Scleraxis is required for maturation of tissue domains for proper integration of the musculoskeletal system

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Scleraxis (Scx) is a basic helix-loop-helix transcription factor that is expressed persistently in tendons/ligaments, but transiently in entheseal cartilage. In this study, we generated a novel ScxCre knock-in (KI) allele, by in-frame replacement of most of Scx exon 1 with Cre recombinase (Cre), to drive Cre expression using Scx promoter and to inactivate the endogenous Scx. Reflecting the intensity and duration of endogenous expression, Cre-mediated excision occurs in tendinous and ligamentous tissues persistently expressing Scx. Expression of tenomodulin, a marker of mature tenocytes and ligamentocytes, was almost absent in tendons and ligaments of ScxCre/Cre KI mice lacking Scx to indicate defective maturation. In homozygotes, the transiently Scx-expressing entheseal regions such as the rib cage, patella cartilage, and calcaneus were small and defective and cartilaginous tuberosity was missing. Decreased Sox9 expression and phosphorylation of Smad1/5 and Smad3 were also observed in the developing entheseal cartilage, patella, and deltoid tuberosity of ScxCre/Cre KI mice. These results highlighted the functional importance of both transient and persistent expression domains of Scx for proper integration of the musculoskeletal components.

During early stages of musculoskeletal development, scleraxis (Scx), a basic helix-loop-helix transcription factor, is expressed in the tendon/ligament cell population and the subpopulation of chondrogenic cells that contribute to establishment of chondro-tendinous/ligamentous junctions. Scx expression occurs only transiently in the chondrogenic lineage because it is rapidly downregulated during the early stages of chondrogenesis. In contrast, Scx is persistently expressed in the tendon/ligament cell lineage, which also includes the diaphragm and cordae tendinae of the heart, during differentiation and maturation. Persistent expression of Scx, in the developing tendons and ligaments in vivo, was also visualised in ScxGFP reporter transgenic mice that faithfully recapitulate endogenous Scx-expressing domains under the control of the promoter/enhancer of mouse Scx. In Scx−/− embryos, force-transmitting and intermuscular tendons are reported to be severely affected and are hypoplastic. However, so far, other Scx-expressing domains have not been explored in detail yet.

Tenomodulin (Tnmd) is an excellent cell surface marker to evaluate differentiation and maturation of dense connective tissues, including tendons and ligaments. Scx positively regulates the expression of type I collagen (Col1) and Tnmd in tenocytes. Transcriptome analysis of mouse embryonic limb tendons also revealed that Tnmd is the second most differentially expressed gene of the top 100 genes differentially expressed between E11.5 and E14.5. Studies on Tnmd null mice demonstrated that Tnmd is necessary for tenocyte proliferation and tendon maturation. Cellular adhesion in the periodontal ligament is decreased on loss of Tnmd and enhanced on Tnmd overexpression. In a rat rotator cuff healing model, fibroblast growth factor-2 enhanced tendon-to-bone healing, in which Tnmd expression was associated with formation of aligned collagen fibres in the repair tissue. Loss of Tnmd results in reduced self-renewal and augmented senescence of tendon stem/progenitor cells. Furthermore, forced expression of Scx resulted in the conversion of human bone marrow-derived mesenchymal

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stem cells into tendon progenitor cells that differentiate into Tnmd-expressing cells\(^{30}\). Additionally in tenocytes, Tnmd expression is markedly upregulated by retroviral overexpression of Scx\(^{25}\).

In this study, we report a novel Scx\(^{Cre}\) knock-in (KI) mouse line that was generated by in-frame replacement of most of the exon 1 on mouse Scx locus with Cre recombinase (Cre), to inactivate endogenous Scx and to drive Cre expression using the endogenous Scx promoter. In this established Scx\(^{Cre}\) KI line, Cre expression was detected using Rosa-CAG-LSL-tdTomato (Rosa-tdTomato) reporter mice expressing tandem dimer Tomato (tdTomato) by Cre-mediated recombination. A number of tdTomato-expressing cells of Scx\(^{Cre}\); Rosa-tdTomato or Scx\(^{Cre}\);Rosa-tdTomato mice were observed in tendons, ligaments, annulus fibrosus of intervertebral discs, and patella cartilage. Loss of Scx resulted in an almost absent expression of Tnmd in tendons, ligaments, and annulus fibrosus of intervertebral discs. Cartilaginous elements transiently expressing Scx were defective in Scx\(^{Cre}\) KI mice lacking Scx. Sox9 expression and phosphorylation of Smad1/5 and Smad3 were markedly decreased in these Scx\(^{+}\) cartilaginous elements. Our results clearly demonstrated the functional importance of Scx in maturation of tissue domains that express Scx both persistently and consistently during development.

Results

Establishment of Scx\(^{Cre}\) KI mice. On mouse chromosome 15, Scx gene, consisting of two exons, is located within the third intron of block of proliferation 1 (Bop1), which is transcribed in the opposite orientation\(^{22}\). The targeting strategy placed Cre expression under the control of Scx promoter and led to the inactivation of Scx allele by in-frame replacement of most of its exon 1, which encodes for most of the coding region, including a b-HLH region. The linearised Scx\(^{CreNeo}\) targeting vector (Fig. 1a) was electroporated into KY1.1 embryonic stem (ES) cell line (C57BL/6\(\times\)129S6/SvEvTac)\(^{23}\). ES cell clone with the correct targeting event was microinjected into blastocysts to obtain chimeric mice. We generated heterozygous mice by crossing these chimeras with C57BL/6 wild type mice and confirmed the successful germ line transmission of Scx\(^{CreNeo}\) targeted allele. Heterozygous Scx\(^{CreNeo}\) KI mice were viable, whereas homozygous Scx\(^{CreNeoCreNeo}\) KI mice were embryonic lethal. This is consistent with the previous report that conventional Scx knockout mice die around embryonic day (E)10.5\(^{24}\). To obtain Scx\(^{Cre}\) KI mice, we deleted the flippase (FLP) recombinase target (FRT)-flanked neomycin-resistance (Neo) cassette by mating Scx\(^{CreNeo}\) KI mice with mice expressing FLP recombinase (FLP)\(^{25}\). The correctly targeted event of Scx\(^{Cre}\) KI mice was confirmed by PCR of tail tip DNA and Southern blot analysis (Fig. 1b,c). Expression of Scx was not detected in a Scx\(^{Cre}\) KI mouse embryo at E12.5 (Fig. 1d). Thus, we successfully disrupted endogenous Scx by inserting a Cre in-frame into the ATG start site of mouse Scx gene.

Cre-mediated recombination in Scx\(^{Cre}\) KI; Rosa-tdTomato. Cre-mediated recombination was examined by crossing Scx\(^{Cre}\) KI mice with Rosa-tdTomato reporter mice\(^{26}\). Although endogenous expression of Scx is detectable around E9.5, Cre-mediated tdTomato expression in Scx\(^{Cre}\); Rosa-tdTomato was first detected around E16.5 (data not shown). In the hind limbs of Scx\(^{Cre}\); Rosa-tdTomato neonates, we detected tdTomato expression in the Achilles tendon and in tendons and ligaments associated with knee joint and heel (Fig. 2a–c). Tail tendons of Scx\(^{Cre}\); Rosa-tdTomato neonates were also positive for tdTomato (Fig. 2d). Moreover, at postnatal day (P)14, more intense expression of tdTomato was observed in body tendons and ligaments (Fig. 2e,f,g). In the trunk of 2-week-old Scx\(^{Cre}\); Rosa-tdTomato mice, intense tdTomato expression was observed in tendons of longissimus muscle (Fig. 2e), central tendon of diaphragm (Fig. 2f), and costal tendons (Fig. 2g). In the hindlimb of Scx\(^{Cre}\); Rosa-tdTomato neonates, tdTomato-positive cells were found in patellar ligament, anterior and posterior cruciate ligaments, quadriceps femoris tendon, patella cartilage, femur cartilage, and meniscus (Fig. 2h–j). In the intervertebral region of Scx\(^{Cre}\); Rosa-tdTomato neonates, tdTomato expression was detected in the outer annulus fibrosus on the ventral side (Fig. 2i). In the hindlimb of Scx\(^{Cre}\); Rosa-tdTomato neonates, the number of tdTomato-positive cells in patella cartilage increased (Fig. 2k). Mosaic Cre-mediated recombination could have occurred because of the heterogeneity in the endogenous Scx mRNA levels and time window of Scx expression in the Scx-expressing cells.

Tendon deficiency and skeletal abnormalities in Scx\(^{Cre}\) KI neonates. Heterozygous Scx\(^{Cre}\) KI mice were viable, fertile, and displayed no apparent developmental defects (Fig. 3a,c,e,g). Consistent with previous findings\(^{5}\), morphological defects in force-transmitting and intermuscular tendons were evident at birth, in Scx\(^{Cre}\) KI neonates (Fig. 3b,d,f,h). The diaphragm of Scx\(^{Cre}\) KI neonates is functional and permits normal breathing. The forelimb autopod of Scx\(^{Cre}\) KI neonates was locked in a dorsal flexure (Fig. 4b), compared to that of Scx\(^{+}\) KI neonates (Fig. 4a). The deltoid tuberosity (DT) and tibial tuberosity observed in Scx\(^{+}\) KI neonates (Fig. 4a,k,m) were missing in Scx\(^{Cre}\) KI neonates (Fig. 4b,l,n). The rib cage, transverse process of lumbar, patella cartilage, and entheseal cartilage of calcaneus of Scx\(^{Cre}\) KI neonates were smaller than that of Scx\(^{+}\) KI neonates (Fig. 4c–j).

Defective maturation of tendons, ligaments, and annulus fibrosus of intervertebral discs in Scx\(^{Cre}\) KI neonates. Tnmd is related to a cartilage-derived angiogenesis inhibitor gene product, chondromodulin (Chmd)\(^{27}\), and is a marker of mature tenocytes and ligamentocytes\(^{10,11,28}\). Tnmd expression is positively regulated by Scx. Scx was heterogeneously expressed in the developing leg tendons (Fig. 5a), whereas uniform expression of Tnmd was observed (Fig. 5b). Higher levels of Scx expression in the tendons were detected near the myotendinous junction (Fig. 5a). We confirmed inactivation of endogenous Scx expression in Scx\(^{Cre}\) at E15.5 by in situ hybridisation (Fig. 5c,d). As previously reported\(^{29}\), in Scx null mice, gene expression of Tnmd and type XIV collagen was not detectable in forelimb tendons. However, during postnatal growth, Tnmd expression in other dense connective tissues, such as ligaments and annulus fibrosus of intervertebral discs, has not been evaluated yet. Thus, we investigated localisation of Col1 and Tnmd in tendons and ligaments of Scx\(^{Cre}\) and Scx\(^{Cre}\);Rosa-Cre KI mice, by in situ hybridisation (Fig. 5e–h) and double immunostaining (Figs 6 and 7). In the hindlimb of a Scx\(^{Cre}\)
neonate, tendons and ligaments were positive for Col1 and Tnmd (Fig. 6a,c,e,g). In the knee joint of a homozygous ScxCre/CreKI neonate, patella ligament, anterior and posterior cruciate ligaments, and anchoring tendons were positive for Col1, but Tnmd expression was almost absent (Fig. 6b,d). Similarly, in the heel of a homozygous ScxCre/CreKI neonate, tendons and ligaments, including the Achilles and extensor digitorum longus tendons, were positive for Col1 but negative for Tnmd (Fig. 6f,h). The meniscus was positive for Col1 but negative for Tnmd at E16.5, despite finding tdTomato positive cells in the meniscus of ScxCre+/+; Rosa-tdTmato mice (Figs 2j and 6i,j). The outer annulus fibrosus of intervertebral discs in ScxCre/+ KI neonates and the tendinous region of the
Figure 2. Cre-mediated recombination in tendons, ligaments, and diaphragm of Scx<sup>Cre<sup>/</sup>+<sup>Rosa-tdTomato</sup></sup> mice. (a–g) Cre-mediated tdTomato expression in P0 (a–d) and P14 (e–g) Scx<sup>Cre<sup>/</sup>+<sup>Rosa-tdTomato</sup></sup> mice. Bright fluorescence was detected in leg tendons (a–c), tail tendons (d), back tendons (e), diaphragm (f), and costal tendons (g). (a–c) Lateral (a,c) and frontal (b) views of skinned whole mount leg of Scx<sup>Cre<sup>/</sup>+<sup>Rosa-tdTomato</sup></sup> mice. White and yellow arrowheads in (a–c) indicate tendons and ligaments of the hindlimb, respectively. Arrowheads in (d) indicate tail tendons. Asterisks in (d) indicate intervertebral region. (e–g) Bright and fluorescent images of dorsal view of lumbar tendons (e), ventral view of diaphragm (f), and lateral view of costal tendons (g) are shown. Arrowheads and asterisks in (e) indicate tendons and spinous processes, respectively. Arrows in (f) indicate central tendon of diaphragm. Arrowheads in (g) indicate ribs. Dotted line in (f) encloses the outer boundary of diaphragm. (h–k) Sagittal sections of knee joint (h,j,k) and vertebral column (i) prepared from Scx<sup>Cre<sup>/</sup>+<sup>Rosa-tdTomato</sup></sup> (h–j) and Scx<sup>Cre<sup>/</sup>+<sup>Cre</sup>; Rosa-tdTomato (k) neonates. An inset in (i) shows magnified image of ventral annulus fibrosus. Arrows in (h,j,k) indicate tdTomato-positive chondrocytes in patella, meniscus, and femur, respectively. acl, anterior cruciate ligament; At, Achilles tendon; cl, cruciate ligament; fe, femur; lu, lung; pa, patella; pcl, posterior cruciate ligament; me, meniscus; np, nucleus pulposus; nt, neural tube; pl, patella ligament; qft, quadriceps femoris tendon; ti, tibia; vb, vertebral body. Scale bars, 200μm (h–k); 100μm (inset in (i)).
Figure 3. Tendon deficiency in Scx\textsuperscript{Cre/Cre} KI mice. (a–h) Tendons and ligaments of 3-week-old Scx\textsuperscript{Cre/+} (a,c,e,g) and Scx\textsuperscript{Cre/Cre} (b,d,f,h) KI mice. Frontal views of knee (a,b), dorsal views of back (c,d), and lateral views of tail (e,f) and heel (g,h) are shown. A black and a white arrow in (a,b) indicate patella ligament and patella, respectively. Arrowheads in (a) indicate collateral ligaments. Asterisk in (a,b) indicates fat pad in the knee joint. Dotted line in (a,b) encloses the patella. Arrowheads in (c,d) indicate back tendons. Arrowheads in (e) indicate tail tendons. Asterisks in (e,f) indicate intervertebral region. At, Achilles tendon; pa, patella; pb, peroneus brevis tendon; pl, peroneus longus tendon.
diaphragm in wild type mice were also positive for Col1 and Tnmd (Fig. 7a,c,e), but Tnmd expression was almost absent in that of ScxCre/Cre KI mice (Fig. 7b,d,f). These results suggest that loss of Scx causes defective maturation of tendons, ligaments, and outer annulus fibrosus of intervertebral discs.

Defective maturation of knee joint of ScxCre/Cre KI mice. Tnmd expression, observed in cruciate ligaments of ScxCre+/+; Rosa-ttdTomato mice, was missing in ScxCre/Cre; Rosa-ttdTomato mice (Fig. 8a,b). In knee joints of 2-week-old ScxCre+/+ KI mice, tendons and ligaments developed well (Fig. 8c), whereas defective maturation was observed in knee joints of homozygous ScxCre/Cre KI mice (Fig. 8d). The size of the patella was also significantly small and enthesal cartilage was defective in a ScxCre/Cre KI mouse (Fig. 8d). To analyse endochondral ossification in the patella, immunostaining using antibodies against type II collagen (Col2), osteocalcin (Ocn), and sclerostin was performed. In a ScxCre+/+ mouse, a central part of the patella was vascularised to be replaced by bone stained with Ocn, but Col2-positive staining indicates the presence of cartilaginous matrix (Fig. 8e,f,h,i). At this stage, the patella of a ScxCre/Cre KI mouse was Col2-positive without any indication of vascularisation and ossification (Fig. 8g,j), suggesting a delay in the endochondral ossification of the patella. In the enthesal region of patella tendon and ligament of a ScxCre+/+ mouse, sclerostin-positive cells were observed in Col2- and Ocn-positive calcified fibrocartilage (Fig. 8e,f,h,i). Col2-positive unmineralised fibrocartilage was also present at an adjacent region in...
patella tendon and ligament of a Scx+/+ mouse (Fig. 8f). Such a structure was not observed in the ScxCre/Cre KI mouse. Taken together, Scx is also required for maturation of tendons, ligaments, and the patella.

Decreased Sox9 expression and phosphorylation of Smad1/5 and Smad3 in the developing enthesal cartilage, patella, and deltoid tuberosity of ScxCre/Cre KI mice. Enthesal cartilage around the tendon/ligament attachment sites and sesamoid bones such as patella are derived from Scx/Sox9 double positive progenitors. Blitz et al. reported that activation of TGF-β and BMP signaling is required for specification and differentiation of these progenitors, respectively. Thus, we examined how loss of Scx affects expression of Sox9 and activation of these signaling pathways in the developing forelimb and hindlimb of heterozygotes and homozygotes at E13.5. Activation of TGF-β and BMP signaling pathways was monitored by phosphorylation of Smad1/5 and Smad3 that are intracellular downstream mediators. As shown in Fig. 9, mature chondrocytes and progenitor cells of the patella and the deltoid tuberosity were visualized as Chmd positive.
and Sox9 positive regions (red), respectively. In a ScxCre/Cre KI mouse, Sox9 expression was markedly decreased in the developing patella and entheseal cartilage (Fig. 9b,h), compared with that in a ScxCre/+ KI mouse (Fig. 9a,g). Sox9 positive progenitors in the developing deltoid tuberosity of a ScxCre/+ KI mouse (Fig. 9g) were

Figure 6. Decreased expression of Tnmd in tendons and ligaments of ScxCre/Cre KI mice. (a–h) Double immunostaining of Chmd (red) and Tnmd (green) (a,b,e,f,i) or Col1 (green) (c,d,g,h,i) was performed in ScxCre/+ (a,b,e,g) and ScxCreCre (b,d,f,h,i) KI neonates and wild type (wt) embryos at E16.5 (i,j). Frozen sagittal sections of knee (a–d,i,j) and heel (e–h) are shown. Tnmd (a,e) and Col1 (c,g) were detected in tendons and ligaments of ScxCre/+; whereas Col1-positive tendons and ligaments of ScxCreCre (d,h) were negative for Tnmd (b,f). Arrows and arrowheads in (a–d) indicate tendons and ligaments respectively; whereas arrows in (i,j) indicate menisci; acl, anterior cruciate ligament; At, Achilles tendon; ca, calcaneus; EDL, extensor digitorum longus tendon; fe, femur; fi, fibula; me, meniscus; pa, patella; pcl, posterior cruciate ligament; pl, patella ligament; qft, quadriceps femoris tendon; ti, tibia. Scale bars, 200 μm.
absent in a ScxCre/Cre KI mouse (Fig. 9h). Similarly, phosphorylation of Smad1/5 and Smad3 observed in a ScxCre/+ KI mouse (Fig. 9c,e,i,k) was markedly decreased especially in the prospective patella and deltoid tuberosity of a ScxCre/Cre KI mouse (Fig. 9d,f,j,l).

Gene expression of Col14a1, Decorin, Mohawk, Tnmd and Egr1 in ScxCre/Cre KI mice. In addition, we investigated the expression of other tendon-associated genes such as Col14a1, Decorin (Dcn), Mohawk (Mkx), Tnmd, and Early growth response 1 (Egr1) in ScxCre/Cre KI embryos at E15.5. Col14a1 is a member of the fibril-associated collagens with interrupted triple helices (FACIT) collagen family. Dcn is a small leucine-rich proteoglycan involved in regulating collagen fibrillogenesis and Mkx is a family member of atypical homeobox genes that are expressed in developing tendons. Egr1 is reported to be a transcription factor regulating the expression of Col1a1 in tendon development. In a ScxCre/Cre KI mouse, Col14a1 expression was almost absent in the developing triceps brachii tendons (Fig. 10a,b). In a wild type mouse, we found expression of Dcn in the developing tendon, dermis, and soft connective tissues (Fig. 10c), which are consistent with previous findings. In ScxCre/Cre KI embryos, we found that Dcn expression was almost absent in triceps brachii tendons (Fig. 10d) and also found generally weak expression of Mkx (Fig. 10e,f). Around the pelvic bone and lumbar at E15.5, Tnmd expressing tendons were also positive for Egr1 (Fig. 10g,i). In ScxCre/Cre KI embryos, expression of Tnmd and Egr1 was lost and decreased in theses tendons, respectively (Fig. 10h,j). These findings suggest that loss of Scx affects the expression of genes related to tendon maturation.

Discussion
We successfully generated ScxCre KI mice that can be used as genetic tools to reveal in vivo function of Scx-expressing tissue domains integrating the musculoskeletal components. In ScxCre KI mice, Cre expression was driven by Scx promoter and endogenous Scx was inactivated. Cre-mediated recombination efficiency of ScxCre KI mice reflected the intensity and duration of endogenous Scx expression. Cre-mediated excision was mainly observed in domains with persistent Scx expression, such as tendons, ligaments, and annulus fibrosus of...
intervertebral discs. In homozygous Scx<sup>Cre/Cre</sup> KI mice, Tnmd, a marker of mature tenocytes and ligamentocytes, was almost absent. In addition, other tendon markers such as Col14a1, Den, Mkx, and Egr1 were downregulated in homozygotes. Moreover, regardless of the short duration of endogenous Scx expression, defective maturation was observed in Scx-expressing domains that are involved in integration of the musculoskeletal components.

Figure 8. Hypoplastic formation of patella and ligaments in knee joint of Scx<sup>Cre/Cre</sup> KI mice. (a,b) Frozen sagittal sections of anterior and posterior cruciate ligaments of Scx<sup>Cre+/+</sup>; Rosa-tdTomato (a) and Scx<sup>Cre/Cre</sup>; Rosa-tdTomato (b) are shown. Cre-mediated tdTomato expression was detected as red fluorescence (a,b). Tnmd-positive cells (green) were visualised by immunostaining using anti-Tnmd antibody. (c,d) Haematoxylin and eosin staining of sagittal sections of knee joint is shown. Paraffin decalcified specimens of 2-week-old Scx<sup>Cre+/+</sup> (c) and Scx<sup>Cre/Cre</sup> (d) KI mice were sliced. (e–j) Double immunostaining of sclerostin (red) and Col2 (green) (e–g) or Ocn (green) (h–j) was performed on frozen sagittal sections of patella prepared from 4-week-old wild type (e,f,h,i) and Scx<sup>Cre/Cre</sup> (g,j) KI mice. Boxed regions in (e,h) are shown at a higher magnification in (f,i), respectively. Arrows in (f) indicate Col2-positive uncalcified fibrochondrocytes. Dotted line in (e,h) encloses a bone region in the patella. Dotted line in (j) encloses patella. acl, anterior cruciate ligament; fe, femur; pa, patella; pcl, posterior cruciate ligament; pl, patella ligament; qfm, quadriceps femoris muscle; ti, tibia. Scale bars, 200 μm.
Figure 9. Decreased Sox9 expression and phosphorylation of Smad1/5 and Smad3 in the developing patella and deltoid tuberosity of ScxCre/Cre KI mouse. Frozen sagittal section of hindlimbs (a–f) and forelimbs (g–l) prepared from ScxCre+/+ (a,c,e,g,i,k) and ScxCre+/Cre (b,d,f,h,j,l) KI embryos at E13.5 are shown. (a–l) Double immunostaining of Chmd (green) and Sox9 (red) (a,b,g,h), p-Smad1/5 (red) (c,d,f,h,j), or p-Smad3 (red) (e,f,k,l). Arrows in (a,c,e,f) indicate developing patella. Arrows and arrowheads in (g–l) indicate the deltoid tuberosity and the insertion sites of tendon of supraspinous muscle, respectively. fe, femur; hu, humerus. Scale bar, 200 μm.
Scx is localised in the intron 3 region of Bop1 gene on chromosome 15. Bop1 and Scx are a pair of bidirectional overlapping coding genes related to cellular proliferation and differentiation, respectively. The first reported Scx knockout failed to form mesoderm and died during early stages of embryogenesis, probably because the neomycin phosphotransferase gene linked to phosphoglycerate kinase promoter (PGK-Neo) cassette affected the expression of Bop1. Later, Murchison et al. reported generation and characterisation of a conditional allele in the Scx locus with FRT-Neo cassette, by flanking the first exon, which includes most of the coding region, with loxP site. As was the case with homozygotes of Scx knockout with a Neo allele, we too failed to obtain viable ScxCreNeo homozygous pups, but we successfully established a new line of ScxCre KI mice by mating a ScxCreNeo heterozygote with a flippase-expressing FLPe mouse. Homozygous ScxCreCre KI mice with tendon defects were viable and similar to Scx knockout mice generated by Murchison et al., suggesting that the replacement of most of the exon 1 by Cre does not cause embryonic lethality, unlike as observed on retention of PGK-Neo cassette in this genomic region.

Figure 10. Expression of genes related to tendon formation in ScxCreCre KI mice. (a–j) In situ hybridisation of Col14a1 (a,b), Dcn (c,d), Mxk (e,f), Tnmd (g,h), and Egr1 (i,j) on sagittal sections of forelimbs (a–f) and frontal sections of trunks (g–j) prepared from Scx+/+ (a,c,e,g,i) and ScxCreCre (b,d,f,h,j) KI embryos at E15.5. Arrows in (a–f) indicate the triceps brachii tendon. Arrows in (g,i,j) indicate Tnmd (g) and Egr1 (i,j) positive tendons. Dotted lines in (a–j) enclose humerus, radius, ulna, and pelvic bone. hu, humerus; nt, neural tube; ra, radius; ul, ulna. Scale bars, 500 μm.
Cre-mediated recombination in a ScxCre<sup>Cre<sup>-/-</sup></sup>; Rosa-tdTomato mouse was less efficient than that in a ScxCre<sup>-/+</sup> or a ScxCre<sup>-H</sup> transgenic mouse mated with a Rosa-tdTomato reporter mouse. Under the control of the endogenous Scx promoter/enhancer in KI mice, Cre expression gradually increased to reach levels sufficient for recombination, thus enabling Cre-mediated tdTomato expression not in the Scx<sup>+/+</sup> progenitor cell population but in the differentiated cell populations of tendons, ligaments, the annulus fibrosus of the intervertebral discs, and patella cartilage. More tdTomato positive cells were observed in ScxCre<sup>Cre<sup>+/</sup>-</sup>; Rosa-tdTomato mice than Scx<sup>Cre<sup>Cre<sup>-/-</sup></sup>;</sup> Rosa-tdTomato, suggesting that the number of cells expressing Cre above the threshold increases in homozygotes due to a gene dosage effect. In the Cre-loxP system, recombination occurs only when Cre protein is accumulated above the recombination threshold. Mosaic and later Cre-mediated recombination is considered to be observed due to heterogeneity in the endogenous Scx mRNA levels and a narrow time window of Scx expression.

We previously reported that Scx positively regulates the expression of Tnmd in tenocytes both in vivo and in vitro<sup>16</sup>. Tnmd expression in Scx<sup>-</sup> positive cells in the periodontal ligament is also upregulated by lentiviral overexpression of Scx and downregulated by knockdown of endogenous Scx<sup>36</sup>. In this study, immunostaining of Scx<sup>Cre<sup>Cre</sup></sup> homozygotes with anti-Tnmd antibody revealed that Scx was necessary for the expression of Tnmd in most parts of the developing tendinous and ligamentous tissues. As previously reported, Tnmd acts as a positive regulator of postnatal tendon growth, maturation of collagen fibres, and cellular adhesion in the periodontal ligament<sup>16</sup>. Loss of Tnmd resulted in abated tenocyte proliferation leading to reduced tenocyte density, greater variation in collagen fibril diameters, and increase in maximal fibril diameters<sup>16</sup>. Thus, ablation of Tnmd expression in tendons, ligaments, and annulus fibrosus of intervertebral discs of homozygous KI mice suggests defective phenotypes in these tissues, other than the previously reported defects in force-transmitting and intermuscular tendons.

In the spinal column, an intervertebral disc lies between adjacent vertebrae and acts as a shock absorber. Intervertebral discs consists of inner and outer annulus fibrosus and nucleus pulposus. The inner annulus fibrosus has cartilaginous matrix associated with Col2 fibres, whereas the outer annulus fibrosus is thick multiple layers of dense connective tissue containing predominantly Col1. We previously reported that Scx<sup>+/Sox9<sup>+</sup></sup> progenitor population gives rise to both inner and outer annulus fibrosus and that Sox9 in this population is indispensable for the formation of inner annulus fibrosus<sup>16</sup>. In this study, we found that Tnmd expression in the outer annulus fibrosus was missing in ScxCre<sup>Cre<sup>+</sup>/KI</sup> neonates. Thus, we demonstrated, for the first time, that Scx is necessary for the maturation of outer annulus fibrosus.

Scx is also transiently expressed in Sox9- expressing entheseal and saemoid cartilage during early stages of chondrogenic differentiation, as previously reported<sup>1,3,37</sup>. This is consistent with our previous findings from lineage analysis using ScxCre transgenic mice that entheseal and patella chondroprogenitors were positive for Scx<sup>37</sup>. In the Scx-expressing domain, mice lacking Sox9 lose the cartilaginous domain that contributes to the establishment of tendon/ligament attachment sites<sup>5,4</sup>. In this study, we found that expression of Sox9 in Scx<sup>+/Sox9<sup>+</sup></sup> chondroprogenitors is markedly decreased. Even though Scx expression in Scx<sup>+/Sox9<sup>+</sup></sup> chondroprogenitors is only transient, loss of Scx caused defective formation of cartilaginous elements arising from the Scx<sup>+/Sox9<sup>+</sup></sup> progenitor population. Moreover, activation of BMP and TGF-β signalling pathways in these cartilaginous elements was diminished as evidenced by decreased phosphorylation of Smad1/5 and Smad3. These findings indicate that transient expression of Scx in chondroprogenitors is functionally important in the process of entheseal cartilage formation.

Muscle contractions in utero generate mechanical forces that are essential for normal embryonic development, through modulation of cell signalling and gene expression. As reported previously, Scx is the mechanical stress responsive gene that is upregulated in the periodontal ligament in response to tensile force and maintains its fibrogenic state by inhibiting mineralisation<sup>36</sup>. Conversely, removal of tensile force in the tendon results in a decrease in Scx expression<sup>16</sup>. The severe defective phenotypes, initially in force-transmitting and intermuscular tendons and later in ligaments of ScxCre<sup>Cre</sup> KI mice lacking Scx, raised the possibility that Scx expression in response to mechanical force during embryonic and postnatal growth might be required for the proper development and structural maintenance of these tissues. Further investigation of target genes regulated by tensile force-responsive Scx may reveal how Scx participates in the regulation of formation and maintenance of tendons and ligaments during development and growth, in the presence or absence of mechanical stress. Such studies are now underway.

Materials and Methods

Animals and embryos. Mice were purchased from Japan SLC (Shizuoka, Japan) or from Shimizu Laboratory Supplies (Kyoto, Japan). The generation and establishment of ScxGFP transgenic strains have been reported previously<sup>3</sup>. For analysis of Cre activity, Rosa-CAG-LSL-tdTomato (Rosa-tdTomato) mice obtained from Jackson laboratory were used<sup>36</sup>. Cre-mediated recombination was monitored by red fluorescence of tdTomato expression driven by CAG promoter. All animal experimental protocols were approved by the Animal Care Committee of the Institute for Frontier Life and Medical Sciences, Kyoto University and the Committee of Animal Experimentation, Hiroshima University, and conformed to institutional guidelines for the study of vertebrates. All methods were carried out in accordance with relevant guidelines and regulations.

Generation of ScxCre<sup>+</sup> KI mouse strain. The ScxCre<sup>Neo</sup> targeting vector was designed to insert the coding region of Cre, along with a translational stop codon followed by FRT-flanked PGK-Neo cassette, in place of the ATG codon of Scx coding region, with simultaneous deletion of most of the Scx exon 1 (Fig. 1a). For construction of the targeting vector, a fused fragment, containing 706 bp Scx genomic region and Cre (706ScxCre)<sup>3</sup>, was cut with BglII from pBluescript SK (+) (Stratagene). 706ScxCre was inserted into the BamHI site of pPE-7neoW-F2LF with PGK-Neo cassette flanked by two FRT sites, so that the PGK promoter was oriented in the opposite direction to the Scx promoter. The 486 bp Scx genomic fragment downstream of BglII site in the first exon and first intron was amplified by PCR using a forward primer with HindIII and BamHI sites (Scx-547F: 5'-AAGCTTGGATCCAGATCTGACCTTCTGCTCAG-3') and a reverse primer with HindIII and NolI sites
(Scx-int2R: 5′-AAGCTTGCGGCGCGGAGGCTCTGACGAC-3′). The BamHI site within Scx-547F was introduced for genotyping using Southern blot analysis. The HindIII-digested amplified fragment was inserted into the HindIII site of pPE7-neo-W-F2L. PKG-Neo cassette with homology arms was then used for modification of bacterial artificial chromosome clone (RP23-415D19, CHORI) by using Red/ET recombination system (Gene Bridges) to generate ScxBAC-CreNeo, in which Cre was introduced in-frame with ATG of Scx exon 1 and replaced with a 548 bp region. The approximately 0.5 kb homology arms, homologous to extreme 5′ and 3′ ends of the genomic region to be retrieved from ScxBAC-CreNeo, were amplified by PCR. The 5′ homology arm was amplified with forward primer (F1: 5′-AAGCTTGCGGCGCGGAGGCTCTGACGAC-3′) and reverse primer (R1: 5′-ATCGATCCTTTGAAACTACCAGCGCTCTAAG-3′). The 3′ homology arm was amplified with forward primer (F2: 5′-ATCGATCAGACCGGCAGCTGCTGTGTTG-3′) and reverse primer (R2: 5′-CTCGAGCCACATGGCCACTTCCTCTTCTCACT-3′). The result amplified 5′ and 3′ homology arms were subcloned into HindIII-ClaI and ClaI-XhoI sites of pMCS-DTA, respectively. For generation of the ScxCreNeo targeting vector, the modified genomic region with 1.7 kb short and 6.3 kb long arms was retrieved from ScxBAC-CreNeo and transferred to pMSC-DTA. The PacI-linearised ScxCreNeo targeting vector was electroporated into K11.1 ES cell line (F1 hybrid of C57BL/6J and 129S6/ScEvTac)23. Total 151 ES cell clones were obtained by positive and negative selection and then screened by PCR using forward primer (F3: 5′-GAGTCCTCTGCGGCCAGGTCTAG-3′) and reverse primer (Cre-R1: 5′-CTTGGACAGCTCATCAGTTGTGCATG-3′) (Fig. 1a). We obtained one correctly targeted ES clone that was microinjected into the blastocysts of BDF1 hybrid to generate chimeric mice.

Genotyping. Mice were genotyped using tail tip genomic DNA by PCR or Southern blot analysis. The wild type Scx allele was detected with forward primer (F4: 5′-CTGGTGTTGAGGCTGTGGG-3′) and reverse primer (R3: 5′-AGGCTCTTGTGCACATGATG-3′). The ScxCre allele was detected with forward primer (Cre-F1: 5′-TCAATTACCTGACCAGCTCACA-3′) and reverse primer (Cre-R2: 5′-CCTGATCCTGCGCAATTTGCTC-3′) (Fig. 1a). Southern blot analysis, 10 μg genomic DNA was digested with HindIII and the following DNA fragments were separated using 0.7% agarose gel electrophoresis. After transfer onto Nytran membranes with Turboblotter system (Schleicher and Schuell), hybridisation was performed using probes labelled with [α-32P]- deoxyctydine triphosphate (dCTP) (PerkinElmer).

Northern blot analysis. Total RNA was extracted from wild type and ScxCre/KI embryos at E12.5. Fifteen micrograms of the total RNA was denatured with 6% formaldehyde, fractionated using 1% agarose gel electrophoresis, and transferred onto Nytran membranes with Turboblotter system. A specific cDNA probe for Scx was labelled with [α-32P]- dCTP. Hybridisations were performed as previously described21.

Histology. ScxCre/KI, Rosa-ttdTomato and ScxCreCre; Rosa-ttdTomato, and ScxCreCre; Rosa-ttdTomato neonates were fixed in 4% paraformaldehyde dissolved in phosphate-buffered saline (PFA/PBS) at 4 °C for 3 h, immersed in 20% sucrose/PBS, frozen, and cryosectioned to a thickness of 8 μm. After washing with PBS, nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma). Cre-mediated tdTomato expression was detected with a fluorescent microscope. For histological evaluation of knee joint during postnatal growth, paraffin sections prepared from 2-week-old ScxCre/KI and ScxCreCre KI mice were rehydrated and stained with haematoxylin and eosin. The images were captured under the Leica DMRXA microscope equipped with the Leica DC500 camera (Leica Microsystems).

Immunostaining. ScxCre/KI, ScxCreCre/KI, ScxCre/KI, ScxCreCre; Rosa-ttdTomato, and ScxCreCre; Rosa-ttdTomato neonates were immersed in 20% sucrose/PBS, frozen, and cryosectioned to a thickness of 8 μm. Sections were fixed in ice-cold acetone prior to double immunostaining of Chmd/Col1, Chmd/Tnmd, and myosin heavy chain (MHC)/Tnmd. For double immunostaining of Chmd and Sox9, p-Smad1/5 or p-Smad3, ScxCre/KI and ScxCreCre KI embryos were fixed in 4% PFA/PBS at 4 °C for 3 h, immersed in 20% sucrose/PBS, frozen, and cryosectioned to a thickness of 6 μm. For undecalced sections, 4-week-old Scx+/+ and ScxCreCre KI mice were anasthetised to perfuse with 4% PFA/PBS containing 20% sucrose. The legs were dissected, fixed in 4% PFA/PBS containing 20% sucrose for 3 h, embedded in SCEM (Leica Microsystems), frozen, and cryosectioned to a thickness of 4 μm, according to Kawamoto’s film method39. The sections were decalcified with 0.25 M EDTA/PBS for 60 min and processed for immunostaining. After washing with PBS, the sections were incubated with 2% skim milk in PBS for 20 min and then incubated overnight at 4 °C with primary antibodies diluted with 2% skim milk in PBS. The sections were incubated with appropriate secondary antibodies conjugated with Alexa Fluor 488 or 594 (Life Technologies) and washed again with PBS. The primary antibodies used were anti-Tnmd (diluted 1:100)22, anti-Col1 (1:500; Rockland), anti-Chmd (diluted 1:500; Cosmos Bio), anti-Col2 (diluted 1:500; Rockland), anti-Ocn (1:800; TαAra), anti-sclerostin (1:500; R&D), anti-MHC (1:400; sigma), anti-Sox9 (1:800; Millipore), anti-p-Smad1/5 (1:50; Cell Signaling), and anti-p-Smad3 (1:250; Rockland). Nuclei were counterstained with DAPI. Images were captured using either a Leica DMRXA microscope equipped with a Leica DC500 camera (Leica Microsystems) or an Olympus IX70 microscope equipped with an Olympus DP80 camera (Olympus).

In situ hybridisation. Antisense RNA probes for each gene were transcribed from linearised plasmids with a digoxigenin (DIG) RNA labelling kit (Roche) as previously described40. For RNA probes, the cDNAs for Col1a1, Dcn, Mkx, and Egr1 were amplified by RT-PCR using the following primers (mCol1a1_F1: 5′-CCCTCAAGAGCCTGACGACGAC-3′), (mCol1a1_R1: 5′-GTATTCTAGAGTACTCGTCTGACGAC-3′), (mDcn_F1: 5′-TGTTGCTGTTCTGATCGTCG-3′), (mDcn_R1: 5′-GACTCACAAGCGGAGGATAGAAGC-3′), (mMkx_F1: 5′-GGGGCCCGCCACATGACACACATCG-3′), (mMkx_R1: 5′-GGGGCCCGCTGAGAAGGCGCTGCACACGACGACGAC-3′), (mEgr1_F1: 5′-TCTCTTCTTACCACCCATGCCAGTCG-3′), and (mEgr1_R2: 5′-TAACTGAAAAGGTATACGATTATAACCATCG-3′). RNA probes of Scx and Tmtd were described previously2,20.
Mouse Col14a1 cDNA was also amplified using primers described previously. For in situ hybridisation on frozen sections, mouse embryos were fixed with 4% PFA/PBS for 3 h, and treated in 20% sucrose before embedding in Tissue-Tek OCT compound (Sakura Finetek). Seven-micron-thick frozen sections of embedded embryos were prepared and postfixed with 4% PFA/PBS for 10 minutes at room temperature and carboxyethoxylated twice in 0.1% DEPC/PBS. Sections were treated in 5 × SSC, and hybridisation was performed at 58 °C with DIG-labelled antisense RNA probes. To detect DIG-labelled RNA probes, immunological detection was performed using an anti-DIG antibody conjugated with alkaline phosphatase (Anti-DIG-AP Fab fragment, Roche) and BM purple (Roche). Images were captured using an IX70 microscope equipped with a DP80 camera (OLYMPUS).

**Skeletal preparation.** Mouse neonates were dehydrated with ethanol. After removing skin and soft tissues, the neonates were stained with 0.015% Alcian blue 8GX (Sigma) and cleared with 2% potassium hydroxide. The neonates were then stained with 0.05% Alizarin Red S (Wako) in 1% KOH and cleared with 1% KOH.

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**Author Contributions**

Y.Y. and C.S. designed the study. Y.Y., A.T., H.W., and G.K. performed the experiments. Y.Y., Y.H., and C.S. analysed the results. Y.Y. and C.S. prepared the manuscript.

**Additional Information**

**Competing Interests:** The authors declare no competing financial interests.

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