Effect of Mechanical Stimulation on Bone Marrow Stromal Cell–Seeded Tendon Slice

Constructs: A Potential Engineered Tendon Patch for Rotator Cuff Repair

Running Title: Engineered Patch for Rotator Cuff Repair

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ABSTRACT

Cell-based tissue engineered tendons have potential to improve clinical outcomes following rotator cuff repair, especially in large or massive rotator cuff tears, which pose a great clinical challenge. The aim of this study was to develop a method of constructing a functional engineered tendon patch for rotator cuff repair with cyclic mechanical stimulation.

Decellularized tendon slices (DTSs) were seeded with BMSCs and subjected to cyclic stretching for 1, 3, or 7 days. The mechanical properties, morphologic characteristics and tendon-related gene expression of the constructs were investigated. Viable BMSCs were observed on the DTS after 7 days. BMSCs penetrated into the DTSs and formed dense cell sheets after 7 days of mechanical stretching. Gene expression of type I collagen, decorin, and tenomodulin significantly increased in cyclically stretched BMSC-DTS constructs compared with the unstrained control group ($P<.05$). The ultimate tensile strength and stiffness of the cyclically stretched tendon constructs were similar to the unstrained control group ($P>0.05$). In conclusion, mechanical stimulation of BMSC-DTS constructs upregulated expression of tendon-related proteins, promoted cell tenogenic differentiation, facilitated cell infiltration and formation of cell sheets, and retained mechanical properties. The patch could be used as a graft to enhance the surgical repair of rotator cuff tears.

ABSTRACT

Cell-based tissue engineered tendons have potential to improve clinical outcomes following rotator cuff repair, especially in large or massive rotator cuff tears, which pose a great clinical challenge. The aim of this study was to develop a method of constructing a functional engineered tendon patch for rotator cuff repair with cyclic mechanical stimulation. Decellularized tendon slices (DTSs) were seeded with BMSCs and subjected to cyclic stretching for 1, 3, or 7 days. The mechanical properties, morphologic characteristics and tendon-related gene expression of the constructs were investigated. Viable BMSCs were observed on the DTS after 7 days. BMSCs penetrated into the DTSs and formed dense cell sheets after 7 days of mechanical stretching. Gene expression of type I collagen, decorin, and tenomodulin significantly increased in cyclically stretched BMSC-DTS constructs compared with the unstrained control group ($P<.05$). The ultimate tensile strength and stiffness of the cyclically stretched tendon constructs were similar to the unstrained control group ($P>0.05$). In conclusion, mechanical stimulation of BMSC-DTS constructs upregulated expression of tendon-related proteins, promoted cell tenogenic differentiation, facilitated cell infiltration and formation of cell sheets, and retained mechanical properties. The patch could be used as a graft to enhance the surgical repair of rotator cuff tears.

Keywords: Bone Marrow Stromal Cells; Stretch; Tendon Slice; Tissue Engineering

22 **Abbreviations**

23 BMSC, bone marrow stromal cell

24 DTS, decellularized tendon slices

25 ECM, extracellular matrix

26 GAPDH, glyceraldehyde 3-phosphate dehydrogenase

27 IACUC, Institutional Animal Care and Use Committee

28 PBS, phosphate-buffered saline

29 RT-PCR, reverse-transcription polymerase chain reaction

30 UTS, ultimate tensile strength

31

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1. INTRODUCTION

Rotator cuff tears are one of the most common causes of shoulder pain [1]. Approximately 50% of the US population has had a degenerative rotator cuff tear by age 60 [2]. Although small rotator cuff tears have better treatment outcomes, surgical repairs of large tears show failure rates as high as 90% because of muscle contraction, decreased range of joint motion, neurovascular damage, or altered shoulder mechanics [3]. High retear rate and poor functional outcomes are common after surgical repair, and currently available scaffolds have limited ability to enhance new tendon formation [4]. Consequently, optimal treatment of rotator cuff tears should focus on preservation or restoration of rotator cuff function. However, designing functional rotator cuff tendon grafts remains a great challenge.

Augmentation of rotator cuff repair is frequently performed with acellular matrices or cell-seeded scaffolds [5-7]. Cell-based strategies in particular seem to be associated with superior results [8]. Previous work has focused on identifying an ideal cell-scaffold framework that could replace the lost tendon. Different scaffold materials such as chitosan constructs [5], injectable hydrogels [6], or knitted silk-collagen scaffolds [7] have mixed results and limited applicability, mostly because the graft strength is substantially lower compared with native rotator cuff tendons. To enhance the strength of engineered tendons, entire tendons can be decellularized and used as scaffolds.

Decellularized tendon slices (DTSs) promise advantages compared with tendon allografts or synthetic materials. First, the immunogenicity and antigenicity of the tissue is reduced by extracting antigenic cells and preserving only the minimally antigenic extracellular matrix

(ECM). Cell disruption and extraction of cell detritus minimizes adverse immunogenic reactions. Second, by preserving other macromolecules besides the collagenous structure, an ideal environment is provided for exogenous (seeded) cells for incorporation, metabolism, and matrix synthesis [17]. By seeding DTSs with bone marrow stromal cells (BMSCs), the process of tendon incorporation and remodeling may be hastened. Further, by preserving the natural collagenous structure, the original biomechanical strength is preserved. This method is suitable for making tendons completely cell free without changing their biomechanical properties [31].

Bioreactors in tissue engineering [9] create a controlled and sterile environment for development of engineered tissues, including tendon [10]. Bioreactors designed to provide in vitro mechanical stimulation are thought to replicate physiologic conditions and promote cell differentiation and tenogenesis [11]. Such bioreactor stimulation has been shown to improve biomechanical properties in decellularized tendon scaffolds reseeded with rabbit tenocytes [12]. However, distribution of the reseeded cells was limited to the surface, with far fewer cells in the core of the scaffold [13]. Whitlock et al [14] increased the porosity and pore size of the tendon scaffold by using a combined solution of TritonX-100 and peracetic acid. Although cells could migrate into deeper layers of tendon tissue, the native structure and ECM microenvironment of the tendon were changed. Omae et al [15] investigated a novel scaffold with multilayer tendon slices, but tendon slices with a thickness of 50 μm are not expected to have enough initial mechanical strength to withstand early motion and rehabilitation. Recently Qin et al [16] demonstrated that native tendon slices with a thickness of at least 300 μm had similar biomechanical characteristics to the intact tendon bundle. DTSs with a thickness of 300 μm

maintained elemental mechanical strength, inherent ultrastructure, specific proteoglycans, and multiple growth factors of tendon ECM [17]. More recent work has shown that grafts of DTSs incorporate into the host tendon, which improves biomechanical performance of the regenerated rotator cuff tendon [18]. However, in that study, the DTSs graft did not host any seeded cells; thus, the performance of cell-seeded DTSs has not yet been characterized.

The aim of this in vitro study was to develop a novel, cell-seeded patch for rotator cuff repair and to test the effect of a mechanical-loading protocol on the cell-seeded patch. The patch was a DTS construct that was seeded with viable BMSCs and then subjected to dynamic stretch culturing. We reviewed morphologic changes, expression of tendon-related genes in BMSCs, and biomechanical properties of the patches after stretch culture. We hypothesized that BMSCs could grow well on the DTS scaffold and that dynamic stretching could stimulate tenogenic differentiation of BMSCs.

2. MATERIALS AND METHODS

2.1. Preparation of DTSs

DTSs were prepared as previously described [17]. Briefly, Achilles tendons (length, 40 mm) were harvested from dogs euthanized for other studies approved by the Mayo Clinic Institutional Animal Care and Use Committee (IACUC). Tendons were soaked in liquid nitrogen for 10 minutes and then thawed at room temperature. The freeze-thaw procedure was repeated 5 times to disrupt all cells in the tendon. Each segment was embedded with optimal cutting temperature compound (Sakura Finetek USA, Inc), fixed on a cryostat (Leica CM1850), and then

longitudinally cut into 300- μ m slices in succession. After washing with phosphate-buffered saline (PBS) for 30 minutes, TDSs were incubated in a nuclease solution (RNase [100 μ g/mL] and DNase [150 IU/mL]; Roche Diagnostic) for 12 hours at 37°C and then washed with PBS. Slices were frozen at -80°C for 60 minutes and then lyophilized in a freeze dryer (Millrock Technology Inc). Finally, the slices were packed in a polyethylene bag and sterilized with ethylene oxide gas.

2.2. Cultivation of BMSCs

Bone marrow was harvested from 10 mixed-breed dogs (weighing 25-30 kg) used in other studies approved by the Mayo Clinic IACUC. Immediately after euthanasia, 4.0 mL of bone marrow was aspirated from the tibia using a 20-mL syringe containing 1.0 mL of heparin (50 IU/mL). The syringe contents were combined with 5.0 mL PBS and centrifuged at 1,500 rpm for 5 minutes at room temperature. After the PBS and heparin were removed, bone marrow cells were cultured onto 4 dishes (100 mm in diameter) in 8 mL of standard medium, which consisted of minimal essential medium with Earle salts (Gibco), 10% fetal calf serum, and 1% antibiotic-antimycotic (Gibco). Bone marrow cells were incubated at 37°C with 5% CO₂ and 100% humidity. After 5 days, the culture medium and floating cells were removed. The remaining adherent cells were defined as BMSCs [19]. The culture medium was changed every other day. BMSCs in the second passage were treated with 0.05% ethylenediaminetetraacetic acid and trypsin to produce a cell suspension and then centrifuged at 1,500 rpm for 5 minutes. The concentrated cell suspension was collected in one tube and cell density was adjusted to 5 $\times$ 10⁵ cells/mL by adding medium.

2.3. Mechanical Stimulation Device

A custom-made mechanical stimulation device was used to apply cyclic uniaxial strain to the cell-seeded constructs (Figure 1a). The device included a component that applied mechanical stretching and a microcontroller that regulated the stepper motors driving the motion. The device included 3 separate wells, and each well was capable of containing up to 4 scaffolds in the culture medium. This unit allowed control over the frequency and amplitude of the strain applied to the scaffolds, and timers could introduce automatic rest periods in the cycle. The instrument was designed to fit inside a standard cell culture incubator.

2.4. Dynamic Culture of Engineered Tendon Constructs Subject to Cyclic Loading

Before seeding BMSCs onto the surface of slices, the ends of each DTS were clamped using the grips that were fixed within the mechanical stimulation device. The cell-slice composites were placed into the device in groups of 4. The composites were clamped such that the exposed length of each slice was 25 mm (Figure 1b). The loading profile consisted of cyclic uniaxial strain with an amplitude of 0.75 mm (equal to 3.0% strain) at 0.2 Hz (12 cycles/minute) with 3.0% elongation for 20 minutes of each hour, 12 hours/day after 2 days of static culture. The 3% strain level was chosen because a strain of 5% or less did not cause structural damage or change the mechanical properties of the tendon slice (300- μ m thick) after fatigue testing with 21,000 cycles [16]. The composites (n=12) were subjected to cyclic strain for 1 day (2,880 cycles), 3 days (8,640 cycles), or 7 days (20,160 cycles) and then collected for analysis. Control composites (i.e., not subject to cyclic loading, n=12) were similarly cultured for 1, 3, or 7 days after 2 days of static culture (Figure 1c).

2.5. Viability of BMSCs on DTSs

To visualize living cells in the engineered tendon constructs, the collected BMSCs (n=2 in each group) were labeled with a fluorescent marker (PKH26 red fluorescent cell linker kit for general cell membrane labeling; Sigma) following the manufacturer's instructions. Briefly, after centrifuging BMSCs and aspirating the supernatant, cells were resuspended gently in fluorescent reagent. Then 2 mL of medium was added, and BMSCs were incubated at 37°C for 30 minutes. The suspension was centrifuged at 1,500 rpm for 5 minutes and cells were washed with PBS. Medium was added and BMSCs were incubated for another 30 minutes before seeding onto the DTSs. A laser scanning confocal microscope (LSM310; Zeiss) was used to observe fluorescence and assess seeding uniformity and cell viability after stretching for 1, 3, or 7 days.

2.6. Morphologic Examinations of BMSC-DTS Constructs

Cell-slice constructs were examined histologically after mechanical stimulation or static incubation. The engineered tendon constructs were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned along the sagittal plane. Two samples in each group were stained with hematoxylin and eosin and examined under a light microscope (BH2, Olympus) to determine alignment and distribution of cells. To understand the morphology of BMSCs on DTSs after dynamic mechanical stimulation, constructs were viewed with scanning electron microscopy. Two samples in each group were transferred from the device after stretch culture and equilibrated in DNase- and RNase-free water at 37°C for 30 minutes; this was followed by fixation in a solution of buffered glutaraldehyde and osmium tetroxide. After dehydration in

graded acetone, specimens were coated with a gold-palladium alloy and examined with a Hitachi 4700 scanning electron microscope at 10 KV (Hitachi Scientific Instruments, Inc).

2.7. Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction

The expression of ECM proteins collagen I, collagen III, decorin, and tenomodulin in the constructs was investigated with real-time reverse-transcription polymerase chain reaction (RT-PCR). After stretch cultivation, the constructs (n=8 for each group) were transferred from the culture dishes and stored at -80°C until RNA extraction. Eight cell-slice composites were withheld before culture to be used as controls. Each specimen was homogenized in TRIzol reagent (Invitrogen) with a MIKRO Dismembrator (B. Braun Biotech). Total RNA was extracted from the specimens according to the manufacturer's protocol. Potential contaminating genomic DNA was digested with DNase (Roche Applied Science) and further removed using the RNeasy Mini Kit (Qiagen). RNA concentration was determined using a RiboGreen RNA quantification kit (Invitrogen), and RNA was reverse transcribed into single-stranded cDNA using the Transcriptor First-Strand cDNA Synthesis Kit (Roche Applied Science). Quantitative RT-PCR was performed with LightCycler 1.5 (Roche Applied Science) to measure the gene expressions of collagen I, collagen III, decorin, and tenomodulin with an annealing temperature of 60°C to 62°C and magnesium concentration of 3.5 mM. The PCR primers designed from canine-specific cDNA sequences are listed in Table 1.

To find a reference gene for RT-PCR, we tested β -actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The β -actin: RNA ratio significantly changed with stretch culture of the constructs. However, there was no significant difference in the GAPDH: RNA ratio between

static and dynamic cultivation. Therefore, GAPDH was chosen as reference gene, as was done in a previous study [20].

2.8. Biomechanical Assessment of Engineered Tendon Patches

DTs were subjected to uniaxial tension testing to characterize mechanical properties. Uncultured DTs controls were rehydrated in saline for 45 minutes prior to testing. Cell-seeded DTs subjected to 1, 3, and 7 days of stretch culture were kept moist in PBS before testing. Before testing, thickness and width of the patches were measured with a digital caliper to determine the cross-sectional area. Both ends of each 40-mm patch were clamped in the grips of a materials testing system (MTS 312; MTS Systems Corp). To prevent patch slippage at the grips, clamps were lined with fine sandpaper. Samples were preconditioned 5 times with 0.2 N of preload and then were loaded to failure at a rate of 12 mm/minute (strain rate of 0.8%/s). The load and displacement of each patch were recorded at a samples rate of 20 Hz. The ultimate tensile strength (UTS) of each patch was calculated by dividing the load at failure by the cross-sectional area of the patch before loading. The point of failure was usually located halfway between the middle of the patch and the bottom of the grip. Stiffness was calculated from the slope of the load-displacement curve.

2.9. Statistical Analysis

Means and standard deviations were calculated for gene expression levels and mechanical parameters (UTS and stiffness). The overall comparisons for gene expression and mechanical parameters among the control group and 2 cell-slice construct groups were analyzed by one-way analysis of variance. The Tukey-Kramer Honestly Significant Difference test was

used as a post hoc test. All statistical analyses were performed with SPSS 17.0 statistical software (SPSS Inc). Differences were considered significant at *P* values less than 0.05.

3. RESULTS

3.1. Mechanical Characteristics of DTSs

The fabricated DTSs were sheet scaffolds measuring about 40×4×0.3 mm³ (Figure 2a). The stress-strain curves of the DTSs showed that each slice had the typical S-shaped curve (Figure 2b) with 4 distinct regions: a toe region, a linear region, a plastic and yield region, and a failure region. The linear regions ranged from 3% to 9% strain.

3.2. Viability of BMSCs after Stretch Culture

TDS alone without cells showed intact, aligned, and densely packed fibers (Figure 3 a). After dynamic stretch cultivation from 1 to 7 days, the number of viable BMSCs labeled with PKH26 increased with time and were uniformly distributed on the DTSs (Figure 3 b-d). Alignment of BMSCs cultured on DTSs also improved with culture time (up through 7 days), and cells elongated along the direction of scaffold fibers. These results suggest that DTSs were capable of supporting cell attachment, inducing cell spreading, and promoting cell alignment.

3.3. Morphologic Observation

Histologically, collagen fibers and the cells seeded on the surfaces of the slices were observed on sagittal sections of the BMSC-DTS constructs (Figure 4). Although most BMSCs were distributed throughout the outside surface of the slices (Figure 4 a-b, e-f), samples subjected to mechanical stimulus showed cells within the slices (Figure 4 c-d, g-h). Scanning

electron microscopy showed that BMSCs slowly but drastically changed from a short spindle or flat cell shape to that of a long spindle when the mechanical stimulus was applied (Figure 5). The unstrained constructs showed the cell sheet grew very well and completely covered the slice scaffold (Figure 5 a-b). With stretch culture, spindle-shaped BMSCs adhered to DTSs and aligned parallel with the direction of the stretch (Figure 5 c-d). After 7 days of stretch culture, dense spindle cell sheets were formed on DTSs (Figure 5 d).

3.4. Gene Expression of ECM Proteins by RT-PCR

The effects of dynamic stretch on the expression of ECM proteins were analyzed by real-time RT-PCR (Figure 6). The mRNA level of collagen I in the strained group was significantly higher than that of the control group after 7 days of culture. Although the amount of collagen I expression in the strained group was higher than that of the unstrained group on days 3 and 7, the differences were not significant (Figure 6 a). Expression of collagen III was not significantly different among control, unstrained, and strained samples (Figure 6 c). The relative expression of decorin and tenomodulin in the strained group was significantly higher than that of the control or unstrained groups after 7 days of culture (Figure 6 b, d).

3.5. Mechanical Properties of the Engineered Tendon Patches

Mechanical stimulation did not significantly alter UTS and stiffness of BMSC-DTS constructs after 7 days in culture (Table 2; $P>0.05$ for all).

4. DISCUSSION

Torn rotator cuff tendons often are repaired surgically to restore shoulder function. However, the results of surgical repair is highly variable, with failure rates ranging from 30% to 90%, depending on the severity of the tendon defect and the stage of degeneration of the joint [21,22]. Mechanical and biological augmentation recently was recommended for rotator cuff repair, especially for large or massive tears [23]. However, currently available materials for augmentation have not achieved satisfactory results [24]. Such challenges make tissue engineering of cell-scaffold constructs an attractive alternative for improving rotator cuff tendon repair. One of the important strategies currently under investigation is in vitro mechanical preconditioning of cell-scaffold constructs before implantation [25,30]. However, a fundamental understanding is lacking with regard to how application of external loads alters mechanical properties, gene expression, and ECM production of engineered constructs. In this study, we evaluated the effect of in vitro mechanical conditioning on BMSC-seeded DTSs. Although 1 week of in vitro cyclic loading did not significantly increase the biomechanical properties (e.g., UTS, stiffness) of these constructs, our histology data have shown that mechanical stimulation did facilitate cell infiltration deeper into DTS scaffolds and improved the ECM protein expression of constructs. Juncosa-Melvin et al used collagen sponge seeded with BMSCs for mechanical stimulation and found increased mechanical properties of collagen sponge under mechanical stimulation [29]. However, the Young's modulus of the DTS scaffold used in the current study (in magnitude around 300 MPa) [16] was much greater than that of gel-sponge scaffold (Young's modulus in magnitude around 0.02 MPa) used in the prior studies [29]. Thus, from the mechanical property perspective, the native tendon slice scaffold should be superior to

the gel-sponge scaffold. In addition, the initial UTS and stiffness of the BMSC-seeded constructs were similar to native tendon bundles [16]. In contrast, the corresponding UTS values of the constructs from the dynamic culture (day 1, 31.7 MPa; day 3, 32.4 MPa; day 7, 34.4 MPa) were similar to or exceeded those of the control, although the difference was not statistically significant.

Mechanical stimulation increased the expression of tendon-related genes (collagen I, decorin, and tenomodulin) after 7 days in culture, which confirmed the benefit of mechanical stimulation of cell-based constructs before implantation. This finding was consistent with Juncosa-Melvin's report that collagen I increased after mechanical stimulation in a collagen-sponge model [29]. Collagen I is the primary protein responsible for load bearing [26], whereas collagen III is generally thought to be involved in the early stages of tendon healing [27]. Decorin is a proteoglycan that dominantly exists in tendon tissue, mediates fibrillogenesis, and regulates collagen remodeling [28]. The increased tenomodulin expression suggests that BMSCs might express a tenocyte phenotype when cyclic loading is applied to the acellular DTS scaffold. Furthermore, mechanical stimulation (3% strain, <21,000 cycles in 1 week) did not change the mechanical properties of the engineered tendon patches. The more interesting finding of the present study was that mechanical stimulation of BMSC-DTS constructs resulted in cell infiltration and dense cell sheets. These findings are consistent with a previous study showing upregulation of collagen I and decorin gene expression with mechanical stimulation [29].

Most strikingly, the BMSCs showed a flat morphology and were randomly aligned (i.e., did not follow the direction of the resident collagen fibers) under static culture. However, after 7 days of stretch culture, the BMSC shape resembled a long spindle and dense cell sheets were formed. The mechanical stimulation induced longitudinal alignment of BMSCs in the direction of stretch. The morphology of BMSCs was similar to that of native tenocytes in situ [13, 32], with cells oriented parallel to the collagen fibers, elongated and engrafted into the scaffold, and able to migrate through the ECM under dynamic stretch culture. The cells were distributed randomly but developed a longitudinal pattern following stretching, which is consistent with the finding that has been observed previously using two-dimensional cell stretching experiments [33, 34].

Currently, a general consensus is that acellular tendon ECM is increasingly used in tissue engineering of rotator cuff tendons. However, seeding cells into deep substance of an intact tendon scaffold is very challenging as the tendon is a dense connective tissue [13]. Increasing tendon porosity by chemical digestion could increase the cell penetration, but also may alter the normal tendon mechanical properties [14]. Continuous perfusion in a bioreactor seemed to improve BMSC seeding, but a lower cell count was observed in perfusion group compared to the static culture group [35]. To overcome the difficulties in cell seeding and penetration, we have disassembled tendon into a unit (tendon slice), which maintained mechanical properties of the whole tendon, and then reassembled them back to an engineered tendon patch for the tendon regeneration [15,16]. This tissue engineered tendon patch not only allowed for much more cell seeding or ingrowth than an intact decellularized tendon scaffold, but also may work well as a

biological graft for surgical repair of rotator cuff tears since the DTS preserved tendon mechanical properties and also tendon ECM bioactive factors [15,16]. In the current work, we have successfully developed a mechanical stimulation regime to further facilitate tenogenesis of engineered tendon patches using BMSC-DTS constructs, and our data supported our hypothesis that BMSC-DTS showed tenocyte potential and increased cell-penetration under mechanical stimulation. Therefore, this engineered tendon composite can be revitalized in the laboratory in a biological and physiological environment.

Future work is needed to overcome several limitations of this study. First, all constructs were evaluated at only one strain level (3% strain). Gene expression of these constructs should be evaluated with different strain levels. Second, the histological and morphological evaluations were non-quantitative due to small sample size. Third, the current study was an in vitro study, thus whether this BMSC-DTC would indeed to increase the tendon healing ability is unknown. Future in vivo animal model will be necessary to validate the BMSC-DTC for rotator cuff healing. Fourth, we did not characterize of the stem cell surface markers on BMSC. However, we used typical BMSC isolation techniques published previously that have been well documented to show stem cell characteristics [19]. Furthermore, analysis of cell behavior (proliferation, migration, and apoptosis assay) would strengthen the study. However, due to its limited scope, in this study we focused on the effects of mechanical stimulation of the cell-seeded scaffold on mechanical properties, morphology and viability of BMSCs and tenocyte related gene expressions (RT-PCR), which indicated that mechanical stimulation of BMSC-DTS constructs upregulated expression of tendon-related proteins, promoted cell tenogenic

differentiation, facilitated cell infiltration and formation of cell sheets, and retained mechanical properties. Finally, protein production was not determined by qualitative or quantitative methods. Future work should focus on identifying temporal relationships between gene expression, protein production, and final mechanical properties. Fourth, an in vivo model is needed to investigate the engineered tendon patch as a graft for repairing rotator cuff tears.

5. CONCLUSIONS

BMSC-seeded engineered tendon patches were developed to potentially serve as a graft for rotator cuff repair. Our results demonstrated that a dynamic mechanical stimulus induced morphologic changes in BMSCs and increased expression of tendon-related genes. The cell-seeded bioactive patch has a potential to accelerate tendon healing and improve the quality of rotator cuff tendon repair.

6. ACKNOWLEDGMENTS

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Legend

Figure 1. A custom mechanical stimulation device. Upper panel, Photograph showing the inside of the incubator and mechanical stimulation device. Up to 12 cell-slice constructs (4 constructs in each of 3 dishes) are connected with a motor-driven frame that generates force and displacement. A microcomputer controls the cyclic strain parameters (ie, stretch amplitude, rate, frequency, and daily stretch time) during each session (**a**). Schematic diagram of a bioreactor unit that includes a cell-slice construct loaded between stainless steel grips within a dish (**b**). Unstrained composites were not subject to cyclic loading and served as controls (**c**).

Figure 2. DTSs (**a**) and representative stress-strain curves of DTSs in 5 samples showing the toe region (**b**) (0%-3% strain), linear region (3%-9% strain), yield region (9%-12% strain), and failure region (>12% strain).

Figure 3. Viability of BMSCs cultured on DTSs after stretch culture. Morphologically, the DTS alone showed intact and aligned fibers (**a**). Laser confocal micrographs for traces of BMSCs on DTSs labeled with the fluorescent marker PKH26-GL (red) (**b-d**). Increasing numbers of labeled BMSCs were observed after 1 day (**b**), 3 days (**c**), and 7 days (**d**) in culture. Arrows indicate the fiber direction, equivalent to the stretch direction. Autofluorescence of DTS fibers (green); cell membrane of BMSCs (red). Scale bar indicates 100 μ m.

Figure 4. Hematoxylin and eosin staining of sagittal sections of bone marrow stromal cell–DTS (BMSC-DTS) constructs in unstrained at day 1 (**a** & **e**), unstrained at day 7 (**b** & **f**), strained at day 1 (**c** & **g**), and strained at day 7 (**d** & **h**). The cell-seeded slices show successful repopulation after culture. Most BMSCs were attached to the outside surface of the DTSs (solid arrows), although a few cells infiltrated the DTSs (dotted arrows).

Figure 5. Scanning electron micrographs of the engineered tendon constructs after dynamic stretch culture (original magnification $\times 400$). With static culture, bone marrow stromal cell (BMSC) have a short spindle or flat cell shape at day 1 (**a**) and day 7 (**b**). BMSCs grew well and covered the slice homogeneously. With stretch culture, BMSC-DTS, assumed a long spindle shape, and aligned parallel to the direction of the stretch at day 1 (**c**) and day 7 (**d**). Note the dense cell sheets on DTSs by day 7. Solid arrow indicates spindle cells; dotted arrow, flat cells; double-headed arrow, the direction of stretch.

Figure 6. Gene expressions of collagen I, collagen III, decorin, and tenomodulin. Significant differences are shown by different symbols. The * indicates expression is significantly different from the control; #, expression is significantly different between strained and unstrained samples.

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Table 1 Primers for Real-Time Reverse Transcriptase Polymerase Chain Reaction

Gene	Bases, No.	Primer Type	Sequence
Collagen I	232	Forward	5'-TGGTTCTCCTGGCAAAGAT-3'
		Reverse	5'-ATCAC CGGGTTCACCTTTA-3'
Collagen III	156	Forward	5'- ACAGCAGCAAGCTATTGAT-3'
		Reverse	5'-GGACAGTCTAATTCTTGTTTCGT-3'
Decorin	174	Forward	5'-CAACAGAGAGGCTTATTTGACTTTA-3'
		Reverse	5'-TGGTACTTTGTCCAGACCC-3'
Tenomodulin	154	Forward	5'-GATCCCATGCTGGATGAG-3'
		Reverse	5'-TACAAGGCATGATGACACG-3'
β -Actin	184	Forward	5'-CTGGCACCACACCTTCTA-3'
		Reverse	5'-GCCAGAGGCATACAGGGA-3'
GAPDH	154	Forward	5'-TATGATTCTACCCACGGCAA-3'
		Reverse	5'- CAGTGGACTCCACAACATAC-3'

Table 2 Mechanical Properties of the BMSC-DTS Constructs during Strained and Unstrained Culture Conditions (n=8 per each group)

Culture Time ^a	Ultimate Tensile Strength, mean±SD, MPa ^b		Stiffness, mean±SD, N/mm ^b	
	Strained	Unstrained	Strained	Unstrained
Day 1	31.7±13.6	29.9±15.2	14.0±6.3	14.5±6.2
Day 3	32.4±10.7	31.7±14.8	13.3±3.2	12.0±2.2
Day 7	34.4±10.4	33.1±15.4	15.0±3.9	13.5±3.0

Abbreviations: BMSC, bone marrow stromal cell; DTS, decellularized tendon slices.

^a Day 0 (control) value for ultimate tensile strength was 30.9±11.6 MPa; for stiffness, 14.6±4.1 N/mm.

^b Ultimate tensile strength and stiffness were not significantly different between unstrained and strained groups from 1 to 7 days' culture.

Abbreviations

BMSC, bone marrow stromal cell

DTS, decellularized tendon slices

ECM, extracellular matrix

GAPDH, glyceraldehyde 3-phosphate dehydrogenase

IACUC, Institutional Animal Care and Use Committee

PBS, phosphate-buffered saline

RT-PCR, reverse-transcription polymerase chain reaction

UTS, ultimate tensile strength