

Research Article

Tendon-derived stem cells undergo spontaneous tenogenic differentiation

Jia Guo^a, Kai-Ming Chan^{a,b,c,d}, Jin-Fang Zhang^{a,b,e,*}, Gang Li^{a,b,c,d,e,**}^a Department of Orthopaedics & Traumatology, The Chinese University of Hong Kong, Prince of Wales Hospital, Hong Kong SAR, PR China^b Lui Che Woo Institute of Innovative Medicine, Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong SAR, PR China^c Stem Cells and Regenerative Medicine Laboratory, Li Ka Shing Institute of Health Sciences, The Chinese University of Hong Kong, Hong Kong SAR, PR China^d Key Laboratory for Regenerative Medicine, Ministry of Education, School of Biomedical Sciences, The Chinese University of Hong Kong, Hong Kong SAR, PR China^e The Chinese University of Hong Kong, Shenzhen Research Institute, Shenzhen, PR China

ARTICLE INFO

Article history:

Received 16 July 2015

Received in revised form

14 January 2016

Accepted 16 January 2016

Available online 18 January 2016

Keywords:

Tendon

Tendon-derived stem cell (TDSC)

Spontaneous tenogenic differentiation

ABSTRACT

Tendon-derived stem cell (TDSC) is a subpopulation of residing stem cells within the intact tendon tissues, with the capacities of self-renewal, clonogenicity, and three-lineage differentiation. Compared with bone marrow derived mesenchymal stem cells (BMSCs), TDSCs are superior for tendon injuries repair as they remain some tendon tissue-specific differentiation properties. In the present study, TDSC was found to undergo spontaneous tenogenic differentiation in which the expression of tenogenic markers were increased while the expression of stemness markers decreased with time in TDSCs culture (without tenogenic induction medium). The further collagen synthesis ability was correspondingly increased during this process. After a longer period of culture, the monolayer of TDSCs formed a “3D” layers with rich extracellular matrices of typical tendon tissues. In addition, the key tenogenic transcription factors, such as Scx, Mlx, Egr1 and Eya1 were all up-regulated in this process. Finally, we compared the spontaneous tenogenic differentiation with TGF- β 1-induced tenogenic differentiation of TDSCs, and the results showed that the spontaneous tenogenic differentiation of TDSCs was general character of TDSCs, similar to but weaker than the effect of TDSCs under tenogenic induction. Taken together, the present study identified that TDSCs had the potential of spontaneous tenogenic differentiation, which may be a better cell source for the treatment of tendon injury.

© 2016 Elsevier Inc. All rights reserved.

Introduction

A tendon (or sinew) is a tough band of fibrous connective tissue that usually connects muscle to bone and is capable of withstanding tension. They can transmit forces from muscle to rigid

bone levers producing joint motion, act as shock absorbers, energy storage sites, and help to maintain posture through their proprioceptive properties [1,2]. As an integral part of a musculotendinous unit, tendon is consisting of 30% collagen and 2% elastin embedded in an extracellular matrix (ECM) containing 68% water and tenocytes [3]. Tenocytes are traditionally considered as the main terminally differentiated cells, responsible for synthesis and formation of tendon fibers and ground substance in a three-dimensional network [3]. Until 2007, tendon stem/progenitor cells were firstly identified as a population of residing stem cell with self-renewal, clonogenicity, and three-lineage differentiation capacity within the human and mouse tendon. These cells express tendon-related genes and are able to form tendon and enthesis-like tissues when implanted in vivo [4]. Our group also isolated and identified this subpopulation from rat tendon and named as tendon-derived stem cell (TDSC) [5].

Tendon injury heals slowly and often results in the formation of inferior fibrotic scar tissue or fibrous adhesions [6,7]. These compromised healed tendons have the risk of reinjury at the repair site

Abbreviations: TDSC, Tendon-derived stem cell; ECM, Extracellular matrix; BMSCs, Bone marrow mesenchymal stem cells; Scx, Scleraxis; Mlx, Mohawk homeobox; Egr1, Early growth response 1; Eya1, Eyes absent homolog 1; NS, Nucleostemin; EphA4, Ephrin type-A receptor 4; Col3a1, Collagen type III; Lox, Lysyl oxidase; TenC, Tenascin C; Col1a1, Collagen type I; Tnmd, Tenomodulin; Dcn, Decorin; Fmod, Fibromodulin; Bgn, Biglycan; PAI-1, Plasminogen activator inhibitor type 1

* Corresponding author at: Department of Orthopaedics & Traumatology, The Chinese University of Hong Kong, Prince of Wales Hospital, Hong Kong SAR, PR China.

** Corresponding author at: Room 904, 9/F, Li Ka Shing Institute of Health Sciences, Prince of Wales Hospital, The Chinese University of Hong Kong, Shatin, Hong Kong, SAR, PR China.

E-mail addresses: zhangjf06@cuhk.edu.hk (J.-F. Zhang), gangli@cuhk.edu.hk (G. Li).

which remains a challenge for clinical doctors. Recent years, the stem cell therapy for tendon repair has displayed positive outcomes in laboratory studies [8–12]. Stem cells from various source have various proliferation and differentiation capacities [13]. Just as bone marrow mesenchymal stem cells (BMSCs), TDSCs remain the tissue-specific differentiation potentials such as osteogenic, adipogenic and chondrogenic differentiation; but they are more easily to form tenocyte than BMSCs [4]. In addition, different from the ectopic bone and tumor formation with BMSC transplantation [14–16], in vitro tenogenic differentiation of TDSCs before transplantation might be a good strategy to promote tendon healing while minimizing the chance of ectopic bone and tumor formation in tendons.

In the present study, TDSCs was found to undergo tenogenic spontaneous differentiation in which the tenogenic markers were increased while the stemness markers were decreased along with TDSCs growing under normal condition. The collagen synthesis ability were correspondingly increased during this process. The key tenogenic transcription factors, Scleraxis (Scx), Mohawk homeobox (Mkx), Early growth response 1 (Egr1) and Eyes absent homolog 1 (Eya1) were demonstrated to be involved in this spontaneous differentiation. In addition, by comparison of spontaneous tenogenic differentiation and TGF- β 1-induced tenogenic differentiation, we found spontaneous tenogenic differentiation was an underlying trend, similar to but weaker than tenogenic inducer's effect.

Materials and methods

Tendon obtainment and TDSCs isolation

SD rats (6–8-week-old, male, weighting 150–220 g) were used for dissection of Achilles tendon. TDSCs isolation was performed as described previously [5]. Briefly, we stripped off the tendon sheath and the surrounding paratenon, cut tendon tissues into small pieces and digested them with 2 mg/ml collagenase type I (Sigma) for 2.5 h at 37 °C. Single-cell suspensions were cultured in DMEM, supplemented with 10% FBS and antibiotics (50 μ g/ml penicillin, 50 μ g/ml streptomycin, and 100 μ g/ml neomycin, Invitrogen) at 5% CO₂. TDSCs from 3rd passage were used for all experiments with triplicate. The clonogenicity and multi-lineage differentiation potential of these cells were identified as described previously before usage [5].

Cell treatment

For spontaneous differentiation, 2×10^5 TDSCs were seeded in 35 mm dishes with normal condition medium and the medium was changed every other day. For morphology observation of long cell culture, pictures were taken by microscope Leica DMIRB. For gene expression analysis, cells were harvested at day 1, day 4, day 7, day 10 and day 30 for quantitative RT-PCR (qRT-PCR) analysis. For TGF- β 1 induction experiment, 2×10^5 cells were seeded in 6-well plates and after 24 h, they were incubated with or without 10 ng/ml TGF- β 1 and harvested at 10 days. All the harvested cells were applied for gene expression assay.

qRT-PCR analysis

Total RNA was isolate from Achilles tendon and TDSCs using TRIzol[®] Reagent (Invitrogen), and reversely transcribed using PrimeScript[™] RT Master Mix (Takara). All of the quantitative real-time polymerase chain reactions (qRT-PCR) were performed using Power SYBR[®] Green Master Mix (Applied Biosystems) on a

StepOnePlus[™] Real-Time PCR System (Life technologies). The primer sequences are shown in Table 1. Rpl 19 was used as endogenous control. The fold changes were calculated by means of relative quantification ($2^{-\Delta\Delta Ct}$).

Sirius red staining

The treated cells were rinsed in cold PBS and fixed in 4% paraformaldehyde (PFA). The cells were then incubated with Picrosirius Red Solution (0.1 g Sirius red F3B in 100 ml 1.3% picric acid solution) for 1 h at room temperature. The reaction substrate was removed and the cells were rinsed thoroughly in acidified water (0.5% acetic acid). Images were taken using microscope Leica DMIRB.

Statistical analysis

Data are expressed as mean \pm SD. Statistical analysis was performed using the two-tailed Student's *t* test. A *p* value less than 0.05 is considered to be statistically significant.

Results

The profile of tendon-related genes with comparison of monolayer TDSCs and intact tendon

In order to screen out the tenogenic markers, the tendon-related genes were investigated in TDSCs (3rd passage) and the intact tendon by qRT-PCR array (Table 2). All the genes listed in Table 2 are divided in two groups: (1) the traditional markers for stem cell such as CD90, CD73 and Nucleostemin (NS) [5,17] and (2) the other genes encoding the main tendon-related collagen and proteoglycan, or expressing in tendon lineage cells. Among these stem cell markers, CD90 was up-regulated by 34-fold and NS was slightly up-regulated by 1.58-fold in monolayer TDSC when compared with tendon. However, CD73 remained unchangeable in the TDSCs and tendon. Therefore, CD90 and NS are considered as the suitable stem cell markers for TDSCs in our study. The expression of Ephrin type-A receptor 4 (EphA4), a tendon-related marker, was showed no significant difference between the tendon and monolayer TDSCs, while the Collagen type III (Col3a1), Lysyl oxidase (Lox) and Tenascin C (TenC) were much higher in TDSCs than that in tendon. On the other hand, Collagen type I (Col1a1) was significantly decreased in TDSCs and the tendon-related genes including Tenomodulin (Tnmd), Decorin (Dcn), and Fibromodulin (Fmod) were also strikingly suppressed in TDSC, suggesting that these factors could be considered as differentiation markers of tendon.

In addition, the key tenogenic transcription factors Scx, Mkx, Egr1 and Eya1 are investigated in TDSCs and tendon. As shown in Fig. 1, these transcription factors were all significantly up-regulated in tendon in comparison with those in TDSCs.

TDSCs had the spontaneous tenogenic differentiation potential

Previous study reported that MSCs had spontaneous osteogenic differentiation [18], so we hypothesize that TDSCs also have the spontaneous differentiation capacity to tenocyte. As shown in Fig. 2A, TDSCs were flat in monolayer pattern before reaching confluence. As the TDSCs were maintained for 30 days, "3D" strands were formed suggesting that the cells had the stronger ability to synthesize the extracellular matrix. And we observed no "3D" strands formation in day 10. Furthermore, we examined the

Table 1
Sequences of PCR primer sets used for qRT-PCR.

Gene	Forward primer sequence	Reverse primer sequence
CD90	CCGTCATGAGAATAACACCA	CCCAACCAGTCACAGAGAAAT
CD73	GCAGCCATCAAAGCAGACAT	AGCGGAGCCATTAGGTAGA
NS	AGGATGGTGATGATCAAGAACATG	GATGGTTACTTGCTGTTGATTGC
Col1a1	TGCTGCCTTTCTGTTCTT	AAGGTGCTGGGTAGGGAAGT
Col3a1	AACGGAGCTCCTGCCCCAT	ATTGCTCGAGCACCTGCGG
Tnmd	CCAGACAAGCAAGCGAGGA	AACTTCCTATTAGACTCTCC
Dcn	TTGCAGGGAATGAAGGGTCT	TGTGGGTGAATTTGCCAATA
Fmod	CTGCTGTATGTCCGGCTGTCTC	GCTGCGCTTGATCTCGTTC
Lox	CAGGCACCGACTGGATATGG	CGTACGTGGATGCTGGATGATG
EphA4	CACCATCATCCATTGCTTTG	AAAGGGTTCAGGCCTTTGAT
TenC	CAGAAGCTGAACCGGAAGTTG	GGCTGTGTGCTATGGCACT
Scx	AACACGGCCTTCACTGCGCTG	CAGTAGCACGTTGCCAGGTG
Mkx	AGTGGCTTTACAAGCACCGT	ACACTGAGCCGCTCGGCGTT
Egr1	GAGCCTGCACCCAACAGTG	TGGGGCTCAGAAAAATGTCA
Eya1	GGTCCTACGCCAACAGATA	GGTCTGTCCATTGCTGCTCT
Rpl 19	CTGAAGTCAAAGGGAATGTG	GGACAGAGTCTTGATGATCTC

Table 2
Comparison of marker gene expression levels between the monolayer TDSCs and the intact tendon by qRT-PCR analysis. The values are expressed as means \pm SD obtained from three independent experiments.

Gene	TDSC/tendon (%)
CD90	3442.427 \pm 55.639
CD73	114.835 \pm 6.911
NS	158.405 \pm 7.991
Col1a1	50.360 \pm 2.206
Col3a1	508.074 \pm 57.318
Tnmd	0.238 \pm 0.027
Dcn	0.019 \pm 0.001
Fmod	6.736 \pm 1.588
Lox	461.850 \pm 6.313
EphA4	94.736 \pm 24.556
TenC	218.761 \pm 5.634

expression profiles of the markers at indicated time points. As shown in Fig. 2B, the stem cell markers CD90 and NS displayed significant downregulation, while the tendon markers including Col1a1, Tnmd, Dcn, and Fmod were all increased during the spontaneous differentiation. The further in situ collagen staining also showed the collagen amounts were increased during this process (Fig. 2C).

Key tenogenic transcription factors were up-regulated during the spontaneous differentiation

Scx, Mkx, Egr1 and Eya1 are the key tenogenic transcription factors to mediate tendon differentiation [19], and we examine their expression during the spontaneous tenogenic differentiation. As shown in Fig. 3, the expression level of Egr1 kept increasing along with spontaneous differentiation; while other three genes expression were up-regulated with reaching the peaks at Day 7. These data demonstrated that TDSCs have spontaneous differentiation potential to tenocyte.

TGF- β 1 promoted TDSCs tenogenic differentiation

TGF- β is well demonstrated as an effective tenogenic inducer [20]. To further confirm the spontaneous tendon differentiation of TDSC, we compared their tenogenic differentiation capacity between spontaneous tenogenic differentiation and TGF- β -induced tenogenic differentiation. The tendon markers including Col1a1, Tnmd, Dcn, and Fmod (Fig. 4A) and tendon transcriptional factors including Scx, Mkx, Egr1 and Eya1 (Fig. 4B) were all up-regulated in the two differentiated groups. More importantly, the Tnmd expression level was all upregulated in both groups, but much higher in spontaneous group than that in TGF- β 1 group. In addition, the collagen amounts were also increased in both groups by in situ collagen staining. The more collagen was synthesized in TGF- β 1 group than that in spontaneous tenogenic differentiation

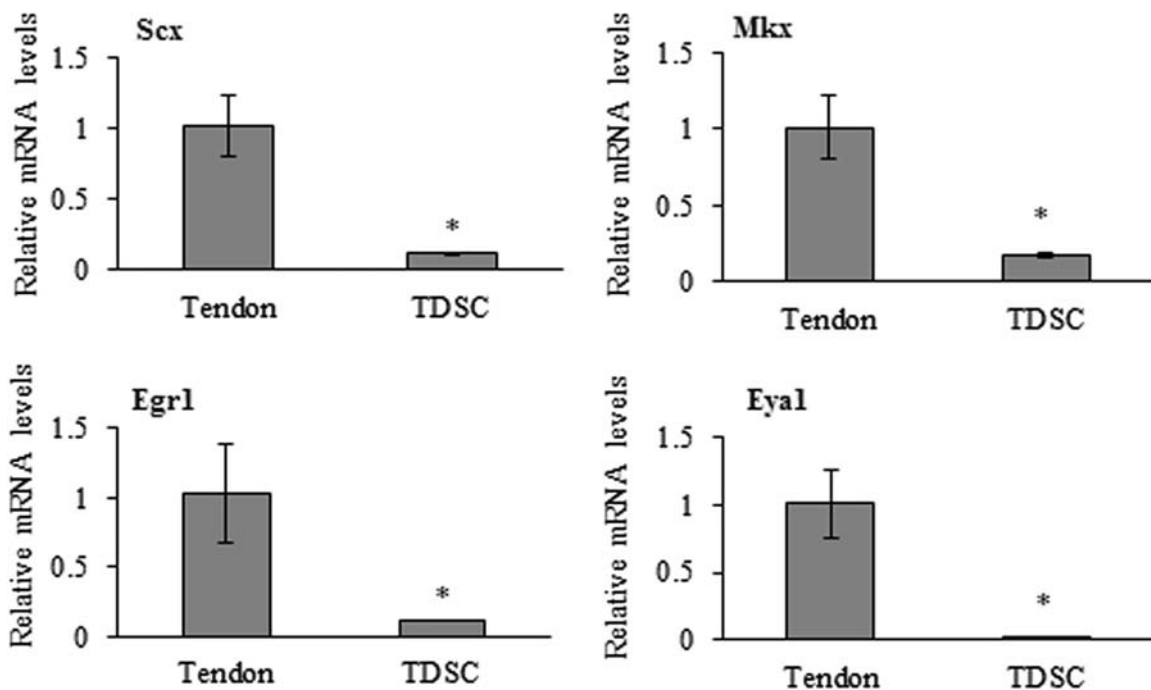


Fig. 1. The expressions of key tenogenic transcription factors are down-regulated in TDSCs compared with those in tendon. TDSCs (3rd passage) were cultured for 2 days and collected for RNA isolation. qRT-PCR analysis was applied to detect the relative mRNA expression levels in monolayer TDSCs and the intact tendon. * $P < 0.05$ vs. tendon.

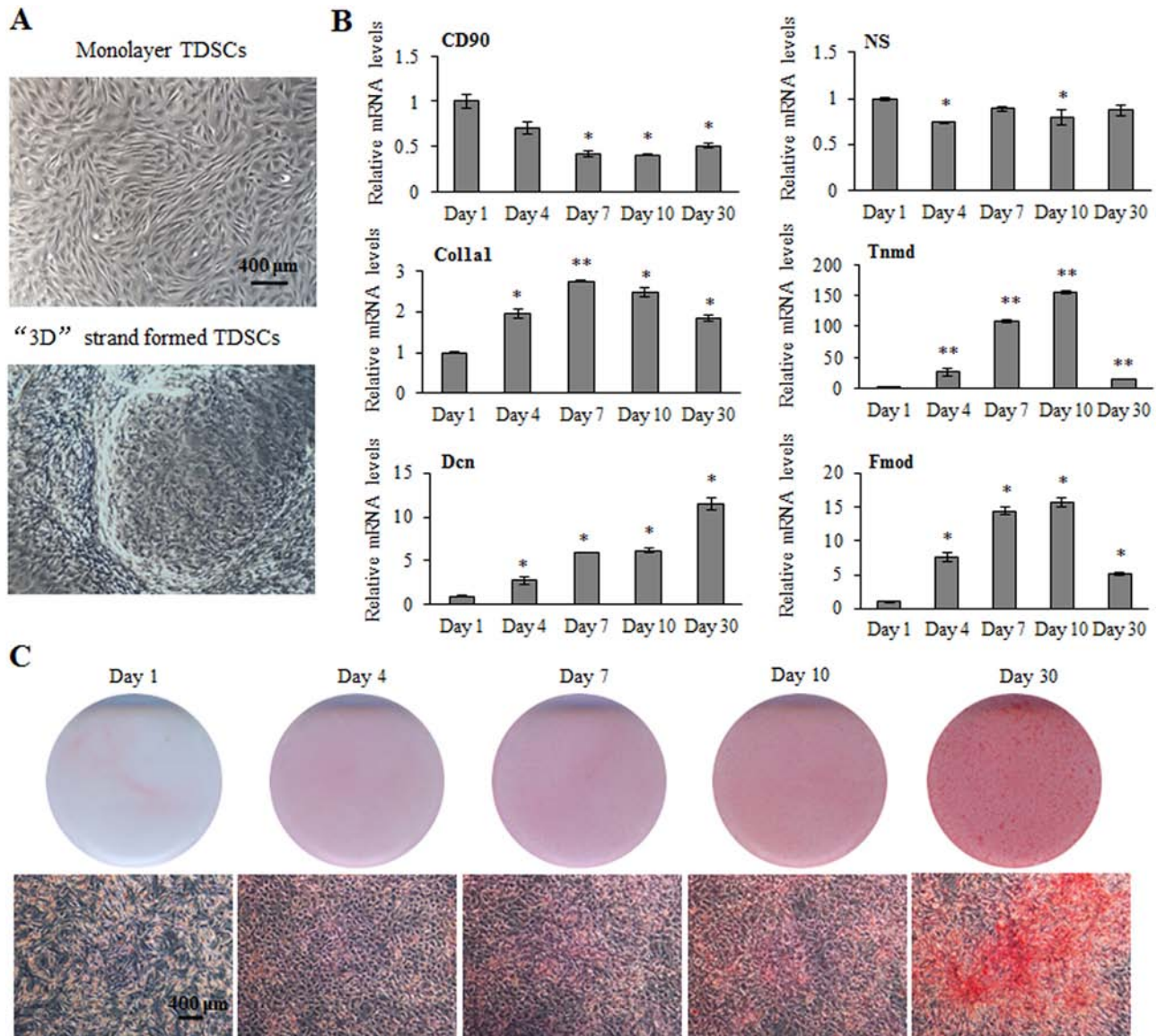


Fig. 2. Morphological changes and spontaneous differentiation of TDSCs. (A) Cultured in growth medium, TDSCs (3rd passage) are flat, showing monolayer before reaching confluent in culture dish (Top), but form 3-dimensional strand after growing 30 days without subculture (Bottom). (B) qRT-PCR analysis shows that the expression of gene markers associated with stemness, such as CD90 and NS decreased, while those related with tenogenic differentiation, such as Col1a1, Tnmd, Dcn and Fmod increased progressively with cell growing. * $P < 0.05$ vs. Day 1 group. ** $P < 0.01$ vs. Day 1 group. (C) Sirius Red staining shows the collagen amounts were increased during this process.

group (Fig. 4C).

Discussion

The discovery of tendon-derived stem cell makes a new era for elucidating the pathology of tendinopathy as well as developing innovative strategies for the tendon injuries. Being heterogeneous cell populations, only a minority of population is TDSCs but the majority is tenocytes. Our previous study also showed that very few (only 1–2%) TDSCs were found in rat flexor tendons [3]. Considering the difficulty for TDSCs isolation, the specific markers for characterizing TDSC identity and defining their biological function must be identified. Zhang and Wang compared TDSCs with tenocytes, and the expression of Oct-4, SSEA-4 and NS was observed in TDSCs [17]. The other factors such as Scx, Tnmd, Tnc, Col1a1, Col3a1 and Dcn were reported as tenogenic markers in previous studies [21,22]. In this study, a group of stem and tenogenic marker genes were compared between TDSC and

intact tendon. The stemness markers, CD90 and NS, were found to be with higher expression in TDSC; while the tenogenic markers including Col1a1, Tnmd, Dcn, and Fmod showed lower expression in monolayer TDSCs.

Tendon injuries can be chronic, known as tendinopathy, or acute, known as tendon rupture. To date, the clinical therapeutic options for tendon injuries were limited to surgical replacement with prosthesis, autografts, allografts, or xenografts. However, tendon healing is accompanied with formation of fibrotically scarring and adhesion, instead of complete regeneration, which may eventually cause partial loss of tendon function [3]. An encouraging effect of TDSCs on tendon injuries repair has been observed in the animal studies [23]. However, the exact niche signals to regulate TDSC fate remain elusive at present. In our study, the “3D” strands were formed after TDSCs were cultured for 30 days, suggesting that the cells had the strong ability to synthesize the extracellular matrix for long time culture. The expression levels of the tenogenic marker were increased while the stemness markers were decreased along with TDSCs growing. The collagen synthesis

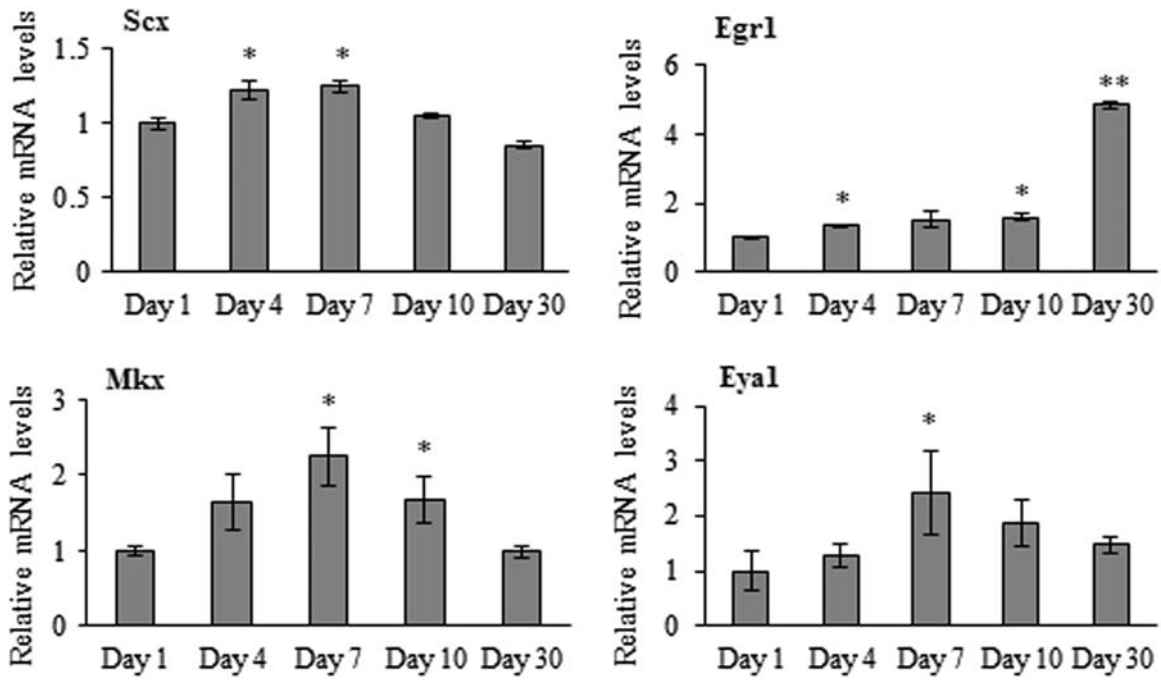


Fig. 3. Key tenogenic transcription factors were up-regulated during the spontaneous differentiation. Cells were cultured with normal condition medium and harvested at indicated days. The qRT-PCR results show the expressions of Scx, Egr1, Mlx and Eya1 were up-regulated along with continuously growing. * $P < 0.05$ vs. Day 1 group. ** $P < 0.01$ vs. Day 1 group.

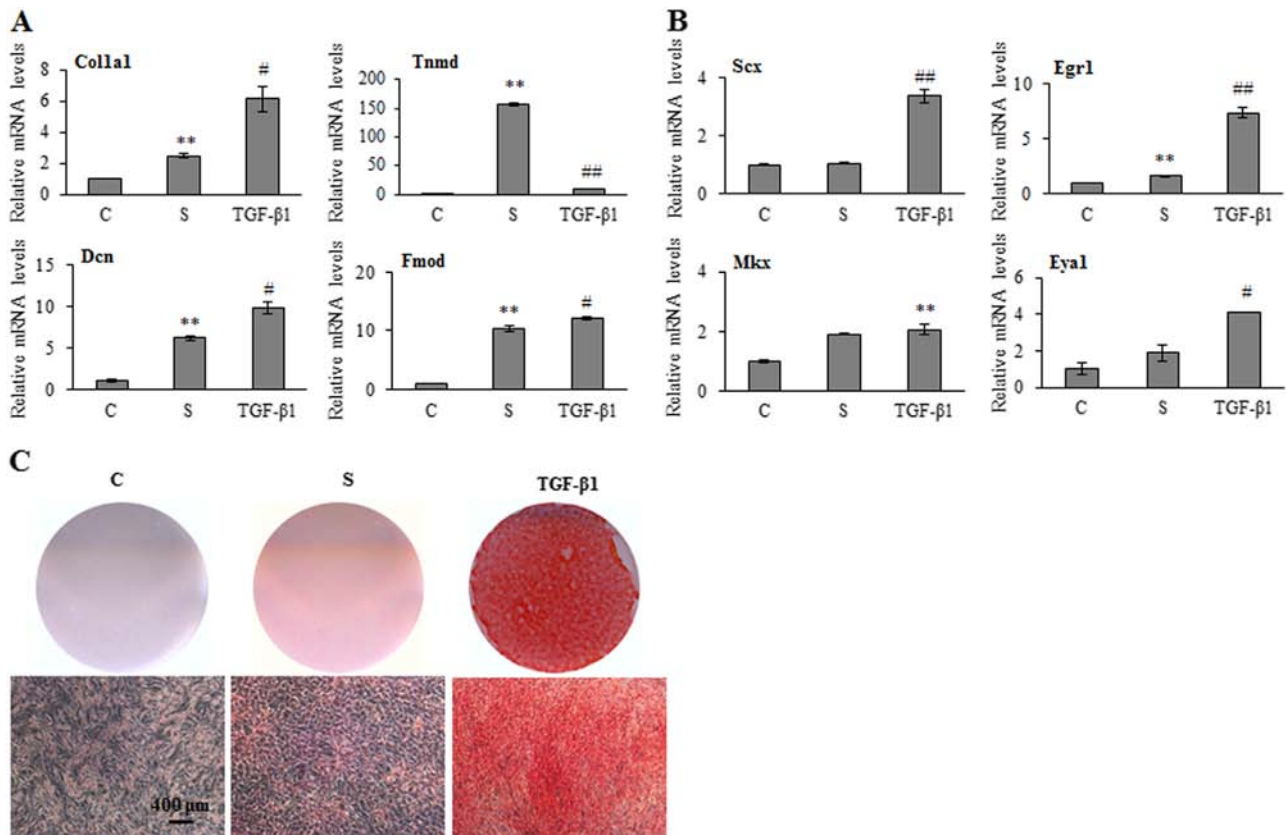


Fig. 4. TGF- β 1 promotes TDSCs tenogenic differentiation. TDSCs were incubated with 10 ng/ml TGF- β 1 for 10 days as the TGF- β 1 group. Cells cultured under normal condition medium for 10 days were considered as the spontaneous group. Cells before induction were considered as the control group. Compared with spontaneous group, the expression levels of both tenogenic markers, Coll1a1, Dcn and Fmod (A) and key tenogenic transcription factors, Scx, Egr1 and Eya1 (B), are much higher in TGF- β 1-induced group. Control group, (C). Spontaneous group, S. TGF- β 1 group, TGF- β 1. ** $P < 0.01$ vs. control group. # $P < 0.05$ vs. spontaneous group. ## $P < 0.01$ vs. spontaneous group. (C). Sirius Red staining shows the more collagen was synthesized in TGF- β 1 group than that in spontaneous tenogenic differentiation group.

ability was correspondingly increased during this process. To date, several studies have been published on spontaneous differentiation of tissue-specific progenitor cells. Somatic stem cells from different origin may have the priority to differentiate spontaneously into the origin-related cell type through “memory” regulation. Li et al. found mesenchymal stem cells have the spontaneous osteogenic differentiated ability through epigenetic dysregulation [18]. Based on our results and the previous reports, we believe that TDSCs has the spontaneous differentiation potential to tenocyte.

Tenogenic transcription factors play important roles in tendon differentiation. As a well-known specific marker for tendons, *Scx* is upregulated during the tendogenic differentiation. It also involves in the generation of tendon progenitors and ECM organization in tendons [24–26]. *Mkx* is required for subsequent tendon growth after tendon progenitor initiation during embryogenesis and is essential for the regulation of the postnatal growth and maturation of collagen fibrils. The transcription factor *Egr1* directs tendon differentiation and promotes tendon repair by activating the injury-induced expression of matrix synthesis genes [27], no abnormal tendon morphology is detectable in *Egr1*-null mice [28]. We suppose that *Egr1* may play marginal roles in tendon differentiation in comparison with *Scx* and *Mkx*. *Eya1*, a muscle transcription factor, also play roles in certain aspects of limb tendon development [29]. We observed the upregulation of these transcription factors in the spontaneous tenogenic differentiation of TDSCs while their expression were much lower in TDSCs than that in tendon tissue.

TGF- β has been widely considered as a tenogenic inducer. In mouse flexor tendon tenocytes, TGF- β 1 up-regulated *Scx*, *Mkx*, *Bgn*, *Col5a1*, *Col12a1*, and *PAI-1* in a dose-dependent manner [30]. TGF- β 2 led to a significantly increase of the mRNA expression level of *Scx* and *Col1a1* [27]. TGF- β 3 also stimulated the *Scx*, *Col1a1*, *Tnmd* and *Dcn* gene expression [31]. Moreover, the transcriptomic analysis of mouse limb tendon cells during development showed that TGF- β signaling was the most strikingly among all the up-regulated pathways [32]. Collectively, these information suggested the crucial role of TGF- β signaling in tendon induction. In our study, TGF- β 1 treatment was considered as a positive control in our study, and we compared the tendon markers expression between the spontaneous differentiation and the TGF- β 1-induced tenogenic differentiation. Except *Tnmd*, all the other tenogenic markers and tenogenic transcription factors expression showed much higher in TGF- β 1 group, suggesting spontaneous tenogenic differentiation is an underlying trend, similar to but weaker than tenogenic inducer's effect. Interestingly, the *Tnmd* expression level was all upregulated in both groups, but much higher in spontaneous group than that in TGF- β 1 group. As well known, the TGF- β 1 signaling pathway is involved in a wide range of cellular process and subsequently regulated a genetic regulatory network, so we suppose some factors mediated by TGF- β 1 may be the inhibitor of *Tnmd*, leading to a suppressive expression of *Tnmd* in TGF- β 1 treatment.

About the *Tnmd*, its exact functions in tenogenesis still remain uncertain [33]. Although *Tnmd* is widely accepted as a tenogenic marker, its knockout mice revealed no severe developmental phenotype, besides slow proliferation of tendon cell in vivo. Recently, other study demonstrated loss of *Tnmd* reduced self-renewal and augmented senescence of TDSCs [34]. In 2006, Shukunami C et al. reported that *Tnmd* was closely associated with the appearance of tenocytes during chick development and was positively regulated by *Scx* in a tendon cell lineage-dependent manner, suggesting its role in tendon formation [35]. Previous studies demonstrated that both their expression were all up-regulated in tendon differentiation or tendon repair [27,30–31]. Moreover, high-density culture was able to induce transient

tenogenic phenotype of dermal fibroblasts likely via cell morphology change and production of pro-tenogenic factors such as *Scx* and *Tnmd* expression [36]. Our results also suggested *Tnmd* might play a specific role in spontaneous tenogenic differentiation. Therefore, digging the deeper mechanism and elucidating the role of *Tnmd* in this process will be our research focus in near future.

Conclusions

The present study identified that TDSCs had the potential of spontaneous tenogenic differentiation through the regulation of the key tenogenic transcription factors. These findings not only open a new door for TDSC tenogenic differentiation, but also provide a novel therapeutics to tendon injury. More importantly, our study provide the idea how to induce or avoid TDSC spontaneous tenogenic differentiation through the proper control of TDSC density and culture time which will facilitate the TDSC study.

Disclosure of conflict of interest

None.

Acknowledgment

This work is supported by a grant from SMART program and Lui Che Woo Institute of Innovation Medicine, The Chinese University of Hong Kong.

References

- [1] L. Robert, M. Moczar, M. Robert, Biogenesis, maturation and aging of elastic tissue, *Experientia* 30 (2) (1974) 211–212.
- [2] M. Kvist, Achilles tendon injuries in athletes, *Ann. Chir. Gynaecol.* 80 (2) (1991) 188–201.
- [3] P. Sharma, N. Maffulli, Biology of tendon injury: healing, modeling and remodeling, *J. Musculoskelet. Neuronal Interact.* 6 (2) (2006) 181–190.
- [4] Y. Bi, D. Ehrlichou, T.M. Kilts, C.A. Inkson, M.C. Embree, W. Sonoyama, L. Li, A. I. Leet, B.M. Seo, L. Zhang, et al., Identification of tendon stem/progenitor cells and the role of the extracellular matrix in their niche, *Nat. Med.* 13 (10) (2007) 1219–1227.
- [5] Y.F. Rui, P.P. Lui, G. Li, S.C. Fu, Y.W. Lee, K.M. Chan, Isolation and characterization of multipotent rat tendon-derived stem cells, *Tissue Eng. Part A* 16 (5) (2010) 1549–1558.
- [6] D.L. Butler, N. Juncosa, M.R. Dressler, Functional efficacy of tendon repair processes, *Annu. Rev. Biomed. Eng.* 6 (2004) 303–329.
- [7] C.S. Proctor, D.W. Jackson, T.M. Simon, Characterization of the repair tissue after removal of the central one-third of the patellar ligament. An experimental study in a goat model, *J. Bone Joint Surg. Am.* 79 (7) (1997) 997–1006.
- [8] H.A. Awad, D.L. Butler, G.P. Boivin, F.N. Smith, P. Malaviya, B. Huijbregtse, A. I. Caplan, Autologous mesenchymal stem cell-mediated repair of tendon, *Tissue Eng.* 5 (3) (1999) 267–277.
- [9] G.S. Kryger, A.K. Chong, M. Costa, H. Pham, S.J. Bates, J. Chang, A comparison of tenocytes and mesenchymal stem cells for use in flexor tendon tissue engineering, *J. Hand. Surg. Am.* 32 (5) (2007) 597–605.
- [10] M.F. Pietschmann, B. Frankewycz, P. Schmitz, D. Docheva, B. Sievers, V. Jansson, M. Schieker, P.E. Muller, Comparison of tenocytes and mesenchymal stem cells seeded on biodegradable scaffolds in a full-size tendon defect model, *J. Mater. Sci. Mater. Med.* 24 (1) (2013) 211–220.
- [11] A.K. Chong, A.D. Ang, J.C. Goh, J.H. Hui, A.Y. Lim, E.H. Lee, B.H. Lim, Bone marrow-derived mesenchymal stem cells influence early tendon-healing in a rabbit achilles tendon model, *J. Bone Joint Surg. Am.* 89 (1) (2007) 74–81.
- [12] X. Chen, X.H. Song, Z. Yin, X.H. Zou, L.L. Wang, H. Hu, T. Cao, M. Zheng, H. W. Ouyang, Stepwise differentiation of human embryonic stem cells promotes tendon regeneration by secreting fetal tendon matrix and differentiation factors, *Stem Cells* 27 (6) (2009) 1276–1287.
- [13] Y. Sakaguchi, I. Sekiya, K. Yagishita, T. Muneta, Comparison of human stem cells derived from various mesenchymal tissues: superiority of synovium as a cell source, *Arthritis Rheum.* 52 (8) (2005) 2521–2529.
- [14] H.A. Awad, G.P. Boivin, M.R. Dressler, F.N. Smith, R.G. Young, D.L. Butler, Repair of patellar tendon injuries using a cell-collagen composite, *J. Orthop. Res.* 21 (3) (2003) 420–431.

- [15] M.T. Harris, D.L. Butler, G.P. Boivin, J.B. Florer, E.J. Schantz, R.J. Wenstrup, Mesenchymal stem cells used for rabbit tendon repair can form ectopic bone and express alkaline phosphatase activity in constructs, *J. Orthop. Res.* 22 (5) (2004) 998–1003.
- [16] R. Tasso, A. Augello, M. Carida, F. Postiglione, M.G. Tibiletti, B. Bernasconi, S. Astigiano, F. Fais, M. Truini, R. Cancedda, et al., Development of sarcomas in mice implanted with mesenchymal stem cells seeded onto bioscaffolds, *Carcinogenesis* 30 (1) (2009) 150–157.
- [17] J. Zhang, J.H. Wang, Characterization of differential properties of rabbit tendon stem cells and tenocytes, *BMC Musculoskelet. Disord.* 11 (2010) 10.
- [18] Z. Li, C. Liu, Z. Xie, P. Song, R.C. Zhao, L. Guo, Z. Liu, Y. Wu, Epigenetic dysregulation in mesenchymal stem cell aging and spontaneous differentiation, *PLoS One* 6 (6) (2011) e20526.
- [19] H. Liu, S. Zhu, C. Zhang, P. Lu, J. Hu, Z. Yin, Y. Ma, X. Chen, H. OuYang, Crucial transcription factors in tendon development and differentiation: their potential for tendon regeneration, *Cell Tissue Res.* 356 (2) (2014) 287–298.
- [20] O.A. Branford, B.R. Klass, A.O. Grobbelaar, K.J. Rolfe, The growth factors involved in flexor tendon repair and adhesion formation, *J. Hand. Surg. Eur.* 39 (1) (2014) 60–70.
- [21] L. Dai, X. Hu, X. Zhang, J. Zhu, J. Zhang, X. Fu, X. Duan, Y. Ao, C. Zhou, Different tenogenic differentiation capacities of different mesenchymal stem cells in the presence of BMP-12, *J. Transl. Med.* 13 (2015) 200.
- [22] H.Y. Nam, B. Pingguan-Murphy, A. Amir Abbas, A. Mahmood Merican, T. Kamarul, The proliferation and tenogenic differentiation potential of bone marrow-derived mesenchymal stromal cell are influenced by specific uniaxial cyclic tensile loading conditions, *Biomech. Model. Mechanobiol.* 14 (3) (2015) 649–663.
- [23] M. Ni, P.P. Lui, Y.F. Rui, Y.W. Lee, Q. Tan, Y.M. Wong, S.K. Kong, P.M. Lau, G. Li, K. M. Chan, Tendon-derived stem cells (TDSCs) promote tendon repair in a rat patellar tendon window defect model, *J. Orthop. Res.*, 30, (2012) 613–619.
- [24] R. Schweitzer, J.H. Chyung, L.C. Murtaugh, A.E. Brent, V. Rosen, E.N. Olson, A. Lassar, C.J. Tabin, Analysis of the tendon cell fate using Scleraxis, a specific marker for tendons and ligaments, *Development* 128 (19) (2001) 3855–3866.
- [25] Y. Asou, A. Nifuji, K. Tsuji, K. Shinomiya, E.N. Olson, P. Koopman, M. Noda, Coordinated expression of scleraxis and Sox9 genes during embryonic development of tendons and cartilage, *J. Orthop. Res.* 20 (4) (2002) 827–833.
- [26] P. Alberton, C. Popov, M. Pragert, J. Kohler, C. Shukunami, M. Schieker, D. Docheva, Conversion of human bone marrow-derived mesenchymal stem cells into tendon progenitor cells by ectopic expression of scleraxis, *Stem Cells Dev.* 21 (6) (2012) 846–858.
- [27] M.J. Guerquin, B. Charvet, G. Nourissat, E. Havis, O. Ronsin, M.A. Bonnin, M. Ruggiu, I. Olivera-Martinez, N. Robert, Y. Lu, Transcription factor EGR1 directs tendon differentiation and promotes tendon repair, *J. Clin. Investig.* 123 (8) (2013) 3564–3576.
- [28] V. Lejard, F. Blais, M.J. Guerquin, A. Bonnet, M.A. Bonnin, E. Havis, M. Malbouyres, C.B. Bidaud, G. Maro, P. Gilardi-Hebenstreit, et al., EGR1 and EGR2 involvement in vertebrate tendon differentiation, *J. Biol. Chem.* 286 (7) (2011) 5855–5867.
- [29] P.X. Xu, J. Cheng, J.A. Epstein, R.L. Maas, Mouse Eya genes are expressed during limb tendon development and encode a transcriptional activation function, *Proc. Natl. Acad. Sci. USA* 94 (22) (1997) 11974–11979.
- [30] Y.M. Farhat, A.A. Al-Maliki, T. Chen, S.C. Juneja, E.M. Schwarz, R.J. O'Keefe, H. A. Awad, Gene expression analysis of the pleiotropic effects of TGF-beta1 in an in vitro model of flexor tendon healing, *PLoS One* 7 (12) (2012) e51411.
- [31] Y. Qiu, X. Wang, Y. Zhang, R. Rout, A.J. Carr, L. Zhu, Z. Xia, A. Sabokbar, Development of a refined tenocyte differentiation culture technique for tendon tissue engineering, *Cells Tissues Organs* 197 (1) (2013) 27–36.
- [32] E. Havis, M.A. Bonnin, I. Olivera-Martinez, N. Nazaret, M. Ruggiu, J. Weibel, C. Durand, M.J. Guerquin, C. Bonod-Bidaud, F. Ruggiero, et al., Transcriptomic analysis of mouse limb tendon cells during development, *Development* 141 (19) (2014) 3683–3696.
- [33] O. Brandau, A. Meindl, R. Fassler, A. Aszodi, A novel gene, tendin, is strongly expressed in tendons and ligaments and shows high homology with chondromodulin-I, *Dev. Dyn.* 221 (1) (2001) 72–80.
- [34] P. Alberton, S. Dex, C. Popov, C. Shukunami, M. Schieker, D. Docheva, Loss of tenomodulin results in reduced self-renewal and augmented senescence of tendon stem/progenitor cells, *Stem Cells Dev.* 24 (5) (2015) 597–609.
- [35] C.I. Shukunami, A. Takimoto, M. Oro, Y. Hiraki, Scleraxis positively regulates the expression of tenomodulin, a differentiation marker of tenocytes, *Dev. Biol.* 298 (1) (2006) 234–247.
- [36] W. Wang, A. He, Z. Zhang, W. Zhang, G. Zhou, Y. Cao, W. Liu, Induction of transient tenogenic phenotype of high-density cultured human dermal fibroblasts, *Connect Tissue Res.* 56 (4) (2015) 288–299.