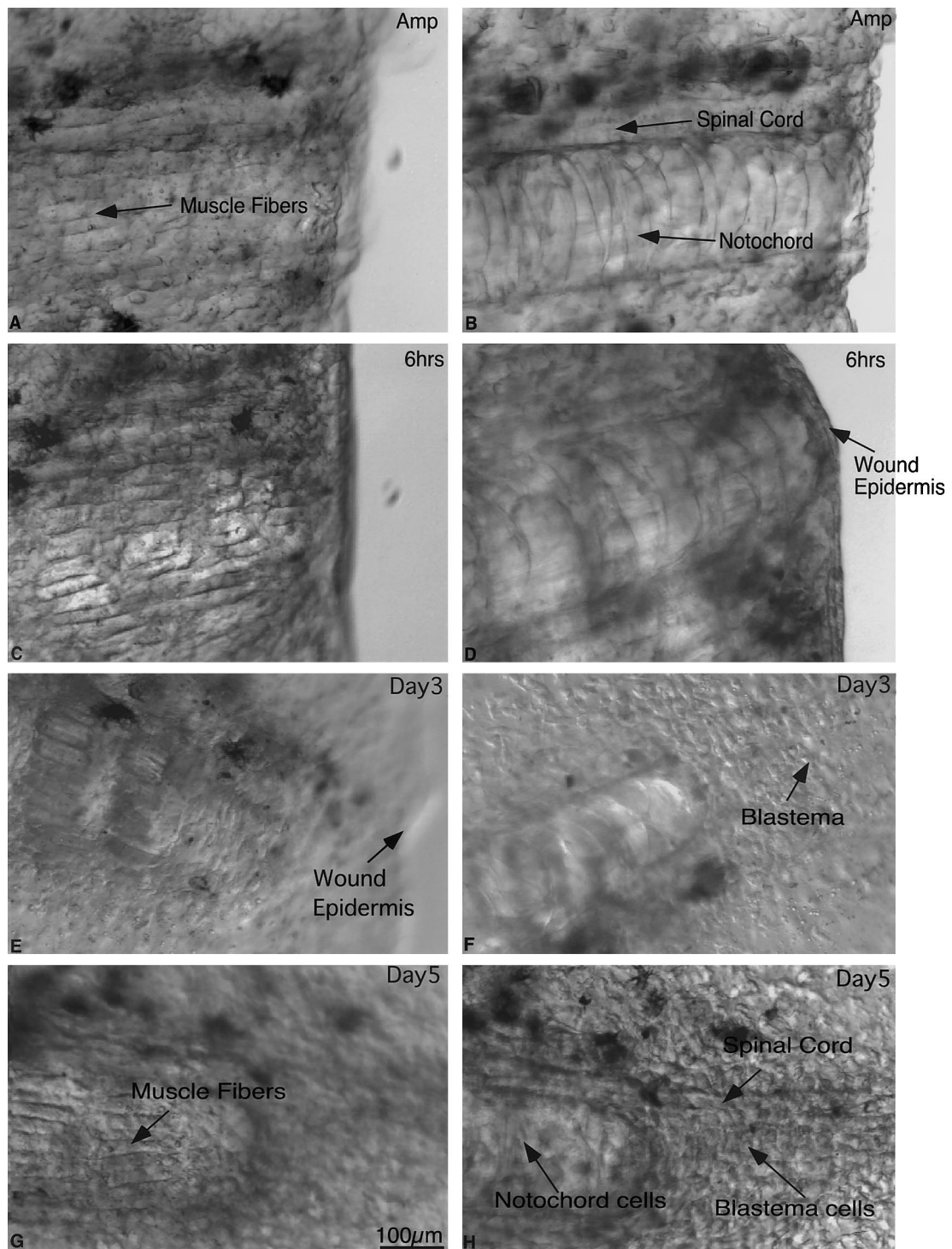


FIG. 1. Characterisation of axolotl tail regeneration using DIC imaging. The left panels show the focal plane of the muscle, and the right panels show the focal plane of the central axis. (A, B) The plane of amputation after the last 5–10 mm of tail was removed with a sterile scalpel. Initially wounding results in contraction of the muscle fibers as shown in (A), and distortion of the regular oval morphology of the notochord cells in (B). Within 6 h, the skin crawled over the wound, forming a wound epidermis as shown in (C) and (D). After a wound epidermis (E) has formed, the next important stage of regeneration is blastema formation (F). The blastema consists of a mound of proliferating undifferentiated cells that will eventually give rise to the lost tissues. In (G) and (H), we see the end of the muscle fibers and notochord cells mark the plane of amputation. A gap now exists between the mature tissue and the wound epidermis. The cells filling this gap are the blastema cells.

with lineage tracer, and then implanted into a regenerating newt limb. After 1 week, the lineage label was found in mononucleate cells. The average number of mononucleate cells increased over time, suggesting that the cells were dividing. The implantation experiments were repeated with an integrated retroviral marker, thus reducing the possibility that mononucleate cells were derived from cytoplasmic transfer of lineage tracer (Kumar, 2000).

Many outstanding questions remain concerning dedifferentiation that cannot be addressed by histological experiments or implantation experiments. Since Lo's experiments relied on the use of cultured myotubes, it remains an open question whether endogenous muscle fibers dedifferentiate and then trans-differentiate to form other cell types. Second, the quantitative contribution of muscle dedifferentiation to the blastema is unknown. It is unclear whether only a small subpopulation of relatively immature myotubes are capable of reversing their differentiation or whether fully formed, large caliber muscle cells are also capable of this transition. Further, it is important to test Hay's proposal that muscle cell nuclei undergo decondensation during dedifferentiation. This may represent a part of the nuclear reprogramming that likely occurs during regeneration. Finally, it is unknown what signals generated from amputation may induce muscle-cell dedifferentiation.

To begin addressing these questions, we have established a system to trace the fate of single cells during tail regeneration of the axolotl (*Ambystoma mexicanum*). The use of *Ambystoma mexicanum* as a system allows us to address the questions of cell fate during regeneration with sufficient precision. Axolotls 2–3 cm in length have a considerable amount of mature tissue, but the animals are still optically clear, allowing easy visualization of fluorescent lineage tracers. Furthermore, larval regeneration occurs in a relatively short period, so it should be possible to trace cells from beginning to late stages of tail regeneration. Using this system, we first focused on visualizing muscle-cell dedifferentiation during regeneration. We found that, 3–5 days postamputation, the muscle cells fragment into mononucleate cells. All nuclei from a single multinucleate muscle fiber “bud off” from the muscle cell, not just the nuclei closest to the amputation site. The mononucleate cells that are generated are morphologically indistinguishable from blastema cells. We further show that direct injury of the muscle fiber as well as an unknown signal released in response to severe tissue damage around the fiber are required to trigger dedifferentiation.

MATERIALS AND METHODS

Animals and Surgery

All experiments were carried out on larval urodele amphibia *Ambystoma mexicanum* (axolotl), supplied by the Indiana Axolotl colony (Bloomington, USA). Axolotls were maintained at 17°C in 40% Holtfreter's and fed daily with artemia. Tail amputations and microinjections were performed on axolotls anaesthetized in 0.01% Ethyl *p*-Aminobenzoate (Sigma).

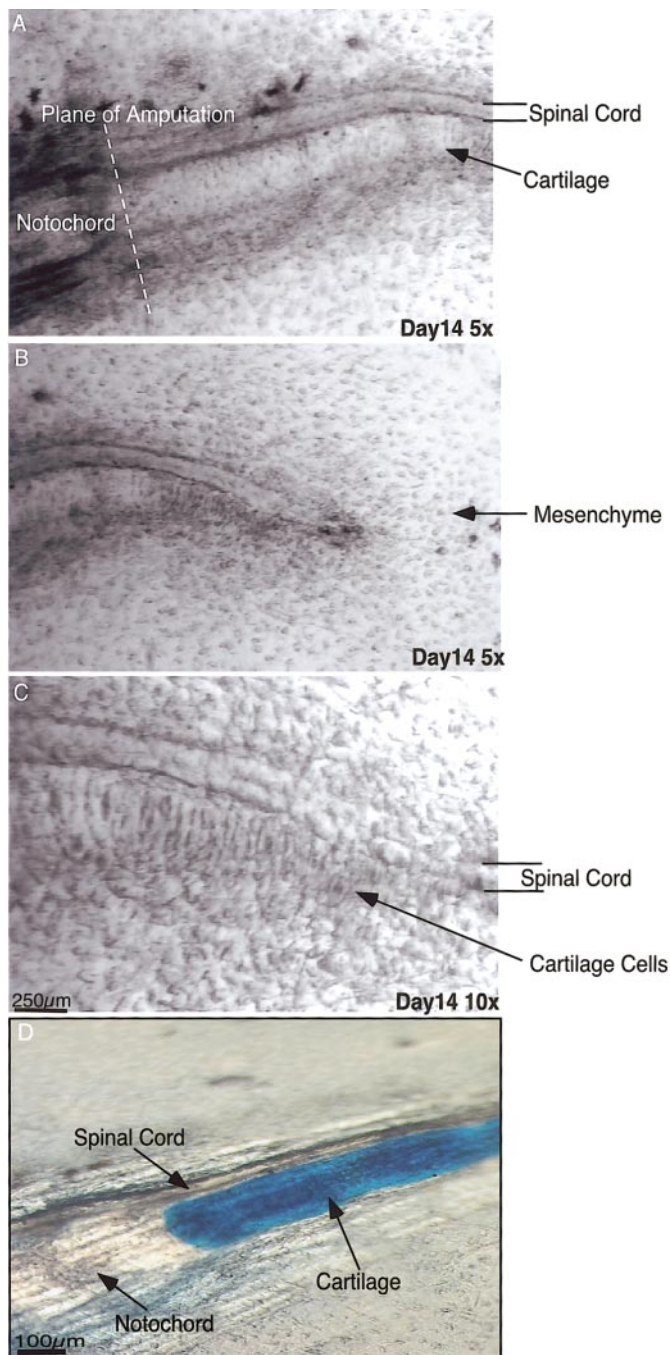


FIG. 2. The morphology of the 14-day regenerating tail as viewed by DIC optics. (A, B) An overall 5× view of the regenerate. (A) A clear boundary is visible between the notochord cells of the mature tissue and the regenerate, where cartilage is formed in place of notochord. (B) The tip of the regenerate. (C) The first morphological signs of differentiation—the round cartilage cells are morphologically distinguishable beneath the regenerating spinal cord. (D) Alcian blue staining confirms the identity of regenerating cartilage cells. The staining clearly shows the boundary between the regenerated cartilage cells (blue) and the notochord cells of the stump that do not take up the stain. Although not shown here, the alcian blue stain is taken up by cartilage cells along the length of the regenerate to the tip of the tail.

Microinjection of Fluorescent Lineage Tracers

For all microinjection experiments, anesthetized animals were stabilized on an optically clear polymer matrix: sylgard (Dow Corning, Wiesbaden, Germany) using insect pins and then covered in 0.01% anesthetic. The first layer of skin was surgically peeled back, creating a replaceable skin flap. Rhodamine or fluorescein dextran 10,000 kDa lysine fixable (Molecular Probes), dissolved in sterile H₂O at 25 mg/ml was pressure injected into single muscle fibers. The pressure injections were carried out by using a Fine Science Tools Pressure Injector mounted along side of an Olympus Stereo SZX12 dissecting microscope with fluorescence attachment. The animals were then allowed to recover in water containing Pen/Strep for 2–3 days before amputation was performed.

The fiber/tail amputations were performed on anesthetized animals placed on a slide that was taped to the stereomicroscope stage. The tail was amputated either close to the labeled fiber, leaving it untouched or just clipping off the end of the fiber. All imaging of the labeled fiber was carried out by using a Zeiss Axioplan 2 microscope controlled by a Metamorph image-acquisition system (Visitron, Munich, Germany).

NLS Dextran

The nuclear localization signal sequence (CGYGVSRRKPRPG-CONH₂) was conjugated to the dextran as follows. Lysine fixable fluorescein dextran (10 kDa; Molecular Probes, Eugene, OR) was dissolved at 25 mg/ml in 1 ml DMSO. Triethylamine (10 μ l) was added. Iodacetic acid succinimidyl ester was dissolved in DMSO at 50 mg/ml. IANHS (160 μ l; Sigma) was added to each of four tubes containing 250 μ l fluorescein dextran, and the mixture was incubated for 30 min at RT. This mixture was then loaded onto equilibrated Nap 5 gel filtration columns (Pharmacia) and eluted in 1 ml 100 mM Hepes, pH7.0. The eluate was then loaded onto a Nap10 column and eluted in 1.5 ml of Hepes, pH7.0. The peptide was dissolved at 50 mg/ml in H₂O, and the following volumes were added to one of the four tubes containing 250 μ l fluorescein dextran: 30, 10, 2.5, and 0 μ l. This mixture was then reacted *o/n* at RT. β -Mercaptoethanol was then added at 5% and the resulting mixture incubated for 20 min RT. This solution was passed through an equilibrated Nap 10 column and eluted in 1.5 ml 100 mM Hepes, pH 7.0. The conjugated NLS-Dextran was subsequently concentrated by using a centricon (Millipore).

Microinjection of Blastema Cells

The posterior 5–10 mm of the tail was amputated by using a sterile scalpel; the animals were then allowed to recover in H₂O containing Pen/Strep. The blastema cells were injected in 2-, 4-, and 6-day regenerates. The animals were anesthetized, pinned onto the sylgard matrix, and then a small hole was made in the first layer of skin to allow easy access of the injection needle. Rhodamine dextran (25 mg/ml) was injected by using a pressure injector, as described for the muscle-fiber injections. The animals were allowed to recover for 2 days and then the blastemas were fixed in 4% PFA, washed extensively in PBS, and then mounted for analysis using a Zeiss Axioplan 2 and a Leica confocal.

RESULTS

DIC Imaging of Axolotl Tail Regeneration

Larval Axolotls 2–3 cm in length have a very flat, optically clear tail, which, although being approximately only

10 cells thick, has considerable differentiation of mature tissue. Nomarski imaging allowed us to optically section through the tail to observe regeneration at all tissue levels. In the larval tail, we were able to discern muscle (Fig. 1A), notochord (Fig. 1B), and the spinal cord (Fig. 1B). At this stage, the larval animal still had its notochord. Amputation was performed by surgically removing the last 5–10 mm of the tail and initially resulted in tissue disruption. The muscle fibers contracted, and the large rounded cells of the notochord became distorted as shown in Fig. 1A. In the first few hours of regeneration, the epithelial skin cells from the sides crawl over the wound forming the wound epidermis (Figs. 1C and 1D).

Between 2 and 5 days, we observed a mound of proliferating cells between the end of the mature tissue (muscle and notochord) and the wound epidermis—this is the regeneration blastema (Figs. 1F and 1H). The exact origin of the blastema remains unclear. At the same time as blastema formation, we also see an independent outgrowth of the spinal cord. Classical studies suggest that the spinal cord is essential for regeneration to occur and it plays an important role in patterning the tissue around it (Holtzer, 1956).

Blastema proliferation and outgrowth continue over several days. We see the first morphological evidence of tissue redifferentiation 14 days postamputation (Fig. 2). The central axis of the regenerating tissue continuing from notochord is, in fact, a central rod of cartilage as seen in the tail regeneration of other species of salamander but not the anuran *Xenopus laevis*, which regenerates a notochord (Holtzer, 1956; Harrison, 1898). We verified that the regenerate was indeed cartilage by using the histological stain, alcian blue (Fig. 2D). The cartilage marker, collagen II, also stains the regenerating rod but not the notochord in these samples (data not shown).

Figure 3 shows a montage of the regenerated tail at 30 days, at which point the muscle close to the amputation plane is almost fully regenerated and organization into myotomal segments can be observed. Whole-mount staining with muscle-specific myosin heavy chain reveals that there is also considerable muscle differentiation in the distal regenerate that has not yet organized into myotomes (data not shown). A rod of cartilage has formed in place of the notochord; the boundary between the two marks the original plane of amputation.

In Vivo Injection of Muscle Fibers

Having documented regeneration using static images, we now wanted to address the role of endogenous muscle fibers during regeneration. To assay this, muscle fibers were pressure injected with rhodamine dextran, NLS-FITC dextran, or a mixture of both into anesthetized animals. We found that the injected fibers in a control unamputated animal (Fig. 4) remained stable and bright for 10 days, after which the fluorescent signal began to gradually decrease. In labeling fibers *in vivo* with both a cytoplasmic and nuclear marker, we observed a wide variety of fiber types differing

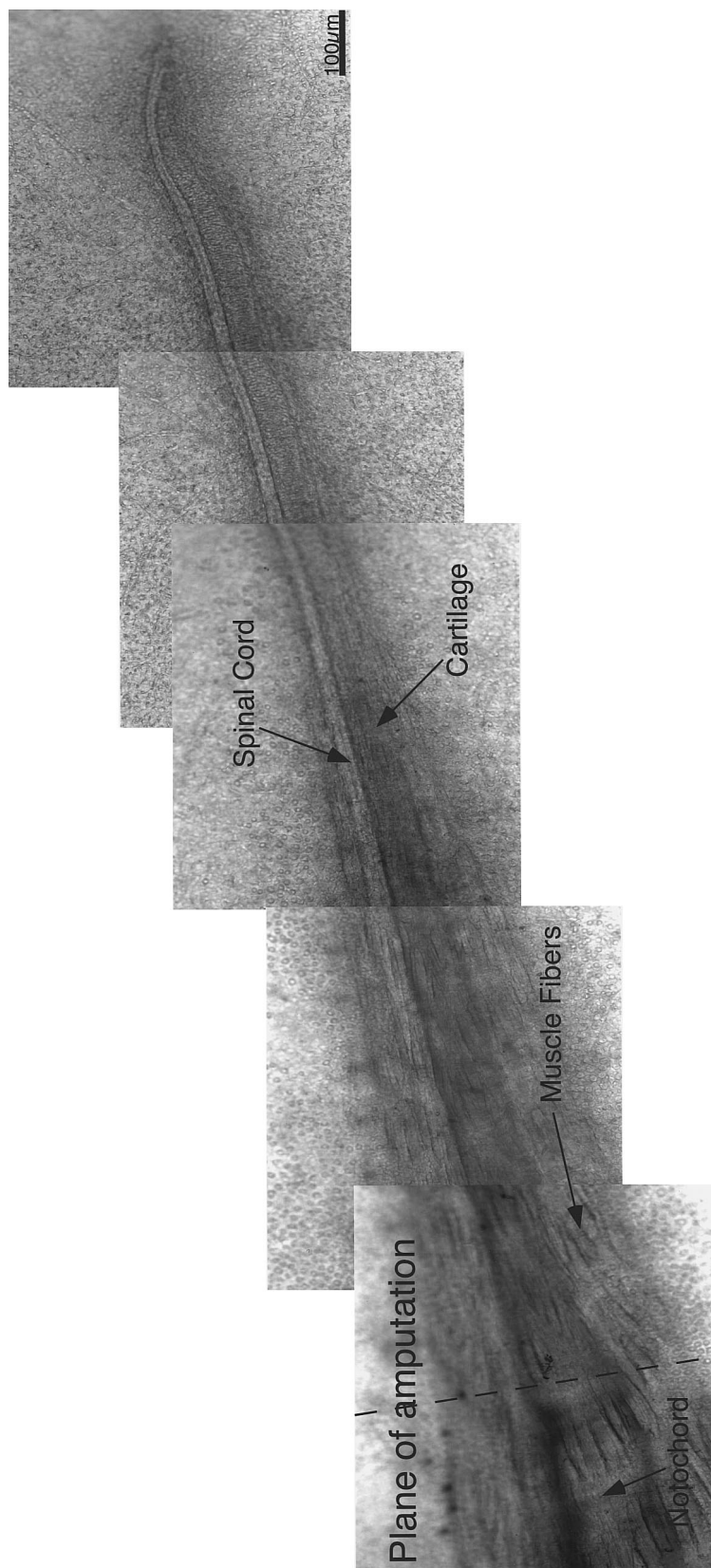


FIG. 3. A montage of the overall tail regenerate after 30 days. The plane of amputation is marked by the boundary between notochord and cartilage cells. The differentiation of muscle was observed by the reformation of myotomes close to the amputation plane. The mesenchymal fin cells also fully regenerate, although this is not clearly shown in this image.

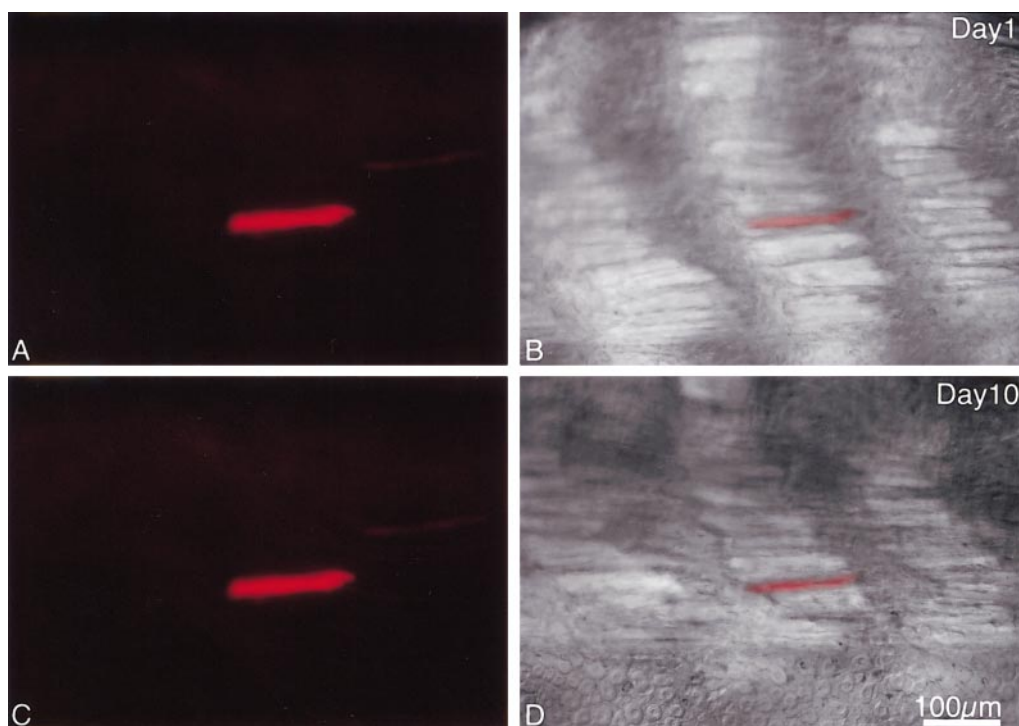


FIG. 4. A rhodamine-dextran-injected muscle fiber remains stable over 10 days in an uninjured animal. (A) Rhodamine dextran-injected fiber in the mature tail on Day 1. (B) Corresponding DIC image of the tail with fluorescent overlay of the muscle fiber. (C) The same injected fiber 10 days later. (D) The matching DIC image with fluorescent overlay.

greatly in size and number of nuclei. We have grouped them into three categories (Fig. 5). Figure 5A shows a small fat fiber usually containing 2–3 nuclei; Figs. 5B and 5D show long, thin fibers with evenly spaced nuclei; and Figs. 5C and 5E illustrate fibers of medium length and width, usually containing in the range of 6–10 nuclei which are located close to the surface of the fiber.

Compared to the cytoplasmic dextran, the frequency of successful injections with the NLS-dextran was quite low since it appeared to stick to the ECM and it did not always accumulate in the nucleus. Therefore, though we were able to use the nuclear label to characterize the different fiber types in the tail, it was not practical to use it when following individual fibers during regeneration.

Dedifferentiation of Mature Muscle Fibers in Vivo Requires Direct Cell Injury

To understand the extent of wounding necessary to induce dedifferentiation, different amputation conditions were examined. Three general classes of muscle cell behavior were found depending on the specific amputation conditions. The results are summarized in Table 1.

Class I. When the tail was amputated close to the labeled fiber (50–100 μm), but no damage was done to the fiber, the injected cell remained stable throughout the

course of regeneration. The fiber did not dedifferentiate and it did not become incorporated into the regenerate ($n = 25$).

Class II. When amputation resulted in removal of approximately 50% of the labeled fiber, the fiber initially retracted, followed by vesiculation of the label, and, within 3 h, the labeled fiber had disappeared. This likely represents death of the cell ($n = 25$).

Class III. When tail amputation resulted in “clipping” of the labeled fiber (Fig. 6), we observed the generation of mononucleate cells from the muscle fiber. “Clipping” involved removing a small portion of cytoplasm at the end of the fiber so that its terminal attachment to the myoseptum was disrupted during amputation. Immediately after clipping, the fiber retracted followed by re-elongation on day 2 after amputation (Fig. 6C). Three to five days postamputation, the fiber had fragmented into 6–10 mononucleate cells, as seen in Fig. 6G. The number of mononucleate cells formed correlated with the number of nuclei we saw in our labeling of larger caliber, unamputated fibers. The mononucleate cells initially appeared round or oval-shaped (Fig. 6G), but, within 1 day after the initial dedifferentiation, the cells extended processes in various directions (Figs. 6I and 8). Dedifferentiation occurred in 15 out of 58 cases of clipping amputation. Taken together, these results indicate that direct injury to the muscle is required to trigger dedifferentiation.

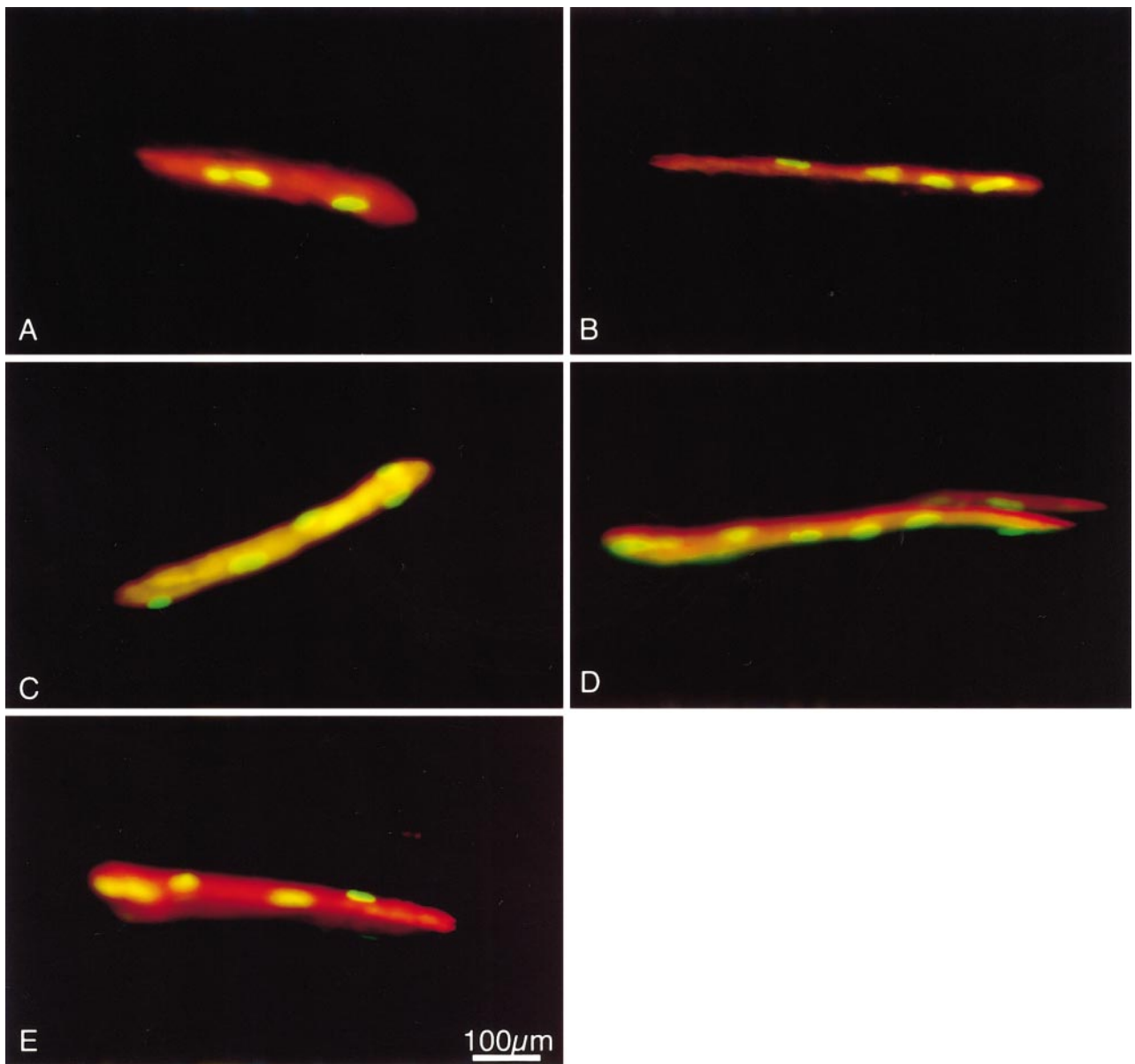


FIG. 5. Injection of single muscle fibers with cytoplasmic rhodamine dextran and a nuclear fluorescein dextran reveal the different types of fibers found in axolotl tails. (A) A short fat fiber with three nuclei. (B) A long thin fiber with four nuclei. (C) A medium-length mature fiber with multiple nuclei close to the surface. (D) A very long fiber with eight nuclei evenly spaced along its length. (E) A medium-length, medium-width fiber with four nuclei randomly dispersed but also quite close to the surface of the fiber.

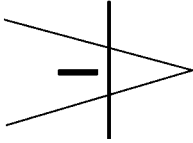
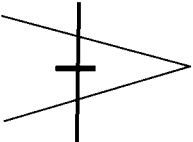
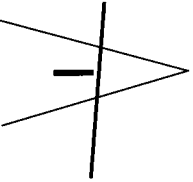
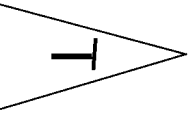
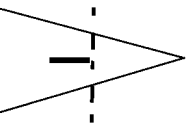
Tissue Damage Is Also Required to Initiate Dedifferentiation

To test whether clipping of the fiber is a sufficient signal to induce dedifferentiation in the absence of tail amputation, we used either a sharp forceps or hypodermic needle to release the end of the muscle fiber from its neighbors in an unamputated tail. Under these circumstances, the muscle fibers re-elongated within 2 days and remained stable over the 15 days for which it was followed. Furthermore, creat-

ing a small tear in the tissue adjacent to the labelled fiber while clipping the fiber did not result in dedifferentiation. The fiber remained stable and unchanged in all cases ($n = 15$).

This left the question of whether complete transection of the tail was required to induce dedifferentiation. To test this, we induced significant injury by cutting halfway through the tail but leaving the distal portion attached so that the two parts healed back together. We refer to this process as "gap regeneration." During gap regeneration, a

TABLE 1
Summary of the "Injury Conditions" Necessary to Induce Muscle Dedifferentiation

	Type of Surgery	Result	No. of Examples
	Amputation plane close to fiber	Fiber stable	25 out of 25
	Amputation plane cuts through the middle of the fiber	Fiber degenerates	25 out of 25
	Amputation plane clips off the end of the fiber	Fiber Dedifferentiates	15 out of 58
	Clipping of the muscle fiber with minimal tissue injury	Fiber stable	15 out of 15
	Severe injury clipping the end of the fiber, the tail remains loosely attached. 'Gap Regeneration'	Fiber Dedifferentiates	10 out of 25

zone of cells resembling a blastema was formed in the middle of two mature pieces of tail. Under these circumstances, the labeled fiber dedifferentiated (10/25 cases) and contributed cells to the forming "blastema" (Fig. 7).

The Comparison of Dedifferentiated Cells to Blastema Cells

Several observations indicate that the mononucleate cells generated from muscle-fiber dedifferentiation are blastema cells. First, we observed cells whose morphology indicated that they were undergoing cytokinesis 1 day after they formed mononucleate cells (Fig. 7G). The total number of visible mononucleate cells increased, doubling within a 24-h period (Table 2). During the time of cell doubling, cells moved laterally and down into the tissue so that they no longer resided in one field of view, and rested in multiple

focal planes (Fig. 7I). Therefore, it is difficult to capture all cells in one picture. Also, this makes it difficult to accurately count 100% of the cells derived from dedifferentiation.

Several days after dedifferentiation, the mononucleate cells that derived from the muscle cells extended numerous processes towards their neighbors (Figs. 6I and 8). To determine whether this striking morphology was characteristic of blastema cells in general, we used rhodamine dextran to label blastema cells *in vivo*. Cells were labeled in dorsal, ventral, and axial positions within the blastema and at the tip of the blastema adjacent to the wound epidermis. This allowed assessment of whether subpopulations of morphologically distinct blastema cells exist. All of the labeled blastema cells that we observed in 10 different blastema samples were morphologically similar and were indistinguishable from dedifferentiated muscle cells (Fig. 8).

We attempted to confirm the identity of the mononucle-

TABLE 2
Mononucleate Cells Generated from Dedifferentiation
of a Labeled Muscle Fiber

Sample	Number of mononucleate cells generated	
	24 h	48 h
Animal 1	8	15
Animal 2	5	11
Animal 3	9	15

Note. Muscle fibers were labeled *in vivo* in the tail and the fate of the cells in response to amputation was monitored. 24 h indicates the number of mononucleate cells counted 24 h postfragmentation of the labeled fiber. 48 h indicates the number of labeled mononucleate cells 48 h postfragmentation.

ate cells generated from dedifferentiation as blastema cells by using the blastema cell marker, RGE 53 (Ferretti, 1991). We found that, in these preparations, this anti-cytokeratin antibody was not a specific marker as it also stained notochord, cartilage, and spinal cord (data not shown). We do not know if this difference from previous descriptions is due to the fact that we are looking in younger animals, tails versus limbs, or because we are looking in a different species. The other blastema marker 22/18 (Kintner, 1984) can only be used under specific fixation conditions that are not compatible with the lineage-tracing experiments.

DISCUSSION

Endogenous Muscle Fibers Dedifferentiate during Regeneration

The results presented here are the first definitive evidence that dedifferentiation of endogenous muscle fibers occurs during regeneration. Previous work has concentrated on illustrating the possibility of dedifferentiation using histological studies (Hay, 1959; Thornton, 1938) or implantation of cultured myotubes. Here, we have followed the changes in a single fiber over time, within the physiological context of the regenerating axolotl tail.

The Features of in Vivo Muscle-Fiber Dedifferentiation

Dedifferentiation of mature muscle fibers occurred between 3 days and 5 days after an amputation or severe wounding that clipped the end of the muscle fiber. Contrary to Hay's observations in the limb, we did not observe budding of mononucleate cells at the end of the fiber closest to the amputation plane. Rather the muscle fiber appeared to fragment giving rise to mononucleate cells approximately equal in number to the original number of nuclei in the mature fiber. This difference may arise from the different morphology of limb muscle fibers which are very long

and thin versus tail muscle fibers, which are relatively short and thick (K.E. and E.M.T., unpublished observations).

The cells derived from the dedifferentiated muscle fiber appeared to rapidly undergo cytokinesis (as seen in Fig. 7), and the mononucleate cell number increased within the first 48 h after formation of mononucleate cells. The timing of the cytokinesis that we observe during dedifferentiation suggests that the nuclei in the mature muscle fiber re-entered S-phase before breaking apart into mononucleate cells since S-phase entry and completion of DNA synthesis takes at least 3 days in urodele cells (Maden, 1976; Tanaka, 1997). This observation is also consistent with experiments showing that urodele myotubes can re-enter S-phase from the multinucleate, differentiated state (Tanaka, 1997; Kumar, 2000). The combined detection of a DNA synthesis marker with our lineage-tracing method to confirm this still poses technical challenges.

We found that the mononucleate cells assumed a striking stellate morphology with multiple processes that wrapped around their neighbors. Direct labeling of blastema cells showed that this is a characteristic morphology of all blastema cells. This unusual morphology has also been observed by EM in preparations from limb blastemas (Egar, 1988). It is interesting to speculate that these blastema cell processes are involved in the patterning of the regenerating structure since previous work has shown that cell migration is activated if blastema cells of different positional identities are juxtaposed (Nardi and Stocum, 1983; Pecorino *et al.*, 1996). The stellate morphology of the blastema cells could allow blastema cells with different positional identities to recognize and move past each other.

Direct Cell Injury Is Necessary but Not Sufficient to Induce Dedifferentiation

The ability to follow a single fiber over time has allowed us to define the conditions that trigger dedifferentiation and the formation of mononucleate cells. Our results clearly show that damage to a single muscle fiber though required, is not sufficient to induce dedifferentiation. The signal, which causes mature differentiated fibers to fragment and produce mononucleate cells, is only released in response to severe tissue damage. This suggests that at least two signals are needed to initiate the process of dedifferentiation: direct cell injury and tissue damage.

In culture it has been shown that urodele myotubes must lose contact inhibition and must be exposed to a component of clotted blood in order to initiate one aspect of dedifferentiation—S-phase re-entry (Tanaka, 1997). However, these culture conditions are not sufficient to induce mononucleate cell formation as occurs *in vivo*. In the future, it will be important to define the molecular nature of the injury-related signals that induce mononucleate cell formation and to determine whether the signals that initiate mononucleate cell formation and cell cycle re-entry overlap.

Dedifferentiation was induced in 25% and 40% of fibers after complete or partial amputation, respectively. It is not clear whether muscle dedifferentiation is only observed in a

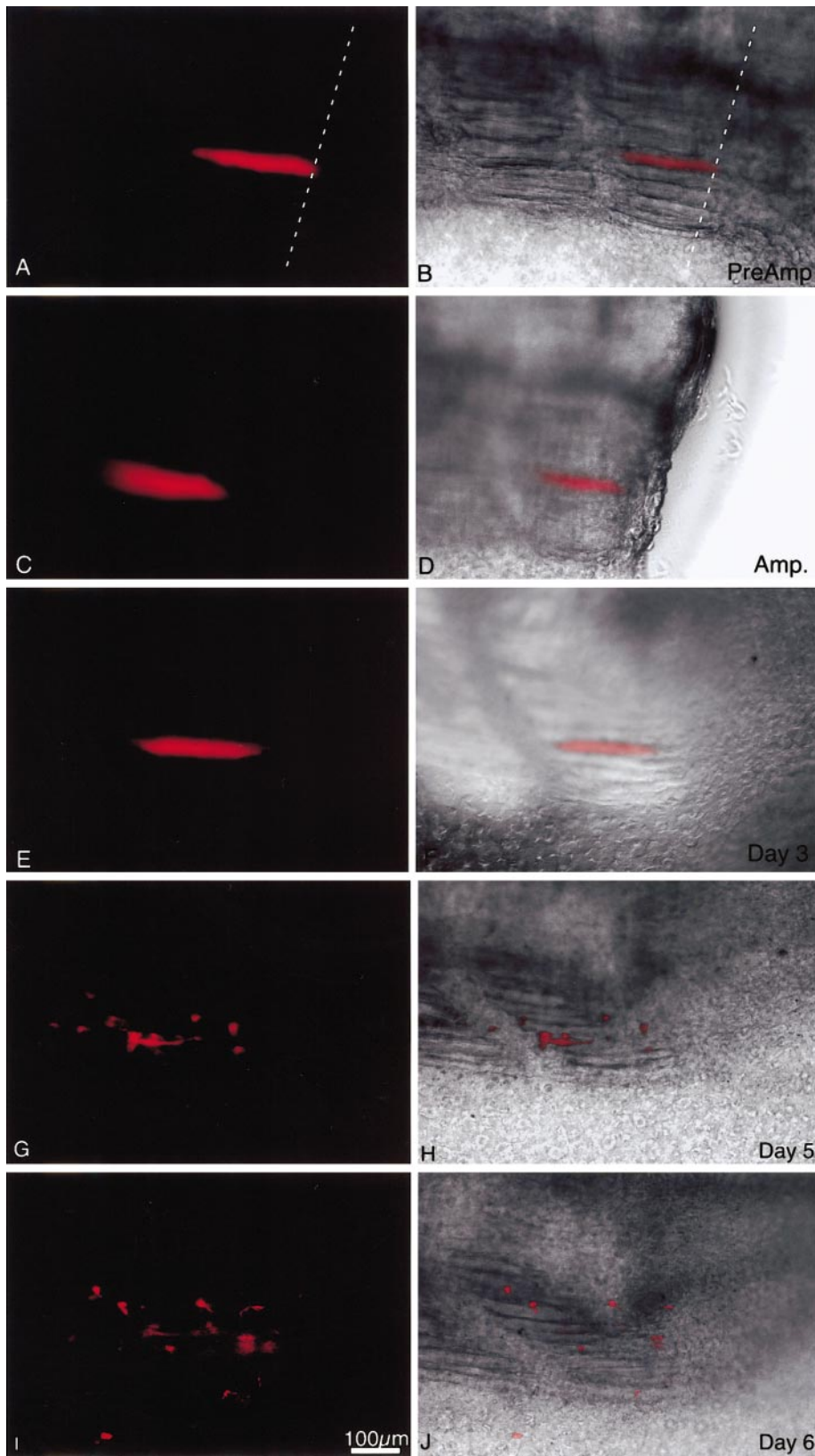
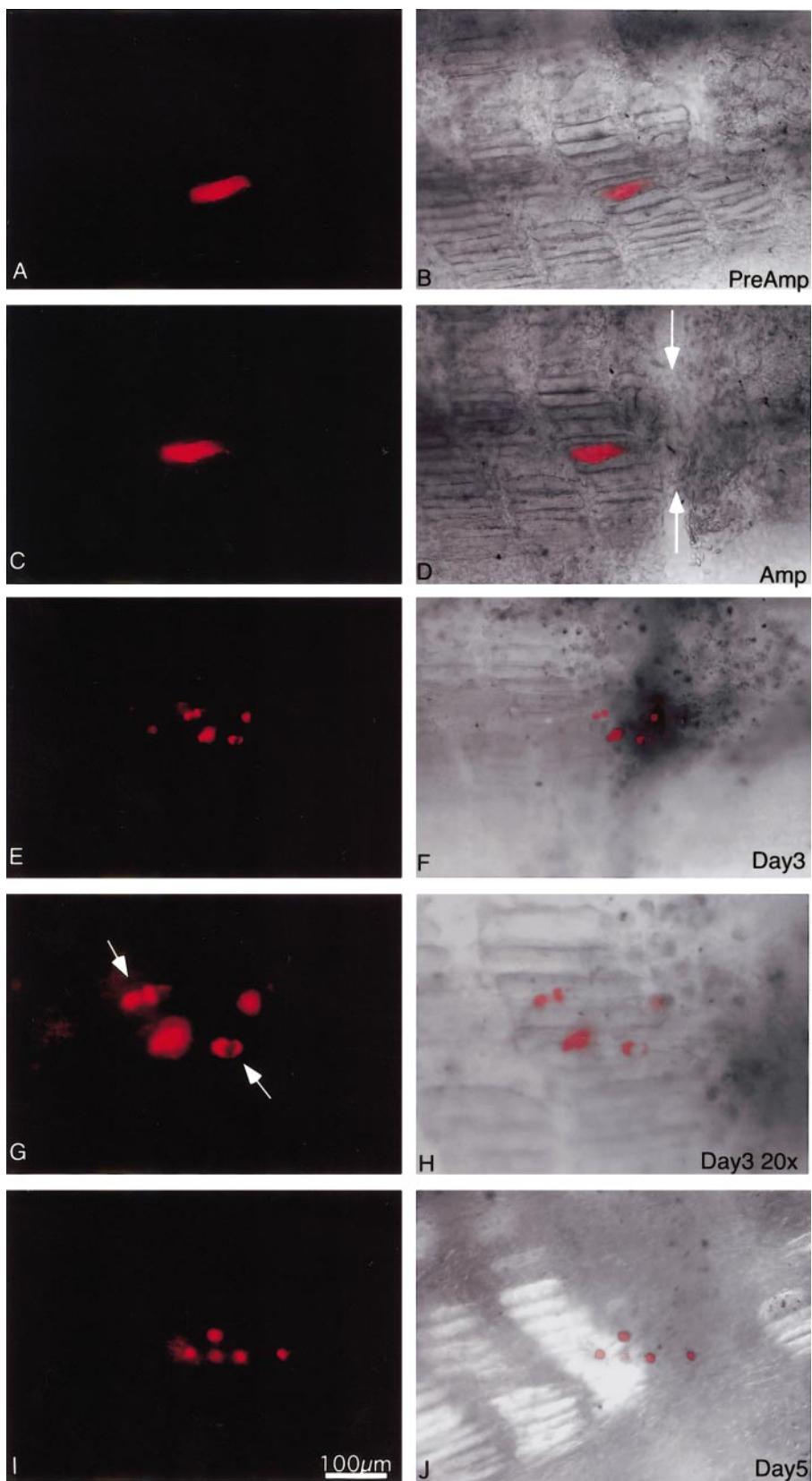


FIG. 6. Tail amputation accompanied by clipping of the fiber results in dedifferentiation of a mature muscle fiber. A single fiber in the tail was labeled with rhodamine dextran (A). (B) The matching DIC image with overlay of the labeled fiber in the tail. The tail was amputated along the dotted line (A, B), clipping the end of the labeled fiber. (C) The retraction of the fiber in response to amputation. The overlay in (D) illustrates the position of the injected fiber in relation to the amputation plane. The fiber then re-elongated as the wound healed over and as the blastema formed (E, F). At 5 days postamputation, the labeled fiber fragmented, giving rise to multiple mononucleate cells (G); the position of the mononucleate cells can be seen clearly in the overlay in (H). During the next 24 h, the initially round mononucleate cells spread out having long processes associated with them (I). The stellate morphology is shown in higher magnification in Fig. 8.



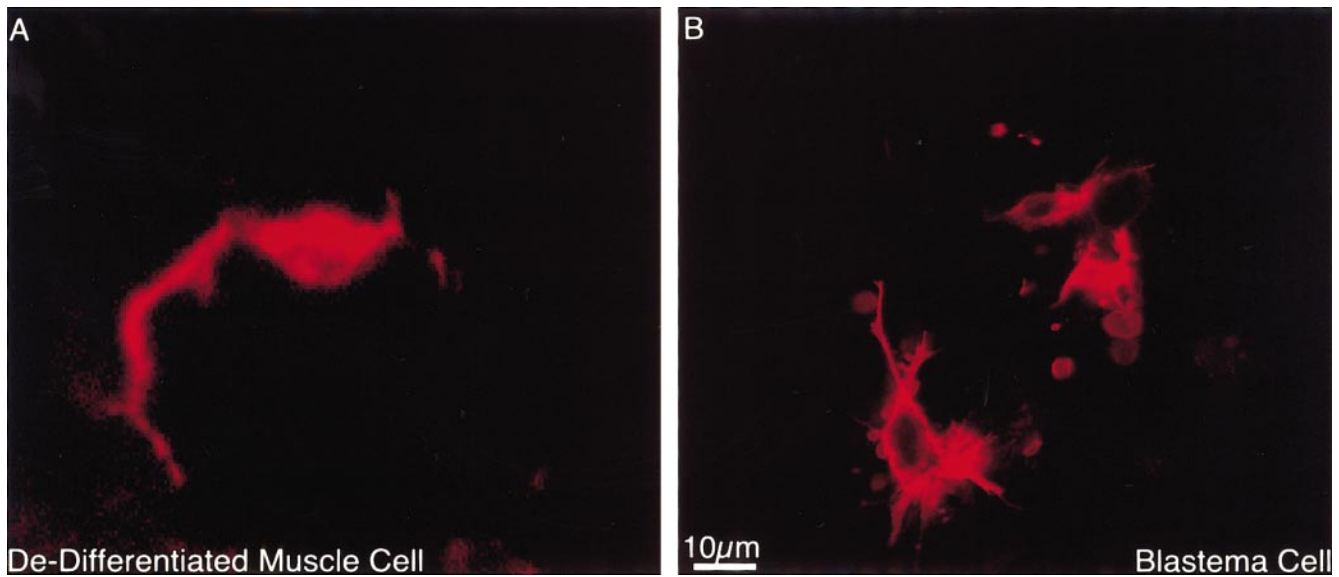


FIG. 8. Cells of dedifferentiated muscle fibers morphologically resemble blastema cells. (A) A high-magnification image of a mononucleate cell with a long, thin process derived from a dedifferentiated muscle fiber. (B) *In vivo*-labeled blastema cells showing similar morphology.

proportion of fibers because all fibers did not get the equivalent signal, or whether different subpopulations of fibers exist—some of which are capable of dedifferentiating and others that are not. Previous histological descriptions of early stages in limb regeneration found that the outer circumference of muscle undergoes larger morphological changes consistent with dedifferentiation compared to inner layers of muscle (Towle, 1901). It is possible that fibers dedifferentiate from discrete locations within the stump to preserve the structure of the mature tissue while still contributing to the blastema. It should, however, also be noted that when Lo *et al.* implanted cultured myotubes into the regeneration blastema, they estimated that 25% of implanted nuclei were found in dedifferentiated mononucleate cells (Lo *et al.*, 1993).

Estimating the Number of Blastema Cells Generated through Muscle Dedifferentiation

The significance of dedifferentiating muscle as a contributor to the blastema has long been disputed (Hay, 1959;

Dinsmore, 1977). Does dedifferentiation of muscle fibers produce a significant proportion of the founding population of blastema cells or is another mechanism, such as activation and proliferation of a resident stem cell the primary mechanism for forming the blastema? From our data, we can estimate the number of blastema cells that have come from the dedifferentiation of muscle. This calculation is based on the number of muscle fibers present in a cross-sectional area of the tail, the frequency at which we see dedifferentiation occurring, and the number of nuclei per muscle cell. Myosin heavy chain staining of muscle fibers in the tail (data not shown) revealed approximately 60 muscle fibers or small muscle bundles within the tail cross-section that could contribute to blastema formation. Our results showed that 25–40% of muscle fibers give rise to mononucleate cells when a labeled fiber is clipped during tail amputation. Taking cell death into consideration, we estimate that probably closer to 25% of the muscle fibers at the plane of amputation would dedifferentiate. These figures imply that about 15 muscle fibers dedifferentiate

FIG. 7. Muscle dedifferentiation during “gap regeneration.” A single fiber was labeled by microinjection with rhodamine dextran (A). (B) An overlay with the DIC image to show its position within the myotome. The tail was severely injured, creating a gap in the tissue as shown by the arrows in (D). During injury, the end of the fiber was clipped (C), but the whole tail was not amputated. At 3 days, the labeled fiber fragmented, forming mononucleate cells (E), the matching DIC image with overlay shows that a blastema-like structure has formed joining the two pieces of mature tissue together (F). (G) and (H) are high magnification views of (E) and (F) to show the mononucleate cells which appear to be just finishing cytokinesis (see arrows). The matching DIC image (H) shows the position of the fragmented cells within the tail. Over subsequent days, these cells increased in number (I) (refer to Results), but all the cells that arose from dedifferentiation are not visible in (I), as the gap blastema is a rounded structure and the cells are on different focal planes. Some cells have also migrated away from the site of the original fiber, as can be seen from the overlay with the DIC image in (J). In the DIC image (J), the zone of cells which forms between the two mature uninjured parts of the tail is clearly visible.

during larval tail regeneration. Since about 10 nuclei were generated from a single dedifferentiating fiber, approximately 150 cells would be generated directly from muscle dedifferentiation, before cell proliferation begins.

Does this number represent a significant proportion of the founding population of the regeneration blastema? Precise quantitative data on the number of blastema cells during early stages of larval tail regeneration is not available but Chalkley (1954) carefully documented the number of cells present in a newt limb blastema. Applying a modified version of Chalkley's method to the tail, we estimate that the 4-day axolotl tail blastema contains 907 cells. Gardiner *et al.* (1986) found that 43% of limb blastema cells come from dermis. Therefore, approximately 516 cells of the tail blastema are likely to be derived from other tissues. Our *in vivo* data shows approximately 150 cells, which is 29% of nondermis-derived blastema cells come from dedifferentiating muscle. From these calculations, we argue that muscle cell dedifferentiation makes a significant contribution to the makeup of the regeneration blastema.

The Role of Dedifferentiation during Regeneration

It is striking that muscle dedifferentiation already occurs efficiently in animals that are still rapidly growing and must still contain many myogenic progenitor cells. The question arises, why do two systems for tissue growth coexist? Commitment to the myogenic lineage occurs during embryonic development and the actual number of uncommitted stem cells may be very small even in these growing animals. Dedifferentiation of muscle and other cell types is likely to be an important mechanism for reprogramming cells to form a multipotent population of progenitor cells that are capable of being repatterned. This idea is supported by studies on *msx1*, a gene that is re-expressed during regeneration (Simon, 1995; Crews, 1995; Koshiba, 1998). When *msx1* is expressed in cultured myotubes, it induces at a low frequency the formation of mononucleate cells (Oldelberg *et al.*, 2000). Strikingly, the mononucleate cells that are formed are capable of differentiating into multiple cell types, such as adipose tissue, cartilage, and osteoclasts—a spectrum of cell types not formed by the myogenic cells that formed the myotubes in the first place.

Our system will require further technical development to determine the exact fate of the dedifferentiated muscle cells. However, our initial data involving bulk transfection of muscle with a CMV-GFP construct followed by amputation leads to a robust incorporation into regenerating cartilage cells (K.E. and E.M.T., unpublished data). These early experiments suggest that dedifferentiated muscle cells do become multipotent during regeneration. Extending the lineage analysis studies presented here should allow us to follow the process of regeneration on the single cell level from the dedifferentiation step to patterning and redifferentiation.

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