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Cell autonomous TGFβ signaling is essential for stem/progenitor cell recruitment into degenerative tendons

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SUMMARY

Understanding cell recruitment in damaged tendons is critical for improvements in regenerative therapy. We recently reported that targeted disruption of transforming growth factor beta (TGFβ) type II receptor in the tendon cell lineage (*Tgfbr2*^{ScxCre}) resulted in resident tenocyte dedifferentiation and tendon deterioration in postnatal stages. Here we extend the analysis and identify direct recruitment of stem/progenitor cells into the degenerative mutant tendons. Cre-mediated lineage tracing indicates that these cells are not derived from tendon-ensheathing tissues or from a *Scleraxis*-expressing lineage, and they turned on tendon markers only upon entering the mutant tendons. Through immunohistochemistry and inducible gene deletion, we further find that the recruited cells originated from a *Sox9*-expressing lineage and their recruitment was dependent on cell autonomous TGFβ signaling. The cells identified in this study thus differ from previous reports of cell recruitment into injured tendons and suggest a critical role for TGFβ signaling in cell recruitment, providing insights that may support improvements in tendon repair.

INTRODUCTION

Response to tissue damage such as injury or pathology commonly involves the activation or recruitment of stem/progenitor cells that help to replenish the tissue and, in many cases, participate in the healing response (Rennert et al., 2012). While some stem/progenitor cells reside within the tissue, e.g., satellite cells in muscle (Yin et al., 2013), in other scenarios they are recruited from an external niche (Jujo et al., 2010; Mathews et al., 2004). Direct detection and investigation of tissue-specific stem/progenitor cells was revolutionized by the advent of Cre technology that facilitated the ability to label specific cell populations and monitor their involvement in tissue homeostasis and repair (Harvey et al., 2019; Kan et al., 2018). The emerging theme is one where, for each tissue, there are various types of stem/progenitor cells that participate in such processes and likely reflect differential responses to different types of tissue damages (Marecic et al., 2015; Rinkevich et al., 2011). Identifying the specific cells that participate in the healing response and the signals involved in their activation and recruitment is critical for progress in efforts to enhance and improve the clinical outcomes of tissue repair. In this study, we identify progenitor cells involved in the response to degenerative changes in mutant tendons that differ from stem/progenitor cells identified so far in tendons.

Tendons are type I collagen-rich tissues that transmit the forces generated by muscle contraction to bone (Kannus, 2000). The considerable mechanical challenges to tendons result in a high frequency of injuries that range from acute damage, e.g., in tendon laceration, to chronic damage due to overuse and tissue degeneration, as in tendinopathy (Sharma and Maffulli, 2005). The considerable burden of tendon injuries to individuals and society is compounded by the slow and frequently poor healing of these tissues, which often results in impaired tissue integrity (Boileau et al., 2005; Gerber et al., 2000). A better understanding of biological processes underlying tendon repair may thus provide insight toward more effective therapies for tendon injuries.

Experimental investigation of tendon repair has mainly focused on acute injury using transected animal tendons (Ferry et al., 2007; Howell et al., 2017). Just like other tissues, it has been suggested that cells involved in tendon healing can be from both intrinsic and extrinsic sources (Harrison et al., 2003; Howell et al., 2017). In the latter scenario, recent studies have shown that some of the recruited cells express stem/progenitor markers (Dyment et al., 2014; Harvey et al., 2019; Runesson et al., 2015). Moreover, several groups have reported that stem/progenitor cells can be isolated from the surrounding peritenon (i.e., epitenon and paratenon) and tendon sheath (Mienaltowski et al., 2013; Wang et al., 2017) and suggest that these tissues may be a source of the recruited cells. Indeed, by taking advantage of *Cre/loxP* reporter system for cell lineage tracing, Dyment et al. (2014) reported that alpha-smooth muscle actin (α-SMA)-positive paratenon cells are the major contributor to the healing response following patellar tendon injury. Other lineage tracing experiments have indicated the potential involvement of Tppp3^{+};Pdgfra^{+} and osteocalcin-expressing cells during tendon repair (Harvey et al., 2019; Wang et al., 2017). More recently, Pagani et al. (2021) reported potential involvement of

Hoxa11CreERT2-expressing mesenchymal stromal cells in ectopic bone formation in injured tendons. Moreover, injured mouse Achilles tendon was found to be infiltrated by stem/progenitor cells that exhibited different regional distribution and temporal expression (Runesson et al., 2015), implying the existence of multiple recruited cell populations. Despite this recent progress in understanding of the healing response in tendons, basic biology of recruited cells, including identity, signals responsible for recruitment, and their role at the injured site, remains largely unknown.

We recently reported that disruption of transforming growth factor beta (TGFβ) signaling in the tendon cell lineage by targeting TGFβ type II receptor gene *Tgfbr2* using the *ScxCre* driver ($Tgfbr2^{ScxCre}$) resulted in tenocyte dedifferentiation in all tendons in early postnatal stages. Tendon cells in $Tgfbr2^{ScxCre}$ mutants appeared normal during embryogenesis, but in early postnatal stages lost all differentiation markers including Scx, tenomodulin, and collagen I (Tan et al., 2020). Extending the analysis of these mutant tendons, we now find that mutant tendons also began to show progressive matrix deterioration or degeneration, a feature that has often been associated with tendinopathy and spontaneous tendon rupture (Kannus and Jozsa, 1991; Longo et al., 2018). Moreover, we find that cells with stem/progenitor features were recruited into the mutant tendons. The recruited cells originated from a *Sox9*-expressing lineage, and we further demonstrate that TGFβ signaling was essential for their recruitment. Additionally, it appears that these cells are different from those reported in other studies of cell recruitment into tendons, suggesting a unique cell population that is implicated in cell recruitment into degenerative tendons.

RESULTS

ScxGFP-expressing cells in $Tgfbr2^{ScxCre}$ mutant tendons are newly recruited

Targeting of the TGFβ type II receptor gene *Tgfbr2* with *ScxCre* resulted in a dramatic tendon phenotype (Tan et al., 2020). In early postnatal stages, a few lateral tendons disintegrated and snapped, while, in the majority of the tendons, the tenocytes lost their cell fate and dedifferentiated (Figure 1A) (Tan et al., 2020). Macroscopically, tendons in postnatal day 7 (P7) mutants appeared gray and thin, in contrast to normal tendons in which the tight organization of the collagen fibers results in a brilliant white color with firm texture (Figure 1B). The underlying changes were examined by transmission electron microscopy (TEM) and histological analyses that revealed intact tendon extracellular matrix in mutant neonates (<P7) and apparent structural deterioration was only observed from P7 onward. These structural changes resemble features that have also been documented in damaged tendons, including disorganization of collagen matrix, severe disruption of the epitenon structure, and paratenon thickening (Figures 1C–1G) (Dyment et al., 2013; Longo et al., 2008).

While all resident tenocytes in the degenerating tendons of $Tgfbr2^{ScxCre}$ mutants lost expression of *ScxGFP* and other tendon differentiation markers by P7 (Figure 2A, black arrowhead) (Tan et al., 2020), we identified a small subset of cells that, contrary to the surrounding cells, expressed *ScxGFP* (Figure 2A, white arrowhead; Figure 2C). The *ScxGFP*-positive cells in mutant tendons also expressed other prototypic tendon markers tenomodulin and *Col1a1* (≥99%; n = 3) (Figures 2F and 2G, white arrowheads). Despite the expression of tendon markers, these cells differ morphologically from normal tenocytes at this stage. P7 wild-type tenocytes display a stellar-like morphology in transverse sections and a rectangular shape in longitudinal sections (Figures 2B and 2E, black arrowheads). In contrast, the *ScxGFP*-positive cells appeared large and rounded in both views (Figures 2A and 2D, white arrowheads). Factors that contribute to the unique morphology of these cells have not been identified to date.

Surprisingly, some of these *ScxGFP*-positive cells exhibited weak or no expression of the Cre reporter *Ai14 Rosa26-tdTomato* (*RosaT*) (Figure 2H). Conversely, nearly all tenocytes in P7 $Tgfbr2^{f/+}$*;ScxCre* heterozygous control pups were marked by robust *RosaT* expression (Figure 2I). *ScxGFP* and *ScxCre* are transgenic mice that utilize the *Scleraxis* enhancer to drive expression of *eGFP* and *Cre* respectively (Blitz et al., 2013; Pryce et al., 2007). In mice that carry both constructs, e.g., the $Tgfbr2^{ScxCre}$ mutant, it is likely that *ScxGFP* signal will be detected first upon *Scx* activation, since detection of *RosaT* requires an intermediate step of protein synthesis. First, Cre activity has to reach threshold levels to induce reporter recombination, followed by a second step in which the reporter signal is accumulated to achieve detectable levels. Based on this logic, we hypothesized that *ScxGFP*-positive but *RosaT*-negative cells in mutant tendons (hereafter called *ScxGFP+;RosaT−*) are cells from a non-*Scx*-expressing cell lineage with a recent activation of the *Scx* enhancer; i.e. they were newly recruited into the mutant tendons. Indeed, when *ScxGFP+;RosaT−* cells were isolated by fluorescence-activated cell sorting (FACS) and cultured, they subsequently also showed expression of the *RosaT* reporter (Figure S1).

Direct detection of cell recruitment in a degenerative tendon, as demonstrated by this observation, is exciting because it may open new directions for analysis of the repair response. We therefore wanted to reinforce this result with an approach that will identify newly recruited cells by a positive signal rather than the absence of

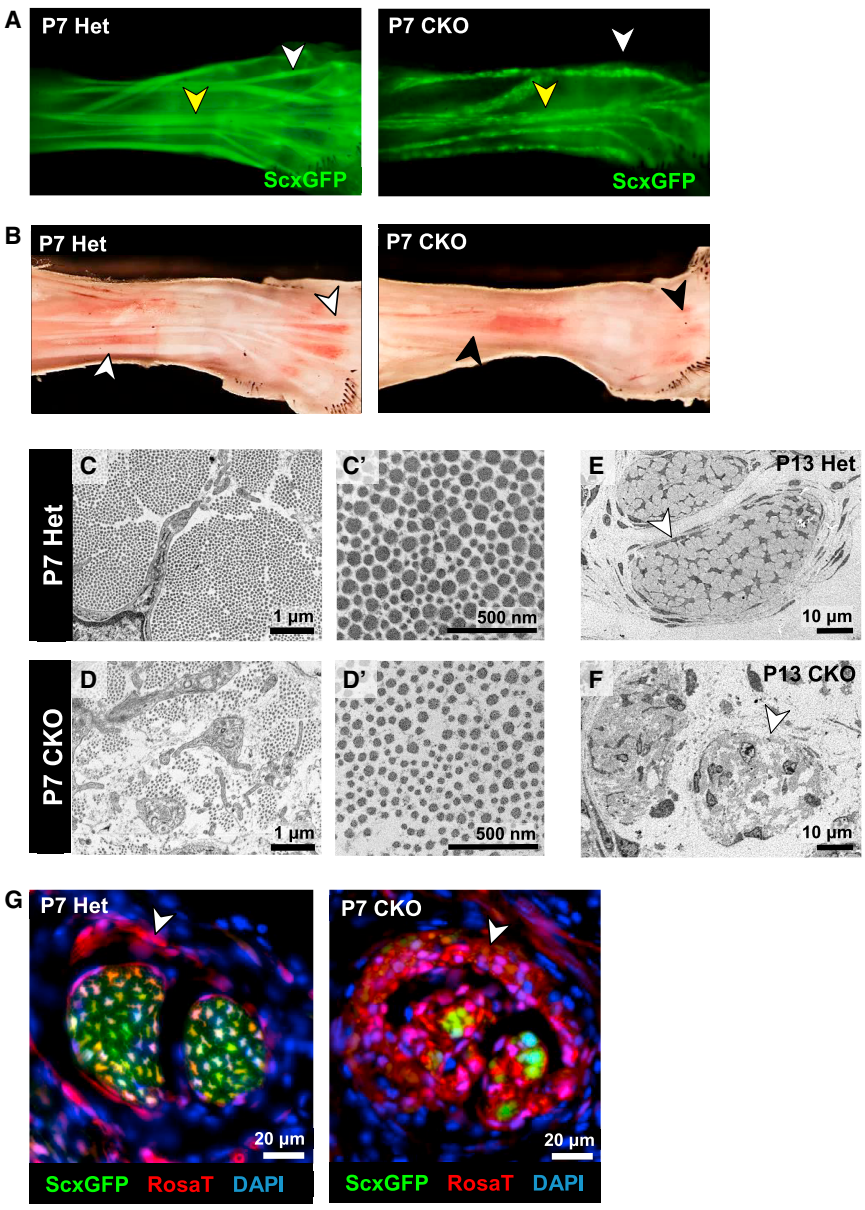

Figure 1. Disruption of tendon structure in *Tgfbr2*^{ScxCre} mutant

(A) Comparison of tendon reporter *ScxGFP* signal in forelimbs from P7 heterozygous control (*Tgfbr2*$^{f/+}$*;ScxCre*) and mutant pups revealed that, in mutants, a few lateral tendons were missing (white arrowhead) and in the other tendons there was a substantial loss of the *ScxGFP* signal (yellow arrowhead).

(B) Brightfield imaging of skinned forelimbs from P7 heterozygous and mutant pups. While normal tendons display a brilliant white color (white arrowheads), mutant tendons had a pale gray appearance (black arrowheads), likely reflecting disruptions to the collagen matrix.

(C–F) TEM analysis of heterozygous and mutant tendons. (C and D) (C′) and (D′) are high-magnification images of collagen fibrils in (C) and (D), respectively. (E and F) By P13, the epitenon (white arrowhead) in some regions of mutant tendons was disrupted and discontinuous.

(G) Cre reporter *Ai14 Rosa26-tdTomato* (*RosaT*) labeling of the paratenon highlights significant thickening of the tissue observed in some mutant tendons (white arrowheads). CKO, mutant; Het, heterozygous.

expression of a reporter. To achieve this goal we repeated the experiment utilizing the *mTmG* dual fluorescent Cre reporter in which the ubiquitously expressed membrane-tomato (*mT*) is replaced by membrane-GFP (*mG*) upon Cre-mediated recombination (Figure 3A) (Muzumdar et al., 2007). The advantage of this reporter system over *RosaT* is that it allows a simultaneous visualization and determination of both the recombined and non-recombined states. As expected, in P7 tendons, *mTmG* cells were labeled red (*mT*) in the absence of Cre activity (Figure 3B), while all tendon cells were recombined and appeared positive for *mG* in *ScxCre;mTmG* pups (Figure 3C). On the other hand, in tendons of the *Tgfbr2*^{ScxCre} mutant, some of the *ScxGFP*-positive cells had a recombined Cre reporter (Figure 3D, white arrowhead), whereas others retained the *mT* signal, indicating they did not recombine the reporter or at least had not yet lost the *mT* signal (Figure 3D, yellow arrowhead).

To further evaluate Cre activity in the *ScxGFP*-positive cells, we attempted to detect expression of the TGFβ type II receptor protein and indeed found expression of the receptor in some of these cells (Figure 3E, black arrowhead). Taken together, these observations reflect a recent induction of *Scx* expression in the *ScxGFP*-positive cells, suggesting these cells are newly recruited into the mutant tendons from a non-*Scx*-expressing niche.

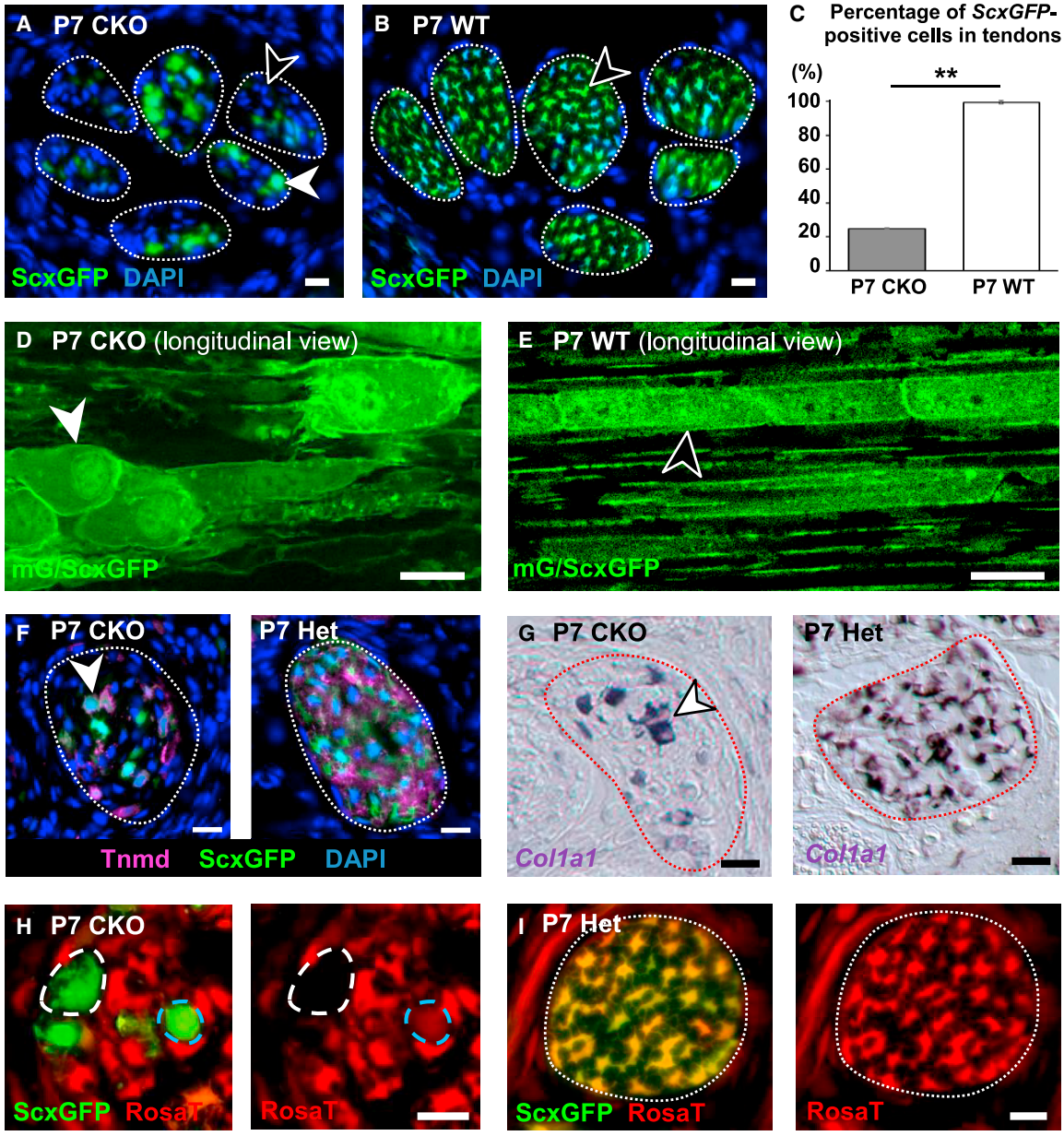

Figure 2. Morphology and marker expression of the *ScxGFP*-positive cells in *Tgfbr2*^{ScxCre} mutant tendons

(A–I) Transverse sections (A, B, and F–I) and longitudinal sections (D and E) of tendons from forelimbs of P7 wild-type and mutant pups.

(A) The majority of cells in mutant tendons lost *ScxGFP* expression (black arrowhead), but a small number of *ScxGFP*-expressing cells were also found in these tendons (white arrowhead).

(B) In wild-type pups, nearly all cells of the extensor digitorum communis tendons expressed the *ScxGFP* reporter (black arrowhead).

(C) Quantitative measurement of *ScxGFP*-positive cells in P7 mutant and wild-type tendons. The results shown are mean ± SD (n = 3 independent samples; **p < 0.01).

(D and E) Extensor digitorum communis tendons of P7 pups carrying the *ScxGFP* and *ScxCre;mTmG* reporters. Wild-type tenocytes were organized in prototypic cell rows and had a rectangular shape in longitudinal view (black arrowhead). However, the *ScxGFP*-positive cells in mutant tendons were rounded and the row organization was disrupted (white arrowhead).

(F and G) The *ScxGFP*-positive cells in mutant tendons also expressed tendon markers tenomodulin (Tnmd) and *Col1a1*, as shown by (F) immunofluorescent staining and (G) *in situ* hybridization (white arrowhead).

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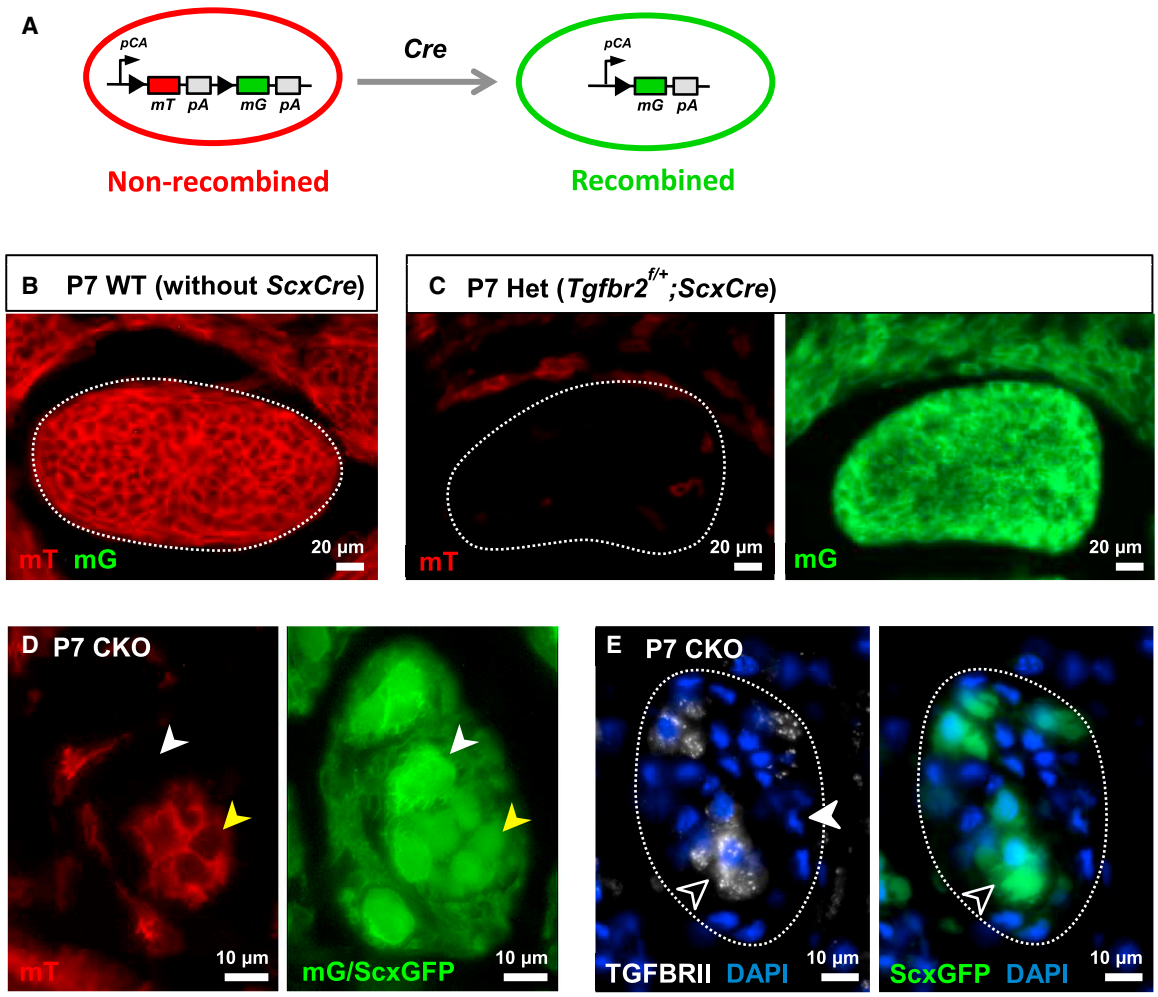

Figure 3. *ScxGFP*-expressing cells in *Tgfbr2*^{ScxCre} mutant tendons are newly recruited

(A) Schematic illustration of the *mTmG* dual-fluorescent Cre reporter (adapted from Muzumdar et al., 2007). Ubiquitously expressed membrane-tdTomato (*mT*) is replaced by membrane-GFP (*mG*) upon Cre-mediated recombination, allowing simultaneous detection of the recombined and the non-recombined states.

(B–E) Transverse sections of P7 forelimb tendons. (B) In the absence of Cre activity, red (*mT*) fluorescence signal is detected in all cells including wild-type tendon cells. (C) In *Tgfbr2*$^{f/+}$*;ScxCre;mTmG* heterozygous pups, tendon cells were recombined and switched the fluorescence from red to green (*mG*). (D) In *Tgfbr2*^{ScxCre} mutant tendons, some of the *ScxGFP*-positive cells were recombined (white arrowheads) and some were not (yellow arrowhead). (E) The resident tenocytes in P7 mutant tendons have completely lost expression of TGFβ type II receptor (TGFBRII) as expected at this stage (white arrowhead), but some of the *ScxGFP*-positive cells still stained positively for TGFBRII (black arrowhead). Tendons are demarcated by dashed lines. CKO, mutant; WT, wild type; Het, heterozygous.

Temporal dynamics of cell recruitment into mutant tendons

An alternative explanation for the presence of *ScxGFP+; RosaT–* cells in mutant tendon could be an outcome of delayed Cre recombination in the existing tendon cells of these mutants. To address this possibility, we analyzed the dynamics of *ScxGFP+;RosaT–* cells in mutant tendons. If the presence of these cells was due to delayed recombination,

(H) Some of the *ScxGFP*-positive cells exhibited weak or no expression of the Cre reporter *RosaT* (blue and white circles respectively), suggesting these cells may be newly recruited with a recent induction of *Scx*.

(I) Lower-magnification image of representative P7 heterozygous control tendons showing all tendon cells were marked by robust *RosaT* signal at this stage. Dashed lines demarcate tendons. Scale bar, 10 μm. CKO, mutant; WT, wild type; Het, heterozygous.

it is expected that the percentage of *ScxGFP+;RosaT−* cells in mutant tendons would decrease over time as these cells will eventually be recombined.

We found that, at P0, ∼99% of total cells in both normal and mutant tendons were *ScxGFP* positive, and ≥98% of these *ScxGFP*-positive cells have recombined the *RosaT* Cre reporter; i.e., *ScxGFP+;RosaT+* (n = 3 for each group). While *ScxGFP+;RosaT−* cells in wild-type tendons remained rare throughout the study (≤0.4% in P0 to P7 samples; n = 3 for each time point), the numbers of *ScxGFP+;RosaT−* cells in mutant pups surged drastically to 6.1% ± 2.4% at P1 (Figure S2). The levels of these cells within mutant tendons peaked between P1 and P3 and stabilized at ∼2% throughout the observation period (Figure S2). These findings reinforce the conclusion that the *ScxGFP+;RosaT−* marker combination provides a robust approach for identifying newly recruited cells. Moreover, they also reflect the dynamic and transient nature of the *ScxGFP+;RosaT−* cells; since these cells eventually recombine the Cre reporter, the initial surge and subsequent steady levels of these cells suggest that new *ScxGFP+;RosaT−* cells are continuously being added into mutant tendons throughout the study period.

There are only a handful of reports of cell recruitment into tendons (Dyment et al., 2013; Harvey et al., 2019; Kaji et al., 2020; Wang et al., 2017), and almost nothing is known about the origin of such cells or the mechanisms of their recruitment. A robust method for detecting newly recruited cells therefore provides a unique opportunity to learn more about this process. Significantly, cell recruitment peaked in early postnatal stages when most mutant tendons were intact and did not show apparent indications of structural damage (Tan et al., 2020). Moreover, since tissue repair involves early recruitment of immune and inflammatory cells to the damaged site (Millar et al., 2010), we wanted to determine if the recruited cells were associated with an immune response and thus examined for the presence of relevant markers. We found only a small number of cells expressing the activated macrophage marker F4/80 in both P1 and P7 mutant tendons (0.6% ± 0.2% and 1.7% ± 0.6%, respectively; n = 3 for each time point) (Figure S3A). Notably, there was no significant difference in their numbers compared with normal tendons (0.8% ± 0.3% and 1.9% ± 0.4% in P1 and P7 normal tendons, respectively; n = 3 for each time point; $p > 0.05$) (Figure S3B). Moreover, mutant tendons stained negatively for the inflammatory marker tumor necrosis factor alpha (TNF-α), as also observed in normal tendons (Figures S3C and S3D). Cell recruitment into the tendons of *Tgfbr2*^{ScxCre} mutants thus initiated prior to any sign of a structural destruction or immune response, suggesting a specific molecular signal and not general tissue damage may be the driver of cell recruitment in this case.

The recruited cells do not originate from peritenon or the tendon sheath

A handful of recent studies identified cell recruitment into tendons mostly in the context of acute injury (Dyment et al., 2013; Runesson et al., 2015; Wang et al., 2017) and possibly also following physiological loading (Mendias et al., 2012). While the origin of such cells remains unclear, it was suggested in a few studies that they may arise from the peritenon (i.e., paratenon and epitenon) or tendon sheath (Dyment et al., 2013; Harvey et al., 2019; Wang et al., 2017). To assess if the recruited cells in *Tgfbr2*^{ScxCre} mutant tendons were derived from peritenon or tendon sheath, we again took advantage of the *RosaT* Cre reporter system. Not much is known about gene expression in these tendon-ensheathing tissues, which clearly does not overlap with gene expression in tenocytes (Harvey et al., 2019; Tan et al., 2020). Interestingly, while peritenon and tendon sheath cells rarely express the tendon reporter *ScxGFP* (0.3% ± 0.2%; n = 3), the majority of these cells (98.6% ± 0.6%; n = 3) are consistently positive for *RosaT* Cre reporter in mice carrying the *ScxCre;RosaT* alleles (Figure 4C). This combination of markers likely represents transient expression of *Scx* in early progenitor cells of the peritenon and tendon sheath that was sufficient for activation of the Cre reporter. Since the newly recruited cells are from a non-*Scx*-expressing lineage and do not express the Cre reporter *RosaT* as noted earlier (Figure 4B), the cells recruited into the mutant tendons were not derived from peritenon and tendon sheath.

Notably, the newly recruited *ScxGFP+;RosaT−* cells could also be detected in the peritenon and tendon sheath of *Tgfbr2*^{ScxCre} mutant pups (7.2% ± 1.2% compared with 0.2% ± 0.1% in P7 wild-type littermates; n = 3) (Figure 4D, blue circles). Since the absence of Cre reporter expression indicates that these are not original peritenon or tendon sheath cells, we postulate that these are either the recruited cells entering the mutant tendons by passing through peritenon and tendon sheath, or cells also being recruited into these tendon-ensheathing tissues in mutant pups.

Additionally, previous studies have demonstrated the invasion of cells expressing α-SMA, also a pericyte marker, into injured tendons with a likely endothelial-perivascular origin (Dyment et al., 2013; Howell et al., 2017). To determine if this may also be the origin of the recruited cells in the tendons of *Tgfbr2*^{ScxCre} mutants, we examined expression of endothelial and pericyte-associated markers (Cathery et al., 2018), but could not detect expression of CD31, CD146, or α-SMA in these cells (data not shown). Taken together, these results suggest that the cells recruited into the tendons of *Tgfbr2*^{ScxCre} mutants are different from cells so far reported to be implicated in tendon injury, implying the existence of multiple sources for stem/progenitor cell recruitment in tendons.

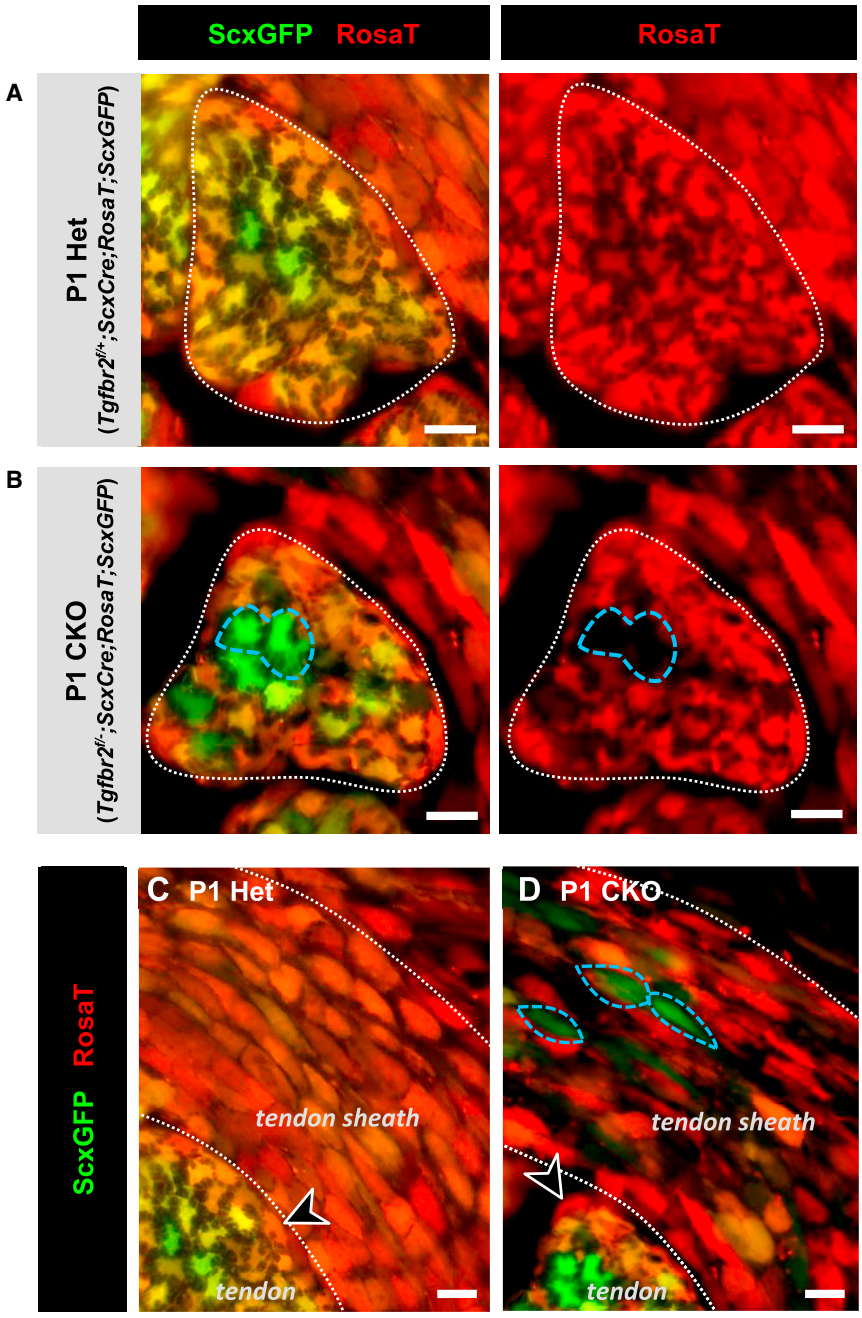

Figure 4. The recruited cells in *Tgfbr2*^{ScxCre} mutant tendons do not originate from peritenon or the tendon sheath

(A–D) Transverse sections of extensor digitorum communis tendons from *ScxGFP* and *RosaT*-carrying mutant and heterozygous control pups.

(A) In tendons of P1 heterozygous mice nearly all cells were positive for both *ScxGFP* and *RosaT*.

(B) Conversely, there was a noticeable presence of *ScxGFP+;RosaT−* cells in mutant tendons starting at P1, suggesting these cells were newly recruited with a recent induction of *Scx* enhancer.

(C) Lineage tracing shows recombination of the *RosaT* reporter in all the cells of the epitenon (black arrowhead) and tendon sheath in *Tgfbr2*$^{f/+}$*;ScxCre* heterozygous pups. The absence of *RosaT* expression in cells newly recruited into the mutant tendons indicates that they are not derived from these regions.

(D) In *Tgfbr2*^{ScxCre} mutant pups, the newly recruited *ScxGFP+;RosaT−* cells could also be detected in the epitenon and tendon sheath (blue dashed circles). Scale bar, 10 μm. CKO, mutant; Het, heterozygous.

The recruited cells have clonogenic features and express stem/progenitor markers

Direct detection and the ability to isolate the newly recruited cells in mutant tendons presented a unique opportunity to characterize the cellular features and possibly origin of the recruited cells. We hypothesized that the recruited cells likely have features of stem/progenitor cells since they were able to differentiate into tendon cells upon entering the mutant tendons and tested for such features. Wild-type tendons contain 2%–4% cells with colony-forming potential, also known as tendon-derived stem/progenitor cells (TSPCs) (Figure 5B) (Bi et al., 2007; Mienaltowski et al., 2013). To test the colony-forming capacity of the recruited cells, we dissociated cells from the tendons of P7 mutant pups and isolated the recruited cells by FACS based on the unique marker combination of these cells (*ScxGFP+;RosaT−*). The cells were seeded at one cell per well in 96-well plates, and colony-forming potential was determined after 9 to 14 days of culture (Figure 5A). We indeed found that 2.9% ± 0.7% of the recruited cells had

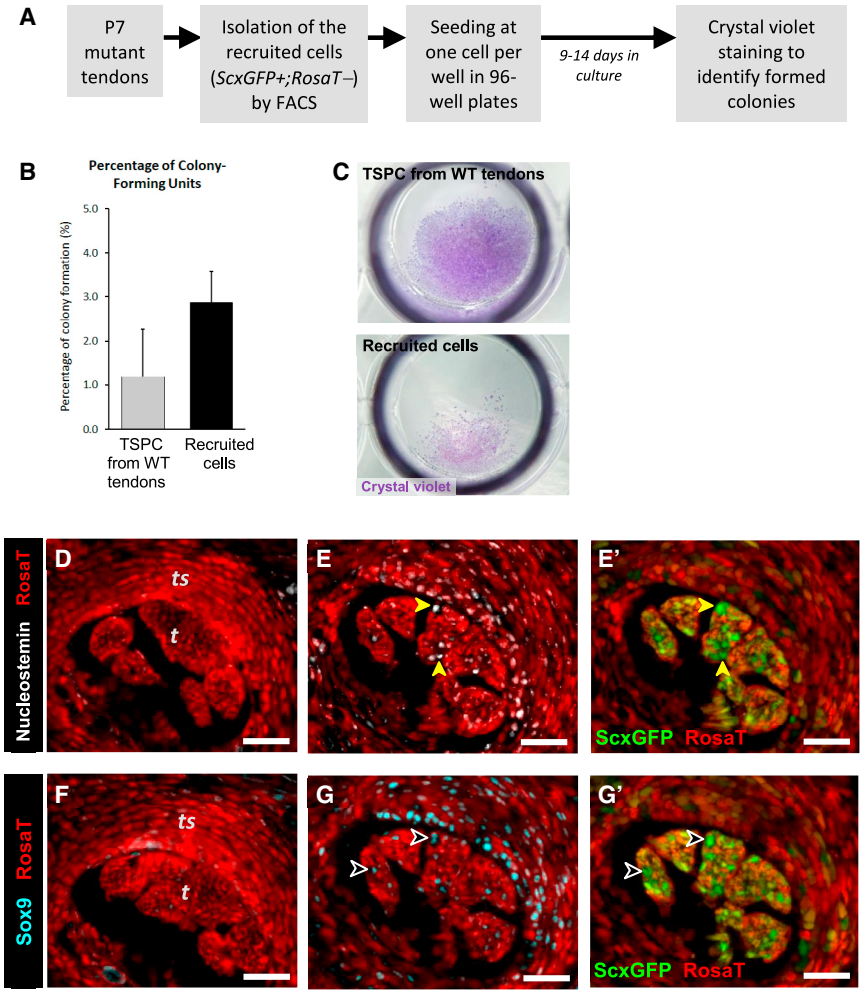

Figure 5. The recruited cells exhibit clonogenic capability in culture and express stem/progenitor markers

(A) Experimental outline for testing the colony-forming capacity of the recruited cells. Briefly, *ScxGFP+;RosaT–* newly recruited cells were FACS-sorted from P7 mutant pups and seeded at one cell per well in 96-well plates. Colony formation was visualized with crystal violet staining and the percentage of wells with colonies served as an indication of stemness.

(B) About 2.9% ± 0.7% of the *ScxGFP+;RosaT–* recruited cells formed colonies in cultures. For a positive control (TSPCs), cells were also harvested from wild-type tendons and evaluated for their clonogenic capability under the same culture conditions. The results shown are mean ± SD (n = 4 independent experiments in duplicate; a total of 768 cells were analyzed).

(C) Colonies formed by the recruited cells isolated from $Tgfbr2^{ScxCre}$ mutants and TSPC.

(D–G) Immunofluorescent staining for stem/progenitor markers on transverse sections of extensor digitorum communis tendons. The *ScxCre* lineage was labeled with the Cre reporter *RosaT*. In $Tgfbr2^{f/+}$;*ScxCre* heterozygous controls, expression of (D) nucleostemin and (F) Sox9 was either undetectable or negligible in tenocytes and the tendon sheath. (E and G) Conversely, numerous cells expressing both nucleostemin (yellow arrowheads) and Sox9 (black arrowheads) were detected within these regions in $Tgfbr2^{ScxCre}$ mutants. (E′) and (G′) represent images from the same field of view in (E) and (G) respectively, and *ScxGFP* signal was captured to identify the *ScxGFP+;RosaT–* newly recruited cells (arrowheads). Scale bars, 50 μm. CKO, mutant; Het, heterozygous; WT, wild type; ts, tendon sheath; t, tendons; TSPC, tendon-derived stem/progenitor cells.

colony-forming potential (n = 4 independent experiments in duplicate) (Figure 5B), but the size of clones formed by the recruited cells varied and in general was smaller than the clones of wild-type TSPCs (7.2 ± 3.5 mm^2 and 21.5 ± 0.7 mm^2, respectively; n = 4 independent experiments) (Figure 5C). The difference presumably reflects the dynamic change of cellular state in the recruited cells, in which some cells were newly recruited and still possessed progenitor stemness features, while others have already advanced in assuming the tendon cell fate and thus lost their proliferative capacity to give rise to large colonies.

Recognizing the progenitor state of the recruited cells, we next tested these cells in P7 mutant tendons for expression of typical markers identified in cultured TSPCs and other established progenitor markers (Bi et al., 2007; Mienaltowski et al., 2013). We found that the recruited cells expressed the stem/progenitor marker nucleostemin, which is not expressed by normal tendon cells (Figures 5D and 5E) (Zhang and Wang, 2010). Surprisingly, we also detected expression of Sox9 protein in the recruited cells (Figure 5G). *Sox9* is most commonly recognized as an early cartilage marker but it is also expressed in various populations of stem/progenitor cells (Furuyama et al., 2011; Scott et al., 2010). Notably, the recruited cells did not express other chondrogenic markers (e.g., collagen II and proteoglycan [results not shown]). It was previously demonstrated that some tendon progenitors express *Sox9*, and some *Sox9CreERT2* activity can be detected in tenocytes even in postnatal stages (Blitz et al., 2013; Huang et al., 2019; Soeda et al., 2010). However, expression of Sox9 protein is undetectable or negligible by immunohistochemistry in normal tenocytes (Figure 5F) and is therefore unique to these recruited cells. Notably, the number of Sox9- and nucleostemin-positive cells was high at P1, coinciding with the peak

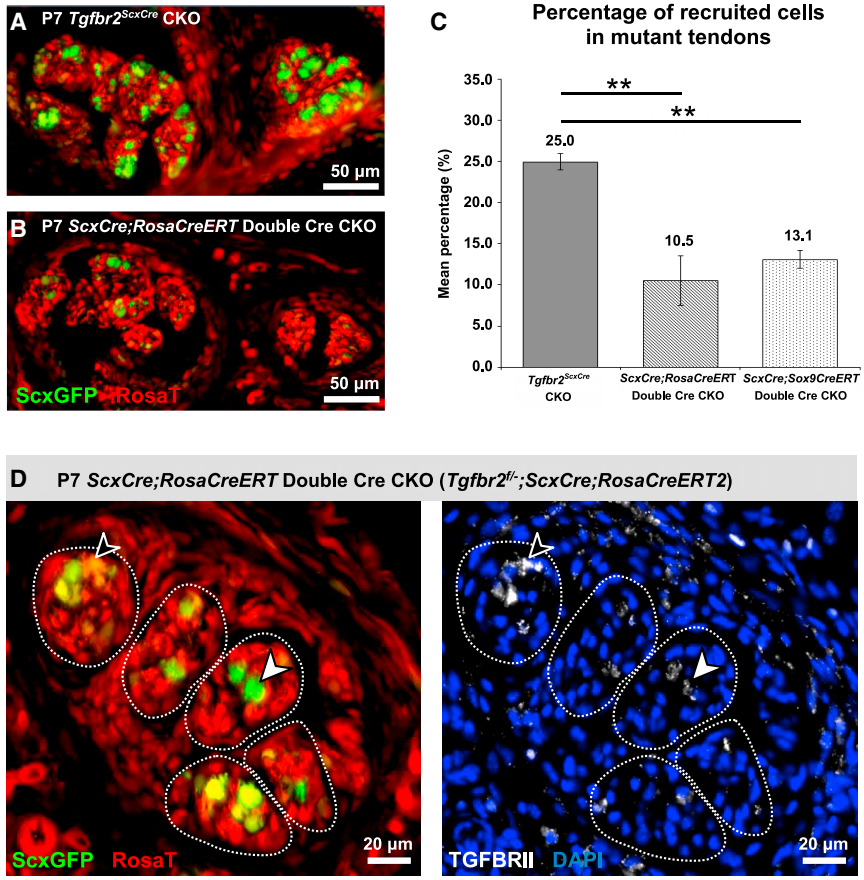

Figure 6. Cell-autonomous TGFβ signaling is essential for cell recruitment into mutant tendons

The ubiquitous *RosaCreERT2* driver was incorporated into the *Tgfbr2*^{ScxCre} mutant background (*Tgfbr2*$^{f/-}$*;ScxCre;RosaCreERT2*, hereafter called *ScxCre;RosaCreERT*) to examine the effect of global loss of the TGFβ signaling on cell recruitment into *Tgfbr2*^{ScxCre} mutant tendons. Tamoxifen was administered at P1 and P2, and the effects on cell recruitment were evaluated at P7.

(A and B) There was a dramatic reduction in the number of recruited cells (*ScxGFP*-expressing cells) in the Double Cre mutant pups (B) compared with *Tgfbr2*^{ScxCre} mutants (A), as shown in transverse sections through the extensor digitorum communis tendons.

(C) Quantitatively, there was a nearly 60% reduction in cell recruitment in the *ScxCre;RosaCreERT* Double Cre mutant pups, suggesting that TGFβ signaling is essential for cell recruitment. For the cell lineage study, the Double Cre strategy was also employed to target *Tgfbr2* specifically in *Sox9*-expressing cells (*Tgfbr2*$^{f/-}$*;ScxCre;-Sox9CreERT2*, hereafter called *ScxCre;Sox9-CreERT*). The results showed about a 48% decrease in recruited cell numbers, suggesting that the recruited cells are from a *Sox9*-expressing cell lineage. The results shown are mean ± SD (n = 3 independent samples, **p < 0.01).

(D) Immunofluorescent staining for TGFBRII on transverse sections of *ScxCre;RosaCreERT* Double Cre mutant forelimbs. The *ScxGFP*-expressing cells that were still recruited into Double Cre mutant tendons were also positive for the receptor (arrowheads), suggesting the dependence of cell recruitment on TGFβ signaling. (Right) For the same section, the expression of the TGFBRII on the background of DAPI nuclear counterstain. CKO, mutant.

recruitment in mutant tendons (Figure 4B; Figure S2) and the prevalence of these cells gradually declined in later stages. Taken together, our results suggest that cells with stem/progenitor features were recruited into mutant tendons. Interestingly, the recruited cells expressing the *ScxGFP* reporter were only detected within or adjacent to mutant tendons (Figure S4), suggesting that, irrespective of their origin, the cells turned on tendon gene expression only upon entering the tissue.

Cell-autonomous TGFβ signaling is essential for cell recruitment into mutant tendons

TGFβ signaling has been implicated in cell motility and recruitment in other systems (Franitza et al., 2002; Tang et al., 2009) and recently also in tendons (Kaji et al., 2020). Since we found that the recruited cells still expressed the TGFβ type II receptor, we next wanted to ask if deletion of *Tgfbr2* in these cells will change their capacity for recruitment. We previously found that the tendon phenotype in *Tgfbr2*^{ScxCre} mutants is dependent on the specific spatio-temporal features of *ScxCre* activity (Tan et al., 2020). To target the *Tgfbr2* receptor before the cells are recruited, we therefore decided to add the ubiquitous inducible Cre deletor (*RosaCreERT2*) (Hameyer et al., 2007) to the *Tgfbr2*^{ScxCre} allele combination. Since the tendon phenotype manifests in *Tgfbr2*^{ScxCre} mutants in postnatal stages, we can use *RosaCreERT2* to induce ubiquitous loss of the receptor at that stage and examine the effect on cell recruitment into mutant tendons.

Pups of the mutant allele combination, *Tgfbr2*$^{f/-}$*;ScxCre;RosaCreERT2* (hereafter called *ScxCre;RosaCreERT* Double Cre mutant), were given tamoxifen at the earliest time point of detectable recruitment at P1 and P2, and harvested at P7. To examine the cumulative effect of TGFβ signaling ablation on cell recruitment, numbers of recruited cells in mutant tendons were counted (see supplemental experimental procedures for further details). Interestingly, we found nearly 60% reduction in the number of recruited cells in

the *ScxCre;RosaCreERT* Double Cre mutant pups compared with $Tgfbr2^{ScxCre}$ mutants (Figures 6A–6C) ($p < 0.01$; $n = 3$). The dramatic reduction in cell recruitment suggests that TGFβ signaling indeed plays a role in cell recruitment. Notably, tamoxifen was administered at P1 and P2, and the disruption of TGFβ signaling therefore could not affect the peak levels of cell recruitment at this stage (Figure S2). The reduction of cell recruitment identified at P7 therefore further demonstrates the transient nature of the newly recruited *ScxGFP+;RosaT−* cell state. By P7, the cells recruited at P1 have likely induced the *RosaT* Cre reporter, and the observed reduction in newly recruited cells at P7 therefore reflects an effect on later waves of cell recruitment.

The partial reduction of recruitment in these experiments may imply the existence of alternative molecular mechanisms or may simply reflect partial Cre activation. Tamoxifen application in neonates has severe deleterious effects. It was therefore not possible to increase the dosage or number of days in which tamoxifen was administered. To test if Cre activity was partial in the *ScxCre;RosaCreERT* Double Cre mutants, we stained mutant forelimb sections with anti-TGFβ type II receptor antibody. Intriguingly, cells that were still recruited into the mutant tendons in the Double Cre experiment were positive for the receptor (Figure 6D, arrowheads), suggesting a complete dependence of cell recruitment on TGFβ signaling. Moreover, the fact that *Tgfbr2*-expressing cells could still be recruited in this scenario demonstrates that the loss of TGFβ signaling did not have a general effect on the capacity of cells to be recruited or on the recruiting signal, but rather TGFβ signaling acts cell autonomously and was required for the ability of individual cells to be recruited in this scenario.

The identity and anatomical origin of the recruited cells is of great importance for future efforts to manipulate and enhance the healing processes. The experimental paradigm used above provided us with a unique tool to test hypotheses regarding the origin of the recruited cells in this experimental model, since we can use various other inducible Cre lines with a more restricted target population to target the *Tgfbr2* receptor and test the effects on cell recruitment. We demonstrated above that most of the newly recruited cells expressed the Sox9 protein (Figure 5G). It was important, however, to determine if *Sox9* expression was induced only during the recruitment process or if it was expressed in the cells prior to their recruitment and therefore may be used as a marker to identify the origin of these cells. We therefore employed the same Double Cre strategy to target *Tgfbr2* but in this time specifically in *Sox9*-expressing cells using a *Sox9CreERT2* driver in combination with the $Tgfbr2^{ScxCre}$ mutant ($Tgfbr2^{f/-}$*;ScxCre;Sox9CreERT2*, hereafter called *ScxCre;Sox9CreERT* Double Cre mutant). The results showed about 48% decrease in recruited cell numbers ($p < 0.01$; $n = 3$) (Figure 6C), suggesting that most, if not all, of the recruited cells indeed expressed *Sox9* prior to their activation.

DISCUSSION

The present studies extend our previous observations where targeted disruption of TGFβ signaling in tendon cells (i.e., *Scx*-expressing cell lineage) led to loss of their cell fate (Tan et al., 2020), and provide three major observations. First, we find in postnatal stages a progressive structural deterioration or degeneration in the mutant tendons, a feature that has often been observed in tendinopathy and spontaneous tendon rupture (Kannus and Jozsa, 1991; Longo et al., 2008). Second, we identify direct recruitment of stem/progenitor cells into the degenerative tendons. The recruited cells turn on tendon markers upon entering the mutant tendons, suggesting possible involvement of these cells in tissue repair. Furthermore, findings from the Cre-lineage tracing indicate that these cells are not derived from surrounding peritenon or tendon sheath, implying the existence of multiple sources for stem/progenitor cell recruitment in tendons. Third, we find that most, if not all, of the recruited cells were from *Sox9* and non-*Scx* lineage, and TGFβ signaling is essential for their recruitment into the mutant tendons. This scenario thus opens an opportunity to directly examine the origin of recruited stem/progenitor cells and the mechanisms of their activation in degenerative tendons.

In mutant tendons, cell recruitment was noticeable already at P1 in the absence of observable structural damage or immune response in these tendons, suggesting that the process of tenocyte dedifferentiation in mutant tendons is also accompanied by the secretion of a specific recruitment signal. We therefore suggest the following model for cell recruitment in this scenario (Figure 7): (1) tenocyte dedifferentiation in neonatal mutants also results in secretion of a stem/progenitor cell activation and/or recruitment signal(s). (2, 3) Activation and/or recruitment of the stem/progenitor cells is dependent on activation of TGFβ signaling in these cells in a cell-autonomous manner. TGFβ ligands may therefore be the recruitment signals in this case. It may, however, also be possible that a different signal is employed for cell recruitment and TGFβ signaling plays an essential role in the activation or motility of the cells toward the degenerative tendons. (4) Expression of *ScxGFP* in the recruited cells is observed only in or near the target tendons, suggesting the induction of the tendon cell fate in these cells is not an integral part of the activation or recruitment process, but rather that an additional local signal or interaction with the tendon cells or environment leads to induction of the tendon cell fate in the recruited cells while they integrate into the mutant tendon.

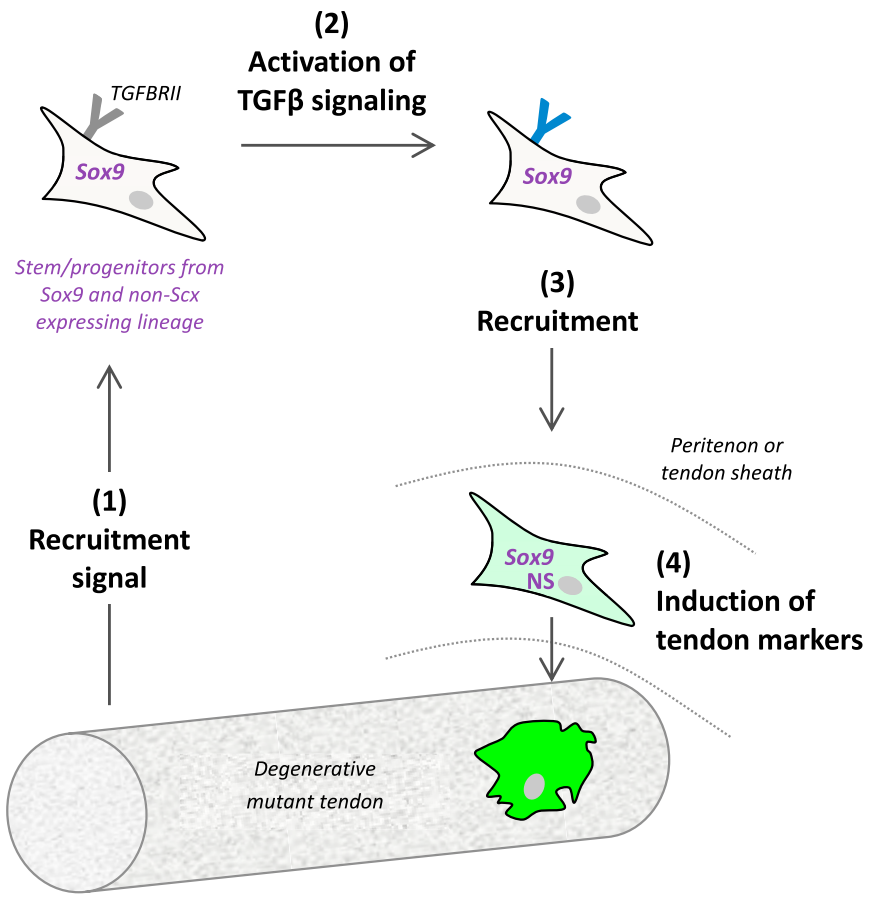

Figure 7. Proposed model for cell recruitment process into *Tgfbr2*^{ScxCre} mutant tendons

(1) The degenerative mutant tendons emit a recruitment signal. (2) The recruitment signal leads to activation of TGFβ signaling and to recruitment of a population of *Sox9*-positive and *Scx*-negative stem/progenitor cells. The *ScxCre*-lineage tracing suggests that these cells are not derived from surrounding peritenon or tendon sheath. (3) The activated cells are recruited toward the mutant tendon. (4) These *Sox9*-expressing cells turned on tendon markers and became *ScxGFP* positive only upon entering the mutant tendons. Notably, the newly recruited cells, identified as *ScxGFP+;RosaT−* cells in this study, could be detected as early as P1 in the mutant tendons and remained detectable throughout the study period. These cells also express nucleostemin (NS), a stem/progenitor marker that has been reported in tendon-derived stem/progenitor cells but not in mature tenocytes. TGFBRII, TGFβ type II receptor.

Tendon damage occurs very frequently, but the tissue tends to heal poorly (Boileau et al., 2005; Gerber et al., 2000). Therefore, there is great interest in the use of stem/progenitor cells to improve tendon repair (Hernigou et al., 2014; Oh et al., 2014). Previous investigations have demonstrated that acute tendon injury involves recruitment of new cells expressing stem/progenitor markers (Dyment et al., 2013; Harvey et al., 2019; Howell et al., 2017; Runesson et al., 2015; Wang et al., 2017). However, not much is known about the possible recruitment of stem/progenitor cells into degenerative tendons. We have previously shown that collagen matrix appeared intact in P3 mutant neonates, and signs of matrix disorganization and deterioration were detected only in mutant pups older than 1 week (Tan et al., 2020). While the degenerative changes seen in these mutants may not mimic clinical scenarios of tendon degeneration, they represent a new model for tendon damage that does share significant similarities with tendon degeneration and may therefore facilitate analysis of stem/progenitor cell recruitment events in such environments, including the stem/progenitor cell identity, sources, and mechanism of activation. Notably, studies have shown differences in regenerative capacity in neonatal tendons compared with adult (Ansorge et al., 2012; Howell et al., 2017). It is therefore not clear if the stem/progenitor cells activated in neonatal stages in this study are also involved in adult tendon damage. However, identifying the unique features of these cells in this study would enable future investigation of their possible involvement in various forms of tendon damage. Moreover, identifying the origin and activation signals for cell recruitment in neonates may open possibilities for isolating or targeting these cells in future studies. In light of the remarkable dearth of animal models for studying degenerative phenotypes in tendons and cell recruitment events in such environments, findings from this study may provide an important foundation for future research.

We show herein that the cells recruited into mutant tendons are clonogenic, and the majority of newly recruited cells express nucleostemin and Sox9. The enhanced colony-forming capacity of the recruited cells may reflect the stem/progenitor origin of these cells. However, we previously found that the dedifferentiated resident tenocytes of the mutant tendons also showed enhanced colony-forming capacity (Tan et al., 2020), which suggested that in this case the enhanced colony-forming capacity may simply be a feature of cells from the mutant tendons.

Nucleostemin is a GTP-binding protein expressed predominantly in the nucleoli of stem/progenitor cells (Beekman et al., 2006). In recent years, nucleostemin has been shown to be expressed by culture-expanded stem/progenitor cells from tendons (Zhang and Wang, 2010). Notably, infiltration of nucleostemin-positive progenitor cells into ruptured rat Achilles tendons has been reported in an earlier study (Runesson et al., 2015). *Sox9* plays an essential role in the development of skeletal tissues (Akiyama et al., 2002), and expression of Sox9 was identified in the stem/progenitor cell populations involved in the repair of various adult tissues, including skeletal tissues such as the ribcage and mandible (Kuwahara et al., 2019; Ransom et al., 2018). Notably, bipotential progenitors that co-express *Sox9* and *Scx* were implicated in the development of tendons, tendon entheses, and ligaments (Blitz et al., 2013; Huang et al., 2019; Soeda et al., 2010). It would therefore be interesting to compare and to determine if the *Sox9*-expressing stem/progenitor cells identified in this study may also be a bipotential progenitor pool that could also participate in cartilage and bone repair. Furthermore, our results suggest that *Sox9* is also expressed in the niche of the stem/progenitor cells identified in this study, and expression of *Sox9* may therefore serve as an initial indicator for possible location and origins of the cells.

Studies have suggested several possible sources of recruited cells into injured tendons, including peritenon and tendon sheath (Dyment et al., 2013; Harvey et al., 2019; Wang et al., 2017). Apparent changes of epitenon cellular activity, e.g., increased proliferation, has been observed in many cases of tendon injuries (Khan et al., 1996). Moreover, recent studies show appearance and subsequent migration of *ScxGFP*-positive cells from paratenon into tendons following injury (Dyment et al., 2013; Sakabe et al., 2018). A similar phenomenon was observed in this study and prompted us to ask if the recruited cells were derived from these tissues. The results from *ScxCre*-lineage tracing indicate that these cells are derived neither from peritenon nor tendon sheath regions. Moreover, the eventual structural destruction of epitenon in our mutant pups may also limit the availability of cells recruited from this region. These results therefore imply the existence of multiple sources of recruited stem/progenitor cells for tendons. Different sources for cell recruitment may reflect specialization of specific cells for different tendon conditions or the concurrent activation of multiple cell populations that may have complementing activities in the healing process. Interestingly, a previous study has shown biphasic infiltration of two different stem/progenitor cell populations into ruptured rat Achilles tendons (Runesson et al., 2015).

To better understand the identity and origin of the recruited cells, further investigation focused on Sox9 because immunostaining demonstrated robust Sox9 expression in these cells during the early phase of recruitment. Results from a Double Cre experiment showed that inducible deletion of TGFβ signaling in *Sox9*-expressing cells significantly reduced the number of recruited cells in mutant pups. The finding not only corroborates our earlier notion that the recruited cells expressed Sox9 but further reveals that at least a subpopulation of the recruited cells is from a *Sox9*-expressing cell lineage. *Sox9CreERT*-lineage tracing shows the presence of *Sox9*-expressing cells in perichondrium and bone marrow in neonates, suggesting the possibility of these tissues as sources of the recruited stem/progenitor cells. Notably, stem/progenitor cells have been identified in the perichondrium and bone marrow, and these cells are involved in tissue repair (Pineault et al., 2019). The possible involvement in these perichondrial cells in tendon healing will be addressed in future studies.

Studies of stem/progenitor cells and their roles in normal development and tissue repair were revolutionized with the advent of Cre technology and the ability to label distinct cell populations with a combination of a tissue-specific Cre driver and a Cre reporter. These studies are typically prospective; a hypothesis regarding the role of a specific cell population is tested by labeling these cells using a tissue-specific Cre (Harvey et al., 2019; Soeda et al., 2010; Wang et al., 2017). In this study, we developed a complementary retrospective approach to identify cell recruitment into tendons. *ScxGFP* is a robust tendon reporter that results in strong GFP expression shortly after activation of the *Scx* enhancer (Pryce et al., 2007). Activation of the *RosaT* reporter in an *ScxCre;RosaT* combination requires two rounds of protein synthesis; first, accumulation of sufficient Cre protein and then, after recombination and activation of the reporter, accumulation of the reporter protein (Madisen et al., 2010). It is therefore likely that, upon induction of the *Scx* enhancer in a cell with no tenogenic history, the *ScxGFP* signal will be the first to be detected, followed by the *RosaT* signal. It should be noted that this approach will not identify cells from the tendon sheathing tissues or intrinsic tendon cells, since these cells are from an *Scx*-expressing cell lineage and thus will have an activated *RosaT* reporter. We suggest, however, that retrospective screening for *ScxGFP+;RosaT−* cells in tendons of *ScxCre;RosaT;ScxGFP*-carrying mice can be used as a general approach for identification of non-*Scx*-expressing cell recruitment into tendons following injury or pathology.

Identifying key players that mediate cell recruitment into damaged tendons is critical to design tendon reparative strategies. At present, almost nothing is known about this process either *in vitro* or *in vivo*. Here we show that TGFβ signaling is essential for the cell recruitment into the degenerative mutant tendon, in which disruption of TGFβ type II receptor in these cells significantly reduced their number in the mutant tendons. In general, the

TGFβ signaling pathway involves ligand-bound TGFβ type II receptors that subsequently activate TGFβ type I receptor. The activated receptor complex will then stimulate nuclear localization of Smad proteins to regulate the expression of target genes. As all TGFβ1–3 ligands bind to a single type II receptor, disruption of this one receptor is sufficient to abrogate all TGFβ signaling (Vander Ark et al., 2018). TGFβ signaling is known to be involved in the recruitment of stem/progenitor cells in various tissues (Tang et al., 2009; Wan et al., 2012). With regard to tendons, activation of the TGFβ signaling pathway has been reported during embryonic tendon cell development (Havis et al., 2014). Moreover, a number of studies have demonstrated increased TGFβ ligand and receptor expression by tendon (Dahlgren et al., 2005; Fenwick et al., 2001) or its adjacent tissues (Khan et al., 1996) in pathological conditions. Interestingly, TGFβ seems to play an important role in mediating tendon repair (Chen et al., 2004), although the exact mechanism remains unclear. More recently, Kaji et al. (2020) reported that TGFβ signaling is required in neonatal tenocytes for their recruitment to the site of tendon transection, and may play a role in promoting neonatal tendon regeneration. Notably, this study identified a role for TGFβ signaling for recruitment of the resident tenocytes into the wound site and suggested a possible additional cell population involved in this process. In the present study, we provide evidence for a distinctly different role for TGFβ signaling in tendons: recruitment of a separate population of stem/progenitor cells from a distant niche into the degenerating tendon. Interestingly, these results highlight repeated involvement of TGFβ signaling in distinct cellular events in tendon biology.

Our results also indicate a cell-autonomous requirement of TGFβ signaling for the recruitment. In the Double Cre experiment with *RosaCreERT2*, the *Tgfbr2* receptor was eliminated from all cells. The failure of cell recruitment in this scenario could therefore be the result of a role for TGFβ signaling in the recruiting tendon, the environment surrounding the tendon, or in the recruited cells themselves. However, as shown in Figure 6D, individual cells that did not lose receptor expression were recruited in this scenario, suggesting that the disruption was not in the tendon or tendon environment, since that would affect all cell recruitment, but rather a direct effect on the stem/progenitor cells that lost *Tgfbr2* expression. Future studies will focus on the mechanism of action at the cellular level of TGFβ signaling by transcriptome analysis of the recruited cells.

Lastly, TGFβ signaling is often associated with collagen matrix production (Leask and Abraham, 2004). However, apparent collagen disorganization was not observed in $Tgfbr2^{ScxCre}$ mutant tendons at the onset of the cellular phenotype (Tan et al., 2020). Instead, collagen disorganization and epitenon deterioration were noted only in pups older than 1 week. The degenerative changes might thus imply a secondary consequence of the cellular changes in these mutants and/or of their movement difficulties. Regardless of the underlying causes, degenerative change has been implicated as a feature of tendinopathy (Kannus and Jozsa, 1991; Longo et al., 2018). Much of what we learn about tendon healing comes from studies on acute tendon injury, and their significance to healing of tendinopathic tendons remains questionable. Moreover, it is difficult to obtain early tendinopathic human tissues because the conditions are often initially asymptomatic. The degenerative phenotype in $Tgfbr2^{ScxCre}$ mutant thus warrants further investigation and may provide a useful animal model for analysis of early degenerative changes in tendons.

EXPERIMENTAL PROCEDURES

Mice

All animal procedures were approved by the by the Animal Care and Use Committee at Oregon Health & Science University (OHSU; TR01-IP00000717). Details for mice used in the study can be found in supplemental experimental procedures.

TEM

Mouse forelimbs were skinned, fixed in 1.5% glutaraldehyde/1.5% formaldehyde, and decalcified in 0.2 M EDTA. TEM was then performed as previously described (Tan et al., 2020). The acquired images were stitched using ImageJ software (https://imagej.nih.gov/ij/) (Preibisch et al., 2009).

In situ hybridization and immunohistochemistry staining

In situ hybridization and immunohistochemistry staining were performed as previously described (Tan et al., 2020). Detailed protocols are provided in supplemental experimental procedures.

Cell isolation and colony-forming unit assay

Cell isolation from mouse tendons and colony-forming unit (CFU) assay were performed using a protocol as previously described (Tan et al., 2020), and detailed protocols are provided in supplemental experimental procedures.

Double Cre experiment to investigate the role of TGFβ signaling in cell recruitment

CreERT2 recombinase was incorporated into the background of $Tgfbr2^{f/-}$*;ScxCre* mutant to enable induction of either ubiquitous (*RosaCreERT2*) or restricted (*Sox9CreERT2*) *Tgfbr2* deletion upon tamoxifen administration. The pups were administered orally with tamoxifen (50 mg/mL in autoclaved corn oil; 15 μL per pup) at P1 and P2 (for $Tgfbr2^{f/-}$*;ScxCre;RosaCreERT* pups) and P1 and P2 and P5 and P6 (for $Tgfbr2^{f/-}$*;ScxCre;Sox9CreERT* pups). Pups were then harvested at P7, a stage at which only recruited cells

are positive for *ScxGFP* in the mutant tendon. Dissected forelimbs were fixed, decalcified, cryosectioned (10 μm), and subjected to cell counting as detailed below.

Quantification of recruited cells in transverse sections of the communis tendons

Changes in recruited cell number in mutant tendons were measured for examining the effect of tamoxifen-inducible TGFβ signaling ablation on cell recruitment. Detailed methods are presented in supplemental experimental procedures.

Statistical analysis

Student's t tests were performed to determine the statistical significance of differences between groups ($n \geq 3$). Unless stated otherwise, all graphs are presented as mean ± standard deviation (SD). A value of $p < 0.05$ is regarded as statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.stemcr.2021.10.018.

AUTHOR CONTRIBUTIONS

Conception and design, G.K.T. and R.S.; methodology, G.K.T. and R.S.; investigation, G.K.T.; acquisition of data, G.K.T., B.A.P., A.S., D.R.K., and S.F.T.; analysis and interpretation of data, G.K.T. and R.S.; writing – original draft, G.K.T.; writing – review & editing, R.S., G.K.T., B.A.P., A.S., D.R.K., and S.F.T.; project supervision, R.S.; funding acquisition, R.S.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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