Studies on the Cellular Origin of Limb Regeneration in the Newt

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Abbreviations

bp: Base pair

CAGGs: Chicken beta actin promoter with CMV-IE

CarA: Frog (*Xenopus laevis*) cardiac actin promoter

Col IV: Collagen type IV

$CreER^{T2}$: Modified Cre recombinase containing the estrogen receptor T2

DAPI: 4,6-Diamidino-2-phenylindole

DAB: 3,3'-Diaminobenzidine-tetrahydrochloride

DMSO: Dimethyl sulfoxide

EGFP: Enhanced green fluorescent protein

FA100: 4-Allyl-2-methoxyphenol

mCherry: Red fluorescent protein

HS4: Chicken beta-globin core insulator

IgG: Immunoglobulin G

MHC: Myosin heavy chain

MPC: Muscle progenitor cell

Pax7: Paired box protein 7

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

SMFC: Skeletal muscle fiber cell

Abstract

The newt, a urodele amphibian, is able to repeatedly regenerate its limbs throughout its lifespan, whereas other amphibians deteriorate or lose their ability to regenerate limbs after metamorphosis. It remains to be determined whether such an exceptional ability of the newt is either attributed to a strategy, which controls regeneration in larvae, or on a novel one invented by the newt after metamorphosis. Here I report that the newt switches the cellular mechanism for limb regeneration from a stem/progenitor-based mechanism (larval mode) to a dedifferentiation-based one (adult mode) as it transits beyond metamorphosis. I demonstrate that larval newts use stem/progenitor cells such as satellite cells for new muscle in a regenerated limb, whereas metamorphosed newts recruit muscle fiber cells in the stump for the same purpose. I conclude that the newt has evolved novel strategies to secure its regenerative ability of the limbs after metamorphosis.

1. Introduction

In general, living organisms have the capacity of self-repair or self-regeneration to maintain their body structure and function throughout their life span. However, this ability is varied among organisms. To understand the difference of regenerative ability between species, it would be necessary to consider both their phylogenetic relationships and developmental stages as well as to understand physiological significance of their regenerative abilities.

In tetrapods (four-limbed vertebrates), the newt, a group of the family *Salamandridae* in urodele amphibians, has been recognized as a useful model organism for the study of self-regeneration of our humans' body parts because of its prominent regenerative ability: the newt can repeatedly regenerate various complex tissues or body parts, such as the limbs, the tail (the spinal cord), the jaws, the eye (the retina and the lens) as well as the brain and the heart, throughout its life span, or regardless of metamorphosis or aging. The age-independent regeneration in the newt was first described by Lazzaro Spallanzani, 18th century Italian naturalist (Spallanzani, 1769), and recently proved by Eguchi et al (2011).

Though almost 250 years have passed since Spallanzani's discovery, we have not had any reasonable answers to a profound question why amphibians (we can extend it to tetrapods these days) that have such amazing regenerative ability are the newt only. Among regeneration systems in the newt, researchers have been especially attracted to limb regeneration because it is visible and dynamic. Moreover, the limb is composed of

various types of tissues, such as the skin, muscle, bone and nerve, that must be highly organized to be controlled physiologically by both the peripheral and central nervous systems, providing the most complicated subject in regeneration biology and regenerative physiology. Elucidation of underlying mechanisms and their medical applications are one of the ultimate goals for researchers in this research area.

When the limb is amputated in the newt, a cell mass covered by the wound epidermis, which is referred to as blastema, is formed on the stump and eventually regenerates the lost limb part. It has been long believed that the newt blastemal cells, which are generated through dedifferentiation of cells in the stump, have the ability to regenerate various types of tissues, i.e., multipotency (for review, see Tsonis, 1996). However, it can be said actually that as yet there is no direct evidence because of technical limitations.

Recently, studies in the axolotl, a neotenic species of another urodele family *Ambystomatidae*, brought new explanation to the newt limb regeneration. First, lineage tracing analyses revealed that the potency of limb tissues was highly restricted during regeneration (Kragl et al., 2009). In other words, basically the tissues in regenerated limb originate from the same type of tissues in the stump (i.e., lineage-restricted manner). Second, it was demonstrated that the muscle in regenerated limb did not originate from muscle fibers, but from satellite cells, the endogenous stem/progenitor cells which is known to contribute to muscle growth and repair in both vertebrates and invertebrates (Sandoval-Guzmán et al., 2014). That is, the axolotl adopts a stem/progenitor-based mechanism for limb muscle regeneration. The same group obtained substantially the same results even when the axolotl was artificially

metamorphosed (Sandoval-Guzmán et al., 2014), although its regenerative ability should have strikingly declined after metamorphosis. On the other hand, in adult newt limb regeneration, they obtained evidence suggesting that muscle fibers contributed to limb muscle regeneration whereas satellite cells did not (Sandoval-Guzmán et al., 2014). From these findings, they concluded that the axolotl regenerates new limb muscle from satellite cells while the newt uses muscle fibers for the same purpose, and that the difference in the cellular origin of regenerated muscle between the newt and the axolotl is attributed to species differences, or diversity of urodele amphibians. The axolotl was thus impressed to be a good regeneration model comparable to the newt. However, it is still not determined whether pre-metamorphosed newt limbs employ dedifferentiation of muscle fibers or not. Although the idea of species differences makes sense, it is imperative that other questions are asked.

In this study, to address this issue, I tried to trace the tissue- and cell-lineage of limb regeneration in the newt. The laboratory to which I belong has already established a transgenic protocol for the Japanese fire-bellied newt *Cynops pyrrhogaster* (Casco-Robles et al., 2011). Applying this protocol, first I made the transgenic newts expressing fluorescent protein in their whole body, transplanted the labeled tissues into wild type animals, and tracked the transplanted tissues during limb regeneration. Subsequently, focusing on the muscle I tracked muscle-originating cells in larvae and metamorphosed newts by using a muscle fiber specific promoter and Cre-loxP system.

2. Materials and Methods

2.1. Animals

Japanese fire-bellied newt, *Cynops Pyrrhogaster* (total body-length: 9-12cm) was used in this study. For transplantation experiments, recipient animals were purchased from commercial supplier (Aqua Grace, Yokohama, Japan). Those newts were captured from Okayama and Miyagi Prefecture. For transgenesis experiments, Toride-Imori was used for collecting fertilized eggs based on two-aquarium-tank system (Casco-Robles et al., 2010). Stock animals were kept in polyethylene containers at 18°C under a natural light condition. They fed with frozen Chironomid larva (*Chironomidae*; Fresh Akamushi; Kyorin, Himegi, Japan) and rearing water was exchanged every day. All experiments were carried out in accordance with the guidelines approved by the University of Tsukuba Animal Use and Care Committee.

2.2. Anesthesia

An anaesthetic FA100 (4-allyl-2-methoxyphenol; DS Pharma Animal Health, Osaka, Japan) dissolved in water was used at room temperature (22°C). Before limb amputation or operations for tissue transplantation, larval (St 56–57; 3 months old; Fig. 1A) and adult newts [~16 months old (Fig. 1B) and older than 3 years] were anaesthetized in 0.05% FA100 for 15 min, in 0.05% FA100 for 1 h and in 0.1% FA100 for 2 h, respectively.

2.3. Limb amputation

After having been anaesthetized, animals were gently rinsed in distilled water and lightly dried on a paper towel, and one side of the forelimbs of each animal was amputated under a dissecting microscope (M165 FC; Leica Microsystems, Wetzlar, Germany) by a blade [for larvae, a tip of the blade (catalogue number: 4991482, Feather Safety Razor, Osaka, Japan); for adults ~16 months old and older than 3 years, a surgical blade (No. 14, Futaba, Tokyo, Japan) and microtome blade (C35, Feather Safety Razor), respectively].

Next, the larval amputees were allowed to recover in 1X penicillin–streptomycin (15140–122; Thermo Fisher Scientific, Yokohama, Japan) containing 0.1X modified Holtfreter's solution (Casco-Robles et al., 2011) at 22°C and then reared in the same condition; adult amputees were placed in a moist container and allowed to recover at 14°C for ~15 h, and then reared in the same container at 18–20°C.

To track the skeletal muscle fiber cells (SMFCs) in swimming larvae, I carried out amputation in the middle of the forearm. In juveniles, I carried out the first amputation in the middle of the forearm; after the blastema had formed (30–40 days after amputation) I carried out the second amputation in the middle of the upper arm of the same limb to collect the blastema for histological analysis. These animals were kept for longer than 3 months to examine later stages of regenerating limbs. The primary reason that I adopted a second amputation on the upper arm is that if I amputated on the forearm, it would be difficult to prove that mCherry+ SMFCs participate in muscle regeneration via dedifferentiation, because similar results can be presumed even when

the cells originating from other tissues fuse to the remnant mCherry+ SMFCs in the stump (see Results). Amputation in the middle of the upper arm ensures discontinuity between muscles in the regenerated forearm and those in the stump of the upper arm. The secondary reason is that animals to be used, which must be mosaic (see Results), were limited.

2.4. Transgene constructs

To track tissues by transplantation, I constructed plasmid vectors, pCAGGs>mCherry or EGFP (I-SceI), which carries a transgene cassette flanked by I-SceI meganuclease recognition sites (Fig. 2), using conventional molecular cloning procedures. To minimize possible *cis* interactions between the construct and functional elements on the chromosome, the construct is flanked by chicken β-globin HS4 core insulators (kindly provided from Dr Gary Felsenfeld at the National Institute of Health, Bethesda, MD, USA). In this system, a fluorescent reporter (mCherry or EGFP) can be expressed universally in the newt under the control of a universal promoter (CAGGs) (Casco-Robles et al., 2011).

To track SMFCs by means of transgenesis, I constructed a plasmid vector, pCreER^{T2}<CarA-CAGGs>[EGFP]mCherry (I-SceI), which carries a transgene cassette flanked by I-SceI meganuclease recognition sites (Fig. 3). This cassette comprises two expression constructs in opposite directions: one (CAGGs>[EGFP]mCherry) is a reporter construct by which EGFP, whose gene is flanked by loxP sites and followed by the mCherry gene, can be expressed under the control of CAGGs; the other

(CreERT2<CarA) was designed so that an inactive form of Cre recombinase [Cre-ER^{T2}: Cre fused to a mutated human oestrogen receptor (ER^{T2})] (Feil et al., 1997) can be expressed under the control of a cardiac actin promoter (CarA; from M11 Cardiac Actin Promoter pCarA, Addgene 17148), which is known to be activated in both SMFCs and cardiac muscle fiber cells (Kroll et al., 1996). Each construct is flanked by 2X HS4 core insulators to minimize possible *cis* interactions within the cassette and between the constructs and functional elements on the chromosome.

2.5. Transgenesis

I prepared transgenic newts by the I-SceI protocol (Casco-Robles et al., 2011): one-cell stage embryos were injected with a construct/enzyme mixture [DNA construct, 80 ng μl^{-1}; I-SceI (catalogue number: R06945; New England Biolabs, Tokyo, Japan), 0.5 U μl^{-1}; I-SceI buffer (New England Biolabs), 1X; phenol red, 0.01%] at 1–2 µl per embryo and reared until a juvenile stage beyond metamorphosis. In this study, I used tail bud embryos (St. 24-26), swimming larvae (St 56–57) that had developed forelimbs with full digits (total body length: ~18 mm, age: 3 months; Fig. 1A) and metamorphosed juveniles (total body length: ~6 cm, age: 16 months; Fig. 1B).

2.6. Transplantation of reporter-expressing tissues

CAGGs>reporter transgenic newts of 1–2 months after metamorphosis were used as donors (Fig. 4). The tissues that exhibited intense mCherry/EGFP fluorescence

uniformly were transplanted into a wild-type animal (recipient) by fine scissors, pins and forceps under a fluorescent dissecting microscope (M165 FC; Leica). The muscle and bone were transplanted individually into the corresponding regions of different recipients 3 years old (Fig. 5 and 6). The nerve (Schwann cells) was implanted in between muscles of a forelimb of a 3-year-old recipient (Fig. 7). The skin was grafted to a recipient of the same age (Fig. 8). In these recipients, after the wound was closed (0.5–1 month after surgery), the limb was amputated so that the grafted tissue remained in the stump. In this study, to track the epidermis, I also transplanted the mCherry+ ectoderm on the presumptive forelimb of a tail bud embryo (St 24–26) to a wild-type embryo at the same stage (Fig. 9). In this case, when the recipient reached 6–9 months of age, the forelimb was amputated.

2.7. Screening of transgenic newts for SMFC tracking and induction of Cre-mediated recombination

For SMFC tracking in larval limb regeneration, I selected swimming larvae that expressed EGFP in their whole body almost evenly and treated them at St 47–53 (age: 1–2 months) in a tamoxifen-containing solution [1–3 μM (Z)-4-hydroxytamoxifen (H7904-5MG, Sigma-Aldrich, St Louis, MO; master mix: 100–300 μM in dimethylsulphoxide) in rearing solution (0.1X Holtfreter's solution) (Casco-Robles et al., 2011)] for a few weeks (Fig. 10). For the study of juvenile limb regeneration, I used mosaic animals, which expressed EGFP in the muscle only, as well as non-mosaic ones. Such mosaic animals were selected at the swimming larval stage. The reason that I used

these mosaic animals is to preclude possible contamination of other kinds of cells in SMFC lineage tracing (see Results).

2.8. Tissue preparation

Larval and adult tissues were fixed in 4% paraformaldehyde in PBS (pH 7.5) at 4°C for 4–6 and for 15 h, respectively, washed thoroughly with PBS at 4°C (for larvae, 5 min x 2, 10 min x 2, 15 min x 2, 30 min x 2 and 1 h x 2; for adults, 15 min x 2, 30 min x 2, 1 h x 2 and 2 h) and then allowed to soak in 30% sucrose in PBS at 4°C. The fixed tissues were embedded into Tissue-Tek O.C.T. Compound (4583; Sakura Finetek USA, Inc., Torrance, CA 90501, USA), frozen at approximately 20°C in a cryostat (CM1860; Leica) and sectioned at 20 μm thickness. The sections were stored at ~20°C until use. We obtained ~30, 50–60 and 120–140 dorsoventral sections from larval, juvenile and adult limbs, respectively. For Pax7 immunohistochemistry, larval limbs were fixed in 2% paraformaldehyde/0.2% picric acid in PBS for 1.5 h at 4°C and sections were obtained in the same way; adult limbs were embedded into Tissue-Tek O.C.T. Compound, immediately frozen in the cryostat, sectioned at 20 μm thickness, fixed in methanol/acetone (1:1; methanol: 134–14,523; acetone: 012–00343; Wako Pure Chemical Industries, Osaka, Japan) at 22°C for 5 min, air dried for 1–3 h and then stored at -20°C until use.

2.9. Antibodies

Primary antibodies were as follows: mouse monoclonal anti-Pax7 antibody (1:200; PAX7-c; Developmental Studies Hybridoma Bank, Iowa, IA 52,242–1,324, USA), anti-myosin heavy chain antibody (1:200–336; MF20-c; Developmental Studies Hybridoma Bank), anti-acetylated tubulin antibody (1:1,000; T6793; Sigma-Aldrich, 63103) and rabbit polyclonal anti-collagen type IV antibody (1:500; Code: 600–401–106–0.1; Rockland Immunochemicals, Gilbertsville, PA 19525, USA).

Secondary antibodies were as follows: Alexa Fluor 488-conjugated goat anti-mouse IgG (H + L) (1:500; A11001; Thermo Fisher Scientific), biotinylated goat anti-mouse IgG (1:400; BA-9200; Vector Laboratories, Burlingame, CA 94010, USA) and rhodamine (TRITC)-conjugated affiniPure goat anti-rabbit IgG (H + L) (1:500; Code: 111-025-003; Jackson ImmunoResearch Laboratories, West Grove, PA 19390, USA).

2.10. Immunohistochemistry

Immunofluorescence and immunoperoxidase labelling of tissues were carried out as described previously (Islam et al., 2014). For immunofluorescence labelling, tissue sections were rinsed thoroughly (PBS, 0.2% Triton X-100 in PBS and PBS; 15 min each), incubated in a blocking solution [3% normal goat serum (S-1000, Vector Laboratories)/0.2% Triton X-100 in PBS] for 2 h, washed twice in PBS and then incubated in primary antibody diluted with blocking solution for 15 h at 4°C. After washing thoroughly, the samples were incubated in secondary antibody diluted with blocking solution for 4 h and washed thoroughly.

For immunoperoxidase labelling, thoroughly rinsed tissues were treated with 3.3% H_2O_2 in methanol for 20 min, washed thoroughly, incubated in a blocking solution containing Avidin D (1:50; Avidin/Biotin Blocking kit, SP-2001, Vector Laboratories) for 2 h, washed twice in PBS and then incubated in primary antibody diluted with blocking solution containing Biotin (1:50; Avidin/Biotin Blocking kit) for 15 h at 4°C. After washing thoroughly, the samples were incubated in biotinylated secondary antibody diluted with blocking solution for 4 h, washed thoroughly, incubated in a mixture of Avidin and Biotin Complex (Vectastain ABC Elite kit, PK-6100, Vector Laboratories) for 2 h, washed thoroughly and then incubated in a DAB solution (DAB substrate kit, SK-4100; Vector Laboratories). In both labelling, after the samples were washed, the nuclei of cells were counterstained with 4,6-diamidino-2-phenylindole (DAPI; 1:50,000; D1306; Thermo Fisher Scientific) or TO-PRO-3 Iodide (1:50,000; T3605; Thermo Fisher Scientific). The tissues were immersed into 90% glycerol in PBS or into VECTASHIELD mounting medium (H-1000; Vector Laboratories) and then mounted under a cover slip.

2.11. Microscopy

EGFP/mCherry fluorescence of tissues in living animals was monitored during development, limb regeneration and tissue transplantation under a dissecting microscope (M165 FC; Leica). Specific filter sets for EGFP (Leica GFP-Plant; Exciter: 470/40 nm; Emitter: 525/50 nml) and mCherry (Exciter: XF1044, 575DF25; Emitter: XF3402, 645OM75; Opto Science, Tokyo, Japan) were applied to avoid bleed-through

artefacts. Images were taken by a digital camera (C-5060; Olympus, Shinjuku, Tokyo, Japan) attached to the microscope and stored in personal computers.

Images of tissue sections were acquired either by a charge-coupled device camera system (DP73; cellSens Standard 1.6; Olympus) attached to a microscope (BX50; Olympus) or through a confocal microscope system (LSM510; LSM 5.0 Image Browser software; Carl Zeiss, Germany).

2.12. Image analysis

Images were analysed by software for the image acquisition systems and Photoshop CS5 Extended (Adobe Systems, San Jose, CA). Figures were prepared using Photoshop CS5 Extended. Image, brightness, contrast and sharpness were adjusted.

3. Results

3.1. Lineage trace in adult limb regeneration by transplantation

In axolotl limb regeneration, principal tissues of the limb give rise to the same type of tissues in a lineage-restricted manner. However, the question was left open whether dermis contributes to the cartilage/bones and perhaps vice versa (Kragl et al., 2009). To examine whether the same is true in the adult newt, I carried out lineage-tracing experiments by transplanting reporter-expressing tissues (Fig. 4–9).

In the skin allograft (n=3), the skin contributed to a new skin comprising epidermal tissues such as the stratified epithelium, mucous glands and dermal tissues, and also gave rise to interstitial cells, which were mostly distributed around the cartilage in the regenerated limb (Fig. 11A). On rare occasions (in three serial sections from one particular animal), I found a small number of reporter+ chondrocytes in the digit's cartilage (Fig. 11B). In animals into which the reporter+ ectoderm of a presumptive limb was transplanted at its embryonic stage (n=2), the epidermis gave rise to a number of interstitial cells and to new epidermal tissues (Fig. 11C, D), but not to cartilage. Therefore, the origin of cartilage might as well be dermal cells as suggested in the axolotl6. In the bone allograft experiment (n=4), bone contributed to cartilage/bones (Fig. 11E), but did not contribute to other tissues. Reporter+ chondrocytes were observed in almost all sections containing cartilage (1–20 cells per section). In the muscle allograft (n=3), as anticipated, the muscle contributed to only the muscle (Fig. 11F). On rare occasions (in two sections from one particular animal), I found a small

number of reporter+ chondrocytes in regenerated cartilage. In this case, the grafted tissues may have contained a type of cell that contributed to the cartilage; thus, I regard this result as inconclusive. In the nerve (that is, Schwann cells) implantation (n=3), Schwann cells contributed to reconstruction of the myelin sheath that covered a regenerated nerve fiber (Fig. 11G).

Consequently, in terms of tissue origin, the overall design of limb regeneration seemed to be conserved between the adult newt and the axolotl: the principal tissues in the limb strictly regenerated the same tissue types (Fig. 11H), with a possible exception of dermis contributing to the bone/cartilage.

3.2. Tracking SMFCs by using a muscle fiber specific promoter and Cre-loxP

3.2.1. Evaluation of the system

Strategy. A plasmid vector, pCreER^{T2}<CarA-CAGGs>[EGFP]mCherry (I-SceI) was designed to track SMFCs by means of transgenesis (Fig. 3). If this system works, SMFCs can be labeled before amputation of the limb to identify cells that originate from these cells during limb regeneration because the CAGGs promoter is activated regardless of cell type or dedifferentiation. In fact, in the newt, promoter activity is persistent throughout limb regeneration (Casco-Robles et al., 2011).

In this system, however, the SMFC (a syncytium or multinuclear cell) after tamoxifen treatment should have both EGFP and mCherry because recombination by tamoxifen is generally known to be imperfect (i.e., not all nuclei undergo

recombination) and both tracers can diffuse throughout the cytoplasm along the fiber (Fig. 12). In fact, in all animals (n=40) examined in this study, SMFCs exhibiting mCherry fluorescence also had EGFP fluorescence (fibers expressing mCherry only were very rare) (Fig. 13). On the other hand, mononucleated cells (or dedifferentiated cells from muscle fibers) in the blastema exhibited, as predicted in Figure 12, either EGFP or mCherry fluorescence (see below), corroborating the binary regulation of gene expression in this system.

If the mCherry+ mononucleated cells fuse with other EGFP+ cells to regenerate a muscle fiber, the fiber should show both EGFP and mCherry fluorescence, although the intensity of mCherry fluorescence is dependent upon the number of recombined nuclei in the fiber and the volume of the fiber. In fact, as we showed below, regenerated muscle fibers exhibited fluorescence of both but with variable intensity of mCherry fluorescence.

mCherry expression patterns. Transgenic newts to track SMFCs were prepared by the I-SceI protocol (Fig. 10; see Methods). For the study of larval limb regeneration, we selected swimming larvae which expressed EGFP in their whole body almost evenly (Fig. 13A). For the study of juvenile limb regeneration, we used mosaic animals, which expressed EGFP in the muscle only (Fig. 13B, C; see Methods), as well as non-mosaic ones.

In swimming larvae which exhibited fluorescence of EGFP in their whole body almost evenly, after induction of Cre-mediated recombination by tamoxifen (Fig. 10) we detected mCherry expression in almost all of the SMFCs in the body (Fig. 13A) as

well as in the heart (not shown), but not in other body parts. Note that EGFP-/mCherry+ mononucleated cells like those observed in late regenerating limbs (see below) were never observed in those animals (n=40; Table 1). After metamorphosis, as the muscle grew, EGFP+/mCherry- striated fibers appeared in the muscle and increased in number. As a result, in juveniles (16 months), EGFP+/mCherry+ fibers became the minority (in the forelimb, only 21-26% of total muscle fibers expressed mCherry; Fig. 13C).

Recombination efficiency. To examine the recombination efficiency of this system, I harvested EGFP+/mCherry+ SMFCs-containing muscles from the forelimbs of juvenile newts instead of those of larvae because the amount of muscles/DNA per animal was very small in the larval stage. We purified genomic DNAs (NucleoSpin® Tissue XS; MACHEREY-NAGEL GmbH & Co., Germany) and carried out PCR with a primer set (forward: 5'-acagctcctgggcaacgtgctggttgt-3'; reverse: 5'-ccggtggagtggcggccctcggcgc-3') designed to amplify a 1,836 bp region containing the floxed EGFP sequence (841 bp) in the transgene cassette. PCR results and DNA sequencing revealed that recombination to ligate the mCherry gene to the CAGGs promoter had taken place in skeletal muscle (Fig. 14), although the ratio of the transgene cassettes, which lost their floxed EGFP sequence, was low (8.2%). Thus, the recombination efficiency seemed to be low but as for one SMFC this value is an underestimate because 74-79% of the juvenile muscle fibers did not express mCherry (see above). It should be noted that in this system recombination had also taken place in the heart (recombination efficiency in cardiac muscle was about 50%), but was not detected in other body parts such as the brain (Fig. 14).

3.2.2. SMFC tracking in larval newt limb regeneration

In larval newts at St 56–57 (~3 months old; Fig. 1A), whose forelimbs had grown with four digits, I found that SMFCs did not contribute to restoration of the missing part of the forelimb after amputation. When mCherry was monitored in living animals, it did not appear in the regenerating part of the limb (neither as cells from fragmented fibers nor as fibers) until ~30 days when the amputated limb structure had almost been restored (Fig. 15, 16; see Movie 1). I subsequently examined satellite cells. I carried out immunohistochemistry with Pax7 antibody (a marker of MPCs including satellite cells) (Sandoval-Guzmán et al., 2014) and found that Pax7-immunoreactive (Pax7+) mononucleated cells appeared in the blastema by day 12 post amputation (Fig. 17A-C; 3–17 cells per blastema, n=3) and increased in number in the next few days (Fig. 17D; 55–144 cells per blastema, n=3), suggesting that MPCs (potentially satellite cells) were recruited for new muscle during limb regeneration. Thus, these results suggest that SMFCs were not the primary source of new muscle in larval newts. Consequently, they also revealed close similarity between the larval newt and the axolotl, at least in terms of muscle regeneration (Fig. 18).

3.2.3. Appearance of mCherry in late limb regeneration

However, as differentiation of digits started taking place, an mCherry+ area became detectable along the ventral side of radius/ulna (Fig. 19; Table 1). This area was characterized by the presence of mononucleated mCherry+ cells (they were Pax7-; Fig. 20). Such a population was never observed in the body of animals after tamoxifen

treatment (n=40). By day 26, these cells occupied the same area as the differentiating flexor muscle for digits. It must be noted here that at this stage there seems to be no overlap in mCherry and the flexor muscle. Therefore, I believe that these mononuclated cells did not give rise to the flexor muscle (Fig. 19A, B). The identity of these cells is not known, but a possibility of mononucleated SMFCs is excluded based on my results of mCherry monitoring throughout the period of limb regeneration (Table 1 and Movie 1). I would like to propose that they might be fibroblasts. My conviction stems from the fact that during axolotl muscle-less limb regeneration the tendons that transduce the action of flexor muscle to the digits are generated, and that condensations of dermal fibroblast are accumulated within the area where normally the flexor muscle would be (Holder et al., 1989). Interestingly, the fibroblast condensations are in an oblique orientation to the flexor muscle, very similar to the one observed in my sections as well. However, by day 48 mCherry+ mononucleated cells were not anymore present, but instead the fibers of the flexor muscle were now positive for mCherry (Fig. 19C, D). This surprising result indicates either that at a later stage these mononucleated cells were differentiated to muscle fibers or that they fused with the fibers. At any rate, my observations clearly indicate that not all muscles derive from one source in newt larvae limb regeneration. My results also suggest that these fibroblast-like cells activate the cardiac actin promoter as well, and undergo recombination in the absence of tamoxifen. The mechanism underlying this tamoxifen-independent recombination is not known, but it is known even in mammals that inducible Cre may spontaneously be activated by endogenous steroid hormones (Feil et al., 1997). Importantly, such a mechanism seems to be elicited specifically in the fibroblast-like cells, because flexor muscle fibers, which

have differentiated before the fibroblast-like cells become mCherry+, do not express mCherry.

3.2.4. SMFC tracking in metamorphosed newt limb regeneration

I performed the same experiments in newts after they metamorphosed (~16 months old or ~10 months after metamorphosis) (Fig. 1B and 21). However, in these experiments, to preclude a possibility of contamination of other kinds of cells (such as those similar to the origin of fibroblast-like cells in larval stages), I adopted mosaic animals in which EGFP/mCherry expression is restricted to SMFCs. In addition, I carried out a first amputation in the middle of the forearm, and after the blastema has been formed (30–40 days after amputation) I carried out a second amputation in the middle of the upper arm of the same limb to collect the blastema for analysis. I then kept these animals for longer than 3 months, to examine later stages of regenerating limbs. Amputation on the upper arm ensures discontinuity between muscles in the regenerated forearm and those in the stump of the upper arm (see Methods).

I found that SMFCs were mobilized to regenerate new muscle (Fig. 22). In this stage, the ratio of muscle fibers expressing mCherry to all muscle fibers in the forelimb was low (21–26%, n=4). Histological examination revealed that fragments of muscle fibers appeared near the stump, and that mononucleated cells expressing mCherry were present in the blastema (Day 36, n=2; Fig. 22A, B; also see Fig. 23 and Movie 2, which shows a three-dimensional image of fragments from EGFP+ fibers near the stump and EGFP+ mononucleated cells in the blastema in another section of the same regenerating

limb). Importantly, a number of mCherry+ SMFCs were found in regenerated muscle (Day 96, n=2, Fig. 22C), suggesting that SMFCs in the stump dedifferentiated to form the blastema and redifferentiated to the SMFCs themselves. It must be noted that the mCherry signal was not detected in other types of cells. Moreover, the muscle in the stump, which could be tracked by EGFP, gave rise to only the regenerated muscle, but not to other tissues. Next, I examined whether satellite cells participate in limb regeneration. Pax7 immunoreactivity was not detected in the blastema even though Pax7+ satellite cells were clearly observed along SMFCs in a more proximal part to the amputation plane (Fig. 24).

Consequently, my results suggest that the origin of muscle changed from stem/progenitor cells to SMFCs in the newt as development proceeded beyond metamorphosis (Fig. 25). This result is different from the one received when axolotls were forced to metamorphose (Sandoval-Guzmán et al., 2014). In that experiment, SMFCs did not contribute to regenerated muscle.

4. Discussion

This study provides evidence that the newt is capable of recruiting both Pax7+ MPCs, such as satellite cells, and SMFCs, to restore muscle during limb regeneration. However, the former was the source in the larval stage and the later in the adult stage. In other words, a stem/progenitor-based mechanism is taken over by a dedifferentiation-based one as development proceeds beyond metamorphosis. Therefore, differences in the origin of new muscle between the newt and axolotl should not be attributed to differences in species but rather explained by differences related to developmental processes. It remains to be studied whether the switching of cell source takes place abruptly or gradually after metamorphosis. It must be noted here that lineage tracing of Pax7+ satellite cells cannot be achieved at this point with the technical limitations in newts. The genome of the newt is not sequenced yet and the Pax7 locus is not available; thus, production of transgenic newts is not possible at this point. The axolotl displays neoteny, heterochronically preserving its larval properties even after sexual maturity. Interestingly, in metamorphosed axolotls, satellite cells still contribute to the blastema but there is no SMFCs contribution (Sandoval-Guzmán et al., 2014), and this could be viewed either as a difference among species, as a difference in time after metamorphosis (in axolotls, maybe <10 months) or as a result from a virtual condition for metamorphosis (hormone injection), which may have been insufficient for the switching of cell source or inhibited it. Importantly, my results clearly indicate a degree of plasticity that urodele amphibians could exhibit to achieve regeneration. It is interesting to note here that in a recent paper it was demonstrated that although preaxial

dominance is characteristic of limb regeneration in the larval newt, it changes after metamorphosis with anterior and posterior digits forming together (Kumar et al., 2015). This is another example of plasticity in limb regeneration mechanism depending on metamorphosis.

Most amphibians studied thus far can regenerate limbs in the larval stage, but after metamorphosis this ability deteriorates or is lost (Tsonis et al., 1996). This is true for the axolotl whose metamorphosis can be induced by thyroid hormone (Monaghan et al., 2014). In contrast, the newt can regenerate limbs regardless of metamorphosis. In general, for animals whose sources of cells for tissue repair and regeneration (for example, stem/progenitor cells and premature cells which still have potency to change their fate) decrease in number and/or activity during development, it would be advantageous to have the ability to recruit comparable cells through dedifferentiation or reprogramming of mature cells. In amphibians, stem/progenitor cells for muscle regeneration may decrease or deteriorate, or specialize to muscle growth/repair during metamorphosis when the body's system is drastically remodelled to adapt to a terrestrial environment. In fact, in adult newt limbs, the ratio of satellite cells to SMFC nuclei decreases (mean ± s.d.: larvae, 14.7 ± 4.9%, n=8; adult, 9.3 ± 4.8%, n=5; Student's *t*-test, P=0.039). However, plenty of satellite cells are still present in the muscle. Therefore, the property of satellite cells and/or surroundings of them that allow them to mobilize into the blastema must change during or after metamorphosis. In a frog (*Xenopus laevis*), it has been suggested that growth factors such as hepatocyte growth factor which recruit satellite cells into the blastema may decrease during metamorphosis (Sato et al., 2005). It may be valuable to examine whether this is true for

metamorphosed newts. Possibly, dedifferentiation of SMFCs evolved to enable SMFCs to regenerate the muscle more efficiently in such conditions of adults. In a recent paper (Yun et al., 2015), it was shown that newts have the ability to clear senescent cells, so that they do not interfere with regeneration. This mechanism could account for the ability of the newt to regenerate many times (Eguchi et al., 2011) at old ages as well. It should also be noted that generation of progenitor cells by dedifferentiation of muscle fibers during adult newt limb regeneration could be attributed to programmed cell death. However, it will be interesting to see whether that is the case in larvae limb regeneration as well (Wang et al., 2015).

In summary, both newts and axolotls seem to have the same basic master plan for limb regeneration: tissues in the regenerating limb originate from the parental ones. However, when it comes to muscle, the newt switches from stem/progenitor cells to SMFC dedifferentiation as it transits from metamorphosis. This could bear significance for the loss of regenerative ability in metamorphosed amphibians, such as frogs and other urodele species. Frogs lose their ability for limb and lens regeneration after metamorphosis (Henry et al., 2010). Axolotls also lose their ability for lens regeneration as they develop (Suetsugu-Maki et al., 2012). Perhaps different animals invent new strategies or modify existing ones to suit their regenerative needs. It is interesting to speculate here that the newt overcame loss of limb regeneration after metamorphosis by resorting in part to dedifferentiation. Delineating the mechanisms of these strategies will undoubtedly provide clues for regeneration in other species including mammals.

5. References

Casco-Robles, M. M., Yamada, S., Miura T. and Chiba, C. (2010) Simple and efficient transgenesis with I-SceI meganuclease in the newt, *Cynops pyrrhogaster*. *Developmental Dynamics* 239: 3275-3284.

Casco-Robles, M. M., Yamada, S., Miura, T., Nakamura, K., Haynes, T., Maki, N., Del Rio-Tsonis, K., Tsonis, P. A. and Chiba, C. (2011) Expressing exogenous genes in newts by transgenesis. *Nature Protocols* 6: 600-608.

Eguchi, G., Eguchi, Y., Nakamura, Y., Yadav M. C., Millán, J. L. and Tsonis, P. A. (2011) Regenerative capacity in newts is not altered by repeated regeneration and ageing. *Nature Communications* 2, 384.

Feil, R., Wagner, J., Metzger, D. and Chambon, P. (1997) Regulation of Cre recombinase activity by mutated estrogen receptor ligand-binding domains. *Biochemical and Biophysical Research Communications* 237, 752–757.

Henry, J. J. and Tsonis, P. A. (2010) Molecular and cellular aspects of amphibian lens regeneration. *Progress in Retinal and Eye Research* 29, 543–555.

Holder, N. (1989) Organization of connective tissue patterns by dermal fibroblasts in the regenerating axolotl limb. *Development* 105, 585–593.

Islam, M. R., Nakamura, K., Casco-Robles, M. M., Kunahong, A., Inami, W., Toyama, F., Maruo, F. and Chiba, C. (2014) The newt reprograms mature RPE cells into a unique multipotent state for retinal regeneration. *Scientific Reports* 4, 6043.

Kragl, M., Knapp, D., Nacu, E., Khattak, S., Maden, M., Epperlein, H. H. and Tanaka, E. M. (2009) Cells keep a memory of their tissue origin during axolotl limb regeneration. *Nature* 460, 60–65.

Kroll, K. L. and Amaya, E. (1996) Transgenic Xenopus embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation. *Development* 122, 3173–3183.

Kumar, A., Gates, P. B., Czarkwiani, A. and Brockes, J. P. (2015) An orphan gene is necessary for preaxial digit formation during salamander limb development. *Nature Communications* 6, 8684.

Monaghan, J. R. Stier, A. C., Michonneau, F., Smith, M. D., Pasch, Bret., Maden, M. and Seifert, A. W. (2014) Experimentally induced metamorphosis in axolotls reduces regenerative rate and fidelity. *Regeneration* 1, 2–14.

Nakamura, K., Islam, M. R., Takayanagi, M., Yasumuro, H., Inami, W., Kunahong, A., Casco-Robles, R. M., Toyama, F. and Chiba, C. (2014) A transcriptome for the study of early processes of retinal regeneration in the adult newt, *Cynops pyrrhogaster*. *PLoS One* 9, e109831.

Sandoval-Guzmán, T., Wang, H., Khattak, S., Schuez, M., Roensch, K., Nacu, E., Tazaki, A., Joven, A. Tanaka, E. M. and Simon, A. (2014) Fundamental differences in dedifferentiation and stem cell recruitment during skeletal muscle regeneration in two salamander species. *Cell Stem Cell* 14, 174–187.

Sato, A., Ide, H. and Tamura, K. (2005) Muscle formation in regenerating Xenopus froglet limb. *Developmental Dynamics* 233, 337–346.

Spallanzani, L. (1769) Prodromo di un opera da imprimersi sopra la riproduzioni animali [An Essay on Animal Reproductions (1769), translated from Italian by M. Matty, 1769, *Kessinger Publishing* (2010).]

Suetsugu-Maki, R., Maki, N., Nakamura, K., Sumanas, S., Zhu, J., Del Rio-Tsonis, K. and Tsonis, P. A., (2012) Lens regeneration in axolotl: new evidence of developmental plasticity. *BMC Biology* 10, 103.

Takaya, H. (1941) Experimental study on limb-asymmetry. *Zoological Science* 20, 181-279.

Tsonis, P. A. (1996) Limb Regeneration. *Cambridge Univ. Press.*

Wang, H., Lööf, S., Borg, P., Nader. G. A., Blau. H. M. and Simon, A. (2015) Turning terminally differentiated skeletal muscle cells into regenerative progenitors. *Nature Communications* 6, 7916.

Yun, M. H., Davaapil, H. and Brockes, J. P. (2015) Recurrent turnover of senescent cells during regeneration of a complex structure. *eLife* 4, e05505.

6. Figures and Legends

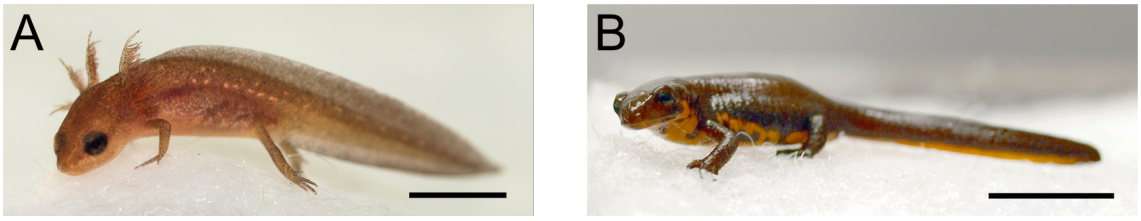

Figure 1. Larval and juvenile newts. (A) Larva (3 months old). It has four limbs, as well as the gills and tail fin. Scale bar, 4 mm. (B) Juvenile (16 months old). Scale bar, 15 mm.

[Adapted from Tanaka H.V., Ng N.C., Yang Yu.Z., Casco-Robles M.M., Maruo F., Tsonis P.A., and Chiba C. (2016) A developmentally regulated switch from stem cells to dedifferentiation for limb muscle regeneration in newts. *Nat Commun*. 7:11069]

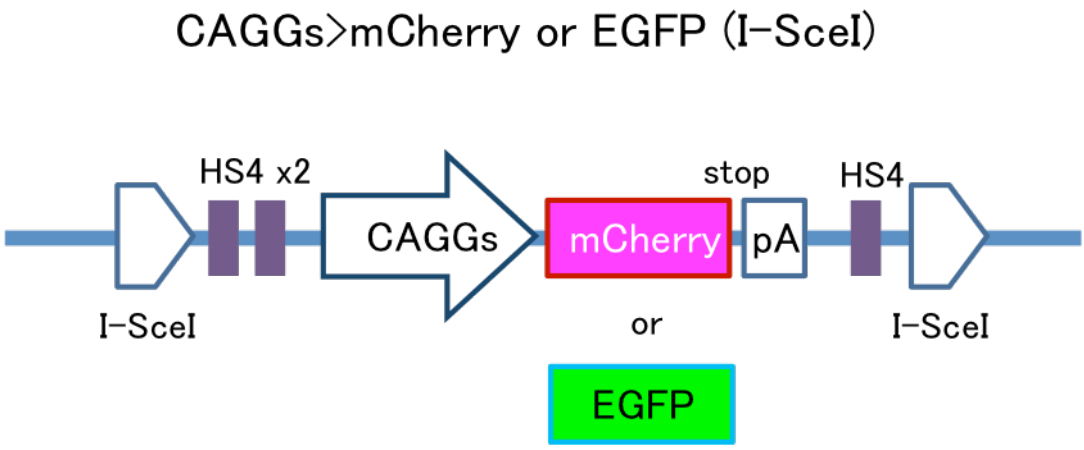

Figure 2. Reporter construct for cell tracking by tissue transplantation. Using this tool, a fluorescent reporter (mCherry or EGFP) can be expressed universally in the newt under the control of the CAGGs promoter (Casco-Robles et al., 2010).

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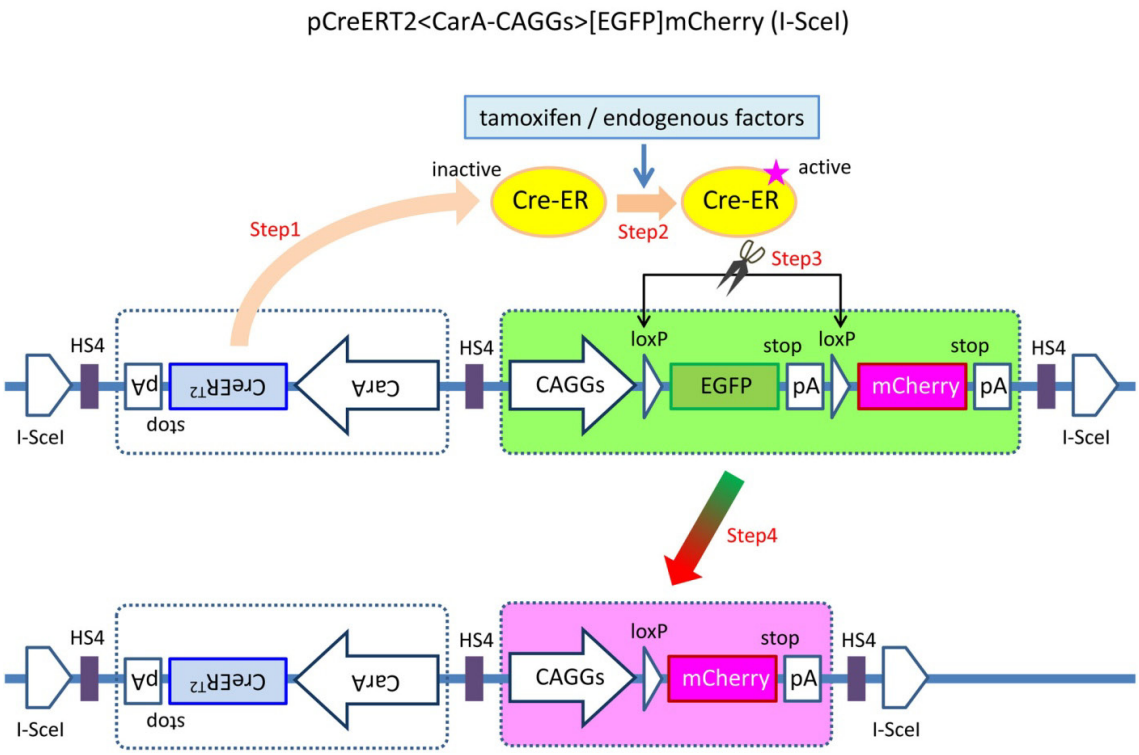


Figure 3. Design of construct for SMFC tracking. I hypothesized that in transgenic newts exhibiting EGFP fluorescence throughout their body, the inactive form of Cre would also be expressed in SMFCs in their limbs (Step 1); when Cre is activated by administration of tamoxifen (Step 2), the EGFP gene in the reporter construct is removed and the mCherry gene is ligated to CAGGs (Step 3), making the SMFCs express mCherry (Step 4).

[Adapted from Tanaka H.V., Ng N.C., Yang Yu.Z., Casco-Robles M.M., Maruo F., Tsonis P.A., and Chiba C. (2016) A developmentally regulated switch from stem cells to dedifferentiation for limb muscle regeneration in newts. *Nat Commun*. 7:11069]

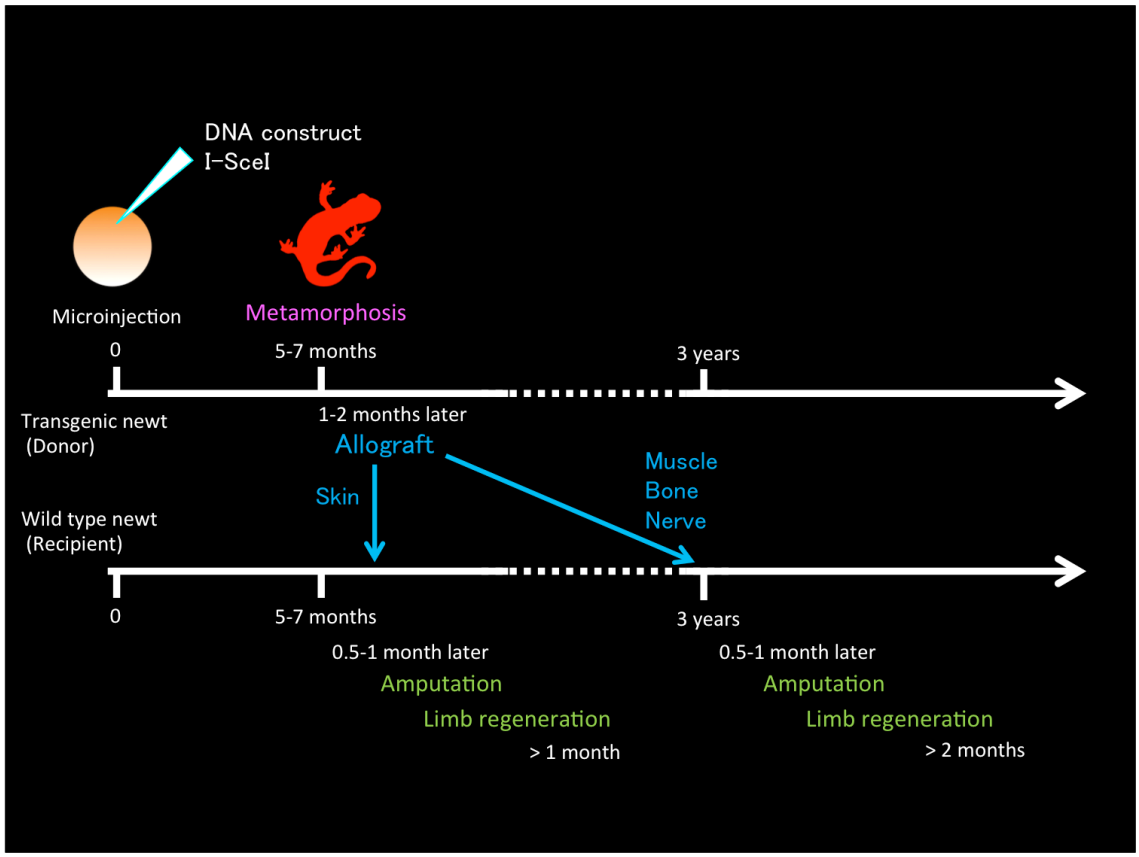


Figure 4. Time schedule of cell tracking by transplantation of reporter expressing tissues. *CAGGs>reporter* transgenic newts of 1-2 months after metamorphosis were used as donors. The skin, muscle, bone and nerve of the forearm, which exhibited intense mCherry/EGFP fluorescence uniformly, were isolated from the donors in a saline solution by fine scissors, pins and forceps under a fluorescent dissecting microscope (Leica M165 FC). The skin was grafted to wild type animal (recipient) of the same age. The muscle and bone were transplanted individually into the corresponding regions of different recipients 3 years old. The nerve (Schwann cells) was implanted in between muscles of a forelimb of a 3-year-old recipient. Details are illustrated in Fig. 5-8.

[Adapted from Tanaka H.V., Ng N.C., Yang Yu.Z., Casco-Robles M.M., Maruo F., Tsonis P.A., and Chiba C. (2016) A developmentally regulated switch from stem cells to dedifferentiation for limb muscle regeneration in newts. *Nat Commun*. 7:11069]

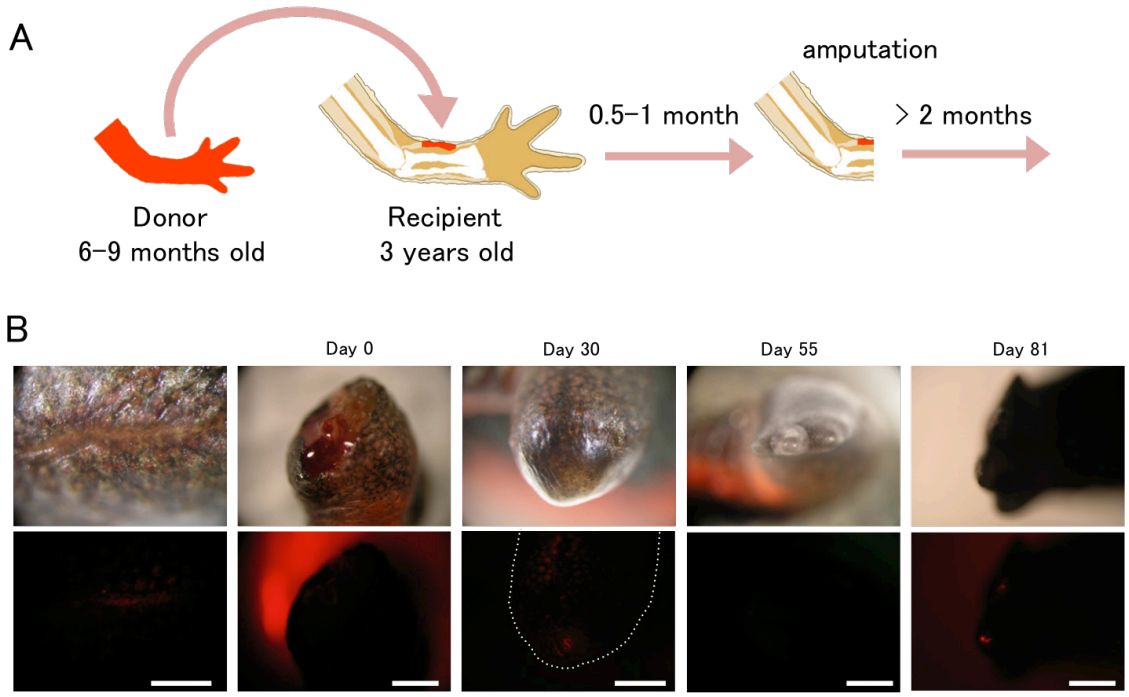


Figure 5. Muscle allograft. (A) Schematic of experiment. An mCherry+ muscle isolated from the forearm of a donor (6-9 months old) was transplanted to the corresponding region of a recipient (3 years old). After the wound was closed (0.5-1 month after surgery), the limb was amputated so that the grafted muscle remained in the stump. (B) Dorsal view of a closed wound from which an mCherry+ muscle was transplanted (left hand column) and apical views of the same limb immediately (Day 0) and on Day 30, 55 and 81 after amputation. mCherry fluorescence was recognized through transparent regions of the skin throughout the process of regeneration. A tissue section of the regenerating limb of this anima on Day 81 is shown in Fig. 11F. Scale bars: 1 mm. In some experiments, animals of the same age as donors were also used as recipients.

[Adapted from Tanaka H.V., Ng N.C., Yang Yu.Z., Casco-Robles M.M., Maruo F., Tsonis P.A., and Chiba C. (2016) A developmentally regulated switch from stem cells to dedifferentiation for limb muscle regeneration in newts. *Nat Commun*. 7:11069]

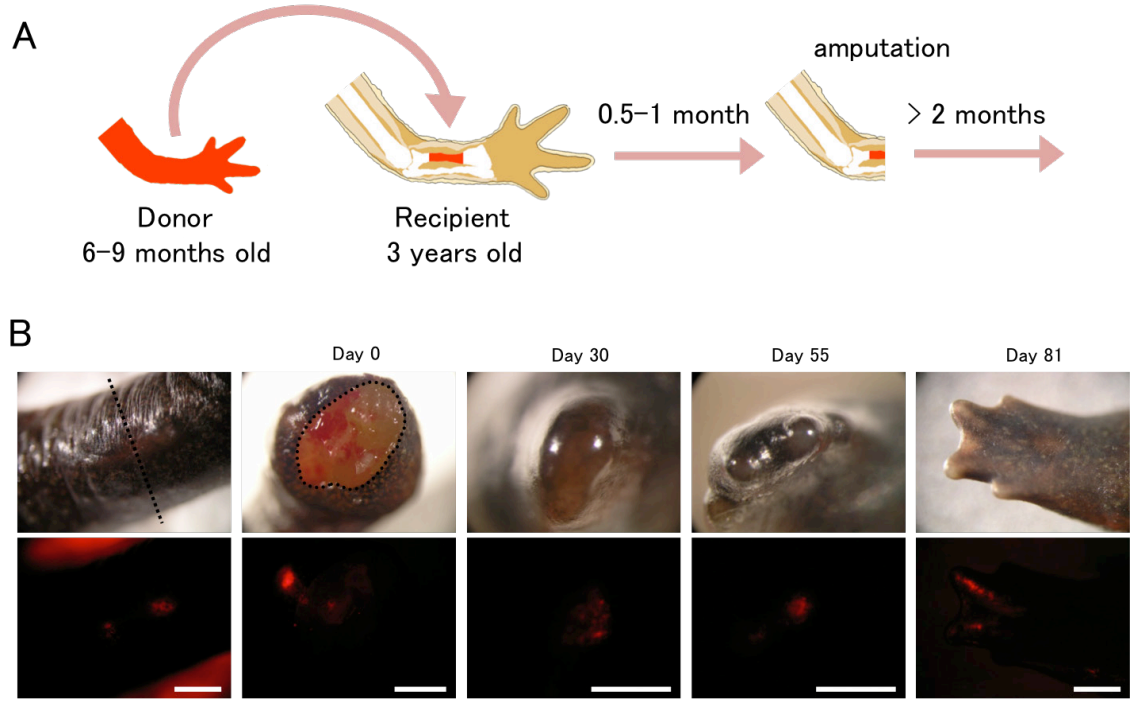


Figure 6. Bone allograft. (A) Schematic of experiment. An mCherry+ bone isolated from the forearm of a donor (6-9 months old) was transplanted to the corresponding region of a recipient (3 years old). After the wound was closed (0.5-1 month after surgery), the limb was amputated so that the grafted muscle remained in the stump. (B) Dorsal view of a closed wound from which an mCherry+ bone was transplanted (left hand column), apical views of the same limb immediately (Day 0) and on Day 30 and 55 after amputation, and a dorsal view on day 81. mCherry fluorescence was recognized through transparent regions of the skin throughout the process of regeneration. A tissue section of the regenerating limb of this animal on Day 81 is shown in Fig. 11E. Scale bars: 1 mm.

[Adapted from Tanaka H.V., Ng N.C., Yang Yu.Z., Casco-Robles M.M., Maruo F., Tsonis P.A., and Chiba C. (2016) A developmentally regulated switch from stem cells to dedifferentiation for limb muscle regeneration in newts. *Nat Commun*. 7:11069]

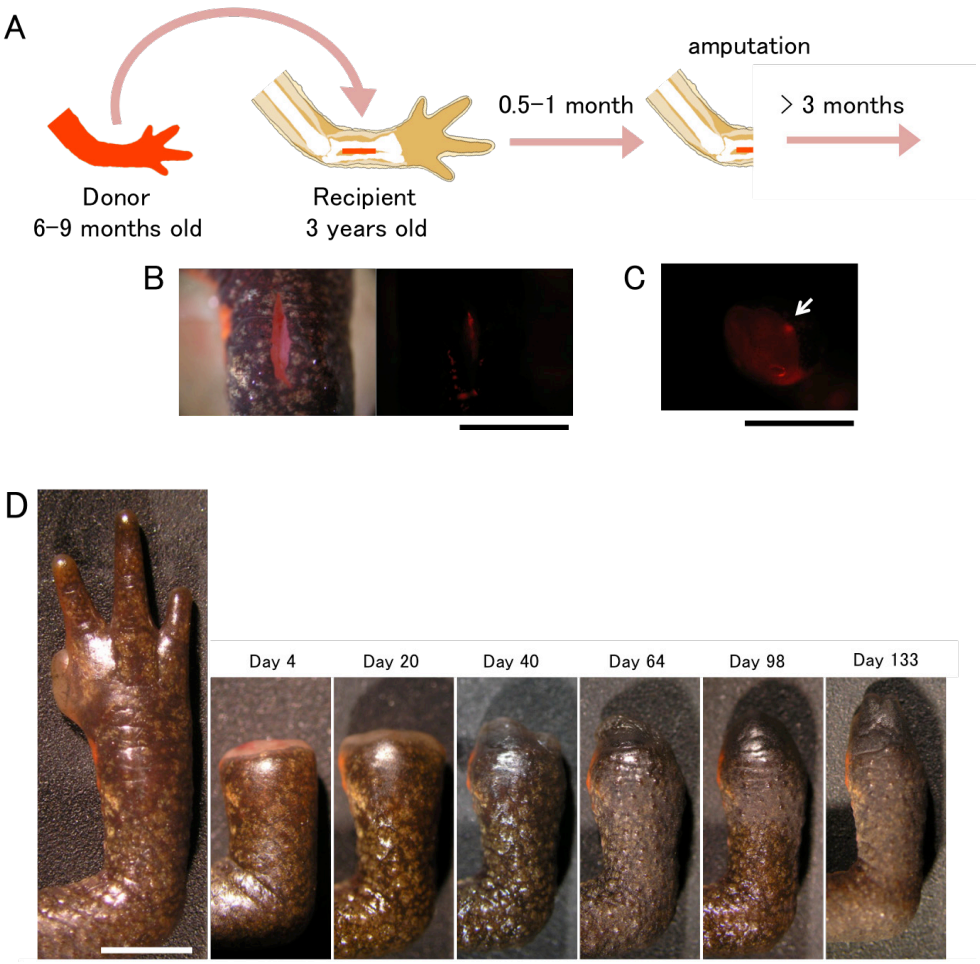


Figure 7. Nerve implantation. (A) Schematic of experiment. A fragment of mCherry+ nerve isolated from a forearm of the donor (6-9 months old) was implanted in between muscles in the dorsal side of a forelimb of the recipient (3 years old). After the wound closed (0.5-1 month after surgery), the limb was amputated so that the implanted nerve remained in the stump. (B) Dorsal view of wound immediately after implantation of an mCherry+ nerve. (C) Apical view of the stump on Day 4. The arrow indicates the implanted nerve. (D) Dorsal views of the same limb during regeneration. In this case, regeneration was slower than in other cases where the implant in the stump could not be recognized. A tissue section of the regenerating limb of this animal on Day 133 is shown in Fig. 11G. Scale bars: 5 mm.

[Adapted from Tanaka H.V., Ng N.C., Yang Yu.Z., Casco-Robles M.M., Maruo F., Tsonis P.A., and Chiba C. (2016) A developmentally regulated switch from stem cells to dedifferentiation for limb muscle regeneration in newts. *Nat Commun*. 7:11069]

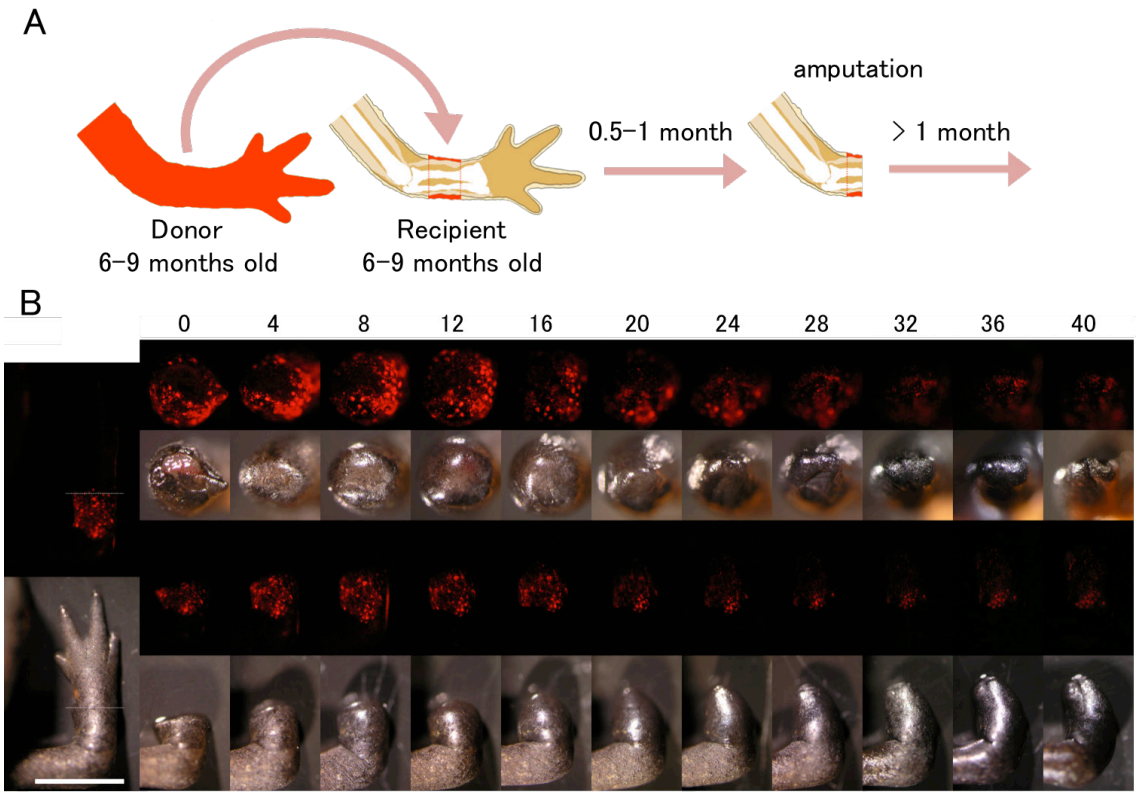
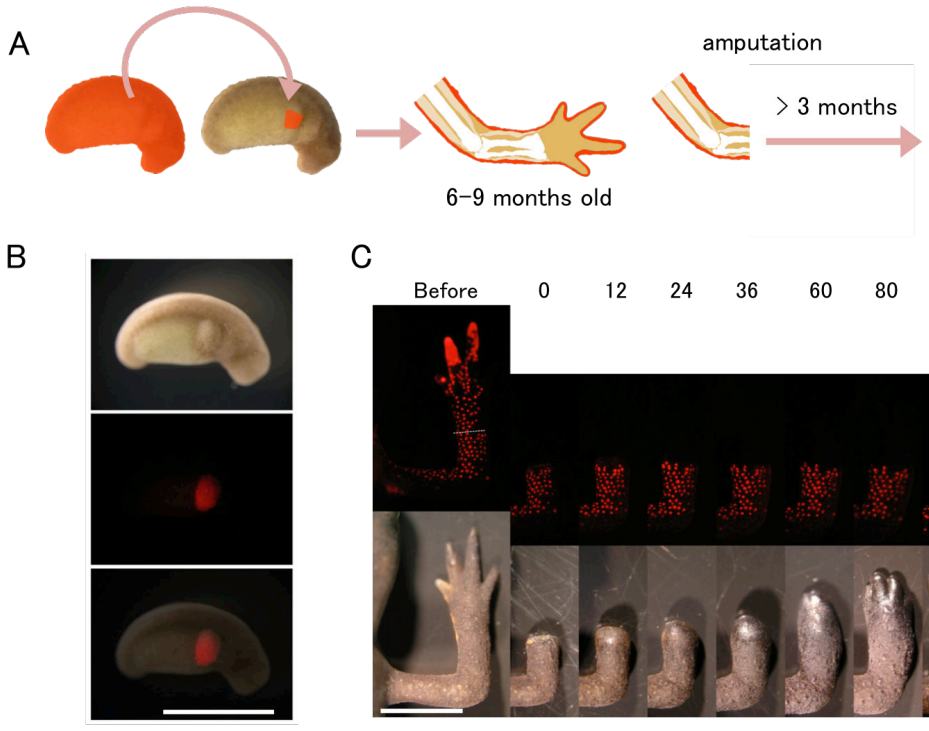


Figure 8. Skin allograft. (A) Schematic of experiment. The skin surrounding the forearm of a recipient was replaced with that (mCherry+) of a donor of the same age (6-9 months old), the mCherry+ skin was allowed to integrate to neighboring tissues for 0.5-1 month, and the limb was amputated along the line across the mCherry+ skin. (B) Apical and dorsal views of an amputated forelimb. The number in each column indicates days after amputation. In this case, the wound was closed by an epithelium with high transparency (i.e., wound epidermis) within 4 days after amputation. In 12 days, the wound epidermis was covered by another highly fluorescent epithelial sheet that contained pigments and the mucous glands. As regeneration proceeded, fluorescence of the new skin declined while its pigmentation increased. Note that most mucous glands were left around the amputation site. Tissue sections of the regenerating limb of this animal on Day 40 are shown in Fig. 11A and B. Scale Bars: 2.5 mm.

[Adapted from Tanaka H.V., Ng N.C., Yang Yu.Z., Casco-Robles M.M., Maruo F., Tsonis P.A., and Chiba C. (2016) A developmentally regulated switch from stem cells to dedifferentiation for limb muscle regeneration in newts. *Nat Commun*. 7:11069]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148

Figure 9. Ectoderm transplantation. (A) Schematic of experiment. The mCherry+ ectoderm on the presumptive forelimb of a tail bud embryo (St. 24-26) was transplanted to a wild type embryo at the same stage as described previously (Takaya H., 1941). (B) A sample embryo after transplantation. When the recipient reached 6-9 months of age, the forelimb was amputated. (C) Dorsal view of a regenerating limb. The number on each column indicates days after amputation. mCherry fluorescence, which was observed throughout the surface of the intact forelimb (except in the region covered by pigmented cells), was obscure in regenerating part of the limb. This was partly due to pigmentation in regenerating skin. Note that most mucous glands did not migrate from the amputation site. Tissue sections of the regenerating limb of this animal on Day 148 are shown in Fig. 4c and d. Scale bars: 2.5 mm. Note: In either the skin allograft (Fig. 8) or ectoderm transplantation (here), tissue sections of regenerated limbs revealed that cells in the new epidermal tissues were not always reporter+ (Fig. 11A-D). Since the CAGGs promoter is not repressed in new epidermal tissues (Casco-Robles et al., 2010), additional cellular sources for skin regeneration may be needed.

[Adapted from Tanaka H.V., Ng N.C., Yang Yu.Z., Casco-Robles M.M., Maruo F., Tsonis P.A., and Chiba C. (2016) A developmentally regulated switch from stem cells to dedifferentiation for limb muscle regeneration in newts. *Nat Commun*. 7:11069]

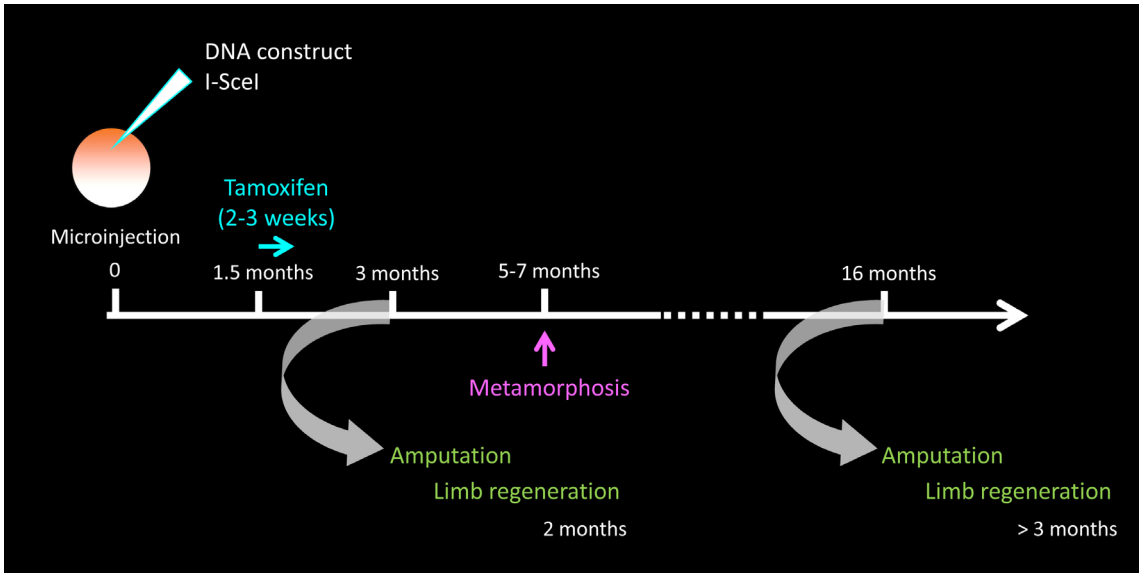
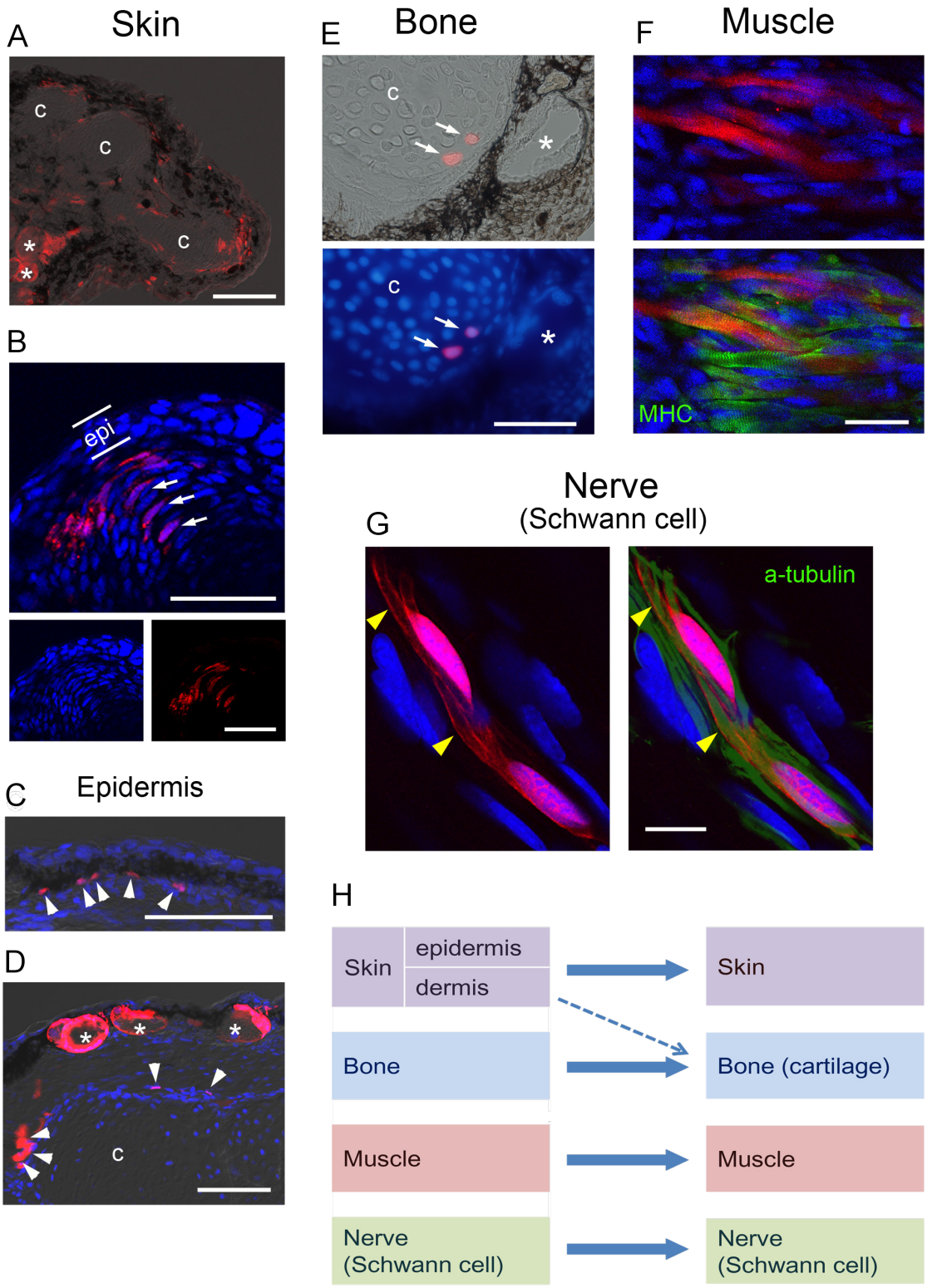


Figure 10. Time schedule of experiments for SMFC tracking. Fertilized eggs (or one-cell stage embryos) were injected with pCreER^{T2}<CarA-CAGGs>[EGFP]mCherry (I-SceI)/I-SceI enzyme mixture. Swimming larvae at St. 47-53 (age: 1-2 months) were

treated in a tamoxifen-containing solution for a few weeks. In this study, I examined swimming larvae (St. 56-57) which had developed forelimbs with full digits (total body length: ~18 mm; age: 3 months; Fig. 1A) and metamorphosed juveniles (total body length: ~6 cm; age: 16 months; Fig. 1B).

[Adapted from Tanaka H.V., Ng N.C., Yang Yu.Z., Casco-Robles M.M., Maruo F., Tsonis P.A., and Chiba C. (2016) A developmentally regulated switch from stem cells to dedifferentiation for limb muscle regeneration in newts. *Nat Commun*. 7:11069]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Muscle
Nerve
(Schwann cell)
Skin
Bone (cartilage)
Muscle
Nerve
(Schwann cell)

Figure 11. Lineage tracing in adult newt limb regeneration by tissue transplantation.

(A, B) Tracing of skin cells by allograft of mCherry+ skin (n=3). (A) mCherry+ cells were distributed along the epidermis and dermis, as well as around the cartilage in a regenerating part of the limb (day 40). c, cartilage. Asterisk, mucous gland. Scale bar, 200 μm. (B) In a growing tip of new digit, mCherry was sometimes observed in chondrocytes (arrows) in the cartilage and in interstitial cells surrounding the cartilage. Epi: epidermis. Scale bars, 100 μm. (C, D) Tracing of epidermal cells by transplantation of mCherry+ ectoderm at embryonic stage (n=2). mCherry was observed in epidermal cells such as those in multilayered epithelium (arrowheads in C) and mucous glands (asterisks in D), as well as in interstitial cells around the cartilage (arrowhead in D) in a regenerating part of the limb (day 148). Scale bars, 200 μm. (E) Tracing of bone cells by allograft of mCherry+ bone (n=4). mCherry was observed in chondrocytes (arrows) in new cartilage of a regenerating limb (day 81). Asterisk: mucous gland. Scale bar, 100 μm. (F) Tracing of muscle cells by allograft of mCherry+ muscle (n=3). mCherry was observed in muscle fibers in a regenerating part of the limb (day 81). MHC, myosin heavy chain immunoreactivity. Scale bar, 50 μm. (G) Tracing of Schwann cells by implantation of a fragment of mCherry+ nerve (n=3). mCherry was observed in Schwann cells along regenerated nerve fibers in a regenerating part of the limb (day 133). Arrowhead: myelin sheath covering a regenerated nerve fiber; a-tubulin, acetylated tubulin immunoreactivity. Scale bar, 20 μm. Blue: nuclei stained by To-pro-3 (B-D, F, G) and DAPI (4,6-diamidino-2-phenylindole) (E). (H) Summary. The skin, bone, muscle and nerve (Schwann cell) regenerated themselves. Exceptionally, dermis contributed to new bone/cartilage.

[Adapted from Tanaka H.V., Ng N.C., Yang Yu.Z., Casco-Robles M.M., Maruo F., Tsonis P.A., and Chiba C. (2016) A developmentally regulated switch from stem cells to dedifferentiation for limb muscle regeneration in newts. *Nat Commun*. 7:11069]

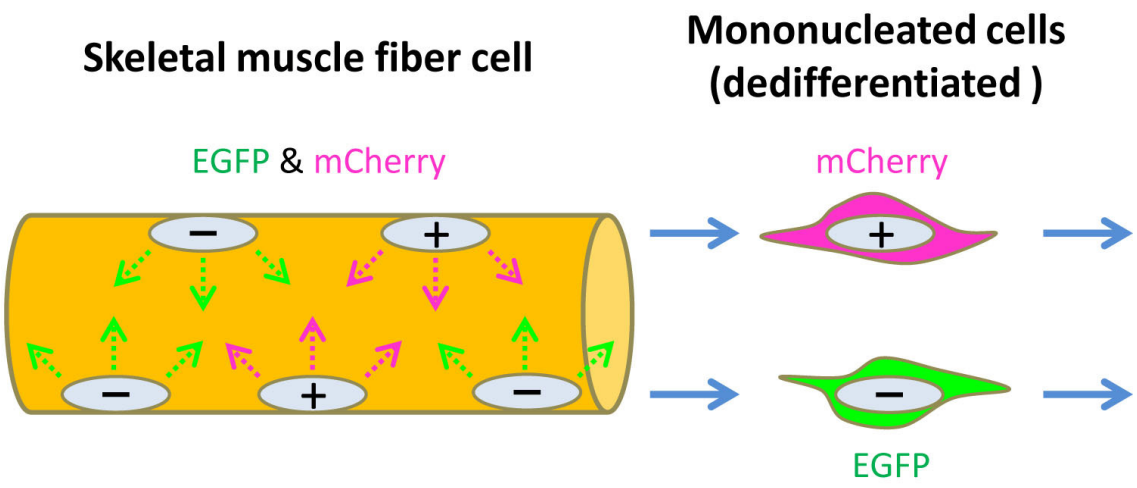


Figure 12. Predicted labeling patterns in SMFC and SMFC-derived mononucleated (dedifferentiated) cells. The SMFC is a syncytium or multinuclear cell. In my labeling system, the cell should express both EGFP and mCherry because of moderate recombination efficiency (see Fig. 14), and exhibit fluorescence of both along the fiber. However, as the cell dedifferentiates into mononucleated cells, these cells should exhibit either EGFP or mCherry fluorescence. Plus (+) indicates a nucleus that has been recombined by tamoxifen-activated Cre, and minus (-) indicates a non-recombined nucleus.

[Adapted from Tanaka H.V., Ng N.C., Yang Yu.Z., Casco-Robles M.M., Maruo F., Tsonis P.A., and Chiba C. (2016) A developmentally regulated switch from stem cells to dedifferentiation for limb muscle regeneration in newts. *Nat Commun*. 7:11069]

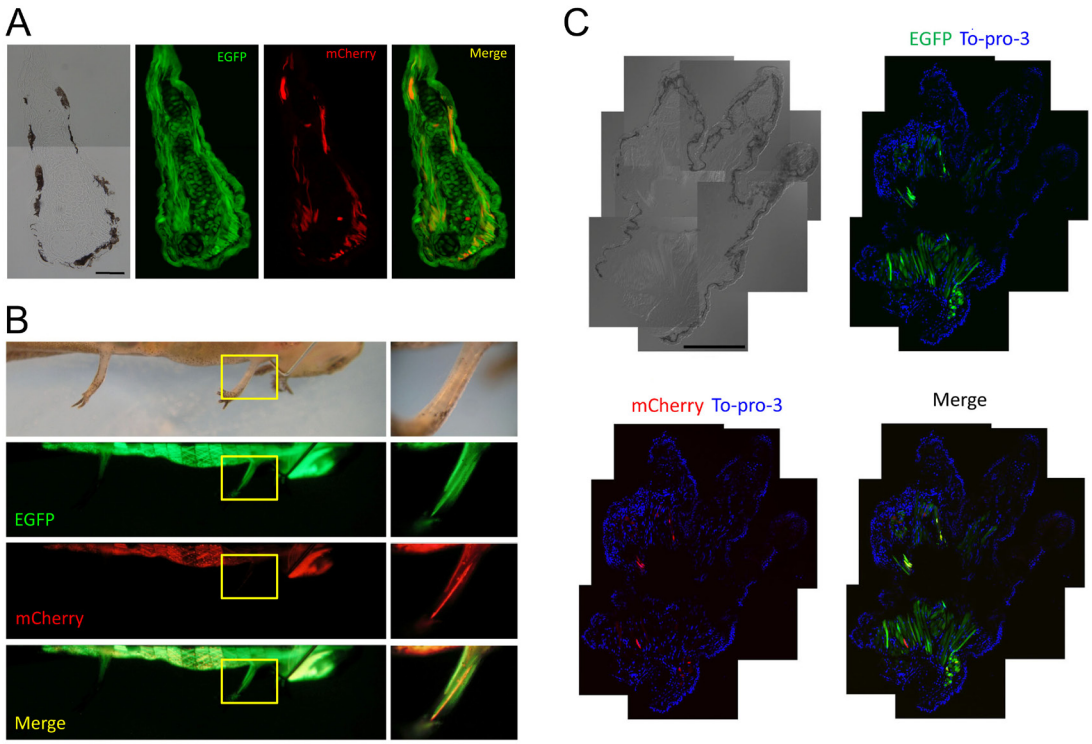


Figure 13. mCherry expression in larval and juvenile muscles. (A) A section of the forelimb of a swimming larva which expressed EGFP in its whole body almost uniformly. Scale bar: 100 µm. (B) Ventral view of another swimming larva. This animal was mosaic and EGFP was expressed exclusively in muscle fibers. Scale bar: 5 mm. Right hand panels are enlargements of the area enclosed by yellow rectangles. In both larvae, almost all of the muscle fibers showed mCherry as well as EGFP fluorescence (A, B). (C) A section of the forelimb (hand) of the animal in B at the juvenile stage. Even though the total number of mCherry+ fibers in the limb that had been labeled in the larval stage (St. 47-53) by tamoxifen did not seem to be different between larvae (St. 56-57) and juveniles (16 months), the ratio of mCherry+ fibers in juvenile limb muscle was obviously low compared to that in larval muscle. This is because new fibers (EGFP+/mCherry-) appeared and increased in number in the muscle as the limb grew after metamorphosis. To-pro-3 shows nuclei. Scale bar: 500 µm.

[Adapted from Tanaka H.V., Ng N.C., Yang Yu.Z., Casco-Robles M.M., Maruo F., Tsonis P.A., and Chiba C. (2016) A developmentally regulated switch from stem cells to dedifferentiation for limb muscle regeneration in newts. *Nat Commun*. 7:11069]

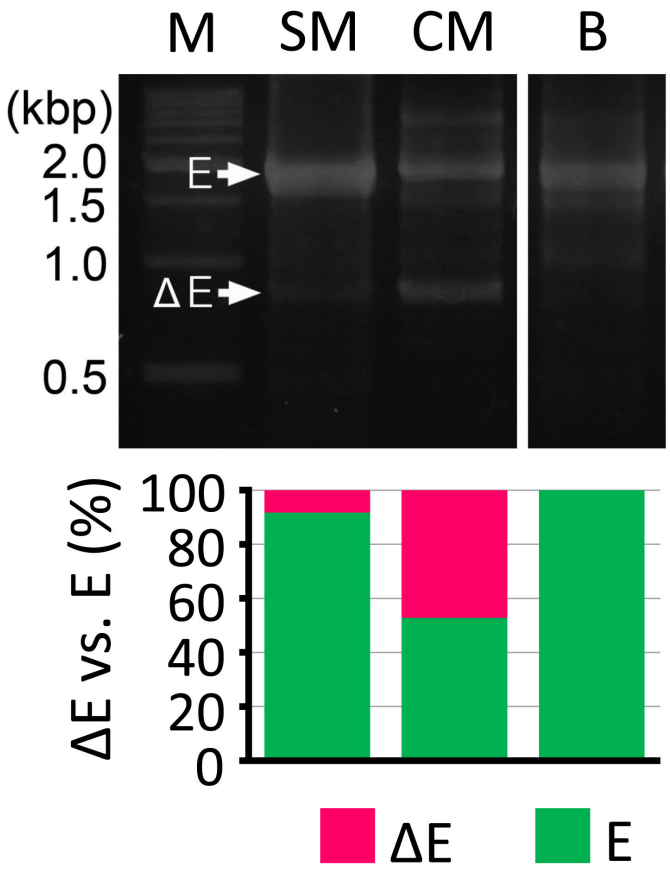


Figure 14. Recombination in the transgene cassette. (Upper panel) PCR detection of the transgene cassette with (E; 1,836 bp) or without floxed EGFP (ΔE; 841 bp) in genomic DNA. (Lower panel) Recombination ratio (%) estimated from the relative intensity of PCR bands (ΔE/E). In juvenile newts, the recombination ratio was 8.2% in the skeletal muscle (SM) of the forelimb and about 50% in the cardiac muscle (CM) of the heart. On the other hand, recombination was not detected in the brain (B). M: marker.

[Adapted from Tanaka H.V., Ng N.C., Yang Yu.Z., Casco-Robles M.M., Maruo F., Tsonis P.A., and Chiba C. (2016) A developmentally regulated switch from stem cells to dedifferentiation for limb muscle regeneration in newts. *Nat Commun*. 7:11069]

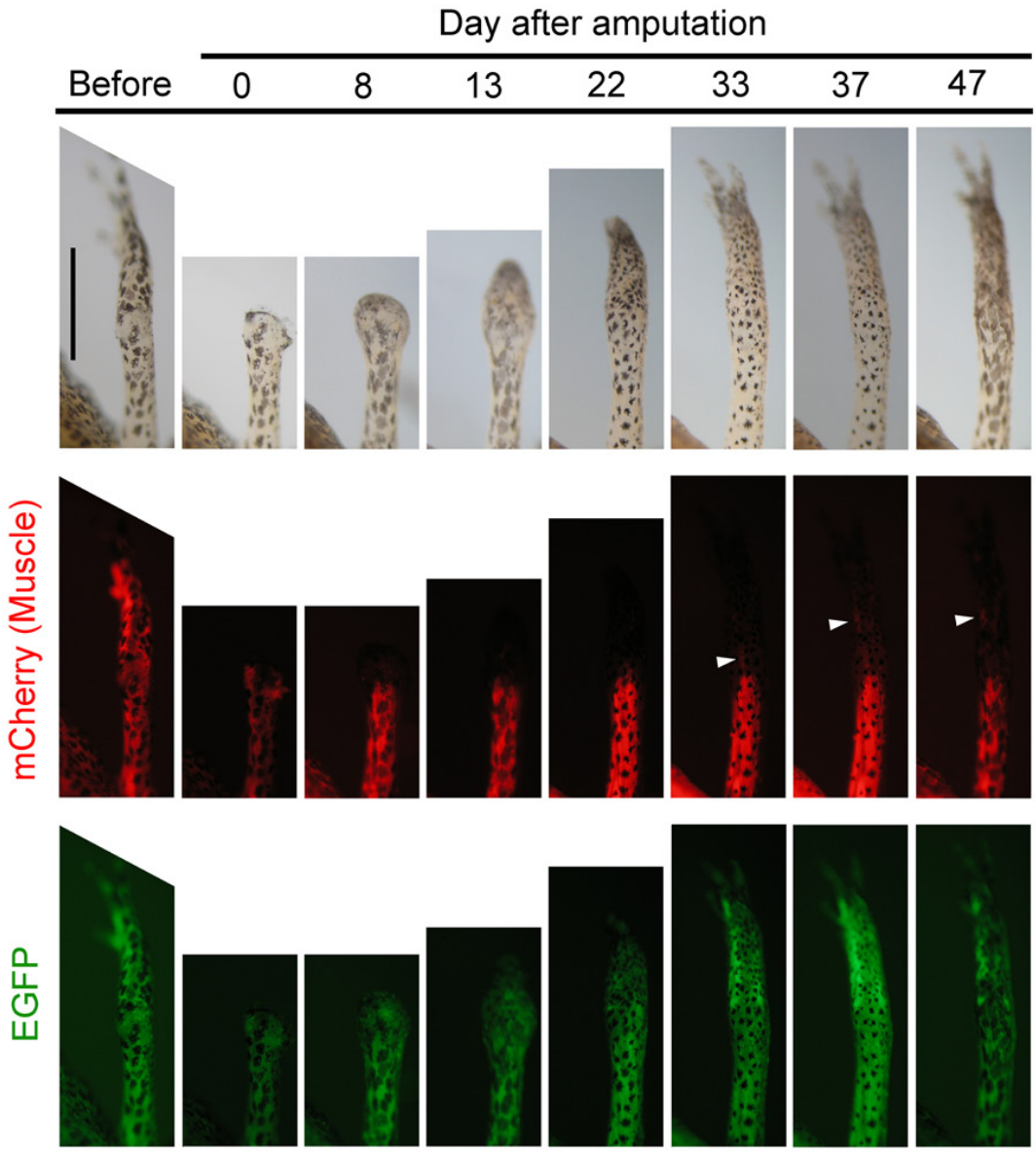


Figure 15. SMFC tracking in larval newt limb regeneration. Monitoring of SMFCs (mCherry+) during limb regeneration (n=6). mCherry was not detected in the regenerating part of the limb until ~30 days when the amputated limb had almost been recovered. Arrowheads: flexor muscle for digits (see Fig. 19). Scale bar, 1 mm.

[Adapted from Tanaka H.V., Ng N.C., Yang Yu.Z., Casco-Robles M.M., Maruo F., Tsonis P.A., and Chiba C. (2016) A developmentally regulated switch from stem cells to dedifferentiation for limb muscle regeneration in newts. *Nat Commun*. 7:11069]

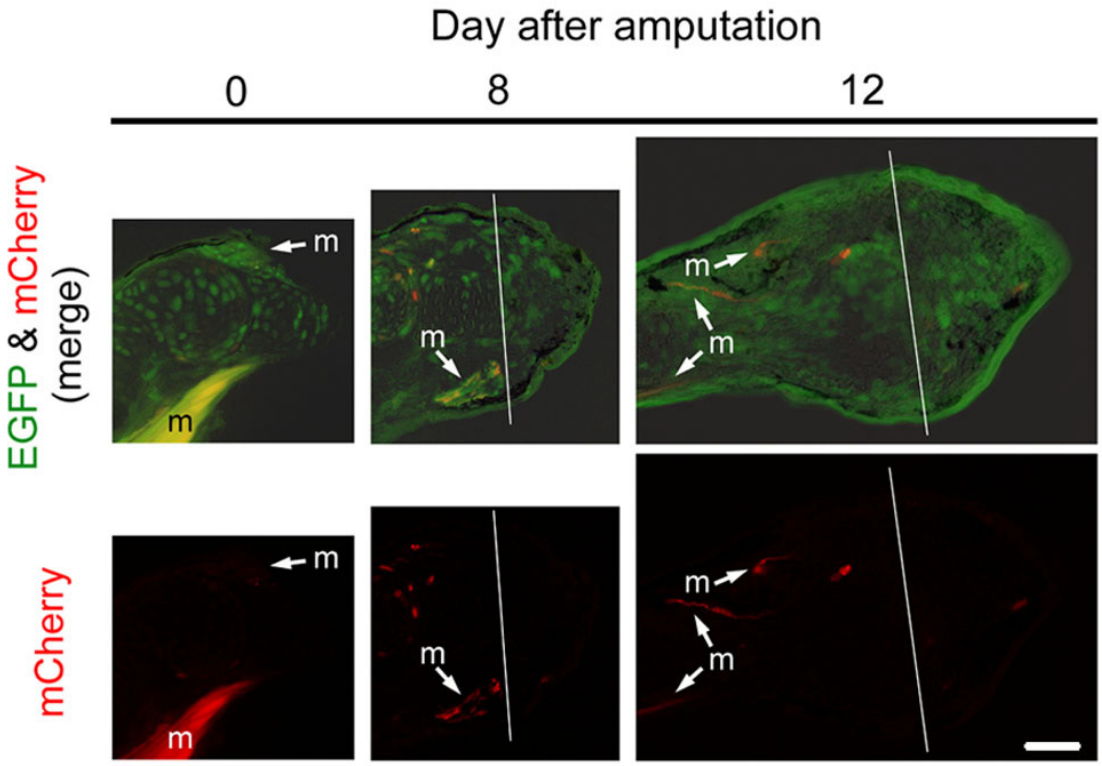


Figure 16. SMFC tracking in larval newt limb blastema. Sections of regenerating limbs (n=3 for each stage). SMFC-derived mCherry+ cells were not observed in the blastema. Lines: amputation site. m: muscle, Scale bar, 100 μm.

[Adapted from Tanaka H.V., Ng N.C., Yang Yu.Z., Casco-Robles M.M., Maruo F., Tsonis P.A., and Chiba C. (2016) A developmentally regulated switch from stem cells to dedifferentiation for limb muscle regeneration in newts. *Nat Commun*. 7:11069]

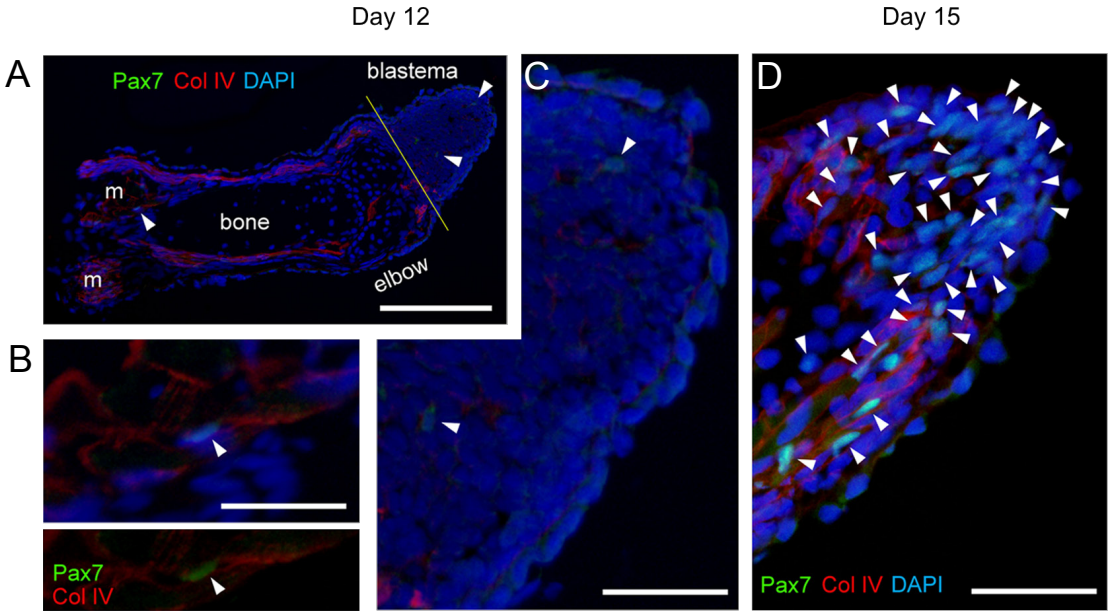


Figure 17. Pax7 immunolabelling of regenerating limbs in larval newt. Pax7 immunolabelling of regenerating limbs on Day 12 (n=3) and Day 15 (n=3) after amputation. (A) On Day 12, a few Pax7+ nuclei (arrowheads) were detected in blastema cells and in satellite cells along the muscle fibers. Col IV, collagen type IV immunoreactivity. DAPI (4,6-diamidino-2-phenylindole), nuclei. Scale bar, 300 µm. The Pax7+ nuclei pointed by arrowheads were enlarged in B and C. Scale bars, 100 µm. (D) On Day 15 when the regenerating part of the limb grew more distally, the number of Pax7+ nuclei (arrowheads) in the blastema was dramatically increased. Scale bar, 100 µm.

[Adapted from Tanaka H.V., Ng N.C., Yang Yu.Z., Casco-Robles M.M., Maruo F., Tsonis P.A., and Chiba C. (2016) A developmentally regulated switch from stem cells to dedifferentiation for limb muscle regeneration in newts. *Nat Commun*. 7:11069]

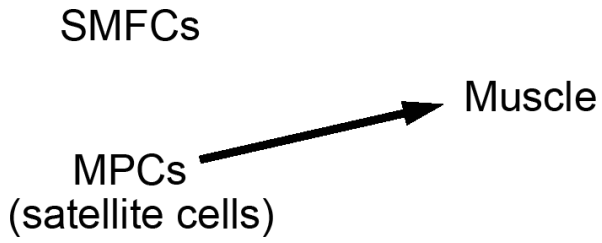


Figure 18. Summary of the origin of limb muscle in larval newts. In larval newts, muscle progenitor cells (MPCs), potentially satellite cells, were recruited for new muscle during limb regeneration, whereas SMFCs were not.

[Adapted from Tanaka H.V., Ng N.C., Yang Yu.Z., Casco-Robles M.M., Maruo F., Tsonis P.A., and Chiba C. (2016) A developmentally regulated switch from stem cells to dedifferentiation for limb muscle regeneration in newts. *Nat Commun*. 7:11069]

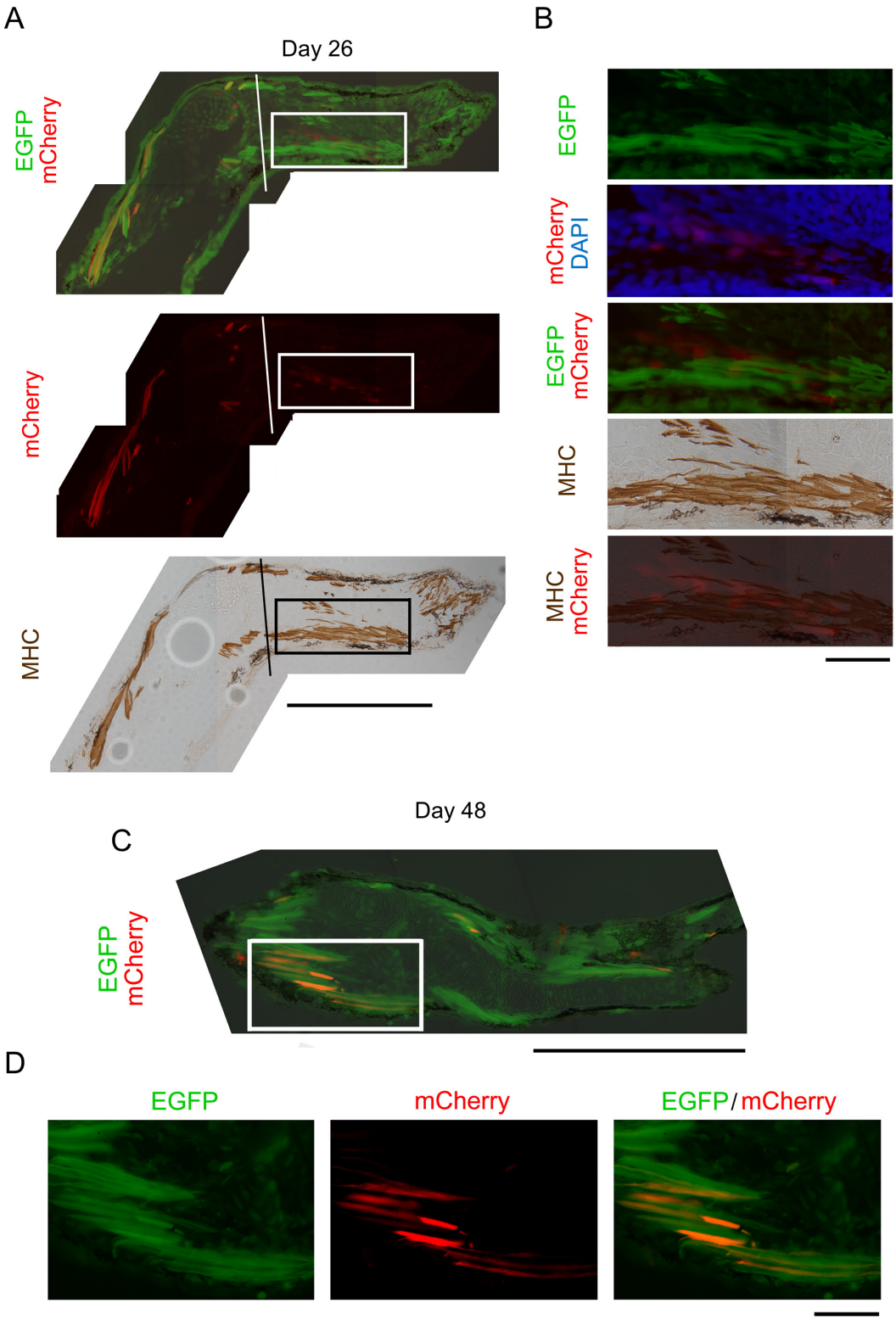

A
Day 26
EGFP
mCherry
mCherry
MHC
B
EGFP
mCherry
DAPI
EGFP
mCherry
MHC
MHC
mCherry
Day 48
C
EGFP
mCherry
D
EGFP
mCherry
EGFP / mCherry

Figure 19. Appearance of mCherry in a late stage of larval limb regeneration. (A) A section of the limb on day 26 after amputation (n=3). mCherry was detected in mononucleated cells, which had occupied the same area as the differentiating flexor muscle for digits (rectangle). Lines: amputation site. MHC, myosin heavy chain immunoreactivity. Scale bar, 500 μm. (B) Enlargement of the area enclosed by the rectangle in A. At this stage, mCherry did not overlap with the flexor muscle. Scale bar, 100 μm. (C) A section of the forearm on day 48 after amputation (n=3). Scale bar, 500 μm. (D) Enlargement of the area enclosed by a rectangle in C. The flexor muscle for digits at this stage exhibited mCherry. Scale bar, 100 μm.

[Adapted from Tanaka H.V., Ng N.C., Yang Yu.Z., Casco-Robles M.M., Maruo F., Tsonis P.A., and Chiba C. (2016) A developmentally regulated switch from stem cells to dedifferentiation for limb muscle regeneration in newts. *Nat Commun*. 7:11069]

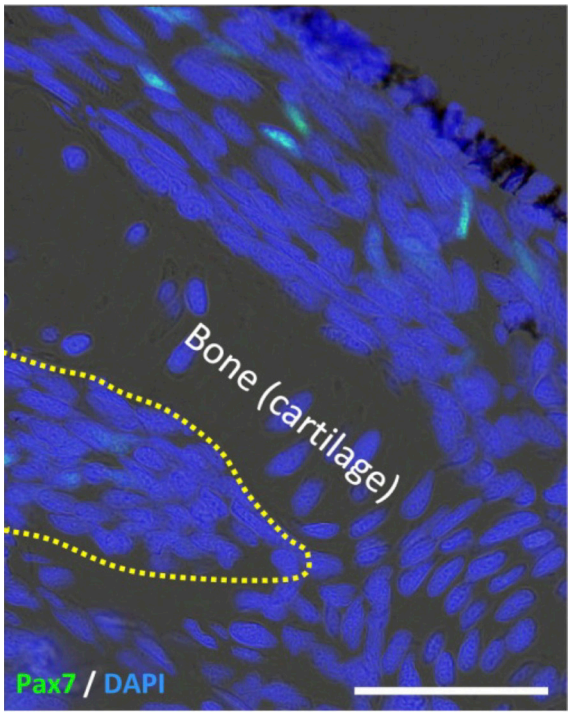


Figure 20. Mononucleated cells in the mCherry positive flexor area (as described in Fig. 19) are Pax7 negative (dotted line). In this image, a few Pax7 positive nuclei are recognized in the same area. These cells are probably satellite cells which have migrated from the stump as described in Fig. 17. Scale bar: 100 µm.

[Adapted from Tanaka H.V., Ng N.C., Yang Yu.Z., Casco-Robles M.M., Maruo F., Tsonis P.A., and Chiba C. (2016) A developmentally regulated switch from stem cells to dedifferentiation for limb muscle regeneration in newts. *Nat Commun*. 7:11069]

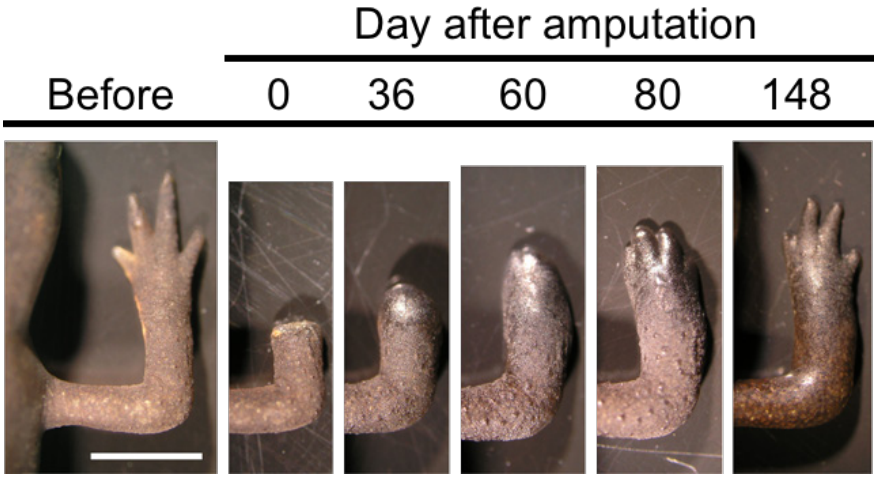
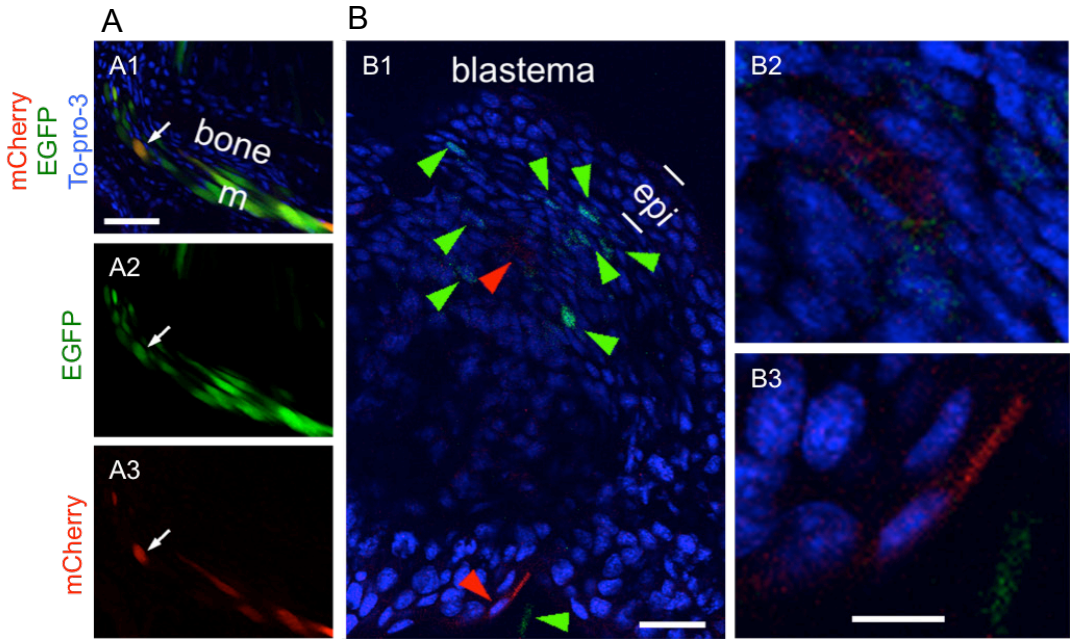


Figure 21. Limb regeneration of a juvenile newt. Scale bar, 5 mm.

[Adapted from Tanaka H.V., Ng N.C., Yang Yu.Z., Casco-Robles M.M., Maruo F., Tsonis P.A., and Chiba C. (2016) A developmentally regulated switch from stem cells to dedifferentiation for limb muscle regeneration in newts. *Nat Commun*. 7:11069]

Day 36


A
B
A1
bone
m
mCherry
EGFP
To-pro-3
A2
EGFP
A3
mCherry
B1
blastema
epi
B2
B3

Day 96

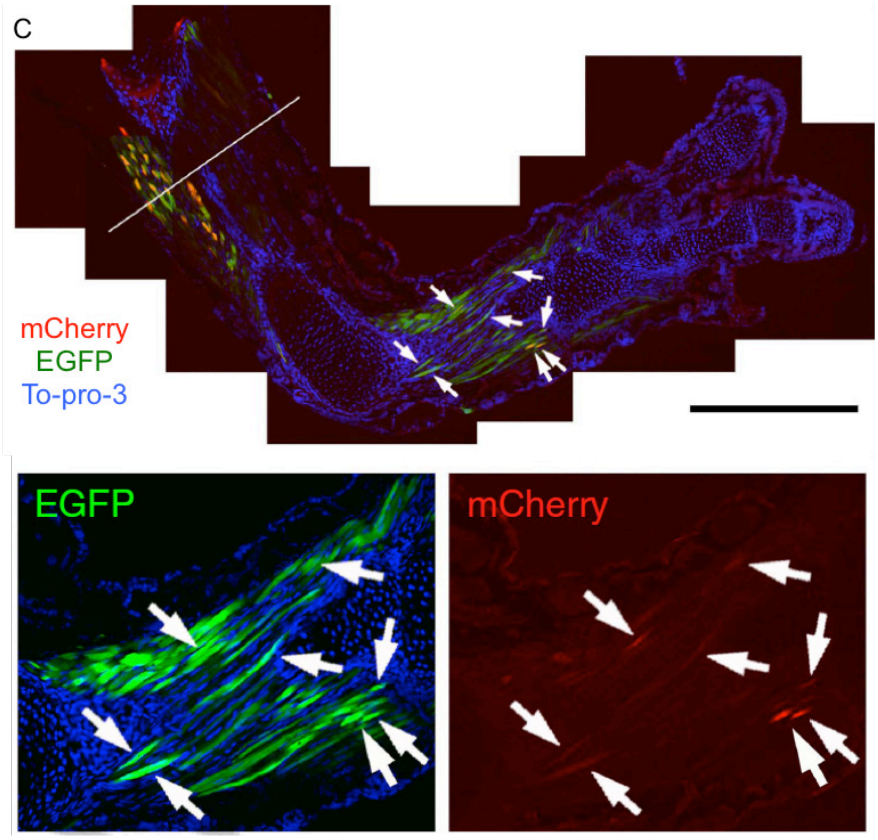

C
mCherry
EGFP
To-pro-3
EGFP
mCherry

Figure 22. SMFC tracking in metamorphosed newt limb regeneration. (A, B) On Day 36 after amputation, fragments of muscle fibers (arrows) were observed in distal regions adjacent to the blastema (A1-3). Scale bar, 100 μm. mCherry+ mononucleated cells (red arrowheads; enlarged in right-hand panels) and EGFP+ cells (green arrowheads) were observed in the blastema (B1-3). Epi, epidermis. To-pro-3: nuclei. Scale bars, 50 μm (left), 10 μm (right-hand panels). (C) In the same limb, at Day 96 after the second amputation in the upper arm (line), mCherry (arrows) and EGFP were observed only in muscle fibers. Scale bars, 1 mm (upper panel), 500 μm (lower panels).
[Adapted from Tanaka H.V., Ng N.C., Yang Yu.Z., Casco-Robles M.M., Maruo F., Tsonis P.A., and Chiba C. (2016) A developmentally regulated switch from stem cells to dedifferentiation for limb muscle regeneration in newts. *Nat Commun*. 7:11069]

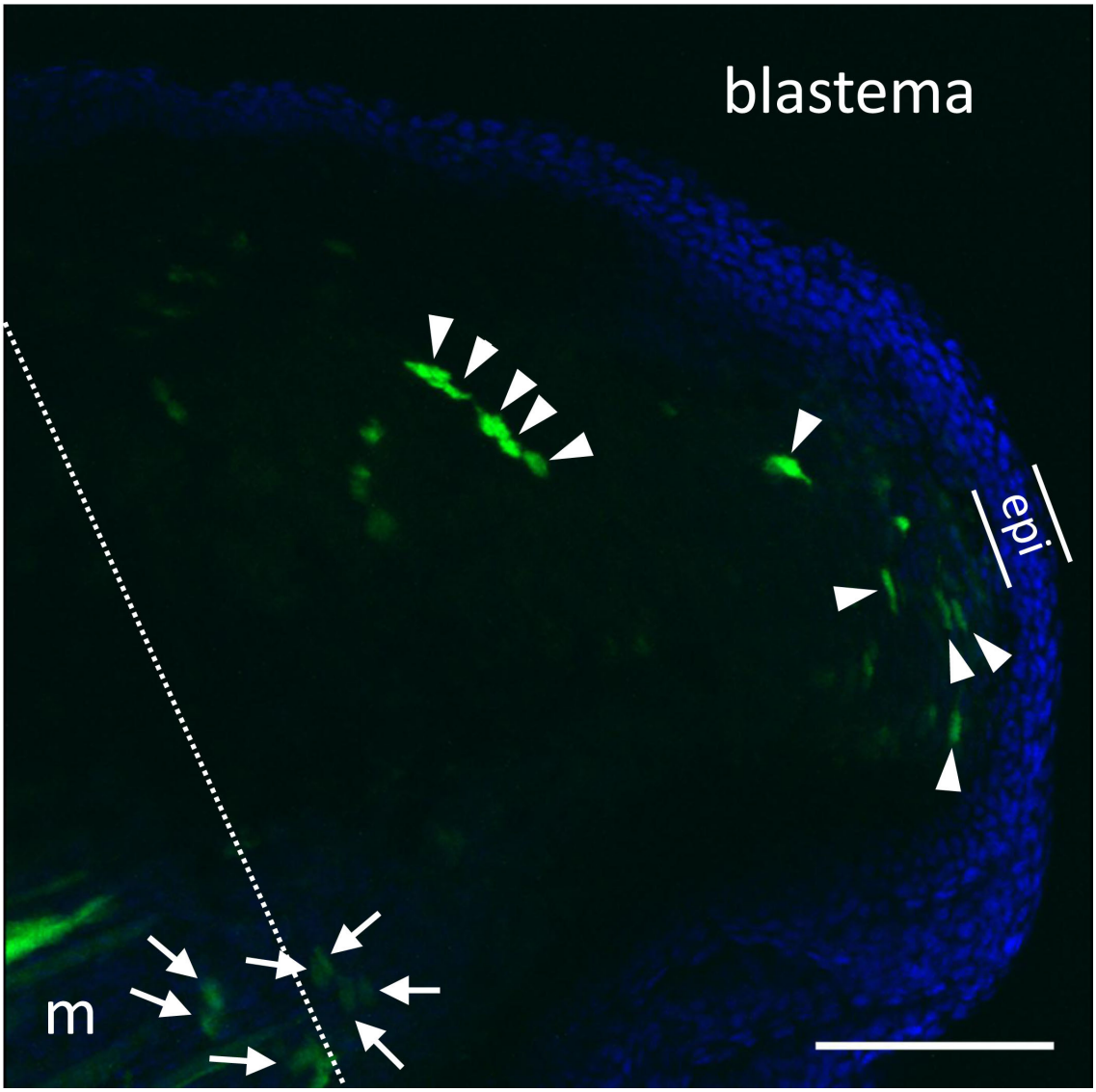


Figure 23. A confocal image of a section that was obtained from the same regenerating juvenile limb (day 36) shown in Fig. 22A and B. 3D-image of this section is shown in Movie 2. In this section, only EGFP+ muscle fiber-derived cells (green) were observed. Fragments of muscle fibers near the stump are indicated by arrows, and mononucleated cells in the blastema are indicated by arrowheads. Dotted line: amputation site. *m*: muscle. *Epi*: epidermis. Scale bar: 200 μm.

[Adapted from Tanaka H.V., Ng N.C., Yang Yu.Z., Casco-Robles M.M., Maruo F., Tsonis P.A., and Chiba C. (2016) A developmentally regulated switch from stem cells to dedifferentiation for limb muscle regeneration in newts. *Nat Commun*. 7:11069]

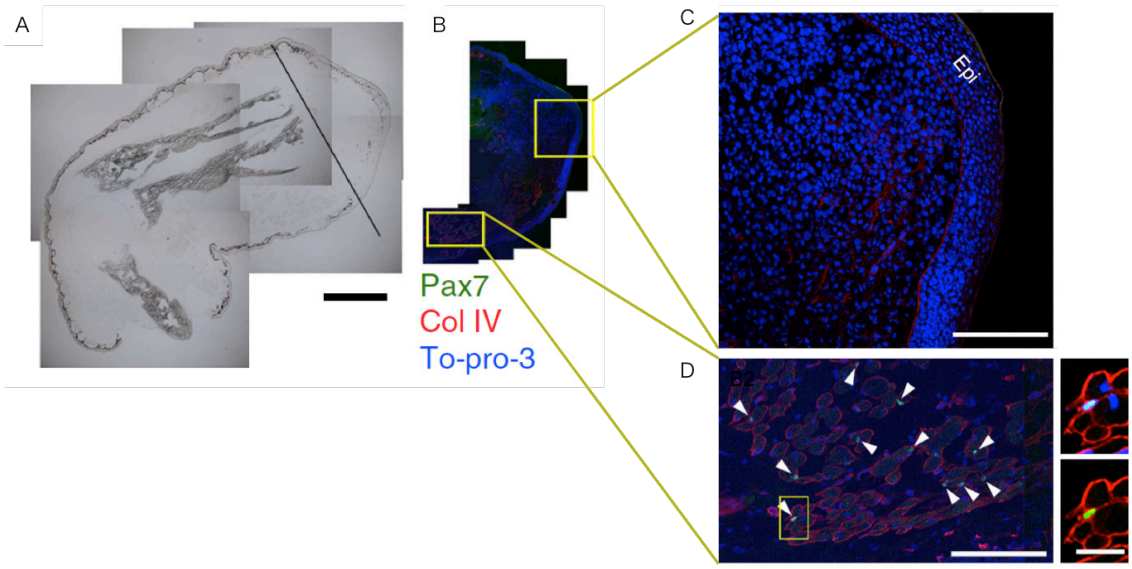


Figure 24. Pax7 immunolabelling of a regenerating limb of adult newt. (A) Translucent image. Line: amputation site. (B) Merged fluorescence image. Col IV, collagen type IV immunoreactivity. To-pro-3: nuclei. Scale bar, 1 mm. (C) Enlargement of a region in the blastema and (D) a region proximal to the amputation site, enclosed by boxes in (B). Scale bars, 250 µm. Arrowheads in (D) Pax7+ nuclei, An example satellite cell (box) is enlarged in the light-hand panels (upper: Col VI/To-pro-3; lower: Col IV/PAX7). Scale bar, 50 µm.

[Adapted from Tanaka H.V., Ng N.C., Yang Yu.Z., Casco-Robles M.M., Maruo F., Tsonis P.A., and Chiba C. (2016) A developmentally regulated switch from stem cells to dedifferentiation for limb muscle regeneration in newts. *Nat Commun*. 7:11069]

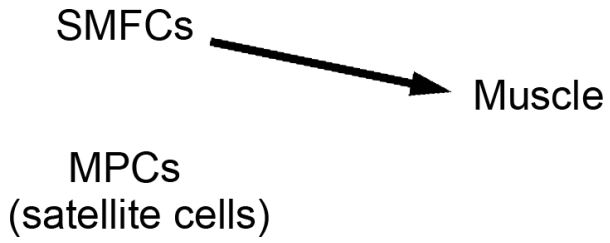


Figure 25. Summary of the origin of limb muscle in metamorphosed newts. In metamorphosed newts, SMFCs were recruited for new muscle during limb regeneration, whereas MPCs such as satellite cells were not.

[Adapted from Tanaka H.V., Ng N.C., Yang Yu.Z., Casco-Robles M.M., Maruo F., Tsonis P.A., and Chiba C. (2016) A developmentally regulated switch from stem cells to dedifferentiation for limb muscle regeneration in newts. *Nat Commun*. 7:11069]

7. Table

Table 1. Appearance of mCherry+ mononucleated cells during larval limb regeneration.

Before amputation	Blastema	Late regenerating limb
0/40 (0%)	0/15 (0%)	9/9 (100%)

* The value is the ratio of animals in which I observed mCherry+ mononucleated cells either in the body before amputation, in the blastema or in the late regenerating limb.

[Adapted from Tanaka H.V., Ng N.C., Yang Yu.Z., Casco-Robles M.M., Maruo F., Tsonis P.A., and Chiba C. (2016) A developmentally regulated switch from stem cells to dedifferentiation for limb muscle regeneration in newts. *Nat Commun*. 7:11069]