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Research Paper

Tenomodulin is Required for Tendon Endurance Running and Collagen I Fibril Adaptation to Mechanical Load

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ABSTRACT

Tendons are dense connective tissues that attach muscles to bone with an indispensable role in locomotion because of their intrinsic properties of storing and releasing muscle- generated elastic energy. Tenomodulin (Tnmd) is a well-accepted gene marker for the mature tendon/ligament lineage and its loss-of -function in mice leads to a phenotype with distinct signs of premature aging on tissue and stem/progenitor cell levels. Based on these findings, we hypothesized that Tnmd might be an important factor in the functional performance of tendons. Firstly, we revealed that Tnmd is a mechanosensitive gene and that the C-terminus of the protein co-localize with collagen I-type fibers in the extracellular matrix. Secondly, using an endurance training protocol, we compared Tnmd knockout mice with wild types and showed that Tnmd deficiency leads to significantly inferior running performance that further worsens with training. In these mice, endurance running was hindered due to abnormal response of collagen I cross-linking and proteoglycan genes leading to an inadequate collagen I fiber thickness and elasticity. In sum, our study demonstrates that Tnmd is required for proper tendon tissue adaptation to endurance running and aids in better understanding of the structural-functional relationships of tendon tissues.

1. Introduction

Tendon biology is under-investigated, yet tendon pathologies are a big burden for affected patients. The poor regenerative ability of tendon tissues (Benjamin and Ralphs, 1997) and unsuccessful surgical attempts to repair tendon tears (Pennisi, 2002) underline the need to develop superior therapies. Identifying key molecular markers would aid in defining the response to therapy (Morse and Gillies, 2010).

Tenomodulin (*TNMD/Tnmd*) is an established gene marker for the mature tendon/ligament lineage in vertebrates (Docheva et al., 2005). So far, limited studies have focused on deciphering its functions in tendon tissues and residing cells (Dex et al., 2016). The gene is abundantly expressed in tendons and ligaments (Brandau et al., 2001; Shukunami et al., 2001; Docheva et al., 2005). Its protein has a highly conserved cleavable C-terminal cysteine-rich domain (Brandau et al., 2001; Shukunami et al., 2001) that has been identified as an important regulator for tendon stem/progenitor cell (TSPC) proliferation and senescence as well as for tendon maturation (Alberton et al., 2015, and reviewed by Dex et al., 2016). A variety of tissues express *Tnmd* mRNA, however Tnmd cleavage and secretion is exclusive to tissues undergoing tension, suggestive of a possible mechano-regulation. The cleaved form is detected in the extracellular matrix (ECM) of mouse Achilles tendons (Docheva et al., 2005) and human chordae tendineae cordis (Kimura et al., 2008), but not in the eye (Oshima et al., 2003). Loss of *Tnmd* transcripts in cultivated human periodontal ligament cells, tenocytes (Itaya et al., 2009; Mazzocca et al., 2011) and in rat tendon fibroblasts has been

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reported (Jelinsky et al., 2010), suggesting its downregulation is a consequence of the lack of mechanical stimuli *in vitro*.

Through a multidisciplinary approach, we aimed to reveal further features and functions of *Tnmd* in tendon tissue. First, we tested whether *Tnmd* gene promotor activity and transcription are responsive to *in vitro* mechanical stretching, which was followed by subjecting wildtype (WT) and *Tnmd* knockout (KO) mice to forced endurance and voluntary running protocols. We performed an ultrastructural analysis using atomic force microscopy (AFM) technology and investigated collagen I fiber topography, diameter distribution, average diameter, and average stiffness from sedentary and trained Achilles tendons. Lastly, we studied the localization of TNMD protein in human tendon and TSPC specimens and examined the effect of *Tnmd* loss-of-function on the expression of collagen I cross-linking genes.

In sum, we report that *TNMD/Tnmd* is a mechanosensitive gene vital for optimal running performance, proper tendon adaptation to running and maintenance of structurally and functionally integral collagen fibrils.

2. Materials and Methods

2.1. Cell Culture

Human Achilles tendon TSPC (3 young healthy donors) were previously isolated (under grant No.: 166-08 of the Ethical Commission of the Medical Faculty of the LMU), and validated to possess multipotential differentiation capacity, self-renewability and clonogenicity, and to express stem cell-, and tendon related gene markers (Kohler et al., 2013; Popov et al., 2015). TSPC were cultured as described in (Kohler et al., 2013; Popov et al., 2015). Cells in passages 1–3 were used for experiments.

2.2. Mouse Strain

The *Tnmd* KO mouse line was established by Docheva et al. (2005). Mouse husbandry, handling and euthanasia were strictly carried out according to the guidelines of the Bavarian authorities. Animals were euthanized with CO_2 and dissected for collection of whole foot and tendon tissues.

2.3. Semiquantitative and Quantitative PCR

Total RNA from human TSPC was isolated with Qiagen RNeasy Mini kit (Qiagen, Hilden, Germany) and used for standard semiquantitative PCR (protocol in Supp. Info.). Total RNA/cDNA from WT and *Tnmd* KO tendons (pool of three animals) was prepared from littermates subjected or not to the forced endurance running protocol. Quantitative PCR of collagen I regulatory and cross-linking genes (asporin, biglycan, decorin, fibromodulin, fibronectin, lysyl hydroxylase, lysyl oxidase and lumican) was performed using RealTime Ready Custom Panel 96 – 32+ plates (https://configurator.realtimeready.roche.com) according to the manufacturer's instructions (Roche, Penzberg, Germany). Briefly, PCR reactions were pipetted on ice and each well contained 10 μl LightCycler 480 probes master mix, 0,2 μl cDNA (diluted 1:5) and 9,8 μl PCR grade water. Plates were subsequently sealed and centrifuged down for 15 s. at 2100 rpm. Crossing points for each sample were determined by the second derivative maximum method and relative quantification was performed using the comparative ΔΔCt method according to the manufacturer's protocol. Four PCR independent repeats were carried out (n = 4).

2.4. Western Blot Analysis

Biopsies from cadaveric human tissue complex gastrocnemius muscle-Achilles tendon-calcaneus (from donors donated to the Department of Anatomy, LMU in Munich) as well as mouse tendons from WT and *Tnmd* KO animals were lysed with 8 M urea, 50 mM Tris-HCl [pH 8.0], 1 mM dithiothreitol, 1 mM EDTA). Protein (25 μg) aliquots were loaded on a 15% SDS- polyacrylamide gel and transferred to Hybond-P membrane (Amersham, GE Healthcare, Waukesha, USA). The membranes were pre-incubated for 4 h at 4 °C in blocking buffer and probed with rabbit polyclonal anti-C-terminal Tnmd antibody, raised against a synthetic polypeptide conforming to amino acids 245 to 252 in mouse and human Tnmd (Docheva et al., 2005). A mouse anti-beta-actin antibody cross-reacting with mouse and human beta-actin (Abcam, Cambridge, United Kingdom) was used as a loading control. Following overnight incubation at 4 °C, membranes were probed with corresponding HRP-conjugated secondary antibodies (Amersham). Protein bands were visualized using an enhanced chemiluminescence system (ECL Plus, Amersham) and film paper. Western blotting was replicated independently three times (n = 3).

2.5. Immunofluorescence

Human biopsies from Achilles tendon (grant No.: 166-08) as well as mouse whole foot were embedded in paraffin and cut in 6 μm thick longitudinal sections. Prior to staining, slides were deparaffinated with xylol and rehydrated *via* ethanol (100–50% EtOH). For antigen retrieval, sections were treated with 0,2% hyaluronidase for 30 min. Following blocking with 1% BSA/PBS for 2 h sections were incubated overnight at 4 °C with primary antibodies against asporin, fibromodulin, lysyl oxidase (All Abcam), lumican (Santa Cruz, Dallas, USA) and Tnmd (Docheva et al., 2005). Corresponding Alexa Fluor 488-labeled secondary antibodies and nuclear dye 4′, 6-diamidino-2-phenylindole (DAPI) (both Life technology, Carlsbad, USA) were applied at room temperature for 1 h and 5 min, respectively. Photomicrographs were taken with an Axiocam MRm camera on Observer Z1 microscope (Carl Zeiss, Oberkochen, Germany). Confocal photomicrographs of Tnmd staining in human and mouse Achilles tendon were taken using a confocal Leica TSC SP2 microscope (Leica, Wetzlar, Germany). Immunofluorescence experiments were repeated thrice independently.

2.6. Mechanical Stimulation

TSPC underwent axial cyclic strain in a six-station stimulation apparatus driven by an eccentric motor as described previously (Popov et al., 2015). For this, 1×10^5 TSPC were cultured for 4 days on FBS-coated flexible silicone dishes (60 mm × 30 mm). Triplicates were then stretched 60 min/day in the long axis at a frequency of 1 Hz and a magnitude of 5% for 3 consecutive days. In parallel, non-stimulated cells were used as controls on day 3. Directly after stimulation, cells were lysed for mRNA isolation as described above. Mechanical stimulation was performed with 2 different TSPC donors (n = 2) (each in triplicates) and repeated twice independently.

2.7. Luciferase Assay

The assay was performed as previously described by us (Alberton et al., 2012). In brief, human TSPC (3×10^4 cells/cm^2) were transfected with 2,5 μg control promoter-less and *Tnmd* promoter-luciferase plasmids using OptiMEM media and Lipofectamine 2000 kit (Invitrogen). After 6 h, transfection media was replaced with complete growth media, and TSPC were cultured for 2 days. Luciferase activity was then measured with the Luciferase Assay E4030 kit (Promega, Madison, USA) according to the manufacturer's instructions. Cells were lysed in 1× lysis buffer, 20 μl of the cell lysates were mixed with 100 μl luciferase reagent and immediately measured on Safire II Luminometer (TECAN, Männedorf, Germany). Two transfected TSPC donors were used for two independent mechanical stimulations (n = 4).

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