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Satellite cells, connective tissue fibroblasts and their interactions are crucial for muscle regeneration

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SUMMARY

Muscle regeneration requires the coordinated interaction of multiple cell types. Satellite cells have been implicated as the primary stem cell responsible for regenerating muscle, yet the necessity of these cells for regeneration has not been tested. Connective tissue fibroblasts also are likely to play a role in regeneration, as connective tissue fibrosis is a hallmark of regenerating muscle. However, the lack of molecular markers for these fibroblasts has precluded an investigation of their role. Using Tcf4, a newly identified fibroblast marker, and Pax7, a satellite cell marker, we found that after injury satellite cells and fibroblasts rapidly proliferate in close proximity to one another. To test the role of satellite cells and fibroblasts in muscle regeneration in vivo, we created *Pax7*^{CreERT2} and *Tcf4*^{CreERT2} mice and crossed these to *R26R*^{DTA} mice to genetically ablate satellite cells and fibroblasts. Ablation of satellite cells resulted in a complete loss of regenerated muscle, as well as misregulation of fibroblasts and a dramatic increase in connective tissue. Ablation of fibroblasts altered the dynamics of satellite cells, leading to premature satellite cell differentiation, depletion of the early pool of satellite cells, and smaller regenerated myofibers. Thus, we provide direct, genetic evidence that satellite cells are required for muscle regeneration and also identify resident fibroblasts as a novel and vital component of the niche regulating satellite cell expansion during regeneration. Furthermore, we demonstrate that reciprocal interactions between fibroblasts and satellite cells contribute significantly to efficient, effective muscle regeneration.

KEY WORDS: Muscle regeneration, Satellite cells, Connective tissue, Fibrosis, Tcf4, Tcf7L2, Pax7, Mouse

INTRODUCTION

Adult vertebrate muscle has a remarkable capacity for regeneration. Because myonuclei within muscle fibers are post-mitotic, regeneration must be mediated by myogenic progenitors or stem cells. To efficiently regenerate an appropriately sized muscle, these cells proliferate, differentiate into muscle, and also replenish themselves. How proliferation versus differentiation of stem cells is carefully balanced is poorly understood, but is known to involve extrinsic signals, including signals from non-muscle cells (Cornelison, 2008). Here, we examine in vivo the role of satellite cells, the cells proposed to be the primary stem cells of adult muscle; muscle connective tissue (MCT) fibroblasts, a cell population in abundance in regenerating muscle, the function of which is largely unexplored; and their interactions during muscle regeneration.

Muscle satellite cells were first identified by their unique anatomical position between the sarcolemma and basement membrane of myofibers and were hypothesized to be the stem cell responsible for adult vertebrate muscle regeneration (Mauro, 1961). Subsequently, satellite cells were found to express the transcription factor Pax7 (Chen et al., 2006; Seale et al., 2000). Functionally, Pax7 is required prior to postnatal day (P) 21 in mice for maintenance of satellite cells (Kuang et al., 2006; Oustanina et al., 2004; Relaix et al., 2006; Seale et al., 2000), but afterwards is not required for regeneration (Lepper et al., 2009). Genetic lineage studies using *Pax7*^{CreER} mice have established that Pax7^{+} cells give rise to regenerated muscle after injury (Lepper et al., 2009; Lepper and Fan, 2010; Shea et al., 2010). Furthermore, transplanted satellite cells can regenerate myofibers in vivo, as first shown by Collins and colleagues (Collins et al., 2005) and subsequently by others (Cerletti et al., 2008; Kuang et al., 2007; Montarras et al., 2005; Sacco et al., 2008). Satellite cells are suggested to be crucial for muscle regeneration because manipulations of important signaling pathways alter both satellite cells and muscle regeneration (for reviews, see Kang and Krauss, 2010; Kuang et al., 2008). However, despite this wealth of studies (Kang and Krauss, 2010; Kuang et al., 2008), surprisingly, the necessity of satellite cells has not been explicitly tested. Furthermore, the finding of other stem cells able to regenerate muscle [e.g. mesoangioblasts and PW1^{+}/Pax7^{-} interstitial cells (PICs)] (Kuang et al., 2008; Mitchell et al., 2010), has called into question the requirement of satellite cells for regeneration.

Satellite cells are regulated by their surrounding niche, which includes multiple cell types. Endothelial and inflammatory cells, particularly macrophages, regulate satellite cell proliferation and differentiation (Christov et al., 2007; Cornelison, 2008; Robertson et al., 1993; Tidball and Villalta, 2010). Another population likely to be an integral component of muscle regeneration is the MCT fibroblasts. Connective tissue fibrosis, an increase in extracellular matrix (ECM), is characteristic of regenerating muscle, as well as many other regenerating tissues (Cornelison, 2008; Goetsch et al., 2003; Huard et al., 2002; Serrano and Munoz-Canoves, 2010; Tomasek et al., 2002; Verrecchia and Mauviel, 2007). This fibrotic ECM is synthesized largely by fibroblasts resident in the MCT (Alexakis et al., 2007; Bailey et al., 1979; Kuhl et al., 1982; Lipton, 1977; Sanderson et al., 1986; Sasse et al., 1981; Zou et al., 2008). However, study of MCT fibroblasts has been hindered by the lack of robust markers for these cells. Recently, we have identified the transcription factor Tcf4 (transcription factor 7-like 2, Tcf7L2) as an excellent marker of MCT fibroblasts (Mathew et al., 2011). Tcf4^{+} fibroblasts are derived developmentally from the lateral plate

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mesoderm and are a separate lineage from somitically derived myogenic cells (Kardon et al., 2003). During development, Tcf4$^+$ fibroblasts are closely associated with developing muscle and regulate muscle fiber type and maturation (Kardon et al., 2003; Mathew et al., 2011). The observation that MCT fibrosis is characteristic of regenerating muscle and our recent finding that fibroblasts regulate muscle development together suggest that Tcf4$^+$ MCT fibroblasts might be important regulators of muscle regeneration.

In this study, we examine in vivo the role of satellite cells, MCT fibroblasts and their interactions in muscle regeneration. Using mouse genetics to genetically label and ablate Pax7$^+$ satellite cells and Tcf4$^+$ MCT fibroblasts, we demonstrate the necessity of satellite cells for muscle regeneration and the regulation of satellite cells by nearby MCT fibroblasts. Importantly, we also demonstrate that reciprocal interactions between satellite cells and fibroblasts ensure efficient and effective muscle regeneration.

MATERIALS AND METHODS

Mice

We generated the *Pax7^{CreERT2}* targeting vector using published methods (Wu et al., 2008). In brief, we recombineered a 7.4 kb fragment of mouse *Pax7* from a BAC library (CHORI clone RP24-128A11) into the pStart plasmid and introduced an *IRES-CreERT2-FRT-Neo-FRT* cassette (from D. Kopinke and L. C. Murtaugh, University of Utah) 8 bp after the endogenous stop codon. The targeting vector was electroporated into G4 ES cells and selected with G418 and FIAU. Twenty-nine of 148 clones exhibited homologous recombination upon Southern blotting with a probe outside the 5′ homology arm. Appropriate recombination was confirmed by a 3′ probe for 28/29 of these, and one of these clones was used to generate chimeras. Subsequent genotyping was performed using the following primers: Forward 1: 5′-GCTGCTGTTGATTACCTGGC-3′; Reverse 1: 5′-GCACTGAGACAGGACCG-3′; and Reverse 2: 5′-CAAAAGACGGCAATATGGTG-3′. PCR products: wild-type, 417 bp; CreERT2, 235 bp. *Neo* was removed by crossing *Pax7$^{iCreERT2+neo}$* mice with *R26R^{Flpe}* mice (Farley et al., 2000). *Tcf4^{CreERT2}* mice were generated (in collaboration with M. L. Angus-Hill and M. R. Capecchi, University of Utah) by replacing the translated part of exon 1 of *Tcf4* by a *CreERT2* cassette (Feil et al., 1997) and GCSF polyA using published methods (Wu et al., 2008). Details of *R26R^{lacZ}*, *R26R^{YFP}*, *R26R^{mTmG}*, *R26R^{DTA}* and *Polr2a^{nlacZ}* mice have been published (Haldar et al., 2008; Muzumdar et al., 2007; Soriano, 1999; Srinivas et al., 2001; Wu et al., 2006). For all experiments, mice were bred onto a C57/Bl6J background and used at 6-8 weeks of age.

Muscle injury, tamoxifen and EdU delivery

Injury was induced by injecting 25 μl of 1.2% $BaCl_2$ or 25 μl of 10 mM cardiotoxin in normal saline into the right tibialis anterior (TA) muscle. The left TA served as the uninjured control. Each dose of tamoxifen was 10 mg in corn oil delivered via gavage (Park et al., 2008). For EdU labeling, mice received 100 μg 5-ethynyl-2′-deoxyuridine (EdU; Invitrogen) in PBS by intraperitoneal injection 8 hours before harvest. All animal protocols were Institutional Animal Care and Use Committee (IACUC) approved.

Immunofluorescence, histology and microscopy

For section immunofluorescence, flash-frozen or OCT-embedded (GFP$^+$) muscles were sectioned at 10 μm, fixed for 5 minutes in 4% paraformaldehyde (PFA), washed in PBS and then, if needed, subjected to antigen retrieval, which consists of heating slides in citrate buffer (1.8 mM citric acid, 8.2 mM sodium citrate in H_2O) in a 2100 PickCell Retriever followed by quenching for 5 minutes in 3% H_2O_2. Cells were fixed for 20 minutes in 4% PFA. Tissue sections and cells were blocked for 30-60 minutes in 5% serum or 0.5% TNB blocking reagent (PerkinElmer) in PBS, incubated overnight at 4°C in primary antibody, washed in PBS, incubated for 2 hours at room temperature in secondary antibody, washed in PBS, then, when needed, incubated for 3 hours in Vector ABC, washed in PBS and labeled for 10 minutes with PerkinElmer TSA Fluorescein or TSA Cy3. Afterwards, slides were washed in PBS, and EdU labeled using the Invitrogen Click-iT Kit per manufacturer instructions. Slides were then washed in PBS, post-fixed for 5 minutes in 4% PFA and mounted with Fluoromount-G (SouthernBiotech) with 2 mg/ml Hoechst. For Sirius Red staining, flash-frozen sections were fixed for 1 hour at 56°C in Bouin's fixative, washed in water, stained for 1 hour in Master*Tech Picro Sirius Red, washed in 0.5% acetic acid, dehydrated, equilibrated with xylene and mounted using Permount (Kiernan, 1990). Nile Blue staining was carried out according to published methods (Kiernan, 1990). Cells and Sirius Red- and Nile Blue-stained sections were imaged in bright-field on a Zeiss Axioplan2 microscope. Immunofluorescent sections were imaged on a Nikon AR1 confocal microscope. Each confocal image is a composite of maximum projections, derived from stacks of optical sections.

For section immunofluorescence, the following antibodies were used and required antigen retrieval: Pax7 [2.4 μg/ml, mouse IgG1, PAX7, Developmental Studies Hybridoma Bank (DSHB)], MyoD (4 μg/ml, mouse IgG1, Sc-32758, Santa Cruz Biotechnology), MyHCemb (3 μg/ml, mouse IgG1, F1.652, DSHB), MyHCI (1.5 μg/ml, mouse IgG1, M8421, Sigma), MyHCperi+II (10 μg/ml, mouse IgG1, M4276, Sigma), laminin (2.5 μg/ml, rabbit polyclonal, L9393, Sigma), Tcf4 (10 μg/ml, mouse IgG2a, 05-511, Millipore; or 0.7 μg/ml, rabbit monoclonal, 2569, Cell Signaling) and phospho-histone H3 (5 μg/ml, rabbit polyclonal, 06-570, Millipore). The following antibodies were used on sections and did not require antigen retrieval: PDGFRα (5 μg/ml, goat polyclonal, AF1062, R&D Systems), F4/80 (2 μg/ml, rat IgG2a, 14-4801, eBioscience) and GFP (20 μg/ml, chick polyclonal, GFP-1020, Aves Labs).

For immunofluorescence of isolated myofibers or fibroblasts, the following antibodies were used, did not require antigen retrieval and were visualized with fluorophore-conjugated secondary antibodies (e.g. 488 or 594 goat anti-mouse IgG2A): Syndecan 4 (1:1500, chick polyclonal, gift of D. D. Cornelison, University of Missouri), CD34 (10 μg/ml, rat IgG2a, 14-0341, eBioscience) and aSMA (5.2 μg/ml, mouse IgG2a, A2547, Sigma), as well as GFP, PDGFRα, Tcf4 (see above).

Cell culture

For myofiber preparations, TAs were digested with 400 U/ml Collagenase I (Worthington) for 90 minutes at 37°C, washed in PBS, fixed for 5 minutes in 4% PFA, washed in PBS and mounted onto slides before being processed for immunofluorescence. Five days post-injury (dpi) with $BaCl_2$, MCT fibroblasts were isolated from TAs by digestion with 2000 U/ml Collagenase I for 60 minutes at 37°C. Cells were filtered, spun at 2500 rpm (1258 ***g***) for 20 minutes and plated (25,000 cells/cm^2) on 6-cm plastic dishes containing gelatin-coated coverslips for 2 hours at 37°C in F12 Hams with 10% FBS. Cells in the supernatant were discarded and 24-48 hours later adherent cells were harvested.

Semi-quantitative PCR

For semi-quantitative PCR, fibroblasts were isolated by pre-plating cells from limb muscles of neonatal wild-type mice and myoblasts isolated by fluorescence-activated cell sorting (FACS) of yellow fluorescent protein (YFP)$^+$ cells from limb muscles of neonatal *Pax7$^{Cre/+}$;R26R$^{YFP/+}$* mice as described by Mathew et al. (Mathew et al., 2011). Total RNA was extracted using the TissueLyser II and Qiagen RNeasy Lipid Tissue Mini Kit and reverse transcribed with Invitrogen Superscript III. Equal amounts of RNA were amplified by 34 cycles of PCR using primers for *Gapdh* (5′-GCACCACCAACTGCTTAGC-3′; 5′-GCCGTATTCATTGTCATACC-3′), *Tcf4* (5′-GGAGGAGAAGAACTCGGAAAA-3′; 5′-AGGTAGGG-GCTCGTCAGGT-3′) and *Col6a3* (5′-ACAAATGCCCTTGCTGCTAC-3′; 5′-ATCGCCCACAATGCCCAGAA-3′).

Quantification and statistics

The number of Pax7$^+$, MyoD$^+$ or Tcf4$^+$ nuclei was determined using the ImageJ Analyze Particles function. Co-labeling of Pax7, MyoD or Tcf4 with phosphohistone-H3 (PHH3) or EdU was determined by additive image overlay in ImageJ. For MyHCemb (embryonic myosin heavy chain) or MyHCtotal (total myosin heavy chain), the total number of MyHCemb$^+$ or MyHCtotal$^+$ pixels was counted. For quantification of MCT extracellular matrix, Sirius Red$^+$ area was quantified by selecting red pixels in Adobe Photoshop, deleting all non-red pixels, converting the resulting image to a

binary image and counting Red$^+$ pixels using the ImageJ Analyze Particles function. For each variable, counts of two to three sections across the entire TA were averaged for three to five individuals of each genotype per time point and analyzed using Student's two-tailed *t*-test.

RESULTS

Tcf4 is highly expressed in MCT fibroblasts during adult muscle regeneration

During development and in the adult, fibroblasts in the MCT endomysium, perimysium and epimysium strongly express Tcf4 (Mathew et al., 2011). To determine whether Tcf4$^+$ MCT fibroblasts are present during muscle regeneration in the adult, we examined the tibialis anterior (TA) muscle of wild-type mice five days post injury (dpi) by $BaCl_2$. $BaCl_2$ causes myofiber hypercontraction and death, but does not affect the surrounding mononuclear populations, such as the satellite cells and fibroblasts (Caldwell et al., 1990; Hansen et al., 1984). In TAs at 5 dpi, there is an abundance of Sirius Red$^+$ MCT surrounding regenerating myofibers (Fig. 1B). Within these MCT regions and outside the laminin$^+$ regenerating myofibers, cells strongly expressing Tcf4 were detected by immunofluorescence (Fig. 1A,C). Because Tcf4$^+$ cells do not co-label with Pax7, MyoD or F4/80 (Fig. 1E,F), they are unlikely to be myogenic progenitors, myoblasts or macrophages. Furthermore, many of these Tcf4$^+$ cells co-label with PDGFRα (platelet-derived growth factor receptor alpha), a receptor expressed on MCT fibroblasts (Fig. 1D) (Joe et al., 2010; Olson and Soriano, 2009; Uezumi et al., 2010).

We characterized Tcf4$^+$ cells further by isolating and growing MCT fibroblasts in culture. MCT fibroblasts were isolated by plating cells freshly dissociated from adult TAs (at 5 dpi by $BaCl_2$) on plastic culture dishes. After two hours, myogenic cells, which do not readily adhere to plastic (Richler and Yaffe, 1970), were discarded and adherent cells were washed and grown for 24-48 hours. The adherent cells were highly enriched with fibroblasts (identifiable morphologically by their pseudopodia and large, round nuclei), and these fibroblasts were Tcf4$^+$ (Fig. 1G,H). In addition, Tcf4$^+$ fibroblasts were PDGFRα$^+$ and αSMA$^+$ (alpha smooth muscle actin, another marker of fibroblasts) (Tomasek et al., 2002) (Fig. 1G,H). MCT fibroblasts have been shown previously to synthesize high levels of ECM and to uniquely synthesize collagen VI, whereas myogenic cells do not synthesize this collagen (Zou et al., 2008). We found by semi-quantitative PCR that fibroblasts, isolated via pre-plating, express Tcf4 and collagen VI whereas myoblasts, isolated by FACS of YFP$^+$ cells from *Pax7$^{Cre/+}$;R26R$^{YFP/+}$* mice (in which myogenic precursors and their descendents are labeled) (Hutcheson et al., 2009) do not (Fig. 1I). In summary, we show that during muscle regeneration MCT fibroblasts express Tcf4, and that Tcf4$^+$ cells are neither myogenic cells nor macrophages.

Pax7$^+$ satellite cells and Tcf4$^+$ fibroblasts rapidly expand in close proximity to one another after muscle injury

To assess the potential role of satellite cells and MCT fibroblasts during muscle regeneration, we characterized the temporal-spatial relationship between Pax7$^+$ satellite cells and Tcf4$^+$ fibroblasts during normal regeneration after $BaCl_2$ injury in wild-type mice. Similar to the findings of others (d'Albis et al., 1988), we observed that regenerating myofibers begin to express the developmental myosin heavy chain isoform MyHCemb at 3 dpi, express peak levels at 5 dpi, and by 14 dpi the mature, regenerated myofibers no longer express MyHCemb, but only mature MyHCI/II isoforms

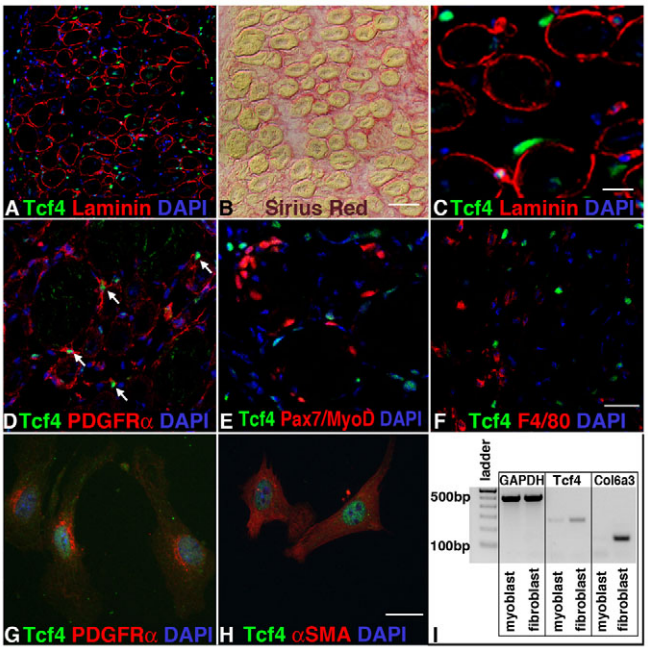

Fig. 1. Tcf4 is highly expressed in muscle connective tissue (MCT) fibroblasts during muscle regeneration. (**A-C**) Tcf4$^+$ cells at 5 dpi ($BaCl_2$) are interstitial to regenerating laminin$^+$ myofibers within Sirius Red$^+$ MCT. A and B show adjacent sections. (**D-F**) In muscle at 5 dpi, some Tcf4$^+$ cells are PDGFRα$^+$ (D, arrows), and all are Pax7$^-$ and MyoD$^-$ (E) and F4/80$^-$ (F). (**G**,**H**) MCT fibroblasts isolated from TAs at 5 dpi are Tcf4$^+$, PDGFRα$^+$ (G) and αSMA$^+$ (H). (**I**) Semi-quantitative PCR shows that neonatal MCT fibroblasts, but not myoblasts, express Tcf4 and Col6a3. Scale bars: in B, 50 μm for A,B; in C, 12.5 μm; in F, 25 μm for D-F; in H, 10 μm for G,H.

(Fig. 2A-G,CC; data not shown). An increase in MCT ECM is also characteristic of muscle regeneration (Huard et al., 2002). By quantifying the amount of Sirius Red$^+$ MCT (Dubowitz and Sewry, 2007), we found that MCT increases to peak levels at 3 dpi, in close proximity to the regenerating myofibers, and then gradually resolves to near-normal amounts by 21 dpi (Fig. 2V-BB,FF). Concentrated in the region of regenerating myofibers, Pax7$^+$ satellite cells rapidly proliferated within 1 dpi (Fig. 2GG) and expanded from 25 cells/mm^2 at 1 dpi to 180 Pax7$^+$ cells/mm^2 by 5 dpi (Fig. 2H-N,DD). Satellite cells either differentiate into myoblasts and myofibers (which downregulate Pax7) or return to quiescence as Pax7$^+$ satellite cells lying under the basement membrane of myofibers (Seale et al., 2000). By 28 dpi, the number of quiescent Pax7$^+$ satellite cells returned to normal, uninjured levels (Fig. 2DD; data not shown). We also found that 3 dpi Tcf4$^+$ fibroblasts rapidly proliferate (Fig. 2HH) and increase from 165 cells/mm^2 at 1 dpi to peak levels of 650 Tcf4$^+$cells/mm^2 at 5 dpi (Fig. 2O-U,EE). These Tcf4$^+$ fibroblasts were in close proximity to satellite cells and regenerating myofibers and lay within the Sirius Red$^+$ MCT (Fig. 2D,K,R,Y). Concomitant with the decrease in MCT, the number of Tcf4$^+$ fibroblasts decreased to normal, uninjured levels by 28 dpi (Fig. 2U,EE; data not shown). Thus, we show for the first time that during muscle regeneration MCT fibroblasts rapidly expand in regions of regenerating myofibers and MCT fibrosis, and in close association with satellite cells. The close temporal and spatial relationship between satellite cells and MCT fibroblasts suggests that interactions between these two cell types might be important for regeneration.

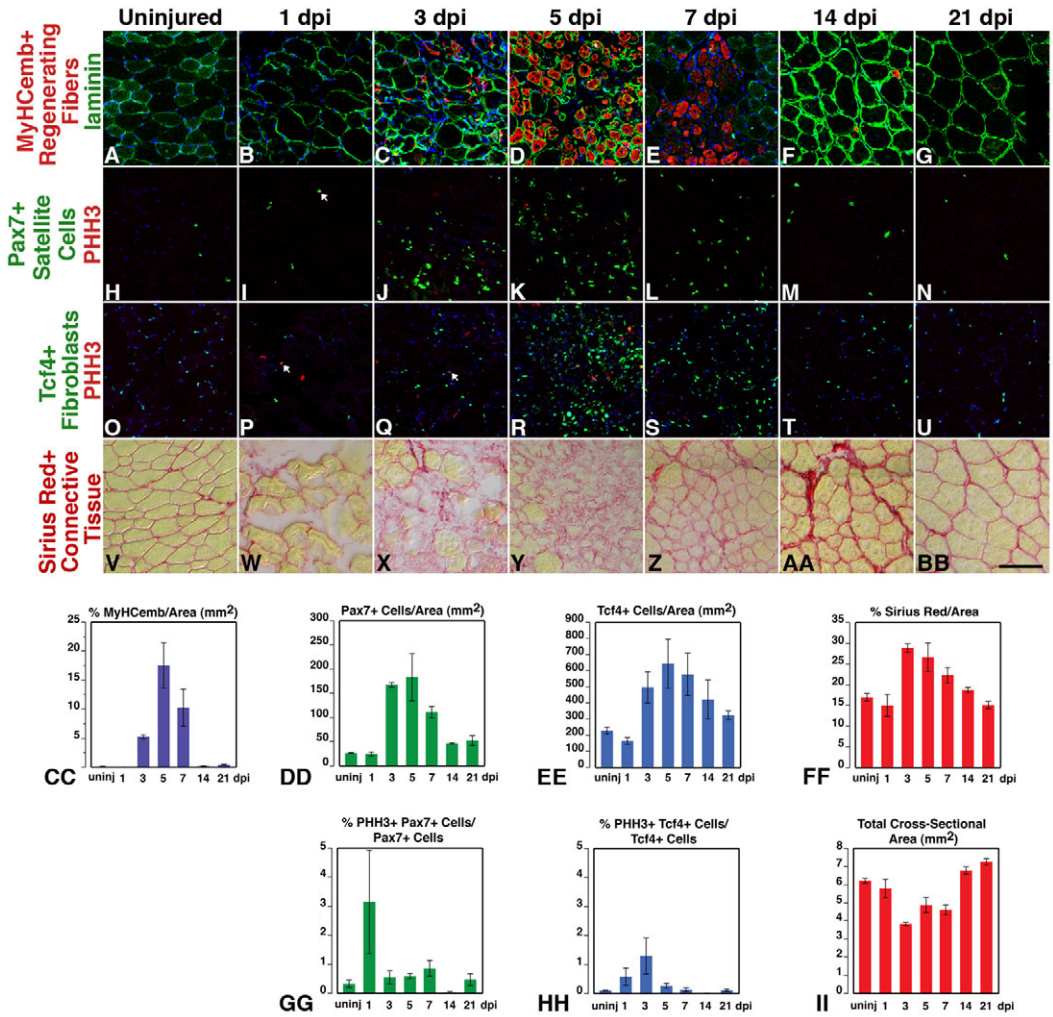

Fig. 2. After $BaCl_2$ injury, Pax7⁺ satellite cells and Tcf4⁺ fibroblasts expand rapidly in close proximity to one another and regenerating myofibers. (**A-G**,**CC**) MyHCemb⁺ regenerating myofibers. (**H-N**,**DD**,**GG**) Pax7⁺ satellite cells. (**O-U**,**EE**,**HH**) Tcf4⁺ muscle connective tissue (MCT) fibroblasts. (**V-BB**,**FF**) Sirius Red⁺ MCT. Arrows in I, P and Q label a few of the PHH3⁺ Pax7⁺ or PHH3⁺Tcf4⁺ cells. (**II**) Tibialis anterior (TA) cross-sectional area measured on Sirius Red-stained sections 30-40 μm from TA origin. At each time point, adjacent sections are shown. Scale bar: 100 μm for A-BB. For all graphs, mean ± s.e.m. are plotted.

Pax7^{CreERT2} mice allow for efficient manipulation of satellite cells

To genetically manipulate satellite cells in the adult, we created tamoxifen-inducible *Pax7*^{CreERT2} mice by homologous recombination. In these mice, an *iresCreERT2* cassette (Feil et al., 1997) is inserted just after the endogenous termination codon, maintaining the 3′UTR and the endogenous poly(A) sequence (Fig. 3A). To test the efficiency of Cre-mediated recombination in Pax7⁺ satellite cells, we crossed *Pax7*^{CreERT2} to *R26R*^{lacZ}, *R26R*^{YFP}, *R26R*^{mTmG} or *Polr2a*^{nlacZ} reporter mice, which express β-galactosidase, cytoplasmic YFP, or membrane-bound GFP in response to Cre (Haldar et al., 2008; Muzumdar et al., 2007; Soriano, 1999; Srinivas et al., 2001). In the absence of tamoxifen, no *Pax7*$^{CreERT2/+}$;*R26R*$^{reporter/+}$ mice ever expressed any of the reporters. We tested whether, in the presence of tamoxifen, *Pax7*^{CreERT2} mice genetically labeled Pax7⁺ cells by harvesting uninjured TAs from adult *Pax7*$^{CreERT2/+}$;*R26R*$^{mTmG/+}$ mice one day after three daily tamoxifen doses. In TA sections, nearly all Pax7⁺ satellite cells lying under the laminin⁺ basement membrane of myofibers were GFP⁺ (Fig. 3F-I). In addition, no other cells (e.g. myonuclei or interstitial cells, such as fibroblasts) other than sublaminar Pax7⁺ cells, were GFP⁺ (Fig. 3J,K-N). To quantify the efficiency of Cre-mediated recombination in satellite cells, we isolated single myofibers from uninjured TAs of *Pax7*$^{CreERT2/+}$;*R26R*$^{YFP/+}$ mice, one day after five daily tamoxifen doses. Ninety-five percent of all Pax7⁺ satellite cells were YFP⁺ (n=764). In addition, we verified that YFP⁺ cells on the myofibers were satellite cells, as YFP⁺ cells were co-labeled with Syndecan4 and CD34, two other satellite cell markers (Fig. 3C,D) (Beauchamp et al., 2000; Cornelison et al., 2001). We also confirmed that Pax7⁺ cells give rise to regenerated muscle by injuring TAs from *Pax7*$^{CreERT2/+}$;*Polr2a*$^{nlacZ/+}$ mice (with five tamoxifen doses) and then staining 14 dpi TAs for β-galactosidase in section or in whole mount (Fig. 3O and Fig. 5B). As expected, all regenerated myofibers were β-galactosidase⁺.

Pax7^{CreERT2} mice were designed to preserve endogenous Pax7 expression and function. To test whether Pax7 expression and function were intact, we generated *Pax7*$^{CreERT2/CreERT2}$;*R26R*$^{YFP/+}$ mice. YFP⁺ CD34⁺ satellite cells were present on myofibers

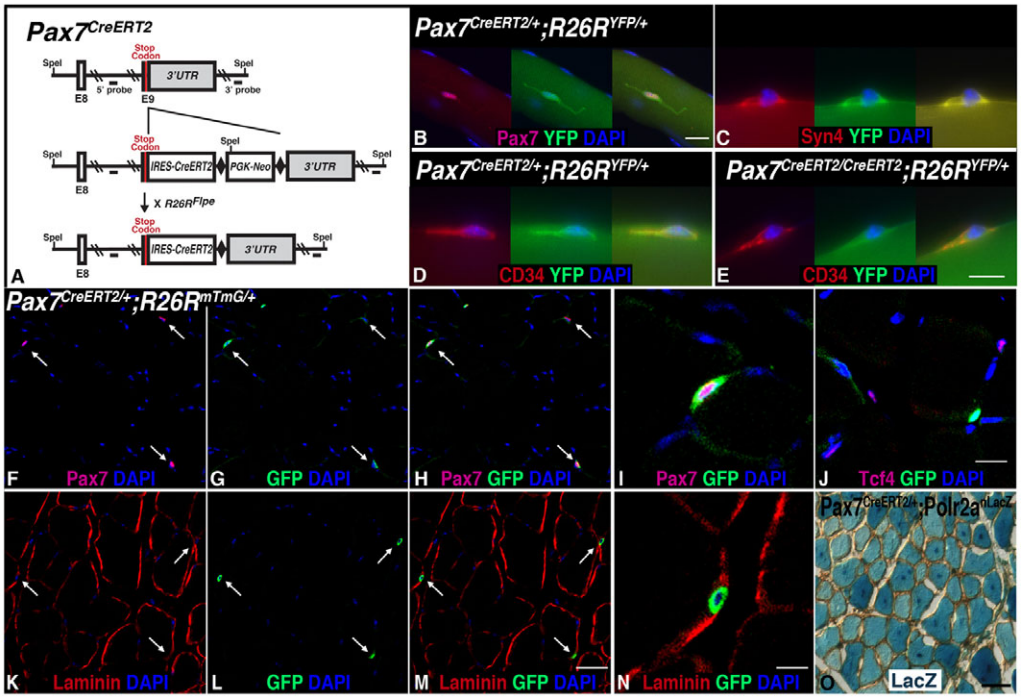

Fig. 3. *Pax7*^{CreERT2} mice efficiently label satellite cells. (**A**) *Pax7*^{CreERT2} targeting strategy. (**B-D**) In myofibers isolated from uninjured tibialis anterior muscles (TAs) from *Pax7*$^{CreERT2/+}$;*R26R*$^{YFP/+}$ mice one day after five daily tamoxifen doses, 95% of Pax7^{+} satellite cells are YFP^{+} (B) and YFP^{+} cells are Syndecan4^{+} (C) and CD34^{+} (D). (**E**) In fibers isolated from uninjured TAs from *Pax7*$^{CreERT2/CreERT2}$;*R26R*$^{YFP/+}$ mice one day after five tamoxifen doses, normal numbers of CD34^{+} satellite cells are present. (**F-O**) In cryosections of uninjured TAs from *Pax7*$^{CreERT2/+}$;*R26R*$^{mTmG/+}$ mice one day after three tamoxifen doses, nearly all Pax7^{+} cells are GFP^{+} (F-I), lie within laminin^{+} myofiber basement membrane (K-N), and are Tcf4^{-} (J). Tomato in Cre-myofibers was quenched by antigen retrieval. Arrows indicate Pax7^{+} satellite cells. (O) At 14 dpi, all regenerating myofibers are β-galactosidase^{+} in *Pax7*$^{CreERT2/+}$;*Polr2a*$^{nlacZ/+}$. Scale bars: in B, 20 μm; in E, 10 μm for C-E; in M, 25 μm for F-H,K-M; in J, 12.5 μm; in N, 6.25 μm for I,N; in O, 50 μm.

isolated from uninjured TAs of *Pax7*$^{CreERT2/CreERT2}$;*R26R*$^{YFP/+}$ mice (1 day after five daily tamoxifen doses; Fig. 3E). In addition, we counted equivalent numbers of Pax7^{+} cells in cryosections of uninjured and injured TAs at 5 dpi ($BaCl_2$) from *Pax7*$^{CreERT2/+}$ and *Pax7*$^{CreERT2/CreERT2}$ mice (data not shown). As the loss of Pax7 prior to P21 has been found to impair satellite cell proliferation and maintenance (Kuang et al., 2006; Lepper et al., 2009; Oustanina et al., 2004; Relaix et al., 2006), the finding of equivalent numbers of Pax7^{+} cells in 6-week-old *Pax7*$^{CreERT2/+}$ and *Pax7*$^{CreERT2/CreERT2}$ mice confirms that Pax7 function is not compromised. Thus, our data demonstrate that *Pax7*^{CreERT2} mice allow for high efficiency Cre-mediated genetic manipulation of satellite cells, without compromising Pax7 expression or function.

Ablation of Pax7^{+} satellite cells leads to a complete loss of muscle regeneration, misregulation of Tcf4^{+} fibroblasts and increased MCT fibrosis

Satellite cells have been proposed to be the primary stem cell responsible for regenerating muscle. To test whether satellite cells are necessary for regeneration, we genetically ablated Pax7^{+} satellite cells using *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice. In these mice, Cre activates expression of diphtheria toxin A (DTA) (Wu et al., 2006) and kills Pax7^{+} satellite cells specifically in response to tamoxifen. We found that in the injured TAs of *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice after five tamoxifen doses (strategy in Fig. 4A-I) and at 5 dpi ($BaCl_2$), 91% of Pax7^{+} cells were ablated (*P=0.02, *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ versus *Pax7*$^{+/+}$;*R26R*$^{DTA/+}$ mice, n=6; Fig. 4A-C). Satellite cell ablation was also confirmed by analyzing the number of Pax7^{+} or Syndecan4^{+} satellite cells (per mm of myofiber length) on isolated uninjured myofibers from *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ and *Pax7*$^{+/+}$;*R26R*$^{DTA/+}$ mice 30 days after five tamoxifen doses. In this assay, 83-84% of satellite cells were ablated (*$P=8\times10^{-5}$ Pax7^{+} cells, n=149 myofibers and three mice or *$P=1\times10^{-7}$ Syn4^{+} cells, n=172 myofibers and three mice; see Fig. S1A-D in the supplementary material).

Satellite cell ablation severely impaired muscle regeneration at 5 dpi, resulting in an 89% reduction in MyHCemb^{+} regenerating myofibers (*P=0.02; Fig. 4D-F) compared with *Pax7*$^{+/+}$;*R26R*$^{DTA/+}$ mice (also with five tamoxifen doses). In addition to impairing muscle, satellite cell ablation affected the expansion of Tcf4^{+} fibroblasts, as their numbers were reduced by 52% (P=0.09; Fig. 4G-I).

Muscle regeneration was dramatically impaired at 28 dpi by satellite cell ablation (but with no other apparent effects on mouse survival or behavior). In *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice (Fig. 4J-V), only a few small, clonal patches of Pax7^{+} cells remained (which presumably escaped Cre-mediated DTA ablation, data not shown). TAs were entirely fibrotic with no visible muscle (except for adjacent uninjured extensor digitorum longus, EDL, muscles) in *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice, compared with fully regenerated muscles in *Pax7*$^{+/+}$;*R26R*$^{DTA/+}$ mice (Fig. 4S,T). Rather than the normal hypertrophy seen after injury (Fig. 2U,V), the weight of injured TAs (normalized to left uninjured TAs) was reduced by 38% (*P=0.01, n=6), and the cross-sectional area reduced by 60% (*P=0.02; Fig. 4L,O,R) in *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ versus *Pax7*$^{+/+}$;*R26R*$^{DTA/+}$ mice. In sections through the entire TA, few myosin^{+} (MyHCI and II) myofibers were present in the injured

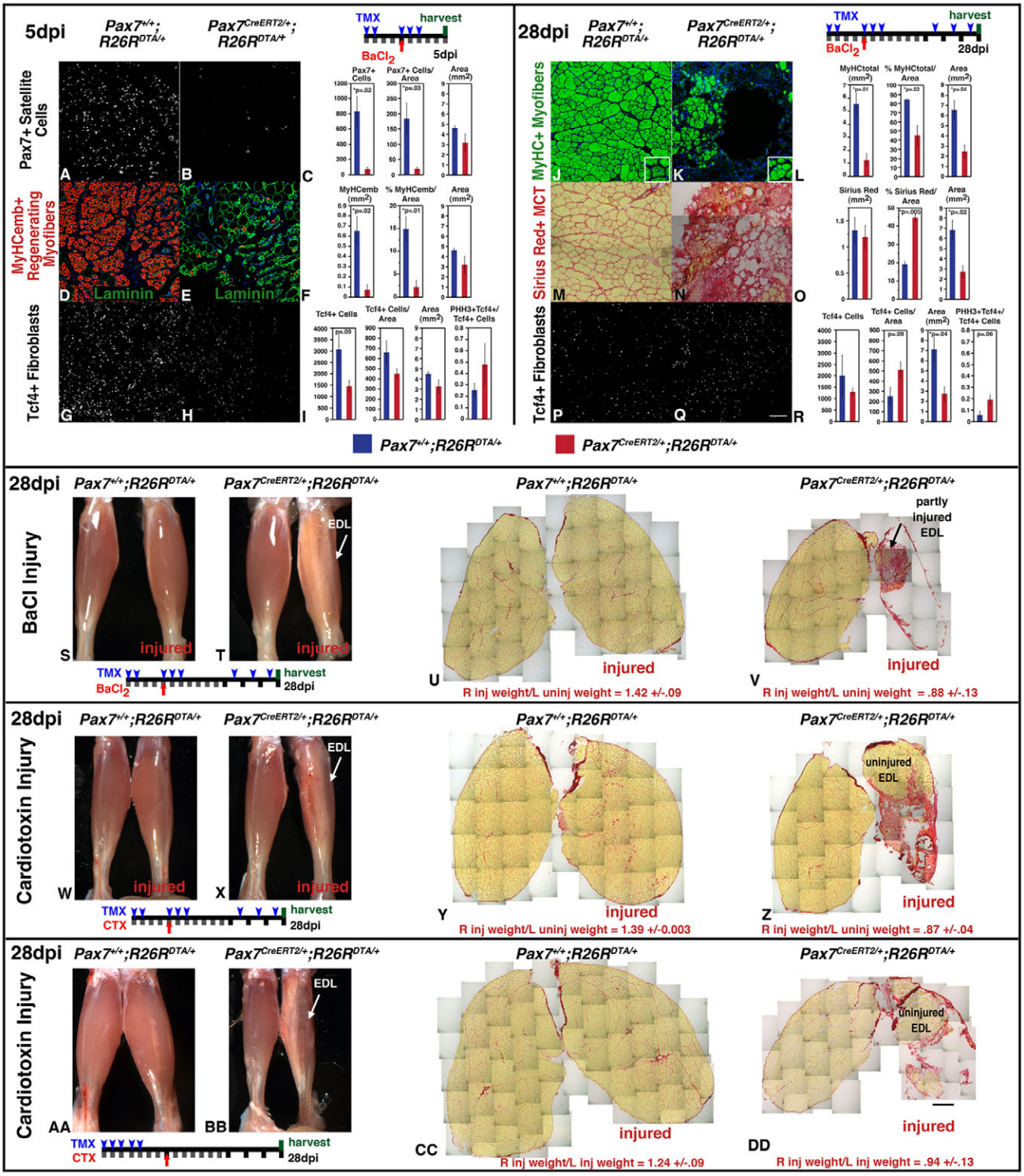

Fig. 4. Ablation of Pax7⁺ satellite cells leads to complete loss of muscle regeneration. (**A-I**) At 5 dpi, 91% of Pax7⁺ cells are ablated (A-C), resulting in fewer MyHCemb⁺ regenerating myofibers (D-F) and Tcf4⁺ fibroblasts (G-I) in *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice. (**J-R**) At 28 dpi, tibialis anterior (TA) cross-sectional area (L,O,R) and MyHCemb⁺ regenerated myofibers (J-L) are reduced, whereas the proportion of Sirius Red⁺ MCT (M-O), and the density of Tcf4⁺ fibroblasts (P-R) is increased in *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice. Insets in J and K show residual, incompletely injured myofibers with peripheral nuclei in *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice compared with regenerated myofibers with centralized nuclei in *Pax7*$^{+/+}$;*R26R*$^{DTA/+}$. (**S-Z**) At 28 dpi, {$BaCl_2$ or cardiotoxin (CTX)], injured TAs are completely fibrotic or edemic in *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice, in whole mount (S,T,W,X) and Sirius Red-stained cross-sections (U,V,Y,Z). (**AA-DD**) Ablation of satellite cells prior to CTX injury leads to loss of regenerated muscle. In all tamoxifen/injury strategy schema, gray bars represent one day and black bars one week, tamoxifen (TMX) administration is indicated by blue arrowheads and $BaCl_2$ or CTX application is indicated by red arrows. Whole mount images have been flipped so the injured limb (R) is on the right. TA weights include attached extensor digitorum longus (EDL). Scale bars: in Q, 100 μm for A,B,D,E,G,H,J,K,M,N,P,Q; in DD, 500 μm for U,V,Y,Z,CC,DD. For all graphs, mean ± s.e.m. are plotted.

region (77% reduction in MyHCI/II area, **P*=0.01; Fig. 4J-L,U-V). The few myofibers in *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice after $BaCl_2$ injury were residual, incompletely injured, small myofibers with peripheral nuclei, whereas myofibers in *Pax7*$^{+/+}$;*R26R*$^{DTA/+}$ mice were regenerated myofibers with characteristic centralized nuclei (insets in Fig. 4J,K). In *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice, muscle was replaced by a 2.3-fold increase in Sirius Red$^+$ MCT (Sirius Red$^+$ MCT area/total area, **P*=0.0002; Fig. 4M-O,U,V), and the number of Tcf4$^+$ fibroblasts/cross-sectional area was increased twofold (*P*=0.09; Fig. 4P-R).

We also tested the requirement of Pax7$^+$ satellite cells for muscle regeneration after injury by cardiotoxin (CTX), a protein kinase C inhibitor that causes cellular apoptosis (Gayraud-Morel et al., 2007; Sinha-Hikim et al., 2007). Twenty-eight days post-injury, muscle had not regenerated in *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice (Fig. 4W-Z). TA weight was reduced by 37% (**P*=0.0003, *n*=6). Muscle was largely replaced by Sirius Red$^+$ MCT (Fig. 4Y,Z) and increased Nile Red$^+$ adipose tissue (see Fig. S1M,N in the supplementary material). To test whether regeneration ever recovered, we harvested cardiotoxin-injured muscles at 56 dpi. Injured TAs still did not regenerate with ablation of satellite cells, and, in fact, the weight of TAs was even more reduced, by 56% (**P*=4×10^{-6}, *n*=6), in *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ versus *Pax7*$^{+/+}$;*R26R*$^{DTA/+}$ mice (see Fig. S1E-H in the supplementary material).

Other cell populations have also been proposed as stem cells important for muscle regeneration. Some of these populations, such as PICs (Mitchell et al., 2010), initially do not express Pax7, but later express Pax7 during their differentiation into muscle. Our analysis of *Pax7*$^{CreERT2/+}$;*R26R*^{mTmG} mice found that in the absence of injury, delivery of tamoxifen caused Cre-mediated recombination only in satellite cells residing beneath the basal lamina of myofibers. Delivery of tamoxifen after injury in *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice could potentially cause Cre-mediated ablation of other stem cells, which after injury express Pax7. To exclude potential ablation of these other cells that express Pax7 after injury, we repeated CTX injury experiments with *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ and *Pax7*$^{+/+}$;*R26R*$^{DTA/+}$ mice, but with five tamoxifen doses prior to injury. Similar to our other experiments, there was a complete absence of regenerated muscle, the weight of injured TAs was reduced by 24% (*P*=0.06, *n*=7), and muscle was replaced by Sirius Red$^+$ MCT in *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice (Fig. 4AA-DD). To determine further whether other stem cell populations might regenerate muscle, we ablated satellite cells, injured muscles via CTX, then at 28 dpi re-injured muscles and assessed regeneration 28 days later. Again, muscle was unable to regenerate, and the weight of injured TAs was reduced by 44% (**P*=0.002, *n*=16; see Fig. S1I-L in the supplementary material) in *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice.

We conclude that genetic ablation of Pax7$^+$ satellite cells resulted in a complete loss of regenerated muscle, demonstrating that satellite cells are required for regeneration. Furthermore, ablation of satellite cells led to misregulation of Tcf4$^+$ fibroblasts and a dramatic increase in MCT, revealing that satellite cells also have an effect on MCT fibroblasts and fibrosis.

Tcf4^{CreERT2} mice allow for manipulation of connective tissue fibroblasts

To genetically manipulate MCT fibroblasts in the adult, we created tamoxifen-inducible *Tcf4*^{CreERT2} mice. Previously, we generated *Tcf4*^{GFPCre} mice and showed that these mice allow for genetic manipulation of MCT fibroblasts (Mathew et al., 2011). To produce a tamoxifen-inducible version, we replaced the translated part of exon 1 and its splice donor with a *CreERT2* cassette placed at the endogenous *Tcf4* start codon (Fig. 5A). This substitution of exon 1 with *CreERT2* resulted in a 'knock-in/knockout allele' such that *Tcf4* is not expressed from the *Tcf4*^{CreERT2} allele. Similar to *Tcf4*^{GFPCre} and other *Tcf4* alleles (Korinek et al., 1998; Mathew et al., 2011), heterozygous *Tcf4*$^{CreERT2/+}$ mice are phenotypically normal and have normal muscle regeneration (data not shown).

We characterized *Tcf4*^{CreERT2} mice by crossing them to *R26R*^{lacZ} or *R26R*^{mTmG} reporter mice. TAs at 5 dpi ($BaCl_2$) were harvested from adult *Tcf4*$^{CreERT2/+}$;*R26R*$^{mTmG/+}$ mice after five tamoxifen doses (tamoxifen scheme in Fig. 6M-X; no reporter was ever expressed in the absence of tamoxifen), cryosectioned and immunolabeled for Tcf4 and GFP. Membrane-bound GFP$^+$ cells were Tcf4$^+$, exhibited a fibroblast morphology, were found interstitial to the regenerating laminin$^+$ myofibers, and did not co-label with the F4/80 macrophage marker (Fig. 5C-K). To characterize further the GFP$^+$ cells, we isolated fibroblasts by pre-plating cells from 5-dpi TAs of *Tcf4*$^{CreERT2/+}$;*R26R*$^{mTmG/+}$ mice after five tamoxifen doses. GFP$^+$ cells exhibited a fibroblast morphology; co-labeled with Tcf4 and the fibroblast markers αSMA and PDGFRα; and did not express the myogenic markers Pax7 or MyoD (Fig. 5L-O). Quantification of the efficiency of the Tcf4^{CreERT2} allele was problematic using *Tcf4*$^{CreERT2/+}$;*R26R*$^{mTmG/+}$ mice as we found that high levels of GFP expressed from the *R26R*^{mTmG} allele were toxic to fibroblasts in culture (data not shown). Based on the efficiency of ablation of fibroblasts in *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice (see next section), we estimate that the *Tcf4*^{CreERT2} allele causes Cre-mediated recombination in ~40% of MCT fibroblasts. To establish that *Tcf4*^{CreERT2} mice never caused Cre-mediated recombination in myogenic cells, we injured the TAs of *Pax7*$^{CreERT2/+}$;*R26R*$^{lacZ/+}$ and *Tcf4*$^{CreERT2/+}$;*R26R*$^{lacZ/+}$ mice that had been given five tamoxifen doses, and harvested TAs at 14 dpi. TAs were collagenase-treated to loosen myofibers (and this treatment also removed interstitial MCT fibroblasts) and processed for β-galactosidase staining. Although all myofibers were β-galactosidase$^+$ in the injured TAs (and some myofibers were β-galactosidase$^+$ in uninjured TAs) from *Pax7*$^{CreERT2/+}$;*R26R*$^{lacZ/+}$ mice, myofibers were never β-galactosidase$^+$ in TAs from *Tcf4*$^{CreERT2/+}$;*R26R*$^{lacZ/+}$ mice (*n*=10; Fig. 5B). This indicates that in *Tcf4*$^{CreERT2/+}$;*R26R*$^{lacZ/+}$ mice Cre-mediated recombination never occurs in satellite cells, myoblasts or myofibers, despite high numbers of these cells during muscle regeneration. Altogether, our data show that *Tcf4*^{CreERT2} mice allow for genetic manipulation of 40% of MCT fibroblasts and do not cause Cre-mediated recombination in satellite cells or macrophages, two other major populations present in regenerating muscle.

Ablation of Tcf4$^+$ MCT fibroblasts alters the expansion of satellite cells and impairs muscle regeneration

To test the potential function of Tcf4$^+$ fibroblasts during muscle regeneration, we ablated fibroblasts during regeneration using *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice. We first assessed the rate of fibroblast ablation by quantifying the decrease in Tcf4$^+$ cells in injured TAs at 5 dpi ($BaCl_2$) of *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ versus *Tcf4*$^{+/+}$;*R26R*$^{DTA/+}$ mice with five tamoxifen doses (strategy in Fig. 6M-X) and found a 67% decrease in Tcf4$^+$ cells (**P*=9×10^{-5}, *n*=10). Potentially, this decrease in Tcf4$^+$ cells could partially reflect the loss of one allele of *Tcf4* in *Tcf4*$^{CreERT2/+}$mice (as *Cre* replaces one *Tcf4* allele) and, thus, our decreased ability to detect Tcf4$^+$ cells. To test this, we quantified the numbers of Tcf4$^+$ cells in *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ versus *Tcf4*$^{+/+}$;*R26R*$^{DTA/+}$ mice with no tamoxifen and found an apparent 25% decrease in Tcf4$^+$ cells. As

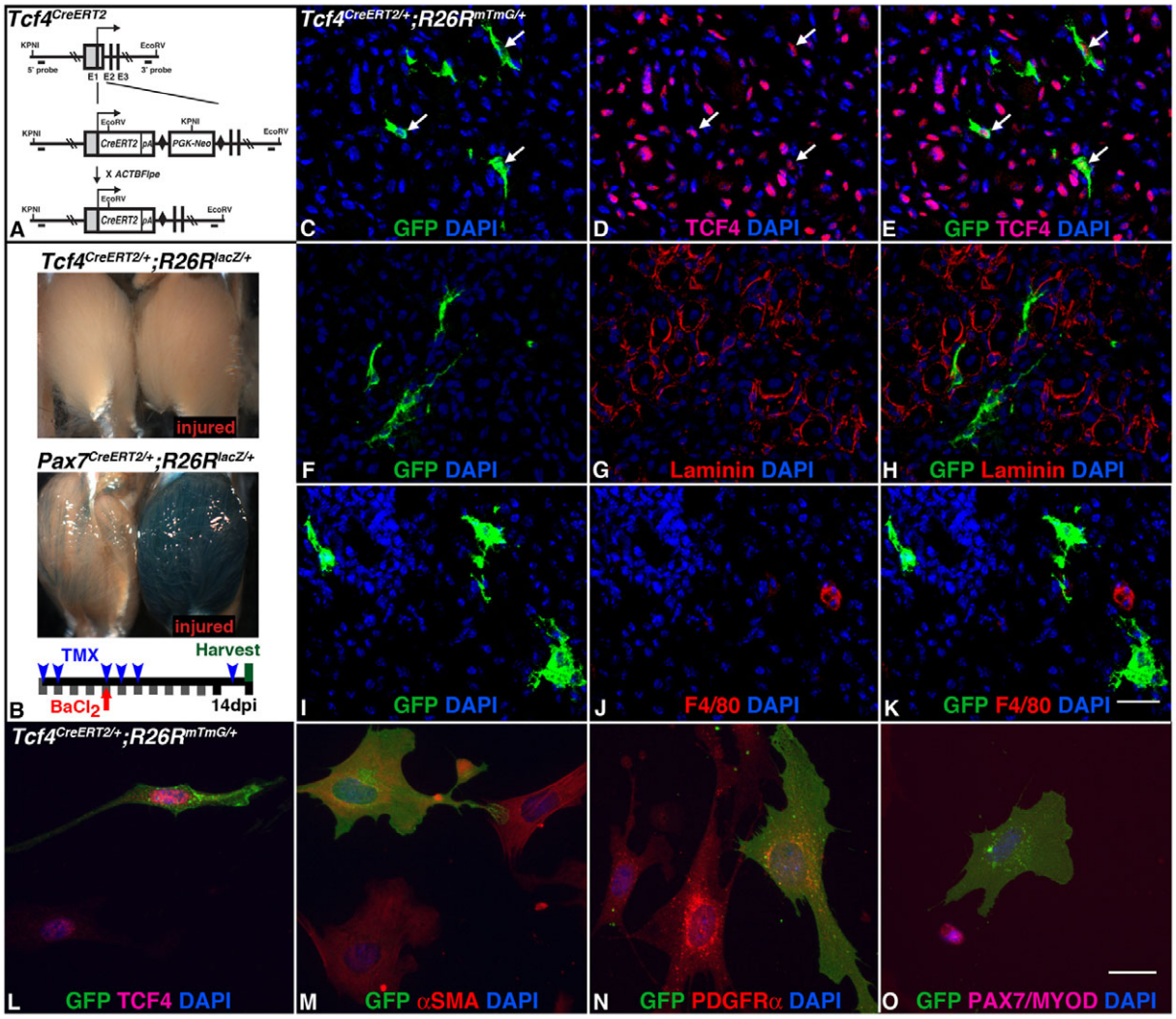

Fig. 5. *Tcf4*^{CreERT2} mice label muscle connective tissue (MCT) fibroblasts. (**A**) *Tcf4*^{CreERT2} targeting strategy. (**B**) At 14 dpi, several myofibers in uninjured tibialis anterior muscles (TAs) and all myofibers in injured TAs of *Pax7*$^{CreERT2/+}$;*R26R*$^{lacZ/+}$ mice are β-galactosidase$^+$, but in TAs of *Tcf4*$^{CreERT2/+}$;*R26R*$^{lacZ/+}$ mice no myofibers are β-galactosidase$^+$. (**C-K**) In cryosections of TAs at 5 dpi ($BaCl_2$) from *Tcf4*$^{CreERT2/+}$;*R26R*$^{mTmG/+}$ mice (five tamoxifen doses), Tcf4$^+$ fibroblasts are GFP$^+$ (F-K), lie in between laminin$^+$ regenerating myofibers (F-H) and are not F4/80$^+$ (I-K). (**L-N**) MCT fibroblasts isolated from TAs at 5 dpi ($BaCl_2$) from *Tcf4*$^{CreERT2/+}$;*R26R*$^{mTmG/+}$ mice (five tamoxifen doses) are GFP$^+$ (L-N), Tcf4$^+$ (L), αSMA$^+$ (M) and PDGFRα$^+$ (N). (**O**) In preparations of all mononuclear cells from the same *Tcf4*$^{CreERT2/+}$;*R26R*$^{mTmG/+}$ mice as shown in L-N, GFP$^+$ cells are Pax7$^-$ and MyoD$^-$. Scale bars: in K, 25 μm for C-K; in O, 10 μm for L-O.

we have not detected decreased numbers of fibroblasts isolated and cultured from *Tcf4*$^{CreERT2/+}$ versus *Tcf4*$^{+/+}$ mice, this decrease in Tcf4$^+$ cells probably results from fibroblasts expressing Tcf4 at levels below the limit of immunofluorescent detection. From these data, we then calculate the ablation rate of Tcf4$^+$ cells to be 42%; 67% decrease in Tcf4$^+$ cells in *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ versus *Tcf4*$^{+/+}$;*R26R*$^{DTA/+}$ mice (five tamoxifen doses) minus the apparent 25% decrease in Tcf4$^+$ cells in *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ versus *Tcf4*$^{+/+}$;*R26R*$^{DTA/+}$ mice (no tamoxifen).

Ablation of 42% of fibroblasts strongly affected satellite cell dynamics during muscle regeneration. At 5 dpi, fibroblast ablation led to a 51% reduction in Pax7$^+$ cells in *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ versus *Tcf4*$^{+/+}$;*R26R*$^{DTA/+}$ mice (*P=0.002; Fig. 6M-O). This reduction in satellite cells was not due to an effect of Tcf4 haploinsufficiency, as levels of Pax7$^+$ cells were equivalent at 5 dpi in *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ versus *Tcf4*$^{+/+}$;*R26R*$^{DTA/+}$ mice in the absence of tamoxifen (data not shown). With ablation of fibroblasts, the reduction in Pax7$^+$ cells was not due to reduced proliferation of Pax7$^+$ cells (Fig. 6M-O). In addition to reduced numbers of Pax7$^+$ cells, there was a 79% reduction in MyoD$^+$ cells (P=0.06; Fig. 6P-R), indicating a decrease in activated MyoD$^+$Pax7$^+$ satellite cells and/or MyoD$^+$ differentiating myoblasts (see Cornelison and Wold, 1997; Yablonka-Reuveni and Rivera, 1994). In addition, there was a 31% decrease in MyHCemb$^+$ regenerating myofibers (P=0.10; Fig. 6S-U). These results indicate that genetically reducing the numbers of fibroblasts negatively affected the initial muscle regenerative process.

To determine the mechanism underlying this impairment in muscle regeneration, we examined *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice at 3 dpi. With five tamoxifen doses (strategy in Fig. 5A-L), there was a 19% reduction in the number of Tcf4$^+$ fibroblasts (calculated as above, but high variance precluded statistical significance, n=10; Fig. 6J-L) in *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice. This ablation of

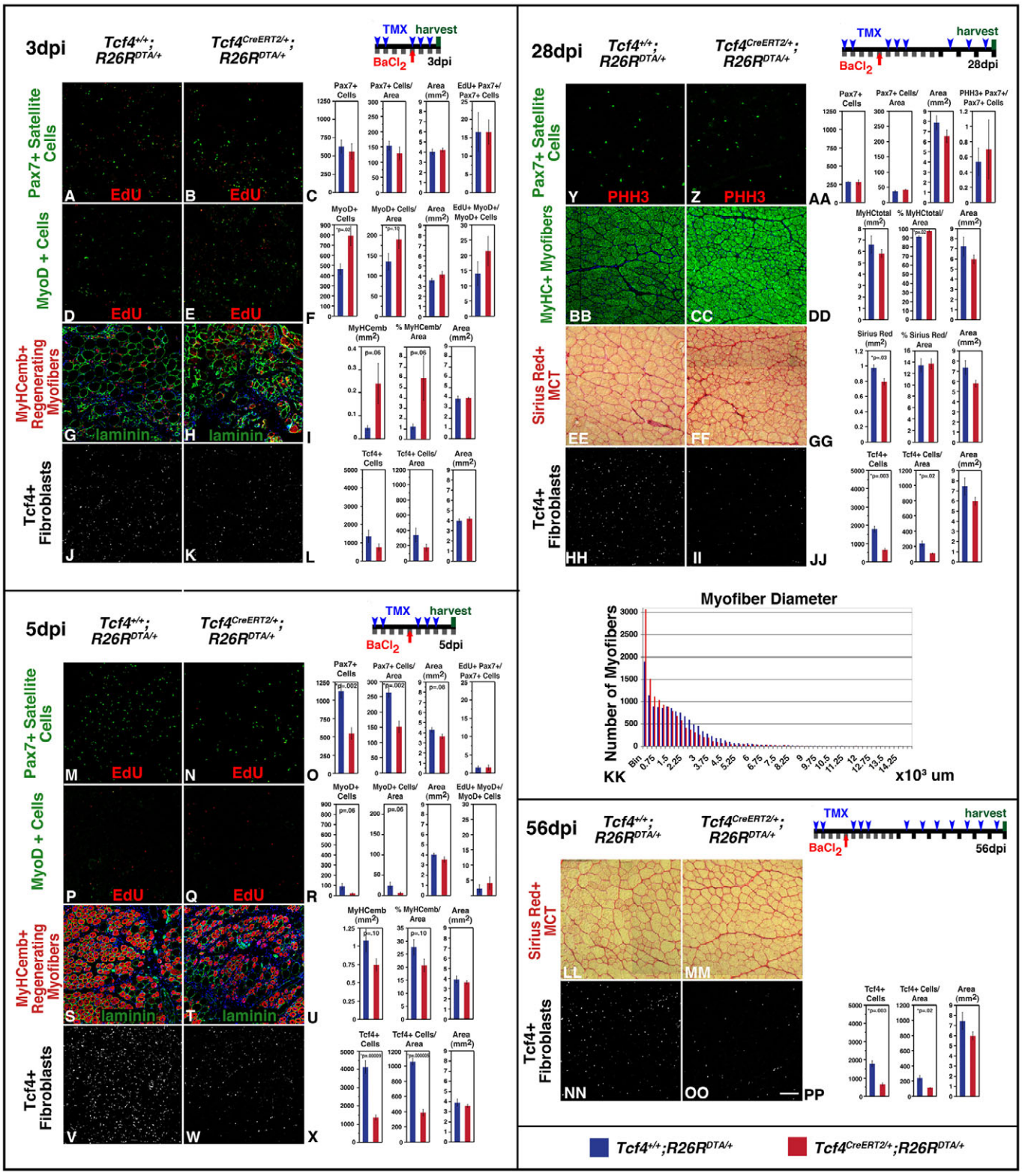

Fig. 6. During muscle regeneration, ablation of Tcf4⁺ fibroblasts leads to premature satellite cell differentiation and smaller regenerated myofibers. (**A-L**) At 3 dpi, Tcf4⁺ cells are reduced (J-L), with no change in Pax7⁺ cells (A-C), but with an increase in MyoD⁺ progenitors/myoblasts (D-F) and MyHCemb⁺ regenerating myofibers (G-I) in *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice. (**M-X**) At 5 dpi, 42% of Tcf4⁺ cells were calculated to be ablated (V-X), resulting in fewer Pax7⁺ cells (M-O), MyoD⁺ progenitors/myoblasts (P-R) and MyHCemb⁺ regenerating myofibers (S-U) in *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice. (**Y-KK**) At 28 dpi, despite Tcf4⁺ fibroblast ablation (HH-JJ), Pax7⁺ cells recover (Y-AA), muscle largely regenerates (BB-GG), but diameter of myofibers is reduced (BB,CC,EE,FF,KK) in *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ versus *Tcf4*$^{+/+}$;*R26R*$^{DTA/+}$ mice. (**LL-PP**) By 56 dpi, regenerated muscle is recovered in *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice. Scale bars: in OO,100 μm for all photomicrographs. For all graphs, mean ± s.e.m. are plotted.

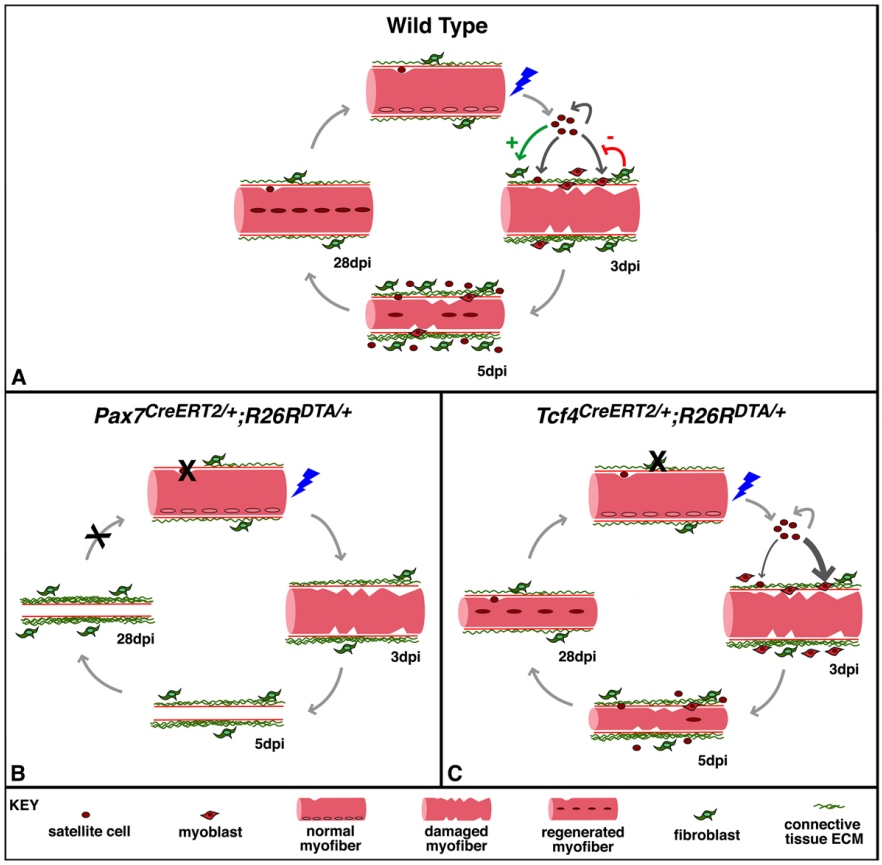

Fig. 7. Model of the role of Pax7$^+$ satellite cells and Tcf4$^+$ muscle connective tissue (MCT) fibroblasts and their interactions during muscle regeneration.
(**A-C**) Summary of cells and their interactions after injury during normal regeneration (A), with ablation of satellite cells (B) and with partial ablation of MCT fibroblasts (C). (A) During normal regeneration, satellite cells and fibroblasts rapidly proliferate. Satellite cells are absolutely required for normal regeneration of myofibers. Reciprocal interactions between satellite cells and fibroblasts are also important for regeneration. (B) With ablation of satellite cells, no regeneration of myofibers occurs and muscle is largely replaced by MCT and fibroblasts. (C) Partial ablation of fibroblasts leads to premature differentiation of satellite cells and results in smaller muscles with smaller myofibers at 28 dpi, when muscle regeneration is normally complete.

fibroblasts did not affect the number or proliferative status of Pax7$^+$ satellite cells (Fig. 6A-C). However, the number of MyoD$^+$ cells was increased 1.7-fold (*P=0.02; Fig. 6D-F) and the area of regenerating myofibers was increased fivefold (P=0.06; Fig. 6G-I) in *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice, indicating that Pax7$^+$ satellite cells had prematurely activated and begun differentiating with ablation of fibroblasts. Altogether, these data indicate that ablation of fibroblasts led to premature activation and differentiation of Pax7$^+$ satellite cells at 3 dpi and resulted in depletion of the pool of satellite cells and MyoD$^+$ cells and a consequent reduction in regenerating myofibers by 5 dpi.

We tested the consequences of this early impairment in muscle regeneration by examining injured TAs from *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice at 28 dpi. Tcf4$^+$ fibroblasts continued to be ablated (38% reduction, calculated as above, *P=0.003, n=6; Fig. 6HH-JJ) in *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice (tamoxifen strategy, Fig. 6Y-JJ). Injured TAs had regenerated, but were somewhat smaller in cross-sectional area (21% reduction, P=0.10; Fig. 6AA,DD,GG,JJ) in *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice. The number of Pax7$^+$ satellite cells had recovered in *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice to levels similar to those found in *Tcf4*$^{+/+}$;*R26R*$^{DTA/+}$ mice (Fig. 6Y-AA). Myofibers had regenerated, although the ratio of MyHC$^+$ myofibers to Sirius Red$^+$ MCT was skewed; there was a 7% increase in the proportion of MyHC (*P=0.02; Fig. 6BB-DD) and a 19% decrease in the amount of Sirius Red$^+$ MCT (*P=0.03; Fig. 6EE-GG). Most striking was the finding that the diameter of the regenerated myofibers was smaller, with a higher frequency of smaller myofibers in *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice (Fig. 6BB-GG,KK). By 56 dpi, with continued ablation of Tcf4$^+$ fibroblasts (Fig. 6LL-PP), injured TAs continued to be slightly smaller in cross-sectional area (27% reduction, but high variance precluded statistical significance, n=6; Fig. 6PP) in *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice. However, the diameter of myofibers was equivalent to that found in control mice (Fig. 6LL-MM).

In summary, genetic ablation of a significant number of Tcf4$^+$ MCT fibroblasts caused Pax7$^+$ cells to differentiate prematurely and thus impaired the early expansion of Pax7$^+$ cells. This alteration in early satellite cell dynamics resulted in smaller muscles with smaller diameter myofibers at 28 dpi, when muscle regeneration is normally complete.

DISCUSSION

Muscle regeneration requires the coordinated interaction of multiple cell types. Our in vivo immunofluorescent, lineage and ablation studies demonstrate that satellite cells, fibroblasts and their interactions are crucial for muscle regeneration (summarized in Fig. 7).

A multitude of studies have implicated satellite cells as the primary stem cell responsible for muscle regeneration. However, the necessity of satellite cells has not been demonstrated previously, and the existence of other stem cells capable of regenerating muscle suggests that multiple types of stem cells might contribute to normal regeneration. To test the role of satellite cells during muscle regeneration, we generated *Pax7*^{CreERT2} mice. *Pax7*^{CreERT2} mice allow for efficient genetic labeling and manipulation of satellite cells without compromising Pax7 function, enabling ‘clean’ manipulation of satellite cells without any potential functional consequence of reduced Pax7 expression or function (Kuang et al., 2006; Lepper et al., 2009; Oustanina et al., 2004; Relaix et al., 2004; Seale et al., 2000). Using these

Pax7^{CreERT2} mice, we show that all regenerated muscle derives from Pax7$^+$ satellite cells and, most significantly, ablation of satellite cells led to a complete and persistent loss of muscle regeneration. Although Pax7 is expressed in the neural crest, we have not seen neural crest-derived glial cells labeled in our lineage experiments or obvious glial defects with ablation of Pax7-derived cells. As *Pax7*^{CreERT2} only labels satellite cells prior to injury (and not myonuclei or other potential myogenic progenitors) and ablation of these satellite cells prior to injury resulted in a complete loss of regenerated muscle, we can conclude that Pax7$^+$ satellite cells are absolutely required for normal muscle regeneration. It should be noted that our experiments do not formally rule out that other stem cells can contribute to muscle regeneration. For instance, other stem cells might contribute to muscle regeneration, but only in the presence of satellite cells (e.g. PICs present in juvenile and regenerating muscle) (Mitchell et al., 2010). In addition, other stem cells might not contribute to normal endogenous muscle regeneration, but could have important therapeutic potential as these cells, when transplanted, are able to regenerate muscle (e.g. mesoangioblasts) (Sampaolesi et al., 2003). Nevertheless, our experiments establish that during normal regeneration, Pax7$^+$ satellite cells are the endogenous stem cell population responsible for regenerating muscle.

Our experiments identify for the first time that MCT fibroblasts are a major cellular component of regenerating muscle. Although transient MCT fibrosis has long been recognized as a feature of regenerating muscle, no previous studies have examined the dynamics of the MCT fibroblasts. We previously showed that Tcf4 is highly expressed in MCT fibroblasts associated with developing, neonatal and adult muscle (Mathew et al., 2011). Here, we demonstrate that Tcf4$^+$ fibroblasts rapidly expand and are present in high numbers in the MCT of regenerating muscle. Recently, two groups have identified by FACS analysis cells from muscle which are PDGFRα^+ and non-myogenic (Joe et al., 2010; Uezumi et al., 2010). In culture, these cells differentiate into fibroblasts and adipocytes and when transplanted into muscle after glycerol injection can contribute to ectopic fat in skeletal muscle. The finding that both fibro-adipogenic cells and Tcf4$^+$ fibroblasts express PDGFRα suggests that these populations might be overlapping. Future experiments will explicitly compare the relationship between Tcf4$^+$ fibroblasts and fibro-adipogenic cells.

Our analysis begins to elucidate the role of Tcf4$^+$ MCT fibroblasts in the transient MCT fibrosis characteristic of muscle regeneration. During regeneration, increased MCT maintains the structural and functional integrity of regenerating muscle (Kaariainen et al., 2000), orients forming myofibers (Sanes, 2004), and sequesters and presents growth factors necessary for satellite cells (Cornelison, 2008). However, excessive ECM during regeneration can impede mechanical function and hinder muscle regeneration (Huard et al., 2002; Sato et al., 2003). Thus, MCT fibrosis must be precisely regulated during regeneration. We found that during normal regeneration Tcf4$^+$ fibroblasts rapidly expand within the MCT, concomitant with the increase in MCT, and later decrease in number, as MCT diminished. This spatial-temporal correlation between the numbers of Tcf4$^+$ fibroblasts and MCT fibrosis was apparent not only during normal regeneration, but also in our ablation experiments. Modest ablation of Tcf4$^+$ fibroblasts led to an effect on fibrosis, with somewhat less MCT and proportionately more muscle at 28 dpi. More striking was the finding that the substantial MCT fibrosis seen with ablation of satellite cells was associated with an increase in the overall density of fibroblasts. Together, these data suggest that Tcf4$^+$ fibroblasts are likely to be an important source of the increased ECM found during regeneration.

Most importantly, our experiments demonstrate that during regeneration Tcf4$^+$ MCT fibroblasts are a significant component of the cellular niche regulating satellite cell-mediated regeneration. During normal muscle regeneration, Pax7$^+$ satellite cells and Tcf4$^+$ fibroblasts rapidly expand in close proximity after injury. Ablation of fibroblasts resulted in premature satellite cell differentiation at 3 dpi, followed by depletion of the satellite cell pool, differentiating myoblasts and regenerating myofibers at 5 dpi, and resulted in smaller muscles and smaller diameter myofibers at 28 dpi, when muscle regeneration is normally complete. Thus, during regeneration, fibroblasts regulate the expansion of satellite cells by preventing their premature differentiation. These results complement our previous findings that during development Tcf4$^+$ fibroblasts promote muscle maturation. In particular, we found in vitro that myoblasts cultured in the presence of Tcf4$^+$ fibroblasts formed larger, more multinucleated myofibers than myoblasts cultured alone (Mathew et al., 2011). In addition, other experiments have shown in vitro that fibroblasts are a source of trophic signals for myogenic cells (Cooper et al., 2004; Joe et al., 2010; Kusner et al., 2010; Melone et al., 2000). However, this is the first demonstration in vivo that fibroblasts regulate muscle regeneration.

Our studies also reveal that fibroblasts are dynamically regulated by satellite cells. Ablation of satellite cells leads to a decrease in the number of Tcf4$^+$ fibroblasts at 5 dpi, but an increase in the overall density of fibroblasts at 28 dpi. Thus, early during the regenerative process, satellite cells and fibroblasts reciprocally and positively regulate the expansion of each other. Subsequently, during the late phases of regeneration the number of fibroblasts is not positively regulated by satellite cells, but is likely to be negatively regulated by satellite cells and other myogenic cells.

Altogether, our experiments demonstrate that satellite cells, fibroblasts and their interactions are crucial for muscle regeneration and suggest the following model of cell interactions during muscle regeneration (Fig. 7A). During regeneration, satellite cells proliferate, differentiate into myofibers, as well as self-renew. Satellite cells are absolutely required for muscle regeneration. Fibroblasts also rapidly proliferate in close association with satellite cells, regenerating myofibers and within the MCT. During the early phase of regeneration, fibroblasts ensure the expansion of satellite cells by preventing their premature differentiation. In turn, satellite cells positively regulate the number of fibroblasts. This positive feedback between satellite cells and fibroblasts ensures efficient and effective muscle repair. Later during the regenerative process, the number of fibroblasts are negatively regulated to prevent excessive MCT fibrosis.

Thus, fibroblast-satellite cell interactions are dynamic. Early reciprocal, positive fibroblast-satellite interactions promote regeneration. Later, negative regulation of fibroblasts, potentially via myogenic cells, inhibits excessive fibrosis. The dynamic nature of these interactions suggest that therapeutic treatments to reduce fibrosis during regeneration, after muscle injury or in the context of muscle diseases will need to be carefully monitored in order to avoid interfering with the early pro-regenerative interaction between MCT fibroblasts and satellite cells. As connective tissue fibrosis and expansion of resident fibroblasts are characteristic of many regenerating tissues (Tomasek et al., 2002; Verrecchia and Mauviel, 2007), dynamic interactions between fibroblasts and stem cells might be important not only for muscle, but for the regeneration of other tissues.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at
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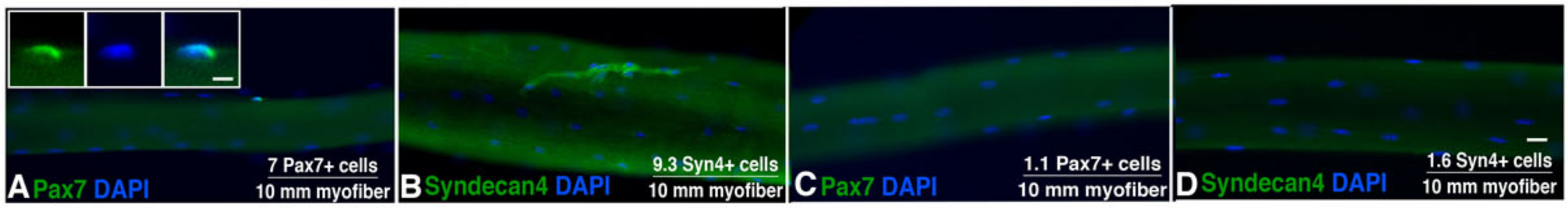
Satellite cells, connective tissue fibroblasts and their interactions are crucial for muscle regeneration

DEV064162 Supplementary Material

Files in this Data Supplement:

Supplemental Figure S1 -

Fig. S1. *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice efficiently ablate satellite cells resulting in long-term loss of regenerated myofibers. (**A-D**) Thirty days after five tamoxifen doses, most Pax7+ and Syndecan4+ satellite cells are ablated in myofibers isolated from *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice. (**E-H**) At 56 dpi (cardiotoxin, CTX), ablation of Pax7+ satellite cells in *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice still results in a complete loss of regenerated myofibers, as shown in whole mount (E,F) and Sirius Red-stained cross-sections (G,H). (**I-L**) After initial ablation of satellite cells, muscle cannot regenerate after two successive CTX injuries. (**M**,**N**) With ablation of satellite cells, at 28 dpi there is an increase in Nile Red+ adipogenic tissue in the tibialis anterior (TA) region in *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$mice. Whole-mount images have been flipped so the injured limb (R) is on the right. Scale bars: in D, 50 μm for A-D; in G, 20 μm for E-G; in R, 500 μm for K-N.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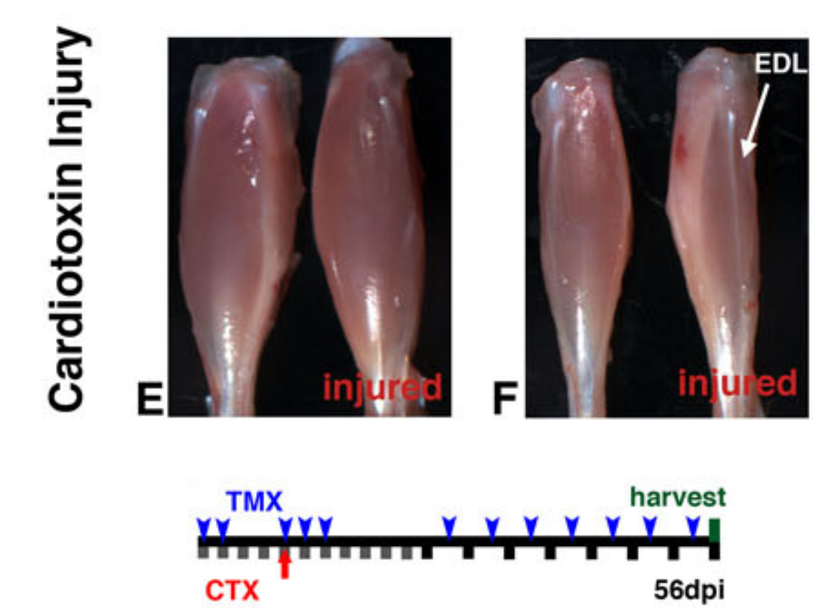
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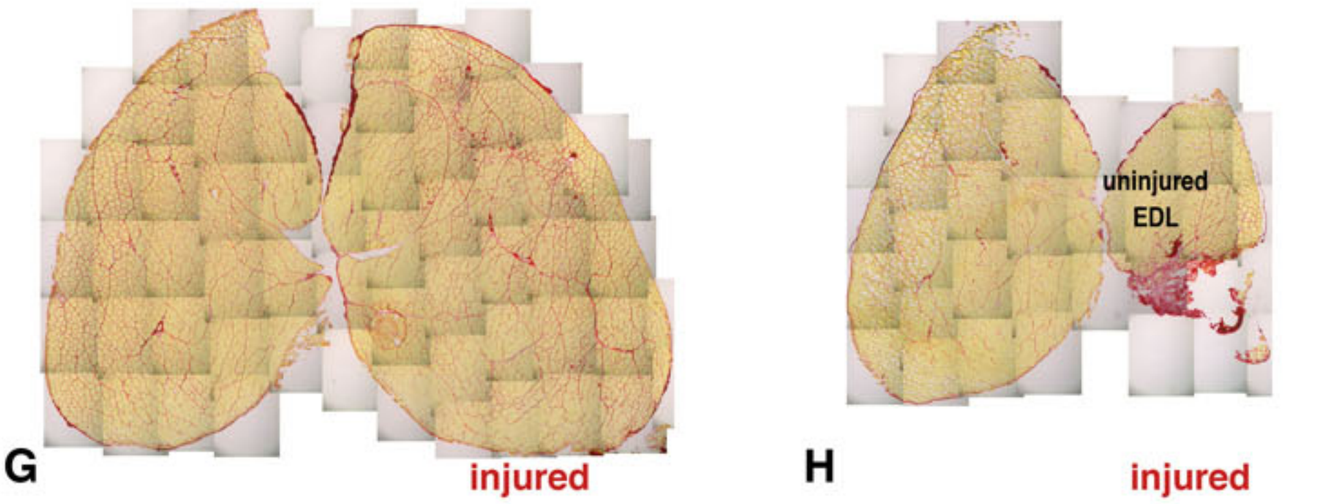
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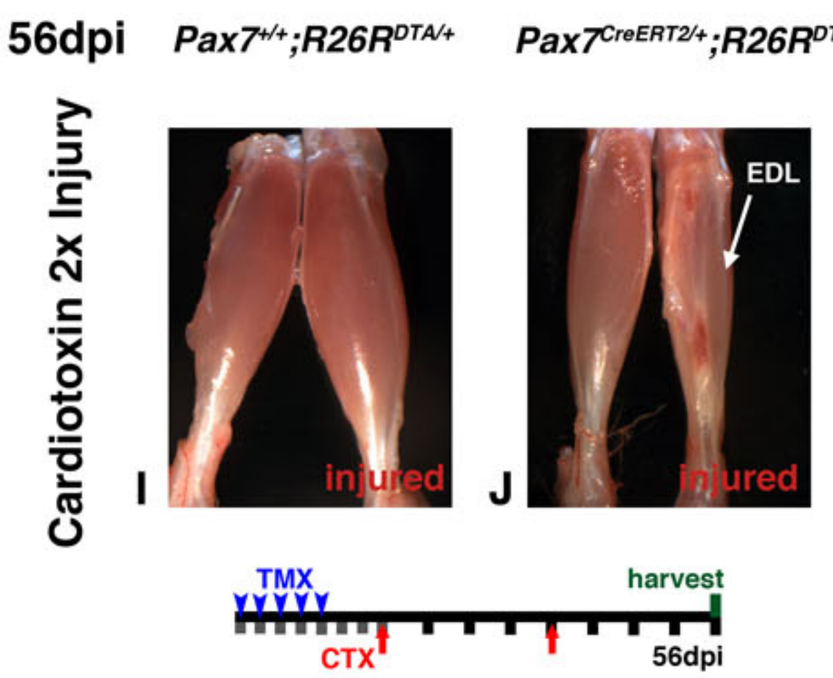
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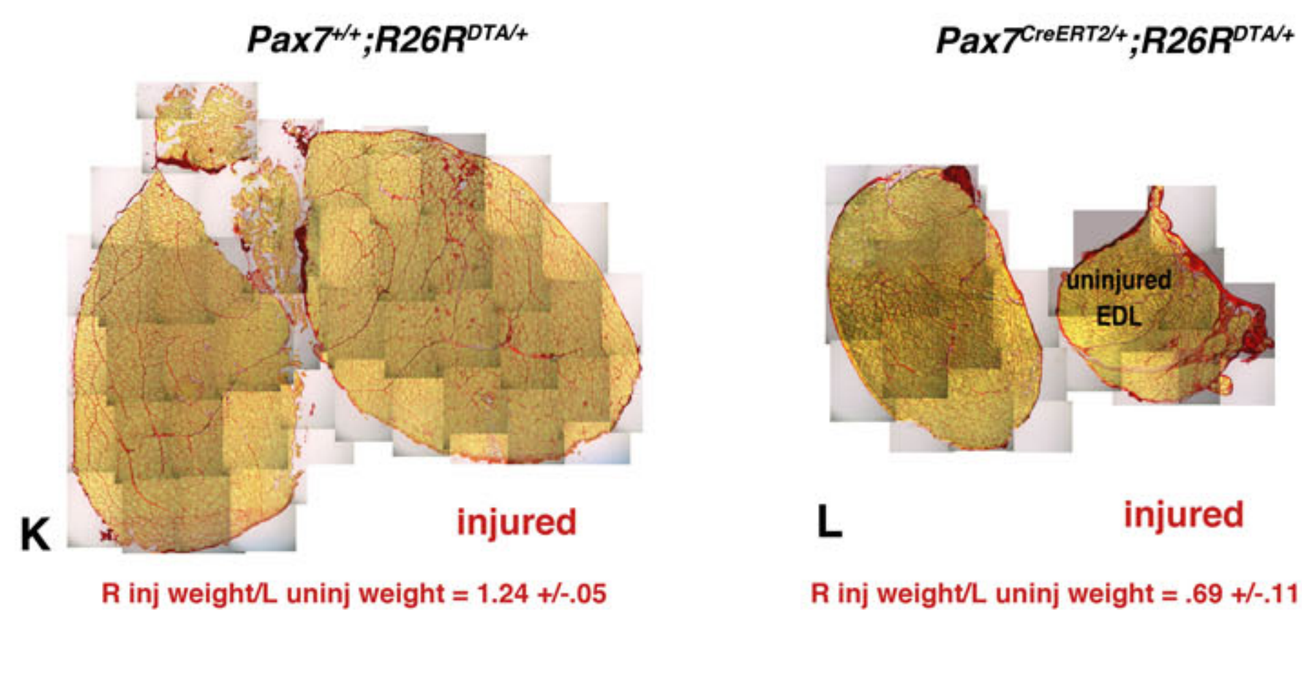
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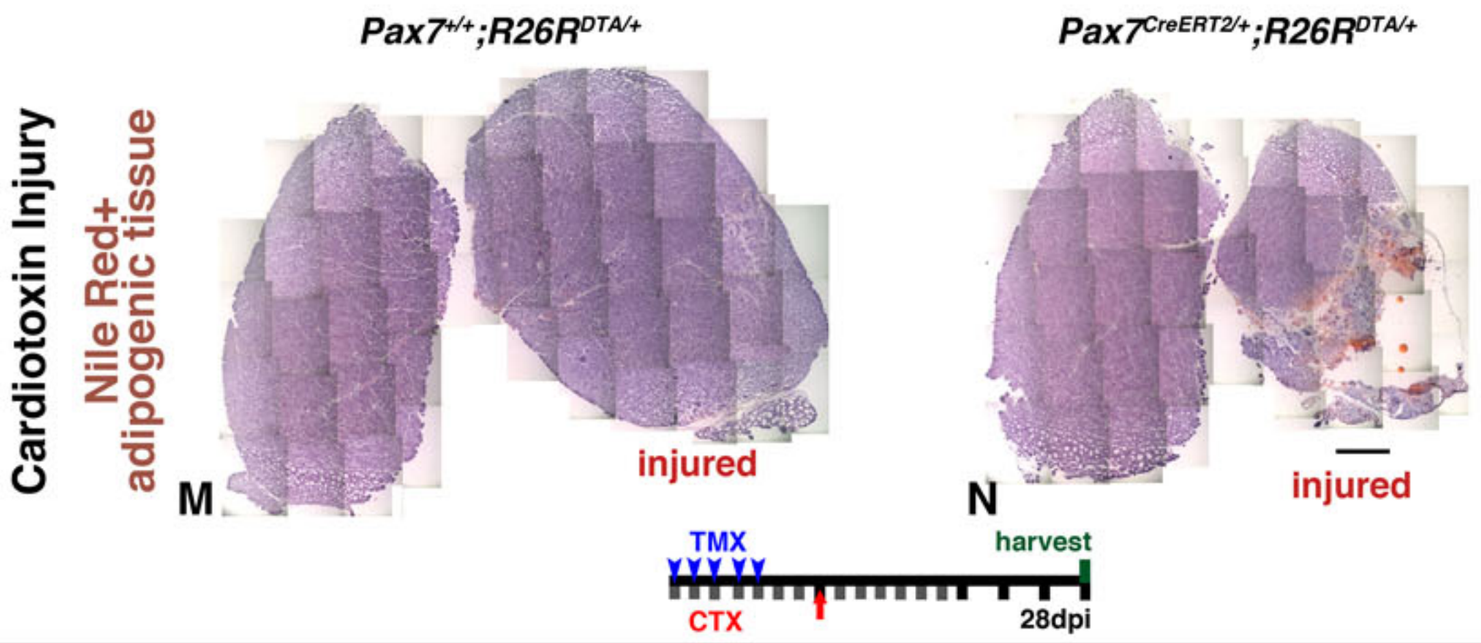
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