

Mechanical force modulates scleraxis expression in bioartificial tendons

A. Scott¹, P. Danielson², T. Abraham³, G. Fong¹, A.V. Sampaio⁴, T.M. Underhill⁴

¹Department of Physical Therapy, University of British Columbia, and Centre for Hip Health and Mobility, Vancouver Coastal Health Research Institute, Vancouver, British Columbia; ²Dept. of Integrative Medical Biology, Anatomy, Umeå University, Umeå, Sweden; ³James Hogg Research Centre-St Paul's Hospital, Vancouver; ⁴Department of Cellular and Physiological Sciences, University of British Columbia

Abstract

Following tendon injury, cartilage, bone and fat metaplasia are often observed, making the optimization of tenocyte differentiation an important clinical goal. In this study we examined the effect of static and cyclic mechanical loading on the expression of genes which play a role in tenocyte differentiation and function, namely scleraxis (Scx) and Type I collagen (Col1a1), and determined the effect of varying mechanical parameters including (1) static vs dynamic load, (2) increasing strain magnitude, (3) inclusion of 10 s rest periods, and (4) increasing cycle number. Cyclic loading resulted in a greater increase of tenocyte gene expression than static loading over 3 weeks in culture. Increasing strain levels potentiated the induction of tenocyte genes. The insertion of a 10 s rest periods further enhanced tenocyte gene expression, as did increasing repetition numbers. These results suggest that mechanical signaling exerts an important influence on the expression of genes which play a role in determining the tendon phenotype. Further work is required to confirm and extend these findings in primary cells such as resident tendon progenitor/stem cells, in order to provide an improved understanding of biology from which optimized rehabilitation programs can be developed.

Keywords: Tenocyte, Mechanotransduction, Tendinopathy

Introduction

Tendon is a dynamic tissue which transmits tensile loads between muscle and bone, thus allowing joint movement¹. It is populated by tenocytes, a specialized, mechanosensitive cell type organized into gap junction-linked arrays². Like osteoblasts, tenocytes are responsible for synthesizing and maintaining a biomechanically competent extracellular matrix, and for mounting an adaptive response to periods of increased loading (i.e. exercise)^{3,4}. Following tendon injury, cartilage, bone and fat metaplasia are often observed, making the optimization of tenocyte differentiation an important clinical goal^{5,6}.

Scleraxis (SCX) is a DNA-binding transcription factor which is present in tendon from the condensation stage and

persists into adulthood⁷. SCX forms heterodimers with NFAT-C (Nuclear factor of activated T-cells, cytoplasmic) and directly regulates transcription of the gene for Type 1 collagen (Col1a1), which encodes the most abundant protein in tendon⁸. Thus, SCX is a key regulator of tenocyte differentiation, and its expression in tenocytes is strongly induced by TGF-beta signaling⁹. Tgfb and Scx knock-outs demonstrate significantly impaired formation of load-bearing tendons⁹.

Mechanical signaling plays an important role in tendon physiology¹⁰. In paralyzed embryos, tendon formation occurs but is markedly inhibited¹¹. During development and in adults, long-term exercise leads to stronger tendons, whereas immobility or injury leads to tendon weakening and metaplasia^{12,13}. Given its apparent role in regulating the tendon phenotype, Scx is a likely candidate for load-induced gene regulation¹⁴. A previous study found that cyclic stretch increased the mRNA levels of Scx, tenascin C, and Types I and III collagen in rat bone marrow-derived mesenchymal stem cells, but the effect of varying mechanical parameters was not specifically examined¹⁵. Zhang and Wang¹⁶ subjected rabbit tendon cells grown in grooved 2D cultures to 4% or 8% strain for 12 hours and found only modest increases in tendon-related genes (Scx was not examined), attesting to the need for further optimization of mechanical loading regimens.

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Corresponding author: Alexander Scott, Department of Physical Therapy, 212-2177 Wesbrook Mall, Vancouver, BC, V6T 1Z3, Canada

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Abbreviations

NFAT-C: Nuclear factor of activated T-cells, cytoplasmic
 TGF-beta: Transforming growth factor beta
 GFP: Green fluorescent protein
 BAT: Bioartificial tendon
 SCX: scleraxis
 COL1a1: Type 1 collagen

In this study we examined the effect of three-dimensional static and cyclic mechanical loading on *in vitro* mRNA levels of key genes related to the tendon phenotype Scx and Type I collagen (Col1a1) in a mesenchymal cell line (C3H10T1/2 cells), and determined the effect of varying mechanical parameters including, (1) static vs dynamic load, (2) increasing strain magnitude, (3) inclusion of 10s rest periods, and (4) increasing cycle number.

Methods

Cell culture

A multi-potent mesenchymal cell line (C3H10T1/2) was obtained from the American Type Culture Collection. Originally derived from embryonic mouse limb bud cultures, this cell line is well characterized for its ability to undergo differentiation into adipocyte, chondrocyte and osteoblast lineages, however there are few published references to their ability to form tendon¹⁷. Primary tenocytes were obtained from the tail tendons of adult (8-10 week old) heterozygous transgenic mice expressing green fluorescent protein (GFP) under the control of the Scx promoter⁷. Cells were grown at 37°C in a CO₂ incubator on standard Sarstedt (Montreal) tissue culture plates in DMEM containing 10% fetal bovine serum, 0.5 mM glutamine, 25 units/ml penicillin and 25 µg/ml streptomycin and passaged 1:5 when confluent using 0.25% trypsin-EDTA. Frozen cell stocks were maintained at -80°C.

Preparation of bioartificial tendons

Bioartificial tendons (BATs) were prepared to allow the application of dynamic mechanical loads to cells in a 3D collagen environment¹⁸. Subconfluent cells were detached in 1 ml of trypsin for 10 minutes, diluted with Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum, passed through a 70 µm filter to exclude cell clumps, and counted with a hemocytometer. 5 x 10⁶ cells were centrifuged at 300 rcf for 7 minutes and resuspended in 5 ml of Type I collagen solution consisting of 3.5 ml Purecol (Sigma-Aldrich, Oakville, Canada), 1 ml of 5 x DMEM, and 0.5 ml fetal bovine serum, brought to 7.4 pH with 0.1 N NaOH immediately prior to use. 150 µl of cell-collagen mixture was pipetted into each well of an untreated TissueTrain[®] plate (FlexCell International, Hillsborough NC) according to the manufacturer's instructions, yielding enough for four 6- well plates to be run in parallel for each experiment. The gel was allowed to set for 2 hours, and the resulting BATs were covered with 2 ml of Dul-

becco's Modified Eagle Medium containing either 1% or 10% fetal bovine serum and 0.25 mM ascorbic acid. In this system, cells develop static tension over the ensuing 48 h as they condense the surrounding matrix.

Mechanical loading regimes

After 48 hours, BATs were either maintained under static load conditions (i.e. no superimposed strain), or subjected to superimposed, cyclic mechanical strain in which single variables were altered in successive iterations (duration, strain level, rest insertion, cycle number). Studies were conducted with multiple replicates, and each study was conducted at least twice. For all studies, we used a frequency of 0.1 Hz in order to minimize the risk of detaching the BATs from their tethers, and because this is similar to the frequency used clinically in tendinopathy rehabilitation emphasizing slow, heavy loading. First, BATs were subjected to static load vs cyclic load (5%, 2 hrs/day) for 1, 2 or 3 weeks. Next, BATs were subjected to strains of varying magnitude (0, 2.5%, 5%, 7.5% or 10%, 2 hours/day for 2 weeks). We then examined the effect of inserting a 10s rest period, while keeping all other parameters constant. (10% strain, 100 cycles, daily for 2 weeks). Finally, the optimized protocol (10% strain with 10s rest periods) was varied by changing the number of repetitions (10, 100, 1000).

Microscopy and image analysis

BATs were removed from the CO₂ incubator and scanned digitally at 1x magnification (EPSON 4990, Toronto) at the start and conclusion of the culture periods. BAT width was measured using the calibrated measuring tool of an imaging software program (Photoshop CS2, Adobe, San Jose CA). BATs were harvested for cryosectioning by detaching from their tethers with scissors, laid flat in a plastic cryomold, covered with Optimal cutting temperature medium (Thermo Scientific, Toronto), frozen on dry ice and stored at -80°C. Cryosections were cut at 10 microns, stained with haematoxylin and eosin, dehydrated and coverslipped with permanent mounting medium. Images were obtained with a digital camera (Retiga EXi, qImaging, Surrey, Canada) attached to a microscope (Carl Zeiss Canada, Toronto). Other BATs were examined in their entirety by detaching from their tethers, rinsing with phosphate buffered saline, fixing in 3.7% paraformaldehyde, permeabilizing in 0.1% Triton, blocking in 1% bovine serum albumin, and labeling with Alexa Fluor 488 phalloidin (Invitrogen, Burlington, Canada) (stains actin). BATs were then placed between two cover slips and examined using multiphoton excitation fluorescence and second harmonic generation as described previously¹⁹. To confirm the expression pattern of GFP in adult transgenic mice, patellar tendons were dissected free, fixed for 1 hr in 2% paraformaldehyde in phosphate buffered saline and examined as above.

RNA isolation and qPCR

Total RNA was extracted from BATs at the indicated time or from musculoskeletal tissues of the adult CD-1 mice (8-10 weeks age), using the TRIzol[®] reagent RNA extraction method according to manufacturer's instructions (Gibco BRL, Life Technologies), after homogenizing tissue in a grinding mill (Mikrodismembrator, Sartorius, Mississauga, Canada). RNA concentration and purity

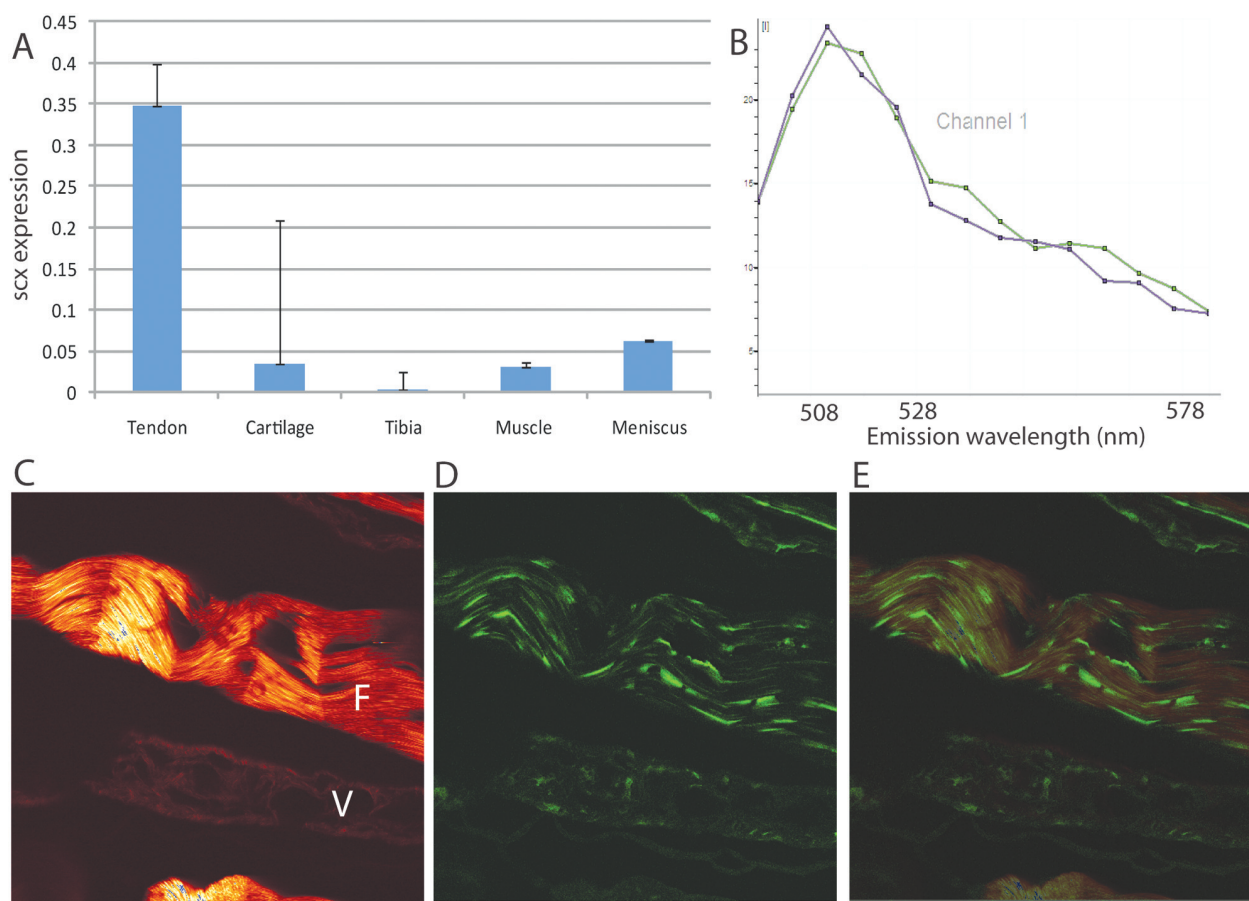


Figure 1. Scx expression in adult tendon. (A) Tendon exhibited the highest expression of Scx among adult musculoskeletal tissues. The same pattern of expression was seen for Colla1 (not shown); n=6 mice. (B) Spectral scanning confirmed the specificity of GFP emission profile from fluorescent cells in Scx-gfp transgenic tendons shown in C-E, confirming relative specificity of Scx in tenocytes. (C) Second harmonic generation microscopy demonstrates the contrast between collagen rich fibre bundles (**F**) and adjacent capillaries (**V**) which occasionally course through adult tendon tissue. (D) The GFP signal is strongest in the load-bearing collagen region, confirming this cell type as adult tenocytes. E. Merged image.

were determined using a spectrophotometer (NanoDrop 2000, Thermo Scientific, Toronto). cDNA was prepared from total RNA using a High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Gene expression was quantitated using qPCR on an ABI 7500 Fast system with TaqMan probe and primer sets. Expression levels were determined using the relative quantitation method with a standard curve. Gene expression was normalized to 18s rRNA abundance.

Gene silencing

Dharmacon[®] gene silencing reagents were obtained from Thermo Scientific (Toronto, Canada). Cells were trypsinized and replated at 150,000 cells per well of a 6 well plate overnight, then exposed to RNAi targeted against Scx, or against a random scrambled sequence for 24 h. Cells were then retransfected. A portion were held back to confirm gene knock-down, and the remainder were used to create BATs as above and the effect of knockdown on collagen remodeling and density determined after 48 hrs using second harmonic generation analysis.

Statistical analysis

To detect differences in gene expression, planned comparisons using the Mann-Whitney U test were conducted, with significance predetermined at $p < 0.05$.

Results

Confirmation of the importance of Scx expression in adult tendon

Scx is a known requirement for embryonic tendon differentiation. We examined the expression of Scx in adult tendon and other closely related collagen-rich musculoskeletal structures (Figure 1A). Scx and Colla1 expression were highest in tendon, compared to cartilage, bone, muscle, or meniscus. Other examined tissues such as lung or liver demonstrated negligible levels of Scx. In adult tendon, Scx expression was highest in load-bearing collagen fibre bundles. A population of vascular-associated cells in tendon also exhibited Scx expression, but this was barely detectable (Figure 1 C,D,E).

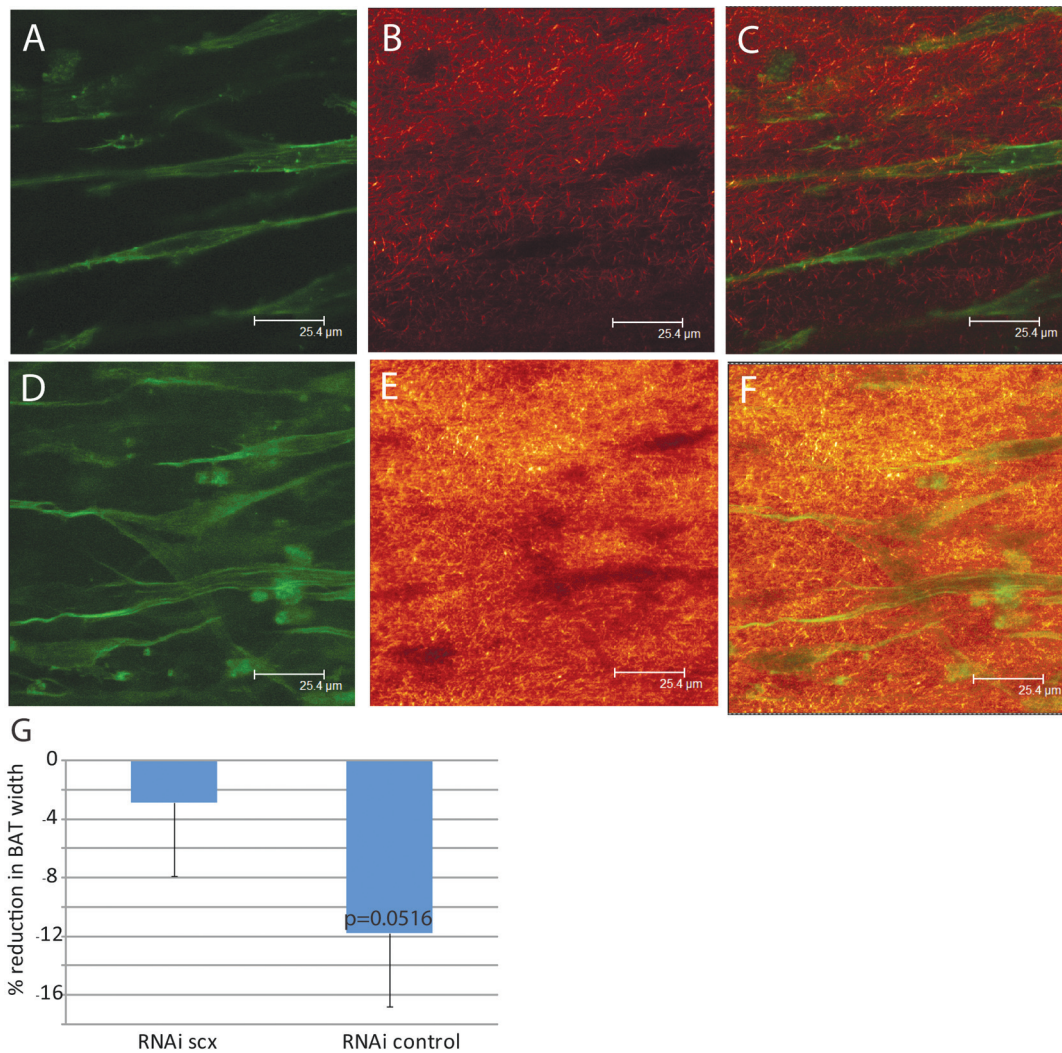


Figure 2. Effect of scleraxis RNAi on collagen remodeling. Primary tenocytes from 8 week old Scx-gfp mice were harvested, expanded in culture to P4, then exposed for 24 hours to Scx RNAi (A-C) or scrambled RNAi (D-F). The panel shows multiphoton excitation fluorescence (A,D), second harmonic generation (B,E) and merged images (C, F). Tenocytes appear as highly elongated cells, although rounded tenocytes can also be seen (particularly panel D). In controls, (D-F), the second harmonic generation signal surrounding tenocytes is bright, indicating a high collagen density. The collagen surrounding tenocytes in which Scx expression has been inhibited emits a much weaker signal. Scx knock-down reduced the extent of tenocyte-driven extracellular matrix remodeling, as gauged by a less extensive reduction in BAT width (G).

Knockdown of Scx expression in mouse adult tenocytes resulted in a reduced collagen density in bioartificial tendons, confirming the importance of Scx in maintenance of the tendon phenotype (Figure 2).

Cyclic mechanical load potentiates, but is not required for, tenocyte differentiation in vitro

Prior to applying cyclic mechanical loads to bioartificial tendons, we examined the influence of the Flexcell culture system on Scx expression. In this system, C3H10T1/2 cells remodel the initial collagen and develop static tension within the BAT if they are allowed to remain longitudinally tethered (Figure 3A). Interestingly, Scx expression increased dramatically over

the first week in culture with static load alone (Figure 3B). The addition of cyclic load potentiated Scx expression (Figure 3C). Col1a1 expression showed a similar but smaller trend in response to cyclic load using this waveform, but it was not statistically significant (1.8 ± 0.14 strained 3 weeks vs 1.42 ± 0.18 , $p=0.107$, Figure 3D).

Strain dependence

Next we examined whether increasing the applied strain level would lead to further gains in Scx and Col1a1 expression. Strains were applied daily for 2 weeks over a range of strain levels to C3H10T1/2 cells grown in the bioartificial tendon system. Increasing strain levels were associated with higher

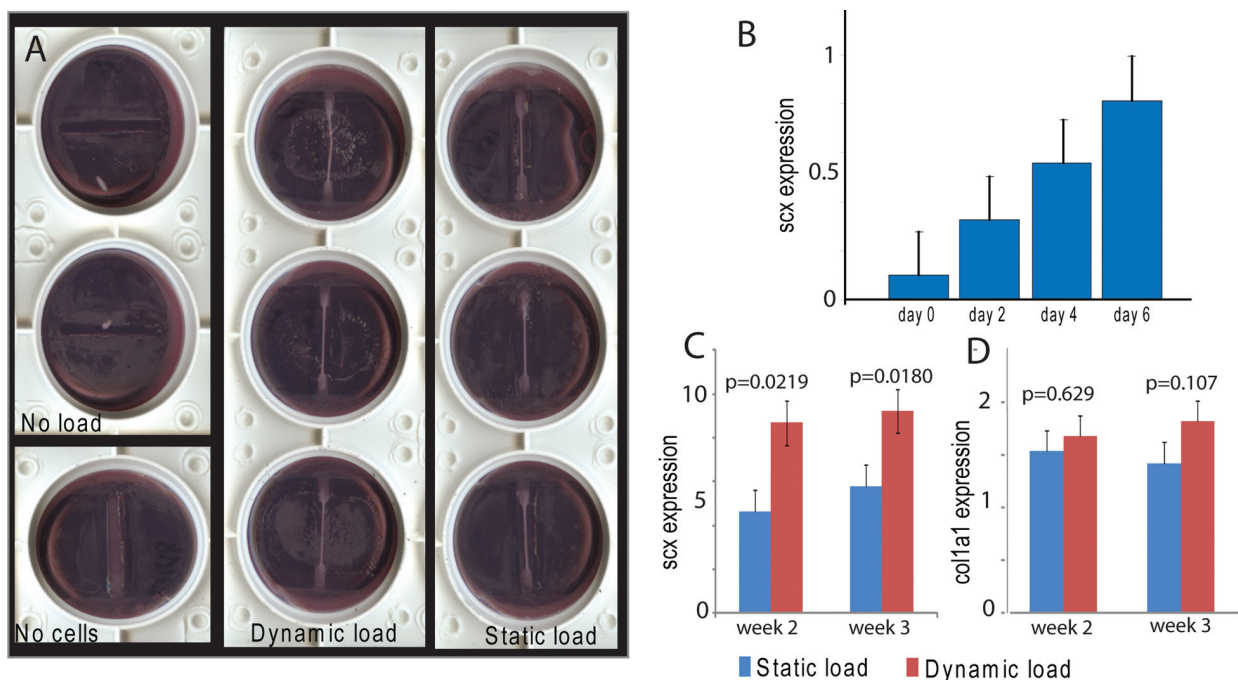


Figure 3. Scx expression is induced by a 3D collagen environment, and is enhanced by cyclic loading. In A, the gross appearance of bioartificial tendons is shown after 3 weeks in culture. In the No Load condition, the tendons have been detached from their tethers to minimize the development of static tension. As a result, the tendons rapidly degenerate (visible as small white remnants). When collagen alone is cultured (No Cells condition, bottom left), the BAT does not change in width and the collagen remains loose and friable. This is in stark contrast to the Dynamic Load and Static Load conditions, where the tendons have become thinner and developed observable tension (i.e. taut appearance). (B) Compared to standard two-dimensional C3H10T1/2 cultures (e.g. day 0), placement of the cells in a three dimensional collagen environment (Static Load) cues this mesenchymal cell type to upregulate Scx mRNA levels in a time-dependent manner. (Representative experiment shown, n=3 per time point). This is particularly evident when an additional, cyclic load (5% strain, no rest cycle) is superimposed, as in C (n=4 per time point). Col1a1 shows a smaller but non-significant trend (D).

levels of Scx and Col1a1, and a more highly differentiated morphology (Figure 4).

Insertion of rest periods potentiates tenocyte differentiation

We conducted a pilot study to examine whether the insertion of rest periods between each loading cycle has the potential to enhance tenocyte differentiation. C3H10T1/2 cells were grown in the bioartificial tendon system with loads applied daily as described in the Methods. The insertion of 10s rest periods appeared to potentiate the induction of Scleraxis and Col1a1 (Figure 5).

Cycle number

Finally, using the optimized protocol (10% strain, 10s rest periods) we examined the effect of increasing repetition numbers on Scx expression in C3H10T1/2 cells (Figure 6). There was a statistically significant increase in Scx expression with highest number of repetitions (p=0.028), but not with the lower repetition numbers. This suggests that a relatively small number of repetitions may be insufficient to induce tenocyte differentiation signaling, and that longer duration loading regimens may improve differentiation.

Discussion

Tendon is a dynamic tissue which, like muscle, is capable of sensing and responding to dynamic loading (reference Kjaer review from same issue). The cell types within tendon capable of responding to mechanical stimuli include tenocytes, mast cells, vascular cell types, and mesenchymal cells which are thought to reside perivascularly in the loose connective tissue linings. It has been suggested for many years that mesenchymal cells may be recruited into tendon tissue following injury, where they participate in the (limited) ability of tendon regeneration²⁰. However, the role and niche of mesenchymal cells in tendon have not been firmly established²¹. While local recruitment of stem/progenitor cells may provide a source of new tenocytes to assist in the body's attempt to regenerate the injured tendon, it may also result in the appearance of poorly differentiated cell types such as cartilage, fat, or bone – a common pathological finding in surgical biopsies of chronically painful tendons⁶. Based on the current results, it is conceivable that exercise following injury, as part of a rehabilitation program, could promote the correct differentiation of local stem/progenitor cells along the tenocyte lineage.

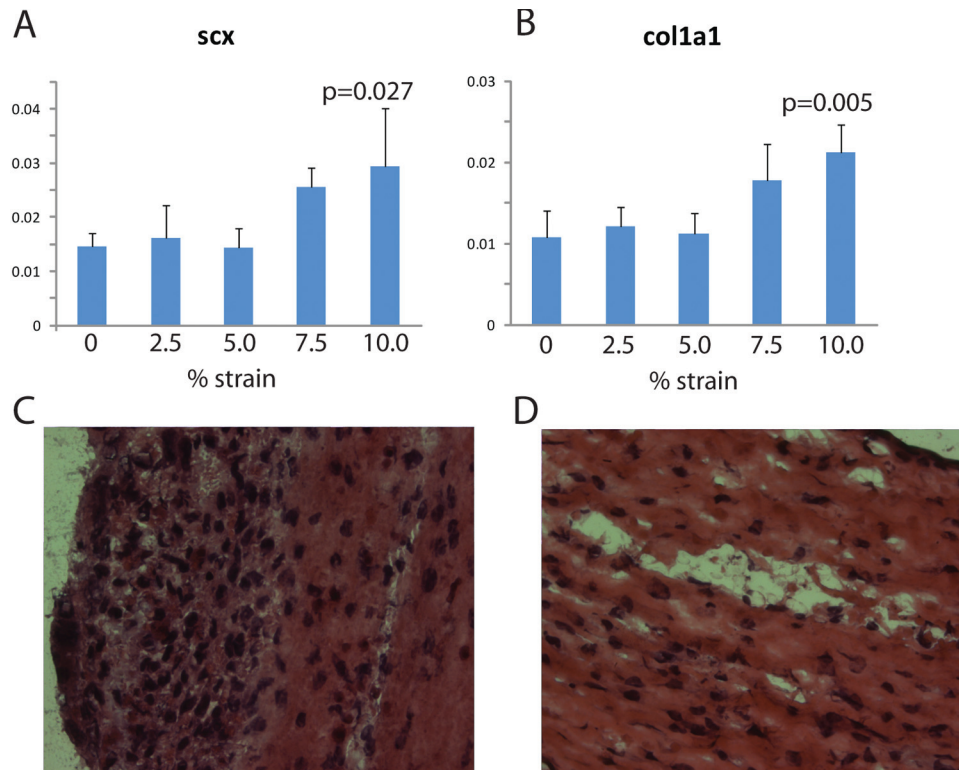


Figure 4. Effect of increasing strain on tenocyte differentiation. (A,B) Tendon-related genes were expressed at higher levels with increasing strain levels in C3H10T1/2 cells (n=4 per strain level). (C,D) The morphology of bioartificial tendons subjected to 0% and 10% strains are shown (haematoxylin and eosin). In C, note the presence of numerous poorly differentiated cells, identified according to their rounded morphology, evidence of cellular proliferation, and their surrounding loose, basophilic extracellular matrix. In D, nuclei appear more elongated.

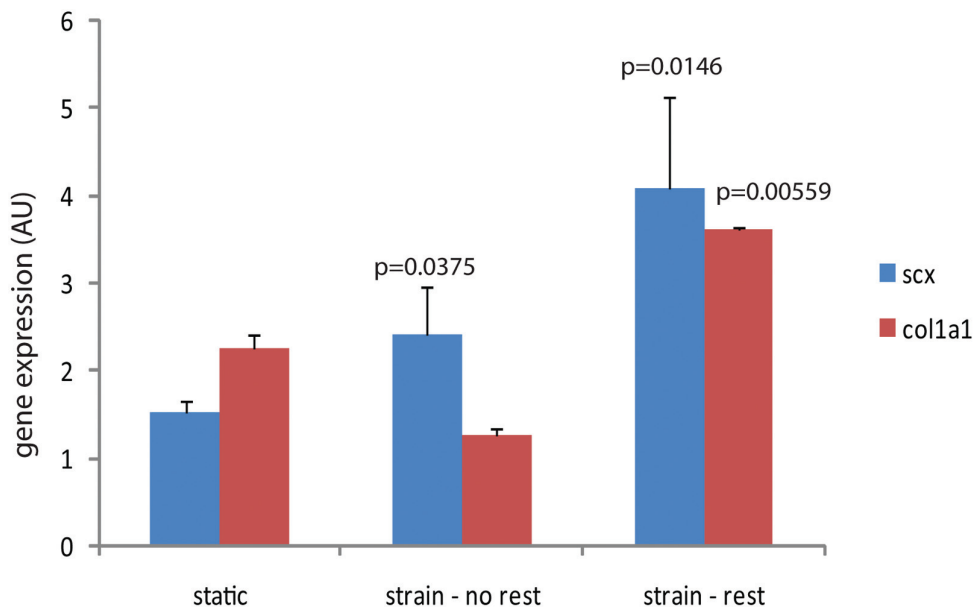


Figure 5. Tenocyte differentiation in response to standard or rest-inserted loading applied daily for 2 weeks. The insertion of 10s rest periods appeared to potentiate the induction of Scx and Col1a1. C3H10T1/2 cells were seeded into BATs, allowed to rest for 48 hours, then cyclically loaded at 10% strain. The only difference between the strain-no rest and strain-rest conditions was the insertion of a 10s rest period in between each repetition (n=8 per condition).

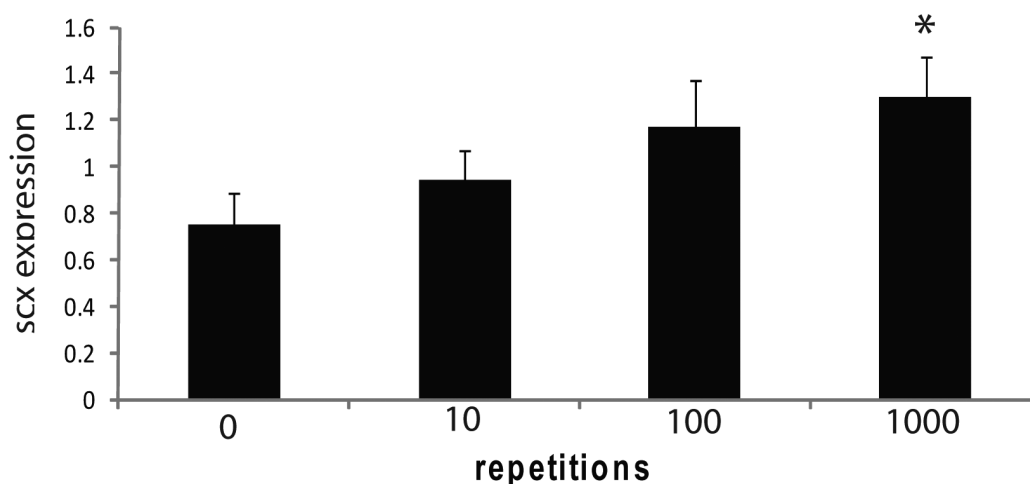


Figure 6. Effect of increasing repetition number on scleraxis expression. C3H10T1/2 cells were seeded into BATs, allowed to rest for 48 hours, then cyclically loaded at 10% strain for the indicated number of repetitions, with 10s rest insertion, once per day (n=4 per condition).

In this study, we used Scx and Col1a1, as indicators of the tenocyte lineage (Figure 1). Scx was selected because Scx knock out mice demonstrate a profound deficit in the formation of load-bearing tendons¹⁴. Other proposed tendon “marker” genes include tenomodulin, tenascin-C and mohawk. We confirmed in adult tenocytes that Scx was localized in load-bearing regions of tendon, and that Scx knock-down resulted in less highly organized three dimensional collagen matrix (Figure 2). However, we also observed a low level of Scx expression in cells associated with the tendons vascularity, hence Scx cannot be viewed as a completely specific tenocyte marker in adult mice. A further limitation of current tendon research is the lack of detailed knowledge of additional pathways which initiate and maintain the tenocyte phenotype. In a recent rat study of the supraspinatus tendon, Scx was not abundantly expressed, and its expression was not increased by overuse, whereas Col1a1 was (Attia et al, unpublished results). Thus, future work may be needed to identify other tenocyte markers for use in other species, particularly as there is no currently available mouse model of overuse tendinopathy reference Warden’s review, this issue²².

The question of how to provide the optimal mechanical loading to promote tendon adaptation and tendon healing is of great importance to sports and vocational rehabilitation clinicians. Currently used rehabilitation regimes, e.g. the Alfredson program for Achilles tendinopathy and related variants, were developed based on concepts of muscle activity (e.g. eccentric loading)^{23,24}. However, the optimal conditions for tenocytes may not be identical to those for skeletal muscle¹. Certainly, higher strains showed the clearest benefit in promoting genes relevant to tenocyte differentiation compared with static or low-strain loading (Figure 4), but caused no change in a cartilage-related gene (Col2a1, data not shown). It will be important to design *in vivo* experiments where varying tendon strains are prescribed and the resultant adaptation, or lack thereof, is

monitored at the tissue and cellular levels. *In vivo* tendon loading involves many variables other than strain level²⁵. Nonetheless, tracking tendon strains and loads during exercise may assist therapists in prescribing exercise regimes with greater accuracy, as well as providing a method to track patient responses to an exercise intervention over time.

This study is limited in that we relied on a mesenchymal cell line (C3H10T1/2) rather than primary tendon-derived progenitor cells²⁶. Currently there is no single cell surface marker for purifying tendon-derived progenitor cells, thus clonal expansion of single cells is perhaps the best currently available protocol for growing these cells²⁶. Zhang and Wang subjected culture-expanded tendon-derived cells to cyclic loading, but the identity of their cells was not confirmed. Thomopoulos et al. subjected mesenchymal stromal cells in 3D culture to cyclic or tensile load, and found that Scx, Col1a1 and cellular alignment were promoted by tensile loading, but not compressive, loading²⁷. Kuo and Tuan subjected human bone-marrow derived mesenchymal cells to cyclic loading in the same three-dimensional model system used here, however the mechanical loading parameter chosen was quite minimal (1% strain for 30 minutes per day at 1Hz). In this study, Scx levels declined over time in non-loaded groups but was maintained (but not increased) by cyclic loading. Thus, our study which employed higher, rest-inserted strain levels may have a greater potential to induce Scx and other tendon-related genes than previously examined protocols. An advantage of the study by Kuo and Tuan is that primary human cells were used. However, the relevance of bone-marrow derived MSCs in tendon healing is not established.

Most interestingly, the insertion of 10s rest periods between loading cycles potentiated tenocyte differentiation in the current experimental model (Figure 5). The mechanisms for this differential response to rest-inserted loading requires further work. It may result, as has been suggested for osteoblasts, from the kinetics of calcium signaling, a key upstream event in

mechanotransduction of both bone and tendon cells^{28,29}. There may be a possibility to further optimize current rehabilitation regimes by the inclusion of rest periods, or by the use of discrete loading periods, as opposed to single daily sessions of loading. These concepts require further research, but the current studies demonstrate the potential for this line of inquiry.

In conclusion, the current study confirms that mechanical loading can potentiate the induction of genes related to the tendon phenotype *in vitro*, and furthermore suggests that this strain-induced gene regulation is, at least in part, mediated by Scx. This biological mechanism may underlie some of the known beneficial effects of exercise during tendon repair and regeneration.

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References

1. Kjaer M. Role of extracellular matrix in adaptation of tendon and skeletal muscle to mechanical loading. *Physiol Rev* 2004;84(2):649-98.
2. McNeilly CM, et al. Tendon cells *in vivo* form a three dimensional network of cell processes linked by gap junctions. *J Anat* 1996;189(Pt3):593-600.
3. Waggett AD, Benjamin M, Ralphs JR. Connexin 32 and 43 gap junctions differentially modulate tenocyte response to cyclic mechanical load. *Eur J Cell Biol* 2006; 85(11):1145-54.
4. Wall ME and Banes AJ. Early responses to mechanical load in tendon: role for calcium signaling, gap junctions and intercellular communication. *J Musculoskelet Neuronal Interact* 2005;5(1):70-84.
5. Clegg PD, Strassburg S, Smith RK. Cell phenotypic variation in normal and damaged tendons. *Int J Exp Pathol* 2007;88(4):227-35.
6. Jarvinen M, et al. Histopathological findings in chronic tendon disorders. *Scand J Med Sci Sports* 1997;7(2):86-95.
7. Schweitzer R, et al. Analysis of the tendon cell fate using Scleraxis, a specific marker for tendons and ligaments. *Development* 2001;128(19):3855-66.
8. Lejard V, et al. Scleraxis and NFATc regulate the expression of the pro-alpha1(I) collagen gene in tendon fibroblasts. *J Biol Chem* 2007;282(24):17665-75.
9. Pryce BA, et al. Recruitment and maintenance of tendon progenitors by TGFbeta signaling are essential for tendon formation. *Development* 2009;136(8):1351-61.
10. Kjaer M, et al. From mechanical loading to collagen synthesis, structural changes and function in human tendon. *Scand J Med Sci Sports* 2009;19(4):500-10.
11. Beckham, C., R. Dimond, and T.K. Greenlee, Jr., The role of movement in the development of a digital flexor tendon. *Am J Anat*, 1977. 150(3): p. 443-59.
12. Kjaer M, et al. Metabolic activity and collagen turnover in human tendon in response to physical activity. *J Musculoskelet Neuronal Interact* 2005;5(1):41-52.
13. Scott A, et al. Human tendon overuse pathology: histopathologic and biochemical findings, in *Tendinopathy in Athletes*, SL Woo, SP Arnoczky and P Renstrom, Editors. 2007, Blackwell Publishing Ltd: Malden, Massachusetts, p. 69-84.
14. Murchison ND, et al. Regulation of tendon differentiation by scleraxis distinguishes force-transmitting tendons from muscle-anchoring tendons. *Development* 2007; 134(14):2697-708.
15. Song G, et al. Mechanical stretch-induced changes in cell morphology and mRNA expression of tendon/ligament-associated genes in rat bone-marrow mesenchymal stem cells. *Mol Cell Biomech* 2010;7(3):165-74.
16. Zhang J and Wang JH. Mechanobiological response of tendon stem cells: implications of tendon homeostasis and pathogenesis of tendinopathy. *J Orthop Res* 2010; 28(5):639-43.
17. Hoffmann A, et al. Neotendon formation induced by manipulation of the Smad8 signalling pathway in mesenchymal stem cells. *J Clin Invest* 2006;116(4):940-52.
18. Triantafillopoulos IK, et al. Nandrolone decanoate and load increase remodeling and strength in human supraspinatus bioartificial tendons. *Am J Sports Med* 2004;32(4):934-43.
19. Abraham T, Carthy J, McManus B. Collagen matrix remodeling in 3-dimensional cellular space resolved using second harmonic generation and multiphoton excitation fluorescence. *J Struct Biol* 2010;169:36-44.
20. Sarkar K and Uthoff HK. Ultrastructure of the common extensor tendon in tennis elbow. *Virchows Arch A Pathol Anat Histol* 1980;386(3):317-30.
21. Salingcarnboriboon R, et al. Establishment of tendon-derived cell lines exhibiting pluripotent mesenchymal stem cell-like property. *Exp Cell Res* 2003;287(2):289-300.
22. Warden S. Development and use of animal models to advance tendinopathy research. *Front Biosci* 2009;14:4588-97.
23. Fahlstrom M, et al. Chronic Achilles tendon pain treated with eccentric calf-muscle training. *Knee Surg Sports Traumatol Arthrosc* 2003;11(5):327-33.
24. Jonsson P, et al. Eccentric training in chronic painful impingement syndrome of the shoulder: results of a pilot study. *Knee Surg Sports Traumatol Arthrosc* 2006; 14(1):76-81.
25. Magnusson SP, et al. Human tendon behaviour and adaptation, *in vivo*. *J Physiol* 2008;586(1):71-81.
26. Bi Y, et al. Identification of tendon stem/progenitor cells and the role of the extracellular matrix in their niche. *Nat Med* 2007;13(10):1219-27.
27. Thomopoulos S, et al. Fibrocartilage Tissue Engineering: The

- Role of the Stress Environment on Cell Morphology and Matrix Expression. *Tissue Eng Part A* 2011;17:1039-1053.
28. Banes AJ, et al. Mechanoreception at the cellular level: the detection, interpretation, and diversity of responses to mechanical signals. *Biochem Cell Biol* 1995;73(7-8): 349-65.
 29. Donahue SW, Donahue HJ, Jacobs CR. Osteoblastic cells have refractory periods for fluid-flow-induced intracellular calcium oscillations for short bouts of flow and display multiple low-magnitude oscillations during long-term flow. *J Biomech* 2003;36(1):35-43.