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Living Nanofiber Yarn-Based Woven Biotextiles for Tendon Tissue Engineering Using Cell Tri-Culture and Mechanical Stimulation

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Abstract

Non-woven nanofibrous scaffolds have been developed for tendon graft application by using electrospinning strategies. However, electrospun nanofibrous scaffolds face some obstacles and limitations, including suboptimal scaffold structure, weak tensile and suture-retention strengths, and compact structure for cell infiltration. In this work, a novel nanofibrous, woven biotextile, fabricated based on electrospun nanofiber yarns, was implemented as a tissue engineered tendon scaffold. Based on our modified electrospinning setup, polycaprolactone (PCL) nanofiber yarns were fabricated with reproducible quality, and were further processed into plain-weaving fabrics interlaced with polylactic acid (PLA) multifilaments. Nonwoven nanofibrous PCL meshes with random or aligned fiber structures were generated using typical electrospinning as comparative counterparts. The woven fabrics contained 3D aligned microstructures with significantly larger pore size and obviously enhanced tensile mechanical properties than their nonwoven counterparts. The biological results revealed that cell proliferation and infiltration, along with the expression of tendon-specific genes by human adipose derived mesenchymal stem cells (HADMSC) and human tenocytes (HT), were significantly enhanced on the woven fabrics compared with those on randomly-oriented or aligned nanofiber meshes. Co-cultures of HADMSC with HT or human umbilical vein endothelial cells (HUVEC) on woven fabrics significantly upregulated the functional expression of most tenogenic markers. HADMSC/HT/HUVEC tri-culture on woven fabrics showed the highest upregulation of most tendon-associated markers than all the other

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mono- and co-culture groups. Furthermore, we conditioned the tri-cultured constructs with dynamic conditioning and demonstrated that dynamic stretch promoted total collagen secretion and tenogenic differentiation. Our nanofiber yarn-based biotextiles have significant potential to be used as engineered scaffolds to synergize the multiple cell interaction and mechanical stimulation for promoting tendon regeneration.

Graphical abstract

Keywords

electrospinning; woven fabric; fiber alignment; cell co- and tri-culture; mechanical stimuli; vascularization

1. Introduction

Tendon-related injuries or diseases are common causes of impaired joint function, with more than 30 million human tendon grafting procedures performed annually worldwide [1]. Tendon grafts are essential for the treatment of various tendon-related conditions due to the inherently poor healing capacity of tendon tissues [2, 3]. Current clinical treatments for the replacement of damaged tendons involve autografts, allografts, and xenografts [4], which still present inevitable disadvantages, such as donor site morbidity, inflammatory response, potential disease transmission, and limited availability of appropriate tendon tissues that closely mimic the size, shape, and physiological characteristics [5]. Thus, there exists a need for tissue-engineered tendon substitutes that better restore the properties of native tissue for improved surgical reconstruction and repair. Recently, significant progress has been made by introducing innovative bio-scaffolds, stem cells, biological growth factors, co-culture techniques, and mechanical stimulus in tendon tissue engineering [6–9]. However, major challenges remain, and further improvement is still needed.

One important aspect for tendon tissue engineering is the appropriate design of fibrous scaffolds that mimic the fibrillary architecture and the mechanical and functional characteristics of native tendon extracellular matrix (ECM) [10, 11]. Such fibrous constructs can potentially be engineered and generated using various fiber-forming techniques and textile processes [12, 13]. Traditional strategies for creating fibrous scaffolds include two steps, in which micrometer-scale fibers are generated through the utilization of wet spinning and dry spinning, and then are assembled into 2D or 3D constructs with tunable properties by textile technology [14]. Many studies have demonstrated that such porous, textile-based scaffolds prepared from weaving, knitting, or braiding techniques uniquely combine

precisely controlled size, shape, and excellent load-bearing and suture-retention strengths for tendon regeneration [15–18]. However, these textiles were usually generated from micro-sized fibers, which differed from the inherent nanoscale fiber organization of native ECM, resulting in reduced cell interaction and low cell stimulation for tissue regeneration [19]. To mimic the fiber scale and topographical cues of native tendon ECM, over the past decade, nanofibrous scaffolds have been fabricated by electrospinning technique [20–23]. Electrospinning can produce fibers with diameters two to three orders of magnitude smaller than those formed by traditional fiber-fabricating processes [24–26]. Electrospun nanofibrous scaffolds or nano/micro hybrid constructions have been demonstrated to promote cell adhesion, proliferation, and differentiation for tendon tissue engineering applications [27–30]. However, such non-woven constructs also have several limitations that prevent broader clinical application [31–33]. The tightly packed fibrous structure, small pore sizes existing between fibers (usually below 3 μm), and inferior controllability of fiber organization may limit cell growth, migration, and infiltration into the inner layers, and are not beneficial for the transportation of oxygen and nutrients throughout the implant site and removal of metabolic waste during tissue regeneration. In addition, electrospun nanofibrous scaffolds possess poor suture-retention strength for surgical reconstruction and usually have insufficient mechanical properties to support primary tendon repair. While some post-treatments and modification, like salt leaching/gas foaming technique, ultrasonication, and freeze drying processes were developed to enhance cellular ingrowth into these scaffolds, achieving high cellular density and infiltration and excellent mechanical properties remains challenging [34, 35].

Another important aspect for tendon tissue engineering is to choose an appropriate cell source and culture system to accelerate the regeneration of the engineered tendon [36, 37]. Mesenchymal stem cells (MSC) are an attractive cell source due to their availability and differentiation capacity for tendon regeneration [38–40]. However, there is still a need to effectively induce and accelerate MSC differentiation and maturation towards tenocytes. Previously published literature has described potential strategies to promote tenogenic differentiation of MSC by using growth factors [41–43] or an MSC/tenocytes co-culture technique [44–46]. Several studies have also shown that mechanical stress is a crucial factor for cellular/ECM alignment and tendon-related ECM protein production [47–49]. Moreover, increasing evidence has shown that the lack of vascularization within engineered tendon grafts can inhibit early tenogenesis and host integration, thus preventing the healing of tendon damage or rupture [50–52].

To overcome the aforementioned challenges, we fabricated polycaprolactone (PCL) nanofiber yarns by utilizing a novel electrospinning approach [19, 53]. The PCL nanofiber yarns were further employed as building blocks for generating nanofibrous, woven textiles for tendon regeneration. This strategy was expected to combine the favorable properties of electrospun nanofibrous scaffolds and textile-based constructs. We fabricated electrospun random and aligned nanofibrous scaffolds, along with nanofiber-yarn based, woven biotextiles. We systematically compared their structures and mechanical properties and compared human adipose derived mesenchymal stem cells (HADMSC) and human tenocytes (HT) response to different scaffolds. We hypothesized that the nanofiber-constructed woven fabrics with aligned microstructures, large pores, better mechanical

stability and flexibility can better promote cell growth and support tenogenic differentiation of MSC. We also co-cultured HADMSC with HT or human umbilical vein endothelial cells (HUVEC), and tri-cultured these three cell types. Then, we implemented a dynamic stretch bioreactor to accommodate the tri-cultured woven biotextile constructs.

2. Materials and methods

2.1 Fabrication of electrospun PCL nonwoven meshes and nanofibrous woven fabrics

We fabricated PCL (Mw = 80 kDa, Sigma, USA) based woven fabrics, and also manufactured electrospun random and aligned meshes as control groups. To fabricate random and aligned PCL nanofibrous nonwoven meshes, a typical electrospinning setup was employed (Fig. 1A). PCL was dissolved in trifluoroethanol (TFE, Sigma, USA) at 10 w/v% as electrospinning solution. The solution was loaded into a syringe, charged with a voltage of 12 kV and fed at a rate of 0.8 mL/h, using a syringe pump. A rotating target located at a distance of 20 cm from the blunt tip of the nozzle was utilized to collect the nanofibers. A low linear velocity of the rotating target (~0.1 m/s) was employed to produce electrospun random nanofibrous meshes, whereas a relatively high linear velocity of ~8 m/s was used to fabricate aligned nanofibrous scaffolds. The morphology of fabricated randomly-oriented and aligned nanofibrous PCL meshes was shown in Fig. 1B and C.

A novel electrospinning setup designed by our groups was employed to continuously fabricate PCL nanofiber yarns as previously published (Fig. 1D) [19, 53]. Briefly, our electrospun nanofiber yarn-forming system employed two oppositely placed metal needles applied with positive and negative voltages to generate nanofibers. Two syringes connected with two digitally controlled syringe pumps were used to supply polymer solutions for the two metal needles. A rotating neutral metal disc (NMD) and a static neutral hollow metal rod (NHMR), placed oppositely in the middle of two needles, were utilized to collect nanofibers and further process them into yarns. The obtained nanofiber yarns then passed through the inner part of the NHMR by the guidance of metal wire and were gathered on the rotating take-up roll. PCL solution was prepared as described for the electrospinning of nonwoven PCL meshes. For the continuous fabrication of PCL nanofiber yarns, the distance between two needles, distance between NMD and NHMR, applied voltages of two needles, solution flow rate of both needles, rotation speed of NMD, and linear velocity of the take-up roll were maintained at 20 cm, 7 cm, ± 9 kV and 1 ml/h, 250 r/min and 1.5 m/min, respectively. With this system, we can easily produce PCL nanofiber yarns in a continuous manner (Fig. 1E), which possess an adequate tenacity capable for the fabrication of woven fabrics. We further employed an electronic jacquard machine to manufacture nanofibrous woven PCL fabrics. PCL nanofiber yarns were used as the weft yarns to pass through poly (L-lactic acid) (PLA) multifilaments (the warp yarns, Shaoxing Zhongfangyuan Co., Ltd, China), which have sufficient mechanical properties for the weaving process, to form the plain weaving structure (Fig. 1H). The filament diameter was about 15 μ m and the filament number was around 20 for each PLA multifilament yarn. The densities of yarns were 55 ends/cm for warp yarns (PLA multifilaments) and 100 picks/cm for weft yarns (PCL nanofiber yarns) respectively in woven fabric.

2.2 Morphological and mechanical characterization of the scaffolds

The morphology of PCL nanofiber yarns and nanofibrous scaffolds were examined via a scanning electron microscope (SEM, FEI Quanta 200, Japan) after gold coating. The fiber diameter, yarn diameter, and angle distribution (relative to the horizontal axis) of the specimens ($n = 3$) were determined from the SEM images using Image J software (National Institutes of Health, USA). The angle distribution and mean diameter were determined from more than 100 randomly selected nanofibers and yarns in the SEM images (Fig. 1K). The mean flow pore size and pore size distribution of the electrospun meshes and woven fabrics were characterized by using a capillary flow porometer (POROLUX TM100FM, IB-FT, Germany) based on the wet/dry flow method. And the results were calculated and obtained by using the LabVIEW software. Uniaxial tension tests were performed using an Instron series testing system (ITW Test & Measurement, USA). The samples were preloaded to 0.5 N to eliminate the “toe” region in the stretch curve and tested with a speed of 30 mm/min. The tests were performed with a gauge length of 30 mm at a constant rate of displacement until failure occurred. The cross-sectional areas of different scaffolds were estimated from measurements made using a digital caliper. The thickness of electrospun random scaffolds was approximately 160 μm , whilst both electrospun aligned meshes and woven fabrics possessed the thickness of about 180 μm . The Young’s modulus at 5%–10% strain, ultimate stress, and strain were determined.

2.3 Cell culture, seeding and differentiation

Established primary human adipose derived mesenchymal stem cells (HADMSC, Lonza, USA) were cultured in growth medium (GM) consisting of DMEM/F12 (Invitrogen, USA), 10% fetal bovine serum (FBS, Invitrogen, USA) and 1% penicillin/streptomycin (P/S, Invitrogen, USA). Tendon differentiation medium (TDM) containing DMEM/F12 medium, 2% FBS, 20 ng/ml Transforming growth factor beta (TGF β)3 (PeproTech, USA) was used for tenogenic differentiation of HADMSC. Human tenocytes (HT) were isolated from the long head of the biceps tendon when the patient had shoulder surgery as approved by the institutional review board at the University of Nebraska Medical Center (#280-16-EP). The HT from three donors (average 65-year old) and were pooled together. HT were cultured in tenocyte growth medium (TGM) containing DMEM medium (Invitrogen, USA), 10% FBS, 1% P/S. Human umbilical vein endothelial cells (HUVEC, Lonza, USA) were cultured in endothelial growth medium (EGM) (EGM-2 BulletKit, Lonza, USA). All of the cells were used at passages 4–6 and cultured in 5% CO_2 at 37 $^{\circ}\text{C}$.

All nanofibrous scaffolds were cut into 1.2 cm $\times$ 1.2 cm squares. Before cell seeding, fibrous scaffolds were sterilized with UV for 2 h, submerged in 70% (v/v) ethanol overnight, washed twice in phosphate buffered saline solution (PBS), and then submerged in GM overnight. Cells were seeded at a density of 1×10^5 cells per fibrous scaffold. For cell co-culture and tri-culture, HADMSC: HT (1:1), HADMSC: HUVEC (4:1), and HADMSC: HT: HUVEC (2:2:1) were cultured respectively in the corresponding mixed media (TDM:TGM=1:1; TDM:EGM=4:1; TDM:TGM:EGM=2:2:1). Medium was replaced every 2 days.

2.4 Dynamic culture of engineered tendon constructs

To investigate the effects of dynamic culture on the tenogenic differentiation, HADMSC: HT: HUVEC (2:2:1) were seeded on PCL nanofibrous woven scaffolds, and the constructs were statically cultured for 2 days. The constructs were then mounted by clamping the two ends, and were further mechanically stimulated in the MechanoCulture T6 Mechanical Stimulation System (CellScale biomaterials testing, Canada) for another 12 days (Fig. S1). The cyclic uniaxial strain with 4% elongation along the direction of PCL nanofiber yarns in the woven fabrics and a frequency of 0.5 Hz were implemented for 2 hours per day for a total of 12 days. Control groups (i.e., not subject to cyclic loading) were cultured for the whole 14 days under static culture.

2.5 Cell viability, morphology and proliferation

The viability and morphology of HADMSC or HT seeded on different scaffolds were investigated by Live/Dead assay (Invitrogen, USA) after 14 days of culture in TDM or TGM as previously described [54]. For the fluorescence imaging of living and dead cells, a confocal laser scanning microscope (CLSM, LSM 710, Carl Zeiss, Germany) was used. An MTT assay was conducted to evaluate the cell proliferation of HADMSC or HT cultured on different scaffolds on days 7 and 14 [19].

2.6 Histological staining and immunofluorescent staining

To quantify the extent of cellular infiltration, HADMSC-seeded scaffolds were cultured in TDM through 14 days, then fixed in 4% paraformaldehyde for 4 hours. After soaking in 20% sucrose overnight at 4 °C, samples were embedded in OCT solution overnight (Fisher Healthcare, USA), and sections of 10 µm thickness were obtained using a cryotome (CM1850, Leica, Germany). Sections were stained with hematoxylin and eosin (H&E). For immunofluorescent staining, cell-seeded scaffolds were fixed in 4% paraformaldehyde, permeabilized in 0.2% Triton X-100, and then blocked with 1% bovine serum albumin (BSA) overnight at 4 °C. The cell-constructs were then treated with primary antibodies to tenomodulin (TNMD, 1:50, Abcam, USA), collagen type I (COL1, 1:100, Santa Cruz Biotechnology, USA), CD31 (1:100, Cell Signaling, USA), or von Willebrand factor (vWF, 1:100, Sigma, USA) overnight at 4 °C. After incubating with secondary fluorescent antibodies for 2 hours, nuclear counterstaining (Draq 5, 1:1000, Thermo Scientific, USA) was conducted for 30 minutes at room temperature. Zeiss 710 CLSM was employed to image the stained samples.

2.7 Total collagen content measurement

On day 14, cell laden constructs were washed in PBS and dried using a vacuum freeze drier (LABCONCO, USA). After the measurements of dry weight, total collagen content was determined using a hydroxyproline assay [55]. The collagen values were calculated assuming 12.5% of collagen is hydroxyproline.

2.8 Western blot analysis

Western blot was performed to measure the protein expression level of TNMD and COL1 in the dynamic culture group and the corresponding control group after 14-day culture. Lysates

of the cell-scaffolds were prepared by extracting proteins with the lysis buffer (RIPA and PMSF, Thermo Scientific, USA). The protein concentrations were determined using the BCA protein assay kit (Thermo Scientific, USA). The tissue lysates were electrophoresed using 10% SDS-PAGE and transferred to a PVDF membrane (Bio-Rad, USA). The detected proteins were probed with fluorescent antibodies against TNMD, COL1 and β -actin (Cell Signaling, USA), followed by the addition of the secondary antibodies (Cell Signaling, USA) at 37 °C for 1 h. The membrane was washed three times and the protein bands were visualized using a scanner (Bio-Rad, USA). The target protein level was quantified and normalized to β -actin bands by densitometry in Photoshop 8.0 (Adobe, USA) and Image J (NIH, USA).

2.9 RNA isolation and qPCR

After 14 days of culture, total cellular RNA was isolated from cell-scaffolds through the sequential use of QIA-Shredder and RNeasy mini-kits (QIAgen, USA). 500 μ g of RNA was reverse transcribed into cDNA by using iScript cDNA synthesis kit (BioRad Laboratories, USA). A quantitative real-time polymerase chain reaction (qPCR) was performed for 40 cycles in a StepOnePlus™ Real-Time PCR System (Thermo Scientific, USA) using SsoAdvanced SYBR Green Supermix (Bio-Rad, USA). The level of expression of each target gene was normalized with 18S rRNA by using a comparative Ct (2^{-C_t}) method. All primers used in this study are listed in Supplementary Table S1.

2.10 Statistical Analysis

All quantitative data were expressed as mean $\pm$ standard deviation (SD). Pairwise comparisons between groups were conducted using ANOVA with Scheffé post-hoc tests in statistical analysis. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Scaffold fabrication

The diameters for as-prepared PCL yarn and its inner fiber were $208.5 \pm 13.7 \mu\text{m}$, and $460.2 \pm 213.0 \text{ nm}$, respectively (Fig. 1F and G). PCL nanofiber yarns, with high weaving density, were further woven repetitively over and under PLA multifilaments, with low density, to form a plain weave structure (Fig. 1H). Only aligned PCL nanofiber yarns could be observed in the obtained woven fabrics, and almost no PLA multifilaments could be observed at right angles (Fig. 1I and J). The woven fabrics are structurally stable and can be easily manipulated during and after weaving. The diameters of the PCL nanofibers of three different fibrous scaffolds were comparable ($484.8 \pm 345.7 \text{ nm}$ in random mesh vs. $452.3 \pm 218.4 \text{ nm}$ in aligned mesh vs. $460.2 \pm 213.0 \text{ nm}$ in weave fabric). The randomly-oriented meshes showed approximately equal distributions at all angles (Fig. 1L). In contrast, most of the fibers in the aligned meshes, most of the fibers in the PCL nanofiber yarns, and most of PCL yarns in the woven fabrics had angles from 80° to 100° with respect to the horizontal axis.

3.2. Woven fabrics displayed significantly larger pore size and enhanced mechanical strength

The pore size distribution measurement was shown in Fig. 2A, and the mean pore size was calculated and presented in Fig. 2B. Woven fabrics possessed a mean pore size of $12.2 \pm 1.1 \mu\text{m}$, considerably larger than that of electrospun random ($2.1 \pm 0.2 \mu\text{m}$) and aligned ($1.4 \pm 0.1 \mu\text{m}$) nanofibrous scaffolds. Uniaxial tensile testing results of three different scaffolds were shown in Fig. 3A–D. The results demonstrated that the mechanical properties of the scaffolds were significantly dependent on the fiber alignment and the overall scaffold structure. Both the woven fabrics and the electrospun aligned meshes possessed notable anisotropic mechanical characteristics. By contrast, homogeneous mechanical properties were observed for electrospun random meshes. Woven fabrics at both the parallel and perpendicular to the direction of PCL nanofiber yarn alignment exhibited significantly higher Young's moduli and ultimate tensile strengths, as compared to electrospun random and aligned meshes. The tensile strength of the woven fabrics at the parallel direction of PCL nanofiber yarn alignment was approximately 5 times higher, and the Young's modulus value was more than 10 times higher than those of the random nanofibrous scaffolds at a random direction (Fig. 3B and C). Meanwhile, the tensile strength of the woven fabrics along aligned direction was more than 2 times higher, and the Young's modulus value was approximately 5 times higher than those of the aligned nanofibrous scaffolds. The ultimate elongation of woven fabrics and electrospun aligned meshes along the aligned direction is comparable, but significantly lower than that of random meshes (Fig. 3D).

3.3. Woven fabrics promoted HT alignment, proliferation, and enhanced their tenogenic phenotype

We first seeded HT on electrospun random and aligned meshes, and woven fabrics, then cultured them in TGM for 14 days to determine how the matrix topography and structure affected their behaviors. Although the HT were isolated from elder donors, the cells had very decent proliferation rate with double time about 48 h and they showed normal tenocyte biomarker expression in the 2D culture and on the scaffold samples. A live/dead assay was employed to measure the cell viability and morphology (Fig. 4A). HT cultured on all three different scaffolds showed high viability (>95%) in TGM throughout 14 days of culture. However, cell morphology varied in response to different scaffold topography. The cells on the random meshes exhibited a haphazard distribution with irregular morphology. In contrast, the cells displayed a spindle-shaped morphology extending parallel to the alignment direction of substrate nanofibers on both electrospun aligned meshes and woven fabrics, demonstrating that HT could sense the nanofiber-based topographic features in the scaffolds and regulate their morphology by contact guidance phenomenon. The cellular metabolism on the woven fabrics was confirmed to be remarkably higher at each time point by MTT assay, compared to the electrospun random or aligned mesh counterparts (Fig. 4B).

The phenotypes of HT were investigated using immunofluorescent staining for tendon-related protein markers (Fig. 5A). All three of the nanofibrous scaffolds expressed TNMD and COL1, which seemingly indicated no significant differences. To further evaluate HT phenotypic markers, tenocyte-specific gene expression was quantified by qPCR (Fig. 5B). Scleraxis (SCX) is a necessary transcription factor for tenogenesis, and tenascin C (TNC) is

an early marker in embryonic tendon [67]. COL1 and COL3 are the primary matrix components of native tendons, and TNMD is recognized as late marker for the mature tenogenic phenotype [68]. HT seeded on woven fabrics and electrospun aligned scaffolds displayed comparable expression of COL3 gene, which was markedly higher than that in the electrospun random scaffolds. Moreover, the expressions of other tenocyte-related genes, including TNC, COL1, and TNMD, were significantly upregulated in the woven fabric group, compared to the electrospun random and aligned scaffold groups.

3.4. Woven fabrics promoted HADMSC alignment, proliferation, infiltration and tenogenic differentiation

We further used HADMSC to determine how these three nanofibrous scaffolds with different architectures affect tenogenic differentiation. After 14-day culture, HADMSC cultured on all the scaffolds showed high viability (>95%) in TDM (Fig. 6A). The attached cells aligned along the direction of PCL nanofibers in both woven fabrics and electrospun aligned meshes. Similarly, MTT assay results showed that the number of cells cultured on all of these three scaffolds increased from day 7 to day 14 (Fig. 6B). Importantly, the cell proliferation rate on the woven fabrics was significantly higher than those on the random and aligned meshes at days 7 and 14. To evaluate the extent of cell migration into the scaffolds, H&E staining of the HADMSC-seeded scaffold cross sections was conducted. After a 14-day culture, the cells infiltrated through the whole scaffold depth from the surface of the woven fabrics, whereas no obvious cell infiltration was observed in both the random and aligned meshes during the same culture period (Fig. 6C). For tenogenic differentiation, robust expression of TNMD and COL1 were detected by HADMSC cultured on all the three nanofibrous scaffolds (Fig. 7A). Importantly, the fiber alignment in the woven fabrics and electrospun aligned meshes could guide the TNMD and COL1 protein formation along the arrangement direction of fibers, resembling the ECM architecture of native tendon tissues. QPCR results showed that HADMSC have significantly higher levels of SCX, COL1, and TNMD gene expression in the woven fabrics, compared to those in the electrospun random and aligned meshes (Fig. 7B). Moreover, TNC gene expression in woven fabrics and electrospun aligned meshes were comparable, which were both significantly higher than electrospun random meshes. Together, these results demonstrated that PCL nanofiber yarn-based woven fabrics provided a more appropriate microenvironment to promote high cell viability, proliferation, infiltration, and tenogenic differentiation of HADMSC.

3.5. Multi-cellular co- or tri-culture promoted *in vitro* tendon-like tissue formation on woven fabrics

Next, we co-cultured HADMSC with HT or HUVEC and tri-cultured HADMSC with HT and HUVEC in the mixed media on woven fabrics. Immunofluorescent staining showed that the protein expression of TNMD and COL1 was detected in the HADMSC alone group and all of the cell co- and tri-culture groups after 14-day culture (Fig. 8A). As expected, very limited CD31 and vWF expressions were detected in both HADMSC alone group and HADMSC/HT co-culture groups, whereas HADMSC/HUVEC co-culture group and HADMSC/HT/HUVEC tri-culture group express obvious CD31 and vWF, probably due to the addition of HUVEC.

The gene expression of tenogenic differentiation markers (SCX, TNC, COL1, COL3, and TNMD) and endothelial cell markers (vascular endothelial growth factor A-VEGFA and ANGPT2) in all four of the groups was evaluated by qPCR (Fig. 8B). The analysis showed that the HADMSC/HT co-culture group significantly upregulated the SCX, TNC, and TNMD gene expression, but downregulated the COL1 and COL3 gene expression, compared to HADMSC alone group (Fig. 8B). The HADMSC/HUVEC co-culture group exhibited significantly higher SCX, COL3, and TNMD gene expression. Moreover, the tri-culture group showed the highest expression of SCX, TNC, and TNMD, indicating that the tri-culture promoted both early and mature tenogenic differentiation. The qPCR results also showed that the endothelial lineage-specific gene expression (i.e. VEGFA and ANGPT2) in the HADMSC/HUVEC co-culture group and tri-culture groups were significantly upregulated compared to the other two groups.

3.6. Mechanical stimulation promoted tenogenic differentiation of tri-cultured HADMSC/HT/HUVEC on woven fabrics

To assess the effects of mechanical stimulation on the tendon-like tissue formation on woven fabrics, we seeded HADMSC/HT/HUVEC on woven meshes and tri-cultured them under static and dynamic environments for 14 days. Immunofluorescent staining showed that tri-cultured cells under dynamic stretch expressed both TNMD and COL1, which were comparable to the static condition (Fig. 9A and 8A). Western blot results showed that dynamic stretch promoted the relative amount of TNMD and COL1 expression normalized to β -actin, compared to the static condition (Fig. 9C–E). Furthermore, mechanical stimulation also significantly increased the total collagen content after 14-day culture (Fig. 9B). The qPCR results showed that the expression levels of SCX, TNC, COL1, COL3, TNMD, and VEGFA genes in the dynamic group were also significantly upregulated, compared to the static group (Fig. 9F). As shown in static co- and tri-culture experiments (Fig. 8B), a significantly lower COL1 gene expression was observed in the co- or tri-culture groups than that in the HADMSC mono-culture group. Mechanical stimulation significantly increased the COL1 expression in HADMSC/HT/HUVEC tri-culture group, which addressed the issue of the COL1 decreasing that happened in static culture condition.

4. Discussion

Native human tendons possess a hierarchical architecture composed of parallel collagen nanofiber bundles oriented along a longitudinal axis, which provide connective flexibility and transmit tensile forces between muscles and bones. Therefore, engineering tendon substitutes requires the utilization of nanofiber-based scaffolds to mimic the native tendon ECM. In our current study, we combined electrospinning nanofiber yarns with textile manufacturing strategies to fabricate woven biotextiles with hierarchical features, aligned fibrous topography, and sufficient mechanical properties to use as tendon tissue engineered scaffolds. Based on our modified electrospinning technique, a PCL nanofiber yarn with uniaxial alignment and reproducible quality was created. Our woven meshes were designed by interlacing PCL nanofiber yarns with notably high weaving density into PLA multifilaments with relatively low weaving density at a right angle. This structure guarantees the cell alignment along the PCL nanofiber yarn direction. Therefore, the PCL nanofiber

yarns-based direction of woven fabrics could better replicate the morphology, structure, and mechanical properties of the parallel collagen nanofiber bundles direction in native human tendons.

Biomimicking the topography of native tendon ECM structure is of significant importance to generate engineered tendon grafts *in vitro* [56]. The electrospun random and aligned meshes and woven fabrics exhibited similar nanoscale fiber diameters, all resembling the nanofiber order of native tendon ECM. Although the electrospun aligned meshes exhibited highly aligned structures similar to the woven fabrics, the PCL microscale yarns twisted by nanoscale fibers in the woven fabrics were similar in structure and organization to the highly-oriented collagen fiber bundles of native tendon ECM. The structure and orientation of the nanofibers greatly influenced the positioning and alignment of cells. Our present results demonstrated that aligned PCL nanofiber yarns in woven scaffolds can guide HADSMC and HT alignment and the synthesis of a tendon-like ECM containing oriented TNMD and COL1 by contact guidance (Fig. 4A, 5A, 6A and 7A). As supported by prior studies, our findings confirm that aligned topography guides cell alignment and organization, and introduces the collagen formation along the arrangement direction of fibers [27, 57, 58].

The scaffold pore size has a profound effect on cell migration, proliferation, and infiltration, which are critically important for the practical application of engineered tissue grafts [59, 60]. Compared to electrospun nonwoven scaffolds with random or aligned nanofiber architecture, our nanofibrous woven fabrics exhibited a larger mean pore size than the random nanofiber and aligned nanofiber scaffolds ($12.2 \pm 1.1 \mu\text{m}$ vs. $2.1 \pm 0.2 \mu\text{m}$ vs. $1.4 \pm 0.1 \mu\text{m}$). The structure of woven fabrics provided more space for cell adhesion and migration, and may also help the transportation of oxygen and nutrients throughout the engineered scaffolds and the removal of metabolic waste during tissue regeneration. Cross-sectional H&E staining of HADSMC-seeded woven fabrics suggested that the cells successfully proliferated and infiltrated throughout the bulk fibrous scaffolds (Fig. 6C). Conversely, the random and aligned nanofibrous scaffolds, with tightly stacked fibers, limited cell growth to only on the superficial surface, rather than infiltrating into the inner layers. Therefore, only the surfaces of the electrospun scaffolds were utilized for cell proliferation. These also explained why HADSMC and HT on woven fabrics showed highest MTT absorbance (Fig. 4B and Fig. 6B). Other studies also demonstrated that a scaffold with a larger pore size supported higher cell metabolic activity due to the effective transportation of oxygen and nutrients and removal of metabolic waste throughout the whole scaffold [61, 62].

Adequate mechanical properties are required for engineered tendon scaffolds, as tendons are subjected to dynamic mechanical forces *in vivo* [32]. Different human ligaments and tendons possess ultimate tensile strengths ranging from several to several hundred MPa and ultimate strain ranging 10 % to 50 %, depending on age, anatomy and measurement method [63–65]. The electrospun random and aligned nanofiber meshes are insufficiently strong to provide mechanical support to primary tendon repairs. Higher ultimate stress values resembling native tendon may be useful to prevent failure before the active mobilization and rehabilitation start. The woven fabrics exhibited enhanced mechanical properties due to the

weaving process and the strengthening of PLA microfilaments (Fig. 3A–D). The tensile strength (10.1 ± 1.4 MPa in the nanofiber yarn direction, and 62.0 ± 8.8 MPa in the multifilament yarn direction) and strain (45.3 ± 8.2 % in the nanofiber yarn direction, and 40.9 ± 4.0 % in the multifilament yarn direction) of the woven fabrics were comparable to the native human tendon [66, 67]. Nowotny et al. reported a mean ultimate stress value of 3.9 ± 0.7 MPa and mean ultimate strain value of 35.8 ± 8.4 % for supraspinatus tendons [66]. Another study measured the tensile properties in different location of human supraspinatus tendons [67]. They found that anterior-bursal and posterior-bursal regions had highest stress (~ 35 MPa) and strain (~ 35 %), which are also comparable to those for our woven fabrics. Importantly, we can also control the anisotropic properties, desired porosity, and mechanical properties of the nanofiber yarn based scaffolds by varying material selection and weaving patterns and yarn weaving density. Such woven fabrics are suitable to serve as a patch for the injured tendon healing. We can also roll the woven fabrics into a cylinder form to repair the totally ruptured tendons.

Hypovascularity and hypocellularity characteristics of native tendon tissues are crucial issues that hinder the clinical healing of tendon-related diseases or injuries [68, 69]. Accumulating evidence suggests that tendon tissues generally possess more vessels than commonly believed, although they are recognized as a poorly vascularized tissue type [70]. Moreover, rich capillary networks in human embryonic tendons are found to promote tendon tissue formation and maturation [71]. Recent investigations have indicated that cell co-culture technique facilitated the cell-to-cell interaction to enhance specific tissue formation, which showed huge potential in tissue engineering applications [72]. In the present study, HT and HUVEC were utilized to co-culture with HADMSC on our nanofibrous woven scaffolds. The HADMSC/HT co-culture system was demonstrated to markedly upregulate the gene expression of SCX, TNC, and TNMD on the woven fabrics compared with the HADMSC mono-culture group (Fig. 8B). Some other studies also confirmed that the indirect and direct co-culture of MSC and tenocytes promoted the tenogenic differentiation of MSC and speeded up the tendon-specific tissue regeneration [44, 73, 74]. Previous studies have demonstrated a high expression of the proangiogenic protein VEGF in cells from fetal human tendons [75, 76]. Our results also showed that co-culture of HADMSC and HUVEC promoted the expression of early tenogenic markers, but not mature markers. We further incorporated HUVEC into HADMSC and HADMSC/HT culture systems and expected HUVEC were beneficial to improve early-stage vascularization during initial tendon-like tissue formation. Our results demonstrated that the incorporation of HUVEC significantly upregulated the vascularization related protein and gene expression (Fig. 8). In our current study, we only controlled the HUVEC to HADMSC (or HADMSC/HT) ratio to be 1:4 to limit the vascularization degree and to mimic the initial vascularization during tendon development and repair. Future study will be done to optimize the HUVEC ratio to maximum the tenogenic differentiation of the co-/tri-culture.

Emerging evidence suggests that mechanical stimulation plays an important role in the development and remodeling of tendons [77–79]. However, it is unclear how mechanical stretch affects the tenogenic differentiation and maturation of tri-cultured HADMSC/HT/HUVEC. We implemented the dynamic stretch regimens that mimic the physiological mechanical stretch of the native tendon. Our study revealed that dynamically cultured

HADMSC/HT/HUVEC-woven fabric constructs promoted total collagen secretion, upregulated tenogenic differentiation markers and tendon related ECM expression (Fig. 9). COL1 is the most abundant tendon ECM protein, which is crucial for repair and regeneration of the fibrous tissues. In other studies, mechanical stimulation was also demonstrated to promote the COL1 protein production in tenocytes [80], dermal fibroblasts [81], MSC [82], and tendon derived stem cells [48]. The changes in gene expression on our dynamically cultured HADMSC/HT/HUVEC-woven fabric constructs were similar to those studies which evaluated the effect of tensile strain on MSC seeded silk fibers [83], MSC-seeded poly(lactide-co-glycolide) nanofiber meshes [77], and tendon derived stem cells seeded poly(L-lactide-co-e-caprolactone)/collagen scaffolds [49], reporting a similar upregulation of SCX and TNC, as well as COL1 and COL3. However, in contrast to these reports, TNMD, the late marker of tendon formation induced by SCX and implicated in collagen organization, was also upregulated with mechanical stimulation in combination with cell tri-culture in the current study. Mechanical stimulation might have a direct or an indirect effect on the cells, upregulating the collagen-binding integrin receptors ($\alpha 1$, $\alpha 2$ and $\alpha 11$) and matrix metalloproteinases (MMP 9, 13 and 14) via ERK and p38 kinase activation and improving nutrient transport, which led to an increase expression in tendon-associated markers [48, 77, 84]. Clinically, it is envisioned that a strategy of combining our PCL nanofiber yarns-constructed woven scaffolds with concurrent stimulation of cell co- or tri-culture and mechanical stimulation could be effectively utilized to generate the newly regenerating tendon tissue constructions for connective tissue engineering. Future studies are still required to elucidate the healing mechanism of the signaling pathway of cell co- or tri-culture and mechanical stimulation influencing the tenogenic differentiation on our nanofibrous woven scaffolds, as well as ensure the *in vivo* validation of these tendon engineered constructions.

Conclusions

In our current study, novel nanofibrous biotextiles, that mimic the ECM of native tendon in terms of structure and inherent nanoscale organization, have been fabricated using electrospun PCL nanofiber yarns and textile weaving technology. The nanofibrous woven scaffolds possessed a 3D aligned microstructure and larger pore size, which were beneficial for cell orientation, proliferation, infiltration, and the expression of tendon fibroblast-associated markers. In addition, this scaffold exhibited desirable mechanical properties, comparable to those of human native tendon tissues. More importantly, our study demonstrated that dynamically cultured HADMSC/HT/HUVEC-woven fabric constructs significantly promoted the tenogenic differentiation by enhancing their collagen production, tendon-related proteins, and gene expression. This study demonstrates that the novel PCL nanofiber yarn based woven fabrics could serve as promising tendon tissue engineered scaffolds and promote tenogenic differentiation by properly combining cell co- or tri-culture and mechanical stimulation for tendon regeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Statement of Significance

Tendon grafts are essential for the treatment of various tendon-related conditions due to the inherently poor healing capacity of native tendon tissues. In this study, we combined electrospun nanofiber yarns with textile manufacturing strategies to fabricate nanofibrous woven biotextiles with hierarchical features, aligned fibrous topography, and sufficient mechanical properties as tendon tissue engineered scaffolds. Comparing to traditional electrospun random or aligned meshes, our novel nanofibrous woven fabrics possess strong tensile and suture-retention strengths and larger pore size. We also demonstrated that the incorporation of tendon cells and vascular cells promoted the tenogenic differentiation of the engineered tendon constructs, especially under dynamic stretch. This study not only presents a novel tissue engineered tendon scaffold fabrication technique but also provides a useful strategy to promote tendon differentiation and regeneration.

Figure 1.

Fabrication of PCL nanofibrous woven fabrics and electrospun nonwoven meshes. (A) Schematic illustration of the typical electrospinning system for fabricating nanofibrous nonwoven meshes. (B) SEM image of the electrospun PCL random-oriented nanofibrous meshes. Scale bar = 20 μm . (C) SEM image of the electrospun PCL aligned nanofibrous meshes. Scale bar = 20 μm . (D) Schematic illustration of the modified electrospinning system for fabricating PCL nanofiber yarns. (E) Photograph of a PCL nanofiber yarn package produced about 2 hours. (F, G) SEM images of the electrospun PCL nanofiber yarns. Scale bars are 100 μm for (F) and 10 μm for (G), respectively. (H) Schematic of the textile weaving process. (I) Photograph and (J) SEM image of the PCL nanofibrous woven fabrics. The plain weave structure includes PCL nanofiber yarns, with a high weaving density, as weft and PLA multifilaments, with a low weaving density as wrap. Therefore, almost no PLA multifilaments could be observed in the as-prepared woven fabrics. Scale bar = 200 μm . (K) Schematic illustration of orientation angular distribution measurement of the nanofibers in the random and aligned nanofibrous meshes, the nanofibers in the nanofibrous yarns, and the nanofiber yarns in the woven fabrics from SEM images. (L) Results of orientation angular distribution measurement.

Figure 2. Pore size characterization of nanofibrous woven PCL fabrics and electrospun nonwoven meshes. (A) Pore size distribution. (B) mean pore diameter. (n=3; *p<0.05, ** p<0.01.)

Figure 3.

Uniaxial tensile testing of PCL nanofibrous woven fabrics and electrospun random and aligned meshes. The tests were performed in the aligned and vertical to the aligned direction for the electrospun, aligned meshes and woven fabrics. The mechanical tests were conducted in a random direction for the random meshes. (A) Representative stress-strain curves. (B) Young's modulus. (C) Tensile strength. (D) Ultimate elongation. (n=6; bars that do not share letters are significantly different from each other, $p < 0.01$).

Figure 4. (A) Live/Dead images for HT-seeded on three different nanofibrous scaffolds and conditioned in TGM for 7 days and 14 days. Scale bars = 100 μ m. (B) MTT results of HT cultured on the random and aligned meshes and woven fabrics in TGM at day 7 and 14 (n=6; **p<0.01).

Figure 5.

Woven fabrics upregulated the expression of tendon-associated gene markers of HT. (A) Immunofluorescent staining for TNMD (green), COL1 (red), and nuclei (blue) on the three different HT-seeded scaffolds in TGM after 14-day culture. Scale bars = 100 μ m. (B) SCX, TNC, COL1, COL3, and TNMD gene expression analysis of HT cultured on electrospun random and aligned meshes and woven fabrics in TGM after 14 days. Relative gene expression is presented as normalized to 18S and expressed relative to the HT cultured on random fibrous scaffolds (n=3; bars that do not share letters are significantly different from each other, $p < 0.05$).

Figure 6.

(A) Representative fluorescent images of living cells (green) and dead cells (red) of HADMSC seeded on three different nanofibrous scaffolds (i.e., electrospun random meshes, electrospun aligned meshes and woven fabrics) conditioned in TDM for 7 days and 14 days. Scale bars = 100 μ m. (B) Cell proliferation quantification by MTT assay at day 7, and day 14 of culture for HADMSC cultured on the three different nanofibrous scaffolds in TDM (n=6; **p<0.01). (C) Cross-sectional H&E staining of HADMSC seeded on the three different nanofibrous scaffolds in TDM for 14 days. The yellow arrows showed the HADMSC infiltration position in the scaffolds. Scale bars = 200 μ m.

Figure 7.

Woven fabrics promoted HADMSC differentiation towards tenocytes in TDM after 14-day culture. (A) Immunofluorescent staining for TNMD (green), COL1 (red), and nuclei (blue) on the three different nanofibrous scaffolds. Scale bars = 50 μ m. (B) qPCR analysis of SCX, TNC, COL1, COL3, and TNMD genes on HADMSC-seeded electrospun random and aligned meshes and woven fabrics. Relative gene expression is presented as normalized to 18S and expressed relative to HADMSC-seeded random fibrous scaffolds (n=3; bars that do not share letters are significantly different from each other, $p < 0.05$).

Figure 8.

Multi-cellular co- or tri-culture promoted *in vitro* tendon-specific marker expression on woven fabrics. (A) Representative immunofluorescent staining for tendon-related markers (TNMD (green), COL1 (red), and nuclei (blue)) and endothelial lineage-specific markers (CD31 (green), vWF (red), and nuclei (blue)) of HADMSC mono-seeded woven scaffolds in TDM, HADMSC/HT co-seeded, HADMSC/HUVEC co-seeded, and HADMSC/HT/HUVEC tri-seeded woven scaffolds in the corresponding mixed medium after 14-day culture. Scale bars = 100 μ m. (B) qPCR analysis of tendon-related genes (SCX, TNC, COL1, COL3, and TNMD) and lineage-specific genes (VEGFA and ANGPT2) of HADMSC mono-seeded woven scaffolds in TDM, HADMSC/HT co-seeded, HADMSC/HUVEC co-seeded, and HADMSC/HT/HUVEC tri-seeded woven scaffolds in the corresponding mixed medium after 14-day culture. Relative gene expression is presented as normalized to 18S and expressed relative to HADMSC mono-seeded woven scaffolds in TDM (n=3; bars that do not share letters are significantly different from each other, $p<0.05$; * $p<0.05$, ** $p<0.01$).

Figure 9.

Dynamic culture enhanced tendon-associated protein and gene expression of HADMSC/HT/HUVEC tri-cultured on woven fabrics. HADMSC/HT/HUVEC were tri-seeded on woven fabrics and cultured in the mixed medium under static and dynamic environment for 14 days. (A) Representative immunofluorescent staining for TNMD (green), COL1 (red), and nuclei (blue). Scale bars = 100 µm. (B) Total collagen content (n=3; **p<0.01). (C–E) Western blotting for TNMD and COL1. Relative protein expression is presented as normalized to β-actin. (F) qPCR analysis for SCX, TNC, COL1, COL3, TNMD, VEGFA, and ANGPT2 gene. Relative gene expression is presented as normalized to 18S and expressed relative to HADMSC/HT/HUVEC tri-cultured woven scaffolds under static environment (n=3; *p<0.05, ** p<0.01).