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Review Article

Molecular characterization and function of tenomodulin, a marker of tendons and ligaments that integrate musculoskeletal components

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Summary Tendons and ligaments are dense fibrous bands of connective tissue that integrate musculoskeletal components in vertebrates. Tendons connect skeletal muscles to the bone and function as mechanical force transmitters, whereas ligaments bind adjacent bones together to stabilize joints and restrict unwanted joint movement. Fibroblasts residing in tendons and ligaments are called tenocytes and ligamentocytes, respectively. Tenomodulin (Tnmd) is a type II transmembrane glycoprotein that is expressed at high levels in tenocytes and ligamentocytes, and is also present in periodontal ligament cells and tendon stem/progenitor cells. Tnmd is related to chondromodulin-1 (Chm1), a cartilage-derived angiogenesis inhibitor, and both Tnmd and Chm1 are expressed in the $CD31^{-}$ avascular mesenchyme. The conserved C-terminal hydrophobic domain of these proteins, which is characterized by the eight Cys residues to form four disulfide bonds, may have an anti-angiogenic function. This review highlights the molecular characterization and function of Tnmd, a specific marker of tendons and ligaments.

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Contents

1. Introduction

Tendons act as transmitters of muscular force and also provide mechanical energy storage *via* reversible stretching of collagen fibers [1]. Ligaments, along with the capsule, seal the joint space to prevent loss of synovial fluid, which lubricates the articular surfaces, and connect one bone to another to stabilize synovial joints by limiting their movement [2]. In the musculoskeletal system, mechanical energy generated from a muscle—tendon unit is used for locomotion, and is stored during deformation of ligaments and the capsule (Fig. 1). The stored energy is then transferred back to the skeletal muscle through the tendon [3]. Tendons and ligaments in the craniofacial, axial, and appendicular regions are specifically marked by tenomodulin (Tnmd), a type II transmembrane glycoprotein [4—6].

Tendons and ligaments, together with fascia of the skeletal muscle, aponeuroses, and the sclera and cornea of the eye, are classified as dense fibrous connective tissue. Such tissue consists of cells, collagen, elastic fibers, proteoglycans, and water. Collagen is the most abundant extracellular matrix protein involved in integration of musculoskeletal components. Tendons and ligaments are primarily composed of type I collagen, which provides mechanical stability and elastic energy storage, but also contain small amounts of types III and V collagen [3]. Other collagens such as types II, VI, XI, XII, and XIV are localized to fibrocartilage at the entheseal junction with bone, but are not present in the midsubstance of the tendon [7,8]. The highly aligned collagen fibers of tendons are arranged along the long axis. In contrast to other vascular rich mesenchymal tissues such as bone, skeletal muscle, and adipose tissue, tendons and ligaments have low vascularity and exhibit a limited distribution of blood vessels [8].

In the early stages of musculoskeletal development, progenitors of musculoskeletal components migrate and settle down in prospective regions to give rise to cartilage, muscle, tendon, and ligament primordia [9]. Each musculoskeletal primordium initially develops as an independent component, but later in development tendons and ligaments integrate each component into a single functional locomotive organ (Fig. 2). The tendon progenitor cell population is derived from the syndetome, lateral plate mesoderm, and neural crest [10,11]. The syndetome is a scleraxis (Scx)-positive subdomain that occupies the dorsolateral portion of the sclerotome to form the axial skeleton [12]. *Scx* also marks the progenitor cells of tendons and ligaments in the appendicular and craniofacial regions [5,13—15].

SRY (sex determining region Y)-box 9 (Sox9), a key regulatory transcription factor involved in chondrogenesis, is also expressed in a subpopulation of the tendon/ligament cell lineage [16,17]. Genetic lineage tracing revealed that tenocytes arise from both $Scx^+/Sox9^+$ and $Scx^+/Sox9^-$ progenitors, whereas ligamentocytes are derived from $Scx^+/Sox9^+$ progenitors [18,19]. The $Scx^+/Sox9^+$ cell population is also present in hyaline cartilage near tendon/ligament attachment sites [14,18]. Conditional knockout studies using *ScxCre;Sox9*$^{flox/flox}$ mice revealed that the $Scx^+/Sox9^+$ cell population is necessary for the establishment of junctions between hyaline cartilage and tendons/ligaments [18].

Various growth factors of the fibroblast growth factor (FGF) family and transforming growth factor beta (TGFβ) superfamily are involved in tendon development and subsequent growth [11]. For example, FGF-8 and FGF-4 positively regulate the expression of *Scx* in the axial and limb tendon progenitors of chicken embryos [20,21]. Induction of early progenitors by FGF signaling is followed by later recruitment and maintenance of tendon progenitors, mediated by TGFβ signaling. Disruption of TGFβ signaling in *Tgfβ2*$^{-/-}$*;Tgfβ3*$^{-/-}$ embryos or mice lacking *TGFβ type II receptor* (*TβRII*$^{-/-}$) results in the loss of most tendons and ligaments throughout the body, although the initial induction of Scx^+ tendon progenitors is not affected [22]. Specification of $Scx^+/Sox9^+$ bone eminence progenitors is also regulated by TGFβ signaling [19], whereas bone morphogenetic protein 4 (BMP4) supplied from developing tendon is required for differentiation of these progenitors into skeletal eminence-forming cells [23].

The cells residing in fully formed tendons and ligaments are elongated fibroblasts called tenocytes and ligamentocytes, respectively. Tenocytes, which reside between the longitudinally aligned collagen fibers, have extended nuclei and complex networks of cytoplasmic processes that extend through the extracellular matrix and connect with neighboring cells *via* gap junctions, allowing cellular communication [8]. The gap junctions of tenocytes are composed of connexin 32 and connexin 43 [24,25]. Tnmd, a specific marker of mature tenocytes and ligamentocytes, is a type II transmembrane protein related to chondromodulin-1 (Chm1), a cartilage-derived growth regulator [4,6,26]. In this review, we focus on functional roles of Tnmd in development, growth, and maintenance of tendons and ligaments.

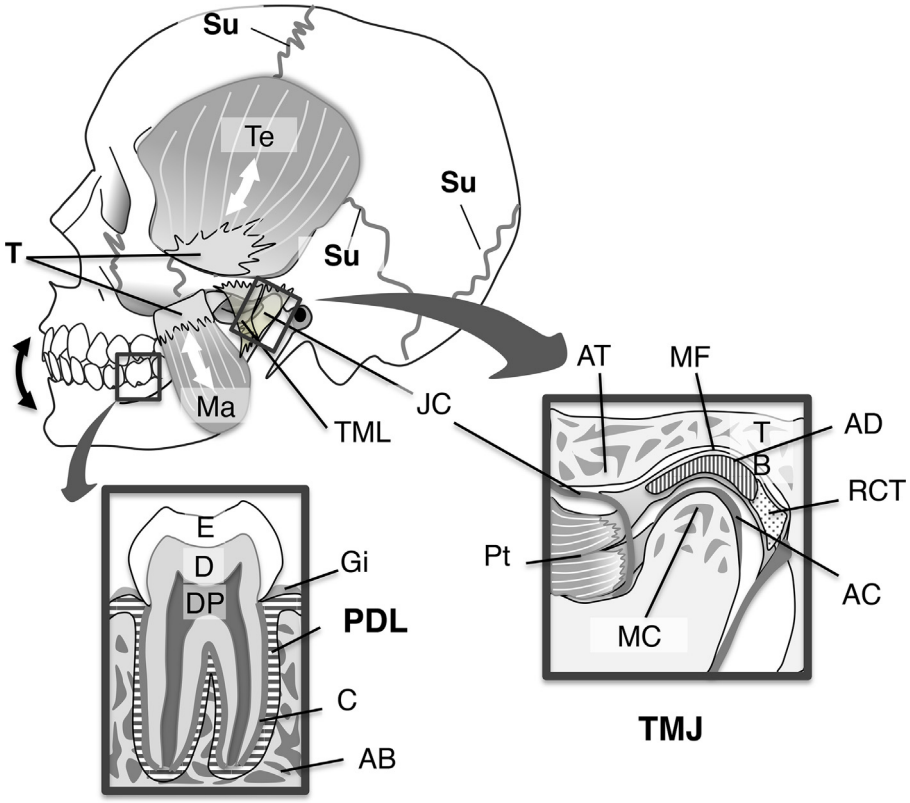

Figure 1 Schematic illustration of musculoskeletal components associated with mastication. Tendons (T) of the masseter muscle (Ma) and temporal muscle (Te) are shown. Temporomandibular joint (TMJ) and periodontium of the mandibular first molar are enlarged. Su, suture; JC, joint capsule; TML, temporomandibular ligament; AT, articular tubercle; MF, mandibular fossa; TB, temporal bone; AD, articular disc; Pt, pterygoid muscle; AC, articular cartilage; MC, mandibular condyle; RCT, retrodiscal connective tissue; E, enamel; D, dentin; DP, dental pulp; Gi, gingiva; PDL, periodontal ligament; C, cementum; AB, alveolar bone.

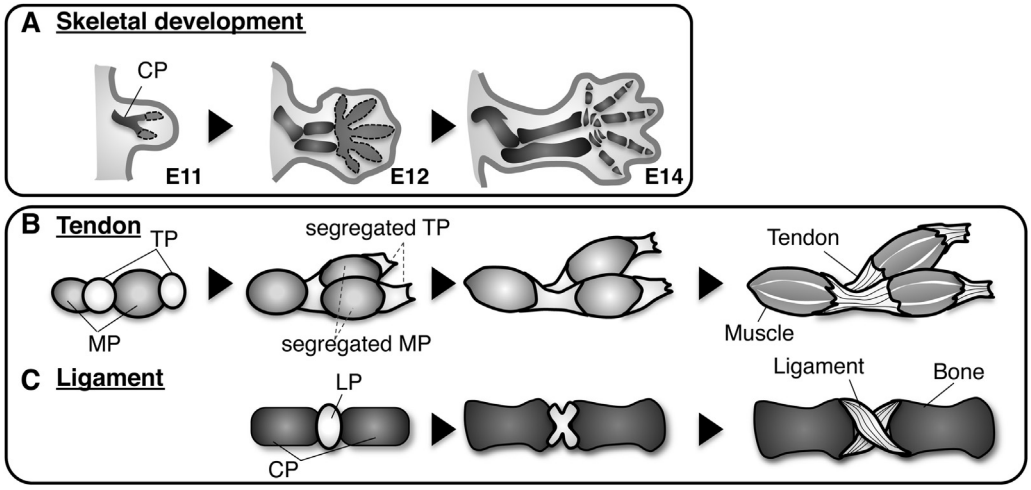

Figure 2 Schematic illustrations of the development of musculoskeletal tissues. (A) Skeletal development of the forelimb of mouse embryo from embryonic day 11 to 14. The regions undergoing precartilaginous condensation are surrounded by a dotted line. (B, C) Simplified models of tendon (B) and ligament (C) development. Tendon primordia segregate into individual tendons, depending on interactions with muscles (B). Ligament primordium forms adjacent to cartilage primordium, in association with joint formation (C). CP, cartilage primordium; LP, ligament primordium; MP, muscle primordium; TP, tendon primordium.

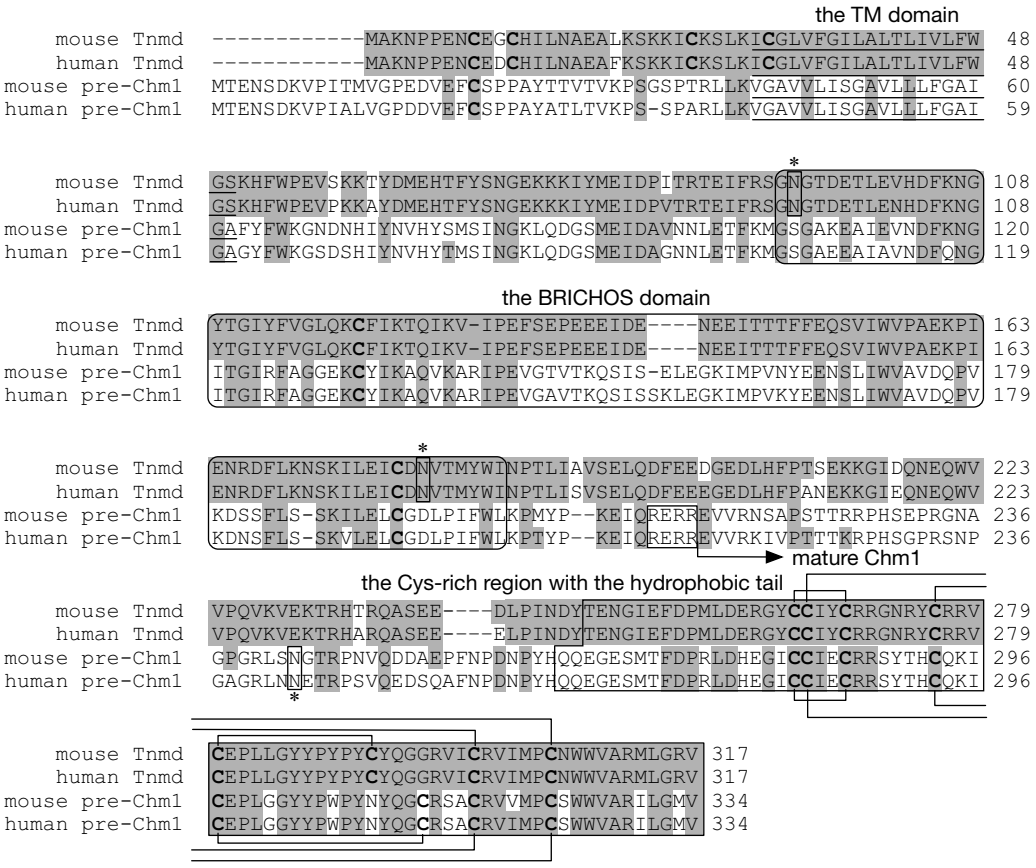

Figure 3 Comparison of the amino-acid sequences of mouse and human Tnmd and Chm1 precursor (pre-Chm1). The amino-acid sequences corresponding to the putative transmembrane (TM) domain are underlined. Asterisks indicate the positions of putative N-glycosylation sites (Asn residues). The furin-like endoprotease cleavage site (Arg-Glu-Arg-Arg) is boxed. The arrow indicates the cleavage product that is secreted from cells as mature Chm1. The C-terminal boxed area shows the conserved Cys-rich region with the hydrophobic tails of Tnmd and Chm1. The connecting lines indicate the configuration of the intramolecular disulfide bonds in the Cys-rich regions of Tnmd and Chm1.

2. Structure of Tnmd and Chm1

As shown in Fig. 3, Tnmd was identified as a type II transmembrane glycoprotein (317 amino acids) with a characteristic hydrophobic domain homologous to Chm1 (120 amino acids) at its C-terminus [4,6,26]. It can be detected by western blotting as glycosylated and non-glycosylated protein bands with molecular masses of 40 and 45 kDa, respectively [27,28].

Chm1 was initially purified from guanidine extracts of bovine epiphyseal cartilage as a growth and maturation factor for cultured chondrocytes [29,30], but was later identified as an angiogenesis inhibitor that blocks the growth of vascular endothelial cells [31]. Purified recombinant human Chm1 protein inhibits VEGF-induced angiogenesis and lymphangiogenesis in corneal micropocket assays as well as tumor angiogenesis and growth [32,33].

Two structural domains are distinguished in Chm1 (Fig. 4A): the N-terminal hydrophilic domain with N- and O-glycosylation sites (domain 1, encoded by exon 6 of the *Chm1* gene), and the remaining C-terminal domain. The C-terminal domain contains all eight Cys residues in the protein (the Cys-rich region), which form four disulfide bridges and are well conserved across vertebrate species, followed by the C-terminal hydrophobic tail (domain 2, encoded by exon 7 of the gene) [34,35]. Systematic Cys-to-Ser mutations and deletion experiments indicated that the single disulfide-bridged cyclic structure (Cys83-Cys99) and the C-terminal tail (Trp111-Val120) in domain 2 represent the principal anti-angiogenic structure of Chm1 (Fig. 4B, upper diagram) [36]. To test this idea, a synthetic Chm1 C-terminal peptide containing this 17-membered cyclic structure was prepared and shown to clearly inhibit VEGF-A-induced migration of cultured vascular endothelial cells [36]. Tnmd is predicted to have a similar but smaller 13-membered disulfide-bridged cyclic structure (Cys280-Cys292) near its C-terminus (Fig. 3). A Tnmd C-terminal peptide containing the smaller cyclic structure (Fig. 4B, lower diagram) was synthesized and shown to be anti-angiogenic as well [36].

Human Chm1 is biosynthesized in cartilage as a type II transmembrane precursor protein of 334 amino acids (Fig. 3). After cleavage at the furin hormone-processing motif (Arg-Glu-Arg-Arg), the C-terminal region (120 amino acids) is secreted as the mature form of Chm1 and stored in the extracellular matrix [30]. No uncleaved Chm1 precursor is present in cartilage. By contrast, Tnmd does not contain

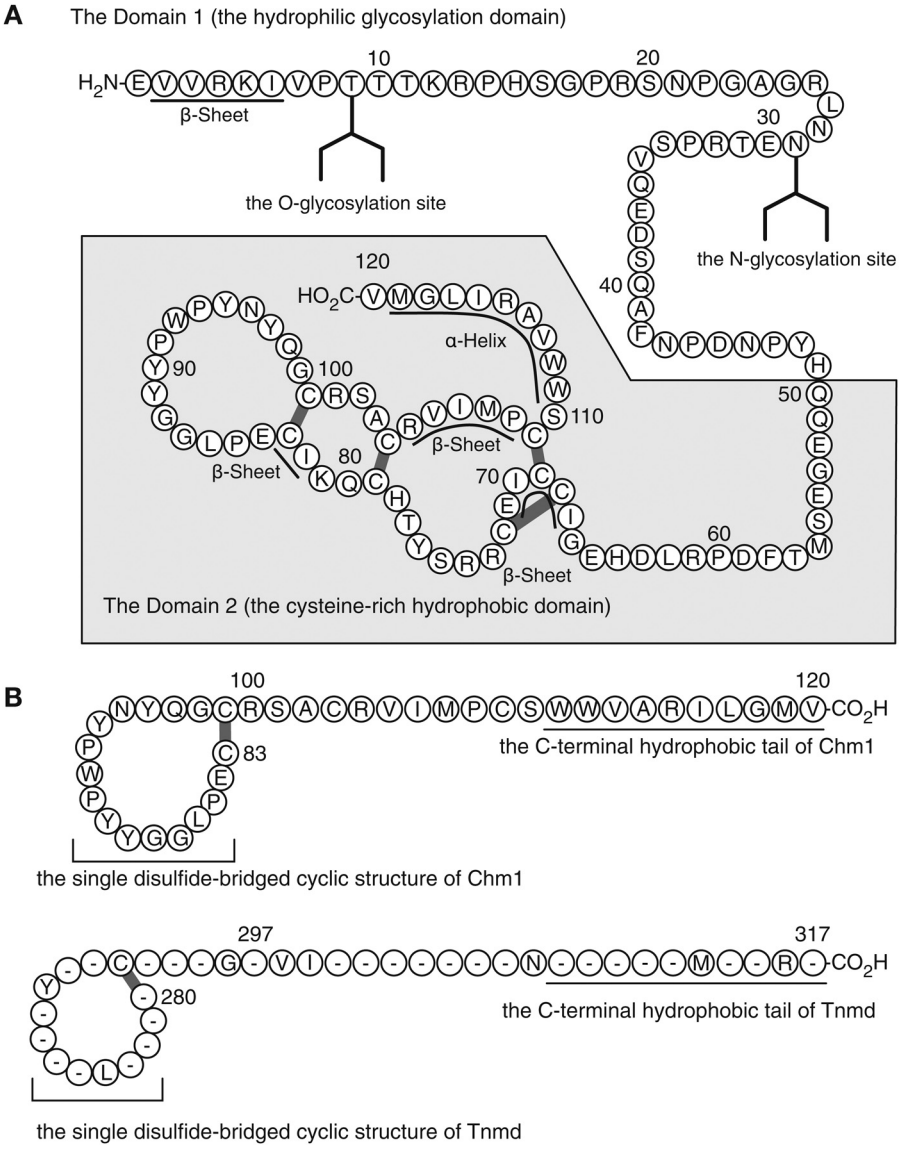

Figure 4 Characteristic domain structures in human Chm1, and the indispensable anti-angiogenic structure in human Chm1 and Tnmd. (A) White circles indicate the amino-acid sequence of human Chm1. Thick bars connecting Cys residues represent intramolecular disulfide bonds predicted from the configurations of disulfide bonds in bovine Chm1. Putative glycosylation sites and the predicted α-helix and β-sheets are also indicated. (B) Comparison of the single disulfide-bridged cyclic structures and C-terminal hydrophobic tails in Chm1 and Tnmd. Conserved amino-acid residues are indicated as (−).

a furin processing signal. Surface labeling experiments of transfected COS7 cells revealed that human Tnmd is indeed expressed as a type II transmembrane protein, with its C-terminus exposed to the exterior of the cell [26]. However, immunohistochemical studies indicated that the C-terminal anti-angiogenic region of Tnmd is stored in the extracellular matrix of a certain type of tendinous tissue [37—39]. Thus, it remains unclear whether the C-terminal part of Tnmd is actually cleaved out.

Both Tnmd and the Chm1 precursor have a BRICHOS domain characterized by a pair of conserved cysteine residues (Fig. 3). The BRICHOS family of proteins has five members; the other three are BRI2, which is mutated in familial British and Danish dementia; CA11, which is involved in stomach cancer; and surfactant protein C, which is involved in respiratory distress syndrome (RDS) [40,41]. The biological role of the BRICHOS domain is not fully understood, although it has been functionally implicated in the post-translational processing of certain types of membrane proteins [42].

3. Distribution of Tnmd during development and postnatal growth

Tnmd is expressed in dense connective tissue including tendons, ligaments, fascia of skeletal muscle, and the sclera and cornea of the eye, all of which are hypovascular [4,5,18,26,28]. In the heart, Chm1 is expressed in valves [43], whereas Tnmd is localized to chordae tendineae, which

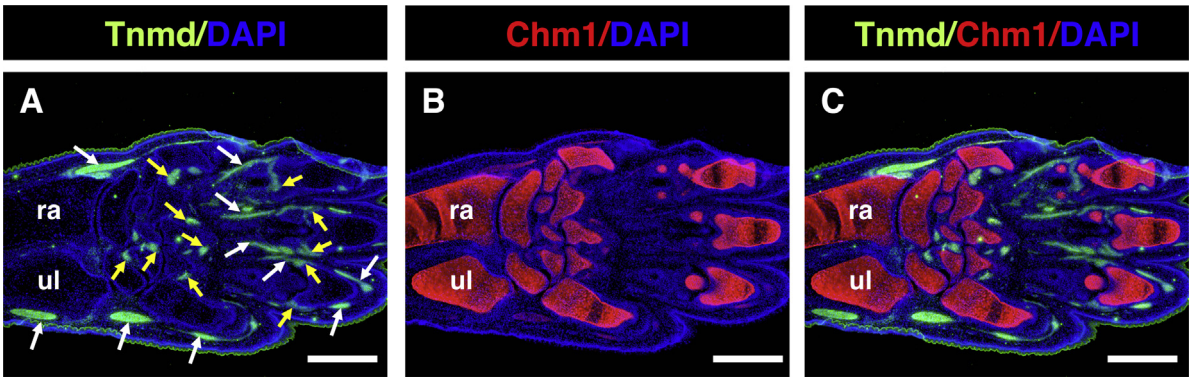

Figure 5 Localization of Tnmd and Chm1. (A—C) A frontal frozen section of the autopod prepared from a *Sox9*$^{flox/+}$ mouse at E18.5. Tnmd^{+} (green) (A, C) and Chm1^{+} (red) (B, C) regions were visualized by double immunostaining of the same section. A merged image is shown in C. White and yellow arrows in A indicate tendons and ligaments, respectively. Radius and ulna are indicated as ra and ul, respectively. Scale bars, 500 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

are similar to tendons that connect skeletal muscles to bone [37—39]. Tnmd is also expressed in human adipose tissue [44], whereas in 5-month-old mice it is expressed at much lower levels in subcutaneous white adipose tissue than in tail tendons [45].

Type I collagen, a major component of the extracellular matrix in fibrous connective tissues, is distributed in both dense and loose connective tissues, but Tnmd is predominantly localized to dense connective tissues containing thick bundles of aligned collagen fibers [5]. In tendons, elongated tenocytes are aligned parallel to thick mature collagen fibers to form regular layers, whereas oval tenocytes are randomly distributed in the interlaminar spaces [5]. Tnmd is expressed in elongated but not oval tenocytes, suggesting that this molecule is a useful marker of mature tenocytes *in vivo*.

During development, Scx expression is associated with formation of tendon/ligament primordium [13], whereas Tnmd expression increases markedly in parallel with maturation of tendons and ligaments [5]. In the periodontal ligament (PDL) that joins the cementum covering the root of the tooth to the alveolar bone, Scx expression is induced during tooth eruption after birth and is significantly upregulated by a tensile force [46]. Similarly, Tnmd is expressed in the PDL of eruptive and post-eruptive teeth, where it promotes maturation or maintenance of the PDL by positively regulating cell adhesion [38]. In contrast to other ligaments connecting two or more adjacent bones, the PDL between the tooth and the alveolar bone is well vascularized and has a high level of alkaline phosphatase (ALP) activity [46,47]. In the PDL, Scx is expressed in ALP-positive PDL fibroblasts, but is not detectable in perivascular cells, which are reported as progenitor cells that ultimately differentiate into osteoblasts, cementoblasts, and fibroblasts [48]. Scx-positive PDL cells contribute to the maintenance of the ligamentous tissues.

Among well-vascularized mesenchymal tissues in the musculoskeletal system, cartilage expressing Chm1 is exceptionally avascular and highly resistant to vascular invasion [49,50]. Dense connective tissue expressing Tnmd is also poorly vascularized [50]. Tnmd and Chm1 are differentially expressed in the CD31-negative avascular mesenchyme in musculoskeletal tissue [50] (Fig. 5). In developing attachment sites for tendons or ligaments, Tnmd- and Chm1-positive cells are derived from Sox9^{+} progenitors and localized to tendons/ligaments and hyaline cartilage, respectively [18].

4. Anti-angiogenic actions of Tnmd

Similarly to Chm1, Tnmd inhibits angiogenesis both *in vitro* and *in vivo* [34]. The soluble form of the C-terminal Cys-rich domain of Tnmd (116 amino acids) inhibits proliferation, migration, and tube formation of human umbilical vein endothelial cells and retinal vascular endothelial cells [26,28]. When malignant melanoma cells overexpressing the soluble form of Tnmd are inoculated into syngeneic mice, tumor growth is significantly suppressed by inhibition of angiogenesis [26]. Although loss of Chm1 or Tnmd in mice does not cause any apparent angiogenesis phenotype during embryonic development [39,51], loss of Chm1 increases expression of vascular endothelial growth factor (VEGF)-A, lipid deposition, and mineralization in the cardiac valve in aged animals [43]. The local absence of Tnmd in the cardiac chordae tendineae induces angiogenesis and matrix metalloproteinase activation [37]. Adenoviral overexpression of the full coding region of Tnmd (317 amino acids) does not inhibit either tube formation *in vitro* or tumor angiogenesis *in vivo*, suggesting that cleavage of the C-terminal Chm1-like domain from Tnmd and subsequent secretion are required for the protein's anti-angiogenic activities [26]. No soluble Tnmd is detected in the conditioned medium of COS7 cells overexpressing Tnmd [26]. Indeed, in tissue extracts of tendons and eye, endogenous Tnmd proteins can be detected by western blotting as a double band between ~40 and ~45 kDa [28]. On the other hand, the short form of Tnmd protein containing the C-terminal hydrophobic region is present in the extracellular matrix of some tendons and chordae tendineae in the heart [37,39]. In addition, 16 kDa Tnmd inhibits retinal pathological angiogenesis in an ischemic retinopathy mouse model [52]. Thus, under some physiological and pathological conditions, the extracellular anti-angiogenic domain of

Tnmd could be cleaved and released from cells in the soluble form by ectodomain shedding, and subsequently modulate angiogenesis.

5. Role of Tnmd in tenocytes/ligamentocytes and tendon stem/progenitor cells

Mice lacking *Tnmd* do not have severe developmental phenotypes, but they do exhibit reduced tendon cell density, reduced tenocyte proliferation, and elevated collagen fibril size in postnatal tendons [39]. Similarly to Chm1, which synergistically stimulates chondrocyte proliferation in the presence of FGF-2 [30], Tnmd positively regulates tenocyte proliferation. Overexpression of Tnmd in a human PDL cell line positively regulates cell adhesion to promote maturation and maintenance of the differentiated phenotype [38].

Tendons contain tendon stem/progenitor cells (TSPCs) involved in tissue homeostasis and regeneration [53,54]. TSPCs contribute to the early healing response in acute injuries, as well as the ectopic cartilage/bone formation observed in chronic tendinopathy. TSPCs reside in a unique molecular niche composed of biglycan and fibromodulin, which modulate BMP signaling and are critical for TSPC function [53,55]. In comparison with human bone marrow-derived mesenchymal stem cells (BMSCs), human TSPCs express *Tnmd* at a higher level [53]. Ectopic expression of Scx induces conversion of human BMSCs into tendon progenitor cells expressing *Tnmd* [56]. In addition, BMP-12 induces *Scx* and *Tnmd* expression in rat BMSCs and mouse C3H10T1/2 cells [57,58].

A recent study showed that loss of Tnmd does not affect multipotency of *Tnmd* KO-derived TSPCs, but instead results in reduced TSPCs clonogenicity and self-renewal together with higher incidence of senescence [59]. Re-expression of either full-length Tnmd or the C-terminal 116 amino acids in *Tnmd* KO-derived TSPCs rescues the proliferation deficit, suggesting that Tnmd is required for TSPCs self-renewal [59].

6. Tnmd expression during tendon repair/healing

Once injured, repair/healing of tendons or ligaments is a slow process due to the hypocellular nature of these tissues and their poor blood supply relative to well-vascularized mesenchymal tissues such as bone and dermis. Damaged or torn tendons or ligaments are often filled with scar tissue, in which collagen fibers are more abundant and haphazardly arranged. Therefore, it is challenging to develop a strategy for inducing complete regeneration of tendons or ligaments. To this end, Tnmd is a useful phenotypic marker for evaluating maturation of tenocytes and ligamentocytes during the healing process. In a rat rotator cuff healing model, FGF-2 enhances tendon-to-bone healing, a process in which increased Tnmd expression is correlated with formation of tendon-like reparative tissues accompanied by extracellular collagen fibers with aligned orientations [15].

7. Regulation of Tnmd expression by transcription factors

Tnmd and Scx are coexpressed in developing tendons and ligaments during development [5]. In *Scx*-null mice, *Tnmd* expression is not detected in the developing tendon [60]. Overexpression of *Scx* in developing chicken hindlimb results in upregulation of *Tnmd* in tenocytes, although Scx does not induce ectopic tendon formation [5]. Upregulation of *Tnmd* is also observed in cultured tenocytes upon overexpression of *Scx* or other b-HLH transcription factors such as *Paraxis* and *Twist* [45]. By contrast, overexpression of *Myogenin*, one of the key transcription factors involved in myogenesis, induces myogenic differentiation in tenocytes and dramatic downregulation of *Tnmd* expression [45]. Likewise, upon Sox9 overexpression, *Tnmd*$^{+}$ tenocytes transdifferentiate into chondrocytes expressing *Chm1* but not *Tnmd* [45]. Pax1, a sclerotomal marker, also downregulates *Tnmd* expression in tenocytes [5]. Mohawk (Mkx), an atypical homeodomain-containing transcription factor, is predominantly expressed in tendons and ligaments [61,62]. In mice lacking *Mkx*, *Tnmd* expression decreases without affecting *Scx* expression, suggesting that Mkx is also involved in regulation of *Tnmd* expression [62]. The low level of *Tnmd* expression in BMSCs increases when these cells are cultured in collagen gel containing glycogen synthase kinase-3 inhibitor [63], suggesting that Wnt/β-catenin signaling also mediates the expression of Tnmd. Together, these observations indicate that *Tnmd* expression is closely associated with the differentiation status of cells in the tendon and ligament lineages and sensitively reflects the maturation level of tenocytes and ligamentocytes.

8. Conclusions and perspectives

Understanding the molecular mechanisms governing development, growth, and maintenance of tendons and ligaments is an important step toward developing functional regenerative therapies for these force-transmitting tissues, which are often replaced by scar tissue after injury. Tnmd is an anti-angiogenic type II transmembrane glycoprotein with a C-terminal Cys-rich domain homologous to that of Chm1. Tnmd specifically marks tendons and ligaments in the craniofacial, axial, and appendicular regions, and the level of Tnmd expression is correlated with avascularity in tendinous and ligamentous tissues. Furthermore, a series of genetic studies using *Tnmd* knockout mice revealed multiple functional roles of Tnmd as a positive regulator of postnatal tendon growth, maturation of collagen fibers, and cellular adhesion in the PDL, as well as in maintenance of stemness in TSPCs. During development and growth, *Tnmd* expression is positively regulated by *Scx* and increases in association with maturation of tendons and ligaments. During the tissue repair/healing process, *Tnmd* expression is expressed at high levels in mature tenocytes, suggesting that it is an excellent marker specific for tenocyte and ligamentocyte differentiation from mesenchymal stem cells. Thus, functional repair of tendons and ligaments can be evaluated in terms of the expression level of *Tnmd* in the healing tissues. Screening of molecules that facilitate upregulation of *Tnmd* during wound healing represents a promising strategy

for identifying agents for future regenerative therapies of tendons and ligaments.

Conflict of interest statement

None declared.

Acknowledgements

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