Myocyte Dedifferentiation Drives Extraocular Muscle Regeneration in Adult Zebrafish

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PURPOSE. The purpose of this study was to characterize the injury response of extraocular muscles (EOMs) in adult zebrafish.

METHODS. Adult zebrafish underwent lateral rectus (LR) muscle myectomy surgery to remove 50% of the muscle, followed by molecular and cellular characterization of the tissue response to the injury.

RESULTS. Following myectomy, the LR muscle regenerated an anatomically correct and functional muscle within 7 to 10 days post injury (DPI). Following injury, the residual muscle stump was replaced by a mesenchymal cell population that lost cell polarity and expressed mesenchymal markers. Next, a robust proliferative burst repopulated the area of the regenerating muscle. Regenerating cells expressed *myod*, identifying them as myoblasts. However, both immunofluorescence and electron microscopy failed to identify classic Pax7-positive satellite cells in control or injured EOMs. Instead, some proliferating nuclei were noted to express *mef2c* at the very earliest point in the proliferative burst, suggesting myonuclear reprogramming and dedifferentiation. Bromodeoxyuridine (BrdU) labeling of regenerating cells followed by a second myectomy without repeat labeling resulted in a twice-regenerated muscle broadly populated by BrdU-labeled nuclei with minimal apparent dilution of the BrdU signal. A double-pulse experiment using BrdU and 5-ethynyl-2'-deoxyuridine (EdU) identified double-labeled nuclei, confirming the shared progenitor lineage. Rapid regeneration occurred despite a cell cycle length of 19.1 hours, whereas 72% of the regenerating muscle nuclei entered the cell cycle by 48 hours post injury (HPI). Dextran lineage tracing revealed that residual myocytes were responsible for muscle regeneration.

CONCLUSIONS. EOM regeneration in adult zebrafish occurs by dedifferentiation of residual myocytes involving a muscle-to-mesenchyme transition. A mechanistic understanding of myocyte reprogramming may facilitate novel approaches to the development of molecular tools for targeted therapeutic regeneration in skeletal muscle disorders and beyond.

Keywords: cell reprogramming, mesenchymal transition, muscle injury, pax7, progenitor cells, satellite cell, skeletal muscle, stem cells

Binocular vision requires intricate control of eye movement by 6 pairs of extraocular muscles (EOMs). Congenital and acquired strabismus secondary to EOM dysfunction cause misaligned binocular inputs, which can lead to amblyopia (neurologic vision loss) or diplopia, collectively affecting as much as 5% to 10% of the US population.^{1,2} More broadly, skeletal muscle injury and degenerative conditions are common and debilitating and represent important causes of morbidity and mortality worldwide.^{3,4} Mammalian myonuclei are defined as being postmitotic, and muscle growth and repair are achieved by myogenic progenitor cells termed satellite cells.^{5–11} These cells are identified by expression of the paired box transcription factor family members Pax7 and/or Pax3. Pax7 is expressed by most adult muscle satellite cells, whereas Pax3 is present only in satellite cells of particular muscles and is transiently expressed by activated satellite cells.^{12–15}

Muscle injury can be a focal injury (e.g., stab wound) or a major injury with significant tissue loss and every degree of injury in between. Response to focal injury would require a local repair process, whereas significant tissue loss would

require more extensive tissue generation (i.e., “whole-organ” regeneration). In mammals, muscle repair following focal injury involves the proliferation of satellite cells to form myoblasts that express the transcription factor MyoD. Some myoblasts maintain MyoD expression, downregulate Pax7, and commit to differentiation by activation of myogenin. These differentiated myoblasts fuse to form myocytes. Other myoblasts maintain Pax7 expression, downregulate MyoD, and eventually withdraw from the cell cycle to maintain the satellite cell population of the muscle.^{14,16,17} In contrast, whole-organ muscle regeneration is much more limited in mammals despite the presence of satellite cells. In salamanders, limb amputation is followed by limb regeneration in which Pax7-positive cells play an important role in some but not all salamander species.¹⁸

Pax7-positive satellite cells have also been described in mammalian EOMs.¹⁹ Additionally, progenitor cells that express *PITX2* have been described.²⁰ EOMs are a specialized form of skeletal muscle derived from branchial and cephalic mesoderm,^{21–23} and EOM satellite cells may have certain distinguishing features compared to their somitic counterparts. However,

published results are somewhat inconsistent. Some results suggest that the EOM stem cell niche is protected from aging and disease^{24,25} and maintains proliferative potential longer than somitic muscles.²⁶ Other results argue that aged EOM stem cells lose their ability to fuse to form multinucleated myotubes.²⁶ This disagreement in published conclusions may be the result of using different species and/or different experimental approaches.

To investigate the potential for adult whole-organ skeletal muscle regeneration, we exploited the well-documented regenerative capacity of adult zebrafish. With its genome nearly fully sequenced and with well-developed molecular tools, the zebrafish (*Danio rerio*) has become an important model for human disease research. In particular, adult zebrafish have robust regenerative capacity in organs as diverse as the brain, retina, fins, and heart.^{27–32} In regenerating complex tissues, zebrafish use both resident stem cells (e.g., melanocyte precursors in fins³³) and lineage-restricted dedifferentiated cells (e.g., cardiac myocytes,³⁴ retinal Müller glia,^{31,35} and osteoblasts^{32,36}), suggesting that zebrafish might be an ideal model for studying de novo whole-organ muscle regeneration.

Our model uses a large EOM myectomy in sexually mature adult zebrafish. We focused on the lateral rectus (LR) muscle because of its anatomic isolation from the other EOMs³⁷ and its surgical accessibility and the ability to assess its function using horizontal gaze eye movement. Here we report that, following myectomy of approximately 50% of the LR muscle, adult zebrafish regenerated an anatomically correct and functional muscle. Unlike embryonic EOM development, the regeneration process appears to be independent of pax7-positive satellite cells, as classic satellite cells are not detectable in zebrafish adult EOMs by electron microscopy or immunofluorescence techniques. Instead, our data reveal that residual EOM myocytes undergo dedifferentiation to form myoblasts, followed by robust myoblast proliferation and redifferentiation into functional muscle fibers. We propose that the ability of residual EOM myocytes to dedifferentiate following severe injury underlies their whole-organ regenerative capacity in zebrafish, compared with other species in which dedifferentiation is actively blocked. Furthermore, adult zebrafish EOM regeneration via dedifferentiation offers a powerful model for studying the mechanistic underpinnings of cellular reprogramming and dedifferentiation.

MATERIALS AND METHODS

Zebrafish (*D. rerio*) Rearing

All animal work was performed in compliance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the University of Michigan Committee on the Use and Care of Animals, protocol 06034. Sexually mature adult (4–18 months of age) wild-type and transgenic (α -actin::EGFP) zebrafish were spawned in our fish facility and raised according to standard protocol at 28°C with a 14-hour light:10-hour dark cycle.

Lateral Rectus Myectomy

Adult zebrafish were anesthetized (0.05% tricaine methanesulfonate) and placed on moist towelleles under a stereomicroscope. The right eye was adducted with a thin blunt probe, and a 0.5- to 1-mm segment of LR muscle was excised (Figs. 1A–C). This manipulation resulted in an immediate loss of LR muscle function (Figs. 1E, 1F, abduction).

Optokinetic Reflex (OKR)

Adult zebrafish were gently immobilized on wet foam in a transparent cylinder containing fresh system water and placed inside a hollow rotating drum marked by alternating white and black stripes.³⁸ In intact animals, drum rotation triggered the horizontal OKR, consisting of smooth pursuit and rapid saccade eye movements. Five animals per time point were used.

EOM Anatomic Analysis

Following euthanasia, LR regeneration was assessed by craniectomy and fluorescence microscopy (Figs. 1G–K). The length of the injured LR muscle was quantified and normalized to the length of the uninjured LR muscle (representing 100%). Five fish were analyzed by this method, either immediately after myectomy or at 3 or 9 days post injury (DPI) (Fig. 1P).

Zebrafish embryos were fixed in 4% paraformaldehyde (PFA) for 2 to 4 hours and cryoprotected in 20% sucrose for frozen sections (12 μ m). Control and experimental LR muscles were evaluated microscopically using frozen or paraffin- or methylacrylate-embedded specimens. Decalcification was performed using either Morse's solution or 10% ethylenediamine-tetraacetic acid (EDTA) in phosphate-buffered saline (PBS, pH 7.2–7.4). Coronal frozen sections (12 μ m) were collected at 0, 12, 24, 36, 48, 72, and 120 hours post injury (HPI). Paraffin sections (5 μ m) were obtained at 3 or 10 DPI. Coronal methylacrylate sections (5 μ m) were collected at 16, 35, 48, 144, and 240 HPI.

Nuclei were counted in 3 to 4 sections of each injured or uninjured muscle sampled. Care was taken not to count nerve and erythrocyte nuclei. Some sections were randomly recounted to assure accuracy, and there was less than 2% variation among the recounts.

BrdU/EdU Incorporation Assays

Cellular proliferation was assessed by intra-peritoneal (IP) injections of 5-bromo-2'-deoxyuridine (BrdU) or 5-ethynyl-2'-deoxyuridine (EdU) and standard detection methods.³⁹

Double labeling experiments were carried out by performing an initial LR muscle myectomy and IP injections of BrdU (20 μ L, 10 mM in PBS) on days 1, 2, and 3 post injury, followed 30 days later by a second myectomy and IP injections of EdU (20 μ L, 10 mM in PBS). In these experiments, fish were killed 30 days after the second myectomy and processed to detect BrdU and EdU incorporation.

For the time-course analysis, myectomized fish were injected with EdU at selected time points and killed 4 hours later. The injured muscles of 4 fish were analyzed at each time point. EdU-positive (EdU⁺) and total 4',6-diamidino-2-phenylindole (DAPI)-stained nuclei were counted from 3 to 11 nonconsecutive sections per muscle (more than 200 sections were analyzed), representing approximately 1900 total nuclei (range, 500–4800 nuclei). Cell proliferation (Fig. 7I) is represented as the percentage of EdU⁺ nuclei in the injured muscle. Percentage was calculated as EdU⁺-stained nuclei-to-DAPI-stained nuclei ratio multiplied by 100.

Transmission Electron Microscopy (TEM)

For TEM, 5 animals were anesthetized and then perfusion fixed in a solution of 4% PFA plus 1.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) followed by decapitation. Fixation of the heads was continued overnight in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, and the heads were then decalcified in 2.5% glutaraldehyde in 7.5% disodium EDTA for 2 days. LR

FIGURE 1. The adult zebrafish LR muscle displays a robust regenerative capability. Expression of EGFP under the control of the α -actin muscle promoter facilitates visualization of the LR muscle in adult zebrafish before surgery (A), after injury (B), and 7 DPI (C), revealing anatomic regeneration of the muscle (image is representative of 5 fish per time). (D) H&E staining of excised LR muscle segment shows typical multinucleated myofibers and sarcomeres. Myectomy of the right LR muscle results in loss of abduction (F) compared to that of an uninjured fish (E). Craniectomy was performed to visualize EGFP-labeled muscle (G–K). At selected time points, zebrafish heads were mounted in agarose (G), and tops of skulls were removed (H). Brain was removed to allow visualization of the skull base (*), where both LR muscles attach to the bone (I). Then the lateral bones of the skull were removed to allow complete visualization of the LR muscles (J). (K) Fluorescent visualization of J. (L–O) Craniectomy of a nonmyectomized fish (L, both of the LR muscles are highlighted), following myectomy (M) at 3 DPI (N) and at 9 DPI (O). Note that the injured muscle is correctly inserted onto the eye (pictures are representative examples of 5 fish per time point). (M, N) The end of the myectomized muscle is shown (arrows). (N) The end of the regrown LR muscle is shown (arrowhead). (P) Quantification of LR muscle regeneration; values are averages

$\pm$ SD ($n = 5$). Different letters indicate significant differences among groups ($P < 0.05$, Newman-Keuls multiple comparisons test). (Q) Diagram of a craniectomized zebrafish head; muscles visualized by this technique are shown, and LR muscles are highlighted in green. *Skull base where the pituitary is located. e, eye; p, pupil.

muscles were dissected from the head, washed in cacodylate buffer, and fixed further for 2 hours in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer. After being washed in cacodylate buffer, muscles were dehydrated in graded ethanol followed by propylene oxide and embedded in EMBED 812 (Electron Microscopy Sciences, Hatfield, PA, USA). Semi-thin sections (0.5 μ m) were cut, mounted on glass slides, and stained in toluidine blue. Subsequently, 70-nm thin sections were cut, mounted on copper slot grids, coated with formvar, and stained with uranyl acetate and lead citrate for examination with TEM (model CM100; Philips/FEI, Hillsboro, OR, USA) at 60 kV. Images were recorded digitally using an ORCA-HR digital camera system (Hamamatsu City, Japan) operated using AMT software (Advanced Microscopy Techniques, Corp., Danvers, MA, USA).

Immunolabeling

Zebrafish immunohistochemistry was carried out as previously described.³⁷ Negative control experiments with no primary or secondary antibodies were performed. Specific antibodies and working concentrations are listed in Supplementary Table S1. In situ hybridization was performed as previously described using digoxigenin-labeled RNA antisense probes.²³ Sense probes were included as negative controls.

RNA Extraction and qRT-PCR

RNA for quantitative RT-PCR (qRT-PCR) analysis was prepared from tissue samples using TRIzol (Invitrogen, Inc., Carlsbad, CA, USA), following the manufacturer's protocol. Uninjured EOMs (24–32 muscles) or dorsal pieces of somitic muscle from the right side were pooled from 3 different fish. Following DNase treatment, the extracted RNA was quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA quality was assessed using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA samples were reverse transcribed using an iScript reverse transcription kit (Bio-Rad Laboratories, Hercules, CA, USA).

TaqMan Master Mixes and probes (Life Technologies, Frederick, MD, USA) were used to analyze *pax3* and *pax7* expression (probes Dr03144248_m1 and Dr03125021_m1, respectively) using CFX96 real-time PCR detection systems (Bio-Rad Laboratories). β -Actin (probe Dr0342610_m1) was used as a reference gene, and gene expression was calculated by the delta-delta method.⁴⁰

Cell Cycle Length Quantification

The cell cycle length (Tc) was estimated following 2 different approaches, both of which used sequential labeling with EdU and BrdU. A schematic is shown in Supplementary Figure S1. For the first approach, a published protocol⁴¹ was followed. EdU and BrdU were injected and detected as described. A 3-hour interval was used for determining the length of S phase (Ts) and an 18-hour interval for determining Tc (Supplementary Fig. S1). Single- and double-labeled nuclei were counted, and Ts and Tc were calculated as described previously.⁴¹

In the second approach, a standard protocol was performed with minor modifications.⁴² IP injections of EdU and BrdU were performed at 42 and 45 HPI, respectively (3-hour interval; Supplementary Fig. S1). Animals were killed at 48 HPI and processed as described above. Single- and double-labeled and

total (DAPI)-stained nuclei were counted, and Tc was calculated.⁴² This method assumed that 100% of nuclei had entered the cell cycle (i.e., growth fraction of 100%).⁴² With this experimental paradigm, the percentage of nuclei that enter the cell cycle can be estimated by dividing the Tc of the first approach⁴¹ by the Tc of the second approach.⁴²

In each experiment, nuclei were counted from 3 to 6 nonconsecutive sections per fish, representing approximately 1000 total nuclei per fish (800–1800 nuclei). For the first approach, 7 fish were analyzed. In the second approach, each calculation was based on 5 fish (Ts) or 6 fish (Tc) per experiment.

Lineage Tracing

To label injured residual myocytes, a pledget soaked in dextran conjugated to Texas Red (10,000 MW; lysine-fixable; Invitrogen) was placed inside the eye orbit for 2 hours immediately following myectomy. The injured muscle was then visualized at selected time points by epifluorescence stereomicroscopy. To validate this technique, several control experiments were performed (Supplementary Fig. S2).

Statistics

Data were analyzed by Student *t*-test or one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparisons test ($P < 0.05$), using Prism version 6.03 software (GraphPad, LaJolla, CA, USA) for Mac OS X (Apple, San Francisco, CA, USA).

RESULTS

Zebrafish Extraocular Muscles Can Regenerate Following a Large Myectomy

To determine whether zebrafish can regenerate lost EOM tissue, the right LR muscle underwent a large myectomy (50% of the muscle). Adult transgenic α -actin::EGFP zebrafish express EGFP in the EOMs, allowing in vivo visualization (Fig. 1A). Following myectomy (Figs. 1B, 1D), these zebrafish unilaterally lost abduction (Figs. 1E–F) and both horizontal pursuit and saccadic eye movements (Supplementary Movies S1, S2). The LR muscle reformed with anatomically correct insertion on the sclera and regained function within 7 to 10 DPI (Fig. 1C and Supplementary Movie S3). Measuring the length of the injured LR muscle in a time course experiment (Figs. 1L–P, 0, 3, and 9 DPI) showed a steady increase in the length of the injured LR muscle from approximately 50% after injury (Fig. 1M) to 100% after 9 days (Fig. 1O).

To characterize the myofibers within the injured muscle, hematoxylin and eosin (H&E) staining was performed (Fig. 2). Transverse sections (Fig. 2J, positions I and II) allowed us to examine the residual muscle origin (proximal stump), whereas sagittal sections (Fig. 2J, position III) allowed us to examine the area previously excised by myectomy. The uninjured control LR muscle contained more muscle fibers (Fig. 2K) and nuclei (Fig. 2L) in the distal part (Fig. 2G, position III) than in the origin (Fig. 2A, position I, and Fig. 2D, position II). This observation suggests that many myofibers do not traverse the entire length of the muscle. At 3 DPI, there was a general hypercellularity and nuclear clustering typical of myoblast

FIGURE 2. H&E staining shows histological analysis of regenerating LR muscle. H&E staining of paraffin sections (5 μ m) of uninjured and injured muscles. (A, D, G) Sections of uninjured muscle at different positions of the LR muscle. (B, E, H) Sections of injured LR muscle at 3 DPI. (C, F, I) Sections of injured LR muscle at 10 DPI, at which time the muscle has correctly inserted again on the sclera and has recovered function. (J) Zebrafish head section showing the 3 positions from which muscle sections were taken; LR muscles are highlighted in green. Quantification of numbers of fibers (K) and nuclei (L) is shown at 10 DPI. Values are average $\pm$ SEM ($n = 4$ fish, 3 sections per fish were averaged). For each position (I, II, or III), differences between uninjured (blue) and injured (white) groups were analyzed by Student's *t*-test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). For each group (uninjured or injured), differences among positions (I vs. II vs. III) were analyzed by ANOVA. Different letters (lowercase for uninjured and uppercase for injured) indicate significant differences among positions ($P < 0.05$, Newman-Keuls multiple comparisons test). Images are representative examples from 4 fish analyzed per position and time point. (A–I) Magnification: $\times 630$ oil. *Skull base, where pituitary is located.

FIGURE 3. Regeneration occurs in the absence of pax7-positive satellite cells. Epifluorescence microscopy was used to detect pax7-positive cells (red) in embryonic somitic tail muscles ([A] magnification $\times 630$ oil) as well as in medial rectus ([B] magnification $\times 400$ oil) and LR muscle ([C] magnification $\times 400$ oil). Arrows indicate ends of the LR muscle; *pax7-positive cell. Neuromuscular junctions (green) and F-actin (yellow) are also noted. In the adult, pax7-positive cells are detectable within the adult zebrafish trunk (somatic) muscles (D), but no pax7-positive cells were detected within the adult EOMs (E) superior oblique; (F) LR) or in the regenerating LR muscle at 24 HPI (G, H). (D–H) Magnification: $\times 630$ oil; nuclei are blue (pictures are representative examples of the 5 fish analyzed per experiment). Gene expression of *pax7* in somitic and intact EOMs was assessed using qRT-PCR (I). In EOMs, *pax7* mRNA (arbitrary units [AU]) was detected at levels that were 25-fold lower than in somitic muscles ($*P < 0.05$).

fusion (Figs. 2B, 2E, 2H). At 10 DPI, despite the observed anatomic recovery by whole-mount analysis and functional recovery by OKR, regenerated myofibers at both the proximal and distal ends appeared small and hyperplastic (Figs. 2C, 2E, 2I), with a significant increase in the number of muscle fibers (Fig. 2K). The greater number of nuclei in the injured muscle (Fig. 2L) and our observations are consistent with ongoing myoblast fusion. Therefore, the injured muscle regenerates its length and function by 10 DPI, although the regeneration process persists beyond 10 days with continuing myoblast fusion. The observed increase in muscle fiber number at 10 DPI may be transient, with myocyte fusion restoring preinjury

fiber numbers or, alternatively, may persist, leading to alterations in posttraumatic muscle physiology. These alternatives suggest a testable hypothesis that we plan to explore in future research.

Adult Zebrafish EOMs Lack Pax7-Positive Satellite Cells

Pax7 is a well-studied marker of muscle satellite cells that are the primary source of myoblasts in muscle repair.^{12,13} Therefore, their role in adult zebrafish EOM regeneration was investigated. Using anti-pax7 antibodies, we evaluated the

FIGURE 4. TEM of LR muscle. Epon sections (0.5 μ m) stained with toluidine blue stain (A) show orientation and anatomy of the dissected LR muscle. The image is oriented so that the *top* of the image is toward the fish's mouth and is viewed in a coronal section orientation. TEM imaging (B–G) of control adult LR muscles showed no “classic” satellite cells in any section examined. Instead, several cells (*arrow*) could be found that had an appearance similar to that of the “postsatellite cells” found in newts (Carlson B, written communication, 2015). (B–G) Scale bars are shown in all panels. Direct magnification: $\times 10500$ (B); $\times 13500$ (C); $\times 19000$ (D, E); $\times 5800$ (F, G). FB, fibroblast cell; MC, myocyte cell; mf, myofibril; nv, nerve.

presence or absence of *pax7*-positive satellite cells in EOMs and somitic muscles of embryonic and adult animals. In embryos, *pax7*-positive nuclei were abundant in somitic muscles (Fig. 3A) and detectable in developing EOMs (Figs. 3B, 3C). In adults, *pax7*-positive nuclei were less abundant but

nevertheless easily identified in somitic muscles (Fig. 3D). In contrast, no *pax7*-positive cells were detected in either uninjured (Figs. 3E, 3F) or regenerating adult EOMs (Figs. 3G, 3H). Transcriptional analysis using qRT-PCR revealed that adult EOMs express *pax7* at a level 25-fold lower than adult somitic

FIGURE 5. Muscle regeneration occurs through a muscle-to-mesenchyme transition. (A) A DIC mosaic (magnification $\times 200$) of an uninjured LR muscle from proximal origin to distal insertion, highlighting the area to be excised (B) and residual muscle at the origin (I). (C–H) The area of the excised segment (C–F) fills gradually with mesenchyme-appearing cells (G) that assume the shape of myocytes by 120 HPI (H). Residual muscle at the origin (I) undergoes major remodeling (J–L) followed by a gradual reappearance of muscle fibers from 48 to 120 HPI (M–O). F-actin staining (phalloidin [purple]) reveals loss of cell polarity at 24 HPI ([Q] DIC image; S, immunofluorescence; magnification $\times 630$ oil) compared to uninjured control ([P] DIC image; R, immunofluorescence; magnification $\times 630$ oil). Expression of the mesenchymal marker vimentin (blue) is induced in injured ([U] DIC image; W, immunofluorescence; magnification $\times 400$) versus uninjured control muscle at 24 HPI ([T] DIC image; [V] immunofluorescence; magnification $\times 400$), along with induction of proliferation (EdU, green). In situ hybridization for *myod* expression (green) at 24 HPI ([Y] magnification $\times 400$) reveals induction of the myogenic pathway in the injured muscle compared to that in the uninjured control LR muscle (X). All pictures are representative examples from 4 to 5 fish examined at each time point or experiment.

muscle (Fig. 3I). Expression of *pax3* in adult EOMs was undetectable by qRT-PCR.

Because EOMs in some species contain muscle progenitor cells that do not express Pax7,²⁰ we used TEM to identify putative satellite cells. Classic satellite cells are defined by their localization between the basal lamina and sarcolemma. However, in the newt, these cells are fully encapsulated by the basal lamina and are sometimes referred to as postsatellite cells.^{43,44} Surprisingly, classic satellite cells were not detected in adult zebrafish EOM (Fig. 4), whereas presumed postsatellite cells (Figs. 4D, 4E, 4F, 4G, arrows) were very rare: quantification from 3 sections of LR muscles prepared from different fish revealed a range of 1 postsatellite cell per 1623 to 7718 μm^2 . We interpreted these collective data to mean that in adult zebrafish, regeneration of EOMs is independent of satellite cells.

Altered Cellular Morphology and Loss of Apical Cell Polarity Support a Muscle-to-Mesenchyme Transition as an Early Step of Regeneration

In the absence of data supporting a contribution by satellite cells to the regenerating EOMs, we sought to identify alternative cellular sources. Differential interference contrast (DIC) microscopy at multiple time points following injury revealed substantial changes in the cellular composition and morphology of myocytes at the residual muscle origin following myectomy (Figs. 5A–O). At this wound margin, the Z-band architecture was lost, and the myofibers became mesenchyme-like in appearance (Figs. 5I–L). As regeneration progressed, the mesenchymal elements were found more and more distally, and by 48 to 72 HPI (i.e., 2–3 DPI), mesenchyme-like cells had bridged the gap created by the myectomy (Figs.

FIGURE 6. Microanatomic analysis of regenerating LR muscle. Methylacrylate sections (5 μ m) stained with Lee's stain show the time course of EOM regeneration from 16 HPI to 10 DPI. Control muscle (A) shows distinct eosin-colored myofibers with sarcomeric banding pattern and non-nuclear basophilic (blue) staining of RNA at the peripheral margins of the fibers. For orientation, e indicates the direction of the eye. Scale bar: 50 μ m. By 16 HPI, the myofiber sarcomeric structure is degrading with loss of the z-band architecture. Small areas of pink degraded muscle material can be noted in many of the mesenchyme-appearing cells (arrows). The blood clot (red blood cells are nucleated in zebrafish) at the left side of image (B) shows the proximal limit of the surgical cut. (C) At 35 HPI, basophilic fiber-like cells are noted (asterisk), representing the onset of myoblast differentiation. At this time point, minimal amounts of degrading sarcomeric muscle remain in the regenerative field. At 2 DPI (D), 6 DPI (E) and 10 DPI (F), distinctly regenerating myofibers are visible first as basophilic muscle fibers, stained intensely blue due to abundant RNA (asterisk). Remodeling of the myofibers continues beyond 10 days. Images were obtained with a 63 $\times$ oil objective and some panels (B, C) are overlapping mosaics. Pictures are representative examples from the 3 fish examined per time point.

5E, 5G). As the gap was closed, the proximal muscle stump gradually recovered its Z-band architecture (Figs. 5M–O). Although there was a slight temporal delay, similar observations were made for the distal regions (Fig. 5H). Importantly, no classic blastema (i.e., mesenchymal progenitor cap) was ever detected at the cut edges of the muscle.

Myocytes have distinct polarity and morphology, which can be assessed through observation, staining for F-actin (phalloidin), and patterns of gene expression. In contrast, mesenchymal cells are defined by a lack of distinct polarity and the expression of vimentin, fibronectin, and smooth-muscle actin (SMA).^{45,46} Therefore, we next characterized the polarity, cellular morphology, and expression of mesenchymal markers in cells that accumulated at the proximal muscle stump in uninjured and regenerating EOMs. Uninjured EOMs have obvious sarcomeric structures (Fig. 5I, P and T), with clear cell polarity of the cytoskeleton (Fig. 5R, F-actin), low levels of vimentin (Fig. 5V), and low mRNA levels of fibronectin (*fn1* and *fn1b*) and *sma* (Supplementary Figs. S3, S4, S5). Following injury, myocytes at the muscle origin lost polarity (Figs. 5L, 5Q, 5U) and F-actin organization (Fig. 5S). Furthermore, there were increased levels of vimentin on immunofluorescence (Fig. 5W) and increased expression of *fn1*, *fn1b*, and *sma* (Supplemen-

tary Figs. S3, S4, S5). In situ hybridization analysis of the control (Fig. 5X) and regenerating muscle (Fig. 5Y) revealed that these mesenchymal cells expressed high levels of *myod*, identifying them as myoblasts. However, no *myod* expression was detected in the control uninjured muscle.

Next, we analyzed control and regenerating LR muscles in thin acrylic sections (Fig. 6). We observed mesenchymal cells consistent with the observed changes in the muscle architecture of the regenerating muscle (Fig. 6A versus Figs. 6B, 6C), followed by the appearance of newly forming basophilic myofibers that indicated the accumulation of mRNA⁴⁷ (Fig. 6D, asterisk). At 6 DPI, myofibers are clearly observed, although even at 10 DPI, the regenerated muscle continued to be hypercellular (Figs. 6E, 6F).

Taken together, these findings are most consistent with the interpretation that, following myectomy, residual myocytes undergo a muscle-to-mesenchymal transition (MMT) within 24 to 36 hours and induce *myod* to form myoblasts.

Cell Proliferation Analysis

Next, we hypothesized that cellular proliferation would be critical to generating sufficient numbers of cells to replace the

FIGURE 7. EOM regeneration involves a proliferative burst that includes postmitotic myonuclei. (A, B, E, F) EdU labeling (*red*) of fish pulsed at 20 HPI and killed at 24 HPI. (C, D, G, H) Higher-resolution detail than the box in A. (I) Cell proliferation quantification of the time course experiment. Values represent average $\pm$ SEM ($n=4$). Different letters indicate significant differences among groups ($P < 0.05$, Student's *t*-test). (J–M) Costaining of EdU and myosin heavy chain of regenerating LR muscle at 24 HPI reveals an elongated myonucleus that has entered the cell cycle. The ability of some myonuclei to proliferate was tested by costaining with EdU and anti-mef2c antibody. Mature somitic muscle (N) as well as EOM (uninjured medial rectus muscle [O]; uninjured LR muscle [P]) express high levels of mef2c in myonuclei. Note that when proliferative nuclei (EdU-labeled)

were detected, they never contained *mef2c* (N, P). Injured LR muscle lost *mef2c* staining almost completely, and only a few *mef2c*-positive nuclei per muscle were found at 24 HPI (Q). Interestingly, some of these *mef2c*-positive nuclei also contained EdU (*asterisk*). Nuclear colocalization of EdU and *mef2c* was confirmed by confocal microscopy (R–U). Cytoplasmic autofluorescence of a zebrafish nucleated red blood cell is shown (*arrow*); note how it does not co-localize with the nuclear DAPI staining. (J–U) Pictures are representative of 5 fish analyzed per experiment.

lost tissue. To characterize cell proliferation, a time course experiment was performed by injecting EdU at selected time points and killing fish 4 hours later (Figs. 7A–H). Cell proliferation over basal levels was first detected between 16 and 20 HPI, increased rapidly at 20 to 24 HPI, remained at high levels at 48 HPI, and persisted beyond 72 HPI, although at lower levels (Fig. 7I). A similar proliferative burst was observed following myectomy of the medial rectus muscle (Supplementary Fig. S6), indicating that this process is not exclusive to the LR muscle.

Having established that EOMs lacked satellite cells and that myectomy induced proliferation, we next sought to determine whether proliferating nuclei derive from residual myocytes. Costaining for EdU and myosin heavy chain at 24 HPI (Figs. 7J–M) revealed elongated EdU-positive myonuclei alongside myosin heavy chain protein, suggesting that myonuclei reenter the cell cycle. To further test this idea, we used an antibody against myocyte enhancer factor-2c (*mef2c*), a transcription factor that is expressed in both muscle and brain^{48,49} and is commonly used as a myonuclear marker.^{50,51} We hypothesized that, although MMT and proliferation would involve loss of myonuclear identity (i.e., loss of *mef2c* expression), at least some of the reprogrammed myonuclei would contain residual *mef2c* protein, allowing us to “catch in-the-act” early proliferating myonuclei (Supplementary Fig. S7A). First, we established that, as expected, myonuclei of mature uninjured somitic muscles (Fig. 7N) and EOMs (Fig. 7O, 7P) contained high levels of *mef2c*. Next, we stained for *mef2c* at 24 HPI while simultaneously colabeling proliferating cells with EdU. Injured LR muscle lost *mef2c* staining almost completely, and only a few *mef2c*-positive nuclei per muscle were found (8–13 nuclei per muscle section, 4–13 sections per fish, 5 fish). Importantly, some of these *mef2c*-positive nuclei also incorporated EdU (1–3 double-labeled nuclei per fish; Fig. 7Q, Supplementary Fig. S7B). Confocal imaging confirmed nuclear colabeling by EdU and anti-*mef2c* antibodies (Fig. 7R–7U, Supplementary Figs. S7C–S7J), revealing the fact that “postmitotic” myonuclei marked by *mef2c* expression proliferate after injury.

Cell Cycle Length Determination

Complete repopulation of the excised muscle field requires a sufficient number of regenerated myonuclei within 5 to 10 days. If the starting population of progenitor cells is small, then the timing would require rapid proliferation and short cell cycle length. Alternatively, if the starting population of progenitor cells is large, then repopulation of the regenerating muscle can take place within the observed time even if proliferation is slower and cell cycle length relatively longer. In order to determine the cell cycle length, a sequential EdU/BrdU double-pulse experiment was performed⁴¹ (Fig. 8A and Supplementary Fig. S1). This revealed a mean $\pm$ SEM Tc of 19.1 (± 0.3 hours; range, 18.1–20.3 hours), and Ts of 6.1 hours (± 0.7 hours; range, 5.4–7.2 hours). In order to determine the fraction of cells that entered the cell cycle, we calculated Tc using a second method that takes into account the total number of nuclei under the assumption that all of them proliferate.⁴² This calculation resulted in a mean $\pm$ SD Tc of 26.43 hours (± 5.17 hours) (Supplementary Table S2). The ratio between the 2 calculations revealed that 72% of nuclei entered the cell cycle in the first proliferative burst. This would

be inconsistent with a small number of stem cells driving the proliferative and regenerative processes, and instead suggests a large starting population of nuclei.

Sequential EOM Regeneration Reveals Myonuclear Dedifferentiation and Proliferation

The data so far suggest that regeneration of EOMs in adult zebrafish uses “postmitotic” myonuclei that dedifferentiate and reenter the cell cycle to provide sufficient cell mass for de novo muscle regeneration, as has been described in regeneration of zebrafish hearts^{34,52} and newt appendages.¹⁸ If myonuclear dedifferentiation is indeed primarily responsible for EOM regeneration, with minimal to no contributions from other sources (e.g., satellite cells, hematopoietic stem cells, and others) to form new muscle fibers, then the same myonuclei would be expected to undergo proliferation during multiple regeneration events. We tested this prediction using sequential myectomy experiments in two different experiments.

In the first experiment, animals were injected with BrdU following an initial myectomy. Thirty days later, a second myectomy was performed. Fish were killed 30 days after the second myectomy. Immunodetection of BrdU revealed a strong and even distribution of staining throughout the twice-regenerated muscle (Fig. 8B). The ubiquity and relative uniformity (i.e., minimal dilution) of BrdU labeling would be consistent with a large starting population and relatively low number of cell divisions. This also suggested that the two regenerative events shared a common progenitor population.

In a second experiment, we tested the prediction of a shared progenitor cell through sequential myectomies with BrdU and EdU labeling. BrdU was provided during the first regeneration process. Thirty days after the initial injury, a second myectomy was performed and dividing cells were labeled with EdU. This resulted in a large population of nuclei within the regenerated muscle that were labeled by either BrdU or EdU. Within the twice-regenerated muscle, 26.6% of labeled nuclei were colabeled with both EdU and BrdU (median: 23%; range, 7%–70%) (Fig. 8C), indicating that these nuclei divided during both regeneration events.

Dextran Lineage Tracing

To further test the role of extant myocyte dedifferentiation in the regeneration of the injured LR using an independent technique, dye-based lineage tracing was used. We specifically took advantage of the fact that the plasma membranes of residual myocytes are injured during myectomy, making these membranes transiently permeable to large molecules that otherwise would never enter the cell. In contrast, small cells, such as satellite cells or fibroblasts, would not be expected to suffer (or survive) such plasma membrane damage from a blunt injury such as a surgical myectomy. Subsequent membrane repair traps these labeled molecules in the cytoplasm, enabling lineage tracing as the muscle regenerates.

To label injured myocytes, EOMs were briefly exposed to dextran conjugated to Texas Red immediately after myectomy (Fig. 9A). Control experiments confirmed that myocytes in uninjured muscle are not labeled when exposed to dextran (Supplementary Figs. S2F–I). As the regenerating muscle grew and the proliferating myoblasts fused to form new

Cell Cycle Measurement

Double Myectomy - Single Labeling

Double Myectomy - Double Labeling

myocytes (Fig. 9B-D), dextran labeling was noted throughout the entire regenerated muscle, including the most distal elements at the insertion onto the eye (Figs. 9E-F).

DISCUSSION

Muscle disorders have a large impact on human health and quality of life. The ability to generate functional muscles *de novo*, as well as to repair injured or atrophic muscle, carries great promise in treating some of the most devastating forms of neuromuscular disease. Long-standing research approaches have made great strides in elucidating the cellular and molecular mechanisms that underlie muscle development. Such studies have identified satellite cells as being important both for muscle development and for muscle remodeling, repair and regeneration.⁵³⁻⁵⁶ However, despite extensive research on mammalian muscles that are rich in satellite cells, the ability of these muscles to undergo regeneration following very extensive damage requiring significant muscle replacement (i.e., “whole-organ” regeneration) is more limited.⁵⁷

To address the current limitations in the field of whole-organ muscle regeneration, we developed a novel model for adult skeletal muscle injury and repair using zebrafish EOMs: a large segment (50%) of the LR muscle is excised while surrounding tissue is left relatively intact (in contrast to salamander limb regeneration models). Characterization of the injured muscle revealed rapid recovery of muscle anatomy and function, although myoblast fusion continued well after anatomic and functional recovery was achieved (Figs. 1, 2), indicating that the regeneration process continues beyond 10 days.

To our initial surprise, EOM regeneration was accomplished with no apparent contribution from classic satellite cells, which were virtually undetectable. The lack of detectable pax7-positive satellite cells in regenerating EOMs might suggest that zebrafish adult EOMs may contain satellite cells that do not express pax7. Indeed, progenitor cells that express *PITX2* have been reported in EOMs.²⁰ We identified rare cells by TEM (Fig. 4) that might represent a type of “postsatellite cells” similar to the cells described in newts.⁴⁴ However, although even these so-called “post” satellite cells in newts express Pax7,⁴³ we

FIGURE 8. Dedifferentiated myocytes are used in muscle regeneration. (A) EdU labeling (red) at 24 HPI, followed by BrdU labeling (green) at 42 HPI (18-hour intervals) was used to assess cell cycle length, as published previously⁴¹; *double-labeled nuclei. Calculating the fraction of nuclei in the population that entered the cell cycle (see Methods and Results sections) reveals that 72% of nuclei entered the cell cycle at this early stage of proliferation. (B) Proliferating cells were labeled with BrdU following an initial myectomy. A second myectomy was performed 30 days later. Injured LR muscles were allowed to regenerate for another 30 days without additional exposure to BrdU. BrdU-labeled nuclei (red) were uniformly distributed throughout the regenerated muscle and displayed similar labeling intensity. (C) A double-myectomy experiment in which the regenerating muscle was exposed to BrdU (red) after the first myectomy and to EdU (green) after the second myectomy. The presence of double-labeled nuclei (yellow/orange, as well as nuclei with punctate red and green labeling) identifies nuclei that participated in both rounds of regeneration events. This image is representative of 15 sections from 7 fish. The average $\pm$ SEM of double-labeled nuclei as a percentage of total EdU and/or BrdU labeled nuclei was $26.2\% \pm 1.21\%$; the median percentage was 23%, and the range was 7% to 70%. Quantification was achieved by averaging the results of 2 independent observers. In control experiments, BrdU failed to label nuclei in the absence of the first myectomy, and EdU failed to label nuclei in the absence of the second myectomy. All images were taken using a Leica confocal microscope with a 63 $\times$ oil objective.

were unable to detect *pax7*-positive nuclei in adult EOMs, whether injured or intact (Fig. 3). The only distinguishing characteristic of newt postsatellite cells appears to be their complete encapsulation by basement membrane.⁴³ Interestingly, newt limb regeneration was also recently noted to be mostly independent of *Pax7*-positive cells.¹⁸

Instead, our zebrafish data are most consistent with myonuclear reprogramming of the residual myocytes to form muscle progenitor cells (i.e., fusion reversal), followed by robust proliferation, cell migration, and redifferentiation to skeletal muscle with myoblast fusion and myofibers formation. This conclusion is clearly at odds with the classic concept that skeletal muscles are composed by “postmitotic nuclei,” and that once myoblasts have fused, they can no longer divide.⁵⁸ Yet, the dedifferentiation model is supported by data obtained through multiple techniques and approaches. Importantly, our conclusions are based not on any one of these experiments but rather on the collective evidence of all data combined. A schematic model is offered in Figure 10.

The following is a summary of the data and our basic interpretation. The residual injured muscle is replaced by a mesenchymal cell mass, as noted by direct microscopic observation as well as use of available mesenchymal markers. Concomitant with the appearance of a mesenchymal cell mass is a robust proliferative wave. We determined that proliferation begins at 16 to 20 HPI, that 72% of the nuclei in the remaining muscle enter the cell cycle over the next 24 hours, and that the cell cycle length is 19.1 hours. EOMs show both anatomical and functional recovery by 7 DPI, although myoblast fusion continues past 10 DPI, indicating that the regeneration process is not yet fully complete at day 10. Taken together, these findings indicate that the EOM regeneration is carried out by a large number of cells that proliferate to regenerate the injured muscle.

To help identify the source of the proliferating mesenchymal cells, two complementary double-myectomy experiments were performed. These revealed that EOM regeneration requires relatively few cell cycles by a large starting population of cells (Fig. 8B), and that the same nuclei can undergo sequential rounds of dedifferentiation-redifferentiation (Fig. 8C). The results of these experiments, combined with the *mef2c*-labeling experiments, indicate that residual myonuclei are the main source of proliferating myoblasts in EOM regeneration. Although the number of colabeled nuclei (*mef2c*⁺/EdU⁺) was low, we interpreted the result of that experiment as reflecting the highly transient moment in which the switch occurs from differentiated to dedifferentiated myonucleus.

To further test our dedifferentiation hypothesis, injured residual myocytes were labeled with dextran and followed over time. At 10 DPI, all regenerated myofibers remained labeled throughout the length of the regenerated muscle (Figs. 9C–F). The most plausible explanation is that the dextran of the regenerated EOM at 10 DPI comes from the myoblasts that are formed by reprogramming of the dextran-labeled residual myocytes. We base this conclusion on the fact that the residual muscle loses myofiber architecture and is completely replaced by a mesenchymal cell mass by 36 HPI (Fig. 5) and that many myofibers do not traverse the entire length of the muscle (based on myofiber counts) (Fig. 2).

During skeletal muscle repair in mammals, proliferating satellite cells, known as myogenic progenitor cells (myoblasts), coexpress *Pax7* and *MyoD* and undergo multiple rounds of cell division.⁵⁹ Our zebrafish model is different in that the muscle is not simply injured but rather half the muscle is surgically excised, necessitating extensive organ regeneration in an adult organism. Although the dedifferentiated and proliferating mesenchymal cells responsible for the injured LR muscle

regeneration do not express *pax7*, they nevertheless express the myoblast marker *myod* (Fig. 5Y).^{60,61} Hence, our interpretation is that these mesenchymal cells of the regenerating muscle are myoblasts. Indeed, H&E-stained paraffin sections analysis of regenerating LR muscle reveals that at 3 DPI, the regenerating muscle is made up of myoblasts and newly-forming myocytes, and by 10 DPI, myoblasts are still actively fusing into myofibers, even in the area of the residual stump. We cannot discard the possibility that other cell types also play a role in the regenerative process, as described in other models of muscle injury.⁶² However, our data are not consistent with such cells making a significant contribution to LR muscle regeneration. Our data collectively support the conclusion that zebrafish EOM regeneration involves residual myocytes dedifferentiation, which includes loss of mature myogenic markers (e.g., myosin, *mef2c*), loss of cell polarity, reversal of myonuclear fusion, reentry into the cell cycle, and activation of muscle progenitor cells (e.g., *MyoD*).

In zebrafish, the ability to dedifferentiate mature myocytes after injury is not unique to EOMs. Genetic fate-mapping experiments reveal that cardiac regeneration in adult zebrafish also involves dedifferentiation.^{34,52} In the powerful model of newt limb regeneration, recent experiments using Cre-lox lineage tracing also reveal a role for myocyte dedifferentiation.¹⁸ Hence, it appears that cellular reprogramming and dedifferentiation may be shared features in organisms that demonstrate robust adult regenerative capacity. It is interesting to note that myocyte dedifferentiation is not observed in axolotl limb regeneration, and even in newt, it represents a partial contributor.¹⁸ In contrast, our findings in adult zebrafish EOM regeneration suggest that dedifferentiated cells are the most significant contributors to regeneration, providing a particularly robust model for studying the dedifferentiation process. We further demonstrate for the first time an MMT process that appears to underlie myocyte dedifferentiation. It is interesting to note that no centrally nucleated myofibers appear to form in regenerating zebrafish EOMs, in contrast to mammalian muscle repair. This may reflect the different mechanisms underlying this process: dedifferentiated myonuclei in zebrafish EOMs versus satellite cells in mammalian skeletal muscle, and may be a worthwhile subject for future research.

Adult zebrafish EOM regeneration also appears to be fundamentally different from zebrafish embryonic EOM development that involves *pax7*-positive cells. It also differs from larval tail muscle repair where dedifferentiation was found to be unnecessary, presumably because larval cells retain a certain level of developmental potency and injury constitutes interrupted development versus a requirement for true regeneration of adult tissue.⁶³ This fundamental difference between larval and adult regenerative mechanisms may underlie the confusion in the research reports about the role of dedifferentiation in zebrafish tissue regeneration. Larvae retain active embryonic pathways and would be expected to use *pax7*-positive progenitor cells. On the other hand, fully grown adults may have a smaller stem cell populations and can no longer rely on this population for extensive regeneration.⁶⁴ It also highlights our opinion that localized muscle repair/remodeling and even small-scale regeneration are distinctly different from the nearly de novo whole-organ muscle regeneration that we observe in our model.

The ability of cells to dedifferentiate to a lineage-restricted progenitor state appears to be broadly shared by a variety of zebrafish tissues, including retina, heart, bone, and cartilage.^{32–36,65} This ability may even underlie the regenerative capacity of zebrafish more broadly by providing a large pool of dedifferentiated progenitor cells that can achieve the necessary proliferative thrust. Indeed, we propose that in most organ-

FIGURE 9. Dye-based lineage tracing. Lineage tracing using dextran conjugated with Texas Red. Labeling the residual myocytes with dextran for 2 hours immediately after surgery was followed by assessment of dextran labeling within the regenerating muscle by whole-mount fluorescent microscopy as described in the legend to Figure 1. At 2 (A), 4 (B), and 10 DPI (C, D), dextran-labeled myofibers can be seen throughout the regenerated portion of the muscle labeling individual fibers. At 10 DPI, transgenic α -actin::EGFP zebrafish were used to visualize the muscles; a perfect colocalization of dextran and EGFP signal was found in the injured muscle (D). (E, F) Detail of the square in D. Pictures are representative examples of 5 fish analyzed per time point. *Skull base. e, eye.

FIGURE 10. Model of EOM regeneration. Following myectomy, the residual muscle undergoes MMT consisting of myocyte reprogramming, dedifferentiation, loss of apical cell polarity, and cell cycle reentry to become myoblasts. These proliferating progenitor cells migrate to repopulate the regenerating muscle, followed by fusion and redifferentiation.

isms, tissue stem cells (such as muscle satellite cells) may not have the proliferative capacity to fully regenerate organs de novo, whereas dedifferentiation can provide the injured or degenerated tissue with sufficient starting material.

The ability of zebrafish to regenerate multiple complex tissues through regulated reprogramming and cellular dedifferentiation raises a conundrum: why can zebrafish do something that humans and other mammals cannot do (i.e., dedifferentiation), and how can this apply to the human condition? We suggest that perhaps humans and other mammals did not lose the capacity to dedifferentiate but rather gained the capacity to block this process in order to prevent tumorigenesis and malignant degeneration. Specifically, we speculate that through evolution, perhaps in the context of the pressure to reduce the risk for cancer in long-lived land-based animals, mammals acquired active mechanisms to block

cell dedifferentiation. Indeed, the recently discovered role of the Arf tumor suppressor in blocking mammalian muscle regeneration provides further support for our hypothesis that an evolutionarily driven balance between regeneration and cancer determines the regeneration potential of different species.⁶⁶ If this is true, then developing the knowledge to temporarily deactivate such a mechanism in a controlled fashion may allow for robust but safe regenerative capabilities that could transform medical care.

In summary, we demonstrated that, following major injury, adult zebrafish EOMs regenerate. To do this, myocytes dedifferentiate, become mesenchymal, and reenter the cell cycle in a process that we described for the first time and named MMT. In this way, dedifferentiated myocytes drive a whole-muscle regenerative process. Understanding the mechanistic underpinnings of skeletal muscle dedifferentiation in

zebrafish will offer new insights into tissue specification, cellular identity, and the potential for using cell reprogramming to repair damaged or missing tissues.

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