JOURNAL OF TISSUE ENGINEERING AND REGENERATIVE MEDICINE
J Tissue Eng Regen Med 2016.
Published online in Wiley Online Library (wileyonlinelibrary.com) **DOI:** 10.1002/term.2150

RESEARCH ARTICLE

Enhanced tenogenic differentiation and tendon-like tissue formation by tenomodulin overexpression in murine mesenchymal stem cells

Yongkang Jiang[1†], Yuan Shi[1†], Jing He[3†], Zhiyong Zhang[1,2], Guangdong Zhou[1,2], Wenjie Zhang[1,2], Yilin Cao[1,2] and Wei Liu[1,2*]

[1]*Department of Plastic and Reconstructive Surgery shanghai 9th People's Hospital, People's Republic of China*
[2]*National Tissue Engineering Centre of China, Shanghai, People's Republic of China*
[3]*Department of Anatomy and Neurobiology, Tongji University School of Medicine, Shanghai, People's Republic of China*

Abstract

As a highly specific marker of tenocytes, tenomodulin (Tnmd) functions remain largely unexplored. We investigated the effect of Tnmd overexpression on tenogenic differentiation of murine mesenchymal stem cells (mMSCs) via plasmid-mediated overexpression in the C3H10T1/2 cell line. The results showed that overexpressed Tnmd could significantly enhance cell proliferation ($p < 0.05$) and the gene expressions of tenogenic-related molecules, including Tnmd, Scleraxis (Scx), collagens I, III and VI and decorin ($p < 0.05$), and significantly inhibit mMSCs differentiation towards the adipogenic, chondrogenic and osteogenic lineages ($p < 0.05$). Upon *in vivo* implantation with rat tail collagen gel subcutaneously in nude mice, Tnmd-overexpressed C3H10T1/2 cells formed neotendon-like tissue, which revealed a histological feature of wave-like dense collagen fibres and cells aligned in parallel. By contrast, a disorganized connective tissue structure with randomly distributed cells was observed in the control group. To further confirm this finding, a conditional Tnmd-overexpressing mouse model was established and the derived primary mMSCs could be induced to overexpress Tnmd with > two-fold upregulated gene expression ($p < 0.05$) by the treatment of doxycycline (Dox). Similarly, conditional overexpression of Tnmd in primary mMSCs also led to faster proliferation ($p < 0.05$), enhanced gene expression of tenogenic markers ($p < 0.05$) and the inhibited expressions of adipogenic and osteogenic markers ($p < 0.05$). The results of enhanced tenogenic differentiation and neotendon formation indicated that Tnmd may serve not only as a tenogenic marker but also as a positive regulator of MSCs tenogenic differentiation, which might be applied to MSCs-mediated tendon regeneration.

Received 23 July 2015; Revised 12 November 2015; Accepted 22 December 2015

Keywords **tenomodulin; overexpression; MSCs; tenogenic differentiation; neotendon formation**

1. Introduction

Tendon engineering offers a promising alternative therapy for tendon repair and regeneration, which remains a clinical challenge in plastic and hand surgery. Regarding tendon engineering, different types of seed cells have been employed previously, such as tenocytes and skin fibroblasts (Cao *et al.*, 2002; Liu *et al.*, 2006; Deng *et al.*, 2009). Of particular interest is the use of mesenchymal stem cells (MSCs) for tendon engineering, because of their multi-differentiation potential (Pittenger *et al.*, 1999), including tenogenic differentiation potential and the capability of regenerating functional tendons (Ouyang *et al.*, 2003; Hankemeier *et al.*, 2009). Although various molecules have been reported to be beneficial for tenogenic differentiation (Hoffmann *et al.*, 2006; Kuo and Tuan, 2008; Sahoo *et al.*, 2010), the search for a specific molecule that can not only serve as a marker of tenocytes but also play a role in tenogenic differentiation is obviously an area to be explored.

**Correspondence to: W. Liu, Department of Plastic and Reconstructive Surgery, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, 639 Zhi Zao Ju Road, Shanghai 200011, People's Republic of China. E-mail: liuwei_2000@yahoo.com*
† These authors contributed equally to this study.

Tenomodulin (Tnmd) has been defined as a highly specific marker for mature tenocytes in the literature (Brandau *et al.*, 2001; Shukunami *et al.*, 2001; Yamana *et al.*, 2001; Jelinsky *et al.*, 2010). It is a type II transmembrane glycoprotein that is characteristically expressed in dense connective tissues, such as tendon and ligament (Brandau *et al.*, 2001; Shukunami *et al.*, 2001; Yamana *et al.*, 2001). Tnmd is mainly composed of a highly conserved C-terminal domain with eight cysteine residues, a BRICHOS domain consisting of a conserved sequence of approximately 100 amino acids, and a putative protease recognition site, RXXR. Cleavage at this site by furin results in the release of the C-terminal cysteine-rich domain from the cell membrane into the extracellular matrix (ECM) (Brandau *et al.*, 2001).

Regarding Tnmd function, this remains largely unexplored. A study (Oshima *et al.*, 2004) has shown that the C-terminal cysteine-rich domain has an anti-angiogenic role, as its overexpression in human umbilical vein endothelial cells (HUVECs) inhibited cell proliferation and tube formation in an *in vitro* assay. Its overexpression in human melanoma cells resulted in suppressing tumour growth *in vivo*, with decreased vessel density (Oshima *et al.*, 2004). Additionally, Tnmd-deficient mice exhibited severely reduced proliferation of derived tenocytes and an altered collagen fibril superstructure, indicating that Tnmd is a regulator of tenocyte proliferation and collagen fibril maturation (Docheva *et al.*, 2005).

This evidence suggests that Tnmd may play a potential role in tendon development and functional maintenance. From a developmental biology point of view, MSC tenogenic differentiation may partially represent the tendon development process (Bruder *et al.*, 1994). Unfortunately, the role of Tnmd in this process has not yet been fully explored. Previously, gene transfection of MSCs was applied to tendon engineering, e.g. *Smad8* (Hoffmann *et al.*, 2006), *Scleraxis* (Alberton *et al.*, 2012) and *BMP12* and *BMP13* (Haddad-Weber *et al.*, 2010). In the present study, we investigated the role of Tnmd in MSC tenogenic differentiation and other lineage differentiations, and neotendon formation primarily via its gene overexpression in an immortalized cell line of murine tendon-derived progenitor TT-D6 cells and a murine MSC cell line, C3H10T1/2. In addition, the effect of Tnmd overexpression on MSC tenogenic differentiation was also validated using murine bone marrow stromal cells (mBMSCs) derived from conditional *Tnmd*-overexpressing transgenic mice.

2. Materials and methods

2.1. Generation of conditional *Tnmd* overexpression mice

This work was performed by Model Animal Research Centre, Nanjing University, as a commercial technical service. Briefly, mouse Tnmd CDS (coding sequence) was excised from pCAGGS–Tnmd plasmid (kindly provided by Dr Yuji Hiraki at Kyoto University, Japan), using *Eco*RI digestion, and then inserted into pTRE vector (pTRE-Tnmd) at the *Eco*RI site, using T4 ligase (New England Biolabs, Ipswich, MA, USA). Linearized pTRE-Tnmd cDNA was microinjected into the pronucleus of fertilized zygotes of C57BL/6 mice, as described (Liu *et al.*, 2013). The eggs were implanted into a pseudo-pregnant ICR foster mother. Founder mice were identified by PCR as described below, using the DNA isolated from the tails and the primer (forward, 5′-GCTCGTTTAGTGAACCGTCAGA-3′; reverse, 5′-CCGTCCTCCTCAAAGTCCTGTA-3′). The pTRE-Tnmd transgenic mice were bred and mated with STOCK Tg(rtTAhCMV) 4Uh/J mice (purchased from the Model Animal Research Centre, Nanjing University, China), which carried the gene encoding the reverse tetracyline-controlled transactivator protein (rtTA) under the control of a human cytomegalovirus (hCMV) early promoter, and conditional overexpression of tenomodulin could be induced with the addition of doxycycline (Dox, Sigma, St. Louis, MO, USA) in the culture medium. The offspring were then bitransgenic and Tnmd overexpression could be induced by treatment with Dox. The transgenic mice were characterized by PCR using above-mentioned primer for pTRE-Tnmd and a new primer for rtTA (forward, 5′-CGCTGTGGGGCATTTTACTTTAG-3′; reverse, 5′-CGTGTCCAGATCGAAATCGTC-3′). All experimental protocols involving mouse experiments were approved by the Animal Care and Experiment Committee of Shanghai Jiao Tong University School of Medicine.

2.2. Cell culture

Cells from the murine tendon-derived cell line TT-D6 (Salingcarnboriboon *et al.*,2003) and the murine MSC cell line C3H10T1/2 (Hoffmann *et al.*, 2006) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. TT-D6 cells needed to be cultured at 33 °C, as the cells harboured a temperature-sensitive mutant of SV40 large T antigen in order to maintain their immortalized status (Salingcarnboriboon *et al.*, 2003). The culture medium was changed every 2 days and the cultured cells were passaged when they reached 80% confluence.

To obtain murine primary BMSCs from the transgenic mice, 10 conditional Tnmd overexpression mice were randomly selected and anaesthetized with pentobarbital (50 mg/kg), and the bone marrow cells were harvested from the femurs by grinding aseptically, as previously described (Zhu *et al.*, 2015). In this study, three or four mouse-derived BMSCs were combined into one cell sample; in total, three cell samples were generated from 10 mice. The derived cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/ml

DOI: 10.1002/term

streptomycin. After culture for 48 h, non-adherent cells were removed by changing the medium. The adherent cells were grown and sorted by magnetic-activated cell sorting (MACS; Miltenyi Biotec, Germany), using a mouse lineage cell depletion kit (Miltenyi Biotec) as previously described (Lamoury *et al.*, 2006). The lineage-negative cells were considered to be mBMSCs. To induce the overexpression of Tnmd, 5 μM Dox was added into the DMEM culture medium, which was changed every 2 days.

To assess the multilineage differentiation of C3H10T1/2 cells and mBMSCs *in vitro*, the cells were plated at a density of 5×10^5 cells/well in six-well plates, followed by a change of differentiation medium every 2 days. Differentiation media were prepared as previously described (Chen *et al.*, 2007): for adipogenic induction, both cell types were cultured in adipogenic medium (0.5 mM isobutylmethylxanthine, 1 μM dexamethasone, 10 μM insulin and 200 μM indomethacin) for 7 days; for osteogenic induction, both cell types were cultured in osteogenic medium (0.01 μM 1,25-dihydroxyvitamin D3, 50 μM ascorbate 2-phosphate and 10 mM β-glycerol phosphate) for 21 days; and for chondrogenic induction, both cell types were cultured in chondrogenic medium [50 μM ascorbate 2-phosphate (all reagents above from Sigma, St Louis, MO, USA), 10 ng/ml transforming growth factor-β1(TGFβ1; R&D Systems, Minneapolis, MN, USA) and 50 ng/ml insulin-like growth factor-1 (IGF-1; R&D Systems)] for 21 days.

To induce chondrogenic differentiation in a pellet culture fashion, C3H10T1/2 cells were resuspended at 5×10^5 cells in 1 ml culture medium, then centrifuged at $240 \times g$ for 5 min to form pellets, according to a previously established protocol (Zhu *et al.*, 2015). These pellets were cultured in the above-mentioned chondrogenic medium, which was changed every 2 days. After 3 weeks of *in vitro* culture, pellet samples were evaluated by qPCR and histochemical analyses.

2.3. Plasmid transfection

To achieve Tnmd overexpression, TT-D6 and C3H10T1/2 cells were transfected with pCAGGS–Tnmd plasmid as the experimental group and an empty control vector, pCAGGS (Yoshimura *et al.*, 2009), as a control, using Fugene HD (Roche, Indianapolis, IN, USA), according to the manufacturer's protocol. Briefly, the cells were plated at a density of 5×10^5 cells/well in six-well plates until they reached >80% confluence. Next day, transfection complex containing 2 μg linearized DNA and 6 μl Fugene HD transfection reagent (1:3 ratio) was added directly into the culture medium. To assure stable expression of Tnmd, the cells were subjected to G418 (Calbiochem, Darmstadt, Germany) selection at a concentration of 800 μg/ml immediately after the transfection. Individual surviving cell clones were picked up by limiting dilution on 96-well plates. The clones were propagated and confirmed for *Tnmd* gene expression by RT–PCR and quantitative PCR (qPCR). Cell clones that demonstrated the highest levels of *Tnmd* gene expression were further confirmed by immunofluorescent staining of Tnmd protein and propagated under the selected pressure of G418 (400 μg/ml), and three cell clones with the highest gene expression level were used for following experiments.

2.4. RNA extraction and RT–PCR analysis

Total RNAs were extracted from TT-D6 and C3H10T1/2 cells transfected with pCAGGS–Tnmd or pCAGGS using Trizol reagent (Invitrogen, Carlsbad, CA, USA), and 1 μg RNA was reverse-transcribed into cDNA, using AMV reverse transcriptase (Promega, Madison, WI, USA) in a 20 μl reaction solution, including 4 μl 5× buffer, 4 μl $MgCl_2$, 1 μl oligo-(dT), 2 μl dNTP, 0.5 μl RNase inhibitor and 0.5 μl AMV reverse transcriptase, using double-distilled water to make up to the final volume. The mixture was then incubated at 30 °C for 10 min, 42 °C for 60 min, 95 °C for 5 min and 5 °C for 5 min. The cDNA was then amplified using a RT–PCR kit (Takara, Shiga, Japan); the primer sequences for detecting tendon-related genes are listed in Table 1. PCR products were separated by agarose gel electrophoresis in TAE buffer containing ethidium bromide and visualized under UV light. β-Actin was used as an internal control. Similarly, total RNAs were also extracted from C3H10T1/2 cells transfected with pCAGGS–Tnmd or pCAGGS and these cells, after multilineage differentiation induction (pCAGGS–Tnmd- or pCAGGS-induced), followed by reverse transcription. RT–PCR was also performed using a RT–PCR kit (Takara); the primer sequences for detecting multilineage differentiation-related genes are listed in Table 2.

Total RNAs were also extracted from mBMSCs derived from conditional *Tnmd*-overexpressing mice, cultured without (TNMD–OE) or with (TNMD–OE + Dox) 5 μM Dox, and from the induced Dox-treated (TNMD–OE + Dox induced) or induced non-treated (TNMD–OE induced) BMSCs after multilineage differentiation, and reverse-transcribed. The cDNA was subjected to qPCR analysis for multidifferentiated genes, including tenogenic genes; the primer sequences are also listed in Tables 1 and 2.

2.5. qPCR

The qPCR procedure was performed using a Power SYBR Green PCR Master Mix (2×; Applied Biosystems, Foster City, CA, USA) in a real-time thermal cycler (Stratagene Mx3000PTM QPCR System, La Jolla, CA, USA), as previously described (Zhu *et al.*, 2010). The mixture of cDNA, PCR master mix, primers and double-distilled water was incubated at 95 °C for 10 min, then with 35 cycles of 95 °C for 30 s, at annealing temperature (see Tables 1, 2) for 30 s, 72 °C for 45 s and 95 °C for 30 s. Analysis of

DOI: 10.1002/term

Table 1. Primers of tendon-related genes used in RT–PCR and qPCR

Gene name	Primer sequence	Annealing temperature (°C)	Size of PCR product (bp)
Scleraxis	F: CCTTCTGCCTCAGCAACCAG R: GGTCCAAAGTGGGGCTCTCCGTGACT	63	156
Tenomodulin	F: TGTACTGGATCAATCCCACTCT R: GCTCATTCTGGTCAATCCCCT	57	115
Collagen I	F: GGCGGCCAGGGCTCCGACCC R: AATTCCTGGTCTGGGGCACC	57	320
Collagen III	F: CAACCAGTGCAAGTGACCAA R: GCACCATTGAGACATTTTGAAG	57	174
Collagen VI	F: AGAACATAGCCTGGACG R: ACAACCCGCCTTAGAG	52	282
Biglycan	F: TGGACCTAGGTCATATTTCTC R: ATATTTGTTTGTGGAGGGACTGG	57	234
Decorin	F: TGTCCATGAGAATGAGATCACCA R: GCCTTCCAGGGACTGAAGAGTCT	50	186
β-Actin	F: CCAAGGCCAACCGCGAGAAGATGAC R: AGGGTACATGGTGGTGCCGCCAGAC	60	592

Table 2. Primers of multilineage differentiation-related genes used in RT–PCR and qPCR

Gene name	Primer sequence	Annealing temperature (°C)	Size of PCR product (bp)
aP2	F: AAATCACCGCAGACGACA R: CACATTCCACCACCAGCT	60	138
Adiponectin	F: GCAGAGATGGCACTCCTGGA R: CCCTTCAGCTCCTGTCATTCC	60	101
C/EBPα	F: GTTAGCCATGTGGTAGGAGACA R: CCCAGCCGTTAGTGAAGAGT	60	353
PPARγ	F: AGACCACTCGCATTCCTTT R: CCCACAGACTCGGCACTCA	58	268
Aggrecan	F: TAGAGGATGTGAGTGGTCTT R: TCCACTAAGGTACTGTCCAC	60	490
SOX9	F: GCTTGACGTGTGGCTTGTTC R: GAGCCGGATCTGAAGATGGA	60	151
Collagen II	F: CACCAAATTCCTGTTCAGCC R: TGCACGAAACACACTGGTAAG	60	124
AKP	F: CCAACTCTTTTGTGCCAGAGA R: GGCTACATTGGTGTTGAGCTTTT	60	110
Osteocalcin	F: CCCAGCGGCCCTGAGTCTGA R: GCTCACACTGCTCCCGGGTG	60	136
Runx2	F: GCCGCTAGAATTCAAAACAGTTGG R: GAATGGCAGCACGCTATTAAATCC	60	103
β-Actin	F: CCAAGGCCAACCGCGAGAAGATGAC R: AGGGTACATGGTGGTGCCGCCAGAC	60	592

each gene expression was conducted in triplicate and repeated in three samples and was normalized to *β*-actin expression, using the $2^{-\Delta\Delta C_T}$ method.

2.6. Immunofluorescent staining

To confirm *Tnmd* overexpression after transfection, TT-D6 and C3H10T1/2 cells transfected with pCAGGS–Tnmd or pCAGGS were fixed in 4% cold paraformaldehyde (PFA; Sigma) in phosphate-buffered saline (PBS) for 10 min and blocked with 5% bovine serum albumin (BSA; Invitrogen) in PBS for 60 min, followed by incubation with rabbit polyclonal anti-Tnmd antibody (1:200; kindly provided by Dr Yuji Hiraki and Dr Chisa Shukunami (Kyoto University, Japan) at 4 °C overnight. After three washes in PBS, PE-labelled anti-rabbit secondary antibody was added and incubated for 60 min at 37 °C. The nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI; 100 nM in PBS; Sigma) for 30 s and visualized under a fluorescence microscope.

2.7. Cell proliferation assay

To evaluate the effect of *Tnmd* overexpression on the proliferation of TT-D6 and C3H10T1/2 cells, the 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide (MTT; Sigma) assay was performed. Both types of cells, transfected with pCAGGS–Tnmd or pCAGGS, were seeded on 96-well plates at a density of 2000 cells/well, cultured for 6 days and tested each day. In each well, 20 μl (5 mg/ml) MTT solution was added and the plates were incubated for 4 h at 37 °C. Then the MTT solution was removed and 150 μl dimethyl sulphoxide (DMSO; Sigma) was added, followed by incubation for 10 min on a low-speed shaking table. The absorbance was measured at 490 nm wavelength, using an enzyme-linked immunosorbent assay reader (Thermo

DOI: 10.1002/term

Scientific, Waltham, MA, USA). The experiment was performed in quadruplicate.

To evaluate the effect of *Tnmd* overexpression on mBMSC proliferation, cell counting kit-8 (CCK-8; Dojindo Laboratories, Japan) was employed, according to the user's manual. Briefly, mBMSCs cultured with or without 5 μM Dox were seeded in 96-well plates at a density of 2000 cells/well. Each group was cultured for 11 days and tested every other day. At the testing time points, 10 μl CCK-8 solution and 90 μl cell culture medium, with or without 5 μM Dox, were added and incubated for 2.5 h at 37 °C under 5% CO_2. Then the absorbance at 450 nm wavelength was measured using the enzyme-linked immunosorbent assay reader. The experiment was performed in quintuplicate and repeated twice.

2.8. Histochemical and immunocytochemical analyses

To detect the effect of *Tnmd* overexpression on the multilineage differentiation of C3H10T1/2 cells and mBMSCs, the related assays were performed including oil red O, alkaline phosphatase (AKP), alizarin red and collagen II staining.

The oil red O staining procedure was performed following the manufacturer's protocol (Genmed Scientific, Arlington, MA, USA). Briefly, cells after adipogenic differentiation were fixed with 4% cold paraformaldehyde for 10 min and then incubated in 2% oil red O reagent for 7 min, followed by washes with PBS, and then observed under a microscope.

The examination of AKP activity was done following the manufacturer's protocol (Shanghai Rainbow Medical Reagent, Shanghai, China). Briefly, cells after osteogenic differentiation were fixed with 4% cold paraformaldehyde for 30 s and then incubated in prepared AKP solution for 45 min. The procedure of alizarin red staining was done following the manufacturer's protocol (Genmed Scientific). Briefly, cells after osteogenic differentiation were fixed with 4% cold paraformaldehyde for 10 min and then incubated in alizarin red solution for 10 min.

To detect the expression of type II collagen (*COL2*), cells after chondrogenic differentiation were fixed with 4% paraformaldehyde and blocked with 5% BSA in PBS, followed by incubation with an anti-mouse type II collagen antibody (1:100 in PBS; Neomarkers, Fremont, CA, USA) at 4 °C overnight. After three washes in PBS, type II collagen was detected using the mouse EnVision$^+$ system (Dako, Denmark).

For semi-quantitative analyses, three fields were randomly selected for observation in each sample under a microscope in all above described procedures and repeated in three samples.

2.9. Ectopic implantation and histological examination

Twelve nude mice, 6–8 weeks old, purchased from the Shanghai Laboratory Animal Centre (Shanghai, China) were involved in this study, and the animal study protocol was approved by the Animal Care and Experiment Committee of Shanghai Jiao Tong University School of Medicine. Briefly, 10 million C3H10T1/2 cells transfected with pCAGGS–Tnmd and pCAGGS were mixed with mouse tail type I collagen gel (1.5 mg/ml; Shengyou, Hangzhou, China) to prepare cell–collagen constructs with length 1 cm and diameter 0.5 cm in a syringe. After anaesthesia with an intraperitoneal injection of chloral hydrate (10%, 100 μl/mouse), an incision was made in the sacral area on each side of the dorsum and a subcutaneous pocket was created. A cell–collagen construct was implanted into the subcutaneous pockets on each side and the incision was sutured with Ethicon 5–0 (Johnson & Johnson, New Brunswick, NJ, USA). At 30 days post-implantation, the mice were euthanized and the implants were harvested for histological examination. Formed tissue samples of each group were fixed in 4% paraformaldehyde at 4 °C overnight. After dehydration in a gradient of ethanols and embedding in paraffin, the tissue samples were sectioned at 5 μm and haematoxylin and eosin (H&E) staining was performed, as described previously (Wang *et al.*, 2008), to examine the tissue structure, especially the collagen fibre formation and alignment.

2.10. Statistical analysis

All quantitative data were presented as mean ± standard deviation (SD). The differences among different groups were analysed using one-way ANOVA and a *post hoc* test for the statistical significance between two groups. Student's *t*-test was also used for statistical analysis of paired groups. SPSS 11.0 software was applied to the analysis; $p < 0.05$ was considered statistically significant.

3. Results

3.1. *Tnmd* overexpression in TT-D6 cells and C3H10T1/2 cells

To confirm the successful overexpression of *Tnmd*, RT–PCR and immunofluorescent staining were conducted. As shown in Figure 1, RT–PCR demonstrated that Tnmd was not expressed in TT-D6 cells and expressed at a very low level in C3H10T1/2 cells when transfected with empty vector pCAGGS, with the current RT–PCR protocol. After transfection with pCAGGS–Tnmd, a high level of *Tnmd* gene expression was observed in both cells (Figure 1, top). Similar results were also shown in immunofluorescent staining, which demonstrated that Tnmd protein was strongly expressed in both TT-D6 and C3H10T1/2 cells transfected with pCAGGS–Tnmd (Figure 1C, D, G, H) when compared to pCAGGS vector-transfected cells (Figure 1A, B, E, F).

DOI: 10.1002/term

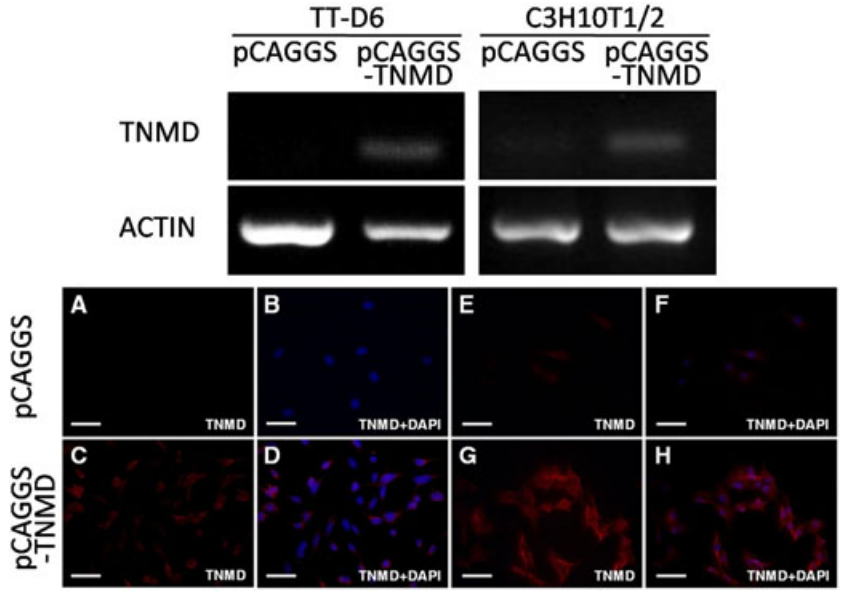

Figure 1. Confirmation of Tnmd overexpression. (Top) RT–PCR examination of overexpressed Tnmd-transfected gene in (left) TT-D6 and (right) C3H10T1/2 cells. (Bottom) Immunofluorescent staining of Tnmd protein production in (A–D) TT-D6 and (E-H) C3H10T1/2 cells. pCAGGS, cell transfected with empty pCAGGS vector; pCAGGS–Tnmd, cell transfected with pCAGGS–Tnmd; TNMD, Tnmd staining (red); DAPI, nuclear counterstaining (blue); bar =100 μm

3.2. Effect of *Tnmd* overexpression on cell morphology and proliferation of the cell lines

TT-D6 and C3H10T1/2 cells transfected with pCAGGS or pCAGGS–Tnmd were observed under reverse phase-contrast microscopy. No obvious differences in cell morphology could be observed between two groups of cells, all exhibiting a spindle shape and fibroblast-like morphology in both TT-D6 and C3H10T1/2 cells (see supporting information, Figure S1).

To evaluate the effect of Tnmd on the proliferation of TT-D6 and C3H10T1/2 cells, a MTT assay was performed. As shown in Figure 2A, the proliferation was obviously faster in *Tnmd*-overexpressed TT-D6 cells than in control TT-D6 cells, with a significant difference between days 2 and 6 ($p < 0.01$). Similarly, *Tnmd*-overexpressed C3H10T1/2 cells also proliferated faster than vector-transfected C3H10T1/2 cells, with a significant difference between days 2 and 6 ($p < 0.01$) (Figure 2B).

3.3. Effect of Tnmd overexpression on tendon related maker expressions in the cell lines

qPCR analysis was performed to examine the gene expression of tendon-related markers. As shown in Figure 3, collagens I (*COL1*), III (*COL3*) and decorin (*DCN*) were all significantly upregulated in their gene expression levels in both *Tnmd*-overexpressed TT-D6 and C3H10T1/2 cells when compared to vector-transfected cells. It was also found that collagen VI (*COL6*) and biglycan (*BGN*) were significantly enhanced for their gene expression in the overexpressed TT-D6 cells but not in the overexpressed C3H10T1/2 cells. By contrast, the gene expression of Scleraxis (*Scx*), a transcript regulator of Tnmd, was significantly upregulated in the overexpressed C3H10T1/2 cells but not in the overexpressed TT-D6 cells (Figure 3). Collectively, these results indicated that *Tnmd* overexpression could promote the expression of tendon-related extracellular components and promote the tenogenic differentiation of C3H10T1/2 cells.

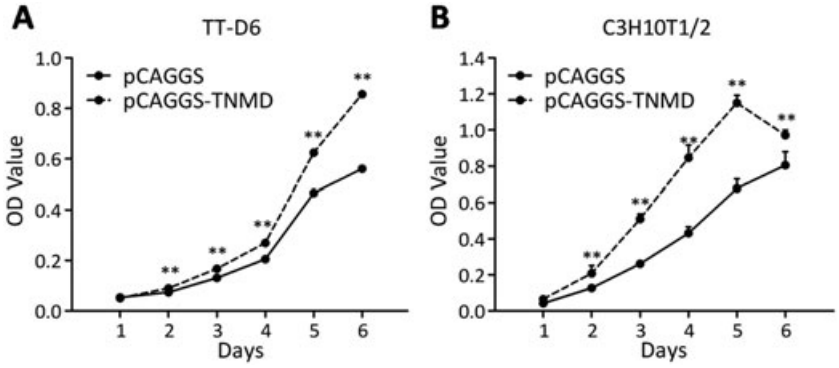

Figure 2. Tnmd overexpression enhanced cell proliferation of both (A) TT-D6 and (B) C3H10T1/2 cells, as revealed by MTT assay. pCAGGS, cell transfected with empty pCAGGS vector; pCAGGS–Tnmd, cell transfected with pCAGGS–Tnmd; $^{**}p < 0.01$

DOI: 10.1002/term

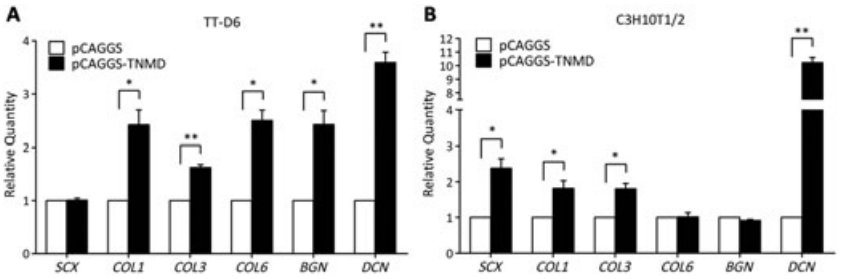

Figure 3. Tnmd overexpression enhanced the gene expressions of tendon-related molecules, as revealed by qPCR analysis: *SCX*, scleraxis; *COL1*, type I collagen; *COL3*, type III collagen; *COL6*, type VI collagen; *BGN*, biglycan; *DCN*, decorin; pCAGGS, cell transfected with empty pCAGGS vector; pCAGGS–Tnmd, cell transfected with pCAGGS–Tnmd; *$p < 0.05$, **$p < 0.01$

3.4. Effect of *Tnmd* overexpression on multilineage differentiation of C3H10T1/2 cells

To investigate the effect of *Tnmd* overexpression on the multilineage differentiation of C3H10T1/2 cells, the cells were subjected to adipogenic, chondrogenic and osteogenic differentiation, following by qPCR analysis and histochemical staining.

As shown in Figure 4A, before induction, adipogenic-related genes, such as adipocyte protein 2 (*aP2*), adiponectin and CCAAT enhancer binding protein-α (*C/EBPα*), were minimally expressed (pCAGGS), but they were downregulated significantly ($p < 0.01$) by overexpressed Tnmd (pCAGGS–Tnmd). Moreover, the above genes increased dramatically in the control cells ($p < 0.01$) after adipogenic induction (pCAGGS induced), but the expressions were inhibited by the overexpressed Tnmd in the induced cells (pCAGGS–Tnmd-induced), which were significantly ($p < 0.01$) lower than those of control cells. Oil red O staining (Figure 4D, a–d) also showed that the adipogenesis in the pCAGGS–Tnmd-induced group was lower than that in the pCAGGS-induced group. The above results indicated that *Tnmd* overexpression could inhibit the adipogenic differentiation of C3H10T1/2 cells.

As shown in Figure 4B, before induction, the expression of chondrogenic-related genes, such as aggrecan, SRY-related high-mobility group box gene 9 (*SOX9*) and type II collagen (*COL2*), were minimally expressed in vector-transfected cells (pCAGGS), but their expressions were significantly downregulated in *Tnmd*-overexpressed cells (pCAGGS–Tnmd), with a significant difference ($p < 0.01$). After chondrogenic induction, the expressions of aggrecan, *SOX9* and *COL2* increased dramatically ($p < 0.01$) in vector-transfected cells (pCAGGS–Tnmd). Nevertheless, the upregulation of aggrecan and *COL2*, but not *SOX9*, gene expressions were significantly inhibited by overexpressed *Tnmd* ($p < 0.01$) (pCAGGS–Tnmd-induced). Immunohistochemical staining also showed that collagen II protein production was only

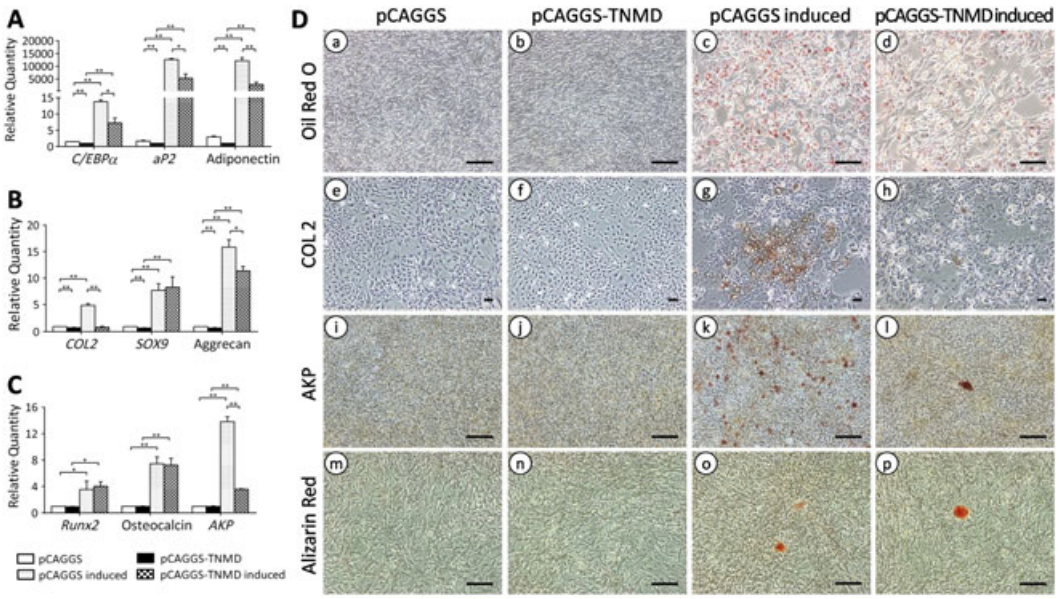

Figure 4. Tnmd overexpression inhibited the differentiation of C3H10T1/2 cells towards adipogenic, chondrogenic and osteogenic lineages, as revealed by (A–C) qPCR and (D) histochemical staining: *aP2*, adipocyte protein 2; *C/EBPα*, CCAAT enhancer binding protein-*α*; *COL2*, type II collagen; *SOX9*, SRY-related high-mobility group box gene 9; *Runx2*, Runt-related transcription factor 2; *AKP*, alkaline phosphatase; pCAGGS, cell transfected with empty pCAGGS vector; pCAGGS–Tnmd, cell transfected with pCAGGS–Tnmd; pCAGGS-induced, cell transfected with empty pCAGGS vector and subject to differentiation induction; pCAGGS–Tnmd-induced, cell transfected with pCAGGS–Tnmd and subject to differentiation induction; bar =100 μm; *$p < 0.05$, **$p < 0.01$

DOI: 10.1002/term

enhanced in induced control cells (pCAGGS-induced) but not in the other three groups (Figure 4D, e–h). Collectively, these results suggested that *Tnmd* overexpression could inhibit the chondrogenic differentiation of C3H10T1/2 cells.

To further confirm the effect of Tnmd on chondrogenic differentiation, a pellet culture model was also applied. As shown in Figure S2 (see supporting information), overexpressed Tnmd formed a tissue mass that had a relatively loose structure, with apparently less matrix production, when examined with both H&E and toluidine blue staining. Furthermore, qPCR revealed that the gene expression levels of *COL2*, *Sox9* and Aggrecan were all significantly downregulated in *Tnmd*-overexpressed cell pellets than in control cell pellets.

As shown in Figure 4C, before induction, expressions of the osteogenic markers AKP, osteocalcin and Runt-related transcription factor 2 (Runx2) were minimally detectable and no significant difference could be observed between the groups, with or without *Tnmd* overexpression ($p > 0.05$). After osteogenic induction, the gene expressions of all these markers were significantly upregulated compared to pre-induction levels ($p < 0.05$). In *Tnmd*-overexpressed C3H10T1/2 cells with osteogenic induction (pCAGGS–Tnmd-induced), upregulation of *AKP* gene expression was significantly inhibited compared to vector-transfected cells ($p < 0.01$) and no significant difference was observed for other two osteogenic markers, Osteocalcin and *RUNX2* ($p > 0.05$). Histochemical staining also confirmed that AKP activity was significantly inhibited in *Tnmd*-overexpressed cells after osteogenic induction (Figure 4D, i–l). However, *Tnmd* overexpression seemed to have no effect on calcium nodule formation, as revealed by alizarin red staining (Figure 4D, m–p).

3.5. Effect of *Tnmd* overexpression on ectopic neotendon formation

To further examine the role of Tnmd in neotendon formation, *Tnmd*-overexpressed and vector-controlled C3H10T1/2 cells were respectively mixed with rat tail collagen gel and implanted *in vivo*. The histology of the tissues 30 days after implantation showed that implants seeded with *Tnmd*-overexpressed cells (pCAGGS–Tnmd) exhibited a porcelain-like white colour and a relatively bigger tissue block, whereas the implants with control cells (pCAGGS) showed a semi-transparent white tissue with a smaller tissue volume (Figure 5A). Histologically, H&E staining revealed that a wave-like dense collagen structure was formed, with the cells aligned in parallel with the direction of the collagen fibres in the experimental implants, similar to tendon tissue structure (Figure 5D, E). By contrast, in the control implants, no dense collagen fibres were observed. Instead, a disorganized connective tissue structure with randomly distributed cells was observed (Figure 5B, C).

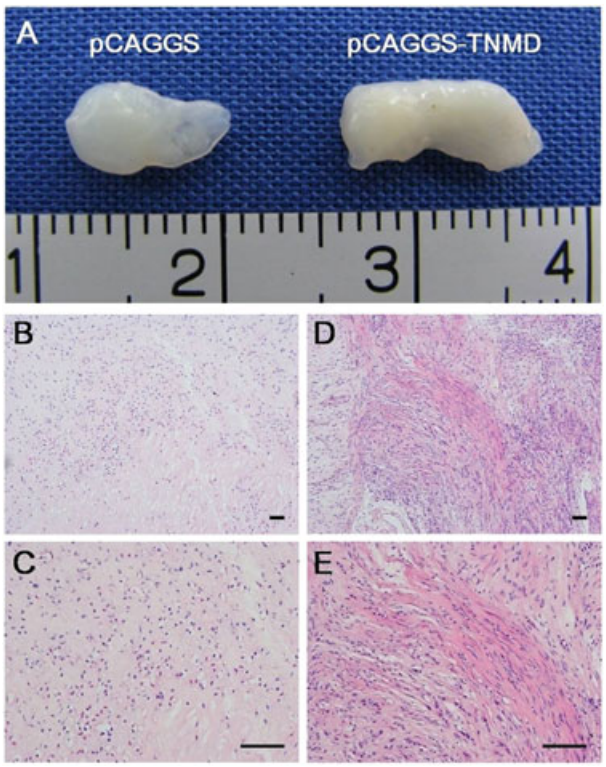

Figure 5. Tnmd overexpression enhanced neotendon tissue formation *in vivo*. C3H10T1/2 cells were transfected with either pCAGGS–Tnmd (overexpression) or pCAGGS vector (control) and mixed with rat tail collagen I gel for *in vivo* implantation. (A) Gross view of harvested tissue 30 days after implantation; (B, C) H&E staining of control tissue with different magnifications; (D, E) H&E staining of overexpression tissue with different magnifications: pCAGGS, cell transfected with empty pCAGGS vector; pCAGGS–Tnmd, cell transfected with pCAGGS–Tnmd; bar =100 μm

3.6. Effect of *Tnmd* overexpression on cell morphology and proliferation of mBMSCs

BMSCs derived from the conditional *Tnmd*-overexpressing mice were cultured either with or without Dox (TNMD–OE+ Dox or TNMD–OE, respectively) and were observed under reverse phase-contrast microscopy. As shown in Figure S3 (see supporting information), no obvious difference in cell morphology was observed between the two groups in passage one cells cultured for 1 and 5 days. However, a CCK-8 assay showed that the proliferation was obviously faster in mBMSCs cultured with Dox (Figure 6A) than in cells without Dox in their culture.

3.7. Effect of *Tnmd* overexpression on tendon-related marker expression in mBMSCs

As shown in Figure 6B, Dox-mediated conditional overexpression of *Tnmd* could significantly upregulate the gene expression of most tendon-related markers, including *SCX*, *COL1*, *COL3*, *BGN* and *DCN*, but not *COL6*. Particularly, *DCN* expression was enhanced about four-fold relative to the control.

DOI: 10.1002/term

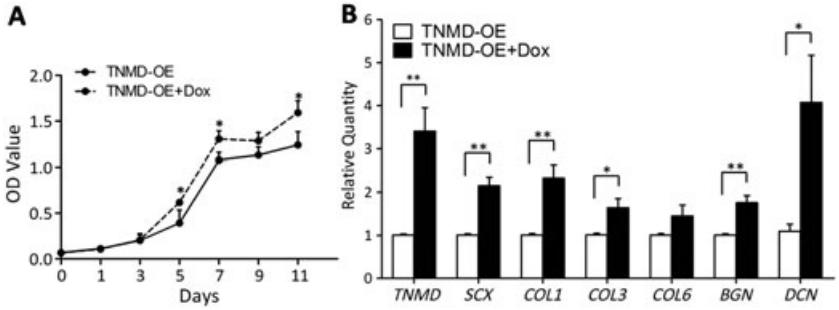

Figure 6. Tnmd overexpression enhanced proliferation and tenogenic marker gene expression of primary murine BMSCs. (A) Cell proliferation; (B) qPCR analysis of tendon-related gene expression: *TNMD*, tenomodulin; *SCX*, scleraxis; *COL1*, type I collagen; *COL3*, type III collagen; *COL6*, type VI collagen; *BGN*, biglycan; *DCN*, decorin; *$p < 0.05$, **$p < 0.01$, between primary BMSCs derived from conditional Tnmd-overexpressing transgenic mice cultured with Dox (TNMD–OE + Dox) or without Dox (TNMD–OE)

3.8. Effect of *Tnmd* overexpression on the multilineage differentiation of mBMSCs

BMSCs derived from the conditional *Tnmd*-overexpressing mice were cultured either with or without Dox and were subjected to adipogenic and osteogenic differentiation. As shown in Figure 7A, after adipogenic induction, the induced gene expression levels of *aP2*, peroxisome proliferator-activated receptor-γ (*PPARγ*) and adiponectin were inhibited significantly in mBMSCs cultured with Dox (with *Tnmd* overexpression) when compared to those of mBMSCs without Dox (control). Meanwhile, oil red O staining (Figure 7C, a, b) also showed that adipogenic potency in mBMSCs cultured with Dox was weaker than that of the non-Dox group, with a significant difference in the staining area ($p < 0.01$; see supporting information, Figure S4 A). These results further provided evidence that *Tnmd* overexpression could inhibit the adipogenic differentiation of MSCs.

As shown in Figure 7B, after osteogenic induction there was no significant difference in the gene expression level of *AKP* between two groups, in agreement with AKP staining, which also showed no difference (Figure 7C, c, d), with no significant difference in the staining area ($p > 0.05$; see supporting information, Figure S4 B). Unlike C3H10T1/2 cells, the gene expression levels of *Runx2* and osteocalcin decreased significantly in the mBMSCs cultured with Dox group (Figure 7B), which also agreed with the finding that alizarin red staining was relatively weaker in the mBMSCs cultured with Dox group than in the mBMSCs without Dox group (Figure 7C, e, f), with a significant difference in the staining area ($p < 0.05$; see supporting information, Figure S4C). Overall, osteogenic potential was inhibited in *Tnmd*-overexpressed mBMSCs.

4. Discussion

Adult stem cells are an important cell source for tissue engineering and regenerative medicine, and BMSCs have already been employed for tendon engineering and

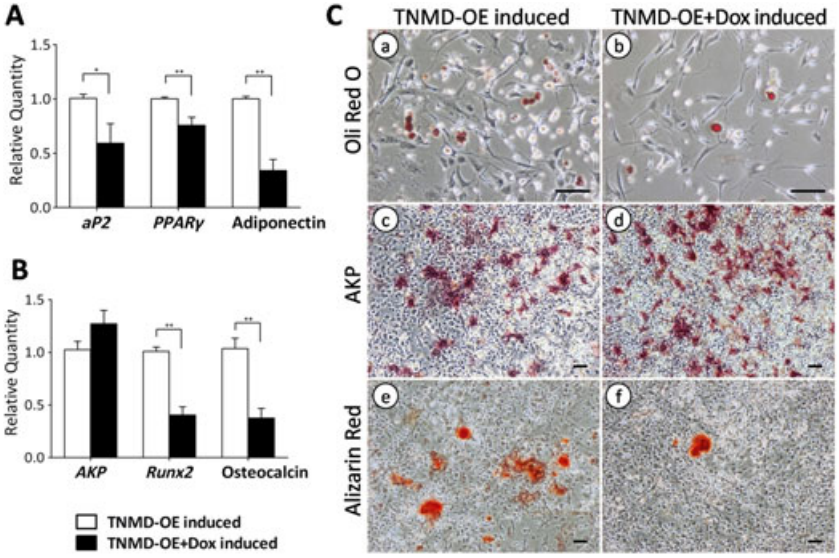

Figure 7. Tnmd overexpression inhibited the differentiation of primary murine BMSCs towards adipogenic and osteogenic lineages, as revealed by (A, B) qPCR and (C) histochemical staining: *aP2*, adipocyte protein 2; *PPARγ*, peroxisome proliferator-activated receptor-γ; *Runx2*, Runt-related transcription factor 2; *AKP*, alkaline phosphatase; TNMD–OE + Dox-induced, primary murine BMSCs derived from the conditional Tnmd-overexpressing transgenic mice cultured with Dox and subjected to differentiation; TNMD–OE-induced, the same BMSCs cultured without Dox and subjected to differentiation; bar =100 μm; *$p < 0.05$, **$p < 0.01$

DOI: 10.1002/term

regeneration (Ouyang *et al.*, 2003; Juncosa-Melvin *et al.*, 2006; Hankemeier *et al.*, 2009). From a developmental biology point of view, MSC differentiation towards a specific lineage partially represents the developmental process of this specific tissue type, such as tendon tissue (Bruder *et al.*, 1994). Therefore, mimicking tendon development might be a good strategy for inducing tenogenic differentiation of MSCs.

Scx and Tnmd are two key molecules that are involved in the process of tendon development. Scx, a basic helix–loop–helix (bHLH) transcription factor, is expressed in tendon progenitor cells and plays a vital role in tendon development (Schweitzer *et al.*, 2001; Brent *et al.*, 2003). *SCX*-null mutant mice exhibited severe disruption of force-transmitting tendons during the differentiation and condensation of tendon progenitors and during the organization of tendon matrix (Murchison *et al.*, 2007). As a transcription factor, its major downstream functional molecules for tendon development remain to be defined, although Tnmd is one of them (Shukunami *et al.*, 2006). The latter authors discovered that *Tnmd* expression was closely associated with the appearance of tenocytes during chick tendon development and was positively regulated by *SCX*, and that retroviral overexpression of Scx resulted in significantly upregulated gene expression of *Tnmd* in cultured tenocytes (Shukunami *et al.*, 2006). Recent research demonstrated that *SCX* delivery via lentivirus led to direct reprogramming of human MSCs into tendon progenitors and a remarkable upregulation of *Tnmd* expression (Alberton *et al.*, 2012). Although *Tnmd* was suggested to be a tenogenic biomarker, its functional role in tendon development and formation remains to be explored.

Studies have shown that *Tnmd* has an anti-angiogenic role, as its overexpression in HUVECs inhibited tube formation in an *in vitro* assay (Oshima *et al.*, 2004), whereas tendon is also considered to be an avascular tissue. Additionally, *Tnmd*-deficient mice exhibited severely reduced tenocyte density and significantly varied collagen fibril diameters, indicating that Tnmd is a regulator of tenocyte proliferation and collagen fibril maturation (Docheva *et al.*, 2005). Tnmd, which is predominantly expressed in tendons and ligaments, is a member of a new family of type II transmembrane glycoproteins, whereas the other family member, chondromodulin I (ChM-I), is highly expressed in cartilage and also has the anti-angiogenic function (Shukunami *et al.*, 2001; Oshima *et al.*, 2004). It is likely that these two molecules may partially compensate for each other's function, and thus tendon remains able to develop in *Tnmd* knockout mice. A similar example is mutual functional compensation between decorin and biglycan; both are members of the small leucine-rich proteoglycans family (SLRP) and can compensate each other in knockout mice (Yoon and Halper, 2005).

In this study, the overexpression method was applied to investigate the functions of *Tnmd*, especially its role in the tenogenic differentiation of adult stem cells. Two cell lines were employed in this study: one was TT-D6, the murine tendon-derived progenitor cell line; the other was C3H10T1/2, the murine MSC cell line. Interestingly, the expression levels of tendon-related markers were significantly enhanced in *Tnmd*-overexpressed cell lines, including collagens I, III and VI, biglycan and decorin and, most importantly, scleraxis. Although there were some differences in the marker expression between two types of cell line (possibly due to different developmental stages), decorin, an important tendon matrix molecule, was upregulated significantly in both TT-D6 and C3H10T1/2 cells (Figure 3). Decorin (*DCN*), as a member of the SLRP family and also the most abundant tendon proteoglycan, plays a key role in regulating matrix assembly because it binds to almost all types of collagen (Yoon and Halper, 2005). Decorin-deficient mice demonstrated coarse and irregular collagen fibril structure and reduced mechanical properties in mature skin and tail tendons (Danielson *et al.*, 1997; Zhang *et al.*, 2006). Biglycan (*BGN*) is also a member of the SLRP family and binds to fibrillar collagens. *BGN*-null mice also developed abnormal collagen fibrils with smaller diameter and weaker tendons, which exhibited reduced stiffness (Ameye *et al.*, 2002). Zhang *et al.* (2006) discovered that biglycan had a comparable spatial localization with decorin and that biglycan expression increased substantially in *DCN*-null tendons, suggesting a potential functional compensation between the two.

Differential expressions of *SCX* and *COL6* were observed in two cell lines. *SCX* was upregulated in C3H10T1/2 cells, whereas *COL6* increased in TT-D6 cells. This was because TT-D6 represents a more mature tenocyte-like cell, which usually has a high level of endogenous *SCX* expression (Salingcarnboriboon *et al.*, 2003). *COL6*, often expressed in mature tendon, is a non-fibrillar collagen and regulates collagen fibrillogenesis via interaction with other extracellular matrix molecules, such as COL1, BGN and DCN. *COL6*-knockout mice displayed a disruption in fibre assembly and fibril formation associated with decreased mechanical strength of tendon tissue (Izu *et al.*, 2011). Comparatively, C3H10T1/2 cells as a MSC cell line began to differentiate into cells with a tenogenic phenotype or immature tenocyte-like cells after *Tnmd* transfection, but they certainly were not mature tenocytes. Therefore, it is reasonable that collagen VI was not significantly upregulated in transfected C3H10T1/2 cells. *SCX* is often considered as a marker for MSC tenogenic differentiation. The upregulated *SCX* expression induced by *Tnmd* overexpression is consistent with the prediction of tenogenic differentiation of C3H10T1/2 cells.

The above results demonstrated that *Tnmd* overexpression could promote MSC tenogenic differentiation by enhanced expression of tenocyte markers. Interestingly, overexpressed *Tnmd* also inhibited other lineage differentiations of C3H10T1/2 cells, including adipogenic, chondrogenic and osteogenic lineages, as shown in Figure 4. The Finnish Diabetes Prevention Study Group reported that expression of Tnmd was strongly correlated with body mass index (BMI) and was downregulated with weight reduction (Tolppanen *et al.*, 2007, 2010). Saiki

DOI: 10.1002/term

et al. (2009) also discovered that Tnmd expression was significantly higher in obese than in lean individuals, and was downregulated during diet-induced weight loss. Considering that Tnmd is an anti-angiogenic factor (Oshima *et al.*, 2004) and it is upregulated by myostatin, an anti-adipogenic factor (Mendias *et al.*, 2008), the increased Tnmd in obesity may become a protective mechanism that restrains the growth of new blood vessels during adipose tissue expansion, as adipose tissue mass can be regulated through the vasculature (Rupnick *et al.*, 2002). It may also inhibit adipogenesis, as shown in this study.

In addition to the experiments performed with cell lines, we also further confirmed the finding with primary mBMSCs derived from conditional *Tnmd*-overexpressing mice. As shown in Figure 6B, Tnmd expression was upregulated > two-fold after Dox treatment; this led to significantly enhanced gene expressions of *SCX*, *COL1*, *COL3*, *BGN* and *DCN*. Similar to the cell lines, overexpressed Tnmd could also significantly inhibit adipogenic and osteogenic differentiations of primary mBMSCs (Figure 7).

We also confirmed that *Tnmd* overexpression promoted cell proliferation in all three different cell types, TT-D6 and C3H10T1/2 cells (Figure 2) and primary mBMSCs of conditional *Tnmd*-overexpressing mice (Figure 6A), which was also consistent with the finding of reduced tenocyte density in *Tnmd*-knockout tendons (Docheva *et al.*, 2005). The mechanism of *Tnmd*-mediated cell proliferation remains unknown. In the future, a further defined signalling role of this molecule may help understanding.

Hoffmann *et al.* (2006) reported that activation of Smad8 in C3H10T1/2 cells promoted tenogenic differentiation and regeneration in an ectopic tendon formation and Achilles tendon defect model. We also employed an *in vivo* ectopic model to investigate the role of *Tnmd* overexpression in neotendon formation in nude mice. As shown in Figure 5, *Tnmd*-overexpressed C3H10T1/2 cells were able to form neotendon-like tissue with a relatively well developed collagen fibre structure 30 days after implantation, along with collagen gel matrix (Figure 5D, E), which was similar to the tissue structure of a previous report in which a neotendon-like tissue was formed ectopically by C3H10T1/2 cells that were genetically modified with *GDF-8* and *BMP-2* (Hoffmann *et al.*, 2006). We also tried the same experiment with primary mBMSCs but failed, and this was likely due to the lack of an effective method to deliver Dox to mBMSCs *in vivo*.

In summary, by applying two cell lines and primary mBMSCs derived from conditional *Tnmd*-overexpressing mice, and *in vitro* and *in vivo* models, this study demonstrated that Tnmd could promote tenogenic differentiation of MSCs, and thus confirmed that Tnmd is not only an important biomarker for tenocytes, but may also play an important role in tendon formation by enhancing MSC tenogenic differentiation, which to our best knowledge has not been reported previously. This finding may have practical importance for tendon regeneration and the translation of tendon engineering in the future. More importantly, the underlying mechanism of this finding needs further investigation. Tnmd is a transmembrane protein and its ligands remain largely unknown, except for vascular endothelial growth factor (VEGF). Whether the intracellular segment of Tnmd has the function of signal transduction is also unknown. The regulation of Tnmd by upstream genes, such as *SCX* and *GDF8*, has been well investigated (Shukunami *et al.*, 2006; Mendias *et al.*, 2008; Alberton *et al.*, 2012). However, how these upstream molecules regulate the gene expression of downstream molecules such as collagens and proteoglycans via tenomodulin remains to be explored in future studies.

5. Conclusions

Tenomodulin is a well-known biomarker for mature tenocytes, but its functions remain unknown. This study demonstrated that *Tnmd* overexpression could promote tenogenic differentiation of MSCs and facilitate MSC-mediated neotendon-like tissue formation *in vivo*. The information gained from this study will further enrich our knowledge in tendon development and promote tendon engineering and regeneration.

Acknowledgements

This study was supported by the National 863 (Grant No. 2012AA020507) and 973 (Grant No. 2005CB522703) Project Foundations and the National Natural Science Foundation (Grant No 31470943 and 81401530). The authors thank Dr Jun-ichi Miyazaki (Kumamoto University, Japan) for kindly providing the pCAGGS vector. We also thank Dr Yuji Hiraki and Dr Chisa Shukunami (Kyoto University, Japan) for kindly providing the pCAGGS–Tnmd plasmid and polyclonal Tnmd antibody.

References

Alberton P, Popov C, Pragert M, *et al*. 2012; Conversion of human bone marrow-derived mesenchymal stem cells into tendon progenitor cells by ectopic expression of scleraxis. *Stem Cells Dev* **21**: 846–858.

Ameye L, Aria D, Jepsen K, *et al.* 2002; Abnormal collagen fibrils in tendons of biglycan/fibromodulin-deficient mice lead to gait impairment, ectopic ossification, and osteoarthritis. *FASEB J* **16**: 673–680.

Brandau O, Meindl A, Fassler R, *et al*. 2001; A novel gene, *tendin*, is strongly expressed in tendons and ligaments and shows high homology with *chondromodulin-I. Dev Dyn* **221**: 72–80.

Brent AE, Schweitzer R, Tabin CJ. 2003; A somitic compartment of tendon progenitors. *Cell* **113**: 235–248.

Bruder SP, Fink DJ, Caplan AI. 1994; Mesenchymal stem cells in bone development, bone repair, and skeletal regeneration therapy. *J Cell Biochem* **56**: 283–294.

Cao Y, Liu Y, Liu W, *et al*. 2002; Bridging tendon defects using autologous tenocyte engineered tendon in a hen model. *Plast Reconstr Surg* **110**: 1280–1289.

Chen FG, Zhang WJ, Bi D, *et al*. 2007; Clonal analysis of nestin$^-$ vimentin$^+$ multipotent fibroblasts isolated from human dermis. *J Cell Sci* **120**: 2875–2883.

Danielson KG, Baribault H, Holmes DF, *et al*. 1997; Targeted disruption of decorin leads to abnormal collagen fibril morphology and skin fragility. *J Cell Biol* **136**: 729–743.

Deng D, Liu W, Xu F, *et al*. 2009; Engineering human neo-tendon tissue *in vitro* with human dermal fibroblasts under static mechanical strain. *Biomaterials* **30**: 6724–6730.

Docheva D, Hunziker EB, Fassler R, *et al*. 2005; Tenomodulin is necessary for tenocyte proliferation and tendon maturation. *Mol Cell Biol* **25**: 699–705.

Haddad-Weber M, Prager P, Kunz M, *et al*. 2010; *BMP12* and *BMP13* gene transfer induce ligamentogenic differentiation in mesenchymal progenitor and anterior cruciate ligament cells. *Cytotherapy* **12**: 505–513.

Hankemeier S, Hurschler C, Zeichen J, *et al*. 2009; Bone marrow stromal cells in a liquid fibrin matrix improve the healing process of patellar tendon window defects. *Tissue Eng A* **15**: 1019–1030.

Hoffmann A, Pelled G, Turgeman G, *et al*. 2006; Neotendon formation induced by manipulation of the Smad8 signalling pathway in mesenchymal stem cells. *J Clin Invest* **116**: 940–952.

Izu Y, Ansorge HL, Zhang G, *et al*. 2011; Dysfunctional tendon collagen fibrillogenesis in *collagen VI*-null mice. *Matrix Biol* **30**: 53–61.

Jelinsky SA, Archambault J, Li L, *et al*. 2010; Tendon-selective genes identified from rat and human musculoskeletal tissues. *J Orthop Res* **28**: 289–297.

Juncosa-Melvin N, Boivin GP, Gooch C, *et al*. 2006; The effect of autologous mesenchymal stem cells on the biomechanics and histology of gel–collagen sponge constructs used for rabbit patellar tendon repair. *Tissue Eng* **12**: 369–379.

Kuo CK, Tuan RS. 2008; Mechanoactive tenogenic differentiation of human mesenchymal stem cells, *Tissue Eng A* **14**: 1615–1627.

Lamoury FM, Croitoru-Lamoury J, Brew J. 2006; Undifferentiated mouse mesenchymal stem cells spontaneously express neural and stem cell markers Oct-4 and Rex-1. *Cytotherapy* **8**: 228–242.

Liu C, Xie W, Gui C, *et al*. 2013; Pronuclear microinjection and oviduct transfer procedures for transgenic mouse production. *Methods Mol Biol* **1027**: 217–232.

Liu W, Chen B, Deng D, *et al*. 2006; Repair of tendon defect with dermal fibroblast engineered tendon in a porcine model. *Tissue Eng* **12**: 775–788.

Mendias CL, Bakhurin KI, Faulkner JA. 2008; Tendons of myostatin-deficient mice are small, brittle, and hypocellular. *Proc Natl Acad Sci USA* **105**: 388–393.

Murchison ND, Price BA, Conner DA, *et al*. 2007; Regulation of tendon differentiation by scleraxis distinguishes force-transmitting tendons from muscle-anchoring tendons. *Development* **134**: 2697–2708.

Oshima Y, Sato K, Tashiro F, *et al*. 2004; Anti-angiogenic action of the C-terminal domain of tenomodulin that shares homology with chondromodulin-I. *J Cell Sci* **117**: 2731–2744.

Ouyang HW, Goh JC, Thambyah A, *et al*. 2003; Knitted poly-lactide-co-glycolide scaffold loaded with bone marrow stromal cells in repair and regeneration of rabbit Achilles tendon. *Tissue Eng* **9**: 431–439.

Pittenger MF, Mackay AM, Beck SC, *et al*. 1999; Multilineage potential of adult human mesenchymal stem cells. *Science* **284**: 143–147.

Rupnick MA, Panigrahy D, Zhang CY, *et al*. 2002; Adipose tissue mass can be regulated through the vasculature. *Proc Natl Acad Sci USA* **99**: 10730–10735.

Sahoo S, Toh SL, Goh JC. 2010; A bFGF-releasing silk/PLGA-based biohybrid scaffold for ligament/tendon tissue engineering using mesenchymal progenitor cells. *Biomaterials* **31**: 2990–2998.

Saiki A, Olsson M, Jernas M, *et al*. 2009; Tenomodulin is highly expressed in adipose tissue, increased in obesity, and down-regulated during diet-induced weight loss. *J Clin Endocrinol Metab* **94**: 3987–3994.

Salingcarnboriboon R, Yoshitake H, Tsuji K, *et al*. 2003; Establishment of tendon-derived cell lines exhibiting pluripotent mesenchymal stem cell-like property. *Exp Cell Res* **287**: 289–300.

Schweitzer R, Chyung JH, Murtaugh LC, *et al*. 2001; Analysis of the tendon cell fate using Scleraxis, a specific marker for tendons and ligaments. *Development* **128**: 3855–3866.

Shukunami C, Oshima Y, Hiraki Y. 2001; Molecular cloning of tenomodulin, a novel chondromodulin-I related gene. *Biochem Biophys Res Commun* **280**: 1323–1327.

Shukunami C, Takimoto A, Oro M, *et al*. 2006; Scleraxis positively regulates the expression of tenomodulin, a differentiation marker of tenocytes. *Dev Biol* **298**: 234–247.

Tolppanen AM, Kolehmainen M, Pulkkinen L, *et al*. 2010; Tenomodulin gene and obesity-related phenotypes. *Ann Med* **42**: 265–275.

Tolppanen AM, Pulkkinen L, Kolehmainen M, *et al*. 2007; Tenomodulin is associated with obesity and diabetes risk: the Finnish diabetes prevention study. *Obesity (Silver Spring)* **15**: 1082–1088.

Wang B, Liu W, Zhang Y, *et al*. 2008; Engineering of extensor tendon complex by an *ex vivo* approach. *Biomaterials* **29**: 2954–2961.

Yamana K, Wada H, Takahashi Y, *et al*. 2001; Molecular cloning and characterization of ChM1L, a novel membrane molecule similar to chondromodulin-I. *Biochem Biophys Res Commun* **280**: 1101–1106.

Yoon JH, Halper J. 2005; Tendon proteoglycans: biochemistry and function. *J Musculoskelet Neuronal Interact* **5**: 22–34.

Yoshimura T, Toyoda S, Kuramochi-Miyagawa S, *et al*. 2009; *Gtsf1/Cue110*, a gene encoding a protein with two copies of a CHHC Zn-finger motif, is involved in spermatogenesis and retrotransposon suppression in murine testes. *Dev Biol* **335**: 216–227.

Zhang G, Ezura Y, Chervoneva I, *et al*. 2006; Decorin regulates assembly of collagen fibrils and acquisition of biomechanical properties during tendon development. *J Cell Biochem* **98**: 1436–1449.

Zhu J, Li J, Wang B, *et al*. 2010; The regulation of phenotype of cultured tenocytes by microgrooved surface structure. *Biomaterials* **31**: 6952–6958.

Zhu Y, Zhang Y, Liu Y, *et al*. 2015; The influence of Chm-I knockout on ectopic cartilage regeneration and homeostasis maintenance. *Tissue Eng A* **21**: 782–792.

Supporting information on the internet

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Figure S1. Cell morphology comparison among the cells of non-transfected (normal) cells transfected with empty vector (pCAGGS) and Tnmd (pCAGGS–TNMD) for both TTD6 cells and C3H10T1/2 cells

Figure S2. Tnmd overexpression inhibited the chondrogenic differentiation of C3H10T1/2 cells in pellet culture

Figure S3. Cell morphology comparison in primary murine BMSCs derived from conditional Tnmd-overexpressing mice between induced (TNMD–OE + Dox) and non-induced (TNMD–OE) cells at days 1 and 5 of culture

Figure S4. Semi-quantitative analysis of staining area for oil red O, alkaline phosphatase (AKP) and alizarin red staining of primary murine BMSCs derived from conditional Tnmd transgenic mice cultured with Dox (TNMD–OE + Dox) or without Dox (TNMD–OE) after induced adipogenic and osteogenic differentiations

DOI: 10.1002/term