Scleraxis is a transcriptional activator that regulates the expression of Tenomodulin, a marker of mature tenocytes and ligamentocytes

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Tenomodulin (Tnmd) is a type II transmembrane glycoprotein predominantly expressed in tendons and ligaments. We found that scleraxis (Scx), a member of the Twist-family of basic helix-loop-helix transcription factors, is a transcriptional activator of Tnmd expression in tenocytes. During embryonic development, Scx expression preceded that of Tnmd. Tnmd expression was nearly absent in tendons and ligaments of Scx-deficient mice generated by transcription activator-like effector nucleases-mediated gene disruption. Tnmd mRNA levels were dramatically decreased during serial passages of rat tenocytes. Scx silencing by small interfering RNA significantly suppressed endogenous Tnmd mRNA levels in tenocytes. Mouse Tnmd contains five E-box sites in the ~1-kb 5′-flanking region. A 174-base pair genomic fragment containing a TATA box drives transcription in tenocytes. Enhancer activity was increased in the upstream region (−1030 to −295) of Tnmd in tenocytes, but not in NIH3T3 and C3H10T1/2 cells. Preferential binding of both Scx and Twist1 as a heterodimer with E12 or E47 to CAGATG or CATCTG and transactivation of the 5′-flanking region were confirmed by electrophoresis mobility shift and dual luciferase assays, respectively. Scx directly transactivates Tnmd via these E-boxes to positively regulate tenocyte differentiation and maturation.

Human and mouse tenomodulin (Tnmd) are type II transmembrane glycoproteins consisting of 317 amino acids1,2. Tnmd is a molecule related to chondromodulin (Chmd), which was previously identified from bovine epiphyseal cartilage as a soluble growth/differentiation-promoting factor for rabbit chondrocytes and later as an anti-angiogenic factor. The C-terminal cysteine-rich domain containing 120 amino acids is secreted from chondrocytes as mature Chmd after cleavage at the processing signal of the human or mouse Chmd precursor protein containing 334 amino acids. Tnmd expression is detected in dense connective tissues, including tendons, ligaments, fascia of skeletal muscle, outer annulus fibrosus of the intervertebral disc, and cornea and sclera of the eye, whereas Chmd is specifically expressed in avascular hyaline cartilage of the cartilaginous bone primordia during development and growth and in articular cartilage. In the postnatal heart, Tnmd and Chmd act as angiogenesis inhibitors and are predominantly localized to the tendinous chord and cardiac valves, respectively.

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Both Tnmd and Chmd exert anti-angiogenic actions through the homologous C-terminal domain containing eight cysteine residues that form four disulphide bonds. In human umbilical vein endothelial cells (HUVECs), proliferation, migration, adhesion to vitronectin, and tube formation on Matrigel were significantly inhibited by adenoviral overexpression of mature Chmd or by overexpression of the C-terminal 116-amino acid fragment of Tnmd containing a secretion signal sequence. The growth of solid tumours, such as malignant melanoma in syngeneic mice, was significantly suppressed because of anti-angiogenic activities of mature Chmd or the C-terminal 116-amino acid fragment of Tnmd. Vascular endothelial growth factor-A stimulated lamellipodial extensions and motility of HUVECs was impaired by Chmd. Although loss of Tnmd does not cause any apparent anti-angiogenic-related phenotype in mouse embryos, surgical removal of the Tnmd-rich layer from the cardiac tendineae cordis induces angiogenesis and matrix metalloproteinase activation. Double immunostaining of CD31, a cell surface marker of vascular endothelial cells, and Tnmd or Chmd in the mouse forelimb at embryonic day (E) 16.5 revealed that Tnmd and Chmd proteins specifically localize to the avascular region of tendon and cartilage, respectively. Thus, both Tnmd and Chmd are anti-angiogenic molecules specifically expressed in association with avascular mesenchymes.

Tendons and ligaments are categorized into typical dense connective tissues characterised by the regular alignment of thick collagen fibres mainly consisting of type I collagen. Tendons bind skeletal muscles to bones to transmit the mechanical force of contraction, whereas ligaments connect bones to correctly align skeletal elements and reinforce flexible joints. During the embryonic development of musculoskeletal components, Tnmd is predominantly expressed in mature tendon fibroblasts (tenocytes) and ligament fibroblasts (ligamentocytes) at high levels. Analysis of Tnmd-deficient mice revealed that Tnmd is necessary for tenocyte proliferation and maturation of tendinous tissue as well as proliferation and senescence of tendon stem/progenitor cells. In the periodontal ligament that connects the cementum of the tooth and the alveolar bone, Tnmd has been demonstrated to enhance the cellular adhesion of fibroblasts. Tnmd expression in chick leg tendons is positively regulated by scleraxis (Scx), a basic helix-loop-helix (bHLH) transcription factor that marks both tendon progenitors and tenocytes during chick and mouse embryonic development. In mice lacking Scx, Tnmd expression is undetectable in tendons; however, it remains unclear whether Scx directly regulates Tnmd expression at the transcription level.

In the present study, we investigated how Scx regulates Tnmd expression under both in vivo and in vitro conditions. Tnmd expression is nearly absent in both the tendons and ligaments of Scx-deficient mice generated by transcription activator-like effector nuclease (TALEN)-induced double-strand DNA break. Silencing of Scx in tenocytes by small interfering RNA markedly suppressed Tnmd mRNA levels. We further analysed the ~1-kb 5′-flanking region and 5′ untranslated region of the mouse Tnmd gene that spans 15 kb and is comprised of seven exons. The investigated region contains a TATA promoter and five E-box sites. We identified transactivation of the promoter and its upstream region by Scx and/or E12 or E47 as well as the preferential binding of Scx/E12 or Scx/E47 heterodimers to CAGATG and CATCTG. Similar transactivation and binding to these E-boxes were observed when we tested Twist1/E12 or Twist1/E47 heterodimers. Hence, Scx directly transactivates Tnmd via these E-box sites to positively regulate tenocyte/ligamentocyte maturation during development and growth.

Results
Expression of Tnmd in tenocytes in vivo and in vitro. Tnmd is predominantly expressed in dense connective tissue, such as tendons and ligaments of mouse embryos at E12.5 and E13.5 by whole-mount in situ hybridisation (Fig. 1a–f). At E12.5, Tnmd was detected in the developing axial tendons along the cervical and thoracic spine, but its expression was low in the limbs (Fig. 1a). In the developing forelimb, Tnmd was detected in the primordia of the triceps brachii tendon and extensor digitorum communis musculii (Fig. 1b), while Scx was widely detected in the tendon primordia of the forelimb (Fig. 1c). At E13.5, Tnmd was expressed in the axial tendons along the entire spine and limbs (Fig. 1d). Tnmd and Scx were coexpressed with the developing tendons (Fig. 1e,f); however, Scx expression was also detected in the developing joint capsules at high levels (Fig. 1f).

We also analysed the expression of Tnmd along with other differentiation markers, including Scx, collagen alpha-2(I) chain (Col1a2), and collagen alpha-1(II) chain (Col2a1), in various mesenchymal cells (Fig. 1g, Supplementary Fig. 1). Tnmd mRNA was detected at high levels in tenocytes and to a lesser extent in C3H10T1/2 cells, whereas Tnmd expression was undetectable in fibroblastic NIH3T3 cells, osteoblastic MC3T3-E1 cells, chondrogenic ATDC5 cells, or primary costal chondrocytes by northern blot analysis (Fig. 1g). In contrast, Scx, a marker of both tendon progenitors and tenocytes, was expressed in tenocytes, C3H10T1/2 cells, MC3T3-E1 cells, differentiated ATDC5 cells, and chondrocytes but not in NIH3T3 cells, suggesting that not only tendon-derived cells but also skeletal cells express Scx in vitro (Fig. 1g). We also detected the expression of Twist1 in rat tenocytes, NIH3T3 cells, and C3H10T1/2 cells (Fig. 1h, Supplementary Fig. 2). Interestingly, the mRNA level of Tnmd dramatically decreased during serial passage of rat tenocytes, while increased mRNA levels of Scx were observed in the fourth or later culture passages of tenocytes and high levels of Col1a2 were detected during serial passages until the 10th culture (Fig. 1i, Supplementary Fig. 3). These results clearly suggest that Tnmd is a specific marker gene for tenocytes both in vivo and in vitro.

Indispensable role of Scx in expression of Tnmd gene and protein in tendons and ligaments.
Mouse Scx consists of two exons (Fig. 2a). To generate Scx-deficient mice, we used TALEN-mediated Technology rather than a conventional homologous recombination-based strategy using embryonic stem cells. To disrupt the Scx gene using TALENs, we designed the TALEN recognition sequences to be within exon 1 of the mouse Scx locus (Fig. 2a), so that most of the Scx protein would be lost due to a frameshift mutation after creation of a double-stranded break by TALENS. TALEN mRNAs produced by in vitro transcription were microinjected into the cytoplasm of fertilized oocytes. After in vitro culture of the injected oocytes overnight, two-cell embryos were...
transferred into pseudopregnant female mice. Fourteen pups with deletion mutations were identified from among 18 newborn mice by genotyping and direct sequencing of the amplified DNA (Fig. 2b). We obtained eight founder mice with deletion mutations that would yield a disruptive frameshift mutation and a premature stop codon (#1, #3, #4, #6, #8, #11, #13, and #14), thereby inactivating the Scx protein (Fig. 2b). We crossed founder #13 with a 11-base pair (bp) deletion, both resulting in frameshift and premature stop codons shortly downstream, with a C57BL/6 wild-type mouse to obtain the heterozygote through germline transmission. Heterozygous Scx<sup>+/-</sup> and homozygous Scx<sup>-/-</sup> mice were viable at two weeks, while Scx<sup>-/-</sup> mice exhibited hypoplastic tendon formation (Fig. 2d,f) compared to wild-type mice (Fig. 2c,e), as previously reported<sup>10,21</sup>.
To examine the expression of Tnmd in Scx\(^{+/−}\) and Scx\(^{−/−}\) mouse tissues, we performed in situ hybridisation in frozen tissue sections (Fig. 3a–o). In the hindlimb of a Scx\(^{+/−}\) embryo at E18.5 and a Scx\(^{+/−}\) neonate at postnatal day 1 (P1), expression of both Col1a1 (Fig. 3a,j) and Scx (Fig. 3b,c) was detected in the tendons and ligaments of the knee (Fig. 3a,b) and ankle (Fig. 3c,j). Similar to Scx, Tnmd was expressed in Col1a1-positive tendons and ligaments of the knee (Fig. 3d,e) and ankle (Fig. 3j,k) of the Scx\(^{+/−}\) neonate at P1. In the Scx\(^{−/−}\) neonate at P1, Col1a1 expression persisted in the tendons and ligaments (Fig. 3g,m), whereas Tnmd expression was nearly absent (Fig. 3h,n). Expression of Tnmd was undetectable in the anterior and posterior cruciate ligaments and distal region of the Achilles tendon of Scx\(^{+/−}\) at P1 (Fig. 3h,n). However, only faint expression of Tnmd was detected in the quadriceps femoris tendon, patella ligament, and proximal portion of the Achilles tendon of Scx\(^{+/−}\) at P1 (Fig. 3h,n). We performed immunostaining to examine the localization of Tnmd proteins in the Achilles tendon (Fig. 3p–s). In the ankle of Scx\(^{+/−}\) and Scx\(^{−/−}\) at P1, Tnmd expression was nearly absent (Fig. 3h,n). In good agreement with the expression pattern of mRNA, the Tnmd protein was localized to the Achilles tendon of Scx\(^{+/−}\) at P1 (Fig. 3q), whereas in the Scx\(^{−/−}\) neonate, faint or no expression of Tnmd was detected in the proximal or distal portion of the Achilles tendon, respectively (Fig. 3s).

We isolated tenocytes from rat limb tendons (Fig. 4a) and depleted Scx in these cells by RNA interference. In tenocytes transfected with siScx-1 or siScx-2, the level of Scx was decreased to less than 25% of that of the control at 72 h after lipofection (Fig. 4b). The level of Tnmd was markedly decreased to 17% and 18% by gene silencing of
Scx with siScx-1 and siScx-2, respectively (Fig. 4b). The expression level of Col1a2 was slightly decreased to 80% by siScx-2, but no significant decrease in Col1a2 expression was detected in cells transfected with siScx-1 (Fig. 4b). These results suggest that Tnmd expression depends on Scx in postnatal mature tenocytes.

**Determination of transcription start sites in mouse Tnmd gene.** Tnmd was cloned as a related gene of Chmd, which was identified as a cartilage-derived angiogenesis inhibitor\(^1\,\text{,}\,^2\). Mouse Tnmd was mapped to chromosome XqE3 (nucleotides 133,851,207–133,865,578 in the UCSC Genome Browser [GRCm38/mm10 assembly]). Based on the genomic and cDNA sequences (GenBank AF219993) of mouse Tnmd\(^3\), we determined the exon-intron boundaries (Table 1) and found that Tnmd consists of seven exons spanning approximately 15 kb (Fig. 5a). Protocadherin (Pcdh19) is located more than 160-kb upstream from the first exon of Tnmd. Tetraspanin 6 (Tspan6) is located immediately downstream of Tnmd, followed by sushi repeat-containing protein, X-linked 2 (SrpX2) and synaptopagmin-like 4 (Sty4) (Fig. 5a).
As an initial step in the identification and characterisation of the mouse Tnmd promoter, we analysed the transcription start site using CapSite hunting technology. We performed nested PCR using CapSite cDNA derived from mouse embryos at E16 and examined six independent cDNA clones. Subsequent sequence analysis revealed two different transcription start sites located at $-58$ and $-84$ bp from the translational start site of mouse Tnmd (Fig. 5b). Designating the distal transcriptional start site as $+1$, a putative TATA box was found $-25$ bp upstream of the distal transcription start site (Fig. 5b). We also found that half of the cDNA clones amplified from CapSite cDNA contained a 90-nucleotide insertion between the cDNA sequences corresponding to exon 2 and exon 3 (data not shown). The inserted region encoding a 30-amino acid sequence was determined to be a single independent exon, located 348-bp upstream of exon 3 in mouse Tnmd. The boundary sequences of this inserted region of the genome follow the GT-AG rule. To date, Tnmd transcripts containing the insertion sequence have only been found in cDNA clones amplified from mouse 16-day embryo CapSite cDNA.

Tnmd expression in tenocytes is positively regulated by Scx, as previously reported. Dimers of various bHLH proteins bind to a short DNA motif known as E-box CANNTG. Five consensus E-box sites are found upstream and downstream of the TATA box of mouse Tnmd (Fig. 5b). We designated these E-boxes as E1 (CACTTG), E2 (CATCTG), E3 (CAATTG), E4 (CAAATG), and E5 (CAGATG).

**Promoter activity of the mouse Tnmd gene in Tnmd-expressing tenocytes.** Sequence analysis of the 5′-flanking region revealed a stretch of 746 bp of repetitive sequences ($-1762$ bp to $-1018$) containing GT-rich repeats, GA- or CA-rich repeats, and AG- and GG-rich repeats. Thus, we analysed the 5′-flanking region from
−1030 to +84 bp containing the TATA box, transcriptional start sites, and five E-boxes (Fig. 5b). To analyse the promoter activity, various lengths of the genomic fragments were cloned into a promoterless pGL4.10[luc2] vector in the forward or reverse orientation (Fig. 6a). The promoter activity of mouse Tnmd was tested by transiently transfecting these constructs into Tnmd-expressing tenocytes (Fig. 1g, Supplementary Fig. 1). The luciferase activities of −90/+84 and −123/+84 in tenocytes were 2.4-fold higher than that of the pGL4.10[luc2] empty vector (Fig. 6b). Increased luciferase activities of −295/+84 (6.2-fold), −443/+84 (7-fold), −525/+84 (4.7-fold), −769/+84 (7.8-fold), and −1030/+84 (7.3-fold) were observed in tenocytes (Fig. 6b). The luciferase activities of +84/−90, +84/−123, +84/−295, +84/−443 and +84/−525 which contained the reverse-oriented genomic fragments were similar or even lower in all tested cells compared to that of the pGL4.10[luc2] empty vector (Fig. 6b), suggesting that this promoter is active in the forward direction but not in the reverse direction. We also performed dual luciferase assays in Tnmd-non-expressing NIH3T3 cells and Tnmd-weakly-expressing C3H10T1/2 cells (Figs 1g and 7). A 174-bp promoter region with a TATA box drove the transcription at a similar basal level in tenocytes, NIH3T3 cells, and C3H10T1/2 cells. However, increased enhancer activity in the upstream region (−1030 to −295) was not observed in these cells. Interestingly, as shown in Fig. 6c, the luc activities of −295/+84, −443/+84, and −525/+84 cloned into the promoter-less pGL4.10[luc2] vector were 4.9-, 6.4-, and 4.0-fold higher than that of the empty vector in tenocytes, whereas the luciferase activities of genomic fragments (+84/−295, +84/−443, and +84/−525) cloned into pGL4.23[luc2/minP] vector with a
minimal promoter (AGACACTAGGGGTATAATGGGAAGCTCGACTTCCAG) were significantly lower than that of the empty vector. When these genomic fragments were cloned into pGL4.10[luc2]/pGL4.10 basic Vector or pGL4.23[luc2/minP]/pGL4.23 with a minimal promoter. The location of the first nucleotide of the upstream transcription start site is denoted as +1. The putative E-box sites and TATA box are shown by grey and black boxes, respectively. (b,c) Promoter activities of the mouse Tnmd gene in Tnmd-expressing tenocytes. Cells were co-transfected with a series of constructs shown in (a) and pGL4.74[hRluc/TK]. Firefly and Renilla luciferase activities were measured 24 h after transfection. The relative luciferase activity normalized to the Renilla luciferase activity is depicted as the fold-induction compared to each activity of the empty vector. All dual luciferase assays were performed in triplicate. The graphs show one representative experiment out of three. Each bar represents the average of three independent transfections (means ± SD.).

Figure 6. Promoter activity of mouse Tnmd in rat tenocytes. (a) Various lengths of DNA fragments of mouse Tnmd (175–1114 bp) in the forward or reverse orientation were cloned into the promoterless pGL4.10[luc2]/pGL4.10 basic Vector or pGL4.23[luc2/minP]/pGL4.23 with a minimal promoter. The location of the first nucleotide of the upstream transcription start site is denoted as +1. The putative E-box sites and TATA box are shown by grey and black boxes, respectively. (b,c) Promoter activities of the mouse Tnmd gene in Tnmd-expressing tenocytes. Cells were co-transfected with a series of constructs shown in (a) and pGL4.74[hRluc/TK]. Firefly and Renilla luciferase activities were measured 24 h after transfection. The relative luciferase activity normalized to the Renilla luciferase activity is depicted as the fold-induction compared to each activity of the empty vector. All dual luciferase assays were performed in triplicate. The graphs show one representative experiment out of three. Each bar represents the average of three independent transfections (means ± SD.).
Transactivation of mouse Tnmd promoter by Scx or Twist1, which dimerizes with E12 or E47.

Scx is a member of the Twist family of bHLH transcription factors, which functions through dimerization with E-proteins and binding to E-boxes (CANNTG)\(^24\). Both Scx and Twist1 were co-expressed with Tnmd in tenocytes (Fig. 1g,h) and upregulated the expression of Tnmd in chick tenocytes, whereas overexpression of Myog, a myogenic bHLH factor, resulted in downregulation of the mRNA levels of Tnmd in chick tenocytes\(^25\). To examine whether Scx is directly involved in the transactivation of mouse Tnmd, we performed dual luciferase assays in rat tenocytes by co-transfecting the luciferase reporter containing five E-boxes (pGL4.10\(-1030/+84\)), three E-boxes (pGL4.10\(-525/+84\)), or two E-boxes (pGL4.10\(-295/+84\)) with various combinations of expression vectors for FLAG-tagged mouse Scx (fmScx) or Twist1 (fmTwi) and/or its heterodimeric partners E12 (fmE12) or E47 (fmE47) tagged with FLAG (Fig. 8). E12 and E47 are products of two alternatively spliced mRNAs and have nearly identical sequences, except in the stretches encoding the DNA-binding domain\(^26\). The luciferase activities of pGL4.10\(-1030/+84\), pGL4.10\(-525/+84\), or pGL4.10\(-295/+84\) cotransfected with fmScx and/or fmE12 or fmE47 were significantly higher than that in the control (Fig. 8a,b,c). Similar luciferase activity of pGL4.10\(-1030/+84\) was observed when cotransfected with fmTwi and/or fmE12 or fmE47 (Fig. 8d).

We then tested whether Scx and E12 or E47 directly interact with these E-boxes identified in the promoter region using electrophoretic mobility shift assays (EMSA). EMSA was performed with nuclear extracts containing fmScx and FLAG-tagged human E12 (fhE12), fmE12, or fmE47 using biotin-labelled oligonucleotides containing the E-box and/or mutated E-box (Table 2). The expected molecular weight of each translated protein was confirmed by western blotting (data not shown). Of the five consensus E-box sequences, a specific shift band was detected when fmScx and fhE12, fmE12 or fmE47 was incubated with a biotin-labelled E1E2 or E5 oligonucleotide (black arrowheads in Fig. 9a–c). In the presence of an anti-FLAG antibody, a supershifted band was clearly detected (hollowed arrowheads in Fig. 9a–c), suggesting that Scx and E12 or E47 heterodimer complexes directly interact with the oligonucleotide E1E2 or E5. To identify which E-box on E1E2 is responsible for the specific binding of Scx/E12, we performed EMSA with mutated oligonucleotides (Table 2, Fig. 9d). Both a specific shift band and supershifted band were detected when fmScx and fhE12 were incubated with the biotin-labelled M1E2 oligonucleotide containing a mutated E1 sequence and normal E2 sequence. No specific bands were detected with the biotin-labelled M1M2, E1M2, or M5 oligonucleotides (Fig. 9d).

We also tested whether Twist1 and/or E12 or E47 bind to E2 (CATCTG) and E5 (CAGATG) by EMSA. EMSA was performed with nuclear extracts containing fmTwi and fhE12, fmE12, or fmE47, using biotin-labelled oligonucleotides containing the E-box and/or mutated E-box (Table 2). Specific shift bands were detected when fmTwi and fhE12 were incubated with a biotin-labelled E1E2 or E5 oligonucleotide (black arrowheads in Fig. 10a,b). In the presence of an anti-FLAG antibody, supershifted bands were clearly detected (hollowed arrowheads in Fig. 10a,b). Both specific shift bands and supershifted bands were detected when fmTwi and fhE12 were incubated with the biotin-labelled M1E2 oligonucleotide containing a mutated E1 sequence and normal E2 sequence. No specific bands were detected with the biotin-labelled M1M2 or E1M2 oligonucleotides (Fig. 10a).
These results suggest that Twist1 and/or E12 or E47 heterodimer complexes directly interact with the oligonucleotide E2 or E5.

Taken together, Scx or Twist1 that dimerizes with E12 or E47 preferentially binds to E2 (CATCTG) and E5 (CAGATG) to transactivate mouse Tnmd.

Discussion

Here, we demonstrated that Scx transactivates the promoter of mouse Tnmd, which consists of seven exons encoding a type II transmembrane protein, marking tenocytes and ligamentocytes. Silencing of Scx caused marked downregulation of Tnmd in cultured tenocytes. Loss of Scx function in vivo nearly abolished Tnmd expression in both tendons and ligaments during musculoskeletal development and growth. Of the five E-boxes around

![Figure 8](image_url)

**Figure 8.** Transactivation of the mouse Tnmd 5′-flanking region containing E-boxes by bHLH factors in rat tenocytes. The mouse Tnmd genomic fragment containing the 5′-flanking region and 5′-UTR with five E-boxes (from −1030 to +84), three E-box sites (from −525 to +84), or two E-box sites (from −295 to +84) was subcloned into a promoterless pGL4.10[luc2] in the forward orientation (pGL4.10 −525/+84 or pGL4.10 −1030/+84). Rat tenocytes were co-transfected with the reporter construct and empty vector pcDNA3 as a control, or expression vectors encoding the FLAG fusion of mouse E12 (fmE12), mouse E47 (fmE47), mouse Scx (fmScx), mouse Twist1 (fmTwi) and pGL4.74[hRluc/TK]. Firefly and Renilla luciferase activities were measured 24 h after transfection. Values were normalized using a pGL4.74[hRluc/TK] and are presented as the fold-induction relative to pcDNA3 (empty vector). Graphs show one representative experiment of at least three. Each bar represents the average of three independent transfections (means ± SD). ***P < 0.05 vs. control.
Table 2. Oligonucleotides for electrophoresis mobility shift assay.

| Sequence |
|----------|
| E1E2 5’ CTCGCCGACATCTGTTAGCCGACCTCACTTGCAACT 3’ |
| 3’ GAGGCCGTTAGCCGACTGAGCTGTAAGTTGTA 5’ |
| M1M2 5’ CTCGCCGACTTTGTAGCCGACCTCACTTGCAACT 3’ |
| 3’ GAGGCCGTTAGCCGACTGAGCTGTAAGTTGTA 5’ |
| E1M2 5’ CTCGCCGATCTGTTAGCCGACCTCACTTGCAACT 3’ |
| 3’ GAGGCCGTTAGCCGACTGAGCTGTAAGTTGTA 5’ |
| E3 5’ GAAAAAAGTCTGACATGCTCTCATTATCTCT 3’ |
| 3’ CTTTACGTGTTAACAGAGATTAAGATAAGG 5’ |
| E4 5’ CTCAGATGTTCGAGCTTTAAGATTTA 3’ |
| 3’ GAGCTCTACAGTTTACAGGCAACTCTTATT 5’ |
| E5 5’ GGGTAAGAGCTGAGATGTGTTTT 3’ |
| 3’ CCCATGTCCTAGAGCTCTACAA 5’ |
| M5 3’ CTTTCTTCTCGAGATGTGTTTT 5’ |

the TATA box, CAGATG and CATCTG are preferential binding sites for Scx, as demonstrated by EMSA. Dual luciferase assays in Tnmd-expressing tenocytes revealed enhancer activity upstream of the promoter region. Thus, Scx directly transactivates Tnmd via these E-boxes to positively regulate tenocyte differentiation and maturation.

We previously reported that chick Tnmd is present at early stages of tendon/ligament formation and is persistently expressed in mature tendons and ligaments at high levels. In mice, our whole-mount in situ hybridisation analysis revealed that Scx expression in the developing tendon and ligament primordia precedes the expression of Tnmd. Later in musculoskeletal development, Scx and Tnmd are coexpressed with mature tendons and ligaments. Thus, in mice, Scx is an early marker gene expressed in both tendon/ligament progenitors and tenocytes/ligamentocytes, whereas Tnmd acts as a late marker gene to indicate mature tenocytes/ligamentocytes.

Consistent with our previous report that Scx is expressed in a subpopulation of skeletogenic Sox9+ progenitor cells that contribute to the formation of future enthesis, Scx was also detected in skeletal cells, including chondrogenic ADTC5, osteogenic MC3T3-E1, and chondrocytes, in vitro. In contrast, Tnmd is not expressed in these skeletogenic cells and is highly specific to mature tenocytes. This is in good accordance with our in situ hybridisation data showing that Tnmd is detected in the developing tendons and ligaments in vivo. Unlike Col1a2, Tnmd expression was dramatically downregulated during serial passaging of tenocytes, suggesting that Tnmd is a good indicator of mature phenotypes in cultured tenocytes. In our rotator cuff injury model using rats, local administration of FGF-2 resulted in biomechanical and histological improvement of the repaired rotator cuff by promoting growth of tenogenic progenitor cells in association with a significant increase in Tnmd-positive cells in the midsubstance of the tendon. A strong positive correlation between the location of the aligned collagen fibre orientation and expression levels of Tnmd mRNA was observed in this model. In chick embryos, Tnmd expression was not detected in oval immature tenocytes, but was observed in elongated mature tenocytes of the embryonic Achilles tendon. Taken together, a high level of Tnmd expression is a good indicator of the maturation of tenocytes localized between aligned collagen fibres under both physiological and pathological conditions.

Interestingly, we also detected the expression of Tnmd in C3H10T1/2 cells, which is an undifferentiated mesenchymal cell line derived from mouse embryonic fibroblasts. C3H10T1/2 cells have the potential to differentiate into tenocytes. As previously reported, Tnmd is also expressed in cultured tendon stem/progenitor cells (TSPC) and Scx-expressing bone marrow stromal cells. Loss of Tnmd results in reduced self-renewal and augmented senescence of tendon/ligament progenitor cells without affecting the multipotential of TSPC. Thus, a low level of Tnmd expression may be required for maintenance of the tenogenic potential in stem/progenitor cells in vitro.

Scx was originally isolated from a mouse E14.5 cDNA library, using the yeast two-hybrid system as a novel partner of bHLH protein that dimerizes with E12. Scx binds to the mouse muscle creatine kinase enhancer (CCAGATGTGGCTGCTC) as a heterodimer with E12. It was also reported that Scx acts as a transcriptional activator for Col1a1 in tendons via binding to the E-box site CAGTG and Aggrecan (Acan) in osteosarcoma-derived ROS17/2.8 cells. Scx/E4 heterodimers bind to CAGGTG to regulate Col1a1 expression together with Sox9 and p300. In this study, we analysed five E-boxes (E1: CATCTG; E2: CATCTG; E3: CAATTG; E4: CAAATG; and E5: CAGATG) upstream and downstream of the TATA box in the mouse Tnmd promoter. EMSA revealed that E2 (CATCTG) and E5 (CAGATG) are Scx-binding E-boxes. Li et al. performed chromatin immunoprecipitation, to show that Scx binds to the region containing both E4 (CAAATG) and E5 (CAGATG). In this study, we determined that Scx does not bind to E4 by our EMSA analysis using nuclear extract of HEK293T cells expressing Scx. We also found that Twist1 binds to these Scx-binding E-boxes and transactivates the genomic region (−1030 to +84). Thus, not only Scx but also Twist1 regulate Tnmd expression via E2 (CATCTG) and E5 (CAGATG) in the promoter region.

Mouse Scx gene consists of two exons and is located within the third intron of block of proliferation 1 (Bop1) transcribed in the opposite orientation. The first reported general knockout mice for Scx were embryonic lethal,
likely because of retention of the neomycin phosphotransferase gene linked to phosphoglycerate kinase promoter (PGK-Neo) in the targeted allele. Subsequently, Murchison et al. generated Scx floxed mice and characterised conditional knockout (CKO) mice inactivating Scx in the Prx1 expressing region to demonstrate severe defects in force-transmitting tendon maturation. Similar hypoplastic tendon formation and other phenotypes including defects in ligament and enthesis maturation were also observed in homozygotes with ScxCre knock-in allele that we generated by in-frame replacement of most of Scx exon 1 with Cre10. Scx−/− mice generated via TALEN-mediated technology in this study were also viable and exhibited similar phenotypes as previously reported Scx CKO and ScxCre knock-in mice. Mutant mice have only an 11-bp deletion that caused a frameshift, leading to premature stop codons shortly downstream without a further footprint. Thus, this is the first viable general knockout mouse line that is easy to use for generating double or triple knockout mice for tendon and ligament research.

In Scx-deficient embryos, severe defects were observed in force-transmitting and intermuscular tendons; however, muscle-anchoring tendons and ligaments were not affected. Homozygous ScxCre knock-in mice exhibited defective maturation of tendons and ligaments as well as enthesal and sesamoid cartilage in which Scx was transiently expressed during development. Similar phenotypes were observed in Scx null mice generated by TALEN-mediated technology. Our in situ hybridisation analysis revealed that Tnmd expression was nearly
absent in both tendons and intracapsular ligaments, such as the anterior cruciate ligaments of Scx null mice at P1. Similarly, Tnmd was minimally detected in the distal portion of the Achilles tendon of P1 neonates lacking Scx.

Silencing of Scx in tenocytes isolated from 2-week old rat limb tendons also resulted in significant downregulation of Tnmd. These results suggest that Tnmd expression in tenocytes is dependent on Scx both in vivo and in vitro.

Gene and protein expression of Tnmd are nearly absent in Scx null embryos, suggesting a crucial role for Scx in Tnmd expression. However, a loss of Tnmd expression was observed in the tenth cultures of tenocytes, although the cells maintained Scx expression at a high level. Scx+ skeletogenic cells, such as MC3T3-E1 and ATDC5 cells, also do not express Tnmd. Enhancer activity was increased in the upstream region (−1030 to −295) in tenocytes, but not in NIH3T3 cells not expressing Tnmd. As shown in whole-mount in situ hybridisation, Scx expression precedes that of Tnmd during embryogenesis. These results suggest that Scx is necessary for the induction of Tnmd expression in mature tenocytes, but not sufficient in immature tenocytes and skeletogenic cells. This is also consistent with our previous finding that retroviral overexpression of Scx in the chick hindlimb resulted in significant upregulation of Tnmd in tendons but did not induce ectopic Tnmd expression outside the tendinous tissue. We speculate that some additional transcription factors are required for the expression of Tnmd. Further studies are underway to elucidate other transcriptional factors that coordinately regulate the transcription of Tnmd with Scx in tendons and ligaments.
Methods

Animals and embryos. Mice and rats were purchased from Japan SLC, Inc. (Hamamatsu, Japan) or CLEA Japan, Inc. (Tokyo, Japan). All animal experimental protocols were approved by the Animal Care Committee of the Institute for Frontier Life and Medical Sciences, Kyoto University, and Committee of Animal Experimentation, Hiroshima University, and conformed to institutional guidelines for the study of vertebrates.

Generation of TALEN-mediated Scx-deficient mice. TALEN plasmids were constructed using the Platinum Gate TALEN Kit (Kit #1000000043, Addgene, Cambridge, MA, USA) as previously described. To prepare TALEN mRNA, TALEN plasmids for mScx-TALEN-A-L and mScx-TALEN-A-R were linearized with SmaI and purified by phenol–chloroform extraction. mScx-TALEN-A-L and -R mRNAs were synthesized and a polyA tail was added using the mMESSAGE mMACHINE T7 ULTRA Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. After purification with the MEGAclear kit (Ambion), mScx-TALEN-A-L and mScx-TALEN-A-R mRNAs were microinjected into the cytoplasm of fertilized eggs obtained from C57BL/6 mice. Injected eggs were transferred into the oviducts of pseudopregnant surrogate ICR female mice. Genomic DNA was extracted from the tail tips of founder mice. A 388-bp fragment of exon 1, which included recognition sites for TALENs, was amplified by PCR using primers (Scx_GT3: 5′-GCCTGTGGGGACCTAAAGAG-3′; Scx_GT4: 5′-TCGGTGGGATGAGTGTGCGCAGGC-3′). The amplified fragment was then used for direct sequencing. Sequencing was performed with the BigDye Terminator Cycle Sequencing kit and an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Cell culture. Mouse tail and limb tendons were isolated from 4-week-old male mice and seeded onto 100-mm cell culture dishes. Tenocytes outgrown from the tendon were passaged once or twice and grown in minimum essential medium Eagle alpha modification (α-MEM) supplemented with 10% foetal bovine serum (FBS). Rat tenocytes were isolated from limb tendons of 7- or 14-day-old male Wistar rats. Minced tendons were incubated with 0.1% ethylenediaminetetraacetic acid (EDTA) (Dojin, Tokyo, Japan) at 37 °C for 20 min and digested with 0.05% trypsin containing 0.53 mM EDTA (Gibco, Grand Island, NY, USA) at 37 °C for 5 min followed by digestion with 0.1% collagenase (Roche, Basel, Switzerland) at 37 °C for 10 min. Tenocytes were grown in α-MEM supplemented with 10% FBS (Cambrex, East Rutherford, NJ, USA) and 50 μg/ml kanamycin (Sigma-Aldrich, St. Louis, MO, USA) on dishes coated with type I collagen (Koken, Tokyo, Japan). Rat chondrocytes were isolated from rib cartilages of 4-week-old Wistar rats. Cartilage minces were incubated with 0.1% EDTA at 37 °C for 20 min and digested with 0.15% trypsin (Difco, Detroit, MI, USA) at 37 °C for 1 h and 0.1% collagenase at 37 °C for 3 h. Chondrocytes were grown in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium (DMEM/F12 medium; Asahi Techno Glass, Haibara, Japan) supplemented with 10% FBS (Sigma-Aldrich). NIH3T3 cells were grown in DMEM (Sigma-Aldrich) supplemented with 10% FBS (Biological Industries, Beit Haemek, Israel) and 50 μg/ml kanamycin. C3H10T1/2 cells were grown in DMEM (Sigma-Aldrich) supplemented with 10% FBS (Sigma-Aldrich). MC3T3-E1 cells were grown in α-MEM supplemented with 10% FBS. ATDC5 cells were grown in DMEM/F12 medium (Asahi Techno Glass) supplemented with 5% FBS (Hana-Nesco Bio Corp., Tokyo, Japan), 10 μg/ml human insulin (Roche), 10 μg/ml human transferrin (Roche), and 3 × 10−7 M sodium selenite (Sigma-Aldrich), as described previously. Cells were incubated at 37 °C in a 5% CO2 atmosphere.

Scx knockdown by RNA interference. Small interfering RNA (siRNA) oligonucleotide duplexes were purchased from GE Healthcare Life Sciences (Little Chalfont, UK). Scx was depleted using siScx-1 (J-113656-09) and siScx-2 (J-113656-10) included in the ON-TARGET plus rat Scx siRNA set (GE Healthcare Life Sciences, LQ-113656-00-002). For the control experiment, siGENOME non-targeting siRNA pool no. 1 (D-001206-13-05) was used. Transfection of siRNA into rat tenocytes was performed with DharmaFECT 1 transfection reagent (GE Healthcare Life Sciences) according to the manufacturer’s instructions.

Quantitative RT-PCR (qRT-PCR) analysis. Total RNA was extracted from rat tenocytes, using an RNeasy Plus Mini kit (Qiagen, Hilden, Germany). Two-hundred nanograms of total RNA was used to synthesize cDNA with a PrimeScript RT reagent kit (Takara Bio, Shiga, Japan). qRT-PCR was performed using SYBR Premix Ex Plus Mini kit (Qiagen, Hilden, Germany). Two-hundred nanograms of total RNA was used to synthesize cDNA with a PrimeScript RT reagent kit (Takara Bio, Shiga, Japan). qRT-PCR was performed using SYBR Premix Ex Taq II (Takara Bio) on a StepOne instrument (Life Technologies, Carlsbad, CA, USA). Relative mRNA expression was normalized to that of 18 S rRNA and calculated using the 2−ΔΔCT method. Specific primers for qRT-PCR are listed in Table 3.

Northern blot analysis. Total RNA was cultured from tenocytes, chondrocytes, NIH3T3 cells, C3H10T1/2 cells, ATDC5 cells, and MC3T3-E1 cells as described previously. Total RNA (15 μg) was denatured with 6% formaldehyde, electrophoresed on a 1% agarose gel, and transferred onto Nytran membranes with Tuboblotter (Schleicher and Schuell, Whatman plc, Maidstone, UK). Rat Tnnl and rat Scx probes were amplified from cDNA prepared from cultured rat tenocytes with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Probes for mouse Tnnl, mouse Sscx, and rat type II collagen alpha 1 (Col2a1) were described previously. A probe for rat type I collagen alpha 2 (Col1a2) was generously provided by Dr. Bjorn Olsen. A probe for mouse Twist1 was amplified from a cDNA clone purchased from Open Biosystems (Lafayette, CO, USA). Hybridisation was performed overnight at 42 °C with an appropriate cDNA probe labelled with [α-32P]dCTP in a solution containing 50% formamide (Wako, Osaka, Japan), 60 μM sodium-sodium phosphate-EDTA buffer (Sigma-Aldrich), 0.1% bovine serum albumin (Sigma-Aldrich), 0.1% Ficoll 400 (GE-Healthcare), 0.1% polyvinylpyrrolidone (Wako), 0.5% sodium dodecyl sulphate (Wako), and 100 μg/ml denatured salmon sperm DNA (Wako).
**Transient transfection and dual luciferase assay.** Rat tenocytes were transiently transfected with pGL4.10[luc2] (Promega, Madison, WI, USA) or pGL4.23[luc2/minP] (Promega) reporters using Lipofectamine LTX (Invitrogen). Each reporter construct was co-transfected with pGL4.74[hRluc/TK]. For co-transfection experiments, reporter and expression vectors were transfected in a 96-well plate format. After 24h, luciferase

| Gene | Forward | Reverse |
|------|---------|---------|
| Col1a2 | ACTGAGCCACCCAGAGTGGAA | CTCTTGCCTTGACGAGTCCTG |
| SceX | GACCATGCTGATGACCCAGGAGAGAAG | CTCTTGGTCTCTCCAGGTGATGAA |
| Tnmd | ATGGGGTTCGCTCCTCAAAGTGCTG | CTCTTGCCTTGAGTGAAGATCTTC |
| 18S rRNA | AGATTTTGACCACATCTGCGGAGTA | TTGCTGAGGTCATGTCTGTTTC |

Table 3. Primers for qRT-PCR.
activities were measured using PicaGene Dual Sea Panys Luminescence kit (TOYO INC MFG CO., LTD., Tokyo, Japan) and microplate luminometer (Berthold Technologies, Bad Wildbad, Germany; Centro XS® LB960). Reporter construct activity was normalized by comparison with the activity of the Renilla luciferase construct. All experiments were performed in triplicate.

**Electrophoretic mobility shift assay (EMSA).** The sequences of the oligonucleotides containing the E-box and/or mutated E-box are shown in Table 2. Double-stranded oligonucleotide probes were labelled with biotin using the Biotin 3’End DNA Labeling kit (Thermo Scientific, Waltham, MA, USA). Nuclear proteins were isolated using a Nuclear Extract kit (Active Motif, Carlsbad, CA, USA) from HEK293T cells or Lenti-X HEK293T Cells (Takara Bio) transfected with pcDNA3 or pcDNA3.1 vectors expressing fmScx, fmTwi, fhE12, fmE12, or fmE47 proteins following the manufacturer’s protocol. pcDNA3.1 vector with fhE12 was kindly provided by Dr. Eric N. Olsson39. DNA–protein binding was assayed with a Gelshift Chemiluminescent EMSA kit (Active Motif) following the manufacturer’s protocol. Briefly, a total of 2 μL of nuclear proteins were incubated with 100 fmol of biotin-labelled DNAs for 30 to 90 min at room temperature in binding buffer containing 1 μg of poly d(I-C), 5 mM MgCl₂, and 2.5% glycerol. For competition experiments, a 200-fold or 400-fold molar excess of unlabelled oligonucleotide was included in the binding reaction. Antibody supershifts were carried out by adding 5 μg of anti-FLAG M2 monoclonal antibody (Sigma–Aldrich) together with nuclear extracts prior to incubation with DNA. Protein–DNA complexes were resolved by electrophoresis on 5% or 4% polyacrylamide gel in Tris-borate-EDTA buffer, transferred onto Nytran SPC membrane (GE Healthcare), and detected with streptavidin-horseradish peroxidase conjugate and chemiluminescent reagent.

**Statistical analysis.** *P*-values were calculated by one-way analysis of variance using the SPSS software package (SPSS 21.0, SPSS, Inc., Chicago, IL, USA). The data were considered statistically significant at a *P*-value < 0.05.

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