Title
Scx[+]/Sox9[+] progenitors contribute to the establishment of the junction between cartilage and tendon/ligament

Author(s)
Sugimoto, Yuki

Citation
Kyoto University (京都大学)

Issue Date
2014-01-23

URL
https://doi.org/10.14989/doctor.r12802

Type
Thesis or Dissertation

Textversion
ETD

Kyoto University
Contribution of the $\text{Scx}^{+}$/Sox9$^{+}$ progenitor cell population to the establishment of the chondro-tendinous/ligamentous junction

Yuki Sugimoto$^{1}$, Aki Takimoto$^{1}$, Haruhiko Akiyama$^{2}$, Ralf Kist$^{3}$, Gerd Scherer$^{4}$, Takashi Nakamura$^{2}$, Yuji Hiraki$^{1}$, and Chisa Shukunami$^{1*}$

$^{1}$Department of Cellular Differentiation, Institute for Frontier Medical Sciences, Kyoto University, Kyoto 606-8507, Japan.
$^{2}$Department of Orthopaedics, Faculty of Medicine, Kyoto University, Kyoto 606-8507, Japan.
$^{3}$Centre for Oral Health Research, School of Dental Sciences, Newcastle University, Newcastle upon Tyne, United Kingdom.
$^{4}$Institute of Human Genetics, Faculty of Medicine, University of Freiburg, Freiburg, Germany.

Running title: Role of Sox9 in the enthesis

Key words: Sox9; Scx; tenocytes; ligamentocytes; chondrocytes.

$^{*}$Address correspondence to: Chisa Shukunami, D.D.S., PhD, 53 Shogoin-Kawahara-cho, Sakyo-ku, Kyoto, 606-8507, Japan.
Tel & Fax: +81-75-751-4633; E-mail: shukunam@frontier.kyoto-u.ac.jp
SUMMARY

Sox9 and Scleraxis (Scx) regulate cartilage and tendon formation, respectively. Here we report that Scx+/Sox9+ progenitors differentiate into chondrocytes and tenocytes/ligamentocytes to form the primordial enthesis, the junction between hyaline cartilage and tendon/ligament. Sox9 lineage-tracing in the Scx+ domain revealed that Scx+ progenitors can be subdivided into two distinct populations with regard to their Sox9 expression history, i.e. Scx+/Sox9+ and Scx+/Sox9− progenitors. Tenocytes are derived from Scx+/Sox9+ and Scx+/Sox9− progenitors. The closer the tendon is to the cartilaginous primordium, the more tenocytes arise from Scx+/Sox9+ progenitors. Ligamentocytes and annulus fibrosus cells of the intervertebral discs are descendants of Scx+/Sox9+ progenitors. Conditional inactivation of Sox9 in Scx+/Sox9+ cells causes defective formation of the enthesal cartilages, tendons, ligaments, and the annulus fibrosus of the intervertebral discs. Thus, the Scx+/Sox9+ progenitor pool is a unique multipotent cell population that gives rise to tenocytes, ligamentocytes, and chondrocytes for the establishment of the chondro-tendinous/ligamentous junction.
INTRODUCTION

In vertebrates, the coordinated body movement is ensured by a close functional and physical association of bones, muscles, tendons, and ligaments. Tendons connect muscles to the skeletal components and function as the force transmitters, while ligaments bind bones together to stabilize joints (Benjamin and Ralphs, 2000; Rumian et al., 2007). Cells in tendons and ligaments are categorized as special types of fibroblasts known as tenocytes and ligamentocytes (Benjamin and Ralphs, 2000). Unlike randomly distributed fibroblasts in loose connective tissues, tenocytes and ligamentocytes in dense connective tissues are highly organized and align in rows between parallel thick fibers mainly consisting of type I collagen that provides the major resistance to tensile forces (Amiel et al., 1984; Canty et al., 2004). By inserting dense regular type I collagen fibers into muscle from the myotendinous junction and into bone from the osteo-tendinous/ligamentous junction called the enthesis, tendons and ligaments integrate each musculoskeletal component to establish a locomotive organ as one functional unit (Benjamin and Ralphs, 1998; Benjamin and Ralphs, 2000). To achieve this integration, progenitors for these cells need to be coordinately distributed at both sides of the junction and then execute each differentiation program there. However, it is still not clear how the coordinated assembly of the skeletal element and/or the muscle component through the tendon/ligament is established during development.

Progenitor cells for tendons, ligaments, cartilage, and bone arise from the sclerotome, the lateral plate mesoderm, and the neural crest (Akiyama et al., 2005; Christ et al., 2004; Mori-Akiyama et al., 2003; Smith et al., 2005), whereas myogenic
progenitors are derived from the myotome (Brent and Tabin, 2002). During the early stages of the musculoskeletal development, these progenitor populations migrate and settle down to the prospective region to give rise to cartilage, muscle, tendon, and ligament primordium (Kardon, 1998). Each primordium for the musculoskeletal component initially develops as an individual unit but later integrated with each other by a previously unknown mechanism.

Sox9, a SRY-related transcription factor containing a high-mobility-group box DNA-binding domain, is an important regulator for cartilage formation. In Sox9-deficient chimeric embryos generated by the injection of Sox9−/− embryonic stem (ES) cells into Sox9+/+ blastocysts, Sox9−/− cells are eliminated from cartilaginous primordia and are instead incorporated into the surrounding connective tissues (Bi et al., 1999). Conditional inactivation studies of Sox9 using Prx1Cre or Col2a1Cre mice have revealed that Sox9 is required for multiple steps of chondrogenic differentiation before and after cartilaginous condensation (Akiyama et al., 2002). In the tendon and ligament cell lineage, Scleraxis (Scx), a basic helix-loop-helix transcription factor, is persistently expressed throughout differentiation (Pryce et al., 2007; Schweitzer et al., 2001). In Scx−/− mice, the intramuscular and force-transmitting tendons in the limbs and tail tendons become hypoplastic, although the short appendicular anchoring tendons and ligaments are not significantly affected (Murchison et al., 2007). Such differential dependence on Scx expression suggests that tendons consist of distinct cell populations that have not been defined yet.

At the early stages of the musculoskeletal development, both Sox9 and Scx are detected in the subpopulation of tendon/ligament progenitors and chondroprogenitors
(Akiyama et al., 2005; Brent et al., 2005; Sugimoto et al., in press). Sox9 is upregulated during chondrogenesis (Zhao et al., 1997), but its expression is downregulated in association with the formation of the cruciate ligaments of the knee joint, Achilles tendon, and patella tendon (Soeda et al., 2010). On the other hand, Scx expression in cartilaginous primordia is transient during chondrogenesis (Cserjesi et al., 1995; Sugimoto et al., in press). Lineage analysis using ScxCre Tg crossing with reporter mice revealed that Scx positive chondroprogenitors differentiate into chondrocytes around the chondro-tendinous/ligamentous junction (CT/LJ) during mouse development (Sugimoto et al. in press). These lines of evidence suggest that Scx and Sox9 expression is coordinately regulated in the cell population bridging between cartilage and tendon/ligament. However, very little is known about the cellular origin or molecular mechanism to regulate the formation of the junction between cartilage and tendon/ligament.

Through the detailed Sox9-lineage tracing in Scx+ cells, we found that the Scx+ cell population can be divided into two distinct populations with or without their Sox9 expression history, i.e. Scx+/Sox9- and Scx+/Sox9+ progenitors. Tenocytes are derived from both Scx+/Sox9- and Scx+/Sox9+ progenitors, while ligamentocytes arise from Scx+/Sox9+ progenitors. Chondrocytes around the CT/LJ are descendants of Scx+/Sox9+ progenitors. The closer the tendon is to the cartilaginous primordium, the more tenocytes arise from Scx+/Sox9+ progenitors. Using loss-of-function approaches, we demonstrate that Scx+/Sox9+ progenitors functionally contribute to the establishment of the junction between hyaline cartilage and tendon/ligament.
MATERIALS AND METHODS

Animals and embryos

Mice were purchased from Japan SLC, Inc. (Shizuoka, Japan) or from Shimizu Laboratory Supplies co. Ltd. (Kyoto, Japan). ROSA26R (R26R) (Soriano, 1999) or Rosa-CAG-LSL-tdTomato (Ai14) (Madisen et al., 2010) strains were crossed to generate the Sox9Cre/+;R26R and Sox9Cre++;Ai14 mice for Sox9 lineage tracing. Ai14 mice harbor a targeted mutation of the Gt(ROSA)26SOR locus with a loxP-flanked STOP cassette preventing transcription of a CAG promoter-driven red fluorescent protein variant, tdTomato. Generation of ScxGFP and ScxCre transgenic strains has been previously reported (Sugimoto et al., in press). To generate Sox9 conditional knock-out mice, Sox9-flox (Kist et al., 2002) and ScxCre transgenic strains were crossed. All of the animal experimental procedures used in this study were approved by the Animal Care Committee of the Institute for Frontier Medical Sciences, Kyoto University and conformed to institutional guidelines for the study of vertebrates.

In situ hybridization

The antisense RNA probes for each gene were transcribed from the linearized plasmids with a digoxygenin (DIG) RNA labeling kit (Roche) as previously described (Takimoto et al., 2009). For RNA probes, the cDNAs for Scx and Myog were amplified by RT-PCR based on its sequence information in GenBank (Scx, S78079; Myog, BC068019). Mouse Sox9 cDNA was previously described (Wagner et al., 1994). For frozen section in situ hybridization, mouse embryos are treated in 20% sucrose without any fixation and then embedded in Tissue-Tek OCT compound (Sakura...
Finetek). The embedded embryos were sliced at 8 µm thickness. Frozen sections were postfixied with 4% paraformaldehyde dissolved in phosphate-buffered saline (PFA/PBS) for 10 minutes at room temperature and then carbethoxylated twice in the 0.1% DEPC/PBS. Sections were treated in 5 x SSC, and hybridization was performed at 58˚C with DIG labeled antisense RNA probes. For whole mount in situ hybridization, mouse embryos were fixed with 4% PFA/PBS overnight. After fixation, embryos were gradually dehydrated with methanol. Prior to hybridization, embryos were rehydrated with PBS and treated with 2-5 µg/ml of proteinase K (Roche) at 25˚C. Hybridization was performed at 65˚C with DIG labeled antisense RNA probe for Tnmd. To detect DIG labeled RNA probes, immunological detection was performed with an anti-DIG antibody conjugated with alkaline phosphatase (Anti-DIG-AP Fab fragment; Roche) and BM purple (Roche).

**Immunostaining**

Embryos were fixed with 4% PFA/PBS at 4˚C for 3 hours, immersed in a series of sucrose solutions (12%, 15%, and 18% sucrose/PBS), frozen, and cryosectioned at a thickness of 8 µm. For Sox9<sup>Cre/+;R26R</sup> mice, specimens were treated with 20% sucrose at 4˚C for 3 hours without prefixation, cryosectioned at a thickness of 10 µm, and then fixed with ice-cold acetone. After washing with PBST (phosphate-buffered saline containing 1% Tween 20), the slides were incubated with 2% skim milk in PBST for 20 min and incubated overnight at 4˚C with primary antibodies diluted with 2% skim milk in PBST. After washing, the sections were incubated with goat anti-rat and anti-rabbit secondary antibodies conjugated to Alexa Fluor 488 or with goat anti-rabbit
and anti-mouse secondary antibodies conjugated to Alexa Fluor 594, and washed again in PBST. The primary antibodies used were anti-GFP (diluted 1:1000; Nakarai), anti-Sox9 (diluted 1:600; Chemicon), anti-Tnmd (diluted 1:1000) (Oshima et al., 2004; Shukunami et al., 2008), anti-Chm1 (diluted 1:1000), and anti-type I collagen (diluted 1:500; Rockland), respectively. The purified ChM-I MoAb (clone: hCHM-05) is commercially available (Cosmo Bio Co., Ltd., Tokyo, Japan). Nuclei were counterstained with 4',6-Diamidino-2-phenylindole (DAPI). The images were captured under a Leica DMRXA microscope equipped with a Leica DC500 camera (Leica Microsystems, Wetzlar).

**Toluidine blue and X-gal staining**

For toluidine blue staining, deparaffinized and/or hydrated sections were stained with a 0.05% toluidine blue solution (pH 4) for 2 to 5 min as previously described (Takimoto et al., 2012). For X-gal staining, embryos were treated with 20% sucrose/PBS at 4°C, and embedded in Tissue-Tek OCT compound (Sakura Finetek). Frozen sections were prepared at a thickness of 14 µm. Before staining, the sections were treated with fixation solution (0.2% glutaraldehyde, 5 mM EGTA, and 2 mM MgCl₂) at 4°C for 5 min. After washing (phosphate buffer containing 2 mM MgCl₂, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40), the sections were incubated with X-gal staining solution (5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 1 mg/ml X-gal) at 37°C overnight.
Skeletal preparations

After fixation with 4% PFA/PBS, mouse embryos were dehydrated with ethanol. Skin and soft tissues were removed, and the embryos were then stained with 0.015% Alcian blue 8GX (Sigma). After clearing with 2% KOH, the embryos were stained with 0.05% alizarin red in 1% KOH and then cleared with 1% KOH.

RESULTS

The Scx\(^+\) cell population in the axial and the appendicular mesenchyme contains two distinct subpopulations: Scx\(^+\)/Sox9\(^+\) and Scx\(^+\)/Sox9\(^-\) progenitors. Scx is expressed in the tendogenic/ligamentogenic regions as well as the chondrogenic regions (Cserjesi et al., 1995; Sugimoto et al., in press). In situ hybridization analysis revealed that Scx\(^+\)/Sox9\(^+\) chondrogenic cells are predominantly distributed in and around the primordial enthesis between cartilage and tendon/ligament (Fig. S1). To compare the expression domains of Sox9 in Scx\(^+\) cells in more detail, we established a transgenic mouse line expressing enhanced green fluorescent protein (EGFP) under the control of promoter and enhancers of mouse Scx (ScxGFP) (Sugimoto et al., in press) and performed double immunostaining using antibodies against Sox9 and GFP in transgenic ScxGFP embryos (Fig. 1).

During axial musculoskeletal development, the paraxial mesoderm separates into the somites that eventually give rise to the vertebrae, ribs, tendons, ligaments, the dermis of the dorsal skin, and muscles (Christ et al., 2004; Christ et al., 2000). In the thoracic somite at E10.5, Sox9 was expressed in the entire sclerotome, notochord, and neural tube (Fig. 1A), while the dorsolateral sclerotome containing tendon
progenitors was composed of Scx$^+$ cells (Fig. 1B). The dorsolateral sclerotome was positive for Sox9, but a small number of Scx$^+$/Sox9$^+$ and Scx$^+$/Sox9$^-$ cells were observed in the dermomyotome (Fig. 1C). At E11.5, Scx$^+$/Sox9$^+$ cells were observed in the vertebral and rib primordia (Fig. S2A,B), and Scx$^-$/Sox9$^+$ cells were surrounded by Scx$^+$/Sox9$^+$ and Scx$^-$/Sox9$^-$ cells (Fig. S2C). At E13.5, Sox9 was detected in the cartilaginous primordia of the vertebral body, neural arch, and ribs (Fig. 1D). In contrast, Scx was exclusively expressed in the vertebral and costal tendon primordia (Fig. 1E). As typically seen in the costal region, Sox9 and Scx exhibited non-overlapping expression patterns at this stage (Fig. 1F).

Appendicular and abdominal muscles are derived from the hypaxial myotome, whereas lateral plate mesoderm gives rise to the skeletal elements, tendons, and ligaments of limbs (Brent and Tabin, 2002). At E10.5, the overlapping expression of Sox9 and Scx was observed in the limb bud mesenchyme except for the distal region (Fig. 1G-I). In the forelimb at E11.5, the primordia of the radius, ulna, carpal, and metacarpal bone express Sox9. Scx$^+$/Sox9$^+$ or Scx$^-$/Sox9$^-$ cells then rearrange into the dorsal and ventral superficial regions surrounding the Sox9$^+$ region (Fig. S2D-F). The Scx$^+$/Sox9$^+$ region was observed at the most proximal region (Fig. S2F). At E13.5, the appendicular cartilaginous elements were single positive for Sox9 (Fig. 1J), but the collateral ligaments and the interzone of the metacarpophalangeal joint were double-positive for Sox9 and Scx (Fig. 1K). At E14.5, Scx$^+$/Sox9$^+$ and Scx$^-$/Sox9$^-$ cells were present in the cartilage of the nasal septum and the fibrous cells of the turbinate primordia, respectively (Fig. 1L). In the vertebral column, the outer layer of the intervertebral discs was shown to be Scx$^+$ (Fig. 1M). Scx$^+$/Sox9$^+$ chondrogenic cells
were found in the entheseal region of the Achilles tendon and the patella (Fig. 1N,O).

Based on these data, we conclude that the Scx$^+$ cell population can be subdivided into two distinct subpopulations: Scx$^+/Sox9^+$ and Scx$^+/Sox9^-$ progenitors, and that Sox9 expression later disappeared in tendons and ligaments as differentiation process proceeds.

The Scx$^+/Sox9^+$ progenitor pool is a unique multipotent cell population that gives rise to chondrocytes, tenocytes, and ligamentocytes.

For lineage-tracing of Sox9$^+$ cells in the Scx$^+$ domains during tendon and ligament formation, we crossed Sox9$^{Cre/+}$ mice (Akiyama et al., 2005) with the Cre reporter line Rosa-CAG-LSL-tdTomato (Ai14) (Madisen et al., 2010) to generate Sox9$^{Cre/+};Ai14$ mice, which were then crossed with ScxGFP mice to obtain Sox9$^{Cre/+};Ai14;ScxGFP$ embryos (Fig. 2A-E, Fig. 3A-G).

In Sox9$^{Cre/+};Ai14;ScxGFP$ embryos at E14.5, cells with the Sox9$^+$ cell lineage were found in the tendons near the vertebral columns, ribs, joints between the ribs and vertebrae, and the developing lung (Fig. 2A). The outer fibrous region of the vertebrae and surrounding membranous regions consisted of Scx$^+$ cells with their Sox9 expression history (Fig. 2B). In the tendinous diaphragm near the heart, most cells were negative for Sox9 and single-positive for Scx (Fig. 2C). Abdominal tendons were single positive for Scx (Fig. 2D). In the tail, the insertion sites of the tendons into the vertebrae were derived from Scx$^+/Sox9^+$ progenitors, whilst tendons located further away from vertebrae were almost exclusively Sox9-negative and Scx single-positive (Fig. 2E).
We then analyzed the contribution of Sox9⁺ progenitors in the lumbar vertebrae and their associated tendons/ligaments of Sox9<sup>Cre/+;Ai14</sup> neonates at the level of the vertebral body, the articular process, or the spinous process (Fig. 2F-H, supplementary material Table S1). At P0, tendons and ligaments in the vicinity of vertebrae were derived from the Sox9⁺ progenitor population (Fig. 2F-H). The lateral region of the thoracolumbar fascia enclosing the erector spinae muscles and tendons anchoring the latissimus dorsi muscle were negative for Sox9 at P0 (Fig. 2H, T4, T3). Thus, Scx⁺/Sox9⁺ progenitors contribute to the formation of ligaments and tendons in the vicinity of ribs and vertebrae, while abdominal tendons are derived from the Scx⁺/Sox9⁻ cell lineage.

In the distal part of the hindlimb of Sox9<sup>Cre/+;Ai14;ScxGFP</sup> embryos at E14.5, the ligaments arose from Scx⁺/Sox9⁺ progenitors, but both Scx⁺/Sox9⁺ and Scx⁺/Sox9⁻ progenitors contributed to tendon formation (Fig. 3A). Scx⁺/Sox9⁺ progenitors contributed to the formation of collateral ligaments (Fig. 3B, L3) and tendons near the entheses (Fig. 3C,D), whereas other parts of tendons mainly arose from Scx⁺/Sox9⁻ progenitors and the proportion of these cells varied between the individual tendons. For instance, extensor digitorum longus tendon was derived from Scx⁺/Sox9⁻ progenitors except for the prospective enthesis (Fig. 3B, T8), while Achilles tendons arose from both the Scx⁺/Sox9⁺ and Scx⁺/Sox9⁻ cell lineage (Fig. 3C-D, T9).

In the knee joint at E14.5, the primordia for cruciate and patella ligaments were visible as the Scx⁺ region within the prospective joint cavity (Fig. 3E). All of the articular components and cartilage were positive for Sox9 (Fig. 3F). The developing cruciate ligaments and capsular ligaments including the patella ligament and tibial
collateral ligament were Sox9-positive (Fig. 3G). In cruciate and patella ligaments at
P0, Sox9 protein was no longer detected in these tendons or ligaments except for
cartilage (Fig. 3H,I). Thus, all appendicular ligamentocytes arise from Scx+/Sox9+
progenitors, whereas appendicular tenocytes are derived from both the Scx+/Sox9+
and the Scx+/Sox9− cell lineage. Taken together with these findings, the Scx+/Sox9+
progenitor pool is a unique multipotent cell population that gives rise to Scx+/Sox9+
chondrocytes and Scx+/Sox9− tenocytes/ligamentocytes.

The closer the tendon is to the cartilaginous primordium, the more tenocytes
arise from the Sox9+ cell lineage.

To investigate how Sox9+ progenitors contribute to limb tendon formation, we
analyzed the distribution of cells with the Sox9+ cell lineage in Tnmd-positive mature
tendons and Chondromodulin-1 (Chm1)-positive mature cartilage in the forearm of the
Sox9Cre/++;R26R mice at P0 (Fig. 4A-L, supplementary material Table S1). Chm1 and
Tnmd are markers of mature chondrocytes and tenocytes/ligamentocytes,
respectively (Oshima et al., 2004; Shukunami et al., 2008; Shukunami et al., 2006).

Within the proximal parts of the ulna and radius, the sheet-like anchoring tendons
consisted of tenocytes derived from Sox9+ progenitors (Fig. 4A,G). In contrast, at the
medial side of the ulna and radius, Sox9+ progeny was not present in the proximal
region of the cord-like force-transmitting tendons that were inserted into the individual
muscles (Fig. 4B,H). However, tenocytes derived from Sox9+ progenitors were found
in the bundled force-transmitting tendons in the dorsal region of the distal ulna and
radius and of the carpal levels (Fig. 4C,D,I,J). The forearm at the wrist level can be
subdivided into several extensor tendon compartments with thick fascia. Within the same compartment, each tendon was derived from Sox9+ and Sox9 progenitors, and the ratio of Sox9+ to Sox9 progenitor-derived tenocytes was similar (Fig. 4I,J). In carpal tendons, more tenocytes derived from Sox9+ progenitors were observed (Fig. 4D,J). Tendons containing many Sox9+ cells were inserted into the proximal edges of the metacarpals or carpals, whereas tendons containing less or no Sox9+ cells were inserted into the middle or distal phalanges, in the more distal region of the autopod (Fig. 4D,J).

Around the metacarpal level, bundled tendons separate into individual tendons that insert into the end point of each digit (Fig. 4E,F). More tenocytes of the Sox9+ cell lineage were observed in the tendons at the palmar side including tendons of the flexor digitorum profundus (T26), flexor digitorum sublimis (T27), and interosseous (T30) (Fig. 4K,L), whilst most dorsal tendons (T18, T19) were negative for Sox9 (Fig. 4E,K). In the collateral ligaments (L9) of the metacarpophalangeal joint, all ligamentocytes were strongly positive for Sox9 (Fig. 4F,L). Although the force transmitting tendons were derived from both Sox9+ and Sox9 progenitors, the anchoring tendons near the elbow wholly arose from Sox9+ progenitors (Fig. 4M). Taken together, the proportion of tenocytes with their Sox9+ expression history varies between the individual force-transmitting tendons but generally, the number of these Sox9+ tenocytes decreases with increasing distance from the skeletal element.

Characterization of the transitional zone between cartilage and tendon/ligament.
We then focused our analysis on the transitional zone between cartilage and tendon/ligament, to reveal the contribution of Sox9⁺ progenitors to the entheses. Entheses are classified into two groups: fibrous and fibrocartilaginous entheses (Benjamin and Ralphs, 2001). Collagen fibers in the fibrous entheses are inserted into bone via the periosteum which gives firmer hold to the tendons and ligament, while the fibrocartilaginous entheses have four zones during the transition from tendon/ligament to bone, consisting of tendon/ligament, fibrocartilage, mineralized fibrocartilage, and bone. Fibrous entheses are mainly present in short ligament or tendons. Since periosteum has been reported to be derived from Sox9⁺ progenitors (Akiyama et al., 2005), we examined the prospective fibrocartilaginous entheses of quadriceps femoris tendon, cruciate ligaments, and the Achilles tendon in Sox9Cre/+;R26R mice (Fig. 5). Type I collagen (Col1) and Chm1 were localized to tendons/ligaments including the prospective enthesal region and hyaline cartilage, respectively (Fig. 5A,D,G), whilst Tnmd was expressed in tendons and ligaments except for the region just adjacent to hyaline cartilage (Fig. 5B,E,H). These Col1+/Tnmd- cells were X-gal positive (Fig. 5C,F,I). Hence, near the joint region, tenocytes, ligamentocytes, and chondrocytes were derived from Sox9+ progenitors, but the prospective enthesal region abutting hyaline cartilage was negative for both Tnmd and Chm1, suggesting the presence of a distinct population in the prospective fibrocartilaginous enthesis bridging between tendons/ligaments and hyaline cartilage.

**Skeletal defects by conditional inactivation of Sox9 in Scx⁺/Sox9⁺ cells.**

We have previously reported generation of two lines of transgenic mouse lines that
express Cre recombinase in the Scx+ domains at high (ScxCre-H) or low (ScxCre-L) levels (Sugimoto et al., in press). Due to the expression gradient and transient expression of Scx around the entheseal cartilage (Fig. S1), more chondrocytes in ScxCre-H are Scx+ than those in ScxCre-L (Sugimoto et al., in press). To investigate the functional role of Sox9 in Scx+/Sox9+ cells by a loss of function approach, we crossed these lines with Sox9-flox mice to inactivate Sox9 in Scx+ cells (Kist et al., 2002). Both ScxCre-L;Sox9flox/+ and ScxCre-H;Sox9flox/+ mice were viable and fertile, but ScxCre-L;Sox9flox/flox and ScxCre-H;Sox9flox/flox mice died after birth. In ScxCre-H;Sox9flox/flox mice, severe skeletal hypoplasia were observed beyond the prospective entheseal cartilage, thus causing the secondary defects in tenocytes derived from Scx+/Sox9- cells (not shown). Thus, we analyzed ScxCre-L;Sox9flox/flox mice with the skeletal defects around the entheseal cartilage in more detail.

In ScxCre-L;Sox9flox/flox neonates, sternum and the ribcage except for the proximal region are missing (Fig. 6A-D). In the vertebral column of ScxCre-L;Sox9flox/flox at E18.5, the vertebral bodies, the intervertebral discs, the articular processes of the neural arch, the transverse processes were hypoplastic (Fig. 6E,F). Severe hypoplasia in the ribcage is expected from the expression of Scx in the early stages of costal cartilage formation (Fig. 6M-O). The appendages of ScxCre-L;Sox9flox/flox are shorter and hypoplastic than that of control and the joint cavity was smaller (Fig. 6A,B, G-L). In the forelimb of ScxCre-L;Sox9flox/flox, hypoplasia of carpal bones at the ulnar side, elbow joint, cartilage around the shoulder joint, deltoid tuberosity of the humerus was evident and the curvature of the wrist was observed (Fig. 6G,H). Interestingly, abnormal mineralization occurred in the olecranon (Fig. 6G,H). In the hindlimb of
ScxCre-L;Sox9\textsuperscript{floxflox}, tarsal bones, cartilage around the hip and the knee joint, and tibial tuberosity were defective (Fig. 6I,J) and the patella within the patella ligament was missing (Fig. 6K,L). Thus, these results suggest that skeletal dysplasia is observed in the Scx\textsuperscript{+} cartilaginous region that is closely associated with tendons and ligaments.

**Defective formation of the junction bridging between cartilage and tendon/ligament by conditional inactivation of Sox9 in Scx\textsuperscript{+}/Sox9\textsuperscript{-} cells.**

Double immunostaining of Tnmd and Chm1 revealed defective formation of the junction between cartilage and tendon/ligament in ScxCre-L;Sox9\textsuperscript{floxflox} at E18.5. Transverse process of the lumber vertebrae (Fig. 7C) and the lateral region of sacral vertebrae (Fig. 7A) provide the attachment sites for axial tendons, but these sites are missing in ScxCre-L;Sox9\textsuperscript{floxflox} (Fig. 7B,D). In control mice, Sox9\textsuperscript{+} cells are scattered in the outer annulus fibrosus near the inner annulus fibrosus (Fig. 7E), while these cells are missing in ScxCre-L;Sox9\textsuperscript{floxflox} (Fig. 7F). In the intervertebral discs of ScxCre-L;Sox9\textsuperscript{floxflox}, formation of the inner annulus fibrosus showing metachromatic staining with toluidine blue was defective, but the outer annulus fibrosus became wider (Fig. 7H), compared to control mice (Fig. 7G). Thus, in ScxCre-L;Sox9\textsuperscript{floxflox}, the prospective entheses were either missing or hypoplastic in the axial skeleton.

In the appendicular skeleton, hypoplastic tendon formation in association with severe defects in skeletal formation at the ulnar side (Fig. 6G,H) was observed in ScxCre-L;Sox9\textsuperscript{floxflox} at E18.5 (not shown). In the knee joint, patella and the frontal region of the femoral condyle are missing (Fig. 7I,J). In the heel, the attachment site
for the Achilles tendon was defective (Fig. 7K-N). Interestingly, cells just adjacent to the tendon attachment site are positive for Sox9 in control mice, whereas such Sox9+ cells were missing in ScxCre-L;Sox9^{flx/flx} (Fig. 7O,P).

DISCUSSION
In our current study, we have demonstrated for the first time that the Scx+ cell population can be subdivided into two distinct populations with regard to their Sox9 expression history, i.e. Scx+/Sox9+ and Scx+/Sox9- progenitors. The Scx+/Sox9+ progenitor pool is a unique multipotent cell population that gives rise to Scx+/Sox9+ chondrocytes and Scx+/Sox9- tenocytes/ligamentocytes (Fig. 8A). The closer the tendon and cartilage are to the prospective enthesis, the more tenocytes and chondrocytes originate from Scx+/Sox9+ progenitors (Fig. 8B). Further analyses of ScxCre;Sox9^{flx/flx} mice revealed that the Scx+/Sox9+ cell population functionally contribute to the establishment of the junction between cartilage and tendon/ligament.

The Scx+/Sox9+ progenitor pool is a multipotent cell population that gives rise to tenocytes, ligamentocytes, annulus fibrosus cells of intervertebral discs, and chondrocytes.

Tenocytes are descendants of Scx+/Sox9+ and Scx+/Sox9- progenitors (Fig. 8A). In general, the number of tenocytes with their Sox9 expression history decreases with increasing distance from the skeletal element. Ligamentocytes and annulus fibrosus cells in intervertebral discs are derived from Scx+/Sox9+ progenitors, whereas chondrocytes are derived from Scx+/Sox9+ and Scx+/Sox9+ progenitors (Fig. 8A). The
closer the cartilage is to the prospective entheses, the more chondrocytes arise from \( Scx^+ / Sox9^+ \) progenitors. Thus, the \( Scx^+ / Sox9^+ \) progenitor cell population is predominantly distributed across the enthesis to form the chondro-tendinous/ligamentous junction during development (Fig. 8B).

In contrast to the axial tendon formation, very little is known about the axial ligament formation. We show that the \( Scx^+ \) axial ligaments are derived from the \( Sox9^+ \) cell lineage. Since axial ligamentocytes are derived from the \( Sox9^+ \) cell lineage, we conclude that these \( Scx^+ \) ligamentocytes originate from the \( Sox9^+ \) sclerotome. However, the timing of \( Scx \) expression in \( Sox9^+ \) ligament progenitors needs to be further investigated to clarify whether the axial ligament progenitors are derived from the \( Scx^+ / Sox9^+ \) dorsolateral domain of the sclerotome or are recruited from the \( Scx^+ \) sclerotome to express \( Scx \) at later stages of development.

The appendicular tendons include a considerable number of tenocytes derived from \( Scx^+ / Sox9^+ \) progenitors, particularly in the distal part of the limbs. Unlike the sclerotome consisting of \( Sox9^+ \) progenitors, both \( Sox9^+ \) and \( Sox9^- \) progenitors are present in the lateral plate mesoderm of limb bud at E10.5. The \( Scx^+ / Sox9^- \) population in the lateral plate mesoderm may represent the prospective distal tendon progenitors, although we cannot exclude the possibility that another as-yet-unknown populations of tendon progenitors are recruited from the surrounding tissue to become \( Scx^+ \) tenocytes later in development.

\( Scx^+ / Sox9^+ \) progenitor cells contribute to the establishment of the CT/LJ.

In \( ScxCre;Sox9^{lox/lox} \) mice, the attachment sites of tendons/ligaments into
cartilaginous primordia and annulus fibrosus of intervertebral discs between vertebrae are severely impaired. The most notable phenotype of ScxCre;Sox9^{flox/flox} embryos is an absence of the ribcage. Chondrogenic cells in the developing costal cartilage have the ability to differentiate into entheseal chondrocytes as evidenced by the expression of Scx in the entire rib cartilaginous primordium. This is compatible with the histological feature that costal chondrocytes are located very close to the tendinous attachment site of the surrounding intercostal muscle to each rib cartilage. Likewise, patella cartilage embedded in the tendon and cartilaginous long bone primordia such as ulna and fibula with their attachment site of the interosseus membrane and anchoring tendons are missing. Thus, our loss of function analysis of Sox9 in the Scx^{+} domain revealed the functional significance of the Scx^{+}/Sox9^{+} progenitor cell population in the establishment of the chondro-tendinous/ligamentous junction, especially in the cartilaginous side.

In Sox9^{flox/flox};Prx1-Cre mice, inactivation of Sox9 in limb bud mesenchyme causes complete absence of cartilage and bone (Akiyama et al., 2002). Severe chondrodysplasia also occurs in Sox9^{flox/flox};Col2a1-Cre by inactivation of Sox9 in precartilaginous condensing cells and chondrocytes (Akiyama et al., 2002). Based on these findings, functional roles of Sox9 in chondrogenesis could be discussed at three critical stages: chondroprogenitor stage, cartilaginous condensation stage, and chondrocyte stage. Similarly, we propose to discuss tendo/ligamentogenesis in three distinct stages: tendon/ligament progenitor stage, tendon/ligament primordium formation stage, and tenocyte/ligamentocyte stage. In ScxCre;Sox9^{flox/flox}, we observed severe hypoplasia of the entheses of tendons/ligaments, annulus fibrosus of
intervertebral discs, and cartilages arising from Scx+/Sox9+ chondroprogenitors. The longer Sox9 expression continues, the severer the defects within the Scx+/Sox9+ domain of ScxCre;Sox9\textsuperscript{flox/flox} embryos become. Unlike chondrogenic cells, which continuously express Sox9, Sox9 was downregulated in the migrating tendon/ligament progenitors before their arrival at the presumptive tendon- or ligamento-forming site. Considering the timing of Sox9 downregulation in the tendon/ligament cell lineages, it is unlikely that the last two stages during tendo/ligamentogenesis critically depend on the function of Sox9. Loss of Sox9 in Scx+/Sox9+ progenitors is likely a main cause of the hypoplastic chondro-tendinous/ligamentous junction in ScxCre;Sox9\textsuperscript{flox/flox}.

Intervertebral discs and joints connect adjacent vertebrae. Each intervertebral disc is composed of an external annulus fibrosus surrounding the internal nucleus pulposus. Cells in the annulus fibrosus of intervertebral discs can be traced back to somitocoele cells included in the central core of the somite, distinct from the progenitor population for the vertebral body (Mittapalli et al., 2005). The annulus fibrosus consists of the inner annulus with chondrocytic cells and the outer annulus with tenocytic cells. We have shown here that both types of cells arise from Scx+/Sox9+ progenitors. In ScxCre;Sox9\textsuperscript{flox/flox} mice, the inner annulus is missing but expansion of the outer annulus takes place on the ventral side. Thus, it is suggested that Sox9 maintains the proper balance between the inner and the outer cell numbers by regulating survival and differentiation of cells in the inner annulus during intervertebral disc formation.

During postnatal growth, entheseal fibrocartilage develops in response to
compressive loads (Benjamin and Ralphs, 1998). Fibrocartilage is an important connective structure between tendon and hyaline cartilage, but its cellular origin remains uncertain. We show that Tnmd and Chm1 are expressed in tendon/ligament and hyaline cartilage, respectively, whereas the transitional region just adjacent to hyaline cartilage or tendon/ligament was negative for Tnmd and Chm1, consistent with our previous observation in rabbits (Yukata et al., 2010). Our lineage analysis further revealed that cells in this Tnmd-/Chm1- zone are positive for Sox9 and Scx. Therefore, it is likely that cells in this transitional zone give rise to fibrochondrocytes during the postnatal development. Further studies to reveal the cellular origin of fibrochondrocytes are now underway by using Sox9CreERT2 and ScxCreERT2 mice.

Acknowledgements

We thank Mr. T. Matsushita and Ms. K. Kogishi for the histological studies and Ms. H. Sugiyama for her valuable secretarial help. This study was partly supported by Grants-in-Aid from the Ministry of Education, Culture, Sport, Science and Technology of Japan.

The abbreviation used are: Scx, Scleraxis; Sox9, Sry-box containing gene 9; Tnmd, Tenomodulin; Chm1, Chondromodulin1; Myog, Myogenin.

References

Akiyama, H., Chaboissier, M. C., Martin, J. F., Schedl, A. and de Crombrugghe, B. (2002). The transcription factor Sox9 has essential roles in successive steps of the


**Murchison, N. D., Price, B. A., Conner, D. A., Keene, D. R., Olson, E. N., Tabin, C.**


Smith, T. G., Sweetman, D., Patterson, M., Keyse, S. M. and Munsterberg, A. (2005). Feedback interactions between MKP3 and ERK MAP kinase control scleraxis
expression and the specification of rib progenitors in the developing chick somite.  


**Zhao, Q., Eberspaecher, H., Lefebvre, V. and De Crombrugghe, B.** (1997).
FIGURE LEGENDS

Fig. 1. Distribution of Sox9+ and Sox9- cells in the Scx+ region of mouse embryos.

(A-O) In ScxGFP transgenic mouse embryos, Sox9+ (red) and Scx+ cells expressing GFP (green) were detected by double immunostaining with antibodies specific for Sox9 and GFP, respectively and nuclei were stained with DAPI (blue). Transverse sections of the thoracic vertebrae at the forelimb level at E10.5 (A-C) and those at the interlimb level on E13.5 (D-F) are shown. Frontal sections of the forelimb at E10.5 (G-I) and sagittal sections of the forelimb at E13.5 (J,K) are also shown. Sagittal sections of the nasal region (L), vertebral column (M), the knee joint (N), and the heel (O) at E14.5 are shown. An arrow in (A-F) indicates the notochord. (C,F,I,J-O) Merged images are presented. Arrowheads in (C) indicate the dorsolateral sclerotome expressing both Sox9 and Scx. An arrowhead and an asterisk in (F) indicate a Scx+ tendon and a Sox9+ rib, respectively. The dotted line in (D), (E), and (F) indicate dorsal root ganglion (drg). Arrowheads in (I) indicate a Scx+/Sox9+ region at the proximal part of the forelimb. Arrowheads in (J) and asterisks in (K) indicate Scx+/Sox9+ regions in the prospective joints of the forelimb. Arrowheads in (M) indicate Scx+ intervertebral regions visualized by GFP expression. An arrow in (N) indicates the developing cruciate ligaments. Femur, tibia, and patella are enclosed by the dotted line. At, Achilles tendon; ca, calcaneus; drg, dorsal root ganglion; fe, femur; me, metacarpal; nc, notochord; ns, nasal septum; nt, neural tube; pa, patella; ph, phalanx; ra, radius; ri, rib; ti, tibia; ul, ulna; vb, vertebral body. Scale bars: 50 μm (K); 100 μm (O); 200 μm (A-C,G-I,L-N); 280 μm (D-F); and 300 μm (J).
Fig. 2. Contribution of Sox9\textsuperscript{+} progenitors to the axial tendon and ligament formation.

(A-E) Transverse or sagittal sections were prepared from Sox9\textsuperscript{Cre/+};Ai14;ScxGFP embryos. Cells with the Sox9\textsuperscript{+} cell lineage were detected by tdTomato reporter expression. Scx\textsuperscript{+} cells were detected with anti-GFP antibody. (A) A transverse section of the trunk at the thoracic level. Arrowheads indicate the axial tendons associating with vertebrae and ribs. (B) A sagittal section of the vertebral column. The developing intervertebral discs are enclosed by the dotted line. Arrowheads, arrows, and asterisks indicate the intervertebral annulus fibrosus, anterior longitudinal ligament, and nucleus pulposus, respectively. (C) The diaphragm is shown. Arrows in (D) indicate the abdominal tendon in the body wall. (E) The sagittal section of tail is shown. Arrows and arrowheads indicate tendons associated and not associated with the vertebrae in the developing tail, respectively. Rostral and caudal sides are indicated by r and c, respectively. (F-H) Frozen frontal sections of the vertebral columns were prepared from Sox9\textsuperscript{Cre/+};Ai14 newborns. Sections at the vertebral bodies (F), the articular process (G), and the spinous process (H) are shown. Sox9\textsuperscript{+} cells were detected through the expression pattern of tdTomato (red). Tnmd was visualized by immunostaining with specific antibodies (green). Arrows in (F-H) and arrowheads in (G) and (H) indicate tendons and ligaments associated with the lumbar vertebral column, respectively. ap, articular process; dp, diaphragm; ht, heart; lu, lung; na, neural arch; nt, neural tube; ri, rib; sp, spinous process; st, sternum; vb, vertebral body. Scale bars: 200 μm.
Fig. 3. Contribution of the \textit{Scx}^+/\textit{Sox9}^+ cell lineage to the formation of appendicular tendons around the developing entheses and ligaments.

(A-D) Distribution of \textit{Scx}-expressing tendons and ligaments (GFP, green) with a \textit{Sox9} expression history (tdTomato, red) were analyzed in a \textit{Sox9}^{Cre/+};\textit{Ai14};\textit{ScxGFP} mouse embryo at E14.5. Arrows and arrowheads indicate tendon and ligaments, respectively. (A) A lateral view of the hindlimb is presented. (B-D) Sagittal sections of the hindlimb are shown. The developing digit with the prospective digital joints is shown in (B). White and yellow arrows in (C) indicate the force-transmitting and the anchoring tendons at the lower leg, respectively. The boxed region in (C) is shown at a higher magnification in (D). (E-I) Sagittal sections of the knee joint prepared from a \textit{Sox9}^{Cre/+};\textit{Ai14};\textit{ScxGFP} embryos at E14.5 (E-G) or a \textit{Sox9}^{Cre/+};\textit{Ai14} newborn mice (H,I) are shown. Developing cartilaginous primordia of the femur and tibia are enclosed by the dotted line. \textit{Scx}^+ cells (E,G, green) and \textit{Sox9}^+ cells (I, green) were detected by immunostaining with GFP and \textit{Sox9} antibodies, respectively. Cells derived from \textit{Sox9}^+ progenitors were detected via the expression of tdTomato (F,G,H, red). Arrowheads in (H) and (I) indicate ligaments of the knee joint. ca, calcaneus; d4, digit 4; d5, digit 5; fe, femur; fi, fibula; me, metacarpal; ph, phalanx; ti, tibia. Scale bars: \(200 \, \mu\text{m}\).

Fig. 4. Distribution of tenocytes derived from the \textit{Sox9}^+ cell lineage in the forearm and digits.

(A-L) Transverse sections of the forearm prepared from a \textit{Sox9}^{Cre/+};\textit{R26R} neonates
are shown. Tnmd\(^+\) (green) and Chm1\(^+\) (red) regions were visualized by double-immunostaining (A-F). Descendants of Sox9\(^+\) progenitors were visualized by X-gal staining (G-L). Black or white arrowheads in (A-L) indicate tendons of the forearm. A yellow arrowhead in (B), (H), (F), and (L) indicates ligaments of the forearm. The boxed regions in (E) and (K) are shown at a higher magnification in (F) and (L), respectively. The region enclosed by a white and a black line in (D) and (J) indicates the carpals. The region enclosed by a red dotted line in (C), (D), (I), and (J) indicates the tendons T21 and T22 as defined in Table S1. The region enclosed by a white or a black dotted line in (B-D) and (H-J) indicates tendons T19 and T25 as defined in Table S1. (M) Schematic illustration of the distribution of tenocytes or ligamentocytes with a Sox9\(^+\) lineage on the dorsal side of the mouse forearm. Bones (white), muscles (pink), and extensor tendons (light green) are shown. The dark blue color indicates tenocytes and ligamentocytes with a Sox9\(^+\) lineage. ca, carpal bones; hu, humerus; me, metacarpal; ra, radius; ul, ulna. Scale bars: 200 μm.

**Fig. 5. Contribution of Sox9\(^+\) progenitors to enthesis formation.**

(A-I) Sagittal frozen sections of the patella (A-C), the knee joint (D-F), the heel joint (G-I), were prepared from Sox9\(^{Cre/++;R26R}\) newborn mice. Col1\(^+\) (green in A,D,G), Tnmd\(^+\) (green in B,E,H), and Chm1\(^+\) (red in A,B,D,E,G,H) regions were visualized by double-immunostaining. The distribution of X-gal-positive cells with a Sox9\(^+\) lineage in the patella (C), the knee joint (F), and the heel joint (I) is also shown. Arrowheads in (D-F) indicate ligaments in the knee joint. Arrowheads and arrows in (G-J) indicate the Achilles tendon (T9) and the superficial digital flexor tendon (T10), respectively. Yellow
arrows in (B), (E), and (H) indicate the Tnmd/Chm1 double-negative region at the developing enthesis of the cruciate ligaments (L5 and L6 in E), the Achilles tendon (T9 in H) and the patella ligament (L4 in B), respectively. Abbreviations: ca, calcaneus; fe, femur; pa, patella; ti, tibia; tl, talus.; Scal bars: 200 μm.

Fig. 6. Skeletal abnormalities by the loss of Sox9 in Scx⁺/Sox9⁺ cells.

(A,B) Lateral views of skeletal preparations of control (A), and ScxCre-L;Sox9floxflox embryos (B) at P0. (C-F) Dorsal views of the ribcage (C,D) and the vertebral bodies of the lumbar vertebrae (E,F) of control (C,E) and ScxCre;Sox9floxflox at P0 (D,F). Arrows in (E) indicate the transverse process. Asterisks in (E) and (F) indicate intervertebral discs of the lumbar vertebrae. (G-L) Appendicular skeletons of control (G,I,K), and ScxCre-L;Sox9floxflox at P0 (H,J,L). Dorsal view of the forelimb (G,H) and lateral view of the hindlimb (I-L) are shown. The elbow joint (G,H), the calcaneous (I,J), and the patella (K) are indicated by an arrow. The dotted line in (K) and (L) encloses the epiphysis of the femur, the tibia, and the patella. (M-P) In situ hybridization of Scx (M,P), Sox9 (N), and Myog (O). Frozen sagittal sections are prepared from wild type embryos at E11.5 (M-O) and E13.5 (P). (M-O) Expression of Scx (M), Sox9 (N), and Myog (O) in the costal region at E11.5 is shown. Scx is detected in Sox9⁺ rib primordia (arrows in M) and in the intercostal region including Myog⁺ muscle primordia (O). (P) Expression of Scx in the costal region at E13.5 is shown. Arrows in (P) indicate the Scx⁺ costal tendons. Ribs are enclosed by the dotted line. cl, clavicle; fe, femur; fi, fibula; hu, humerus; pa, patella; ra, radius; sc, scapula; ti, tibia; ul, ulna; vb, vertebral body. Scale bars: 200 μm.
Fig. 7. Defective formation of the junction between cartilage and tendon/ligament by the loss of Sox9 in Scx+/Sox9+ cells.

(A-F) Frontal sections of the sacral (A,B) and the lumbar (C-F) vertebrae of control (A,C,E) and ScxCre;Sox9^floxfloxflox at E18.5 (B,D,F). Tnmd+ (green) and Chm1+ (red) regions were visualized by double-immunostaining (A-D). The transverse processes in control are indicated by arrows in (A) and (C), but those of ScxCre;Sox9^floxfloxflox are missing as indicated by arrows in (B) and (D). Asterisks in (A-D) indicate the nucleus pulposus of the intervertebral discs. Sox9+ cells are visualized by immunostaining (E,F). Arrows in (E) indicate Sox9+ cells in the outer annulus fibrosus of control mice. The dotted line in (E) or (F) encloses the outer annulus fibrosus. (G,H) Toluidine blue staining of the sagittal sections of the lumbar vertebrae of control (G) and ScxCre;Sox9^floxfloxflox at E18.5 (H). Asterisks in (G) and (H) indicate the nucleus pulposus of the intervertebral disc. Black and gray boxes show width of the inner (in) and the outer (out) annulus fibrosus of the intervertebral disc, respectively. Red bars show width of the vertebral body regions. An arrow indicates intervertebral region. (I-P) Sagittal sections of the hindlimb of control (I,K,M,O) and ScxCre;Sox9^floxfloxflox E18.5 (J,L,N,P). Tnmd+ (green) and Chm1+ (red) regions were visualized by double immunostaining (I,K,J,L). Knee joint (I,J) and the attachment site of the Achilles tendon to the calcaneus (K,L) are shown. Arrows in (K) and (L) indicate the junction between hyaline cartilage and the Achilles tendon. Toluidine blue staining (M,N) and Sox9+ (red) region visualized by immunostaining (O,P) are shown. The dotted line in (O) or (P) encloses the calcaneus and the Achilles tendon. Arrows in (O) indicate
Sox9+ entheseseal cells just adjacent to the hyaline cartilaginous calcaneus. Acl, anterior cruciate ligament; At, Achilles tendon; ca, calcaneus; fe, femur; in, inner annulus fibrosus; out, outer annulus fibrosus; pa, patella; pcl, posterior cruciate ligament; pl, patella ligament; qft, quadriceps femoris tendon; vb, vertebral body; ti, tibia; sdf, superficial digital flexor tendon. Scal Bars: 100 μm (G,H,M-P), 200 μm (A-F,I-L).

**Fig. 8. Establishment of the junction between cartilage and tendon/ligament along the Scx/Sox9 axis.**

(A) Schematic representation depicting differentiation of the tendogenic, ligamentogenic, and chondrogenic cell lineages along the Scx/Sox9 axis. The differentiation pathways of Scx+/Sox9+ chondroprogenitors (CP), Scx+/Sox9+ teno-/ligamento-/chondro-progenitors (TLCP), and Scx+/Sox9+ tenoprogenitors (TP) are shown. (B) Schematic illustration of the establishment of the chondro-tendinous/ligamentous junction (CTJ/CLJ) to form the osteo-tendinous/ligamentous junction (OTJ/OLJ). Scx+/Sox9+ progenitors give rise to the primordial CTJ/CLJ. The established CTJ/CLJ further develops to form the OTJ/OLJ during postnatal growth. Sox9 and Scx expression levels are shown by the dark gray-colored and light gray-colored regions, respectively.

**Fig. S1. Predominant distribution of Scx+/Sox9+ cells around the primordial enthesis, the transitional zone from tendon/ligament to cartilage.**

(A-G) In situ hybridization of Scx (A,D,E,G), Sox9 (B,F), and Myog (C). Frozen sagittal
sections are prepared from wild type embryos at E13.5 (A-F) and E16.5 (G). Expression of Scx, Sox9, or Myog in the forelimb (A-D) and hindlimb (E-G). An arrowhead in (A) indicates the expression of Scx at the myotendinous junction. Arrows in (A) and (B) indicate the insertion sites of tendons of triceps brachii muscles into the olecranon. Humerus and ulna are enclosed by the dotted line (C). Arrows in (D) indicate the Scx+ ligaments of the digit. Arrows in (E) and (F) indicate the Scx+/Sox9+ epiphyseal regions. (G) Expression of Scx in the knee joint at E16.5 is shown. Femur, tibia, and patella are enclosed by the dotted line. An arrow in (G) indicates cruciate ligament. ca, calcaneus; fe, femur; fi, fibula; hu, humerus; pa, patella; pl, patella ligament; qtf, quadriceps femoris tendon; ti, tibia; ul, ulna. Scale bars: 200 µm.

**Fig. S2. Distribution of Sox9+ and Sox9- cells in the Scx+ region of ScxGFP mouse embryo at E11.5.**

(A-F) In ScxGFP transgenic mouse embryos, Sox9+ (red) and Scx+ cells that are labeled by GFP (green) were detected by double immunostaining with antibodies specific for Sox9 and GFP, respectively; nuclei were stained with DAPI (blue). Merged images are presented in (C) and (F). Transverse section of a thoracic level (A-C) and forelimb (D-F) of ScxGFP mouse are shown. Arrows in (A)-(C) indicate notochord. Arrowheads in (C) and (F) indicate Scx+/Sox9+ region in the proximal rib (C) and proximal forelimb (F), respectively. The dotted line in (A) and (B) indicate dorsal root ganglions. drg, dorsal root ganglion; nc, notochord; nt, neural tube; ri, rib. Scale bars: 200 µm.
### Fig. 1

<table>
<thead>
<tr>
<th></th>
<th>Sox9</th>
<th>ScxGFP</th>
<th>Sox9/ScxGFP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E10.5</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>B</td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>C</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
</tr>
<tr>
<td><strong>E13.5</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
<tr>
<td>E</td>
<td><img src="image13.png" alt="Image" /></td>
<td><img src="image14.png" alt="Image" /></td>
<td><img src="image15.png" alt="Image" /></td>
</tr>
<tr>
<td>F</td>
<td><img src="image16.png" alt="Image" /></td>
<td><img src="image17.png" alt="Image" /></td>
<td><img src="image18.png" alt="Image" /></td>
</tr>
<tr>
<td><strong>E10.5</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td><img src="image19.png" alt="Image" /></td>
<td><img src="image20.png" alt="Image" /></td>
<td><img src="image21.png" alt="Image" /></td>
</tr>
<tr>
<td>H</td>
<td><img src="image22.png" alt="Image" /></td>
<td><img src="image23.png" alt="Image" /></td>
<td><img src="image24.png" alt="Image" /></td>
</tr>
<tr>
<td>I</td>
<td><img src="image25.png" alt="Image" /></td>
<td><img src="image26.png" alt="Image" /></td>
<td><img src="image27.png" alt="Image" /></td>
</tr>
<tr>
<td><strong>E13.5</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J</td>
<td><img src="image28.png" alt="Image" /></td>
<td><img src="image29.png" alt="Image" /></td>
<td><img src="image30.png" alt="Image" /></td>
</tr>
<tr>
<td>K</td>
<td><img src="image31.png" alt="Image" /></td>
<td><img src="image32.png" alt="Image" /></td>
<td><img src="image33.png" alt="Image" /></td>
</tr>
<tr>
<td>L</td>
<td><img src="image34.png" alt="Image" /></td>
<td><img src="image35.png" alt="Image" /></td>
<td><img src="image36.png" alt="Image" /></td>
</tr>
<tr>
<td>M</td>
<td><img src="image37.png" alt="Image" /></td>
<td><img src="image38.png" alt="Image" /></td>
<td><img src="image39.png" alt="Image" /></td>
</tr>
<tr>
<td>N</td>
<td><img src="image40.png" alt="Image" /></td>
<td><img src="image41.png" alt="Image" /></td>
<td><img src="image42.png" alt="Image" /></td>
</tr>
<tr>
<td>O</td>
<td><img src="image43.png" alt="Image" /></td>
<td><img src="image44.png" alt="Image" /></td>
<td><img src="image45.png" alt="Image" /></td>
</tr>
</tbody>
</table>
Fig. 2

**Sox9^{Cre/+};Ai14;ScxGFP (E14.5)**

**Sox9/ScxGFP**

**Tnmd/Sox9**

(A) Sox9^{Cre/+};Ai14;ScxGFP (E14.5)

(B) Sox9/ScxGFP

(C) Tnmd/Sox9

(D) Sox9^{Cre/+};Ai14 (P0)

(E) Sox9/ScxGFP

(F) Tnmd/Sox9

(G) Sox9^{Cre/+};Ai14 (P0)

(H) Sox9/ScxGFP

Fig. 3

Sox9<sup>Cre</sup>+/Ai14;ScxGFP (E14.5)

Sox9/ScxGFP

A

B

C

D

E

F

G

H

I

ScxGFP

Sox9

Sox9/ScxGFP

Sox9

Sox9/Cre+/Ai14 (P0)
Fig. 4

**Sox9^{Cre/+};R26R (P0)**

<table>
<thead>
<tr>
<th>Tnmd/Chm1</th>
<th>Sox9^{lineage}</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong></td>
<td></td>
</tr>
<tr>
<td><strong>B</strong></td>
<td></td>
</tr>
<tr>
<td><strong>C</strong></td>
<td></td>
</tr>
<tr>
<td><strong>D</strong></td>
<td></td>
</tr>
<tr>
<td><strong>E</strong></td>
<td></td>
</tr>
<tr>
<td><strong>F</strong></td>
<td></td>
</tr>
<tr>
<td><strong>G</strong></td>
<td></td>
</tr>
<tr>
<td><strong>H</strong></td>
<td></td>
</tr>
<tr>
<td><strong>I</strong></td>
<td></td>
</tr>
<tr>
<td><strong>J</strong></td>
<td></td>
</tr>
<tr>
<td><strong>K</strong></td>
<td></td>
</tr>
<tr>
<td><strong>L</strong></td>
<td></td>
</tr>
</tbody>
</table>

- **Fig. 4**: Extensor tendons in forearm
  - **Autopod**
  - **Zeugopod**
  - **Stylopod**

- **A, B, C, D, E, F, G, H, I, J, K, L**: Images showing Sox9^{lineage} and Tnmd/Chm1 expression at ulna and radius level, carpal level, and metacarpal level.
Fig. 5

**Sox9\textsuperscript{Cre+};R26R (P0)**

<table>
<thead>
<tr>
<th>Patella</th>
<th>Knee joint</th>
<th>Heel joint</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>D</td>
<td>G</td>
</tr>
<tr>
<td>B</td>
<td>E</td>
<td>H</td>
</tr>
<tr>
<td>C</td>
<td>F</td>
<td>I</td>
</tr>
</tbody>
</table>

- **Col1/Chm1**
- **Tnmd/Chm1**
- **Sox9\textsuperscript{+} lineage**
Fig. 7

ScxCre-\textsuperscript{-}L; Sox9\textsuperscript{flox/flox} Control Tnmd/Chm1

ScxCre-\textsuperscript{-}L; Sox9\textsuperscript{flox/flox} Tnmd/Chm1 Sox9 TB

Control

I

J

K

L

M

N

O

P

qft

fe

acl

ti

pa

pcl

At

sdf

in

out

in

out

in

out

in

out

p

c

l

M

O

P

ca
Fig. 8

A

Scx+/Sox9+ CP
Sox9↑

Other Chondrocytes

Sox9↑

Entheseal Chondrocytes

Scx+/Sox9+ TLCP
Sox9↓

Scx↑

Tenocytes

Ligamentocytes

Scx↓

Scx↑

B

Scx+/Sox9+ Progenitors

Primordial CTJ/CLJ

Sox9↓

Scx↑

Cartilage

Tendon/Ligament

OTJ/OLJ

CTJ/CLJ

Sox9↓

Scx↑

Cartilage

Tendon/Ligament
Supplemental Fig. 1
Supplemental Fig. 2

<table>
<thead>
<tr>
<th>E11.5</th>
<th>Sox9</th>
<th>ScxGFP</th>
<th>Sox9/ScxGFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>nt</td>
<td>ri</td>
<td>nc</td>
</tr>
<tr>
<td>B</td>
<td>nt</td>
<td>ri</td>
<td>nc</td>
</tr>
<tr>
<td>C</td>
<td>nt</td>
<td>nc</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>nt</td>
<td>nc</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>nt</td>
<td>nc</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>nt</td>
<td>nc</td>
<td>nc</td>
</tr>
</tbody>
</table>
Table S1

<table>
<thead>
<tr>
<th>No.</th>
<th>Nomenclature of tendons in this study</th>
<th>Nomenclature of ligaments in this study</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>Thoracolumbar fascia (anterior layer)</td>
<td>L1</td>
</tr>
<tr>
<td>T2</td>
<td>Origin of longissimus muscle</td>
<td>L2</td>
</tr>
<tr>
<td>T3</td>
<td>Origin of latissimus dorsi muscle</td>
<td>L3</td>
</tr>
<tr>
<td>T4</td>
<td>Thoracolumbar fascia</td>
<td>L4</td>
</tr>
<tr>
<td>T5</td>
<td>Origin of longissimus muscle</td>
<td>L5</td>
</tr>
<tr>
<td>T6</td>
<td>Origin of iliocostalis lumborum muscle</td>
<td>L6</td>
</tr>
<tr>
<td>T7</td>
<td>Origin of multifidi muscle</td>
<td>L7</td>
</tr>
<tr>
<td>T8</td>
<td>Extensor digitorum longus tendon</td>
<td>L8</td>
</tr>
<tr>
<td>T9</td>
<td>Achilles tendon</td>
<td>L9</td>
</tr>
<tr>
<td>T10</td>
<td>Superficial digital flexor tendon</td>
<td></td>
</tr>
<tr>
<td>T11</td>
<td>Origin of tibialis anterior muscle</td>
<td></td>
</tr>
<tr>
<td>T12</td>
<td>Quadriceps femoris tendon</td>
<td></td>
</tr>
<tr>
<td>T13</td>
<td>Origin of pronator muscle</td>
<td></td>
</tr>
<tr>
<td>T14</td>
<td>Common extensor tendon</td>
<td></td>
</tr>
<tr>
<td>T15</td>
<td>Common extensor tendon</td>
<td></td>
</tr>
<tr>
<td>T16</td>
<td>Origin of pronator muscle</td>
<td></td>
</tr>
<tr>
<td>T17</td>
<td>Extensor carpi ulnaris tendon</td>
<td></td>
</tr>
<tr>
<td>T18</td>
<td>(a) Extensor digiti quarti tendon</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(b) Extensor digiti quinti tendon</td>
<td></td>
</tr>
<tr>
<td>T19</td>
<td>Extensor digitorum communis tendon</td>
<td></td>
</tr>
<tr>
<td>T20</td>
<td>Origin of extensor pollicis muscle</td>
<td></td>
</tr>
<tr>
<td>T21</td>
<td>Extensor carpi radialis brevis tendon</td>
<td></td>
</tr>
<tr>
<td>T22</td>
<td>Extensor carpi radialis longus tendon</td>
<td></td>
</tr>
<tr>
<td>T23</td>
<td>Extensor pollicis tendon</td>
<td></td>
</tr>
<tr>
<td>T24</td>
<td>Flexor carpi radialis tendon</td>
<td></td>
</tr>
<tr>
<td>T25</td>
<td>Extensor indicis proprius tendon</td>
<td></td>
</tr>
<tr>
<td>T26</td>
<td>Flexor digitorum profundus tendon</td>
<td></td>
</tr>
<tr>
<td>T27</td>
<td>Flexor digitorum sublimis tendon</td>
<td></td>
</tr>
<tr>
<td>T28</td>
<td>Palmaris longus tendon</td>
<td></td>
</tr>
<tr>
<td>T29</td>
<td>Flexor carpi ulnaris tendon</td>
<td></td>
</tr>
<tr>
<td>T30</td>
<td>Interosseous</td>
<td></td>
</tr>
</tbody>
</table>

T1-T30 indicate tendons in this study.
A/F indicates anchoring or force-transmitting tendons.
L1-L9 indicate ligaments in this study.