Novel System for Engineering Bioartificial Tendons and Application of Mechanical Load[*][†]

JOANNE GARVIN,[1,2] JIE QI,[3] MELISSA MALONEY,[3] and ALBERT J. BANES, Ph.D.[1–4]

ABSTRACT

Cells cultured in three-dimensional collagen gels express a more native state phenotype because they form a syncytial network that can be mechanically loaded. Moreover, cells remodel their matrix by eliminating water, and by reorganizing and aligning the collagen fibrils. Last, the ability to subject cells to mechanical loading in a native matrix is desirable because cells, in tissues as well as the matrix, bear strains and alter their expression profile consistent with either immobilization, moderate activity, or repetitive loading. This is the first report of a model bioreactor system to fabricate and culture tendon cell-populated, linear, tethered matrix constructs that can be mechanically loaded by a computer-driven, pressure-controlled system. Bioartificial tissues (BATs) as tendon constructs were molded in a novel, rubber bottom Tissue Train culture plate bearing nonwoven nylon mesh anchors at the east and west poles of each culture well. Mechanical loading was achieved by placing an Arctangle loading post (an Arctangle is a rectangle with curved short ends) beneath each well of the six-well culture plate and using vacuum to displace the flexible membrane downward, resulting in uniaxial strain on the BAT. BATs populated with avian flexor tendon cells expressed collagen genes I, III, and XII as well as aggrecan, fibronectin, prolyl hydroxylase, and tenascin, consistent with expression levels of cells grown on collagen-bonded two-dimensional surfaces or in native, whole, avian flexor tendon. Likewise, cells in BATs established a morphology of linearly arranged cells aligned with the principal strain direction as in fascicles of whole tendons. Last, BATs that were mechanically loaded had an ultimate tensile strength that was nearly 3-fold greater than that of nonloaded BATs in the first week of culture. Taken together, these results indicate that tendon cells fabricated in a mechanically loaded, linear collagen gel construct assume a phenotype that is similar to that of a native tendon in terms of appearance and expression and are stronger than nonexercised counterparts yet far weaker than native adult tendons. This technique represents a novel approach to culturing cells in a mechanically active, three-dimensional culture environment that can be readily used for the fabrication of tissue simulates for drug testing or tissue engineering.

[1]Curriculum of Applied and Materials Sciences, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina.

[2]Department of Orthopedics, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina.

[3]Flexcell International, Hillsborough, North Carolina.

[4]Department of Biomedical Engineering, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina.

INTRODUCTION

ORTHOPEDIC TISSUE ENGINEERING involves a combination of technologies derived from cell biology, materials science, and mechanical engineering. In the United States, more than 100,000 patients per year undergo surgery to repair tendon or ligament injuries.[1] The current "gold standard" for surgical repair is to use autologous tendon. However, one caveat is that during repair, the mechanical strength and structural characteristics of the host tissue are permanently altered. During anterior cruciate ligament (ACL) reconstruction, often with the use of patellar tendon, an initial loss of strength in the host tissue is typically observed from the time of implantation. A gradual increase in strength may occur, but typically never reaches the original magnitude.[2,3] Therefore, an ideal treatment has yet to be defined as a long-term solution for tendon or ligament repair. Research in connective tissue engineering has currently been focused on using natural materials as a matrix into which cells are seeded[4–7] or acellular synthetic materials such as Dacron,[8,9] polytetrafluoroethylene,[10] polypropylene,[11] and carbon fibers.[12] Each approach has its advantages and disadvantages. Most of these synthetic materials do not approximate the material properties of tendon or ligament, resulting in stress shielding in the natural tissue. Moreover, wear debris can result in an immunological response that ultimately leads to implant failure, resulting in additional surgery. In other cases, degradation products result in acidification of the surrounding tissue, cell death or growth stasis, and implant failure.

A natural material such as fibrillar collagen can act as a scaffold allowing cells to integrate it into host tissue. This material can be formulated to approximate the host tissue's collagen type (generally type I collagen) and material properties and is minimally antigenic. In addition, it would be advantageous to use a material seeded with native tendon cells because it is these cells that are responsible for normal tissue maintenance, remodeling, and metabolism. Together, these ideas are the basis for the hypothesis that mechanically conditioned tendon internal fibroblasts grown in a tethered, three-dimensional collagenous matrix will mimic native tendon in appearance, genetic expression, and biomechanical strength. This is the first attempt to create a bioartificial tendon using native tendon cells in a molded, type I collagen matrix that can be subjected to a mechanical loading regimen.

MATERIALS AND METHODS

Cell culture

Avian tendon internal fibroblasts (ATIFs) were isolated from the flexor digitorum profundus tendons of 52-day-old White Leghorn chickens ($n = 3$ different isolates). Chicken feet were obtained from a Purdue processing plant (Robbins, NC). Legs were washed with soap and cold water before tendon isolation. The flexor digitorum profundus tendons were removed from the middle toes after transection at the proximal portion of the metatarsal and distal portion of the tibiotarsus. Using sterile technique, tendons were dissected from their sheath and placed in a sterile dish of phosphate-buffered saline (PBS) with 20 mM HEPES, pH 7.2, with $1\times$ penicillin–streptomycin ($1\times$ p/s: penicillin at 100 U/mL, streptomycin at 100 μg/mL). Cells were subsequently isolated by sequential enzymatic digestion and mechanical disruption.[13,14] Cells were cultured until confluent in Dulbecco's minimal essential medium-high glucose (DMEM-H) with 10% fetal calf serum (FCS), 20 mM HEPES (pH 7.2), 100 mM ascorbate 2-phosphate, and $1\times$ p/s.

Fabrication of three-dimensional bioartificial tendon

Avian tendon internal fibroblasts were enzymatically removed from a polystyrene culture plate with 0.025% trypsin. Cells were collected into a 15-mL conical tube, sedimented, washed in PBS, resuspended in 10 mL of medium, and counted. Type I collagen (Vitrogen; Cohesion Technologies, Palo Alto, CA) was mixed with growth medium and FBS and neutralized to pH 7.0 with 1 M sodium hydroxide. Two hundred thousand cells per 170 μL of collagen mixture were suspended and apportioned into each well of a Tissue Train culture plate (Flexcell International, Hillsborough, NC) (Fig. 1c). Linear, tethered, three-dimensional (3-D) cell-populated matrices were formed by placing the Tissue Train culture plate atop a four-place gasketed baseplate with planar-faced cylindrical posts inserted into centrally located, rectanglar cutouts (six-place Loading Station with Trough Loaders; Flexcell International) beneath each flexible well base[15,16] (Fig. 1a). The Trough Loaders had vertical holes in the floor of the rectangle through which a vacuum could be applied to deform the flexible membrane into the trough. The trough provided a space for delivery of cells and matrix (Fig. 2). The baseplate was transferred into a 5% CO_2, humidified incubator at 37°C, where the construct was held in position under vacuum for 1.5 h until the cells and matrix formed a gelatinous material connected to the anchor stems.[15,16] Bioartificial tendons (BATs; Flexcell International) were then covered with growth medium (3 mL/well), cultures were digitally scanned (see below, BAT Contraction Index), and plates were returned to the incubator.

The construct assumed an elongated cylindrical shape, differentiating it from a traditional 2-D monolayer culture (Fig. 3a). After 24 h in culture the matrix and cell attachments to the anchor points were mechanically bonded and secure.
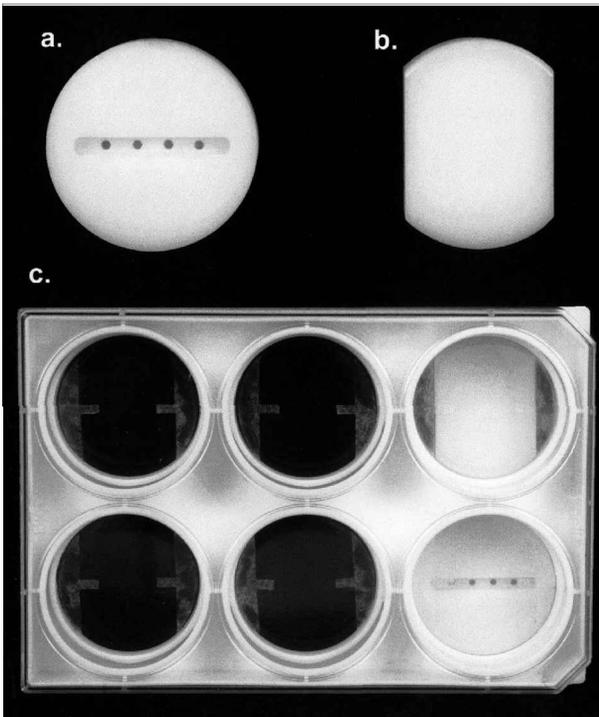
FIG. 1. (**a**) A Delrin Trough Loader insert that is 35 mm in diameter and completely fills the space beneath a well of a Tissue Train culture plate. The trough is 25 × 3 × 3 mm. The four holes are 1 mm in diameter and communicate with the reservoir beneath the culture plate so that vacuum can draw the overlying rubber membrane into the trough, creating a space into which cells and gel can be cast. Once the gel is cast, the Trough Loader is removed. To mechanically load the bioartifical tissue (BAT), an Arctangle loading post (**b**) is placed beneath the Tissue Train well so that the linear sides correspond to the east and west poles of the anchors to which the linear gel is attached. Vacuum draws the flexible but inelastic anchors downward, resulting in uniaxial strain on the BAT. (**c**) A Tissue Train culture plate with linear anchors in each well and two wells with a Trough Loader and Arctangle loading post.

Mechanical loading

BATs were uniaxially loaded by placing Arctangle loading posts (Flexcell International; rectangular with curved short ends) beneath each well of the Tissue Train plates in a gasketed baseplate and applying vacuum to deform the flexible membranes downward at the east and west poles (Figs. 1b and 3b). The flexible but inelastic nonwoven nylon mesh anchors deformed downward along the long sides of the Arctangle loading posts thus applying uniaxial strain along the long axis of each BAT. The loading regimen was 1 h/day at 1% elongation and 1 Hz, using a Flexercell (Flexcell International) Strain Unit to control the regimen. This regimen was selected after performing a dose–response experiment in which elongation and time were varied. Greater strains and du-

rations caused matrix failure and gross cell elongation at strains and durations lower than the failure point. Moreover, *in vivo* data indicate that the natural strain in flexor tendons, such as the flexor digitorum profundus tendon, is about 1% with a normal operating limit up to 3–4%. In addition, the cell-populated collagen gel construct can be broken at less than 0.7 to 1 mPa. The selected load duration was sufficient time to stimulate gene expression changes.

Growth curves

Cell numbers in replicate 2-D cultures ($n = 3$/group) were determined every 24 h. Three-dimensional BATs were removed from culture with forceps, placed into 15-
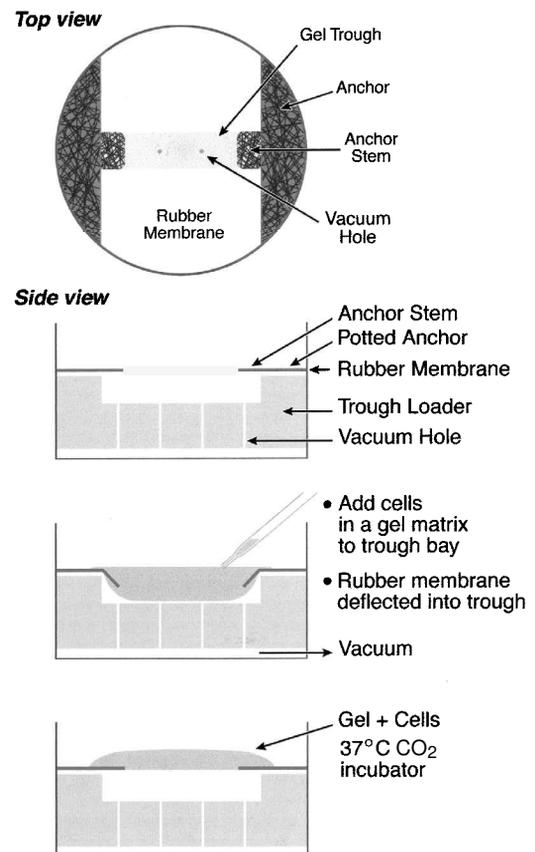

FIG. 2. Schematic diagram of one well of a Tissue Train six-well culture plate (top view) shown from above, the gel trough into which the rubber membrane is drawn by vacuum, the nonwoven nylon mesh anchor bonded to the rubber in the sector portion, and the anchor stem with collagen bonded to it. Side view: The anchor stem is shown free of the rubber bottom connected to the potted nylon anchor. Vacuum drawn through the Trough Loader holes pulls the rubber membrane downward to closely conform to the trough bay dimensions. Cells in a collagen gel are then added to the trough bay and the constructs are gelled at 37°C in a CO_2 incubator. After gelation, vacuum is released and the cultures receive culture medium.
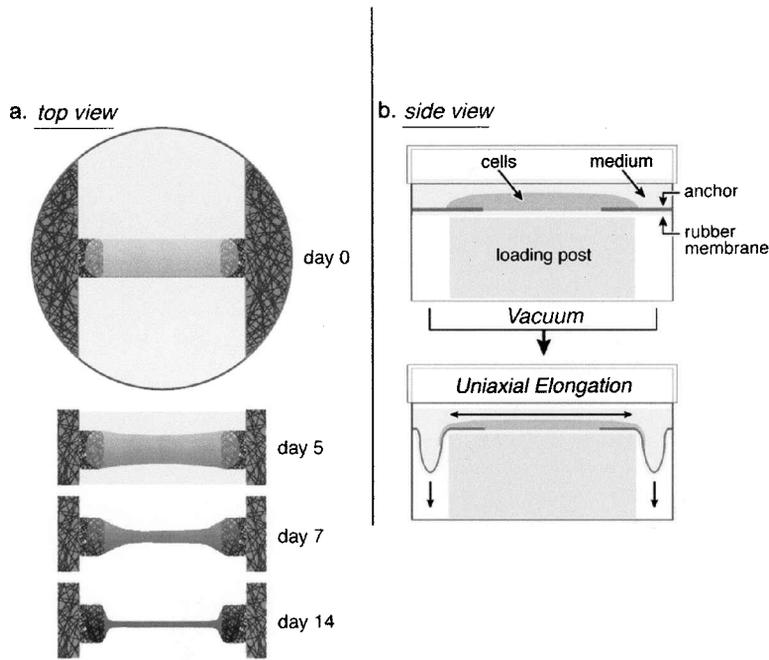
FIG. 3. (**a**) Top view: Dimensions of a typical bioartificial tendon (BAT) from the initial molding on day 0 through contraction phases on days 5, 7, and 14. The BAT assumes an hourglass shape (days 5 and 7) and finally a cylindrical shape (day 14). (**b**) Side view: One well of a Tissue Train culture plate with a molded linear BAT immersed in culture medium. The rubber membrane faces an apposing lubricated Arctangle shaped loading post (rectangle with curved short ends). When vacuum is applied to the well bottom the rubber membrane deforms downward at the east and west poles, resulting in uniaxial elongation of the BAT.

mL conical tubes containing 1.5 mL of 0.1% collagenase each, and incubated at 37°C, 5% CO_2 ($n = 6$ per group). Cells were sedimented and resuspended in an equal volume of PBS, and cell numbers ($n = 3$/group) were determined with a Nubauer hemocytometer.

BAT contraction index

BATs were cultured for up to 8 days. The overall reduction in construct area and volume (defined as remodeling or matrix contraction) as well as the width of the narrowest horizontal region of each BAT were determined every 24 h ($n = 6$). Each plate of BATs was imaged with a Hewlett-Packard (Palo Alto, CA) scanner at 600-dpi resolution. Images were analyzed with IMAQ Vision software by National Instruments (Austin, TX). The periphery of each BAT was outlined to determine the overall area. Each BAT was then outlined again to determine the width of the narrowest horizontal region, and a measurement calculated. The width of each BAT was measured three times and averaged.

Histology

Three-dimensional BAT preparations were fixed *in situ* with 3.7% paraformaldehyde for 30 min at 25°C in the wells of a Tissue Train culture plate. After fixation the BATs were placed in O.T.C. embedding medium and frozen at -20°C. BATs were sectioned into 5-μm-thick sections, using a cryostat, and applied to a glass microscope slide. Sections were stained with hematoxylin and eosin (H&E). Sections were observed and imaged at $\times 10$ and $\times 40$ magnification using a BH61 light microscope (Olympus America, Melville, NY).

Actin and nuclear staining

The BATs were fixed, while attached to the anchor points, with 3.7% paraformaldehyde at 25°C for 30 min (three BATs per group). After removal of the fixative, 0.2% Triton X-100 and 0.5% bovine serum albumin (BSA) were added to the BATs at 25°C for 30 min. The solutions were aspirated and the BATs were washed three times with PBS. Cells were stained at room temperature for 1 h with rhodamine–phalloidin (200 U/mL, dissolved in methanol) (Molecular Probes [Eugene, OR], 1:400 dilution) to stain polymerized actin and with 1 μg/ml of 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI, 1 μg/mL; Sigma, St. Louis, MO) (Sigma) to stain nuclei.[17,18] Fluorochromes were diluted in 0.2% Triton X-100 and 0.5% BSA. After 1 h, the fluids were discarded and the constructs were washed three times with PBS. Cells were imaged at $\times 40$ magnification, using an Olym-

pus BH61 microscope with a ×40 objective lens and AnalySIS 3.0 (Soft Imaging System, Munich, Germany).

Gene expression profile of 2-D cultures, 3-D constructs, and native tendon

Comparative gene expression profiles for cells grown in 2-D monolayer cultures, 3-D BATs, and native whole tendon were created using a quantitative reverse transcriptase polymerase chain reaction (RT-PCR) (n = 3/group). The experiment was repeated twice. On day 8 of culture, total RNA was isolated from each population, using a Qiagen (Hilden, Germany) minikit system. RNA was isolated from whole avian tendon, using phenol–chloroform–isoamyl alcohol (PCI) extraction and ethanol precipitation.[19] The optical density (OD) of each sample was determined with a DU640B spectrophotometer (Beckman Coulter, Fullerton, CA) to determine the total RNA concentration and purity. RNA samples having an OD from 1.9 to 2.1 were used.

Reverse transcriptase and quantitative polymerase chain reaction

The reverse transcriptase reaction was conducted with 1.1 μg of total RNA for each sample (n = 3/group) (Invitrogen, Carlsbad, CA). Each reaction tube was subjected to the following conditions: 25°C for 10 min, 42°C for 2 h, 99°C for 5 min, and 5°C for 5 min (Table 1). Primers were designed with GeneFisher software and synthesized by MWG Biotech (High Point, NC). Table 1 includes the primer sequences and PCR product length for each gene. cDNAs were separated in 1.5% agarose gels and identities were confirmed by sequence analysis. Expression levels for collagen I, collagen III, collagen XII, decorin, tenascin, fibronectin, prolyl hydroxylase, and β-actin were quantitated.

Material properties of BAT constructs

Engineering stress–strain curves were generated for the bioartificial tendon constructs (BATs) at 7 days. Tensile

TABLE 1.   PCR CONDITIONS USED FOR EACH GENE

Gene	Primer sequence	Product length (bp)	Cycle conditions	Cycles
Collagen I	5′-GGTCCTCAGGGTCTTCTTGG-3′ 3′-CACCAGGAGCACCGTTGACT-5′	184	94°C, 5 min 94°C, 1 min; 45°C, 1 min; 72°C, 30 s 72°C, 5 min	28
Collagen III	5′-AGGTGAACGTGGTCCACAAGGT-3′ 3′-GCACCAGCTGGTCCAGTCTCT-5′	300	94°C, 5 min 94°C, 1 min; 65°C, 1 min; 72°C, 5 min 72°C, 5 min	26
Collagen XII	5′-AGTATCAGTCTGGGCCTGGCAA-3′ 3′-TTTCTCCCTCTCCAGAAAGGGCTT-5′	300	94°C, 5 min 94°C, 1 min; 65°C, 1 min; 72°C, 1 min 72°C, 5 min	35
Decorin	5′-CATCCCTTACTGAGCTTCACCTT-3′ 3′-ACTCACACCAGAATAGGTTGCCTG-5′	300	94°C, 5 min 94°C, 1 min; 65°C, 1 min; 72°C, 1 min 72°C, 5 min	22
Tenascin	5′-TGTCTACAACATCAAGCTGCCTGT-3′ 3′-AGCCTGCCTTACCTTCTGCTGT-5′	298	94°C, 5 min 94°C, 1 min; 65°C, 1 min; 72°C, 1 min 72°C, 5 min	37
Prolyl-hydroxylase	5′-AACAGCCAATGAAGTAGAGGCAGT-3′ 3′-ACGACAATGCGTTGGGTTACTCA-5′	300	94°C, 5 min 94°C, 1 min; 60°C, 1 min; 72°C, 1 min 72°C, 5 min	22
β-Actin	5′-GCCATCCTGCGTCTGGACCGGGCT-3′ 3′-GTGATGACCTGGCCGTCAGGCAGC-5′	227	94°C, 5 min 94°C, 1 min; 60°C, 1 min; 72°C, 30 s 72°C, 5 min	20

tests were performed using an ElectroForce 3200 (ELF) mechanical tester (EnduraTEC Systems, Minnetonka, MN), equipped with custom-fabricated, soft-foam covered microtissue grips. The modulus of elasticity for each BAT was determined by measuring the slope of the linear portion of the engineering stress–strain curve. Ultimate tensile strength was determined by finding the peak stress from this curve.

Each BAT subjected to a tensile test was removed from its Tissue Train anchor point with metal forceps and placed in the center of the grips with approximately one-third of the material secured at each end. Each BAT was loaded in tension for a total of 5 mm of displacement. All BATs failed at less than 5 mm of elongation.

The initial cross-sectional area (A_0) of each BAT was required to calculate engineering stress (σ_e). This was obtained through the detection of the minimal cross-sectional area, along the length of the BAT, before test initiation (time = 0). A custom LabVIEW (National Instruments) program was used to obtain diameter data from two cameras focused on the front and the side, 90° to the front view of the BAT. The following formulas were used in the program to calculate the engineering stress–strain curve.

Initial cross-sectional area:

$$A_0 = \left[\frac{\pi}{4} \left(D_{\text{camera 1}} \times D_{\text{camera 2}} \right) \right]_{0,\text{min}}$$

Engineering stress (σ_e):

$$\sigma_e = \frac{F_t}{A_0}$$

where F_t is force at time t and A_0 is the initial cross-sectional area.

Engineering strain (ϵ_e)

$$\epsilon_e = \frac{y_{\text{displacement}}}{L_0}$$

where $y_{\text{displacement}}$ is the displacement of the cross-head at time t and L_0 is the original length of the BAT.

RESULTS

Growth curve

Cells were cultured for up to 11 days. Analyses of AT-IFs grown in BATs with an initial seeding density of 200,000 cells, and of cells grown in 2-D monolayers, demonstrated the typical lag, log, and stationary phases of a traditional growth curve ($n = 3$/group per time period). Both culture conditions also reflected similar generation times: 2-D, 33 h; 3-D (200,000 cells), 31 h. However, BATs with an initial seeding density of 500,000 cells did not demonstrate a typical log phase, but rather remained in a stationary phase (Fig. 4). Both 3-D cultures contained the same number of cells after 11 days.
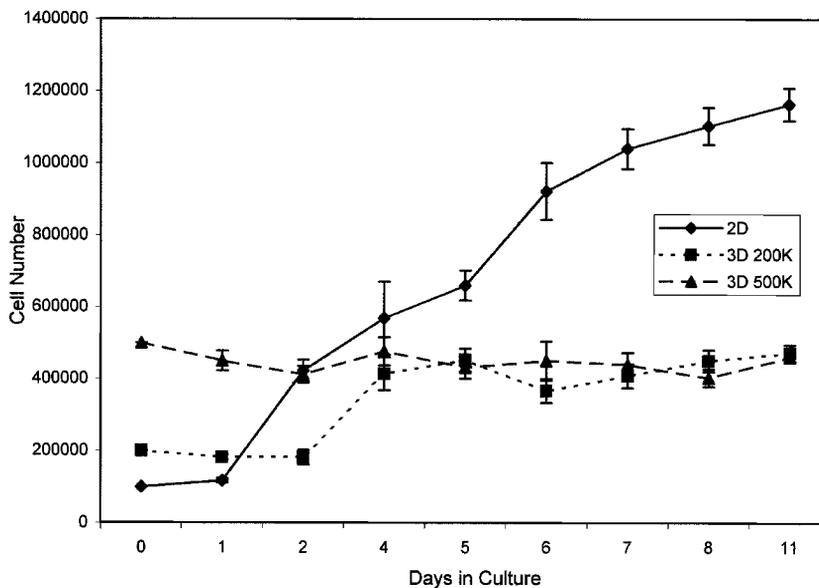

FIG. 4. Growth curves for avian internal fibroblasts grown in 2-D polystyrene culture dishes covalently bonded with type I collagen and BATs plated at 200K or 500K cells in collagen gels in Tissue Train culture plates. Cells in 2-D cultures entered log phase and passed through several division cycles whereas cells in 3-D gels plated at 200K cells per gel divided once and those plated at 500K cells per gel did not divide.

These data indicated that a comparable cell-to-matrix ratio was maintained although the initial seeding densities differed.

Contraction index

ATIFs in a linear collagen gel attached to matrix-bonded anchor ends to form a 3-D "tendinous" construct ($n = 6$/group). The BATs were cultured for up to 11 days and initially assumed a rectangular to cylindrical shape (Fig. 5, inset). As the cells reorganized and remodeled the collagen matrix, macroscopic radial contraction of the construct was evident. Over an 8-day period, image analysis revealed that the ATIFs contracted the overall area of the construct by 82% (mean $\pm$ SD; $p < 0.001$), with a reduction in midsection width by 89% ($p < 0.001$) (Fig. 5). Contraction parameters were compared by one-way ANOVA and least-square means post-hoc multiple comparisons ($\alpha = 0.05$).

Histology

BATs stained with hematoxylin and eosin appeared tendon-like, demonstrating a multicellular top layer resembling an epitenon and deeper cells aligned in the direction of the long axis of the BAT (Fig. 6). Mechanically loaded BATs had similarly aligned cells with even more elongate nuclei and cytoplasmic extensions. As with whole tendon, cells were spread and stacked throughout the collagenous matrix. An epitendinous sheath surrounds native whole tendon. This is observed by the more intense hematoxylin nuclear staining of the surface cells. This epitendinous staining is also observed as a dense, basophilic stain in the bioartificial tendons. Together, these data indicated that the appearance of the bioartificial tendon mimicked the histologic appearance of whole native tendon.

Cytoskeletal and nuclear staining

Staining with rhodamine–phalloidin (for filamentous actin) and DAPI (for nuclei) showed a three-dimensional view of the cellular architecture of the bioartificial tendons. The cells were elongated and stacked throughout the matrix, similar to the appearance of the heyatoxylin and eosin (H&E)-stained BATs. Moreover, numerous cell-to-cell contacts were observed. Cells residing in the midsection of the construct were aligned parallel to neighboring cells. Cells residing toward the end points of the BATs were spread in a more random fashion (Fig. 7). This effect occurs because of an increase in intrinsic strain in the central portion of the BAT. This region of the BAT had a smaller cross-sectional area compared with that at the end attachment points. At the initial time of plating, the cells in BATs were rounded and demonstrated minimal attachment to the surrounding matrix. Cell spreading increased as time in culture increased.
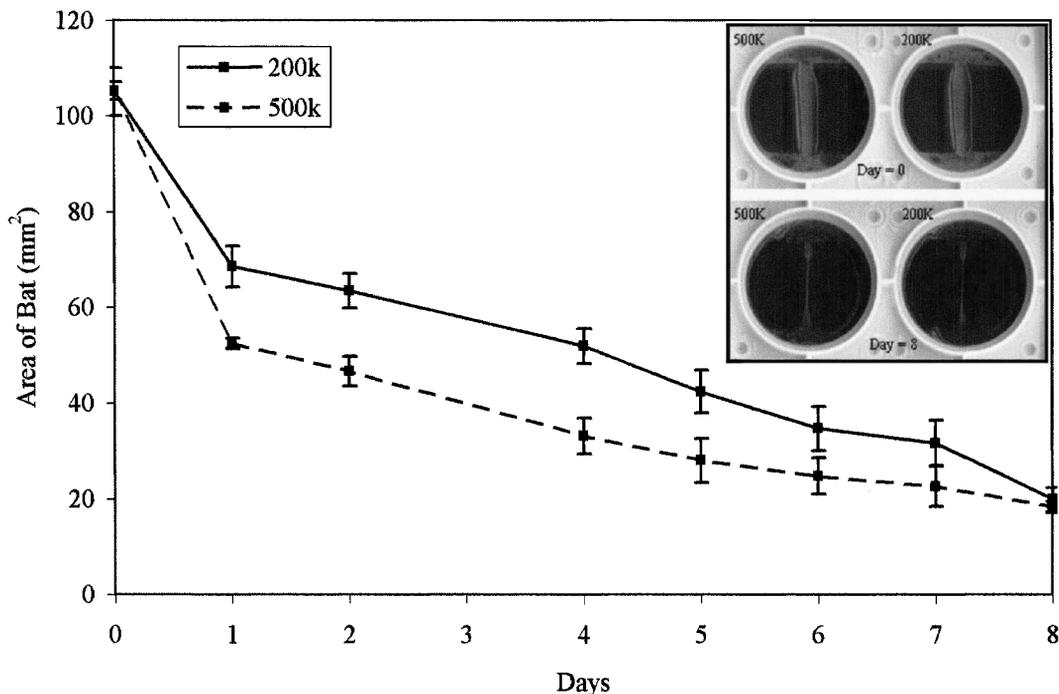

FIG. 5. Dimensional analyses of bioartificial tendons (BATs) fabricated from 200K or 500K avian tendon internal fibroblasts per BAT. A higher ratio of cells to gel matrix increased the contraction rate.
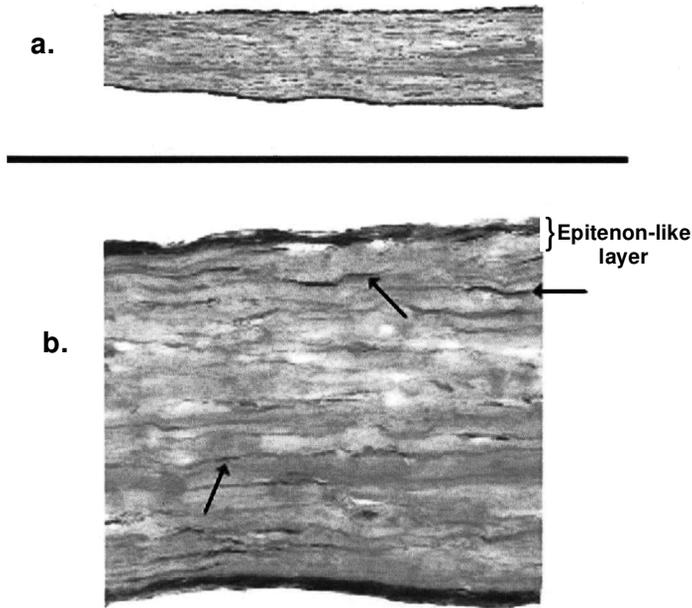
FIG. 6. (**a**) Longitudinal cross-section of a bioartificial tendon cultured for 10 days in a Tissue Train culture well and then harvested, fixed, sectioned, and stained with hematoxylin and eosin (H&E). (**b**) A higher magnification shows an epitenon-like surface layer that is two or three cells thick as well as longitudinally aligned tenocytes with elongate basophilic nuclei. Arrows indicate elongate nuclei and cells. Original magnification: (**a**) ×10; (**b**) ×40.

Cells stained at the time of initial plating until approximately day 2 showed minimal polymerized actin cytoskeletons. By day 7 the cell processes were fully extended and formed attachment points to the collagen matrix and surrounding cells. Furthermore, by day 7 in culture, the cells contracted the collagenous matrix substantially. By day 14, gross macroscopic radial contraction was evident. Moreover, microscopically, the cells assembled into a more tendon-like anatomic appearance. The midsection of the BATs contained TIFs that were well spread throughout the matrix. The periphery of the BAT contained a more organized aggregation of TIFs that resembled an epitenon.

Gene expression profile

Results of gene expression analyses indicated that all genes tested for were expressed in BATs as well as in whole tendon and 2-D monolayer cultures (Fig. 8, $n = 3$/group; experiment repeated twice). These data indicated that ATIFs cultured in the 3-D collagenous matrix retained their phenotypic expression profiles for the predominant collagens found in tendon. Cells grown in 2-D monolayer cultures with a collagenous substrate also retained genetic expression of the predominant collagens found in tendon cells and did not vary from the expression levels in BATs. Some explanations for this include a low passage number (p3) and that the 2-D tissue culture plate growth surface was treated with type I collagen. The means of these samples passed a Student's t test and showed no statistically significant difference ($p > 0.05$; $\alpha = 0.05$). The only statistically significant difference in values between samples isolated from whole tendon and those isolated from BATs was for genes encoding collagen XII (60% greater expression in whole tendon) and tenascin (10% less expression in whole tendon) ($p < 0.001$). Mechanical loading increased the mRNA levels of collagen XII on day 3 by 33% ($p < 0.05$) (Fig. 9). The mRNA level of prolyhydroxylase were increased at day 3 by 61% and by 33% on day 5 ($p < 0.05$).

Mechanical properties

The modulus of elasticity for control and mechanically loaded BATs composed of type I collagen and 200,000 chick TIFs was determined on day 7. At initial plating (day 0), the BATs could not be subjected to tensile testing because of their weak, gelatinous nature. It was assumed that the modulus at this time point was approximately equal to zero. The modulus of elasticity of the BATs increased over time and increased with mechanical conditioning (Table 2). The average modulus for control BATs on day 7 was 0.49 MPa whereas that for mechanically conditioned BATs was 1.8 MPa. BATs subjected to a cyclic mechanical load of 1% elongation at 1 Hz for 1 h/day for 7 days had a 2.9-fold greater ultimate tensile strength compared with nonloaded controls (Table 2; $p < 0.22$).
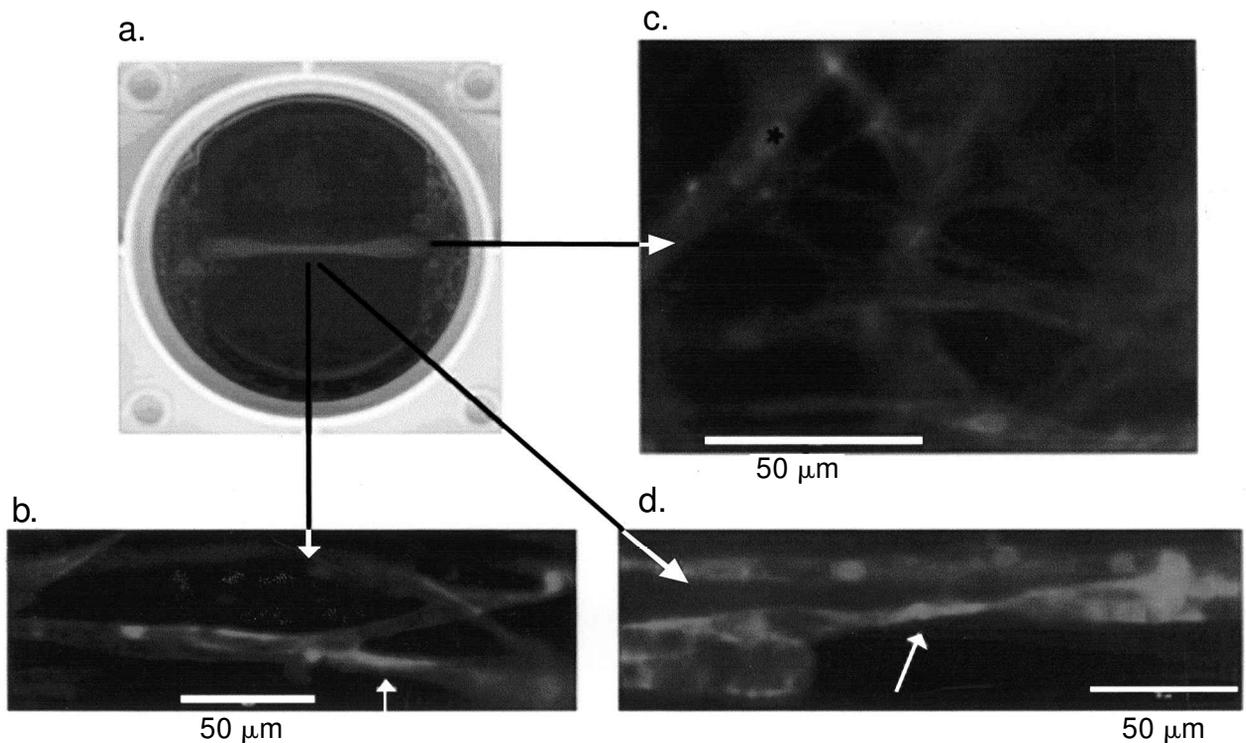
FIG. 7. (**a**) A BAT in one well of a Tissue Train culture plate well with a tendon cell-populated collagen gel on day 10. The gel construct is contracted and cells are aligned with the principal strain direction except at the stress-shielded anchor ends. The arrow pointing toward 3:00 indicates cells in the collagen gel at the anchor stem region that are stress shielded and therefore not aligned with the principal strain axis (**c**). The asterisk in (**c**) is atop the DAPI-stained nucleus of a tenocyte. The arrow pointing toward 4:00 (**d**) shows cells within the collagen gel matrix at the anchor stem–gel matrix border. These cells are aligned with the long axis of the gel construct and in the principal strain direction. The arrow pointing toward 6:00 (**b**) depicts cells in the midsection of the BAT that are aligned with the principal strain direction. Nuclei are stained with DAPI and the actin cytoskeletons are stained with rhodamine–phalloidin.

DISCUSSION

A three-dimensional tenocyte-populated linear bioartificial tendon was created by a novel molding process.[15,16] The goal was to use a 3-D cell culture approach to create a tissue replacement that mimicked the biological behavior and material properties of native tendon. This approach has been explored for creating bioartificial muscle tissue.[20,21] We observed that the tenocytes possessed mitotic ability, functioned to remodel their surrounding matrix, and retained their intrinsic phenotypic mRNA expression patterns and appearance. Thus, the hypothesis that tendon internal fibroblasts grown in a tethered, three-dimensional collagenous matrix mimic native tendon in appearance and genetic expression was validated.

The tenocytes dispersed in a collagen gel remodeled and contracted their matrix by an 82% reduction in area over an 8-day period. This confirms what has previously been reported in other systems: that matrix contraction by fibroblasts is typically rapid in the first week of culture.[6,22,23] *In vitro* cell-populated matrix cultures that are fabricated by combining cells, matrix components, and nutrients or other growth factors has been previously reported.[22–27] Fibroblasts incorporated into a collagen gel remodel their matrix in a process that simulates a wound repair sequence.[24,28] It has been proposed that developmental matrix remodeling may be regulated through cell attachment to the collagen and other matrix molecules.[29,30] During this remodeling process, fibroblasts remodel the collagen matrix to form a uniaxially oriented material in response to the appropriate orientation cues, such as mechanical stress or magnetic fields.[6,22,31] The alignment of fibroblasts throughout the BATs supports the hypothesis that forces exerted by cells alter the surrounding collagen matrix. This gradual alignment, in turn, can provide the mechanical cues to neighboring cells to orient in a similar pattern.

The immobilized end-point anchors for the BATs created the mechanical stresses necessary to develop a uniaxially oriented material with histology resembling that of tendon. As the fibroblasts exerted traction on the collagen matrix, the matrix was consolidated in the uncon-
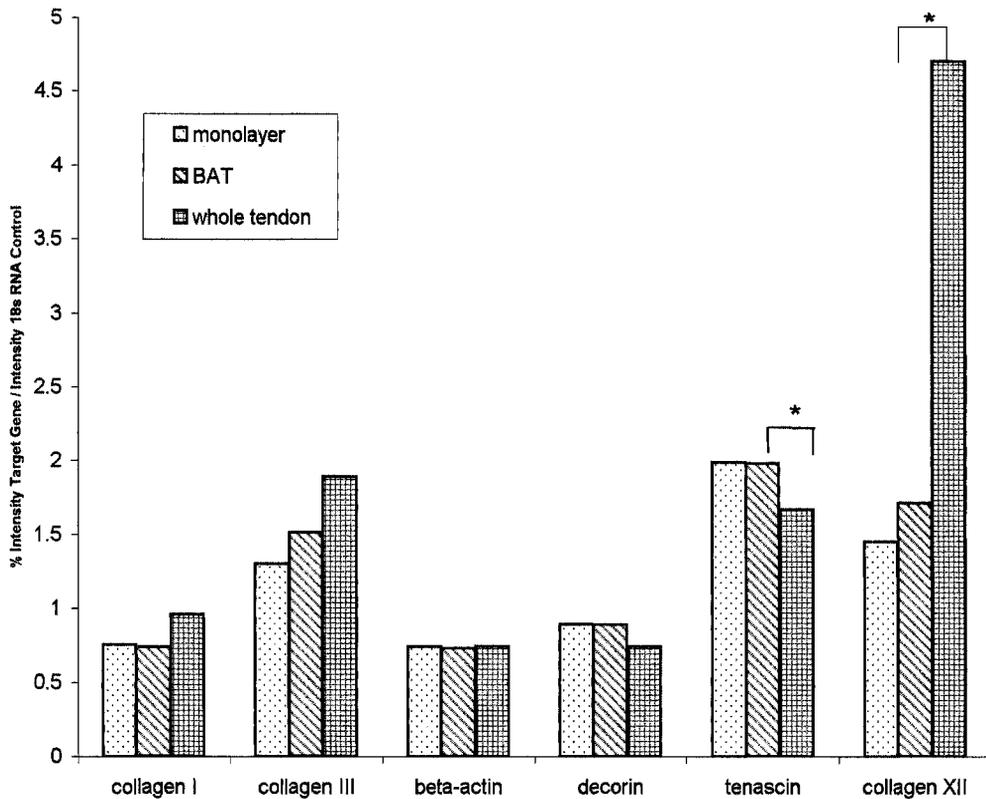
FIG. 8. Gene expression levels for collagen I, collagen III, collagen XII, decorin, tenascin, and β-actin as markers that are highly expressed in tendon cells. Expression levels were similar for cells grown in 2-D cultures on collagen-bonded surfaces in BATs in collagen gels or in whole tendon. Cells in native tendon expressed slightly less tenascin and about 2.2-fold more collagen XII than 2-D and 3-D counterparts ($p < 0.05$).

strained portions of the culture. Moreover, the collagenous matrix increased in alignment and stiffness along the axis between the two anchored end points. The increasing stiffness in the BATs may have been the signal for the cells to orient in a direction parallel to the principal strain. It can also be assumed that the intrinsic strain at the central two thirds of the construct was greater because the construct assumed an hourglass-shaped appearance at that location ($\sigma = F/A$). There was a 7% greater reduction of the cross-sectional area in this central region when compared with the end-point regions.

Tenocytes in the BATs were mitotic, which is consistent with other reports of fibroblasts in three-dimensional collagen matrices.[6,32] However, this is the first report of a growth curve comparing tenocytes grown in two dimensions (monolayer) versus those grown in three dimensions (BATs). The cells grown in a monolayer and those grown in BATs share similar generation times. However, one difference between the two groups was that the cells grown in three-dimensional culture entered into the stationary phase of the growth curve on day 5, whereas the cells grown in a monolayer continued in the exponential phase of the growth curve.

The mitotic halt may be a result of contact inhibition with neighboring cells. Staining cells in BATs with rhodamine–phalloidin at the same time point (day 5) showed an overlap between adjacent cells. This probable cellular junction was an indication that intracellular communication may have been established, allowing for transmission of the mechanical signals to exit the cell cycle. Cellular communication occurs through gap junctions. This hypothesis could further be investigated by immunohistochemical staining with anti-connexin 43 antibody, the protein involved in forming gap junctions in both human and avian tenocytes.[33]

A profile of gene expression for some of the principal genes expressed by tenocytes was created. This approach evaluated the RNA expression profile of tenocytes in BATs compared with that expressed by cells maintained in a monolayer culture in whole tendon. This evaluation was performed to ensure that tenocytes grown in the 3-D BATs retained their genotypic expression patterns.
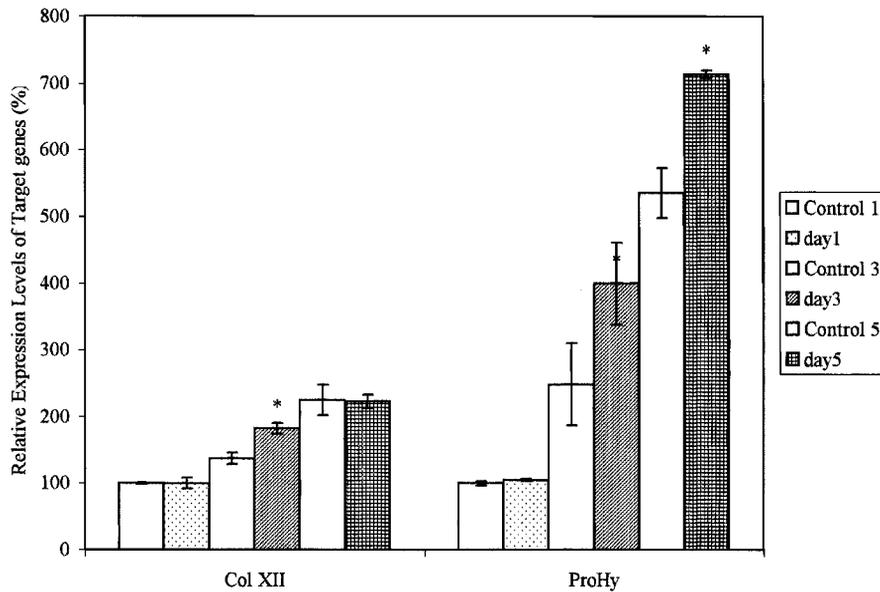
FIG. 9. Cells in BATs that were mechanically loaded at 1 Hz, 1% elongation for 1 h/day for up to 5 days increased expression levels of collagen XII (Col XII) on day 3 (15%, $p = 0.05$). Prolyl hydroxylase (ProHy) expression was increased 32% on day 3 and more than 2-fold on day 5 in loaded cultures ($p < 0.05$).

The expression patterns of genes encoding collagen I, collagen III, β-actin, and decorin were the same when comparing the RNA isolated from cells in BATs with that of cells in either a 2-D monolayer or whole tendon. Expression patterns of the genes encoding for tenascin, fibronectin, and collagen XII were the same when compared with cells grown in either monolayer or 3-D BAT cultures. There was a statistically significant difference between expression profiles for RNA isolated from whole tendon and from BATs for genes encoding collagen XII and tenascin. However, loading increased expression of type XII collagen and prolyl hydroxylase. Increased hydroxylase activity could be responsible for greater stability in the collagen fibrils and hence greater ultimate tensile strength. These findings were based on data from experiments with BATs that were maintained in culture for 7 days. Lysyl oxidase expression did not change, suggesting that aldehyde creation from ϵ-amino groups of lysine or hydroxylysine and subsequent formation of Schiff base cross-links was unlikely to be the cause of increased matrix strength (data not shown). It would be worthy of investigation to determine whether time in culture would yield a less significant difference between the expression of tenascin and collagen XII in BATs. Tenascin is an extracellular matrix (ECM) protein that is highly expressed during organogenesis and active turnover of the ECM.[34] This may be a plausible reason why the expression of this message was greater in the developing BATs than in the adult whole tendon. Collagen XII is a protein that is known to associate with and increase stability of collagen fibrils.[35]

Young's modulus was determined for mechanically conditioned and for control BATs on day 7. Conditioning the BATs[TM] drove their moduli toward that of mesenchymal stem cells seeded onto a collagen matrix (31.7 MPa).[4] Moduli for various native whole tendons have been reported to average 1.5 GPa for *in vitro* testing[36] and 1.2 GPa at maximum forces *in vivo*.[37] The elastic moduli of the BATs were significantly lower than that of native tendon, but a trend of strengthening with loading was demonstrated. A qualitative but significant increase in stiffness and decrease in elasticity was observed for

TABLE 2. COMPARISON OF MODULUS OF ELASTICITY AND ULTIMATE TENSILE STRENGTH
RESULTS FOR MECHANICALLY CONDITIONED AND CONTROL SPECIMENS ON DAY 7

	Load	No load
Day 7 elasticity (MPa)	1.80 ± 1.82	0.49 ± 0.24
Day 7 ultimate tensile strength (kPa)	327.65 ± 172.03	112.20 ± 6.07

each BAT over the duration of the testing period. It can be hypothesized that a quantitative increase in stiffness would occur over time and could approach a modulus of elasticity close to that of whole tendon.

The biomechanical strength and moduli of the BATs was increased by applying cyclic mechanical strain *in vitro* (Table 2). Moreover, findings indicate that an anabolic steroid, nandrolone, in conjunction with cyclic load, can increase the strength of BATs [TM] populated with human supraspinatus tenocytes.[38]

Tendons are in a continuous state of dynamic remodeling. Soft musculoskeletal tissues adapt to their mechanical environment by atrophying and weakening in response to immobilization, and strengthening in response to exercise.[39] Application of daily, cyclic mechanical strain can enhance the biomechanical properties of bioartificial tendons. This approach may be useful in tissue engineering such constructs for future clinical applications.

CONCLUSION

This is the first report describing the fabrication and characterization of a bioartificial tendon using native tendon cells suspended in a type I collagen matrix that can be readily subjected to regulated, cyclic, mechanical loading. Furthermore, this is the first study to characterize a tissue-engineered tendon construct histologically, genetically, and biomechanically. Tendon internal fibroblasts grown in a tethered, three-dimensional collagenous matrix mimic native tendon in appearance and genetic expression but are weaker in biomechanical strength.

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Address reprint requests to:
Albert J. Banes, Ph.D.
253 Burnett-Womack Building
Campus Box 7055
Orthopedics
University of North Carolina
Chapel Hill, NC 27599-7055

E-mail: AJBVAULT@med.unc.edu