Differential expression and cellular localization of novel isoforms of the tendon biomarker tenomodulin

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Abstract

Tenomodulin (Tnmd), also called Tendin, is classified as a type II transmembrane glycoprotein and is highly expressed in developing as well as in mature tendons. Along with scleraxis (scx), Tnmd is a candidate marker gene for tenocytes. Its function is unknown, but it has been reported to have anti-angiogenic properties. Results in a knockout mouse model did not substantiate that claim. It has homology to chondromodulin-I. Single nucleotide polymorphisms of TNMD have been reported to be associated with obesity and Alzheimers disease in patients. In the present study, we showed the existence of multiple Tnmd isoforms in human tendons and a role in regulation of tenocyte cell proliferation. Results of experiments with intracellular localization of green fluorescent protein (GFP)-linked Tnmd constructs shows that isoforms I and II localize to the cell tubule formation on Matrigel in vitro, inhibition of endothelial cell tubule formation on Matrigel in vitro, inhibition of endothelial cell migration in vitro, and failure to develop capillaries in the retina, following post-oxygen-induced retinopathy in vivo (16, 31, 32). However, data from a Tnmd mouse knockout did not support the involvement of Tnmd in anti-angiogenesis, although, in retrospect, only isoforms 1 and 2 were actually knocked out (8). Two recent reports also associated six, single nucleotide polymorphisms (SNPs) in Tnmd with macular degeneration and with obesity (43, 45, 46). A report of a myostatin (MSTN−/−, GDF-8) knockout mouse model has indicated that expression of Tnmd as well as collagen 1 and scleraxis (Scx) are decreased (27). Although Tnmd is accepted as a major tenocyte biomarker, its function in tendons is still disputed and unknown.

These nonconcordant findings between in vivo and in vitro studies indicate that the understanding of Tnmd is incomplete. In the present study, we showed the existence of multiple Tnmd isoforms in human tendons and a role in regulation of tenocyte cell proliferation. Results of experiments with intracellular localization of green fluorescent protein (GFP)-linked Tnmd constructs shows that isoforms I and II localize to the nuclear envelope while isoform III is mainly cytoplasmic. Functional prediction models suggest that each Tnmd isoform may play distinct roles in vivo.

METHODS

Tendon collection from patients. Flexor carpi radialis (FCR, mid-tendon specimen location), biceps brachii tendons (BT, long head of the biceps brachii tendon proximal to the rotator cuff), flexor digitorum profundus tendons (FDP from proximal to a repair end in the digit), synovium (from the flexor tendon complex in the wrist), and adhesion (from the FDP) were collected at surgery from discarded tissue (deidentified male and female patients ages 26–67 yr, UNC Memorial Hospital, Institutional Review Board approved protocol). Specimens for nucleic acid extraction were placed in labeled plastic tubes and snap-frozen in liquid nitrogen in the operating room and then transferred to a −80°C freezer until processed. Specimens for cell isolation were placed in DMEM-H medium with 20 mM HEPES, pH 7.4, and antibiotics in preparation for cell isolation (1). Porcine Achilles tendons were collected at a local abattoir, and cells were isolated and cultured as for human cells. COS-7 cells were obtained from the University of North Carolina (UNC) cell repository (Cell Culture facility, UNC, Chapel Hill, NC) and cultured as for tenocytes.

Isolation and maintenance of tenocytes. Tenocytes from the deep collagenous material were freed by collagenase digestion followed by trypsin then collagenase digestion, washed, collected, and then transferred to medium (1, 2, 10, 35). Cells were washed in complete DMEM-H medium with 15 mM glutamine, 20 mM sodium pyruvate, 20 mM anti-angiogenesis activity (9, 16, 31, 32). Tnmd is also expressed in the eye, in cornea, retina, and sclera, where it is thought to act as an angiogenesis inhibitor (32). Data supporting the anti-angiogenesis concept include blocking endothelial cell tubule formation on Matrigel in vitro, inhibition of endothelial cell migration in vitro, and failure to develop capillaries in the retina, following post-oxygen-induced retinopathy in vivo (16, 31, 32). However, data from a Tnmd mouse knockout did not support the involvement of Tnmd in anti-angiogenesis, although, in retrospect, only isoforms 1 and 2 were actually knocked out (8). Two recent reports also associated six, single nucleotide polymorphisms (SNPs) in Tnmd with macular degeneration and with obesity (43, 45, 46). A report of a myostatin (MSTN−/−, GDF-8) knockout mouse model has indicated that expression of Tnmd as well as collagen 1 and scleraxis (Scx) are decreased (27). Although Tnmd is accepted as a major tenocyte biomarker, its function in tendons is still disputed and unknown.

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HEPES, pH 7.2, 0.1 mM ascorbate 2-phosphate, and antibiotics (1% sodium penicillin G and streptomycin sulfate and 0.1% Fungizone). Cells were plated on type 1 collagen-coated polystyrene culture dishes at 25,000 cells/cm² and grown to 90% confluence before passage with 0.1% trypsin. Cells were used from passages 1 to 3.

**Immunohistochemistry.** Two-millimeter cubed tendon segments of each tendon type were harvested at surgery, oriented in plastic histologic block containers so that a longitudinal cross section could be sectioned, embedded in optimum cutting temperature medium, and frozen at −20°C. Five-micrometer sections were cut using a microscopy and placed on an albumin-coated glass slide (A.M. Laboratory, Baltimore, MD). Tenocytes from FCR or biceps tendons were grown to passage two and plated on collagen-coated cover slips. Tnmd was stained with anti-NH2- or COOH-terminal Tnmd antibodies (Sigma Chemical, St. Louis, MO; Santa Cruz Biotechnology) at a 1:200 dilution at 4°C overnight. After washing three times with PBS (pH 7.2), Tnmd was visualized with an Alexa 568-conjugated goat anti-rabbit IgG at 1:500 dilution (Molecular Probes). Images were taken with an epifluorescence microscope (Olympus BX60; OPELCO, Dulles, VA) or a Leica SP2 AOBS laser scanning confocal microscope at 0.16-μm optical section (Leica Microsystems, Exton, PA) with a ×40 or ×60 oil immersion objective.

**Molecular modeling.** The I-TASSER molecular modeling program developed at the University of Michigan, Ann Arbor, was used to produce the three-dimensional structures shown in Fig. 2, A-C (the laboratory of Y. Zhang, Center for Computational Medicine and Bioinformatics, University of Michigan, Ann Arbor, MI; http://zhang.bioinformatics.ssw.ku.edu/I-TASSER). The program has updated algorithms for secondary structure, binding energies, including neural network solvent accessibility predictions and two round progressive assembly simulations (28, 50).

**Quantitative PCR.** Total RNA was isolated using an RNAeasy mini kit (QIAGEN, Valencia, CA), according to the manufacturer’s protocol. cDNA was synthesized with SuperScriptII (Invitrogen, Carlsbad, CA). The expression levels of target genes were determined by SYBR green real-time PCR using 18S rRNA as an internal control (Ambion, CA). The PCR conditions were as follows: 30 cycles of 94°C for 30 s, 60°C for 60 s, and 72°C for 30 s (optimized for each major reaction set). Tenomodulin isoforms I and II share the same NH2-terminal sequence, and isoforms I and III share the same COOH-terminal sequence. Because of the overlap in sequence between each isoform set, four pairs of primers were designed to determine the expression levels of each isoform. To reduce the bias caused by the location of primers, we used two different sets of primers to amplify all three isoforms; primer set 1, located in exons 3 and 5, amplified isoforms 1 and 3; and primer set 2, located in exons 3 and 5, amplified all three isoforms. Primer set 3, located in exons 3 and 5, amplified isoforms 1 and 3; and primer set 4, located in exons 3 and 5, amplified all three isoforms. Primer sequences were as follows: primer set 1, 5′-ATTCAAGAAGG-GAAATGCACTGTA-3′ (forward) and 5′-TAGGCTTTCTGCTTGGACCCCAAC-3′ (reverse); primer set 2, 5′-ACT TCT GGC CGG AGG TACCCA A-3′ (forward) and 5′-TGC TGG GAC CCA AAT CAC TCA CTG TA-3′ (reverse); primer set 3, 5′-TTG GGT CCC AGG AGA AAA GCC TA-3′ (forward) and 5′-TCT CTC ATC CAG CAG GGG ATC AAA-3′ (reverse); and primer set 4, 5′-CGG AGT AGA GGA GA-3′ (forward) and 5′-CAG TCT GTG TAG CTG TAT GGA-3′ (reverse). Primers for scx and MSTN were 5′-AAG AAA AGC CAG CGC AGA AAC TTCC-3′ (forward) and 5′-GAT GAC GAT-3′ (reverse). After preincubation with blocking buffer (10% nonfat milk, 0.1% Tween 20 in TBS), the membrane was probed with the specific polyclonal anti-Tnmd antibody against either NN2-terminal or COOH-terminal peptide sequences in Tnmd at 4°C overnight, followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody. Immunoreactive signals were detected by enhanced chemiluminescence, and blots were digitally recorded and analyzed by image analysis for molecular weight determinations.

To determine if Tnmd was secreted from the cells, the medium of transfected cells was replaced with serum-free medium at 24 h posttransfection. Cells were further cultured for 16 h, and the supernatant fluids were collected. Cell lysates were collected in RIPA buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% SDS) containing protease inhibitors. Aliquots (20 μg total protein/lane) of the samples were mixed with 2× SDS-PAGE sample buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 10% β-mercaptoethanol, and 20% glycerol), separated in a 12.5% SDS-PAGE, and then transferred to a nitrocellulose membrane. After preincubation with blocking buffer (10% nonfat milk, 0.1% Tween 20 in TBS), the membrane was probed with the specific polyclonal anti-Tnmd antibody against either NN2-terminal or COOH-terminal peptide sequences in Tnmd at 4°C overnight, followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody. Immunoreactive signals were detected by enhanced chemiluminescence, and blots were digitally recorded and analyzed by image analysis for molecular weight determinations.

**Overexpression of Tnmd isoforms I, II, and III.** cDNA of human Tnmd isoforms I, II, and III were amplified from human tendon total RNA using primers listed as follows: isoform I, 5′-aact cGA Gct ATG GCA AAG AAT CCT CCA GAG AA-3′ (forward) and 5′-aact ATC GCA CCC TCC CCA GAG TGC GGG C-3′ (reverse); isoform II, 5′-aact cTA Gct ATG GCA AAG AAT CCT CCA GAG AA-3′ (forward) and 5′-aact aGG ATC CTA CTG TGT GGA ATG AAT AAG AGG ATG AT-3′ (reverse); and isoform III, 5′-aCT CGA Gct ATG GAG CAC ACT TCT TAC AGC AAT-3′ (forward)
Fig. 2. Predicted structures of tenomodulin isoforms from the I-TASSER molecular modeling program. The tenomodulin gene is located on the X chromosome at q22.1 (a in A–C). There is a BRICHOS (British dementia, chondromodulin, surfactant protein) domain at different locations in each isoform [BRICHOS domain (B) and amino acid sequence for each isoform (C)]. The deduced amino acid sequence is given for each isoform (D). For the three-dimensional (3D) models, the blue ball is the NH2-terminal end, and the red ball is the COOH-terminal end; the magenta balls represent the ligand, and the green pattern is the ligand binding site in the protein.

A: TNMD isoform I modeled as a cytosine methyltransferase. Isoform I is a 317-amino-acid protein with a BRICHOS domain between residues 93 and 186.

B: isoform II modeled as a SUMO-1-like SENP-1 protease that was 207 amino acids with a BRICHOS domain between residues 93 and 186.

C: isoform III modeled as a /H9251-syntrophin adaptor protein that was 254 amino acids with a BRICHOS domain between residues 30 and 123.
and 5′-aaa aGG ATC CGA CCC TCC CCA GCA TGC GGG C-3′ (reverse). PCR products were purified with a QIAquick PCR purification kit (QIAGen) and cut with Xho I and BamH I. The digested PCR products were purified and ligated with Xho UbamH I-digested pEGFP-C1 vector with T4 DNA ligase, respectively. The cloning constructs were confirmed with DNA sequencing. Representative human tenocytes from the listed tendons, from porcine Achilles tendon cells, or COS-7 cells were transfected with the constructs for isoforms 1, 2, or 3. Cell images were taken with an epifluorescence microscope (Olympus BX60; OPELCO) or a LeicaSP2 AOBS laser scanning confocal microscope at 0.2 micron optical section (Leica microsystem) with a ×40 oil immersion objective.

**Knockdown of Tnmd isoforms with small-interfering RNA.** A small-interfering RNA (siRNA) targeting human Tnmd exon 5 was purchased from Ambion (S 34442, CAAUCCCACUCUAAUAUCAtt, catalog no. 4392420; Grand Island, NY), which knocked down all three human Tnmd isoforms. siRNAs at a final concentration of 40 nM were transfected into human tenocytes with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. The control group was transfected under the same conditions but with scrambled siRNA. Cells were collected at 48 h posttransfection for qPCR analysis.

**Cell proliferation assay.** A cell proliferation assay was carried out with a bromodeoxyuridine (BrdU) cell proliferation assay kit (Millipore, NJ). Twenty-four hours posttransfection, 0.4 μl of 500× BrdU reagent was added to each well in a six-well culture plate, and cells were incubated for another 24 h. Cells were fixed with the fixing solution and stained with anti-BrdU antibody and DAPI. BrdU-positive cells (proliferating cells) were visualized with Alexa 568-conjugated goat anti-mouse IgG. Triplicate assays were performed, and >500 cells/well were counted. The overall percentage of proliferating cells divided by the total cell number was calculated for each RNA interference experiment.

**Statistical analysis.** All experiments were repeated at least three times. Statistical analyses were performed using a one-way ANOVA and Student’s t-test using SigmaStat. A value of *P* < 0.05 was deemed as significant.

## RESULTS

**Three Tnmd transcripts.** Three distinct Tnmd transcripts were expressed in tendon tissue from multiple human specimens and tendon types as well as porcine and equine (equine data not shown) and from isolated tenocytes from the tissue sources (Fig. 1). Data mining in the Genecard database revealed that Tnmd had its locus on chromosome X at q22.1 and was expressed in tendon tissue from multiple human species and tendon types as well as porcine Achilles tendon cells, or COS-7 cells were transfected with the constructs for isoforms 1, 2, or 3. Cell images were taken with an epifluorescence microscope (Olympus BX60; OPELCO) or a LeicaSP2 AOBS laser scanning confocal microscope at 0.2 micron optical section (Leica microsystem) with a ×40 oil immersion objective.

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**Molecular models of tenomodulin isoforms.** Using the I-TASSER molecular modeling program, we were able to develop three-dimensional models for each of the tenomodulin isoforms. Tnmd isoform I modeled as a cytosine methyltransferase (Fig. 2A). Tnmd isoform II modeled as a SUMO-1-like SENP-1 protein (Fig. 2B). Tnmd isoform three modeled as an α-syntrophin, plextrin homology domain protein (Fig. 2C). RMSDα is the root mean square deviation between models and the Protein Data Bank structure in the structurally aligned regions by transmembrane domain (TM)-align. IDENTα is the percentage sequence identity in the structurally aligned region. Cov. represents the coverage of the alignment and is equal to the number of structurally aligned residues divided by the length of the model.

A consensus prediction of gene ontology (GO) terms is derived from the structural analogs that have a functional homology (Fh) score of ≥1.0. The GO score associated with each prediction is defined as the average weight of the GO term, where the weights are assigned based on the Fh score of the template from which the GO term is derived. A prediction with a GO score >0.5 signifies a prediction with high confidence and vice versa.

**Immunostaining and localization of tnmd in human tendon tissue.** Immunostaining of Tnmd in tenocytes in human tendon sections from six different patients showed that Tnmd was highly expressed in nonpathologic, adult, human FCR (Fig. 3, A–C) and pathologic BT (Fig. 3, D–F). Tnmd immunostaining showed perinuclear and cytoplasmic localization (Fig. 3). Some signal in the nucleus was observed in patient c. Unexpectedly, no obvious plasma membrane localization was seen given the transmembrane spanning sequence in isoforms one and two.

**Localization of GFP.** Tnmd in cultured cells in COS-7 cells showed different and more distinct distribution patterns (Fig. 4A). GFP-Tnmd I showed robust cytoplasmic puncta, most of which

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*Fig. 3. Immunostaining of tenomodulin in human tendon sections. Tenomodulin was stained with an anti-tenomodulin COOH-terminal antibody (red) that mainly recognized isoforms I and III (based on Western blot data), and nuclei were stained with DAPI (blue). A–C: tenocytes within fascicles in human flexor carpi radialis tendon tissue. D–F: tenocytes within fascicles in human flexor carpi radialis tendon tissue. Tenomodulin was detected immunohistochemically within all cells from each specimen examined. Scale bar is 8 μm. A Leica SP2 confocal microscope was used to take these images at 0.16 optical sections, with a ×63 objective and ×4 zoom.*
Tenomodulin is green, blue is nuclei stained with DAPI. Scale bar is 40 μm. Also large puncta at the nuclear envelope while isoform III was cytoplasmic. Isoform I as green puncta in the cytoplasm and at the nuclear envelope; isoform II was diffusely distributed for isoform III (Fig. 4B). Porcine tenocytes showed Tnmd isoform III was cytoplasmic. Images (0.16-μm optical sections) were obtained with a Leica SP2 confocal microscope with a 40× objective.

Western blot of Tnmd isoforms in transfected COS-7 cells, tendon tissue, and cultured human tenocytes. Figure 5, A and B, was from the same blot on GFP-Tnmd construct-transfected COS-7 cells whose lysates were probed with anti-GFP (A) and anti-tenomodulin COOH-terminal-recognizing (B) antibodies, respectively. Lane 1 is a COS-7 cell alone control (plasmid, no GFP); lane 2 is GFP transfected alone (37-kDa band); lane 3 is GFP-fused TNMD isoform I transfected (75-kDa band); and lane 4 is GFP-fused TNMD isoform II transfected (58-kDa band) and GFP-fused TNMD isoform III transfected (58-kDa band). There was concordance in identification of TNMD-positive bands from GFP-stained to anti-TNMD antibody-stained bands in lanes 3, 4, and 5. In the overexpression experiments, GFP-fused Tnmd isoforms were transfected in COS-7 cells, and expression was confirmed with anti-GFP Western blots (Fig. 5A). Reprobing of the same membrane with anti-Tnmd COOH-terminal antibody showed immunopositive bands at the same positions as in the anti-GFP blot, indicating concordance between the GFP signal and the immunohistochemical identity of the tenomodulin proteins (Fig. 5B). The reprobing result also indicated that the COOH-terminal-recognizing antibody did not recognize Tnmd isoform II to the same degree as for isoforms I and III. The membrane was also probed with an anti-NH2-terminal antibody, but high levels of nonspecific bands were detected (results not shown). The anti-Tnmd COOH-terminal antibody also detected two bands in COS-7 cell lysates (Fig. 5B, lane 1), which are most likely Tnmd isoforms I and III based on their locations on the gel. Because the antibody does not recognize isoform II to a great degree, the bands shown in Fig. 5C are most likely Tnmd isoforms I and III. One single major band at 42 kDa was detected in cultured human biceps and FCR tenocytes. Three different protein sizes (33, 45, and 64 kDa) of Tnmd were detected in human tendon tissue with an anti-Tnmd COOH-terminus-recognizing antibody in a Western blot (Fig. 5C). These results indicate that the processing of Tnmd mRNA and posttranslational modification of Tnmd proteins may be altered in cultured tenocytes in vitro and tendon tissue in vivo.

Lack of secreted TNMD isoforms in supernatant fluids. Tenocytes were cultured in serum-free medium for 16 h, and the supernatant fluids were collected, concentrated ~20-fold, and subjected to Western blot analysis by probing with anti-GFP antibody as well as anti-COOH-terminal tenomodulin antibody (Fig. 5D, lanes 1, 2, and 3). Results showed that no detectable Tnmd isoforms were detected in the supernatant fluids, indicating that Tnmd was not secreted under the conditions of the experiment. These results cast doubts on a possible secreted form of tenomodulin that might have an anti-angiogenic role.
Fig. 5. Western blot of human tenomodulin in transfected COS-7 cells, tendon tissue, and cultured human tenocytes. A and B were from the same blot on GFP-Tnmd construct-transfected COS-7 cells whose lysates were probed with anti-GFP (A) and anti-tenomodulin COOH-terminal-recognizing (B) antibodies, respectively. Lane 1, COS-7 cells alone control (plasmid, no GFP); lane 2, GFP-transfected alone (37-kDa band); lane 3, GFP-fused TNDM isoform I transfected (75-kDa band); lane 4, GFP-fused TNDM isoform II transfected (58-kDa band) and GFP-fused TNDM isoform III transfected (58-kDa band). There was concordance in identification of TNMD-positive bands from GFP-stained to anti-TNMD antibody-stained bands in lanes 3, 4, and 5. Cultured human FCR tenocytes were from a same blot on GFP-transfected COS-7 cells whose lysates were probed with anti-GFP (A) and anti-tenomodulin COOH-terminal-recognizing antibody. Lanes 1 and 2 were cell lysates from two human FCR patient’s cultured tenocytes (bands at 42 kDa); lane 3 was from an FCR tendon tissue lysate (bands at 64, 45, and 33 kDa). Lanes 1, 2, and 3 in D were from concentrated supernatant fluids from cultured FCR tenocytes as a test for secreted tenomodulin. No bands as evidence of secreted Tnmd were detected under the conditions of the experiment.

Differential expression of Tnmd isoforms in human tendon tissues. To determine the relative expression levels of the three Tnmd isoforms in human tendons, four pairs of primers were used in qPCR to amplify all three isoforms simultaneously and each of two other sets simultaneously so that a value for each specific isoform could be calculated as described in METHODS. Primer sets 1 and 4 yielded the expression level of all seven isoforms. Expression levels of isoforms II and III were determined by subtracting the signal for primer sets 2 or 3 from that for primer set 1 or 4, respectively. Differential expression of Tnmd isoforms was found in different human tendons (Table 1 and Fig. 6). FCR tendon specimens represented a “control” tendon specimen group, given they were collected from intact, nonpathologic tendons that were surgically divided to provide a tendon transfer to actuate the thumb. For FCR tendon specimens, isoform II was the dominant isoform (61%) with isoform I representing 10% and isoform III representing 29% (Table 1, within the FCR group: P < 0.017 isoform II to isoform I; isoform II to isoform III P < 0.025; FCR vs. BT: FCR isoform II to BT isoform II, P < 0.05; FCR isoform II to BT isoform I or isoform III, P < 0.05; not significant (NS) among isoform amounts for BT alone). Biceps BT are generally more pathologic due to partial tears that accrue. For biceps tendon specimens from five patients, isoform III was the dominant isoform (37% of total Tnmd), followed by isoform II (36% total) and then full-length isoform I (27%) (Table 1 and Fig. 6). However, there were no significant differences among the amounts of isoforms for the biceps BT samples (NS). Therefore, the pathologic biceps tendon specimens had only 59% of the control level of the dominant isoform II Tnmd isoform, 266% of isoform I, and 127% of isoform III. The ratios for Tnmd in two FDP specimens were more like FCR while the ratios for synovium and tendon adhesion differed mainly in the amount of isoform III.

Knockdown of tmnd decreased cell proliferation. Reduced cell number in patellar tendon was reported in Tnmd knockout mice (8). However, Tnmd isoform III was likely still functional in the knockout mice due to lack of targeting to isoform III exons. Results of a cell proliferation assay (Brdu insertion) performed on Tnmd siRNA-treated human tenocytes, in which each of the three isoforms of Tnmd were suppressed, showed a reduction in cell proliferation (from 24 to 18%, a 25% reduction; Fig. 7A), which confirmed the in vivo finding in a knockout mouse model (8). The qPCR results also showed that

Table 1. Tenomodulin mRNA isoform relative amounts in tendon tissue types

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Isoform I</th>
<th>Isoform II</th>
<th>Isoform III</th>
</tr>
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<tbody>
<tr>
<td>FCR (n = 5)</td>
<td>10.3 ± 3.04*</td>
<td>60.9 ± 6.87*#</td>
<td>28.8 ± 8.32*</td>
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<tr>
<td>Biceps (n = 11)</td>
<td>27.4 ± 5.47#</td>
<td>36.0 ± 6.04#</td>
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<tr>
<td>FDP (n = 2)</td>
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<td>64.4</td>
<td>15.5</td>
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<tr>
<td>Adhesion</td>
<td>24.4</td>
<td>71.4</td>
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</tr>
<tr>
<td>Synovium</td>
<td>26.5</td>
<td>27.4</td>
<td>46.1</td>
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Values for flexor carpi radialis (FCR), biceps, and flexor digitorum profundus (FDP) are means ± SE; n, no. of experiments. Relative amounts of each tenomodulin isoform were calculated as described in METHODS. Isoform II was the dominant isoform in nonpathologic FCR tendons and was statistically different in amount compared with isoforms I and III in the FCR group (P < 0.017, isoform II to isoform I; isoform II vs. isoform III, P < 0.25). FCR isoform II was also statistically in greater amount than that in the biceps tendon group and compared with isoforms I and III (#P < 0.05). There were no significant differences in isoform amounts within the biceps tendon group and compared with isoforms I and III (#P < 0.05). There were no significant differences for isoform levels within the BT group (NS).
porters of the Tnmd gene.

**DISCUSSION**

Tnmd is a highly expressed, candidate marker gene for tenocytes given its clear immunochemical localization in embryonic, juvenile, and adult tenocytes and colocalization with Scx, an acknowledged tenocyte marker whose expression is suppressed by Sox9 (4). However, it must be noted that Tnmd has been identified in cells of other tissues such as bone, ligament, cardiac muscle, striated skeletal muscles, eye, and brain (4). Other accepted tenocyte markers include Mohawk (18), MSTN (27), and tenascin-C (4, 27, 34, 40). However, the function of tenomodulin in tendons is still unclear. Tnmd, similar to chondromodulin I (ChM-I), contains two extracellular domains: BRICHOS and a COOH-terminal cysteine-rich domain (16). The BRICHOS domain has been found in eight different protein families with functions associated with cancer, dementia, and respiratory diseases (39). The BRICHOS domain was initially defined from sequence alignments of the Bri protein associated with familial dementia, chondromodulin associated with chondrosarcoma, and surfactant protein C precursor (proSP-C) associated with respiratory distress syndrome and interstitial lung disease (ILD) (47). The BRICHOS domain has been identified in 12 protein families. Mutations in the Bri2 and proSP-C genes result in familial dementia and ILD, respectively, conditions associated with amyloid formation. The domain itself is thought to have a chaperone function, but the mechanism of action is still unclear. The proSP-C BRICHOS domain has been suggested to act as a chaperone that targets the SP-C region of proSP-C and prevents its aggregation while assisting its safe membrane insertion as a TM helix (20). The COOH-terminal cysteine-rich domain is only found in ChM-I and Tnmd (4). In ChM-I, this domain can be released from the plasma membrane by furin; however, no definitive furin cleavage site has been identified (41). In in vitro experiments, the COOH-terminal cysteine-rich domain from both ChM-I and Tnmd increased chondrocyte proliferation and inhibited endothelial cell proliferation and tube formation (31, 30). However, the cleavage of the COOH-terminal cysteine-rich domain from Tnmd in vivo has not been proven, although a potential furin cleavage site (RXXR) was predicted (3, 16, 49). In vivo, knockout of ChM-I did not affect the proliferation of endothelial cells or chondrocytes (8). However, adult tenons had a reduced cell number likely due to decreased cell proliferation in the newborn (8). There are no other data to confirm this finding yet. Moreover, no effects on angiogenesis were found in Tnmd knockout mice (8). The association of Tnmd SNPs with human brain pathology in Alzheimer’s disease fits with the BRICHOS domain association and dementia (15, 44, 47). However, the links to obesity and macular degeneration, although intriguing, are more difficult to explain (44).

Data mining results showed three different Tnmd transcripts in human tissues from the GeneCard database (36). Multiple Tnmd protein bands, concordant with the predicted molecular weights, given the isoform sizes, were also verified in COS-7 cells transfected with each GFP-Tnmd isoform construct, human tenocytes from tendon tissue, and cultured human tenocytes. However, the functions of each Tnmd isoform are still unknown. Predictions of Tnmd isoform structures in a three-dimensional molecular modeling program (I-TASSER program) showed different structures and predicted functions for each isoform (Fig. 2, A–C). The I-TASSER (threading/assembly/refinement) molecular modeling program uses threading, or comparisons of a target sequence to the conformation and folding of peptides of known sequences, energy of binding considerations, as well as iterative algorithms for structure prediction (37, 50, 51). TNMD I was predicted to be a cytosine-specific methyltransferase, TNmd II was classified as a SUMO protease, SENP1, and Tnmd III was predicted to be similar to α-syntrophin (37). A potential cytosine methyltransferase function for Tnmd I suggests a gene regulatory function that is concordant with its role in the developmental stage of growth (52). Methylation of DNA can block or deblock genes and hence regulate expression epigenetically (21). The nuclear envelope localization, presence of a type II transmembrane sequence, and entry into the nucleus of Tnmd I was also consistent with a role in gene regulation. Methytransferases are known to bind to chromatin at nucleosomes (19). Results of strain experiments on porcine tenocytes indicate that Tnmd translocates to the nucleus in response to substrate deformation and cell stretching. Interestingly, none of the Tnmd isoforms contains a known nuclear leading sequence that would home the protein to the nucleus. Further analysis of domain searches indicated that Tnmd isoforms contain only a BRICHOS domain in each isoform at different locations. Moreover, isoform

Fig. 7. Tenomodulin RNA interference assay. Knockdown of tenomodulin in human FCR cells reduced cell proliferation (A) and upregulated the expression of myostatin (MSTN) and scleraxis (Scx) (B). MSTN expression was increased by >60%, and Scx was increased by >90%. siRNA, small-interfering RNA. *Statistically significant, P < 0.05.
one does not have a sequence that binds DNA as one would expect in a methylase enzyme. SUMO proteases (small ubiquitin-like modifier) such as SENP-1 (in a family of SUMO-specific proteases such as SENP1–6) cleave the bonds between SUMO and their cognate receptors, such as SUMO-1 and androgen receptors, in the nucleus (19). The nuclear envelope localization, presence of the transmembrane sequence, and role in protein regulation are consistent with a SENP-1-like protein for Tnmd isoform II. α-Syntrophin is a scaffolding protein found in myocytes and is part of the dystrophin complex comprised of dystroglycans, syntrophins, dystrobrevins, sarcoglycans, and sarcospan, each comprised of isoforms (12, 22). One representative member of this group, α-syntrophin, acts as an adapter protein to recruit signaling proteins to the sarcolemmal dystrophin scaffold in mature muscle (22). α-Syntrophin has been shown to bind ion channels (7, 11, 23), G protein-coupled receptors (6), water channels (30), kinases and associated proteins (14, 17, 25, 26), and neuronal nitric oxide synthase (5). However, it does not have a significant presence in the nucleus but mainly is found in the cytoplasm (22). Tnmd isoform III does not have the transmembrane sequence found in Tnmd isoforms I and II and is largely found in the cytoplasm as well and not in the nucleus. Experiments are underway to test its possible role as a scaffolding protein. In addition, the relative amounts of each isoform in tendon samples suggest that there are intrinsic differences in expression of Tnmd in different tendon types as well as pathologic tendon states. These latter data speak to the issue of intrinsic differences between or among different tenocytes in types of tendons or pathologic change in ratios of isoforms as biochemical factors, or changes in strain in the tissues act on the tenocytes driving pathology. For instance, some tendinopathies demonstrate increased proliferation of tendon tissue (increased cell number or increased matrix). An altered tenomodulin ratio could shed light on loss of control of cell proliferation.

The FCR specimens were collected from patients undergoing a tendon transfer to provide movement via the reconstructed FCR to relieve nerve paralysis in an arthritic thumb unable to provide adequate grip strength for function due to pain levels (29). The FCR specimen is collected in nonpathologic tendon distal to the wrist. The tendon is split, longitudinally, maintaining the proximal half intact and the split distal portion to suture to the thumb phalanx to facilitate movement. This type of specimen is as close to “normal” as a tendon specimen can be in a control patient population. The biceps tendon specimens were taken from adult males at the time of a supraspinatus tendon repair due to a rotator cuff injury. The long head of the biceps tendons is generally ruptured, or partially ruptured, and is surgically severed to reduce pain and allow space for motion at the rotator cuff (24).

These specimens fall in the category of “pathologic.” The FDP specimens are generally taken from “freshened” ends of a ruptured digital flexor tendon and are normal except they were proximal to a rupture. A comparison of tenomodulin isoform ratios for these various specimens indicates that the percentages of Tnmd isoforms II and III are reversed compared with the normal FCR ratios (Table 1 and Fig. 6). The biceps tendons are abnormal and are expressing more of the cytoplasmic Tnmd isoform III that is the scaffold protein α-syntrophin-like, and less of the SENP-1, SUMO protease that could regulate specific protein degradation. These results suggest potential functional differences among Tnmd isoforms that are clearly altered in pathologic tissue. Scientific knowledge in the tendon field will be advanced as the functions of the splice variants of this highly expressed tendon marker gene are revealed.

Because of the lack of specific antibodies for each Tnmd isoform, we were unable to determine the cellular localization of each protein isoform immunohistochemically. However, overexpression of each GFP-linked Tnmd isoform showed different cellular distributions, which supports the hypothesis that each

Fig. 8. Predicted regulatory pathway of tenomodulin.
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Tenomodulin (Tnmd) isoforms may play distinct functions in tenocytes and/or cells in other tissues. Isoforms I and II were more associated with the nuclear envelope, and isoform III was mainly cytoplasmic. The predictions of structure and functions from the I-TASSER program meshed reasonably well with the intracellular localization data. An analysis of the differential expression of Tnmd isoforms will help us better understand possible functional differences among different tendons, possibly due to their mechanical environment and especially pathology. Because of the difference in the stability of mRNA and PCR amplification efficiency, qPCR is not an accurate assay to determine the expression levels of each isoform. A Northern blot result would be a better approach when sufficient quantities of human tissue specimens are available.

Cell proliferation was reduced 20% in Tnmd knocked down cells, consistent with the knockout mouse result (8). SMYD3, a histone methyltransferase, is associated with increased cell proliferation in colorectal and hepatocarcinomas, likely by complexing with RNA polymerase and regulating transcription of cell cycle genes (13). Tnmd isoform I models as a cytosine methyltransferase and may act in similar fashion to regulate cell proliferation. A SUMO-specific SENP-1 protease can regulate colon cancer cell proliferation in vitro (48). Knocking the mRNA down blocked cell division in DLD-1 colon cancer cells. Tnmd isoform II modeled as a SUMO-I-like SENP-1 protease and could act in similar fashion. α-Syntrophin has no identifiable role in regulation of cell division as yet.

The Western blot data for the GFP-Tnmd isoform constructs transfected in COS-7 cells indicated concordance between the anti-GFP-recognizing antibody binding to bands of 75, 58, and 58 kDa for Tnmd isoforms I, II, and III, respectively. When the mass of GFP was subtracted from the estimated mass of each isoform band, the molecular weights were 38, 21, and 21 kDa, respectively, for isoforms I, II, and III. Importantly, the same blot when stripped and reprobed with anti-COOH-terminal-recognizing anti-Tnmd antibody revealed the same bands as with the anti-GFP antibody. However, the reprobing result also indicated that the COOH-terminal-recognizing antibody did not recognize Tnmd isoform II to the same degree as for isoforms I and III. The membrane was also probed with an anti-NH2-terminal antibody, but high levels of nonspecific bands were detected. The anti-Tnmd COOH-terminal antibody also detected two bands in COS-7 cell lysates (Fig. 5B, lane 1), which were most likely Tnmd isoforms I and III based on their locations in the gel. Because the antibody does not recognize isoform II to a great degree, the bands shown in Fig. 5C are most likely Tnmd isoforms I and III. One single major band at 42 kDa was detected in cultured human biceps and FCR tenocytes. Three different protein sizes (33, 45, and 64 kDa) of Tnmd were detected in human tendon tissues with an anti-Tnmd COOH-terminus-recognizing antibody in a Western blot (Fig. 5C). More human specimens must be analyzed to confirm protein sizes from control and pathologic specimens. These results indicate that the processing of Tnmd mRNA and post-translational modification of Tnmd proteins may be altered in cultured tenocytes in vitro and tendon tissue in vivo.

Tenocytes were cultured in serum-free medium for 16 h, and the supernatant fluids were collected, concentrated ~20-fold, and subjected to Western blot analysis by probing with anti-GFP antibody as well as anti-COOH-terminal tenomodulin antibody (Fig. 5D, lanes 1, 2, and 3). Results showed that no detectable Tnmd isoforms were detected in the supernatant fluids, indicating that Tnmd was not secreted under the conditions of the experiment. These results cast doubt on a possible secreted form of tenomodulin that might have an anti-angiogenic role.

Expression of MSTN and scleraxis was reduced in MSTN knockout mice, and addition of MSTN protein to the culture medium increased the expression of scx and Tnmd (27). Therefore, we hypothesized that a negative feedback relationship may exist between Tnmd and MSTN. Knockdown of Tnmd increased the expression of MSTN and scx, likely through the upregulation of MSTN. A potential regulatory pathway of Tnmd is depicted based on the reports and data in the present study (27, 33, 38) (Fig. 8). Further study on the localization to cytokines, effector molecules, as well as cell proliferation, matrix expression, cell migration, and response to wounding and strain, of each Tnmd isoform in tenocytes will uncover the functions and the regulatory pathways of Tnmd in the development and maintenance of tendons. Tnmd isoforms may serve as new biomarkers and, more importantly, as novel therapeutic targets in tendon injury and diseases, given the effect on cell proliferation.

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DISCLOSURES

A. J. Banes is president of Flexcell Intl Corp. (FIC) and receives compensation as such.

AUTHOR CONTRIBUTIONS


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