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Fibrin Gels Exhibit Improved Biological, Structural, and Mechanical Properties Compared with Collagen Gels in Cell-Based Tendon Tissue-Engineered Constructs

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The prevalence of tendon and ligament injuries and inadequacies of current treatments is driving the need for alternative strategies such as tissue engineering. Fibrin and collagen biopolymers have been popular materials for creating tissue-engineered constructs (TECs), as they exhibit advantages of biocompatibility and flexibility in construct design. Unfortunately, a few studies have directly compared these materials for tendon and ligament applications. Therefore, this study aims at determining how collagen versus fibrin hydrogels affect the biological, structural, and mechanical properties of TECs during formation *in vitro*. Our findings show that tendon and ligament progenitor cells seeded in fibrin constructs exhibit improved tenogenic gene expression patterns compared with their collagen-based counterparts for approximately 14 days in culture. Fibrin-based constructs also exhibit improved cell-derived collagen alignment, increased linear modulus (2.2-fold greater) compared with collagen-based constructs. Cyclic tensile loading, which promotes the maturation of tendon constructs in a previous work, exhibits a material-dependent effect in this study. Fibrin constructs show trending reductions in mechanical, biological, and structural properties, whereas collagen constructs only show improved tenogenic expression in the presence of mechanical stimulation. These findings highlight that components of the mechanical stimulus (e.g., strain amplitude or time of initiation) need to be tailored to the material and cell type. Given the improvements in tenogenic expression, extracellular matrix organization, and material properties during static culture, *in vitro* findings presented here suggest that fibrin-based constructs may be a more suitable alternative to collagen-based constructs for tissue-engineered tendon/ligament repair.

Introduction

New treatment modalities are needed for frequent and costly tendon and ligament injuries given that conventional treatments often lead to nonfunctional repairs. These injuries account for more than one-third of all musculoskeletal medical treatments annually in the United States.[1] Surgeons typically treat these injuries with tissue allografts or autografts to enable patients to quickly return to normal activities of daily living. However, recent evidence suggests that these procedures do not dramatically improve long-term patient outcomes.[2–5] Tissue engineering is an alternative approach that seeks to promote functional healing through the design and implantation of constructs containing stem/progenitor cells seeded in a biocompatible scaffold.

Creating effective tissue-engineered constructs (TECs) requires knowledge of normal tendon and ligament structure and function during growth and development. Tendons and ligaments begin developing about embryonic day 10 on induction of the tissue-selective transcription factor Scleraxis (*Scx*), which continues to be expressed throughout tendon development, growth, and maturation.[6,7] SCX has been shown to regulate expression of extracellular matrix (ECM) proteins that are critical to tendon and ligament function.[8–11] During later stages of development, the ECM matures and is composed mostly of aligned type I collagen fibrils, which function to transmit large and impulsive mechanical loads that affect joint movement. In addition, these tissues are mechanosensitive, requiring external forces to properly mature.[12,13] Tendon and ligament progenitor (TLP) cells respond to these mechanical stimuli by enhancing SCX

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expression and other ECM proteins.[7,14–16] These cell-ECM interactions during development are important in defining the composition and organization of these tissues. Similarly, understanding how cell-material interactions affect TEC formation will lead to more effective strategies for soft tissue repair.

Various biocompatible materials have been used in TEC designs. Type I collagen gels have been extensively studied, because they provide a compliant three-dimensional environment that supports cellular remodeling of TECs *in vitro*[17,18] and can serve as a cell delivery vehicle for surgical implantation.[19,20] Cells in collagen gels tension the extracellular material and further respond to mechanical stimulation by expressing tendon-specific markers.[7,14,21] Previous studies in our lab have shown that these TECs improve the stiffness and modulus of repairs *in vivo*,[20,22] and mechanical stimulation of these TECs before implantation further enhances repair biomechanics.[23,24] Alternatively, fibrin gels have been recently proposed for creating tissue constructs *in vitro*, as fibrin promotes greater collagen synthesis than collagen gels and more readily remodels according to the physical boundary conditions of the construct apparatus.[25–27] Cells in fibrin gels also appear to recapitulate aspects of native tendon development *in vitro*[28,29] and exhibit improved cell-derived collagen organization in response to mechanical stimulation.[30,31] However, research has not yet been conducted that directly compares how collagen and fibrin materials affect the ability of TLPs to respond to mechanical stimuli and produce an organized ECM.

In this study, we evaluated how the scaffold material and cyclic mechanical stimulation affect the biological, structural, and mechanical properties of TECs *in vitro*. Given the aforementioned benefits of fibrin gels to remodel and promote collagen synthesis, we expected fibrin-based TECs to exhibit improved formation compared with collagen TECs. Here, we tested the hypotheses that for approximately 14 days in culture, murine TLPs in fibrin TECs will exhibit significantly increased (1) tenogenic gene expression, (2) collagen alignment, and (3) TEC linear modulus compared with collagen TECs. Furthermore, we hypothesized that mechanically stimulating fibrin TECs will result in even greater increases in these measures compared with mechanically stimulated collagen TECs.

Materials and Methods

Experimental design

TLP cells were isolated from ScxGFP mice kindly provided by Dr. Ronen Schweitzer.[32] ScxGFP+ cells (referred to as TLPs) were harvested and pooled from embryonic day 17.5 (E17.5) litters (Supplementary Fig. S1A, B; Supplementary Data are available online at www.liebertpub.com/tea), as these cells are actively differentiating and poised for the significant matrix production that occurs after birth.[10,11,33] Four separately pooled litters were used to create TECs to account for any biological variability, and technical replicates were created across these biological replicates to achieve sample sizes at each time point described next and shown in Supplementary Figure S1C. TECs were evaluated for approximately 2 weeks in culture, as fibrin-based TEC mechanical properties plateau by this time.[28] Fibrin and collagen TECs were allowed 6 days to initiate matrix formation around dish posts before initiating mechanical stimulation, as TECs begin to plateau in their contraction kinetics by this time (Fig. 1). Quantitative real-time polymerase chain reaction (qPCR) ($n=4$) was used to assess cellular phenotype (relative to normal TLPs) for chondrogenic (*Sox9*) and osteogenic (*Runx2*) transcription factors as well as for tenogenic genes (*Scx*, *Mkx*, *Egr1*, *Tnmd*, *Col1a1*, *Col3a1*, *Fmod*, and *TnC*) known to be important in the development and maintenance of tendon tissue (see qPCR methods below for gene nomenclature).[10,11,34–37] ScxGFP expression and collagen organization were analyzed using multi-photon (MP) fluorescence microscopy, and immunohistochemistry (IHC) was performed to evaluate localization and organization of ECM proteins COL1, COL3, and TNC ($n=2$). TECs were failed in tension to determine the temporal changes in biomechanical properties ($n=12$) during TEC formation and maturation between days 6 and 14, as TECs were far too fragile to be reliably tested in tension on day 2 or earlier. Only linear stiffness and linear modulus were measured, as TECs failed at the testing grips, thus precluding analysis of failure properties. Transmission electron microscopy (TEM) was used to quantify collagen fibril diameter and fibril area fraction (FAF, $n=2$), which have been previously correlated to biomechanical properties.[38,39]

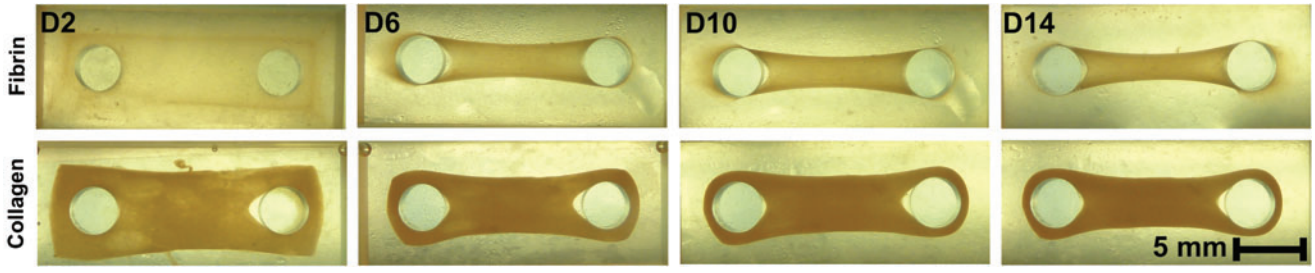

FIG. 1. TEC contraction shows differences in early formation of fibrin and collagen TECs. Fibrin and collagen TECs show the greatest changes in contraction over the first 6 days in culture. At day 2, fibrin TECs show a more translucent, less dense matrix compared with the collagen TECs at day 2. By day 6, however, the density of fibrin TECs increased between the posts. Collagen TEC density also increased between days 2 and 6, but these differences were not as extensive as for fibrin. Note that both fibrin and collagen TECs contracted around silicone dish posts, resulting in reduced cross-sectional area over time, but retained their full length by continuing to anchor between the silicone dish posts between days 6 and 14. TEC, tissue-engineered construct. Color images available online at www.liebertpub.com/tea

Cell isolation and expansion

All reagents were purchased through Life Technologies (Carlsbad, CA) unless otherwise indicated. Impregnated heterozygous ScxGFP+ females were euthanized with CO_2 and cervical dislocation according to procedures approved by the University of Cincinnati IACUC (Supplementary Fig. S1A). E17.5 ScxGFP+ embryos were isolated and genotyped using a fluorescent lamp (460–495 nm; BLS Ltd., Budapest, Hungary) and goggles (500–550 nm; BLS Ltd.) for GFP expression. Limbs and tails were dissected from ScxGFP+ and ScxGFP− pups, dissociated at 37°C in 0.25% trypsin for 30 min followed by centrifugation with 1× phosphate-buffered saline (PBS). Tissue was then digested in collagenase D (3 mg/mL; Fisher Scientific, Pittsburgh, PA) and dispase (2 mg/mL) in cell culture medium (MEM α, 10% fetal bovine serum [FBS], and 1% antibiotic/antimycotic) for 30 min. at 37°C. Resulting cell suspension was prepared for fluorescence-activated cell sorting (FACS) in buffer consisting of 1% heat-inactivated FBS, 2.5 mM HEPES solution, 1 mM EDTA, and 1% penicillin/streptomycin in 1× Mg/Ca-free phosphate-buffered saline. Instrument voltages (BD FacsAria II) were set using ScxGFP− control cells, and ScxGFP+ cells were subsequently collected for cell culture. TLPs were suspended in culture medium (MEM α, 10% FBS, and 1% antibiotic/antimycotic), plated in monolayer (passage 0, P0), grown to 90% confluency, and passaged every other day until P4 to obtain sufficient cell numbers for generating TECs.

Three-dimensional TEC generation

TLPs were encapsulated at a density of $0.5e^6$ cells/mL in fibrin or collagen gel. A total of 1.4 mL of cell-gel mixture was pipetted into each well of custom-made silicone dishes,[40] yielding a TEC that initially measured 12.0 × 11.0 × 5.2 mm (gage length × width × thickness). Collagen gels (bovine hide; Life Technologies) were created according to the manufacturer's protocol at a final collagen concentration of 2.4 mg/mL. Fibrin gels were created as previously described[28] at a final concentration of 3.4 mg/mL bovine fibrinogen (Sigma-Aldrich; St. Louis, MO) and 4 U/mL bovine thrombin (Sigma). TECs were fed every 2 days with culture medium (MEM α, 10% FBS, and 1% antibiotic/antimycotic) supplemented with 200 μM l-ascorbic acid 2-phosphate (Sigma) and 20 ng/mL TGFβ-3 (Peprotech; Rocky Hill, NJ) to stimulate collagen synthesis[41–43] and incubated at 37°C, 5% CO_2, and 95% relative humidity.

Mechanical stimulation

TECs were stimulated in displacement-limited control using a custom-made, pneumatically controlled mechanical stimulation system[23] and a previously optimized stimulation regime of 2.4% peak strain (1 Hz stretch, 0.12 Hz rest) for 3000 cycles per day.[44]

RNA isolation and qPCR

RNA was isolated in TriReagent and extracted according to the manufacturer's specifications. RNA was quantified using a UV spectrophotometer and converted to cDNA using the High-Capacity RNA-to-cDNA kit (4387406; Applied Biosystems, Grand Island, NY). Real-time reactions (10 ng) were performed using Taqman® Gene Expression Mastermix (4369510; Applied Biosystems) and Taqman probes (assay ID is indicated in parentheses for each gene) for scleraxis (*Scx*, Mm01205675_m1), Mohawk homeobox (*Mkx*, Mm00617017_m1), early growth response 1 (*Egr1*, Mm00656724_m1), tenomodulin (*Tnmd*, Mm00491594_m1), type I collagen α1 chain (*Col1a1*, Mm00801666_g1), type III collagen α1 chain (*Col3a1*, Mm01254476_m1), fibromodulin (*Fmod*, Mm00491215_m1), tenascin-C (*TnC*, Mm00495662_m1), runt-related transcription factor 2 (*Runx2*, Mm00501584_m1), and SRY-box 9 (*Sox9*, Mm00448840_m1). The relative amount of mRNA for each gene of interest was computed using delta Ct values normalized to ribosomal RNA, *18S* (Hs99999901_s1), which was found to be stably expressed across treatments within this study. Gene expression was normalized to freshly FACS sorted (''normal'') E17.5 ScxGFP cells using the delta-delta Ct method. While expression of these markers varies during development, we elected to use ''normal'' E17.5 TLPs to benchmark *in vivo* expression levels and evaluate how gene expression of TLPs in TECs could be compared with their initial *in vivo* condition.

MP fluorescence and immunohistochemical imaging

Sample preparation. TECs were fixed in 4% paraformaldehyde for 1.5 h at 4°C, washed in 1× PBS for 10 min, preserved in 30% sucrose for 1 h, embedded in OCT media (Andwin Scientific, Addison, IL), and stored at −80°C. To prepare samples for IHC, TECs were cryosectioned longitudinally using cryofilm (Type 2C; Hiroshima, Japan)[45] at 150–200 μm from the top surface of the TECs. Sections were hydrated in 1× PBS, blocked (Power Block; Biogenix, Fremont, CA) for 30 min at room temperature (RT). Sections were then incubated in separate primary antibodies for type I collagen (1:100, AB758; Millipore, Billerica, MA), type III collagen (1:200, AB7778; Abcam, Cambridge, MA), and tenascin-C (1:500, AB6346; Abcam) overnight at 4°C. Sections were then washed thrice in 1× PBS for 15 min and incubated in secondary antibodies for 1 h at RT. Finally, sections were washed and counterstained with Hoechst 33352 (Life Technologies) in 50% glycerol. To prepare whole-mount samples for MP imaging, TECs were thawed, washed with 1× PBS for 10 min at RT, and then cut longitudinally to expose the interior of the TEC. TECs were whole-mounted on glass slides in 1× PBS just before imaging.

Imaging technique. For IHC imaging, sections were imaged on a Zeiss Axio Imager Z1 fluorescence microscope (Jena, Germany) using filters for DAPI, ScxGFP, Cy3, and Cy5 secondary antibodies. Images were captured at 5× and 20× magnification with equal exposure times across all treatment groups. For MP imaging, the interior, longitudinal face of TECs was imaged using a TiSapphire laser and a Nikon A1R upright MP laser scanning microscope (Melville, NY) to assess localization of ScxGFP-expressing cells and collagen organization using second harmonic generation (SHG).[46] Images were captured in galvanometric mode using a 25× water-immersion objective for ScxGFP (525–575 nm) and SHG signal (400–450 nm), and laser settings were held constant across all treatment groups. This technique yielded images with ~500-μm wide field of view

along the thickness (top to bottom) of each TEC through a 100-μm depth using a 2-μm step size. Image files were imported into FIJI (v. 1.47) using the Bio-Formats plug-in (v. 4.4.9), and images were generated using maximum-intensity z projections.

Transmission electron microscopy

Sample preparation. TECs were prepared for TEM as previously described.[28]

Imaging and sampling technique. TEC transverse cross-sections were imaged on an FEI Tecnai 12 Twin Transmission Electron Microscope using a 2k×2k cooled CCD camera (F214A; Tietz Video and Image Processing Systems, Gauting, Germany). A thorough sampling of each section was performed on at least three sections from each sample. Magnifications of 11,000× were used to measure fibril diameter and FAF, resulting in 30 views per sample. Image calibration was completed with a cross-grating replica grid (2160 lines/mm; Agar Scientific, Stansted, United Kingdom).

Image quantification. Gray-scale images were imported into FIJI for image quantification and processed to compute fibril diameters and FAF as previously described.[47,48] Briefly, gray-scale images were thresholded into binary images; then, FIJI's analyzed particle function was used to compute fibril diameter from the minor axis of the best-fit ellipse around each fibril. Diameters of poorly aligned fibrils were further separated based on their major:minor axis ratio exceeding 2:1. FAF was calculated as the percentage of matrix area occupied by collagen fibrils (irrespective of fibril orientation) according to the formula $FAF = \sum \frac{A_i}{A}$, where A_i is the cross-sectional area (CSA) of an individual fibril and A is the CSA of the total ECM in the image.

Biomechanical testing

TECs were loaded in a tensile failure system (Model 100R; TestResources, Inc., Shakopee, MN), gripped at the interior edges of the loop ends by fine-grit sand paper, and tensioned under minimal load (~5 mN). TEC gage length (grip-to-grip distance), width, and thickness were calculated from the average of three measurements taken across the TEC using calibrated digital images and FIJI. Elliptical CSA was computed from averaged thickness and width measurements. TECs were failed in tension at a moderate strain rate (10%/s) in the same tensile system, and stiffness and modulus were calculated from the linear region of the failure curve.[23]

Statistical analysis

Data were normal and homoscedastic, and all values reported in this article are (mean ± SEM) unless otherwise indicated. Due to the high-dimensional data produced by examining expression of numerous genes, principal component analysis (PCA) was used to identify trends in gene expression patterns across treatments using the R statistical package.[47] PCA is a statistical method that reduces data dimensionality into independent, linear combinations (principal components [PCs]) of the original data in which each component describes a proportion of variability in the dataset.[49–51] The PCs that combined for the majority of variability in the data were statistically tested using analysis of variance (ANOVA) methods, and results were interpreted by evaluating which genes significantly ($p<0.01$) and strongly correlated ($R>0.6$) with each of these components.[51]

Due to the unbalanced design created by delaying the onset of mechanical stimulation, two statistical designs were evaluated: (1) PC scores, TEC dimensions, biomechanical parameters, and FAF of the statically cultured TECs were tested via two-way ANOVA with material and time as fixed factors ($p<0.05$); (2) to evaluate the effects of mechanical stimulation, these measures were tested via three-way ANOVA with material, stimulation, and time as fixed factors for days 10 and 14 only ($p<0.05$). Delta Ct values were similarly tested via multivariate analysis of variance (MANOVA). Collagen fibril diameter distributions of each treatment replicate were pooled for analysis. Since fibril diameter distributions are non-Gaussian in nature, treatment groups were analyzed via Mann–Whitney U tests. In both statistical designs, multiple *post hoc* comparisons were made using Fisher's protected least significant difference ($p<0.05$). Bivariate linear regression analyses were independently performed for each TEC material ($p<0.05$) to identify potential relationships between biological/structural measurements and mechanical properties.

Results

TEC contraction and dimensions

Material and time in culture significantly affected TEC width, thickness, and CSA ($p<0.05$, Fig. 1 and Table 1). However, gage length was not affected by material ($p=0.073$) or time ($p=0.949$). Although both materials showed decreased CSA over time, fibrin TECs ranged from 34% to 47% smaller in CSA compared with collagen TECs across all time points. Mechanical stimulation did not significantly affect width ($p=0.243$), thickness ($p=0.864$), or CSA ($p=0.389$), but it significantly reduced the gage length of both TEC materials ($p<0.05$, Table 1).

TEC tenogenic expression

PCA, which was used to summarize gene expression data and to characterize the phenotype of TLPs relative to normal E17.5 TLPs, revealed that fibrin enhances the tenogenic phenotype of TLPs for longer culture periods than collagen (Fig. 2). The first (PC1) and second principal (PC2) components accounted for 54% and 20% of the data variability, respectively. Subsequent components accounted for less than 6% each and were not further analyzed. PC1 scores strongly correlated with most genes investigated (*Scx*, *Mkx*, *Col1a1*, *Col3a1*, *Tnmd*, *TnC*, *Fmod*, and *Sox9*), whereas PC2 described expression of Runx2 and Egr1 (Supplementary Table S1). Time in culture significantly affected PC1 scores ($p<0.01$), material significantly affected PC2 scores ($p<0.05$), and both PC1 and PC2 scores showed significant interactions between material and time ($p<0.05$). Although expression levels for each material approached those of normal ScxGFP cells for the PC1-associated genes, each material exhibited distinctly different temporal patterns (Fig. 2). While PC1 scores for fibrin TECs (-4.0 ± 1.1)

Table 1. Tissue-Engineered Construct Dimensions and Biomechanics

Treatment			Response measure					
Stimulation	*Material*	*Time point (days)*	*Linear stiffness (N/mm)*	*Linear modulus (MPa)*	*Gage length* (mm)*	*Width* (mm)*	*Thickness* (mm)*	*Cross-sectional area (mm^2)*
Static	Fibrin	6	0.09 ± 0.02	0.27 ± 0.05	7.03 ± 0.18	2.83 ± 0.11^{a}	1.17 ± 0.08^{a}	2.66 ± 0.26^{a}
		10	$0.19 \pm 0.02^{a,b}$	$0.64 \pm 0.05^{a,b}$	7.32 ± 0.21^{c}	2.48 ± 0.06	1.11 ± 0.07^{a}	2.18 ± 0.18^{a}
		14	$0.27 \pm 0.02^{b,bb}$	$1.34 \pm 0.19^{a,b,bb}$	7.57 ± 0.23^{c}	$1.85 \pm 0.12^{a,b,bb}$	1.15 ± 0.07^{a}	$1.71 \pm 0.19^{a,bb}$
	Collagen	6	0.05 ± 0.01	0.09 ± 0.02	7.91 ± 0.22	3.65 ± 0.12^{a}	1.55 ± 0.04^{a}	4.44 ± 0.19^{a}
		10	$0.12 \pm 0.02^{a,b}$	0.26 ± 0.04^{a}	7.55 ± 0.24	2.84 ± 0.14^{b}	1.48 ± 0.10^{a}	$3.31 \pm 0.26^{a,b}$
		14	$0.24 \pm 0.02^{b,bb}$	$0.60 \pm 0.09^{a,b,bb}$	7.44 ± 0.23^{c}	$2.51 \pm 0.18^{a,bb}$	1.67 ± 0.05^{a}	$3.25 \pm 0.19^{a,bb}$
Mech. stimulated	Fibrin	10	0.19 ± 0.02	0.65 ± 0.08	6.63 ± 0.26^{c}	2.45 ± 0.10	1.07 ± 0.05	2.08 ± 0.16
		14	0.21 ± 0.03	0.95 ± 0.17	6.89 ± 0.27^{c}	2.22 ± 0.14	1.04 ± 0.06	1.85 ± 0.20
	Collagen	10	0.22 ± 0.02	0.43 ± 0.05	7.41 ± 0.15	2.94 ± 014	1.65 ± 0.02	3.82 ± 0.18
		14	0.27 ± 0.01	0.59 ± 0.06	6.73 ± 0.24^{c}	2.54 ± 0.20	1.62 ± 0.06	3.16 ± 0.15

*Initial tissue-engineered construct dimensions were $\sim 12.0 \times 11.0 \times 5.2$ mm (gage length × width × thickness).
[a]Significantly different w.r.t. material at corresponding time point ($p < 0.05$).
[b]Significantly different w.r.t. at day 6 ($p < 0.05$).
[bb]Significantly different w.r.t. at day 10 ($p < 0.05$).
[c]Significantly different w.r.t. mechanical stimulation (corresponding material and time point, $p < 0.05$).

exhibited initial expression patterns different from normal (2.0 ± 0.6, $p < 0.01$) at day 2, these scores continually increased over the 2-week culture period, achieving normal expression by day 14 (1.9 ± 0.4, $p = 0.949$). Conversely, PC1 scores for collagen TECs (-1.3 ± 1.1, $p < 0.01$) increased more rapidly than for fibrin, reaching near normal levels by day 6 (1.6 ± 0.2, $p = 0.713$). However, these scores then decreased back toward day 2 levels with additional time in culture, becoming significantly different from normal by day 14 (-0.9 ± 0.9, $p < 0.05$). PC2 scores revealed significant deviation for two genes, which was caused by the significant upregulation of *Runx2* ($p < 0.05$) and significant downregulation of *Egr1* ($p < 0.01$) in all TEC conditions when compared with normal cells by MANOVA (Supplementary Table S1). Fibrin TECs also exhibited significant upregulation of tenogenic markers (*Scx*, *TnC*, and *Fmod*) relative to collagen TECs at day 14 (Supplementary Table S1). *Col1a1, Col3a1,* and *TnC* expression was significantly downregulated relative to normal for both fibrin and collagen TECs (Fig. 3). While these markers also illustrated temporal increases, only *Col1a1* and *TnC* achieved normal expression levels by day 14 or earlier.

There was a significant interaction between material and mechanical stimulation for PC1 ($p < 0.05$), indicating a material-dependent response to mechanical stimulation (Fig. 2). Although the expression patterns did not appear to mature toward normal in fibrin TECs exposed to mechanical stimulation, this effect did not reach statistical significance ($p = 0.08$). Conversely, collagen TECs appeared to benefit from mechanical stimulation, as their PC1-associated gene expression patterns remained similar to normal for approximately 14 days in culture (0.9 ± 1.3, $p = 0.444$).

Cellular and matrix organization

Fibrin TECs exhibited improved collagen alignment compared with collagen TECs at later time points in culture (Figs. 4–6). Staining for COL1, COL3, and TNC proteins revealed differences in ECM composition and organization between fibrin and collagen TECs (Fig. 3A). Collagen TECs had more type I collagen present under all conditions. While collagen TECs showed temporal increases in COL3 and TNC (mostly at the TEC surfaces), all three proteins were better aligned throughout the full width of fibrin TECs at days 6 and 14 (Fig. 4). Furthermore, fibrin TECs showed increased expression of COL3 and TNC toward the center of the constructs. MP imaging revealed Scx-expressing cells homogenously distributed through the depth of TECs in both materials at day 2 (Fig. 5A, E). Although cells and collagen showed better alignment at the surface of both materials over longer culture periods, fibrin TECs showed better collagen alignment compared with collagen TECs at greater depths from the surface (Fig. 5C, G). There were no observed effects of mechanical stimulation on the organization of COL1, COL3, or TNC in either collagen- or fibrin-based TECs as revealed by IHC (Fig. 4); however, there was an apparent reduction in the thickness of the layer of ScxGFP cells at the surface of mechanically stimulated TECs compared with static controls (Fig. 5C, D; G, H).

TEM images additionally revealed improvements in collagen fibril alignment and packing density (Fig. 6). Collagen fibrils that were aligned parallel to the axis of tension were sectioned at right angles to the fibril axis and were, therefore, recognized by their circular cross-sectional profile. In contrast, fibrils that were poorly aligned with the axis of tension were sectioned obliquely. Both materials exhibited improvements in the alignment of collagen over time, although fibrin TECs appeared to have better alignment overall. Average fibril diameters for both fibrin (36.8 ± 0.12 nm) and collagen (39.02 ± 0.32 nm) TECs decreased over time (35.19 ± 0.07 and 31.10 ± 0.20 nm, respectively; $p < 0.05$). However, mechanical stimulation reduced this effect in both fibrin (37.49 ± 0.10 nm; $p < 0.01$) and collagen (36.27 ± 0.21 nm; $p < 0.01$) by day 14. Fibrin TECs exhibited reduced FAF compared with collagen at day 6 ($8.8\% \pm 0.4\%$ vs. $11.2\% \pm 0.5\%$, respectively; $p < 0.01$). However, fibrin TECs experienced significant increases in FAF by day 14 ($15.8\% \pm 0.7\%$; $p < 0.01$), unlike collagen TECs ($12.54\% \pm 0.6\%$; $p =$

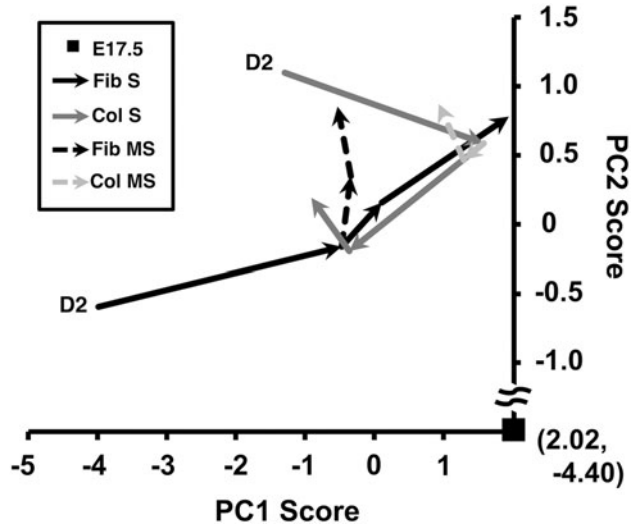

FIG. 2. Collagen TECs show improved tenogenic expression patterns early, but fibrin TECs exhibit continual improvement in expression patterns over the 14-day culture period. Shown are the temporal changes in the first (PC1) and second (PC2) principal component (PC) scores for fibrin and collagen under static (S) and mechanically stimulated (MS) conditions (see text for description of principle component analysis). Note that PC1 correlates to the expression of most tenogenic genes, whereas PC2 correlates to *Runx2* and *Egr1* expression (Supplementary Table S1). Treatment groups are compared with the expression patterns of normal E17.5 Scx-expressing cells (graph origin). At day 2, PC1 scores for static collagen TECs are initially closer to E17.5 values than for fibrin TECs. Although quickly ''maturing'' to normal tenogenic expression patterns by day 6, PC1 values for collagen TECs then decline toward day 2 values by day 14. By contrast, PC1 scores for static fibrin TECs continually increase toward normal tenogenic expression values by day 14. Initiating mechanical stimulation at day 6 slows the increase in PC1 score for fibrin TECs, whereas stimulation of collagen TECs slows the reduction in PC1 scores away from normal tenogenic expression. PC2 scores for all TEC conditions significantly differ from normal, primarily caused by significant downregulation of *Egr1* (tenogenic) and significant upregulation of *Runx2* (osteogenic) levels *in vitro*. Mean PC scores are shown beginning at day 2 with subsequent *arrowheads* corresponding to days 6, 10 and 14, respectively. Col, collagen; Fib, fibrin; MS, mechanically stimulated; S, static.

0.413). Mechanical stimulation also had a material-dependent effect on FAF, as stimulated fibrin TECs showed decreased FAF ($12.03\% \pm 0.5\%$; $p < 0.01$), whereas stimulated collagen TECs did not ($13.4\% \pm 0.8\%$; $p = 0.946$).

TEC mechanical properties

Material and time in culture significantly affected TEC biomechanics, and both materials exhibited significant increases in linear stiffness and modulus over time ($p < 0.05$; Fig. 7 and Table 1). Although fibrin TECs (0.19 ± 0.02 N/mm) showed increased linear stiffness compared with collagen TECs at day 10 (0.12 ± 0.02 N/mm; $p < 0.05$), both fibrin and collagen TECs approached similar stiffness values by day 14 (0.27 ± 0.02 vs. 0.24 ± 0.02 N/mm, respectively; $p = 0.326$). Fibrin TECs exhibited a 2.2-fold greater linear modulus compared with collagen TECs by day 14 (1.34 ± 0.19 vs. 0.60 ± 0.09 MPa, respectively; $p < 0.01$). Failure curves showed that by day 14, both TEC materials developed toe and linear regions during early deformation that are characteristic of viscoelastic tendon tissues. Mechanical stimulation did not significantly affect linear stiffness ($p = 0.23$) or modulus ($p = 0.471$) (Table 1). However, material and mechanical stimulation showed significant interactions for linear stiffness ($p < 0.01$). Linear regression analysis did not reveal any genes or fibril characteristics that significantly correlated with TEC biomechanical properties across all treatment groups ($p > 0.05$).

Discussion

This study compared how fibrin and collagen hydrogels influence cellular phenotype, ECM organization, and biomechanical properties in TECs created using TLPs. Our results suggest that fibrin-based TECs more appropriately regulate the tenogenic phenotype compared with collagen-based TECs over extended culture periods, as they exhibited a continued progression toward the tenogenic expression patterns of normal E17.5 Scx-expressing TLPs. The temporal changes in tenogenic markers during development are only recently becoming elucidated. *Scx* is initially expressed in the limb mesenchyme of TLPs as early as E10.[6] As tendon further develops, these cells begin producing an organized ECM (composed of collagens, proteoglycans, and glycoproteins).[8–11] In fact, mid-embryonic tendon development is marked by dramatic temporal increases in the expression of numerous collagens and collagen-regulating transcription factors (e.g., *Egr1*),[11] which appears to persist during matrix production in late embryonic and early postnatal development.[33] The expression of more mature tendon markers such as *Mkx*, *Fmod*, and *Tnmd* subsequently follows these earlier developmental events.[34,35,52,53] TLPs in fibrin TECs also show a temporal increase in Scx expression over the 2-week culture period, followed by enhanced expression of *Col1α1*, *Col3α1*, *Mkx*, *Fmod*, and *Tnmd* by later time points (Fig. 3 and Supplementary Table S1). While expression of these markers also initially increased in collagen TECs, they actually declined by day 14 to levels well below normal tenogenic expression (Fig. 2). However, despite these improvements of fibrin over collagen, it is important to note that a number of genes investigated (e.g., *Egr1*, *Tnmd,* and *Runx2*) were misregulated compared with normal. These data suggest that TLPs were either lacking some appropriate signals or receiving inappropriate cues, and further research is warranted to identify how to more effectively regulate the expression of these markers *in vitro*. Nevertheless, such temporal differences in tenogenic expression between fibrin and collagen suggest that these materials might differentially affect ECM composition and organization and, ultimately, TEC biomechanical properties.

The initial composition of the TEC material had a significant effect on collagen organization with fibrin-based TECs producing a more aligned ECM than collagen-based TECs. Fibrin appeared to be greatly degraded by day 14 (Supplementary Fig. S2A, B). In fact, rapid loss of fibrin-based constructs was evident in the absence of TGFβ supplementation (Supplementary Fig. S2C); however, acellular

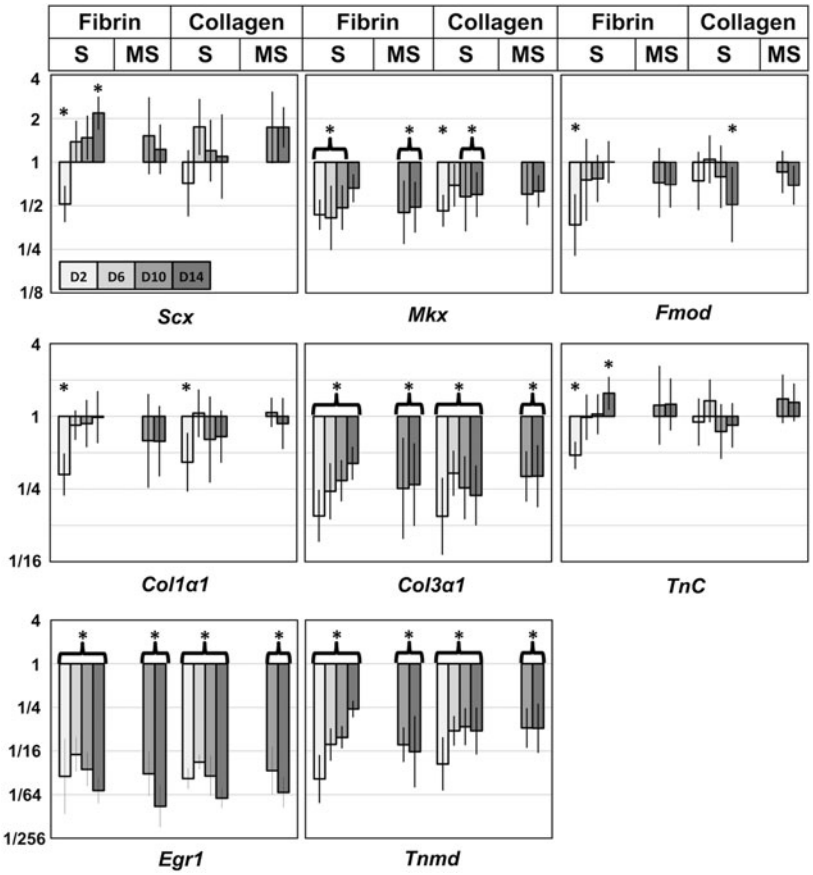

FIG. 3. Tenogenic expression increases with culture time in fibrin but not collagen TECs. Gene expression fold change plots (normalized to E17.5 Scx-expressing cells) illustrate temporal changes in individual genes for each TEC material. Consistent with principal component analysis illustrated in Figure 2, static fibrin TECs exhibit continual temporal increases from day 2 through 14 for most tenogenic markers investigated, whereas collagen shows upregulation of these markers early (days 2 and 6) followed by moderate decreases by day 14. In fibrin TECs, some genes are only moderately overexpressed (*Scx*, *TnC*) or underexpressed (*Col3α1*, *Tnmd*) by day 14. *Different from E17.5 ScxGFP + sorted cells ($p < 0.05$).

fibrin TECs remain intact and are minimally degraded over the 2-week culture period (data not shown). These findings are consistent with other reports showing that smooth muscle cells can actively degrade fibrin even in the presence of a fibrinolytic inhibitor.[54,55] In our study, TLPs counteracted the degrading fibrin matrix by synthesizing and organizing their own ECM. This cell-derived ECM showed improved packing of aligned collagen through the full TEC thickness by day 14 (Figs. 4 and 5A–C). Taken together, these findings suggest that the cell-derived matrix was largely responsible for the mechanical function of fibrin-based TECs at the end of culture. By contrast, collagen TECs retained the exogenous collagen matrix at each time point, showing an abundance of amorphous collagen (Figs. 4 and 5E–H) and larger diameter fibrils (> 60 nm), which were not found in the fibrin TECs at any time point (Fig. 6). These results suggest that the mechanical properties of collagen-based TECs depend on both cell-derived matrix and the exogenous, residual collagen still present from the initial material. Furthermore, our results show that collagen alignment only appreciably improved at the surfaces of collagen TECs (Figs. 4 and 5G). TNC is a protein associated with tissue remodeling during development and healing,[33,50,56] and our data illustrate that the interior of fibrin-based TECs showed greater expression of this marker compared with collagen under all treatment conditions (Fig. 4). However, TNC expression was increased at the edges of collagen TECs by day 14, thus suggesting that greater matrix remodeling occurred in the collagen TEC periphery. Moreover, small-diameter (presumably cell-derived) collagen fibrils adjacent to the cell membrane in collagen TECs were less well aligned with the tension axis than corresponding fibrils in fibrin TECs (Fig. 6), consistent with findings that the presence of disorganized collagen in the ECM hinders subsequent deposition of organized cell-derived collagen.[57] Although previous reports have illustrated positive correlations between the average fibril diameter and biomechanical properties of tendon tissues,[38,58] this was not evident in our TECs. In fact, average fibril diameter actually decreased over time (Fig. 6), whereas linear modulus and stiffness increased (Fig. 7). The reduction in average fibril diameters was caused by the increased prevalence of smaller-diameter fibrils (15–30 nm) present at day 14 (Fig. 6). In the case of fibrin-based TECs, this increased prevalence of fibrils (in concert with matrix contraction) led to increases in fibril packing density (i.e., FAF), which has also been shown to positively affect the mechanical properties of tendon tissues.[39] Since the alignment and packing of collagen along the loading axis is critical for the mechanical function of tendons and ligaments, our results strongly suggest that the improved density of collagen, aligned along the axis of tension, contributed to the greater than 2.2-fold increased linear modulus of fibrin TECs compared with collagen TECs at day 14 (Fig. 7B). Although the small size of tendons/ligaments from which TLPs were harvested precludes measurement of tissue mechanics in the embryonic mouse, TEC modulus at day 14 (~ 1 MPa) approximates the modulus of developing embryonic and postnatal

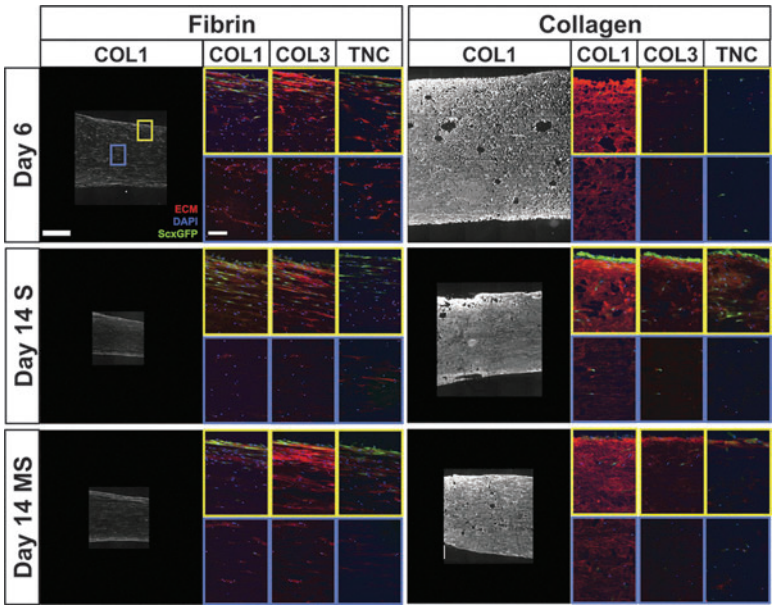

FIG. 4. Fibrin TECs show improved alignment of extracellular matrix (ECM) proteins compared with collagen TECs. Immunostaining of longitudinal cryosections ($\sim$200 µm from the top surface) shows the localization and organization of ECM proteins for fibrin- (*left column*) and collagen-based (*right column*) TECs. 5× magnification images show COL1 (*gray*), and 20× magnification *insets* show COL1, COL3, or TNC (*red*, as indicated), ScxGFP-expressing cells (*green*), and cell nuclei (DAPI, *blue*) at the edges (*yellow boxes*) and center (*blue boxes*) of TECs. Collagen TECs show greater presence of COL1 throughout their entire width than fibrin TECs at all time points, due in part by the staining of the bovine collagen in the gel. Both materials show a greater intensity of COL1, COL3, and TNC at the TEC edges; however, fibrin TECs show increases in COL3 and TnC at the TEC center compared with collagen TECs. Despite the increased presence of COL1 in collagen TECs, all markers are better aligned in fibrin TECs. Mechanical stimulation did not create any notable effects on ECM proteins. 5×, scale bar = 1 mm. 20×, scale bar = 200 µm.

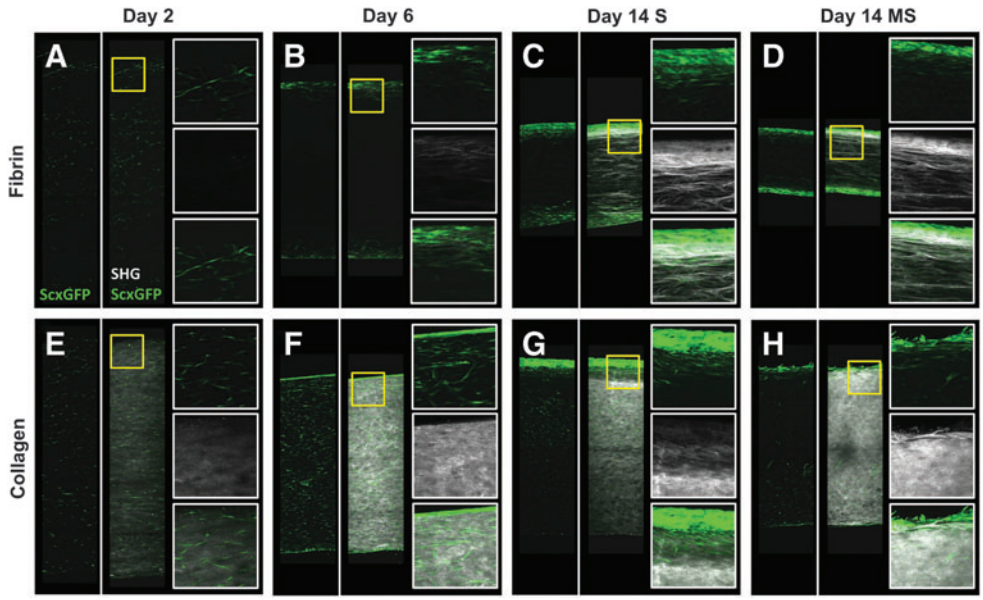

FIG. 5. Fibrin TECs exhibit temporal improvements in collagen alignment throughout the TEC thickness, whereas collagen TECs show only minimally improved alignment at the TEC surface. Shown in Z-projections (100 µm thick optical sections) at the center of TECs are spatiotemporal changes in the localization of Scx-expressing cells (*green*) and alignment of collagen (*gray*; second harmonic generation, SHG) in fibrin **(A–D)** and collagen **(E–H)** TECs. Vertically aligned, *yellow box insets* correspond to a magnified (300 × 300 µm) region as depicted at the TEC surface, illustrating ScxGFP, SHG, or ScxGFP/SHG (merged images), respectively. Scx-expressing cells elongate along the axis of tension (left to right in images) and are relatively homogenously distributed by day 2 in both fibrin **(A)** and collagen **(E)** TECs. Higher numbers of Scx-expressing cells are concentrated at the surface by day 14 with fewer cells located toward the center of the TECs **(C, G)**. SHG signal illustrates that collagen is aligned along the axis of tension throughout the depth of fibrin TECs **(C)**, whereas collagen TECs only exhibit alignment of collagen near their surface **(G)**. Mechanical stimulation reduces the thickness of the Scx-expressing cell layer at the surface of fibrin **(D)** and collagen **(H)** TECs.

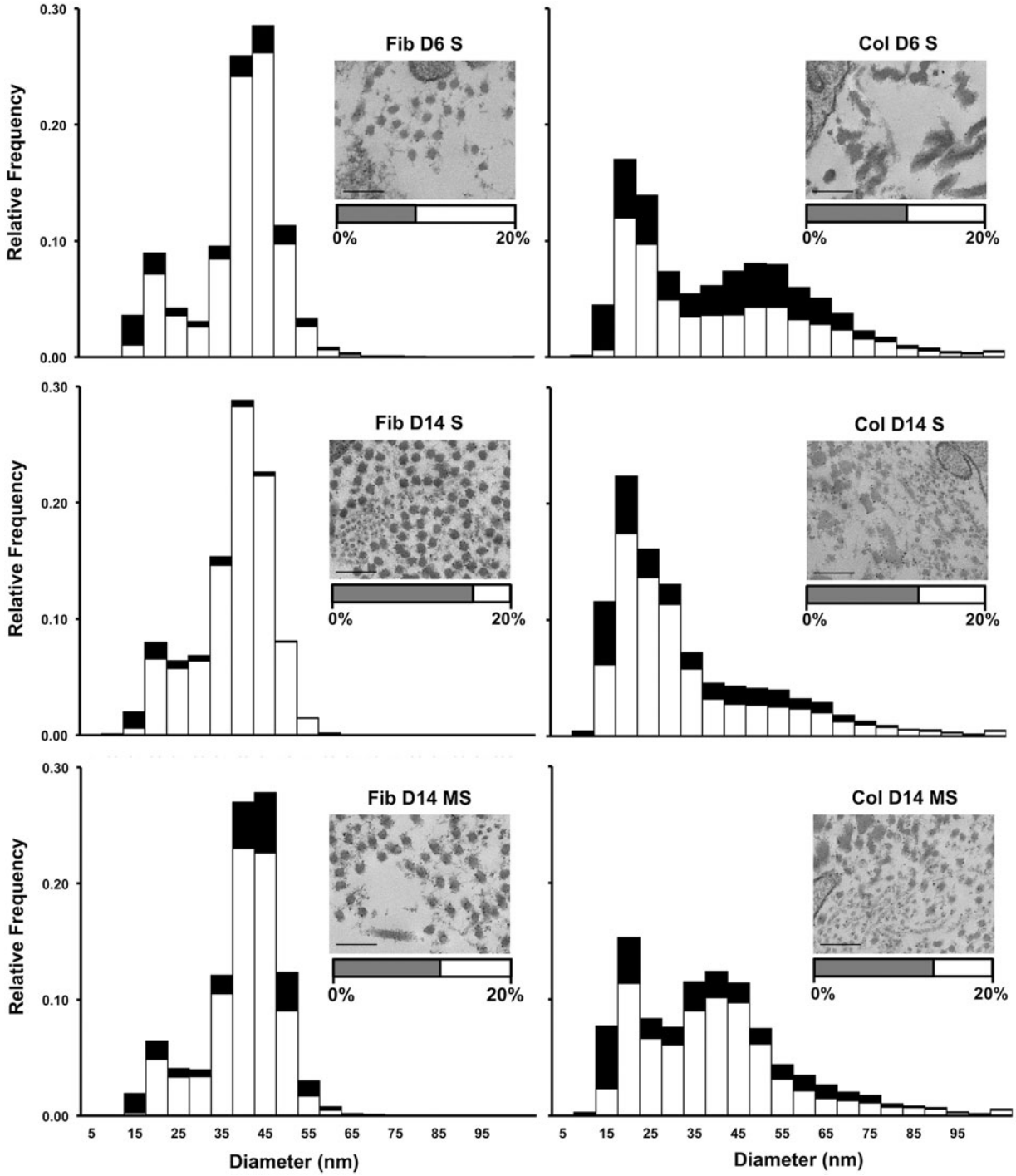

FIG. 6. Fibrin TECs exhibit improved alignment and increased packing density of collagen fibrils compared with collagen TECs by day 14. The relative frequencies of fibril diameters (nm) are shown for fibrin (*left column*) and collagen (*right column*) for static TECs at days 6 and 14 and for mechanically stimulated TECs at day 14 (note that mechanical stimulation was initiated at day 6). These fibril diameter distributions illustrate how material, time, and mechanical stimulation affect both aligned (*white vertical bars*) and poorly aligned (*black vertical bars*) collagen fibrils. Micrograph *insets* depict representative images of each treatment group, including *gray bars* that represent the fibril area fraction (FAF, relative to full scale of 20%). Fibrin TECs show a more aligned and narrower distribution of fibril diameters compared with collagen TECs for all treatment conditions. Although collagen TECs include larger-diameter fibrils (>65 nm) not seen in fibrin TECs, many of these collagen fibrils are poorly aligned. Average fibril diameter for both materials decreases slightly over time, although mechanical stimulation slows this reduction. FAF, indicative of collagen fibril packing density, increases 79.5% in fibrin TECs with little change seen for collagen TECs. *Inset* scale bar = 200 nm.

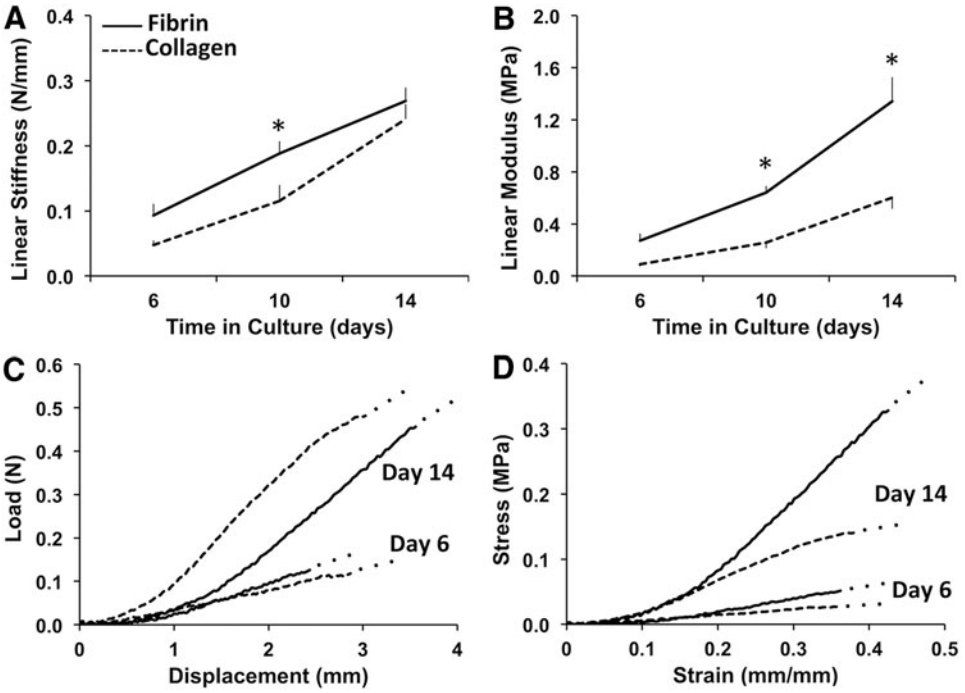

FIG. 7. Fibrin TECs show increased material properties compared with collagen TECs by day 14. Linear stiffness (explicitly shown in **A** and computed from the slope of the curve in **C**) increases with time in culture for both materials. While fibrin TECs do have significantly greater stiffness than collagen TECs at day 10, this effect is no longer apparent by day 14. However, fibrin TECs show significantly greater increases in linear modulus **(B, D)** due to their smaller cross-sectional areas at all time points (Fig. 2 and Table 1). The sub-failure load-displacement **(C)** and stress-strain **(D)** curves illustrate that both materials developed the toe, linear, and failure regions, which is characteristic of normal tendon tissue. *Significance with regard to material ($p < 0.05$).

tendons as measured in studies of chick tenogenesis (ranging from ~0.1 to 2.0 MPa during mid- to late-stage development).[59,60] While moduli of tendons rapidly increase for approximately three orders of magnitude during postnatal maturation,[61–63] we and others have shown that implanting TECs *in vivo* increases their linear modulus 2–3 orders of magnitude,[23,24,64] suggesting that these TECs may be suitable for injury repair.

Since efficient transduction of mechanical stimuli in tendon depends on an organized ECM, we also evaluated how our TECs responded to mechanical stimulation and found a significant interaction between material and mechanical stimulation for linear stiffness, PC1-associated gene expression, and FAF. Our findings indicate that collagen rather than fibrin TECs respond positively to mechanical stimulation. While pilot data showed that both materials reach ~85% contraction by day 6 during static culture, the cell-derived collagen matrix in statically cultured fibrin TECs improves in density and alignment between days 6 and 14 (Fig. 5B, C). Therefore, we hypothesize that the cell-derived collagen matrix in fibrin TECs at day 6 is not yet sufficient to appropriately respond to this stimulus and that delaying the onset of mechanical stimulation might prove beneficial. However, other studies have shown that peak strains for approximately 10% are required to increase collagen production and mechanical properties of fibrin-based TECs,[30,31,65] suggesting that the 2.4% peak strain used in our study may have been too low to stimulate beneficial effects. By contrast, mechanical stimulation improved tenogenic expression patterns in collagen TECs, as they retained near normal expression for approximately 14 days in culture. Mechanical stimulation has been found to upregulate tenogenic markers in other progenitor cells in collagen TECs,[14,21,66] illustrating that such a stimulus can regulate tenogenic expression when appropriately applied. Future research will examine how modifying the mechanical stimulus (e.g., time of initiation, strain amplitude, and frequency) differentially affects fibrin and collagen TECs.

This study is not without limitations. (1) Due to the small size of embryonic mice, we were not able to harvest tendon/ligament tissue alone. Instead, we sorted for cells that express the tendon/ligament-selective transcription factor Scleraxis. While we verified that these cells reside in the tissues of interest during late embryonic development (Supplementary Fig. S1B), other non-Scx-expressing cell populations might also contribute to tendon and ligament formation. (2) The initial protein concentrations of collagen and fibrin were not equal but selected to be consistent with numerous previous studies.[22,26,28,30,31,67] However, other studies have illustrated that varying the concentrations of these materials can affect parameters such as collagen synthesis, cell proliferation, TEC contraction, and biomechanics.[40,55,68] Systematically varying the concentration of collagen or fibrin used in future studies will be necessary to evaluate whether the effects measured here have been influenced by these differences. (3) We stimulated TECs with TGFβ-3 supplemented medium, because fibrin TECs failed to form and collagen TECs failed to contract without this growth factor. Since TGFβ-3 regulates collagen synthesis and other tendon markers,[42,69,70] its presence may have affected interactions among material and time or masked the effects of mechanical stimulation. Future research will focus on how TGFβ-3 and other growth factors influence expression of such tenogenic markers in progenitor cells. (4) Evaluating TECs for approximately 14 days in culture may have missed longer-term maturation effects. While this endpoint corresponds to previous *in vitro* and *in vivo* TEC studies using these materials,[23,24,28] further research may be warranted to understand how fibrin-based TECs respond to

longer culture periods. For instance, do they retain their normal tenogenic expression and improve mechanically or do they lose their normal tenogenic expression similar to collagen TECs at day 14?

By comparing fibrin and collagen biopolymers for tendon/ligament tissue engineering, this study has identified that fibrin TECs exhibit improved tenogenic expression patterns, collagen alignment, and linear modulus compared with collagen TECs for approximately 2 weeks in culture. Although these findings suggest that fibrin may be a more suitable scaffold material than collagen to create TECs for tendon and ligament repair, unanswered questions remain. Do these *in vitro* improvements in TEC formation translate to improved tendon repairs *in vivo*? We previously found that TEC linear stiffness and linear modulus were positively correlated with *in vivo* tendon repair stiffness and modulus, respectively.[23,24] However, it is not yet clear whether the quantity (stiffness) or the quality (modulus) of the collagenous matrix implanted at surgery is more critical for improving tendon repair. Although fibrin-based TECs clearly show improved collagen organization and linear modulus relative to collagen TECs at day 14, collagen TECs are larger and exhibit similar linear stiffness to fibrin TECs during the culture period. Furthermore, we do not know how the phenotype of the cells at implantation affects subsequent healing. Does promoting tenogenic differentiation *in vitro* improve the repair? Although we have identified *in vitro* improvements in fibrin-based TECs, these findings have not been evaluated using a more mature, clinically relevant stem/progenitor cell source. However, research using adult cells in fibrin suggests that stimulation with growth factors can be used to significantly enhance collagen deposition and mechanics,[25,71] which could be used to generate TECs sufficient for implantation. Clearly, the debate about which material is more effective for tissue-engineered repairs has only just begun, and future research should identify the extent to which enhancing *in vitro* properties of fibrin- and collagen-based TECs improves tendon repair. Significant advancements in tissue engineering strategies will be made as we identify how *in vitro* modulation of TEC biological, structural, and mechanical properties contributes to positively affecting repair *in vivo*.

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Disclosure Statement

No competing financial interests exist for any of the authors.

References

1. United States Bone and Joint Initiative. The Burden of Musculoskeletal Diseases in the United States. Rosemont, IL: American Academy of Orthopaedic Surgeons, 2008.
2. Lohmander, L.S., Ostenberg, A., Englund, M., and Roos, H. High prevalence of knee osteoarthritis, pain, and functional limitations in female soccer players twelve years after anterior cruciate ligament injury. Arthritis Rheum **50,** 3145, 2004.
3. Streich, N.A., Zimmermann, D., Bode, G., and Schmitt, H. Reconstructive versus non-reconstructive treatment of anterior cruciate ligament insufficiency. A retrospective matched-pair long-term follow-up. Int Orthop **35,** 607, 2011.
4. Sajovic, M., Strahovnik, A., Dernovsek, M.Z., and Skaza, K. Quality of life and clinical outcome comparison of semitendinosus and gracilis tendon versus patellar tendon autografts for anterior cruciate ligament reconstruction: an 11-year follow-up of a randomized controlled trial. Am J Sports Med **39,** 2161, 2011.
5. Stener, S., Ejerhed, L., Movin, T., Sernert, N., Papadogiannakis, N., and Kartus, J. The reharvested patellar tendon has the potential for ligamentization when used for anterior cruciate ligament revision surgery. Knee Surg Sports Traumatol Arthrosc **20,** 1168, 2012.
6. Schweitzer, R., Chyung, J.H., Murtaugh, L.C., Brent, A.E., Rosen, V., Olson, E.N., Lassar, A., and Tabin, C.J. Analysis of the tendon cell fate using Scleraxis, a specific marker for tendons and ligaments. Development **128,** 3855, 2001.
7. Mendias, C.L., Gumucio, J.P., Bakhurin, K.I., Lynch, E.B., and Brooks, S.V. Physiological loading of tendons induces scleraxis expression in epitenon fibroblasts. J Orthop Res **30,** 606, 2012.
8. Shukunami, C., Takimoto, A., Oro, M., and Hiraki, Y. Scleraxis positively regulates the expression of tenomodulin, a differentiation marker of tenocytes. Dev Biol **298,** 234, 2006.
9. Léjard, V., Brideau, G., Blais, F., Salingcarnboriboon, R., Wagner, G., Roehrl, M.H.A., Noda, M., Duprez, D., Houillier, P., and Rossert, J. Scleraxis and NFATc regulate the expression of the pro-α1(I) collagen gene in tendon fibroblasts. J Biol Chem **282,** 17665, 2007.
10. Murchison, N.D., Price, B.A., Conner, D.A., Keene, D.R., Olson, E.N., Tabin, C.J., and Schweitzer, R. Regulation of tendon differentiation by scleraxis distinguishes force-transmitting tendons from muscle-anchoring tendons. Development **134,** 2697, 2007.
11. Léjard, V., Blais, F., Guerquin, M., Bonnet, A., Bonnin, M., Havis, E., Malbouyres, M., Bidaud, C.B., Maro, G., Gilardi-Hebenstreit, P., Rossert, J., Ruggiero, F., and Duprez, D. EGR1 and EGR2 involvement in vertebrate tendon differentiation. J Biol Chem **286,** 5855, 2011.
12. Thomopoulos, S., Kim, H., Rothermich, S.Y., Biederstadt, C., Das, R., and Galatz, L.M. Decreased muscle loading delays maturation of the tendon enthesis during postnatal development. J Orthop Res **25,** 1154, 2007.
13. Schwartz, A.G., Lipner, J.H., Pasteris, J.D., Genin, G.M., and Thomopoulos, S. Muscle loading is necessary for the formation of a functional tendon enthesis. Bone **55,** 44, 2013.
14. Scott, A., Danielson, P., Abraham, T., Fong, G., Sampaio, A.V., and Underhill, T.M. Mechanical force modulates scleraxis expression in bioartificial tendons. J Musculoskelet Neuronal Interact **11,** 124, 2011.

15. Benhardt, H.A., and Cosgriff-Hernandez, E. The role of mechanical loading in ligament tissue engineering. Tissue Eng Part B Rev **15,** 467, 2009.
16. Riehl, B.D., Park, J.H., Kwon, I.K., and Lim, J.Y. Mechanical stretching for tissue engineering: two-dimensional and three-dimensional constructs. Tissue Eng Part B Rev **18,** 288, 2012.
17. Awad, H.A., Butler, D.L., Harris, M.T., Ibrahim, R.E., Wu, Y., Young, R.G., Kadiyala, S., and Boivin, G.P. *In vitro* characterization of mesenchymal stem cell-seeded collagen scaffolds for tendon repair: effects of initial seeding density on contraction kinetics. J Biomed Mater Res Part A **51,** 233, 2000.
18. Abraham, T., Carthy, J., and McManus, B. Collagen matrix remodeling in 3-dimensional cellular space resolved using second harmonic generation and multiphoton excitation fluorescence. J Struct Biol **169,** 36, 2010.
19. Awad, H.A., Butler, D.L., Boivin, G.P., Smith, F.N.L., Malaviya, P., Huibregtse, B., and Caplan, A.I. Autologous mesenchymal stem cell-mediated repair of tendon. Tissue Eng Part A **5,** 267, 1999.
20. Juncosa-Melvin, N., Boivin, G.P., Gooch, C., Galloway, M.T., West, J.R., Dunn, M.G., and Butler, D.L. The effect of autologous mesenchymal stem cells on the biomechanics and histology of gel-collagen sponge constructs used for rabbit patellar tendon repair. Tissue Eng Part A **12,** 369, 2006.
21. Juncosa-Melvin, N., Matlin, K.S., Holdcraft, R.W., Nirmalanandhan, V.S., and Butler, D.L. Mechanical stimulation increases collagen type I and collagen type III gene expression of stem cell-collagen sponge constructs for patellar tendon repair. Tissue Eng Part A **13,** 1219, 2007.
22. Awad, H.A., Boivin, G.P., Dressler, M.R., Smith, F.N.L., Young, R.G., and Butler, D.L. Repair of patellar tendon injuries using a cell-collagen composite. J Orthop Res **21,** 420, 2003.
23. Juncosa-Melvin, N., Shearn, J.T., Boivin, G.P., Gooch, C., Galloway, M.T., West, J.R., Nirmalanandhan, V.S., Bradica, G., and Butler, D.L. Effects of mechanical stimulation on the biomechanics and histology of stem cell-collagen sponge constructs for rabbit patellar tendon repair. Tissue Eng Part A **12,** 2291, 2006.
24. Shearn, J.T., Juncosa-Melvin, N., Boivin, G.P., Galloway, M.T., Goodwin, W., Gooch, C., Dunn, M.G., and Butler, D.L. Mechanical stimulation of tendon tissue engineered constructs: effects on construct stiffness, repair biomechanics, and their correlation. J Biomech Eng **129,** 848, 2007.
25. Neidert, M.R., Lee, E.S., Oegema, T.R., and Tranquillo, R.T. Enhanced fibrin remodeling *in vitro* with TGF-beta1, insulin and plasmin for improved tissue-equivalents. Biomaterials **23,** 3717, 2002.
26. Grassl, E.D., Oegema, T.R., and Tranquillo, R.T. Fibrin as an alternative biopolymer to type-I collagen for the fabrication of a media equivalent. J Biomed Mater Res Part A **60,** 607, 2002.
27. Sander, E.A., Barocas, V.H., and Tranquillo, R.T. Initial fiber alignment pattern alters extracellular matrix synthesis in fibroblast-populated fibrin gel cruciforms and correlates with predicted tension. Ann Biomed Eng **39,** 714, 2011.
28. Kalson, N.S., Holmes, D.F., Kapacee, Z., Otermin, I., Lu, Y., Ennos, R.A., Canty-Laird, E., and Kadler, K.E. An experimental model for studying the biomechanics of embryonic tendon: evidence that the development of mechanical properties depends on the actinomyosin machinery. Matrix Biol **29,** 678, 2010.
29. Kalson, N.S., Holmes, D.F., Herchenhan, A., Lu, Y., Starborg, T., and Kadler, K.E. Slow stretching that mimics embryonic growth rate stimulates structural and mechanical development of tendon-like tissue *in vitro*. Dev Dyn **240,** 2520, 2011.
30. Balestrini, J.L., and Billiar, K.L. Equibiaxial cyclic stretch stimulates fibroblasts to rapidly remodel fibrin. J Biomech **39,** 2983, 2006.
31. Nieponice, A., Maul, T.M., Cumer, J.M., Soletti, L., and Vorp, D.A. Mechanical stimulation induces morphological and phenotypic changes in bone marrow-derived progenitor cells within a three-dimensional fibrin matrix. J Biomed Mater Res Part A **81,** 523, 2007.
32. Pryce, B.A., Brent, A.E., Murchison, N.D., Tabin, C.J., and Schweitzer, R. Generation of transgenic tendon reporters, ScxGFP and ScxAP, using regulatory elements of the scleraxis gene. Dev Dyn **236,** 1677, 2007.
33. Liu, C., Aschbacher-Smith, L., Bathelery, N.J., Dyment, N., Butler, D.L., and Wylie, C. Spatial and temporal expression of molecular markers and cell signals during normal development of the mouse patellar tendon. Tissue Eng Part A **18,** 598, 2012.
34. Liu, W., Watson, S.S., Lan, Y., Keene, D.R., Ovitt, C.E., Liu, H., Schweitzer, R., and Jiang, R. The atypical homeodomain transcription factor Mohawk controls tendon morphogenesis. Mol Cell Biol **30,** 4797, 2010.
35. Docheva, D., Hunziker, E.B., Fassler, R., and Brandau, O. Tenomodulin is necessary for tenocyte proliferation and tendon maturation. Mol Cell Biol **25,** 699, 2005.
36. Kjaer, M. Role of extracellular matrix in adaptation of tendon and skeletal muscle to mechanical loading. Physiol Rev **84,** 649, 2004.
37. Mackie, E.J., Ahmed, Y.A., Tatarczuch, L., Chen, K., and Mirams, M. Endochondral ossification: how cartilage is converted into bone in the developing skeleton. Int J Biochem Cell Biol **40,** 46, 2008.
38. Christiansen, D.L., Huang, E.K., and Silver, F.H. Assembly of type I collagen: fusion of fibril subunits and the influence of fibril diameter on mechanical properties. Matrix Biol **19,** 409, 2000.
39. Goh, K.L., Holmes, D.F., Lu, H.-., Richardson, S., Kadler, K.E., Purslow, P.P., and Wess, T.J. Ageing changes in the tensile properties of tendons: influence of collagen fibril volume fraction. J Biomech Eng **130,** 021011, 2008.
40. Juncosa-Melvin, N., Boivin, G.P., Galloway, M.T., Gooch, C., West, J.R., Sklenka, A.M., and Butler, D.L. Effects of cell-to-collagen ratio in mesenchymal stem cell-seeded implants on tendon repair biomechanics and histology. Tissue Eng Part A **11,** 448, 2005.
41. Hata, R., and Senoo, H. L-ascorbic acid 2-phosphate stimulates collagen accumulation, cell proliferation, and formation of a three-dimensional tissue-like substance by skin fibroblasts. J Cell Physiol **138,** 8, 1989.
42. Murata, H., Zhou, L., Ochoa, S., Hasan, A., Badiavas, E., and Falanga, V. TGF-β3 stimulates and regulates collagen synthesis through TGF-β1-dependent and independent mechanisms. J Invest Dermatol **108,** 258, 1997.
43. Kapacee, Z., Yeung, C.Y.C., Lu, Y., Crabtree, D., Holmes, D.F., and Kadler, K.E. Synthesis of embryonic tendon-like tissue by human marrow stromal/mesenchymal stem cells requires a three-dimensional environment and transforming growth factor β3. Matrix Biol **29,** 668, 2010.
44. Nirmalanandhan, V.S., Shearn, J.T., Juncosa-Melvin, N., Rao, M., Gooch, C., Jain, A., Bradica, G., and Butler, D.L. Improving linear stiffness of the cell-seeded collagen

sponge constructs by varying the components of the mechanical stimulus. Tissue Eng Part A **14,** 1883, 2008.
45. Ushiku, C., Adams, D.J., Jiang, X., Wang, L., and Rowe, D.W. Long bone fracture repair in mice harboring GFP reporters for cells within the osteoblastic lineage. J Orthop Res **28,** 1338, 2010.
46. Chen, X., Nadiarynkh, O., Plotnikov, S., and Campagnola, P.J. Second harmonic generation microscopy for quantitative analysis of collagen fibrillar structure. Nat Protoc **7,** 654, 2012.
47. R Core Team. R: a language and environment for statistical computing. www.R-project.org, 2013.
48. Starborg, T., Kalson, N.S., Lu, Y., Mironov, A., Cootes, T.F., Holmes, D.F., and Kadler, K.E. Using transmission electron microscopy and 3View to determine collagen fibril size and three-dimensional organization. Nat Protoc **8,** 1433, 2013.
49. Ringnér, M. What is principal component analysis? Nat Biotechnol **26,** 303, 2008.
50. Dyment, N.A., Liu, C., Kazemi, N., Aschbacher-Smith, L., Kenter, K., Breidenbach, A.P., Shearn, J.T., Wylie, C., Rowe, D.W., and Butler, D.L. The paratenon contributes to scleraxis-expressing cells during patellar tendon healing. PLoS One **8,** e59944, 2013.
51. Jackson, J.E. A User's Guide to Principal Components. Hoboken, NJ: John Wiley & Sons, Inc., 2003.
52. Ito, Y., Toriuchi, N., Yoshitaka, T., Ueno-Kudoh, H., Sato, T., Yokoyama, S., Nishida, K., Akimoto, T., Takahashi, M., Miyaki, S., and Asahara, H. The Mohawk homeobox gene is a critical regulator of tendon differentiation. Proc Natl Acad Sci U S A **107,** 10538, 2010.
53. Ezura, Y., Chakravarti, S., Oldberg, A., Chervoneva, I., and Birk, D.E. Differential expression of lumican and fibromodulin regulate collagen fibrillogenesis in developing mouse tendons. J Cell Biol **151,** 779, 2000.
54. Grassl, E.D., Oegema, T.R., and Tranquillo, R.T. A fibrin-based arterial media equivalent. J Biomed Mater Res Part A **66,** 550, 2003.
55. Ahmann, K.A., Weinbaum, J.S., Johnson, S.L., and Tranquillo, R.T. Fibrin degradation enhances vascular smooth muscle cell proliferation and matrix deposition in fibrin-based tissue constructs fabricated *in vitro*. Tissue Eng Part A **16,** 3261, 2010.
56. Imanaka-Yoshida, K., Hiroe, M., and Yoshida, T. Interaction between cell and extracellular matrix in heart disease: multiple roles of tenascin-C in tissue remodeling. Histol Histopathol **19,** 517, 2004.
57. Saeidi, N., Guo, X., Hutcheon, A.E.K., Sander, E.A., Bale, S.S., Melotti, S.A., Zieske, J.D., Trinkaus-Randall, V., and Ruberti, J.W. Disorganized collagen scaffold interferes with fibroblast mediated deposition of organized extracellular matrix *in vitro*. Biotechnol Bioeng **109,** 2683, 2012.
58. Dressler, M.R., Butler, D.L., Wenstrup, R., Awad, H.A., Smith, F., and Boivin, G.P. A potential mechanism for age-related declines in patellar tendon biomechanics. J Orthop Res **20,** 1315, 2002.
59. McBride, J.,D.J., Trelstad, R.L., and Silver, F.H. Structural and mechanical assessment of developing chick tendon. Int J Biol Macromol **10,** 194, 1988.
60. Marturano, J.E., Arena, J.D., Schiller, Z.A., Georgakoudi, I., and Kuo, C.K. Characterization of mechanical and biochemical properties of developing embryonic tendon. Proc Natl Acad Sci U S A **110,** 6370, 2013.
61. Ansorge, H.L., Meng, X., Zhang, G., Veit, G., Sun, M., Klement, J.F., Beason, D.P., Soslowsky, L.J., Koch, M., and Birk, D.E. Type XIV collagen regulates fibrillogenesis: premature collagen fibril growth and tissue dysfunction in null mice. J Biol Chem **284,** 8427, 2009.
62. Ansorge, H.L., Adams, S., Birk, D.E., and Soslowsky, L.J. Mechanical, compositional, and structural properties of the post-natal mouse achilles tendon. Ann Biomed Eng **39,** 1904, 2011.
63. Zhang, G., Ezura, Y., Chervoneva, I., Robinson, P.S., Beason, D.P., Carine, E.T., Soslowsky, L.J., Iozzo, R.V., and Birk, D.E. Decorin regulates assembly of collagen fibrils and acquisition of biomechanical properties during tendon development. J Cell Biochem **98,** 1436, 2006.
64. Calve, S., Lytle, I.F., Grosh, K., Brown, D.L., and Arruda, E.M. Implantation increases tensile strength and collagen content of self-assembled tendon constructs. J Appl Physiol (Bethesda, MD: 1985) **108,** 875, 2010.
65. Syedain, Z.H., Weinberg, J.S., and Tranquillo, R.T. Cyclic distension of fibrin-based tissue constructs: evidence of adaptation during growth of engineered connective tissue. Proc Natl Acad Sci U S A **105,** 6537, 2008.
66. Garvin, J., Qi, J., Maloney, M., and Banes, A.J. Novel system for engineering bioartificial tendons and application of mechanical load. Tissue Eng Part A **9,** 967, 2003.
67. Kuo, C.K., and Tuan, R.S. Mechanoactive tenogenic differentiation of human mesenchymal stem cells. Tissue Eng Part A **14,** 1615, 2008.
68. Vavken, P., Joshi, S.M., and Murray, M.M. Fibrin concentration affects ACL fibroblast proliferation and collagen synthesis. Knee **18,** 42, 2011.
69. Kuo, C.K., Petersen, B.C., and Tuan, R.S. Spatiotemporal protein distribution of TGF-betas, their receptors, and extracellular matrix molecules during embryonic tendon development. Dev Dyn **237,** 1477, 2008.
70. Pryce, B.A., Watson, S.S., Murchison, N.D., Staverosky, J.A., Dünker, N., and Schweitzer, R. Recruitment and maintenance of tendon progenitors by TGFbeta signaling are essential for tendon formation. Development **136,** 1351, 2009.
71. Hagerty, P., Lee, A., Calve, S., Lee, C.A., Vidal, M., and Baar, K. The effect of growth factors on both collagen synthesis and tensile strength of engineered human ligaments. Biomaterials **33,** 6355, 2012.

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