Multilayered Electrospun Scaffolds for Tendon Tissue Engineering

Abby Chainani,^{1,2,*} Kirk J. Hippensteel, MD,^{1,*} Alysha Kishan, BS,^{1,2} N. William Garrigues, PhD,^{1,2}
David S. Ruch, MD,¹ Farshid Guilak, PhD,^{1,2} and Dianne Little, BVSc, PhD¹

Full-thickness rotator cuff tears are one of the most common causes of shoulder pain in people over the age of 65. High retear rates and poor functional outcomes are common after surgical repair, and currently available extracellular matrix scaffold patches have limited abilities to enhance new tendon formation. In this regard, tissue-engineered scaffolds may provide a means to improve repair of rotator cuff tears. Electrospinning provides a versatile method for creating nanofibrous scaffolds with controlled architectures, but several challenges remain in its application to tissue engineering, such as cell infiltration through the full thickness of the scaffold as well as control of cell growth and differentiation. Previous studies have shown that ligament-derived extracellular matrix may enhance differentiation toward a tendon or ligament phenotype by human adipose stem cells (hASCs). In this study, we investigated the use of tendon-derived extracellular matrix (TDM)-coated electrospun multilayered scaffolds compared to fibronectin (FN) or phosphate-buffered saline (PBS) coating for use in rotator cuff tendon tissue engineering. Multilayered poly(ϵ -caprolactone) scaffolds were prepared by sequentially collecting electrospun layers onto the surface of a grounded saline solution into a single scaffold. Scaffolds were then coated with TDM, FN, or PBS and seeded with hASCs. Scaffolds were maintained without exogenous growth factors for 28 days in culture and evaluated for protein content (by immunofluorescence and biochemical assay), markers of tendon differentiation, and tensile mechanical properties. The collagen content was greatest by day 28 in TDM-scaffolds. Gene expression of type I collagen, decorin, and tenascin C increased over time, with no effect of scaffold coating. Sulfated glycosaminoglycan and dsDNA contents increased over time in culture, but there was no effect of scaffold coating. The Young's modulus did not change over time, but yield strain increased with time in culture. Histology demonstrated cell infiltration through the full thickness of all scaffolds and immunofluorescence demonstrated greater expression of type I, but not type III collagen through the full thickness of the scaffold in TDM-scaffolds compared to other treatment groups. Together, these data suggest that nonaligned multilayered electrospun scaffolds permit tenogenic differentiation by hASCs and that TDM may promote some aspects of this differentiation.

Introduction

SHOULDER PAIN is the leading cause of musculoskeletal pain in people over the age of 65,¹ and rotator cuff and subacromial bursa pathologies contribute most to shoulder pain.² The functional rotator cuff is a composite of several tendons, ligaments, and muscles, but tears involving the supraspinatus and infraspinatus tendons are the most commonly identified injuries.³ Rotator cuff tears account for over 4 million physician visits and 300,000 surgical repairs annually,^{4,5} and have a similar impact on individual quality of life as diseases such as congestive heart failure, diabetes, or

clinical depression.⁶ Suture repair to reattach the torn tendon to bone using an arthroscopic or open approach is the most common repair technique and is highly cost effective.⁷ However, up to 30% of rotator cuff tears are irreparable,⁸ and retear rates of 34%–94% are reported after suture repair.⁹ Efforts to improve clinical outcomes have focused on the use of extracellular matrix scaffold allograft or xenograft patches for mechanical augmentation of rotator cuff repair. These patches are thought to improve healing in the critical early postoperative period because they contain bioactive molecules. However, these approaches have limitations for a variety of reasons, including poor cellularization, immunogenic

Abstract previously presented by Hippensteel KJ, Chainani A, Kishan A, Garrigues NW, Ruch DS, Guilak F, Little D. Tendon-Derived Matrix Coated Multi-Layered Hydrospun Scaffolds for Tendon Tissue Engineering. *Trans Orthop Res Soc* 2012.

Departments of ¹Orthopaedic Surgery and ²Biomedical Engineering, Duke University Medical Center, Durham, North Carolina.

*These authors are cofirst authors.

responses, insufficient mechanical properties to support physiological loading postoperatively, and poor suture retention.^{10–17}

Tissue engineering affords the opportunity to overcome some of these limitations by providing functional, cell-based scaffolds for rotator cuff repair. In particular, nanofibrous scaffolds are suited for this application because they can be engineered to mimic the ultrastructure and properties of the native tendon, using techniques such as electrospinning.¹⁰ Several such approaches are under investigation, including the use of nanofibrous scaffolds for interfacial regeneration of the tendon-to-bone interface^{18–21} as an over-the-top tendon augmentation scaffold,^{22,23} and as interposition grafts.²⁴ One challenge in the use of electrospun scaffolds for rotator cuff repair has been the lack of cell infiltration through the full thickness of the scaffold.^{25–27} Several approaches have been taken to mitigate this problem. For example, electrospun scaffolds based on biological materials, such as collagen, demonstrate more rapid cell infiltration than synthetic polymers.²⁷ However, mechanical properties of pure biopolymers are generally inferior to synthetic polymers,^{25,28,29} although composite fibers of synthetic and biopolymers may have improved tensile properties compared to the synthetic polymer especially at low concentrations.^{30–32} Physical methods have been used to improve the porosity and therefore cell infiltration, including the use of sacrificial fibers, which enhance cell infiltration and matrix deposition *in vitro* and in subcutaneous pouch models.^{25,26} However, in an over-the-top rotator cuff augmentation animal model, this approach was less successful possibly because the scaffold with sacrificial fibers was less resistant to compression during handling and in a challenging *in vivo* environment.²³ Other methods to improve porosity include the use of salt leaching techniques,³³ combined micro- and nanofiber scaffolds,^{34,35} and laser ablation.³⁶

Multilayered electrospinning has been reported previously, either using a layer-by-layer approach with simultaneous wet electrospinning and cell-seeding,³⁷ by alternating layers of nanofibers and microfibers,³⁸ by alternating polymers,³⁹ or by alternately coating electrospun layers with collagen.⁴⁰ With wet electrospinning, electrospun fibers can be aligned by depositing nonaligned fibers onto an aqueous collecting medium, and then drawing out the fibers using a rotating mandrel.^{41–43} Alternatively, others have reported intermittent collection of nonaligned layers of electrospun biopolymers from the surface of a saline bath, termed “hydrospinning,” followed by vacuum desiccation of the scaffold.⁴⁴ Cell-seeding by direct deposition onto the surface of these scaffolds after vacuum desiccation resulted in rapid cell infiltration throughout the scaffold, in contrast to similar scaffolds prepared by standard electrospinning techniques onto a solid ground plate.⁴⁴

In addition to early and complete cell infiltration into the scaffolds, the rapid synthesis and accumulation of the tendon-specific extracellular matrix is also likely to be crucial to the ultimate success of a patch designed for rotator cuff augmentation or interposition. We have previously shown that a ligament-derived matrix can enhance development of a ligamentous phenotype by human adipose stem cells (hASCs) compared to type I collagen gel alone,⁴⁵ and others have found that the engineered tendon matrix enhances proliferation, maintenance of tendon stem cell stemness, and

tendon-related gene expression compared to tissue culture plastic.⁴⁶ These studies align with work in other musculoskeletal fields, where tissue-specific extracellular matrices have potent ability to induce tissue-specific neotissue formation without the use of exogenous growth factors.^{47–51} Extracellular matrix proteins such as small intestinal submucosa, collagen, and silk fibroin have been incorporated into electrospun synthetic polymer fibers to improve hydrophilicity, cell attachment, early proliferation, and neotissue organization.^{52–54} However, there is controversy as to how well the native structure of collagen is preserved during electrospinning,^{28,55} and recent work confirms that collagen is unfolded in fluorinated solvents and only partially refolded during the electrospinning process itself.⁵⁶ As an alternative, coating of electrospun fibers with collagen has been used to circumvent the problem of collagen denaturation, while retaining the beneficial effects of collagen on cell attachment.⁴⁰

The primary objective of this study was to develop and evaluate the use of multilayered electrospun scaffolds fabricated by sequential collection of individual layers from the surface of an aqueous ground substrate as a single scaffold for potential use in rotator cuff tissue engineering. A second aim of the project was to evaluate if there was additional benefit to coating the scaffolds with the tendon-derived extracellular matrix (TDM) compared to fibronectin (FN), previously used to improve cell attachment⁴⁴ or phosphate-buffered saline (PBS)-coated controls.

Materials and Methods

Tendon-derived matrix preparation

Fresh digital flexor and extensor tendons were dissected from adult female porcine hindlimbs ($n = 10$) obtained from a slaughterhouse. Tissue was minced, lyophilized (Labconco Freezone 2.5L; Labconco), and pulverized using a 6750 Spex SamplePrep Freezer Mill (Spex CertiPrep). The resulting powder was sieved to pass through 106- μm wire mesh openings and stored at -80°C until use.

Multilayered electrospun scaffold preparation

Multilayered electrospun scaffolds were prepared in a similar manner as previously described.⁴⁴ Poly(ϵ -caprolactone) (PCL) (Mn = 80,000) (Sigma-Aldrich, St. Louis, MO) was dissolved at 100 mg/mL in 70% dichloromethane/30% ethanol for 24 h before use. The resulting solution was electrospun through a 25G needle fitted with a round wire mesh focusing cage (3 cm diameter, needle tip protruding 4 mm from bottom of cage) at 2.5 mL/h and 17 kV with a 17-cm needle-ground distance. The ground collector was a saline bath (1.25 g/L NaCl in distilled water), and nonaligned layers were collected sequentially from the surface of the saline bath every 90 s using a 5 $\times$ 7.5-cm glass slide, for a total of 70 layers/scaffold. Relative humidity was 20%–40% and ambient temperature was 18°C–25°C. Each scaffold was allowed to dry at room temperature, and then stored at room temperature and protected from light until use. Scaffolds were cut into individual test strips and sutured to a 3.2-mm-outer diameter Teflon ring-shaped holder to maintain static tension and suspension in media. Each scaffold was rehydrated and sterilized in a graded series of ethanol baths

before a final 30-min rinse in PBS pH7.4. Scaffolds were randomized into three treatment groups: PBS coated, FN coated, or TDM coated. Scaffolds were coated on each side by direct pipetting with human FN in PBS (BD Biosciences) at $4\text{ }\mu\text{g}/\text{cm}^2$, TDM in PBS at $2\text{ mg}/\text{cm}^2$, or an equal volume of PBS and allowed to dry. The surfaces of scaffolds were sterilized under ultraviolet light for 10 min on each side and prewetted before cell seeding.

Analysis of fiber diameter

One 6-mm biopsy punch was harvested from the center of each of three representative scaffolds, critical point dried in CO_2 , and then sputter coated with gold. Each sample was viewed with a Philips 501 Scanning Electron Microscope. Three representative images were taken of each sample and the diameter of 50 fibers within each image was measured using GNU Image Manipulation Program (GIMP) 2.8.4 freeware.

Cell culture and seeding

The hASCs used in this study were isolated by collagenase digestion⁵⁷ of lipoaspirate surgical waste from five deidentified female donors (age 36–59, body mass index 19.6–33.1) with approval of the Duke University Institutional Review Board. Cells were expanded in monolayer on tissue culture plastic through passage 4 as described previously.⁴⁵ Cells were used at passage 4 and seeded at a density of 0 or 1×10^6 hASCs/ cm^2 for each treatment group (scaffolds coated with PBS, FN, or TDM). Scaffolds were seeded on one side of the scaffold with half of the cells and allowed to incubate for 15 min. Scaffolds were then turned over and seeded with the remaining cells. The specimens were then incubated for a further 15 min before transfer to 6-well plates coated with 2% agarose, and addition of culture media. Scaffolds were maintained without growth factors at 37°C and 5% CO_2 in Advanced DMEM (Life Technologies), 10% fetal bovine serum (Zen-Bio), 1% penicillin–streptomycin–fungizone (Life Technologies), 4 mM L-glutamine (Life Technologies), and 15 mM L-ascorbic acid-2-phosphate (Sigma-Aldrich), which was changed every other day for the designated culture periods.

Biochemical assays

On days 0 (unseeded), 1, 14, and 28 (hASC-seeded), scaffolds from each treatment group (PBS, FN, and TDM) ($n=5$) were harvested and lyophilized to obtain dry weight. Samples were pulverized using a freezer mill, and digested for 1 week in a papain solution ($125\text{ }\mu\text{g}/\text{mL}$) at 60°C . The dsDNA content was quantified using the Picogreen Assay (Life Technologies). The sulfated glycosaminoglycan (s-GAG) content was quantified spectrophotometrically using the 1,9-dimethylmethylene blue (DMMB) dye (pH 3.0).⁵⁸ The hydroxyproline assay was used to determine the total collagen content using a conversion factor of 1:7.46 to convert hydroxyproline to collagen.⁵⁹ All results were normalized to dry weight (mean $\pm$ SD).

RNA isolation and real time quantitative polymerase chain reaction

RNA was extracted from hASC-seeded scaffolds in each of the three treatment groups ($n=5$) after 4, 7, and 14 days of

cell culture. Scaffolds were pulverized and RNA extraction was performed using the QiaShredder column (Qiagen) followed by the RNeasy Mini kit (Qiagen) with on-column DNAase treatment. Equal amounts of RNA were reverse transcribed using the Superscript VILO cDNA Synthesis Kit (Life Technologies). Real-time polymerase chain reaction was performed on an iCycler (Biorad) using Express qPCR SuperMix (Life Technologies). Commercially available primers and probes (Applied Biosystems) were used to compare transcript levels for seven different genes: 18S rRNA (endogenous control, assay ID Hs99999901_s1), type I collagen (COL1A1, assay ID Hs00164004_m1), type III collagen (COL3A1, assay ID Hs00164103_m1), biglycan (BGN, assay ID Hs00959143_m1), decorin (DCN, assay ID Hs00266491_m1), tenomodulin (TNMD, assay ID Hs00223332_m1), and tenascin C (TNC, assay ID Hs00233648_m1). Data from each gene of interest for each sample were corrected for efficiency and normalized to expression of 18S. These data were then expressed as fold-change relative to the level of gene expression in 1 million P4 hASCs before cell seeding from each donor at day 0.⁶⁰

Histology

Unseeded and hASC-seeded scaffolds from each of the three treatment groups ($n=5$) were harvested after 28 days of culture, embedded in optimal cutting temperature gel (Sakura), and frozen at -80°C . Samples were cut into 7–10- μm sections and mounted on slides and evaluated by light microscopy after staining with hematoxylin and eosin and safranin-O/fast green or examined under a Zeiss LSM 510 Confocal Microscope (Carl Zeiss) after immunofluorescence labeling of human type I and III collagen, as described previously.⁴⁵

Mechanical testing

After harvest at day 0 or 28, hASC-seeded dog-bone samples ($n=5$) were wrapped in gauze soaked in PBS and stored at -80°C until analysis. Samples were marked in the center and at 5 mm proximal and distal to the central mark using India ink to allow regional strain analysis of the central centimeter of the dog-bone. The ends of each sample were sandwiched and glued between fine-gauge waterproof sandpaper and then clamped and mounted in the load frame. The specimens were tested wet in tension at a strain rate of 1%/s with 0.5 g preload using an electromechanical testing system (Bose Enduratec Smart Test Series; Bose Corporation) with a 2.27 kg load cell (Sensotec Model 31; Honeywell International), using the full displacement limits of the transducer. Initial scaffold thickness was measured using a digital camera (Allied Vision Technologies, Inc.) and digital calipers in GIMP 2.8.4 freeware. Midsubstance strains were calculated from digital images acquired at 20 Hz and interpolated to load frame data using custom MATLAB (MathWorks) code. The Young's modulus of the linear region and stress and strain at yield were calculated in Excel (Microsoft Office).

Statistical analysis

Data are reported as mean $\pm$ SD, tested for normality, transformed using Box-Cox transformation if necessary,

and then evaluated for the effect of scaffold coating and time using factorial analysis of variance (ANOVA). The Newman-Keuls *post hoc* test was used to determine differences between treatments following ANOVA. Significance was reported at the 95% confidence level for all analyses ($\alpha=0.05$).

Results

Scanning electron micrographs of surface and edge of unseeded and hASC-seeded scaffolds after 28 days of culture are shown in Figure 1. The thickness of the PBS-, FN-, and TDM-scaffolds were 0.80 ± 0.06 , 0.76 ± 0.07 , and 0.73 ± 0.04 mm, respectively, at day 0 and did not differ among groups. The median fiber diameter was 2235 nm (interquartile range 1998–2468 nm). The DNA content of all scaffolds significantly increased after cell seeding and with culture until day 14, after which the DNA content did not increase further; there was no effect of scaffold coating on DNA content at any time point (Fig. 2A). The s-GAG content significantly increased after cell seeding at all time points, but there was no effect of scaffold coating (Fig. 2B). At day 0, TDM-scaffolds had significantly increased collagen content compared to the other groups, but by day 1, there was no effect of coating on collagen content between groups. The collagen content returned to day 0 levels in the TDM-coated groups by day 14, and by day 28, the collagen content of TDM-scaffolds was greater than FN- or PBS-scaffolds and all scaffolds at all other time points (Fig. 2C).

COL1A1 expression was significantly increased compared to P4 cells from day 4 and remained elevated (Fig. 3). *COL3A1* expression was increased at day 7 of culture only, and *DCN* expression increased in all scaffold groups from day 7 after seeding (Fig. 3), but *BGN* expression levels did

not change (data not shown). *TNMD* expression showed a trend ($p=0.09$) to increase at day 4 compared to other time points (data not shown). *TNC* expression increased in all scaffold groups at day 4 and remained elevated (Fig. 3). There was no significant effect of scaffold coating on gene expression.

Immunofluorescence for human type I collagen revealed a positive signal through the full thickness of the scaffold in all three cell-seeded treatment groups, but appeared to be more robust in the TDM-scaffolds (Fig. 4). Immunofluorescence for human type III collagen demonstrated robust positive signals through the full thickness in all scaffold groups (Fig. 5). Safranin O/fast green of unseeded and seeded scaffolds demonstrated synthesis of a new extracellular matrix through the full thickness of the scaffold in seeded scaffolds (Fig. 6A, B), and hematoxylin and eosin staining confirmed cellular infiltration through the full thickness of the scaffold (Fig. 6C, D).

There was no effect of time in culture or scaffold treatment on Young's modulus (Fig. 7A); however, yield strain increased from day 0 to 28 (Fig. 7B). Yield stress increased over time in culture for the FN-scaffold group only (Fig. 7C). No scaffold failed within the maximum displacement limits permitted by the actuator and transducer used in this study, representing a strain in excess of 2.5, well beyond the limits of physiological relevance, thus failure properties and mode were not determined.

Discussion

The findings of this study show that multilayered electrospun PCL scaffolds formed by electrospinning onto a saline ground solution and sequential collection of multiple layers as a single scaffold permitted complete cellular

FIG. 1. Scanning electron micrographs of edge (A, B) and surface (C, D) of unseeded (A, C) scaffolds, and phosphate-buffered saline (PBS)-coated scaffolds seeded with human adipose stem cells and cultured for 28 days (B, D).

FIG. 2. DNA (A), sulfated glycosaminoglycan (s-GAG) (B), and collagen (C) content (normalized to dry weight) of tendon-derived extracellular matrix (TDM)-, fibronectin (FN)-, and PBS-coated multilayered electrospun scaffolds at day 0 (unseeded), 1, 14, and 28, after seeding with human adipose-derived stem cells. Groups having different letters are significantly different from each other ($p \leq 0.05$, $n = 5$).

infiltration and formation of the tendon-like extracellular matrix by hASCs by measures of gene expression, protein synthesis, biochemical assays, and immunofluorescence. Additionally, coating each side of these scaffolds with TDM enhanced the synthesis and accumulation of collagen as compared to FN- or PBS-scaffolds. The mechanism of en-

hanced induction of tenogenesis by TDM in the absence of exogenous growth factors remains to be determined. Multilayered scaffolds containing TDM may solve current challenges associated with achieving cell infiltration through the full thickness of electrospun scaffolds and therefore represent a novel approach for rotator cuff tendon tissue engineering.

FIG. 3. Expression of type 1 collagen (*COL1A1*), type III collagen (*COL3A1*), decorin (*DCN*), and tenascin C (*TNC*) by human adipose-derived stem cells after seeding on TDM-, FN-, and PBS-coated multilayered electrospun scaffolds at day 4, 7, and 14 of culture, normalized to 18S expression and unseeded cell pellets at day 0. Groups having different letters are significantly different from each other ($p \leq 0.05$, $n = 5$).

FIG. 4. Immunofluorescence of human type I collagen of (-) unseeded and (+) human adipose-derived stem cell-seeded TDM-, FN-, and PBS-coated multilayered, electrospun scaffolds cultured for 28 days. Scale bar=50 μ m. Color images available online at www.liebertpub.com/tea

FIG. 5. Immunofluorescence of human type III collagen of (-) unseeded and (+) human adipose-derived stem cell-seeded TDM-, FN-, and PBS-coated multilayered, electrospun scaffolds cultured for 28 days. Scale bar=50 μ m. Color images available online at www.liebertpub.com/tea

FIG. 6. Safranin O/fast green (**A, B**) and hematoxylin/eosin (**C, D**) micrographs of unseeded (**A, C**) and human adipose-derived stem cell-seeded (**B, D**) TDM-, FN-, and PBS-coated multilayered, electrospun scaffolds cultured for 28 days. Scale bar = 100 μ m. Color images available online at www.liebertpub.com/tea

FN- and PBS-scaffolds were evaluated in this study as controls, since previously electrospun PCL scaffolds have been either left untreated,²⁵ or treated with FN to improve cell attachment.⁴⁴ We found no benefit in this study to any of the scaffold coatings on cell proliferation or ultimate infiltration, as assessed by dsDNA content and histology. Similarly, scaffold coating did not have an effect on s-GAG content, but TDM-scaffolds had increased collagen content by day 28 of cell culture compared to other groups. Not surprisingly, the collagen content was greater in the unseeded TDM-scaffolds compared to other coating groups on day 0 of culture (harvested after 2 h of time in culture media), but then decreased by 24 h after cell seeding, probably as a result of loss of the un-crosslinked TDM coating from the scaffold into the media. This finding suggests that hASCs require only a short early period of exposure or low concentrations of TDM for beneficial effects on collagen synthesis to be seen at much later time points in these scaffolds. The mechanism and effects of more stable incorporation of TDM into or onto the electrospun fibers on collagen synthesis and tenogenic differentiation are the subject of ongoing investigations in an effort to circumvent the problems associated with coating PCL nanofibers.⁵³ Further, in this study, the TDM coating was most likely to be superficial and not contained within the core of the scaffold. This issue is

also being addressed in ongoing investigations in an attempt to further enhance tenogenesis through the full thickness of the scaffold. We did not evaluate for markers of osteogenesis or chondrogenesis in this study. However, in similar studies, to evaluate the chondrogenic response of hASCs on uncoated multilayered electrospun PCL scaffolds, we have found weak initial gene expression of aggrecan and type II and X collagen by hASCs, which does not change significantly after seeding, and negative immunohistochemistry for type II or X collagen in the absence of chondrogenic growth factors or cartilage-derived matrix (data not shown).

Expression of type III collagen as assessed by immunofluorescence was not noticeably different between scaffold groups, however, type I collagen immunofluorescence was markedly increased in TDM-scaffolds after 28 days of culture compared to other groups, and surprisingly FN-scaffolds appeared to have the lowest content of new type I collagen. Interestingly however, at the mRNA level, no difference between scaffold groups was identified in *COL1A1* expression.

The Young's modulus was not different between scaffold groups and did not increase after 28 days in culture, whereas the yield strain increased. Values reported in this study using a 1%/s strain rate for the Young's modulus and yield stress and strain were greater than those reported previously for nonaligned electrospun PCL using a 0.1%/s strain rate, but

FIG. 7. The Young's modulus of linear region (A), yield strain (B), and yield stress (C) of human adipose-derived stem-cell seeded TDM-, FN-, and PBS-coated multilayered electrospun scaffolds at day 0 and after 28 days of culture. Groups having different letters are significantly different from each other ($p \leq 0.05$, $n = 5$).

values for yield strain were similar to those reported previously.²⁹ Fiber diameters in the current study ($\sim 2 \mu\text{m}$) were greater than in the previously reported study ($< 1.5 \mu\text{m}$).²⁹ Previous studies have found an increase in the modulus with an increasing fiber diameter, but opposite effects on elongation at break and yield strength for aligned⁶¹ and non-aligned⁶² fiber patterns. Further, the degree of strain rate dependence on tensile mechanical properties in electrospun nanoscaffolds is unknown, and may account for some of the differences in mechanical properties observed, as has been seen in other tissues.⁶³

The degradation profile of the multilayered electrospun PCL scaffolds in the current study is not yet known; however, decreases in the Young's modulus and elongation at break were detected in 15- μm -thick PCL nanofibrous scaffolds after 1-month of incubation in a physiological solution.⁶² Therefore, in the current study, it is possible that the lack of change in Young's modulus over time in culture represented load-sharing between the newly formed tendon-like tissue and the degrading electrospun PCL scaffold. Not surprisingly, the Young's modulus of this nonaligned scaffold was lower than all regions of the human rotator cuff, except for the relatively nonaligned posterior aspect of the supraspinatus tendon.⁶⁴ Therefore, we are continuing to explore ways to improve initial mechanical properties using this electrospinning technology. Aligned electrospun scaffolds are more attractive for tendon tissue engineering than nonaligned scaffolds because they recapitulate the mechanical anisotropy of the target tissue,⁶⁵ and enhance collagen production.⁶⁶ However, there are conflicting reports as to the benefit of alignment on tendon-related gene expression. Compared to randomly aligned nanofibers, expression of *COL1A1*, *DCN*, and *TNMD* by mesenchymal stem cells was increased on aligned nanofibers,⁶⁷ but conversely, there was no beneficial

effect of alignment on the expression of *COL1A1*, *DCN*, or scleraxis (*SCX*) by embryonic fibroblasts.⁶⁸ Further, the human supraspinatus tendon demonstrates heterogeneous regional anisotropy, being highly anisotropic medially, but more isotropic on the bursal side close to its insertion.⁶⁹ Regional matching of augmentation patch properties to the underlying rotator cuff is likely to be important, since mismatch of patch material properties to the underlying tendon may result in stress concentrations and increased likelihood of postoperative repair failure.⁷⁰ Thus, given the problems associated with cellular infiltration throughout aligned nanoscaffolds, and the regional anisotropy of the target tissue, multilayered electrospun scaffolds afford the possibility of introducing regional lamellar anisotropy to improve baseline mechanical properties and maintain collagen production, without a negative impact on the overall ability of cells seeded on the scaffolds to differentiate toward a tendon phenotype or to fully infiltrate. Lamellar regional anisotropy can be introduced within an electrospun scaffold, using several techniques,^{71–73} and future studies will evaluate this potential in multilayered electrospun scaffolds.

The fiber diameters obtained in this study were larger than the 800-nm-diameter fibers previously reported for nanofibrous scaffolds produced using this technique.⁴⁴ This larger fiber size is likely attributable to the greater flow rate and the different solvents used in this study, since the needle size and electric field strength were comparable. The effect of the larger fiber diameters ($\sim 2 \mu\text{m}$) observed in this study on the degree of cell infiltration and differentiation compared to smaller fiber diameters is unknown, although larger fiber diameters can improve cell infiltration in electrospun scaffolds.³⁸ Nonetheless, the sequential collection of multiple layers into a single scaffold likely still improves cell infiltration compared to scaffolds composed of similar diameter

fibers collected as a single layer, since culture in a flow perfusion bioreactor was required to achieve complete cell infiltration in scaffolds composed of 5- μ m fibers.³⁸ The effect of the fiber diameter on tenogenic differentiation of rotator cuff fibroblasts, mesenchymal stem cells, and embryonic fibroblasts has recently been examined.^{61,67,68} As aligned fiber diameter increases to the microfiber scale, rotator cuff fibroblasts elongate and align to a greater extent, demonstrate increased tendon-like gene expression, and form a more tendon-like tissue in contrast to the scar-like tissue formed on nanofibers.⁶¹ Similarly, as the diameter increased on non-aligned fibers, the aspect ratio also increased for mesenchymal stem cells, and expression of *COL1A1*, *DCN*, and *TNMD* or *SCX* increased at later time points.^{67,68} These studies all found a detrimental effect on cell numbers associated with microfibers compared to nanofibers, but in most cases, differences in cell numbers resolved after several weeks in culture.^{61,67,68}

The reason for the beneficial effect of TDM treatment on the total collagen content after 28 days of culture and on type I collagen production through the full thickness of the scaffold is unknown, but is consistent with similar findings previously using hASCs seeded in a type I collagen gel system.⁴⁵ The extracellular matrix provides a complex environment that regulates many aspects of cellular behavior.⁷⁴ Therefore, the most likely mechanism is via matricellular interactions with tendon-specific components of the extracellular matrix.^{45,75–79} Importantly, however, the use of tissue-specific extracellular matrix avoids the use of exogenous growth factors for tenogenic differentiation, where identifying the safest and most appropriate physiological dose to realize the preferred biomechanical and morphological properties at the repair site may be challenging,⁸⁰ and likely to be more difficult where combinations of growth factors are used. In this study, the enhanced type I collagen production exhibited on TDM-scaffolds did not improve the mechanical properties of the constructs. We did not evaluate neocollagen alignment, which may have been disorganized in the static loading conditions used in this study. Thus, future studies may wish to examine the synergistic effect of TDM and dynamic loading on the collagen content,⁸¹ and potentially, the development of mechanical properties.

In summary, our findings indicate that multilayered electrospun scaffolds incorporated with TDM show increased levels of collagen accumulation by ASCs as compared to FN- or PBS-coated scaffolds. This approach provides a novel means for tissue engineering of the rotator cuff. While the exact mechanism of action of TDM is under investigation, future studies will additionally focus on improving the stability of TDM within the scaffolds, reducing potential immunogenicity,⁸² and on developing regional anisotropy and alignment, while maintaining cellular infiltration and tenogenic differentiation.

Acknowledgments

This work was supported by NIH grants AR59784 (D.L.), AR48852 (F.G.), AG15768 (F.G.), AR48182 (F.G.), and AR50245 (F.G.) and by Synthes USA (D.L., D.S.R., F.G.).

Disclosure Statement

No competing financial interests exist.

References

1. Taylor, W. Musculoskeletal pain in the adult New Zealand population: prevalence and impact. *N Z Med J* **118**, U1629, 2005.
2. Lewis, J.S. Rotator cuff tendinopathy. *Br J Sports Med* **43**, 236, 2009.
3. Patte, D. Classification of rotator cuff lesions. *Clin Orthop Relat Res* **254**, 81, 1990.
4. Physician Visits—National Ambulatory Medical Care Survey 1998–2006. US Department of Health and Human Services, Centers for Disease Control and Prevention, National Center for Health Statistics. http://www.cdc.gov/nchs/ahcd/ahcd_reports.htm. Accessed August 5, 2013.
5. Hospitalizations—National Hospital Discharge Survey 1998–2006. US Department of Health and Human Services, Centers for Disease Control and Prevention, National Center for Health Statistics. http://www.cdc.gov/nchs/data/nhds/10Detaileddiagnosesprocedures/2006det10_numberalldiagnoses.pdf. Accessed August 5, 2013.
6. Gartsman, G.M., Brinker, M.R., Khan, M., and Karahan, M. Self-assessment of general health status in patients with five common shoulder conditions. *J Shoulder Elbow Surg* **7**, 228, 1998.
7. Vitale, M.A., Vitale, M.G., Zivin, J.G., Braman, J.P., Bigliani, L.U., and Flatow, E.L. Rotator cuff repair: an analysis of utility scores and cost-effectiveness. *J Shoulder Elbow Surg* **16**, 181, 2007.
8. Warner, J.J. Management of massive irreparable rotator cuff tears: the role of tendon transfer. *Instr Course Lect* **50**, 63, 2001.
9. Neri, B.R., Chan, K.W., and Kwon, Y.W. Management of massive and irreparable rotator cuff tears. *J Shoulder Elbow Surg* **18**, 808, 2009.
10. Zhang, X., Bogdanowicz, D., Eriskien, C., Lee, N.M., and Lu, H.H. Biomimetic scaffold design for functional and integrative tendon repair. *J Shoulder Elbow Surg* **21**, 266, 2012.
11. Cheung, E.V., Silverio, L., and Sperling, J.W. Strategies in biologic augmentation of rotator cuff repair: a review. *Clin Orthop Relat Res* **6**, 1476, 2010.
12. Ricchetti, E.T., Aurora, A., Iannotti, J.P., and Derwin, K.A. Scaffold devices for rotator cuff repair. *J Shoulder Elbow Surg* **21**, 251, 2012.
13. Longo, U.G., Lamberti, A., Maffulli, N., and Denaro, V. Tendon augmentation grafts: a systematic review. *Br Med Bull* **94**, 165, 2010.
14. Shea, K.P., McCarthy, M.B., Ledgard, F., Arciero, C., Chowaniec, D., and Mazzocca, A.D. Human tendon cell response to 7 commercially available extracellular matrix materials: an *in vitro* study. *Arthroscopy* **26**, 1181, 2010.
15. McCarron, J.A., Derwin, K.A., Bey, M.J., Polster, J.M., Schils, J.P., Ricchetti, E.T., and Iannotti, J.P. Failure with continuity in rotator cuff repair “healing”. *Am J Sports Med* **41**, 134, 2013.
16. Shea, K.P., Obopilwe, E., Sperling, J.W., and Iannotti, J.P. A biomechanical analysis of gap formation and failure mechanics of a xenograft-reinforced rotator cuff repair in a cadaveric model. *J Shoulder Elbow Surg* **21**, 1072, 2012.
17. Derwin, K.A., Badylak, S.F., Steinmann, S.P., and Iannotti, J.P. Extracellular matrix scaffold devices for rotator cuff repair. *J Shoulder Elbow Surg* **19**, 467, 2010.
18. Moffat, K.L., Kwei, A.S., Spalazzi, J.P., Doty, S.B., Levine, W.N., and Lu, H.H. Novel nanofiber-based scaffold for rotator cuff repair and augmentation. *Tissue Eng Part A* **15**, 115, 2009.
19. Moffat, K.L., Zhang, X., Greco, S., Boushell, M.B., Guo, X.E., Doty, S.B., Soslowsky, L.J., Levine, W.N., and Lu, H.H. In vitro and in vivo evaluation of a bi-phasic nanofiber scaffold for integrative rotator cuff repair. *Trans Orthop Res Soc* **35**, 482, 2011.

20. Xie, J., Li, X., Lipner, J., Manning, C.N., Schwartz, A.G., Thomopoulos, S., and Xia, Y. "Aligned-to-random" nanofiber scaffolds for mimicking the structure of the tendon-to-bone insertion site. *Nanoscale* **2**, 923, 2010.
21. Xie, J., Ma, B., Michael, P.L., and Shuler, F.D. Fabrication of nanofiber scaffolds with gradations in fiber organization and their potential applications. *Macromol Biosci* **12**, 1336, 2012.
22. Taylor, E.D., Nair, L.S., Nukavarapu, S.P., McLaughlin, S., and Laurencin, C.T. Novel nanostructured scaffolds as therapeutic replacement options for rotator cuff disease. *J Bone Joint Surg Am* **92(Suppl 2)**, 170, 2010.
23. Beason, D.P., Connizzo, B.K., Dourte, L.M., Mauck, R.L., Soslowky, L.J., Steinberg, D.R., and Bernstein, J. Fiber-aligned polymer scaffolds for rotator cuff repair in a rat model. *J Shoulder Elbow Surg* **21**, 245, 2012.
24. Inui, A., Kokubu, T., Mifune, Y., Sakata, R., Nishimoto, H., Nishida, K., Akisue, T., Kuroda, R., Satake, M., Kaneko, H., and Fujioka, H. Regeneration of rotator cuff tear using electrospun poly(D,L-lactide-co-glycolide) scaffolds in a rabbit model. *Arthroscopy* **28**, 1790, 2012.
25. Baker, B.M., Gee, A.O., Metter, R.B., Nathan, A.S., Marklein, R.A., Burdick, J.A., and Mauck, R.L. The potential to improve cell infiltration in composite fiber-aligned electrospun scaffolds by the selective removal of sacrificial fibers. *Biomaterials*, **29**, 2348, 2008.
26. Baker, B.M., Shah, R.P., Silverstein, A.M., Esterhai, J.L., Burdick, J.A., and Mauck, R.L. Sacrificial nanofibrous composites provide instruction without impediment and enable functional tissue formation. *Proc Natl Acad Sci USA* **109**, 14176, 2012.
27. Telemeco, T.A., Ayres, C., Bowlin, G.L., Wnek, G.E., Boland, E.D., Cohen, N., Baumgarten, C.M., Mathews, J., and Simpson, D.G. Regulation of cellular infiltration into tissue engineering scaffolds composed of submicron diameter fibrils produced by electrospinning. *Acta Biomater* **1**, 377, 2005.
28. Mathews, J.A., Wnek, G.E., Simpson, D.G., and Bowlin, G.L. Electrospinning of collagen nanofibers. *Biomacromolecules* **3**, 232, 2002.
29. Li, W.J., Cooper, J.A., Jr., Mauck, R.L., and Tuan, R.S. Fabrication and characterization of six electrospun poly(alpha-hydroxy ester)-based fibrous scaffolds for tissue engineering applications. *Acta Biomater* **2**, 377, 2006.
30. Hong, S., and Kim, G. Electrospun micro/nanofibrous conduits composed of poly(epsilon-caprolactone) and small intestine submucosa powder for nerve tissue regeneration. *J Biomed Mater Res B Appl Biomater* **94**, 421, 2010.
31. Lee, H., and Kim, G. Biocomposites electrospun with poly(epsilon-caprolactone) and silk fibroin powder for biomedical applications. *J Biomater Sci Polym Ed* **21**, 1687, 2010.
32. Lee, S.J., Liu, J., Oh, S.H., Soker, S., Atala, A., and Yoo, J.J. Development of a composite vascular scaffolding system that withstands physiological vascular conditions. *Biomaterials* **29**, 2891, 2008.
33. Nam, J., Huang, Y., Agarwal, S., and Lannutti, J. Improved cellular infiltration in electrospun fiber via engineered porosity. *Tissue Eng* **13**, 2249, 2007.
34. Levorson, E.J., Raman Sreerekha, P., Chennazhi, K.P., Kasper, F.K., Nair, S.V., and Mikos, A.G. Fabrication and characterization of multiscale electrospun scaffolds for cartilage regeneration. *Biomed Mater* **8**, 014103, 2013.
35. Shalumon, K.T., Chennazhi, K.P., Tamura, H., Kawahara, K., Nair, S.V., and Jayakumar, R. Fabrication of three-dimensional nano, micro and micro/nano scaffolds of porous poly(lactic acid) by electrospinning and comparison of cell infiltration by Z-stacking/three-dimensional projection technique. *IET Nanobiotechnol* **6**, 16–25, 2012.
36. McCullen, S.D., Miller, P.R., Gittard, S.D., Gorga, R.E., Pourdeyhimi, B., Narayan, R.J., and Lobo, E.G. *In situ* collagen polymerization of layered cell-seeded electrospun scaffolds for bone tissue engineering applications. *Tissue Eng Part C Methods* **16**, 1095, 2010.
37. Yang, X., Shah, J.D., and Wang, H. Nanofiber enabled layer-by-layer approach toward three-dimensional tissue formation. *Tissue Eng Part A* **15**, 945, 2009.
38. Pham, Q.P., Sharma, U., and Mikos, A.G. Electrospun poly(epsilon-caprolactone) microfiber and multilayer nanofiber/microfiber scaffolds: characterization of scaffolds and measurement of cellular infiltration. *Biomacromolecules* **7**, 2796, 2006.
39. Kidoaki, S., Kwon, I.K., and Matsuda, T. Mesoscopic spatial designs of nano- and microfiber meshes for tissue-engineering matrix and scaffold based on newly devised multilayering and mixing electrospinning techniques. *Biomaterials* **26**, 37–46, 2005.
40. Truong, Y.B., Glattauer, V., Briggs, K.L., Zappe, S., and Ramshaw, J.A. Collagen-based layer-by-layer coating on electrospun polymer scaffolds. *Biomaterials* **33**, 9198, 2012.
41. Khil, M.S., Bhattarai, S.R., Kim, H.Y., Kim, S.Z., and Lee, K.H. Novel fabricated matrix via electrospinning for tissue engineering. *J Biomed Mater Res B Appl Biomater* **72**, 117, 2005.
42. Shang, S., Yang, F., Cheng, X., Walboomers, X.F., and Jansen, J.A. The effect of electrospun fibre alignment on the behaviour of rat periodontal ligament cells. *Eur Cell Mater* **19**, 180, 2010.
43. Smit, E., Buttner, U., and Sanderson, R.D. Continuous yarns from electrospun fibers. *Polymer* **46**, 2419, 2005.
44. Tzezana, R., Zussman, E., and Levenberg, S. A layered ultra-porous scaffold for tissue engineering, created via a hydro-spinning method. *Tissue Eng Part C Methods* **14**, 281, 2008.
45. Little, D., Guilak, F., and Ruch, D.S. Ligament-derived matrix stimulates a ligamentous phenotype in human adipose-derived stem cells. *Tissue Eng Part A* **16**, 2307, 2010.
46. Zhang, J., Li, B., and Wang, J.H. The role of engineered tendon matrix in the stemness of tendon stem cells *in vitro* and the promotion of tendon-like tissue formation *in vivo*. *Biomaterials* **32**, 6972, 2011.
47. Urist, M.R. Bone: formation by autoinduction. *Science* **150**, 893, 1965.
48. Covey, D.C., and Albright, J.A. Clinical induction of bone repair with demineralized bone matrix or a bone morphogenetic protein. *Orthop Rev* **18**, 857, 1989.
49. Cheng, N.C., Estes, B.T., Awad, H.A., and Guilak, F. Chondrogenic Differentiation of adipose-derived adult stem cells by a porous scaffold derived from native articular cartilage extracellular matrix. *Tissue Eng Part A* **15**, 231, 2009.
50. Yang, Q., Peng, J., Guo, Q., Huang, J., Zhang, L., Yao, J., Yang, F., Wang, S., Xu, W., Wang, A., and Lu, S. A cartilage ECM-derived 3-D porous acellular matrix scaffold for *in vivo* cartilage tissue engineering with PKH26-labeled chondrogenic bone marrow-derived mesenchymal stem cells. *Biomaterials* **29**, 2378, 2008.
51. Cheng, N.C., Estes, B.T., Young, T.H., and Guilak, F. Genipin-crosslinked cartilage-derived matrix as a scaffold for human adipose-derived stem cell chondrogenesis. *Tissue Eng Part A* **19**, 484, 2013.
52. Yoon, H., and Kim, G. Micro/nanofibrous scaffolds electrospun from PCL and small intestinal submucosa. *J Biomater Sci Polym Ed* **21**, 553, 2010.
53. Lim, J.S., Ki, C.S., Kim, J.W., Lee, K.G., Kang, S.W., Kweon, H.Y., and Park, Y.H. Fabrication and evaluation of

- poly(epsilon-caprolactone)/silk fibroin blend nanofibrous scaffold. *Biopolymers* **97**, 265, 2012.
54. Powell, H.M., and Boyce, S.T. Engineered human skin fabricated using electrospun collagen-PCL blends: morphogenesis and mechanical properties. *Tissue Eng Part A* **15**, 2177, 2009.
 55. Zeugolis, D.I., Khew, S.T., Yew, E.S., Ekaputra, A.K., Tong, Y.W., Yung, L.Y., Hutmacher, D.W., Sheppard, C., and Raghunath, M. Electro-spinning of pure collagen nanofibres—just an expensive way to make gelatin? *Biomaterials* **29**, 2293, 2008.
 56. Bärck, J., Heissler, S., Geckle, U., Ardakani, M.F., Schneider, R., Ulrich, A.S., and Kazanci, M. Resemblance of electrospun collagen nanofibers to their native structure. *Langmuir* **29**, 1562, 2013.
 57. Estes, B.T., Diekman, B.O., Gimble, J.M., and Guilak, F. Isolation of adipose-derived stem cells and their induction to a chondrogenic phenotype. *Nat Protoc* **5**, 1294, 2010.
 58. Enobakhare, B.O., Bader, D.L., and Lee, D.A. Quantification of sulfated glycosaminoglycans in chondrocyte/alginate cultures, by use of 1,9-dimethylmethylene blue. *Anal Biochem* **243**, 189, 1996.
 59. 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.
 60. Pfaffl, M.W. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**, e45, 2001.
 61. Eriskin, C., Zhang, X., Moffat, K.L., Levine, W.N., and Lu, H.H. Scaffold fiber diameter regulates human tendon fibroblast growth and differentiation. *Tissue Eng Part A* **19**, 519, 2013.
 62. Bolgen, N., Menciloglu, Y.Z., Acatay, K., Vargel, I., and Piskin, E. *In vitro* and *in vivo* degradation of non-woven materials made of poly(epsilon-caprolactone) nanofibers prepared by electrospinning under different conditions. *J Biomater Sci Polym Ed* **16**, 1537, 2005.
 63. Clemmer, J., Liao, J., Davis, D., Horstemeyer, M.F., and Williams, L.N. A mechanistic study for strain rate sensitivity of rabbit patellar tendon. *J Biomech* **43**, 2785, 2010.
 64. Lake, S.P., Miller, K.S., Elliott, D.M., and Soslowky, L.J. Effect of fiber distribution and realignment on the nonlinear and inhomogeneous mechanical properties of human supraspinatus tendon under longitudinal tensile Loading. *J Orthop Res* **27**, 1596, 2009.
 65. Li, W.J., Mauck, R.L., Cooper, J.A., Yuan, X., and Tuan, R.S. Engineering controllable anisotropy in electrospun biodegradable nanofibrous scaffolds for musculoskeletal tissue engineering. *J Biomech* **40**, 1686, 2007.
 66. Lee, C.H., Shin, H.J., Cho, I.H., Kang, Y.M., Kim, I.A., Park, K.D., and Shin, J.W. Nanofiber alignment and direction of mechanical strain affect the ECM production of human ACL fibroblast. *Biomaterials* **26**, 1261, 2005.
 67. Bashur, C.A., Shaffer, R.D., Dahlgren, L.A., Guelcher, S.A., and Goldstein, A.S. Effect of fiber diameter and alignment of electrospun polyurethane meshes on mesenchymal progenitor cells. *Tissue Eng Part A* **15**, 2435, 2009.
 68. Cardwell, R.D., Dahlgren, L.A., and Goldstein, A.S. Electrospun fibre diameter, not alignment, affects mesenchymal stem cell differentiation into the tendon/ligament lineage. *J Tissue Eng Regen Med* 2012. DOI: 10.1002/term.1589.
 69. Lake, S.P., Miller, K.S., Elliott, D.M., and Soslowky, L.J. Tensile properties and fiber alignment of human supraspinatus tendon in the transverse direction demonstrate inhomogeneity, nonlinearity, and regional isotropy. *J Biomech* **43**, 727, 2010.
 70. Chaudhury, S., Holland, C., Thompson, M.S., Vollrath, F., and Carr, A.J. Tensile and shear mechanical properties of rotator cuff repair patches. *J Shoulder Elbow Surg* **21**, 1168, 2012.
 71. Garrigues, N.W., Little, D., O'Connor, C.J., and Guilak, F. Use of an insulating mask for controlling anisotropy in multi-layer electrospun scaffolds for tissue engineering. *J Mater Chem* **20**, 8962, 2010.
 72. Nerurkar, N.L., Mauck, R.L., and Elliott, D.M. Modeling interlamellar interactions in angle-ply biologic laminates for annulus fibrosus tissue engineering. *Biomech Model Mechanobiol* **10**, 973, 2011.
 73. Nerurkar, N.L., Baker, B.M., Sen, S., Wible, E.E., Elliott, D.M., and Mauck, R.L. Nanofibrous biologic laminates replicate the form and function of the annulus fibrosus. *Nat Mater* **8**, 986, 2009.
 74. Huxley-Jones, J., Pinney, J.W., Archer, J., Robertson, D.L., and Boot-Handford, R.P. Back to basics—how the evolution of the extracellular matrix underpinned vertebrate evolution. *Int J Exp Pathol* **90**, 95, 2009.
 75. Maquart, F.X., Bellon, G., Pasco, S., and Monboisse, J.C. Matrikines in the regulation of extracellular matrix degradation. *Biochimie* **87**, 353, 2005.
 76. Maquart, F.X., Pasco, S., Ramont, L., Hornebeck, W., and Monboisse, J.C. An introduction to matrikines: extracellular matrix-derived peptides which regulate cell activity. Implication in tumor invasion. *Crit Rev Oncol Hematol* **49**, 199, 2004.
 77. Rapraeger, A.C., Krufka, A., and Olwin, B.B. Requirement of heparan sulfate for bFGF-mediated fibroblast growth and myoblast differentiation. *Science* **252**, 1705, 1991.
 78. Swindle, C.S., Tran, K.T., Johnson, T.D., Banerjee, P., Mayes, A.M., Griffith, L., and Wells, A. Epidermal growth factor (EGF)-like repeats of human tenascin-C as ligands for EGF receptor. *J Cell Biol* **154**, 459, 2001.
 79. Walker, A., Turnbull, J.E., and Gallagher, J.T. Specific heparan sulfate saccharides mediate the activity of basic fibroblast growth factor. *J Biol Chem* **269**, 931, 1994.
 80. Hee, C.K., Dines, J.S., Dines, D.M., Roden, C.M., Wisner-Lynch, L.A., Turner, A.S., McGilvray, K.C., Lyons, A.S., Puttlitz, C.M., and Santoni, B.G. Augmentation of a rotator cuff suture repair using rhPDGF-BB and a type I bovine collagen matrix in an ovine model. *Am J Sports Med* **39**, 1630, 2011.
 81. Kuo, C.K. and Tuan, R.S. Mechanoactive tenogenic differentiation of human mesenchymal stem cells. *Tissue Eng Part A* **14**, 1615, 2008.
 82. Rowland, C.R., Little, D., and Guilak, F. Factors influencing the long-term behavior of extracellular matrix-derived scaffolds for musculoskeletal soft tissue repair. *J Long Term Eff Med Implants* **22**, 181, 2012.

Address correspondence to:

Dianne Little, BVSc, PhD

Department of Orthopaedic Surgery

Duke University Medical Center

Box 3093, 375 MSRB1

Durham, NC 27710

E-mail: dianne.little@duke.edu

Received: March 7, 2013

Accepted: June 20, 2013

Online Publication Date: August 23, 2013