Scleraxis and collagen I expression increase following pilot isometric loading experiments in a rodent model of patellar tendinopathy

Danielle Steffen [a], **Michael J. Mienaltowski** [b] **and Keith Baar** [a,c,d]

***a* - Department of Neurobiology,** *Physiology & Behavior, University of California Davis, 1 Shields Avenue, 195 Briggs Hall, Davis, CA 95616, USA*
***b* - Department of Animal Science,** *University of California Davis, Davis, CA, USA*
***c* - Physiology and Membrane Biology,** *University of California Davis, Davis, CA, USA*
***d* - VA Northern California Health Care System,** *Mather, CA 95655, USA*

Corresponding author at: *Department of Neurobiology, Physiology & Behavior, University of California Davis, 1 Shields Avenue, 195 Briggs Hall, Davis, CA 95616, USA. kbaar@ucdavis.edu.*
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Abstract

The effect of mechanical load on tendinopathic tissue is usually studied in the context of identifying mechanisms responsible for tendon degradation. However, loading is also one of the most common treatments for tendinopathy. It is therefore possible that different loads result in different cellular responses within a tendon. To test this hypothesis, we first established a rodent model of tendinopathy that has a transcriptional signature similar to human tendinopathy. Tendinopathy was modeled in the rat by producing a lesion in the central core of the patellar tendon using a biopsy punch, followed by two weeks to allow scar formation. We performed 3' Tag RNA-Seq to identify genes that were differentially expressed between the native and scarred rat patellar tendon. Genes involved in extracellular matrix (ECM) structure and turnover were increased, mitochondrial genes were decreased, and there was no inflammatory signature in the tendinopathic tissue. These transcriptional changes phenocopy previously published whole transcriptome analysis in human tendinopathy. After validating the model, the initial response to injury and loading was determined. Two weeks after creation of the patellar tendon lesion, the tendon was loaded using either 4 × 30s isometric or a time-under-tension matched (360 × 0.33s) dynamic protocol. Injured +/- loading and contralateral control tendons were collected eighteen hours after loading, RNA was extracted, and gene expression was quantified using qRT-PCR of the scar with or without loading. The expression of scleraxis and type I collagen increased following isometric loading relative to those loaded dynamically. By contrast, the expression of type II collagen increased in the dynamic samples relative to those loaded isometrically. These data suggest that dynamic loading of a central core tendon injury increases fibrocartilage markers, whereas long isometric loads stimulate markers of tendon regeneration.

Introduction

Persistent patellar tendon pain and loss of function is diagnosed as patellar tendinopathy [1]. The incidence of anterior knee pain, which includes patellar tendinopathy, in the general population has been reported at 23%, whereas in adolescents this number rises to 29% [2]. The prevalence of patellar tendinopathy is highest in elite volleyball and basketball players, with well over one-third of players affected [3]. In fact, structural abnormalities in the patellar tendon are present in over 60% of elite basketball players [4]. Increased training load correlates with disruption of the tendon structure [5], strongly supporting the idea that overuse contributes to the development of tendinopathy. Even though injury resulting from excessive chronic loading, or an acute overload are thought to initiate the clinical condition, loading though rehabilitation exercises is also thought to be required for healing an injured adult tendon. However, the molecular basis of this contradiction remains poorly understood.

One reason why the molecular basis of tendinopathy and the response to subsequent loading is poorly understood is the lack of a validated rodent model of tendinopathy. There are a small number of rodent physiological patellar tendon injury models [6] and animal models for other tendinopathies [7,8]; however, the repeatability and similarity of these models to the human condition has yet to be determined. Given that overuse is thought to contribute to tendinopathy, an uphill running protocol has been successfully used to damage Achilles tendon structure in some [6,9] but not all instances, highlighting the variability in many of the existing "physiological" models of tendinopathy. In contrast, Beason and colleagues [8] have demonstrated that a biopsy punch creates a highly reproducible tendon injury, but whether the resulting tendon lesion is similar to human tendinopathy has yet to be determined.

Further, while pain is a defining feature of human tendinopathy, tendons can have structural damage without being painful [10] and this structural damage precedes rupture [11]. Chronic human tendinopathy lacks an inflammatory signature [12−14], shows increased matrix turnover [12,13,15−17], a larger population of smaller collagen fibrils [13,18,19], and increased intratendinous vascularity [12,14,20]. More specifically, the increased matrix turnover seems to result from increased expression of the mRNA for type I and III collagen [12,13] and either no change [16] or increased [13] expression of certain matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), the proteins that regulate the resorption of tendon matrix. There are also more large proteoglycans [15,16] with negatively charged glycosaminoglycan (GAG) side chains that increase the water content of the tendinopathic tissue [15] and the presence of these molecules correlates with pain [21].

Cook and colleagues state that in degenerative tendinopathy, interventions influencing tendon structure are less critical, as the pathology [of tendinopathy] appears to have limited reversibility [22]. We hypothesize that the "limited reversibility" of tendinopathy is due to stress shielding of the injured region. The rationale underlying this hypothesis is as follows: (1) Injury to a tendon weakens the material properties of the damaged region [23]; (2) Since the damaged area is mechanically inferior, stress preferentially passes through neighboring regions of the tendon that are stiffer (shielding the injured area from stress); (3) Experimentally, stress shielding the entire healthy patellar tendon produces a tissue with the hallmarks of a scar [24]: increased cellularity, collagen disorganization, and reduced mechanics [25,26]; and (4) Injured tendons with lower material properties, such as tendons from fetuses [27,28] and β-aminopropionitrile (BAPN) treated adult tendons [29], heal with relatively good collagen alignment since stress shielding is not possible. These data suggest that stress shielding in injured adult tendons results in scar formation and tendinopathy.

If tendon scar formation is the result of stress shielding and lack of tensile load, applying directional load through the scar should increase genes enriched in developmental or mature tendons and accelerate tendon regeneration. One way to overcome stress-shielding and apply a tensile load to a viscoelastic material like a tendon [30] is to hold it at a constant length under tension. Isometric contractions have been used in the past to treat tendinopathy and provide immediate pain reduction [31,32]. During a long (30+ s) isometric hold, stress relaxation occurs and the stress within the tissue decreases with time. As the stiffness of the stronger/healthy part of the tendon decreases over time, it dips below that of the of the injured region resulting in directional load passing through the scar. Given that tenocytes deposit collagen along the line of force [33], we hypothesize that this tensile load signal is essential for repair. However, whether isometric loading provides a sufficient molecular signal to promote recovery has yet to be tested. The current study was designed to test the hypothesis that isometric loading a central core tendinopathy would increase the expression of tendon markers (*COLIα1, SCX, MKX, TNMD, FMOD*), whereas an equal time-under-tension using dynamic loading would increase the expression of cartilage markers (*ACAN, COMP, COLIIα1*).

Results

Rat Model of Patellar Tendinopathy

Two weeks following removal of the central third of the rat patellar tendon using a biopsy punch (Fig. 1B), the gross appearance of the patellar tendon to the surgeon was that vascularity increased (Fig. 1C). Fifteen days after injury, there was a visible scar that did not differ between groups at the injured site in the patellar tendon (Fig. 1C). Hematoxylin and Eosin (H&E) stains of the uninjured patellar tendon showed collagen fibers aligned with the direction of force and elongated fibroblasts between collagen fibers (Fig. 1D). Two weeks after injury, there was a notable collagen disorganization with the fibers wrapping around the injured site. There was also an increase in cellularity and loose connective tissue that formed distal to the injury (Fig. 1E and F). The loss of collagen organization, increased cellularity and vascularity are hallmarks of tendinopathy [34,35].

Transcriptomics in healthy and injured rat PTs

We performed 3'TAG-seq of adult healthy and injured tissue to assess which genes change

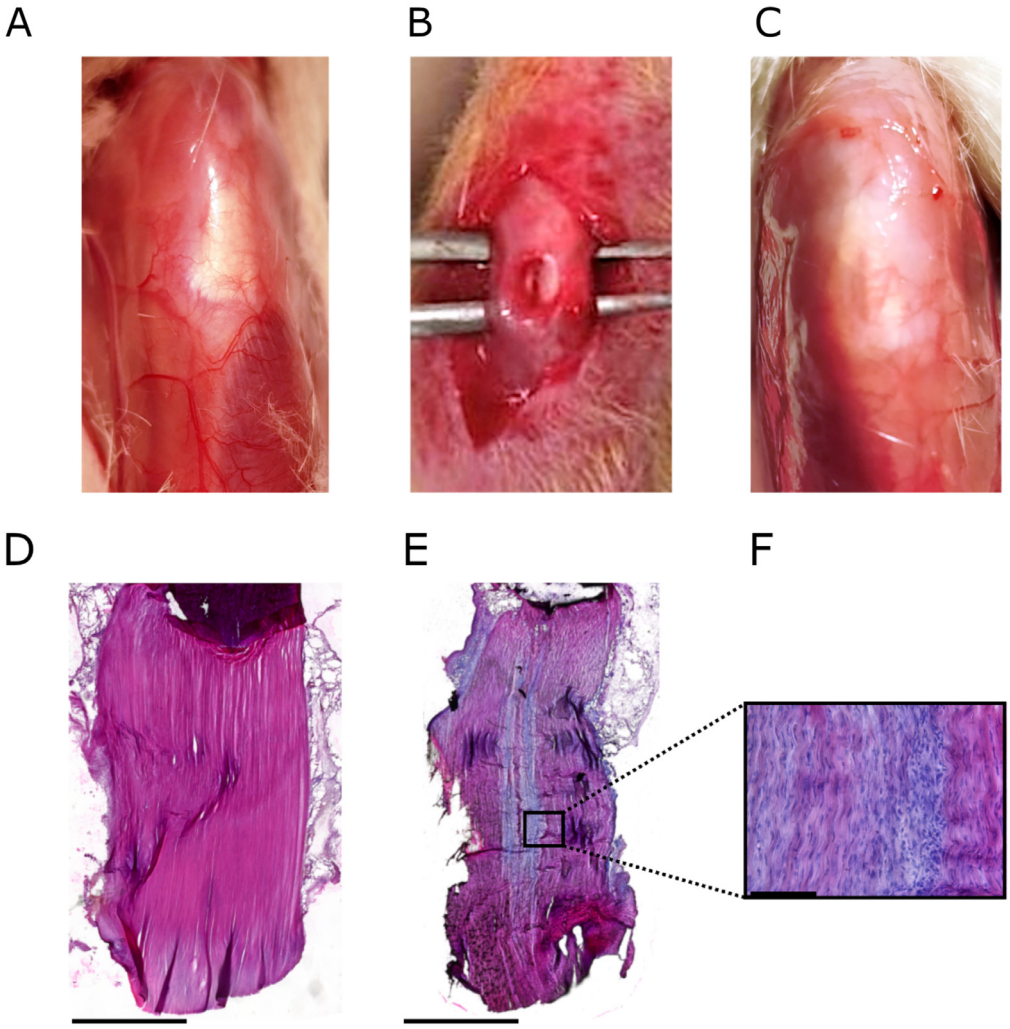

Fig. 1. Model of patellar tendinopathy. **(A)** Intact patellar tendon with synovial sheath. **(B)** Injury was induced to the central core of the patellar tendon with a 2 mm biopsy punch. **(C)** Fifteen days after injury there was noticeable vascularization and enlargement of the patellar tendon. H&E stained patellar tendon at 10X: **(D)** healthy and **(E)** injured scale bar is 1.8mm; and **(F)** 20X injured, scale bar is 150μm.

expression in response to injury. Approximately 6% of genes were differentially regulated with 12,041 identified and 707 classified as differentially expressed (0.6 log fold change/ 1.5-fold change and $p < 0.05$).

Heat Map (rat scar vs healthy): We created a heat map to describe the relationship of all genes with significant (no minimum absolute fold change, $q < 0.05$) differences in expression between the healthy adult (A) and scar tendon (S). The dendritic branches at the top of the figure describe the relatedness of the samples based on the expression of these genes. Each sample is a column, while each row is a gene. Gene set 1 has higher expression in the scar group than the healthy adult. Gene set 2 has lower expression in all the scar samples, yet a higher expression in two but not all four healthy adult samples (Fig. 2).

Gene Ontology- Cellular Compartment (rat scar vs healthy). Differentially regulated transcripts in the rat patellar tendon tendinopathic tissue can be classified into gene ontology categories based on the cellular compartment in which proteins encoded by those transcripts are located. In our analysis, we decided to rank cellular compartments that contained a minimum of 200 detected genes by the ratio of differentially expressed genes to total genes. In this case, the largest regulated gene set relative to gene set size belonged to the mitochondrial membrane (Table 1). Most genes belonging to the mitochondrial membrane were downregulated in expression in the injured patellar tendon

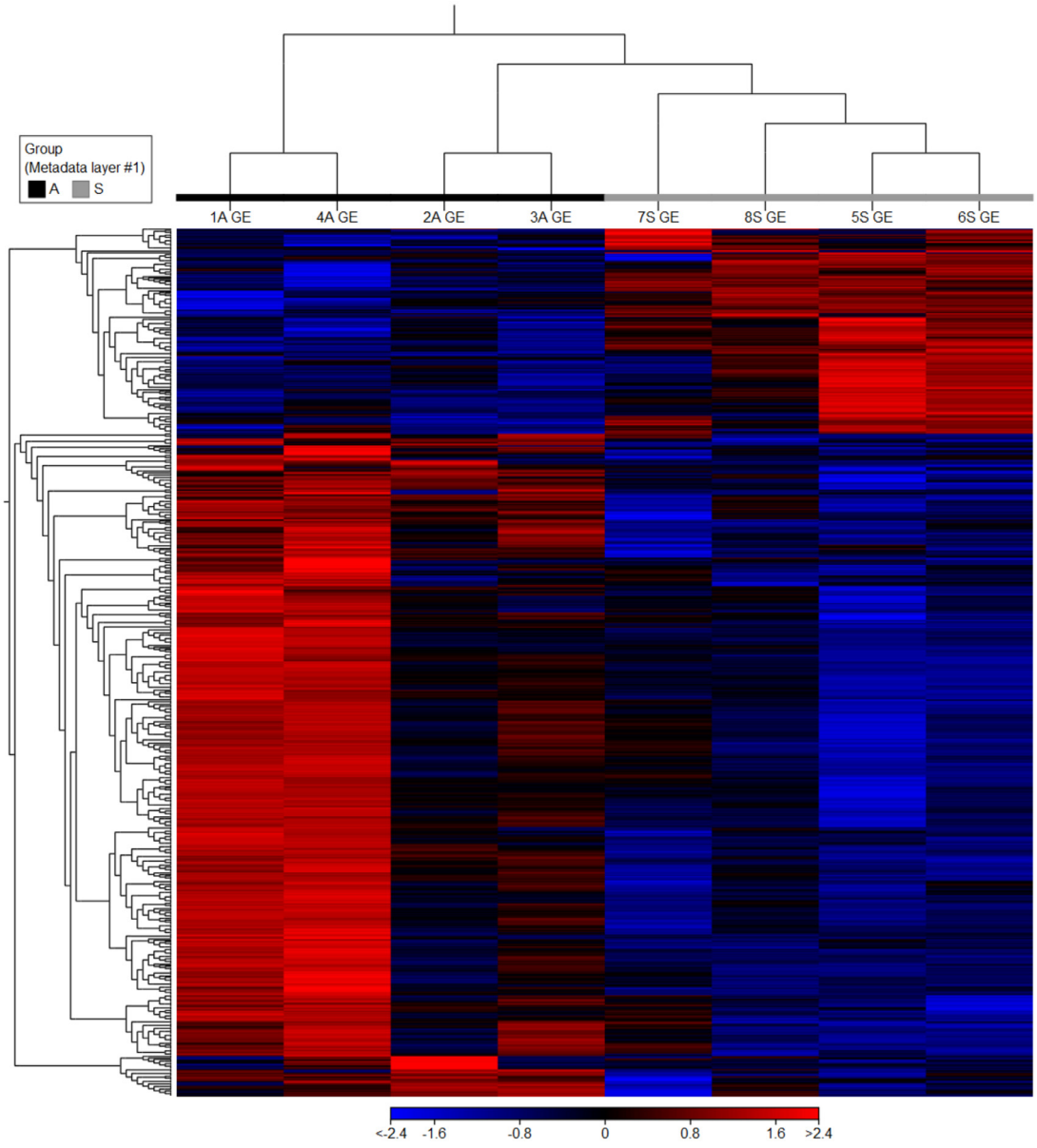

Fig. 2. Heat Map of 3' TagSeq transcriptome analysis for all significant (FDR $p<0.05$) differences in expression between the healthy and adult scar rat patellar tendon. The uninjured adult (A) samples are the left four columns, while the injured scar adult (S) are to the right. The colors indicate levels of expression with red representing higher expression and blue lower expression of a particular gene.

(Supplemental Table 3). Extracellular matrix genes were the second most regulated group (Table 1) and expression of most of these genes were upregulated in the injured tendon (Supplemental Table 4). Membrane protein complexes were among the most regulated groups, yet 12/50 DEGs were shared between membrane protein complex and mitochondrial membrane (Supplemental Tables 3, 5, and 6).

Rat vs Human Tendinopathy: We next compared our RNAseq findings to microarray data of human tendinopathy because we were interested in the transcriptional similarity between the rodent model of tendinopathy and the human condition. The human tendinopathy data consisted of male (n=15, age: 32−66) and female (n=8, age: 46−65) samples from supraspinatus (n=15), extensor carpi radilis brevis (n=3), flexor/pronator (n=2), patellar (n=2),

Table 1. Top selected cellular compartments regulated in rat scar vs healthy.

Description	DEG/ Detected Genes	Detected Genes	DEG	FDR p-value
Mitochondrial membrane	0.11	289	32	1.34E-05
Extracellular matrix	0.09	206	19	0.022921
Membrane protein complex	0.09	573	50	1.21E-05
Adherens junction	0.08	260	22	0.027486
Anchoring junction	0.08	269	22	0.0422
Cytoskeleton	0.07	601	45	0.001951
Mitochondrion	0.07	1132	84	1.36E-06
Extracellular space	0.07	962	65	0.001063
Cytoplasmic part	0.05	6894	327	6.87E-05

Descriptions of cellular compartments with >200 detected genes and FDR<0.05 were selected. Cellular comparments displayed in decending order of DEG/ Detected Genes.

and distal biceps (n=1) tendons. Twelve of the patients also had a history of at least one corticosteroid injection. The microarray of human tendinopathy identified that 5% of genes were differentially regulated (paired t-test; $p<0.01$, fold change ≥ 1.5).

The genes presented in Table 2 were determined to have an orthologous relationship between human and Norway rat species using NCBI Orthologs. The top regulated transcripts shared between the human and rat tendinopathy data sets were: *BNC2, CTHRC1*, and *MARCKS*. Increased matrix turnover and a lack of inflammation were characteristic of both human tendinopathy and the rat model. Expression of *COL1A1, COL5A1, COL5A2, COL8A2*, and *COL12A1* were significantly increased in both groups. *COL3A1* was only significantly increased in human tendinopathy, yet was elevated (FC=2.1, p=.209) in the injured rat patellar tendon. Expression of *MMP2* and *MMP14*, but not *TIMP1* or *TIMP2*, increased in human tendinopathy and the rat model. The kinases *JAK3* and *DCLK1* were also both expressed at greater levels in scar tissue. Interestingly, *JAK3* transcripts were only detected in the scar tissue in the rat tendinopathy model (Table 2). Notably, inflammatory pathways were not regulated in human tendinopathy [12] or the rat patellar tendinopathy model (Supplemental Table 7).

Gene expression and loading

Patellar tendons from the isometric and dynamic groups were loaded fourteen days after injury and all tissues collected eighteen hours later. The unexercised contralateral control tendons were collected at the same time. There were no group differences in baseline body weight, patellar tendon wet weights, or RNA content. There was a significant increase in the amount of RNA per tissue wet weight of the

Table 2. Comparison of expression patterns of rat central core tendinopathy model with human tendinopathy.

Gene Symbol	Gene Name	Human q	Human FC	Rat q	Rat FC
Top regulated transcripts (Table 3)					
BNC2	Basonuclin 2	0.000315	2.9	0.0205	2.6
CTHRC1	Collagen triple helix repeat containing 1	0.0011	4.4	6.72E-13	24.4
MARCKS	Myristoylated alanine-rich kinase C substrate	0.0045	2.8	0.0216	2.8
Matrix metallopeptidase and TIMP genes (Table 6)					
TIMP1	Tissue inhibitor of metalloproteinase 1	0.3	1.6	0.94	1.1
TIMP2	Tissue inhibitor of metalloproteinase 2	0.5	1.1	0.16	1.9
MMP2	Matrix metalloproteinase 2	0.0482	1.8	0.00421	2.9
MMP14	Matrix metalloproteinase 14	0.0166	1.9	0.000505	3.8
Collagen genes (Table 7)					
COL1A1	Collagen, type I, alpha 1	0.0396	1.7	0.00218	4.1
COL3A1	Collagen, type III, alpha 1	0.0428	2	0.209	2.1
COL5A1	Collagen, type V, alpha 1	0.0428	2	0.0019	2.8
COL5A2	Collagen, type V, alpha 2	0.0316	2.1	0.000836	3.6
COL8A2	Collagen, type VIII, alpha 2	0.0428	1.8	0.00686	2.9
COL12A1	Collagen, type XII, alpha 1	0.0316	2.1	5.06E-06	8.1
Kinases (Table 8)					
JAK3	Janus kinase	0.0783	1.8	N/A*	29.4
DCLK1	Doublecortin-like kinase 1	0.00783	28	0.045	2

Table adapted from Tables 3, 6, 7, and 8 of: Jelinsky et al. *Regulation of gene expression in human tendinopathy*. BMC Musculoskeletal Disorders 2011, 12:86. Microarray data compared to RNAseq findings for rat patellar tendinopathy model. * Not detected in control tissue.

Table 3. Baseline body weight, patellar tendon weight, total patellar tendon RNA.

	Body Weight (g)	PT control (mg)	RNA control (ng)	RNA/ PT (ng/ mg)	PT scar (mg)	RNA scar (ng)*	RNA/ PT scar (ng/mg)*
Scar	438 ± 8.66 (n=5)	65 ± 5.61 (n=5)	17398 ± 4856 (n=4)	263.7 ± 83.00 (n=4)	72.6 ±15.42 (n=5)	31644 ± 20633 (n=5)	455.9 ± 326.9 (n=5)
Dynamic	440 ± 8.30 (n=6)	66.5 ± 14.43 (n=6)	22636 ± 5874 (n=5)	341.5 ± 95.74 (n=5)	59.6 ±16.94 (n=5)	37272 ± 14880 (n=5)	615.3 ± 140.0 (n=5)
Isometric	426 ± 16.83 (n=6)	51.33 ± 13.79 (n=6)	18489 ± 6463 (n=5)	353.8 ± 65.76 (n=5)	57 ±13.47 (n=5)	30652 ± 14089 (n=5)	531.5 ± 209 (n=5)

Values are mean ± standard deviation (number of measures). Patellar tendon weight is the wet weight.
* Significant scar vs control (mixed-effects analysis). RNA (ng) p = 0.0215. RNA/ PT (ng/mg) p= .0042

injured patellar tendons in comparison to contralateral control tissue (Table 3).

Expression of genes enriched in developing tendons: Isometric loading increases scleraxis expression

The expression of tendon associated genes, SCX, TNMD, or FMOD was greater in the injured leg regardless of group (Fig. 3A–D). Following isometric exercise, there was an increase in SCX expression relative to contralateral control leg in the isometric group (Fig. 3E). There were no statistically significant differences in expression between loading groups for MKX, TNMD, or FMOD. However, for each of those genes the isometric group tended to show a greater relative increase (Fig. 3F–H).

Collagen gene expression: collagen Ia1 increases with isometric, whereas collagen IIa1 expression levels increase with dynamic loading

The expression of collagen Ia1, IIa1, and IIIa1 all increased in the in the injured patellar tendon (Fig. 4A–C). There was a significant increase in the expression relative to contralateral control leg of

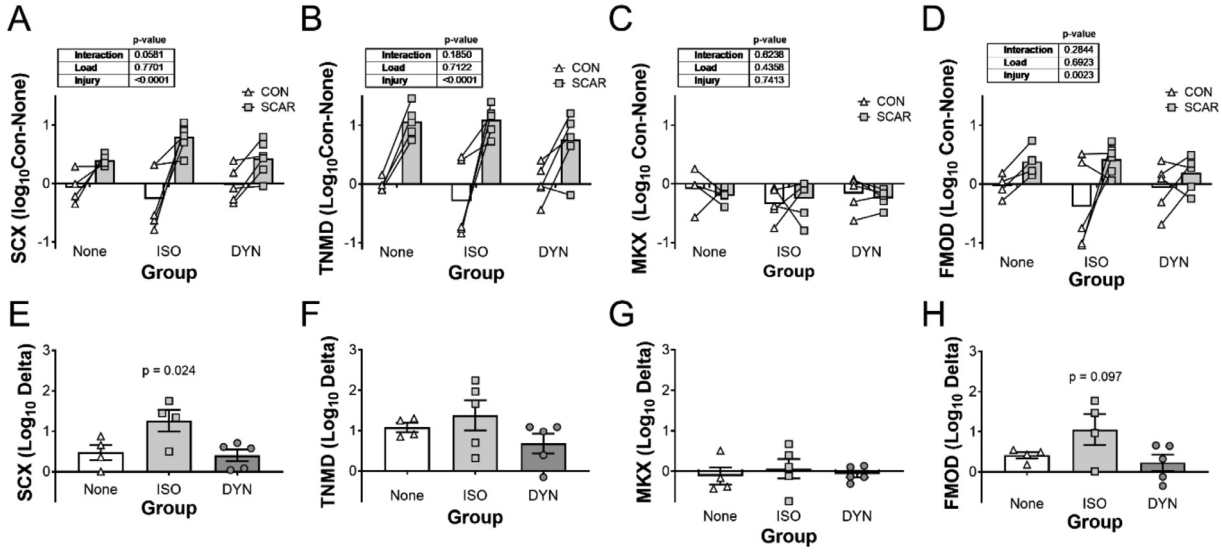

Fig. 3. Expression of tendon enriched markers following injury and no load (none), isometric (ISO: 4 × 30 s), or dynamic (DYN: 360 × 0.33 s) loading. **(A–D)** Expression levels relative to contralateral uninjured control patellar tendon. **(E–H)** Gene expression in control (white bars) and injured (grey bars) legs for each loading group: none, isometric (ISO), and dynamic (DYN), relative to the no loading control expression. **(A,E)** The expression of scleraxis increased in the tendon scar and further increased with an isometric load (Mixed effects model: exercise x scar p=.0279. Tukey's multiple comparisons test: none scar vs isometric scar, p= .0018; scar isometric vs scar dynamic, p=.0207). **(B,F)** Mohawk expression was unaffected by injury or load. **(C,G)** Tenomodulin expression increased in the tendon scar. **(D,H)** Fibromodulin expression increased in the tendon scar.

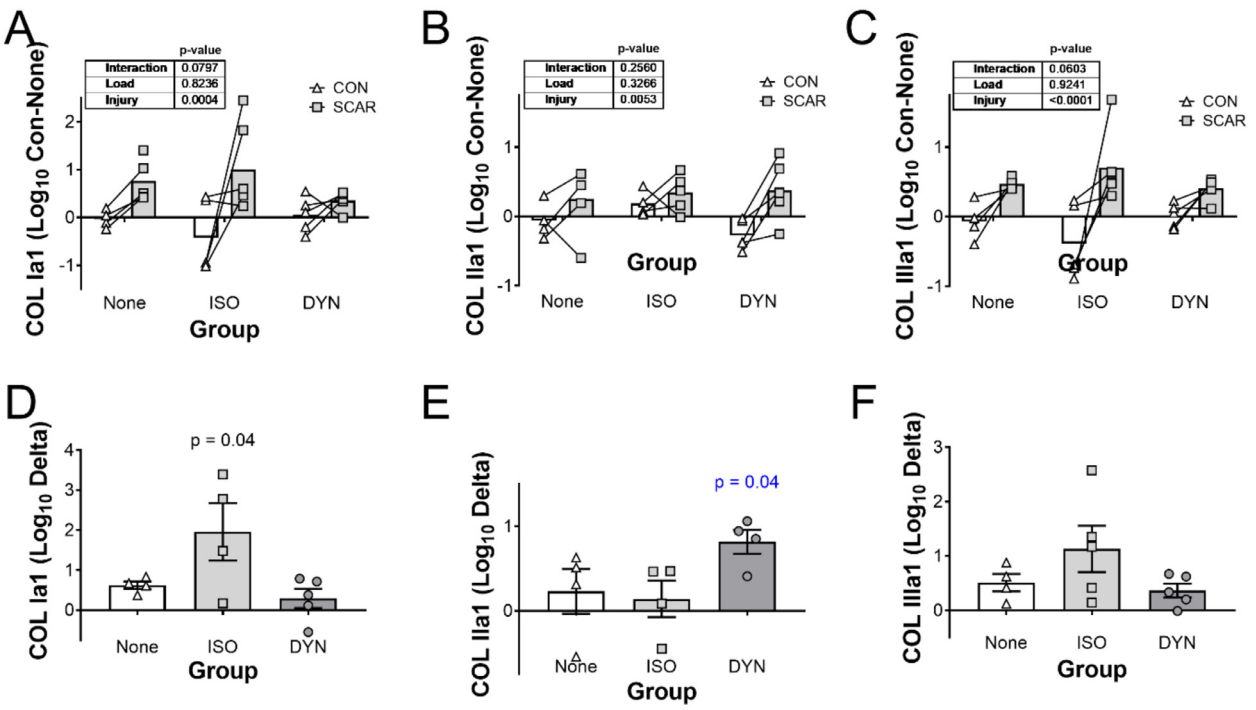

Fig. 4. Expression of collagens Iα1, IIα1, and IIIα1. **(A,D)** There were no differences in collagen Iα1 expression, but isometric loading increased the expression (D) relative to the no load control and (A) relative to the contralateral patellar tendon. **(B,E)** Collagen IIα1 (E) expression increased in the tendon scar and the (B) expression was significantly elevated in the dynamic load group relative to the control limb compared to the isometric group and substantially increased compared to the scar (Ordinary one-way ANOVA, p=.0298; Tukey's multiple comparisons: isometric vs dynamic, p= .0405; none vs dynamic, p= .0615). **(C,F)** Expression of collagen IIIα1 was increased in the tendon scar.

collagen Ia1 in the isometrically loaded group (Fig. 4D). The expression of collagen IIa1 increased in the dynamic group relative to the isometric group (t-test), whereas type III collagen tended to be higher in the isometric group; however, this was not statistically significant (Fig. 4D–F).

Expression of cartilage associated genes: ACAN and COMP increase following injury

The expression of aggrecan and cartilage oligomeric matrix protein (COMP) was increased in the injured patellar tendon regardless of group (Fig. 5A, B). Expression of the SOX9 was not changed with injury (Fig. 5C). There were no differences in expression of these genes relative to contralateral control following loading (Fig. 5D–F).

Discussion

Tendinopathy is one of the most common musculoskeletal diseases without known treatment [36]. Here, we use transcriptomics to validate a rodent model to study tendinopathy and suggest that isometric loading improves the molecular response of a tendinopathic tissue. Two weeks of cage activity following removal of the middle third of the patellar tendon showed similar changes in gene expression, including increased matrix turnover, decreased mitochondrial gene expression, and a lack of inflammatory signature, as human tendinopathy. Isometric loading of the injured patellar tendon increased the expression of tendon genes like scleraxis and collagen Ia1, whereas a time under tension matched dynamic loading protocol increased expression of collagen IIa1.

Fifteen days after injury to the central core of the patellar tendon, there was collagen disorganization with the fibers wrapping around the injured site, increased in cellularity, loose connective tissue, and increased vascularity within the injured region (Fig. 1). These are all hallmarks of human tendinopathy [34,35]. Similarly, the amount of RNA per gram of patellar tendon increased in the injured tendon. In humans, an increase in RNA/mg tissue has been observed in biopsies from patients with patellar tendinopathy resulting either from chronic overload [37] or an acute injury due to insertion of a biopsy punch [38]. Since the ratio of RNA/DNA does not change [38], this suggests that the increase in RNA in the tendinopathic tissue reported here and in the work of Olesen is the result of an increase in cellularity. In

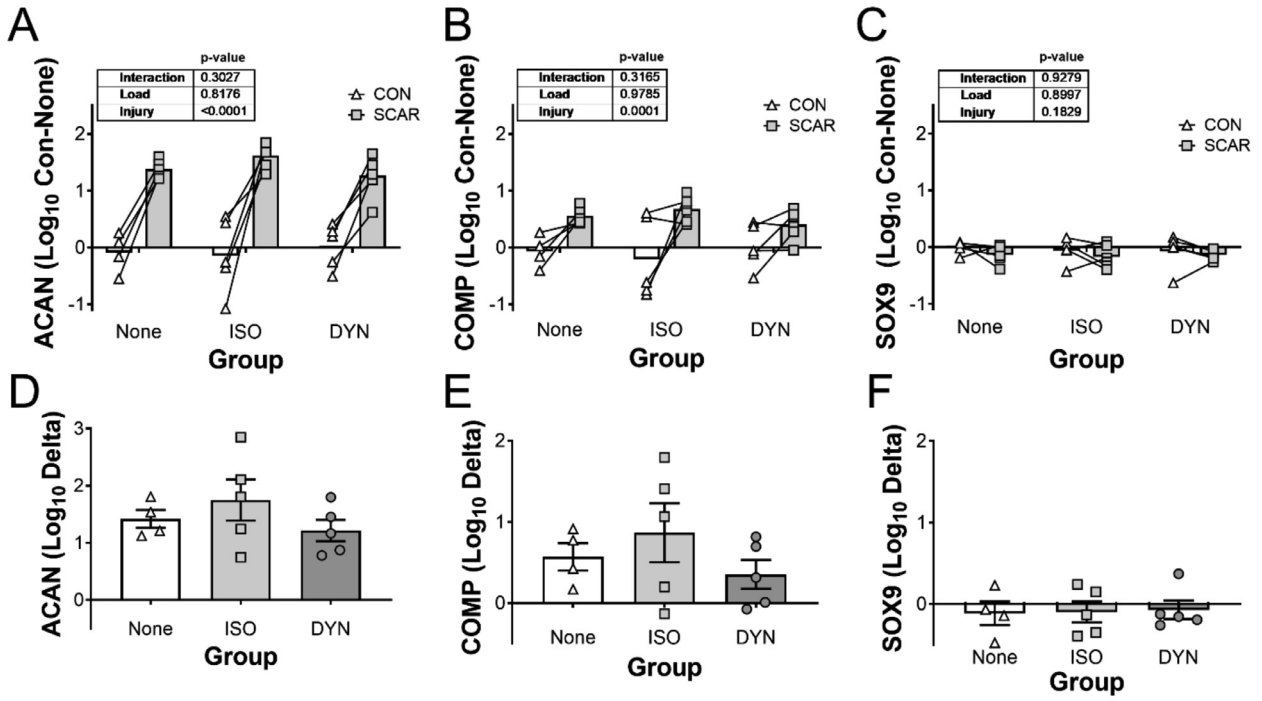

Fig. 5. Expression of selected fibrocartilage markers. **(A,D)** Expression of aggrecan increased in the patellar tendon scar. **(B,E)** COMP expression increased in the patellar tendon scar and was unaffected by load. **(C,F)** SOX9 expression did not change.

support of this hypothesis, Fig. 1 shows an increase in cellularity at the site of tendon injury. Both chronic tendinopathy and the biopsy punch also increased collagen Ia1 and IIIa1 expression in the injured tendon [38]. Similarly, Corps and colleagues [39] found that COLIa1 expression increased (8-fold) in painful human tendinopathy but decreased (on average) in samples from ruptured tendon. The 4.13-fold increase (FDR p-value = .002) in COLIa1 expression that we observe in the rat model of patellar tendinopathy correspond nicely with the human tendinopathy data.

Beyond the targeted analysis above, when comparing our transcriptomic data to the only published transcriptomic analysis of human tendinopathy [12] many important similarities were noted. First, the global pathway analysis performed by Jelinsky and colleagues identified genes encoding for the electron transport chain and oxidative phosphorylation as two of the most downregulated pathways in human tendinopathy. Our analysis likewise shows that the largest regulated gene set relative to gene set size was mitochondrial genes and like the human tendinopathy samples we found that most of the mitochondrial genes were downregulated in the injured patellar tendon. Second, Jelinsky and colleagues found that expression of extracellular matrix related (focal adhesion, integrin signaling, and collagen) and cell cycle progression genes were upregulated [12]. Extracellular matrix genes were the second most regulated group in our transcriptomic analysis and as in human tendinopathy, we found that expression of these genes was increased in the injured tendon. Third, the top regulated transcripts shared between the human and rat tendinopathy data sets were: *BNC2, CTHRC1*, and *MARCKS*. Basonuclin 2 is one of the top regulated TFs in fibroblasts during early tendon development (E11 -> E13) [40]. CTHRC1 is located in the ECM and may negatively regulate collagen synthesis [41]. The myristoylated alanine-rich C-kinase substrate (MARCKS) is important in fibroblast migration [42]. These data suggest that in both human tendinopathy and the rat patellar tendon injury model there is a signature of fibroblast migration into the tendinopathic region (MARCKS), early tendon development (BNC2), and the acute inhibition of collagen synthesis (CTHRC1). Fourth, the change in collagen gene expression was almost exactly the same in the rat patellar tendon model and human tendinopathy in general [12] and patellar tendinopathy more specifically [37]. Taken together, these data suggest that the rat patellar tendon injury model shows a change in gene expression that is characteristic of human tendinopathy.

Having established the relevance of the rodent model, we next addressed the effect of loading on tendinopathic tissue. The resulting data suggest that isometric loading (four 30 s contractions, each separated by two minutes of rest) is beneficial to treat

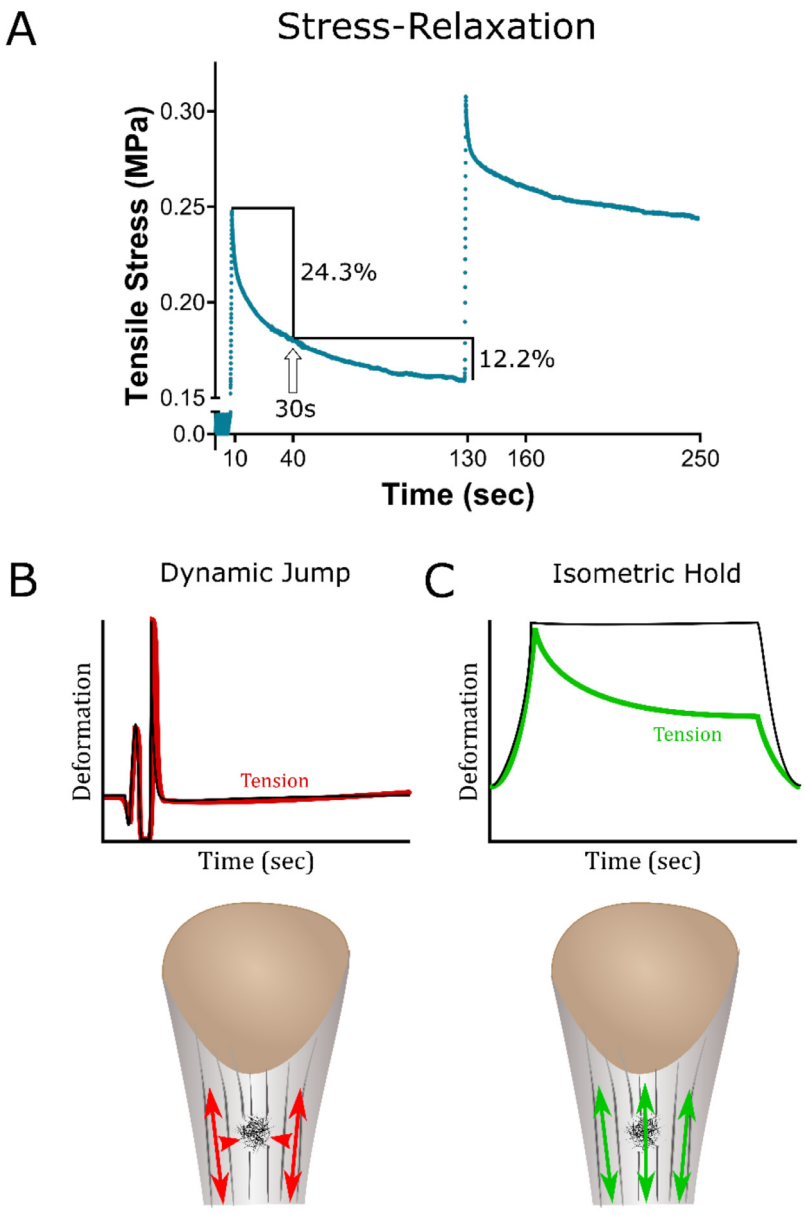

Fig. 6. Measuring and modelling stress shielding and stress relaxation. **(A)** Following 10 pre-conditioning cycles, rat PT underwent a stress relaxation test (2 min hold following a 0.2MPa stress). Note that 75% of total relaxation occurs in the first 30 s. **(B)** Dynamic loading (such as during running or jumping) occurs quickly causing load to be shunted around the injured region. With a central core injury, the injured region is loaded in radial compression as the tendon undergoes isovolumetric elongation. **(C)** Stress-relaxation decreases stiffness of the surrounding collagen below that of the injured region allowing load to pass though the injured region. Uniaxial load passing though the injured region provides a signal that increases the expression of genes enriched in mature and developing tendons.

tendinopathy because we found that expression of the tendon-enriched marker, scleraxis, increased 2.62-fold relative to an injured unexercised patellar tendon (6.8- versus 2.6-fold higher than their respective contralateral patellar tendon group) and the primary matrix gene, collagen Ia1, increased 3.12-fold relative to an injured unexercised patellar tendon 18 h after isometric loading. By contrast, dynamic loading (360 contractions over eight minutes with each contraction lasting 333 ms so that both the exercise duration and the time under tension were matched) tended to decrease scleraxis and collagen Ia1 expression and increased collagen IIa1 expression relative to the isometric group. These data suggest that different forms of loading can result in diametric changes in gene expression in the same tissue.

One possible explanation for the increase in scleraxis and collagen Ia1 expression following isometric loading is that this form of contraction resulted in a uniaxial strain across the cells within the injured region of the tendon. Following injury, the damaged region of a tendon can be shielded from further injury through a process called stress-shielding. While stress-shielding is widely appreciated in bone [43], it is less well characterized in tendon. In tendon, stress-shielding occurs when the strong, aligned, healthy matrix shields a damaged region from load (stress). Preventing stress from passing through the damaged matrix may prevent catastrophic injury; however, without a uniaxial tensile force, proper matrix synthesis and orientation is not possible [25,33]. Isometric contractions can overcome stress-shielding through a process called stress-relaxation. Stress-relaxation is a property of viscoelastic materials, such as a tendon, whereby a material undergoes a time-dependent decrease in stress at a constant strain [30]. In large human tendons, stress decreases by ~60% in the first 30 s [30]. Similarly, in the rat patellar tendon we have seen that within the first 30 s after applying a static load stress in the patellar tendon approaches a nadir after decreasing ~25% (Fig. 6). We hypothesize that as the strong portions of the tendon relax (become less stiff), the injured region becomes stiffer than the healthy tendon and a directional tensile strain passes through the scar and is sensed by the cells (Fig. 6).

Collagen Ia1 expression following isometric loading increased significantly more relative to the contralateral control limb than seen in the dynamic group. In humans, one hour of leg extensions increased tendon collagen protein synthesis (measured by radiolabeled proline) that peaked at 24 h post exercise and remained elevated 42 and 72 h later [44]. This increase in collagen protein occurs in spite of an acute (4h) decrease in collagen Ia1 mRNA that returns to baseline levels 24 h after resistance exercise regardless of sex [45]. In animals, a similar increase in collagen protein synthesis is seen with increased load. For example, two days of overload of the chicken anterior latissimus dorsi increased collagen synthesis rates 5-fold [46]. These data suggest that loading increases collagen protein; however, whether this is the result of an increase in transcription as observed here or increased collagen translation remains unclear. It is possible that increases in collagen Ia1 mRNA are not the only way to increase collagen protein following loading.

Fibrocartilage forms as a musculoskeletal adaptation to compression. A tendon placed under compressive loads increases expression of markers of fibrocartilage such as the large proteoglycan aggrecan [47] and type II collagen [48]. The dynamic loading group showed a 6.6-fold increase in collagen IIa1 expression, whereas in the isometric group collagen IIa1 was unchanged. These data suggest that dynamic loading of a central core tendon injury may cause local compression of the cells within the scar. Increased type II collagen expression is seen in mid-portion Achilles tendinopathy [49]. Other markers of fibrocartilage, such as aggrecan were increased in the tendinopathic tissue regardless of loading. Aggrecan is a large negatively charged proteoglycan that pulls water into the tissue to help resist compression. Within a tendon, aggrecan is synthesized by cells in compressed regions [47,50]. In the current work, aggrecan expression increased more than 30-fold in the injured tendon, again suggesting that after a central core tendon injury compression increases within the injured region during normal cage activity. However, aggrecan expression did not increase further with acute loading. Interestingly, the cartilage-specific marker SOX9 was unchanged in the injured tendon even though it acts in an upstream activator for both collagen IIa1 [51] and aggrecan [52]. This might reflect the timepoint of collection, 18 h after loading where a load-induced increase may have subsided, and 15 days after injury, when a mature scar is expected to have already formed [24].

There were significant limitations to the current study. First, even though the loading protocols were matched for duration and time under tendon, the dynamic loading (with repeated shortening contractions) was more metabolically costly, and more muscle fatigue was noted. This suggests that there may have been greater load across the tendon in the isometric group. Second, we measured only changes in gene expression. It is possible that if protein levels/phosphorylation had been analyzed a different molecular response would have been found. Third, the greater change in gene expression between the isometrically loaded and contralateral control limb was driven by an apparent decrease in expression in the control patellar tendon in 2-3 animals in the isometric group. It is possible that there was a systemic effect of exercise that decreased gene expression in the healthy limb; however, it is also possible that this reflects an issue with the quality of RNA from those subjects. Any future work should include an uninjured group to account for any potential systemic effect. Fourth, the data does not distinguish the type or location of the cells that contributed to the expression data reported. Even though the tendons were trimmed to include the injured region, how much of the surrounding healthy tissue was taken likely varied between samples. Last, the timeline of collection was likely not ideal. A single time after loading is likely to have missed (either too early or too late) the peak expression of the genes measured. Despite these limitations, the data suggest that isometric loading promotes a tendon cell fate (Figs. 3A/E and 4D), whereas dynamic loading promotes a more

fibrocartilage expression (Fig. 4E) pattern in central core patellar tendinopathy.

Perspective

Prior work by our lab used a combined nutrition and isometric loading protocol to effectuate complete regeneration of a central core patellar tendinopathy in an elite basketball player [32]. The current work sought the molecular response to load in a rodent model of patellar tendinopathy. We propose that a scar forms within the core of the patellar tendon in part due to stress-shielding at the injured site. This stress shielding prevents the uniaxial stretch across the tenocytes within the scar that is necessary for aligned collagen deposition [33] and scar revision. Data from the current work support the hypothesis that isometric loading decreases stress-shielding, providing a tensile load stimulus to the cells within the scar, and increases the expression of genes enriched in mature and developing tendons (COL Ia1 and SCX). However, it is essential to better understand the time course of the response, where changes in expression occur, and whether there are systemic effects of loading before isometric loading can be used as a tool for resolving tendinopathy.

Materials and methods

Animals

All experiments were approved by the University of California Davis Institutional Animal Care and Use Committee under Protocol #21306. Wistar rats were obtained from Charles River Laboratories and housed in 12 h light and dark cycle with two to three per cage and *ad libitum* access to food and water throughout the experiment. For the sequencing experiments, patellar tendons were collected from four adult and four injured adult (4.5 months) rats. For the injury and loading experiments, eighteen adult (4 month) male rats had a patellar tendon injury induced using a 2mm biopsy punch (described in detail below) and recovered for 14 days with normal cage activity. The biopsy punch caused a complete tendon rupture in one rat, so seventeen rats were assigned to the experimental groups: scar (5), isometric (6), and dynamic (6).

Patellar tendon injury

Rats were anesthetized with isoflurane (2.5%). The right distal hindlimb was shaved and skin prepared aseptically. The patellar tendon was exposed using blunt dissection though a small longitudinal incision on the lateral aspect of the knee. To induce injury, a flat stainless-steel probe was placed underneath the tendon and a 2 mm biopsy punch was inserted into the central core of the patellar tendon distal to the patella (Fig. 1). Following injury, the wound was closed with sterile sutures and an intraperitoneal (IP) injection of buprenorphine (0.05-0.1 mg/kg) was administered as analgesic. Rats resumed normal walking within 15 min and were monitored daily for fourteen days post-op.

Loading protocol

Fourteen days post-injury, the rats in the isometric and dynamic groups were loaded using separate protocols that were matched for duration and time-under-tension. The “scar” only group was not loaded and only received the injury to the right patellar tendon. Exercised rats were anesthetized with isoflurane (2.5%) and placed in a 3D printed device with the right knee secured at 120°. The quadriceps muscle was stimulated using a Dual-Mode Muscle Level System (Aurora Scientific) to place a load across the patellar tendon. Both protocols used a pulse frequency of 100 Hz to elicit a near maximal contractile force and a pulse width of 0.2 ms. The isometric loading protocol consisted of 4 $\times$ 30 s isometric contractions each separated by two minutes of rest because most stress relaxation within a rat PT occurs in the first 30 s (Fig. 6). The dynamic loading protocol was 360 $\times$ 0.333 second contractions separated by one second of rest between contractions. Even though the contraction duration is much shorter in the dynamic group, ground contact time during running is $\sim$0.24 s. Therefore, the dynamic contraction protocol is similar to what an average runner would experience on an 8 min run. The loading protocols totaled 8 min under tension because tendon cells are maximally activated within 10 min of loading [53]. Following exercise, rats were once again injected with buprenorphine for analgesia and allowed to recover before being returned to their cage.

Tissue collection

Patellar tendon tissue was collected eighteen hours after loading, on day fifteen after injury. Rats were anesthetized and patellar tendon tissue surgically removed. The patellar tendon was trimmed to exclude enthesis regions. Patellar tendon tissue was weighed and then immediately snap-frozen in liquid nitrogen. Tendons were stored at -80°C until RNA extraction.

3’ Tag RNA-Seq and DGE analysis

RNA was isolated using an Animal Tissue RNA Extraction Kit (Norgen, Ontario CA). Sequencing libraries were generated using a 3′-Tag sequencing method (QuantSeq 3′ mRNA-Seq Library Prep Kit

FWD for Illumina; Lexogen, Greenland, NH) and sequenced using a HiSeq 4000 (Illumina, San Diego, CA) with single end 90 bp reads at the UC Davis Genome Center similarly to previous techniques described [54,55]. For each sample, approximately 10M single end reads were generated. Sequencing data were analyzed using CLC Genomics Workbench 21.0.3 (QIAGEN) similarly to a previous analysis [56]. Within Workbench, the quality of raw reads were checked with a Workbench program QC for Sequencing Reads 0.2 based upon FastQC and then trimmed with Trim Reads 2.5 within Workbench. Reads were mapped to the *Rattus novegicus* (Ensembl Rnor_6.0) annotated reference genome using Workbench's RNA-Seq Analysis 2.3 program. The Differential Expression for RNA-Seq 2.5 program within Workbench was used for evaluating gene expression differences between uninjured and scarred tendon tissue. Statistical significance in expression differences was determined by student's t test with false discovery rate multiple test correction $q < 0.05$. To generate a dendritic heat map of expression levels amongst all 8 samples, all differentially expressed genes ($q < 0.05$) were applied. Gene enrichment analysis based upon cell compartment gene ontology categories and $\log_2$ fold change of expression >0.6 and $q < 0.05$ was performed using Workbench's Gene Set Test 1.2 program. Moreover, pathway analysis was performed by applying the same list of genes, $\log_2$ fold changes, and q values to Advaita's iPathwayGuide (Advaita, Ann Arbor, MI) [57,58]. Relationships between the rat tendinopathy model and human tendinopathy were made by comparing gene symbols, fold changes, and q-values to a previously published human tendinopathy microarray expression study by Jelinsky and colleagues [12] (relative to matched normal human tendon) to scarred patellar tendon versus uninjured patellar tendon rat expression data.

Total RNA and gene expression- injury and loading

RNA was isolated using guanidinium thiocyanate-chloroform-isopropanol method. Prior to homogenization, patellar tendon tissues were finely minced on a glass plate on dry ice.

Samples were homogenized in TRIzol (Ambion, ThermoFisher, Waltham, MA). Phase separation was performed with chloroform (Millipore, ThermoFisher, Waltham, MA). The RNA containing aqueous phase was isolated. Glycogen (RNA grade, ThermoFisher, Waltham, MA) was added (0.4 μg/μL) to improve RNA precipitation and 100% isopropanol added to aid in the precipitation of RNA. RNA was washed twice with 70% ethanol, dried, and resuspended in water. RNA concentration was measured by spectrophotometry (Epoch Microplate Spectrophotometer, BioTek Instruments Inc., Winooski, VT). cDNA was synthesized by combining one microgram of RNA and a cDNA synthesis mix (MultiScribe RT, 10x RT buffer, 10x Primers, dNTPs, RNase inhibitor; Applied Biosystems, Foster City, CA) and running an RT reaction using the settings provided by the manufacturer. cDNA was diluted (1 μg/ 100 μL) prior to qPCR. Each sample was amplified in triplicate with 3 μL cDNA, 5 μL SYBR green (Bio-Rad, Hercules, CA; PCRbio.com, Wayne, Pennsylvania), and 2 μL of 10 uM primer (Invitrogen, ThermoFisher, Waltham, MA) (Supplemental Table). qPCR reactions were performed on a CFX384 Touch Real-Time PCR Detection System (BidRad, Hercules, CA). GAPDH was used as housekeeping control. Absolute C_T for GAPDH was not different between groups (Supplemental Fig. 1). Gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method [59].

Statistical analysis- injury and loading experiments

RNA in the injured and contralateral control tissues was compared using a paired t test since the analysis compared the uninjured and injured leg from the same animal. These fold comparisons could not be made for three rats because RNA of adequate quality could not be isolated from contralateral control leg. Outlier tests were performed with ROUT with Q = 1%. Gene expression relative to contralateral control tissue in the scar, isometric, and dynamic groups was performed as follows: 2^(-ΔΔCt) values were log transformed, fold change comparisons made where applicable (injured vs contralateral control), and then statistical tests performed. Gene expression in the scar and control legs for each loading group (no load, isometric, and dynamic) was compared using mixed model analysis, due to the data missing from the excluded callouses. Multiple comparisons were made using Tukey's multiple comparisons test. Within animal changes due to the injury were determined between the injured and contralateral control leg raw data and then log-transformed. Following log transformation, the groups were analyzed using one-way ANOVA. All statistical analysis was performed with GraphPad Prism software (version 8.3.1 for Windows).

Data and materials availability

The 3' TAG Seq data will be deposited in the Gene Expression Omnibus (GEO) and/or Sequence Read Archive (SRA) maintained by the NIH's National Center for Biotechnology Information

(NCBI). Metadata will be maintained to characterize each sample included in genomic analysis; these will include study design details, sample details (e.g., rodent age at time of experimentation, samples collected with anatomical information), and any relevant sample processing details as required and recommended by GEO, SRA, and NIH in general. All other data is available in the main text or supplementary materials.

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Declaration of competing interests

KB has received an honorarium, support for travel, and a research contract from PepsiCo on a nutritional intervention tangentially related to the current work. KB is paid by professional sports and Olympic teams to consult on tendon issues. DS and MM have no competing interests.

CRediT authorship contribution statement

Danielle Steffen: Conceptualization, Methodology, Formal analysis, Data curation, Writing – original draft, Writing – review & editing. **Michael J. Mienaltowski:** Formal analysis, Data curation, Writing – review & editing. **Keith Baar:** Conceptualization, Methodology, Formal analysis, Writing – review & editing.

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Supplementary materials

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