

Scleraxis-Lineage Cells Contribute to Ectopic Bone Formation in Muscle and Tendon

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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 postnatal, 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 coexpress markers of endochondral ossification (Osterix, *SOX9*). Furthermore, these adult preinjury restricted cells coexpressed mesenchymal stem cell markers including *PDGFR α* , *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. Postnatal 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 BMP receptor (e.g., *caACVR1*) activity. *STEM CELLS* 2017;35:705–710

SIGNIFICANCE STATEMENT

Heterotopic ossification (HO) is the formation of pathologic extra-skeletal bone. A significant area of inquiry in this field has been the source of progenitor cells which contribute to HO. In this article, we demonstrate that a population of mesenchymal cells resident to tendon and muscle contribute to both trauma-induced and genetic HO.

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 [1]. 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 [2, 3]. These *Tie2*⁺ cells have been shown to be muscle-resident *CD31*⁻/*CD45*⁻/*PDGFR α* ⁺/*Sca1*⁺ cells suggesting they are mesenchymal stem cells and not endothelial cells as initially thought [4]. The

contribution of *Glast-cre* cells that reside within the muscle interstitium has also been evaluated. These cells coexpress mesenchymal markers including *PDGFR α* and *S100A4*, and also appear to contribute to different stages of HO [5]. 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 transcription factor, is expressed in connective tissues including tendon, ligaments, and perimysium between muscle fibers [6–9]. Furthermore, scleraxis-expressing cells collected after

tendon injury exhibit robust chondrogenic potential both in vitro and when implanted in vivo [10].

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.

MATERIALS AND 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 60°C applied to the depilated dorsum for 18 seconds [11]. Separately, mice underwent implantation of a human r-BMP loaded collagen scaffold into the gastrocnemius muscle with injection of cardiotoxin [12]. A 3 × 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/caAcvr1^{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 4°C. Sections were embedded in paraffin or optimal cutting temperature compound (Tissue-Tek; Sakura Finetek) and 5-7 µm 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 three 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 Analysis

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

Statistical Analysis

Experiments were performed with $n \geq 3$ 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 SD.

RESULTS

Scleraxis-Lineage Cells Contribute to All Phases of Trauma-Induced HO

We first demonstrated that the Achilles tendon, which is transected in the burn/tenotomy model of trauma-induced HO (tHO) (Supporting Information 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; Supporting Information 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 (Supporting Information 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 ($95.1 \pm 1.2\%$) and chondroid ($65.8 \pm 13.0\%$) regions of developing tHO (Fig. 1B, Supporting Information Fig. S3). To exclude any contribution of trauma-induced activation of scleraxis we then used postnatal, tamoxifen-inducible, scleraxis-lineage restricted reporter mice (*Scx-creERT2/tdTomato^{fl/fl}*) with tamoxifen activation ending four days prior to injury (Fig. 1C; Supporting Information 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 ($50.0 \pm 25.6\%$), chondroid ($31.0 \pm 7.8\%$), and endosteal (49.4 ± 18.6) regions of tHO (Fig. 1D, 1E).

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

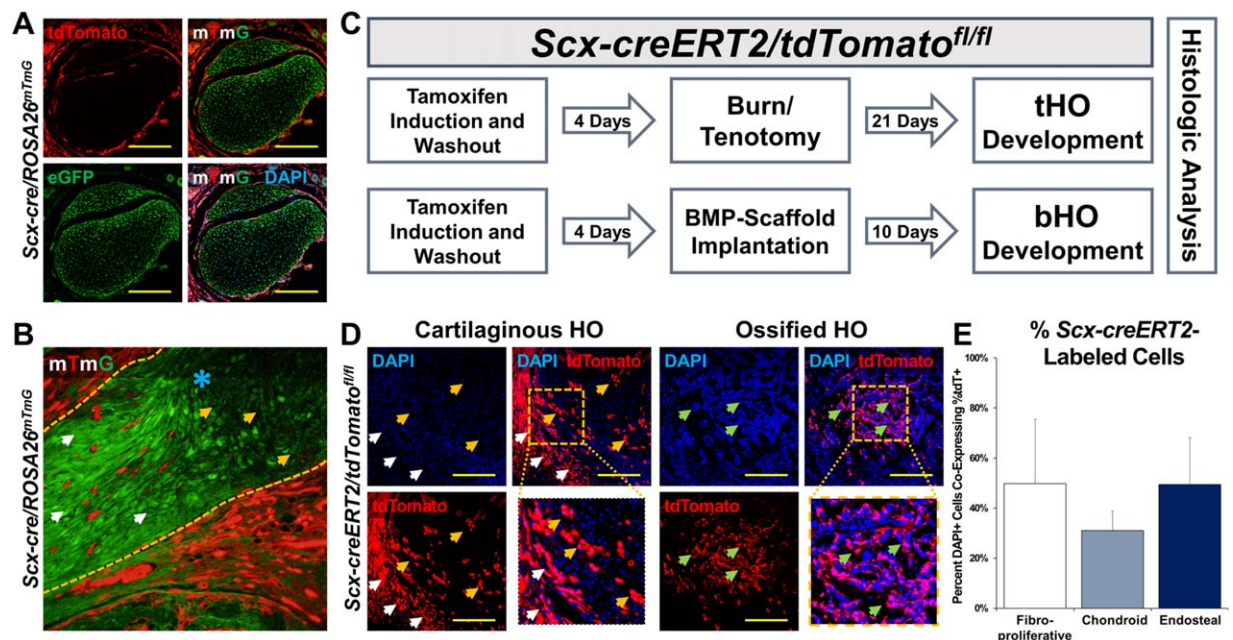


Figure 1. Scleraxis-lineage cells contribute to all phases of tHO. **(A):** Scleraxis lineage (*Scx-cre/ROSA26^{mTmG}*) defines the Achilles Tendon (eGFP+) prior to injury. **(B):** Scleraxis lineage (*Scx-cre/ROSA26^{mTmG}*) defines both fibroproliferative (eGFP+; white arrow) and chondroid (eGFP+;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

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-2D). Importantly, tdTomato+ cells in this model expressed both SOX9 and OSX in tHO (Fig. 2B-2D). Similar findings were observed in our bHO model, although bHO was predictably more ossified with comparatively diminished markers of active endochondral ossification versus tHO (Fig. 2E-2H).

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 PDGFR α and S100A4, which are expressed by Glast-cre cells [5], and Sca1 which is expressed by Tie2+ nonendothelial cells [4]. In both tHO (Fig. 3A-3D) and bHO (Fig. 3E-3H), 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 (Supporting Information 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-4D), although no HO was detectable within muscle (Supporting Information 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 (Supporting Information 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 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 (Supporting Information Fig. S8). To exclude any contribution of cardiotoxin-induced activation of scleraxis, we then designed a postnatal, 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

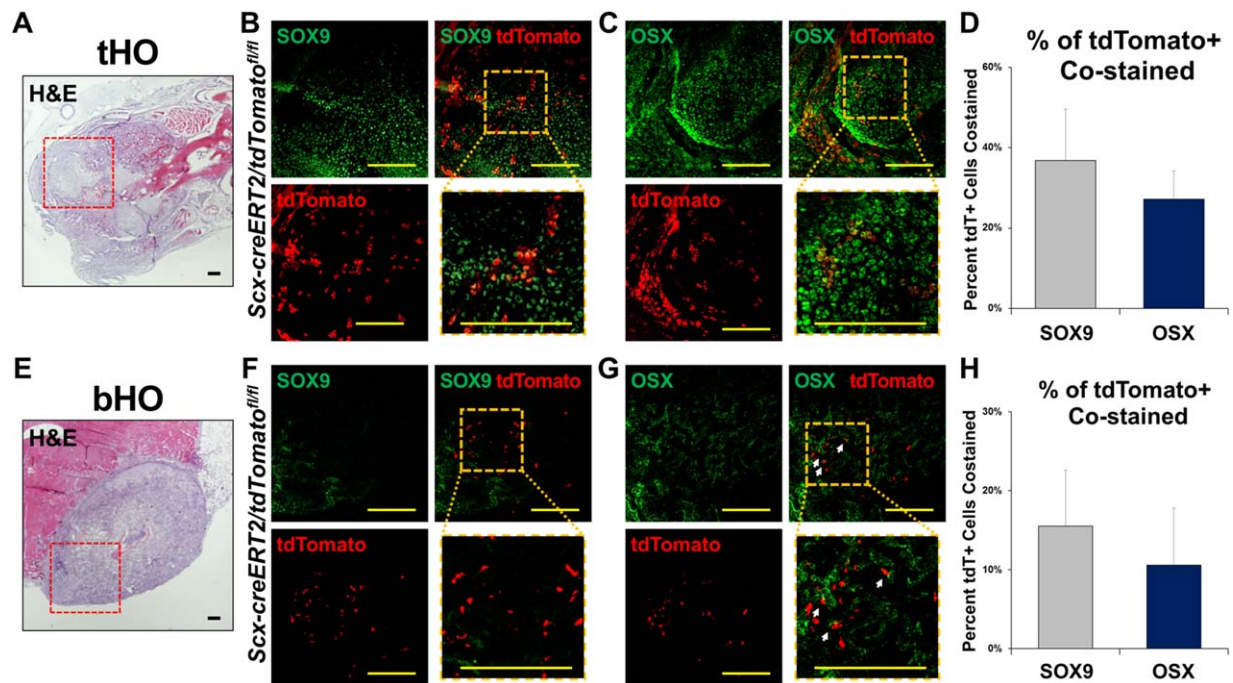


Figure 2. Scleraxis-lineage cells defined in the uninjured adult (tdTomato+) contribute directly to the endochondral anlagen in both trauma-induced (tHO) and BMP-scaffold driven (bHO) heterotopic ossification. **(A):** Representative H&E demonstrating areas of tHO in *Scx-creERT2/tdTomato^{fl/fl}* mice. **(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 μ m. Abbreviations: bHO, BMP-loaded scaffold; H&E, hematoxylin and eosin; tHO, trauma induced heterotopic ossification.

postcardiotoxin (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 postcardiotoxin 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 nonendothelial Tie2+ cells and *Glast-Cre* cells [3–5, 13, 14]. Using markers such as PDGFR α , *Sca1*, and *S100A4*, others have shown that Tie2+ and *Glast-cre* cells which contribute to HO are likely mesenchymal cells [3–5, 13, 14]. Our findings confirm the coexpression 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 [15]. 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, preinjury 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 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

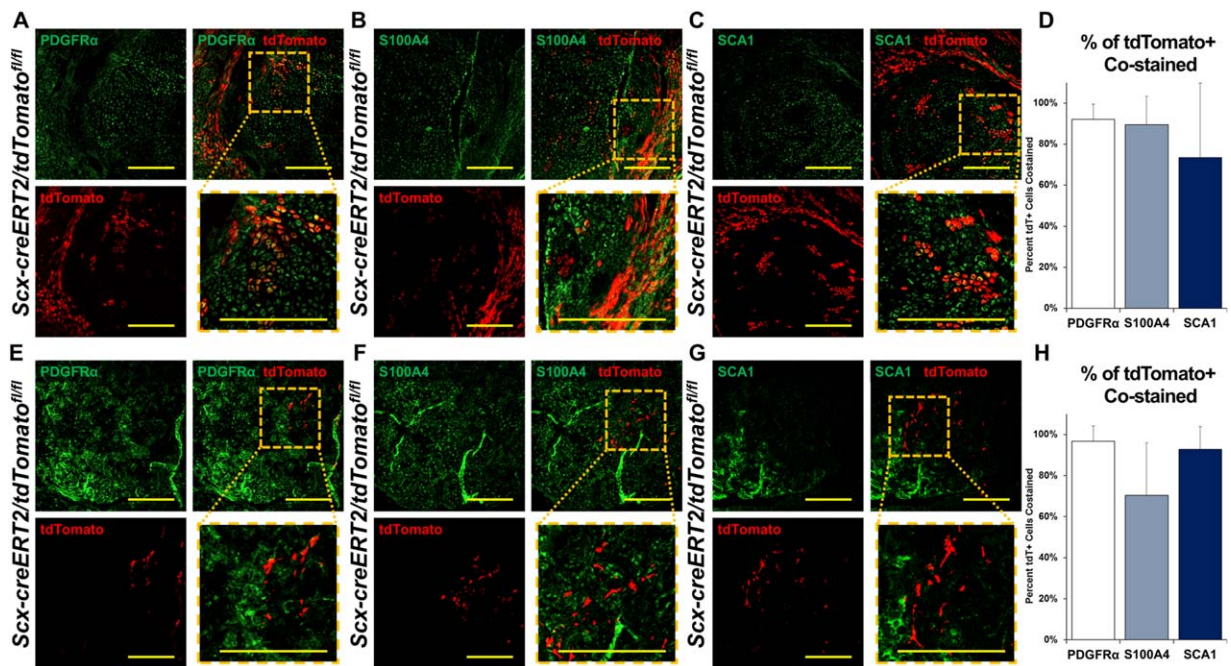


Figure 3. Scleraxis-lineage cells defined in the uninjured adult (*Scx-creERT2/tdTomato^{fl/fl}*) display mesenchymal cell markers in both trauma-induced (tHO) and BMP-scaffold (bHO) heterotopic ossification. **(A):** *Scx-creERT2* cells (tdTomato+) express PDGFRα in tHO. **(B):** *Scx-creERT2* cells (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.

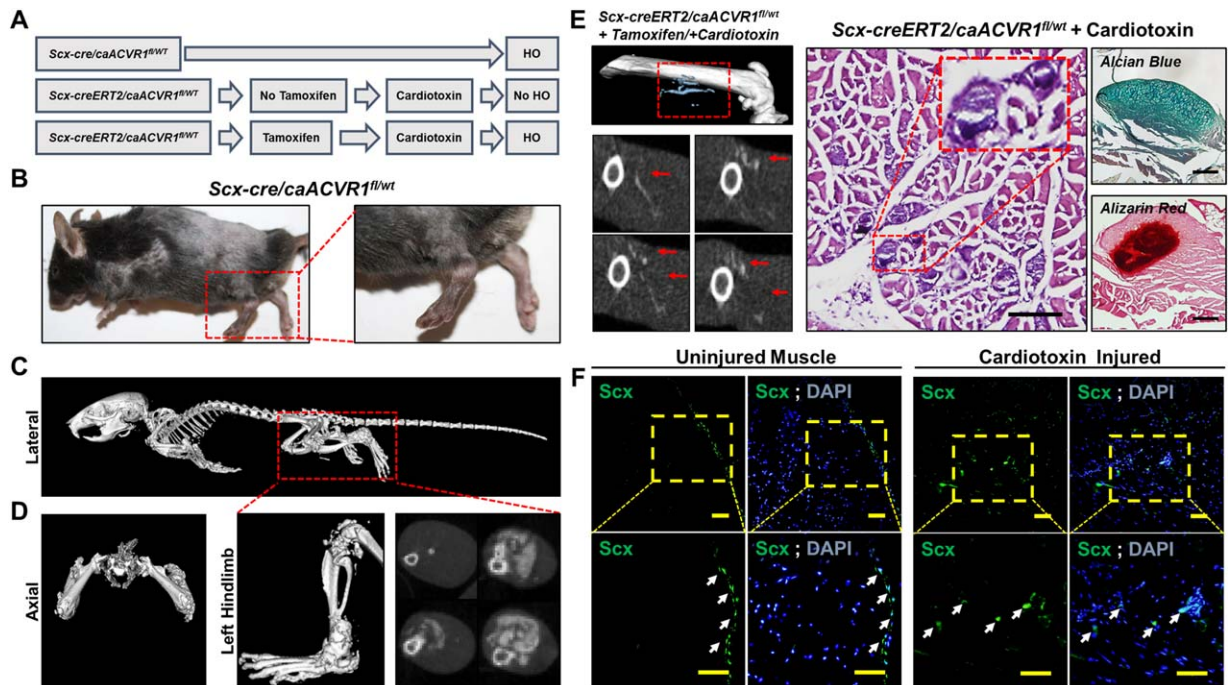


Figure 4. Scleraxis lineage-restricted *caACVR1* expression causes tendon and intramuscular HO. **(A):** Schematic showing spontaneous HO generation by scleraxis-lineage cells expressing *caACVR1* (*Scx-cre/caACVR1^{fl/wt}*) and by preinjury scleraxis-lineage cells after cardiotoxin injury (*Scx-creERT2/caACVR1^{fl/wt}*). **(B):** Photograph of *Scx-cre/caACVR1^{fl/wt}* mouse showing ectopic bone formation at the hindlimb. **(C):** Whole body three-dimensional (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 nondecalcified 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. Abbreviation: HO, heterotopic ossification.

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 postnatal, preinjury Scleraxis expression are capable of chondrogenic and osteogenic differentiation after local injury or with hyperactive BMP receptor activity.

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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.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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