

Research Article

Sex differences in tendon structure and function[†]

Dylan C Sarver¹, Yalda Ashraf Kharaz², Kristoffer B Sugg^{1,3,4}, Jonathan P Gumucio^{1,3}, Eithne Comerford², Christopher L Mendias^{1,3,*}

Departments of ¹Orthopaedic Surgery, ³Molecular & Integrative Physiology and ⁴Surgery, Section of Plastic Surgery, University of Michigan Medical School, Ann Arbor, MI, USA

²Department of Musculoskeletal Biology, Institute of Ageing and Chronic Disease, University of Liverpool, Liverpool, UK

**Corresponding Author*

Christopher L Mendias, PhD
Department of Orthopaedic Surgery
University of Michigan Medical School
109 Zina Pitcher Place
BSRB 2017
Ann Arbor, MI 48109-2200
734-764-3250
734-647-0003 fax
cmendias@umich.edu

Author Contribution Statement

DS, EC and CM designed the experiments. DS, YAK, KS, JG, EC and CM conducted experiments, evaluated the results, and prepared the manuscript. All authors have read and approved the final submitted manuscript.

Running Title: Sex differences in tendons

[†]This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: [10.1002/jor.23516]

Additional Supporting Information may be found in the online version of this article.

Received 14 September 2016; Revised 21 December 2016; Accepted 6 January 2017

Journal of Orthopaedic Research

This article is protected by copyright. All rights reserved

DOI 10.1002/jor.23516

Abstract

Tendons play a critical role in the transmission of forces between muscles and bones, and chronic tendon injuries and diseases are among the leading causes of musculoskeletal disability. Little is known about sex-based differences in tendon structure and function. Our objective was to evaluate the mechanical properties, biochemical composition, transcriptome, and cellular activity of plantarflexor tendons from four month old male and female C57BL/6 mice using in vitro biomechanics, mass spectrometry-based proteomics, genome-wide expression profiling, and cell culture techniques. While the Achilles tendons of male mice were approximately 6% larger than female mice ($P < 0.05$), the cell density of female mice was around 19% larger than males ($P < 0.05$). No significant differences in mechanical properties ($P > 0.05$) of plantaris tendons were observed. Mass spectrometry proteomics analysis revealed no significant difference between sexes in the abundance of major extracellular matrix (ECM) proteins such as collagen types I ($P = 0.30$) and III ($P = 0.68$), but female mice had approximately two-fold elevations ($P < 0.05$) in less abundant ECM proteins such as fibronectin, periostin, and tenascin C. The transcriptome of male and female tendons differed by only 1%. In vitro, neither the sex of the serum that fibroblasts were cultured in, nor the sex of the ECM in which they were embedded, had profound effects on the expression of collagen and cell proliferation genes. Our results indicate that while male mice expectedly had larger tendons, male and female tendons have very similar mechanical properties and biochemical composition, with small increases in some ECM proteins and proteoglycans evident in female tendons. This article is protected by copyright. All rights reserved

Keywords: tendon; sex; mechanics; proteomics; transcriptomics

Introduction

Tendons play an important role in the storage and transmission of forces in the musculoskeletal system. Injuries and diseases of tendons are a significant healthcare problem for both physically active and inactive individuals. There are sex-based differences in the burden and disabilities associated with tendon disorders, with several studies indicating that women have greater disability and reduced function following tendon injury, even after patients have been treated for these disorders¹⁻⁵. Unlike other musculoskeletal conditions, even though there appears to be sex-based differences in the response of tendon to mechanical loading and in the recovery from injury⁶, there are currently limited to no sex-specific guidelines on the treatment and rehabilitation of patients with tendon disorders. One reason for a lack of sex-specific guidelines in the treatment of tendon disorders is likely due to a lack of understanding of fundamental biological differences between male and female tendons.

It is possible that sex-based differences in tendon morphology, composition and mechanical properties may contribute to the greater susceptibility that women have in developing tendinopathies. Mice are a commonly used animal model in the study of tendon biology and mechanics⁷. To gain greater insight in sex-based differences in tendon structure and function, we evaluated the histology, mechanical properties, biochemical composition and transcriptome of plantarflexor tendons from adult male and female C57BL/6 mice using in vitro tissue staining techniques, biomechanical testing, mass spectrometry-based proteomics and genome-wide expression profiling. The plantarflexor tendons were selected since they are major load bearing tendons in the hindlimb of mice. Based on epidemiological data, we tested the hypothesis that female tendons would demonstrate greater fatigue than male tendons when subjected to cyclical mechanical loading, and that this would be accompanied by a reduction in the content and expression of the major fibrillar collagens, type I and type III. To further address mechanism, we also conducted a series of in vitro studies to determine the effect of the sex of the serum in culture medium, or the sex of the extracellular matrix, on the behavior of male and female tendon fibroblasts in a cell autonomous or non-cell autonomous fashion.

This article is protected by copyright. All rights reserved

Methods

Animals. This study was approved by the University of Michigan IACUC. Four month old male and female C57BL/6 mice were purchased from Charles River (Wilmington, MA) and used in experiments within two weeks of purchase. Mice were housed two per cage and separated by sex in specific pathogen free conditions, and provided with food and water *ad libidum*. Prior to removing hindlimb plantarflexor tendons for analysis, mice were anesthetized with ketamine and xylazine. After tissues were surgically removed, mice were sacrificed by cervical dislocation and induction of a bilateral pneumothorax.

Histology. Tissue preparation for histology was performed as modified from a prior study⁸. Briefly, Achilles tendons were rinsed, snap frozen, and stored at -80°C until sectioned with a cryostat at 10 µm. Tissue sections, obtained from the mid-belly of the tendon, were stained with wheat germ agglutinin lectin conjugated to AlexaFluor 488 (WGA-AF488; Life Technologies) and DAPI (Sigma Aldrich, St. Louis, MO) to visualize extracellular matrix and cell nuclei, respectively. Images were captured on an Olympus BX-51 microscope and camera. ImageJ software (NIH, Bethesda, MD) was used to quantify tendon cross-sectional area (CSA) and cell density, which was determined as the number of nuclei per cross-sectional area.

Mechanical Properties Testing. Mechanical testing of plantaris tendons was performed as modified from prior studies^{9,10}. Plantaris tendons were removed by cutting the muscle belly just proximal to the myotendinous junction, and cutting the calcaneus distal to the enthesis. Tendons were wrapped in saline-soaked gauze and stored at -20°C until use. Prior to mechanical tests, tendons were thawed at room temperature and then submerged in PBS maintained at 25°C. The tendon was held at just taut length, and CSA was calculated from 10 evenly spaced width and depth measurements from high-resolution digital photographs of both top and side views of the tendon. Side views were obtained by using a prism embedded in the side of the bath. These measurements were fit to an ellipse, and the average ellipse area was used as the tendon CSA for mechanical properties measurements. The tendon

was then transferred to a bath for mechanical properties testing, containing PBS maintained at 25°C. Using braded silk sutures passed around the tissue and secured with square knots, the distal end of the tendon was secured by affixing the calcaneus to a 10N dual-mode servomotor/force transducer (model 305LR, Aurora Scientific, Aurora, ON, Canada), while the proximal end of the tendon was secured to a hook attached to a micropositioner. GLUture (Abbott Laboratories, Abbott Park, IL) was used to reinforce the proximal tendon attachment to the hook. Tendon length was adjusted to just taugt (approximately 1-3 mN of load), and recorded as L_0 . Each tendon was subjected to 300 load-unload cycles¹¹ at a constant velocity of 0.10 L_0/s , and a length change that was 10% of L_0 . Data was recorded using custom LabVIEW software (National Instruments, Austin, TX). Load, stress, tangent modulus (calculated from the linear region of the curve), and energy loss were determined for each load-unload cycle.

Proteomics. Mass spectrometry (MS) proteomic analysis was conducted as modified from previous studies^{12,13}. The Achilles and plantaris tendons from each animal were combined, homogenized, and proteins were extracted with 4M guanidine-HCl and subjected to in-solution trypsin digestion followed by reduction and alkylation. Four male and four female mice were used. LC-MS/MS analysis was performed using an Ultimate nanosystem (Dionex/Thermo Fisher Scientific, Loughborough, UK) coupled on-line to a Q-Exactive Quadrupole-Orbitrap instrument (Thermo-Scientific, Waltham, MA). Aliquots of tryptic peptides, equivalent of 1.7 μ g protein per sample, were loaded on column and run on a 1 hour gradient with an inter-sample 30 minutes blank as described previously^{12,13}.

Raw MS data files were analyzed with PEAKS (Version 7, Bioinformatics Solutions, Waterloo, ON, Canada) to identify protein composition. Searches were performed against the UniProt mouse proteome database (proteome UP000000589) and parameters used were: 10 ppm peptide mass tolerance and 0.01 Da fragment mass tolerance; one missed cleavage; one nonspecific cleavage; fixed modification, carbamidomethylation; variable modifications, methionine oxidation, proline oxidation, This article is protected by copyright. All rights reserved

and lysine oxidation. Search results were adjusted to 1% false discovery rate (FDR), unique peptides > 2 and confidence score > 50%. Label free quantification was also performed using Progenesis^{QI} LC/MS software (Waters, Elstree Hertfordshire, UK) where the spectra for each feature was exported to PEAKS7 against the UniProt mouse database and filtered using the same parameters. The resulting peptide-spectrum matches were re-imported into Progenesis^{QI}. The proteomics data set for this study has been deposited in the ProteomeXchange Consortium via the PRIDE¹⁴ partner repository (identifier PXD004612).

RNA Isolation, Microarray and Quantitative PCR. RNA isolation, microarray analysis and quantitative PCR (qPCR) were performed as previously described^{15,16}. A total of 6 animals were used for each sex. The Achilles and plantaris tendons from each animal were combined, homogenized in QIAzol (Qiagen, Valencia, CA), and RNA was isolated using a miRNeasy micro kit (Qiagen) supplemented with DNase treatment (Qiagen). After reverse transcription of RNA with iScript supermix (Bio-Rad, Hercules, CA), quantitative PCR (qPCR) was conducted in a CFX96 real time thermal cycler using iTaq SYBR green supermix reagents (BioRad). The $2^{-\Delta\Delta C_t}$ method was used to normalize the expression of mRNA transcripts to the stable housekeeping genes PPID and B2M, for cell culture and whole tissue qPCR respectively. A further normalization was performed from the female to the male group. A listing of RNA transcripts and primer sequences is provided in Supplemental Table S-1.

Microarray measurements of male and female tendon RNA were performed by the University of Michigan DNA Sequencing Core following manufacturer recommendations. Equal amounts of RNA isolated from three individual wells was pooled into a single sample for microarray analysis, and two pooled samples of male RNA and two pooled samples of female RNA were analyzed. RNA was pooled as gene expression from a pooled RNA sample is similar to the average from the individual samples comprising the pooled sample^{17,18}. RNA was prepared for microarray analysis using a GeneChip Pico WT kit (Affymetrix, Santa Clara, CA) and hybridized to Mouse Gene ST 2.1 strips (Affymetrix). Raw
This article is protected by copyright. All rights reserved

microarray data was analyzed using the oligo package of Bioconductor in the R statistical environment. ArrayStar version 14 (DNASTAR, Madison, WI) was used to generate a heat map using hierarchical clustering with Euclidean clustering and centroid linkage. The full microarray dataset is available through the NIH GEO database (ascension number GSE85415).

Cell Culture. Tendon fibroblasts were isolated from tail tendons as described^{16,19}. Male and female tail tendons were dissected separately, minced finely, and placed in a solution containing 0.2% type II collagenase (Life Technologies, Carlsbad, CA) in Dulbecco's Modified Eagle Medium (DMEM) for 2 hours at 37°C with agitation. Cells were then pelleted, resuspended in DMEM supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic (Life Technologies), and filtered through a 70 µm strainer. Male and female cells were plated separately on 100 mm type 1 collagen-coated dishes (BD Biosciences, San Jose, CA) and grown to 70% confluence in a humidified incubator maintained at 37°C and 5% CO₂. Fibroblasts were lifted with TrypLE (Life Technologies) and resuspended in 3-dimensional gels consisting of type I collagen that was extracted from the tail tendons of either male or female mice as described¹⁶. To prepare collagen gels, tails were removed from euthanized animals, washed with 70% ethanol, and tail tendon fascicles were extracted and placed in 0.2% acetic acid for 5 days at 4°C with intermittent agitation. The excess tissue was then pelleted, and the supernatant removed and lyophilized, before resuspension in 0.2% acetic acid for a final concentration of 2.7 mg/mL. Collagen gels were formulated to contain either 100% male, 100% female, or a 50%/50% male/female mixture of collagen, along with 10× Minimal Essential Medium (Life Technologies), and 0.34 NaOH in an 8:1:1 ratio. Approximately 5×10^5 male or female cells were embedded in a sex-specific or mixed-sex collagen gel, and placed in a 24-well tissue culture plate (BD Biosciences) to set. Collagen gels containing cells were cultured with growth media that contained DMEM and 1% antibiotic/antimitotic (Life Technologies), and either 10% male serum, 10% female serum, or 5% male and 5% female mouse serum (Biochemed, Winchester, VA). Media was changed daily for 3 days prior to RNA isolation.

This article is protected by copyright. All rights reserved

Statistics. Sample size was calculated from energy absorption values of tendons from a previous study⁹. To detect a 30% difference in energy absorption between males and females, using a power of 80% and an $\alpha=0.05$, required $N=6$ per each group. We accounted for an additional 3-4 mice to account for any animals that are lost prematurely prior to completion of the study. Differences between male and female tendons were tested using unpaired t-tests ($\alpha=0.05$) in Prism 7.0 software (GraphPad, La Jolla, CA). For fatigue testing, significance was adjusted for multiple observations using the Holm-Sidak method. Microarray data were tested using t-tests, and genes with a false discovery rate adjusted p-value of <0.05 were considered significant. For MS proteomics, statistical analysis on label-free datasets was performed by Progenesis^{Q1} on all detected features using transformed normalized abundances. Identification of proteins with two or more peptides, greater than 1.5-fold change in abundance, and a false discovery rate adjusted p-value of <0.05 were considered significant.

Results

Compared to males, the Achilles tendons of female mice had a 6% smaller cross-sectional area (CSA), while cell density was 19% larger (Fig. 1A-D). We then measured mechanical properties of plantaris tendons over 300 load-unload cycles. A representative stress-strain curve and the fatigue data for all 300 cycles is shown in Supplemental Figure S-1. Based on the overall shape of the force-loss versus cycle number curve, representative cycle numbers were picked to best represent the decline in load across the cycling protocol (Table 1). Tendon L_0 and cross-sectional area did not differ between the two groups (Table 1). Throughout the testing protocol, none of the measured parameters, including peak load, peak stress, tangent modulus, and energy loss, differed between the sexes at any specific cycle number (Table 1).

After testing mechanical properties, we sought to evaluate the protein composition of tendons using mass spectrometry-based proteomics. PEAKS software identified 240 proteins in male tendons and 279 proteins in female tendons. The entire dataset is available through the ProteomeXchange Consortium database as described in the methods, and subset of proteins of interest that are important components of the extracellular matrix (ECM) or play a known role in tendon cell biology are presented in Fig. 2. No significant sex-based differences were observed in the abundance of type I collagen, type III collagen, type XII collagen, biglycan, decorin, fibromodulin, lumican, versican, cartilage oligomeric matrix protein (COMP), thrombospondin-4 (Tsp4), and tenomodulin (Fig. 2A-J, N-O). However, compared to males, females had a 140% increase in fibronectin, a 182% increase in periostin, and an 89% increase in tenascin C (Fig. 2K-M).

We next evaluated differences in the transcriptome between male and female tendons. Of the over 41,000 genes measured, 408 transcripts had 2-fold differential expression between sexes, and many of these transcripts were not annotated or were non-coding RNAs (Supplemental Table S-2). The similarities between the transcriptome of male and female tendons is apparent in the heat map (Fig. 3).

We then evaluated the expression of specific transcripts of importance to tendon structure and

Accepted Article
fibroblast biology with qPCR. There were no differences in the expression for type I and III collagen, while there was an upregulation of scleraxis and a downregulation of elastin and tenomodulin (Fig. 4).

Finally, we sought to determine whether the sex of the extracellular matrix or the sex of the serum in the medium that fibroblasts were cultured in could impact the expression of collagen or genes associated with fibroblast proliferation or differentiation. In general, while some statistically significant differences were observed, the effects were not profound. Female cells had a downregulation in type I collagen when cultured in male ECM with mixed serum, and also when cultured in mixed ECM with female serum (Fig. 5A). Type III collagen was elevated in female fibroblasts compared to males no matter the sex of the ECM or serum (Figure 5B). For the cell proliferation marker Ki67, female cells had a downregulation in this gene when cultured in male ECM with mixed serum, and also when cultured in mixed ECM with female serum (Fig. 5C). Female cells had a downregulation in scleraxis when cultured in male ECM with mixed serum, female ECM with mixed serum, and also when cultured in mixed ECM with male serum (Fig. 5D). Regardless of the sex of the serum, female cells upregulated tenomodulin expression when cultured in mixed ECM (Fig. 5E).

Discussion

Tendon is an adaptive tissue that plays an important role in force transmission during locomotion. Despite well-documented sex-based differences in the burden and disabilities associated with tendon disorders¹⁻⁴, we have a limited understanding of biological and biomechanical differences in male and female tendons. A previous study in rats reported a greater load to failure in male Achilles tendons compared to females, although this appeared to be due to the larger size of male tendons, as no differences in failure stress were detected²⁰. Further, no differences in stiffness were detected, although female rats had a greater linear modulus than males²⁰. In human subjects, there are mixed reports in the literature about whether or not sex plays a role in determining tendon mechanical properties. Some have found males to exhibit larger tendon loads during plantarflexion compared to females and have related this to the increased incidence of Achilles tendon injury in males²¹. Others have reported females to have reduced tendon stiffness and efficiency during mechanical testing and accredit this to the differences in athletic performance and injury onset^{6,22}. Part of the discrepancies in results may be due to the different modes of testing, which include inverse dynamics, ultrasound, or measurements made from isolated tendon fascicles. Therefore, the purpose of this study was to investigate the functional and structural differences between male and female tendons *ex vivo* using non-destructive testing to better understand how sex influences the mechanical properties, proteome and transcriptome of tendons, as well as in what way the sex of the environment modulated fibroblast gene expression. Overall, our study has shown that although there were some subtle differences in the size, cellular composition, proteome and transcriptome, there were very little differences in the structure and function of male and female tendons.

One part of the central hypothesis tested in this study was that female tendons would demonstrate greater fatigue than male tendons when subjected to cyclical mechanical loading. To our surprise, the mechanical properties of plantaris tendons of male and female mice were nearly identical, demonstrating similar stress, stiffness, and fatigue profiles. Consistent with the mechanical properties

This article is protected by copyright. All rights reserved

Accepted Article

findings, type I and III collagen proteins, which are the primary load bearing structure for longitudinal force transduction in tendon^{23,24}, were present at a similar level between males and females. For other proteins that can determine the mechanical properties of tendons, a decrease in proteoglycan content is often concomitant with a weaker structure and reduced modulus^{25,26}. In agreement with the mechanical properties findings, the levels of the main proteoglycans in tendon, decorin, biglycan, fibromodulin, lumican, and versican were not different between the two sexes. COMP, a member of the thrombospondin family, plays an important role in the biomechanical properties of collagenous tissue due to its functions in collagen fibril organization and maturation, and compared with wild-type controls, mice deficient in COMP show significantly greater loss in collagen fibril organization during a stretch²⁷. COMP therefore likely plays a role in protecting the ECM from fatigue damage during repeated mechanical loading, which is consistent with the biochemical and mechanical findings in the current study. Additionally, no differences were found between male and female mice for Tsp4, which has a similar role to COMP in organizing the ECM²⁸. Tenomodulin is a type II transmembrane protein that is expressed in mature fibroblasts and may play a role in fibroblast differentiation²⁹, and was also present in similar levels between the two sexes.

Of the proteins that were present at elevated levels in female tendons compared to male tendons, three have been associated with an increased inflammatory response and decreased quality of tissue following repair. Fibronectin is a glycoprotein that interacts with cells via integrins to support cell adhesion, migration, growth, and differentiation, as well as collagen fibrogenesis^{30,31}. However, in a variety of ECM types, local elevations of fibronectin have been linked to a greater fibrotic response and decreased healing of tissue to injury^{32,33}. Periostin is a matricellular protein that helps to organize the ECM and participate in integrin-mediated signaling³⁴, but high amounts of periostin are associated with excessively fibrotic tissue repair^{34,35}. Tenascin C is a glycoprotein associated fibroblast migration and proliferation³⁶, and increased levels of tenascin C have been associated with inflammation and fibrosis during tissue repair³⁷. Fibronectin, periostin, and tenascin C proteins were all highly enriched

in female tendons compared to male tendons. Based on epidemiological and clinical studies of patients with tendinopathies, we hypothesized that female tendons from mice would have inferior mechanical properties to males during fatigue loading. Although speculative at this point, it is possible that the greater burden that female patients have compared to males for certain tendon disorders may not be due to greater mechanical damage, but rather a difference in the response of female cells to injury, repetitive stress, or functional recovery. Additionally, the greater abundance of tenascin C in females may explain the increase in cell density observed in these tendons.

In this study we profiled both the proteome and the transcriptome of adult mice. In humans, tendons appear to grow robustly until approximately 17 years of age, at which time protein synthesis is reduced dramatically³⁸. The cell density values in the current study are consistent with previous data from adult mice and rats^{39,40}. In mice, a similar response appears to occur between 2-3 months of age^{39,41}. As tendon is a relatively hypocellular tissue with low rates of protein turnover, changes in the transcriptome may take time to be reflected in the proteome. For example, although no difference in tenomodulin protein abundance was detected in our study, the transcript was downregulated in female tendons. Alternatively, posttranslational modifications may also contribute to differences in the relationship between transcript levels and protein abundance. In either case, it is interesting to note some differences between the proteome and transcriptome of adult tendons.

Sex has been reported to be a factor in the behavior of cells from male and female animals of the same species⁴². We sought to determine if tendon fibroblasts exhibited sex-based differences in the expression of genes involved in ECM production, including type I and III collagen, and cell proliferation and differentiation, including Ki67, scleraxis, and tenomodulin^{8,29,43}. In order to determine whether there were any cell autonomous or non-cell autonomous effects of sex on the behavior of fibroblasts, we used an in vitro 3D culture system that permitted us to control for the sex of the serum in the culture medium, as well as the sex of the matrix in which male and female fibroblast cells were embedded. For most of the genes that were evaluated, while statistically significant

Accepted Article

differences were observed in some cases, the effect was generally small. The only consistent pattern that emerged was that fibroblasts from female mice expressed slightly elevated levels of type III collagen than males without regard to the sex of the medium or the ECM. This is consistent with previous findings in human subjects⁴⁴, although the reason for the consistent induction in type III collagen in female cells is not clear. However, women are more susceptible to the development of keloid scars than men, and these scars are highly enriched in type III collagen^{45,46} and it is possible that female fibroblasts make type III collagen at higher levels than male fibroblasts.

Our study is not without limitations. While the plantaris tendon has a uniform morphology and arises from a relatively unipennate muscle, and is a useful model for certain types of tendons, the sex-based differences in the mechanical properties of the plantaris tendon might not reflect other tendons with a more complex geometry or those that exist in synovial sheaths. For in vitro studies, although we measured the expression levels of important ECM and cell proliferation genes, it is possible that sex as a biological factor has a greater impact on the expression of other genes important in tendon fibroblast function. Additionally, some of the ECM proteins are likely to be lost during the collagen extraction process. The collagen gels were not subjected to repeated mechanical loading, which differs from the mechanical loading environment that fibroblasts would experience in vivo. Cell density was calculated from sections obtained from the mid-belly of the tendon, and it is possible that there are regional differences in cell density along the length of the tendon. We did not control for the reproductive cycle of female mice in this study, however female mice were housed separately from males which limits the release of reproductive hormones, and ovariectomy had little effect on the mechanical properties of adult rat tendons²⁰. While we performed fatigue testing, we did not perform failure testing, and it is possible that more pronounced disparities in mechanical properties are present between male and female mice at higher strain levels. Despite these limitations, we think our study provides novel insight into structural and functional features of male and female tendons.

There are well-documented disparities in the burden and incidence of tendon injuries and diseases in men and women ^{21,47 48,49}. While females make up over half of participants in clinical research, many studies that use animal models or cells do not include sex as a factor in study design or interpretation ⁵⁰. In the current study, using C57BL/6 mice, which are the most common strain of mice in contemporary biomedical research studies and have the most extensively sequenced genome ⁵¹, we evaluated sex-specific differences in the structure, mechanical properties, proteome, and transcriptome of tendons in an integrated fashion. While some differences were detected, overall there was a high degree of similarity in the morphology and behavior of tendons between male and female mice. Although our findings in a murine model do not indicate substantial differences in tendon biology between male and female animals, due to the greater burden that women suffer for certain types of tendon disorders, further studies that use a translationally-relevant injury model would provide additional insight into sex as a biological factor in tendon biology.

Acknowledgments

This work was supported by NIH grants R01-AR063649, F31-AR065931 and F32-AR067086.

Accepted Article

References

1. Razmjou H, Davis AM, Jaglal SB, et al. 2011. Disability and satisfaction after rotator cuff decompression or repair: a sex and gender analysis. *BMC Musculoskelet Disord* 12:66.
2. Chung SW, Oh JH, Gong HS, et al. 2011. Factors affecting rotator cuff healing after arthroscopic repair: osteoporosis as one of the independent risk factors. *Am J Sports Med* 39(10):2099–2107.
3. Chung SW, Park JS, Kim SH, et al. 2012. Quality of life after arthroscopic rotator cuff repair: evaluation using SF-36 and an analysis of affecting clinical factors. *Am J Sports Med* 40(3):631–639.
4. Garner MR, Gausden E, Berkes MB, et al. 2015. Extensor Mechanism Injuries of the Knee: Demographic Characteristics and Comorbidities from a Review of 726 Patient Records. *J Bone Joint Surg Am* 97(19):1592–1596.
5. Galasso O, Riccelli DA, De Gori M, et al. 2016. Quality of Life and Functional Results of Arthroscopic Partial Repair of Irreparable Rotator Cuff Tears. - PubMed - NCBI. *Arthroscopy: The Journal of Arthroscopic & Related Surgery*.
6. Magnusson SP, Hansen M, Langberg H, et al. 2007. The adaptability of tendon to loading differs in men and women. *International Journal of Experimental Pathology* 88(4):237–240.
7. Hast MW, Zuskov A, Soslowsky LJ. 2014. The role of animal models in tendon research. *Bone and Joint Research* 3(6):193–202.
8. Schwartz AJ, Sarver DC, Sugg KB, et al. 2015. p38 MAPK signaling in postnatal tendon growth and remodeling. *PLoS ONE* 10(3):e0120044.
9. Mendias CL, Lynch EB, Gumucio JP, et al. 2015. Changes in skeletal muscle and tendon structure and function following genetic inactivation of myostatin in rats. *J. Physiol. (Lond.)* 593(8):2037–2052.
10. Gumucio JP, Flood MD, Phan AC, et al. 2013. Targeted inhibition of TGF- β results in an initial improvement but long-term deficit in force production after contraction-induced skeletal muscle injury. *J. Appl. Physiol.* 115(4):539–545.
11. Shepherd JH, Riley GP, Screen HRC. 2014. Early stage fatigue damage occurs in bovine tendon fascicles in the absence of changes in mechanics at either the gross or micro-structural level. *Journal of the Mechanical Behavior of Biomedical Materials* 38:163–172.
12. Peffers MJ, Thorpe CT, Collins JA, et al. 2014. Proteomic Analysis Reveals Age-related Changes in Tendon Matrix Composition, with Age- and Injury-specific Matrix Fragmentation. *J. Biol. Chem.* 289(37):25867–25878.
13. Kharaz YA, Tew SR, Peffers M, et al. 2016. Proteomic differences between native and tissue-engineered tendon and ligament. *Proteomics* 16(10):1547–1556.
14. Vizcaíno JA, Csordas A, del-Toro N, et al. 2016. 2016 update of the PRIDE database and its

related tools. *Nucleic Acids Res.* 44(D1):D447–56.

15. Oak NR, Gumucio JP, Flood MD, et al. 2014. Inhibition of 5-LOX, COX-1, and COX-2 increases tendon healing and reduces muscle fibrosis and lipid accumulation after rotator cuff repair. *Am J Sports Med* 42(12):2860–2868.
16. Hudgens JL, Sugg KB, Grekin JA, et al. 2016. Platelet-Rich Plasma Activates Proinflammatory Signaling Pathways and Induces Oxidative Stress in Tendon Fibroblasts. *Am J Sports Med* 44(8):1931–1940.
17. Kendzioriski C, Irizarry RA, Chen KS, et al. 2005. On the utility of pooling biological samples in microarray experiments. *Proc. Natl. Acad. Sci. U.S.A.* 102(12):4252–4257.
18. Chaillou T, Jackson JR, England JH, et al. 2015. Identification of a conserved set of upregulated genes in mouse skeletal muscle hypertrophy and regrowth. *J. Appl. Physiol.* 118(1):86–97.
19. Mendias CL, Gumucio JP, Lynch EB. 2012. Mechanical loading and TGF- β change the expression of multiple miRNAs in tendon fibroblasts. *J. Appl. Physiol.* 113(1):56–62.
20. Pardes AM, Freedman BR, Fryhofer GW, et al. 2016. Males have Inferior Achilles Tendon Material Properties Compared to Females in a Rodent Model. *Ann Biomed Eng.*
21. Greenhalgh A, Sinclair J. 2014. Comparison of achilles tendon loading between male and female recreational runners. *Journal of Human Kinetics* 44:155–159.
22. Kubo K, Kanehisa H, Fukunaga T. 2003. Gender differences in the viscoelastic properties of tendon structures. *Eur J Appl Physiol* 88(6):520–526.
23. Willett TL, Labow RS, Aldous IG, et al. 2010. Changes in collagen with aging maintain molecular stability after overload: evidence from an in vitro tendon model. *J Biomech Eng* 132(3):031002.
24. Wood LK, Kayupov E, Gumucio JP, et al. 2014. Intrinsic stiffness of extracellular matrix increases with age in skeletal muscles of mice. *J. Appl. Physiol.* 117(4):363–369.
25. Yoon JH, Halper J. 2005. Tendon proteoglycans: biochemistry and function. *J Musculoskelet Neuronal Interact* 5(1):22–34.
26. Gordon JA, Freedman BR, Zuskov A, et al. 2015. Achilles tendons from decorin- and biglycan-null mouse models have inferior mechanical and structural properties predicted by an image-based empirical damage model. *Journal of Biomechanics* 48(10):2110–2115.
27. Schulz J-N, Nüchel J, Niehoff A, et al. 2016. COMP-assisted collagen secretion--a novel intracellular function required for fibrosis. *Journal of Cell Science* 129(4):706–716.
28. Frolova EG, Drazba J, Krukovets I, et al. 2014. Control of organization and function of muscle and tendon by thrombospondin-4. *Matrix Biol.* 37:35–48.
29. Huang AH, Lu HH, Schweitzer R. 2015. Molecular regulation of tendon cell fate during development. *J. Orthop. Res.* 33(6):800–812.

- Accepted Article
30. French-Constant C. 1995. Alternative splicing of fibronectin--many different proteins but few different functions. *Exp. Cell Res.* 221(2):261–271.
 31. Pankov R, Yamada KM. 2002. Fibronectin at a glance. *Journal of Cell Science* 115(Pt 20):3861–3863.
 32. Chiang H-Y, Korshunov VA, Serour A, et al. 2009. Fibronectin is an important regulator of flow-induced vascular remodeling. *Arterioscler. Thromb. Vasc. Biol.* 29(7):1074–1079.
 33. To WS, Midwood KS. 2011. Plasma and cellular fibronectin: distinct and independent functions during tissue repair. *Fibrogenesis Tissue Repair* 4:21.
 34. Conway SJ, Izuhara K, Kudo Y, et al. 2014. The role of periostin in tissue remodeling across health and disease. *Cell. Mol. Life Sci.* 71(7):1279–1288.
 35. Ontsuka K, Kotobuki Y, Shiraishi H, et al. 2012. Periostin, a matricellular protein, accelerates cutaneous wound repair by activating dermal fibroblasts. *Exp. Dermatol.* 21(5):331–336.
 36. Jones FS, Jones PL. 2000. The tenascin family of ECM glycoproteins: structure, function, and regulation during embryonic development and tissue remodeling. *Dev. Dyn.* 218(2):235–259.
 37. Cohen C, Leal MF, Belangero PS, et al. 2016. The roles of Tenascin C and Fibronectin 1 in adhesive capsulitis: a pilot gene expression study. *Clinics (Sao Paulo)* 71(6):325–331.
 38. Heinemeier KM, Schjerling P, Heinemeier J, et al. 2013. Lack of tissue renewal in human adult Achilles tendon is revealed by nuclear bomb (14)C. *FASEB J.* 27(5):2074–2079.
 39. Mendias CL, Gumucio JP, Bakhurin KI, et al. 2012. Physiological loading of tendons induces scleraxis expression in epitenon fibroblasts. *J. Orthop. Res.* 30(4):606–612.
 40. Couppe C, Svensson RB, Heinemeier KM, et al. 2016. Quantification of cell density in rat Achilles tendon: development and application of a new method. *Histochem. Cell Biol.*
 41. Suominen H, Kiiskinen A, Heikkinen E. 1980. Effects of physical training on metabolism of connective tissues in young mice. *Acta Physiol. Scand.* 108(1):17–22.
 42. Zanotti S, Kalajzic I, Aguila HL, Canalis E. 2014. Sex and genetic factors determine osteoblastic differentiation potential of murine bone marrow stromal cells. *PLoS ONE* 9(1):e86757.
 43. Docheva D, Hunziker EB, Fässler R, Brandau O. 2005. Tenomodulin is necessary for tenocyte proliferation and tendon maturation. *Mol. Cell. Biol.* 25(2):699–705.
 44. Sullivan BE, Carroll CC, Jemiolo B, et al. 2009. Effect of acute resistance exercise and sex on human patellar tendon structural and regulatory mRNA expression. *J. Appl. Physiol.* 106(2):468–475.
 45. Thompson CM, Hocking AM, Honari S, et al. 2013. Genetic risk factors for hypertrophic scar development. *J Burn Care Res* 34(5):477–482.
 46. Gauglitz GG, Korting HC, Pavicic T, et al. 2011. Hypertrophic scarring and keloids: pathomechanisms and current and emerging treatment strategies. *Mol. Med.* 17(1-2):113–125.

47. Järvinen TAH, Kannus P, Maffulli N, Khan KM. 2005. Achilles tendon disorders: etiology and epidemiology. *Foot Ankle Clin* 10(2):255–266.
48. Maffulli N, Testa V, Capasso G, et al. 2008. Surgery for chronic Achilles tendinopathy produces worse results in women. *Disabil Rehabil* 30(20-22):1714–1720.
49. Silbernagel KG, Brorsson A, Olsson N, et al. 2015. Sex Differences in Outcome After an Acute Achilles Tendon Rupture. *Orthop J Sports Med* 3(6):2325967115586768.
50. Clayton JA, Collins FS. 2014. Policy: NIH to balance sex in cell and animal studies. *Nature* 509(7500):282–283.
51. Fontaine DA, Davis DB. 2016. Attention to Background Strain Is Essential for Metabolic Research: C57BL/6 and the International Knockout Mouse Consortium. *Diabetes* 65(1):25–33.

Figure Legends

Figure 1. Representative cross-sections of male and female Achilles tendons stained with WGA and DAPI. Quantitative analysis of (A) cross-sectional area (CSA) and (B) cell density. Values are mean \pm SD, N = 6 tendons for each group. Representative images for (C) male and (D) female tendons are shown. Scale bar is for both (C) and (D). Differences between groups were tested using t-tests (*, $p < 0.05$).

Figure 2. Proteomic analysis of male and female plantarflexor tendons measured with mass spectroscopy. Values are mean \pm SD, N = 4 for each group. Identification of proteins with two or more peptides, greater 1.5-fold abundance and FDR adjusted p-value of < 0.05 (*) were considered significant.

Figure 3. Heatmap of global gene expression data subjected to hierarchical clustering from microarrays of male and female plantarflexor tendons. N=2 pooled samples of three separate wells of RNA for each group.

Figure 4. Gene expression analysis of male and female plantarflexor tendons. Quantitative analysis of qPCR for (A) type I collagen, (B) type III collagen, (C) scleraxis, (D) elastin, and (F) tenomodulin. Target gene expression values were normalized to the stable housekeeping gene beta-2 microglobulin and then to relative male expression levels. Values are mean \pm SD, N = 5 for each group. Differences between groups were tested using t-tests (*, $p < 0.05$).

Figure 5. Sex-specific extracellular matrix and serum effects on male and female fibroblast gene expression. For each ECM and serum combination, male and female cells were used in separate collagen gels. Gene expression analysis of (A) type I collagen, (B) type III collagen, (C) Ki67, (D) scleraxis, and (E) tenomodulin for male and female fibroblasts cultured in extracellular matrix (ECM) from male mice, female mice, or both sexes, and serum from male mice, female mice, or both sexes. Target gene expression values were normalized to the stable housekeeping gene PPID and

then to relative male expression levels. Values are mean \pm SD, N = 5 for each group. Differences between groups were tested using t-tests (*, $p < 0.05$).

Accepted Article

Table 1. Mechanics and morphology of plantaris tendons from male and female mice. Values are mean±SD. N=9 for males, N=10 for females. Differences tested using t-tests ($\alpha=0.05$), and for stretches, Holm-Sidak corrections were performed to account for multiple observations. No differences were observed between male and female tendons.

	Male	Female
Cross-sectional area (mm ²)	0.07±0.01	0.06±0.02
Length (mm)	5.00±0.89	4.87±0.56
<i>Stretch 1</i>		
Peak load (mN)	407±115	398±94
Peak stress (MPa)	5.93±1.48	6.38±1.54
Tangent modulus (MPa)	79.0±15.7	90.8±19.9
Energy loss (μJ/mg)	110±38.5	111±28.2
<i>Stretch 20</i>		
Peak load (mN)	339±91	320±78
Peak stress (MPa)	4.94±1.15	5.16±1.39
Tangent modulus (MPa)	93.3±21.0	120±34.6
Energy loss (μJ/mg)	51.2±14.5	50.0±11.6
<i>Stretch 50</i>		
Peak load (mN)	321±88	300±79
Peak stress (MPa)	4.66±1.10	4.83±1.34
Tangent modulus (MPa)	95.8±21.9	121±35.1
Energy loss (μJ/mg)	44.1±12.4	42.7±10.0
<i>Stretch 100</i>		
Peak load (mN)	307±86	286±79
Peak stress (MPa)	4.48±1.08	4.60±1.30
Tangent modulus (MPa)	97.7±22.8	122±35.9
Energy loss (μJ/mg)	39.4±9.1	38.2±9.1
<i>Stretch 300</i>		
Peak load (mN)	288±86	264±84
Peak stress (MPa)	4.20±1.09	4.26±1.28
Tangent modulus (MPa)	101±24.9	125±37.4
Energy loss (μJ/mg)	33.0±9.1	32.1±8.3

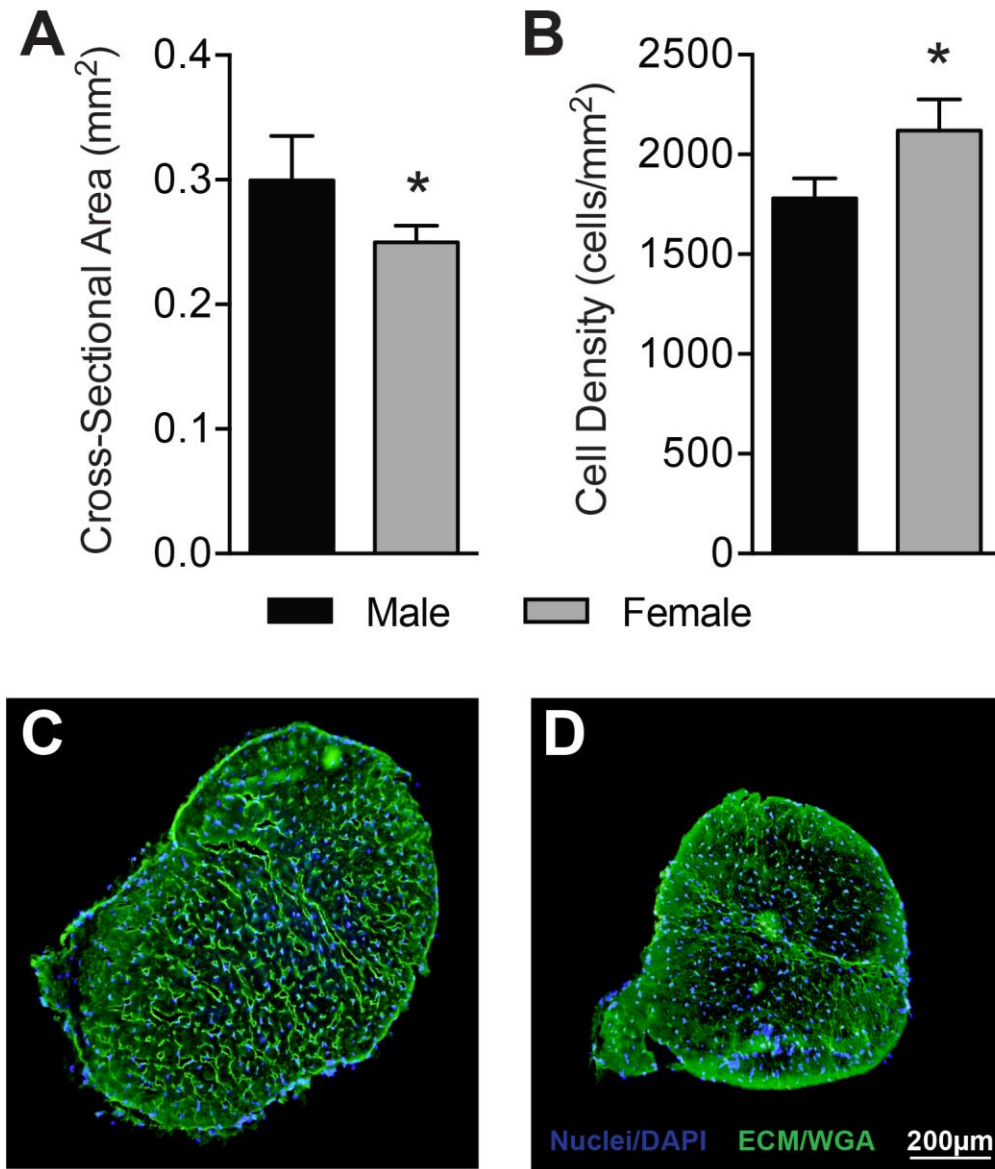


Figure 1

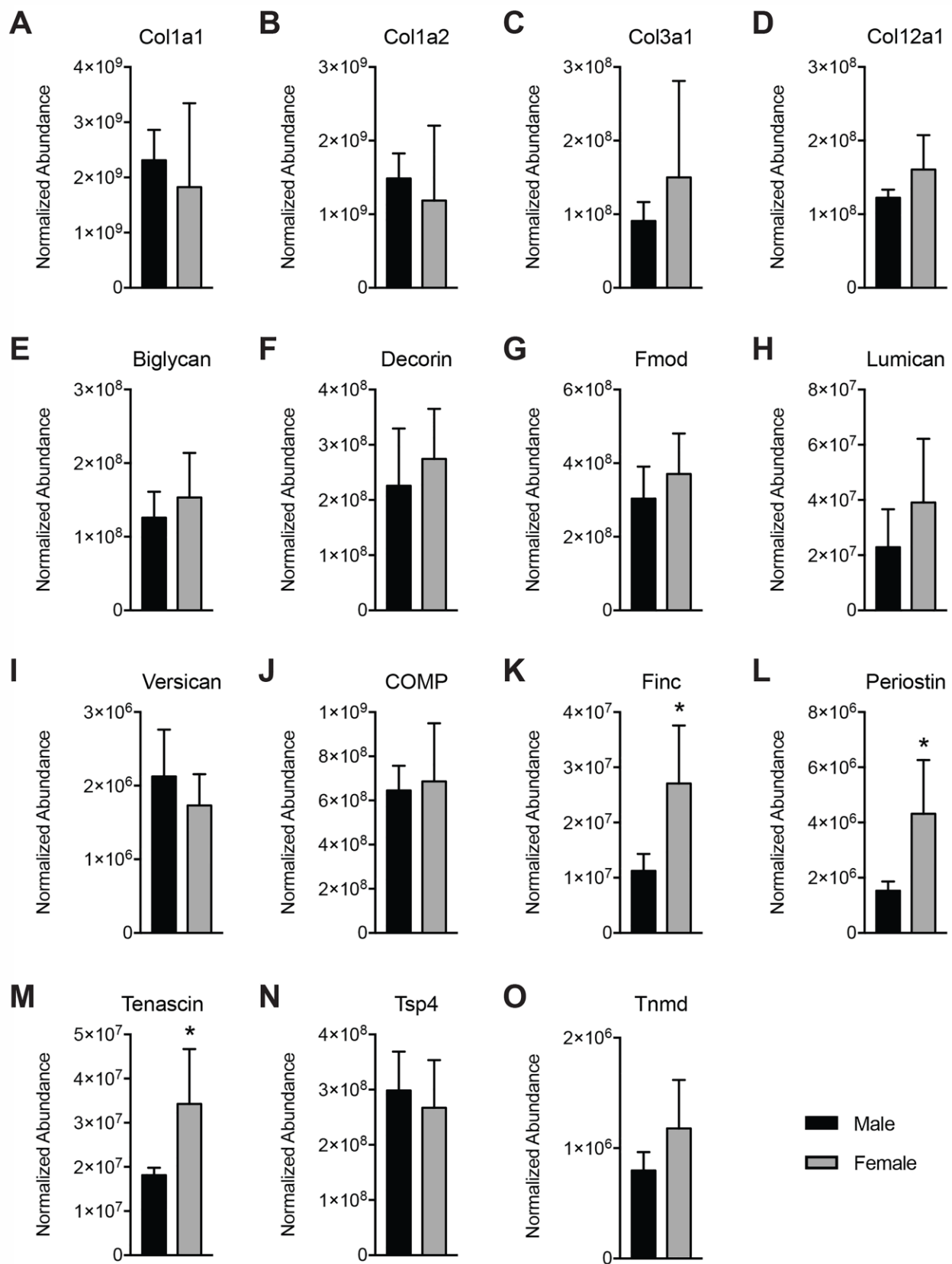


Figure 2

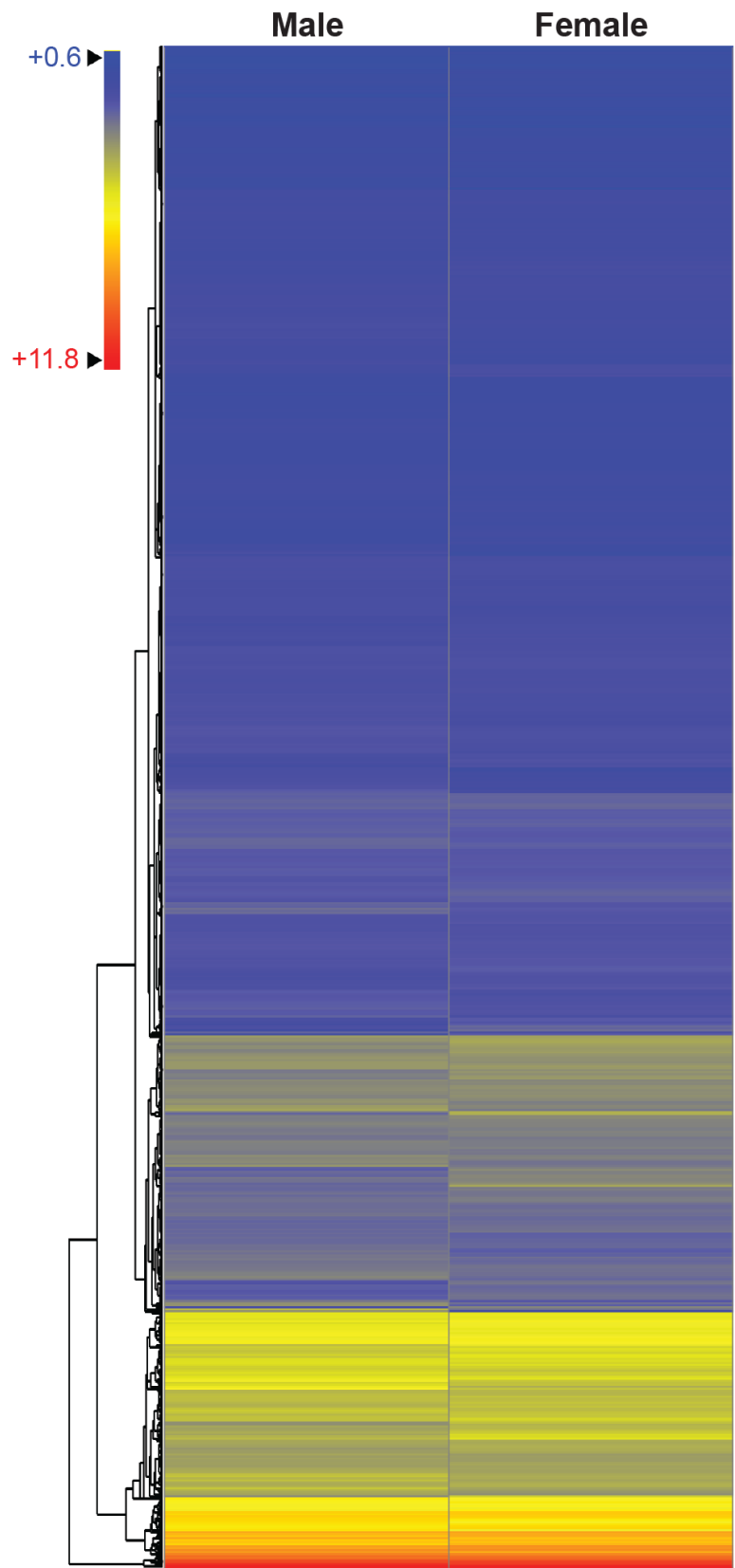


Figure 3

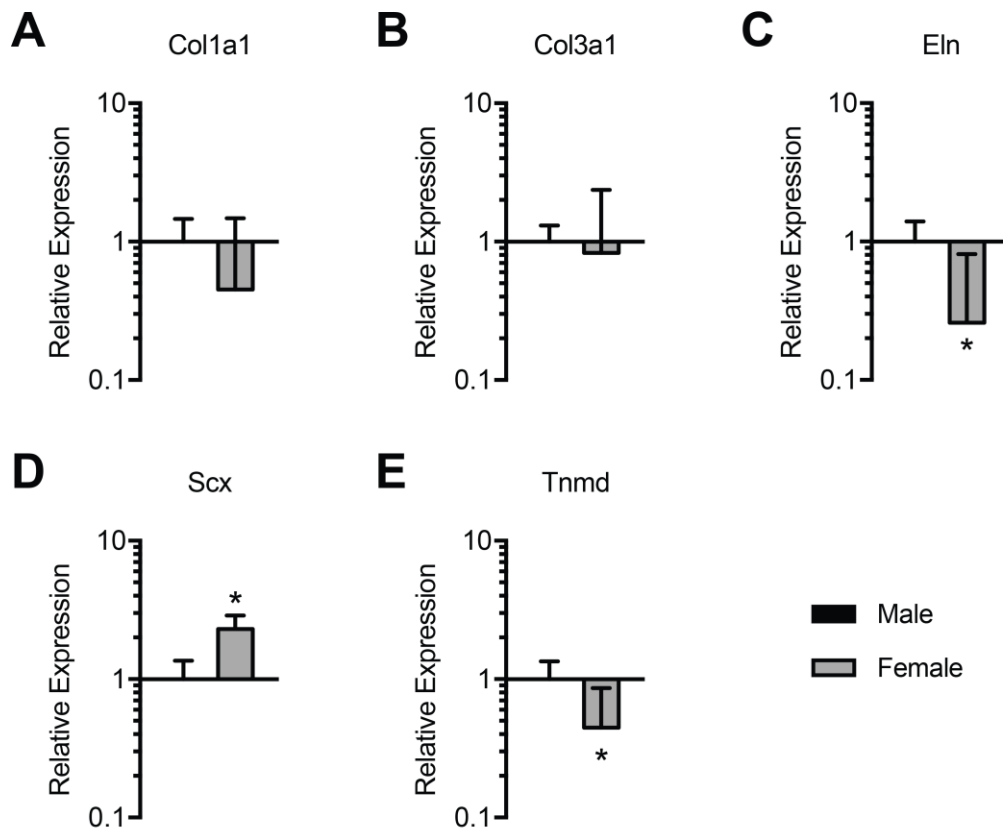


Figure 4

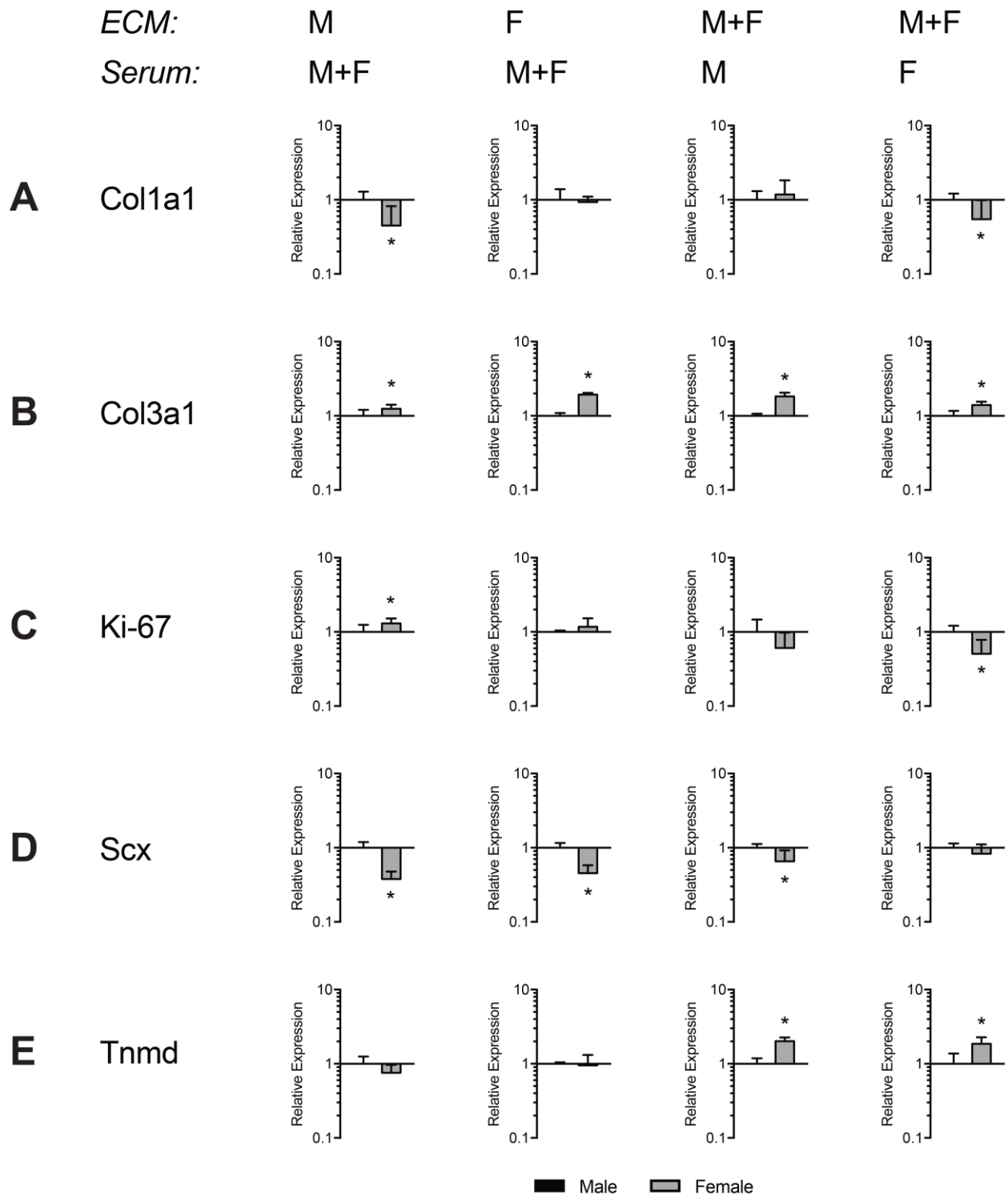


Figure 5