Research Article

Sex differences in tendon structure and function†

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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.

Keywords: tendon; sex; mechanics; proteomics; transcriptomics

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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 disorders [1-5]. 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 injury [6], 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 mechanics[7]. 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.

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 study [8]. 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 agglutin 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 -20ºC 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 taught 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 taught (approximately 1-3 mN of load), and recorded as L_o. Each tendon was subjected to 300 load-unload cycles [11] at a constant velocity of 0.10 L_o/s, and a length change that was 10% of L_o. 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 1.7µg 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,

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 PRIDE [14] 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 $2^{-\Delta\Delta Ct}$ 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

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 Biosceinces, San Jose, CA) and grown to 70% confluence in a humidified incubator maintained at 37°C and 5% CO_2. 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 described [16]. 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 Biosceinces) 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.

Statistics. Sample size was calculated from energy absorption values of tendons from a previous study [9]. To detect a 30% difference in energy absorption between males and females, using a power of 80% and an α=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 (α=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^{QI} 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_o 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

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 disorders [1-4], 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 detected [20]. Further, no differences in stiffness were detected, although female rats had a greater linear modulus than males [20]. 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 males [21]. 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

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 stretch [27]. 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 ECM [28]. Tenomodulin is a type II transmembrane protein that is expressed in mature fibroblasts and may play a role in fibroblast differentiation [29], 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 signaling [34], but high amounts of periostin are associated with excessively fibrotic tissue repair [34,35]. Tenascin C is a glycoprotein associated fibroblast migration and proliferation [36], and increased levels of tenascin C have been associated with inflammation and fibrosis during tissue repair [37]. 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 dramatically [38]. 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 species [42]. 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

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 subjects[44], 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 tendons [20]. 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.

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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 ± 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 ± 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 ± 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 ± SD, N = 5 for each group. Differences between groups were tested using t-tests (*, $p<0.05$).

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 (α=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
Stretch 1		
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
Stretch 20		
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
Stretch 50		
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
Stretch 100		
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
Stretch 300		
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

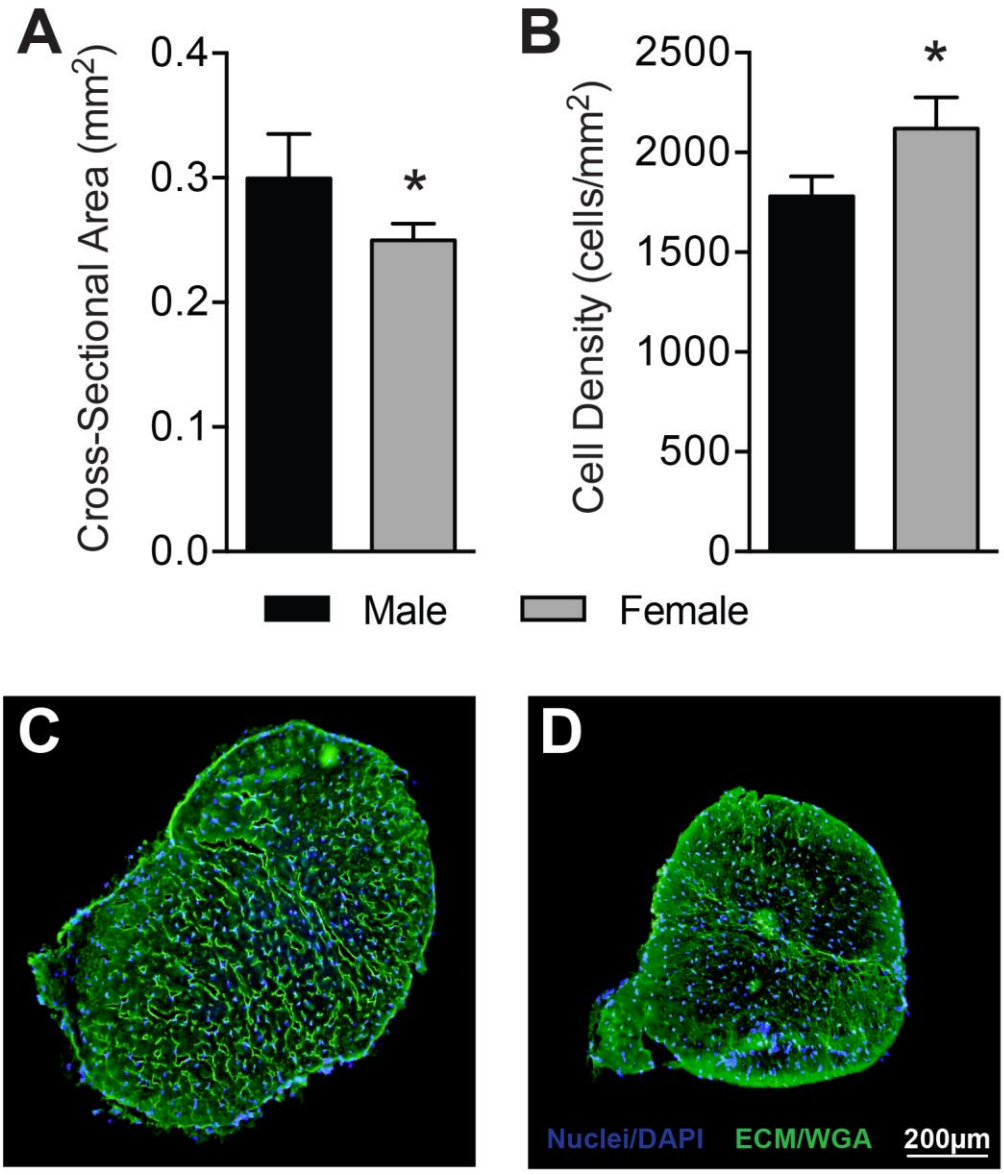

Figure 1

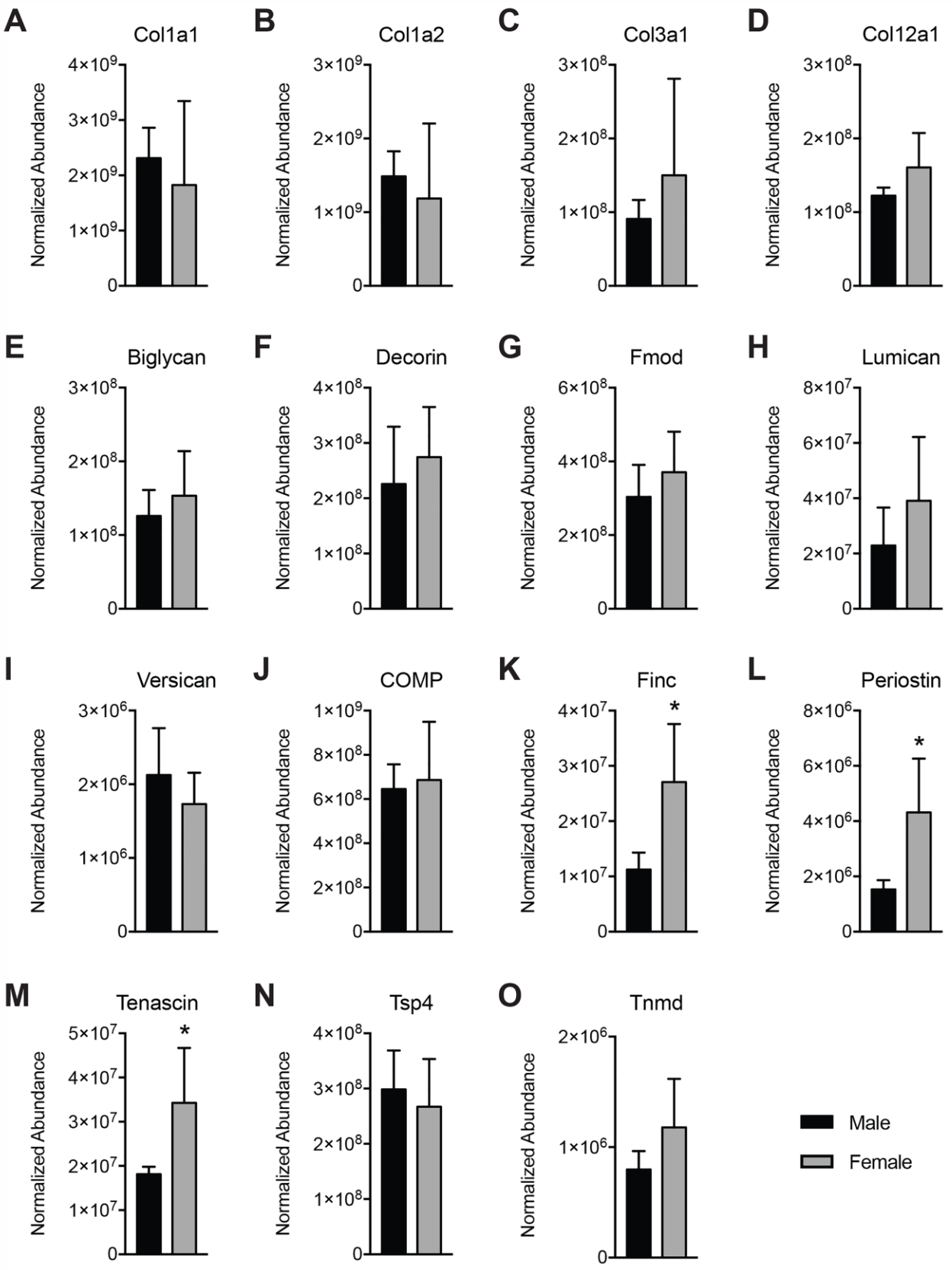

Figure 2

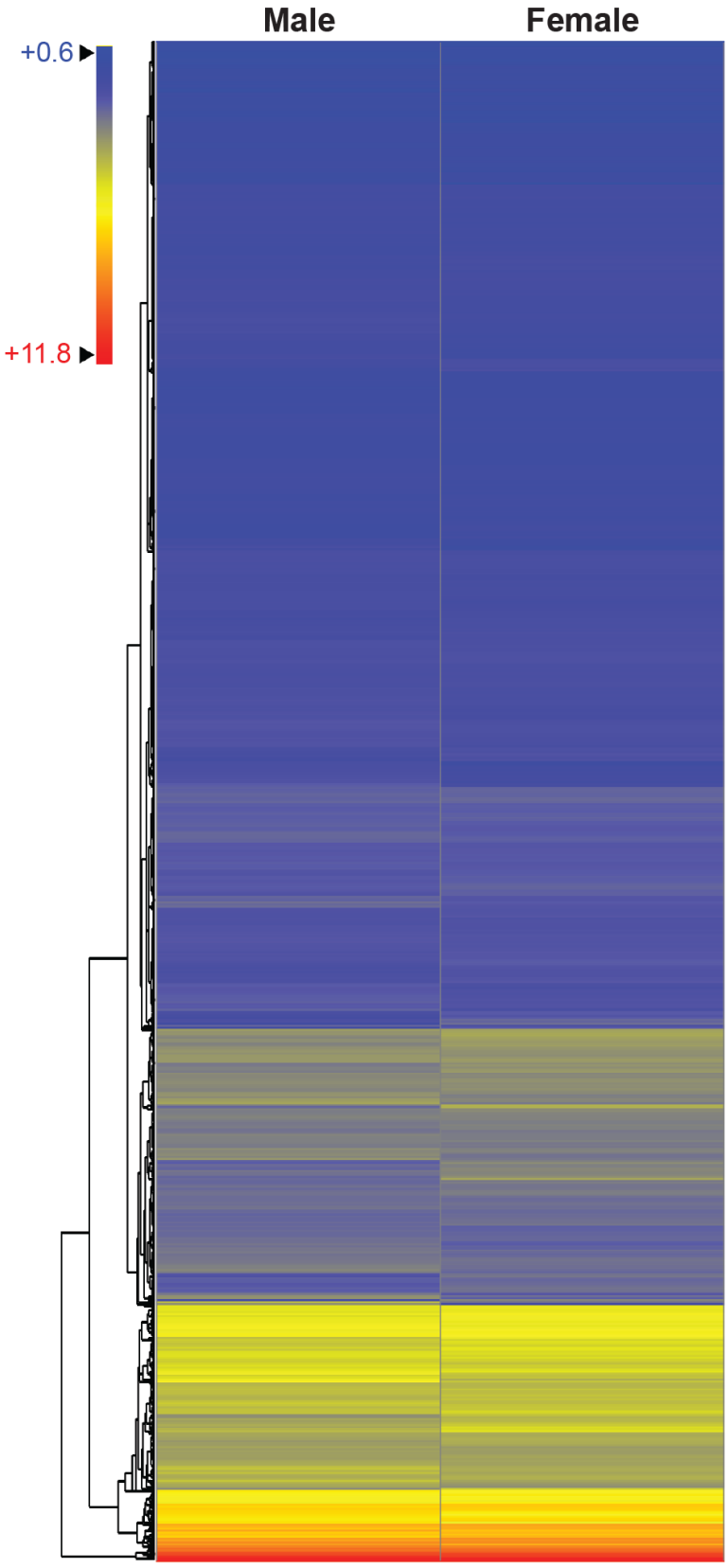

Figure 3

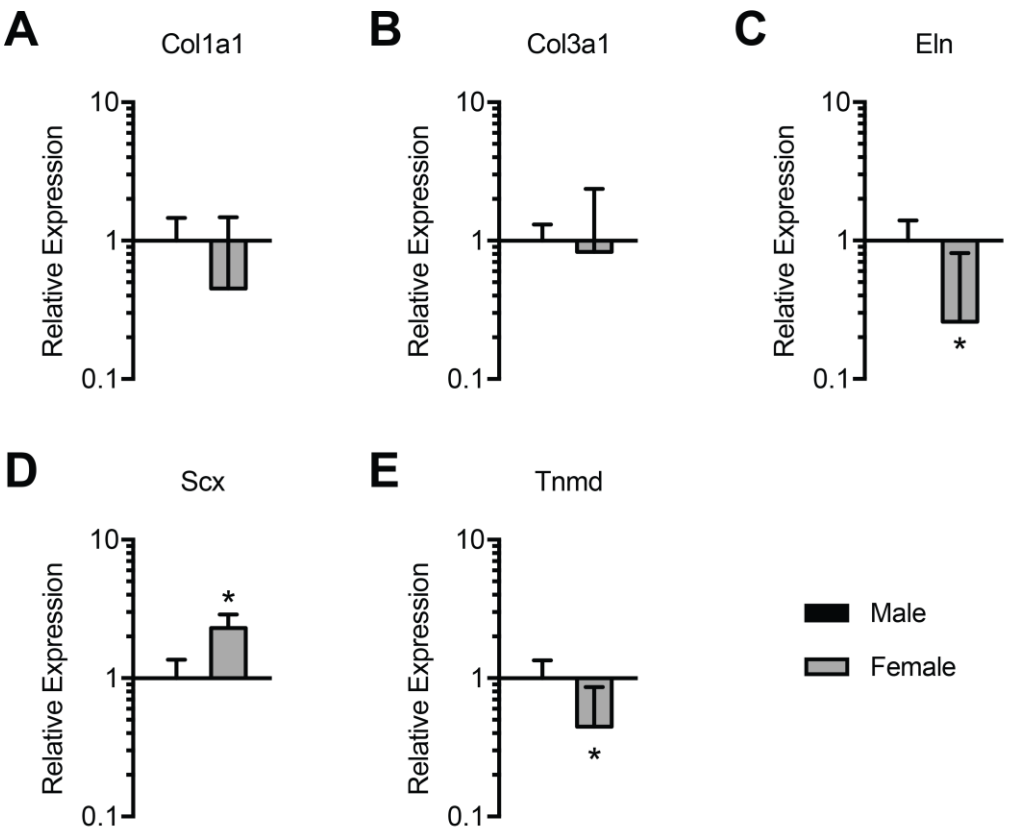

Figure 4

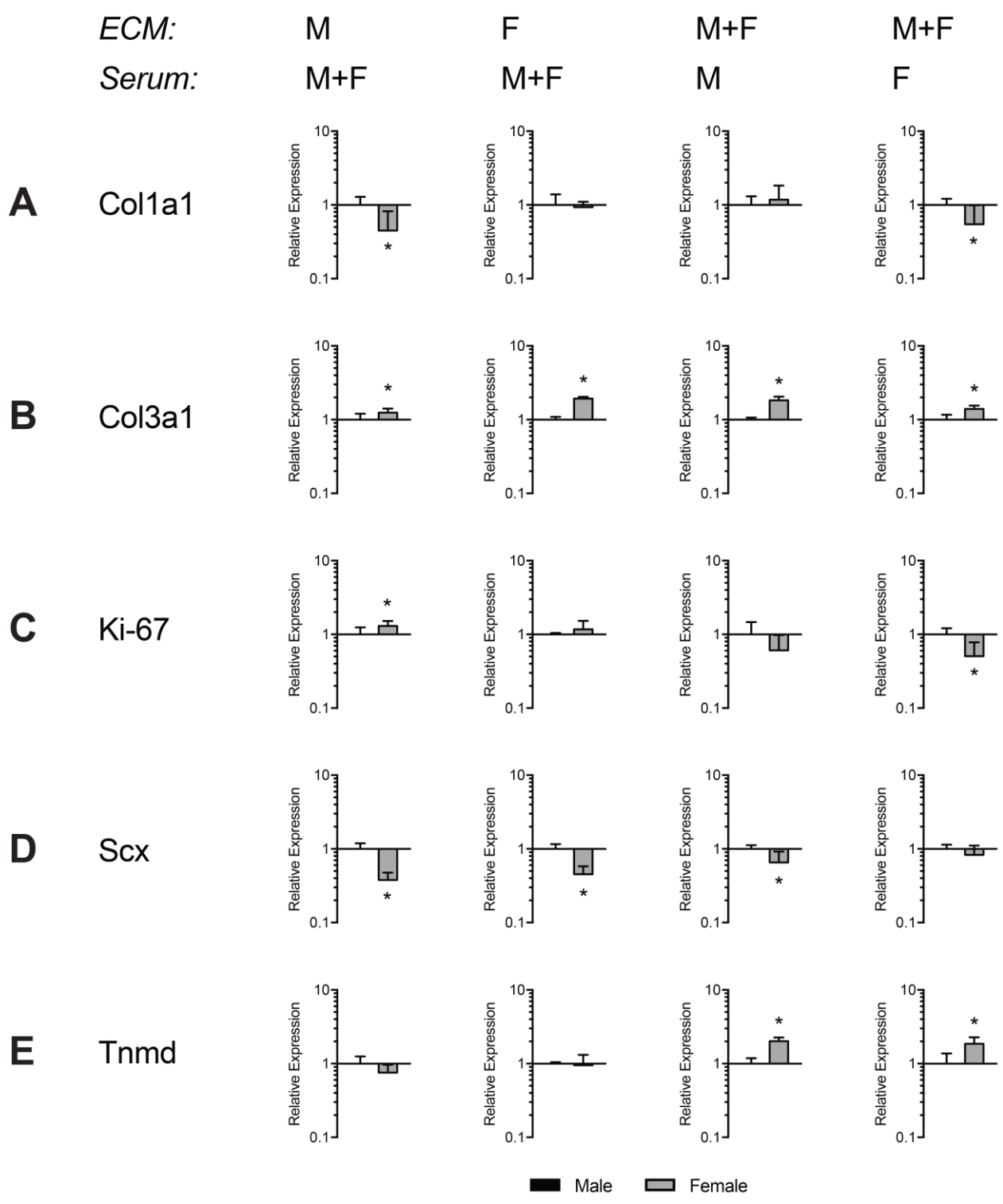

Figure 5