The utilization of decellularized tendon slices to provide an inductive microenvironment for the proliferation and tenogenic differentiation of stem cells

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Running headline: Decellularized tendon slices for stem cell differentiation

Abstract

The extracellular matrix (ECM) microenvironment for the stem cell niches, including but not limited to the biochemical composition, matrix topography, and stiffness, is crucial to stem cell proliferation and differentiation. The purpose of this study was to explore the capacity of the decellularized tendon slices (DTSs) to induce stem cell proliferation and tenogenic differentiation. Rat adult stem cells, including tendon-derived stem cells (TDSCs) and bone marrow-derived stem cells (BMSCs), were identified to have universal stem cell characteristics. The DTSs were found to retain the native tendon ECM microenvironment cues, including the inherent surface topography, well-preserved tendon ECM biochemical composition and similar stiffness to native tendon. When the TDSCs and BMSCs were cultured on the DTSs respectively, the LIVE/DEAD assay, alamarBlue® assay, scanning electron microscopy examination and qRT-PCR analysis demonstrated that the DTSs have the capacity to support these stem cells homogeneous distribution, alignment, significant proliferation and tenogenic differentiation. Taken together, the findings of this study indicate that the DTSs can provide a naturally inductive microenvironment for the proliferation and tenogenic differentiation of TDSCs and BMSCs, supporting the use of decellularized tendon ECM as a promising and valuable approach for tendon repair/reconstruction.

Keywords: Decellularized tendon slices; Tendon derived stem cells; Bone marrow derived stem cells; Microenvironment; Proliferation; Differentiation.

1. Introduction

The repair/reconstruction of injured tendons remains a great challenge for orthopaedic surgeons. Due to its poor blood supply and limited self-repair capability, damaged tendon tissue heals very slowly and rarely attains the structural integrity and mechanical strength of normal, undamaged tendon [1]. The current treatment modalities, such as autografts, allografts, synthetic and natural extracellular matrix (ECM)-derived grafts [2-3], can be used to repair the damaged tendons. However, the clinical outcomes of these treatment modalities are suboptimal owing to inevitable donor site morbidity, risk of disease transmission or rejection, and high failure rates [2-4]. Promisingly, regenerative medicine has attracted increasing attention as a potential strategy for the treatment of tendon injuries. More promisingly, it has become increasingly apparent that for tissue regeneration, bioscaffolds are not just to serve as a simple cell carrier but also to provide an important source of information that drives cell fate determination, such as cell attachment, proliferation and differentiation [5, 6].

As a tissue-specific bioscaffold for tendon regeneration, the decellularized tendon tissues have received growing research interest due to their great potential for future clinical and current research application. While most studies related to the decellularized tendons have focused on the decellularization protocols of different species and different tissue sources [7-11], a study of Omae et al reported that the decellularized multilayer sliced tendon scaffold might provide an environment for directing the differentiation of bone marrow derived stem cells (BMSCs) into the tenogenic lineage [12]. Also, an engineered tendon matrix film from decellularized tendon tissues was found to promote the proliferation and differentiation of rabbit tendon-derived stem cells (TDSCs) [13]. More recently, Yang et al demonstrated that incorporation of native tendon ECM components enhanced the bio-functionality of 3D tension-bearing scaffold [14]. In the same study, the human adipose stem cells seeded in tendon ECM-supplemented scaffold exhibited significantly increased proliferation and tenogenic differentiation [14].

However, using bovine Achilles tendon as a proof-of-concept model, Tong et al discovered that it was the precise orientation of collagen fibrils, not the amount of collagen protein, that effected BMSCs alignment, elongation and differentiation [15]. Most importantly, it also highlighted the importance of mimicking native tendon tissue microenvironment cues (including the precise topographical pattern, correct ECM components and stiffness characteristics) on the design of artificial materials that possess the capability to guide BMSCs to commit to the tenogenic lineage [15]. Nevertheless, no studies to date have taken all of these microenvironment cues into account simultaneously when the tendon ECM scaffold was prepared. Fortunately, previous work in our lab has successfully developed decellularized tendon slices (DTSs) with a thickness of 300 μm , which were thin sheet scaffolds with the elemental mechanical strength, inherent ultrastructure, specific proteoglycans, and multiple growth factors of tendon ECM [11]. Unlike the tendon slices with the thickness of 50 μm [12] or 80 μm [16], the DTSs with 300 μm or more in thickness had similar mechanical characteristics to the intact tendon bundle [17], and the DTSs had been more systematically evaluated [11]. In contrast to the other studies based on detergent decellularization [7-10], the DTSs were prepared using repetitive freeze/thaw of the intact tendons, frozen section as well as nuclease treatment, which led to nearly complete removal of cells and DNA without the worries about the hazardous effects of residual cytotoxic chemicals [11]. Moreover, the biochemical composition of the tendon ECM, including proteoglycans (fibromodulin, biglycan) and growth factors (TGF- β 1, IGF-1, VEGF, and CTGF), were well preserved in the DTSs [11]. However, it remains an unanswered question whether the DTSs that preserved these native tendon ECM microenvironment cues possess the capacity to promote stem cell proliferation and tenogenic differentiation.

In the current study, it was hypothesized that the DTSs may provide an excellent substrate for promoting stem cell proliferation and tenogenic differentiation. To test this hypothesis, the identified TDSCs and BMSCs were cultured on the DTSs respectively and the effect of the DTSs on cell viability and proliferation, cell morphology and

alignment, and tenogenic differentiation was investigated.

2. Materials and methods

2.1 Isolation and culture of rat TDSCs and BMSCs

Sprague Dawley (SD) rats (4~5 weeks old; male) weighing 100 to 120 g were used in this study. All experiments were conducted in accordance with the approved guidelines set out by the Sichuan University Animal Care and Use Committee. After sacrifice, Achilles tendons and flexor tendons were excised from both limbs of each rat. For the isolation of TDSCs, the tendon sheath and the surrounding paratenon were removed, and the middle tendon portion tissues were minced into small pieces and digested with 3 mg/ml collagenase type I (Worthington) and 4 mg/ml dispase (Sigma) in PBS for 1.5 h at 37°C. Then, the obtained suspensions were passed through a 70 µm cell strainer (BD, Falcon, USA) to yield single-cell suspension. The released cells were washed in PBS by centrifugation at 1200 rpm for 5 min and resuspended in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 20% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine (all from Invitrogen, Carlsbad, CA). For the isolation of BMSCs, the femurs and tibiae were carefully dissected free of adherent muscle and connective tissue. The tip of each bone was removed with a rongeur, and the marrow was harvested by inserting a 27-gauge syringe needle into one end of the bone and flushing with DMEM. The bone marrow cells were filtered through 70 µm cell strainer, centrifuged at 1200 rpm for 5 min and resuspended in complete medium supplemented with 20% FBS. Fresh medium was replaced every 3 d. Cells from the passage 3 were used in the subsequent assays.

2.2 In vitro self-renewal potential of rat TDSCs and BMSCs

A colony-forming assay was used to characterize the self-renewal potential of rat TDSCs and BMSCs. For this assay, 500 cells and 1000 cells at passage 3 were plated and cultured for 7 d in a 25-cm² flask. Upon harvesting, the cells were fixed with methanol for 5 min and stained with 0.5% crystal violet (Sigma) for 30 min at 37°C. Then, the number of colonies was counted. Colonies less than 2 mm in diameter and faintly stained

were ignored.

2.3 Flow cytometry assay

For flow cytometry analysis of cell surface antigens, TDSCs and BMSCs (total of 1×10^6 cells for each) were incubated with 1 μ g of R-phycoerythrin (PE) and fluorescein isothiocyanate (FITC)-conjugated mouse anti-rat monoclonal antibodies at 4°C for 30 min. These antibodies included PE-conjugated mouse anti-rat CD44 (Abcam), CD90, CD106 (BD Pharmingen) and also FITC-conjugated mouse anti-rat CD34 (Santa Cruz Biotechnology) and CD45 (Biolegend). PE-or FITC-conjugated isotype-matched IgG₁ (BD Pharmingen) were used as controls. After incubation, the stained cells were washed with PBS and resuspended in 500 μ L of PBS (with 10% FBS) and subjected to FACS analysis (Beckman Coulter).

2.4 Immunocytochemistry analysis of stem cell markers

To characterize the stemness of TDSCs and BMSCs, the stem cell markers including octamer-binding transcription factor 4 (Oct-4), Nanog and stage-specific embryonic antigen-1 (SSEA-1) were examined by immunocytochemistry. Cells were seeded on the coverslips in the six-well plates at a density of 2×10^4 cells/coverslip with 2.5 ml medium and cultured 37°C with 5% CO₂ for 2–3 d. For immunofluorescence staining, cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature and treated with 0.5% Triton X-100 for 15 min. After washing in PBS three times, the fixed cells were blocked with 1% BSA in PBS for 30 min at room temperature. Then, cells were incubated with either rabbit anti-rat Oct-4 (1:50, Abcam), rabbit anti-rat Nanog (1:50, Abcam) or mouse anti-rat SSEA-1 (1:50, Millpore) or overnight at 4°C. After subsequent washing with PBS for three times, the cells were again incubated with Cy3-conjugated goat anti-rabbit IgG antibody (1:200 for Oct-4; 1:400 for Nanog, Jackson ImmunoResearch Laboratories, PA) or Alexa Fluor® 488-conjugated goat anti-mouse IgG antibody (1:50, Jackson ImmunoResearch Laboratories, PA) for 1 h at room temperature in the dark. The cells were also counter-stained with DAPI (Sigma) staining. The stained cells were examined using fluorescence microscopy.

2.5 Multi-differentiation potential

The multi-differentiation potential of the TDSCs and BMSCs to differentiate into osteogenic, adipogenic and chondrogenic lineages was examined. For osteogenesis, TDSCs and BMSCs (4×10^3 cells/cm²) were cultured in osteogenic induction medium consisting of basic growth medium (DMEM plus 10% FBS) added with 0.1 μ M dexamethasone, 10 mM β -glycerol phosphate, and 50 μ g/ml ascorbate-2. After 21 or 28 d of culture, cells were fixed with 75% ethanol for 20 min, washed with PBS twice and stained with 0.1% Alizarin red solution (Sigma) for 30 min. For adipogenesis, TDSCs and BMSCs (8×10^3 cells/cm²) were cultured in basic growth medium with the addition of 0.5 μ M dexamethasone, 10 μ g/ml insulin, 50 μ M indomethacin, and 50 μ M isobutylmethylxanthine (IBMX). After 14 d of culture, cells were fixed with 10% formalin and stained with 0.5% fresh Oil red O solution (Sigma) for 30 min. For chondrogenesis, a pellet culture system was used. About 5×10^5 cells were pelleted into a micromass by centrifugation at 1000 rpm for 10 min in a 15-mL conical polypropylene tube and cultured in basic growth medium or chondrogenic medium, which consisted of basic growth medium supplemented with 10^{-7} M dexamethasone, 40 μ g/ml proline, 10 ng/ml TGF- β 3, 50 μ g/ml ascorbate2-phosphate, 100 μ g/ml sodium pyruvate, and 1% (ITS) + Premix (Becton Dickinson). After 21 d of culture, the pellets were fixed by 4% paraformaldehyde for 30 min at room temperature. For microscopy, the pellets were embedded in paraffin, cut into 5 μ m thick sections, and stained with Safranin O/Fast Green (Sigma).

2.6 Preparation and characterization of the DTSs

DTSs were fabricated using our previously published protocol [11]. Briefly, the harvested Achilles tendons from adult beagle dogs (15–20 kg) were trimmed into segments roughly 40 mm in length. After washing in cation-free phosphate buffered saline (PBS) (3 $\times$ 30 min), the tendon segments (TSs) were subjected to repetitive freeze-thaw treatment (liquid nitrogen for 2 min and then thawed in saline solution at 37°C for 10 min) for five times. Then, the TSs were fixed to the cutting base plate of a

cryostat (Leica CM3050S, Nussloch, Germany) and longitudinally cut into tendon slices with a thickness of 300 μ m. Following washing three times in PBS for 30 min each, the tendon slices were incubated in nuclease solution (including RNase 100 μ g/mL and DNase 150 IU/ mL) (Roche Diagnostic, Indianapolis, IN) for 12 h at 37°C. Finally, the tendon slices were rinsed for 30 min in PBS at room temperature with gentle agitation and the rinsing was repeated three times. Then, the prepared DTSs were lyophilized and sterilized by ethylene oxide for use. In addition, the TSs were directly performed to longitudinally cut into tendon slices with a thickness of 300 μ m, as the native tendon slices (NTSs).

For surface topography characterization, the samples including NTSs and DTSs (n=3 for each) were fixed in 2.5% glutaraldehyde for 2 h at 4°C. After dehydration in graded ethanol, the specimens were conducted to critical point drying, gold sputter coating, and then their ultrastructure was observed under scanning electron microscopy (SEM) (FEI Inspect F50) at an accelerating voltage of 30 kV. Afterwards, the average collagen fibril diameter was measured from the high resolution SEM micrographs (at a magnification of 40000 $\times$) of the NTSs and DTSs by Image J software. For each sample, a total of 10 collagen fibrils were randomly selected for measuring.

To characterize the nanomechanical properties of the microenvironment provided by the DTSs, the stiffness (as measured by the Young's modulus) measurements were performed using an atomic force microscopy (AFM). The TSs and NTSs were used as controls. Prior to testing, these samples were immersed into PBS at room temperature. Then, samples were mounted on an Agilent 5500 series AFM (Agilent Technologies, USA) and indented by a pyramid-tipped probe (Nanosensors Inc., Neuchatel, Switzerland) with typical radius < 10 nm and spring constant of 0.2 N/m. Eight measurements at different positions of each sample (n=3 for each group) were conducted. The recorded AFM cantilever deflection-indentation curves were used to derive force-indentation curves, and the Young's modulus was calculated from the force-indentation relation using the Hertz cone model [18, 19].

2.7 Cell viability and proliferation assay

To investigate the effect of DTSs on cell viability, TDSCs and BMSCs were seeded at a density of 2×10^5 cells/cm² based on the area of the sterilized DTSs, respectively. The seeded cells were allowed to attach for 2h before being immersed in complete medium. During this process, medium of $\sim 5 \mu\text{L}$ was added to cells-seeded DTSs every 30 min in case of cell death [20]. The cell viability over a period of 3 d was assessed qualitatively by LIVE/DEAD staining assay. Briefly, the cells-seeded DTSs samples were washed with PBS for three times, and incubated in $2 \mu\text{M}$ calcein AM (staining live cells) and $4 \mu\text{M}$ PI (staining dead cells) in PBS for 30 min at 37°C and washed again with PBS. A phase contrast fluorescence inverted microscope (Olympus, Japan) was used to view the samples.

Further, to quantitatively assess the effect of DTSs on cell proliferation, the sterilized DTSs (0.2 cm^2 for each slice) were placed in wells of 96 well plates. Then, the TDSCs (4×10^3 cells/well) or BMSCs (5×10^3 cells/well) were seeded in wells of 96-well plates, with or without DTSs. Cells not exposed to the DTSs served as the control. A sample size of $n=6$ wells/group/time point was used. At periodic intervals (1, 2, and 3d), cell proliferation was quantified using alamarBlue® (Invitrogen) assay by following the manufacturer's instructions. Briefly, at the end of the incubation, the culture medium was replaced with fresh medium without FBS ($100 \mu\text{L}/\text{well}$). Afterward, $10 \mu\text{L}$ of alamarBlue® was added into each well. After a 4 h incubation at 37°C , $50 \mu\text{L}$ of 3% SDS was directly added into each well to stop the reaction. Then, the DTSs were removed and the absorbance of alamarBlue® was measured at 570 nm, using 600 nm as the reference wavelength (normalized to the 600 nm value).

2.8 Cell morphology and alignment

To observe the effect of DTSs on cell morphology and alignment, both types of stem cells were seeded onto the sterilized DTSs at a density of 2×10^5 cells/cm². After seeding for 6h, 1d, and 3d, the cell morphology and alignment were visualized with SEM as described above, except for the use of a 20 kV acceleration voltage.

2.9 Quantitative TaqMan® real-time PCR analysis

To evaluate the effect of DTSs on tendon-lineage differentiation, the expression of tendon-specific markers at the mRNA level was examined in TDSCs or BMSCs cultured on the DTSs in complete culture media (10%FBS) for 3,7, and 14 d. TDSCs and BMSCs grown in 6-well plates at different time points served as control. At each time point, total cellular RNA was isolated by lysis in TRIzol (Invitrogen). The mRNA was reversely transcribed to cDNA by the RevertAid™ First Strand cDNA synthesis kit (Fermentas, Lithuania, USA). The newly synthesized cDNA was amplified by Quantitative TaqMan® real-time PCR. The rat specific primers and TaqMan® probes for tendon-specific genes, including scleraxis (SCX), tenomodulin (TNMD), and thrombospondin-4 (THBS4), and tendon related genes, collagen types I (COL I) and III (COL III), as well as the internal control, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were designed by Sango Biotech (Shanghai, China). The sequences of the primers and probes were shown in Table 1. A total of 2 µl cDNA of each sample were amplified in a 30 µl reaction mix using the TaqMan® probes with specific primers on an FTC-3000 qPCR system (Funglyn Biotech Inc. Canada). Cycling conditions were: denaturation at 94°C for 2 min, 40 cycles at 94°C for 20 s, optimal annealing temperature (shown in Table 1) for 20 s and 60°C for 30 s. The expression of target gene was normalized to that of GAPDH gene. Relative gene expression was calculated with the $2^{-\Delta Ct}$ formula.

2.10 Statistical analysis

Data were presented as mean $\pm$ standard deviation (SD). A one-way analysis of variance was performed with Dunnett's T3 comparison test to determine the statistical differences between the different groups for stiffness characterization. A two-tailed Student's t-test was used to determine statistical significance of cell proliferation and differentiation assays. Results with $p < 0.05$ were considered statistically significant.

3. Results

3.1 Identification and characterization of rat TDSCs and BMSCs.

During the primary culture, the adherent cells derived from tendon tissue or bone

marrow were present in a heterogeneous population and became more homogeneous with subsequent passaging (Fig.1A and Fig.2A). From passage 0 to passage 3, the tendon-derived cells mainly displayed two different types of morphologies: spindle shape fibroblast-like morphology and cobblestone-like morphology (Fig.1A), while the bone marrow-derived cells maintained elongated spindle shape morphology (Fig.2A).

To determine whether tendon-derived cells and bone marrow-derived cells were clonogenic, the cells at passage 3 isolated from rat tendon tissue and bone marrow were generated and cultured as single-cell suspensions. A portion of cells attached onto the plate and remained quiescent for 3-5 d before they started rapidly dividing to form colonies. After 7 d, colonies that had formed from single cells were visualized using crystal violet staining (Fig.1B and Fig.2B). About 4–6% of tendon-derived cells formed adherent cell colonies (Fig.1B), while approximately 2–3% of bone marrow-derived cells formed colonies (Fig.2B). Morphologically, these colonies were heterogeneous in size and cell density (Fig.1B and Fig.2B), potentially indicating unequal rates of cell proliferation.

The TDSCs and BMSCs were examined by flow cytometric analysis for cell surface antigens. The results revealed that these cells were uniformly positive for the mesenchymal stem cell (MSC) marker CD44 and fibroblastic marker CD90, respectively (Fig.1C and Fig.2C). They were negative for the hematopoietic stem cell marker CD34, for the leukocyte marker CD45, and for the endothelial cell markers CD106, thus verifying the lack of contaminating hematopoietic cells and endothelial cells (Fig.1C and Fig.2C).

Immunocytochemistry staining for stem cell markers showed that both TDSCs and BMSCs at passage 3 were positive for Oct-4, Nanog, and SSEA-1, further confirming the unique phenotype of the isolated TDSCs and BMSCs (Fig.1D and Fig.2D).

Multi-lineage differentiation assays exhibited that the isolated TDSCs and BMSCs were able to differentiate toward osteogenesis, adipogenesis, and chondrogenesis after induction, as demonstrated by Alizarin Red S, Oil-red O, and Safranin O /Fast Green

staining respectively (Fig.1E and Fig.2E).

3.2 Surface topography and stiffness of the DTSSs

The high magnification SEM micrographs revealed that the surface topography of the DTSSs was very similar to that of the NTSSs (Fig.3A). The nanotopographic cues of native tendon tissue microenvironment, such as the precise orientation of collagen fibrils and the characteristic banding patterns of native collagen, were well preserved in the DTSSs. The collagen fibril diameter of the NTSSs and DTSSs was comparable (128 ± 44 nm vs. 112 ± 33 nm) (Fig.3B). AFM measurements showed that the stiffness (as measured by the Young's modulus) of the DTSSs was 1.208 ± 0.940 MPa and was similar to that of the TSs at 1.211 ± 0.771 MPa or the NTSSs at 1.295 ± 1.106 MPa ($p > 0.05$, Fig.3C).

3.3 Cell viability and proliferation induced by the DTSSs

After 6 h incubation of cells-DTSSs, both TDSCs and BMSCs attached well to the surface of the DTSSs as demonstrated by fluorescence staining with a LIVE/DEAD assay (Fig.4A, B), where live cells and dead cells were fluorescently labeled green and red, respectively. Obviously, these cells showed a high rate of viability. From 1 to 3 d, a high percentage of viable cells were seen in the surface of DTSSs, with very few dead cells (Fig.4A, B). Moreover, alignment of these live cells on the DTSSs was apparent from the results of LIVE/DEAD staining (Fig.4A, B).

AlamarBlue® assay revealed that significant higher cell viability of TDSCs was determined when the cells were seeded on the DTSSs for 2 d (Fig.5A), as compared with media alone. Although no significant difference was found at 1 d and 3 d, higher cell viability was also observed in DTSSs group when compared with the control (Fig.5A). For BMSCs, although significant lower cell viability was found at 1 d when compared with the control, significant higher cell viability was observed when the cells were seeded on the DTSSs for 2 d (Fig.5B). And higher cell viability was also observed in DTSSs group at 3 d when compared with the control but no significant difference was found (Fig.5B). These results confirmed that the DTSSs were capable of promoting cell viability and proliferation.

3.4 Cell morphology and alignment induced by the DTSs

The SEM micrographs indicated that the native ultrastructure of tendon tissue (i.e. the precise orientation of collagen fibrils) was well preserved in the DTSs (Fig. 6A and H). After 6 h of incubation, both TDSCs and BMSCs were well attached on the surface of the DTSs and exhibited spherical morphology (Fig.6B, E, I and L). At 1 d, these cells displayed elongated spindle morphology and were aligned along the direction of collagen fibrils, which were more pronounced in higher magnification micrographs (Fig. 6C, F, G and M). By 3 d, a confluent and dense cell layer was observed suggesting significant cell proliferation on the DTSs (Fig.6D, G, K and N). Additionally, the SEM results also revealed homogeneous cell distribution on the surface of the DTSs (Fig.6B-G, and I-N).

3.5 Cell differentiation induced by the DTSs

The TDSCs seeded on the DTSs group demonstrated an up-regulation of SCX at all three time points compared to the pure TDSCs group, but no significant difference was found between the two groups (Fig.7A). The expression of SCX was found significantly higher in the BMSCs-DTSs group than the pure BMSCs group at 3 d, but the difference between the two groups was negligible at 7 d or 14 d, in spite of relatively higher at 7 d or relatively lower at 14 d (Fig.7F). Both TNMD and THBS4 at 7 d were expressed at significantly higher levels in TDSCs-DTSs group, and no significant difference was found at the other two time points (Fig.7B, C). However, at all three time points, the expression of TNMD and THBS4 demonstrated significantly higher in the BMSCs seeded on the DTSs group compared to the pure BMSCs group (Fig.7G, H). An unexpected finding is that only the expression of COL I at 14 d was significantly higher in the TDSCs-DTSs group than the pure TDSCs group (Fig.7D). The expression of COL III exhibited relatively higher levels in the TDSCs-DTSs group at 7 d and 14 d (Fig.7E). The BMSCs seeded on the DTSs showed relatively lower expression levels of both COL I and III at all three time points in the DTSs group (Fig.7I and J). Nevertheless, there was no significant difference for COL I or III between the stem cells-DTSs group and pure stem cells group (Fig.7E, I and J). The lack of significant differences in the

expression of COL I and III, from 3 d to 14 d, which could be attributed in part to the fact that they are also tendon matrix components. Taken together, these results indicated that the DTSSs promoted tenogenic differentiation of TDSCs and BMSCs.

4. Discussion

Tendon tissue represents a rich and valuable source of both tendon derived stem cells and ECM for tendon tissue engineering and regenerative medicine applications. Natural decellularized allogenic or xenogenic tendon ECM have recently started to attract more and more attention because of the fact that tendon ECM combines the advantages of its intrinsic mechanical competence with that of providing tenogenic stimuli for immigrating cells mediated, for example, by the growth factors and other mediators entrapped within the natural ECM [21]. Meanwhile, it was pointed out that the critical challenge in utilizing decellularized tendon ECM for tendon reconstruction is to develop mild decellularization strategies which maintain ECM structure, composition, and biomechanics, but allow complete removal of tissue donor cell components and good recellularization results [21]. Promisingly, we recently developed the DTSSs, in which all cellular and nuclear materials were efficiently removed while the intrinsic ultrastructure, elemental mechanical strength, specific proteoglycans and multiple growth factors of the native tendon ECM were well preserved [11]. Moreover, the current study further demonstrated that the DTSSs possessed the inherent surface topography and similar stiffness to native tendon based on the SEM examination and AFM measurements. Yet, the existing decellularized tendon ECM research suggests that many different types of acellular tendon scaffolds are capable of supporting cellular attachment [7-10], and/or the tenogenic differentiation of varying fractions of the seeded cells [12, 13, 16]. Due to differences in the experimental design and cell sources, it is challenging to directly compare the results of these studies [7-10, 12, 13, 16] to determine the optimal substrate for cell fate determination or tendon repair/reconstruction. However, to date, no studies have taken all of these microenvironment cues into account simultaneously when the tendon ECM scaffold was prepared. Hence, DTSSs appear to be more preferable to other

decellularized tendon matrix. In this study, we demonstrated that: (1) the DTSs promote the proliferation, distribution and alignment of rat TDSCs and BMSCs; and (2) the DTSs promote tenogenic differentiation of rat TDSCs and BMSCs.

TDSCs, a multipotent MSC population derived from tendon tissues, possess universal stem cell characteristics, including clonogenicity, multipotency, and self-renewal capacity [22]. Ever since TDSCs were first identified and successfully isolated in 2007 [22], as a brand new member in MSC families, they have been increasingly regarded as a promising cell type for musculoskeletal regeneration, particularly for tendon repair/construction. In addition, among the many kinds of MSC, BMSCs are the most well studied for tendon repair/construction because they are easily accessible and possess the potential of tenogenic differentiation [12, 15]. Also, it is noteworthy that TDSCs themselves highly expressed tendon-related factors, such as SCX and TNMD [22, 23]. Thus, in order to more objectively evaluate the ability of the DTSs to induce tenogenic differentiation of stem cells, both TDSCs and BMSCs were chosen for our study. We have successfully isolated and identified the TDSCs and BMSCs derived from adult rat tendon tissues and bone marrow, on the basis of several universal criteria of stem cells. Consistent with previous study [22], our data suggested that TDSCs were closely related to BMSCs, but not identical. However, the differences between TDSCs and BMSCs were beyond the scope of our investigation. Collectively, our study demonstrated that the isolated TDSCs and BMSCs possess universal stem cell characteristics, including clonogenicity, multi-differentiation potential, and the expression of stem cell markers.

To date, reseeding of the decellularized tendon ECM remains a challenge because the tendon ECM is dense and does not allow sufficient cell infiltration. Moreover, in comparison to native tendon, cells remain mostly inhomogeneously distributed in the reseeded ECM and do not align [19]. Hence, major milestones in the recellularization of decellularized tendon ECM are to gain a homogeneous cell distribution and to achieve sufficient cell survival and infiltration. Encouragingly, in the current study, when the stem cells were seeded on the DTSs, both TDSCs and BMSCs showed homogeneous

distribution and alignment along the direction of collagen fibrils of the DTSs, as seen by the LIVE/DEAD staining and SEM assay. In addition, the results of LIVE/DEAD staining revealed that these stem cells maintained highly cell viability on the DTSs in a time-frame of 3 d. Our previous results indicated that a few cells penetrated into DTSs after 4 d of culture in vitro [11]. Hopefully, the DTSs would allow for greater cellular ingrowth than the decellularized intact tendon scaffold. As expected, the results of cell viability analysed by alamarBlue® demonstrated that the DTSs promote the proliferation of TDSCs and BMSCs. These results further imply that those proteoglycans and growth factors retained in the DTSs may have biological activity and play a facilitating role in the growth and proliferation of these stem cells. Consistent with our results, rabbit TDSCs on engineered tendon matrix grew more quickly than on plastic culture substrate, which serves as a “gold standard” in cell culture nowadays [13].

Another encouragingly, the gene expression results clearly indicated that the DTSs provide an inductive microenvironment for the tenogenic differentiation of rat TDSCs and BMSCs based on the tendon-specific gene expression. The expression of TNMD and THBS4, is significantly higher in the TDSCs seeded on the DTSs group than the pure TDSCs group only at 7 d, whereas significantly higher in the BMSCs seeded on the DTSs group than the pure BMSCs group at all three time points. These differences may be attributed to the fact that although the TDSCs were confirmed to possess the characteristics of stem cells on the basis of a number of different criteria, the current method for isolation and culture of TDSCs is unable to obtain a highly purified population of tendon stem cells. Moreover, it is difficult to isolate pure tenocytes or TDSCs even using the separation procedures [24]. The unavoidable existence of tenocytes, which was used to induce tenogenic differentiation of other MSCs, such as amniotic epithelial cells [25] and adipose-derived stem cells [26], may lead to tenogenic differentiation of TDSCs in the pure cell group. Maybe for this reason, TDSCs exhibited higher tenogenic differentiation markers than BMSCs at basal state, as were shown in previous studies [22, 23]. Recently, of the 19 tendon-specific genes derived from both

human and rat, TNMD and THBS4, were found to have the highest tendon-selective expression compared to other tissues examined [27]. Accordingly, TNMD and THBS4 were selected as the tenogenic differentiation markers of stem cells in our study. Interestingly, the well-established tendon marker SCX was found significantly higher in the BMSCs-DTSs group than the pure BMSCs group only at 3 d, but no significant difference was found between the two groups at 7 d and 14 d, as well as the TDSCs-DTSs group and the pure TDSCs group at all three time points. Although SCX has been selected as one of tendon specific markers [14, 28-30] or even the only indicator of tenogenic differentiation [16] in many studies, it appears not to be a more specific marker for tendon as compared to TNMD and THBS4 in our study. These findings are consistent with a previous study suggesting that the expression of the widely referenced tendon marker SCX was not specific for adult tendon tissue [27]. It was also expressed in other tissues, including muscle and heart [27]. Although, another possible reason is that SCX, as an early tendon differentiation marker, may play an important role in the onset of tenogenic differentiation. As such, SCX has been reported to positively regulate the expression of TNMD [31]. Consistent with previous studies [28,31], an initial up-regulation of SCX at 3 d in the stem cells-DTSs group was followed by a significant increase in the expression of TNMD at the later time-points. Interestingly, while a previous study has demonstrated that the expression of COL I was significantly up-regulated in rabbit TDSCs cultured on engineered tendon matrix compared to those on plastic surfaces [13], in this study, we found that there was no significant difference for COL I or COL III between the stem cells-DTSs group and pure stem cells group, except for the significantly higher expression of COL I in TDSCs-DTSs group at 14 d. This discrepancy may be caused by the tendon ECM from different species and tissues, method of decellularization, as well as the differences of stem cell populations. The lack of significant differences in the expression of COL I and III in the current study, may be attributed in part to the collagen-rich microenvironment in the DTSs. It is possible that the collagen-rich microenvironment initiated a negative feedback mechanism that

repressed collagen type I and III synthesis. Similar to our results, it was found that there was no significant difference for COL I or COL III between the tendon ECM-supplemented group and the pure collagen group [14]. In another study, the expression of COL I was significantly higher in BMSCs before being seeded on the decellularized multilayer sliced tendon scaffold, even higher than that in the intact tendon [12]. Taken together, as significantly enhanced expression of tendon-specific markers in stem cells-DTSs group, the DTSs were confirmed to provide a naturally inductive microenvironment for the tenogenic differentiation of rat TDSCs and BMSCs.

Increasing evidence has demonstrated that the biochemical and biophysical cues from the ECM microenvironment can regulate and direct stem cell behaviors. For example, mouse embryonic stem cells acquired the osteogenic differentiation fate in the decellularized bone-specific ECM microenvironment created by osteogenic cell line MC3T3-E1[32]; human adipose stem cells seeded in the scaffold supplemented with tendon ECM extracts exhibited significantly increased proliferation and tenogenic differentiation [14]. These studies suggest that ECM composition play an important role in deciding stem cell fate. Specifically, it was reported that fibromodulin and biglycan as two critical components that organize the tendon ECM niche, their absence could alter the fate of the tendon stem/progenitor cells from tenogenesis to osteogenesis [22]. Moreover, topographical cues of ECM microenvironment have also been reported to play important roles in regulating stem cell behaviors, such as cell morphology, alignment, proliferation, and differentiation [33, 34]. More recently, the biomaterials that mimic the alignment character of tendon collagen fibrils induced the tenogenic differentiation of MSCs without the addition of any exogenous growth factors [28, 35]. Notably, the manipulation of MSCs into a specific cell shape and arrangement is not necessarily accompanied by differentiation into a specific lineage [15, 35]. In addition, another important biophysical cue: the stiffness of the matrix microenvironment, has been shown to play key roles in determining stem cell fate. The work by Engler et al. discovered that on soft substrates mimicking the elasticity of brain tissue, the MSCs started to show a

neuronal phenotype; on substrates of intermediate stiffness resembling striated muscle, the same stem cells developed into the myoblast lineage; and on stiff substrates resembling the matrix precursor to bone (osteoid), the stem cells began to resemble osteoblasts [19, 36]. Similarly, the bioimprint with similar stiffness to bovine tendon was found to promote tenogenic differentiation of BMSCs, whereas the softer bioimprint with much less stiffness can still support BMSCs alignment and elongation, but failed to promote an up-regulation of tenogenic marker TNMD [15]. Overall, these studies suggest the importance of mimicking the biochemical and/or the biophysical signals cues of native tissue microenvironment. Accordingly, when the DTSSs were prepared and developed, the native tendon ECM microenvironment cues were much more faithfully preserved. In the current study, the DTSSs possess the inherent surface topography (i.e. the precise orientation of collagen fibrils), well-preserved tendon ECM biochemical composition and similar stiffness to native tendon. Hence, the DTSSs were believed to provide an optimized microenvironment for stem cell fate determination. In the present study, to our knowledge, the enhanced proliferation of TDSCs and BMSCs in the stem cells-DTSSs group is most likely ascribed to the roles of those well-preserved tendon ECM bioactive factors in DTSSs. The orientation alignment and tenogenic differentiation of these stem cells on the DTSSs may involve a myriad of factors including, but not limited to, the inherent surface topography, biochemical composition and stiffness of decellularized tendon ECM. However, it is not yet clear to what extent these factors regulate tenogenic differentiation of stem cells. Future studies are needed to determine whether there is a dose-dependent effect of tendon ECM biochemical composition on the tenogenic differentiation of stem cells, or the influence of the absence of any one factor in the tendon ECM microenvironment.

While the present study has demonstrated DTSSs were capable of promoting the proliferation and tenogenic differentiation of TDSCs and BMSCs, there were several limitations in this study. First, the differences between TDSCs derived from rat Achilles tendons and flexor tendons were not distinguished. Second, the current sample size for

the stiffness characterization is relatively small, resulting in low power for some comparisons. Finally, now that the resident cells of tendons are constantly subjected to mechanical load, the effects of mechanical loading on stem cells seeded on the DTSs should be investigated in future studies. Also, further investigation will focus on the optimization of the DTSs strategy, to better understand the effects of the tendon ECM microenvironment on stem cell lineage specification and to enhance the bioactive and tenogenic inductive properties.

5. Conclusions

In summary, our study demonstrates that the DTSs possess the native tendon ECM microenvironment cues, including the inherent surface topography, well-preserved tendon ECM biochemical composition and similar stiffness to native tendon, which endow the DTSs with the capacity to support homogeneous distribution, alignment, proliferation and tenogenic differentiation of rat TDSCs and BMSCs. Our results further support the use of decellularized tendon ECM as a promising and valuable approach for tendon repair/reconstruction

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Fig.1. Isolation and characterization of TDSCs. (A) Morphological features of tendon-derived cells at different passages. During the culturing process, three different types of adherent cells were observed: spindle shape fibroblast-like morphology (solid arrow), cobblestone-like morphology (dotted arrow), and polygonal morphology (arrowhead). Scale bar=100 μm . (B) The colony-forming efficiency of tendon-derived cells. The results showed the statistical analysis of No. of colonies formed by tendon-derived cells at the initial seeding density of 500 and 1000 cells/T-25 flask ($n=3$). Scale bar=500 μm . (C) Flow cytometry analysis of the expression of indicated cell surface markers related to mesenchymal stem cells, hematopoietic stem cells, and endothelial cells on TDSCs. (D) Immunocytochemistry staining of stem cell markers for TDSCs. (E) Multi-differentiation potential of TDSCs toward osteogenesis (Alizarin Red S; Scale bar=100 μm), adipogenesis (Oil Red O; Scale bar=100 μm), and chondrogenesis (Safranin O/Fast Green; Scale bar=100 μm) in vitro. The inset shows the TDSCs in the regular medium were not found to exhibit such a multi-differentiation potential.

Fig.2. Isolation and characterization of BMSCs. (A) Morphological features of bone marrow-derived cells at different passages. During the culturing process, two different types of adherent cells were observed: elongated spindle shape morphology (solid arrow) and polygonal morphology (arrowhead). Scale bar=100 μm . (B) The colony-forming efficiency of bone marrow-derived cells. The results showed the statistical analysis of No. of colonies formed by bone marrow-derived cells at the initial seeding density of 500 and 1000 cells/T-25 flask ($n=3$). Scale bar=500 μm . (C) Flow cytometry analysis of the expression of indicated cell surface markers related to mesenchymal stem cells, hematopoietic stem cells, and endothelial cells on BMSCs. (D) Immunocytochemistry staining of stem cell markers for BMSCs. (E) Multi-differentiation potential of BMSCs toward osteogenesis (Alizarin Red S; Scale bar=100 μm), adipogenesis (Oil Red O; Scale bar=100 μm), and chondrogenesis (Safranin O/Fast Green; Scale bar=100 μm) in vitro. The inset shows the BMSCs in the regular medium were not found to exhibit such a multi-differentiation potential.

Fig.3. (A) SEM characterization for the surface topography of NTs and DTs. Scale bar=3 μm . (B) The collagen fibril diameter of NTs and DTs ($p>0.05$). (C) Stiffness of TSs, NTs and DTs as determined by AFM ($p>0.05$).

Fig.4. LIVE/DEAD staining analysis of TDSCs (A) and BMSCs (B) seeded on the DTs at 6 h, 1 d and 3 d by fluorescence microscopy, with live cells stained green (calcein AM) and dead cells stained red (PI). Scale bar = 200 μm .

Fig.5. The proliferation of TDSCs (A) and BMSCs (B) seeded on the DTs at 1 d, 2 d, and 3 d as determined by the alamarBlue® assay. *, signifies a p value of < 0.05 as compared to the pure cell group.

Fig.6. SEM micrographs of morphology, alignment and distribution of TDSCs (B-D, I-K) and BMSCs (E-G, L-N) on the surface of the DTSs after 6 h, 1 d and 3 d of culture. (A, H) Unseeded DTSs. The dotted arrows indicate the orientation of collagen fibers of the DTSs. The solid arrows indicate the elongation and alignment of the TDSCs and BMSCs on the DTSs. Scale bar = 200 μ m in images A-G; Scale bar = 50 μ m in images H-N.

Fig.7. Quantitative real-time PCR analysis of tenogenic-related gene expression of the TDSCs (A-E) and BMSCs (F-J) seeded on the DTSs at different time points. The gene expression levels are normalized with the housekeeping gene, GAPDH. * signifies a p value of < 0.05, ** signifies a p value of < 0.01 compared to the pure stem cells group, N=6.

Table 1

The primer and probe sequences, product size and annealing temperature used for TaqMan® qPCR.

Genes	5'-3'	Primer and probe sequences	Production size (bp)	Annealing temperature (°C)
GAPDH	Forward	AAGCTCATTTCCTGGTATGACA	86	56
	Reverse	TCTTACTCCTTGGAGGCCATGT		
	Probe	CAACAGGGTGGTGGACCTCA		
SCX	Forward	AGAACACCCAGCCCAAACA	67	52
	Reverse	CGGTCTTTGCTCAACTTTCT		
	Probe	CACCTTCTGCCTCAGCAACC		
TNMD	Forward	GTCACATTCTAAATGCAGAAG	115	52
	Reverse	CTCCCCCAAACAGGACAAT		
	Probe	GTGTTTGGTATCCTGGCCTT		
THBS4	Forward	ACCCTAACCAGTCGGATGT	100	54
	Reverse	CAGTTGTCTGTGCTGTCCT		
	Probe	CTGGTTGGGGACTCCTGTGAC		
COL I	Forward	GTGCTAAGGGTGAAGCTGGT	126	52
	Reverse	CATCAGCACCAGGGTTTCCAG		
	Probe	CACCACGCACACCCTGGGGAC		
COL III	Forward	CTGGAGATAAGGGTGAAGGT	189	56
	Reverse	GAGGGCCTCCTTCACCTTTCT		
	Probe	CTTCCAGGTATAGCTGGACCTC		