ORIGINAL ARTICLE

Tendon-Derived Extracellular Matrix Enhances Transforming Growth Factor- β 3-Induced Tenogenic Differentiation of Human Adipose-Derived Stem Cells

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Because of the limited and unsatisfactory outcomes of clinical tendon repair, tissue engineering approaches using adult mesenchymal stem cells are being considered a promising alternative strategy to heal tendon injuries. Successful and functional tendon tissue engineering depends on harnessing the biochemical cues presented by the native tendon extracellular matrix (ECM) and the embedded tissue-specific biofactors. In this study, we have prepared and characterized the biological activities of a soluble extract of decellularized tendon ECM (tECM) on adult adipose-derived stem cells (ASCs), on the basis of histological, biochemical, and gene expression analyses. The results showed that tECM enhances the proliferation and transforming growth factor (TGF)- β 3-induced tenogenesis of ASCs in both plate and scaffold cultures *in vitro*, and modulates matrix deposition of ASCs seeded in scaffolds. These findings suggest that combining tendon ECM extract with TGF- β 3 treatment is a possible alternative approach to induce tenogenesis for ASCs.

Keywords: decellularized tendon matrix, adipose stem cells, aligned scaffold, differentiation, tendon regeneration

Introduction

TENDON INJURIES OCCUR frequently in sports and daily activities due to excessive load or overuse. In the United States alone, >120,000 tendon and ligament surgical repairs are performed annually.¹⁻³ The natural healing process of tendons is slow and insufficient, resulting in fibrotic scar formation and inferior mechanical strength at the injured sites.⁴ Current clinical outcomes of tendon repair remain unsatisfactory due to limitations including autograft donor site morbidity, risk of injury recurrence, and limited long-term function recovery.⁵⁻⁷ Therefore, tissue engineering approaches, which use a combination of cells, scaffolds, and bioactive molecules, are gaining increasing research interest as a promising alternative strategy to treat tendon injuries.

The use of adult mesenchymal stem cells (MSCs) as the cellular component for tendon tissue engineering has been increasingly explored in recent years.⁸⁻¹⁰ Compared with other cell sources, adipose-derived stem cells (ASCs) are abundant and can be isolated by minimally invasive ap-

proaches.^{11,12} To date, a variety of growth factors have been reported to be tenogenic for ASCs. For instance, bone morphogenetic protein (BMP)-14 and BMP-12 were shown to induce strong tenogenic differentiation of mouse and canine ASCs, respectively.^{13,14}

However, no single growth factor has been found to exclusively promote tenogenic differentiation: BMP-14 is also capable of stimulating ASC differentiation toward other mesenchymal lineages, such as osteogenesis^{15,16} and chondrogenesis.¹⁷ Likewise, transforming growth factor (TGF)- β 1 exhibits both tenogenic and chondrogenic effect for ASCs.^{18,19} Rather, the evolving microenvironment produced by progenitor cells plays an important role in cell response to growth factor to induce proliferation and tissue-specific differentiation during development or tissue repair.²⁰⁻²² In terms of tendon tissue, the influence of biochemical and biophysical cues presented by tendon microenvironment on growth factor bioactivity to promote tendon-specific effects is not well understood.

Studies in tendon development have revealed the complexity of tendon differentiation. As shown across several

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animal models, members of the TGF- β superfamily are actively involved in tendon development and healing in a spatiotemporally specific manner. For example, mouse patellar tendon cells were found to respond to TGF- β signaling at developmental stages starting at gestation day 17.5 and ending at postnatal day 14.²³ Consistent with this finding, micromass culture of chick embryonic limb bud mesodermal cells with TGF- β demonstrated significant upregulation of tendon markers, scleraxis (SCX), and tenomodulin (TNMD), with concurrent reduction in cartilage markers.²⁴ Conversely, disruption of TGF- β signaling resulted in the loss of most tendons and ligaments in an SCX-GFP mouse model.²⁵ When injured, high levels of TGF- β expression and activity were seen throughout the healing period.^{26–28}

Concurrently, recent research has illustrated the pivotal role of the extracellular matrix (ECM) in tendon differentiation.^{29,30} ECM is composed of the structural and signal molecules secreted by the resident cells of each tissue. While the ECM of most tissues share highly conserved structural proteins (e.g., collagen, proteoglycans), it is the unique biophysical arrangement of these proteins and the highly orchestrated deposition and presentation of soluble cues that serve to promote and maintain a particular cell phenotype.^{31,32} Tendon is rich in ECM components, and many of the tendon ECM proteins have been found to play important roles in tendon differentiation and organization.^{33–35} As proof, tendon-derived stem/progenitor cells (TSPCs) seeded on decellularized tendon/ligament ECM demonstrated improved proliferation and tendon cell phenotype.³⁶ Taken together, it is reasonable to assume that the presence of native tendon ECM may be beneficial to TGF- β -induced tenogenic differentiation of MSCs for tendon repair.

In addition to biochemical cues, scaffolds are also utilized in tendon tissue engineering to provide mechanical support as well as topographical cues that mimic the architecture of native tendon. Because tendon is primarily composed of aligned collagen fibers, scaffold anisotropy is an important topographical characteristic to consider in tendon tissue engineering. For instance, human tendon fibroblasts seeded on aligned microfibrillar scaffold exhibited promoted expression of phenotypic markers.³⁷ In this study, we have therefore prepared and employed aligned poly- ϵ -caprolactone (PCL) scaffolds to partially reproduce the biophysical feature of native tendon ECM.

The objective of this study was to investigate the effect of native tendon ECM components and TGF- β on the tenogenesis of human ASCs (hASC). A soluble extract of decellularized tendon ECM (tECM) was prepared as described previously.³⁵ The individual and combined effects of tECM and TGF- β 3 on hASC behavior, including proliferation and differentiation, were analyzed by using tECM as a medium supplement for cell culture with or without TGF- β 3 for up to 2 weeks in tissue culture plates, and by ASCs seeded on aligned PCL scaffolds. We hypothesized that tECM is able to enhance the proliferation and TGF- β 3-induced tenogenesis of ASCs *in vitro* and tECM modulates matrix deposition and organization of ASCs on scaffolds.

Materials and Methods

Cell isolation and culture

hASCs were obtained from lipoaspirate-derived fat tissue of two donors (34-year-old male and 38-year-old female)

using an automated cell isolation system (Tissue Genesis, Inc.), with University of Pittsburgh Institutional Review Board approval. Isolated hASCs were cultured in a growth medium (GM) consisting of DMEM-high glucose (Gibco), 10% fetal bovine serum (FBS), and penicillin/streptomycin (P/S). hASCs between passage 2 and 4 (P2 and P4) were used for experiments.

Colony-forming unit-fibroblast assay

The colony-forming unit-fibroblast (CFU-F) assay was performed using an established method described elsewhere with the culture time span extended to 14 days.³⁸ hASCs from each donor at P2 were plated separately in 100 mm dishes (Falcon) in triplicate at densities of 100 cells per dish and cultured in GM. The cultures were stained with 0.5% crystal violet solution in methanol and visible colonies scored.

Flow cytometry

hASCs at P2 were detached by trypsin-EDTA and incubated with propidium iodide (PI) and PE- or FITC-conjugated mouse (IgG1, κ) anti-human antibodies for 30 min at 4°C. Antibodies include mouse anti-human CD31, CD34, CD44, CD45, CD73, CD90, and CD105 (BD Biosciences). Dead cells were excluded by positive PI staining. PE- or FITC-conjugated isotype-matched IgGs (BD Biosciences) were used as controls. After washing, the cells were sorted using the FACSaria II SORP flow cytometer (BD Biosciences) and data analyzed with DiVa v6 software.

Preparation of tendon ECM

A soluble fraction of tECM was prepared using our previously reported protocol.³⁵ The proximal part of superficial digital flexor tendons were harvested from hind legs of 2–3-month old calves purchased from a commercial abattoir (Research 87, Inc.), pulverized, and decellularized by 1% Triton X-100 (Sigma-Aldrich). After nuclease treatment (200 U/mL DNase, Worthington), the acellular tissue was extracted in 3 M urea (Sigma-Aldrich) for 3 days. Urea was removed by dialysis in 3500 MWCO cassettes (Thermo Scientific) against water for 2 days and then the tECM extract was spin-concentrated, sterilized using 0.25 μ m PVDF syringe filter units (Millipore), and stored as 1 mg/mL stock at –20°C until use.

Preparation of scaffold

Microfibers were fabricated by electrospinning. A solution of PCL (MW = 70,000–90,000; Sigma-Aldrich) prepared at 18% w/v in 1:1 (v/v) dimethylformamide (DMF) and tetrahydrofuran (THF) (Fisher Scientific) was loaded into a 10 mL syringe and extruded at 2 mL/h through a 22-gauge blunt-tip needle using a syringe pump (PY2 70-2209; Harvard Apparatus). A 10 kV DC potential (Gamma High Voltage) was applied to create an electrostatic field with a distance of 15 cm between the needle tip and a custom-designed rotating mandrel. Electrospinning was performed for 2 h per scaffold to form the scaffold sheet, which was trimmed to 4 cm in width and dried in vacuum overnight to remove residual organic solvent. Scaffolds were cut into 20 $\times$ 5 mm rectangular pieces, hydrated, and sterilized in

75% ethanol, and then soaked in GM overnight. The scaffolds were secured to the bottom of culture wells in customized incubators for cell seeding.

Scaffold characterization

Both aligned and nonaligned scaffolds were dried in vacuum, mounted on aluminum stubs, sputter-coated with 3.5 nm gold, and examined by a scanning electron microscope (SEM, field emission, JSM6335F; JEOL) operated at 3 kV accelerating voltage and 8 mm working distance. The external surface of the central part of the constructs was selected for imaging. Fiber diameter and degree of alignment were quantified from the SEM images ($n=4/\text{group}$). Briefly, the diameters of 50 randomly selected fibers in each image were measured by ImageJ, and average fiber diameter calculated. The angle between fiber and horizontal orientation was measured by ImageJ (50 fibers counted in each image). The thickness of the scaffolds was measured by a digital caliper ($n=30$).

Differentiation of hASCs

Differentiation along mesenchymal lineages, including osteogenesis, adipogenesis, and chondrogenesis, was performed to assess the multipotency of the isolated hASCs using an established protocol with slight modifications³⁸: 10 ng/mL BMP-6 was added into the standard chondrogenic medium to improve TGF- β -driven chondrogenesis of hASCs.³⁹ To induce tenogenesis, hASCs at P3 were serum-starved overnight at a density of 1×10^4 cells/cm² in plate culture and at 6×10^4 cells/cm² in scaffold culture, respectively. Cells were then treated with or without 10 ng/mL TGF- β 3 (PeproTech) in a basal medium (BM) consisting of high-glucose DMEM, 1 $\times$ Insulin-Transferrin-Selenium-X (ITS), and P/S (Gibco) supplemented with 10% v/v of 1 mg/mL tECM, 1 mg/mL collagen type I solution (Col I; PureCol Advanced Biomatrix), or FBS for up to 14 days. The tECM and Col I groups were also treated with 2% FBS to maintain robust cell attachment and vitality for tenogenesis of scaffold culture.

Cell proliferation tests

hASCs at P3 were plated on culture plastic and scaffolds at a density of 0.5×10^4 and 4×10^4 cells/cm², respectively. Twenty-four hours after initial seeding, cells were fed with BM containing one of the following supplements at 10% v/v: 1 mg/mL tECM, 1 mg/mL Col I solution, or FBS. DMEM supplemented with 10% v/v Hanks' Balanced Salt Solution

(HBSS; Gibco) was used as a negative control. On days 0, 3, and 7, MTS assay (CellTiter 96 Assay; Promega) was performed to spectrophotometrically determine the metabolic activity of cells from each group. In addition, cells were nuclear stained by 4, 6-diamidino-2-phenylindole, dilactate (DAPI; Life Technologies) at each time point and imaged using an Olympus CKX41 inverted fluorescent microscope equipped with a CCD camera to reflect cell nuclei density.

Real-time polymerase chain reaction analysis of gene expression

Total cellular RNA was isolated at days 3, 7, and 14 after differentiation treatment (Rneasy; Qiagen) and first-strand cDNA synthesized using the SuperScript III First-Strand cDNA synthesis kit (Invitrogen). Real-time polymerase chain reaction (PCR) was performed using SYBR green Supermix in a Step One Plus real-time PCR system (Applied Biosystems; Life Technologies). The targets and sequences of primers are shown in Table 1. Relative expression level of each gene was normalized to that of 18S rRNA and calculated using the $\Delta\Delta C_t$ method.

Protein extraction and Western blot assay

Eight days after differentiation induction, total protein was extracted from each group by the TM buffer (Total Protein Extraction Kit; Millipore) and concentration measured by the BCA assay. Equal loads of reduced protein samples of the same concentration ($\sim 800 \mu\text{g/mL}$) were electrophoretically separated in NuPAGE Bis-Tris Mini Gel (Life Technologies) and transferred onto PVDF membranes (iBlot dry blotting system; Invitrogen) for incubation with rabbit anti-scleraxis (SCX) or anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primary antibody (Abcam) at 4°C overnight. Western blots were developed using horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG secondary antibodies (GE Healthcare Bio-Sciences) and West Dura Extended Duration Substrate (Thermo Scientific).

Mechanical testing

Tensile properties of scaffolds were analyzed using the Bose 3230 mechanical tester. Scaffolds were securely mounted between two clamps at 10 mm and loaded with uniaxial force applied at a displacement rate of 0.2 mm/s until 10 mm displacement. The tensile force and the displacement were recorded, and the slope of the linear portion of the stress-strain curve was calculated as Young's modulus.

TABLE 1. PRIMER SEQUENCES OF GENES ANALYZED BY REAL-TIME POLYMERASE CHAIN REACTION

Gene		Primer sequence (5'-3')	Product size (bp)
18S rRNA	Forward	GTAACCCGTTGAACCCCAT	151
	Reverse	CCATCCAATCGGTAGTAGCG	
SCX	Forward	ACACCCAGCCCAAACAGA	65
	Reverse	GCGGTCCTTGCTCAACTTTC	
TNC	Forward	GGTGGATGGATTGTGTTCTGAGA	328
	Reverse	CTGTGTCCTTGTCAAAGGTGGAGA	
SOX9	Forward	CTGTAGGCGATCTGTTGGGG	85
	Reverse	AGCGAACGCACATCAAGA	

Matrix deposition and characterization

hASCs were seeded on PCL scaffolds at a density of 6×10^4 cells/cm² and cultured with BM supplemented with either only 2% FBS or 2% FBS plus 10% tECM (v/v) for 3 weeks. Ascorbate-2-phosphate (50 µg/mL; Sigma-Aldrich) was added to each group to optimize endogenous ECM synthesis. Negative controls consisted of cell-free scaffolds treated under the same conditions. Collagen content in each group was quantified using the Chloramine T-based hydroxyproline assay. Briefly, cell-seeded scaffolds were papain digested at 60°C overnight, reacted with 4N NaOH, and then neutralized with HCl. The samples were then reacted with Chloramine T reagent (Fisher Scientific) and subsequently Ehrlich reagent (Sigma-Aldrich). Absorbance at 550 nm was measured spectrophotometrically by a microplate reader (BioTek).

Immunofluorescent staining

Cell-seeded scaffolds were washed in PBS, fixed in 4% paraformaldehyde, and blocked with 1% bovine serum albumin and 22.52 mg/mL glycine in PBS-T. Primary antibodies used included goat anti-tenomodulin (Tnmd, 1:50, sc49325, Santa Cruz), or rabbit anti-Col I (1:500, ab34710; Abcam), with overnight incubation at 4°C. Alexa Fluor 488 chicken anti-goat or Alexa Fluor 488 goat anti-rabbit was used as secondary antibodies at 1:500 dilution (Life Technologies). For F-actin staining, fixed cells were permeabilized in 0.1% Triton X-100 and then incubated with Alexa Fluor 488 phalloidin for 30 min at room temperature (Life

Technologies). After nuclear counterstaining with DAPI (Life Technologies), cells were imaged using a confocal microscope (Olympus FluoView 1000).

Statistical analysis

Data are presented as mean $\pm$ standard deviation. All quantitative assays were performed for no less than thrice independently (N equals to the number of independent tests in figure legends). In each replicate, cells from two donors were treated and analyzed separately in duplicate, and data were combined. One-way ANOVA with Bonferroni *post hoc* test and Student's *t*-test was performed with SPSS (SPSS Statistics software 21; IBM) to determine statistical significance. Significance was considered at $p < 0.05$.

Results

Characterization of human ASCs

After 14 days of culture, $28.83\% \pm 3.31\%$ of the adherent cells isolated from the stromal vascular fraction of adipose tissue were found to be proliferating by the CFU assay, indicating replication capability within the cell population. Upon differentiation induction, the cultured cells at P2 were able to undergo differentiation toward multiple mesenchymal lineages, including adipogenesis, osteogenesis, and chondrogenesis (Fig. 1A). Moreover, the phenotypic analysis by flow cytometry suggested a relatively homogeneous population that expressed mesenchymal cell markers (CD44, CD73, CD90, and CD105), which was free of hematopoietic and endothelial markers (CD31, CD34, and

FIG. 1. Characterization of hASCs. (A) Histological detection of ASC multipotency: Adipogenesis by Oil Red staining (red), osteogenesis by Alizarin Red staining (red), and chondrogenesis by Safranin-O staining (red). (B) Flow cytometry analysis of the cell surface markers characteristic for mesenchymal stem cells and hematopoietic and endothelial cells. ASC, adipose-derived stem cell; hASCs, human ASCs. Color images available online at www.liebertpub.com/tea

CD45) (Fig. 1B).⁴⁰ Taken together, these results confirmed that the cell population used for subsequent experiments exhibited characteristics consistent with ASCs derived from subcutaneous liposiphate.

Effect of tendon ECM on ASC behavior in tissue culture plates

To investigate the role of tendon ECM in regulating ASC behavior, we extracted the soluble fraction of decellularized tECM from juvenile bovine Achilles tendons. As previously reported, the tECM solution prepared by this method is cell free and rich in noncollagenous ECM proteins, with a constant yield rate and consistent composition (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/tea).³⁵ tECM or Col I solution was used at 1 mg/mL as medium supplements at 10% v/v to treat ASCs on tissue culture plastic for up to 7 days. ASCs cultured with HBSS-supplemented medium or FBS-supplemented medium (10% v/v) were used as negative and positive control, respectively (Fig. 2A). At days 0, 3, and 7, cell density and metabolic activity were determined by DAPI staining and MTS assay. After 7 days of culture, higher cell density in tECM and FBS-treated groups was clearly visualized by DAPI staining (Fig. 2B). ASCs cultured with tECM for 7 days demonstrated a significantly higher metabolic activity than those with Col I, the most abundant structural ECM protein in tendon tissue, but slightly lower than those cultured with FBS (Fig. 2C).

The individual and combined effect of tECM and TGF- β 3 on hASC tenogenesis was analyzed at the mRNA and protein level. Three types of media (BM supplemented with 10% v/v FBS, Col I, or tECM) were prepared, with or without 10 ng/mL TGF- β 3, in which ASCs were cultured for up to 14 days (Fig. 3A). Real-time PCR analysis showed that treatment with tECM alone did not significantly increase the expression of SCX, the primary marker for early tendon differentiation. However, tECM combined with TGF- β 3 gave rise to significantly higher levels of SCX expression than all other groups tested at days 3 and 7 (Fig. 3B). The difference in SCX expression among the groups was confirmed at the protein level by Western blot

(Fig. 3D). Interestingly, unlike SCX, TNC expression was upregulated by tECM treatment in the absence of TGF- β 3 after 7 and 14 days of culture, while the combined treatment of tECM and TGF- β 3 led to the highest level of TNC mRNA among all groups (Fig. 3C). ASCs treated with TGF- β 3 in the presence of Col I again showed delayed upregulation of tendon marker compared to the tECM plus TGF- β 3 treatment (Fig. 3C). Taken together, these data suggested that tECM treatment in plate resulted in partial adoption of the tendon cell phenotype in the absence of other inductive cues, and enhanced tenogenesis of ASCs induced by TGF- β 3. Moreover, tECM exhibited no such inductive effect on chondrogenesis of ASCs in plate culture, suggesting a tissue-specific functionality of the tECM (Supplementary Fig. S2).

Characterization of the aligned microfiber scaffolds

We next attempted to generate a physical scaffold environment that mimics the structural feature of tendon. A microfibrillar PCL scaffold was fabricated by electrospinning (Fig. 4A). Aligned scaffolds exhibited highly uniaxial fiber orientation: most fibers were oriented at between 80° and 100° with respect to cross axis (Fig. 4D, F), in contrast to the random orientation seen in the nonaligned scaffolds (Supplementary Fig. S3). The mean thickness of aligned scaffold was $103.3 \pm 18.9 \mu\text{m}$ ($n=30$). No significant difference in fiber diameter was found between aligned and random scaffold (Fig. 4E, $1.26 \pm 0.51 \mu\text{m}$ vs. $1.29 \pm 0.34 \mu\text{m}$).

When tension was applied in the direction of fibers, the aligned scaffolds displayed 2.5-fold higher tensile strength compared to the randomly oriented scaffolds (Fig. 4B, C). Anisotropy of the aligned scaffold was confirmed by tensile testing along two planes: the elastic modulus along the axis of fibers (longitudinal) was 10-fold higher than that in the perpendicular direction (cross), as expected from the uniform orientation of fibers (Fig. 4B, C). hASCs seeded on aligned scaffolds adopted elongated morphology and were orientated in the direction of fibers after 3 days of culture. In contrast, hASCs seeded on random scaffolds exhibited polygonal shape without uniformity in orientation (Supplementary Fig. S3).

FIG. 2. Assay of hASC proliferation in plate cultures. (A) hASCs were plated on 2D tissue culture plastic and treated with BM containing one of the following supplements at 10% v/v: HBSS, 1 mg/mL tECM solution (tECM), 1 mg/mL collagen type I solution (Col I), or FBS. (B) After 7 days of culture, DAPI nuclear staining showed high cell density in tECM- and FBS-treated groups. (C) MTS assay revealed an elevated cellular metabolic activity in tECM- and FBS-treated groups. * $p < 0.05$; ** $p < 0.01$; $N=3$. BM, basal medium; 2D, two dimensional; FBS, fetal bovine serum; HBSS, Hanks' Balanced Salt Solution; tECM, tendon extracellular matrix. Color images available online at www.liebertpub.com/tea

FIG. 3. Tenogenesis of hASCs in plate cultures. (A) hASCs were plated on 2D tissue culture plastic, and treated with or without 10 ng/mL TGF- β 3 in BM containing one of the following types of supplements at 10% v/v: FBS, 1 mg/mL tECM solution (tECM), or 1 mg/mL collagen type I solution (Col I). (B, C) Real-time PCR analysis of (B) scleraxis (SCX) and (C) tenascin-C (TNC) expression levels. tECM treatment up-regulated SCX expression in the presence of TGF- β 3, and increased TNC expression with or without TGF- β 3. (D) Western blot assay showed consistent difference in SCX protein. * p < 0.05; ** p < 0.01; N = 3. PCR, polymerase chain reaction; TGF, transforming growth factor. Color images available online at www.liebertpub.com/tea

Effect of tendon ECM on ASC behavior in scaffolds

ASCs seeded on aligned scaffolds were treated for 1 week in BM supplemented with 10% v/v FBS, Col I, or tECM. No observable difference in cell shape was found among groups: most ASCs cultured on the aligned scaffolds were elongated and aligned in the direction of the surrounding fibers regardless of treatment method, as indicated by immunofluorescent staining of F-actin (Fig. 5A). Nevertheless, the cell metabolic activity differed greatly among groups: the tECM-treated group exhibited an enhanced metabolic activity compared to Col I- or HBSS-treated group. On day 7, the metabolic activity of tECM-treated cells remained significantly higher than Col I- or HBSS-treated cells, and was comparable to FBS-treated cells (Fig. 5B).

Given the established positive influence of fiber alignment on tenogenic differentiation, analysis of gene expression and matrix deposition was only performed on aligned microfibrillar scaffolds. When cultured in BM with TGF- β 3 and 2% FBS, SCX expression in hASCs was increased by the presence of tECM on days 3, 7, and 14, whereas in the Col I group, the upregulation in SCX was not seen until day 14 (Fig. 6A). Similarly, tECM supplementation resulted in significantly higher TNC levels compared to controls at all three time points tested. In contrast, Col I treatment did not significantly upregulate TNC level, although there was a trend of increase (Fig. 6A). To further investigate the extent of tECM-mediated tenogenesis, we analyzed the presence of Tnmd, a tendon-specific membrane glycoprotein found in the late phase of differentiation, by immunofluorescent staining. Compared to other treatment groups, an evidently higher density and intensity of staining for Tnmd (green) was seen in the tECM-treated group (Fig. 6B).

We next examined the influence of tECM on the synthesis and organization of collagen, the primary structural protein of tendon tissue. Cells seeded on PCL scaffolds were treated with L-ascorbate 2-phosphate for 3 weeks to accelerate ECM synthesis. Immunofluorescent staining of Col I on the surface of scaffolds qualitatively confirmed the presence of newly synthesized matrix in both the control and tECM-treated groups, revealing arrays of collagen fibrils extended in the direction of the PCL fibers. Interestingly, denser collagen fibrils were found on scaffolds treated with tECM (Fig. 7A) compared to those treated with FBS only, while the amount of collagen presented by tECM alone on the acellular scaffolds was negligible (Fig. 7A). This was expected as tECM is composed of a high ratio of noncollagenous proteins (Supplementary Fig. S1).

The observed difference in collagen content was confirmed quantitatively by the hydroxyproline assay. In the presence of tECM, hASCs produced a 2.4-fold higher amount of collagen per scaffold than controls (86.54 ± 7.46 μ g/scaffold vs. 36.79 ± 2.49 μ g/scaffold). This pattern persisted when collagen content was normalized against double-stranded DNA (dsDNA) content, with a 1.8-fold higher collagen content per unit weight of dsDNA (145.05 ± 17.46 μ g/ μ g vs. 80.02 ± 17.77 μ g/ μ g DNA) in the tECM-treated group versus control group.

Discussion

The goal of this study was to investigate the modulatory effect of soluble tECM on known biochemical (i.e., TGF- β 3)

FIG. 4. Characterization of PCL scaffold. (A) Gross appearance of scaffold. (B) Strain–stress curves indicated dramatic difference in tensile strength between aligned and random scaffolds; anisotropy in tensile strength was noticed in aligned scaffolds (Aligned longi vs. Aligned cross). (C) In aligned scaffolds, the elastic modulus was the highest in longitudinal direction (Aligned longi vs. Aligned cross), which was also significantly higher compared with randomly oriented scaffolds (Random longi, Random cross). * $p < 0.05$; $N = 3$. (D) SEM image of aligned microfibrillar PCL scaffolds. (E) Distribution of fiber diameters. (F) Most fibers in the aligned scaffold were oriented at between 80° and 100° with respect to cross axis. PCL, poly- ϵ -caprolactone; SEM, scanning electron microscope. Color images available online at www.liebertpub.com/tea

and biophysical cues that promote tendon differentiation, to advance the development of functional engineered tendon grafts. Human ASCs were prepared and characterized, and individual and combined effects of tECM and TGF- β 3 on cell behavior, including proliferation and differentiation, were examined. We found that (1) tECM enhanced TGF- β 3-induced tenogenesis of hASCs in both plate and scaffold culture, and (2) tECM favorably modulated matrix deposition and organization by hASCs seeded on microfibrillar scaffolds.

In the native tendon microenvironment, dense ECM surrounds the tendon cell. TSPCs exhibited impaired proliferative and tenogenic potential in the absence of critical ECM

proteins,³⁰ and showed reduced TNMD expression when seeded on tissue culture plastic than on tendon ECM.⁴¹ The tECM prepared in our study increased hASC proliferation and TNC expression when used as a culture supplement in the absence of other inductive cues. Expression of both TNC and SCX was further enhanced when treated concomitantly with TGF- β 3, as shown in both plate and scaffold cultures. Our findings indicate that the regulatory effects of tECM on tendon cell behavior also apply to ASCs. Consistent with our results, Little *et al.*⁴² found increased proliferation and partial adoption of tendon phenotype by ASCs seeded on acellular tendon/ligament matrix. The tECM contains not only collagen but also a number of

FIG. 5. Behavior of hASCs seeded on aligned scaffolds. Cultures were treated with BM containing 10% v/v FBS, Col I, or tECM. (A) After 3 days of culture, most ASCs cultured on aligned scaffolds were elongated and aligned regardless of treatment methods. Green: F-actin; blue: DAPI. (B) MTS assay showed an enhanced cellular activity in the tECM-treated group compared to Col I- or HBSS-treated groups, which was comparable to FBS-treated cells at day 7. * $p < 0.05$; $N = 3$. Color images available online at www.liebertpub.com/tea

FIG. 6. Tenogenic differentiation of hASCs seeded on aligned scaffolds. (A) Real-time PCR assay showed that scleraxis (SCX) and tenascin-C (TNC) expression was significantly increased in the presence of tECM. (B) Immunofluorescence showed that staining for tenomodulin (Tnmd) was denser and more intense (green) in tECM-treated group. * $p < 0.05$; ** $p < 0.01$; $N = 4$. Color images available online at www.liebertpub.com/tea

noncollagenous proteins, including fibronectin, fibromodulin, biglycan, and decorin, all of which are known to regulate MSC activities, such as adhesion, proliferation, stemness, and differentiation.^{21,43–45} In addition, a variety of growth factors, such as TGF-β, IGF-1, vascular endothelial growth factor (VEGF), and connective tissue growth factor (CTGF), were found embedded in the decellularized tECM.⁴⁶ How these bioactive molecules, along with other yet-to-be-identified

components in the tECM, contribute to the bioactivity of the tECM in our results remains to be investigated. The enhancement of ASC tenogenesis by tECM combined with TGF-β3 treatment exemplifies the complexity in the fine control of tissue differentiation: the tECM may serve as a reservoir of signals by itself or, not mutually exclusively, exert a regulatory effect on exogenous inductive cues.²¹ Moreover, we found little inductive effect of tECM on chondrogenesis.

FIG. 7. Matrix deposition by ASCs cultured on aligned scaffolds. (A) Immunofluorescent staining for collagen type I (green) found denser collagen fibrils deposited by cells treated with tECM compared to tECM-free group. (B) Hydroxyproline assay showed higher collagen content in the tECM-treated group. Collagen content of each group was normalized to that of the corresponding cell-free group. ** $p < 0.01$; $N = 3$. Color images available online at www.liebertpub.com/tea

Substantially lower SOX9 expression in two-dimensional culture of ASCs treated with TGF- β 3 in BM, regardless of medium supplement, was found when compared to standard chondrogenic treatment for pellet culture. However, despite all the aforementioned findings, further mechanistic studies are needed to elucidate whether the observed protenogenic effect of tECM is attributable to its unique composition or a less-specific feature. For instance, we cannot exclude the possibility that the observed difference is due to changes in osmolarity. Mass spectrometry-based proteomic technologies for high-throughput ECM composition analysis and ECM microarray platforms will be employed in future studies to identify the specific combination of proteins that impart the unique tenogenic effect of tECM.

To date, ASCs are widely used for scaffold repopulation and as a means to improve vascularization, matrix deposition, and implant integration,^{47–50} whereas optimization of tendon-specific differentiation of seeded ASCs remains elusive. On one hand, ASCs isolated from a variety of animal species have been reported to upregulate tenogenic markers *in vitro* under specific treatment,^{13,14,18,20,51} suggesting the tenogenic potential of ASCs. On the other hand, Eagan *et al.* questioned the suitability of hASCs for tendon tissue engineering and reported the lack of any significant and consistent upregulation in the expression of COL I, TNC, or SCX, in hASCs treated for up to 4 weeks with TGF- β 1 or IGF-1.⁵² In addition, hASCs showed lower SCX expression compared to TSPCs when cultured *in vitro*.⁵³ Incorporating tissue-specific ECM molecules into differentiation protocols represents an alternative approach to develop a robust tenogenesis strategy for ASCs to better exploit the regenerative potential of ASCs applied to tendon tissue engineering. In future studies, more tendon phenotypic markers, such as Mohawk (MKX), and ECM protein encoding genes should be analyzed to advance our knowledge in the protenogenic effect of tECM.⁵⁴

As noted above, the scaffold is another key component in tendon tissue engineering by creating proper microenvironment for mechanical support and tissue regeneration. Therefore we prepared electrospun, aligned fibrous PCL scaffolds consisting of microfibers ($\sim 1.3\ \mu\text{m}$), to simulate the size of collagen fibrils in natural tendon tissue ($1\text{--}300\ \mu\text{m}$).⁵⁵ The diameter of fibers has been found to have an influence on seeded cells: compared with nanofibers, microfibers promoted the expression of phenotypic markers of tendon fibroblasts, possibly due to the resemblance of the healthy, mature matrix with micron-sized collagen bundles.^{37,56}

A promising future prospect based on this work is the application of dynamic/cyclic stretch to cell-seeded scaffolds to simulate the loading of native tendon during motions.⁵⁷ Moreover, the PCL scaffold used in this study may be further modified to address some of these limitations. For instance, although aligned scaffold demonstrated anisotropy similar to that of tendon tissue, improvements in tensile strength ($\sim 26\ \text{MPa}$) are clearly needed if intended to be used as a clinical tendon graft ($\sim 550\ \text{MPa}$).⁵⁸ In addition, functional *in vivo* implant likely requires a scaffold that sufficiently retains bioactive agents. For this purpose, scaffold surface modification may be carried out to immobilize bioactive macromolecules contained in tECM.⁵⁹ Likewise, scaffold thickness and porosity may need to be improved to allow sufficient cell infiltration.⁶⁰

We examined the influence of tECM on collagen synthesis and organization by hASCs seeded on aligned scaffolds. Consistent with previous studies, collagen fibrils were aligned in the direction of the PCL fibers.⁶⁰ More abundant and homogenous distribution of collagen fibrils were found in cells treated with tECM. The tECM-induced enhancement of collagen fibrillogenesis may be due to the bioactivity of small leucine-rich proteoglycans (SLRPs) and glycoproteins in tECM, such as decorin, biglycan, lumican, and collagen oligomeric matrix protein (COMP). These proteins are able to bind noncovalently to collagen molecules at specific sites in the gap region of fibrils and therefore facilitate collagen fibrillogenesis and stabilization.^{34,61–63} Acellular scaffolds possessed negligible collagen content when treated with tECM. This confirms that the tECM acts by promoting collagen production in ASCs rather than by merely presenting collagen to the scaffold.

Conclusions

In this study, a bioactive, soluble fraction of tECM was prepared, characterized and incorporated into growth/differentiation medium to treat ASCs. We demonstrated that tECM treatment enhanced the proliferation and tenogenic capacity of hASCs. Moreover, when cultured on scaffolds that mimic the architecture of native tendon tissue, hASCs treated with tECM exhibited an increased Col I matrix synthesis and improved organization. These findings provide new insights into the role of tissue-specific ECM in guiding site-appropriate cell responses in terms of connective tissue differentiation and healing. In addition to serving as an *in vitro* differentiation model, the design attributes of the scaffold culture system developed in this study are applicable to functional tendon tissue engineering that aims at simultaneous induction of phenotypic markers and enhanced matrix deposition. Our findings highlight the importance of reproducing the native tissue microenvironment as a design principle for eliciting desired cellular responses for tissue regeneration.

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Disclosure Statement

No competing financial interests exist.

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