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 (Tgfbr2ScxCre) 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 Sca1+ lineage and their recruitment was dependent on cell autonomous TGFβ signaling. The cells identified in this study 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/floxP 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...
ScxCre-driver (structural deterioration was only observed from P7 onward. 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 Tgfb2ScxCre 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 Tgfb2f/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 Tgfb2ScxCre 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
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 Tgfb2ScxCre 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 Tgfbr2ScxCre 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).

(legend continued on next page)
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.

Figure 3. ScxGFP-expressing cells in Tgfbr2ScxCre mutant tendons are newly recruited

(A) Schematic illustration of the mTmG dual-fluorescent Cre reporter (adapted from Muzumdar et al., 2007). Ubiquitously expressed membrane-tomato (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 Tgfbr2f/+; ScxCre;mTmG heterozygous pups, tendon cells were recombined and switched the fluorescence from red to green (mG). (D) In Tgfbr2ScxCre 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.
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 Tgbr2ScxCre 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 Tgbr2ScxCre 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 Tgbr2ScxCre mutant pups (7.2% ± 1.2% compared with 0.2% ± 0.1% in P7 wild-type littersmates; 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, 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 Tgbr2ScxCre 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 Tgbr2ScxCre 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.
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/C0). 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...
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 mm² and 21.5 ± 0.7 mm², 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 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 mm² and 21.5 ± 0.7 mm², 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.

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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 ten-
dons. 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 Tgfb2 in these cells will change their capacity for recruit-
ment. We previously found that the tendon phenotype in

![Figure 6: Cell-autonomous TGFβ signaling is essential for cell recruitment into mutant tendons](image)

The ubiquitous RosaCreERT2 driver was incorporated into the Tgfb2ScxCre mutant background (Tgfb2f-/ScxCre;RosaCreERT2, hereafter called ScxCre;RosaCreERT) to examine the effect of global loss of the TGFβ signaling on cell recruitment into Tgfb2ScxCre mutant tendons. Tamoxifen was adminis-
tered 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 Tgfb2ScxCre 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 Tgfb2 specifically in Sox9-expressing cells (Tgfb2f-/ScxCre;Sox9CreERT2, hereafter called ScxCre;Sox9-
CreERT). The results showed about a 48% decrease in recruited cell numbers, suggest-
ing 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.

Tgfb2ScxCre mutants is dependent on the specific spatio-
temporal features of ScxCre activity (Tan et al., 2020). To
target the Tgfb2 receptor before the cells are recruited, we
therefore decided to add the ubiquitous inducible Cre dele-
tor (RosaCreERT2) (Hameyer et al., 2007) to the Tgfb2ScxCre
allele combination. Since the tendon phenotype manifests
in Tgfb2ScxCre mutants in postnatal stages, we can use Rosa-
CreERT2 to induce ubiquitous loss of the receptor at that
stage and examine the effect on cell recruitment into mut-
ant tendons.

Pups of the mutant allele combination, Tgfb2f-/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 pro-
cedures 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 Tgfbr2ScxCre 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 or number of days in which tamoxifen was administered. 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 Double Cre experiment, 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 Tgfb2-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 Tgfb2 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 Tgfb2 but in this time specifically in Sox9-expressing cells using a Sox9CreERT2 driver in combination with the Tgfb2ScxCre mutant (Tgfb22/−;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.
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<sup>ScxCre</sup> 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 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<sup>ScxCre</sup> 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 decalified 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<sup>Wnt1</sup>;<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<sup>Wnt1</sup>;<ScxCreRosaCreERT pups) and P1 and P2 and P5 and P6 (for Tgfbr2<sup>Wnt1</sup>;<ScxCreSox9CreERT 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 ≥ 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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