Tenomodulin regulates matrix remodeling of mouse tendon stem/progenitor cells in an ex vivo collagen I gel model

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Abstract

Tenomodulin (Tnmd) is predominantly expressed in tendon and ligament tissues. Loss of Tnmd in mice leads to a profound phenotype in vitro, characterized by reduced self-renewal but increased senescence of mouse tendon stem/progenitor cells (mTSPCs), as well as in vivo, by significantly impaired early tendon healing. Interestingly, injured Achilles tendons from Tnmd-deficient mice showed inferior tendon repair, which was characterized by less contracted fibrovascular scars with disorganized matrix composition in comparison to wild type (WT) mice on day 8 after injury. To better understand Tnmd role in tendon repair, here we implemented an ex vivo three-dimensional (3D) collagen gel model and investigated whether Tnmd knockout affects the collagen contraction of mTSPCs. TSPCs were isolated from WT and Tnmd knockout (KO) tendons at 6, 9, and 12 months of age. Adhesion assay demonstrated that loss of Tnmd in mTSPCs resulted in reduced adhesion to collagen type I. Quantitative time-dependent analysis revealed that Tnmd-deficient mTSPCs of all ages have significantly reduced capacity to contract collagen matrix in comparison to WT cells. Furthermore, 18 months old mTSPCs of both genotypes showed lower collagen contractility than cells obtained from 6, 9, and 12 months old animals, demonstrating an overall effect of organismal aging on matrix remodeling. Nevertheless, both cell types had a similar survival rate for the 5 days of cultivation within the gels. Lastly, quantitative PCR for 48 different genes revealed that the knockout of Tnmd majorly affected the gene expression profile of mTSPCs, as several transcription factors, tendon matrix, collagen cross-linking, and lineage maker genes were down-regulated. Taken together, our results clearly demonstrated that loss of Tnmd in mTSPCs led to profoundly altered gene expression profile, insufficient adhesion to collagen type I, and impaired ability to contract the extracellular matrix.

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1. Introduction

Tendons are dense connective tissues with high collagen type I content, and are critical components of our musculoskeletal system that integrate and transmit forces from skeletal muscle to bone [1,2]. Musculoskeletal injuries are common in our society and increase with aging. Furthermore, about 45% of all injuries in orthopaedic and trauma surgery departments are due to rupture of tendons and/or ligaments [3,4]. Moreover, most of these injuries tend to become chronic conditions, and rarely ruptured tendons fully restore their initial strength and function [3,4]. Due to the lack of key molecular markers, the precise mechanisms behind tendon healing are still not fully understood and therefore, tendon-specific agents that can steer the repair to better and quicker outcomes are still not available in medical practice [5,6].

Tenomodulin (Tnmd) protein is classified as a type II transmembrane glycoprotein containing a highly conserved C-terminal cysteine-rich domain that upon cleavage is co-localized with collagen type I fibrils into the tendon extracellular matrix (ECM) [7,8]. Tnmd has been proved to be strongly expressed in tendons and ligaments, and widely accepted as a specific marker for the mature tendon/ligament lineage [7,8]. In the last decades, many
laboratories isolated and characterized tendon stem/progenitor cells (TSPCs) by their self-renewal ability, clonogenicity, and multipotency, but also by expressing high levels of tendon-related gene markers, such as Tnmd and a master transcription factor, Scleraxis (Scx) [1,2], a feature distinguishing this cell type from other adult mesenchymal stem/progenitor cells. Previously, we have obtained TSPC from WT and Tnmd KO tail tendons and demonstrated that loss of Tnmd results in reduced self-renewal capacity and augmented cell senescence [9]. In our recent study focusing on early tendon repair, Tnmd KO mice clearly showed inferior tendon repair which was characterized by less contracted fibrovascular scars with disorganized matrix composition and large adipocyte and vessel accumulations in comparison to WT mice [10].

Motivated by the above data, we investigated in the present study, the Tnmd role in matrix remodeling by cultivating mTSPCs ex vivo in a three dimensional (3D) collagen type I model. mTSPCs were isolated from WT and Tnmd KO tail tendons of 6, 9, 12, and 18 months old animals and analyzed by genotype validation, collagen type I adhesion assay, gel contractile kinetics up to day 5, cell survival and cytoskeleton analyses, and quantitative PCR for gene expression profiling.

2. Material and methods

2.1. Isolation and expansion of mTSPCs

Primary mTSPCs were isolated from WT and Tnmd KO tail tendons of 6, 9, 12, and 18 months old animals using an enzyme digestion protocol and cultivated as previously described by Alberton et al [9]. In order to gain a sufficient number of cells for experiments, tail tendons of three animals (n = 3) per genotype and per developmental time point were pooled for cell isolation. The above time points were selected for the following reasons: i) skeletal maturation; ii) 6 and 9 months correspond to the beginning and end time points of the Achilles injury model and iii) 12 and 18 months reflect animal aging. Prior to cell isolation, animals were housed and sacrificed in strict accordance with the regulations and guidelines of the Lower Franconia authorities. Cells at passage 3 were used in all experiments.

2.2. Genotyping of mTSPCs

Genomic DNA was isolated from WT and Tnmd KO TSPCs and genomic PCR was performed with CFX96 Real-Time System (BioRad Laboratories, Munich, Germany) with denaturation at 95 °C for 10 min followed by 35 cycles of 95 °C for 30 s, 49 °C for 30 s, and 72 °C for 60 s with a final extension at 72 °C for 10 min. The primers and reaction mix are listed in Table S1.

2.3. RNA isolation and PCR

Total RNA from mTSPC of each genotype was extracted using Qiagen RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s guideline. For cDNA synthesis, 1 μg total RNA and Transcriptor First-Strand cDNA Synthesis Kit (Roche, Mannheim, Germany) were used. Semiquantitative PCR was performed with Taq DNA Polymerase (Qiagen, Hilden, Germany) and the implemented primer pairs are listed in Table S2. The RealTime PCR plates with 48 different genes (Table S3) were custom designed with format 96-well/32 + by BioRad (BioRad Laboratories, Cat. Nr. 10025218, USA) and used in accordance to the manufacturer’s instructions and our well-established protocol [7,16]. Gene expression was analyzed with the ΔΔCT method and presented as fold change of Tnmd KO TSPCs to WT. Three independent repeats per genotype were carried out.

2.4. Tnmd western blotting (WB) and enzyme-linked immunosorbent assay (ELISA)

WB and ELISA were performed as follows: eight collagen gels loaded with WT or Tnmd KO mTSPCs were harvested to extract proteins using RIPA buffer (Sigma, St. Louis, Missouri, USA). For WB, 30 μg of total protein was loaded on 12% Bis-Tris Plus gels (Hoeffer SE 260), and then transferred onto an Immobilon Transfer Membranes (Millipore, Burlington, Massachusetts, USA). Then, the protein membranes were blocked with 5% milk (Carl Roth, Karlsruhe, Germany), and incubated with primary rabbit anti-mouse antibody against Tnmd (Metabion, 1:500 dilution) or β-actin (Abcam, Cambridge, UK, 1:10000 dilution) overnight. The next day, the membranes were incubated with anti-rabbit HRP-conjugated secondary antibody (Thermo Scientific, Waltham, Massachusetts, USA) for 1 h at RT. Protein was visualized by SuperSignal West Dura Extended Duration Substrate and detected using the ChemiDoc XRS + Molecular Imager (BioRad). For ELISA analysis, Tnmd ELISA kit (CusaBio, China) was implemented according to the manufacturer’s instructions. Per well, 30 μg of total protein, isolated from WT or Tnmd KO mTSPCs cultivated in collagen gels, was loaded.

2.5. Assessment of mTSPC metabolic activity

Resazurin assay was performed to assess metabolic activity of WT and Tnmd KO mTSPC as previously described [4]. Briefly, mTSPCs were plated in triplicates at a density of 1 × 10³ cells/well in 96-well plates. Cells were incubated with Resazurin reagent for 3 h, and then the fluorescence intensity was measured at an excitation of maximum wavelength of 545 nm and an emission maximum of 590 nm.

2.6. Three-dimensional (3D) cell culture in collagen I gels

3D cell culture in collagen I gels was carried out as previously described [4] at a cell density of 1 × 10⁴ cells/ml. Collagen gels (70 μl total volume) with 5 mm diameters were prepared using a sterile cylindrical mould. mTSPC survival and cytoskeletal organization in the gels were revealed by Live/Dead staining using calcein and ethidium homodimer-1 (Eth) kit (Molecular probes, Eugene, Oregon, USA) and phalloidin-AF480 staining (Life technologies, Carlsbad, USA) for F-actin as previously described [4].

2.7. Cell adhesion assay

Cell adhesion assay was conducted on collagen I-coated 96-well plates (Millipore, Burlington, Massachusetts, USA). Forty minutes prior to cell seeding, mTSPCs were labelled with 5 μM calcein added in the culture media and incubated at 37 °C. Next, mTSPCs were plated in triplicates at a density of 6 × 10⁴ cells/well and incubated from 15 to 120 min at 37 °C. Non-adherent cells were removed by washing with PBS. Next, the fluorescence intensity was measured at an absorbance maximum of 494 nm and an emission maximum of 517 nm. For calculation of the percentage of adherent cells during the investigated time period, aliquot of 6 × 10⁴ calcein-labelled cells was measured and set as a maximum value.

2.8. Gel contraction assay

To analyze the gel contraction by WT and Tnmd KO mTSPCs, gels were imaged daily till day 5, and gel area was measured with the “polygonal tool” of Image J program (National Institutes of Health, Bethesda, USA). Three different gels were analyzed per genotype and per developmental time point. The rate of gel contraction was expressed as a percentage of the initial gel size.
2.9. Statistics

Data was presented as mean ± standard deviation (SD). Unpaired Student's t-test was used to detect statistical differences between genotypes using GraphPad Prism5 software (GraphPad). *P < 0.05 and **P < 0.01 were considered statistically significant.

3. Results

3.1. mTSPC validation and Tnmd protein deposition

TSPCs, isolated from WT and Tnmd KO tail tendons at 6, 9, 12, and 18 months of age, (Fig. 1a) were initially validated by genotyping. Wild type allele shows a band of 136 bp, amplifying the segment of gDNA between Tnmd 5'-UTR and exon 2. Knockout allele was detected by 290 bp band, corresponding to the gDNA segment of the inserted knockout cassette (in exon 1) and exon 2 (Fig. 1b). Next, a super pool of WT and a super pool of Tnmd KO mtSPCs of different animal ages were verified by semi-quantitative PCR for Tnmd mRNA. As expected Tnmd transcript (amplicon of 249 bp) was observed only in WT cells (Fig. 1c). To examine Tnmd protein production, mtSPCs cultivated in collagen I gels for 5 days, were lysed for WB and ELISA analyses at day 5. WB results demonstrated a 16 kDa protein band corresponding to the cleaved Tnmd C-terminal domain (Fig. 1d), whilst this was absent in Tnmd KO samples. The Tnmd protein deposition was further confirmed by ELISA, showing approximately 0.9 ng/ml Tnmd protein concentration in WT mtSPC gels. In contrast, empty gels or loaded with Tnmd KO mtSPCs showed only background levels (Fig. 1e).

3.2. Tnmd KO mTSPC exhibited reduced metabolic and proliferation activities

Short-term Resazurin assay (1.5 h after cell plating) demonstrated that Tnmd KO mTSPCs have a significantly lower metabolic activity than WT cells. Moreover, long-term Resazurin assay (1–5 days) showed that WT mtSPCs have significantly higher cell proliferation ability than Tnmd KO cells (Fig. 1f).

3.3. Tnmd KO mTSPCs exhibited reduced adhesion to collagen type I and impaired ability to contract 3D collagen matrix

Prior to quantitative analyses, we performed Live/Dead stainings of WT and Tnmd KO TSPCs cultivated in 3D collagen I gels for up to 5 days. mtSPCs of both genotypes of all ages had similar survival, monitored by the incidence of calcine-positive cells (labelled in green color) in the given culture period (Fig. 2a). To visualize cell morphology, F-actin stainings were conducted at the end time point. Interestingly, we observed a pronounced morphological difference between WT and Tnmd KO mtSPCs when cultivated in the 3D matrix. WT mtSPCs exhibited elongated and spindle-like shapes, whilst Tnmd KO cells were larger, more flattened and star-like (Fig. 2b). Next, quantitative adhesion assay clearly showed that Tnmd KO mtSPCs have significantly lower adhesion efficiency to collagen I matrix than WT cells at all examined time points (30–120 min) (Fig. 3 a). Since integrin
receptors are critical mediators of cell to ECM binding, we then studied if Tnmd KO mTSPCs exhibit altered integrin expression. Semi-quantitative PCR analyses revealed that both cell types are comparable in the expression of the collagen I binding integrins namely α1, α2, α10, α11, and β1 (Fig. S1). To examine if Tnmd-deficient mTSPCs have ECM contractile phenotype, the 3D collagen gels were photographed every 24 h for 5 days and the contraction rate was calculated (Fig. 3b–f). The obtained kinetic curves revealed that Tnmd KO mTSPCs contracted significantly less the 3D ECM than their WT counterparts for all animal ages and all examined points (Fig. 3c–f). In addition, we detected that the contraction rate of mTSPCs isolated from very old animals (18 months) of both genotypes was profoundly reduced when compared to mTSPCs isolated from younger animals. However, the genotype difference persisted also at this time point. These data suggests that in aged animals ECM contraction is impaired and the contractile phenotype of Tnmd KO mTSPCs is further accelerated (Fig. 3f).

3.4. Knockout of Tnmd in mTSPC resulted in abnormal gene expression profile

Quantitative PCR screening of 48 different genes was performed. Some genes (Col15a1, Acan, Ibsp, Lpl, Myod1, Myog, Nanog, Pou5f1, Sp7, and Tjap2a) were not detectable, several genes (Col5a1, Egr2, Fas4, and Sox9) were not differentially expressed between WT and Tnmd KO mTSPCs, and only genes with significant change in expression were plotted in Fig. 4. Our results demonstrated that Tnmd-deficient mTSPCs exhibited significant down-regulation of multiple genes, namely collagens - Col1a1, Col3a1, Col6a1, Col12a1, and Col14a1 and the ECM genes - Comp, Prg4 and Thbs4, whilst

![Fig. 2. mTSPCs viability and morphology in 3D collagen gels. (a) Live/Dead staining and (b) actin staining of WT and Tnmd KO mTSPCs derived from 6, 9, 12, and 18 months old animals and cultured in 3D collagen gels up to 5 days. Live cells are labelled in green, while dead cells are in red. Actin fibres are visible in green. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)](https://doi.org/10.1016/j.bbrc.2019.03.063)
several tendon-related genes - *Acta2*, *Epha4*, *Tgf-β1*, *Thbs2* and *Tnc* were significantly up-regulated (Fig. 4a). Furthermore, the well-accepted tendon-specific transcription factors - *Egr2*, *Eya1*, *Eya2*, *Mkx*, and *Six2* were also significantly down-regulated, whereas *Scx* and *Six1* were significantly up-regulated in *Tnmd* KO mTSPCs (Fig. 4b). Regarding the collagen cross-linking genes, loss of *Tnmd* resulted in significant down-regulation of *Dcn*, *Fmod*, *Lum*, and *Lox*, whilst *Aspn*, *Bgn*, *Fn*, *Tgm2*, and *Plod1* were significantly up-regulated (Fig. 4c). Lastly, with respect to the expression of gene markers related to other cell lineages, significant down-regulation of chondrogenic (*Col2a1*), osteogenic (*Runx2*), and adipogenic (*Pparγ*) genes was observed in *Tnmd* KO mTSPCs. Interestingly, one myogenic lineage marker - *Des* was found upregulated in this cell type (Fig. 4d).

4. Discussion

Understanding the modes of action of key molecular players involved in tendon healing can be employed for the development of medicinal cues that can boost this process to more satisfactory clinical outcomes. *Tnmd* is one of the few known gene markers of the mature tendon/ligament lineage [7,8] and our previous studies have shown that it regulates TSPCs self-renewal and senescence, as well as is important for proper maturation and adaptation of collagen fibrils in tendon tissue upon mechanical loading [9,11]. More recently, by investigating surgically challenged Achilles tendons of WT and *Tnmd* KO mice, we found that *Tnmd* expression is critical for the early phase of tendon repair [10]. In order to further clarify the exact roles of *Tnmd* in the healing process, we carried out 3D ex vivo collagen I gel model and studied whether loss of *Tnmd* impacts the matrix contractility of mTSPCs.

Matrix contraction is fundamental for tissue remodeling and wound healing, as a great variety of pathologies are associated with abnormal contraction and scarring [12,13]. Based on the contractile and remodeling activities of tendon-resident cells, collagen fibers become aligned and proper matrix organization and maturation is achieved over time after tendon injury. 3D gel models have been already applied to study matrix remodeling and wound healing by fibroblasts, as well as tenocytes [13–16].

Collagen type I is the major ECM protein of the tendon matrix and during healing, it is deposited and extensively remodeled at the site of injury [1,2]. Therefore, we implemented in our study, hydrogels composed of collagen type I. We performed an initial adhesion assay towards collagen type I, which showed that the *Tnmd*-deficient mTSPCs have significantly reduced attachment. Our results were consistent with the previous observations by Komiyama et al., who reported that *Tnmd* overexpression in NIH3T3 and human periodontal ligament cells enhances cell adhesion to collagen type I and fibronectin, whilst fibroblasts derived from neonatal connective tissues of *Tnmd* KO mice results in decreased cell adhesion to collagen type I and fibronectin, when compared to WT cells [17].
Next, we cultivated mTSPCs in collagen gels and validated Tnmd protein expression by WB and ELISA. Specifically, the WB results pointed out that the cleaved 16 kDa C-terminal domain of Tnmd being produced by the WT mTSPCs in the gels and as expected not by Tnmd KO cells. In earlier studies, this domain was detected in the ECM of mouse and human tendons [7,11], as well as human and porcine chordae tendineae cordis [18] suggesting that the collagen gels are a suitable ex vivo tendon model. Following our validation, we calculated the gel contraction rate by imaging the area of the gels every 24 h up to day 5. The obtained data demonstrated that Tnmd-deficient mTSPCs, derived from 6, 9, 12 and 18 months old animals, exhibit significantly reduced capacity to contract the ECM in comparison to mTSCPs isolated from their WT counterparts. One possible reason for the observed phenotype is a difference in cell survival between the WT and KO mTSPCs. However, Live/Dead stainings revealed that both genotypes had similar survival rate within the gels at each examined time point. Interestingly, we observed a pronounced morphological aberrance between WT cells, having elongated and spindle-shaped appearance, and Tnmd KO mTSPCs, being star-like and flattened. Kohler et al. reported similar changes in morphology of human TSPCs derived from aged/degenerative Achilles tendons [19], hence, the above novel findings indicate that Tnmd-deficient TSPCs resemble an aged cell phenotype. For this reason, as well as to fish out potential molecular shift in the KO cells, we screened 48 different genes by quantitative PCR. Our results revealed that the loss of Tnmd, majorly affects the gene expression profile of mTSPCs as most of the transcription factors, ECM, collagen cross-linking, and lineage maker genes were significantly downregulated. Interestingly, we detected increased Scx expression in Tnmd-deficient mTSPCs, which is consistent with previous quantitative Scx PCR performed with WT and Tnmd KO mTSPCs cultured in vitro [9]. There is a fine interplay between Scx and Tnmd expression [20,21]; for example, Shukunami et al. showed that Scx overexpression in chicken tenocytes leads to up-regulation of Tnmd expression [21]. Another study by Murchison et al. demonstrated that tendons in Scx-deficient mice have significantly reduced Tnmd expression [22]. These observations indicate first, that Tnmd is a direct target of Scx and second, that there might a feedback loop due to loss of Tnmd. However, the exact signaling pathways underlying Tnmd function in regulation of collagen matrix remodeling by TSPCs have to be clarified in future. Specifically, follow up studies should aim at identifying the binding partners of Tnmd and the related molecular cascade. Another aim should be to further optimize the 3D collagen gel model so that it can more closely mimic the native tendon matrix. Taken together, our findings clearly demonstrated that loss of Tnmd in mTSPCs leads to profoundly altered gene expression profile and cell morphology, reduced adhesion to collagen type I, and impaired ability to contract the ECM. We believe that our study contributes to the better understanding of molecular factors involved in the tendon healing processes and provides an innovative idea for development of medicinal boosters for quicker and complete tendon repair.

Conflicts of interest

The authors declare no conflict of interests.
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Transparency document

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.03.063.

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