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Research paper

Conditional tenomodulin overexpression favors tenogenic lineage differentiation of transgenic mouse derived cells

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ABSTRACT

Tenomodulin (TNMD) is a type II transmembrane protein that is widely expressed in a variety of avascular connective tissues and fat tissue. Its function remains largely unknown except for a marker for mature tenocytes. This study reports the generation of tetracycline (Tet)-on driven conditional TNMD overexpressing mice and thus to provide a tool for systemic investigation of its role in regulating functions of various tissues. The current study focuses on in vitro comparison of tenogenic differentiation potentials induced by doxycycline (Dox) treatment among bone marrow derived stem cells (BMSCs), adipose derived stem cells (ASCs), dermal fibroblasts (DFs) and tenocytes (TCs) of the same transgenic mice. The results showed that BMSCs exhibited the best tenogenic potential than other three cell types ($p < 0.05$ for majority of markers), whereas ASCs and DFs revealed similar potentials ($p > 0.05$ for majority of markers). TCs were found the least capable of being induced for tenogenic gene expression. In addition, TNMD overexpression also significantly inhibited the differentiation towards osteogenic and chondrogenic lineages in both BMSCs and ASCs ($p < 0.05$). However, the inhibition of adipogenic differentiation was not observed in ASCs ($p > 0.05$), suggesting different gene regulation mechanisms may involve in different tissue types and thus leading to different functions, which is likely to be revealed with a transgenic mouse model.

1. Introduction

Tenomodulin (TNMD) is a type II transmembrane surface molecule that is composed of a highly conserved C-terminal domain with eight cysteine residues, a BRICHOS domain which is composed 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 C-terminal cysteine-rich domain from the cell membrane into the extracellular matrix (ECM) (Brandau et al., 2001). TNMD is mainly expressed in avascular tissues in general and particularly in dense connective tissue such as tendon/ligament, heart valve and eye (Oshima et al., 2003). Interestingly, this molecule is also highly expressed in fat tissue (Saiki et al., 2009), an anatomic location exceptional from its principal distributed tissue types.

The functions of TNMD are relatively unexplored. Previous reports indicated that the extracellular part of this molecule, C-terminal cysteine-rich domain, has an anti-angiogenesis effect. Its overexpression in human umbilical vein endothelial cells (HUVECs) inhibited cell proliferation and tube formation in an in vitro assay (Oshima et al., 2003; Oshima et al., 2004). In addition, this molecule is also expected to

Abbreviations: aa, amino acid; AKP, alkaline phosphatase; aP2, adipocyte protein 2; ASCs, adipose derived stem cells; BMSCs, bone marrow derived stem cells; CDS, coding sequences; COL1, collagens I; COL2, collagens II; COL3, collagens III; COL6, collagens VI; DCN, decorin; DFs, dermal fibroblasts; DMEM, Dulbecco's modified Eagle's medium; Dox, doxycycline; ECM, extracellular matrix; FBS, fetal bovine serum; hCMV, human cytomegalovirus; HUVECs, human umbilical vein endothelial cells; IGF-1, insulin-like growth factor-1; MACS, magnetic-activated cell sorting; MSCs, mesenchymal stem cells; PCR, polymerase chain reaction; PPAR-γ, peroxisome proliferator-activated receptor-γ; qPCR, quantitative PCR; rtTA, reverse tetracycline transcriptional activator; Runx2, Runt-related transcription factor 2; SCX, scleraxis; Sox9, SRY-box 9; SRY, sex determining region; TCs, tenocytes; Tet, tetracycline; TGF-β3, transforming growth factor-β3; TNC, tenascin-C; TNMD, tenomodulin.

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participate in fat metabolism regulation (Tolppanen et al., 2008; Saiki et al., 2009; Senol-Cosar et al., 2016).

Tenogenic differentiation is one of the areas of mesenchymal stem cells (MSCs) research, which may potentially be translated into tendon regeneration (Yin et al., 2013; Deng et al., 2014; Jiang et al., 2016; Lu et al., 2016; Yin et al., 2016). MSCs are also the potential source of cell therapy for tendon injury (Docheva et al., 2015; Gaspar et al., 2015). In addition to MSCs, certain types of mature and differentiated somatic cells, such as dermal fibroblasts (DFs), are also possible to be transdifferentiated into a tenogenic phenotype and thus potentially be used for tendon regeneration (Liu et al., 2006; Chen et al., 2016; Wang et al., 2016). And despite the disadvantages such as limited resource and poor proliferation, functional tenocyte-based tendon engineering had been successfully produced (Wang et al., 2008; Chen et al., 2016). It thus would be interesting to investigate how overexpressed TNMD affects the behavior of multiple cell types and what is the difference in their functions given the same stimulator to different cell types.

Transgenic mouse model is a widely used methodology to investigate the role of a single molecule in directing functions of multiple cell types. However, overexpression of certain molecule including TNMD will have an embryonic lethal effect and also the difficulty to regulate the transgene expression level, and thus limits its application. The development of conditional overexpressing transgenic mouse driven by an inducible promoter systems such as tetracycline (Gossen and Bujard, 1992), ecdysone (No et al., 1996), steroids (Burcin et al., 1999) and rapamycin (Ye et al., 1999) are likely to overcome the undesirable shortcomings of the traditional transgenic mouse model. The tetracycline (Tet)-inducible gene regulation system is a powerful tool that allows for temporal and dose-dependent regulation of target transgene expression in vitro (Gossen and Bujard, 1992; Rabiee et al., 2014) and in vivo (Furth et al., 1994; Jazwa et al., 2013). For example, Runt-related transcription factor 2 (Runx2) overexpressing mice displayed multiorgan abnormalities, including ectopic calcification and induction of the osteogenic program in a limited number of tissues, such as muscle and lung (He et al., 2011). The Tet-On system comprises two key components: the doxycycline (Dox)-dependent regulator, such as reverse tetracycline Transcriptional Activator (rtTA), and the response element containing the target gene, such as pTRE-X. The Tet-On system usually contains integrated copies of the regulator and response plasmids in order to create a double-stable transgenic mice line (Kistner et al., 1996).

Previously, we described a Tet-On transgenic TNMD mice that ubiquitously expresses rtTA driven by human cytomegalovirus (hCMV) early promoter (Jiang et al., 2016), which was used to support the role of TNMD overexpression in driving MSC tenogenic differentiation. We hypothesized that overexpression of TNMD gene in an overexpressing animal model will exert different functions among different cell types. In this study, we employed the same model to examine the difference in tenogenic lineage differentiation potentials among bone marrow derived stem cells (BMSCs), adipose-derived stem cells (ASCs) and DFs using tenocytes (TCs) as a positive control, given the same overexpressed gene, TNMD.

2. Materials and method

2.1. Generation the pTRE TNMD transgenic mouse line

Generation of conditional TNMD overexpression mice was briefly mentioned previously (Jiang et al., 2016) and more detailed description was provided in this study. In the process, pCAGGS-TNMD plasmid was kindly provided by Dr. Yuji Hiraki at Kyoto University, Japan. After sufficient amplification, mouse TNMD coding sequences (CDS) were excised from pCAGGS-TNMD plasmid using *Eco*RI digestion, and then inserted into pTRE vector at *Eco*RI site using T4 ligase (New England Biolabs, Ipswich, MA) resulting in pTRE-TNMD plasmid. Then, the plasmid was linearized followed by the microinjection into the pronucleus of fertilized zygotes of C57BL/6 mice as described (Conner, 2004; Liu et al., 2013). A pseudo-pregnant ICR foster mother served as a recipient for the egg implantation (This work was performed by Model Animal Research Center, Nanjing University as a commercial technical service) to generate the founder pTRE-TNMD mice. To identify founder (F0) transgenic mice, tail biopsies were collected from the animals aged from two to three weeks for genomic DNA isolation using Gentra Puregene Mouse Tail Kit (Qiagen). The generated founder mice were further screened by polymerase chain reaction (PCR) analysis of the DNA derived from pTRE-TNMD mouse tails using specially designed primers (forward: 5-GCTCGTTTAGTGAACCGTCAGA-3 and reverse: 5-CCGTCCTCCTCAAAGTCCTGTA-3). Each founder (F0) mouse was given a specific number and bred with wild type C57BL/6 mouse but never crossed each other. Then the tails of each founder's offspring were collected and identified by PCR as previous described (Jiang et al., 2016). The positive offspring mice of each founder were mated each other to reproduce the next generation and the process was repeated until the positive rate reached 100%. 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. Generation of conditional TNMD overexpressing mice

Subsequently, conditional TNMD overexpressing mice were generated by breeding pTRE-TNMD mice with STOCK Tg (rtTAhCMV) 4Uh/J mice (purchased from Model Animal Research Center, Nanjing University, China). To identify the conditional TNMD overexpressing mice that carried the gene encoding rtTA under the control of a hCMV early promoter, the finally generated bitransgenic mice were characterized by PCR using above mentioned primer for pTRE-TNMD and a new primer for rtTA (forward: 5-CGCTGTGGGGCATTTTACTTTAG-3 and reverse: 5-CATGTCCAGATCGAAATCGTC-3). All experimental protocols involving mouse experiments were approved by the Animal Care and Experiment Committee of Shanghai Jiao Tong University School of Medicine.

2.3. Induction of TNMD overexpression with Dox treatment

In all experiments, four types of cells derived from the conditional TNMD overexpressing mice were tested for conditional TNMD gene overexpression with the addition of 5 μM Dox (Sigma, St. Louis, MO, USA) in culture medium.

2.4. Harvest and in vitro culture of BMSCs

To obtain murine primary BMSCs from the transgenic mice, 10 conditional TNMD overexpressing mice aged from 3 to 4 weeks old were randomly selected and sacrificed via excessive anesthesia, and the bone marrow cells were harvested from the femurs by grinding and rinsing aseptically as previously described (Zhu et al., 2015). In this study, BMSCs derived from 3 to 4 mice were combined into one cell sample, total 3 cell samples were generated from 10 mice. The derived cells were cultured in low glucose Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Logan City, UT) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY), 100 U/ml penicillin and 100 mg/ml 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, Germany) as previously described (Zhu et al., 2015). The lineage negative cells were considered as BMSCs and seeded at a density of 2×10^4 cells/cm^2. To induce the overexpression of TNMD, 5 μM Dox was added into the DMEM culture medium with medium change every 2 days.

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2.5. Harvest and in vitro culture of ASCs and tri-lineage differentiation

To obtain murine primary ASCs from the transgenic mice, 20 transgenic mice aged from 6 to 8 weeks old were randomly selected and sacrificed as previously described. ASCs derived from 6 to 7 mice were combined into one cell sample, total 3 cell samples were generated from 20 mice. Briefly, subcutaneous adipose tissues were harvested from the inguinal and armpit areas, cut into small fragments and digested with 0.075% (w/v) collagenase (NB4, Serva, France) in serum-free low glucose DMEM at 37 °C for 2 h. The cell suspension was harvested and filtered through a 40-μm nylon filter mesh (BD Falcon, Bedford, MA) to remove tissue residues. The cells were seeded onto 10-cm dishes at a density of 3×10^5 cells/cm^2 for primary culture and 2×10^4 for subculture. Cell culture medium was consisted of DMEM plus 10% FBS with or without 5 μM Dox, and changed every 2 days.

The assessing of multi-lineage differentiation of ASCs in vitro was described previously (Xiong et al., 2015). The cells were seeded at a density of 1×10^5 cells per well in six-well plates followed by the change of differentiation media every 2 days. The differentiation media were prepared as below described.

For osteogenic induction, non-induced or Dox-induced cells were cultured in osteogenic medium (0.01 μM 1,25-dihydroxy vitamin D3,Sigma, St Louis, MO), 50 μM ascorbate-2-phosphate (Sigma, St Louis, MO) and 10 mM β-glycerol phosphate (Sigma, St Louis, MO) for 21 days. Total 5 views (×100) were randomly selected from 3 cell samples and subjected to quantitative analysis of Alizarin Red staining area with Image-Pro Plus processing software (version 6.0, Media Cybernetics, Silver Spring, MD). Mean plus standard deviation was derived from the data of five views.

To induce chondrogenic differentiation in a pellet culture fashion, ASCs were resuspended to 5×10^5 cells in 1 ml culture medium, then centrifuged at 240*g* for 5 min to form pellets according to a previously established protocol (Zhu et al., 2015). These pellets were cultured in chondrogenic medium (50 μM ascorbate-2-phosphate, Sigma, St Louis, MO), 10 ng/ml transforming growth factor-β3 (TGF-β3, R&D Systems, Minneapolis, MN) and 50 ng/ml insulin-like growth factor-1 (IGF-1, R&D Systems, Minneapolis, MN) and the medium was changed every 2 days. After 3 weeks of in vitro culture, pellet samples were evaluated by qPCR and histochemical analyses. Total 3 histology samples respectively derived from 3 pellet samples were subjected to quantitative analysis of Alcian Blue staining area with Image-Pro Plus processing software. Mean plus standard deviation was derived from the data of three views.

For adipogenic induction, both Dox treated and non-treated cells were cultured in adipogenic medium (0.5 mM isobutylmethylxanthine (Sigma, St Louis, MO), 1 μM dexamethasone (Sigma, St Louis, MO), 10 μM insulin (Gibco, Grand Island, NY) and 200 μM indomethacin (Sigma, St Louis, MO)) for 10 days. Total 5 views (×100) were randomly selected from 3 cell samples and subjected to quantitative analysis of Oil Red staining area with Image-Pro Plus processing software. Mean plus standard deviation was derived from the data of five views.

2.6. Harvest and in vitro culture of DFs

DFs derived from 6 to 7 mice aged from 6 to 8 weeks were combined into one cell sample, and total 3 cell samples were generated from 20 sacrificed transgenic mice. Skin tissue from back of the mice was harvested and cut into strips in the width of 5 mm. To separate epidermis and dermis, the skin tissue was treated with 0.2% dispase (Roche Diagnostics, Indianapolis, IN) at 4 °C overnight followed by mechanical separation of dermis and epidermis. The dermis was then cut into small pieces and treated with 0.2% collagenase in serum-free high glucose DMEM on a rotator at 37 °C for 2 h. The cells were cultured with high glucose DMEM culture medium containing 10% FBS at a density of 3×10^4 cells/cm^2. Culture medium with or without 5 μM Dox was changed every 2–3 days for cell expansion and further experiments.

2.7. Harvest and in vitro culture of TCs

Total 20 transgenic mice aged from 6 to 8 weeks were included. TCs derived from 6 to 7 mice were combined into one cell sample, and total 3 cell samples were generated from 20 mice. Briefly, flexor tendons were harvested from hind limbs, collected, and surrounding soft tissues were carefully removed. The harvested tendons were minced and subjected to enzyme digestion with 0.2% collagenase in serum-free high glucose DMEM on a rotator at 37 °C for 2 h. After being filtered through a 40-μm filter mesh, the isolated primary TCs were seeded with high glucose DMEM culture medium containing 10% FBS at a density of 1×10^6 cells/cm^2 for primary culture, and 5×10^5 cells/cm^2 for subculture. Culture medium with or without 5 μM Dox was changed every 2–3 days for cell expansion and further experiments.

2.8. Cell morphology analysis

As previously described (Wang et al., 2016), both Dox-induced and non-induced cells of all four cell types were observed under phase-contrast microscope (Nikon, Japan) and 3 views of high power magnification (×100) were randomly selected from each group, in which 20 cells were randomly selected from each view and analyzed with Image-Pro Plus processing software (version 6.0, Media Cybernetics, Silver Spring, MD). The analyzed characters included cell area, cell body aspect (length/width ratio) cell length and cell width. All measurements were performed on 20 cells per view and total 3 views of each group were measured to generate means and standard deviation.

2.9. Cell proliferation assay

Passage 3 of all four different cell types were seeded in 24-well plate at a density of $(1–2) \times 10^4$ cells/well. Culture medium with or without 5 μM Dox was changed every 2 days. At days 1, 3, 5, 7, 9 and 11, cells in quadruplicate wells of each group were digested with 0.05% or 0.25% trypsin (Gibco, Grand Island, NY) and counted separately. The assay was repeated in 3 cell samples for each group.

2.10. RNA extraction and qPCR assay

Total RNAs were extracted respectively from different types of cells at passage 3, which were cultured with or without Dox for 7 days using Trizol reagent (Invitrogen, Carlsbad, CA) for examining tenogenic gene expression. Or the cells were harvested at desired time points for tri-lineage differentiation as described in Section 2.5. Briefly, 2 μg RNA was reversely transcribed into cDNA with AMV reverse transcriptase (Promega, Madison, WI) in a 20-μl reaction solution including 4 μl 5× buffer, 1 μl oligo-(dT), 2 μl dNTP, 0.5 μl RNase inhibitor and 0.5 μl AMV reverse transcriptase, using ddH_2O to meet the final volume. To transcribe cDNAs, the mixture was incubated at 30°Cfor 10 min, 42 °C for 60 min, and 95 °C for 5 min (Wang et al., 2015).

A Power SYBR Green PCR master mix (2×) (Applied Biosystems, Foster City, CA) and a real-time thermal cycler (stratagene Mx3000PTM QPCR System, La Jolla, CA) were employed to perform quantitative PCR (qPCR) analysis. The mixture of cDNA, PCR master mix, primers and ddH_2O was incubated at 95 °C for 10 min, then with 40 cycles of 95 °C for 30 s, at annealing temperature (see Table 1) for 30 s, 72 °C for 45 s and 95 °C for 30 s. Analysis of each gene expression was conducted in triplicate, repeated in three samples and was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression by using $2^{-\Delta\Delta C(t)}$ method.

2.11. Comparison of relative gene expression levels among four cell types

To compare the tenogenic differentiation or transdifferentiation potential among four cell types, the gene expression levels of each Dox-induced cell group were normalized to non-induced group with the same

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Table 1
Primers used in qPCR analysis.

Gene name	Primer sequence	Annealing temperature (°C)	PCR product size (bp)
GAPDH	F: GACTTCAACAGCAACTCCCAC R: TCCACCACCCTGTTGCTGTA	60	125
Tenomodulin	F: TGTACTGGATCAATCCCACTCT R: GCTCATTCTGGTCAATCCCCT	60	115
Scleraxis	F: CCTTCTGCCTCAGCAACCAG R: GGTCCAAAGTGGGGCTCTCCGTGACT	60	156
Collagen I	F: GGCGGCCAGGGCTCCGACCC R: AATTCCTGGTCTGGGGCACC	60	320
Collagen III	F: CAACCAGTGCAAGTGACCAA R: GCACCATTGAGACATTTTGAAG	60	174
Collagen VI	F: AGAACATAGCCTGGACG R: ACAACCCGCCTTAGAG	60	282
Decorin	F: CTACCTTCACAACAACAACATCTC R: GCAGAACGCACATAGACACATC	60	165
Tenascin-C	F: CAAGGGAGACAAGGAGAGTGAT R: AGGCTGTAGTTGAGGCGGTAAC	60	159
AKP	F: CCAACTCTTTTGTGCCAGAGA R: GGCTACATTGGTGTTGAGCTTTT	60	110
Runx2	F: GCCGCTAGAATTCAAAACAGTTGG R: GAATGGCAGCACGCTATTAAATCC	60	103
Osteocalcin	F: CCCAGCGGCCCTGAGTCTGA R: CCCAGCGGCCCTGAGTCTGA	60	136
Sox9	F: GCTTGACGTGTGGCTTGTTC R: GAGCCGGATCTGAAGATGGA	60	151
Collagen II	F: CACCAAATTCCTGTTCAGCC R: TGCACGAAACACACTGGTAAG	60	124
Aggrecan	F: TAGAGGATGTGAGTGGTCTT R: TCCACTAAGGTACTGTCCAC	60	490
aP2	F: AAATCACCGCAGACGACA R: CACATTCCACCACCAGCT	60	138
PPARγ	F: AGACCACTCGCATTCCTTT R: CCCACAGACTCGGCACTCA	60	268
Adiponectin	F: GCAGAGATGGCACTCCTGGA R: CCCTTCAGCTCCTGTCATTCC	60	101

cell type separately. The relative gene level of non-induced group of each cell type was set as 1, so that relative expression level of one gene could be compared among four cell groups that were induced for TNMD overexpression with Dox.

2.12. Cell colony forming efficiency assay

Colony forming efficiency assay was performed as previously described (Xiong et al., 2015). Passage 3 ASCs with or without Dox-induction were respectively seeded with DMEM plus 10% FBS on 10-cm dishes (10 dishes for each group) at a density of 2000 cells per dish. Culture medium was changed every 3 days. After 2 weeks of culture, the cells were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet and the number of colonies with diameter ≥2 mm was counted.

2.13. Statistical analysis

The statistical analyses were performed using the statistical software SPSS (version 19.0, SPSS, Chicago, IL). Data are presented as mean and standard deviation. Student's *t*-test was used for statistical analysis of all paired comparisons and one-way ANOVA test was used to analyze differences among different groups. Student-*t*-test was also used to analyze the difference of paired groups. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Characterization of TNMD transgenic mouse

The pTRE-TNMD plasmid (Fig. 1A) was injected into pronucleus of fertilized zygotes of C57BL/6 mice. The injected embryos divided and then produced 2-cell embryos after overnight culture and these were transferred to the oviducts of 3 pseudopregnant female C57BL/6 mice. These transferred embryos resulted in 41 live progeny and PCR results indicated that 8 (19.5%) of these carried pTRE-TNMD transgene including founders No. 3, No. 7, No. 8, No. 9, No. 28, No. 29, No. 31 and No. 37 (Fig. 1B, Table 2). During the further breeding, some founders failed to give offspring mice that carried the transgene due to various causes, and only founders No. 7 and No. 9 succeeded (Table 2). Following the progress of the mice reproduction, the positive rate of pTRE-TNMD detected by RT-PCR increased gradually, and reached 100% positive after ninth generations of reproduction derived from No. 7 and No. 9 founder mice. The finally generated conditional TNMD overexpressing mice positive for bitransgenes (pTRE-TNMD and rtTA) revealed no obvious abnormal phenotype when compared to pTRE-TNMD transgene mice (Fig. 1C, D). More importantly, all four types of cells (BMSCs, ASCs, DFs and TCs) derived from the double-transgene mice exhibited conditional TNMD overexpression temporally induced by Dox and well controlled in a dose-depended manner in vitro (data not showed), and 5 μM was selected as the optimal dosage for various experiments based on primary tests.

3.2. No effect on cell morphology by conditional TNMD overexpression

To observe the effect of induced TNMD overexpression on cell morphology, four types of cells were cultured on the dishes. As shown in Fig. 2A, after 7 days of culture without or with 5 μM Dox-induction, microscopic observation revealed no obvious cell morphological difference between induced and non-induced cells. The BMSCs exhibited relatively heterogeneous cell shapes in both Dox-induced and non-induced groups. Among them, most cells exhibit polygon cell shape along with some small sized and light reflected cells. The cultured ASCs revealed relatively homogenous cells, which are relatively smaller. By contrast, both TCs and DFs showed homogenous morphology of spindle shape with no difference between induced and non-induced groups.

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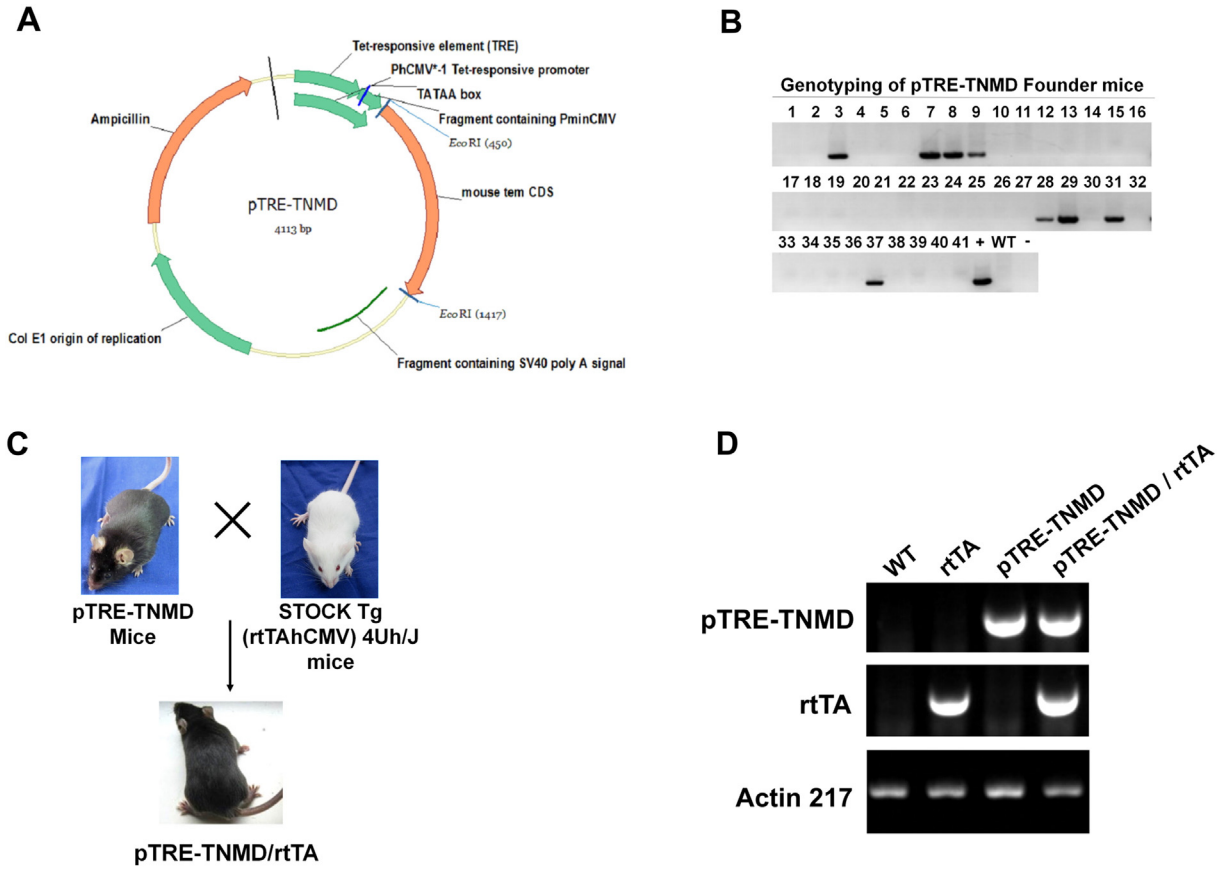

Fig. 1. The procedure of generating conditional tenomodulin (TNMD) overexpressing mice. (A) The plasmid construct containing pTRE responsive element and TNMD coding cDNA (pTRE-TNMD plasmid). (B) The genotyping of pTRE-TNMD founder mice. The symbol "+" indicates a positive control using pTRE-TNMD plasmid; "WT" is C57BL/6 wild type mice serving as a negative control, and "−" is ddH_2O as a blank control. (C) The conditional TNMD overexpressing transgenic mice were generated by hybridizing pTRE-TNMD transgenic mice with STOCK Tg (rtTAhCMV) 4Uh/J transgenic mice. (D) The genotyping of C57BL/6 wild type mice (WT), STOCK Tg (rtTAhCMV) 4Uh/J mice (rtTA), pTRE-TNMD mice (pTRE-TNMD), and conditional TNMD overexpressing transgenic mice (pTRE-TNMD/rtTA).

To further provide quantitative supporting data, Dox-induced and non-induced cells of each cell type were analyzed for their morphological parameters. As shown in Fig. 3, there was no significant difference between Dox-induced and non-induced cells in cell area, cell body aspect, cell length and cell width for all four cell types ($p > 0.05$), except for cell area of TCs ($p < 0.05$).

3.3. Conditional TNMD overexpression promoted cell proliferation

Passage 3 cells were cultured in 24 well-plate at the density of (1–2) × 10^4 cells/cm^2 and cultured in DMEM plus 10% FBS, and the cells were collected every other days for total 11 days and counted. As shown in Fig. 2B–E, all cell types except for DFs revealed enhanced cell proliferation after Dox induction, with significant difference in total cell number between Dox-induced and non-induced groups ($p < 0.05$) at most of the examined time points.

3.4. Conditional TNMD overexpression promoted differentiation and transdifferentiation towards tenogenic lineage

In this study, *scleraxis (SCX)*, *collagens I (COL1)*, *III (COL3)* and *VI (COL6)*, *decorin (DCN)* and *tenascin-C (TNC)* were used as the teongenic markers to examine this particular lineage differentiation. As shown in Fig. 4, after Dox induction for 7 days, both BMSCs (Fig. 4A) and ASCs (Fig.4B) showed more than two folds of up-regulated gene expression level of *TNMD* when compared to non-induced cells ($p < 0.01$), indicating the success of this transgenic animal model. Relatively, the *TNMD* gene expression levels were less up-regulated in both TCs ($p < 0.01$,

Table 2
The summary of positive ratio of pTRE-TNMD transgene mice.

Founder (no.)	Total (positive)									Results
	F1	F2	F3	F4	F5	F6	F7	F8	F9	
3 (male)	32 (3)	55 (31)	48 (37)	8 (6)						Reproductive failure
7 (male)	12 (8)	60 (47)	93 (79)	57 (50)	81 (70)	120 (112)	104 (98)	113 (113)	143 (143)	Found new mice line
8 (male)	20 (6)									Reproductive failure
9 (male)	23 (10)	62 (48)	57 (43)	45 (32)	89 (78)	69 (59)	95 (89)	101 (100)	112 (112)	Found new mice line
28 (female)	0									Accidental death
29 (female)	27 (6)	50 (31)	83 (46)							Reproductive failure
31 (female)	23 (8)									Reproductive failure
37 (male)	40 (0)									Accidental death
In total	117 (41)	227 (157)	281 (205)	110 (88)	170 (148)	189 (171)	199 (187)	214 (213)	255 (255)	
Positive ratio	23.0%	69.2%	73.0%	80.0%	87.1%	90.5%	94.0%	99.5%	100.0%	

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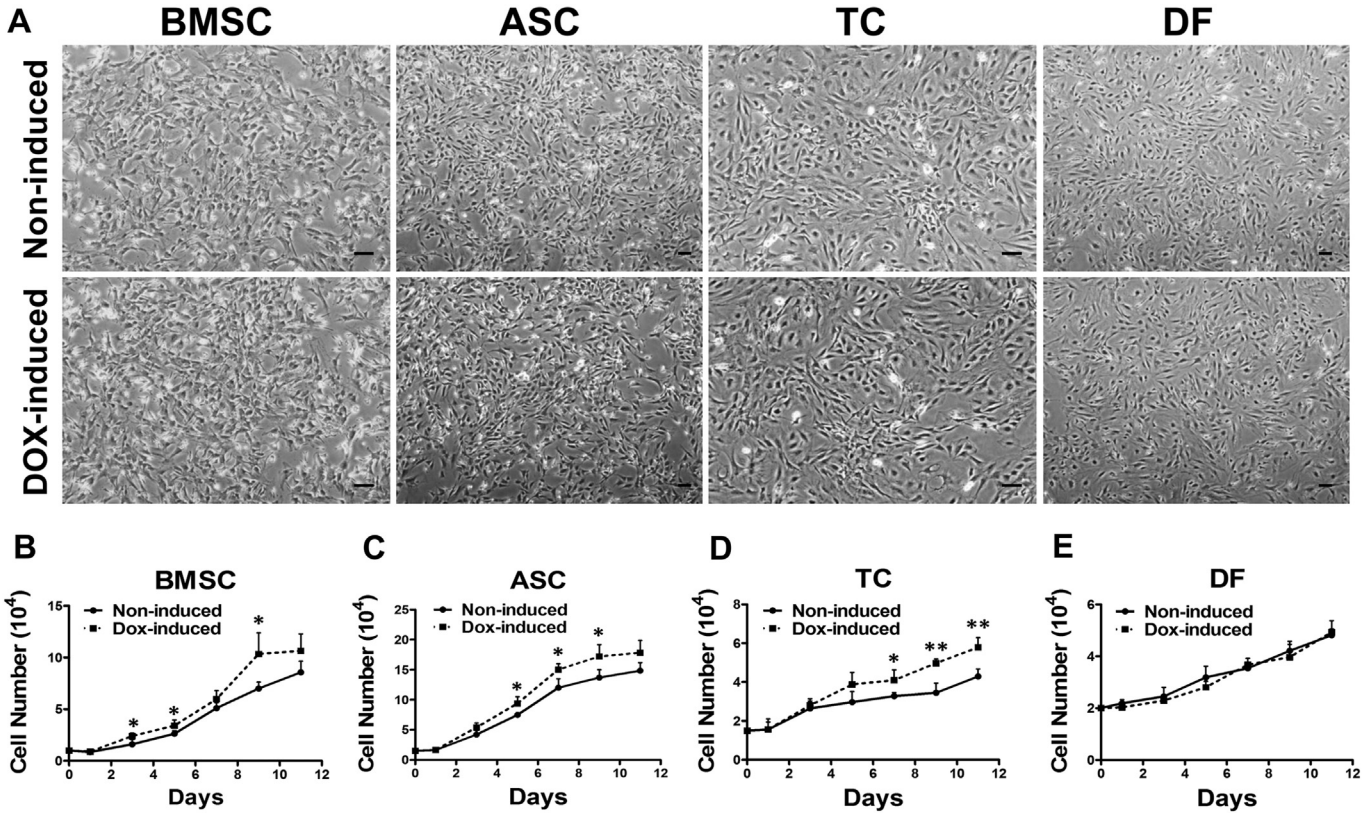

Fig. 2. TNMD overexpression promotes cell proliferation but has no effect on cell morphology. No significant difference in cell morphology change is observed in all four cell types between their respective Dox treated and non-treated paired groups (A). TNMD overexpression induced by Dox treatment enhances the proliferation of BMSCs (B), ASCs (C) and TCs (D) but not DFs (E). Scale bar = 100 μm; *$p < 0.05$, **$p < 0.01$.

Fig. 4C) and DFs ($p < 0.01$, Fig. 4D), suggesting that differential expression levels induced by Dox among different types of tissues.

Even so, up-regulated *TNMD* gene expression could drive tenogenic differentiation of BMSCs (Fig. 4A) and ASCs (Fig. 4B) and DF tenogenic transdifferentiation (Fig. 4D), as the gene expression of all these markers was significantly enhanced ($p < 0.01$, or $p < 0.05$) except for *DCN* in ASCs ($p > 0.05$) and *COL3, TNC* in DFs ($p > 0.05$). In addition, most markers, except for *COL6* and *DCN* ($p > 0.05$), were enhanced for their gene expression in TCs ($p < 0.01$, or $p < 0.05$, Fig. 4C).

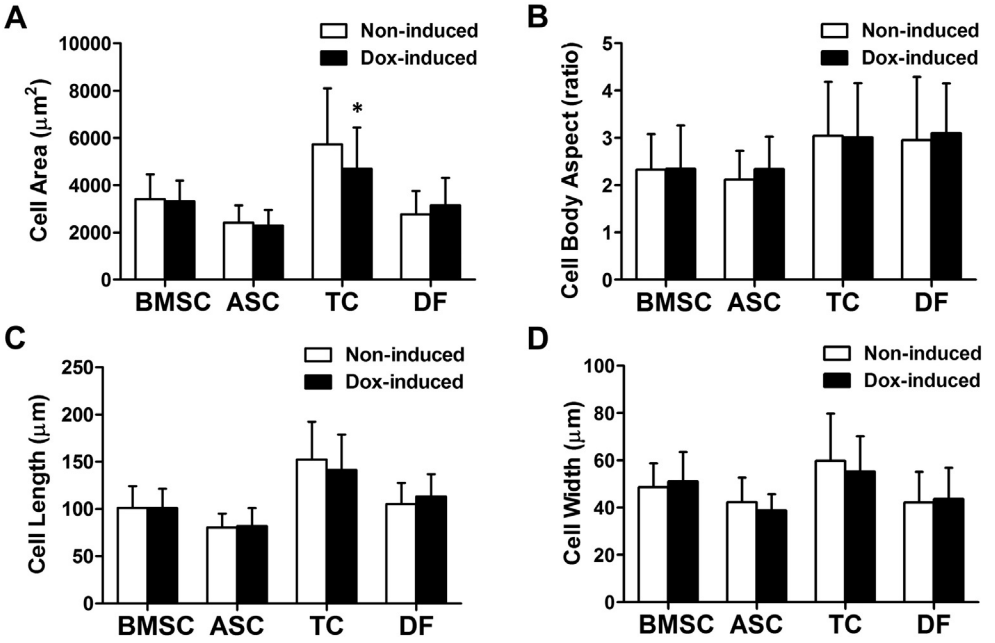

Fig. 3. Cell morphology analysis showed similarity between Dox-induced and non-induced cells of all cell types. (A) Cell area analysis; (B) Cell body aspect analysis; (C) Cell length analysis; (D) Cell width analysis.*$p < 0.05$.

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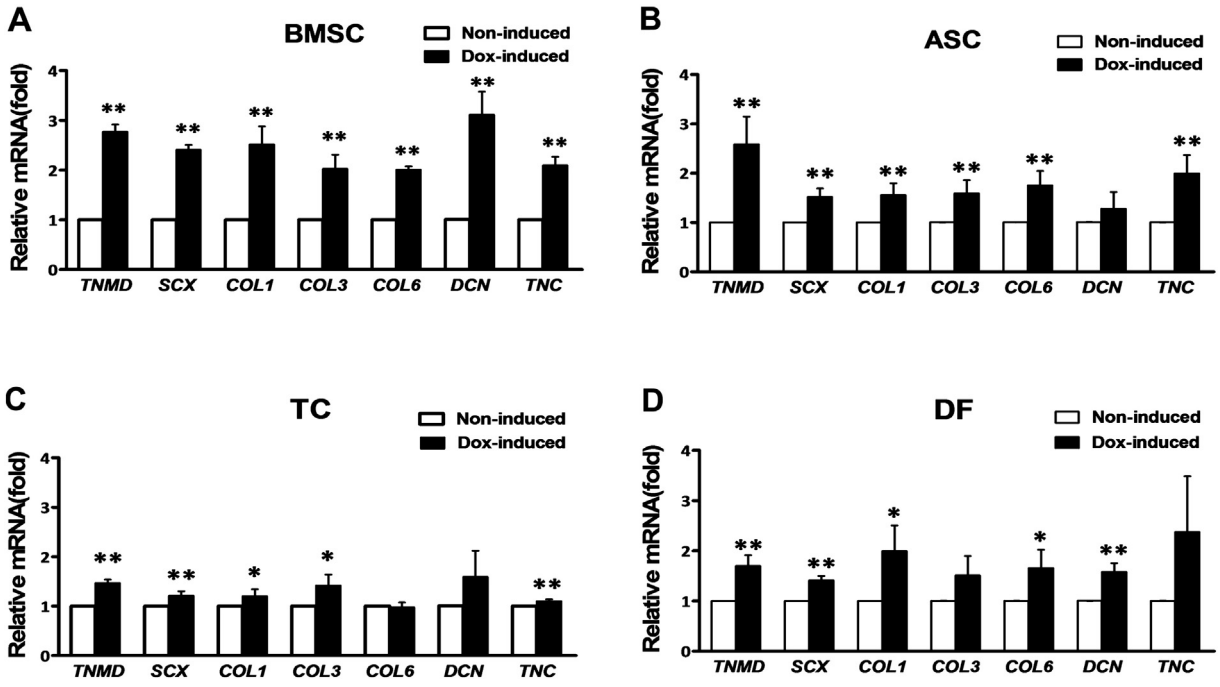

Fig. 4. Conditional TNMD overexpression promotes differentiation and transdifferentiation towards tenogenic lineage. qPCR analysis of the gene expression of tenogenic markers in BMSCs (A), ASCs (B), TCs (C) and DFs (D). Abbreviation: *TNMD* = tenomodulin; *SCX* = scleraxis; *COL1* = collagen I; *COL3* = collagen III; *COL6* = collagen VI; *DCN* = decorin; *TNC* = tenascin-C. *$p < 0.05$; **$p < 0.01$.

3.5. MSCs exhibited more potent tenogenic differentiation potential than mature differentiated cells

The relative gene expression levels of tenogenic markers were compared among four different cell types. As shown in Fig. 5, among these cell types, BMSCs could be best induced for the expression of *TNMD* as well as other tenogenic markers ($p < 0.05$), indicating that BMSCs may have better potential for induced differentiation towards a particular lineage in a transgenic model. ASCs, as another type of MSCs, exhibited better induced expression of tenogenic markers than TCs for *TNMD, TNC* and *COL6* ($p < 0.05$). However, DFs exhibited the expression levels of certain induced markers equivalent to those of ASCs, such as *SCX, TNC, DCN, COL1, COL3* and *COL6* ($p > 0.05$).

3.6. Conditional TNMD overexpression promoted ASCs colony forming efficiency

As shown in Fig. 6A–C, conditional TNMD overexpression led to significantly enhanced colony forming efficiency. In the total 10 tested

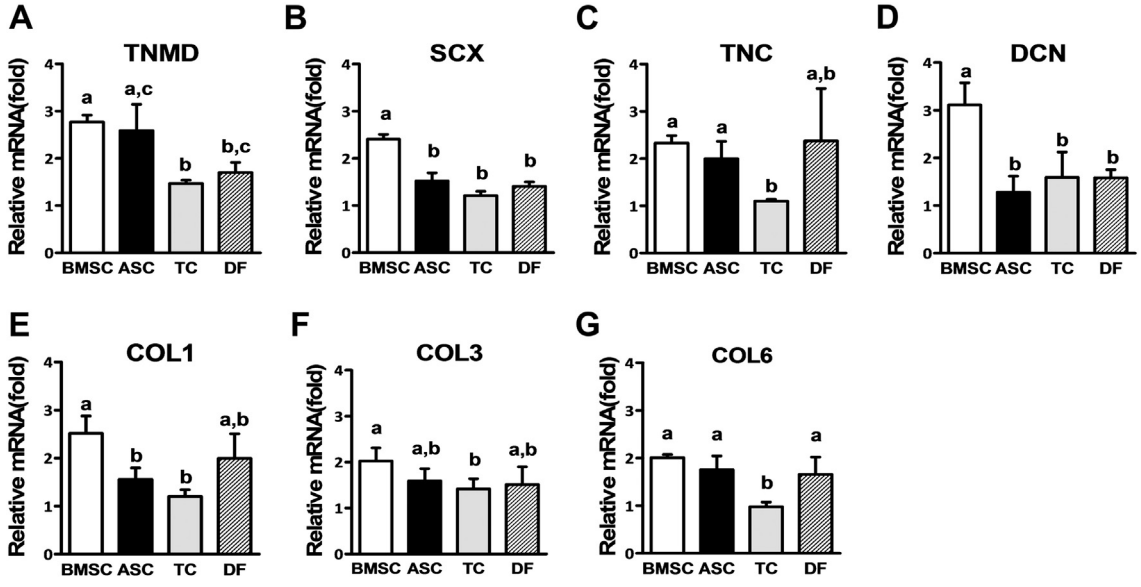

Fig. 5. Tenogenic differentiation potential comparison among four cell groups. The Dox induced gene expression levels relative to their non-induced levels were compared for BMSC, ASC, TC and DF groups for *TNMD (Tenomodulin)*, *SCX (scleraxis)*, *COL1(collagen I)*, *COL3(collagen III)*, *COL6 (collagen VI)*, *DCN(decorin)* and *TNC(tenascin-C)*. Groups labeled with different letters indicate statistically significant difference ($p < 0.05$), whereas no significant difference is found between the groups labeled with the same letter ($p > 0.05$).

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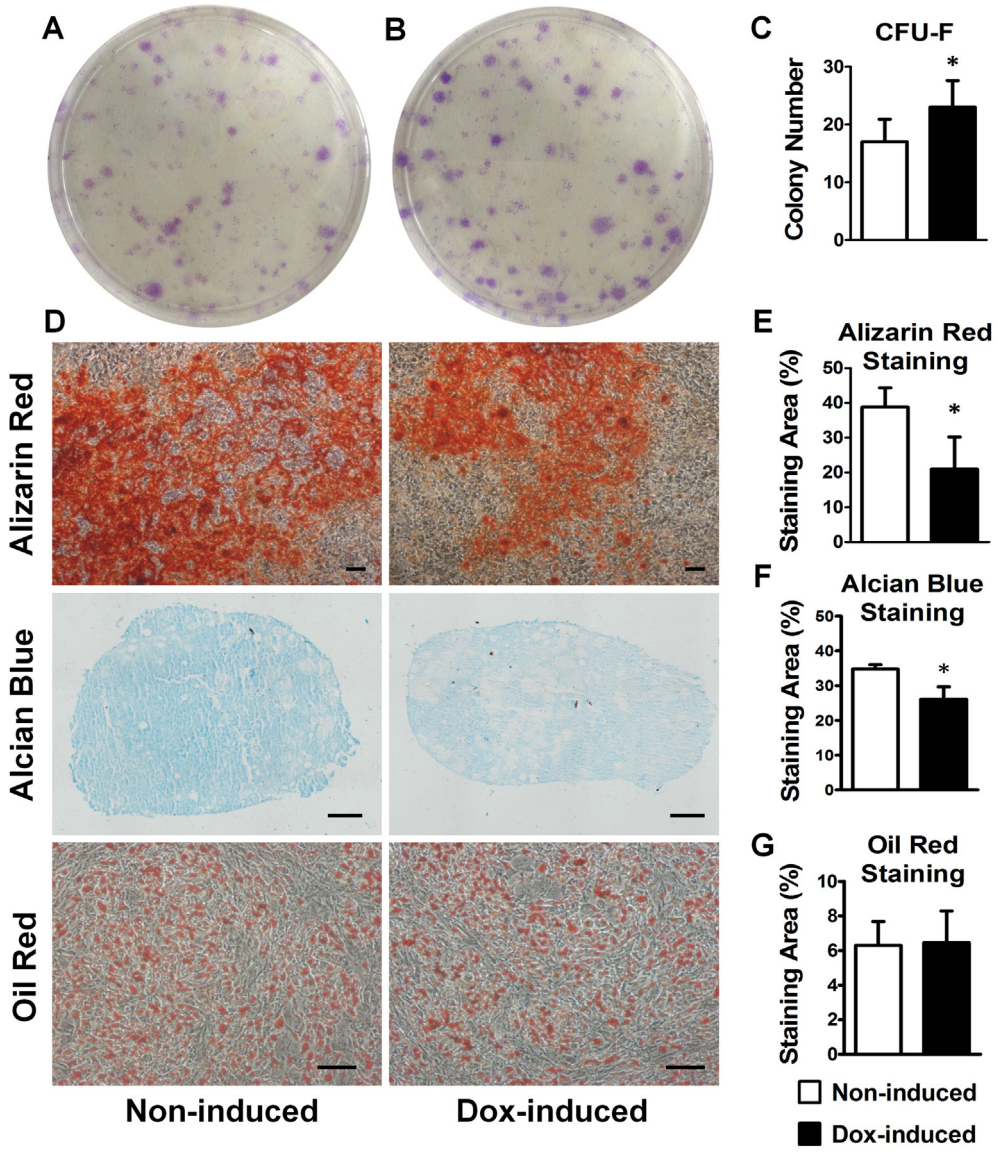

Fig. 6. Conditional TNMD overexpression promotes ASC colony forming efficiency and inhibits other lineage differentiation potentials. Gross view of formed cell colonies of non-induced (A), Dox-induced (B) cells and their quantitative analyses (C). Specific histochemical staining of the ASCs under induced multi-lineage differentiation with or without Dox (D) and their respective semi-quantitative analyses of staining areas of Alizarin Red (E), Alcian Blue (F) and Oil Red (G). *$p < 0.05$.

culture dishes for each group, cell cultures at the density of 2000 cells per 10 cm dish along with Dox induction formed averaged 23 ± 4.6 colonies per dish, which was significantly higher than that of the non-induced cells (17 ± 3.9 colonies per dish, $p < 0.05$).

3.7. Conditional TNMD overexpression inhibited ASCs differentiation potentials towards other lineages

Passage 3 ASCs derived from the transgenic mice were cultured with or without 5 μM Dox and were subjected to osteogenic, chondrogenic and adipogenic differentiation as described in the method. As shown, conditional overexpression of TNMD significantly inhibited the gene expression of *alkaline phosphatase (AKP)* and *Osteocalcin* ($p < 0.05$), but not *Runx2* ($p > 0.05$) under osteogenic induction condition (Fig. 7A). Relatively weaker Alizarin Red staining was found in Dox-induced group than in non-induced group with significant difference in the staining area between two groups (Fig. 6D and E, $p < 0.05$).

Similarly, conditional TNMD overexpression led to significantly reduced gene expression levels of *SRY (sex determining region Y)-box 9 (Sox9)*, *collagen II (COL2)* and *Aggrecan* when compared to the levels of non-induced cells ($p < 0.05$, Fig. 7B). Additionally, relatively weaker Alcian Blue staining was found in induced group than in non-induced group with significant difference in the staining area between two groups ($p < 0.05$, Fig. 6D and F).

However, conditional overexpression of *TNMD* seemed to have no effect on adipogenic differentiation of ASCs. As shown in Fig. 7C, no significant difference in the gene expression levels of *adipocyte protein 2(aP2)*, *peroxisome proliferator-activated receptor-γ (PPARγ)*, and *Adiponectin* between two groups ($p > 0.05$). Additionally, there was also no significant difference in the Oil Red staining intensity and staining area between two groups (Fig. 6D and G, $p > 0.05$).

4. Discussion

Cell functions according to the signals triggered by a certain molecule. Many examples have demonstrated such an important biological process which determines the eventual function of a given cell type. For example, transfection of BMP into cells could not only drive osteogenic differentiation of MSCs (Meinel et al., 2006), but also induce transdifferentiation of DFs into osteoblasts (Ishihara et al., 2015). By

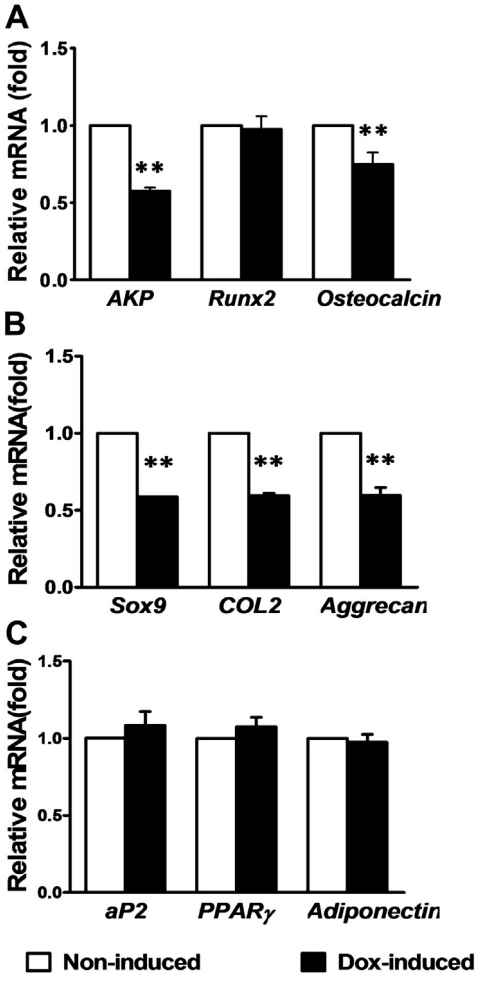

Fig. 7. TNMD overexpression inhibits the expression of osteogenic and chondrogenic genes of ASCs under the induction of multi-lineage differentiation. qPCR analysis reveals differential gene expression between non-induced and Dox-induced cell groups for osteogenic (A) and chondrogenic (B) markers, but not for adipogenic markers(C). Abbreviation: *AKP* = alkaline phosphatase; *Runx2* = Runt-related transcription factor 2; *Sox9* = SRY (sex determining region Y)-box 9; *COL2* = collagen II; *aP2* = adipocyte protein 2; *PPARγ* = peroxisome proliferator-activated receptor-γ. $^{**}p < 0.01$.

overexpressing exogenous MyoD, a fibroblast cell line could be induced towards a skeletal muscle linage (Qin et al., 2007).

Tendon is an important part of musculoskeletal system, which is an essential tissue type that transfers muscle contraction force to limb movement. Additionally, TNMD is considered as an important molecule for tendon development, because it not only serves as a tenogenic lineage marker, but also regulates tendon matrix maturation and guides tendon stem/progenitor cell destiny (Shukunami et al., 2001; Docheva et al., 2005; Jiang et al., 2016). As a type II transmembrane glycoprotein, it is composed of extracellular domain which contains a highly conserved C-terminal with eight cysteine residues region, a transmembrane domain from amino acid (aa) 31 to aa 49 (Brandau et al., 2001; Dex et al., 2016). Thus far, its functions remain to be explored in addition to being as a specific marker for mature TCs. It was found that TC number significantly reduced and collagen superstructure was disrupted in the tendon of TNMD knock-out mice, indicating its potential role in tendon development (Docheva et al., 2005; Alberton et al., 2015).

In our previous study, we specifically invested the role of TNMD overexpression in MSC tenogenic differentiation. It was demonstrated that the overexpression not only enhanced tenogenic differentiation of MSC line, but also promoted neotendon formation in vivo (Jiang et al., 2016). However, it remains unknown if such an approach will have similar effect on tenogenic lineage differentiation of other cell types. With the advances in genetic manipulation technologies, the best way to study gene function might be through the generation of "gain-of-function" (transgenic) or "loss-of-function" (knock-out, knock-down) animal models. Previous report already revealed the importance of TNMD in tendon development via loss-of-function model (Docheva et al., 2005; Alberton et al., 2015), this study further investigated its function for cell lineage switch using gain-of-function way via TNMD overexpression transgenic mouse model.

Tet-On transgenic mouse is an animal model that can efficiently investigate the function of a certain molecule, because the expression of desired gene can be regulated in a temporal and dose-dependent way (Kistner et al., 1996). As shown in Fig. 1, in the current study, a Dox-dependent regulator, rtTA was employed to activate TNMD gene expression by its binding to pTRE-TNMD in the presence of Dox induction. During the process, it was found that this transgenic mouse line has been bred to homozygosity, and only 2 out of 8 founders were able to be propagated to the ninth generation without silencing of transgene expression (Table 2). Through further breeding with STOCK Tg (rtTAhCMV) 4Uh/J mice, we were able to generate Tet-On conditional TNMD transgenic mice, and the TNMD expression could be induced by Dox temporally and in a dose-dependent manner (data not shown).

Although this animal model is potentially valuable for investigating TNMD overexpression in tissue development, the current study mainly focused on the effect of its overexpression on tenogenic lineage differentiation of adult cells in an in vitro model.

In this study, BMSCs, ASCs and DFs were chosen because the former two represent MSCs, whereas DFs represent mature and differentiated somatic cell type. In addition, we also used TCs as a positive control. As shown in Fig. 4, it was observed that Dox induction could significantly enhance TNMD expression in all four cell types along with significantly up-regulated gene expression of all tenogenic markers in general, indicating that overexpressed TNMD not only enhanced tenogenic lineage differentiation of MSCs, but was also able to promote transdifferentiation of DFs.

To further compare the potentials among different cell types towards tenogenic lineage, we artificially grouped the relative gene expression levels of a certain molecule from four cell types to make a horizontal comparison. As shown in Fig. 5, although similar level of TNMD was observed between BMSCs and ASCs (Fig. 5A), a significantly higher level of induced SCX expression, a transcription factor driving MSC towards tenogenic lineage, was observed in BMSCs than in ASCs (Fig. 5B), indicating greater tenogenic potential in BMSCs than in ASCs.

Interestingly, all tenogenic genes were induced in TCs with relatively less extent. Although the mechanism remains unknown, we assumed this was because that TCs themselves already exhibited a potent tenogenic phenotype with relatively higher endogenous expression levels and thus exogenous gene expression would not be able to further greatly induce the expression of tenogenic markers.

Although DFs represent fully differentiated somatic cells, the relative gene expression comparison indicates that DFs can achieve tenogenic differentiation potential similar to that of ASCs. As shown in Fig. 5, there was no significant difference in the gene expression levels of *SCX, TNC, DCN, COL1, COL3* and *COL6* between ASCs and DFs, suggesting that DFs may become a possible cell source for tendon regeneration. Indeed, it has been shown that DFs could be induced into a tenogenic phenotype via artificial cell elongation (Wang et al., 2016). In our previous report, we also showed that DFs could replace TCs to engineer and repair tendon defect in vivo (Liu et al., 2006).

A previous study from our group showed that TNMD overexpression could reduce BMSCs differentiation towards osteogenic, chondrogenic and adipogenic lineage (Jiang et al., 2016). Similar to BMSCs, TNMD overexpression also led to significantly reduced differential potentials towards osteogenic and chondrogenic lineages of induced ASCs, as the gene expression levels of *AKP*, *osteocalcin* (osteogenic markers), *Sox 9, collagen 2* and *Aggrecan* (chondrogenic markers) were significantly

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down-regulated after Dox induction (Fig. 7A and B). This was also supported by the reduced staining area of Alizarin Red (Fig. 6D and E) and Alcian Blue (Fig. 6D and F).

However, in contrast to our expectation, TNMD overexpression had no effect on adipogenic differentiation potential in ASCs. Actually, previous studies revealed that TNMD may also participate in fat metabolism (Tolppanen et al., 2007; Tolppanen et al., 2010). TNMD expression increases in human preadipocytes during differentiation, whereas silencing TNMD blocks adipogenesis (Senol-Cosar et al., 2016). All these reports suggest that TNMD may act as a protective factor in visceral adipose tissue to alleviate insulin resistance in obesity (Senol-Cosar et al., 2016). Therefore, we assume that no effect of TNMD overexpression on adipogenic lineage differentiation is likely caused by two reasons. One is the already highly expressed endogenous TNMD gene when compared to other vascularized soft tissues (Brandau et al., 2001), which renders ASCs relatively insensitive to the driving effect of exogenous TNMD gene expression. The other may rely on the great adipogenic lineage potential naturally preserved in ASCs, which is less influenced by exogenous gene when compared to other types of MSCs such as BMSCs.

Overall, transgenic mouse model via inserting TNMD gene into a fertilized egg is theoretically able to distribute the gene evenly to all tissue types in a fully developed animal body. This method provides a means of ideal comparison among different cell types of the same animals, because the variations in gene transfection efficiency, gene insert place and activation/inactivation of the inserted gene can be avoided in general. Interestingly, this horizontal comparison provides the valuable information that BMSCs are more potent than ASCs for tenogenic lineage differentiation and DFs exhibit the tenogenic potential similar to or perhaps even better than ASCs. These findings may provide valuable rationales for the selection of proper cell source for tendon regeneration. In the future, revealing the detailed mechanism how the TNMD overexpression triggers the tenogenic differentiation pathway in BMSCs will be essential for both basic and translational works.

Declaration of interest

The authors have no conflict of interests to disclose.

Acknowledgments

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