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Axin2-lineage cells contribute to neonatal tendon regeneration

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Abstract

Purpose: Tendon injuries are a challenging clinical problem with few treatment options. Identifying the molecular regulators of tendon is required for the development of new therapies. While the Wnt pathway is critical for the maintenance and differentiation of many tissues, the role of Wnt signaling in tendon cell biology remains largely unexplored.

Methods: The effects of Wnt activation were tested *in vitro* using neonatal tendon-derived cells cultured in 2D and 3D conditions. The inducible Axin2CreERT2 was then used to label Axin2+ cells in vivo and cells were traced during neonatal tendon regeneration.

Results: We showed that activation of Wnt signaling results in proliferation of neonatal tendon cells. While tendon marker expression was inhibited by Wnt activation under 2D conditions, *Scx* expression was not affected under 3D uniaxial tension, suggesting that the microenvironment contextualizes tendon cell response to Wnt signaling. Using an *in vivo* model of neonatal tendon regeneration, we further showed that Wnt signaling cells comprise a sub-population of tenocyte and epitenon cells that proliferate after injury and are recruited during regeneration.

Discussion: Collectively, these studies suggest that Wnt signaling may play a role in tendon cell proliferation, differentiation, and regeneration.

Keywords

tendon; Wnt; TGFβ; 3D culture; regeneration

Introduction

Tendons are connective tissues that integrate muscle to their proper skeletal sites to enable motion. This function is mediated by highly aligned type I collagen-rich fascicles that are synthesized and maintained by resident tenocytes expressing characteristic markers such as *Scleraxis* (*Scx*), *Tenomodulin* (*Tnmd*), and *Mohawk* (*Mkx*) [1,2]. Surrounding tendon and tendon fascicles are the epitenon and endotenon, respectively, which are maintained by cells expressing laminin, α-smooth muscle actin (αSMA), and platelet-derived growth factor receptor α (PDGFRα) [3–6].

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Disclosures

The authors declare no conflicts of interest.

Tendon function is often permanently compromised by injuries, which are common and clinically challenging. These injuries can occur due to mechanical over-use or acute trauma [7,8]. While damage can range from mild inflammation to complete tear, disorganized scar formation typically accompanies adult tendon injuries, and native structure is not restored [9,10]. To date, treatment to address tendon pain and damage is largely limited to corticosteroids, physical therapy, or surgical repair. However, none of these treatments restore native tendon structure or function. Biologic treatments, such as platelet-rich plasma and stem cells, remain controversial with mixed clinical efficacy [11,12]. A better understanding of the cell and molecular regulators underlying basic tendon biology is therefore required for the development of effective therapies.

One cell type of interest with therapeutic relevance is tendon stem/progenitor cells (TSPCs) [13]. The first characterization of these cells demonstrated expression of *Scx* in addition to cell surface markers associated with mesenchymal stem cells [14]. Further, TSPCs undergo clonal expansion and can differentiate toward cartilage, bone, fat, and tendon fates. While TSPCs were initially proposed to reside within the tendon fascicle, recent research identified TSPC-like cells near blood vessels (CD146+) and in the epitenon (*Tppp3*+/PDGRα+, αSMA+), which are activated by tendon injury [3,5,15–17]. That TSPCs may originate from multiple sources is supported by experiments showing that both epitenon and tendon fascicle cells can be isolated, expanded in culture, and induced toward multiple lineages [18]. While these studies were carried out using juvenile (8–10 week old) or older animals, other studies showed that tendon-derived cells from neonatal postnatal day 7 (P7) rat have improved proliferative and differentiation potential compared to 8 week old rats [19]. We previously showed that functionally regenerative healing in neonatal P5 mice compared to adult mice (4–6 months) is driven by proliferation and recruitment of *Scx*-lineage tenocytes and non-*Scx*-lineage cells that adopt a tenogenic fate [20,21]. However, the molecular cues and signaling pathways that regulate tendon regeneration and TSPC populations have not yet been fully elucidated.

To date, the transforming growth factor beta (TGFβ) signaling family remains the best studied pathway for tendon. TGFβ signaling is critical for induction and maintenance of tendon progenitors during development as loss of signaling results in loss of tendons or progressive degeneration [22,23]. During healing, TGFβ can play contextually different roles.: While TGFβ signaling is strongly associated with fibrotic scar formation in adults, in neonates, it is required for tenocyte recruitment during regeneration [21,24–26]. Although a number of other pathways have been identified for tendon healing, including fibroblast growth factor (FGF), connective tissue growth factor (CTGF), and platelet-derived growth factor (PDGF), these pathways are relatively less studied [5,15,16,27–30]. One intriguing pathway is the Wnt pathway, which is well-established in the maintenance of tissue-specific stem cells in many tissues including intestine, mammary glands, skin [31–34]. However, the role of Wnt signaling in tendon is largely known. To address this question, we combine *in vitro* and *in vivo* models to test the hypothesis that Wnt signaling may identify TSPCs in neonatal tendon, which have regenerative potential. We find that while Wnt activation increases cell proliferation, tendon differentiation was context-specific, and labeling of Wnt signaling cells by Axin2CreERT2 identified a subset of recruited tenogenic cells in the regenerated neonatal neotendon.

Materials and Methods

Mice

The following mouse lines were used: ScxGFP [35], ScxCreERT2, Axin2CreERT2 [31], and ROSA26-TdTomato Ai14 [36]. Labeling of Axin2-expressing, Wnt signaling cells was performed via delivery of tamoxifen in corn oil by oral gavage (1.25 mg/pup) at postnatal days 2 and 3. Proliferating cells were labeled by subcutaneous EdU injection (0.05 mg/pup) 2 hours prior to harvest. All animal procedures were approved by the Institutional Animal Care and Use Committee at Icahn School of Medicine at Mount Sinai.

Tendon and mesenchymal stromal cell isolation and culture

Achilles tendons were dissected from postnatal day 7 (P7) mice in ice cold 1X HBSS+1% Pen/Strep. Tendons were enzymatically digested in 0.25% Trypsin without EDTA (Gibco) for 30 mins at 37°C, followed by centrifugation at 2,000 rpm for 5 minutes and resuspension in 0.5 mg/ml Collagenase type 2 (Worthington), 0.25% Trypsin without EDTA in 1X HBSS+1% Pen/Strep. The digest was incubated at 37°C for one hour with 15 minute vortex intervals. Enzymes were inactivated by adding serum-containing media (DMEM+10% FBS+1% Pen/Strep), and the tendon digest was filtered through a 70um filter to remove debris. Cells were plated on tissue culture treated dishes, cultured in DMEM+10% FBS+1% Pen/Strep in hypoxic culture (5% O_2) until confluent, and used for experiments at passage 2–4.

Bone marrow-derived mesenchymal stromal cells (MSCs) were isolated from the long bones of 6–8 week old mice. Briefly, bone marrow was flushed from tibias and femurs with cold PBS+1% Pen/Strep using a 26G needle, red blood cells were lysed in ice cold RBC Lysis Buffer (eBioscience), strained through a 70 μm cell strainer and resuspended in serum containing media (DMEM+10% FBS+1% Pen/Strep). Cells were plated on a tissue culture treated dish and media was changed the following day to remove any unattached or dead cells. Cells were subsequently cultured in DMEM+10% FBS+1% Pen/Strep in hypoxic culture (5% O_2) until confluent and used for experiments at passage 2–4.

Flow Cytometry

Cultured tendon cells were stained using antibodies against CD45, CD31, CD11b, Sca1, CD29,, CD90, CD105, and CD140a (Biolegend). After gating out dead cells and debris, isotype controls were used to determine $CD45^-$ $CD31^-$ $CD11b^-$ Triple Negative (TN), non-hematopoietic cells for further analysis. Experiments were run on an LSRFortessa X-20 (BD Biosciences) and Attune NxT (ThermoFisher) and data was analyzed using FCSExpress software (DeNovo) and FlowJoV10 (TreeStar).

Cell proliferation assay

To assay cell proliferation, 2,500 cells were plated in triplicate for each time point in DMEM+10% FBS+1% Pen/Strep supplemented with 10 ng/mL TGFβ2 or 5 μM CHIR. The Cell Counting Kit-8 (CCK-8, Dojindo) was used to measure cell proliferation by absorbance according to manufacturer's directions. Cells were incubated with CCK-8 solution for 2 hours and absorbance detected using the SpectraMaxi3 plate reader at 450nm. Phenol

red-containing media was used as a negative control and media absorbance was subtracted from the absorbance values of experimental samples.

Osteogenic and chondrogenic differentiation assays

For osteogenic differentiation, cells were seeded at 5.3×10^4 cells/cm^2 in αMEM+10% FBS+1% Pen/Strep. After 24 hours, cells were switched over to differentiation media containing 10mM betaglycerophosphate and 50 μg/ml ascorbic acid in αMEM+10% FBS+1% Pen/Strep and cultured for 14 days. Media was refreshed twice a week. Cells were fixed in 4% paraformaldehyde and stained with Alizarin Red (1%, aqueous) (Poly Scientific R&D) for 20 minutes. Wells were then washed in deionized water, air dried, and imaged using a Leica M165FC stereoscope.

For chondrogenic differentiation, cells were cultured as micromasses (2×10^5 cells in 10 μl of DMEM+10% FBS+1% Pen/Strep. After 2 hours at 37°C to allow adherence, cell culture media was added to the micromasses. After 24 hours, cells were incubated in serum-free differentiation media containing 1% ITS, 1X sodium pyruvate, 50 μg/ml ascorbic acid, 0.1 μM dexamethasone, 40 μg/ml L-proline and 10 ng/ml TGFβ3 in high glucose DMEM+1%Pen/Strep and cultured for 14 days. Media was refreshed twice a week. Cells were fixed in 4% paraformaldehyde and stained with Alcian Blue (0.5% in 3% acetic acid, pH 1.0, Poly Scientific R&D) overnight. Wells were washed in 3% acetic acid (pH 1.0) followed by 2 washes in 3% acetic acid (pH 2.5), air dried, and imaged using a Leica M165FC stereoscope.

Fabrication of 3D engineered tendon constructs under uniaxial tension

Neonatal tendon cells were trypsinized and embedded into 3D type I collagen gels at 250,000 cells per 2 mg/mL PureCol as previously described [37]. Cell-seeded gels were then cast into sterile PDMS dishes between pinned prolene sutures [37]. Gels were placed in a humidified 37°C incubator for 1 hr to allow the gel to set before flooding with DMEM+10% FBS+1% Pen/Strep only or DMEM+10% FBS+1% Pen/Strep supplemented with 10 ng/mL TGFβ2, 5μM CHIR, or 10 ng/mL TGFβ2+5μM CHIR for 21 days. Media was refreshed twice per week.

RNA extraction, reverse transcription, and real time quantitative polymerase chain reaction

Cells were lysed in Trizol (Life Technologies) and total RNA was extracted using Trizol/chlorofrom. Total RNA was quantified using NanoDrop 2000 and 0.5–1 μg of RNA was reverse transcribed to generate cDNA using the Super Script VILO mastermix (Invitrogen). Gene expression was determined by qPCR using PowerUp SYBR Green Mastermix (Applied Biosystems) and validated primers (Table 1) by the Mount Sinai qPCR Core Facility. Product specificity was confirmed by melt curve analysis, and relative expression was determined using $2^{-\Delta\Delta CT}$ analysis after normalization to β actin expression.

Achilles tendon transection surgeries

Full transection of the Achilles tendon was carried out without repair at postnatal day 5 (P5) as previously described [20]. Mice were sacrificed and hindlimbs collected at 3 and 28 days post injury. All animals were included in analyses.

Histology

Hindlimbs were fixed overnight in 4% paraformaldehyde in PBS at 4°C, decalcified in 50mM EDTA in PBS for 14 days at 4°C, followed by 1 hour in 5% sucrose then overnight in 30% sucrose at 4°C. Hindlimbs were then embedded in Optimal Cutting Temperature media (OCT) (Fisher). Alternate transverse sections (12 μm) were collected on charged slides through the entire length of the Achilles tendon from the calcaneal to muscle insertion. EdU labeled cells were detected using the Click-iT EdU detection kit (Life Technologies) as per manufacturer's instructions. Tendon constructs were fixed in 4% paraformaldehyde in PBS at room temperature for 30 minutes, embedded in OCT, and transverse sections (12 μm) collected. Immunostaining for proliferating cells was carried out using antibody against Ki67 (Biolegend). For all staining, DAPI counterstain was used to visualize cell nuclei. All fluorescent imaging was carried out using the Zeiss Axio Imager microscope with Apotome optical sectioning.

Statistical analyses

Data are reported as mean ± standard deviation and analyzed using paired or unpaired t-tests where appropriate. For experiments with multiple comparisons, one-way and two-way ANOVAs with Tukey's posthoc analyses were used. Significance was determined at $p<0.05$. Sample size was chosen based on previous data. All statistics were performed using GraphPad Prism software.

Results

Neonatal tendon cells express MSC markers and demonstrate reduced differentiation potential toward chondrogenesis and osteogenesis compared to bone-marrow MSCs

To characterize tendon cells derived from neonatal mice and cultured in 2D, we used a panel of common mesenchymal stromal cell (MSC) markers previously used to define tendon stem progenitor cell (TSPC) populations [13,14]. After gating for singlets and live cells, the majority of cells (>98%) were triple negative for hematopoietic, endothelial, and myeloid markers CD45, CD31, and CD11b, respectively (Figure 1A). Consistent with previous studies from juvenile or adult murine TSPCs, a large percentage of these triple negative cells expressed MSC markers Sca1, CD90, CD29, and CD140a (~60–80%) (Figure 1B). However, few cells expressed CD105 (~10%) (Figure 1B). To determine the proportion of the cultured cells that were derived from tenocytes versus epitenon, we labeled *Scx*-expressing cells at days 2 and 3 after birth by tamoxifen administration of ScxCreERT2/Rosa26-TdTomato pups and isolated tendon cells at 7 days after birth. After passage 3, flow analysis of TdTomato+ cells indicated that 13% of cells were derived from tenocytes while the majority of the cells were likely of epitenon origin (Figure 1C). Analysis of ScxGFP+ cells 24 hours after initial isolation and plating showed that the initial population of plated cells were 80±6% ScxGFP (ie. intrinsic tenocytes, n=3 mice), indicating that epitenon cells likely out-proliferate tenocytes with continued 2D culture and passage (Supplemental Figure 1).

Since previous studies suggested that adult CD105-negative TSPCs give rise to ectopic cartilage and heterotopic ossification in response to injury [38], we tested the chondrogenic and osteogenic capacities of neonatal tendon cells to further characterize the cells.

Compared to bone marrow-derived MSCs (bMSCs), neonatal tendon cells showed comparable differentiation potential toward chondrogenesis but minimal differentiation toward osteogenesis, as evidenced by similar Alcian Blue and poor Alizarin Red staining, respectively (Figure 1D). Gene expression confirmed lower expression of osteogenic differentiation markers *Osx* in neonatal TSPCs, while *Sox9* expression was comparable between groups with reduced *Col2a1* (Figure 1E). This data suggests that chondrogenic potential may be incomplete in neonatal tendon cells compared to bMSCs.

Effect of Wnt signaling on neonatal tendon cells is contextualized by the microenvironment.

Since Wnt signaling is essential in the maintenance of multiple stem cell populations, we next asked whether Wnt signaling is required to maintain neonatal tendon cells. Using the Wnt agonist CHIR, we tested the effect of Wnt activation on neonatal tendon cells proliferation and differentiation. Cells were used after 2D expansion, similar to Figure 1 experiments above. Since TGFβ signaling is a known regulator of proliferation and tenogenic/chondrogenic differentiation, TGFβ2 was used as a positive control. In 2D culture, TGFβ2 or CHIR treatment resulted in enhanced neonatal tendon cell proliferation at all timepoints, compared to basal media conditions (no treatment, NT) (Figure 2A). Gene expression analysis showed reduced expression of tendon markers *Scx* and *Mkx* with CHIR treatment at day 15 with no difference in cartilage markers *Sox9* and *Col2a1* (Figure 2B). No difference in gene expression was observed with TGFβ2 treatment compared to NT.

Although these results suggested that CHIR suppresses tendon differentiation of neonatal tendon cells, it is well established that proper tendon differentiation depends on culture microenvironment (previous studies have demonstrated the loss of tendon marker expression in tendon cells in 2D culture while tenogenesis is enhanced under 3D uniaxial tension [39–42]. To test whether these effects of Wnt activation is maintained in a more physiologic environment, we therefore embedded neonatal tendon cells (after 2D expansion) in 3D collagen gels held under static tension (Figure 3A). Differences in cell-mediated gel contraction were detectable by 14 days of culture by whole-mount imaging of constructs. The presence of CHIR resulted in enhanced contraction relative to TGFβ2 or no treatment (Figure 3B). Qualitatively, ScxGFP expression was most intense at day 21 for all constructs, and full contraction was observed with CHIR treatment (Figure 3B).

Consistent with whole mount imaging, quantitative analysis of transverse cryosections showed significantly reduced cross-sectional area between CHIR relative to NT or TGFβ2 treatment at day 21, indicative of enhanced contraction (Figure 4A, B). The number of DAPI+ cells was increased with CHIR treatment relative to NT and TGFβ2, however staining for Ki67+ proliferating cells at day 21 did not reveal any difference in proliferation between groups (Figure 4C, Supplemental Figure 2). Analysis of ScxGFP expression showed that almost all of the cells within 3D constructs expressed ScxGFP, independent of treatment condition (Figure 4D). CHIR treatment however, exhibited a distinctive dense ring of ScxGFP+ cells in the periphery, that was not observed in NT or TGFβ2. Gene expression analysis by qPCR confirmed no difference in *Scx* expression between groups, but *Mkx* and *Sox9* were minimally expressed with CHIR treatment compared to NT and TGFβ2 (Figure

4E). *Col2a1* expression was not detected in any samples (not shown). Collectively, these data indicate that Wnt activation improved cell number in both 2D and 3D environments. While Wnt activation suppressed tendon markers in 2D culture with no effect on cartilage markers, ScxGFP was induced and maintained under 3D tension, suggesting the importance of the biomechanical microenvironment in contextualizing the effect of Wnt signaling.

Neonatal tendon cells are derived from both Axin2^{lin} and non-Axin2^{lin} cells

To determine whether Wnt signaling cells represent a population of stem/progenitor cells in neonatal tendons, we used the tamoxifen-inducible Axin2CreERT2/Rosa26-TdTomato line to label Wnt-signaling cells at P2 and P3 after birth (Figure 5A). Since Axin2 is a Wnt target gene, all cells undergoing active Wnt signaling would be labeled at time of tamoxifen administration. At passage 0 confluency, we observed the presence of both ScxGFP+ and ScxGFP- cells within the cultured population. Similarly, both Axin2^{lin}+/ScxGFP+ and Axin2^{lin}+/ScxGFP- cells were identified (Figure 5B). After 2 passages, flow cytometry showed that nearly 40% of the cultured population were Axin2^{lin} cells, indicating that non-Axin2^{lin} cells were also capable of expansion. Analysis of MSC marker expression showed that the majority of cultured cells expressed Sca1, CD90, and CD29 with minimal expression of CD105 (Figure 5C). The only notable difference in surface marker expression was CD140a, which was expressed by ~80% of non-Axin2^{lin} cells compared to ~60% of Axin2^{lin} cells. CD140a is expressed by epitenon cells [43], indicating that Axin2^{lin} cells may be derived from both the tendon fascicle as well as the epitenon. This hypothesis is supported by our Scx^{lin} cell data, (Figure 1C), which only comprised 13% of the culture population. Overall, these data suggest few differences in stemness marker profiles of Axin2^{lin} versus non-Axin2^{lin} cells in culture.

Axin2^{lin} cells proliferate and contribute to neo-tendon formation after neonatal tendon injury

The presence of Axin2^{lin}+/ScxGFP+ and Axin2^{lin}+/ScxGFP- cells suggested that Axin2CreERT2 may label both neonatal tenocytes and epitenon cells *in vivo*. Since our previous studies showed that Scx^{lin} and non-Scx^{lin} cells contributed to neo-tendon formation during neonatal tendon regeneration [21], we next tested whether the recruited cells were derived from Axin2^{lin} sub-populations (Figure 6A). Analysis of transverse sections of uninjured contralateral tendon at 3 days post-injury (DPI) confirmed Axin2^{lin}+/ ScxGFP+ tenocytes, localized mostly to the Achilles tendon periphery (Figure 6B). A few Axin2^{lin}+/ScxGFP- epitenon cells were also detected. With injury at 3DPI, the proportion of Axin2^{lin}+/ScxGFP+ cells was unchanged, while the number of proliferating EdU+ cells increased relative to uninjured control (Figure 6B–D). Most of the proliferating cells were Axin2^{lin}-, but proliferating Axin2^{lin}+ cells were also observed (Figure 6E). By 28 DPI, abundant Axin2^{lin}+ cells were observed within the forming neo-tendon (Fig 6F, 6G). Transverse sections through the neo-tendon showed that the majority of Axin2^{lin}+ cells were also ScxGFP+, however numerous Axin2^{lin}-/ScxGFP+ cells were also detected (N=1). This data suggests that Axin2^{lin} cells are one of the regenerative cell sources that proliferate and contribute to neonatal tendon repair in response to injury.

Discussion

In this study, we characterized neonatal tendon-derived cells after expansion and found that expanded cells included a mixed population of Scx^{lin} and non-Scx^{lin} cells that expressed most MSC markers including Sca1, CD90, CD29, and CD140a. While cells were capable of chondrogenic and tenogenic differentiation, osteogenic differentiation was quite limited, suggesting that neonatal tendon cells may have restricted potential or may be more akin to progenitor cells rather than multipotent stem cells. We also found that activation of Wnt signaling enhanced neonatal tendon cell proliferation and differentiation in two *in vitro* models. While the proliferative effects of Wnt activation were observed in both 2D and 3D culture, regulation of tendon gene expression was contextualized by the culture environment. Under 2D conditions, we showed that Wnt signaling suppressed tendon markers, similar to previous reports [44]. Under 3D uniaxial tension, however, ScxGFP and *Scx* expression were maintained. The tendon differentiation and maturation marker *Mkx* was suppressed in both 2D and 3D, however, indicating that Wnt activation may be promoting a tendon progenitor fate rather than a differentiated or mature tendon fate. The importance of the cell microenvironment in differential signal transduction is well established. For example, cells maintained in rounded morphologies adopt chondrogenic or adipogenic phenotypes, while cells maintained in elongated morphologies adopt fibrochondrogenic or osteogenic phenotypes under similar media conditions [45–47]. Other microenvironmental cues, such as substrate stiffness, can also modulate cell differentiation independent of chemical cues [48]. Our data support the use of 3D uniaxial culture conditions to better represent the physiologic microenvironment of tendon cells.

In 2D culture, it was previously reported that Wnt activation antagonizes TGFβ signaling in tendon stem/progenitor cells via inhibition of Smad2/3 phosphorylation [44]. Future studies will test potential interactions by using Wnt and TGFβ inhibitors in 3D culture and combining Wnt and TGFβ activation. Surprisingly, treatment with TGFβ2 did not result in increased proliferation under 3D conditions, in contrast to previous findings in mouse embryonic fibroblasts or MSCs [40,49]. This may be due to differences in cell type or the use of serum in the media, as serum is known to contain unidentified growth factors. Significant research in chondrogenic cultures, for example, suggests that the presence of serum introduces variability as well as impairs chondrogenic induction, compared to serum-free formulations [50]. To date, a chemically-defined media formulation has not been established for tenogenic differentiation. Although increased DAPI+ cells was observed with CHIR treatment in 3D culture, analysis of cell proliferation at the final timepoint did not reveal differences between groups. This may suggest that differences in proliferation occurred at earlier timepoints or that the reduced cell numbers stemmed from apoptosis. The dynamics of proliferation and apoptosis with CHIR and TGFβ treatment will be determined in future studies.

Numerous studies *in vitro* and *in vivo* showed that Wnt signaling maintains undifferentiated stem cells in many tissues [51] including hair follicle [52], intestines [53 54], lungs [55,56], neural crest [57], eyes [58], and uterus [59]. A recent study identified novel tissue-specific and common human Wnt target genes using bulk RNA sequencing on human endoderm tissue-derived organoids [60]. By removing Wnt *in vitro*, this study also demonstrated that Wnt signaling is

necessary for stem cell maintenance in a 3D culture microenvironment. However, the role of Wnt signaling in tendon is less clear. Our findings showed Wnt-mediated maintenance of ScxGFP expression *in vitro* and labeling of Axin2^{lin}+/ScxGFP+ tenocytes and Axin2^{lin}+/ScxGFP-neg cells in neonatal control tendons *in vivo*. Upon injury, these cell populations proliferate at 3 days post injury and contribute to tendon repair at 28 days post injury. These data indicate that, in neonatal mice, Wnt signaling may have multiple functions. Since Axin2^{lin}+ and Axin2^{lin}- cells were detected in roughly equal proportions with culture, future studies will determine the proportion of Axin2^{lin}+ cells at initial isolation, to determine whether shifts may occur with 2D expansion.

While the Axin2^{lin}+ subpopulation within the tendon fascicle and epitenon may represent a pool of progenitor cells, we also identified a population of ScxGFP+ cells in the neo-tendon at 28 days post-injury that were Axin2^{lin}-neg. This may be due to incomplete recombination or suggest that Axin2^{lin} cells do not represent the sole source of regenerative cells. Quantification after injury showed proliferation of both Axin2^{lin} and non-Axin2^{lin} cells. The proliferating non-Axin2^{lin} population likely includes other stem/progenitor subpopulations as well as immune cells, including tissue-resident and bone marrow-derived macrophages, which we and others previously showed to be present at day 3 after injury [61]. Other cells may include non-Axin2^{lin} tenocytes or aSMA+ myofibroblasts. One limitation to these studies is we infer that Axin2+/ScxGFP- cells are epitenon cells due to their localization to the tendon periphery. However these may very well comprise an additional population of cells that are not derived from either tendon fascicle or epitenon. Emerging single cell RNA sequencing datasets will allow more precise phenotyping of the various cell populations present in tendon and their functions.

Future studies will determine whether Wnt signaling is required for functional neonatal tendon regeneration, using genetic deletion or *in vivo* delivery of small molecules. We will also test whether the Axin2+ TSPC subpopulation becomes more restricted with adult tendon maturation. Insights gained from these studies can be applied to adult tendon injury models with an objective of informing better therapeutic strategies to shift adult tendon repair from fibrotic to regenerative.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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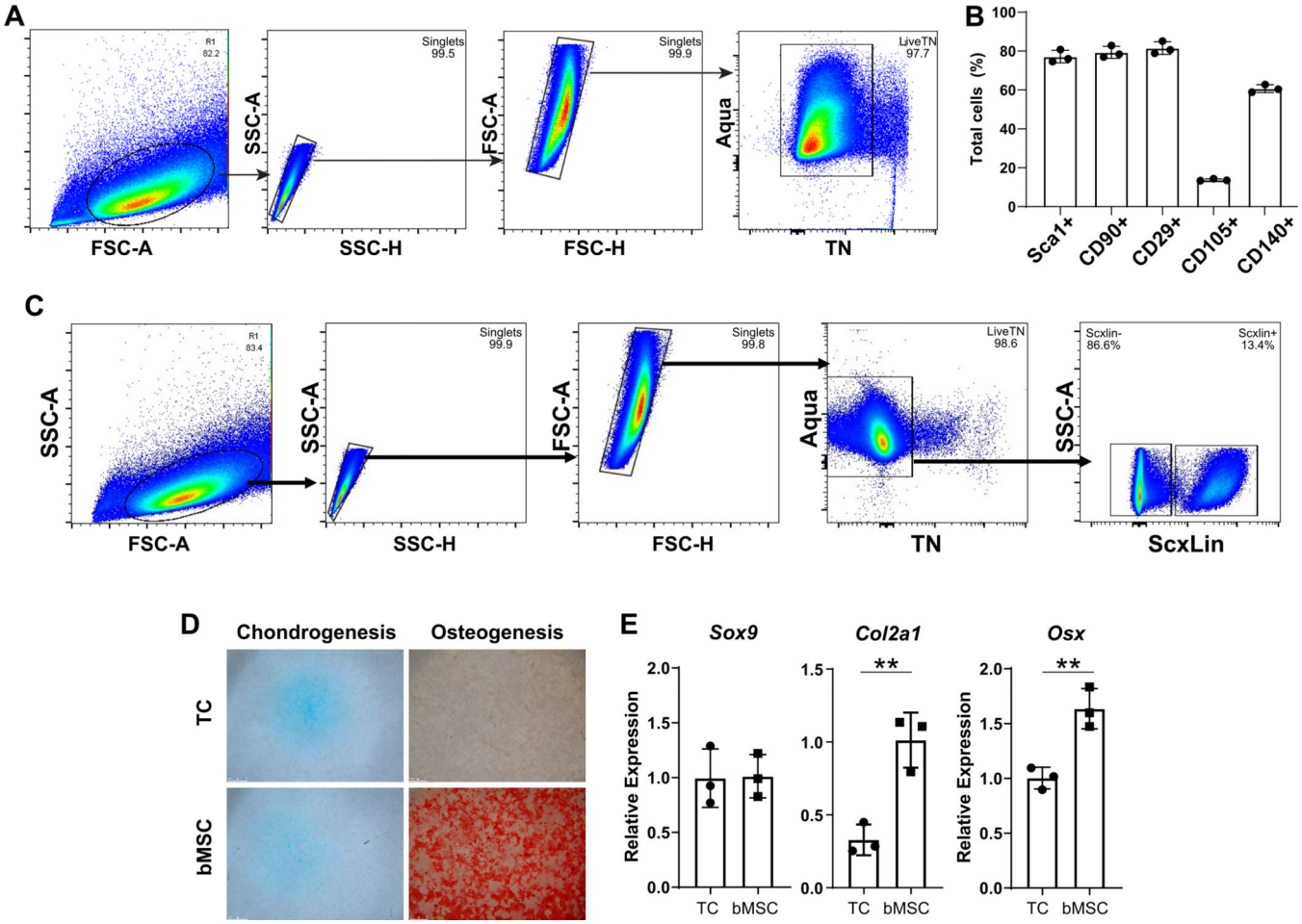

Figure 1: Characterization of neonatal tendon-derived cells.

(A) Flow cytometry gating and (B) quantification of established MSC and tendon stem/progenitor cell markers. (C) Flow cytometry gating and quantification of Scx^{lin} and non-Scx^{lin} cells after culture. (D) Day 21 Alcian Blue and Alizarin Red staining and (E) gene expression for neonatal tendon cell (TC, top) and juvenile bMSCs (bottom) undergoing chondrogenesis and osteogenesis, respectively. n=3 ** indicates $p<0.01$.

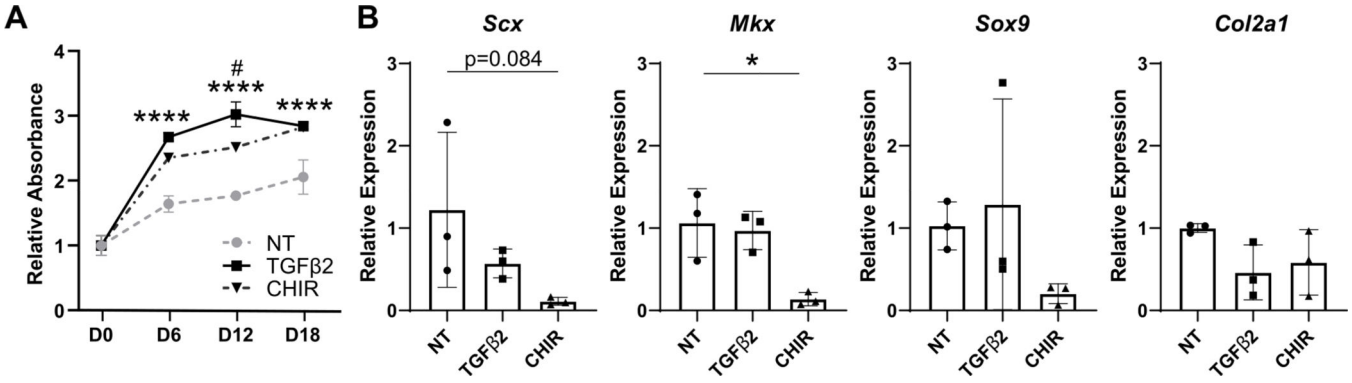

Figure 2: Activation of Wnt signaling by CHIR treatment increases neonatal tendon cell proliferation but inhibits tendon gene expression in 2D.

(A) TGFβ2 and CHIR treatment increases proliferation compared to no treatment (NT). (B) Tendon and cartilage gene expression with TGFβ2 and CHIR treatment at D15. n=3 **** $p<0.0001$ TGFβ2, CHIR vs NT # $p<0.05$ TGFβ2 vs CHIR. *$p<0.05$.

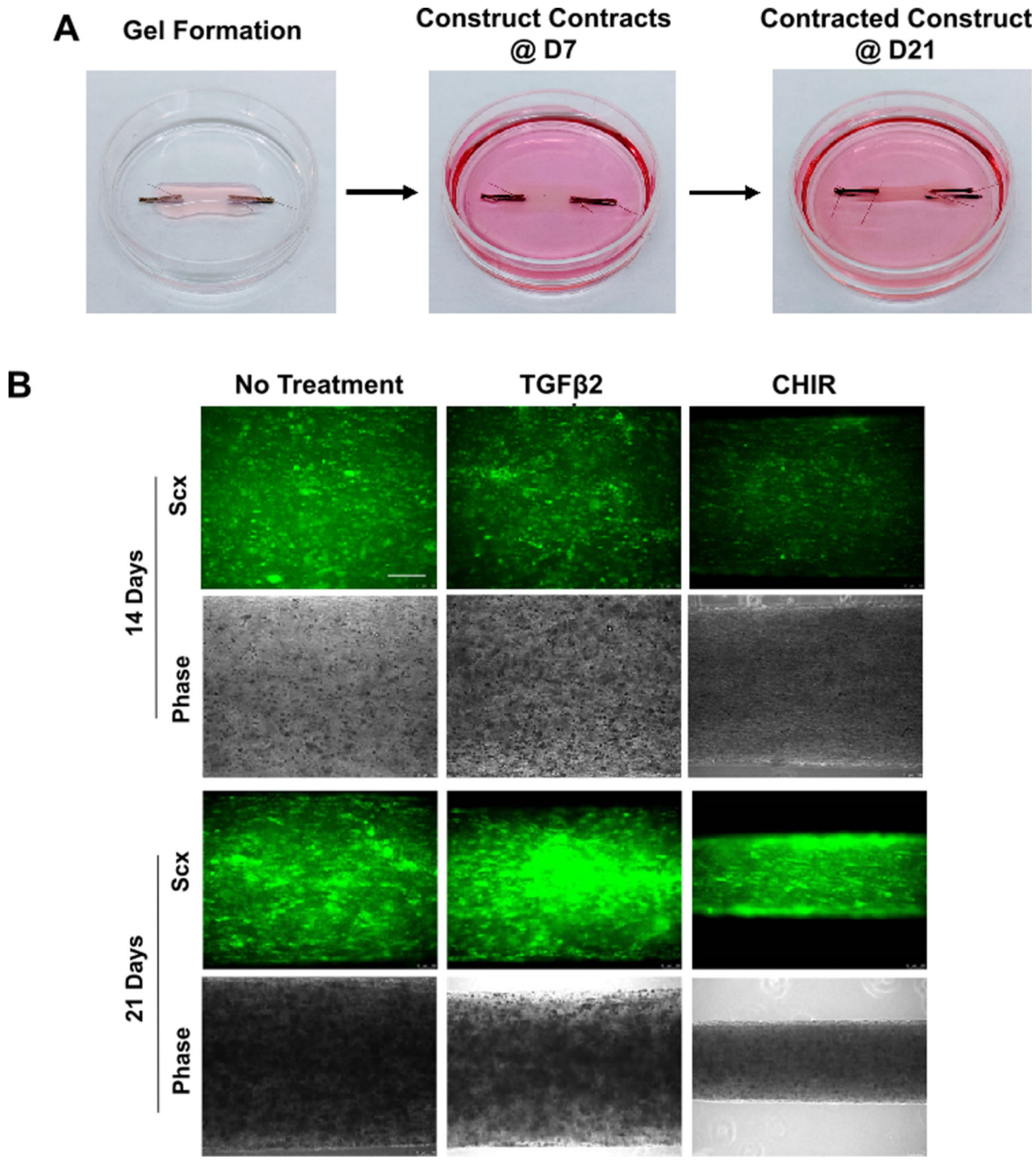

Figure 3: ScxGFP expression is induced and maintained in neonatal tendon cells under 3D uniaxial tension.

(A) Cell-seeded collagen gels contract to form tensioned linear constructs by 21 days. (B) ScxGFP expression and phase contrast imaging of whole mount constructs after 14 and 21 days of culture in 3D. Scale bar: 200 μm.

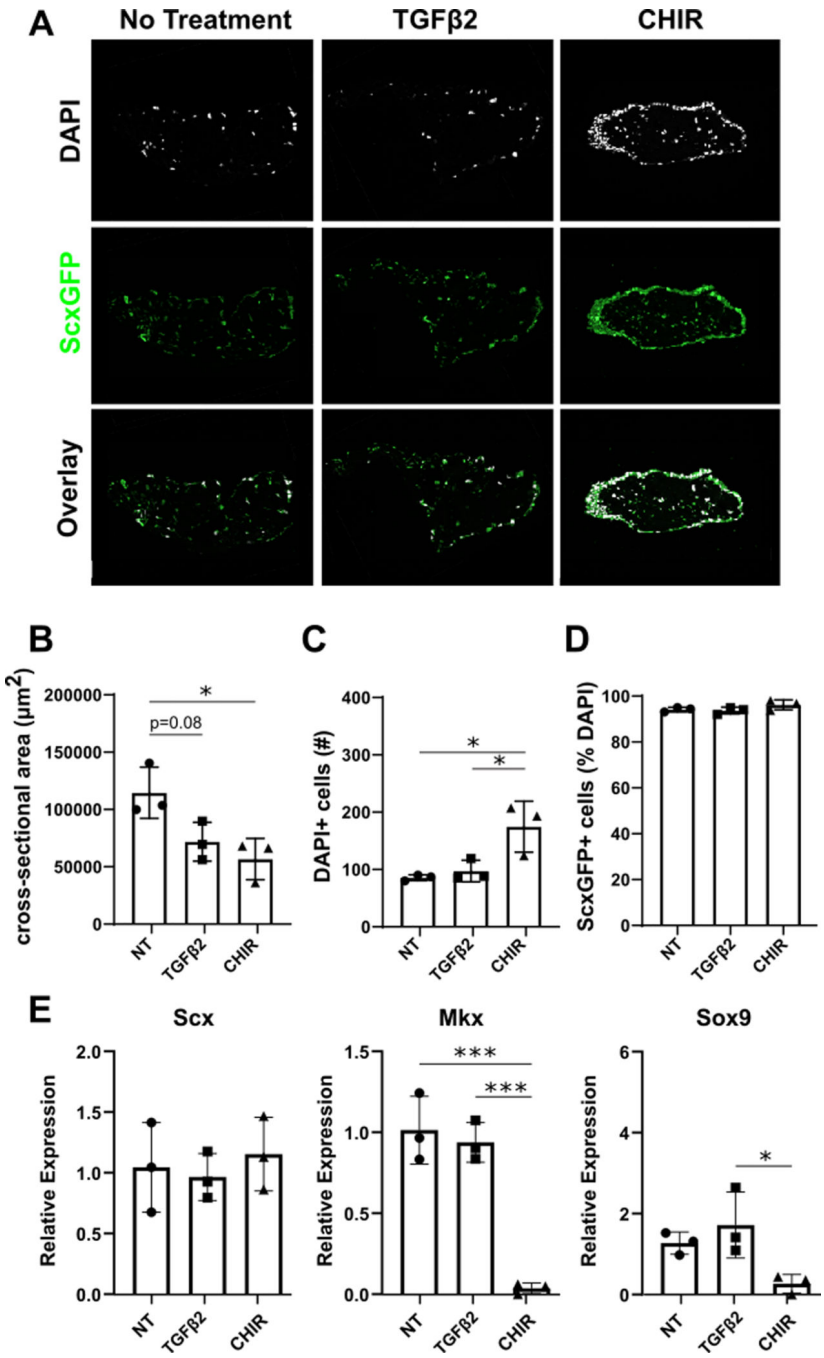

Figure 4: CHIR treatment enhances neonatal tendon cell contraction and proliferation in 3D.

(A) Transverse cryosections with 21 days of TGFβ2 and CHIR treatment. Quantification of (B) cross-sectional area, (C) DAPI+ cells, and (D) ScxGFP+ cells in transverse cryosections with treatments. (E) Gene expression analysis of tendon and cartilage markers. n=3 *p<0.05 *** p<0.001.

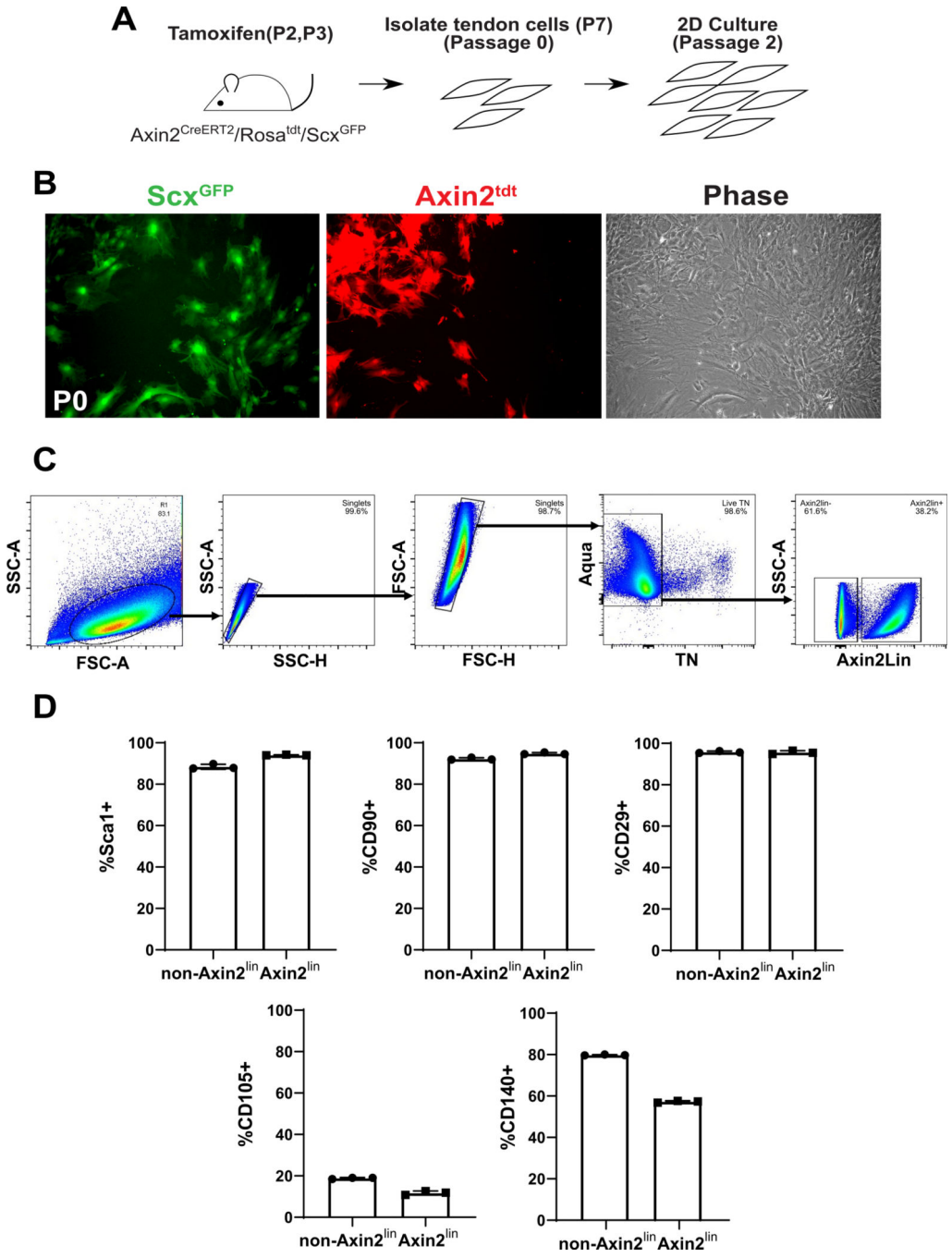

Figure 5: Characterization of Axin2^{lin} cells isolated from neonatal tendons.

(A) Schematic describing tamoxifen labeling of Axin2^{lin} cells, cell isolation, and 2D culture. (B) Fluorescence and phase contrast imaging of cells at passage 0 (P0). (C) Flow cytometry gating for Axin2^{lin} and non-Axin2^{lin} tendon cells with culture. (D) Flow cytometry analysis of Axin2^{lin} cells using established mesenchymal stem cell and tendon stem/progenitor cell markers.

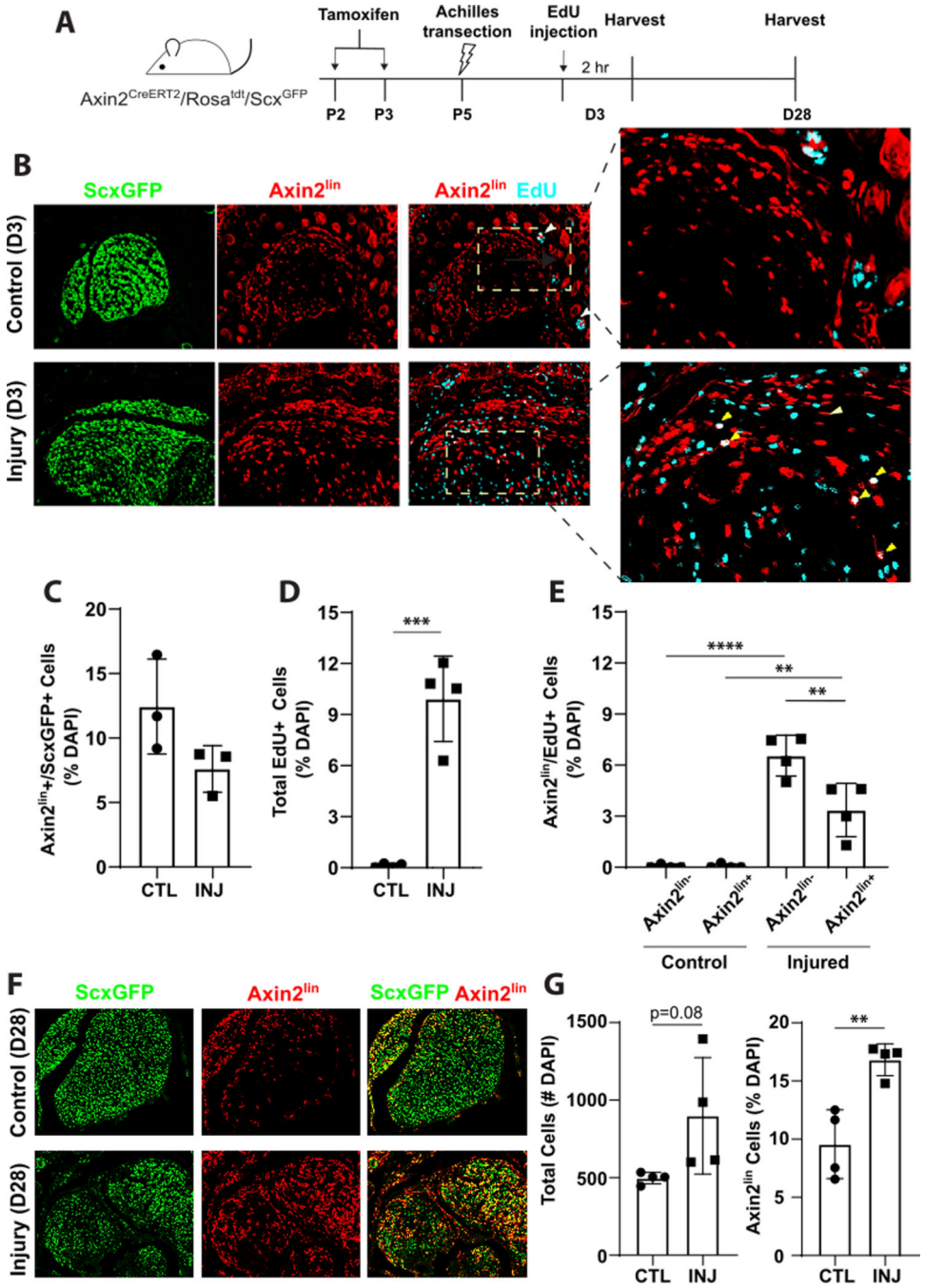

Figure 6: Axin2^{lin} cells proliferate and are recruited during neonatal tendon regeneration.

(A) Schematic describing tamoxifen labeling of Axin2^{lin} cells and Achilles transection injury. (B) EdU detection and (C-E) quantification of Axin2^{lin} and ScxGFP cells at D3 post-injury in transverse cryosections of contralateral control (CTL) and injured (INJ) hindlimbs. (F) Fluorescence imaging and (G) quantification of Axin2^{lin} cells at D28 post-injury in transverse cryosections. n=4 **p<0.01 ***p<0.001 ****p<0.0001.

Table 1:

List of primers used.

Target Name	Forward Primer	Reverse Primer
Osterix	GATGGCGTCCTCTCTGCTTG	GCCATAGTGAGCTTCTTCCTCAA
Sox9	CGTGCAGCACAAGAAAGACC	GGACCCTGAGATTGCCCAGA
Col2a1	ATCTTGCCGCATCTGTGTGT	GGCCCTAATTTTCCACTGGC
Scleraxis	AGAAAGTTGAGCAAAGACCGTG	TCAGTGGCATCCACCTTCAC
Mohawk	CGTGACAACCCGTACCCTAC	TTTGACACCTGCACTAGCGT