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Mechanical load stimulates expression of novel genes *in vivo* and *in vitro* in avian flexor tendon cells

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Summary

Objective: Our experiments were designed to test the hypothesis that tendon cells might respond differently to applied strain *in vitro* than *in vivo*.

Design: We tested cells in whole tendons from exercised chickens and from isolated surface (TSC) and internal tendon (TIF) *in vitro* that were subjected to mechanical strain. We hypothesized that tendon cells differentially express genes in response to mechanical loading *in vivo* and *in vitro*.

Methods: We utilized an in-vivo exercise model in which chickens were run on a treadmill in an acute loading regime for 1 h 45 min with the balance of time at rest to 6 h total time. Gene expression was analyzed by a differential display technique. In addition, isolated avian flexor digitorum profundus TSC and TIF cells were subjected to cyclic stretching at 1 Hz, 5% average elongation for 6 h, ± PDGF-BB, IGF-I, TGF-β1, PTH, estrogen, PGE_2, or no drug and/or no load. mRNA was then collected and samples were subjected to differential display analysis.

Conclusions: Load with or without growth factor and hormone treatments induced expression of novel genes as well as some known genes that were novel to tendon cells. We conclude that the study of gene expression in mechanically loaded cells *in vivo* and *in vitro* will lead to the discovery of novel and important marker proteins that may yield clues to positive and negative cell strain responses that are protective under one set of conditions and destructive under another.

Key words: Mechanical load, Novel genes, Tendon cells, Exercise.

Introduction

Tendons are fibrous connective tissues designed to transmit the force of muscle contraction to bone to effect limb movement. They have a complex structure: tendon is comprised of a highly aligned matrix containing 70–80% type I collagen to provide tensile strength, 10–40% elastin yielding compliance and elasticity, proteoglycans as pulse dampeners, and lipids, whose presence in the tendon epitenon may reduce shear stress-induced friction [1–7]. Two cell populations are represented in the major compartments of tendon: the surface epitenon contains large, polygonal cells (tendon surface epitenon cells, TSC), in syncytia embedded in a lipid and proteoglycan-rich matrix containing 25% collagen, while the internal portion of tendon contains fibroblasts (tendon internal fibroblasts, TIF) in syncytial layers amidst linear and branching collagen fascicles and bundles [4, 5, 8–11]. TSC store in their matrix types I and III collagens, fibronectin, TGF-β, as well as positive (IGF-I) and potentially negative modulators of cell division (IGF-I binding proteins, IGF-bp3, 5) [4–6, 12, Mohapatra, unpublished]. Seventy-five per cent of the protein secreted by cultured TSC is fibronectin [4]. Fibronectin is also prominent immunochemically in the epitenon of avian and human flexor tendons [4]. In fact, fibronectin is a marker for the epitenon in tendon since the internal cells do not express this protein to a high degree. TSC are most active in migrating into and populating a wound bed in tendon following injury [13, 14]. TIF migrate and divide less in response to injury. TIF and TSC in mature tendon are present as syncytia situated in layered longitudinal sheets, intimately connected to each other [18]. We have shown that

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within a syncytium, cells are connected by connexin cx43 and cx32 gap junctions but between syncytia are connected by only cx43 [18]. Cells within the epitenon communicate with cells in the internal compartment via cx43 gap junctions. TIF express IGF-I mRNA as detected by PCR, in-situ hybridization and ELIZA assay for protein (Tsuzaki, submitted). TIF and TSC cache stores of IGF-I in the tendon epitenon and internal compartment that appears to be utilized during and after trauma (Tsuzaki, submitted). Moreover, the epitenon and internal compartment of rat and avian tendon express mRNA for IGF binding protein (IGF-BP5), particularly in the epitenon TSC cells (Mohapatra *et al.*, in preparation).

Tendons in the equine model are subjected to strains in excess of 12% and strain rates of 200% per second, in agreement with others [19, 20]. However, tendon overuse can be destructive resulting in focal necrotic lesions, pain, and disorganized and biomechanically weak matrix [21]. Tendon cells also respond to load by instantly releasing intracellular calcium stores, altering their cytoplasmic filament content, increased actin/tubulin ratio and polymerizing [4, 22, 23]. However, TSC and TIF do not always respond in the same way to load. It is likely that all cells in mechanically active tissues detect, process and relay load signals to surrounding cells in a feedback loop designed to provide tissue maintenance functions (proper matrix type and cell/matrix ratio). The diversity of the tendon cell responses may be related to their functions and the way they receive and process chemical and mechanical signals [24].

Given that no specific markers for tendon cells have been reported to date, our goal was to use a modified method of differential display to search for potential genes specific to TSC or TIF that are regulated by mechanical load, growth factors, hormones or the combination of treatments. The important modifications to the technique included pooling of isolated RNAs prior to reverse transcription and subsequent pooling of cDNAs to improve the reproducibility of differential display polymerase chain reaction products. Differentially expressed genes were selected only if a band was present or absent and of equal strength in lanes from duplicate reactions. This technique has not been used on tendon cells or whole tendon to date. We show the power of this technique in selecting genes that are differentially expressed in cultured cells and whole tendons subjected to mechanical load *in vitro* and *in vivo*, and the approach taken to identify a novel gene sequence.

Methods

TENDON CELL ISOLATION AND CULTURE

Briefly, TSC and TIF were isolated by the method of Banes and co-workers [4] and used at passage 3. Cells were isolated from Flexor digitorum profundus tendons (FDP) from the legs of 52 day-old chickens by a combination of enzymatic treatments with collagenase and trypsin to remove surface epitenon cells, followed by scraping with a rubber policeman to remove vestiges of the epitenon. This was followed by another trypsinization and wash to assure removal of surface cells, and by mincing of the remaining tendon sample and collagenase treatment to free internal fibroblasts [4]. Epitenon surface synovial cells (TSC) and internal fibroblasts (TIF) were propagated in 83 mm diameter N_2 gas plasma-treated, polystyrene culture plates in DMEM-H with 10% fetal calf serum, 0.5 mM ascorbate, 20 mM HEPES, pH 7.2, 100 µg streptomycin and 100 units penicillin/ml and passaged every 3 days using 0.01% trypsin in PBS. Cells were plated in six-well Bioflex® culture plates with 35 mm diameter rubber membrane bottom wells bonded with a genetic type I collagen surface (Flexcell Intl. Corp., Bioflex®) [25–29]. Cells were cultured to quiescence by plating cells at near confluence (25 k cells/cm^2), growing the cells in complete medium for 72 h, halving the growth medium with DMEM-H without serum on days 3 and 5 and using the cells on day 6 [30]. These culture conditions result in a majority of TSC and TIF attaining the G_0 state in the cell cycle where they can be released from G_0 by serum or growth factors [30].

COLLECTION OF TOTAL RNA FROM CELLS AND TISSUES

Total RNA (20–30 µg) was collected from cultured cells from single, six-well BioFlex culture plates with 35 mm diameter wells (9.63 cm^2 growth surface area/well, 57.75 cm^2 GSA/plate) in 250 µl/plate guanidine isothiocyanate lysis buffer (GI buffer) [31]. The cell lysate was added to a QiaShredder column then sedimented in a microcentrifuge for 1 min at 13 k × g (Qiagen, Inc., Chatworth, CA). A volume of 70% ethanol was added, the mixture added to an RNeasy spin column and the sample sedimented for 15 sec at 8 k × g. Total RNA was washed free of DNA and protein on the column with 700 µl RW1 buffer (DNA and protein eluted here), followed by two washes with RPE buffer, then total RNA eluted with 30 µl DEPC water (diethylpyrocarbamate water). Sample RNA quantitation was performed at A260 nm (1 OD 260 nm = 40 µg/ml). If the absorbance ratio (A260/280) was less than 1.9, the RNA

was purified further using the phenol-chloroform isoamyl alcohol method and precipitation with sodium acetate (Message Clean Kit, Genhunter Corp., Brookline, MA). Samples were extracted with vortexing in 40 µl phenol-chloroform (PC) in gel-containing phase-lock tubes (5 Prime-3 Prime, Inc., Boulder, CO). After sedimentation, the supernatant fluid was collected and nucleic acid precipitated with 5 µl 3M NaOAC and 200 µl absolute ethanol for 1 h at −80°C. The 14 k × g pellet was washed with 500 µl ethanol and dried in a Speed-Vac drying centrifuge. RNA pellets were dried, reconstituted in diethylpyrocarbonate-treated water (DEPC) and absorbance determined at 260 and 280 nm. Samples were lyophilized and reconstituted at 0.1 µg RNA/µl DEPC H_2O. Tissue samples from 52 day-old White Leghorn chickens were collected at a chicken processing plant (a generous gift from Golden Poultry Inc., Sanford, NC). Chicken organs (1.6–5 g each of brain, pectoralis muscle, spleen, kidney, lung, heart, tendon, bone, liver, cartilage) were collected within 2.5 minutes of death, placed in liquid nitrogen, then on dry ice, and transported to the laboratory. Samples were weighed (1 g tissue each), pulverized with a pestle in liquid nitrogen in a chilled (−80°C) mortar, and 20 ml RNA Stat-60 was added to each sample to solubilize the tissue (Tel-Test B, Friendwood, TX). Samples were vortexed, subjected to a Polytron treatment for 1 min, then 4 ml chloroform were added and the mixture was vortexed. After sedimentation at 4°C for 15 min at 900 × g, the supernatant fluids were transferred and further extracted with 10 ml RNA Stat-60; 4 ml CCl_4, 0.7 volumes isopropanol, were then vortexed, sedimented at 900 × g for 30 min and supernatant fluid was collected. Pellets were reconstituted in 1 ml DEPC water, $OD_{260\,nm}$ was measured, samples were treated with DNase I as above, extracted with PC, and RNA was purified as above. Samples were reconstituted in DEPC water at 0.1 µg/µl.

DIFFERENTIAL DISPLAY METHOD

Pooled total RNA was used as a template for all differential display analyses following a modification of the protocol recommended by the protocol in the RNAmap differential display kit (GenHunter Corp., Nashville, TN). Briefly, 1 µg of total RNA was incubated in the presence of MMLV reverse transcriptase for one hour at 42°C with T12-MA, MG, MC or MT [32]. These 14-base oligonucleotide primers consist of a 12 nucleotide polyT primer with a degenerate base (M) in the penultimate position, and a specific ultimate base (A, G, C, or T) at the 3′ end. Reverse transcription reactions were set up in triplicate and cDNAs were pooled to reduce variability between differential display reactions. Differential Display PCR amplification was performed in duplicate reactions using pooled cDNAs as the template. The primers used for PCR amplification consisted of the original primer used to generate cDNA (T12-MA, etc.) and AP-1, 2, 3 or 4, 10-nucleotide primers which hybridize at an arbitrary location in the cDNA template. Amplification was performed with a Perkin Elmer PE9600 thermocycler in the presence of ^{33}P-dCTP according to the following profile: one cycle of 95°C for 2 minutes followed by 30 cycles of 95°C for one minute. Duplicate samples were separated side by side on a 6% polyacrylamide sequencing gel. Bands of interest which appeared to be differentially expressed in both duplicate lanes were excised from the dried gel. DNA was extracted in water, and re-amplified using the same primer set. Because one band can represent several different amplification products of the same size, PCR re-amplification products were subcloned in the PCRII TA cloning vector system (Invitrogen Corp., San Diego, CA) for subsequent DNA sequence analysis by an automated fluorescent, dideoxy sequencing method (Applied Biosystems, model 373 sequencer, CA). Sub-clones were sequenced in both directions with primers directed against the M13 or T7 vector flanking sequence and identified using the BLAST algorithm at the National Center for Biotechnology Information (NCBI, 33).

NORTHERN BLOT

Poly(A)$^+$ RNA was isolated from a portion of the total RNA using Oligo(dT) cellulose columns. Either total RNA (10 µg) or Poly(A)$^+$ (1 µg) samples were separated in a 1% agarose/formaldehyde gel and capillary blotted on to a nylon membrane, to which the RNAs were cross-linked. The cloned cDNA fragment of C8 ligated in pCR™II vector was amplified using M13 and T7 primers, separated in a 1.8% agarose gel, and purified. Fifty nanograms of probe cDNA was labeled with ^{32}P-dCTP (3000 Ci/mmol) using a random priming kit (Prime-It® RmT, STRATAGENE, La Jolla, CA, U.S.A.) and hybridized with the blot in formamide prehybridization/hybridization solution at 42°C overnight, followed by a low stringency washing (with 2 × SSC/0.1% SDS for 5 min twice, then with 0.2 × SSC/0.1% SDS for 5 min twice) and the blot exposed to radiographic film for 24 to 72 h at −80°C [34]. The C8 mRNA bands were quantitated after normalizing to that of avian G6PDH by image analysis.

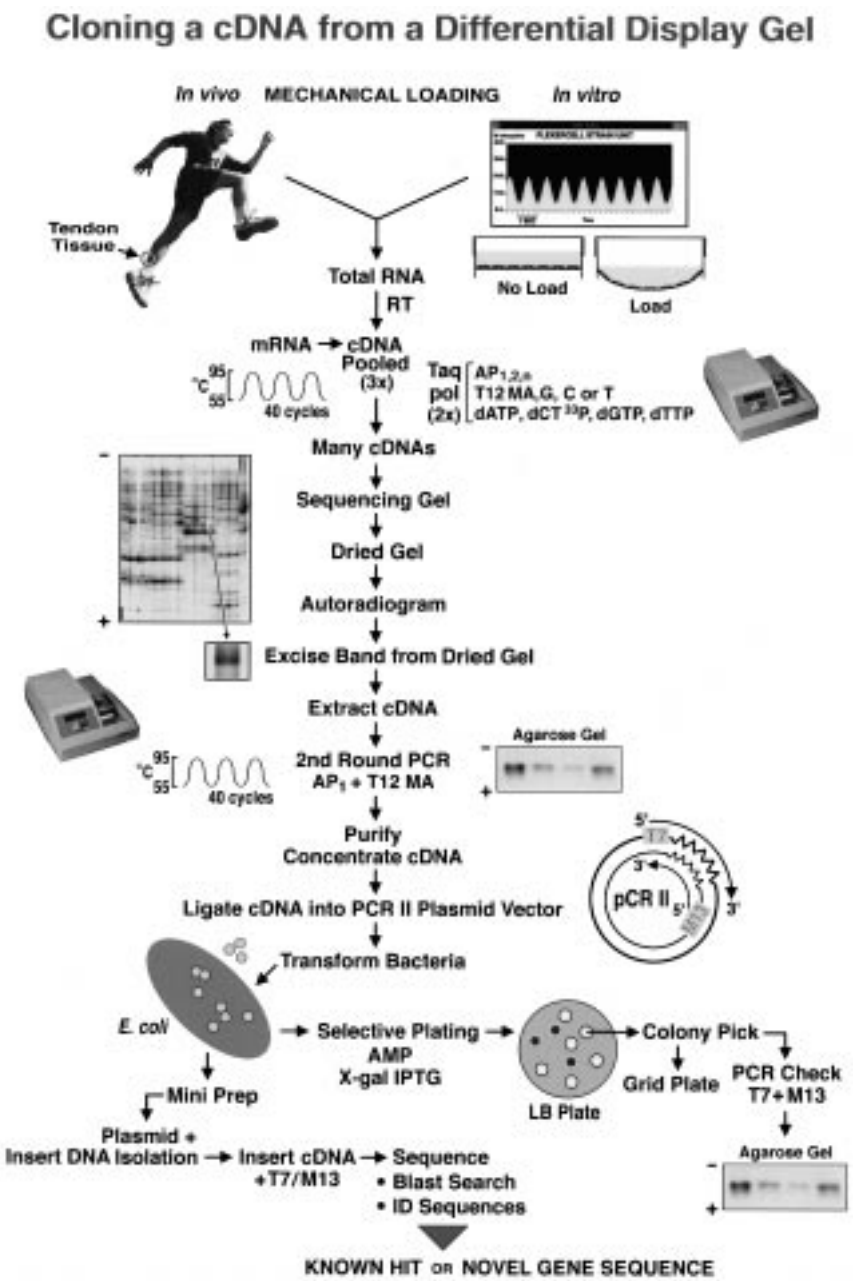

FIG. 1. Depicts the schematic showing how tendons or cells are processed to RNAs, messages converted to cDNAs, cloned, and sequenced, then compared to sequences registered in GenBank.

TREADMILL RUNNING OF ROOSTERS, TENDON CELL EXTRACTION AND DIFFERENTIAL DISPLAY

Nine-week-old roosters were exercised at 2–2.5 Hz for 1 h and 45 min on a treadmill then killed 4 h 15 min later (total 6 h). Flexor tendons were collected, then frozen, and mRNA was extracted for differential display analysis. Total RNA was collected from duplicate samples of tendon and muscle in GI buffer, and total RNA was purified after DNAse treatment and precipitation. Samples were reverse transcribed and subjected to differential display analysis using a GeneHunter kit and AP-1, AP-2, AP-3 or AP-4 and T12MA primers as above. Individual bands representing partial gene sequences were excised from the blot, amplified by PCR, T/A cloned and sequenced in both directions (Fig. 1).

Results

We have implemented a number of modifications to the differential display technique in order to reproducibly identify genes (known and unknown) which are differentially expressed in avian tendon,

a tissue which has historically proven resistant to molecular analysis (Fig. 1). It is now well-documented that one of the major shortcomings of the differential display technique is the poor reproducibility of banding patterns from experiment to experiment. We propose that the root of the problem with reproducibility in differential display begins at the level of cDNA synthesis and is exacerbated at each level of amplification. Different efficiencies in cDNA synthesis reactions can give rise to significantly different populations of template molecules for subsequent differential PCR amplification. To address this problem, in all of our experiments, cDNA syntheses were performed in triplicate, then reaction products were pooled, thereby creating a more homogeneous population of template material for subsequent amplification reactions. By pooling these reaction products, cDNAs, which are commonly represented (in high or low copy number) in each of the reactions are 'enriched' while effectively diluting those cDNAs which are not common to each cDNA reaction. Subsequent PCR amplification of this pooled cDNA material with various primer combinations was performed in duplicate reactions and separated side by side on sequencing gels. Bands which were differentially expressed in both duplicate reactions were subsequently excised for further analysis.

Figure 2 shows the level of reproducibility in duplicate reactions from this amplification strategy. Tendon TSC and TIF cell culture samples in either control or mechanical load series were separated on a sequencing gel after being subjected to the differential display strategy described above. The same samples were subjected to PCR amplification with 4 different arbitrary primer sets (AP-1, 2, 3 or 4). It is evident from Fig. 2 that the vast majority of cDNAs represented in these 4 reactions are equally expressed independent of cell type or mechanical stimulation. Furthermore bands are equally represented in duplicate lanes with a very low level of 'single bands' (present in one lane and not the duplicate). Bands 5a, 5b, 20 and 23 (AP-2 TSC L) are clear examples of cDNAs which appear to be load-induced and are represented in duplicate reactions from loaded TSC and TIF cells. In other instances an increase in apparent intensity of a band is less clear. For example, bands 11, 12 and 24 appear to have load-induced increases in expression even though there is significant background representation in the non-loaded samples. In fewer cases, cDNAs are down-regulated in response to mechanical stimulation. Bands 1, 2, 27 and 28 are examples of cDNAs whose band intensity decrease with load. While these apparent changes in mechanical load-induced gene expression are provocative, Northern blot analysis must be performed with the cloned PCR products to confirm the differential expression of these cDNAs.

To address the question of tissue specificity, differential display was performed on 10 normal avian tissues from 52 day-old White Leghorn chickens and that of cultured epitenon cells (TSC) and internal fibroblasts (TIF) with and without mechanical load (data not shown, see Tables). The mRNA display patterns of tendon and bone were most similar to those of cartilage and muscle indicating a level of conservation of gene expression in connective tissues. Significantly, the mRNA display pattern of cells in control 52 day-old normal primary avian tendon tissue shared similarities with expression of cultured cells. Ten bands that were differentially expressed in mechanically loaded TIF that were between 72–362 nucleotides in length were cloned and sequenced. Six novel sequences were identified (Table I). Four known sequences were found, one of which was a 72 nucleotide sequence homologous to the human mRNA KIAA0183 catalogued by the St Louis human genome project group (Table II). The second known sequence was a 214 nucleotide length sequence with high homology to avian cystatin, a cysteine proteinase inhibitor. A third sequence was that of cyclophilin, a DNA regulatory molecule and a fourth was pro-alpha collagen I.

The patterns of gene expression in growth factor and hormone-treated TSC and TIF with or without load were detected by differential display (TSC data not shown, Fig. 3). The autoradiogram in Fig. 3 shows the results of differential display following in-vitro loading of TIF cells. In addition we studied the synergistic effect of load with a spectrum of known modulators of biological function [platelet-derived growth factor (PDGF); insulin-like growth factor 1 (IGF-I); transforming growth factor beta1 (TGFβ1); parathyroid hormone (PTH(1-34)); β-estradiol and Prostaglandin E2 (PGE2)] (Table III).

The second bracket in the control lane shows bands which may represent cDNAs expressed more highly in the load than the non-load group. The lowest bracket shows bands which represent cDNAs which are expressed at a lower level in load than in non-loaded counterparts. Cells treated with PTH and load demonstrated regulated expression of a band at the middle of the gel in brackets (Fig. 3, band #11) which was cloned and identified as pro-alpha collagen type I (Table II). Several sequences were up- or down-regulated in β-estradiol-treated and loaded cells (in brackets).

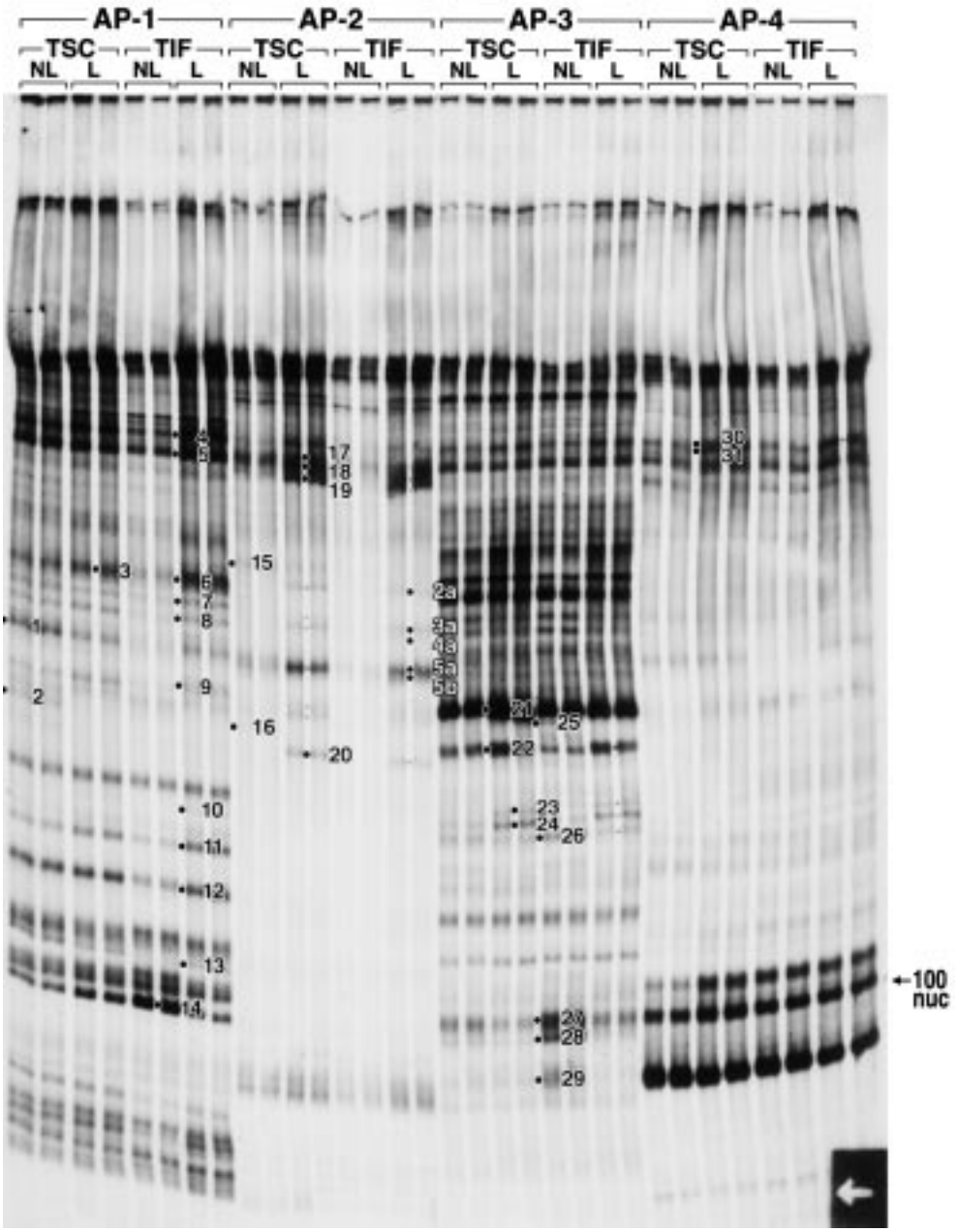

FIG. 2. Shows a differential display autoradiogram whose samples were amplified with 4 different arbitary primers (AP-1, 2, 3 and 4) and one poly A tail primer (T12-MA) using a GeneHunter Differential Display Kit. Thirty-one sequences were cloned from the gel by the outlined procedure. AP-1 and AP-3 in conjunction with T12MA yielded the most complex pattern of amplimers.

Only a few sequences were noticeably altered in cells treated with PGE_2 and load vs. PGE_2 alone. Notice that a whole sequence of bands was up-regulated in treated and loaded cells compared to non-loaded, non-treated cells (location of the bracket in control cells + load and just below the last highly expressed band in the control load bracket).

Multiple sequence differences were detected between exercised and control FDP tendon groups from trained roosters (Table IV). Five novel sequences were cloned comprised of 90, 134, 276, 330 and 383 base pairs. Muscles actuating the flexor tendons were taken from control and exercised groups and subjected to differential display analysis. In contrast, many sequences in muscle appeared to undergo moderate down-regulation in response to treadmill running (E25L load samples compared to control C2R non-loaded samples). One clone (C8, 383 bp) was enriched in tendon. Its message size appears in Fig. 4.

Table I
Known mRNAs expressed in isolated tendon epitenon, internal fibroblasts or whole tendon +/− mechanical load

Clone	bp	Protein	Increase
TIFL 5acl3#8	214	Cystatin	TIFL, TSCL
TIF L 4cl1-	345	Cyclophilin	TSC TIF
Tendon 1cl4	293	Serp I P. falciparum	Tendon
Tendon 1cl3	322	Titin	Tendon
Tendon 1cl4	249	Calmodulin	Tendon
TSCNL#16cl1	290	Proalpha type I collagen	TSC NL
TIFL PTH#11	223	Cardiac myosin light chain	TIF

(1) Autoradiogram 1-26-96, AP2, T12MA; Autoradiogram 1-31-96, AP1, T12MA; Autoradiogram 3-19-96; Autoradiogram 6-12-96, AP1, T12MA.
(2) Clone is the name given to the isolated clone containing the cDNA sequence; bp is size of the cloned fragment in base pairs; protein is the closest match to a known protein in GenBank; increase is an approximate amount by which the mRNA is increased based on density of the band in the differential display autoradiogram.
(3) SC is avian tendon surface epitenon cell; TIF is avian tendon internal fibroblast; mechanical load conditions are 1 hz, 0.05 average strain for 6 h *in vitro* using a Flexercell Strain Unit; NL is no mechanical load; L is mechanical load.

Table II
Novel mRNAs expressed in isolated avian tendon epitenon or internal fibroblasts +/− mechanical load

Clone	bp	Sequence/ Homology	Increase
TIFL 2acl3S1	257	ELK 1 protein	TIFL
TIFL 5bcl2S10	72	hmRNA KIAA0183 protein	TIFL
TIFL 3acl1S2	240	Novel	TIFL
TIFL 3acl3S3	219	Novel	TIFL
TIFL 5bcl7S12	220	Novel	TIFL
Tendon C8cl2	202	Novel	Tendon, spleen bone
Tendon C8cl4	372	Novel	Tendon, spleen bone
Tendon cl2	424	Novel	Avian tendon
TSCNL 1cl1	205	Novel	TSC, TIF
TIFNL 8cl2	362	Novel	TIFL
TSCNL 15cl2	187	Novel	TSCNL

(1) Autoradiogram 1-26-96, AP2, T12MA; Autoradiogram 1-31-96, AP1, T12MA; Autoradiogram 3-19-96; Autoradiogram 6-12-96, AP1, T12MA.
(2) Clone is the name given to the isolated clone containing the cDNA sequence; bp is size of the cloned fragment in base pairs; protein is the closest match to a known protein in GenBank; increase is an approximate amount by which the mRNA is increased based on density of the band in the differential display autoradiogram.
(3) SC is avian tendon surface epitenon cell; TIF is avian tendon internal fibroblast; mechanical load conditions are 1 hz, 0.05 average strain for 6 h *in vitro* using a Flexercell Strain Unit; NL is no mechanical load; L is mechanical load.

Discussion

The differential display technique is a powerful tool to study the expression of messages that are produced in either high or low levels [32]. About 50–100 mRNAs can be amplified given the correct set of PCR primers. This is a convenient number of cDNA amplimers given that the discrimination of an average sequencing gel is limited to about this number of bands. Generally, a typical experiment with several different primer combinations will yield amplimers from 1–2 kb down to less than 100 nucleotides in length. As seen in Fig. 2, duplicate PCR reactions of tendon cell mRNA result in good reproducibility from sample to sample with little lane to lane variability. Others have shown that 95% reproducibility is possible [32]. Detection of rare messages is possible with starting material in abundance as low as 30 copies per cell. Our contribution to the differential display technique is that of a strategy to pool starting RNAs from multiple control or treatment groups, pool reverse transcription products to minimize artifacts that might

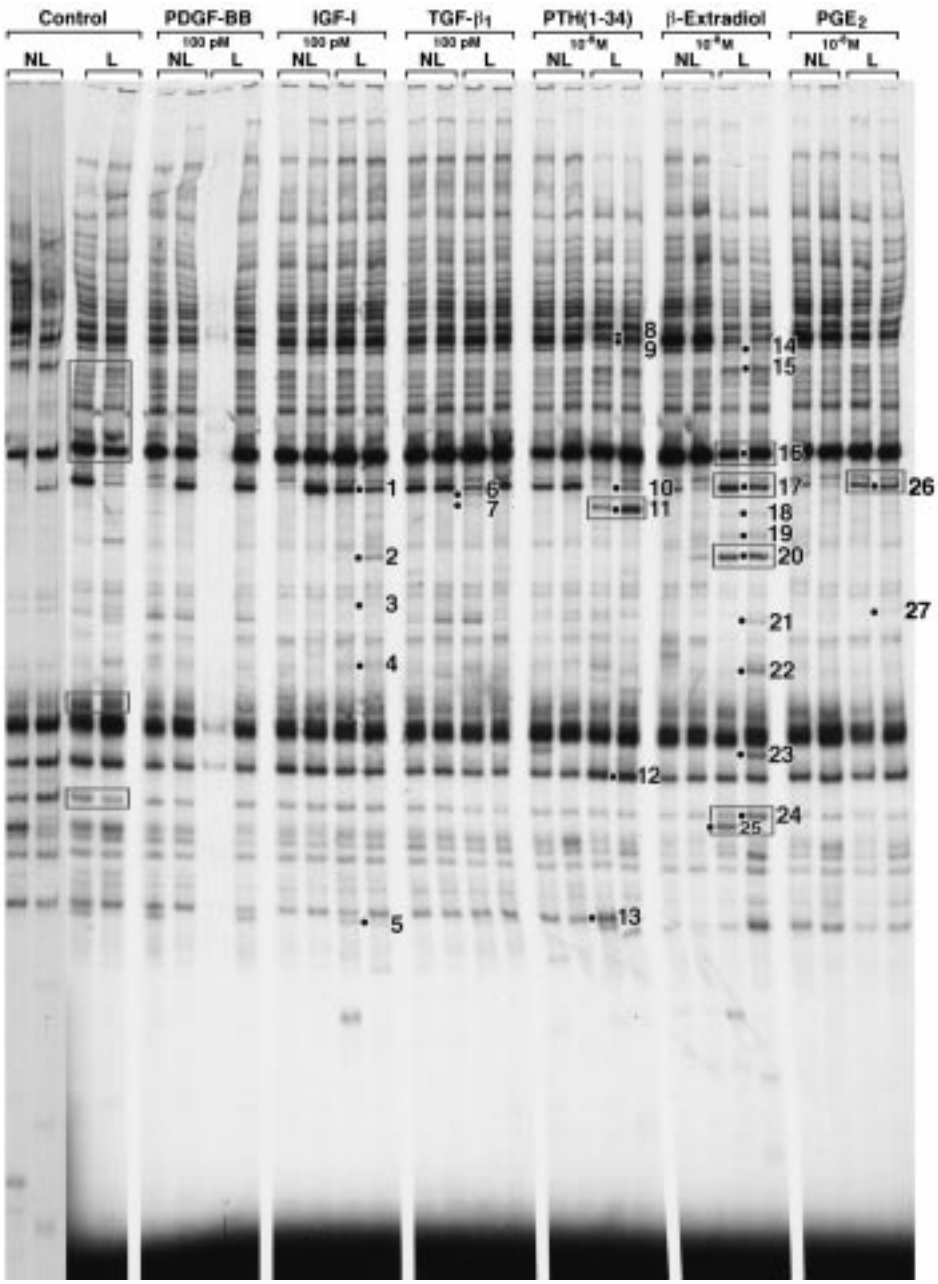

FIG. 3. Shows the differential display autoradiogram of avian tendon cell (TIF) message expression with and without PDGF-BB, IGF-I, TGF-b1, PTH, estrogen or PGE2 with and without 6 h of 1 hz 0.05 average strain as a mechanical load regimen. Twenty-seven clones were sequenced from this gel. These are the first data showing growth factor and hormone sensitivity of tendon cells with load.

be created by over- or under-amplification of any single mRNA, and pooling reaction product cDNAs thereafter in the differential display PCR reactions. The last 'filter' consisted of cloning sequences from bands that were represented equally in duplicate samples run on the differential display gel. This strategy of sample pooling at several steps along the way yielded a more homogeneous pattern and cloning result than is usually reported for this technique. Moreover, this strategy made it more likely that similarities and differences might be detected between cultured cells and cells from tissue *in vivo* than might otherwise be possible.

A goal of the current study was to identify potential marker genes for tendon cells since there are no accepted markers published for tendon cells to date. Our lab has reported that surface epitenon cells express an abundance of fibronectin (FN) whereas internal fibroblasts do not, and it has been suggested that FN might be a nonspecific marker for epitenon cells [5]. However, we wanted

Table III
Known and novel mRNAs expressed in growth factor and hormone-treated, isolated tendon cells +/− mechanical load

Clone	bp	Sequence/ Homology	Increase	Decrease
TFL estrogen S17	239	Novel	TIFL	—
TIFL estrogen cl1S20	197	Novel	TIFL	—
TIFL estrogen cl1S22	150	Novel	TIFL	—
TIFL estrogen cl2S22	155	Novel	TIFL	—
TSCL PDGF-BB S27	258	Novel	TSCL	—
TSCL PDGF-BB S29	229	Novel	TSCL	—
TSCL PDGF-BB S30	209	Novel	TSCL	—
TSCL PDGF-BB S30	209	Novel	TSCL	—
TIFL IGF-I S2	197	Novel	TIFL	—
TSCL PDGF-BB S31	173	Novel	TSCL	—
TSCL PDGF-BB S32	89	Novel	TSCL	—
TIFL estrogen S16	255	Novel		TSCL

(1) Autoradiogram 6-12-96, AP1, T12MA.
(2) Clone is the name given to the isolated clone containing the cDNA sequence; bp is size of the cloned fragment in base pairs; protein is the closest match to a known protein in GenBank; increase is an approximate amount by which the mRNA is increased based on density of the band in the differential display autoradiogram.
(3) SC is avian tendon surface epitenon cell; TIF is avian tendon internal fibroblast; mechanical load conditions are 1 hz, 0.05 average strain for 6 h *in vitro* using a Flexercell Strain Unit; NL is no mechanical load; L is mechanical load.

Table IV
Novel mRNAs expressed in flexor digitorum profundus tendons from control and exercised chickens

Clone	bp	Sequence/ Homology	Increase	Decrease
FLEXTENCPL S33	276	Novel	—	Tendon
FLEXTENCPL S33	330	Novel	—	Tendon
FLEXTENCPL S39	90	Novel	Tendon load	—
FLEXTENCPL S38	134	Novel	Tendon load	—
FLEXTENL SC8	182	Novel	Tendon, bone, spleen	—

(1) Autoradiogram 6-13-96, AP1, T12MA.
(2) Clone is the name given to the isolated clone containing the cDNA sequence; bp is size of the cloned fragment in base pairs; protein is the closest match to a known protein in GenBank; increase is an approximate amount by which the mRNA is increased based on density of the band in the differential display autoradiogram.
(3) SC is avian tendon surface epitenon cell; TIF is avian tendon internal fibroblast; mechanical load conditions are 1 hz, 0.05 average strain for 6 h *in vitro* using a Flexercell Strain Unit; NL is no mechanical load; L is mechanical load.

to identify markers that are specific to each principal tendon cell type (epitenon vs. internal fibroblast cell) in addition to markers expressed by cells that were mechanically loaded. Lastly, we searched for unique messages expressed by tendon cells that were treated with growth factors or hormones with and without load. We found that cultured tendon epitenon and internal fibroblasts retained multiple similarities in their mRNA phenotypic expression compared to that of cells in resting, normal avian tendon. However, the differences in patterns between cultured cells and whole tendon may be attributable to the fact that RNA from whole flexor tendons reflect a complex mixture of all cell types in tendon at the 52 day-old period whereas isolated cells represent more pure cell populations in a quiescent state.

Some genes whose sequence was known but whose expression in tendon had not been previously reported were identified in whole tendon and/or in cultured cells (Table I). Titin, also known as connectin, is the largest protein reported to date. A titin-like sequence was cloned several times independently from whole tendon. Titin is a 3000 kDa protein that links the Z line to the myosin filament in striated muscle sarcomeres [35]. It is an elastic protein usually found in striated skeletal muscle, whose role may be as a shock cord to keep sarcomeres from pulling apart. There have been few reports of this protein detected in

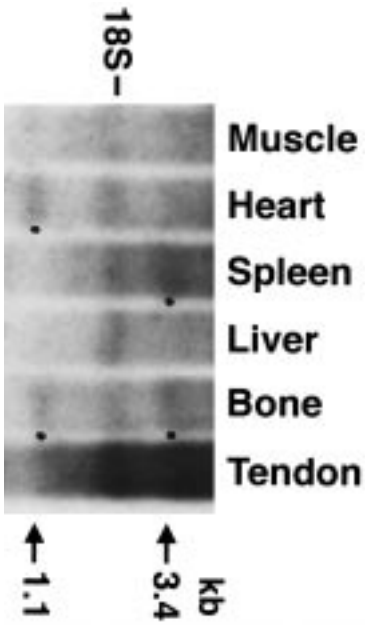

FIG. 4. Shows a Northern blot of 1.1 and 3.4 kb messages with which the C8 probe hybridized. A 383 bp probe that was cloned into a P. gem plasmid propagated in *E. coli* was used to hybridize to the unknown message. This message was expressed to a high degree in tendon and to a lesser extent in bone and spleen. The 3.4 kb band may be a splice variant of the 1.1 kb band.

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          10         20         30         40         50
5' TAGCCAGCG AAACGATG TT AATACTTTC TGGTAATGCA TTTCTGGTTT
          60         70         80         90        100
AAATAAATTA AGGATGTTTT CTAGTTGTGC ATGAATGCAG ACAACTTAGT
         110        120        130        140        150
AAGTTTTGAC AAATGTTTAA ATGTGTAATG TAAGCAAAAG GTTAACAAAA
         160        170        180        190        200
GTGAAGCCAG TAAGCTGGGT CTTTTGTCAT TTGC TAAAAA AAAAAATTAA
         210        220        230        240        250
TTCAAAATGA ATAAATGCTG ATGTTTGTGG CTGTTTTCAT AAATATCACC
         260        270        280        290        300
TTTAGGTTTT GTTTAACTTT TGGTTACTGC AAATTAGGAC AACTTTTTTC
         310        320        330        340        350
TTTTTTTACT TTTTTCTTTT TTCTTTTTTT TTTAATTATT TTTTTYTATG
         360        370
TAGATTATTC TGTTTTCGCT GGCTA -3'
```

FIG. 5. Shows the sequence of clone C8, a novel gene sequence enriched in tendon.

non-muscle cells [36–38]. Its presence in tendon could serve to prevent cell syncytia from pulling apart under dynamic loads during repetitive motion.

Cyclophilins A, B and C include a class of nucleases that have homology to an apoptotic nuclease (NUC18) [39, 40]. These proteins might be induced in response to excessive mechanical load to stimulate apoptosis in specific cells to create more space in tendon or release tension in a syncytium. Cyclophilins have also been implicated in recruiting leukocytes to an area of inflammation [41]. Cyclophilin A has been identified in the synovial fluid of patients with rheumatoid arthritis. Excessive load could induce cell death via cyclophilins and apoptosis. This does not appear to be the case for the bulk of cells in tendon cell cultures subjected to cyclic load since these cells flourish when subjected to cyclic stretching and divide when given growth factors and load.

Cystatin represents a class of cysteine protease inhibitors that counteract the destructive action of cysteine proteases [42]. It is thought that excessive mechanical loading can lead to a syndrome known as repetitive motion disorder with accompanying matrix degradation. If mechanical loading induces cysteine protease activity which could result in matrix degradation, then at the same time, load-induced cystatin could reduce or inhibit the potential destructive effects and spare tendon. Cystatin expression could act as a modulator of degradation in tendons that are subjected to repetitive motion.

Calmodulin is an important calcium binding protein that in turn binds to other proteins, such as enzymes, to affect their activities. Earlier studies with stretching cells and resolution of proteins in two dimensional gels had indicated that calmodulin protein expression increased after 5 days of mechanical loading of tendon cells (Banes, unpublished data). Activated calmodulin can also bind to myosin light chain kinase, another protein whose message was up-regulated by load.

Collagen I, the principal component of tendon matrix, was also up-regulated by load as was Serp I, a transcription factor.

At least 23 additional cDNAs were cloned and sequenced from TSC or TIF and identified as potentially novel components due to a lack of an identifiable counterpart in GenBank. Some of these sequences were specific to loaded TIF and some to loaded TSC. These bands are numbered in Figs 2 and 3 and appear in Tables II, III and IV. Five additional novel sequences were identified from tendon or muscle of exercised chickens (Fig. 3, Table IV, Fig. 5).

The loading regimen chosen was brief, involving only 6 hours of cyclic tension at 1 Hz and 5% average elongation (0.05 strain). The experiments performed on live chickens involved a similar strain regimen. We found that one hour and 45 minutes of exercise over a period of 6 h was sufficient to induce differential gene expression in cells from whole tendon in trained roosters compared with unexercised control birds. Moderate exercise induced dramatic gene expression changes in flexor tendons of adult chickens. Overall, brief exercise or mechanical loading to tendon cells resulted in a dramatic change in gene expression. *In vitro*, TSC responded differently than TIF did.

Treatment of cells with growth factors also elicited dramatic changes in gene expression. PDGF-BB produced the most dramatic changes in either TSC or TIF. However, IGF-I did not evoke as dramatic a change in expression as did load alone in either cell type. Surprisingly, TGF-β1 evoked the least profound change of any treatment in either cell type.

Treatment of TIF with parathyroid hormone (PTH 1-34) induced some change in loaded cells. Band 11 in Fig. 3 is pro-alpha 1 type I collagen. Many changes were induced by treatment of cells with estrogen and load with many novel sequences identified. Lastly, several novel sequences were identified in prostaglandin E_2-treated cells.

These results speak to the power of the differential display technique to reveal known and novel sequences expressed under a variety of conditions. The differential display technique is faster and more efficient than subtractive hybridization for this purpose because differential display requires far less sample (0.1 μg total RNA per reaction), is quick (about 7 days to sequence determination) and costs less. However, one needs to generate about 20–30 potentially novel sequences in order to identify one to five sequences that are truly novel, are specific and highly expressed in a given cell or tissue of interest. At this time, we have many candidate genes for markers for either tendon epitenon cells or internal fibroblasts as well as for cells subjected to load and/or growth factor and hormone treatment. We expect to identify such highly expressed messages and to utilize these to select conditions that allow us to culture tendon cells in a tissue engineered construct that simulates an in-vivo tendon. We also hope to use tendon cell markers to stage tendon disease such as those involving overuse, disuse and synovitis.

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References

1. Merrilees MJ, Flint MH. Ultrastructural study of tension and pressure zones in a rabbit flexor tendon. Amer J Anat 1980;157:87–106.
2. Oakes BW, Bialkower B. Biomechanical and ultrastructural studies on the elastic wing tendon from the domestic fowl. J Anat 1977;123:369–87.
3. Vogel K, Evanko SP. Proteoglycans of fetal bovine tendon. Trans Orthop Res Soc 1988;13:182.
4. Banes AJ, Donlon K, Link GW *et al.* A simplified method for isolation of tendon synovial cells and internal fibroblasts: Conformation of origin and biological properties. J Orthoped Res 1988;6:83–94.
5. Banes AJ, Link GW, Bevin AG *et al.* Tendon synovial cells secrete fibronectin in vivo and in vitro. J Orthoped Res 1988;6:3–82.
6. Tsuzaki M, Yamauchi M, Banes AJ. Tendon collagens: Extracellular matrix composition in shear stress and tensile components of flexor tendons. Connect Tissue Res 1993;29:141–52.
7. Brigman BE, Yin H, Tsuzaki M, Lawrence WT, Banes AJ. Fibronectin in the tendon-synovial complex: Quantitation in vivo and in vitro by ELISA and relative mRNA levels by polymerase chain reaction and northern blot. J Orth Res 1994;12:253–61.
8. Greenlee TK, Ross R. The development of rat flexor digital tendon, a fine structure study. J Ultrastructure Research 1967;18:354–76.
9. Rowe RWD. The structure of rat tail tendon fasicles. Conn T Res 1985b;14:21–30.
10. Rowe RWD. The structure of the rat tail tendon. Conn T Res 1985a;14:9–20.
11. Riederer-Henderson MA, Gauger A, Olson L, Robertson C, Greenlee TK Jr. Attachment and extracellular matrix differences between tendon and synovial fibroblastic cells. In Vitro 1983;19:127–33.
12. Clemmons DR. Insulin-like growth factor binding protein control secretion and mechanisms of action. Adv Exp Med Biol 1991;293:113–23.
13. Gelberman RH et al. Flexor tendon healing and restoration of the gliding surface. An ultrastructural study in dogs. J Bone Joint Surg [Am] 1983;65:70–80.
14. Gelberman RH, Steinberg D, Amiel D, Akeson W. Fibroblast chemotaxis after tendon repair. J Hand Surg 1991;16:686–93.
15. Hughes FJ, Aubin JE, Hersche JNM. Differential chemotactic responses of different population of fetal rat calvaria cells to platelet-derived growth factor and transforming growth factor. Bone and Mineral 1992;19:63–74.
16. Banes AJ, Hu P, Xiao H *et al.* Tendon cells of the epitenon and internal tendon compartment communicate mechanical signals through gap junctions and respond differentially to mechanical load and growth factors. AAOS Repetitive Motion Meeting, Bethesda, MD, June 1994.
17. Banes AJ, Sanderson M, Boitano S *et al.* Mechanical load ± growth factors induce [Ca2+]i release, cyclin D1 expression and DNA synthesis in avian tendon cells. In: Van C Mow, Guilak F, Tran Son Tay R, Hochmuth R, Eds. Cell Mechanics and Cellular Engineering. Springer Verlag, New York, Inc.; 1994. 210–32.
18. McNielly C, Benjamin M, Banes AJ, Ralphs J. Immunochemical localization of multiple connexins in flexor tendons. J Anatomy 1996;189:593–600.

19. Abrahams M. Mechanical behavior of tendons in vitro. A preliminary report. Med Biol Engin 1967;5:433–43.
20. Herrick WC, Kingsbury HB, Lou DYS. A study of the normal range of strain, strain rate and stiffness of tendon. J Biomed Matls Res 1978;12:877–94.
21. Archambault JM, Wiley JP, Bray RC. Exercise loading of tendons and the development of overuse injuries. Sports Med 1995;20 [2]:77–89.
22. Banes AJ, Gilbert J, Taylor D, Monbureau O. A new vacuum-operated stress-providing instrument that applies static or variable duration cyclic tension or compression to cells in vitro. J Cell Sci 1985;75:35–42.
23. Banes AJ, Horesovsky G, Tsuzaki M *et al*. Connexin 43 is mechanosensitive in avian flexor tendon cells. In: Caterson B, Archer C, Benjamin M, Ralphs J, Eds. The Biology of the Synovial Joint. 1996. in press.
24. Banes AJ, Tsuzaki M, Hu P, Brigman B, Brown T, Almekinders L, Lawrence WT, Fischer T. Cyclic mechanical load and growth factors stimulate DNA synthesis in avian tendon cells. Special Issue on Cytomechanics in J Biomechanics 1995;28:1505–13.
25. Pederson DR, Bottlang M, Brown TD, Banes A. Hyperelastic constitutive properties of polydimethylsiloxane cell culture membranes. BED Ad Bioengin ASME 1993;26:607–9.
26. Pederson DR, Brown TD, Banes AJ. Mechanical behavior of a new substratum for strain-induced cell culture experiments. Proc NACOB II, Second North American Congress on Biomechanics 1992; 355–6.
27. Gilbert JA, Banes AJ, Link GW, Jones GJ. Surface strain of living cells in a mechanically active in vitro environment. ANSYS, pp. 13.2–13.7, ANSYS Conf Proc, ed. David E. Dietrich, Swanson Analysis Systems, Houston, PA, 1989.
28. Gilbert JA, Banes AJ, Link GW, Jones GL. Video analysis of membrane strain: An application in cell stretching. Exp Tech 1990;Sept/Oct:33–45.
29. Gilbert JA, Banes AJ, Weinhold PS, Link GW, Jones GL. Strain profiles for circular plates and membranes employed to mechanically deform cells in vitro. J Biomechanics 1994;27, #9:1169–77.
30. Banes AJ, Tsuzaki M, Yamamoto J, Fischer T, Brown TD, Miller L. Mechanoreception at the cellular level: The detection, interpretation and diversity of responses to mechanical signals. Special Issue on Cytomechanics, Biochemistry and Cell Biology 1995;73:349–65.
31. Gilman M. Preparation of cytoplasmic RNA from tissue culture cells. In: Ausubel FM, et al., Ed. Current protocols in molecular biology. NY: Greene. pp. 4.1.2–4.1.6.
32. Liang P, Pardee A. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science 1992;257:967–71.
33. Altschul SF, Gish W, Miller W, Myers E, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990;215:403–10.
34. Chirgwin JM, Pryzbyla AE, MacDonald RJ, Rutter WJ. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 1979;18:5294–9.
35. Maruyama K. Connectin/titin, giant elastic protein of muscle. FASEB J 1997;11:341–5.
36. Eilertson KJ, Keller TC. Identification and characterization of two huge protein components of the brush border cytoskeleton: evidence from a cellular isoform of titin. J Cell Bio 1992;119:549–57.
37. Schaart G, Pieper FR, Kuijpers HJ, Bloemendal H, Ramaekers FC. Baby hamster kidney [BHK-21/C13] cells can express striated muscle type proteins. Differentiation 1991;26 [2]:105–15.
38. Mencarelli C, Magi B, Marzocchi B, Armellini D, Pallini V. Evolution of the ''titin epitope'' in neurofilament proteins. Comp Biochem Physiol 1991;100 [4]:741–4.
39. Montague J, Gaido M, Frye C, Cidlowski J. A calcium-dependent nuclease from apoptotic rat thymocytes is homologous with cyclophilin. J Bio Chem 1994;269:18 877–80.
40. Montague JW, Hughes FM, Cidlowski JA. Native recombinant cyclophilins A, B and C degrade DNA independently of peptidylprolyl cis-trans-isomerase activity. Potential roles of cyclophilins in apoptosis. J Bio Chem 1997;272 [10]:6677–84.
41. Billich A, Winkler G, Aschauer H, Rot A, Peichl P. Presence of cyclophilin A in synovial fluids of patients with rheumatoid arthritis. J Exp Med 1997;185 [5]:975–80.
42. Henskens YM, van de Keijbus PA, Veerman EC et al. Protein composition of whole and parotid saliva in healthy and periodontitis subjects. Determination of cystatins, albumin, amylase and IgA. J Perio/Res. 1996;31 [1]:57–65.

Appendix A

ABBREVIATIONS

A	Adenosine
$A_{260/280\ nm}$	Absorbance at 260 and 280 nanometers
AP-1, 2, 3, 4	Arbitrary primers 1, 2, 3 and 4
BLAST	'Basic Local Alignment Search Tool' software program to perform data-base searching of nucleotide or protein sequence comparisons to known sequences
C	Cytidine
cDNA	Complementary deoxyribose nucleic acid
cx32	Connexin 32
cx43	Connexin 43
DEPC	Diethylpyrocarbonate
DMEM-M	Dulbecco's Minimal Essential Medium with high glucose
DNA	Deoxyribose nucleic acid
Dnase I	Deoxyribonuclease I enzyme
ELIZA	Enzyme linked immuno assay
FDP	Flexor digitorum profundus tendon
G	Guanosine
G_0	Stop point in the G_1 (first) growth phase of the cell cycle

GI	Guanidine isothiocyanate
GSA	Growth surface area
HEPES	N-2 Hydroxyethylpiperazine-N-2-Ethane Sulfonic Acid
IGF-I	Insulin-like growth factor-I
IGF-Lp3	Insulin-like growth factor binding protein 3
IGF-Lp5	Insulin-like growth factor binding protein 5
M	Degenerate base
M13	M1 phage promoter sequence
MMLV	Moloney murine leukemia virus
mRNA	Messenger ribose nucleic acid
N_2	Nitrogen
NUC 18	Apoptotic nuclease
OLIGO (dT)	Polymer of deoxythymidylate attached to beads to hybridize Poly (A+)mRNA
^{33}P-d CTP	Phosphorus 33 labeled deoxycytidine triphosphate
PC	Phenol chloroform
PCR	Polymerase chain reaction
PCR™ II	Proprietary cloning designation for a plasmid vector
PDGF-BB	Platelet-derived growth factor-BB homodimer
PGE_2	Prostaglandin E_2
PTH	Parathyroid hormone
Poly $(A)^+$	Polyadenylate polymer sequence at the 3′ end of mRNA
Poly T	Poly-thymidylate polymer
RnA	Ribose nucleic acid
RW1	First wash buffer
SDS	Sodium lauryl sulfate
SSC	Saline sodium citrate
Sec	Seconds
T	Thymidine
TIF	Tendon internal fibroblasts
TSC	Tendon surface cells from epitenon
T7	T7 phage promoter sequence
T12-MA	Oligonucleotide primer comprised of 12 thymidylate nucleotides linked to a 5′ terminal deoxyadenylate residue
T12-MC	Oligonucleotide primer comprised of 12 thymidylate nucleotides linked to a 5′ terminal deoxycytodylate residue
T12-MG	Oligonucleotide primer comprised of 12 thymidylate nucleotides linked to a 5′ terminal deoxyguanylate residue
T12-MT	Oligonucleotide primer comprised of 12 thymidylate nucleotides linked to a 5′ terminal deoxythymidylate residue

Appendix B

SYMBOLS

µg	microgram mass designation
cm2	centimeters squared
l	microliter volume designation
× g	times gravity
× k	times thousand
c	degrees Centigrade
g	gram
Ci	Curie
µmol	millimole
h	hour