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Corresponding Author

Benjamin Levi
University of Michigan - Surgery
1500 E. Medical Center Dr.
Ann Arbor, Michigan 48109
United States
T: 734-936-4000
E: benlevimd@gmail.com

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Scleraxis-lineage cells contribute to ectopic bone formation in muscle and tendon

Shailesh Agarwal, MD*[,1]; Shawn J Loder, BS*[,1]; David Cholok, BS[1]; Joshua Peterson, BS[1]; John Li, MD[1]; Christopher Breuler, BS[1]; R. Cameron Brownley, BS[1]; Hsiao Hsin Sung, MD, DDS[1]; Mike Chung, MD[1], Nobuhiro Kamiya, MD[2], Shuli Li, PhD[1], Bin Zhao, PhD[3]; Vesa Kaartinen, PhD[4]; Thomas A. Davis PhD[5]; Ammar T. Qureshi, PhD[5]; Ernestina Schipani, MD, PhD[6]; Yuji Mishina, PhD[4]; Benjamin Levi, MD[1]

* These two authors contributed equally to this manuscript
[1] Burn/Wound and Regenerative Medicine Laboratory, Department of Surgery, Section of Plastic Surgery, University of Michigan
[2] Department of Sports Medicine, Tenri University, Japan
[3] Albert Einstein College of Medicine, Department of Genetics, Bronx, New York
[4] Biologic and Materials Sciences, University of Michigan School of Dentistry
[5] Regenerative Medicine Department, Naval Medical Research Center, Silver Spring, MD
[6] Orthopaedic Research Laboratories, Department of Orthopaedic Surgery, University of Michigan

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DISCLOSURES

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ABSTRACT

The pathologic development of heterotopic ossification (HO) is well described in patients with extensive trauma or with hyperactivating mutations of the bone morphogenetic protein (BMP) receptor *ACVR1*. However, identification of progenitor cells contributing to this process remains elusive. Here we show that connective tissue cells contribute to a substantial amount of HO anlagen caused by trauma using post-natal, tamoxifen-inducible, scleraxis-lineage restricted reporter mice (*Scx-creERT2/tdTomato*$^{fl/fl}$). When the scleraxis-lineage is restricted specifically to adults prior to injury marked cells contribute to each stage of the developing HO anlagen and co-express markers of endochondral ossification (Osterix, SOX9). Furthermore, these adult pre-injury restricted cells co-expressed mesenchymal stem cell markers including PDGFRα, Sca1, and S100A4 in HO. When constitutively active *ACVR1* (*caACVR1*) was expressed in scx-cre cells in the *absence* of injury (*Scx-cre/caACVR1*$^{fl/fl}$), tendons and joints formed HO. Post-natal lineage-restricted, tamoxifen-inducible *caACVR1* expression (*Scx-creERT2/caACVR1*$^{fl/fl}$) was sufficient to form HO after directed cardiotoxin-induced muscle injury. These findings suggest that cells expressing scleraxis within muscle or tendon contribute to HO in the setting of both trauma or hyperactive bone morphogenetic protein receptor (*e.g.* caACVR1) activity.

INTRODUCTION

Heterotopic ossification (HO) is a pathologic process in which ectopic bone forms within muscle, tendons, or other soft tissues. Clinically, HO lesions develop at sites of musculoskeletal injury or burns, and in patients with hyperactive BMP receptor signaling (Shore et al., 2006). Because HO forms in several tissue types, identification of common progenitors has been an area of growing interest.

Several different progenitor cells have been evaluated in the context of BMP-induced HO models within muscle. Using lineage-tracing mice, Tie2+ cells have been noted to undergo osteogenic differentiation *in vivo* (Lounev et al., 2009; Medici et al., 2010). These Tie2+ cells have been shown to be muscle-resident CD31-/CD45-/PDGFRα+/Sca1+ cells suggesting they are mesenchymal stem cells and not endothelial cells as initially thought (Wosczyna et al., 2012). The contribution of Glast-cre cells that reside within the muscle interstitium has also been evaluated. These cells co-express mesenchymal markers including PDGFRα and S100A4, and also appear to contribute to different stages of HO (Kan et al., 2013). In these models, however, BMP activity is not limited to the contributing MSCs, and it is not clear whether BMP is required to engender a niche supportive of ectopic bone.

Clinically, HO is often observed in close proximity to connective tissue within myofascial planes and joints. Additionally, transection of the Achilles' tendon in murine models leads to robust ectopic bone through endochondral ossification. Scleraxis, a basic helix-loop-helix (bHLH) transcription factor, is expressed in connective tissues including tendon, ligaments, and perimysium between muscle fibers (Blitz et al., 2013; Dyment et al., 2014; Schweitzer et al., 2001; Sugimoto et al., 2013). Furthermore, scleraxis-expressing cells collected after tendon injury exhibit robust chondrogenic potential both *in vitro* and when implanted *in vivo* (Asai et al., 2014).

Given the shared presence of scleraxis-expressing cells in both muscle and tendon, we hypothesized that these cells contribute to HO formed after trauma or due to hyperactive BMP signaling.

RESULTS

Scleraxis-lineage cells contribute to all phases of trauma-induced HO (tHO)

We first demonstrated that the Achilles tendon, which is transected in the burn/tenotomy model of trauma-induced HO (tHO) (**Fig S1A**), is marked almost completely by both Scx-cre lineage cells (*Scx-cre/ROSA26*^{mTmG}) and by active expression of Scx (*Scx-GFP*) prior to injury (**Fig 1A; Fig S1B**). By comparison, intramuscular regions demonstrate a more restrictive pattern with both Scx-cre lineage and active Scx expression limited to areas of connective tissue (**Fig S2**). These regions are the site of ectopic bone formation in our second model of HO formation utilizing an intramuscular BMP-loaded scaffold (bHO).

Using our burn/tenotomy model of tHO, we found that Scx-cre cells contribute to a majority of the fibroproliferative (95.1±1.2%) and chondroid (65.8±13.0%) regions of developing tHO (**Fig 1B, Fig S3**). To exclude any contribution of trauma-induced activation of scleraxis we then used post-natal, tamoxifen-inducible, scleraxis-lineage restricted reporter mice (*Scx-creERT2/tdTomato*$^{fl/fl}$) with tamoxifen activation ending four days prior to injury (**Fig 1C; Fig S4**). These animals were then evaluated for the formation of either tHO or bHO (**Fig 1C**). We found that *Scx-creERT2*-labeled cells (tdTomato+) contribute to all stages of HO development and could be found in fibroproliferative (50.0±25.6 %), chondroid (31.0±7.8%), and endosteal (49.4±18.6) regions of tHO (**Fig 1D,E**).

Scx-creERT2 cells contribute directly endochondral ossification in both tHO and bHO.

Given the observed contribution of *Scx-creERT2* cells to tHO we wanted to identify whether these cells contribute directly to the process of endochondral ossification by which HO transitions from cartilage to bone. First we identified areas of tHO in *Scx-creERT2/tdTomato*$^{fl/fl}$ mice demonstrating robust endochondral ossification (**Fig 2A**). These tissues are enriched for chondrogenic (SOX9) and osteogenic (OSX) differentiation markers (**Fig 2B-D**). Importantly, tdTomato+ cells in this model expressed both SOX9 and OSX in tHO (**Fig 2B-D**). Similar findings were observed in our BMP-loaded scaffold model, although bHO was predictably more ossified with comparatively diminished markers of active endochondral ossification versus tHO (**Fig 2E-H**).

Scx-creERT2 cells display a mesenchymal cell phenotype

Next, we explored the relationship between Scx-creERT2 cells and MSC populations which have previously been shown to contribute to HO. Specifically, we examined PDGFRα and S100A4, which are expressed by Glast-cre cells (Kan et al., 2013), and Sca1 which is expressed by Tie2+ non-endothelial cells (Wosczyna et al., 2012). In both tHO (**Fig 3A-D**) and bHO (**Fig 3E-H**) we noted a high percentage of tdTomato+ cells expressing these markers, however, not all MSC marker+ cells were tdTomato+ consistent with the heterogeneity of HO. Interestingly, when we evaluated the uninjured tissues, while these markers were present, there was no evidence of enrichment in either tendinous or muscular bodies (**Fig S5**).

Expression of caACVR1 within Scx-cre cells results in HO at the joints

To clarify whether Scx-cre cells are capable of producing HO we bred mice with Scx-cre lineage-restricted *caACVR1* expression (*Scx-cre/caACVR1*$^{fl/wt}$). Strikingly, *Scx-cre/caACVR1*$^{fl/wt}$ mutant mice developed HO at the joints, with 100% of hindlimbs showing HO at the Achilles' tendon, in the absence of traumatic insult (**Fig 4A-D**), though no HO was detectable within muscle (**Fig S6**). Generation of mutant mice conditionally expressing *caACVR1* in muscle- or osteoblast-cells (*Ckmm-cre/caACVR1*$^{fl/wt}$ and *Col1.creERT/caACVR1*$^{fl/wt}$) did not form HO suggesting that BMP receptor hyperactivity in differentiated muscle or osteoblasts is unable to cause HO (**Fig S7**).

Expression of caACVR1 in Scx-creERT cells results in intramuscular HO after trauma

Notably, intramuscular HO lesions in patients with fibrodysplasia ossificans progressiva (FOP) form after muscle trauma. Because HO was *not* observed within the muscle of mice with *caACVR1* in *Scx-cre* cells without injury, we investigated the effect of injury on *caACVR1* expression in these cells.

First, to evaluate the effect of intramuscular inflammation on this system we utilized a separate method of injury from our tHO and bHO, injection of cardiotoxin directly into the muscle body. Using our *Scx-GFP* reporter we demonstrated that this was sufficient to drive an increase in Scx expression in areas adjacent to connective tissue (**Fig S8**). To exclude any contribution of cardiotoxin-induced activation of scleraxis we then designed a post-natal, tamoxifen-inducible, scleraxis-lineage restricted model of caACVR1 expression (*Scx-creERT2/caACVR1*$^{fl/wt}$). This system restricted caACVR1 expression to only tamoxifen activated Scx-creERT2 cells. After tamoxifen washout *Scx-creERT2/caACVR1*$^{fl/wt}$ mice received cardiotoxin-induced injury. MicroCT demonstrated the presence of ectopic bone within the hamstring 20 days post-cardiotoxin (**Fig 4E**). Routine histology confirmed the presence of heterotopic bone with robust Alcian blue and Alizarin red staining (**Fig 4E**). Immunofluorescent staining for Scx confirmed a normal restricted staining pattern prior to injury with presence of scattered intramuscular Scx+ cells present post-cardiotoxin consistent with regions of new HO (**Fig 4F**).

DISCUSSION

These findings indicate that scleraxis-lineage restricted cells have the capacity to form HO in the settings of trauma and with hyperactive BMP receptor activity. Recent studies have identified additional candidate HO including endothelial or non-endothelial Tie2+ cells and Glast-Cre cells (Kan and Kessler, 2014; Kan et al., 2013; Lemos et al., 2015; Medici et al., 2010; Wosczyna et al., 2012). Using markers such as PDGFRα, Sca1, and S100A4, others have shown that Tie2+ and Glast-cre cells which contribute to HO are likely mesenchymal cells (Kan and Kessler, 2014; Kan et al., 2013; Lemos et al., 2015; Medici et al., 2010; Wosczyna et al., 2012). Our findings confirm the co-expression of these mesenchymal cell markers in Scx-creERT2 cells, in both trauma or BMP-scaffold implantation. Interestingly, while these markers are not highly enriched in uninjured tissue, they are expressed throughout the early anlagen and persist into regions of hypertrophic chondrocyte formation in both models of HO suggesting that these markers pointing to a conserved role for mesenchymal cells in both processes.

The physical source of cells contributing to HO is also of interest. Previous studies identified Scx-cre cells in muscle interstitium and along fascial planes (Mendias et al., 2012). Our data were consistent with this demonstrating restriction of both active scleraxis and scleraxis lineage to areas of tendon and connective tissue within the muscle. In the setting of trauma and BMP-induced HO, pre-injury scleraxis-lineage restricted cells contributing to HO may originate locally and it is possible that the differences in regional sources of Scx-cre cell in uninjured tissue contributes to differences in the prevalence of Scx-cre cells in the HO anlagen between our models. Our findings using the tamoxifen inducible model of *caACVR1* expression (*Scx-creERT2/caACVR1*$^{fl/wt}$) demonstrate that hyperactive BMP signaling restricted to these cells is sufficient to form HO consistent with the idea that these cells form a local/regional pool of prospective HO progenitors.

Interestingly using *Scx-cre/caACVR1*$^{fl/wt}$ mice we found that HO was restricted to the tendons, with no evidence of intramuscular HO in the absence of injury. However, we have also found that Scx-cre cells are located intramuscularly and that intramuscular injury was sufficient to cause enrichment of Scx expressing cells in regions adjacent to pre-existing connective tissue planes. Interestingly, even when we controlled for trauma-induced Scx-activation using conditional *Scx-creERT2/caACVR1*$^{fl/wt}$ mice, injury was still sufficient to cause intramuscular HO in the background of caACVR1 expression. This suggests either these contributing cells are derived from another source, or that the injury stimulus causes these cells to form HO and highlights the possibility that these cells proliferate in response to trauma, causing an identifiable lesion.

Our findings are just as important for what they are unable to show – the existence of a single progenitor cell which contributes to tHO. Identification of a single progenitor cell responsible for HO has posed a challenge to researchers, and our findings lead us to believe that although a single scleraxis-progenitor is likely *not* entirely responsible for HO. It is possible that a broader lineage inclusive of the Scx lineage is responsible for HO, although identification of this broader lineage may not be realistic. Our findings do indicate that a subset of connective tissue cells identified by post-natal, pre-injury Scleraxis expression are capable of chondrogenic and osteogenic differentiation after local injury or with hyperactive BMP receptor activity.

FIGURE LEGEND

Figure 1. Scleraxis-lineage cells contribute to all phases of trauma-induced heterotopic ossification (tHO). (A) Scleraxis lineage (*Scx-cre/ROSA26^{mTmG})* defines the Achilles Tendon (eGFP+) prior to injury; (B) Scleraxis lineage (*Scx-cre/ROSA26^{mTmG})* defines both fibroproliferative (eGFP+; white arrow) and chondroid (eGFP+; yellow arrow) tHO after injury; (C) Experimental set up of tamoxifen induction in *Scx-creERT2/tdTomato$^{fl/fl}$* mice with burn/tenotomy (tHO) or BMP-induced models (bHO); (D) Localization of adult pre-injury scleraxis-lineage restricted cells (tdTomato+) cells in the fibroproliferative and (tdTomato+; white arrow), chondroid (tdTomato+; yellow arrow) cells of cartilaginous tHO and in the endosteal cells (tdTomato+; green arrow) of late-ossified tHO; (E) Quantification of the adult pre-injury scleraxis-lineage restricted fraction of in the fibroproliferative, chondroid, and endosteal cells in tHO. Scale bars represent 200 μm.

Figure 2. Scleraxis-lineage cells defined in the uninjured adult (tdTomato+) contribute directly to the endochondral anlagen in both trauma-induced (tHO) and BMP-scaffold driven (bHO) heterotopic ossification. (A) Representative H&E demonstrating areas of tHO in *Scx-creERT2/tdTomato$^{fl/fl}$* mice; (B) Expression of SOX9 (green) by tdTomato+ cells in tHO; (C) Expression of OSX (green) by tdTomato+ cells in tHO; (D) Percent of Scx-creERT2 cells (tdTomato+) expressing SOX9 or OSX in tHO (5 high power fields); (E) Representative H&E demonstrating areas of bHO in *Scx-creERT2/tdTomato$^{fl/fl}$* mice; (F) Expression of SOX9 (green) by tdTomato+ cells in bHO; Expression of OSX (green) by tdTomato+ cells in bHO; (G) Percent of Scx-creERT2 cells (tdTomato+) expressing SOX9 or OSX in bHO (5 high power fields). Scale bars represent 200 μm.

Figure 3. Scleraxis-lineage cells defined in the uninjured adult (*Scx-creERT2/tdTomato$^{fl/fl}$*) display mesenchymal cell markers in both trauma-induced (tHO) and BMP-scaffold (bHO) heterotopic ossification. (A) Scx-creERT2 cells (tdTomato+) express PDGFRα in tHO; (B) Scx-creERT2 cells (tdTomato+) express S100A4 in tHO; (C) Scx-creERT2 cells (tdTomato+) express SCA1 in tHO; (D) Percent of Scx-creERT2 cells (tdTomato+) which express mesenchymal markers in tHO (5 high power fields); (E) Scx-creERT2 cells (tdTomato+) express PDGFRα in bHO; (F) Scx-creERT2 cells (tdTomato+) express S100A4 in bHO; (G) Scx-creERT2 cells (tdTomato+) express Sca1 in bHO; (H) Percent of Scx-creERT2 cells (tdTomato+) expressing mesenchymal markers in bHO (5 high power fields). Scale bars represent 200 μm.

Figure 4. Scleraxis lineage-restricted *caACVR1* expression causes tendon and intramuscular HO. (A) Schematic showing spontaneous HO generation by scleraxis-lineage cells expressing *caACVR1* (*Scx-cre/caACVR1$^{fl/wt}$*) and by pre-injury scleraxis-lineage cells after cardiotoxin injury (*Scx-creERT2/caACVR1$^{fl/wt}$*); (B) Photograph of *Scx-cre*/*caACVR1$^{fl/wt}$* mouse showing ectopic bone formation at the hindlimb; (C) Whole body 3D MicroCT reconstruction confirming ectopic bone formation at the hindlimbs *Scx-cre/caACVR1$^{fl/wt}$* mouse; (D) Axial, sagittal, and serial cross sections of microCT showing ectopic bone at the distal Achilles' tendon; (E) 3D MicroCT reconstruction showing HO in the hamstring muscle of *Scx-creERT2/caACVR1$^{fl/wt}$* mice induced with

tamoxifen and later injected with cardiotoxin with serial cross sections; H&E, Alcian blue, and Alizarin red staining of HO after cardiotoxin injection in non-decalcified sections of intramuscular HO in *Scx-creERT2/caACVR1*$^{fl/wt}$ mouse; (F) immunofluorescent staining for scleraxis in muscle of *Scx-creERT2/caACVR1*$^{fl/wt}$ mice in the presence or absence of cardiotoxin. Scale bars represent 200 μm.

Supplemental Figure 1. Injury to Scleraxis expressing tendon is sufficient to form heterotopic ossification. (A) Hindlimb Achilles' tendon transection forms HO at the tendon transection site; Skin incision over the Achilles' tendon does not form HO. Yellow arrow = tibia; orange arrow = fibula; red circle = heterotopic ossification; cm = calf muscle. (B) Active scleraxis expression (*Scx-GFP*) is present in the Achilles Tendon (eGFP+) prior to injury. Scale bars represent 200 μm.

Supplemental Figure 2. Scleraxis is restricted to connective tissue within muscle. (A) Scleraxis lineage (*Scx-cre/ROSA26*^{mTmG}*)* defines the intramuscular connective tissue (eGFP+) but not myofibrils prior to injury. (B) Active scleraxis expression (*Scx-GFP*) is present in intramuscular connective tissue (eGFP+) but not myofibrils prior to injury. Scale bars represent 200 μm.

Supplemental Figure 3. Scleraxis-lineage cells contribute to the development of cartilaginous tissue in heterotopic ossification (tHO). Scleraxis lineage (*Scx-cre/ROSA26*^{mTmG}*)* defines a majority chondroid cells (eGFP+) in tHO after injury. Scale bars represent 200 μm.

Supplemental Figure 4. Expression of tdTomato in tamoxifen induced conditional reporters (*Scx-creERT2/tdTomato*$^{fl/fl}$) is limited areas of tendon and connective tissue. Scale bars represent 200 μm.

Supplemental Figure 5. Expression of mesenchymal progenitor markers are limited in uninjured muscle and tendon. Scale bars represent 200 μm.

Supplemental Figure 6. *Scx-cre/caACVR1*$^{fl/wt}$ mice do not form intramuscular HO in the absence of injury. MicroCT demonstrating absence of HO in the hamstring or calf muscles of *Scx-cre/caACVR1*$^{fl/wt}$ mice after 6 weeks with serial cross sections.

Supplemental Figure 7. *caACVR1* expression restricted to osteoblasts or myocytes does not result in HO. (A) Western blot confirming that *Ckmm-cre*$^{+/0}$*/caACVR1*$^{fl/wt}$ overexpress pSmad 1/5 when compared with littermate Cre(-) control; (B) Plain film radiography showing absence of HO in *Col1.creERT*/*ROSA26*^{LacZ}/*caACVR1*$^{fl/wt}$; (C) Immunohistochemistry showing LacZ expression indicating recombination with concordant pSmad 1/5/8 expression. Scale bars represent 200 μm.

Supplemental Figure 8. Intramuscular scleraxis expression (*Scx-GFP*) is present in regions adjacent to connective tissue after cardiotoxin injection. Scale bars represent 200 μm.

Graphical Abstract. Scleraxis lineage cells contribute to genetic and traumatic induced HO. (A) Scleraxis lineage (*Scx-cre/ROSA26*^{mTmG}*)* characterizes Achilles tendon. (B) Co-expression of scleraxis lineage (*Scx-creERT2/tdTomato*$^{fl/fl}$*)* cells with markers of chondrogenic and osteogenic differentiation (Osx and Sox9). (C) Gross imaging and μCT reconstruction of *de novo* HO formation in *Scx-cre/caACVR1*$^{fl/wt}$ mouse. (D) Induction of intramuscular HO following ctx injection in *Scx-creERT2/caACVR1*$^{fl/wt}$ mouse. Scale bars represent 200 μm.

METHODS

Ethics Statement

Procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of Michigan (PRO0005909) or the Walter Reed Army Institute of Research/Naval Medical Research Center Institutional Animal Care and Use Committee (12-OUMD-20s).

Animals

All animals were housed in standard conditions. All mice used for burn/tenotomy or BMP sponge implantation were young adult male (6-8 weeks old) C57BL/6 background. *Scx-cre/ROSA26^{mTmG}, Scx-cre/ROSA26^{tdT}* and *Scx-creERT2/ROSA26^{tdT}* mice were used for lineage-tracing experiments. Mice with burn/tenotomy underwent transection of the left hindlimb Achilles' tendon with 30% burn using a metal block heated to 60C applied to the depilated dorsum for 18 seconds (Peterson et al., 2014). Separately, mice underwent implantation of a human r-BMP loaded collagen scaffold into the gastrocnemius muscle with injection of cardiotoxin (Shimono et al., 2011). A 3 mm × 1 mm–thick collagen discs are prepared by punched out of collagen sponge sheets (Resorbable collagen AceSurgical) with 10 μL of recombinant human BMP-2 (1 μg/10 μL; Sigma, St. Louis, MO). An intramuscular pocket in the calf muscles was dissected and one rBMP-2–collagen disc was placed into each pocket. The surgical site was then injected with 100 μL cardiotoxin (0.3 μg/10 μL; Sigma, St. Louis, MO). Mice were euthanized at the indicated timepoints.

Transgenic Animals

Transgenic mice used in this study were: *Scx-cre/ROSA26^{mTmG}, Scx-cre/ROSA26^{tdT}* and *Scx-creERT2/ROSA26^{tdT}, Ckmm-cre/caAcvr1$^{fl/wt}$*, or *Col1.CreERT/ caAcvr1$^{fl/wt}$*. *Col1.CreERT/ ca-Acvr1$^{fl/wt}$* underwent tamoxifen induction during weanling stage at P2 through nursing.

Histology and Imaging

Hind limbs were fixed overnight in 10% neutral buffered formalin and subsequently decalcified in 19% EDTA solution for 4-6 weeks at 4C. Sections were embedded in paraffin or optimal cutting temperature compound (OCT; Tissue-Tek; Sakura Finetek, USA) and 5-7 um sections were prepared. Immunofluorescence was carried out using primary antibodies directed against the following: SOX9 (Santa Cruz, Cat No. 17341 or 20095), Osterix, pSmad 1/5 (Santa Cruz, Cat No. 12353), Sca1, PDGFRa, or S100A4. Late timepoint *ScxGfp/Scx-creERT2/ROSA26^{tdT}* sections were stained with anti-tdT (Abcam, Cat No. 62341).

All fluorescently stained images were taken using a Leica Upright SP5X Confocal Microscope or Olympus BX-51 upright light microscope equipped with standard DAPI, 488 nm, and TRITC cubes attached to an Olympus DP-70 high resolution digital camera. Each site was imaged in all channels and overlaid in DPViewer before examination in Adobe Photoshop.

Quantification of Costaining

To quantify staining/costaining, we performed counts on n=5 high powered fields (hpfs) chosen at random from across n=3 distinct sections. Each high powered field is hand counted and results are tallied from 3 separate reviewers to ensure consistency. Immunofluorescent staining was regarded as positive, when red/green channel signal overlapped with or was immediately proximal to DAPI positive staining in the blue channel. For costaining of tdTomato+ with one of more of the fluorescent stains described above, we counted only a triple positive of DAPI+, tdTomato+, and whichever immunofluorescent stain is being evaluated.

Computed Tomography (CT) Analysis

Mouse hindlimbs were imaged with microCT (Siemens Inveon, using 80 kVp, 80mA, and 1100 ms exposure). Images were reconstructed to demonstrate sites of ectopic bone formation.

Statistical Analysis

Experiments were performed with n≥3 using Student's t-test. Means and SDs were calculated from numerical data, as presented in the text, figures, and figure legends. All bar graphs are presented as means. Error bars represent 1 standard deviation (s.d.).

AUTHOR CONTRIBUTIONS

S.A., S.J.L, and B.L. designed the experiments. S.A., J.R.P., A.T.Q., J.A.F., L.M., and T.A.D. performed all murine traumas; S.A., S.J.L., D.C., J.P., R.C.B., J.L., C.B., M.C., and H.H.S., collected data; N.K., E.S., and Y.M. provided transgenic mice and helped with study design and analysis; S.L., T.D., A.Q., E.S., Y.M., and B.L. reviewed data and edited manuscript; S.A., S.J.L, and B.L. drafted the manuscript.

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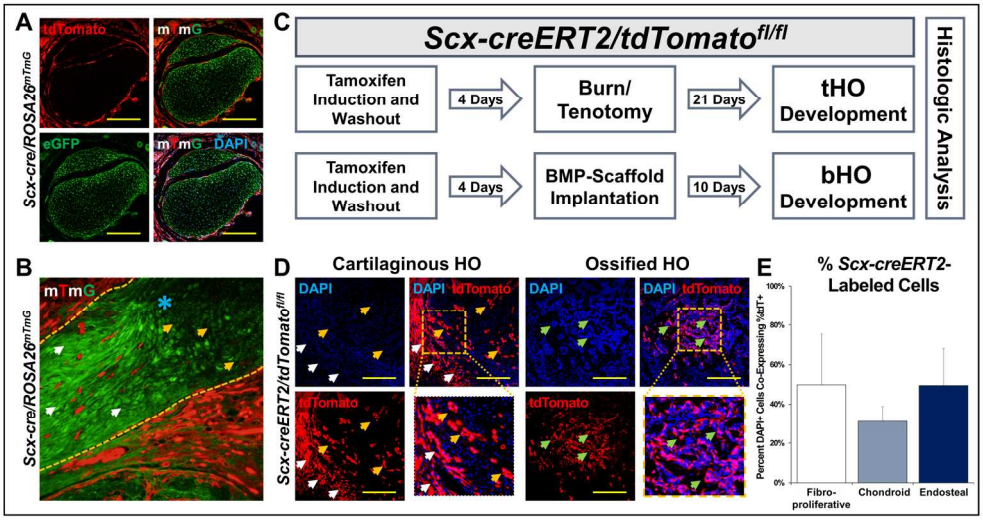

Figure 1

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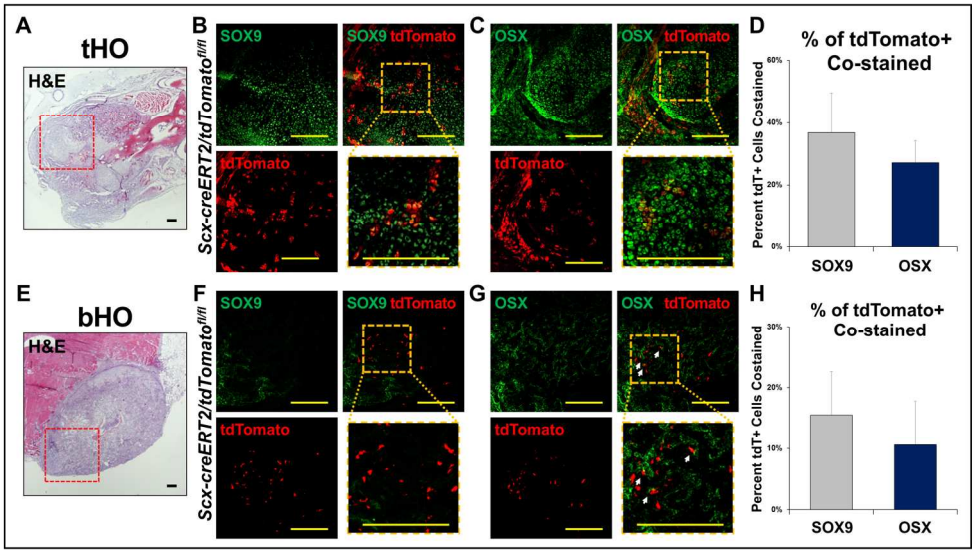

Figure 2

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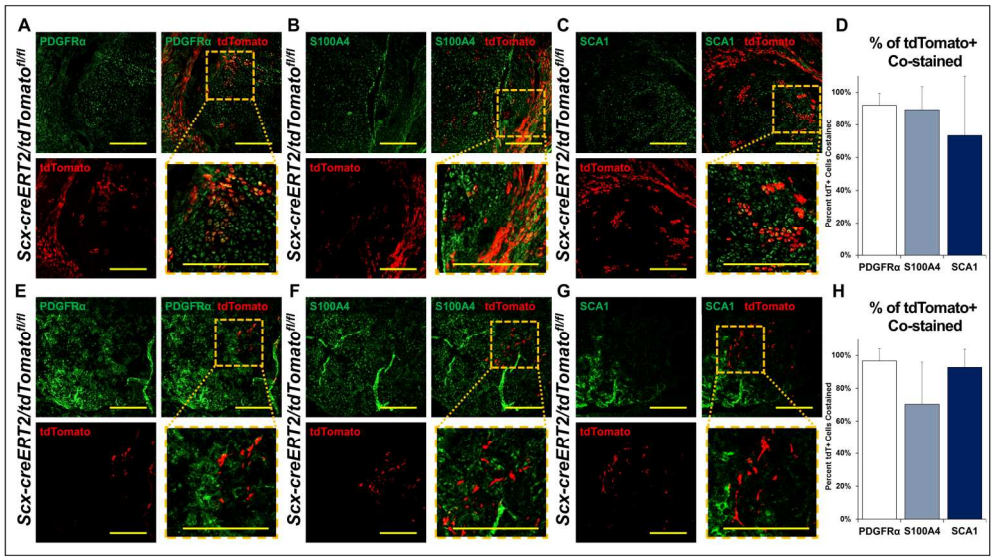

Figure 3

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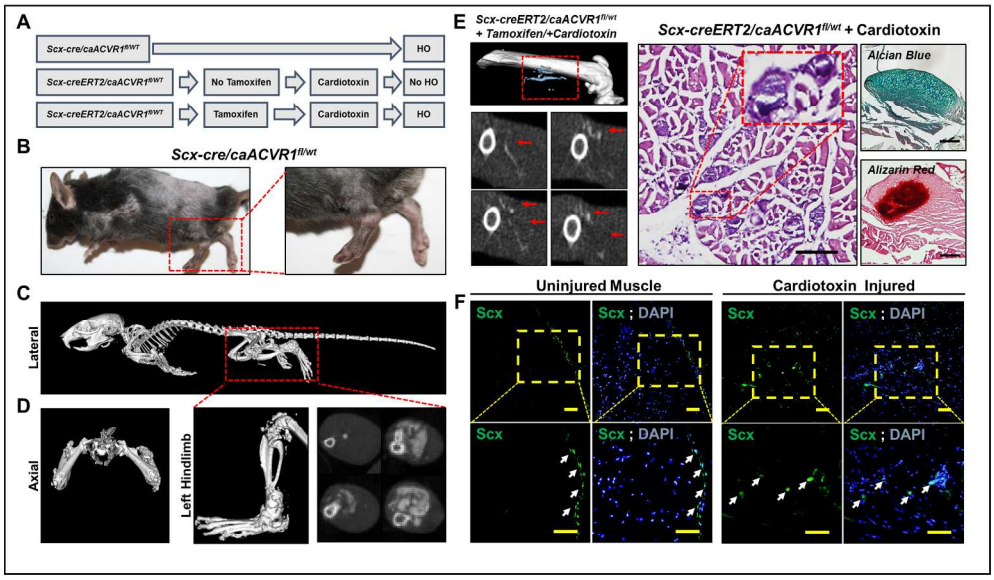

Figure 4

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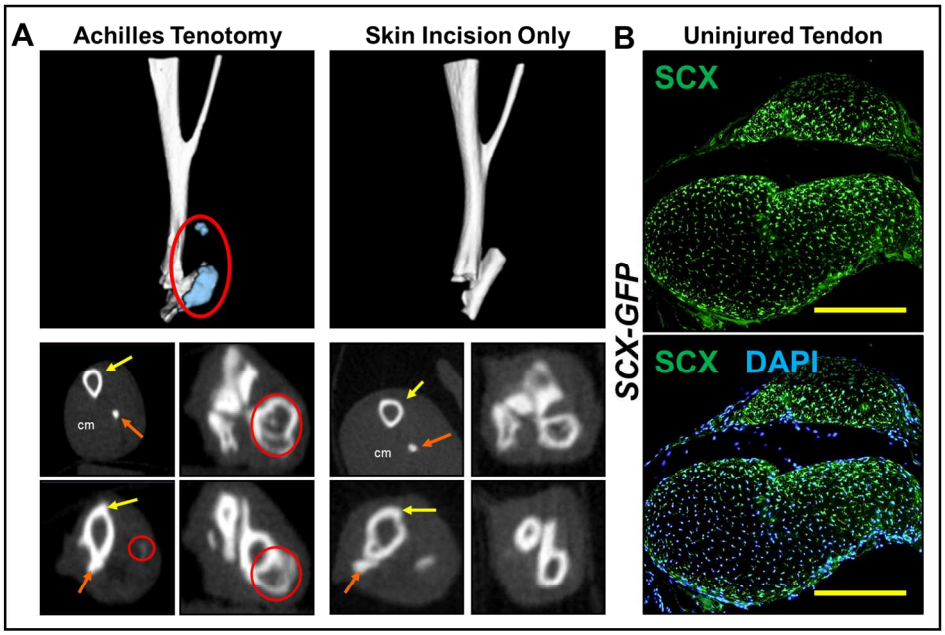

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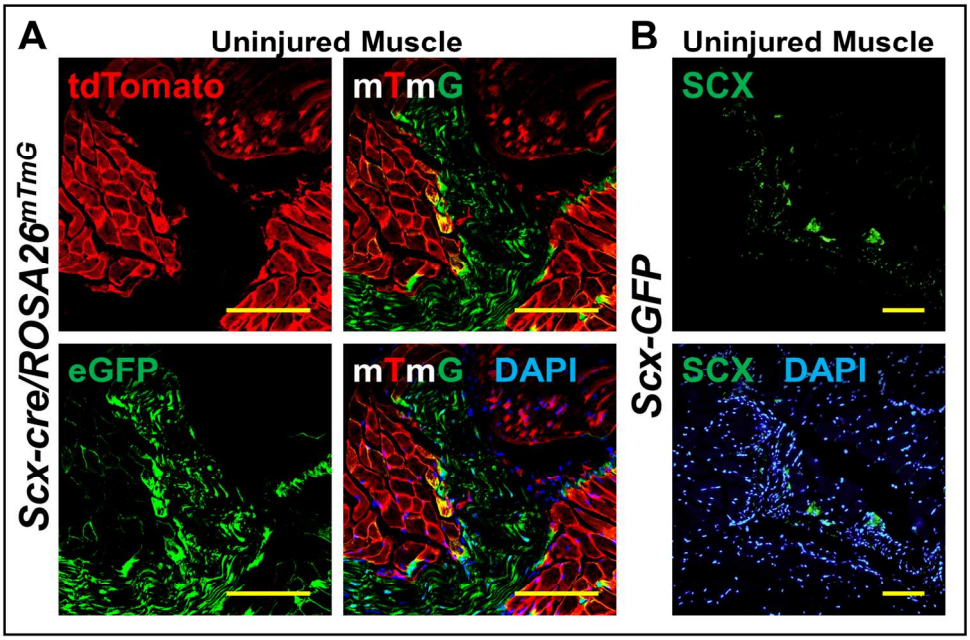

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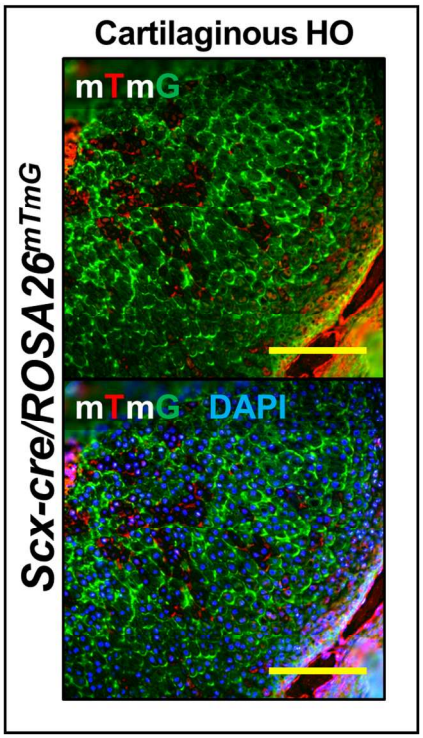

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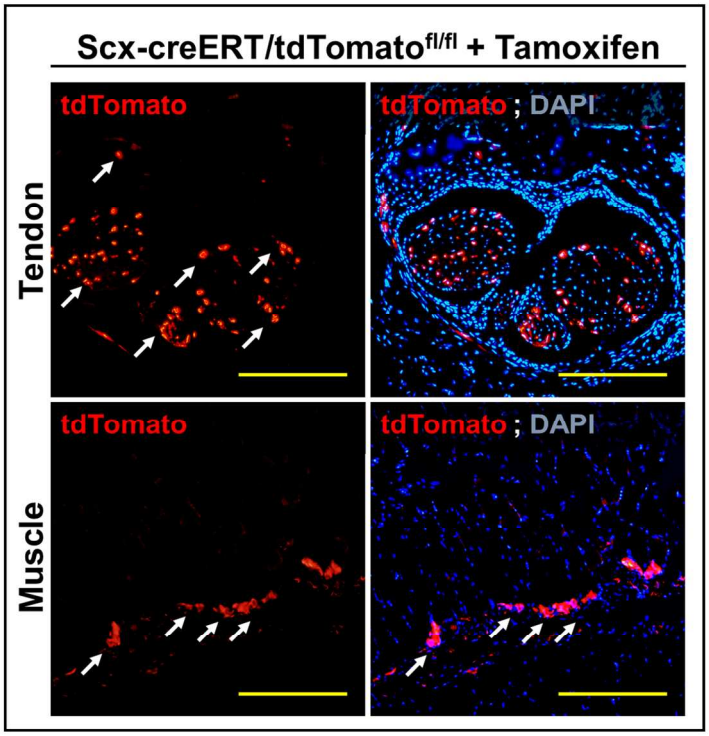

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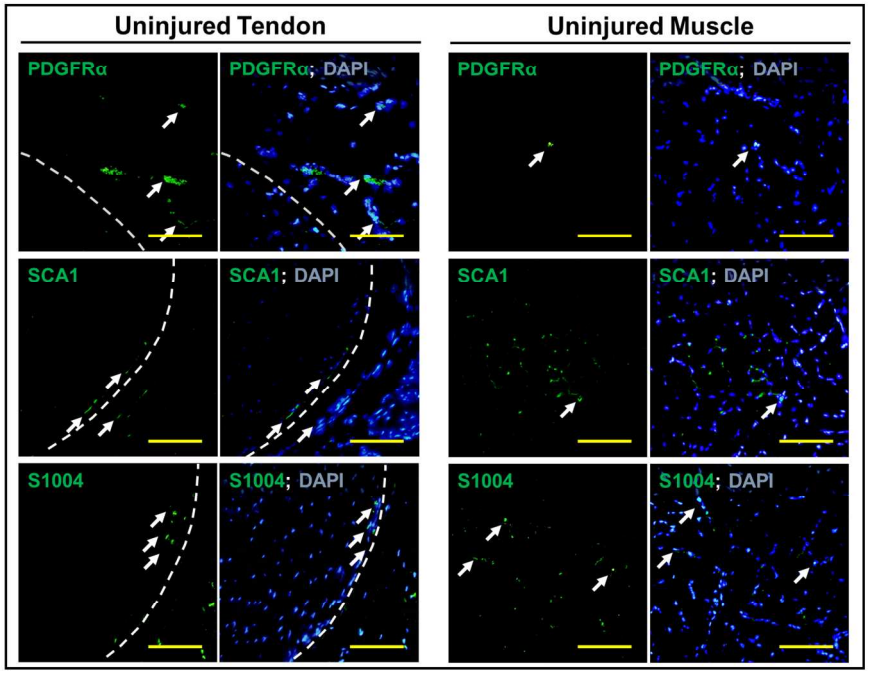

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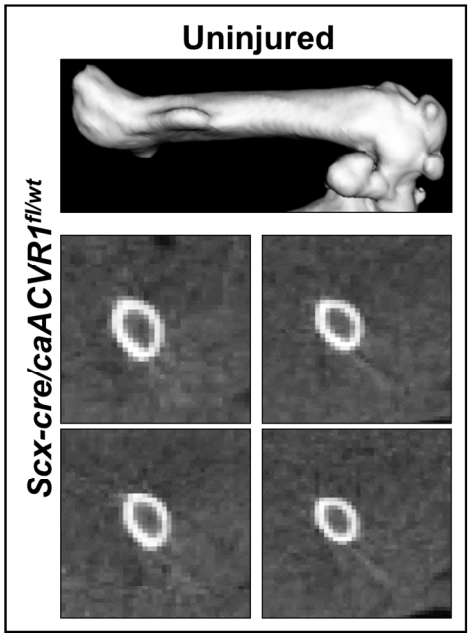

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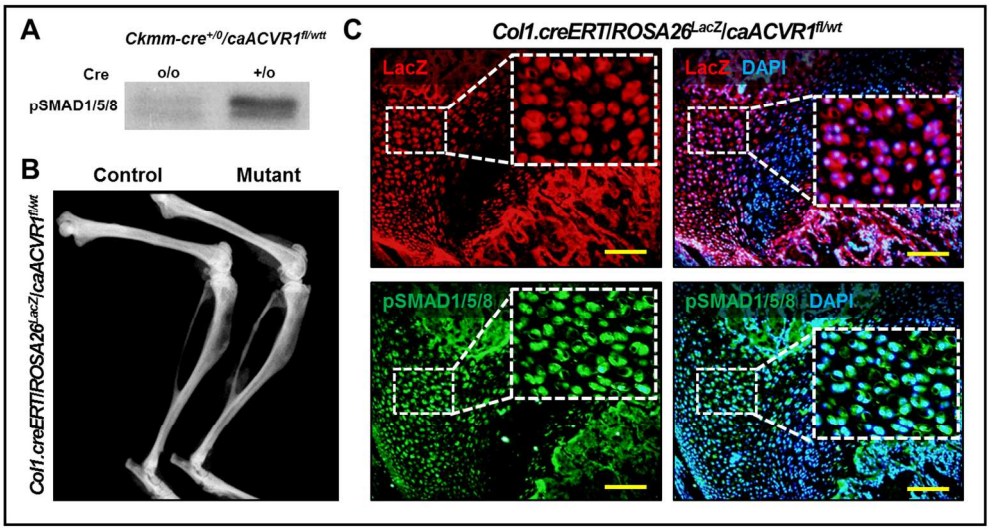

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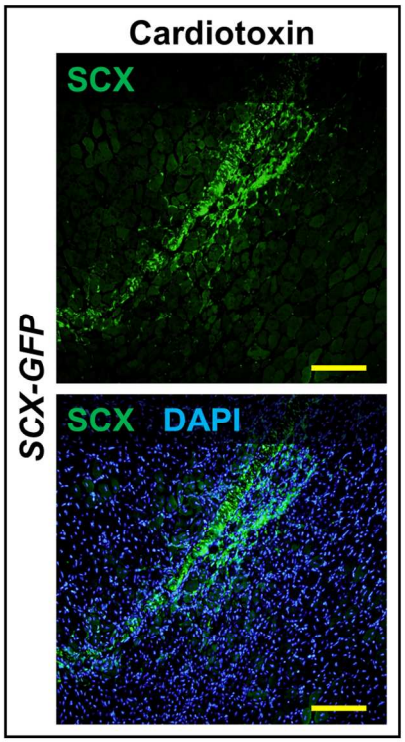

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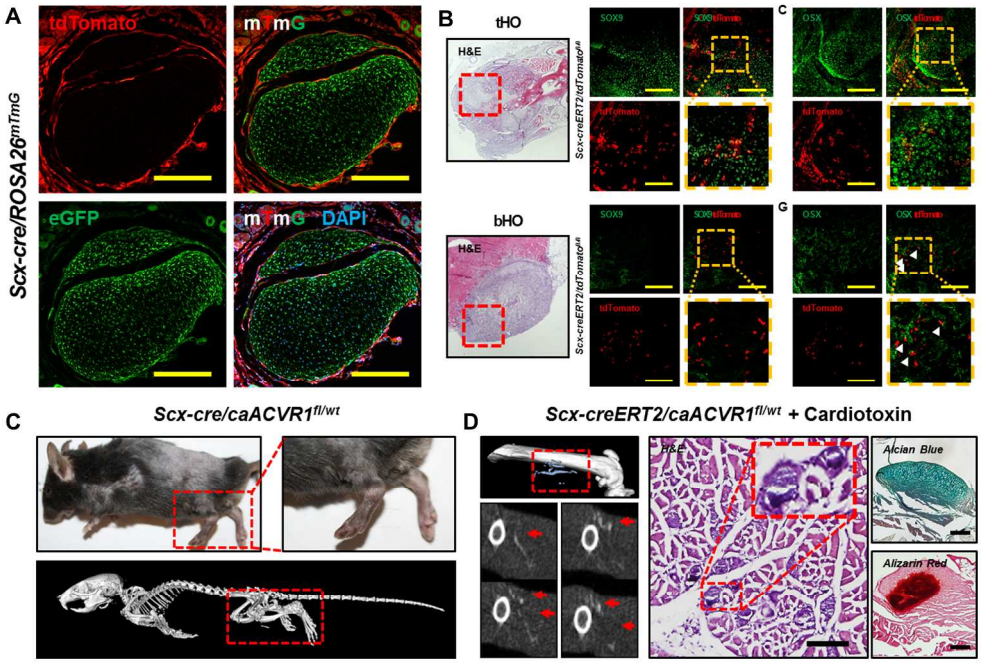

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