Fundamental Differences in Dedifferentiation and Stem Cell Recruitment during Skeletal Muscle Regeneration in Two Salamander Species

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SUMMARY

Salamanders regenerate appendages via a progenitor pool called the blastema. The cellular mechanisms underlying regeneration of muscle have been much debated but have remained unclear. Here we applied Cre-loxP genetic fate mapping to skeletal muscle during limb regeneration in two salamander species, Notophthalmus viridescens (newt) and Ambystoma mexicanum (axolotl). Remarkably, we found that myofiber dedifferentiation is an integral part of limb regeneration in the newt, but not in axolotl. In the newt, myofiber fragmentation results in proliferating, PAX7+ mononuclear cells in the blastema that give rise to the skeletal muscle in the new limb. In contrast, myofibers in axolotl do not generate proliferating cells, and do not contribute to newly regenerated muscle; instead, resident PAX7+ cells provide the regeneration activity. Our results therefore show significant diversity in limb muscle regeneration mechanisms among salamanders and suggest that multiple strategies may be feasible for inducing regeneration in other species, including mammals.

INTRODUCTION

In salamanders, limb amputation causes the formation of a proliferative progenitor cell zone called the blastema that faithfully regenerates the original limb (Stocum and Cameron, 2011). Whether blastema cells arise from resident adult stem cells or by cellular dedifferentiation has long been debated and skeletal muscle has been an intense focus of such studies (Slack, 2006). Conclusive, quantitative evidence for skeletal muscle dedifferentiation was lacking, due to the inability to long-term fate map endogenous muscle fibers. Histological and short-term cell labeling studies suggested that multinucleated myofibers and implanted myotubes dedifferentiate into mononuclear, proliferative cells in the first weeks of limb and tail regeneration (Calve et al., 2010; Duckmanton et al., 2005; Kumar et al., 2004; Odelberg et al., 2000). On the other hand, PAX7+ satellite cells exist in salamander muscle tissue and become proliferative upon amputation (Morrison et al., 2006). Implantation of cultured satellite cells contributed to regeneration but the fate of endogenous PAX7+ cells was not clear (Kragl et al., 2009; Morrison et al., 2006, 2010).

Here we describe Cre-loxP-based genetic fate mapping of muscle during limb regeneration in two salamander species, Notophthalmus viridescens and Ambystoma mexicanum. Surprisingly, in the newt, Notophthalmus viridescens, muscle dedifferentiation makes a significant contribution to muscle regeneration, while in the axolotl, Ambystoma mexicanum, myofibers make no contribution to limb regeneration and PAX7+ satellite cells are the main contributor to axolotl limb muscle regeneration. These results reveal an unexpected evolutionary diversity in muscle dedifferentiation among closely related species.

RESULTS

Myofibers Contribute to Regenerated Muscle in the Newt Limb

Our aim was to permanently mark muscle fibers in the newt limb and follow them through regeneration. To genomically integrate a Cherry-to-nlsYFP loxP reporter, we flanked the loxP expression cassette with ToI2 transposon sites and coelectroporated limb tissue with a ToI2 transposase expression vector (Figure 1A) (Kawakami, 2007). In addition, the electroporation mix contained a Cre expression plasmid: either a muscle-specific Cre expression vector, MCK-Cre (Jaynes et al., 1989); a ubiquitously expressed CMV-Cre plasmid; or control empty PUC19 vector (Figure 1A). Importantly, the transposase and the Cre-driver vectors did not contain transposase sites, and therefore would be transiently expressed. Therefore,
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only the loxP expression cassette would integrate long-term into the genome of the limb cells.

We first confirmed that YFP expression from the loxP reporter depended on CRE activity and that expression lasted through regeneration. When the loxP reporter was coexpressed with empty PUC19 in the mature limb, no YFP+ cells were observed (Figures 1A and 1B). In contrast, when the ubiquitously expressed CMV:Cre was used, nuclei both within and outside of muscle were labeled (Figures 1A and 1C). Upon amputation of CMV:Cre limbs, we detected nuclear YFP signal in multiple cell types of the 2-month-old regenerates, including skeletal muscle (Figures S1B and S1C, available online), epidermis (Figures S1D and S1E), and cartilage (Figures S1E and S1F). These results showed that we could track the progeny of stump cells into the regenerate long-term.

We next assessed the specificity of the muscle Cre-driver, MCK:Cre, which yielded nuclear YFP+ expression exclusively in skeletal muscle as determined by coimmunostaining with Myosin Heavy Chain (MHC) (Figure 1D). YFP+ nuclei in muscle were only detected within the laminin+ basement membrane (Figure 1E) and they colocalized with the myogenic transcription factor MEF2C (Figure 1F). Of 642 YFP+ nuclei (n = 5 limbs), all were found within the basement membrane, and 561 out of 563 YFP+ nuclei were MEF2C+.

It was also important to confirm that PAX7+ satellite cells were not targeted by our labeling method. The electroporation conditions we employed were in fact unable to access satellite cells, as PAX7+ cells never expressed Cherry from the loxP reporter when the loxP reporter was electroporated alone (Figure S2A, n = 208 PAX7+ nuclei). We also did not find any YFP+PAX7+ cells after coelectroporation of the loxP reporter with CMV:Cre (data not shown). Consistent with these observations, electroporation of the loxP reporter and MCK:Cre yielded no YFP+PAX7+ nuclei (833 YFP+ nuclei in five limbs) (Figure S2B). We further confirmed these results in an in vitro culture of dissociated limb myofibers (Figures S2C and S2D). In such preparations, we counted 249 YFP+ nuclei out of 4,399 DAPI nuclei associated with myofibers and none were targeted by our labeling method. The electroporation driver, when the loxP reporter was coexpressed with CMV:Cre recombinase is under the control of the ubiquitous CMV promoter.

An important question is whether the injured myofibers dedifferentiate during the regeneration process. To test this, we first asked if YFP+ cells in the 2-week-old blastema had lost the muscle marker MHC (Figure 2A). Proximal to the amputation plane, we found YFP+ nuclei both within and around MHC+ myofibers (Figures 2B and 2E). At the base of the blastema, close to the amputation plane, we detected evidence of skeletal muscle fragments that were positive for MHC and contained YFP+ nuclei (Figures 2B and 2D). Importantly, in the distal blastema we observed YFP+ cells that were negative for MHC expression, indicating that muscle cells dedifferentiate to form mononuclear, blastema cells (Figures 2B and 2C).

To determine if the YFP+MHC+ blastema cells were proliferative, we double immunostained for YFP and the proliferating cell nuclear antigen (PCNA). 16.7% ± 2.3% (n = 4 limbs) of the YFP+ nuclei in the blastema were double positive, indicating that they

Having shown the specificity and durability of the labeling method, we used it to trace myofibers during limb regeneration by coelectroporating upper arms with MCK:Cre, the loxP reporter, and the transposase constructs 14 days before amputation. To map the long-term fate of the myofiber-derived progeny, we analyzed limbs at the late palette and the late digit stages of regeneration (Iten and Bryant, 1973). Figure S3A shows the developing humerus, ulna, and radius, outlined by collagen-II staining in late palate stage regenerate where myogenesis was not yet complete. We detected YFP+ nuclei both proximally and distally to the level of amputation. Double immunostaining against collagen-II showed that YFP+ nuclei never colocalized with regenerating cartilage (Figure S3B), which was in contrast to the observations after CMV:Cre-mediated recombination (Figure S1F). When late digit stage regenerates were analyzed, we detected YFP+ nuclei in myofibers along the entire proximal-distal axis, except at the digit tips (Figures 1G–1L). On average 5.33% ± 1.37% of the myofibers were labeled throughout the limb excluding the fingertips (Figure 1M) and YFP+ nuclei were not found in myofibers. It is likely that the lack of YFP+ nuclei in the fingertips is due to the fact that there is almost no muscle found in this region. These data showed that myofibers quantitatively contributed to new muscle formation during newt limb regeneration.

**Myofibers Dedifferentiate into Proliferative Blastema Cells during Newt Limb Regeneration**

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**Figure 1. Long-Term Contribution by Myofibers to Limb Regeneration in Newts**

(A) Schematic outline of the experimental paradigms. Letters within parenthesis indicate the panels depicting the outcomes of the alternative procedures.

(B) Only Cherry+ cells are visible when no Cre recombinase is expressed.

(C) Cells within and outside of skeletal muscle are YFP+ when Cre recombinase is under the control of the ubiquitous CMV promoter.

(D) Only cells in skeletal muscle are YFP+ when Cre is under the control of the muscle-specific MCK promoter. Dual Cherry and YFP expression indicates that not all copies of the loxP construct have converted to YFP.

(E) YFP+ nuclei are located within the laminin+ basement membrane.

(F) YFP+ nuclei are MEF2C+. Arrows point to colocalization.

(G) Drawing of the location of the transverse sections shown in (H)–(L).

(H) YFP+ nuclei in stump muscle.

(I–K) YFP+ nuclei at the indicated levels (in G) along the proximo-distal axis.

(L) Lack of YFP+ nuclei in the fingertip region.

Dotted lines in (I)–(K) indicate the cartilage boundary. The inserts in (H)–(L) are shown in (H)+-(L) and (H)+-(L).

(M) Graph showing the fraction of labeled myofibers at the indicated levels (in G) along the proximo-distal axis. Data are presented as mean ± SEM (n = 4).

Scale bars: 20 μm. See also Figures S1, S2, and S3.
Figure 2. Myofibers Contribute Proliferating Cells to the Regenerating Newt Limb
(A) Schematic outline of the experimental paradigms. Letters within parenthesis indicate the panels depicting the outcomes of the alternative procedures.
(B) Overview in longitudinal section of a 14-day regenerate shows typical figures of fragmenting skeletal muscle during blastema formation. Dashed line indicates the amputation plane. Asterisks indicate fragmenting myofibers.
(C) Close-up of region C in the overview (B) shows examples of YFP+MHC− mononuclear cells in the distal blastema.
(D) Close-up of region D in the overview (B) shows YFP+ nuclei within and outside of MHC− fragments.
(E) YFP+MHC− and YFP−MHC+ nuclei in a representative cross-section around the stump/blastaema boundary. Arrowheads in (C)–(E) point to YFP+MHC− cells.
(F and G) Example of a PCNA+YFP+ cell (arrowhead) in the blastema.
(H and I) EdU+YFP+ (arrowheads) mononuclear cells in the blastema and EdU−YFP+ (arrows) nuclei within the muscle.
(J) Examples of YFP+EdU+ and YFP−EdU+ myonuclei in MHC− myofiber in the hand region (region K in Figure 1).
(K) Examples of a YFP+EdU+, YFP−EdU−, and YFP−EdU+ nuclei within the laminin+ basal membrane in a myofiber in the hand region.
In (J) and (K), arrows point to YFP+EdU+, arrowheads point to YFP−EdU−, and asterisks point to YFP−EdU+ myonuclei.
Scale bars, (B), (F), (H), (J), and (K): 200 μm; (C–E), (G), (I), (J′), and (K′): 20 μm.
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Muscle Dedifferentiation Does Not Contribute to Limb Regeneration in Axolotl

The axolotl (*Ambystoma mexicanum*), another salamander species commonly used to study limb regeneration, presented additional opportunities to study muscle dedifferentiation. To initially assess whether axolotl myofibers contribute to limb regeneration similarly to the newt, we performed electroporation-based muscle labeling. The MCK::Cre, CAGGS::loxP-ChERRY-STOPloxP-H2B::YFP and CMV::ToI2::transposase expression plasmids were coelectroporated into the axolotl limb (Figure 3A). Electroporated limbs showed robust nuclear YFP expression closely associated with myofibers as visualized by immunocytochemistry against MEF2C (Figures 3B–3D) or muscle-specific MHC (Figure 3E). Labeled limbs were amputated in the upper arm, allowed to regenerate fully, and then visualized both by whole-mount imaging at 33 dp (n = 5, Figure 3F) and by sectioning of entire regenerated limbs at 90 dp. In contrast to the results from the newt, nuclear counts along the sectioned regenerate showed that all visible YFP nuclei (732) were restricted to the upper arm stump of the host animal of the other genotype (“Axolotl LB-transplant,” Figure 4A). We therefore counted Cherry+ nuclei that showed very strong versus weaker signal. Out of 879 counted nuclei, two were found to be positive for the nuclear muscle marker MEF2C or the satellite cell marker PAX7. For a total of 513 Cherry+ nuclei counted, 509 were found to be MEF2C+ (Figures S5E–S5H, n = 11 limbs). Conversely, for a total of 861 Cherry+ nuclei counted, two were found to be positive for Pax7 (Figures S5I–S5L, n = 9 limbs). These results indicate that the cell labeling based on fusion of blastema cells with host cells during limb redifferentiation is muscle specific.

We then assessed the percentage of nuclei in the Cherry+ myofibers that had the genotype CAGGS::loxP Cherry. In mature myofibers, transcripts and proteins show enrichment close to the nucleus producing a given transcript (Rossi et al., 2000). We therefore counted Cherry+ myofiber nuclei that showed very strong versus weaker signal. Out of 879 counted nuclei (six animals), 43% ± 10% of nuclei showed very high nuclear Cherry+ signal (Figures S5M–S5P). This data indicates that we had an efficient conversion of the loxP cassette and a good yield in Cherry-expressing nuclei in limb myofibers.

To determine the contribution of myofibers to the limb regenerate, we amputated the labeled limbs in the upper arm and allowed regeneration to occur. Limbs were visualized by whole-mount microscopy and cross-section. Whole-mount visualization by widefield microscopy indicated that the visible Cherry+ myofibers were restricted to the upper arm of the regenerate and no signal was observed in the lower arm and hand (Figures 4D and 4E; Figures S6A–S6C, n = 4). Fluorescence intensity measurements along the proximal-to-distal limb axis showed high levels of Cherry+ fluorescence in the upper limb segment up to the amputation plane, while in the lower arm and hand, fluorescence levels dropped to those matching the contralateral
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**Muscle Dedifferentiation in Limb Regeneration**

**Figure A**

- **CMV Transposase**
  - Tol2 CAG 
  - MCK Cre 
  - Electroporation in larvae

- **Analysis**
  - Amputation day 0
  - Wholemount analysis day 33
  - Sections analysis day 90

- **Electroporation in metamorphic animals**
  - Amputation analysis day 3
  - Analysis day 90

**Legend**

- **CMV:Transposase+CAG:loxPCherry-STOPloxP-h2bYFP + MCK:Cre**

**Figure B**

- YFP MEF2C DAPI
- YFP ME2FC
- YFP MHC DAPI

**Figure F**

- Direct Electroporation
- 33 dpa

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control limb that had not received a blastema transplant (Figure 4F; Figure S6D). We also cross-sectioned regenerated limbs and quantitated the percentage of MHC* myofibers that were Cherry* at different levels along the amputated limb (Figures 4G–4K, n = 3). At the amputation plane, an average of 21% ± 15% of MHC* myofibers strongly expressed Cherry. In contrast, we observed no MHC-Cherry myofibers in the lower arm and hand in the regenerates. These tracing results using germline transgenic animals confirm that myofibers make no detectable contribution to the regenerated axolotl limb. Though we had not observed any labeled muscle in the regenerated limb, we examined the limb blastema for any possible evidence of muscle-derived mononucleate, proliferative cells as was found in the newt. We examined longitudinal sections of 10-day blastemas derived from LB-transplant animals. In contrast to the newt data, we found no labeled cells in the distal portion of the axolotl limb blastema (Figure S7A, n = 9). Among nine samples examined, all Cherry* fragments that potentially represented mononucleate cells were located close to the amputation plane and none were found in the mid or distal blastema. Only 164 out of 580 Cherry* fragments colocalized with a Hoechst* nucleus (Figures S7A–S7C). Furthermore, none of the Cherry* signal was associated with a PCNA* nucleus (Figures S7A–S7C) and no Cherry* signal was found colocalizing with Pax7* blastema cells (Figures S7D–S7F). In summary, our results indicate that axolotl myofibers undergo considerable morphological changes at the amputation plane, but we found no evidence for their contribution to proliferative progenitor cells.

PAX7* Cells Regenerate Muscle in Axolotl

Considering the lack of myofiber contribution to the axolotl limb regenerate, we searched for the source of cells for muscle regeneration. Previously, labeling of limb myofibers plus satellite cells via embryonic transplantation (green fluorescent protein labeled presomatic mesoderm transplant; GFP-PSM) resulted in robust contribution of GFP* cells to the regenerated limb muscle (Kragl et al., 2009; Nacu et al., 2013). We confirmed here that the GFP* nuclei were MEF2C* (83% ± 2.7%) and Pax7* (12% ± 2.3%) (n = 4, 277 ± 33 cells per section) (Figures 5A–5E). Since our above data indicated that myofibers make no contribution to the limb regenerate, this observation indicates that PAX7* satellite cells are a major contributor to muscle regeneration in axolotl. To confirm the participation of Pax7* cells in regeneration, we traced cells from the GFP-PSM labeled limbs (myofibers + satellite cells) into the blastema and found many GFP* cells in the blastema (Figures 5F–5H, n = 5). Out of a total of 834 GFP* blastema cells, 809 expressed Pax7 protein. This is in contrast to the labeling of Cherry* myofibers alone (from the LB-transplants) that gave no colocalization of muscle-derived Cherry* signal with Pax7* cells in the blastema (Figures S7D–S7F). These results indicate that Pax7* cells are quantitatively the major contributors to muscle regeneration in the axolotl.

To further assess the proliferating status of the PSM-derived GFP* cells in the blastema, we injected EdU in pulses prior to blastema collection, resulting in significant incorporation of the nucleotide analog in the blastema (Figure 5I). We corroborated colocalization of the nucleotide analog EdU with GFP in different areas of the blastema (Figures S5J and S5K). Furthermore, we immunostained for PCNA and found abundant GFP* cells expressing this proliferation marker (Figure 5L). Taken together our data indicate that Pax7* satellite cells from the mature limb produce proliferative, Pax7* muscle progenitors of the limb blastema. In the axolotl, proliferating Pax7* cells are found abundantly and broadly distributed in the midbud limb blastema, consistent with their role as the muscle progenitors for limb regeneration (Figures S6G and S6H). In contrast, by midbud blastema stages the adult and larval newt limb blastema was devoid of Pax7* cells (Figures 6A–6F), although Pax7* cells had been described in the very early stages of newt limb regeneration (Morrison et al., 2006), probably representing satellite cells activated by muscle injury. We further compared the molecular profile of axolotl versus newt cells by examining myogenic determinants in isolated YFP* and GFP* cells from the newt and axolotl blastemas, respectively, by RT-PCR. These experiments confirmed that Pax7 was not expressed in YFP* cells arising from dedifferentiation in the newt blastema while Pax7 was expressed in GFP* cells isolated from satellite-cell-derived axolotl limb blastema cells (Figure 6I). Interestingly, cells from both axolotl and newt blastemas expressed Myf5 but not two other myogenic determinants, Myogenin and Mhf4 (Figure 6I). These results highlight fundamental differences in the cellular composition of the axolotl and newt limb blastema.

To address whether the difference between the two species reflects the special neotenic character of the axolotl, we forcibly metamorphosed axolotls and then analyzed limb muscle regeneration (Figure 7). Postmetamorphic axolotls were electroporated to specifically label limb myofibers as described for Figure 1 and Figure 3. We examined cross-sections of electroporated mature limbs and confirmed that only myofiber nuclei were expressing YFP. When we examined regenerated limbs for contribution of labeled muscle to the regenerate, we only observed YFP* cells proximally to the amputation plane (n = 3, 190 nuclei) (Figures 7A–7C). To confirm this result, we metamorphosed animals that had been LB transplanted and tamoxifen-injected as described in Figure 4 (Figure 7D). Upon amputation, the
regenerated limb showed no muscle labeled in lower limb or hand (Figure 7E, n = 4). Longitudinal sections of the limb regenerate confirmed the lack of Cherry⁺ myofibers in the lower limb or hand muscle (Figures 7F–7H).

**DISCUSSION**

Our fate mapping experiments showed an unexpected difference in the occurrence of myofiber dedifferentiation during limb regeneration in two salamander species. In the newt, labeled myofibers generated PAX7⁺, proliferative cells in the blastema that contributed exclusively to regenerated myofibers. Indeed, the newt limb blastema is essentially devoid of PAX7⁺ cells except for proximal regions at very early stages after amputation. In axolotl, labeled myofibers gave rise neither to proliferative cells in the blastema nor to regenerated myofibers at later stages. In contrast, our muscle grafting data indicate that axolotl limb muscle regeneration occurs by the recruitment of abundant PAX7⁺ cells from the mature tissue into the blastema, where they proliferate. The vast majority of myogenic blastema cells express PAX7 and are derived from PAX7⁺ satellite cells.

We have performed parallel electroporation experiments in the two species and obtained clearly different contributions of myonuclei to the regenerating limb. The lack of myofiber contribution in axolotl was confirmed using germine transgenically integrated cassettes where the efficiency of muscle labeling was at least as high as in the newt experiments: 9% of MHC⁺ myofiber nuclei expressed the labeling cassette in the axolotl experiments, compared to 5% in the newt. Another consideration was the life cycle of the animals. The axolotl is a neotenic animal in which larval features such as the gills are retained throughout life, and a question was whether the axolotl tracing results reflected a larval mode of regeneration. However, no contribution of labeled myofibers to the regenerate was observed in the postmetamorphic axolotl. Complementarily, no PAX7⁺ cells were found in the larval newt midbud blastema, revealing concrete molecular differences in the composition of the limb blastema between the two species.

In newt, myofiber-derived cells contributed to regenerated muscle and we so far found no evidence of contribution to cartilage, consistent with our RT-PCR data showing that dedifferentiated YFP⁺ cells in the newt blastema express Myf5 mRNA. Previous experiments tracking clonally cultured newt satellite cell progeny after limb implantation described contribution to cartilage in addition to skeletal muscle (Morrison et al., 2010). On the other hand, tracking of endogenous muscle and satellite cells in the axolotl showed no contribution to cartilage. We currently do not know whether newt satellite cells truly have a unique, broad potential to form cartilage, or whether experimental circumstances due to the culturing and implantation of the newt satellite cells could have influenced their properties. In vivo tracing studies specifically targeting endogenous satellite cells would clarify this issue. At present it is technically not possible in the newt because we were unable to transfekt/ label endogenous satellite cells in the newt limb. Similarly, in the future, it would be important to exclusively lineage trace satellite cells in axolotls to confirm their role in muscle regeneration, and to characterize the active transcriptional programs in these cells.

Newts and axolotls were separated from each other approximately 100 million years ago (Steinfartz et al., 2007). Although the ability of adult limb regeneration is a unique feature of salamanders among tetrapods (Simon and Tanaka, 2013), our observations suggest that microevolutionary selection pressures have led to divergent implementation of muscle dedifferentiation in these two species. It is important to note that adult newts can regenerate the lens of the eye by dedifferentiation of the pigmented epithelial cells of the iris (Grogg et al., 2005). Unlike newts, axolotls are able to regenerate the lens only during an early developmental time window of 2 weeks starting at the limb bud stage (Suetsugu-Maki et al., 2012).

The capacity to regenerate complex body parts is limited among vertebrates but not exclusive to salamanders: zebrafish can regrow amputated fins and larval frogs regenerate their limbs and tails. Cell tracking experiments in these animals showed varying manifestation of cellular dedifferentiation. New muscle arises from satellite cells and not from preexisting myofibers during tail regeneration in tadpoles (Gargioli and Slack, 2004; Rodrigues et al., 2012). In contrast, fin regeneration involves dedifferentiation of osteoblasts and heart regeneration involves...
Figure 5. Tracing Using Presomitic Mesoderm Transplants in Axolotls Shows that PAX7+ Cells Contribute to the Limb Blastema

(A) Labeling and tracking of PAX7+ cells during limb regeneration. To label limb myofibers plus satellite cells, presomitic mesoderm (PSM) was transplanted from a GFP transgenic embryo to a white (nontransgenic) host and allowed to develop limbs. Schematic image of a PSM transplanted animal is shown. After amputation through a labeled region, a limb with green muscle and satellite cells is regenerated.

(B–E) Cross-section of the mature limb from PSM-labeled animal immunostained for MEF2C, a transcription factor expressed in differentiated muscle cells, and PAX7, a transcription factor found in the muscle satellite cells. Arrows label PAX7+GFP+ cells while arrowheads label MEF2C+GFP+ cells. Asterisks show nuclei negative for any labeling.

(F–H) A 10-day upper arm blastema (longitudinal section) from a PSM-labeled animal. All GFP+ cells in the blastema are PAX7+ (arrowheads). GFP (F), PAX7 (G).

(I) Longitudinal section of a 10-day blastema from a PSM transplant immunostained for GFP and EdU. Solid line shows amputation plane.

(J–J”) High magnification of inserts in (I) showing colocalization of GFP+ cells with EdU (arrowheads).

(K–K”) Colocalization of GFP+ cells and PCNA in the 10-day blastema (arrowheads).

Scale bars: (B)–(H) and (J)–(L): 50 μm; (I): 100 μm.
Figure 6. Abundance versus Lack of PAX7+ Cells in Axolotl and Newt Blastemas, Respectively

(A–E) Distribution of PAX7+ cells in the adult newt limb mesenchyme 14 days after amputation shows lack of PAX7 expression in the blastema. Note the background staining in the skin. (B–E) Distribution of PAX7+ cells in relation to myofibers (indicated by laminin staining) and to YFP+ cells deriving from muscle (C, blastema, D and E, limb stump). Arrows point to PAX7+ nuclei and arrowheads point to YFP+/PAX7− nuclei. Dashed lines indicate the amputation plane. (F) Absence of PAX7+ cells in the premetamorphic, larval newt limb blastema. Images show the distribution of PAX7+ cells in a 12-day-old blastema. Dashed lines indicate the amputation plane. (G) Abundance of PAX7+ cells in the premetamorphic, larval axolotl limb blastema. Images show the distribution of PAX7+ cells in a 10-day-old blastema. Dashed line indicates the amputation plane. (H) PCNA-expressing PAX7+ in the axolotl limb blastema. (I) Difference in molecular profile of myofiber-derived blastema cells from newt with PSM-derived blastema cells from axolotl. PCR was performed with cDNA from the tail, stump muscle, and blastema YFP+ or GFP+ cells from newt and axolotl, respectively. Pax7 is not expressed in myofiber-derived cells in the newt blastema, whereas it is expressed in axolotl muscle-derived blastema cells. Other myogenic factors show similar regulation in newt and axolotl.

Scale bars: (A), (F), and (G): 200 μm; (C)–(E) and (H): 20 μm.
proliferation of cardiomyocytes in zebrafish (Jopling et al., 2010; Kikuchi et al., 2010; Knopf et al., 2011; Singh et al., 2012; Sousa et al., 2011). These observations along with recent cell tracing work in other vertebrate and invertebrate model organisms underscore the existence of a variety of cellular processes for blastema formation during regeneration (Eisenhoffer et al., 2008; Wagner et al., 2011). Understanding the underlying mechanisms may have significant implications for regenerative medicine (Blau and Pomerantz, 2011).

Taken together, our results demonstrate the flexibility and diversity of cellular mechanisms, even among salamanders, used to arrive at successful regeneration, implying that multiple strategies are feasible for inducing muscle and limb regeneration in adult tetrapods.

**EXPERIMENTAL PROCEDURES**

**Animals and Procedures**

Red-spotted newts, Notophthalmus viridescens, were supplied by Charles D. Sullivan Co. (Nashville, TN, USA). Axolotls, Ambystoma mexicanum, were bred in our CRTD facility. Animals were anesthetized by being placed in an aqueous solution of ethyl 3-aminobenzoate methanesulfonate (Sigma). The CAGGS:loxP-GFP-STOPloxP-Cherry and CAGGS:ert2-cre-ert2-T2A-nucGFP

Figure 7. No Contribution of Myofiber-Derived Cells to Regeneration in Postmetamorphic Axolotl

(A–C) Colocalization of YFP* nuclear signal with MEF2C in the upper limb proximal to the amputation plane in regenerated limbs in postmetamorphic animals. (D–D') Animals with limbs labeled by LB transplants were injected with thyroxine to induce metamorphosis and amputated in the upper limb. Fluorescence and brightfield image of a limb from a metamorphic animal with myofibers labeled is shown. White line marks the amputation plane. (E and E') Fluorescence and brightfield image of the regenerated limb. (F–H) Longitudinal section of the metamorphic regenerated limb immunostained for MHC. Cherry* myofibers are found only in the upper arm and not in the lower arm or hand.

Scale bars, (A)–(C): 50 μm; (D)–(H): 1 mm.
transgenic axolotls were generated via Scel meganuclease assisted plasmid injection, as described in Khattak et al. (2009), Sobkow et al. (2006), and the Supplemental Experimental Procedures. All surgical procedures were performed according to the European Community and local ethics committee guidelines.

**Injections and Electroporations**

Plasmid preparation and injection procedures are provided in the Supplemental Experimental Procedures.

**Blastema Transplants**

**Donor Blastema Formation**

The left or right forelimb of each animal was cut in the midupper arm and bone was trimmed to allow wound epithelium to form properly and to allow blastema to form.

**Blastema Transplantation**

The donor blastema was sliced off at day 6 and transplanted to an ipsilateral amputated upper limb. The transplanted blastema naturally adhered to the host due to the presence of clotted blood at the transplantation site. Limbs were allowed to fully regenerate. Different adhered to the host due to the presence of clotted blood at the ipsilateral amputated upper limb. The transplanted blastema naturally formed according to the European Community and local ethics committee guidelines.

**Muscle Dedifferentiation in Limb Regeneration**

**Tissue Dissociation and RT-PCR**

Newt limbs were dissociated according to Morrison et al., 2006. Newt and axolotl blastemas were dissociated into single cells (Kragl et al., 2009), and YFP+ and GFP+ cells were picked up for further RT-PCR analysis. The tissue dissociation and RT-PCR procedures are provided in the Supplemental Experimental Procedures.

**Supplemental Information**

Supplemental Information for this article includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2013.11.007.

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**References**


