

Recruitment and maintenance of tendon progenitors by TGF β signaling are essential for tendon formation

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Tendons and ligaments mediate the attachment of muscle to bone and of bone to bone to provide connectivity and structural integrity in the musculoskeletal system. We show that TGF β signaling plays a major role in the formation of these tissues. TGF β signaling is a potent inducer of the tendon progenitor (TNP) marker scleraxis both in organ culture and in cultured cells, and disruption of TGF β signaling in *Tgfb2*^{-/-};*Tgfb3*^{-/-} double mutant embryos or through inactivation of the type II TGF β receptor (TGFBR2; also known as β RII) results in the loss of most tendons and ligaments in the limbs, trunk, tail and head. The induction of scleraxis-expressing TNPs is not affected in mutant embryos and the tendon phenotype is first manifested at E12.5, a developmental stage in which TNPs are positioned between the differentiating muscles and cartilage, and in which *Tgfb2* or *Tgfb3* is expressed both in TNPs and in the differentiating muscles and cartilage. TGF β signaling is thus essential for maintenance of TNPs, and we propose that it also mediates the recruitment of new tendon cells by differentiating muscles and cartilage to establish the connections between tendon primordia and their respective musculoskeletal counterparts, leading to the formation of an interconnected and functionally integrated musculoskeletal system.

KEY WORDS: TGF β , Connective tissue, Ligaments, Tendons, Mouse

INTRODUCTION

Tendons transmit the force generated by muscle contraction to the skeleton, thereby determining the specific connections between muscles and their skeletal insertions (Benjamin et al., 2008). Recent studies identified scleraxis (*Scx*), a bHLH transcription factor gene, as a unique marker for the tendon cell fate (Cserjesi et al., 1995; Schweitzer et al., 2001), and used *Scx* expression to describe tendon induction and differentiation during embryogenesis (Brent et al., 2003; Schweitzer et al., 2001) (reviewed by Tozer and Duprez, 2005). In somites, tendon progenitors (TNPs) are found in the syndetome, a dorsolateral stripe of the sclerotome at the junction between adjacent myotomes (Brent et al., 2003). In limb buds, TNPs are induced in mesenchyme directly under the ectoderm, in locations that follow the proximal-to-distal outgrowth of the limb bud (Schweitzer et al., 2001); by E12.5, the TNPs align as loosely organized progenitors between the differentiating muscles and corresponding cartilage condensations. The TNPs later condense and differentiate to give rise to overtly distinct tendons by E13.5 (Murchison et al., 2007).

A molecular framework for tendon induction and differentiation is also beginning to emerge. FGF signaling plays an important role in the induction of TNPs (reviewed by Tozer and Duprez, 2005). In somites, FGFs emanate from the myotome to induce adjacent sclerotomal cells to become TNPs (Brent et al., 2003; Brent and Tabin, 2004). In limb buds the source and identity of FGFs that

direct the induction of TNPs has not been established to date, but expression of FGF4 has been reported in limb muscles (Edom-Vovard et al., 2002). The subsequent condensation and differentiation of TNPs is dependent on the transcriptional activities of *Scx* (Murchison et al., 2007).

TGF β s comprise a small subfamily within the TGF β superfamily (Massague et al., 2000; Shi and Massague, 2003). The regulation and function of TGF β signaling has been the subject of numerous studies, leading to the targeting in mice of all the unique participants in TGF β signaling, notably the ligands *Tgfb1-3* (Kulkarni et al., 1993; Proetzel et al., 1995; Sanford et al., 1997) and their receptors (Dudas et al., 2006; Oshima et al., 1996). Significantly, TGF β s use a single type II receptor, TGFBR2 (also known as β RII), which implies that targeted recombination of a conditional allele, *Tgfb2*^{fllox}, is sufficient for the disruption of all TGF β signaling, circumventing complications due to redundancy of ligands or receptors (Chytil et al., 2002). The analysis of such mutants has established a role for TGF β signaling in numerous developmental processes (Dunker and Kriegelstein, 2000; Serra and Chang, 2003), including crucial roles in skeletogenesis, the disruption of which can manifest in reduced chondrocyte proliferation, cleft palate, disrupted skeletal boundaries, fused joints and the failure of sternum development (Baffi et al., 2006; Proetzel et al., 1995; Sanford et al., 1997; Seo and Serra, 2007; Spagnoli et al., 2007). TGF β signaling has also been associated with the connective tissues because of its capacity to induce extracellular matrix (ECM) proteins and an involvement in the development of fibrosis (reviewed by Mauviel, 2005). More recently it was demonstrated that disruption of TGF β signaling resulted in the loss of *Scx* expression in cranial tissues, suggesting a role for TGF β signaling in tendon development (Oka et al., 2008).

We show that disruption of TGF β signaling results in the loss of most tendons and ligaments – the first demonstration of a molecular activity with an essential role in formation of these tissues. The induction of TNPs was not affected in mutant embryos and tendon loss was apparent only at E12.5, concurrent with the organization of

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tendon primordia that align between the differentiating muscles and the prechondrogenic mesenchymal condensations. Moreover, we have found that TGF β signaling is a potent inducer of *Scx* both in organ culture and in cultured cells, suggesting a role for TGF β signaling in tendon induction. TGF β signaling is thus essential for maintenance of the early TNPs, and we propose that it also mediates recruitment of additional tendon cells by the adjacent muscles and cartilage condensations to establish the connections of tendon primordia with these tissues, an essential event for the subsequent differentiation and growth of mature tendons.

MATERIALS AND METHODS

Mice and histology

Existing mouse lines were previously described: *Tgfb2* (Sanford et al., 1997), *Tgfb3* (Proetzel et al., 1995), *Tgfb2^{lox}* (Chytil et al., 2002), *Prx1Cre* (Logan et al., 2002) and *ScxGFP* (Pryce et al., 2007). *Tgfb1^{-/-}* mice were generated using the stochastic germline activity in *Prx1Cre* females (Logan et al., 2002), and the colony was expanded to verify recombination in the germline.

Tgfb2^{-/-}; *Tgfb3^{-/-}* embryos were retrieved at the expected ratio in harvests performed up to E12.5 (10/164 embryos), but the frequency decreased sharply in later stages (3/297 embryos at E14.5 and older).

In situ hybridization, antibody staining, BrdU and TUNEL assays were performed as previously described (Murchison et al., 2007).

Organ culture

Organ culture was performed as previously described (Zuniga et al., 1999). Embryos were harvested in DMEM and limb buds or trunks were dissected and placed on metal grids in six-well plates containing Nutrient Medium (Zuniga et al., 1999). Affigel beads (BioRad) were soaked in 20 μ g/ml TGF β 2 or TGF β 3, or 25 μ g/ml hFGF4 recombinant proteins (R&D Systems) for 1 hour on ice and grafted, and the plates were incubated at 37°C, 5% CO₂. We found a progressive loss of endogenous mRNAs for E12.5 limbs (but not E10.5 trunks) incubated for 2 hours and longer and therefore limited the duration of these experiments.

Tissue culture

C3H10T1/2 cells (ATCC) were seeded in six-well plates (2.5 \times 10⁶ cells/well) in DMEM-10% FBS; after 24 hours the medium was supplemented with 20 ng/ml TGF β 2 protein (R&D Systems). Activation medium was maintained till harvest or replaced by DMEM-10% FBS after 1 hour. Cells were trypsinized in duplicate, RNA was prepped using RNeasy mini (Qiagen) and 1 μ g RNA was used for cDNA synthesis (Invitrogen Superscript III).

Qualitative PCR primers were:

5'*Scx*Exon1a, GAGACGGCGGCGAGAACACCC;

3'*Scx*Exon2a, GCGTGTCTTGGGGACCTGCG;

TNCExon12-5', GAACACCGATGCTCTCTACTGACG; and

TNCExon13-3', ATGTGGGCAGTCCGTTCAGCA.

Quantitative RT-PCR was performed using ABI 7900HT with SYBR green. Results were normalized to GAPDH and four samples were used for each time point. Primers were: *Scx*-Q5'-1 AGAGACGGCGGC-GAGAACAC and *Scx*-Q3'-2 GTGGGGCTCTCCGTGACTCTTC.

RESULTS

TGF β signaling is essential for the formation of limb tendons

A report of TGF β gene expression in tendons of chick embryos (Merino et al., 1998) prompted us to check whether the same was true in mouse, and we detected expression of both *Tgfb2* and *Tgfb3* in limb tendons at E14.5 (Fig. 5A,B). A functional link between TGF β signaling and limb tendons was recently proposed in an analysis of the role TGF β signaling plays in the development of the deltoid tuberosity, a lateral outgrowth of the humerus (Seo and Serra, 2007). The deltoid tuberosity was not affected when *Tgfb2^{lox}* was targeted in chondrocytes (Baffi et al., 2004), but it did not develop

in *Tgfb2^{-/-}* embryos (Sanford et al., 1997) or when TGF β signaling was disrupted throughout the limb mesenchyme (Seo and Serra, 2007; Spagnoli et al., 2007). As growth of the deltoid tuberosity is dependent on biomechanical activation (Dysart et al., 1989), it was suggested that the phenotype might be attributed to a loss of muscles or tendons in these mutants (Seo and Serra, 2007). To address this possibility, *Tgfb2^{-/-}* mice were bred with the tendon reporter *ScxGFP* (Pryce et al., 2007). The deltoid tuberosity and deltoid tendon were apparent in sagittal sections through the humerus of a wild-type embryo at E16.5, but missing in sections from a *Tgfb2^{-/-}* littermate (Fig. 1A,B, white arrowheads and arrows, respectively). Moreover, antibody staining for myosin heavy chain (MHC) revealed that the muscles around the humerus were also significantly reduced in the *Tgfb2^{-/-}* embryo, suggesting a defect in tendons or muscles as being the underlying cause for the loss of biomechanical activity in *Tgfb2^{-/-}* embryos.

Detection of *ScxGFP* in whole limbs revealed a more severe tendon phenotype, most of the extensor tendons were missing in the forelimb of E15.5 *Tgfb2^{-/-}* embryos, but segments of the extensor tendons persisted in the digits (Fig. 1C,D, white arrows and arrowheads, respectively). To study the phenotype in greater detail, we analyzed cross sections through the forelimbs of E14.5 embryos. In digit sections, the extensor tendons appeared similar in wild-type and *Tgfb2^{-/-}* embryos, but in more proximal positions the extensor tendons were missing or rudimentary in mutant embryos in agreement with the tendon loss we saw in whole limbs (Fig. 1F, a1-b3, white arrows). Surprisingly, the flexor tendons appeared normal in the mutant embryos (Fig. 1F, a1-b3, yellow arrows).

We found expression of *Tgfb3* in tendons as well, but tendons were not disrupted in *Tgfb3^{-/-}* embryos (not shown). However, an allelic series, combining mutations in both TGF β genes resulted in a dramatic phenotypic series. In *Tgfb2^{-/-}*; *Tgfb3^{-/-}* embryos, the loss of extensor tendons was enhanced relative to the loss in *Tgfb2^{-/-}* embryos and flexor tendons were severely reduced as well (Fig. 1F, c1-c3, white and yellow arrows, respectively). Remarkably, in double mutant *Tgfb2^{-/-}*; *Tgfb3^{-/-}* embryos, no tendons were detected at all limb levels (Fig. 1F, d1-d3), except for a remnant of the flexor profundus tendon in the digit, whose signal is disproportionately enhanced in Fig. 1F, d1. Some *ScxGFP*-expressing cells were also found at the level of the metacarpals, encircling the muscles in a pattern that was not related to tendons in this position (Fig. 1F, d2).

Double mutant *Tgfb2^{-/-}*; *Tgfb3^{-/-}* embryos were retrieved at a very low frequency (see Materials and methods), prompting us to study some aspects of the phenotype using the conditionally targeted *Tgfb2^{lox}* allele (Chytil et al., 2002) in conjunction with *Prx1Cre* (Logan et al., 2002), a limb-specific Cre deleter that shows early activity through the limb mesenchyme, resulting in the targeting of all tendon cells (see Fig. S1 in the supplementary material). This combination leads to a complete disruption of TGF β signaling in limb mesenchyme and will be denoted *Tgfb2^{Prx1Cre}* (Seo and Serra, 2007; Spagnoli et al., 2007). Similar to *Tgfb2^{-/-}*; *Tgfb3^{-/-}* embryos, *ScxGFP* could not be detected in limbs from *Tgfb2^{Prx1Cre}* embryos at E14.5 (not shown) and in situ hybridization (ISH) analysis showed a complete loss of *Scx* expression in these limbs as well (Fig. 1G,H).

Tgfb2^{-/-}; *Tgfb3^{-/-}* and *Tgfb2^{Prx1Cre}* embryos represent a broad disruption of TGF β signaling, hence we cannot rule out the possibility that the effects on *Scx* expression were secondary to effects on other tissues. It is, however, important to note that cartilage condensation appeared normal in these mutants (Seo and Serra, 2007), and ISH with a *Myod* probe showed that early muscle differentiation was not affected either (Fig. 1I,J). Subsequent aspects

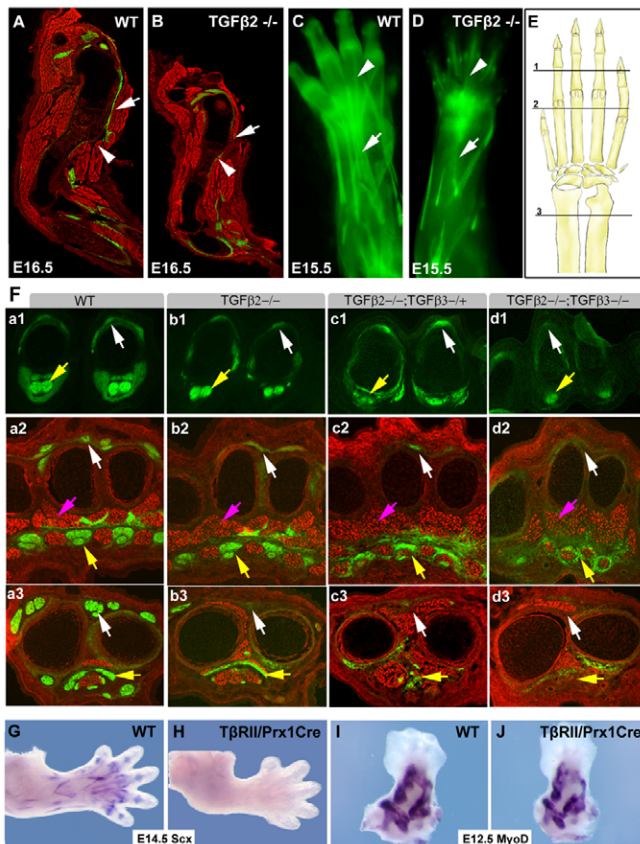


Fig. 1. Loss of *ScxGFP* tendon signal in an allelic series of *Tgfb2*- and *Tgfb3*-null alleles. Tendons were visualized using the *ScxGFP* transgenic reporter and sections were counterstained with an MHC antibody. **(A,B)** Sagittal sections through the forelimbs of an E16.5 wild-type mouse embryo and a *Tgfb2*^{-/-} littermate. White arrowheads indicate the normal and missing Deltoid tuberosity in A and B, respectively; white arrows indicate the Deltoid tendon and missing tendon in A and B, respectively. **(C,D)** The extensor tendons of an E15.5 wild-type embryo and a *Tgfb2*^{-/-} littermate visualized using the *ScxGFP* tendon reporter. White arrows indicate the extensor digitorum tendon in C and missing extensors in D; white arrowheads indicate extensor tendons in the digits. **(E)** Schematic drawing of the forelimb. The positions of sections shown in F are marked, 1, digits; 2, metacarpals; 3, proximal to the wrist. **(F)** Transverse sections through the forelimb of E14.5 embryos from an allelic series of *Tgfb2* and *Tgfb3* mutant mice. The panels are sub-indexed with a letter to denote the genotype: a, wild type; b, *Tgfb2*^{-/-}; c, *Tgfb2*^{-/-};*Tgfb3*^{-/-}; d, *Tgfb2*^{-/-};*Tgfb3*^{-/-}. The numeral in each panel denotes the corresponding plane of section in E. White arrows indicate extensor tendons; yellow arrows, flexor tendons; pink arrows, muscles. **(G-J)** Whole-mount ISH on forelimbs from wild-type and *Tgfb2*^{-/-};*Tgfb3*^{-/-} embryos with a *Scx* probe at E14.5 (G,H) and a *Myod* probe at E12.5 (I,J).

of skeletal differentiation were, however, affected when TGF β signaling was disrupted (Seo and Serra, 2007; Spagnoli et al., 2007). Although muscle pattern was not drastically altered, the positions of some muscles were changed and the muscles appeared less compact, with increased spaces between myotubes (Fig. 1F, a2,b2,c2,d2, pink arrows). We did not determine at this time if these effects represent a requirement for TGF β signaling in muscles or a secondary consequence of the tendon phenotype.

The complete loss of *Scx* expression in TGF β signaling mutants prompted us to evaluate the expression of other tendon markers. Expression of tenomodulin (*Tnmd*), encoding a tendon-specific transmembrane protein (Brandau et al., 2001), could not be detected in *Tgfb2*^{-/-};*Tgfb3*^{-/-} embryos (Fig. 2A,B), nor did we detect expression of *Colla1*, the most robust ISH probe in tendon cells, which encodes the most abundant protein in tendons, collagen I (Murchison et al., 2007). In transverse sections of forelimbs from a *Tgfb2*^{-/-};*Tgfb3*^{-/-} embryo (Fig. 2C,D), or sagittal sections from a *Tgfb2*^{-/-};*Tgfb3*^{-/-} forelimb (Fig. 2E,F, white and yellow arrows), the ISH signal for *Colla1* did not highlight tendon cells, even when the ISH reactions were over-developed and other cells, notably osteoblasts, showed a robust *Colla1* signal. Moreover, even in Hematoxylin and Eosin stained sections, in which tendons have a distinct condensed morphology, tendons could not be detected in mutant embryos (Fig. 2G,H, yellow arrow). Finally, in studies of the tendon phenotype in *Scx*^{-/-} embryos, we found that some tendon rudiments that lost expression of *Scx*, *Tnmd* and *Colla1*, retained the capacity to attach tissues and expressed tendon matrix proteins such as collagen XII and tenascin C (Murchison et al., 2007). However, in limbs from *Tgfb2*^{-/-};*Tgfb3*^{-/-} embryos, we could not detect any tendon-related expression of tenascin C or collagen XII (Fig. 2I-L). Taken together, these results imply that tendon cells do not exist in the limbs of embryos in which TGF β signaling is disrupted, the first mutant combination in which a complete loss of limb tendons is identified.

Tendons and ligaments throughout the body are missing in mutants that disrupt TGF β signaling

The essential role of TGF β signaling in the development of limb tendons prompted us to examine whether other tendons were also affected when TGF β signaling was disrupted. Although limb tendons are derived from the lateral plate mesoderm, the axial tendons originate in a distinct somitic domain, the syndetome (Brent et al., 2003), and cranial tendons originate from cranial neural crest cells (Chai et al., 2000; Kontges and Lumsden, 1996). The different embryonic origins of axial, cranial and appendicular tendons suggest that there may also be divergent aspects to the regulation of their differentiation and development (reviewed by Tozer and Duprez, 2005).

The distinctive structures of trunk tendons seen in wild-type skinned embryos was missing in *Tgfb2*^{-/-};*Tgfb3*^{-/-} littermates (Fig. 3A,B, white arrows). Moreover, in transverse sections through the head of embryos at E14.5, in which staining for collagen II and MHC was used to highlight the neck muscles and cartilage, *Tnmd*-expressing tendons cells could not be detected in sections from a *Tgfb2*^{-/-};*Tgfb3*^{-/-} embryo (Fig. 3C,D). The same transverse sections extended also through the jaws and the masseter, the major cranial muscle (Fig. 3G,H). *Tnmd*-expressing tendons anchor the masseter to the jaws, but *Tnmd* could not be detected next to any of the cranial muscles in the *Tgfb2*^{-/-};*Tgfb3*^{-/-} embryos (Fig. 3G,H, arrows). Interestingly, *Scx*-expressing progenitors of the masseter tendons could not be detected in heads of *Tgfb2*^{-/-};*Tgfb3*^{-/-} mutant embryos already at E12.5 (Fig. 3E,F, arrows).

The most robust axial tendons in the mouse are the tail tendons that originate in sacral muscles and extend through the tail to insert at the dorsal and ventral sides of tail vertebrae (Fig. 3I, white arrow) (see also Murchison et al., 2007). In cross section, these long tendons appear as peripheral dots in each quadrant of the tail (Fig. 3M, white arrows). Minor aspects of tail movement are regulated by intrinsic muscles that extend across a single vertebra and anchor via short tendons (Fig. 3M,O, yellow arrows) (see also Shinohara, 1999). The long tendons of the tail were entirely missing in *Tgfb2*^{-/-} mutant

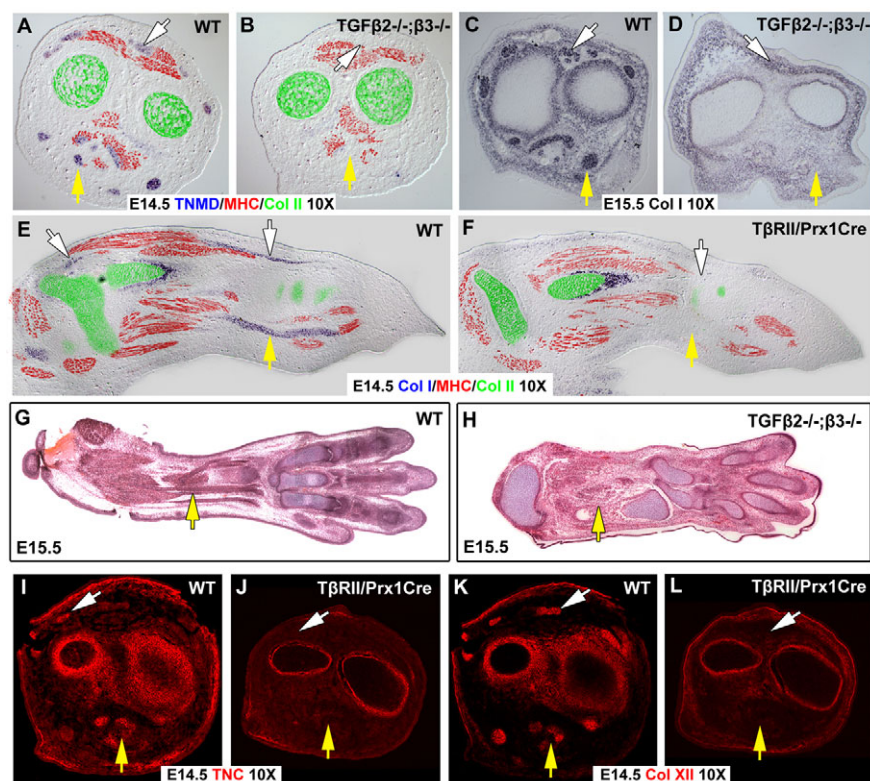


Fig. 2. All limb tendons are lost in *Tgfb2*^{-/-};*Tgfb3*^{-/-} and *Tgfb2*^{Prx1Cre} mutant embryos. Comparison of tendon markers in sections from the limbs of wild-type and TGFβ signaling mutants. In all panels white arrows indicate extensor tendons, yellow arrows indicate flexor tendons. (A-D) ISH with a *Tnmd* probe (A,B) and a collagen I probe (C,D) on transverse sections through the zeugopod of wild-type embryos and *Tgfb2*^{-/-};*Tgfb3*^{-/-} littermates at E14.5 and E15.5. Sections in A and B were subsequently stained with antibodies for MHC (red) and collagen II (green). (E,F) ISH with a collagen I probe on sagittal sections through the limbs of an E14.5 wild-type embryo and a *Tgfb2*^{Prx1Cre} littermate was followed by antibody staining for MHC (red) and collagen II (green). (G,H) Hematoxylin and Eosin staining of planar sections through limbs of an E15.5 wild-type embryo and a *Tgfb2*^{-/-};*Tgfb3*^{-/-} littermate. (I-L) Antibody staining for tenascin C (I,J) and collagen XII (K,L) on transverse sections through the zeugopod of a wild-type embryo (I,K) and a *Tgfb2*^{Prx1Cre} littermate (J,L).

embryos (Fig. 3I,J,M,N, white arrows), but the intrinsic muscles of the tail and their tendons were intact (Fig. 3M-P, yellow arrows); these tendons persisted even in double mutant *Tgfb2*^{-/-};*Tgfb3*^{-/-} embryos (not shown).

Ligaments, connecting bones across joints to provide structural integrity during joint movement, are similar to tendons in ultrastructure and cellular composition, suggesting they may also be affected by a disruption of TGFβ signaling. It was recently shown that in *Tgfb2*^{Prx1Cre} embryos the joints in the autopod do not undergo cavitation and remain fused (Seo and Serra, 2007; Spagnoli et al., 2007). Ligaments do not form in these joints (not shown), but that may be a secondary consequence of the failure of joint formation. We therefore focused on the knee, in which cavitation does occur in these embryos. ISH with a *Coll1a1* probe (Fig. 3K,L) on a sagittal section through the hindlimb, highlighted the unique features of the knee, the cruciate ligaments that connect the femur and the tibia, and the patella, connected to the tibia with the patellar ligament and connected to the quadriceps muscles with a short tendon (Fig. 3L). Limited cavitation could be seen in the knees of *Tgfb2*^{Prx1Cre} embryos, but no ligaments or tendons were found in these knees (Fig. 3M). The muscles were also drastically atrophied in the mutant knee, but at E17.5 that was most likely to be a secondary consequence of the loss of tendons. TGFβ signaling is thus the first molecular activity shown to be essential for the formation of almost all tendons and ligaments.

TGFβ signaling is required for TNP expansion and reorganization at E12.5

Having established an essential role for TGFβ signaling in tendon genesis, we next wanted to identify the onset of the tendon phenotype in TGFβ signaling mutants. Contrary to the dramatic tendon phenotype described above, at E11.5 *Scx* expression appeared normal in *Tgfb2*^{Prx1Cre} embryos (not shown) and

Tgfb2^{-/-};*Tgfb3*^{-/-} embryos (Fig. 4A,B). As it is possible that residual TGFβ signaling exists in these mutants, we generated a null allele of the type II receptor, *Tgfb2*^{+/+}, by recombining the *Tgfb2*^{lox} allele in the germline (see Materials and methods). *Tgfb2*^{+/+} embryos die at E10.5 (Oshima et al., 1996), but we found that at E10.5 *Scx* expression was not affected, even in *Tgfb2*^{-/-} embryos (Fig. 4C), demonstrating that the initial induction of TNPs was not dependent on TGFβ signaling.

The first indication of tendon loss was seen in mutant embryos at E12.5, *Scx* expression in the somites of *Tgfb2*^{-/-} embryos was markedly reduced and was almost completely lost in *Tgfb2*^{-/-};*Tgfb3*^{-/-} embryos (Fig. 4D-F). Interestingly, in limbs of *Tgfb2*^{-/-} embryos, *Scx* expression was dramatically reduced on the dorsal side but only partially reduced on the ventral side, corresponding to the loss of extensor but not flexor tendons in *Tgfb2*^{-/-} embryos at later stages (Fig. 4G,H,J,K). Moreover, *Scx* expression could hardly be detected in *Tgfb2*^{Prx1Cre} limbs (not shown) and *Tgfb2*^{-/-};*Tgfb3*^{-/-} limbs at E12.5 (Fig. 4I,L). The full scope of the tendon phenotype was thus reflected in the loss of TNPs already at E12.5, a stage in which the TNPs undergo expansion and reorganization to form loosely organized tendon primordia between the differentiating muscles and the cartilage condensations.

The dramatic loss of *Scx* expression between E11.5 and E12.5 could be the result of apoptosis of the TNPs in the absence of TGFβ signaling. However, TUNEL staining on frontal sections from trunks of *Tgfb2*^{-/-} mutants at E11.5, E12.5 and E13.5, showed no cell death in the *ScxGFP*-positive TNPs, but robust TUNEL activity in the sclerotome (Fig. 4M,N; data not shown). Ectopic TUNEL labeling was also not found in sections from limb buds of *Tgfb2*^{Prx1Cre} embryos at E11.5 and E12.5 (not shown). The loss of *Scx*-expressing cells could also be caused by a failure of TNP proliferation, but BrdU labeling in *ScxGFP*-expressing cells appeared normal in limb buds of *Tgfb2*^{Prx1Cre} embryos at E11.5 (Fig. 4O). The loss of TNPs

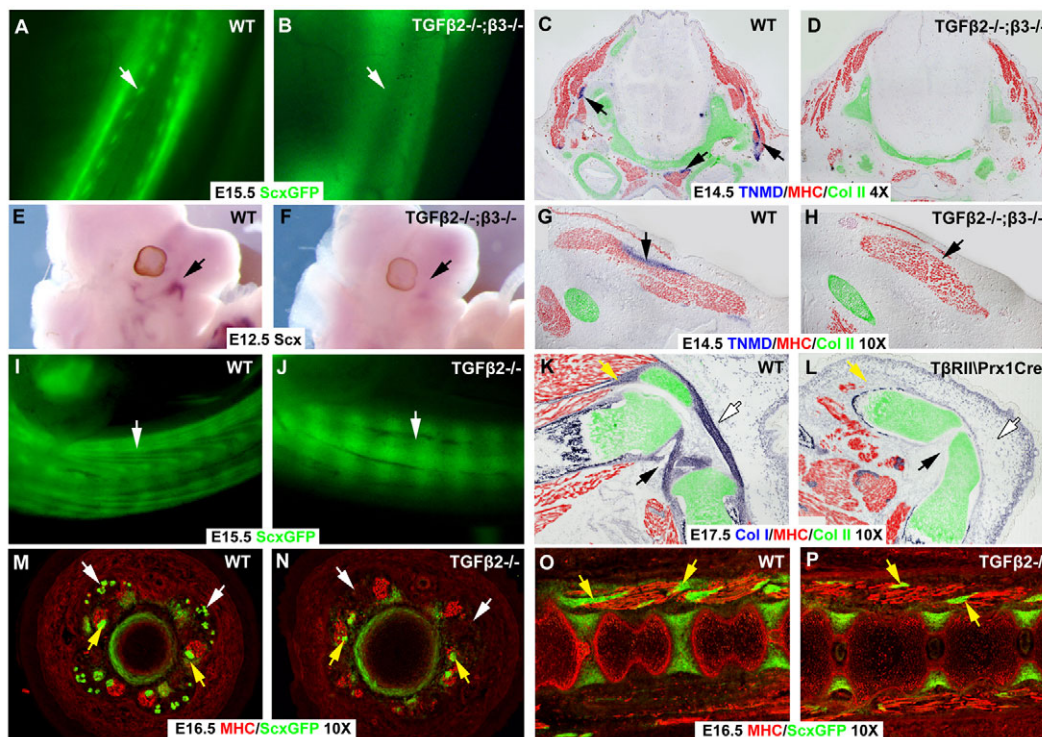


Fig. 3. Tendons and ligaments throughout the body are lost in TGF β signaling mutants. (A,B) The *ScxGFP* tendon reporter in trunks of skinned E15.5 wild-type embryos and *Tgfb2*^{-/-};*Tgfb3*^{-/-} littermates. Arrows indicate tendons. (C,D,G,H) Transverse sections through the heads of a wild-type embryo (C,G) and a *Tgfb2*^{-/-};*Tgfb3*^{-/-} littermate (D,H) at E14.5 were processed for ISH for *Tnmd*, followed by immunostaining with antibodies to MHC (red) and collagen II (green). (C,D) Neck muscles. (G,H) The masseter muscle of the jaw. Arrows indicate actual and missing *Tnmd* signal. (E,F) Whole-mount ISH for *Scx* on heads of an E12.5 wild-type embryo and a *Tgfb2*^{-/-};*Tgfb3*^{-/-} littermate. Arrows indicate *Scx* in the masseter. (I,J) *ScxGFP* in tails from an E15.5 wild-type embryo and a *Tgfb2*^{-/-};*Tgfb3*^{-/-} littermate. Arrows indicate tendons. (K,L) Sagittal sections through the knees of E17.5 wild-type and *Tgfb2*^{Prx1Cre} embryos processed for *Col1a1* ISH followed by immunostaining with antibodies to MHC (red) and collagen II (green). Yellow arrows, patellar tendons; white arrows, patellar ligament; black arrows, cruciate ligaments. (M-P) *ScxGFP* tendon reporter and antibodies to MHC (red) in transverse sections (M,N) and sagittal sections (O,P) through the tails of E16.5 wild-type (M,O) and *Tgfb2*^{-/-} (N,P) embryos. White arrows, extrinsic tail tendons; yellow arrows, intrinsic muscles and tendons.

in TGF β mutants is thus not caused by cell loss and is likely to represent a failure in maintenance of the tendon cell fate when TGF β signaling was disrupted.

TGF β 2 and TGF β 3 are expressed in TNPs, and in the differentiating muscles and cartilage, in early embryonic stages

The loss of TNPs in TGF β signaling mutants prompted us to examine the expression of TGF β 2 and TGF β 3 in the relevant embryonic stages. In somites, *Scx* is expressed in the syndetome, a dorsolateral stripe at the junction between adjacent myotomes (Brent et al., 2003), which can be conveniently visualized in frontal sections (illustrated in Fig. 4A), wherein the myotomes can be highlighted by antibody staining for MHC expression (see Fig. 6A,B). Expression of *Tgfb3* in somites was very faint in embryos between E10.5 and E12.5, but low levels of *Tgfb3* were detected in the syndetome (Fig. 6E,F). Interestingly, *Tgfb2*, which plays a more central role in axial tendon development, was not expressed in the syndetome, although expression was detected in condensed medial domains, the initial sites of cartilage condensation (Fig. 6I,J, black arrows), and in a triangular domain overlapping with MHC expression at the center of the differentiating myotome (Fig. 6I,J, red arrows).

In limb buds, *Tgfb2* was again expressed in prechondrogenic mesenchymal condensations as they emerge in a proximal to distal progression, resulting in transient expression in digit condensations at E12.0 and a later restriction to the presumptive joints by E13.0 (Fig. 5C,D). Expression of *Tgfb2* was also seen in differentiating muscles in the limb buds, but the expression was less distinct than that seen in somites (Fig. 6K,L, red arrows). Finally, a comparison of *Tgfb2* and *Scx* expression in alternating sections at E12.5 showed that *Tgfb2* was expressed in some TNPs as well; for example, in the TNPs that connect the pronator quadratus muscle to the radius and ulna (Fig. 5D,L, black arrows), and in other TNPs demarcated more clearly in sagittal sections through limbs at this stage (Fig. 5C,K, black arrows). However, not all TNPs expressed *Tgfb2*; for instance, the TNPs in the digits, the last TNPs induced at this stage, were positive for *Scx* but did not express *Tgfb2* (Fig. 5C,K, white arrows). *Tgfb2* and *Scx* expression were also overlapping in prospective joints, although in this case *Tgfb2* expression was much broader than that of *Scx*, as seen in the presumptive wrist joint (Fig. 6C,K, yellow arrows). Expression of *Tgfb3* in limb buds at these stages was again very faint, but similar to the expression in somites, *Tgfb3* transcripts were detected in TNPs, e.g. in the pronator quadratus tendons (Fig. 6H, black arrows).

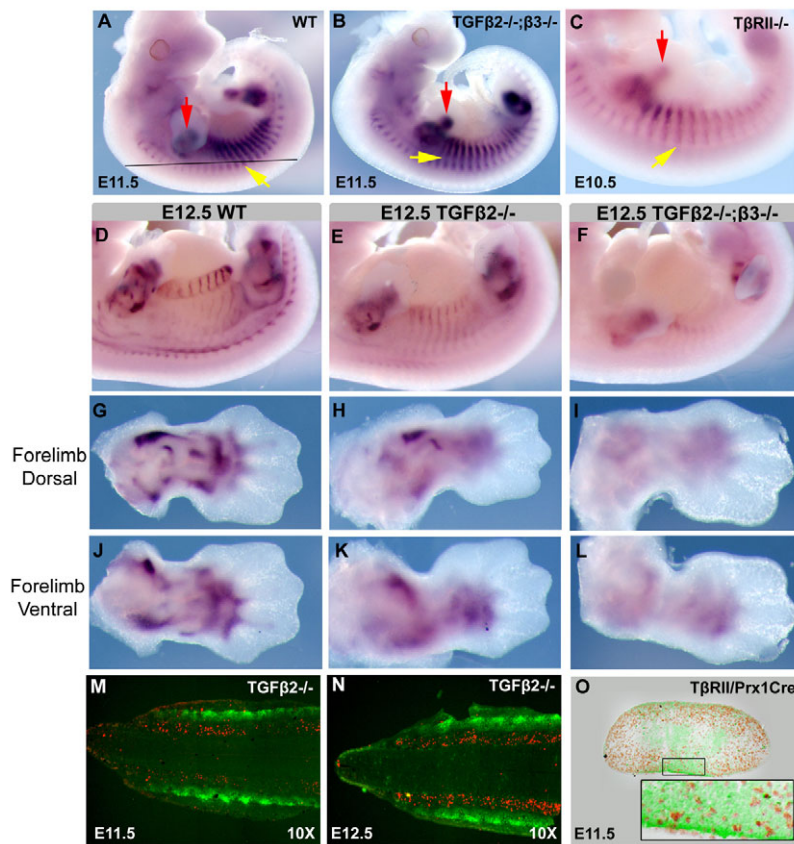


Fig. 4. TNPs are lost in TGF β signaling mutants at E12.5. (A–C) Whole-mount ISH for *Scx* on E11.5 wild-type (A), E11.5 *Tgfb2*^{-/-};*Tgfb3*^{-/-} (B) and E10.5 *Tgfbr2*^{-/-} (C) embryos. Yellow arrows, *Scx* expression in somites; red arrows, *Scx* expression in limb buds. The black line in A indicates the level of a frontal section through the trunk. (D–L) Whole-mount ISH for *Scx* on E12.5 whole embryos and dorsal and ventral forelimbs from wild-type (D,G,I), *Tgfb2*^{-/-} (E,H,K) and *Tgfb2*^{-/-};*Tgfb3*^{-/-} (F,I,L) embryos. (M,N) TUNEL staining (red) superimposed on *ScxGFP* signal (green) on frontal sections through the back (illustrated in A) of *Tgfb2*^{-/-} embryos at E11.5 (M) and E12.5 (N). (O) BrdU staining superimposed on a *ScxGFP* signal in a transverse section through the limb bud of a E11.5 *Tgfbr2*^{Prx1Cre} embryo. The inset is an enlargement of the boxed ventral *Scx*-positive domain.

The combined expression of *Tgfb2* or *Tgfb3* in the stages relevant for the tendon phenotype therefore encompasses the TNPs, the muscles and the prechondrogenic skeletal condensations, suggesting a possible role for TGF β signaling in the interaction that is established at this stage between the forming tendons and their musculoskeletal counterparts. In an attempt to identify the cells that can activate TGF β signaling, we examined the expression of *Tgfbr2*, finding low and very broad expression in the undifferentiated mesenchyme (Fig. 6G). Interestingly, *Tgfbr2* was not expressed at the distal parts of the limb bud, the site of the most recent induction of TNPs at this stage (Fig. 6C,G, white arrows).

Robust induction of tendon markers by TGF β signaling

The abrupt loss of *Scx* expression in TGF β signaling mutants prompted us to test whether TGF β signaling has a direct effect on *Scx* expression. Affigel beads loaded with recombinant TGF β 2 protein were grafted into limb buds in organ culture and caused a robust and highly reproducible induction of *Scx* expression at all stages from E10.5 to E13.5 (Fig. 7A–D, yellow arrows; data not shown). *Scx* induction was detected already after 1.5–2 hours of incubation and up to 16 hrs of incubation (not shown), and *Scx* expression was also induced by beads loaded with recombinant TGF β 3 protein (not shown). Moreover, *Scx* expression was also induced by grafting TGF β 2-loaded beads in somites of E10.5 embryos (Fig. 7G, yellow arrows).

In limb buds, endogenous *Scx* expression is induced in mesenchymal cells directly under the ectoderm (Schweitzer et al., 2001), but *Scx* expression induced by a TGF β 2 bead extended much deeper in the mesenchyme (Fig. 7E,F). Interestingly, *Scx* induction was limited to undifferentiated mesenchyme and *Scx* expression was

never detected in the ectoderm or prechondrogenic condensations. Endogenous expression of *Scx* is also constrained in the proximodistal axis, extending at E12.5 only up to the forming metacarpal-phalangeal joint (Fig. 7H, red arrow). Interestingly, a TGF β 2 bead grafted at the level of the metacarpal-phalangeal joint resulted in induction of *Scx* expression proximal but not distal to the bead (Fig. 7I, yellow arrow), and the introduction of two TGF β 2 beads resulted in robust and symmetric induction of *Scx* around the proximal bead, but *Scx* was not induced around the distal bead. The distal restriction of *Scx* induction by TGF β -loaded beads is likely to be related to the fact that expression of *Tgfbr2* could not be detected in the distal mesenchyme at this stage (Fig. 6G, white arrows).

Scx induction has been previously associated with FGF signaling and has been shown to be regulated by modulation of the MAP kinase cascade (Brent et al., 2003; Brent and Tabin, 2004; Edomovovard and Duprez, 2004; Smith et al., 2005). We therefore used UO126, a specific inhibitor of ERK1/2 phosphorylation (Favata et al., 1998; Yamamoto et al., 2003), to investigate whether *Scx* induction by TGF β signaling occurred through an indirect activation of the MAP kinase cascade. To verify inhibition of the MAPK cascade, we tested induction of *Sprouty2* (*Spry2* – Mouse Genome Informatics), a common target of FGF signaling (Minowada et al., 1999), and found that the induction of *Sprouty2* by a bead loaded with FGF4 protein was completely blocked by the addition of 50 μ M UO126 to the medium (Fig. 7K,L). However, induction of *Scx* by a TGF β 2 bead was not affected in the presence of the same concentration of UO126 (Fig. 7M), demonstrating a MAPK-independent pathway for *Scx* induction by TGF β signaling.

Robust induction of *Scx* in organ culture led us to test the capacity of TGF β signaling to induce *Scx* expression in cultured cells as well. Induction was initially tested in mouse embryonic fibroblasts

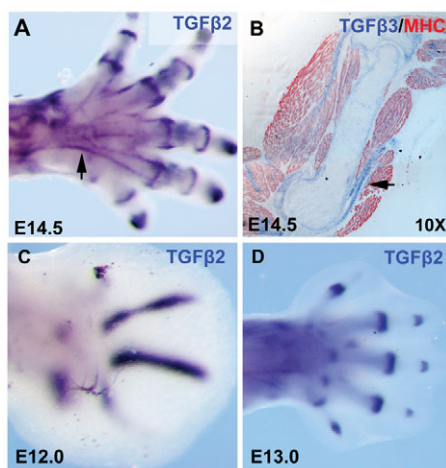


Fig. 5. Expression of *Tgfb2* and *Tgfb3*. (A) Whole-mount ISH for *Tgfb2* on an E14.5 hindlimb. (B) Section ISH for *Tgfb3* followed by immunostaining with antibodies to MHC (red) on a sagittal section through the humerus of an E14.5 embryo. (C,D) Whole-mount ISH for *Tgfb2* on forelimbs from embryos at E12.0 (C) and E13.0 (D).

(MEFs) extracted from *ScxGFP* embryos. Propagation of *ScxGFP* MEFs in culture resulted in complete loss of the *ScxGFP* signal (Fig. 8A), but incubation with 0.3 nM TGF β 2 in the medium for 24 hours resulted in a considerable induction of *ScxGFP* (Fig. 8A). The *ScxGFP* signal level was variable in the induced MEFs, highlighting the importance of cellular context for *Scx* induction. To examine the induction in a more homogenous system, we next tested the effects

of TGF β signaling in C3H10T1/2 cells, a murine cell line considered to represent a mesenchymal progenitor state (Pinney and Emerson, 1989).

Non-induced C3H10T1/2 cells expressed *Scx* at a moderate level, and in semi-quantitative RT-PCR we found that addition of TGF β 2 to the medium resulted in a significant increase in *Scx* mRNA that continued to accumulate up to 24 hours after induction (Fig. 8B). To gain a better appreciation for the dynamics of *Scx* induction, we next exposed the cells to TGF β 2 just for one hour and monitored *Scx* expression for up to 48 hours (Fig. 8C). Quantitative RT-PCR of these samples shows induction of *Scx* expression already 30 minutes after the addition of TGF β 2 and maintenance of high levels of *Scx* up to 24 hours after induction. The moderate measure of the ‘fold change’ of *Scx* expression in this experiment is likely to be a numeric reflection of the significant expression of *Scx* in non-induced cells and not a reflection of absolute levels of *Scx* induction.

To distinguish between a simple induction of *Scx* and an induction of TNPs, we wanted to evaluate the expression of other tendon markers following TGF β activation. Tenascin C, an extracellular matrix protein, is expressed distinctly in tendons (Fig. 2I) (Chiquet-Ehrismann et al., 1991), and the expression has previously been used as a good marker for early tendon cells (Edom-Vovard et al., 2002; Kardon, 1998). The induction of tenascin C by TGF β signaling has been previously reported (Pearson et al., 1988), and was also detected in our TGF β 2-induced C3H10T1/2 cells (Fig. 7B), but interestingly we did not detect induction of tenascin C by TGF β beads in organ cultures (not shown), highlighting the importance of the cellular context for these activities.

A small number of other tendon markers have been identified, including *Tnmd* (Brandau et al., 2001), collagen XII and collagen XIV (Walchli et al., 1994; Young et al., 2000), and mohawk

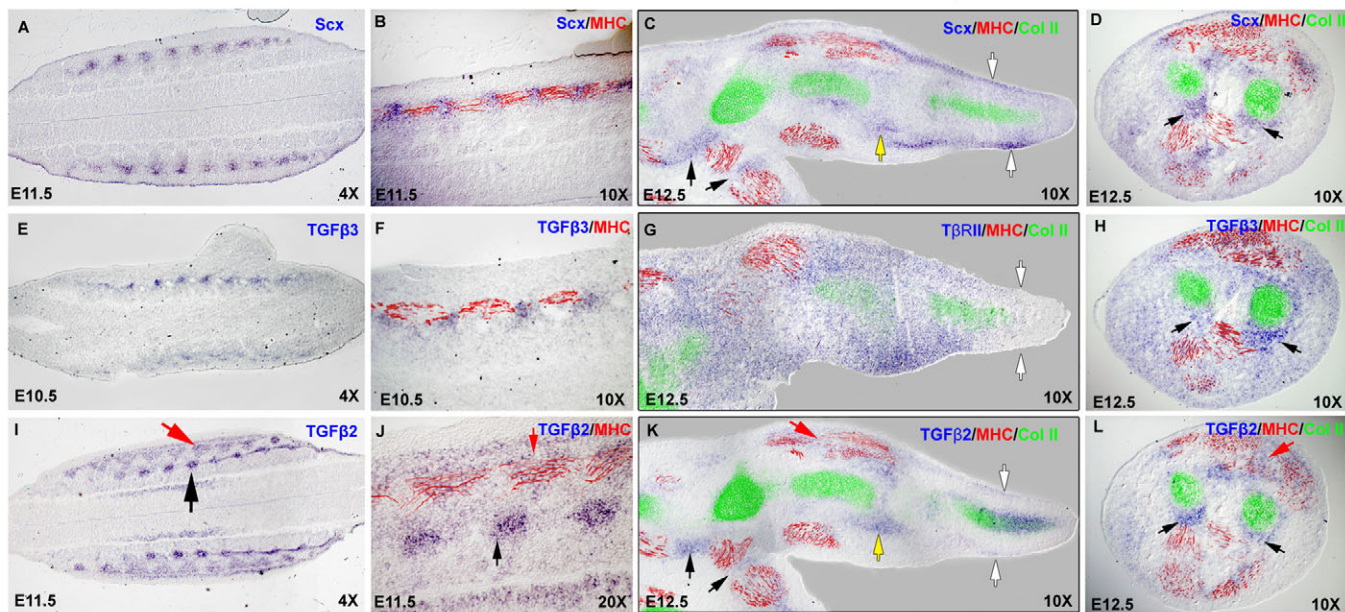


Fig. 6. Expression of *Tgfb2* and *Tgfb3* in and around the TNPs. (A-L) Section ISH for *Scx* (A-D), *Tgfb3* (E,F,H), *Tgfb2* (G) and *Tgfb2* (I-L) followed by immunostaining with antibodies to MHC (red) and collagen II (green). (A,B,E,F,I,J) Frontal sections through the back (illustrated in Fig. 4A) from embryos at E11.5 (A,B,I,J) and E10.5 (E,F). B, F and J are higher magnification images of A, E and I, respectively. Black arrows, early cartilage condensations; red arrows, myotome. (C,G,K) Sagittal sections through the forelimbs of an E12.5 embryo. White arrows, TNPs in the digits; red arrows, expression in muscle; black arrow, TNPs; yellow arrow, the wrist. (D,H,L) Transverse sections through the zeugopod of an E12.5 embryo at the level of the pronator quadratus muscle. Black arrows, TNPs; red arrow, expression in muscle.

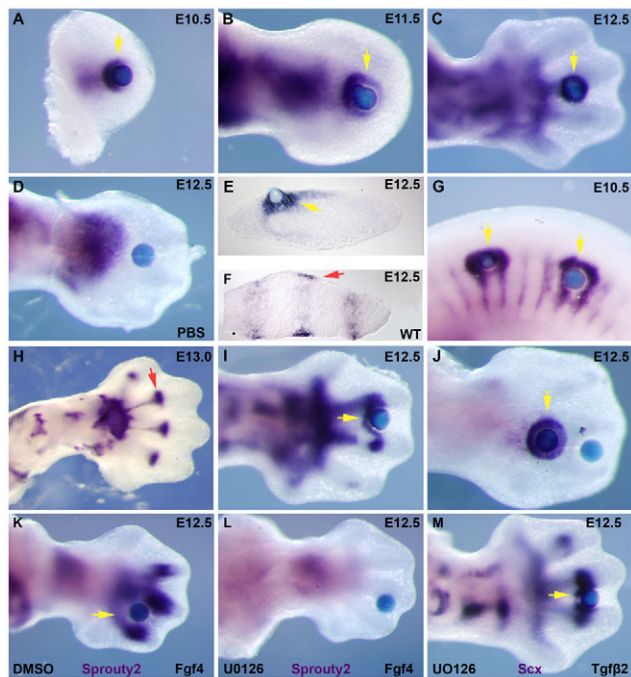


Fig. 7. Induction of *Scx* by TGF β signaling in organ culture.

(A–J) Whole mount ISH for *Scx* after 4–6 hours of incubation with Affigel beads saturated with 0.02 mg/ml TGF β 2 protein or PBS. Yellow arrows, induced *Scx* expression; red arrows, endogenous *Scx* expression. (A–C) TGF β 2 beads in forelimb buds from embryos at E10.5 (A), E11.5 (B) and E12.5 (C). (D) PBS control beads in a forelimb from an embryo at E12.5. (E) Transverse cryosection after *Scx* whole-mount ISH for an E12.5 limb incubated with a TGF β 2 bead. (F) Section ISH for *Scx* in a transverse section from an E12.5 wild-type forelimb in a position corresponding to the section in E. (G) TGF β 2 beads in the trunk of an E10.5 embryo. (H) Normal expression of *Scx* at E13.0. The red arrow highlights the sharp boundary for *Scx* expression at the metacarpal-phalangeal joint. (I) TGF β 2 beads at the level of the metacarpal-phalangeal joint at E12.5. (J) Distal and proximal TGF β 2 beads in an E12.5 limb. (K, L) Whole-mount ISH for *Sprouty2* after a 6-hour incubation with an FGF4-loaded bead in the presence of 50 μ M UO126 dissolved in DMSO (L), or just DMSO as control (K). (M) Whole-mount ISH for *Scx* after a 6-hour incubation with a TGF β 2 bead in the presence of 50 μ M UO126.

(Anderson et al., 2006). None of these genes has been linked to a progenitor state and some clearly represent a later stage of tendon differentiation (Murchison et al., 2007; Shukunami et al., 2006). The induction of any of these genes by TGF β signaling could not be detected in organ culture or in C3H10T1/2 cells (not shown), suggesting that additional molecular events following the activation by TGF β might be required for tendon differentiation.

DISCUSSION

We show that TGF β signaling plays a major role in the genesis of tendons and ligaments. TGF β signaling is a potent inducer of tendon markers in mesenchymal cells, and tendons and ligaments are entirely missing in embryos in which TGF β signaling is disrupted. Our data provide insight but no conclusive answer to two major aspects of TGF β signaling in tendon genesis. Are the essential TGF β s secreted by the TNPs or from the muscle and cartilage? And does TGF β signaling promote just maintenance of existing TNPs or also recruitment of new TNPs? The analysis presented below of the

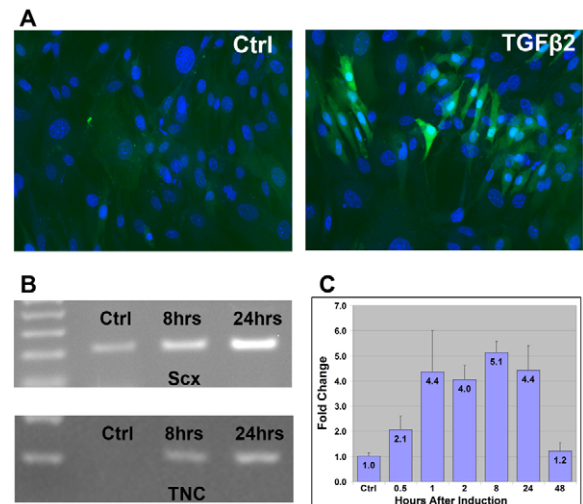


Fig. 8. Induction of early tendon markers by TGF β signaling in tissue culture.

(A) Mouse embryonic fibroblasts (MEFs) from *Scx*^{GFP} embryos after a 24-hour incubation in culture medium alone (control) or supplemented with 0.3 nM TGF β 2 protein, counterstained with DAPI to detect the cell nuclei (blue). (B) Induction of *Scx* and tenascin C in C3H10T1/2 cells by TGF β signaling. C3H10T1/2 cells were incubated in six-well plates with culture medium supplemented with 0.3 nM TGF β 2 protein for 8 hours or 24 hours. Semi-quantitative RT-PCR amplification (25 cycles) was performed to detect the relative levels of mRNA for *Scx* and tenascin C. (C) Changes in the levels of *Scx* transcript following a pulse of TGF β activation. C3H10T1/2 cells incubated in six-well plates were supplemented with 0.3 nM TGF β 2 protein for one hour, after which the cells were washed and returned to regular medium. Cells were harvested at the indicated times after the initiation of the induction. Levels of *Scx* mRNA were determined by QRT-PCR and normalized to the levels of GAPDH, results of four separate experiments were averaged. Levels of *Scx* transcript are represented as fold change relative to non-induced cells.

phenotypic series of *Tgfb2* and *Tgfb3* null alleles in the context of the expression of these genes provides a strong argument for an essential role for TGF β s that emanate from the muscles and cartilage, and a more minor role for the TGF β s that originate in the TNPs. Whereas the induction and loss of TNPs in mutant embryos highlights an obvious role for TGF β signaling in maintenance of TNPs, the involvement of TGF β s that emanate from the interacting tissues implies that other mesenchymal cells are inevitably also exposed to TGF β signaling and thus are likely to be recruited to the growing tendon primordium, supporting its attachment to the muscles and cartilage (Fig. 9). These findings provide a new framework for understanding the integration and attachment of the forming tendons with their respective muscles and cartilage elements, and present TGF β signaling as an obvious candidate in efforts to manipulate tendons and ligaments for experimental and clinical purposes.

Maintenance of the TNP cell fate is dependent on TGF β signaling

TNPs, identified as *Scx*-expressing cells, are induced between E9.5 and E12.5 in the syndetome and limb bud mesenchyme, and later differentiate to overtly distinct tendons by E13.5 (Brent et al., 2003; Schweitzer et al., 2001). The tendon phenotype in *Tgfb2*^{-/-}; *Tgfb3*^{-/-} and *Tgfb2*^{Prx1Cre} embryos highlights a crucial stage in tendon development between E11.5 and E12.5; in mutant embryos, the

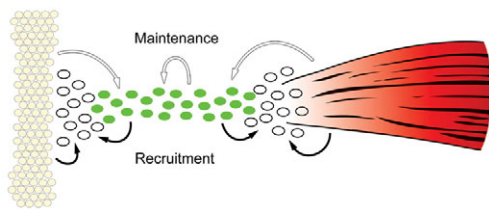


Fig. 9. TGF β signaling promotes maintenance and recruitment of tendon progenitors. Tendon progenitors (green cells) that align between the cartilage condensations (yellow cells) and differentiating muscles (red) at E12.5 are dependent at this stage on TGF β signaling (white arrows). This essential role for TGF β s from the muscles and cartilage (e.g. TGF β 2 which is expressed exclusively in these tissues in the somites) suggests also an affect on adjacent mesenchymal cells (white), possibly by recruiting them to the tendon cell fate (black arrows). Autocrine activity of TGF β s from the tendon progenitors plays a more minor role in progenitor maintenance. Although the loss of TGF β 3, which is expressed exclusively in tendon progenitors in the somites, does not result in tendon loss, the enhanced phenotype in double mutant *Tgfb2*^{-/-};*Tgfb3*^{-/-} embryos shows that autocrine activity does contribute to progenitor maintenance (white arrow). It is also possible that TGF β s from the tendon progenitors contribute to the recruitment of neighboring mesenchymal cells (black arrows).

TNPs appeared normal up to E11.5 and were then lost by E12.5, demonstrating a role for TGF β signaling in maintenance of the tendon cell fate at this stage. Significantly, the loss of *Scx* expression was not accompanied by cell death, suggesting that in the absence of TGF β signaling the progenitors assumed a different cell fate.

Loss of TNPs in slightly later stages has recently been associated with the tendon phenotype in two other mutations. In *Scx*^{-/-} embryos, TNPs appeared normal up to E12.5, but the progenitors were lost by E13.5 in tendons that failed to differentiate (Murchison et al., 2007). TNP loss was also seen in muscleless limbs in chick embryos (Edom-Vovard and Duprez, 2004; Kardon, 1998), and in muscleless limbs of mouse embryos homozygous for the splotch delayed (*Spd*) mutation, in which *Scx* expression in the stylopod and zeugopod was reduced by E12.5 but lost only at E13.5 (Bonnin et al., 2005; Tremblay et al., 1998) (T. J. Riordan and R.S., unpublished). TNP loss in all of these cases was not associated with cell death. Taken together, these results imply that early *Scx* expression does not represent a commitment to the tendon cell fate and that putative TNPs are dependent on endogenous and exogenous activities to persist on the path of tendon differentiation, including TGF β signaling at E11.5, a signal from muscles by E12.5 and the transcriptional activities of *Scx* by E13.5. The emergence of overtly distinct tendons at E13.5 is likely to be associated with differentiation of TNPs to committed tenocytes.

TGF β s from the muscles and cartilage are essential for tendon formation

The robust induction of tendon markers by TGF β signaling represents an obvious mechanism for maintenance of the tendon cell fate. However, although expression of *Tgfb2* or *Tgfb3* in the TNPs suggests an autocrine TGF β function, expression of these genes in the muscles and cartilage suggests an alternative in which TGF β signaling plays a role in communication between the differentiating tissues of the musculoskeletal system. In somites, *Tgfb2* is expressed in the differentiating muscles and cartilage, and *Tgfb3* is expressed in the TNPs. The extensive loss of TNPs in the somites and tail of *Tgfb2*^{-/-} embryos therefore demonstrates that the essential signal for

maintenance of the TNPs comes from the muscles and cartilage. Interestingly, although *Tgfb3* expression in the TNPs is not sufficient for their maintenance, the accelerated loss of TNPs in *Tgfb2*^{-/-};*Tgfb3*^{-/-} embryos shows that *Tgfb3*, and by inference signals from the TNPs, also plays a maintenance role (Fig. 9, white arrows).

A similar analysis cannot be applied to the tendon phenotypes in limb buds because of the overlap between the expression domains of TGF β 2 and TGF β 3, but a comparison of the tendon phenotypes in muscleless limbs highlights a similar paradigm. In *Spd* embryos, *Scx* expression in the limb bud is normal at E11.5, but expression in the presumptive stylopod and zeugopod is decreased at E12.5 and lost completely by E13.5 (Bonnin et al., 2005) (T. J. Riordan and R.S., unpublished). A signal from the muscles, probably TGF β , is therefore essential for maintenance of the TNPs. The loss of TNPs is, however, accelerated in TGF β signaling mutants, with complete loss of TNPs already at E12.5, showing that TGF β s from sources other than the muscles, probably the expression of *Tgfb2* and *Tgfb3* in the TNPs, also contribute to the maintenance of TNP cell fate. We therefore conclude that TGF β s from the muscles and cartilage are essential for tendon formation at E12.5, and that TGF β s from the TNPs also contribute to the maintenance of tendon markers in these cells (Fig. 9, white arrows).

Recruitment of new tendon cells by TGF β signaling

The robust induction of tendon markers by TGF β signaling suggests also a possible role in the recruitment of new tendon cells. By E12.5, the TNPs align between the differentiating muscles and cartilage as tendon primordia that later connect with these tissues (Brent et al., 2003) (T. J. Riordan and R.S., unpublished). Dependence of tendon formation on TGF β signaling is thus concurrent with the integration of the musculoskeletal system, and the essential role of TGF β s from the muscles and cartilage implicates TGF β signaling in the cross talk between the tissues of the musculoskeletal system. TGF β s from the muscles and cartilage thus inevitably affect adjacent mesenchymal cells as well and are likely to recruit these cells to the tendon cell fate, leading to the generation of a continuous tendon primordium between the muscles and cartilage (Fig. 9, black arrows).

An important implication of this model is the notion that tendons may not be derived exclusively from committed early progenitors. The assertion that early *Scx*-expressing cells are tendon progenitors was based on the continuity of *Scx* expression, but a direct lineage from early *Scx*-expressing cells to all of the tenocytes in mature tendons has not been shown to date (Brent et al., 2003; Schweitzer et al., 2001; Tozer and Duprez, 2005). The results in this study suggest a wave of tendon progenitor recruitment between E11.5 and E12.5, and dynamic expression of the TGF β genes in tendons through embryogenesis (not shown) suggests that TGF β signaling may be involved in the recruitment of tendon cells in later stages as well. Continuous recruitment of tendon cells is further supported by the recent identification of progenitor or stem cells for tendons that can be isolated from human and mouse tendons (Bi et al., 2007).

Induction of TNPs by FGF and TGF β signaling

A pulse of TGF β signaling in C3H10T1/2 cells led to an early induction of *Scx* expression that occurred within 30 minutes and which therefore was most likely caused by direct mediators of TGF β signaling. Elevated levels of *Scx* persisted for 24 hours after activation, suggesting that *Scx* expression might also be regulated secondarily by early transcriptional targets of TGF β signaling.

Interestingly, previous studies have shown a role for FGF signaling in the induction of TNPs. Both FGF and TGF β signaling might thus be involved in tendon induction, and because TGF β signaling is not essential for the early induction of TNPs, it is possible that the two cascades are essential in complementary phases of tendon induction – early progenitors being induced by FGF signaling and later recruitment of tendon cells being mediated by TGF β signaling.

Combined functions of TGF β and FGF signaling that manifest in synergism or epistasis of the two signaling cascades have been reported in a number of developmental and disease processes, including survival of dopaminergic neurons (Roussa et al., 2004), development of lens cataracts (Cerra et al., 2003), chondrocyte proliferation (Mukherjee et al., 2005) and the development of calvarial bones (Sasaki et al., 2006). As both FGF and TGF β signaling have now been shown to induce early tendon markers, it will be important to establish in future studies the relationships between these signaling cascades in tendon induction.

The role of TGF β signaling in differentiation of the connective tissues

The connective tissues comprise a heterogeneous group of tissues that combine to generate complex ECM structures, and that have so far received limited attention at the cellular and molecular levels. The fate of connective tissues in mutant or manipulated embryos has not often been addressed, largely owing to the paucity of distinct molecular markers. A possible role for TGF β signaling in the formation of these tissues was suggested previously because of the capacity of TGF β signaling to induce the accumulation of ECM proteins (Mauviel, 2005). Indeed, a recent study shows expression of TGF β isoforms in healing tendons and their capacity to promote tendon healing (Chan et al., 2008). The tendons and ligaments are classified as dense regular connective tissues, and a recent study has demonstrated a disruption of another of these tissues, the annulus fibrosus of the intervertebral disc, when *Tgfb2^{fllox}* was targeted using the *Col2Cre* mouse (Baffi et al., 2006). TGF β signaling might also be involved in the induction or differentiation of other connective tissues. For example, the less compact appearance of muscles in mutant embryos suggests a partial disruption of the connective tissues of the muscles. Finally, the demonstration that the skeletal phenotype in the deltoid tuberosity of *Tgfb2^{-/-}* mutants was a secondary consequence of the loss of limb tendons and biomechanical stimulation, suggests that unrecognized effects on the development of connective tissues may underlie other phenotypes identified in TGF β signaling mutants.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/136/8/1351/DC1>

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