

Salamander limb regeneration involves the activation of a multipotent skeletal muscle satellite cell population

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In contrast to mammals, salamanders can regenerate complex structures after injury, including entire limbs. A central question is whether the generation of progenitor cells during limb regeneration and mammalian tissue repair occur via separate or overlapping mechanisms. Limb regeneration depends on the formation of a blastema, from which the new appendage develops. Dedifferentiation of stump tissues, such as skeletal muscle, precedes blastema formation, but it was not known whether dedifferentiation involves stem cell activation. We describe a multipotent Pax7⁺ satellite cell popula-

tion located within the skeletal muscle of the salamander limb. We demonstrate that skeletal muscle dedifferentiation involves satellite cell activation and that these cells can contribute to new limb tissues. Activation of salamander satellite cells occurs in an analogous manner to how the mammalian myofiber mobilizes stem cells during skeletal muscle tissue repair. Thus, limb regeneration and mammalian tissue repair share common cellular and molecular programs. Our findings also identify satellite cells as potential targets in promoting mammalian blastema formation.

Introduction

Amputation or tissue removal can lead to the regeneration of lost structures in some vertebrate species, such as the salamanders (e.g., the newt and the axolotl; Stocum, 1997; Tanaka, 2003; Brockes and Kumar, 2005). For example, adult newts can rebuild entire limbs, tails, and jaws through an epimorphic regeneration process that leads to the restoration of complete and functional tissue architecture (Brockes and Kumar, 2002). Epimorphic limb regeneration proceeds by rapid wound closure and is critically dependent on the formation of a multipotent mesenchymal growth zone, the blastema, which gives rise to the newly formed limb (Wallace, 1981).

Data show that mature tissues in the stump (e.g., bone, cartilage, and skeletal muscle) respond to amputation by disorganization, histolysis, and increased cellular proliferation. This process is generally referred to as the dedifferentiation step leading to the formation of blastema progenitors (Iten and Bryant, 1973). However, the resolution of our picture on the contributing tissues at the cellular level is low at present. It is unclear to what extent differentiated cells reverse mature phe-

notypes and to what extent undifferentiated cells, such as stem cells, residing within differentiated tissues become activated, followed by their incorporation into the blastema. The lack of molecular markers has also obstructed the prospective isolation of blastema progenitors.

Skeletal muscle is an important contributor to blastema formation (Brockes, 1997). The skeletal muscle fiber is a syncytial (multinucleate) cell type, whose differentiation during embryonic development is characterized by the cellular fusion of somite-derived precursors (Buckingham, 2001; Tajbakhsh, 2005). An intriguing aspect of the regenerating salamander appendages is the reversal of differentiation. Both static analyses and dynamic *in vivo* tracing showed that skeletal muscle fibers break up, the syncytium becomes fragmented as a response to limb or tail removal, and muscle-derived mononucleate progeny significantly contribute to the blastema (Thornton, 1938; Hay, 1959, 1962; Lentz, 1969; Echeverri et al., 2001). Isolated salamander myotubes can also undergo a cellularization process by which the syncytium turns into mononucleate progeny after reimplantation into the regenerating limb (Lo et al., 1993; Kumar et al., 2000).

Although adult mammals do not form a blastema after limb amputation, their skeletal muscle tissue regenerates after injury (Charge and Rudnicki, 2004). However, mammalian

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Abbreviation used in this paper: H3P, phosphorylated histone 3.

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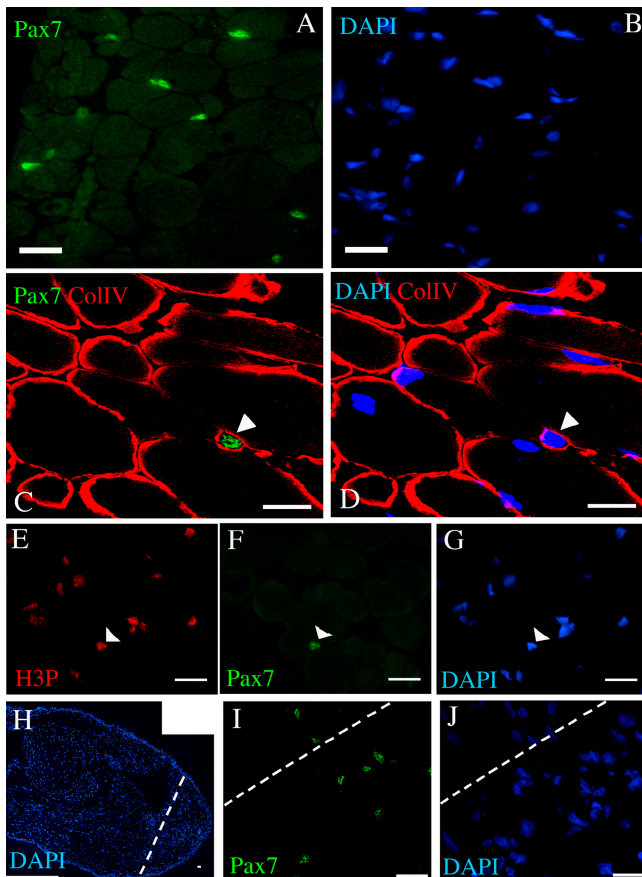


Figure 1. Pax7⁺ cells are present in newt limb skeletal muscle. (A and B) Immunostaining of limb skeletal muscle identifies Pax7⁺ cells within skeletal muscle tissue. DAPI staining shows the nuclei in the tissue section. (C and D) Photomicrographs showing a typical Pax7⁺ cell being surrounded by basement membrane. Arrowheads point to a Pax7⁺ cell nucleus. (E–G) Photomicrographs showing a mitotic Pax7⁺ cell 4 d after amputation. Arrowheads point to a Pax7/H3P double-positive cell. (H–J) Pax7⁺ cells appear in an early bud stage blastema. Dotted line marks the level of amputation. Bars, 50 μ m.

skeletal muscle regeneration does not involve cellularization of the syncytium. Instead, a stem cell population called satellite cells, which express markers such as Pax7, M-cadherin, and Myf5, reenters the cell cycle, proliferates, and incorporates into nascent or into preexisting myofibers during mammalian muscle regeneration (Cornelison and Wold, 1997; Collins et al., 2005). Mammalian satellite cells reside between the basal lamina and the sarcolemma of the myofiber (Seale et al., 2000). Earlier studies identified a cell population that is closely apposed to the myofiber in the adult newt limb as well. But in

Table 1. Number of mitotically active Pax7⁺ cells in amputated and non-amputated limbs

Limb	Pax7	Pax7/H3P	Percentage
Control	420	16	3.8
4 d after amputation	195	61	31.3
7 d after amputation	342	29	8.5
11 d after amputation	592	68	11.5

The number of mitotic H3P⁺/Pax7⁺ cells peaks at 4 d after amputation.

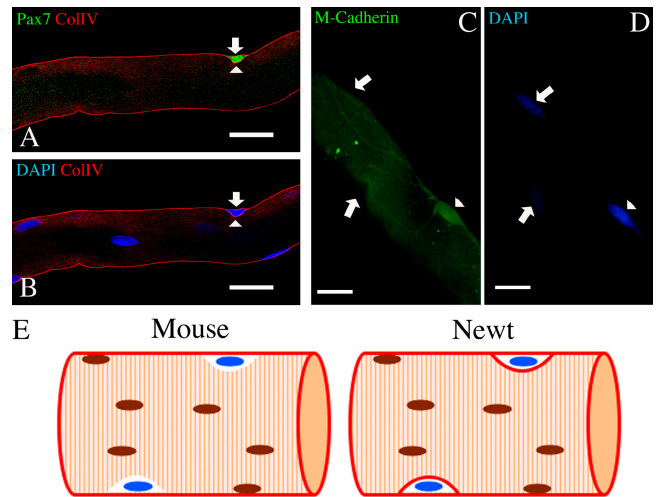


Figure 2. Satellite cells can be copurified with isolated single skeletal muscle fibers. (A–D) Satellite cells are attached to the myofiber after isolation and plating. (A and B) Arrows point to a Pax7⁺ satellite cell, and arrowheads point to the collagen IV⁺ basal lamina. (C and D) Arrows point to two myonuclei, and arrowheads point to an M-cadherin⁺ satellite cell. (E) Schematic model of mouse and newt myofibers. Satellite cells (blue nuclei) are tightly attached to both mouse and newt myofibers, but an additional basement membrane (maroon) separates the satellite cells from the sarcolemma. Bars, 50 μ m.

contrast to mammals, these cells were shown to be completely encapsulated by a basement membrane (Popiela, 1976; Cameron et al., 1986), and it has remained unsettled whether adult newts possess a cellular population that is equal to mammalian satellite cells. In addition, it has not been established whether dedifferentiation of skeletal muscle leads to the activation of a stem cell population within the tissue and if such cells could contribute to the new limb.

To start addressing these questions we combined histological analyses and in vitro culture of single newt myofibers, along with implantation and tracing of labeled myofiber-derived cells. We find that the salamander myofiber contains a satellite cell population. As we can distinguish between the process of cellularization of the syncytial myofiber on one hand and satellite cell activation on the other, the quantitative aspects of these two separate events can be examined. Satellite cell activation prevails in our model of skeletal muscle plasticity, leading to the production of a multipotent progeny population. Therefore, the data highlight the possibility of promoting blastema formation by the activation of cellular and molecular programs that also operate in mammals.

Results

To test whether newt skeletal muscle in the limb contains a satellite cell population, we used a monoclonal antibody against Pax7, which is a specific marker of skeletal muscle satellite cells. As shown in Fig. 1 (A and B), similar to mammalian muscle, Pax7⁺ cells are present in newt limb skeletal muscle. However, a basement membrane surrounds the Pax7⁺ cells (Fig. 1, C and D). No Pax7⁺ cells were detected outside the skeletal muscle tissue (unpublished data). To test whether Pax7⁺ cells reenter

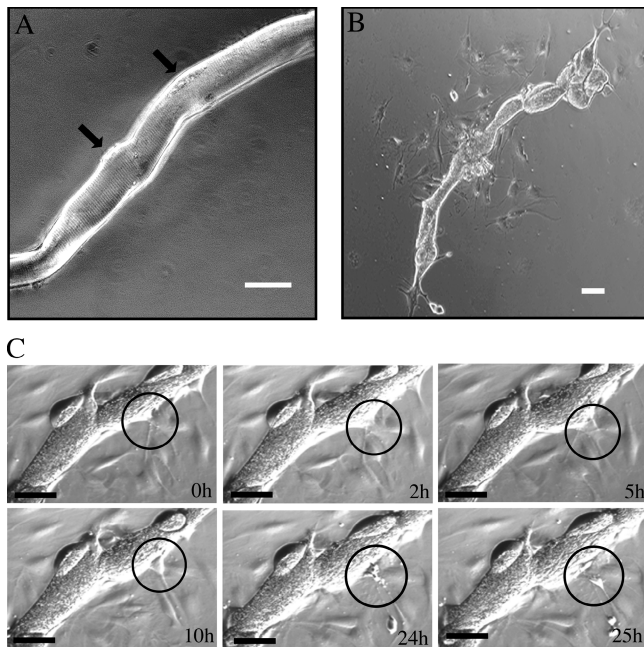


Figure 3. Progeny cells bud off the myofiber and proliferate. (A) Photomicrograph showing an isolated single newt skeletal muscle fiber directly after attachment. Note the visible striation demarking the sarcomeres. Arrows point to two visible nuclei, which could either be myonuclei or located in satellite cells. (B) Photomicrograph showing the same 15-d-old myofiber in culture. The myofiber morphology has changed and several lobular structures are seen while mononucleate progeny has been produced. (C) Time-lapse photomicrographs showing a sequence of one representative budding event, which leads to the derivation of a mononucleate cell. Note the protrusion of the myofiber in the circled area, which is concomitant with the appearance of a mononucleate progeny. Time points indicate the duration of the one specific budding event. A longer movie capture is shown in Video 1. Video 1 is available at <http://www.jcb.org/cgi/content/full/jcb.200509011>. Bars, 50 μm .

the cell cycle after limb amputation, we immunostained limb sections with an antibody raised against phosphorylated histone 3 (H3P), which marks mitotic cells (Ajiro et al., 1996). We found that Pax7⁺ cells are largely quiescent in the uninjured limb, but become mitotic after limb removal (Fig. 1, E–G; and Table I). We saw Pax7⁺ cells outside of skeletal muscle tissue 4 d after amputation, and detected Pax7⁺ cells within the blastema upon formation (Fig. 1, H–J). These data show that quiescent satellite cell activation is a response to limb removal and the findings suggest that satellite cells leave their niche to incorporate into the blastema.

To understand the cellular basis of the plasticity of skeletal muscle fibers, we established an ex vivo culture of living, intact single newt myofibers. We isolated and plated single myofibers that were viable and displayed characteristic morphology, such as Z band striation marking the boundaries of the sarcomeres (Fig. 3 A). This technique has previously been used to establish single myofiber culture from both mammalian and salamander species with no contamination from other tissues or cell types (Rosenblatt et al., 1995; Kumar et al., 2004). As indicated by the presence of Pax7⁺ (Fig. 2, A and B) and M-cadherin⁺ cells (Fig. 2, C and D), muscle fibers from the newt limb could be copurified with a satellite cell population after isolation

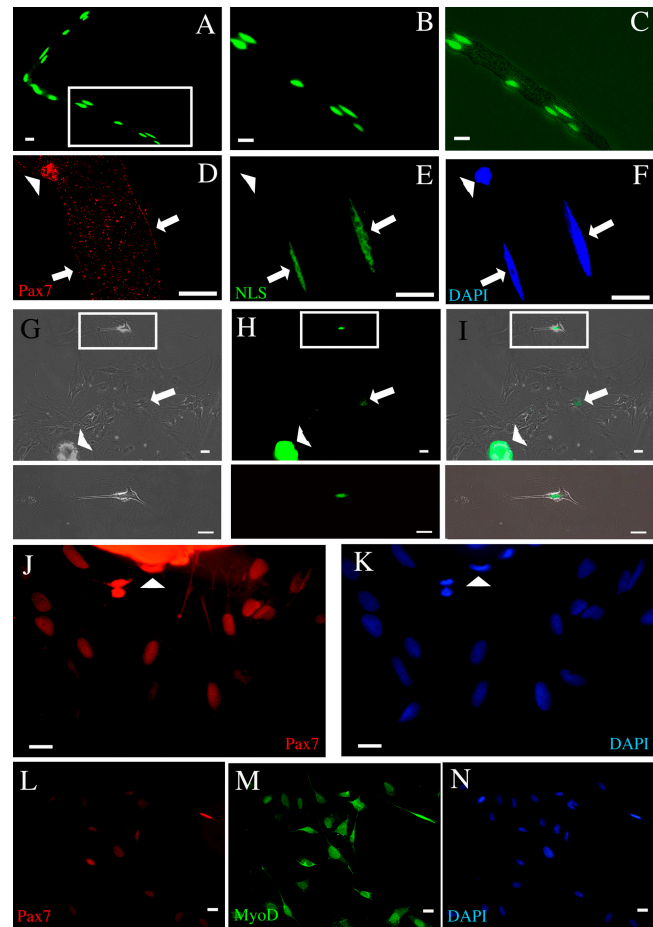
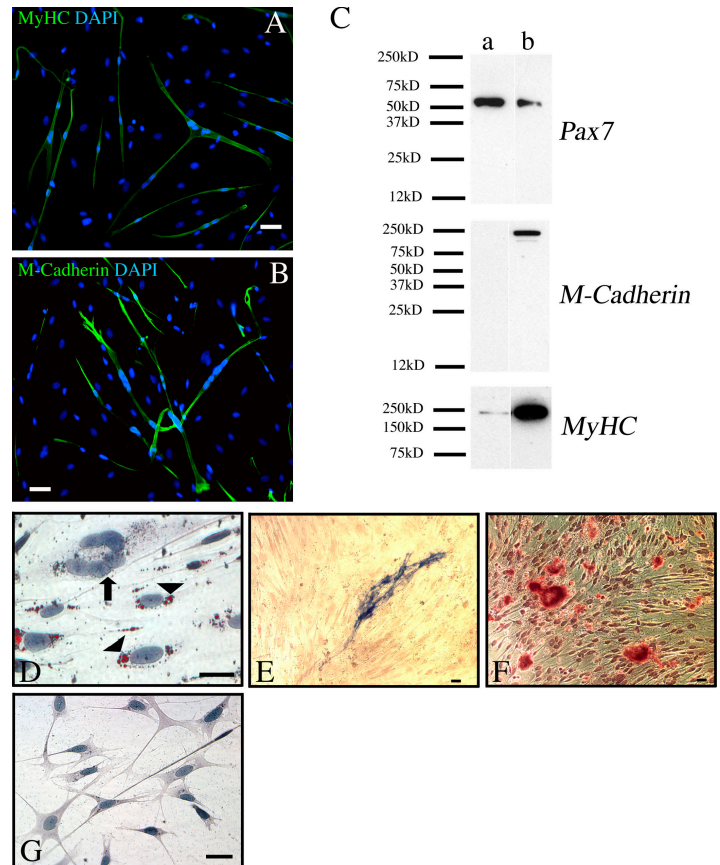


Figure 4. Myofiber-derived proliferating cells are satellite cell progeny. (A–C) Photomicrographs showing that injected fluorescent NLS-dextran marks the nuclei of the myofiber. B and C are high power magnifications of the boxed area in A. C is an overlay of the fluorescent and light microscopy images. (D–F) Photomicrographs showing that the fluorescent dextran exclusively labels myonuclei in the syncytium, but not the nuclei in satellite cells. Arrows point to mononuclei, arrowheads point to satellite cell. (G–I) Photomicrographs showing that the vast majority of the myofiber progeny lack the NLS-dextran lineage tracer. Although no fluorescent dextran-containing progeny were seen in 69 of 70 single myofiber cultures, these images show the single occasion when two fluorescent dextran⁺ cells were detected (arrows and boxes), but these cells did not proliferate. Arrowheads point to the myofiber, which has hypercontracted. The pictures underneath G–I are enlarged images of the boxed area. (J and K) Most of the myofiber-derived progeny remain Pax7⁺ (red) directly after activation, but the intensity of the staining is strongest closest to the hypercontracted myofiber (arrowheads). (L–N) Satellite cell progeny express Pax7 (red) and MyoD (green) for several generations. Bars, 50 μm .

and plating. Similar to the in vivo analyses, we found an additional basement membrane between the myofiber itself and the satellite cells, as indicated by the Pax7–collagen type IV double immunostaining (Fig. 2, A and B). Thus, newt single myofibers can be isolated containing the myofiber proper, along with the tightly associated satellite cells. The model in Fig. 2 E shows the location of newt satellite cells compared with their mammalian counterparts. We observed that on average 7.7% of the 2,167 nuclei in a representative sample of 55 single fibers were found to be in satellite cells, and 9 myofibers were devoid of satellite cells. Three-dimensional confocal microscopic analyses showed the complete absence of cells outside of the basal

Figure 5. Newt satellite cell progeny are multipotent. (A and B) Newt satellite cell progeny form myotubes in myogenic media. Note the myosin heavy chain⁺ myotubes in A and the M-cadherin⁺ (MyHC) myotubes in B. (C) Western blot analyses show the increased amount of M-cadherin and myosin heavy chain and the reduced amount of Pax7 proteins as a result of myogenic differentiation (lane a, proliferation medium; lane b, after 6 d in myogenic differentiation medium). (D) Satellite cell progeny can enter an adipogenic pathway, as revealed by Oil red staining in lipid droplets (arrowheads). The arrow points to a myotube that is devoid of lipid droplets. The cultures were counterstained by hematoxylin. (E and F) Satellite cell progeny can enter an osteogenic pathway. An alkaline phosphatase⁺ focus is shown in E, and Alizarin red marks calcium deposits produced by osteogenic cells in F. (G) Lack of Alizarin red staining in cells cultured in proliferation media. Bars, 50 μ m.



lamina, indicating that the myofiber cultures did not contain contaminating cells from elsewhere. All satellite cells were encased by basement membrane directly after attachment, and 99% of the cells in satellite cell positions were Pax7⁺.

On average, after 7 d in culture the myofibers started to produce proliferating progeny cells. Fig. 3 (A and B) shows a myofiber directly after attachment and with proliferating progeny after ~15 d in culture. Video 1 (available at <http://www.jcb.org/cgi/content/full/jcb.200509011/DC1>) illustrates the budding of single cells from the myofiber, and Fig. 3 C shows the single frame sequence of one budding event taken from the time-lapse movie capture in Video 1. Budding of cells continued until the myofiber hypercontracted and detached from the substrate. Myofiber-derived cells migrated onto the surrounding substrate and proliferated. At this stage it was unclear whether the proliferating progeny cells were derived by cellularization of the myofiber itself and/or by activation of quiescent satellite cells. To distinguish between these two events, we injected a fluorescein-conjugated nuclear-localizing dextran (NLS-dextran) into the myofibers directly after the attachment of the myofiber to the substrate (Fig. 4, A–C). This lineage tracer cannot be transferred between cells and, therefore, should only label myonuclei. In agreement with this, none of the Pax7⁺ satellite cells were labeled with NLS-dextran. Conversely, none of the NLS-dextran-labeled myonuclei were Pax7⁺ (Fig. 4, D–F). Out of the 70 single myofibers that we observed, we were only able to detect two mononucleate cells at one occasion that appeared to contain NLS-dextran (Fig. 4, G–I), and these two cells

did not proliferate. All other proliferating cells were NLS-dextran negative. Because the fluorescent NLS-dextran signal was easily detectable in all of the myonuclei and we analyzed the myofiber-derived progeny at 12-h intervals, we can exclude the possibility that the NLS-dextran signal was diluted because of rapid proliferation. These data show that satellite cell activation, rather than cellularization of the syncytium, resulted in a proliferating cell progeny population in our culture system. These proliferating satellite cells retained Pax7 expression and were also positive for MyoD for several generations (Fig. 4, J–N). In accordance with earlier observations on mammalian myofiber cultures (Zammit et al., 2004), Pax7 expression became heterogeneous in prolonged newt satellite cell progeny cultures (unpublished data).

Because the blastema is a multipotent tissue, we tested whether newt satellite cells were able to adopt anything other than myogenic fates. When cultured in myogenic medium, satellite cell progeny readily formed myotubes, which expressed M-cadherin and myosin heavy chain (Fig. 5, A and B). Western blot analyses confirmed the up-regulation of myosin heavy chain and M-cadherin during myogenesis, which was concomitant with the increased number of myotubes and the decreased number of myoblasts in the culture (Fig. 5 C). Simultaneously, Pax7 levels dropped in the protein extracts (Fig. 5 C). When cells were exposed to adipogenic media, we detected that at least 30% of the cells contained lipid droplets and displayed adipocyte morphology. In contrast, the few myotubes that were visible in the adipogenic media did not contain lipid droplets

(Fig. 5 D). Cells that were not cultured in adipogenic media were negative for Oil Red staining (Fig. 5 G). When satellite cell progeny were cultured in osteogenic media, we saw that 10% of the cells produced alkaline phosphatase-positive foci (Fig. 5 E) and that the cells produced calcium deposits stained by Alizarin red (Fig. 5 F). In addition, clonal analysis also indicated that the progeny are multipotent, displaying myogenic (not depicted), adipogenic, and osteogenic potential (Fig. S2, available at <http://www.jcb.org/cgi/content/full/jcb.200509011/DC1>). These data show that skeletal muscle satellite cell progeny can adopt nonmyogenic fates and indicate that satellite cells could represent a multipotent blastema progenitor population.

To test whether satellite cells are able to contribute to newly formed limb tissues, we injected labeled satellite cell progeny intramuscularly before amputation. Satellite cell progeny were labeled with BrdU before injection, during their *in vitro* expansion. As a control, we injected the contralateral limbs with PBS before amputation at the same axial level. As blastema formation and regeneration occurred we saw that a large number of the injected, BrdU-labeled cells appeared in clusters within the blastema at all analyzed stages of the regeneration. At the medium bud stage, BrdU-labeled cells were found within both the blastema (Fig. 6, A and B) and, strikingly, the epidermis (Fig. 6, J–M). BrdU-labeled cells were not detected in the contralateral regenerate, which was injected with PBS before amputation (Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200509011/DC1>). There was no difference in the speed and morphology of regeneration between cell- and PBS-injected limbs. BrdU-labeled cells were also clearly visible in the late bud stage regenerate, although the intensity of the BrdU label varied more, compared with the medium bud stage regenerate (Fig. 6, C–F). The contralateral PBS-injected regenerate was also devoid of BrdU-labeled cells at this stage (Fig. S1). All four injected limbs developed cartilage at this stage, and BrdU-labeled cells were detected within newly formed cartilage tissue in all four cases (Fig. 6, G–I). These results show that implanted satellite cell progeny can give rise to new tissues during limb regeneration and indicate that metaplasia may occur during salamander limb regeneration.

Discussion

The ability to form a regeneration blastema, which leads to the epimorphic regeneration of complex body structures, is restricted to some amphibians and fish among vertebrates (Poss et al., 2003). A conundrum of regenerative biology is why mammals, with a few exceptions, do not form a blastema or a blastema-like structure despite the fact that they can functionally repair some tissues, such as skeletal muscle (Charge and Rudnicki, 2004) and liver (Fausto and Campbell, 2003). Of particular interest is whether the generation of progenitor cells during epimorphic regeneration in salamander and during mammalian tissue repair proceeds by the activation of different or overlapping mechanisms. A unique feature of blastema formation in salamanders is the process of dedifferentiation of stump tissues that follows appendage removal. The possibility

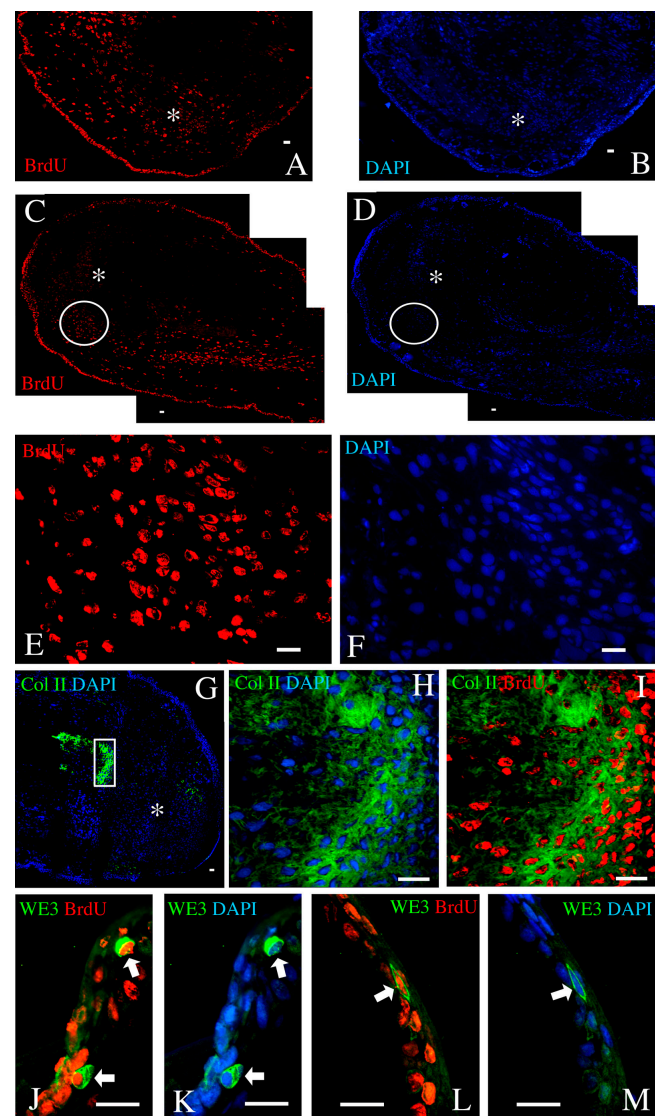


Figure 6. Injected BrdU-labeled satellite cell progeny incorporate into new tissues during limb regeneration. (A and B) Photomicrographs showing a medium bud stage regenerate. BrdU-labeled satellite cell progeny are found in the blastema (*) and, notably, also in the epidermis. (C and D) Photomicrographs showing a late bud stage regenerate. BrdU-labeled cells are still seen in the blastema and in the epidermis. (E and F) High resolution images of the circled area in C and D, showing significant amounts of BrdU-labeled cells in the blastema. (G) Photomicrographs showing cartilage. Boxed area is shown at high magnification in H and I. (H and I) Collagen type II⁺/BrdU-labeled cells are present in cartilage. (J–M) WE3⁺ (Tassava et al., 1986)/BrdU-labeled cells are present in the epidermis (arrows). Bars, 50 μ m.

to induce blastema formation and regeneration in mammals through the activation of a comparable dedifferentiation program has been proposed (Hughes, 2001; Bryant et al., 2002; Stocum, 2004). This is especially valid for skeletal muscle tissue because dedifferentiating skeletal muscle is a significant source of blastema progenitors. Although the potential role of stem cells in blastema formation has been suggested (Corcoran and Ferretti, 1999; Carlson, 2003; Odelberg, 2004), no such cells have been previously identified in the newt limb. Hence, it is still not clear whether the term dedifferentiation solely refers

to the reversal of the differentiated state of mature cells, to the activation of stem cells in the disorganizing tissues, or to a combination of these two definitions. If both processes coexist, the quantitative aspects of their relative contribution in vivo remain to be elucidated.

Our data clearly show that satellite cells, which are comparable to mammalian skeletal muscle stem cells, exist in newt skeletal muscle as well. First, we found that newt satellite cells or their progeny express molecular markers, such as Pax7, M-cadherin, and MyoD, all of which are expressed by mammalian satellite cells or their progeny as well (Zammit and Beauchamp, 2001). Second, when we isolated single myofibers a satellite cell population was copurified, despite the presence of an additional basal lamina between the satellite cell and sarcolemma. Third, similar to the mammalian myofiber cultures, we observed that satellite cell activation occurred that was characterized by cell cycle reentry and proliferation of the satellite cell progeny population. Finally, we showed that the satellite cell progeny population in newts is multipotent, which has also been observed in mammals (Asakura et al., 2001; Wada et al., 2002; Shefer et al., 2004).

Thus, the results indicate that newts do not represent an exception in the vertebrate phyla, and like other amphibians (Mauro, 1961; Gargioli and Slack, 2004) and mammals they also contain Pax7⁺ stem cells in their skeletal muscle tissue. However, the additional basement membrane that separates newt satellite cells from the sarcolemma may reflect that newt satellite cells are in some respect evolutionary intermediates between interstitial stem cells and satellite cells, which were found to be separate populations in mammals (Asakura et al., 2002; Tamaki et al., 2002). Identification of further stem cell populations in newt skeletal muscle, along with functional studies, could address this issue.

The satellite cell progeny population was able to adopt nonmyogenic fates in vitro and they incorporated into the regeneration blastema after intramuscular injection before amputation. We also noted a contribution to the epidermis and detected satellite cell progeny within newly formed cartilage tissue. The observed multipotentiality of satellite cell progeny does not directly address the question of whether activated satellite cells adopt divergent fates without in vitro expansion. However, the onset of tissue-specific molecular differentiation programs and the large number of satellite cell progeny within various tissues, which did not alter the speed and mode of regeneration, suggest that the integrated satellite cell progeny are functional. Furthermore, lineage shifting across germ layer boundaries has been shown to occur during salamander tail regeneration (Echeverri and Tanaka, 2002). Clearly, additional experiments are required to assess the plasticity of satellite cells in vivo and to establish whether metaplasia characterizes salamander limb regeneration. Nevertheless, in light of the available observations, a plausible hypothesis is that skeletal muscle dedifferentiation results in a significant contribution by satellite cells to the blastema and to the regenerate. Pax7⁺ cells are also found in the blastema of the regenerating axolotl tail (Schnapp et al., 2005) and tail regeneration in the *Xenopus laevis* tadpole also involves satellite cell activation (Gargioli

and Slack, 2004). These observations further suggest an important role of satellite cells in the regeneration of missing body parts in vertebrates.

In a study similar to our own, Kumar et al. (2004) showed that limb myofibers isolated from axolotl larvae undergo cellularization and fragmentation. The authors noted that only 3.5% of the myofibers contained the satellite type of cells and that these were not observed in their skeletal muscle fiber plasticity model. We saw that 86% of the isolated myofibers contained satellite cells and that only satellite cell progeny proliferated in our culture system, although we could not detect any sign of proliferating progeny that could have been derived by cellularization of the myofiber. At present, it is unclear whether the discrepancies between our observations and the model presented by Kumar et al. (2004) reflect phylogenetic or ontogenetic differences, or are caused by dissimilarities in the experimental paradigms. However, both studies underpin the necessity to further assess the quantitative aspects and functional relevance of satellite cell activation that leads to multipotent progeny on one hand and cellularization and/or fragmentation of the syncytium on the other during limb regeneration.

Our results show that epimorphic limb regeneration activates such programs, which lead to regeneration of muscle tissue in mammals after injury. Mammalian skeletal muscle responds to various challenges, such as stretching or mechanical damage, by activating a proliferation program in satellite cells that is followed by differentiation and fusion into myotubes and into myofibers. In this context, it is interesting to note the study by Echeverri et al. (2001), which showed that amputation as such was not sufficient to produce blastema progenitors. Instead, a mechanical stimulus (minor clipping of the muscle fiber) was required for the generation of progeny from dedifferentiating axolotl tail muscle in vivo (Echeverri et al., 2001). The exact identity of signals that link tissue injury to blastema formation needs to be elucidated, as it may reveal key aspects of blastema formation involving both myofiber fragmentation and concomitant stem cell activation. Formation of a blastema-like structure, although a rare event, is possible in mammals, as exemplified by the healing capacity of MRL mice and by the seasonal regeneration of deer antlers (Gourevitch et al., 2003; Price et al., 2005). The question is how blastema formation is induced in mammals and how it can be promoted. We propose skeletal muscle satellite cells as a potential target in the promotion of mammalian blastema formation.

Materials and methods

Antibodies

The following primary antibodies were used: mouse monoclonal anti-Pax7 IgG (Developmental Studies Hybridoma Bank), mouse monoclonal anti-myosin heavy chain IgG (MF20; Developmental Studies Hybridoma Bank), mouse monoclonal anti-M-cadherin IgG (Clone 1B11 used for immunofluorescence; nanotools GmbH), rabbit polyclonal anti-collagen type IV antibody (Rockland Immunochemicals, Inc.), rabbit polyclonal anti-M-cadherin antibody (used for immunoblotting; Invitrogen), rabbit polyclonal anti-H3P antibody (Upstate Biotechnology), rat monoclonal anti-BrdU IgG (Trichem ApS), rabbit polyclonal anti-MyoD antibody (Santa Cruz Biotechnology, Inc.), anti-WE3 monoclonal IgG (Developmental Studies Hybridoma Bank), mouse monoclonal anti-collagen type II IgG (CHEMICON International, Inc.). For immunofluorescence studies, primary antibodies were detected

with appropriate species-specific Alexa Fluor-conjugated secondary antibodies (Invitrogen).

Animals and procedures

All experiments were performed according to European Community and local ethics committee guidelines. Adult red-spotted newts, *Notophthalmus viridescens*, were supplied by Charles D. Sullivan Co., Inc. and maintained in a humidified room at 15–20°C. Animals were anesthetized by placing them in an aqueous solution of 0.1% ethyl 3-aminobenzoate methanesulfonate salt (Sigma-Aldrich) for 15 min. Forelimbs were amputated by cutting just proximal to the elbow or wrist, and the soft tissue was pushed up to expose the bone. The bone and soft tissue were trimmed to produce a flat amputation surface. Animals were left to recover overnight in an aqueous solution of 0.5% sulfamerazine (Sigma-Aldrich) before being placed back into a 25°C water environment. At specified time-points, the regenerating limbs were collected after anesthetization.

Immunohistochemistry

Tissue samples were mounted on cork using Gum Tragacanth (Sigma-Aldrich), snap frozen in isopentane (VWR), and cooled to freezing point in liquid nitrogen. 5- μ m-thin frozen sections were thawed at room temperature and immediately fixed in acetone/methanol (1:1) for 5 min at –20°C. Sections were blocked with 20% normal goat serum (DakoCytomation) diluted in PBS for 30 min at room temperature. Sections were incubated with a relevant primary antibody overnight and with secondary antibodies for 1 h at room temperature. Antibodies were diluted in blocking buffer and sections were mounted in mounting medium (DakoCytomation) containing 100 ng/ml DAPI (Sigma-Aldrich).

Newt single myofiber isolation and culture

Newts were anesthetized and decapitated. The skin was removed from the underside of the forelimbs, exposing the musculature. Excess fat and connective tissue was carefully removed from around the musculature. A group of muscles located between the elbow and wrist were isolated with forceps and carefully dissected away from the bone, handling only the tip of the muscle to prevent damage. Digestion with type I collagenase (Sigma-Aldrich) solution (0.2% wt/vol in DME; Invitrogen) supplemented with 1% Glutamax (Invitrogen) and 1% penicillin/streptomycin (Invitrogen) was performed in a water bath at 25°C for 3–4 h. All media used in this and subsequent cell cultures were diluted 24% with distilled water. After digestion, myofibers were disaggregated as previously described (Rosenblatt et al., 1995). Single myofibers were placed in 35-mm Falcon culture dishes (BD Biosciences) coated with 1 mg/ml Matrigel (BD Biosciences) in DME supplemented with 13% FCS (Invitrogen), 1% Glutamax, 1% penicillin/streptomycin, and 1% insulin (Sigma-Aldrich) and cultured at 25°C. Myofiber cultures were fixed in 2% PFA at various time points and processed for immunofluorescence studies.

Immunofluorescence staining of cultured cells

The protocols for immunofluorescent staining of cells and newt single myofibers were followed as previously described (Beauchamp et al., 2000), with the exception that cells and myofibers were fixed with 2% PFA.

Lineage tracing of myofiber-derived cells

A synthetic polypeptide containing the NLS of the polyomavirus large T antigen, CGYGVSRKRPRPGC, was synthesized by Thermo Electron Corporation. The peptide was covalently linked to fluorescein-conjugated dextran (70 kD; Invitrogen) via the COOH-terminal cysteine residue, using the heterobifunctional cross-linker sulfo-SMCC (Pierce Chemical Co.) as described previously (Broder et al., 1997; Maroto et al., 2004). Myofibers were injected with NLS-conjugated fluorescein-dextran directly after their attachment, using a Femtojet in combination with an Injectman (Eppendorf AG). Myofiber cultures were analyzed using both brightfield and fluorescence microscopy at 12-h intervals before fixation or passaging of the myofiber-derived cells.

Differentiation studies

For myogenic differentiation, satellite cell progeny were grown to 90–100% confluency and incubated in DME supplemented with 0.5% horse serum (Invitrogen), 1% Glutamax, 1% penicillin/streptomycin, and 1% insulin. After 3 and 6 d in differentiation medium, cells were fixed with 2% PFA and processed for immunofluorescence studies. For immunoblotting, cells were lysed with RIPA buffer supplemented with a protease inhibitor cocktail (Roche). 2 μ g of each cell lysate was separated on a 10% PAGE gel and transferred to nitrocellulose membrane. The membrane was blocked with

5% dry milk fat and 0.1% Tween 20 (Sigma-Aldrich) in PBS and subsequently probed with primary antibodies. Primary antibodies were recognized with species-specific streptavidin-conjugated secondary antibodies (GE Healthcare). Membranes were developed using an ECL detection kit (GE Healthcare). For adipogenic and osteogenic differentiation, cells were grown to 90–100% confluency and incubated in adipogenic and osteogenic media as described previously (Colter et al., 2001). Cells in adipogenic medium were stained with Oil red (Colter et al., 2001). Cells in osteogenic medium were stained with Alizarin red (Digirolamo et al., 1999), and alkaline phosphatase was detected using kit 85 (Sigma-Aldrich) according to the manufacturer's instructions. For clonal analyses, cells were cultured at a density of 0.5–1.0%, so that single cells were clearly discernible. Single cells were isolated with cloning cylinders (Sigma-Aldrich) and incubated for 30 s in trypsin-EDTA (0.05% trypsin and 0.53 mM EDTA; Invitrogen) at room temperature. Trypsinized single cells were transferred to one well of a 24-well culture plate that contained a 1:1 ratio of normal and conditioned proliferation media (13% FCS, 1% Glutamax, 1% insulin, and 1% penicillin/streptomycin). Proliferating clonal cells were maintained at a confluency of no more than 60% to avoid spontaneous differentiation before being subjected to differentiation studies.

In vivo injection studies

Satellite cell progeny were grown in the presence of 10 μ M BrdU for 6 d before injection. 20,000 cells were suspended in 4 μ l PBS diluted with 24% water. Animals were anesthetized and cells were injected using a Hamilton syringe intramuscularly in the upper forelimb halfway between the elbow and shoulder. 30 min after injection the limb was removed just above the elbow as described in Animals and procedures. Contralateral limbs were injected with PBS to serve as control. The regenerates were harvested at different time points and processed for immunohistochemistry.

Microscopy and image processing

An LSM 510 Meta laser microscope with LSM 5 Image Browser software (both Carl Zeiss MicroImaging, Inc.) was used for confocal analyses. A microscope (Axioplan 2; Carl Zeiss MicroImaging, Inc.) with Openlab 3.1.7 software (Improvision Ltd.) was used for brightfield and fluorescence microscopy analyses. Images were taken at room temperature and were further processed using Photoshop (Adobe) according to the JCB guidelines.

Online supplemental material

Fig. S1 shows that the progeny of injected BrdU-labeled satellite cells are found in the regenerate, but not in the contralateral regenerate. Fig. S2 shows a multipotent satellite cell progeny clone. Video 1 shows the derivation of proliferating mononucleate cells from a 10–14-d-old newt myofiber in vitro. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200509011/DC1>.

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