

## Plasticity and recovery of skeletal muscle satellite cells during limb regeneration

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**ABSTRACT** Salamander limb regeneration depends on local progenitors whose progeny are recruited to the new limb. We previously identified a Pax7<sup>+</sup> cell population in skeletal muscle whose progeny have the potential to contribute to the regenerating limb. However, the plasticity of individual Pax7<sup>+</sup> cells, as well as their recovery within the new limb, was unclear. Here, we show that Pax7<sup>+</sup> cells remain present after multiple rounds of limb amputation/regeneration. Pax7<sup>+</sup> cells are found exclusively within skeletal muscle in the regenerating limb and proliferate where the myofibers are growing. Pax7 is rapidly down-regulated in the blastema, and analyses of clonal derivatives show that Pax7<sup>+</sup> cell progeny are not restricted to skeletal muscle during limb regeneration. Our data suggest that the new regeneration blastema is not entirely a composite of lineage-restricted progenitors. The results demonstrate that except for a transient and subsequently blunted increase, skeletal muscle satellite cells constitute a stable pool of reserve cells for multiple limb regeneration events.—Morrison, J. I., Borg, P., Simon, A. Plasticity and recovery of skeletal muscle satellite cells during limb regeneration. *FASEB J.* 24, 750–756 (2010). [www.fasebj.org](http://www.fasebj.org)

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AQUATIC SALAMANDERS, FOR EXAMPLE, newts, possess a lifelong ability to regenerate body parts and tissues (1). Following removal, the new limb originates from local progenitor cells in the stump tissues. These progenitors give rise to a mesenchymal growth zone, called the blastema. The blastema is a central structure as it develops into the new tissues of the regenerate (2).

Skeletal muscle can produce blastema cells in two different ways. First, the multinucleated myofiber can fragment into mononucleate progeny, a process usually referred to as cellularization (3–5). Cellularization indicates a remarkable cellular plasticity, since the blastema progenitor is a fully differentiated postmitotic cell type. Alternatively, quiescent reserve cells within the muscle tissue become activated and produce cells, which incorporate into the blastema. We recently identified such a cell population in the newt limb, which is closely apposed to the newt myofibers. These cells express paired box transcription factor Pax7 and the cell adhesion molecule M-cadherin, indicating that they correspond to the satellite stem-cell population

found under the basal lamina in mammalian skeletal muscle (6). However, the additional basement membrane surrounding salamander Pax7<sup>+</sup> cells indicates some degree of evolutionary divergence from satellite cells in other vertebrates, such as mammals (7).

As a response to amputation, Pax7<sup>+</sup> cells reenter the cell cycle, and the progeny of the Pax7<sup>+</sup> cell population has the capacity to contribute to various tissues during limb regeneration. At present, it is unclear whether cellularization and Pax7<sup>+</sup> cell activation are quantitatively comparable processes, nor is it understood whether the resulting progeny cells are equivalent within the blastema.

Not only does the regeneration capacity of a newt persist throughout the entire life of the animal, the limb can be rebuilt after several amputations. The gross morphology of the new limb is indistinguishable from the original (8, 9), but it is not established whether each cell type reappears in the regenerates. One consequence of this is that consecutive regeneration events may involve different mechanisms by which blastema cells are produced, if one particular progenitor type is missing in the rebuilt limb. It has also been proposed that dedifferentiation of postmitotic cells is one way to ensure that reserve cells are not exhausted and that progenitors derive local cues from their parental cells (10, 11). Furthermore, the newt limb blastema is a multipotent structure defined at the tissue level, but the extent of lineage restriction of individual cells needs further assessment (12, 13). Blastema cells may be committed to contribute to tissues corresponding to their origin, alternatively the blastema cells may develop into several tissue types during regeneration.

To address these questions, we studied skeletal muscle satellite cells in order to determine whether and where they are found in the regenerating limbs and whether individual satellite cells are restricted to myogenic lineage. We saw that the satellite cell population

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regenerates within the new limb. Furthermore, Pax7<sup>+</sup> expression is rapidly down-regulated in the blastema, indicating that satellite cell identity in the newly forming limb is not constantly maintained. We also provide new evidence suggesting that satellite cell progeny may contribute to cartilage during limb regeneration.

## MATERIALS AND METHODS

### Antibodies

The following primary antibodies were used: mouse monoclonal anti-Pax7 IgG (Developmental Studies Hybridoma Bank, Iowa City, IA, USA), mouse monoclonal anti-myosin heavy chain IgG (MF20; Developmental Studies Hybridoma Bank), rabbit polyclonal anti-collagen type IV (Rockland Immunochemicals, Gilbertsville, PA, USA), rat monoclonal anti-BrdU IgG (Trichem ApS, Skanderborg, Denmark), mouse monoclonal anti-collagen type II IgG (Chemicon International, Temecula, CA, USA), rabbit polyclonal anti-syndecan-4 IgG (HI40; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-GFP-IgG (Millipore, Beverly, MA, USA), and mouse monoclonal anti-PCNA IgG (Chemicon). For immunofluorescence studies, primary antibodies were detected with appropriate species-specific Alexa Fluor-conjugated secondary antibodies (Invitrogen, Carlsbad, CA, USA).

### Animals and procedures

All experiments were performed according to European Community and Stockholm ethics committee guidelines. Adult red-spotted newts, *Notophthalmus viridescens*, were supplied by Charles D. Sullivan Co. (Nashville, TN, USA) 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, St. Louis, MO, USA) for 15 min. Forelimbs were amputated by cutting just proximal to the elbow, 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. For animals undergoing two rounds of regeneration, the amputation procedure was repeated 11 wk following the primary amputation. At specified time points, the regenerating limbs were collected after anesthetization. For BrdU-labeling experiments, animals were injected intraperitoneally with 0.1 ml of 1 mg/ml BrdU at 24 and 48 h before sacrifice.

### Immunohistochemistry

Tissue samples were mounted on cork using gum tragacanth (Sigma-Aldrich), snap-frozen in isopentane (VWR International, West Chester, PA, USA), and cooled to freezing point in liquid nitrogen. Frozen sections (5 µm thickness) were thawed at room temperature and immediately fixed in 4% formaldehyde for 5 min at room temperature. Sections were blocked with 10% normal goat serum (DakoCytomation, Glostrup, Denmark) diluted in PBS for 60 min at room temperature. Sections were incubated with a relevant primary antibody overnight at 4°C 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). Sections being labeled for BrdU were fixed in 4%

formaldehyde for 5 min at room temperature, incubated for 20 min in permeabilization solution (0.2% Triton-X100, 2 M HCl, and PBS) at 37°C, followed by a 5-min incubation in 0.1 M Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub> at room temperature, prior to blocking and subsequent antibody incubations.

### Newt single myofiber isolation

Newts were anesthetized and decapitated. The skin was removed from the underside of the forelimbs, exposing the musculature. Excess fat and connective tissue were carefully removed from around the musculature. A group of muscles located between the elbow and wrist was 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% w/v 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. After digestion, myofibers were disaggregated, as described previously (14). Single myofibers were placed in Falcon culture dishes (BD Biosciences, San Jose, CA, USA) 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 immunohistochemical studies (described above).

### Fate mapping of clonal derivatives

Satellite cells derived from cultured newt myofibers were plated at low density in a 10-cm culture dish, so individual cells could be identified and followed. Individual cells were isolated with a clonal ring, trypsinized, and transferred to one well of a 24-well plate. Individual colonies were followed daily to ensure that the clonal colonies were derived from one cell. Cells were cultured and passaged as described previously (6). Cells were labeled using a CMV promoter-driven eGFP adenovirus (a kind gift from Dr. J. C. Belmonte, The Salk Institute for Biological Studies, La Jolla, CA, USA; ref. 15). Cells were grown to ~50% confluency in 10-cm dishes. Virus (19.5 µl) was added to each dish in the presence of 5 µg/µl polybrene for 18 h. Fresh medium was added, and cells were cultured for an additional 48 h.

For injections, newts were sedated with 0.1% tricane 2 wk after amputation and positioned so the ventral side of the newt was facing upward. Using a 10-µl Hamilton syringe (Hamilton, Reno, NV, USA), 3 µl of cell suspension was injected into the limb blastema. The numbers of cells injected for clone 1 and clone 2 were 30,000 and 15,000, respectively. The newts were left to recover overnight in 0.5% sulfamerazine.

### Comparative counts for Pax7 in uninjured and regenerating forelimbs

Tissue samples were mounted, sectioned, and stained for Pax7 as stated. Forelimb tissue that had regenerated from the amputation site was mounted for sectioning. For each forelimb collected, serial sections were made and collected on 5 glass slides so each slide represented the entire aspect of the forelimb. Pax7<sup>+</sup> cells were cataloged for each section using images obtained by an ×10 objective positioned over several regions representing the entire section. Because of irregularities in sectioning and limb sizes, the number of sections per slide for each sample varied. To gain a comparative and representative measure of Pax7<sup>+</sup> cells, we decided to count ≥6 sections for each forelimb sample, as indicated in the

legend for **Fig. 2**. The number of limbs was as follows: uninjured limb,  $n = 3$ ; 7-wk regenerate,  $n = 2$ ; 8-wk regenerate,  $n = 2$ ; 11-wk regenerate,  $n = 1$ ; 7-wk reamputated regenerate,  $n = 1$ . For statistical analysis, a  $\chi^2$  test was used to compare the frequency distribution of Pax7<sup>+</sup> cells in uninjured and regenerating limbs. To compare the group means from uninjured and injured samples, a Student's  $t$  test and Mann-Whitney  $U$  test was performed. Variations between groups were measured using the  $F$  test, in order to assign the grouped data to the appropriate conditions in the Student's  $t$  test. Statistical significance for all analyses was accepted at  $P < 0.05$ .

### Microscopy and image processing

An LSM 510 Meta laser microscope with LSM 5 Image Browser software (both Carl Zeiss MicroImaging, New York, NY, USA) was used for confocal analyses. A microscope (Axioplan 2; Carl Zeiss MicroImaging) with Openlab 3.1.7 software (Improvision, Waltham, MA, USA) was used for bright-field and fluorescence microscopy analyses. Images were taken at room temperature and were further processed using Photoshop (Adobe Systems, San Jose, CA, USA) by linear adjustments.

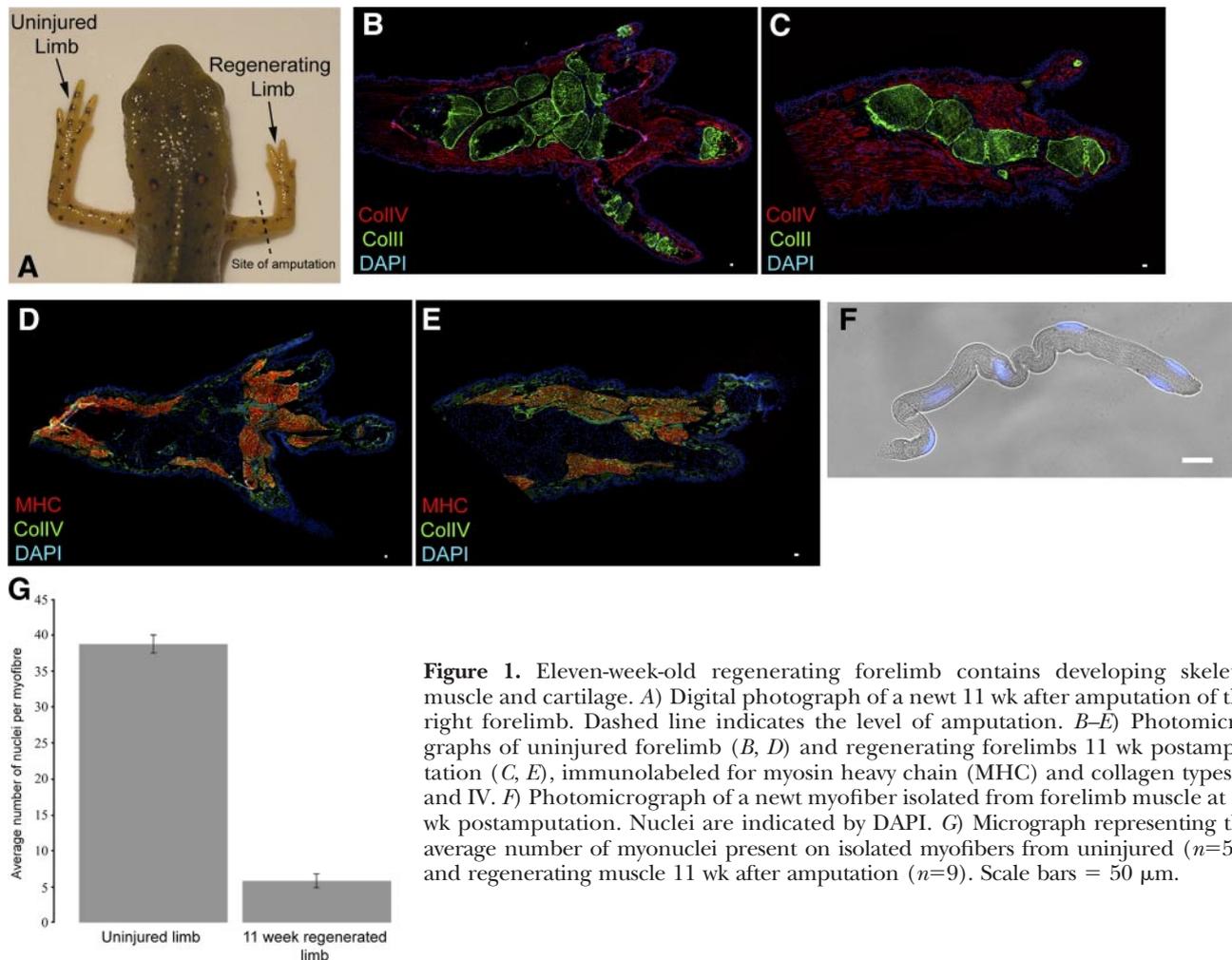
## RESULTS

To test whether the satellite cell population is regenerated in the new limb, we first amputated the forelimb

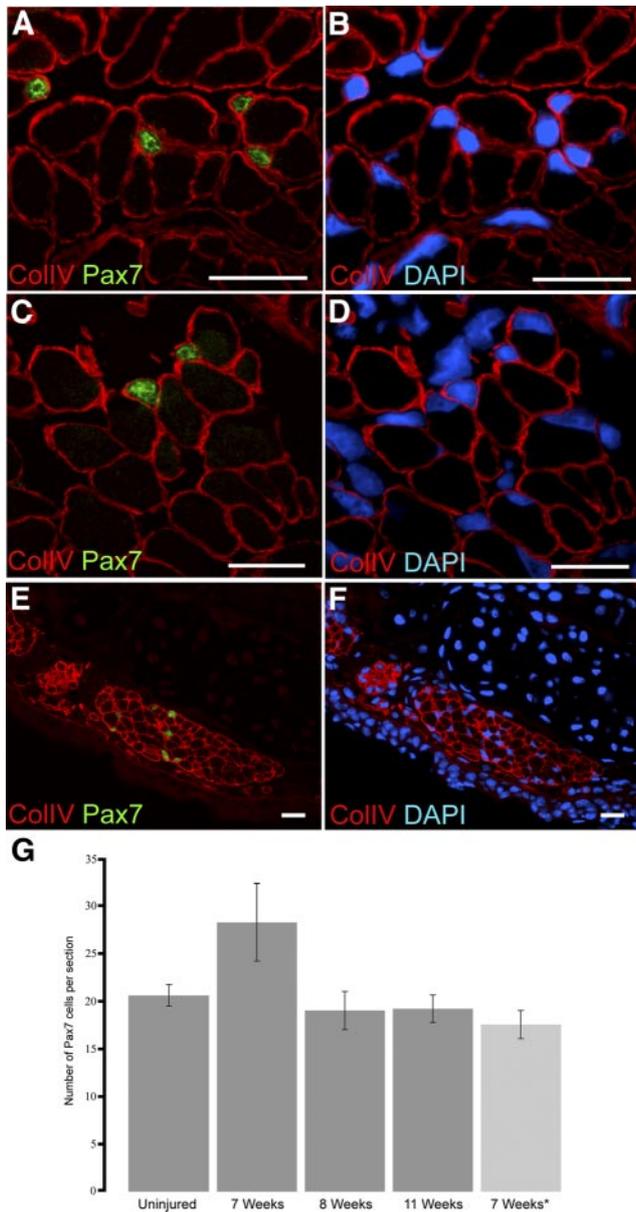
above the elbow. **Figure 1A** shows a representative limb 11 wk after amputation. The regenerating limb has at this stage developed significant amounts of skeletal elements and skeletal muscle, as revealed by myosin heavy chain, collagen IV, and collagen II expression (**Fig. 1C, E**). The tissues also display an overall morphology that is comparable with the contralateral, uninjured control limb (**Fig. 1B, D**). Eleven weeks after amputation, the new limb has not reached its original size and is still growing. In accordance with this observation, isolated myofibers are significantly smaller and contain fewer nuclei than their counterparts, which were obtained from control limbs (**Fig. 1F, G**).

We tested whether the growing limbs contain satellite cells, which could contribute to the developing tissues. Skeletal muscle in the growing limb contained Pax7<sup>+</sup> cells, which were encapsulated by a basement membrane, similarly to the uninjured limb (**Fig. 2A–D**, Supplemental Fig. 2A–H). Notably, Pax7<sup>+</sup> cells were only found within skeletal muscle and not elsewhere (**Fig. 2E, F**). Occasional Pax7<sup>+</sup> cells were visible only in the proximal region of the blastema during the first 4 d after amputation (data not shown and ref 6). As in uninjured limbs, Pax7<sup>+</sup> cells also are positive for Syndecan-4 (16) in the regenerate (Supplemental Fig. 1).

Next, we compared the number of satellite cells in uninjured and regenerating limbs. Given the different



**Figure 1.** Eleven-week-old regenerating forelimb contains developing skeletal muscle and cartilage. **A)** Digital photograph of a newt 11 wk after amputation of the right forelimb. Dashed line indicates the level of amputation. **B–E)** Photomicrographs of uninjured forelimb (**B, D**) and regenerating forelimbs 11 wk postamputation (**C, E**), immunolabeled for myosin heavy chain (MHC) and collagen types II and IV. **F)** Photomicrograph of a newt myofiber isolated from forelimb muscle at 11 wk postamputation. Nuclei are indicated by DAPI. **G)** Micrograph representing the average number of myonuclei present on isolated myofibers from uninjured ( $n=56$ ) and regenerating muscle 11 wk after amputation ( $n=9$ ). Scale bars = 50  $\mu$ m.



**Figure 2.** Pax7<sup>+</sup> cell population is restored in the skeletal muscle of the regenerating limb. *A–D*) Photomicrographs of immunolabeled Pax7<sup>+</sup> cells encapsulated by collagen type IV<sup>+</sup> basement membrane in uninjured (*A, B*) and 11-wk-old regenerate (*C, D*) forelimb muscle. *E, F*) Low-power magnification photomicrographs of transverse tissue sections showing that Pax7<sup>+</sup> cells are only present within skeletal muscle (*E*). Nuclei are indicated by DAPI (*F*). *G*) Micrograph representing the average number of Pax7<sup>+</sup> satellite cells/section in uninjured forelimbs and regenerating forelimbs at indicated time points. Numbers of sections counted for the uninjured, 7-, 8-, and 11-wk and 7 wk after second amputation time points were 61, 16, 13, 26 and 15, respectively. Asterisk indicates that the forelimb has been amputated twice. Scale bars = 50  $\mu$ m.

limb sizes and that satellite cells were only found in muscle whose representation varied in different sections, the frequency distribution of satellite cells varied in the different samples (Supplemental Table 1). However, when we compared the relative number of satellite cells/section by quantifying the average number of

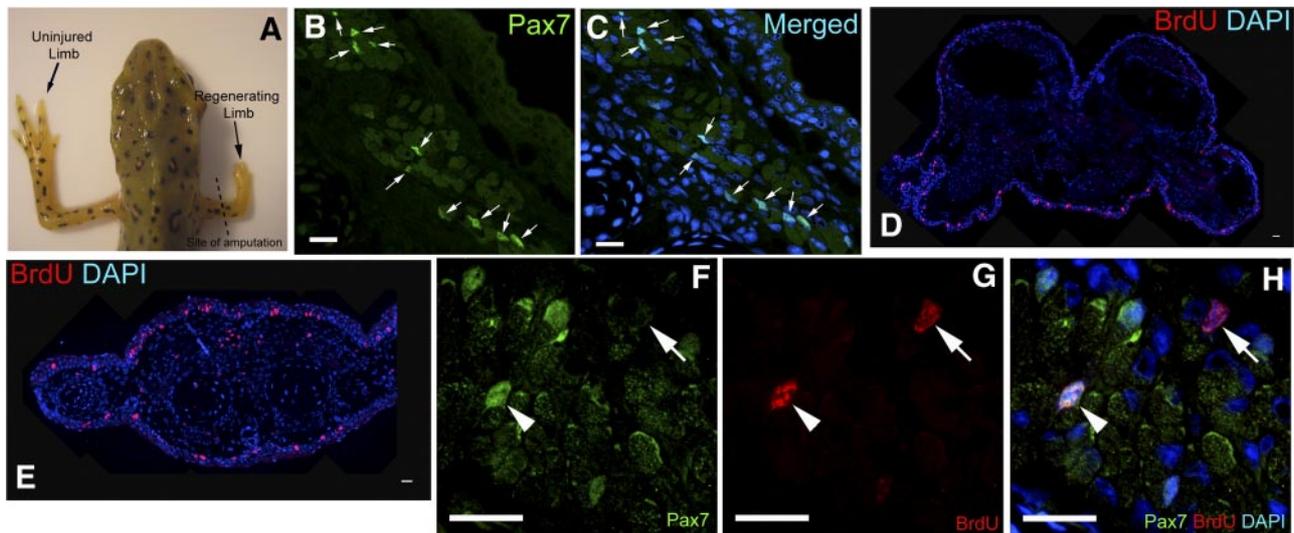
Pax7-positive cells in uninjured and regenerating forelimbs at various time points, we did not detect statistically significant differences between the samples (Fig. 2G; Supplemental Table 2). These data show that satellite cells replenish their compartment after amputation.

To test that satellite cells may serve as limb progenitors in subsequent regeneration events, we reamputated the regenerating limb after 11 wk of regeneration. The second regenerate grew at a similar rate as the first regenerate (Fig. 3A). Seven weeks after removal of the second limb, the new limb muscle contained satellite cells (Fig. 3B, C). To detect proliferating cells, we administered the nucleotide analog bromodeoxyuridine (BrdU), which incorporates into cells that are replicating their DNA before cytokinesis (17). Three intraperitoneal injections of BrdU within 48 h resulted in no labeled cells in the mesenchymal tissues in the control limbs (Fig. 3D). However, the skin contained a number of cycling cells. In contrast to this, BrdU<sup>+</sup> cells were found in the mesenchymal tissues in addition to the skin in regenerating limbs (Fig. 3E).

To test whether satellite cells were quiescent or cycling at this stage, we used double immunolabeling against Pax7 and BrdU. In contrast to the control limbs (data not shown), we could detect a number of cycling Pax7-expressing cells within skeletal muscle (Fig. 3F–H). These data indicate that proliferating Pax7<sup>+</sup> cells contribute to the growing muscle.

To further test the potential of satellite cells during limb regeneration, we analyzed the fate of clonal derivatives after injection into the blastema. Our previous data (6) indicated that the satellite cell population was not restricted to skeletal muscle during limb regeneration. However, the study did not address potentiality at single-cell resolution nor did the labeling technique allow long-term tracing. We extended these studies by genetically labeling clonal derivatives of satellite cells. We isolated two independent satellite cell clones from single myofiber preparations and labeled the progeny using adenovirus, expressing enhanced green fluorescent protein (GFP). In accordance with our previous observations (6), satellite cell progeny isolated from single myofiber preparations retained Pax7 expression after activation and multiple doublings. Eighty-two (clone 1) and eighty percent (clone 2) of the cells expressed Pax7 before injection (Fig. 4A, B), and an additional 9% (clone 1) and 12% (clone 2) of the nuclei were found in myosin heavy chain (MHC)-expressing mononucleated or multinucleated cells. These data indicated that the injected cells were myogenic and they were derived from single satellite cells.

One day after injection, we found numerous GFP/Pax7<sup>+</sup> expressing cells in the blastemas (Fig. 4C–F) but not in PBS injected blastemas (Supplemental Fig. 3A, B). A quantitative assessment showed that 55% of the GFP<sup>+</sup> cells expressed Pax7, indicating that a substantial proportion of the injected cells down-regulated Pax7. Twenty percent of the GFP<sup>+</sup> cells expressed the proliferating cell nuclear antigen (PCNA), indicating that the injected cells are viable and actively dividing in the limb (Supplemental Fig. 4). Four days after injection, we could detect GFP<sup>+</sup> cells in the blastemas (Fig. 4G)



**Figure 3.** Pax7<sup>+</sup> cells proliferate in the regenerating muscle after reamputation. *A*) Digital photograph of a 7-wk-old regenerate after reamputation of a regenerating right forelimb. Dashed line indicates level of amputation. *B*, *C*) Photomicrographs demonstrating the presence of Pax7<sup>+</sup> cells in the regenerating skeletal muscle 7 wk after reamputation of the forelimb. *D*, *E*) BrdU labeling of an uninjured limb (*D*) and of a 7-wk-old regenerate (*E*). *F–H*) Photomicrographs showing a proliferating Pax7<sup>+</sup> cell (arrowhead) in the skeletal muscle of a 7-wk-old regenerate. The arrow indicates a BrdU<sup>+</sup>/Pax7<sup>−</sup> cell in the regenerating muscle. Nuclei are indicated by DAPI. Scale bars = 50 μm.

for both clones. However, we could not find Pax7/GFP double-positive cells, indicating a continued rapid down-regulation of Pax7 expression. At this time point, both clonal populations have given rise to progeny that showed colabeling with MHC in myotube- and myofiber-like structures (Fig. 4*H*, *I*; Supplemental Fig. 3*C*, *D*), while no GFP<sup>+</sup> cells were detected in stump or uninjured control limb muscle (Supplemental Fig. 3*E–H*). At this time point, we also found GFP-expressing cells in cartilage, indicated by morphology and by collagen type II staining (Fig. 4*J*). We next analyzed the regenerating limbs 27 d after injection. Both clones showed contribution to cartilage (Fig. 4*K*, *L*) at this time point. Of the 43 representative cartilage sections that we counted, 10 contained clusters of GFP<sup>+</sup> cells showing substantial contribution to cartilage. However, we could not find any GFP-expressing muscle fibers at this time point, due to the fact that the relative contribution of injected cells to the multinucleated fibers may not be sufficient to ensure detectable levels of GFP expression in the syncytium. Taken together, both clonal populations gave rise to both myogenic (2/2 cases 4 d postinjection) and chondrogenic progeny (2/2 cases 4 d postinjection; 5/5 cases 27 d postinjection).

## DISCUSSION

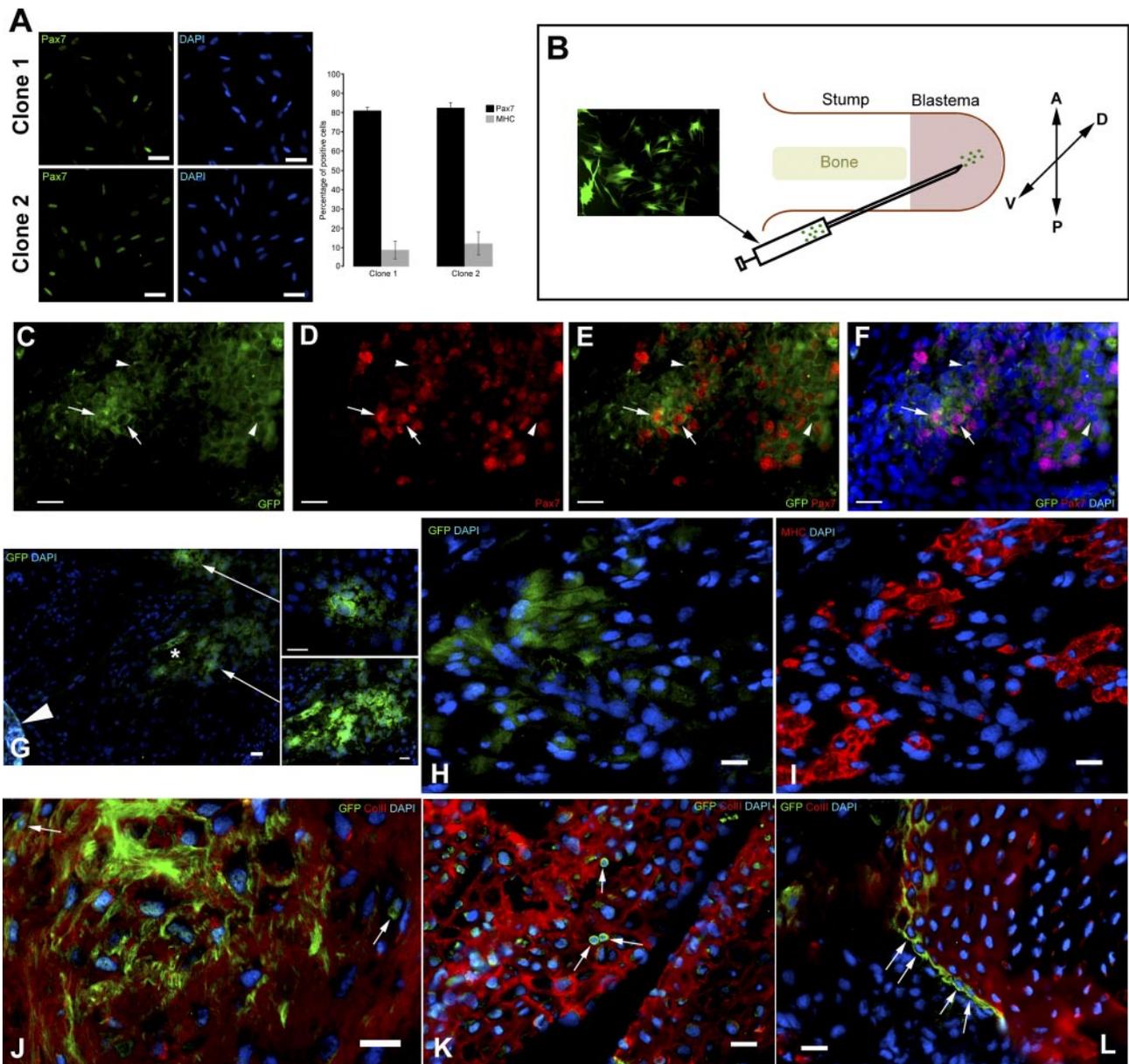
The identification of a satellite cell population in newt limb skeletal muscle and its activation after amputation showed that limb regeneration in salamanders and skeletal muscle tissue repair in mammals share common cellular and also molecular mechanisms (6, 18–20). Satellite cell contribution to the blastema complements the model in which blastema cells are generated by

dedifferentiation of the postmitotic myofibers through a cellularization process (21).

One question we set out to address in this work was whether the satellite cell population becomes restored or depleted during limb regeneration. While it has been clear that functional skeletal muscle develops in the regenerated limb, it was not determined whether the regenerated limb also harbors satellite cells. Hence, it was possible that cellularization of the myofibers would be a mechanism by which salamanders ensure that they have an indefinite pool of limb progenitors for multiple rounds of regeneration when the alternative source is exhausted. Since satellite cells reappear in comparable quantities in skeletal muscle after 2 rounds of amputations, we predict that the relative contribution of satellite cells to the regenerate remains constant.

The origin of satellite cells in the regenerate is unclear. Satellite cells in adult mammals can differentiate into muscle fibers and also produce new satellite cells (22–25). Available data, including the present work, do not address the origin of the restored Pax7<sup>+</sup> population. However, on the basis of the situation in mammals, it is tempting to speculate that one source of the satellite cells found in the regenerate is the Pax7-expressing satellite cells or a subpopulation of those in the newt limb as well. This hypothesis is supported by previous experiments, which indicated that cells from neither dermis nor cartilage contribute to myoblasts (26–28).

Pax7<sup>+</sup> cells were only found in skeletal muscle and not elsewhere showing that this marker is highly specific to satellite cells also during limb regeneration not only in the uninjured limb. Satellite cells were proliferating in developing muscle, indicating that they contribute to the growing muscle at the stages when the



**Figure 4.** Clonal satellite cell progeny are not restricted to muscle during limb regeneration. *A*) Clone 1 and clone 2 immunolabeled with Pax7 antibody. Graph depicts the percentage of cells in each clone that were Pax7<sup>+</sup> and MHC<sup>+</sup>. *B*) Diagram showing how the adenovirally eGFP-labeled cells were injected into the blastema. *C–F*) Photomicrographs of Pax7<sup>+</sup> (arrows) and Pax7<sup>-</sup> (arrowheads) GFP-labeled clone 2-derived satellite cell progeny 1 d postinjection. *G*) Photomicrograph showing the eGFP labeled clone 1-derived cells in the blastema 4-d postinjection. Insets: magnified images showing GFP<sup>+</sup> cells within the blastema. Arrowhead and asterisk indicate wound epidermis and blastema, respectively. *H, I*) Photomicrograph of GFP<sup>+</sup> clone 1-derived cells 4 d postinjection expressing MHC. *J*) Photomicrograph of GFP<sup>+</sup> clone 2-derived cells (arrows) 4 d postinjection within collagen type II-producing cartilage. *K*) Photomicrographs of GFP<sup>+</sup> clone 2-derived cells (arrows) 27 d postinjection residing in the collagen type II producing cartilage. *L*) Photomicrographs of GFP<sup>+</sup> clone 1-derived cells (arrows) 27-d postinjection residing in the collagen type II-producing cartilage. Scale bars = 50  $\mu$ m.

new limb has not reached its final size. Several other markers, such as Syndecan-4 and FoxK1 (16, 29), are expressed by newt Pax7<sup>+</sup> cells, but these are not specific for satellite cells in contrast to Pax7 (Supplemental Fig. 1; and data not shown).

Whether satellite cells produce progeny that switch lineage during newt limb regeneration is still an open question. Our results show the potential of satellite cells to contribute to nonmuscle tissues during limb regeneration and indicate that not all blastema cells have

restricted potential. A recent, very elegant study using an integrated GFP transgene in a closely related salamander (the axolotl) showed no evidence for satellite cells crossing over lineage boundaries during limb regeneration in juvenile, premetamorphic animals (30). Equivalent studies in newts are required to see whether the discrepancy between the results in newts and axolotls reflects differences in the experimental settings or species variations, or whether the cellular dynamics during regeneration diverge in developing

compared to fully mature, adult animals. In contrast to the situation in axolotl, we see that Pax7<sup>+</sup> cells are not found in the newt blastema except very early after amputation (data not shown and ref. 6), and these cells probably represent the activated and migrating satellite cells into the blastema. Later, Pax7 expression ceases, which is likely due to its down-regulation in satellite cell progeny. In line with this model is our finding that although the vast majority of clonally derived cells in culture expressed Pax7, we were unable to detect Pax7 protein in these cells 4 d later. In addition, the progeny of clonal satellite cell derivatives was not only found in skeletal muscle but also in cartilage. We detected contribution to muscle 4 d but not 27 d after injection. This is likely to be due to the low relative contribution of injected cells, which is not sufficient enough to ensure detectable level of GFP expression in the syncytium. Because of the high nonspecific fluorescence in skin, we were unable to address whether the injected cells also contributed to dermis and potentially epidermis (6). Recently, it was shown that the limb blastema reprogrammed cardiomyocytes, resulting in the transdifferentiation of cardiomyocytes into skeletal muscle and cartilage (31). Furthermore, the expression of pluripotency-inducing factors is triggered during newt limb regeneration (32). These observations and our results further support the model that the newt limb blastema has a reprogramming activity and that not all blastema cells are restricted to one particular cell type during adult limb regeneration. **[F]**

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