Comparative analysis of gene expression in normal and degenerate human tendon cells: effects of cyclic strain

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Directed by Professor Jin Woo Lee

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I shall be a humble and humane doctor making my best both in academic research and clinical practice.

June 2013

Written by the author.

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ABSTRACT

Comparative analysis of gene expression in normal and degenerate human tendon cells: effects of cyclic strain

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Tendinopathy is a persistent clinical problem which has been treated with mixed results. Many of the limitations in the treatment of chronic painful tendinopathy are based on lack of knowledge of the molecular mechanisms of tissue regeneration. As the understanding of the key mediators of tissue regeneration and treatment effects develop, we believe that molecular gene expression signatures early in the mechanotransduction pathway will accurately predict risk stratification and correlate with different clinical outcomes. Studies aimed at elucidating the mechanism of tendinopathy have focused on small cohorts of genes which leave an incomplete picture of the tendon degeneration process. This study aimed to investigate tendinopathy via a comprehensive panel of mechanotransduction-related genes and cytoskeletal tensional homeostasis with and without the application of cyclic strain, which has been demonstrated to disrupt the normal homeostasis of connective tissue. Tendon samples of “normal” and “degenerate” portion were obtained from patients undergoing surgical procedures to treat chronic painful tendinopathy. A separate, but identical cyclic strain model was established in order to measure cytoskeletal

tensional homeostasis. Before the cyclic strain, the normal tendon cells exhibited varying patterns of elevated expression of 7 genes compared with the degenerate tendon cells. In response to cyclic strain, cytoskeletal gene expression was up-regulated in normal tendon cells, especially in the expression of COL1A1, ITGA6, CTNNA1, and CLEC3B; however, cyclic strain had no effect on degenerate tendon cells. In addition, cyclic strain exacerbated the inhibition of protein expression in either type of cells, especially in the degenerate tendon cells. These data provide a more complete picture of tendinopathy, and a way to evaluate various promising treatments.

Key words : tendinopathy, tensional homeostasis, cyclic strain

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I. INTRODUCTION

Tendinopathy is a common problem among athletes and workers and constitutes a high proportion of referrals to orthopaedic surgeons.[1-3] Tendinopathy can be disabling and frequently results in lost productivity, reduced physical activity, and early retirement from sports or labor.[4-6] Despite the prevalence and recalcitrant nature of tendinopathy, its pathogenesis remains poorly understood, since few studies have examined its earliest development.[7] Biopsy samples obtained at end-stage disease from patients undergoing surgery for longstanding tendon pain typically reveal variable tendon cell density, increased hyaluronan and chondroitin sulfate content, increased collagen turnover with decreased type I collagen, and neurovascular proliferation.[8-11] In addition to internal variations within tendon, there are variations in structure, composition and cell phenotype between tendons from different sites, and, increasingly, it is recognized that tendons are 'engineered' according to the functional demands on them in specific anatomic locations.[12] There are variations in the content of

proteoglycan and collagen, and there is evidence of different rates of matrix turnover. Highly stressed tendons, such as the supraspinatus in the rotator cuff, show increased levels of collagen remodeling compared with those that are not under high stress, for example the distal biceps tendon in the forearm, which has much lower rates of collagen turnover.[13, 14] The continual process of matrix remodeling is a constitutive activity in normal tendons, affecting proteoglycans in addition to collagen,[15, 16] and is thought to be primarily mediated by metalloproteinases acting in the extracellular environment, such as matrix metalloproteinase (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS).

The strain induced signaling of cells through mechanotransduction pathways play a significant role in maintaining the normal homeostasis of connective tissues.[17, 18] In tendon cells, cyclic strain activates a wide array of cellular machinery, including DNA synthesis, mitosis, and cell differentiation.[17, 19-21] While cyclic loading is known to be beneficial to tendon health,[22] repetitive tissue strain has also been implicated in the etiology of repetitive stress (overuse) injuries of tendons.[23, 24] Although the precise mechanism by which repetitive strain initiates tissue injury is still unknown, it is likely to be a cell-mediated event. Tendon "overuse" has been proposed as a destructive mechanism that precedes overt pathologic development, implying that repeated strains below the injury threshold induce changes in the tendon-matrix composition and organization.[25, 26] Since metalloproteinase expression in tendon cells is known to be modulated by mechanical loading,[27, 28] it is possible, given the absence of inflammation in most specimens, that at least some of the changes in gene expression are induced by an altered mechanical environment. Remodeling of the tendon matrix may be induced

by increased levels of strain and shear or compressive forces acting on the tissue. Alternatively, there may be a catabolic response to the local loss of strain as a result of microscopic fiber damage. In support of this, it has been demonstrated that stress-shielded and immobilized ligaments and tendons rapidly lose their mechanical properties,[29, 30] an effect requiring viable cells and mediated via the activity of metalloproteinases such as collagenase.

Studies of ruptured tendons from humans have shown that a number of molecular changes occur within the degenerate tendon, including altered content and expression of collagen and proteoglycan and expression of matrix-degrading enzymes.[1, 8, 10, 11, 31] However, which if any of these changes precede and lead to rupture, and which are a consequence of tearing is unknown. There is an emerging hypothesis that excessive mechanical strain on tendons modulates the metabolism and/or phenotype of the resident tendon cells, disrupting the balance of matrix synthesis and degradation.[32] Partial tendon tearing then occurs through abnormal tissue, and this result in increased strain on the remaining tendon and further deterioration. Stress deprivation may also play a role in progressive degeneration, with recent studies showing that tendon cells in ruptured (stress-deprived) fibrils increase synthesis of collagenolytic MMPs that, when activated, could degrade and weaken surrounding fibrils.[33] It has also been used to describe the pathological features of the extracellular matrix (ECM) network in tendinopathy.[18]

Numerous *in vitro* studies have investigated the effects of cyclic and continuous mechanical strain on gene expression by human tendon cells. Most have focused on

alterations in the expression of a small number of cytokine and cell proliferation,[34-36] but microarrays which allow the activity of numerous genes to be monitored simultaneously have never been reported. Alterations in cell-matrix and cell to cell adhesion play important roles not only in maintaining the structural integrity of connective tissues, but also in sensing changes in the biomechanical environment of cells and mediating the transmission of bidirectional forces across their plasma membranes.[37-39] The relationship between internal cytoskeletal tension and gene expression would appear to be a key factor in understanding the ability of cells to adapt their ECM to changing external stresses. Although mechanotransduction signals are mediated through a variety of means (focal adhesions, integrins, cytoskeleton).[40]

A further understanding of the mechanisms of tendinopathy at a molecular and cellular level will provide the first step toward pharmaceutically targeting the disease either in conjunction with or in place of our current surgical interventions. Work-to-date has focused on the cell culture level and has used cultures derived from normal tendons. This study will focus on how cytoskeletal transformation leads to changes in gene expression and compare the discrepancies found in normal and degenerate adult human tendons from the same patients to further establish the mechanisms underlying onset and potential repair of tendinopathy. Expanding analysis to the whole-tendon tissue level and including data from tendons with existing tendinopathy should provide further insight into the problem.

The objective of this study is to determine if changes in the cytoskeletal tensional homeostasis of human tendon cells are related to the control of gene

expression and to compare the ability of tendon cells cultured from normal and degenerate tissue to re-establish their cytoskeletal tensional homeostasis in response to a changing mechanical environment. Human tendon cells in monolayer culture were screened for adhesion-related genes using targeted RT-PCR microarrays, and this study report, for the first time, the effect that a predominantly tensile cyclic mechanical strain has on their expression. This study further show that a definitive differing gene expression both with and without cyclic strain in normal tendon cells compared to degenerate.

II. MATERIALS AND METHODS

1. Tissue collection

The tendon specimens analyzed were as follows: 1) tissue from patients with painful tendinopathy for more than 6 months, obtained from the site of the lesion (in the tendon midsubstance) during surgery and having an abnormal histologic appearance; 2) macroscopically normal specimens from patients undergoing tendon transfer, trimmed from the distal end of the residual tendon after debridement. All procedures were conducted using facilities and protocols approved by Institutional Review Board of Yonsei university (IRB # 4-2011-0489) and all patients signed informed consent forms. Samples from a total of 3 patients (one man and two women), were included in the study, and comprised two Achilles tendons (donor 1 and 2) and one posterior tibial tendon (donor 3). Mean age of patients at the time of tissue collection was 42 years (range, 38-49 years). Tissue collected in the operating room was immediately rinsed with normal saline solution to remove adherent blood. Sharp dissection was used to remove all fatty tissue and any synovial covering, such that only an isolated portion of tendon tissue was used for the study. Specimens were transported to the laboratory in ice-cold balanced saline solution. Dissected pieces of midtendon (between 10 mg and 70 mg wet weight) were rinsed in sterile RNase-free phosphate buffered saline, blotted on a filter paper, frozen in liquid nitrogen, and stored at -70 ℃ until use.

All harvested tendon specimens were coded with a sample number, as well as a specimen number identifying the type of tendon harvested. We collected the following information about the patient's medical history on an IRB approved form that have the patient's specimen number on it along with the date of collection, but not the patient's name, age, height and weight, cause of the tendon problem, duration of symptoms, and name of the doctor who performed the surgery. We also noted the condition of the degenerate tendon intra-operatively and noted whether the tendon was stretched or completely ruptured. All data analyses were performed with knowledge of only those items listed on the medical history form described above, without knowledge of any personal identifying information. Harvested tendons were destroyed immediately upon completion of study.

2. Histologic examination

Portions of tendon used for histologic examination were marked with Alcian blue on one surface to aid orientation. Tissue was fixed in neutral buffered formalin for 48 hours and then transferred to 70% (volume/volume) ethanol. Dehydration was performed over 24 hours, concluding with 4 changes of 100% (v/v) ethanol, once every 6 hours. Specimens were cleared in methyl benzoate for 3 days and infiltrated with 1% (weight/volume) celloidin in methyl benzoate for 3 days and 4% (w/v) celloidin in methyl benzoate for 3 weeks. After rinsing with chloroform (3 changes), paraffin wax infiltration was performed with 6 changes over 3 days, with the last 4 changes

under vacuum. Specimens were embedded in paraffin blocks and oriented so that longitudinal sections were cut from the side closest to the tissue used for RNA extraction. Serial sections from the normal and degenerate regions were stained with hematoxylin and eosin using standard methods. Images were captured using a Leica microscope, camera, and Image Manager software (all from Leica Microsystems, Wetzlar, Germany).

3. Cell culture

Tendon cells (fibroblast-like cells) were cultured from both normal and degenerate tendon tissue. Using sterile technique, tendons were dissected from their sheath and placed in a sterile dish. The cells were expanded to passage two (P2) in 75 cm^2 tissue culture flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 0.01 mg/ml gentamicin, 1% antibiotic/antimycotic solution at 37 ℃ in a 5% CO_2 atmosphere. When cells reached confluence, they were seeded onto 6 well, flexible bottomed culture plates (Bioflex® culture plates, Flexcell International Corp., Hillsborough, NC, USA) at a density of 6 X 10^5 cells/well (Figure 1). The cells were incubated for 24 hours to allow adherence prior to mechanical stimulation.

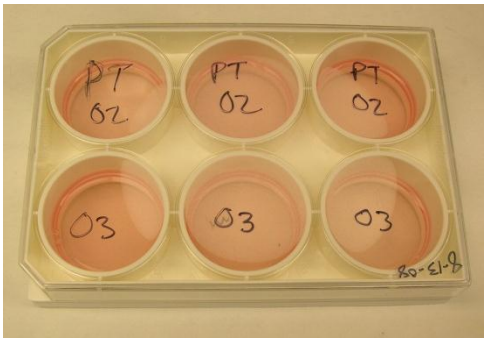

Figure 1. Flexible bottomed 6-well culture plate (57.75 cm^2 total growth surface area, or 9.62 cm^2/well) used with the Flexcell® Tension System for providing equibiaxial strain to cells in monolayer culture.

4. Cyclic strain application

The cells were loaded to 15 or 60 minutes of cyclic mechanical strain in a computer-controlled, pressure driven apparatus (Flexcell® Strain Unit, Flexcell International Corp., Hillsborough, NC, USA) placed in an incubator set at 37 ℃, and 5% CO_2 (Figure 2).

Figure 2. Flexcell® Tension System. A computer-regulated bioreactor that applies cyclic or static tensile strains to cells cultured in vitro. Uses regulated vacuum pressure to deform cells cultured on flexible-bottomed culture plates, producing up to 33% elongation.

Bioflex® 6-well culture plates with 35 mm flexible, 0.5 mm thick membrane were placed over the cylindrical loading post in a gasketed baseplate and vacuum was applied to deform the flexible membranes downward (Figure 3).

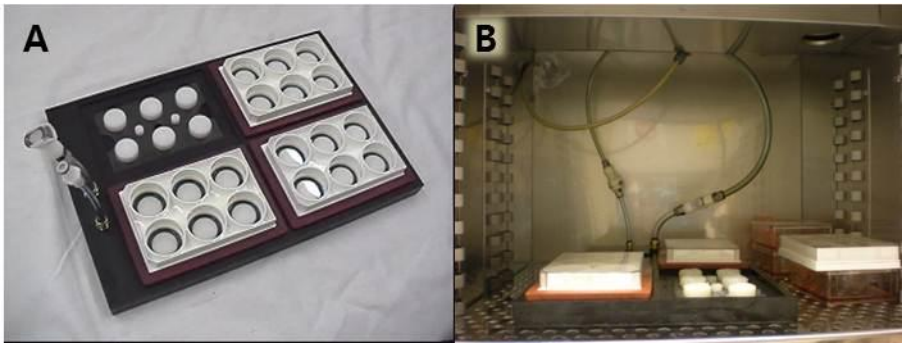

Figure 3. Bioflex® loading station. Bioflex® baseplate and four gaskets with 25 mm diameter loading posts (A) and vacuum was applied to deform the flexible membranes downward in an incubator (B).

Cyclic mechanical loading applied in a sinusoidal pattern with a frequency of 0.5 Hz and a maximum elongation of 1% (Figure 4). Control cells not receiving cyclic mechanical loading were placed in the same incubator next to the loading station of the Flexcell® apparatus. Experimental and control plates were allocated to each of the two time points (15 and 60 minutes) and separate experiments performed for light microscopy, cell viability assay, RNA isolation, and protein extraction. Final measurements were made 6 hours after cyclic strain. This regimen was based on *in vivo* data and a dose-response experiment in which elongation and time were varied.[36, 41, 42] At the end of the experiment period, Bioflex® culture plates were examined by light microscopy to determine changes in the orientation of the cells and evidence of cellular detachment.

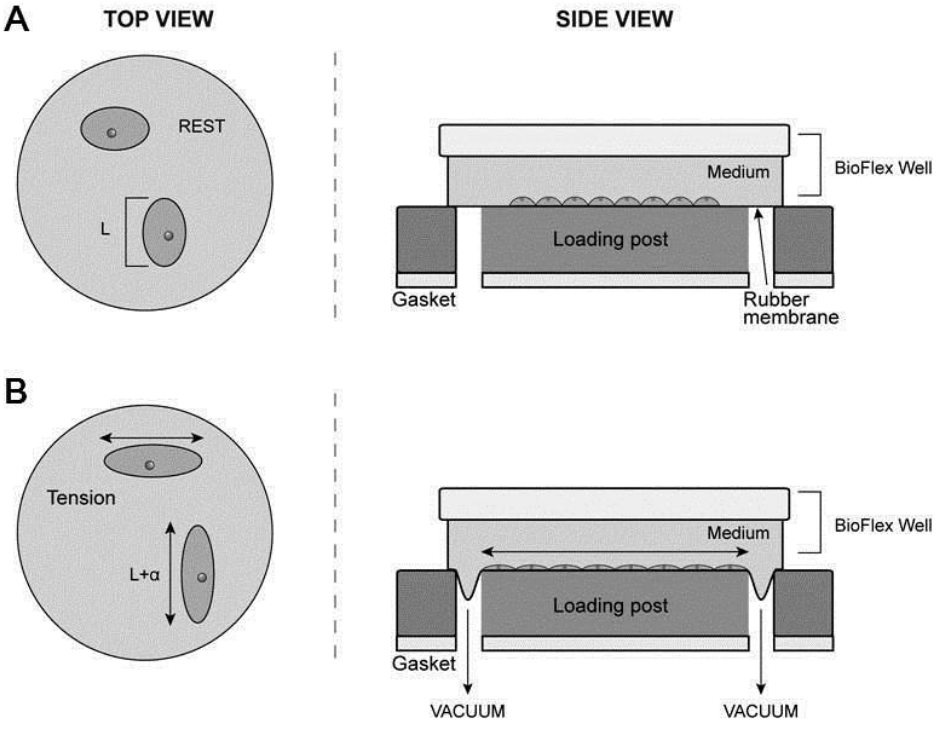

Figure 4. Schematic diagram of one well of Bioflex® culture plate. Vacuum traction applied to the rubber membrane on the undersurface of the well provided mechanical load to the cell-matrix culture; (A) before vaccum, (B) after vacuum.

5. Cell proliferation assay

Cell activation was measured by the MTT assay, a colorimetric assay for estimating mammalian cell survival, proliferation and activation based on the ability of viable cells to reduce yellow 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase.[43, 44] At the end of each time point the cells were harvested by trypsinization and resuspended in fresh media; 50 μL of this cell suspension was added to 96-well plates and spun at 1800 g for 5 min. The supernatant was removed and 50 μL of the MTT solution (4 mg/mL in DMEM) added to each well. The cells were incubated at 37℃ for 2 hours in the dark. Following incubation, the plate was centrifuged and the supernatant discarded. The resulting formazan crystals were dissolved by the addition of 200 μL of dimethylsulfoxide (Sigma-Aldrich, St.Louis, MO, USA), and absorbance was measured at 570 nm in a microplate reader (Tecan SpectrophorPlus, Singapore).

6. RNA isolation

Culture media were removed from the wells and total RNA was isolated from the normal and degenerate tendon cell following cyclic strain by modification of the method of Chomczynski and Sacchi.[45] Briefly, 0.5 mL of TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) was added to each well of the Bioflex® culture plate. After a 5 minute incubation period, the cell lysate

was added to a tube containing 0.1 mL of chloroform and shaken vigorously by hand for 15 seconds. A further incubation of 2–3 minutes at room temperature was required prior to centrifugation of the samples at 12,000 g for 15 minutes at 4℃. Following centrifugation, 300 μL of the clear aqueous phase was added to an equal volume of 100% isopropanol and vortexed to disperse the precipitate. Total RNA samples were eluted from the columns in 50 μL of RNase-free water and stored at -80℃. The concentration and purity (based on the A260/280 absorbance ratio) of the samples was determined using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Rockland, DE, USA). This combined extraction technique yielded 180–250 ng RNA from six wells with an A260/280 value of 1.96 ± 0.07; an A260/280 value > 1.9–2.0 corresponds to a pure sample free of contaminating protein (Applied Biosystems, Foster City, CA, USA). Samples were stored at -80℃ until array analysis.

7. Real-time PCR array analysis

Total RNA samples (donor 1) were assessed for degradation status by denaturing agarose gel electrophoresis, prior to analysis. Contaminating genomic DNA was removed from total RNA samples by DNase I digestion prior to first strand synthesis. First strand synthesis was performed using the RT^2 PCR array First Strand Kit (SABiosciences, Fredrick, MD, USA). Samples were then screened for the expression of 96 genes encoding ECM

and adhesion molecules using the RT^2 Profiler PCR Array System (SABiosciences, Fredrick, MD, USA). SABiosciences PCR arrays are sets of optimized PCR primer assays that perform gene expression analysis using the principle of real-time PCR. They achieve a multigene profiling capability similar to that of microarray or SuperArray technology by setting up multiple PCRs in a 96-well plate format. Experimental and control samples at each of the two time points were analysed in triplicate to allow for biological variation between samples and provide a statistically sound data set. Comparisons were then made between normal and degenerate tendon samples and a gene was said to be differently regulated if there was a four-fold difference in expression. Assays with cycle threshold (Ct) values greater than 35 cycles were considered not expressed. The PCR array data were analyzed using R software (R Development Core Team, 2008) and we performed a clustering analysis and produced a heatmap with dendrograms of genes differentially expressed between the normal and degenerate tendon.

8. Quantitative real-time PCR analysis

One μg of extracted RNA from both normal and degenerate tendon samples in each donor was reverse-transcribed and amplified by PCR as described as follows. The resulting cDNAs were diluted 20 times and individually analyzed. PCR reaction mixtures consisted of SYBR Green PCR premix (Applied Biosystems, Foster City, CA, USA), 10 pM specific primers, and 2 mL cDNA in the ABI PRISM® 7500HT Sequence Detector

(Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed for 40 amplification cycles. Mean cycle threshold values from triplicate (n = 3) measurements were used to calculate gene expression. The relative increase in fluorescent dye emission was monitored in real-time during PCR using an appropriate detection system. A Ct value was used to access an index mRNA level. Variability in amplification due to different mRNA starting concentration was controlled by the internal standard, GAPDH. Primers and fluorescently labeled probes were designed using Primer Express™ 2.0 (Applied Biosystems, Foster City, CA, USA) based on sequences available through GenBank. The list of primer stets for specific gene amplification is listed on Table 1.

Table 1. Primer sequences for quantitative real-time PCR

Gene	Strand	Primer Sequence
COL1A1	S	5'ACATGTTCAGCTTTGTGGACC3'
	AS	5'CATGGTACCTGAGGCCGTTC3'
COL11A1	S	5'ATGGACCAGCAGGATTACGTGG3'
	AS	5'TGTACCTGCTGACCCACGTTCT3'
CTNNA1	S	5'GGACCTGCTTTCGGAGTACATG3'
	AS	5'CTGAAACGTGGTCCATGACAGC3'
ITGA4	S	5'TCTAGTGGTTGCCTACACCTG3'
	AS	5'AAATGCCAGCCACAAACTCG3'
ITGA6	S	5'CGAAACCAAGGTTCTGAGCCCA3'
	AS	5'CTTGGATCTCCACTGAGGCAGT3'
LAMA2	S	5'GGCAATCTGAATACACTCGTGAC3'
	AS	5'TGTGTTGGTCCTCTCAGCATCC3'
CLEC3B	S	5'AGCTCAAGAGCCGTCTGGACAC3'
	AS	5'GGAAGGTCTTCGTCTGGGTGAA3'

S: Sense Strand; AS: Antisense Strand

9. Western Blot analysis

To determine the protein level of candidate gene, a standard Western blotting technique was used. Tissue from normal and degenerate tendon were washed with PBS and centrifuged at 3,000 rpm for 2 minutes, dissolved in RIPA buffer (10 mM PBS, 1% NP40, 0.5% sodium deoxycholate and 0.1% SDS) containing proteinase inhibitors [10 mL / mL PMSF (10 mg / mL), 30 mL / mL Aprotonin (Sigma, cat # A6279) and 10 mL / mL sodium orthovanadate (100 mM)]. The obtained samples were treated with electrophoresis loading buffer [1.0 mL glycerol, 0.5 mL 2-mercaptoethanol, 3.0 mL 10% SDS, 1.25 mL 1.0M Tris-HCL (pH6.7), 1-2 mg bromophenol blue] and simmered for 3 minutes. With 10% SDS-PAGE, electrophoresis was performed on nitrocellulose membranes (Millipore Co., Bedford, MA, USA). Membranes were blocked with 10 mM Tris-buffered saline (TBS, pH8.0) containing 5% nonfat dry milk at 4 ℃ overnight. After anti-COL11A1, ITGA6, CTNNA1 diluted into 1:1,000 and anti-CLEC3B (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted into 1:2,000 and incubated overnight at room temperature, it was washed 3 times with PBS and treated with anti-rabbit as 1:5,000 diluted secondary antibody (Amersham, UK) for 1 hour. After anti-COL1A1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted into 1:1,000 and incubated overnight at room temperature, it was washed 3 times with PBS and treated with anti-goat as 1:5,000 diluted secondary antibody (Amersham, UK) for 1 hour. After anti-GAPDH, β-actin diluted into 1:3,000 and incubated overnight at room temperature, it was

washed 3 times with PBS and treated with anti-mouse as 1:5,000 diluted secondary antibody (Amersham, UK) for 1 hr. Then, chromophores from the ECL system (Amersham, UK) made a light-emitting with the X-ray film. The level of expression of different proteins was analyzed by using the public domain software Image J [a Java image processing program inspired by National Institutes of Health (NIH) Image for Macintosh] by working in a linear range.

10. Statistics

Difference between groups was determined by Mann-Whitney U test and Wilcoxon signed rank test using Statistical Package for Social Sciences (SPSS, version 18.0, Chicago, IL, USA) and the level of significance set at $p < 0.05$. For the gene expression study, expression profiles of the target genes were measured relative to the mean critical threshold (Ct) values of two different calibrator genes (GAPDH and β-actin) using ΔΔCt method described by Livak and Schmittgen.[46] Mann-Whitney U tests were used for statistical comparison of the control and experimental groups using mean Ct values derived from the triplicate samples.[47]

III. RESULTS

1. Characterization of tendon tissue samples

Degenerate tendons had a thickened and inelastic morphology. In the transected area, there was little tendon-like tissue remaining filled with a very cellular gelatinous matrix, with few collagenous fibers visible. The outer surface of the tendon, both dorsal and ventral, was also surrounded by a thickened, scar-like tissue.

2. Histologic examination

A histologic analysis was conducted to characterize the normal and degenerate tendon samples. Three normal tendons (two Achilles and one posterior tibial tendon) showed a mostly normal histologic appearance, although there were some variations in cell shape and density. The sections consisted of mostly longitudinally oriented fibrous matrix, and the majority of cells were long, thin, and aligned with the collagen fibers, essentially as described elsewhere.[48-51] Degenerate tendons showed histologic features characteristic of painful tendinopathy, with loss of the normal fibrillar structure, loss of cell orientation, an increase in the number of fibroblasts, an increased proportion of ovoid or rounded cells (sometimes clustered or in small rows). Similar changes were reported in a large study of painful Achilles tendinopathy.[48] Most specimens showed some blood vessel infiltration into the fibrillar matrix, a feature that has been associated with the onset of clinical symptoms in the tendon (Figure 5).[52]

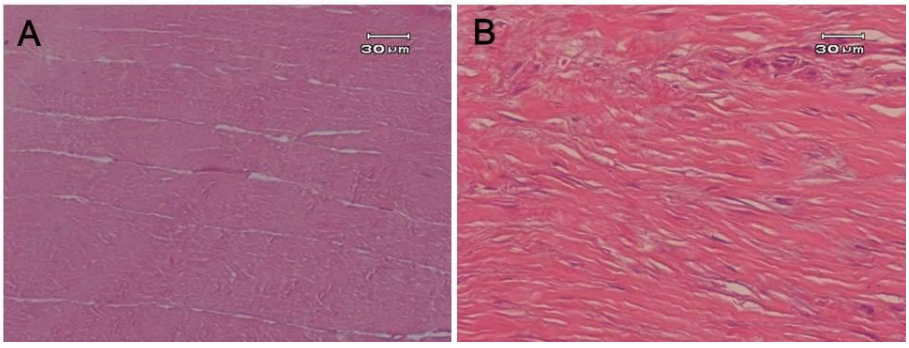

Figure 5. Histopathologic changes seen in tendinopathy demonstrating a lack of an inflammatory response. (A) normal tendon with scattered elongated cells. (B) pathologic tendinous tissue with islands of high cellularity and disorganization.

Under light microscopy, a difference in cellular orientation was not observed after the application of cyclic strain, with no evidence of cell detachment (Figure 6).

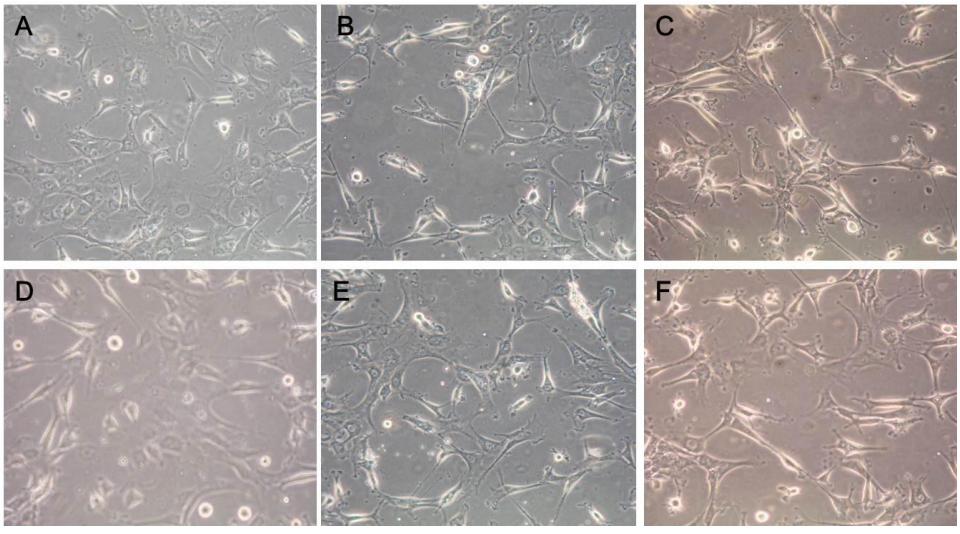

Figure 6. Light microscopy. (A) normal control, (B) normal 15 minutes, (C) normal 60 minutes, (D) degenerate control, (E) degenerate 15 minutes, and (F) degenerate 60 minutes strained tendon cells.

3. Cell proliferation activity

Cell proliferation on the normal and degenerate tendon samples during the 15 or 60 minutes of cyclic mechanical strain was assessed with the MTT assay (Figure 7). Both normal and degenerate tendon cells exhibited increased proliferation with cyclic strain. In the control group, the cell viability was statistically lower in the degenerate tendon cells (0.18 ± 0.02) than that in the normal tendon cells (0.22 ± 0.02, $p < 0.05$), while this difference became more obvious after cyclic strain for 15 minutes (0.27 ± 0.02 in the degenerate vs. 0.35 ± 0.02 in the normal, $p < 0.01$) and 60 minutes (0.23 ± 0.02 in the degenerate vs. 0.32 ± 0.02 in the normal tendon cells, $p < 0.01$).

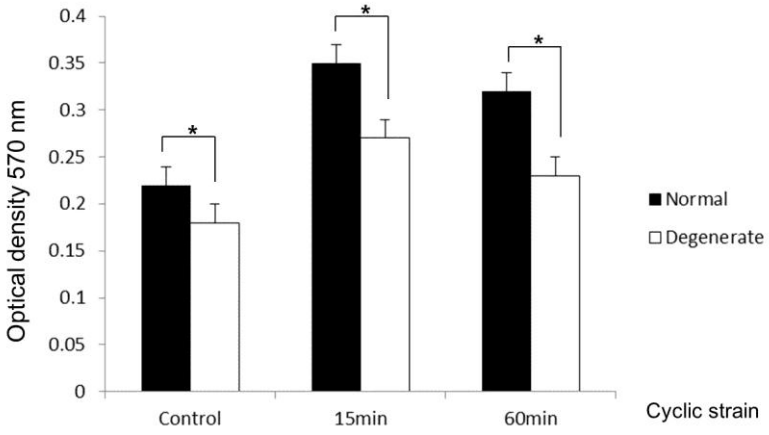

Figure 7. Following the application of a cyclic strain of 1% for 15 and 60 minutes, the colorimetric MTT (tetrazolium) assay was used to detect living cells; absorbance was measured at 570 nm. Data are from three separate experiments. Asterisk (*) indicates $p < 0.05$.

4. Real-time PCR microarray analysis

We carried out a quantitative real-time PCR using an RT^2 Profiler plate. The plate contained a set of primers for 96 genes expressed for ECM and adhesion molecules. We compared gene expression profiles between the normal and degenerate tendon cells. All of the RNA obtained from the normal tendon cells (control, 15 minute-strained, and 60 minute-strained), and degenerate tendon cells (control, 15 minute-strained, and 60 minute-strained) (n = 6 samples/patients) was used for RNA amplification. Representative gene arrays from a single patient (donor 1) are shown comparing gene expression in the normal and degenerate tendon cells. We determined signal intensity of each gene and, after global normalization,[53] compared gene expression in the normal and degenerate tendon cells at a p value of 0.05. To provide a graphical representation of all the results, we converted our data into a "heat" map (Figure 8). The color gradient indicates normalized transcription signal values (fold changes above or below expression values detected in the normal and degenerate tendon cells). Genes that had mean expression signals that were within four-fold of baseline controls were considered to be not significant. At this level of significance, 7 genes (COL1A1, COL11A1, CTNNA1, ITGA4, ITGA6, LAMA2 and CLEC3B) were up-regulated in the normal tendon cells compared with the degenerate tendon cells. To better delineate the differences in the gene expression patterns between the normal and degenerate tendon cells, we recalculated 7 up-regulated gene expression (C/N; signal ratio of each gene between the

normal and degenerate tendon cells) and rearranged them based on their signal densities. Genes noted to be increased in the normal tendon cells after cyclic strain included those that contribute to collagen α-chains (COL1A1, COL11A1), cell adhesion (CTNNA1, ITGA4, ITGA6, LAMA2), and cell attachment (CLEC3B). None of gene expression was decreased in the normal tendon cells compared with the degenerate tendon cells.

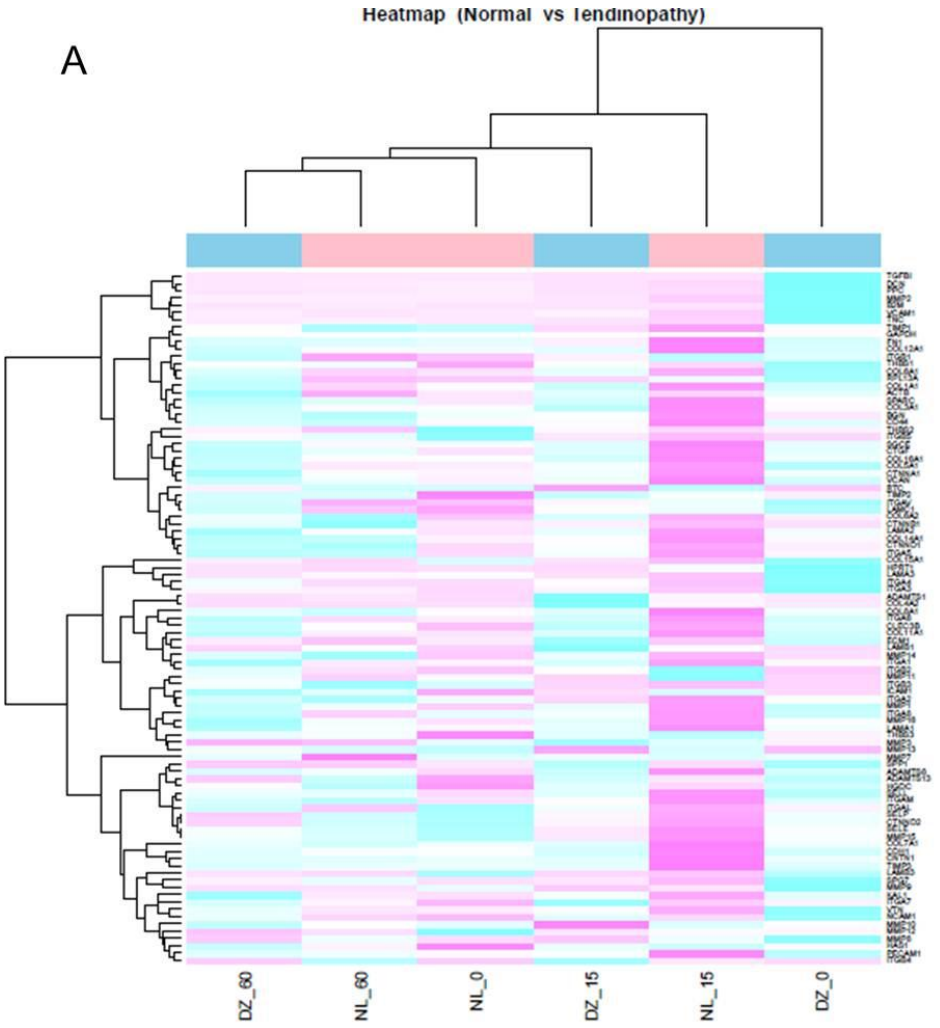

Figure 8. Heat map generated from real-time PCR microarray data. The expression level of 96 human extracellular matrix and adhesion molecule related gene targets whose expression was statistically significantly different from the normal and degenerate tendon cells has been converted to a "heat" map with *light pink* representing up-regulation of transcripts, and *light blue* representing down-regulation of transcripts (A). *(continued on next page)*

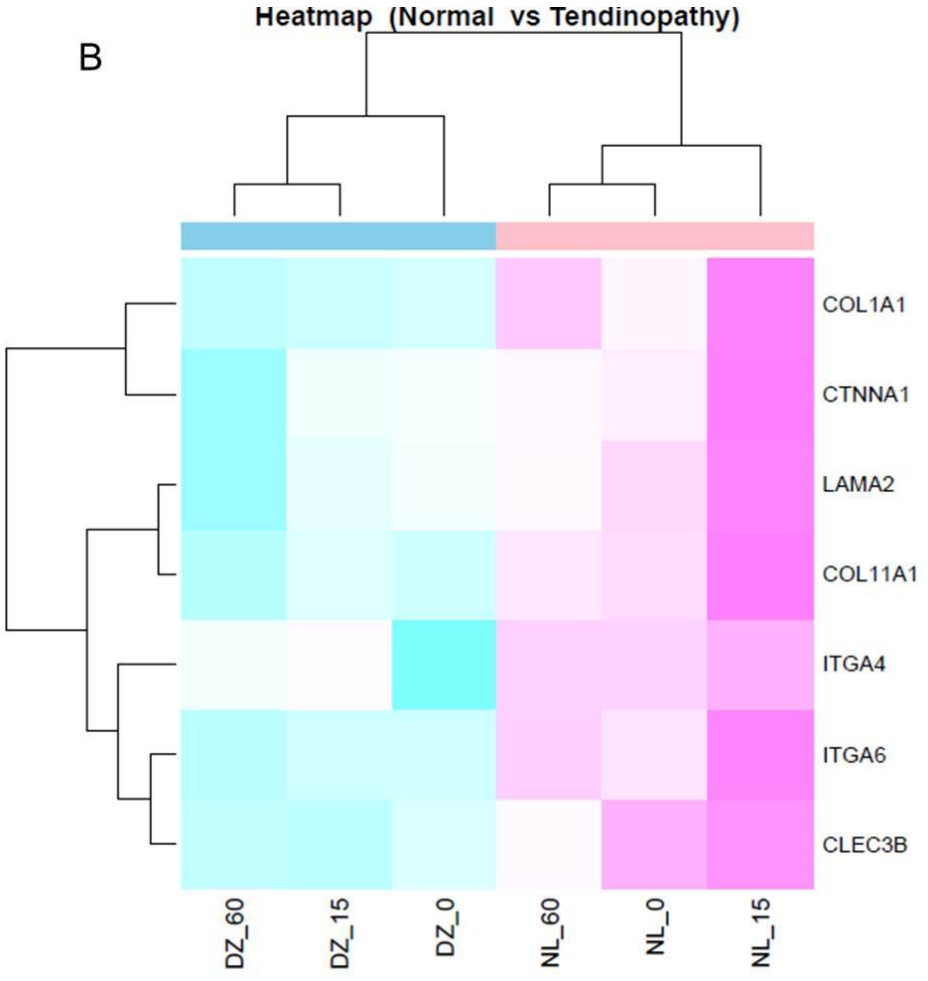

Figure 8. Heat map generated from real-time PCR microarray data. Gene transcripts that had $2^{-\Delta\Delta Ct}$ values ≥ 2 in the normal tendon cells compared to degenerate include COL1A1, COL11A1, CTNNA1, ITGA4, ITGA6, LAMA2 and CLEC3B (B).

5. Quantitative real-time PCR analysis

The mRNA expression levels for all of the 7 up-regulated genes (COL1A1, COL11A1, CTNNA1, ITGA4, ITGA6, LAMA2 and CLEC3B) after 15 and 60 minutes of cyclic strain were determined by quantitative real-time PCR. The gene expression patterns were relatively uniform among three donors. We identified that all 7 genes were up-regulated in the control group of the normal tendon cells when compared to the degenerate tendon cells. In the 15-minute strain group, we observed an elevated and prolonged up-regulation of COL1A1, COL11A1, CTNNA1, ITGA6, LAMA2 and CLEC3B in the normal tendon cells. After 60 minutes strain, lower mRNA level occurred compared with 15 minutes strain. However, mRNA expression of ITGA4 was markedly decreased after both 15 and 60 minutes strain in the normal tendon cells. In the degenerate tendon cells, mRNA expression of all 7 genes were significant different from the normal tendon cells. Surprisingly, all 7 genes were not expressed or underexpressed during the whole observation condition in the degenerate tendon cells. As a result, the effect of cyclic strain was much more pronounced in the normal tendon cells. Cyclic strain induced expression of the genes resulted in a spike at 15 minutes strain, and either remained slightly elevated, or waned towards baseline values by 60 minutes strain. However, there was no effect with cyclic strain in the degenerate tendon cells (Figure 9).

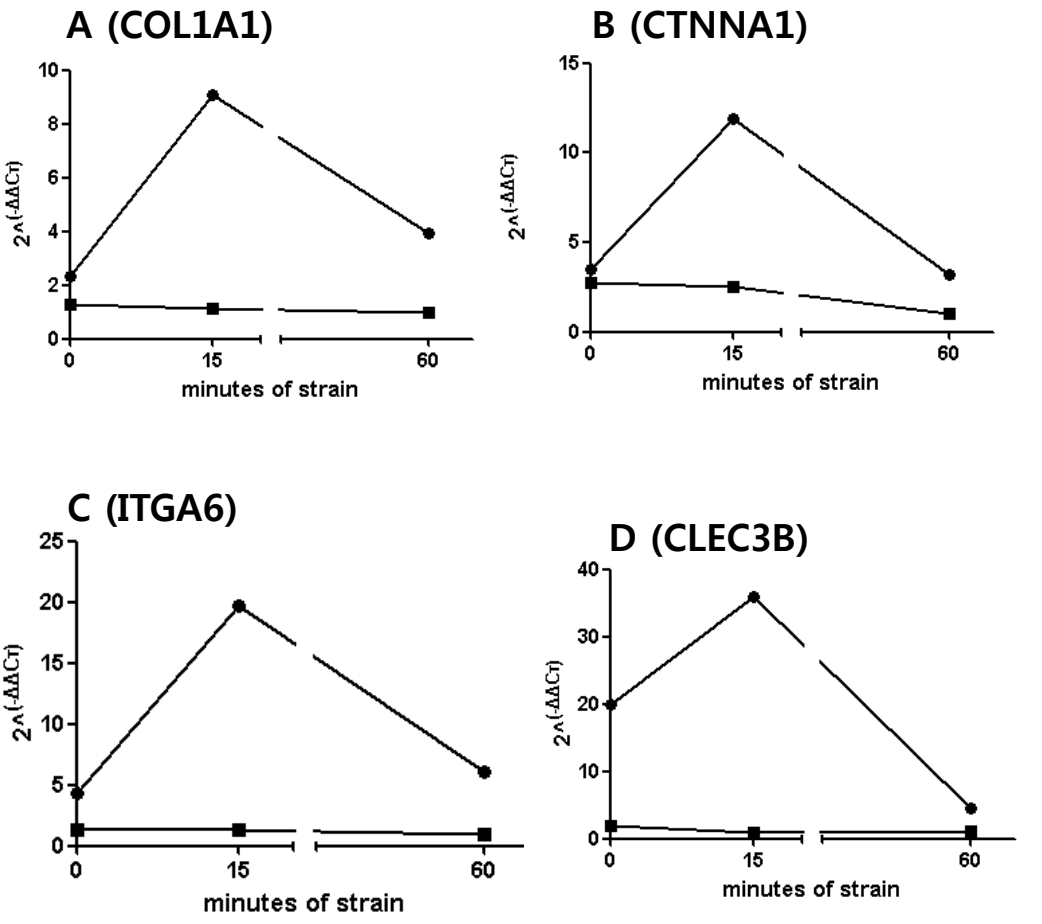

Figure 9. Real-time PCR analysis of 7 candidate gene expression in the normal [black filled circle], and degenerate tendon cells [black square]. *(continued on next page)*

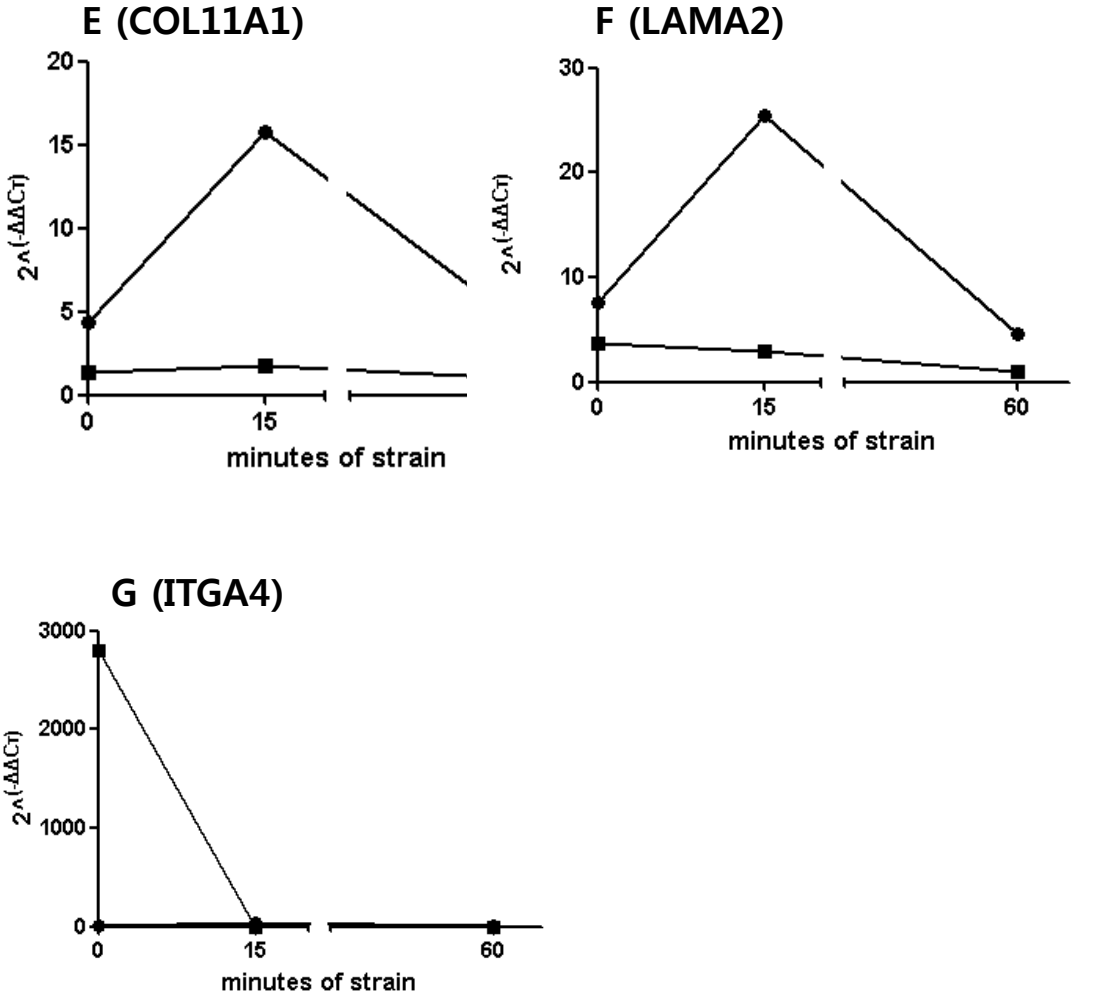

Figure 9. Real-time PCR analysis of 7 candidate gene expression in the normal [black filled circle], and degenerate tendon cells [black square]. Gene expression for COL1A1 (A), CTNNA1 (B), ITGA6 (C), CLEC3B (D), COL11A1 (E), LAMA2 (F), and ITGA4 (G) was analyzed at different time points after cyclic strain. Values represent the mean data are from three separate experiments with the same batch of cells.

6. Protein expression analysis by Western blotting

For Western blotting, we used protein (3-7 μg) extracted from the samples; due to limitations of sample size, we could only repeated the experiment twice. Protein from the normal and degenerate tendon cells was analyzed for COL1A1, COL11A1, CTNNA1, ITGA6 and CLEC3B expression; the blots were stripped and reprobed for β-actin to control for protein loading. Without cyclic strain, COL1A1, CTNNA1, ITGA6, and CLEC3B, which demonstrated increased gene expression in the normal tendon cells compared with the degenerate counterparts, also showed significantly increased protein expression. Cyclic strain (15 and 60 minutes of strain) significantly decreased protein synthesis in the normal and degenerate tendon cells. Interestingly, normal tendon cells at 15 and 60 minutes of strain have very modest decrease of COL1A1 and ITGA6 protein production, while cyclic strain markedly deteriorated these two protein production to a baseline level in the degenerate tendon cells. In contrast, a significant decrease in CTNNA1 and CLEC3B protein production was observed in the normal and degenerate tendon cells. Western blot analysis for tissue protein expression confirmed the enhanced production of COL1A1 and ITGA6 in the normal tendon cells even after cyclic strain (Figure 10). Researcher also tried to examine the protein expression of LAMA2 and ITGA4, but could not get clear results practically because of the extremely low sensitivity of anti-LAMA2 and anti-ITGA4 antibody in Western blotting.

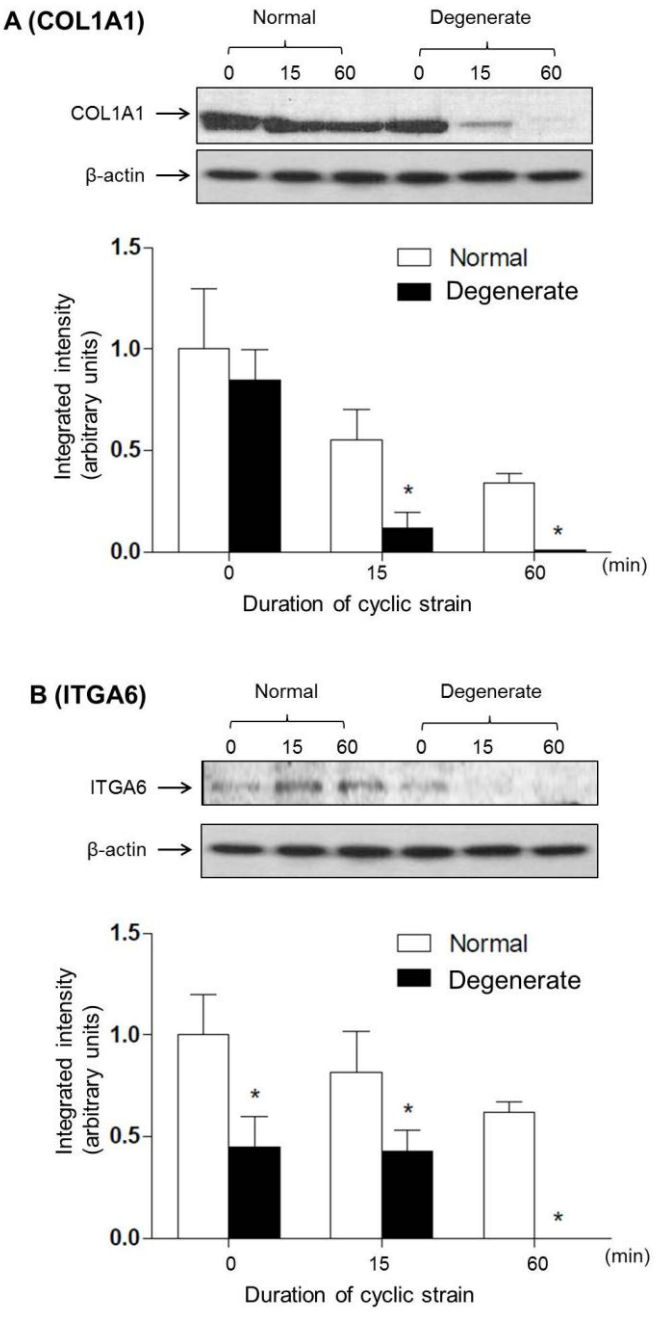

Figure 10. Representative Western immunoblot analysis and quantitative assessment of COL1A1, ITGA6, CTNNA1, and CLEC3B protein expression in comparison of the normal and degenerate tendon cells given cyclic strain. *(continued on next page)*

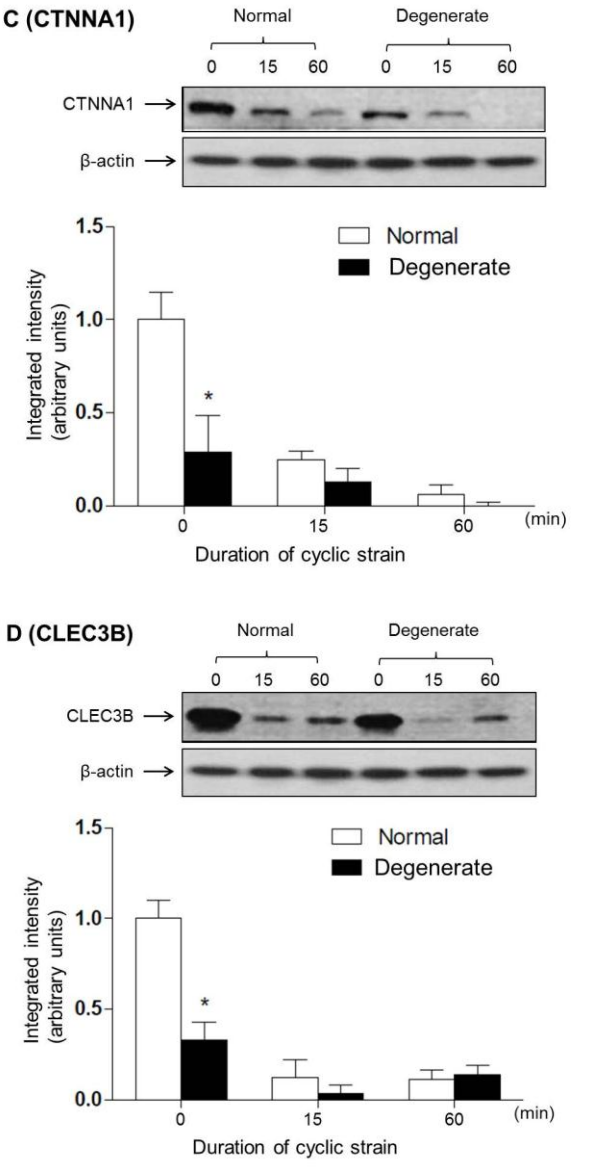

Figure 10. Representative Western immunoblot analysis and quantitative assessment of COL1A1 (A), ITGA6 (B), CTNNA1 (C), and CLEC3B (D) protein expression in comparison of the normal and degenerate tendon cells given cyclic strain. Relative quantities and data were normalized from scanned immunoblots using the β-actin values and intensity levels were quantified using Image J software. Each points represents mean ± SEM and data are from two separate experiments with the same batch of cells. Asterisk (*) indicates $p < 0.05$.

IV. DISCUSSION

This study is the first to investigate the alterations of gene expression levels after application of cyclic strain in the human tendon cells from patients with chronically painful tendinopathy, as compared with the normal tendon cells. Tendon cells are major cellular constituents of the tendon architecture and are responsible for synthesizing all components of the ECM.[54, 55] As a result, tendon cells are the likely mediator in maintaining the balance between ECM production and degradation.[54, 55] Several lines of evidence suggest that tendinopathy is controlled by mechanical stimulation of inflammatory mediators and subsequent degenerative enzyme production from the tendon cells themselves. At the cellular level, several methods for applying mechanical load to tendon cells within cell cultures have been devised and investigated.[19, 56, 57] Application of cyclic mechanical strain has been shown to increase levels of IL-1β expression and IL-6 secretion in human flexor tendon cells.[58] Additionally, Wang et al. have demonstrated that mechanical stretching increases levels of cyclooxygenase expression (COX-1, COX-2) and production of prostaglandin-E_2 (PGE-2).[35] This over-expression of inflammatory mediators has subsequently been shown to up-regulate expression of matrix-degrading enzymes. Tsuzaki et al.[59] demonstrated the up-regulation of MMP-1, MMP-3, and MMP-13 in response to treatment of human tendon cell cultures with IL-1β.[59] Furthermore, Archambault demonstrated the combination of a stretch stimulus and IL-1β led to MMP production in a cultured rabbit fibroblast model.[27] A reasonable working model from the cell culture data, then, appears to be that a mechanism exists in which load-stimulated tendon cells produce inflammatory cytokines and mediators, including IL-1β, which

can then create a positive feedback loop inducing further production of IL-1β and MMPs which act to degrade the tendon matrix.

The expression of growth factors and other potential modulators of tendon cell activity has been shown to increase in tendinopathy, including transforming growth factor β, platelet-derived growth factor receptor and neurotransmitters, such as glutamate and substance P.[60-64] These changes might, however, be part of the healing response of the tissue and a requisite for tendon repair.[65, 66] Garvin et al.[42] reported that the biomechanical strength and moduli of the bioartificial tendons was increased by applying cyclic mechanical strain *in vitro*. Moreover, findings indicated that an anabolic steroid, nandrolone, in conjunction with cyclic load, can increase the strength of the tendon populated with human supraspinatus tendon cells. A previous study has suggested that dermal fibroblasts can respond to increases in opposing external matrix strains by a reciprocal decrease in contractile force, which maintains a homeostatic tension.[67] The same may hold true for tendon cells. However, the upper and lower limits of the external forces against which the cell can maintain tensional homeostasis is likely dependent on a myriad of factors including cell type and local ECM composition as well as the frequency and rate of external stress application.

The pattern of gene expression across the complete gene set related to cellular adhesion and ECM composition was distinct for each tendon group, suggesting that each represented a distinct tissue state. Findings in this study show that cytoskeletal tensional homeostasis in the degenerate tendon is impaired and characterized by changes in the tendon-matrix composition and organization, and the absence or

reduced expression of key cytoskeletal mediators. A marked difference in patterns of mechanotransduction-related genes was seen after application of cyclic strain between the normal versus degenerate tendon cells. This study identified 7 transcripts whose expression varied significantly. In the normal tendon cells, cyclic strain caused a significant initial increase ($p < 0.05$) in gene expression at 15 minutes, and thereafter followed modest decline at 60 minutes of cyclic strain. However, there was no effect with cyclic strain in the degenerate tendon cells. Moreover, protein synthesis in the normal and degenerate tendon cells decreased in response to cyclic strain at either 15 or 60 minutes. While cyclic strain markedly brought down protein production of CTNNA1 and CLEC3B in both the normal and degenerate tendon cells, we observed modest decline in the protein production of COL1A1 and ITGA6 in the normal tendon cells. It is conceivable tensional homeostasis in the normal tendon cell may be due to a prolonged aberrant expression of these two mediators (COL1A1 and ITGA6). These data suggest that the expression of key genes related to tensional homeostasis and tissue recovery are suppressed or not activated in tendinopathy.

Collagen type I is a key element of the ECM whose molecular architecture is ideally suited primarily for the transmission of tensile load. In pathologic human tendon, there is increased expression of the mRNA of collagen types I and III, and increased amounts of type III collagen protein are found in the tendon matrix.[68] Collagen type XI is essential for the formation of cartilage collagen fibrils, as well as for the differentiation and spatial organization of growth plate chondrocytes.[69] Its expression, however, is not limited to cartilage, but belongs to the group of fibrillar collagen genes.[70] Wescott et al. previously reported that cyclic tensile strain down-

regulates collagen type XI expression in the periodontal ligament cells.[71] In this study, of the thirteen genes encoding collagen α-chains that were constitutively expressed, two were significantly up-regulated in the normal tendon cells comparing with degenerate tendon cells, the short-chain collagens type I and XI. Although the function of these collagens is not well understood, their binding activity suggests that they have an anchoring function and play a role in cell adhesion and mechanotransduction.

Cells in culture are not uniformly attached to their substrate, but are 'tack-welded' at focal adhesions, where integrin receptors physically link actin-associated cytoskeletal proteins (talin, vinculin, α-actinin and paxillin) to the ECM,[38] as well as to adhesion molecules on the surface of adjacent cells. Integrin-mediated adhesive interactions play key roles in cell migration, proliferation and differentiation, and also regulate intracellular signal transduction pathways that control adhesion-induced (outside-in) changes in cell physiology.[37, 39] Integrins thus function both as cell adhesion molecules and intracellular signaling receptors, and it is likely that the previously reported changes in cell signaling in response to mechanical deformation are downstream of events mediated by integrins.[72] Previous studies introduced numerous integrin receptors involved in cell attachment to collagen and other substrate adhesion molecules and function to provide cell–matrix linkage. Integrin α3β1 is one of the integrin heterodimers that binds to collagen type I, while the adhesion of fibroblastic cells to laminin is mediated by both α3β1 and α6β1 integrins.[73] Integrin α8 also forms heterodimers with integrin β1 and functions as a receptor for tenascin, fibronectin and vitronectin.[74] Functions of integrin α6 have been

studied using a GoH3 neutralizing antibody directed against integrin α6. It is suggested that α6β1 is involved in adhesion to the E8-cell binding site of laminin in non-muscle cells.[75, 76] Integrin α6 is expressed during early mouse development at the stages of laminin containing basement formation, which remains during embryogenesis.[77] Here, delamination of Myf5 expressing muscle progenitor cells formed laminin-rich myotome mediated by integrin α6β1 expression.[78, 79] Reduction in the expression of these genes is therefore consistent with detachment and reorientation of the cells observed microscopically.

E-cadherin and a group of tightly associated cytoplasmic proteins called α- and β-catenin mediate cell to cell adhesion and transmembrane control of adhesion.[80-83] α-Catenin links cadherins to actin, and this may be important in regulating actin dynamics at cell to cell contacts.[84] It is up-regulated in the normal tendon cells. However, α-Catenin showed decreased protein synthesis after cyclic strain in both normal and degenerate tendon cells, which suggested that this adhesion molecule is dispensable for tensional homeostasis and /or regeneration of tendon tissue and that its loss can be compensated by other actin-binding proteins.

Numerous ECM components have been reported on the tendon side of the myotendinous junction. These include fibronectin, laminin, tenascin (formerly known as myotendinous antigen), tetranectin, and a heparan sulfate-rich proteoglycan.[85-88] Tetranectin is enriched at developing myofascial junctions, which are structurally similar to the myotendinous junctions.[89] During regeneration after muscle injury, tetranectin protein was seen at the stumps of the damaged myofibers. Likewise, tetranectin immunostaining was seen at the cell surface at apparent focal

adhesion points *in vitro*. This is keeping with the possibility that tetranectin is involved in cell-matrix interactions, which has implications for the present study. The increase in mRNA expression with decrease of protein production after mechanical strain in the normal tendon cells is strongly indicative of a causal relationship, and suggest that tetranectin functions in the tendon to maintain the fibroblast phenotype.

A number of investigators have studied the effect of tensile stress on cells *in vitro*.[41, 90-94] Increasing tensile stress causes an increase in cellular activity. The nature of this activity varies with the cells under investigation and the methods used. Sutker et al.[95] examined the effects of cyclic strain on DNA and collagen synthesis in rat medial collateral ligament fibroblasts. They reported an increase in cell number, DNA synthesis, and collagen production in response to cyclic load. Almekinders et al.[19] investigated the *in vitro* effects of repetitive motion and nonsteroidal antiinflammatory medication on human tendon fibroblasts. Repetitive motion (108 hours) was associated with an increased DNA content, protein synthesis, and release of prostaglandin. Neidlinger-Wilke et al.[41] examined the effect of different cyclic strains (1.0%, 2.4%, 5.3%, and 8.8% surface strains) on human osteoblasts. Cyclic strains were applied for 15 minutes per day on 3 consecutive days. The results showed a significant increase in proliferation after applied strains of 1%, while higher strain magnitudes had lesser effects or even decreased the mitotic activity of the cells.

Previous studies of the effect of mechanical strain on gene expression by tendon cells have been performed with normal tendon fibroblast, most commonly from human patellar tendon or avian tendon.[34, 35, 42] By comparing expression profiles between the normal and degenerate tendon cells, the present study represents an

important advance. Most of the studies investigated the effects of longer motion period on cells. Little knowledge exists about the influence of short-term strain and different duration of stress time. This study showed for the first time that even a short-term strain of only 15 minutes causes change in gene expression. Unexpectedly, a cyclic strain of 60 minutes resulted in a decrease in gene expression compared with the 15 minutes of strain. Moreover, comparing the same observation between the normal and degenerate tendon cells revealed distinct results, that 15 and 60 minutes of stretching exerted a positive effect on gene expression in the normal tendon cells. However, this phenomenon could not be detected in the degenerate tendon cells. When the normal tendon cells were exposed to 60 minutes of cyclic strain, decrease in gene expression was observed compared with the 15 minutes of strain. Maybe this duration of stress induces cell damage. This lack of increase is in contrast to other *in vitro* investigations showing only a positive effect on cellular proliferation after mechanical load. In accordance with *in vivo* studies, *in vitro* mechanical load can be both beneficial and detrimental. Little is known about the mechanisms that link mechanical stimulation and cellular response. Studies from flow-mediated endothelial mechanotransduction showed that effects occur within seconds and include a variety of electrophysiological and biochemical responses in the cells.[96] Delayed responses are associated with cytoplasmic and nuclear changes linked to transcription factor activation and the regulation of gene transcription. Nuclear changes include activation of cell cycle, differentiation, and activation of apoptosis, the so-called programmed cell death.[97] King and Cidlowski[98] showed that cell proliferation and apoptosis share a number of morphologic and biochemical similarities. Apoptosis can occur during any

phase of the cell cycle. Apoptosis also plays a substantial role in cardiac reperfusion injury, muscular dystrophy, and exercise-induced injury.[97] Concerning why cyclic strain resulted in decreased protein synthesis in the degenerate tendon cells, we hypothesize that strain may lead to programmed cell death and consequent decrease in cellular proliferation. However, normal tendon cells maintained cellular proliferation after cyclic strain. Maybe a longer strain time deteriorates protective processes. It is known that heat shock protein 72 provides protection against diverse forms of environmental and physiologic stresses to cells. During phases of stress-induced cell proliferation, heat-shock proteins might modify the levels and stability of certain proteins important for cell proliferation.[99] Strain regimen in this study was selected based on a dose-response experiment in which elongation and time were varied.[41] Greater strain and durations caused matrix failure and gross cell elongation at strains and durations lower than the failure point. The selected load duration was sufficient time to stimulate gene expression changes. 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%.[42]

Although the most widely used commercially available apparatus for delivering controlled mechanical strain to cells *in vitro*, the Flexcell® system does have limitations, particularly when it comes to determining the physical characteristics of the strain. First, the mechanical in-plane deformation applied to Bioflex® plates does not produce a purely tensional strain because tension in one plane is always accompanied by compressive and shear strains due to the Poisson effect.[100, 101] Second, cells cultured on a Bioflex® plate will experience about half the

applied substrate strain programmed into the computer.[102] And third, the amount of cellular deformation will vary with the distance from the middle of the membrane; cells near the perimeter of the field will experience greater deformation.[100] Nevertheless, despite the shortcomings of all existing model systems in precisely defining the strain profile, *in vitro* methodology still remains the most effective way to screen cells for the expression of mechanoresponsive genes. Another consideration is that the traditional culture systems in widespread use for investigating the response of cells to mechanical strain *in vitro* have a number of limitations, not least the fact that tendon cells are normally surrounded by a complex network of collagens, proteoglycans and noncollagenous proteins, which are not grown on tissue culture plastic or films of matrix proteins, such collagen or fibronectin. Some attempt has been made to address this shortcoming for compressive force application by seeding tendon cells into type I collagen gels,[42] but collagen is only one of the major structural macromolecules found in extracellular matricies. For the field to progress, therefore, it is clear that future *in vitro* analyses of mechanoresponsive gene and protein expression require well characterized three-dimensional models incorporating tendon cells into hydrogel matrices designed to produce a tissue construct resembling more closely the tendon *in vivo*.

A major observation of this study was the remarkable up-regulation of COL1A1, COL11A1, CTNNA1, ITGA4, ITGA6, LAMA2 and CLEC3B in the normal tendon cells. In comparison to degenerate tendon cells, our analysis revealed a sustained protein synthesis in COL1A1 and ITGA6 during mechanical strain in the normal tendon cells. Surprisingly, cyclic strain markedly decreased protein synthesis

in the degenerate tendon cells without having any measurable effect in gene expression.

V. CONCLUSION

This investigation has demonstrated a complex set of interactions between human tendon cells cultured *in vitro* and a mechanical strain. Alterations in the pattern of expression are suggestive of a homeostatic mechanism whereby cell adhesion molecules are predominately up-regulated to facilitate cellular reorientation in response to their altered functional environment. Some of these genes are also involved in cellular mechanosensing, and changes in their pattern of expression carry implications for the activation of a number of downstream mechanotransduction pathways. Nevertheless, additional studies will be necessary to reveal the importance of the differentially expressed transcripts, and their expression kinetics. Results from this study might have future applications, including the identification of markers for early diagnosis, targets for drug design, and indicators for treatment responsiveness and prognosis.

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ABSTRACT (IN KOREAN)

기계적 자극에 대한 인체 정상 건 세포와 퇴행성 건 세포간의 유전자 발현 비교 분석

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최 우 진

퇴행성 건증(tendinopathy)은 주로 하지 건에서 발생하는 질환으로서 젊은 연령에서 노령층까지 다양하게 나타나므로 그 임상적 중요성이 매우 크다. 현재까지 건증에 대한 수많은 약물 요법과 치료 방법 등이 꾸준히 제시되고 있으나, 아직까지 건증의 발병 기전이 정확하게 정립되지 않은 상태이다. 최근 들어 조직의 재생과 치유에 관여하는 핵심 매개체에 대한 연구가 발전함에 따라, 외부 자극에 대한 세포신호변환(mechanotransduction) 경로에서 초기에 나타나는 유전자 발현이 건증의 유발 인자를 정확하게 예측하고 치료의 예후와 관련이 있을 것으로 생각되고 있다. 본 연구의 목적은 건증을 가진 인체 건 조직에서 배양한 건 세포에 기계적 부하를 가한 후 나타나는 유전자 발현을 정상 건과 비교함으로써, 여러 가지 원인에 의해 발생 된 건증에서의 세포 내 변화 기전을 조사하여, 분자생물학적으로 건증의 발생 기전을 밝히고자 하였다.

지속되는 통증을 가진 만성 건증에 대한 수술적 치료를 시행한 환자들에게서 “정상” 및 “퇴행성” 건 세포를 추출하여 세포골격의 긴장 항상성(cytoskeletal tensional homeostasis)을 측정하기 위한 기계적 자극(cyclic strain) 모델을 만들었다. 기계적 자극을 가하기 전에, 정상 건 세포는 퇴행성 건 세포와 비교하여 세포 외 기질 및 세포간 접촉에 관여하는 7 개 유전자의 발현이 증가되어 있었다. 기계적 자극 후에, 정상 건 세포에서 COL1A1, ITGA6, CTNNA1 및 CLEC3B 의 유전자 발현이 증가되었으나, 퇴행성 건 세포는 아무런 변화가 없었다. 게다가, 기계적 자극은 두 군 모두에서, 특히 퇴행성 건 세포에서 단백질의 생성을 억제하였다. 이 연구를 통하여 건 병증의 발병 기전에 대한 보다 완전한 이해와, 다양한 치료 방법을 제시하는데 도움을 줄 수 있을 것으로 사료된다.

핵심되는 말: 건증, 긴장 항상성, 기계적 자극