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Multi-omics analysis reveals sex-specific etiology of human muscle weakness following musculoskeletal injury

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Abstract

Background Musculoskeletal injuries comprise a growing source of disability worldwide, and the recovery of muscle strength following injury is a critical determinant of patient reported outcomes. Females experience exacerbated muscle atrophy, poorer outcomes, and higher re-injury rates, necessitating a comprehensive interrogation of sex-specific skeletal muscle differences. Our purpose in the current study was to perform an unbiased transcriptomic profiling of muscle samples to identify putative sex-specific molecular targets to enhance recovery in patients who underwent anterior cruciate ligament reconstruction (ACLR).

Methods We performed cellular phenotyping, bulk and single nucleus RNA-sequencing on muscle biopsy samples obtained from thirty-six participants (18 M, 18F). Muscle samples were obtained from the ACLR and contralateral limb with follow-up tissue collection of the injured limb also occurring at seven days and four months post-ACLR. Transcriptomic analyses illuminated putative molecular mechanisms through which sex influences muscle recovery following acute injury.

Results Females exhibited greater muscle atrophy relative to males at 4 months post-ACLR compared to the uninjured limb. Bulk and single nucleus paired-limb transcriptomic analyses revealed the emergence of sex-specific myonuclear signaling cascades that demonstrate impaired reactive oxygen species scavenging in females. Females exhibited attenuated *SOD2* expression that was associated with increased indices of oxidative stress and protein damage. Within females, angiogenesis signaling was also impaired and associated with capillary rarefaction after reconstructive surgery.

Conclusions These findings reveal inherent sex-based differences in muscle pathology that likely necessitate unique clinical treatments following musculoskeletal injury.

Keywords Skeletal muscle, ACL, Reactive oxygen species, Capillary, Anterior cruciate ligament reconstruction

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Background

Incidence rates of traumatic joint injury continue to climb worldwide contributing to chronic musculoskeletal disorders and disability [1, 2]. The burden and incidence of musculoskeletal injury and disorders is much higher in females [2, 3], who also have poorer recovery and long-term outcomes [4, 5]. Among the most common are injuries to the anterior cruciate ligament (ACL) [1], where females show exacerbated muscle atrophy, weakness, and poorer functional recovery [6–10]. Muscle deficits resultant from ACL injury are concentrated within the knee extensor quadriceps muscles with a distinct etiology that includes, but is not limited to, depressed protein anabolism, fiber atrophy, diminished intrinsic fiber contractile mechanics, oxidative stress, myostatin induction, neurophysiological dysfunction, and poor muscle quality [11–20]. There is limited understanding of sex-specific muscle alterations after ACL injury. This critical knowledge gap represents a significant opportunity to improve post-injury care as treatment is often a "one size fits all" approach that does not target the unique injury response in females [21–23].

Thus, our goal was to delineate sex-specific molecular effectors of skeletal muscle maladaptation following injury and ACL reconstruction (ACLR). We performed bulk and single-nucleus RNA-sequencing in patient biopsies, which we combined with cellular phenotyping approaches to define sex-specific transcriptional contributions to muscle deficits. We found that the transcriptomic response to injury and reconstruction is only ~ 40% shared between sexes. Female-specific pathways pointed to deficits in antioxidant and angiogenic capacities which likely contribute to poor functional outcomes following injury. This study offers the first large-scale transcriptomic assessment of this patient population at single-nucleus resolution, available for clinicians and the scientific community. Overall, we have identified unique myonuclear transcriptional signatures that need to be considered when optimizing treatment approaches specific to the needs of both sexes.

Methods

Please see Additional file 1: Supplemental Methods for complete methods (including: isometric dynamometry [24], immunohistochemistry [25–30], RNA-sequencing, single nucleus RNA-sequencing [31–35], deconvolution [36], and immunoblotting [13]).

Ethical approval.

This study was approved by the Institutional Review Board (#43046) at the University of Kentucky and performed in accordance with the ethical standards of the 1964 Declaration of Helsinki. Informed written consent was obtained from each patient or guardian before participation in this study. Additionally, all minors in this study assented to participation.

Subjects

Thirty-six (18F, 18 M) participants participated in this longitudinal observational study (Additional file 1: Fig. S1 and Additional file 1: Table S1, S2). Inclusion criteria included age between 15–40 years, ACL tear occurring no more than 6 months prior to enrollment, registering at least a 5/10 on the Tegner Activity Scale, and have sustained a non-contact sport-related ACL injury. Exclusion criteria included complete prior ACL injury/reconstruction, total knee dislocation, spinal fusion, taking anticoagulant medication, recent inflammation or infection of the lower limb(s), diabetes, pregnancy, diminished capacity to provide informed consent, or inability to attend study visits. The diagnosis of the ACL injury was made by one of two orthopedic surgeons via magnetic resonance imaging (MRI) and physical examination. All subjects were confirmed to have a complete tear of the ACL. Subject demographic information can be found in Additional file 1: Tables S1 and S2. Sample number is denoted in all figure legends. Bone-patellar-bone graft ACL surgeries were completed by one of two orthopedic surgeons. To document activity levels, participants completed the Cincinnati Sports Activity Scale [37]. All participants were classified as Level I (participating in sports 4 to 7 days/week). All participants participated in sports that required running, twisting, and/or turning (e.g. racquet sports, baseball, hockey, skiing, wrestling), and most participated in sports that involve jumping, hard pivoting, cutting (e.g. basketball, volleyball, football, soccer, gymnastics). There were no sex specific differences in activity level or demographic and anthropometric data.

Muscle Biopsies

Muscle biopsy specimens from the non-involved contralateral (Uninjured) vastus lateralis were collected prior to ACL surgical reconstruction (ACLR). Muscle biopsy specimens (~ 150 mg) from the vastus lateralis were obtained after application of local anesthesia (lidocaine) using a modified Bergström technique [38, 39]. Follow-up biopsies of the injured limb were obtained seven days post- reconstructive surgery (ACLR) and four months post-reconstructive surgery (4mo post-ACLR) at the UK Center for Clinical and Translational Science. The ACLR and Uninjured biopsies were collected at between 150 and 250 mm from the mid patella. The 4mo post-ACLR biopsy site was located between 30 and 40 mm proximal to the ACLR biopsy. Samples were dissected free of fat and connective tissue and were quickly blotted to remove excess blood; approximately 50 mg each were 1) immediately flash frozen in liquid nitrogen for transcriptomic

(bulk sequencing: $n = 13$ F, 14 M, single nucleus sequencing: $n = 2$ F, 2 M) and protein ($n = 9$ F, 9 M) analyses and 2) mounted in OCT on cork and flash frozen in liquid nitrogen–cooled 2-methylbutane ($n = 18$ F, 18 M). Samples were stored at −80 °C until processing. Tissue results presented encompass the Uninjured limb prior to surgical reconstruction, and the injured limb at 7 days post-ACLR and 4 months post-ACLR.

Muscle cryosectioning and immunohistochemistry

Performed as published previously [28, 30].

RNA fluorescent in situ *hybridization*

RRAD mRNA location in muscle cross sections was determined with single-molecule RNA fluorescent in situ hybridization using similar methods [40]. This method allows for the detection and visualization of a specific RNA molecule (*RRAD*) within tissue samples. Briefly, 7 µm thick were fixed in 4% paraformaldehyde for 15 min, ethanol dehydrated (50%, 70%, 100% ethanol for 5 min each), and incubated in 3% hydrogen peroxide for 10 min to quench endogenous peroxidases per manufacturer instructions (ACDBio). Antigen retrieval was performed using a protease (#322,336, ACDBio). Target mRNA was hybridized with a human *RRAD* probe (#1,587,641-C2, ACDBio), amplified, and detected using the TSA vivid 570 fluorescent reagent (#323,272, ACDBio). Samples were incubated overnight in a laminin primary antibody (#L9393, Sigma). The next day, slides were incubated in goat anti-rabbit AF488 (#A-11034, ThermoFisher) for 1 h. Slides were co-stained with DAPI (#D3571, ThermoFisher) prior to being mounted with fluorescent mounting media (#H-1000, Vector).

Statistical analysis

Statistical analysis of the RNA-sequencing and snRNA-sequencing data was performed using R and is detailed in the supplemental methods. For all other outcomes, statistical analysis was performed on continuous data using a two factor repeated measures ANOVA with a sphericity assumption. Šídák's multiple comparisons tests were performed with individual variances computed for each comparison to specifically compare post-injury data with the Uninjured limb value when significant sex x injury interactions were noted ($p < 0.05$). Pairwise comparisons of interest via Šídák's are denoted in figures with asterisks defined for given p-value references. Sex x injury interaction p-values are provided for all comparisons in the respective figure legends. For instances where two independent samples were compared, we performed two-sample t-tests. No data points were excluded from any analysis. Statistical testing is denoted in all figure legends. Data are presented as mean ± SD with individual data points overlaid on bar charts. No assumption violations, particularly normality and equal variance violations, were observed for parametric statistical testing. P-values less than 0.05 were considered statistically significant. All analyses were performed with GraphPad Prism 10.2.0.

Results

Disproportionate muscle atrophy in females after ligament injury and reconstruction is accompanied by sex-specific transcriptome alterations

We identified significantly greater atrophy in female muscle compared to male muscle 4 months following ligament injury and reconstruction (ACLR; Fig. 1A-D and Additional file 1: Fig. S1; $n = 18$ F, 18 M). In females, atrophy occurred in both muscle fiber types with greater atrophy in Type 2 fibers (Females: −19% (Type 1), −31% (Type 2), −26% (pooled); Males: −5% (Type 1), −8% (Type 2), −7% (pooled), Fig. 1C). Muscle weakness (both peak isometric torque and rate of torque development) was present in both sexes 4 months following reconstruction (Fig. 1E-F), but a trend emerged with greater relative muscle weakness in females (Torque: males: −36%,

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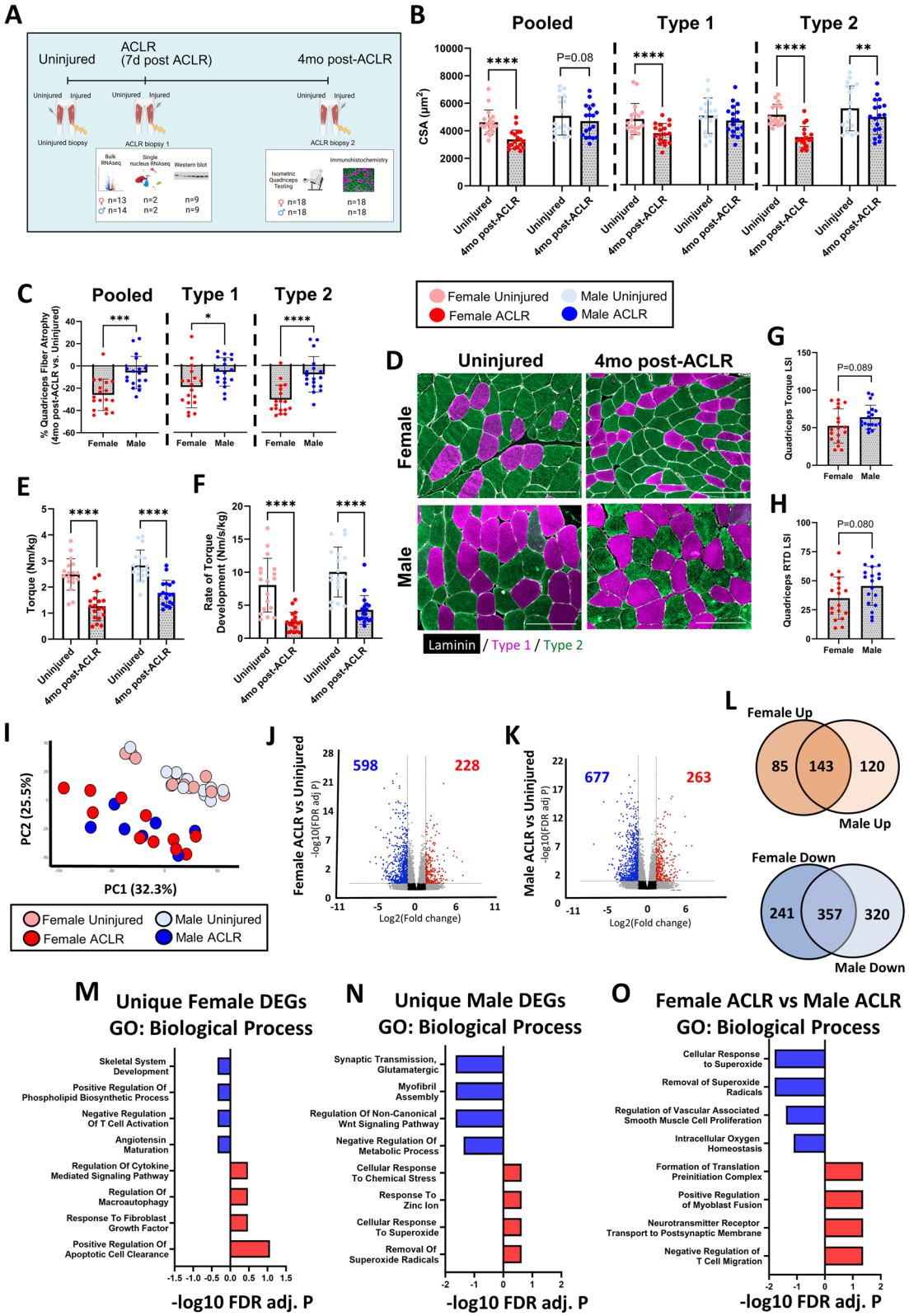
Fig. 1 Disproportionate muscle atrophy in females after ligament injury and reconstruction is accompanied by sex-specific transcriptome alterations. **A** Study diagram. **B** Pooled and fiber type-specific myofiber cross-sectional area (CSA; sex x injury interaction: Pooled CSA: $p = 0.0016$, Type 1 CSA: $p = 0.0246$, Type 2 CSA: $p = 0.0007$). **C** Percent atrophy comparing the 4 month post-ACLR to the Uninjured limb. **D** Representative immunohistochemical image of muscle fiber types 1 (pink), 2a (green), and laminin (white). Scale bar = 100 µm. **E** Muscle torque in ACLR and Uninjured limbs (sex x injury interaction $p = 0.3868$; main effect of sex $p = 0.0115$). **F** Rate of torque development in ACLR and Uninjured limbs (sex x injury interaction $p = 0.8747$; main effect of sex $p = 0.0279$). **G** Muscle torque limb symmetry index (LSI) displaying ACLR peak torque values as a percentage of their respective Uninjured-limb torque values. **H** Rate of torque development LSI displaying ACLR rate of torque development values as a percentage of their respective Uninjured-limb values. **I** Principal component (PC) analysis generated from transcriptomic data from female and male muscle. **J**-**K** Volcano plots displaying significantly different genes (FDR adjusted $p < 0.05$, log2FC ± 1.5) between ACLR and Uninjured muscle in females (**J**) and males (**K**). **L** Venn diagrams depicting overlap between upregulated or downregulated genes in female and male ACLR muscle. **M**–**O** Enriched upregulated and downregulated pathways after injury assessed via gene ontology unique to females (**M**) and males (**N**). **O** Enriched pathways directly comparing the ACLR limb between sexes. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.001$ vs Uninjured via Šídák's multiple comparison post hoc tests performed when significant interactions were detected via 2 factor ANOVA. *$p < 0.05$, ***$p < 0.005$, ****$p < 0.001$ per independent t-test. $n = 36$ (18F, 18 M) for panels **B**-**H** and n = 27 (13F, 14 M) for panels **I**-**O**. Study diagram created with BioRender



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females: −48%, $p = 0.089$, Fig. 1G; Rate of torque development: males: −54%, females: −65%, $p = 0.080$, Fig. 1H).

We sought to determine the muscle transcriptomic signature at an early time point following injury (7 days post-ACLR) to investigate molecular drivers underlying this sex disparity ($n = 13$ F, 14 M). Principal component analysis (PCA) revealed global differences in the muscle transcriptome of males and females after injury; however, a greater proportion of the transcriptome variance was explained by ACLR than by sex (F ig. 1I). We identified 826 differentially expressed genes (DEGs) in the female injured limb and 940 DEGs in the male injured limb (Fig. 1J-K). Sex-divergent transcriptional responses to ACLR were assessed by contrasting lists of significantly up and downregulated genes in each sex (Fig. 1L). Transcriptional responses were heterogenous between sexes, with only ~ 40% of up- and down-regulated genes conserved between the males and females. Gene ontology analysis revealed genes associated with superoxide removal and zinc ion response were uniquely upregulated in males (Fig. 1M-N). We observed a downregulation of genes involved in glutamatergic synaptic transmission and non-canonical WNT signaling in males, while both sexes shared a downregulated set of genes related to extracellular matrix remodeling and muscle contraction (Additional file 1: Fig. S2A). To further assess sex specific changes in the ACLR limb, we ran a direct comparison between the female ACLR limb and the male ACLR limb (Additional file 1: Fig. S2B). Similar to our within-subject sex comparison, gene ontology analysis revealed genes associated with superoxide removal and vascular cell proliferation were uniquely upregulated in males (Fig. 1O and Additional file 1: Fig. S2C-E).

Single-nucleus RNA-sequencing reveals divergent myonuclear transcriptome response after injury between sexes

To further dissect these transcriptional differences at a cell-specific level, we employed single nucleus RNA-sequencing on paired male and female (snRNA-seq; 2 F, 2 M) quadriceps biopsies from the non-injured Uninjured and ACLR limbs (Additional file 1: Fig. S3A-C). We subsetted myonuclei from our dataset (Fig. 2A; 19,039 myonuclei) and split by respective injury and sex status (Fig. 2B-C). Transcriptionally, sex differences were more pronounced in Type 1 than Type 2 myonuclei (Fig. 2D-K). Pathway analysis of sex-unique Type 1 myonuclear DEGs displayed significant downregulation of genes associated with insulin signaling pathways in females (Fig. 2F) and muscle contraction in males (Fig. 2G). In Type 2 myonuclei, we observed similar downregulation of genes associated with insulin signaling and nitric oxide response in females (Fig. 2J), whereas males exhibited downregulation of calcium-handling genes (Fig. 2K).

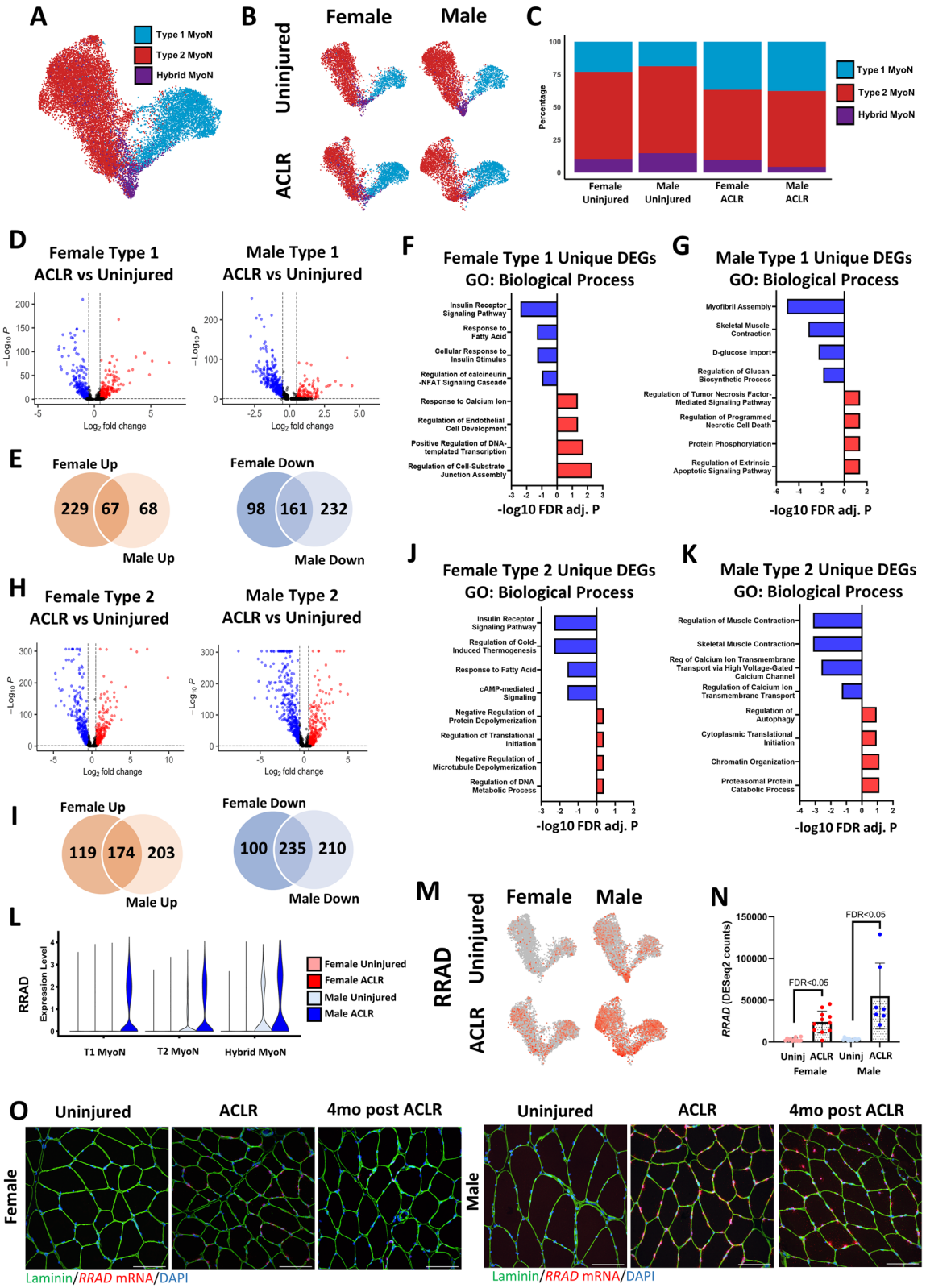
Given persistent strength deficits in the relative absence of atrophy in males, coupled with uniquely altered calcium-handling pathways, we sought to identify unique male DEGs that may explain this divergence. We identified RRAD (Ras-Related Glycolysis Inhibitor And Calcium Channel Regulator), a strong excitation–contraction uncoupler in striated muscle [41], as among the most differentially induced genes in male myonuclei that did not change following ACLR in female myonuclei (Fig. 2L-M). Bulk RNA-seq analysis supports greater induction of *RRAD* expression in males (Fig. 2N). Myonuclear *RRAD* induction through 4 months after ACLR in males was confirmed via fluorescent in situ hybridization of *RRAD* RNA (RNA-FISH; Fig. 2O). Deconvolution of our bulk RNA-seq analysis supported myonuclear induction of RRAD following ACLR (Additional file 1: Fig. S4A-C). Elevated and sustained *RRAD* induction may explain the persistent weakness in the absence of atrophy observed in males (Fig. 1A-E).

Deficient free radical scavenging in female muscle after injury

Bulk and snRNA-seq pathway analyses identified genes associated with free radical scavenging as elevated in

(See figure on next page.)

Fig. 2 Single nucleus RNA-sequencing reveals divergent myonuclear transcriptome response after injury between sexes. **A**-**B** Uniform manifold approximation and projection (UMAP) visualization of (**A**) integrated body myonuclei (excluding specialized myonuclei, MyoN) and (**B**) split by sex and injury. **C** Relative myonuclear proportion across sex and injury. **D** Volcano plot for Type 1 myonuclei between ACLR and Uninjured male or female muscle. **E** Venn diagrams depicting overlap between upregulated or downregulated genes in type 1 myonuclei between female and male ACLR muscle. **F**-**G** Enriched upregulated and downregulated pathways in type 1 myonuclei assessed via gene ontology after ACLR unique to females (**F**) and males (**G**). **H** Volcano plot for type 2 myonuclei between ACLR and Uninjured male or female muscle. **I** Venn diagrams depicting overlap between upregulated or downregulated genes in type 2 myonuclei between female and male ACLR muscle. **J**-**K** Enriched upregulated and downregulated pathways in type 2 myonuclei assessed via gene ontology after ACLR unique to females (**J**) and males (**K**). **L** Violin plot depicting *RRAD* expression (Male ACLR vs Uninjured body myonuclei: FDR adj $p < 0.05$, Female ACLR vs Uninjured body myonuclei: FDR adj $p > 0.05$). **M** Feature plots depicting *RRAD* expression across sex and injury. **N** *RRAD* expression assessed via RNA-seq across sex and injury. FDR adj $p < 0.05$ in both groups. **O** Fluorescent in situ hybridization of *RRAD* mRNA (RNA-FISH) in Uninjured, ACLR, and 4 months post-ACLR. Scale bar = 50 µm. $n = 4$ (2F, 2 M) for panels **A**-**M** and $n = 27$ (13F, 14 M) for panel **N**



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males (Fig. 1N) and downregulated in females (Fig. 1O). Given the well-characterized role of oxidative stress in muscle atrophy we speculated these observations may underscore differences in muscle atrophy between sexes [42, 43]. Manganese superoxide dismutase 2 (*SOD2*) was upregulated only in males in bulk RNA-seq, myonuclear snRNA-seq, and deconvoluted bulk RNA-seq datasets (Fig. 3A-C and Additional file 1: Fig. S2C and S4D-E). At the protein level, SOD2 declines in females, but not males, following ACLR (Fig. 3D-E), which may be due to male-specific declines in the expression of the SOD2-specific ubiquitin ligase, *AURKA* (Fig. 3F) [44]. Male participants likely also exhibited potentially greater superoxide buffering capacity through injury-induced elevated *NQO1* expression that did not occur in females (Fig. 3G and Additional file 1: Fig. S2D) [45]. Of note, the increase in *SOD2* expression positively correlated with preservation of muscle fiber size at 4 months post-ACLR (Fig. 3H).

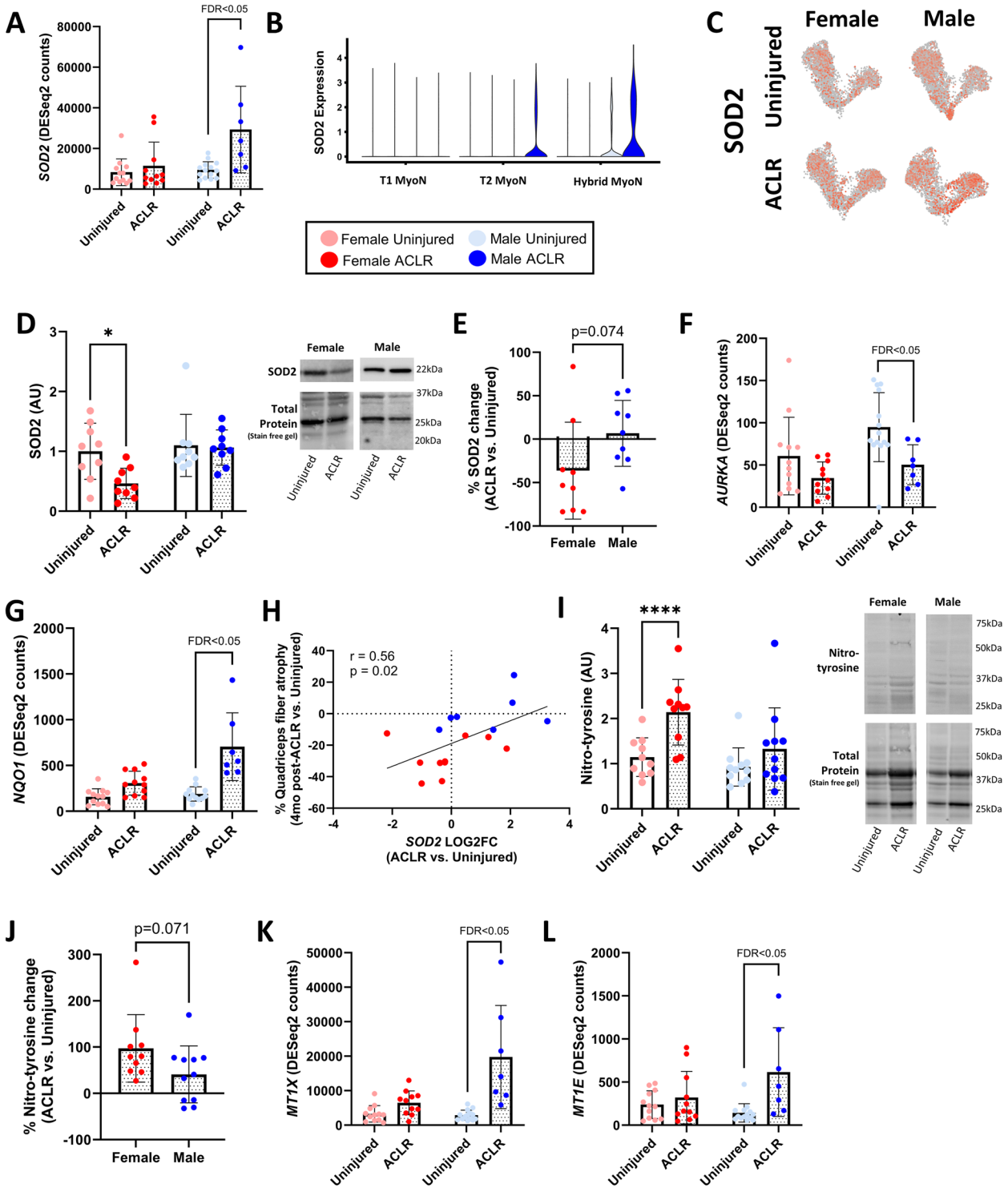
In line with these findings, females showed significantly higher muscle nitro-tyrosine levels, indicating greater oxidative damage ($p < 0.001$, F ig. 3I-J). Nitro-tyrosine is a marker for oxidative stress and peroxynitrite and is associated with SOD2 dysfunction [46, 47]. Two antioxidant metallothionein isoforms [48], *MT1X* and *MT1E*, also showed male-specific upregulation following injury and reconstruction (Fig. 3K-L and Additional file 1: Fig. S2E). These differences in free radical scavenging enzymes, particularly the male-specific upregulation of *SOD2*, *NQO1*, and *MT1X*, suggest a heightened capacity for oxidative stress management in males, potentially contributing to their relative preservation of muscle size following injury.

Inadequate angiogenic signaling and capillary rarefaction in female muscle after injury

To further assess global myonuclear sex specific changes in the ACLR limb, we pooled all myonuclei in our snRNA-seq dataset and ran a direct comparison between the female ACLR limb and the male ACLR limb (Fig. 4A). Females exhibited downregulation of angiogenesis-related pathways (Fig. 4B). Adequate capillarization of skeletal muscle is mediated via myofiber-endothelium cross talk and is imperative for gas exchange, nutrient delivery, and anabolic responsiveness [26]. To determine if angiogenic communication was disrupted in females, we performed cell communication analysis using CellChat on myonuclei and vascular (endothelial and arteriole) nuclei in female and male ACLR muscle (Fig. 4C-D). We examined the changes in signal strength between sexes to infer their correlation with muscle capillarization. We found downregulation of myonuclear – endothelial cell communication in females, with VEGF and PECAM1 pathways decreasing information flow in females (Fig. 4E). *VEGFA* ligand was significantly upregulated in male type 1 and type 2 myonuclei after injury (Fig. 4F and Additional file 1: Fig. S5A-B) and the VEGF receptor (*FLT1*) was also upregulated in male endothelial cells after injury (FDR-adjusted p value < 0.05; Fig. 4G and Additional file 1: Fig. S5C). Additionally, we identified six signaling pathways unique to female ACLR with the greatest induction of thrombospondin (THBS) in females (Fig. 4E). Thrombospondins were among the first identified endogenous protein inhibitors of angiogenesis through their ability to suppress VEGF bioavailability and activity, inhibit endothelial cell migration, and suppress nitric oxide signaling [49, 50]. Furthermore, *TYMP*, an endothelial growth factor that stimulates angiogenesis in ischemic muscle [51, 52], exhibits upregulation in muscle following injury in males but not females (Fig. 4H and Additional file 1: Fig. S5D). No changes in endothelial or smooth muscle cell population numbers were observed in our snRNA-seq dataset (Additional file 1: Fig. S3A-B), but we did observe an increase in arteriole proportion in males after ACLR via deconvolution analysis of our bulk RNA-seq data (Additional file 1: Fig. S4A). To define potential phenotypic consequences of these early molecular changes, we quantified capillary abundance in male and female muscle 4 months after ACLR. Capillaries per fiber density (C:Fi) significantly declined in female muscle while remaining unchanged in males (F ig. 4I-K). Deficits in capillary content exhibited a

(See figure on next page.)

Fig. 3 Deficient free radical scavenging in female muscle after injury. **A** *SOD2* expression assessed via RNA-seq. **B** Violin plot depicting *SOD2* expression (Male ACLR vs Uninjured body myonuclei: FDR adj $p < 0.05$, Female ACLR vs Uninjured body myonuclei: FDR adj $p > 0.05$). **C** Feature plots depicting *SOD2* expression. **D** SOD2 protein expression normalized to total protein abundance (sex x injury interaction $p = 0.0430$). **E** SOD2 percent change (ACLR vs. Uninjured). **F** *AURKA* expression assessed via bulk RNA-seq. FDR adj $p < 0.05$ in males but not females. **G** *NQO1* expression assessed via bulk RNA-seq. FDR adj $p < 0.05$ in males but not females. **H** Pearson correlation analysis comparing LOG2 fold change in SOD2 expression [ACLR vs. Uninjured] and quadriceps muscle fiber atrophy [4mo post-ACLR vs. Uninjured]. **I**) Nitrosylated tyrosine abundance normalized to total protein abundance (sex x injury interaction $p = 0.0235$). **J** Nitro-tyrosine percent change (ACLR vs. Uninjured). **K** *MT1X* expression assessed via bulk RNAseq. FDR adj $p < 0.05$ in males but not females. **L** *MT1E* expression assessed via bulk RNAseq. FDR adj $p < 0.05$ in males but not females. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.0001$ vs Uninjured via Šídák's multiple comparison post hoc tests performed when significant interactions were detected via 2 factor ANOVA. $N = 27$ (13F, 14 M) for panel A, F-G, K-L; $n = 4$ (2F, 2 M) for panels **B**-**C**, $n = 18$ (9F, 9 M) for panels **D**-**E** and **I**-**J**



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correlation to muscle fiber atrophy 4 months post-ACLR as well (Fig. 4L).

Discussion

Females are at higher risk of musculoskeletal injury and have poorer long-term outcomes and functional recovery than males [4, 5, 53]. Here, we aimed to identify a biological and molecular basis for this sex-specific disparity by conducting transcriptomic and muscle phenotyping analyses. Females undergo disproportionate muscle atrophy compared to males at the conclusion of standard of care treatment, and we identify that only ~ 40% of the muscle transcriptomic response to injury was shared between sexes. Single nucleus RNA-sequencing showed that a large portion of this variance was of myonuclear origin. Transcriptomic signature analyses displayed injury-responsive upregulation of enrichment pathways related to handling of reactive oxygen species in males that were absent in females. These data were confirmed with muscle oxidative damage unique to females following injury. Recent work demonstrated that oxidative stress is a causative factor of muscle atrophy following ligament injury, and SOD2 overexpression is sufficient to attenuate muscle atrophy in a mouse ligament injury model [15]. SOD family proteins are among the most critical antioxidant enzymes in skeletal muscle [54] and are known to be less abundant in females [55]. Our data demonstrate this difference is exacerbated following injury, suggesting critical sex-based differences in free radical handling. We have previously shown reduced complex I and II-driven mitochondrial respiration in muscle one week after ACLR, but those studies were insufficiently powered to detect sex-specific differences [15]. In pre-clinical models of disuse atrophy, evidence exists for exacerbated depression of mitochondrial gene expression in females [56]; however, our results do not indicate a similar response in females following ACLR. Additionally, our female bulk RNA-seq data suggest a larger inflammatory burden following ACLR. Together, increased inflammation and poorer free-radical scavenging in females may underscore the greater atrophic burden experienced by females.

Moreover, we found reduced myofiber-derived angiogenic signaling in females compared to males following injury. Inferred myofiber-vascular endothelium communication revealed VEGF and PECAM1 signaling as among the most downregulated pathways in the female injured limb compared to the male injured limb. We also observe reduced capillary density in female muscle following ACLR. Thus, it is possible diminished myofiber-vascular cross-talk contributes to capillary loss in females. Previous preclinical work has shown that promotion of angiogenesis is one of the most efficacious strategies to promote functional muscle restoration after injury [57]. Further, low capillary abundance and disrupted myofiber VEGF signaling diminish exercise adaptation [26, 28, 58, 59]. Limited research has explored sex-based differences in muscle capillary supply during health or injury [60], and assessment of these outcomes in the recovery from injury is needed to optimize rehabilitative approaches in females.

Despite the exacerbated muscle atrophy in females following ACLR, we observed relative parity in strength losses between sexes. Within type 2 myonuclei, males showed a unique decline in genes associated with calcium transport and strong induction of *RRAD* expression. RRAD is an inhibitor of the L type calcium channel [61] that significantly decreases calcium transients and excitation–contraction coupling in skeletal muscle [62]. Disrupted calcium transport may explain weakness in males with minimal myofiber atrophy.

This study is not without limitations. The Uninjured limb was used as a comparator to the ACLR limb, and all data collection within the Uninjured limb occurred after ACL injury. The injury itself may have influenced physical activity levels that could impact gene expression and fiber size within the Uninjured limb that serves as our between-limb comparison. However, we have shown the majority of quadriceps atrophy occurs following reconstructive surgery [63], making the non-injured limb a useful genetic comparator in this context. Additionally, in prior work we have shown muscle strength within the Uninjured limb does not exhibit changes, suggesting that

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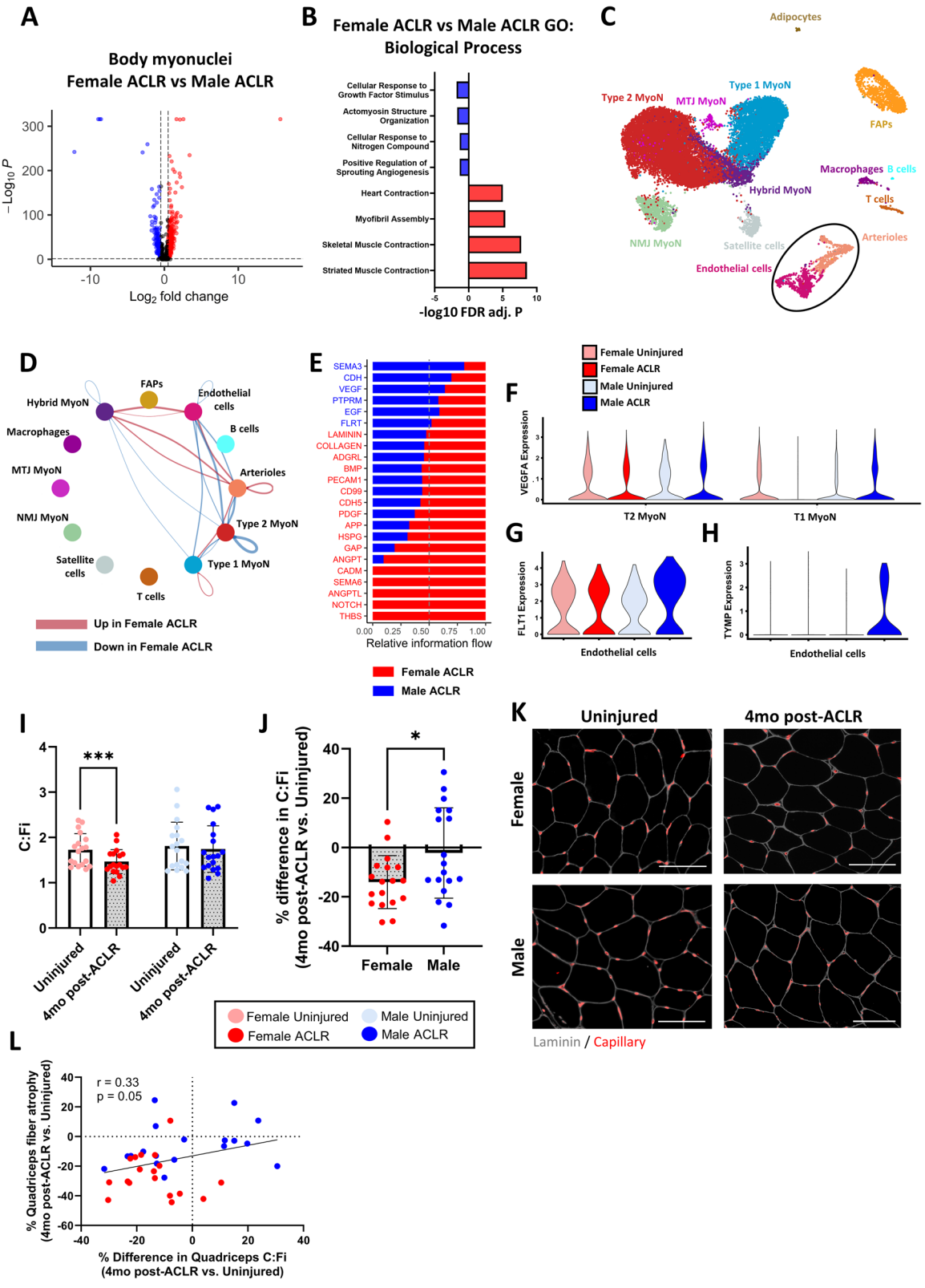
Fig. 4 Deficient angiogenic signaling and capillary rarefaction in females after injury. **A** Volcano plot for body myonuclei (MyoN) in ACLR male or female muscle (FDR adj $p < 0.05$, log2FC ± 1.5). **B** Enriched upregulated and downregulated pathways between females and males in body myonuclei assessed via gene ontology in ACLR limb. **C** Uniform manifold approximation and projection (UMAP) including all nuclei with endothelial cells and arterioles encircled. **D** CellChat interaction strengths using body myonuclei, endothelial cells, and arterioles compared between female and male ACLR limbs. **E** Information flow plot depicting cell–cell interactions that were ranked based on their differences in overall information flow between females and males. **F** Violin plot depicting *VEGFA* expression. **G** Violin plot depicting *FLT1* expression. **H** Violin plot depicting *TYMP* expression. **I**) Capillaries per fiber (C:Fi; sex x injury interaction $p = 0.0416$). **J** Capillaries per fiber percent change comparing the Uninjured to 4 months post-ACLR. **K** Representative immunohistochemical images of capillaries (red) and muscle fiber borders (grey), scale bar = 100 µm. **L** Pearson correlation analysis comparing the percent change in capillaries per fiber [4mo post-ACLR vs. Uninjured] and quadriceps muscle fiber atrophy [4mo post-ACLR vs. Uninjured]. ***$p < 0.005$ vs Uninjured via via Šídák's multiple comparison post hoc tests performed when significant interactions were detected via 2 factor ANOVA. $n = 4$ (2F, 2 M) for panels **A**-**H** and $n = 36$ (18F, 18 M) for panels **I**-**L**



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even if there are molecular changes within the Uninjured limb, molecular changes are insufficient to meaningfully alter muscle function [63]. Further, our participants all indicated a goal to return to competitive sport. Objective return-to-sport criteria often utilize between-limb normalized measures of quadriceps strength (limb symmetry index), underscoring the clinical utility of our comparison to the non-involved limb. Additionally, our single nucleus sequencing data are exploratory, as we only have an $n=4$ (2 paired men and women); cell-level analyses in particular should be interpreted with this in mind.

Collectively, these findings suggest that disrupted oxidative stress and capillary rarefaction are unique pathways that may underlie atrophy and weakness in females after injury while males show preferential disruption in calcium transport. Our findings reveal robust sex-specific diversity in the muscle transcriptome following injury, similar to those present in the ACL itself [64]. These data underscore the necessity of sex considerations in prescription of treatments and rehabilitative exercise to enhance recovery outcomes following musculoskeletal injury.

Conclusions

Women have poorer recovery of muscle strength and higher re-injury rates after anterior cruciate ligament injury. Here, we investigated skeletal muscle bulk and single nucleus gene expression changes in both men and women after injury and surgical reconstruction. This showed a number of differences between men and women. Namely, reduced oxidative stress buffering capacity and capillarity may explain sex-divergent outcomes following ACLR. These findings could help guide treatment decisions, enabling female-specific personalized therapies that are more likely to be effective and improve outcomes in women.

Abbreviations

ACL	Anterior Cruciate Ligament
ACLR	Anterior Cruciate Ligament Reconstruction
MRI	Magnetic Resonance Imaging
PCA	Principal Component Analysis
DEGs	Differentially Expressed Genes
SOD2	Manganese Superoxide Dismutase 2
RRAD	Ras-Related Glycolysis Inhibitor And Calcium Channel Regulator
FISH	Fluorescent In-Situ Hybridization

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12916-026-04818-8.

Additional file 1.

Additional file 2.

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Authors' contributions

Conceptualization: ARK, CSF Methodology: ARK, AMO, NTT, SG-V, CRB, TB, CSF Clinical Study Administration and Sample Procurement: DEL, CEC, PAK, AVS, DLJ, BN, CSF Analysis: ARK, CSF Bioinformatics: ARK, YW Funding acquisition: BN, CSF Supervision: CSF Writing – original draft: ARK, AMO, CSF Writing – review & editing: ARK, AMO, NTT, SG-V, CRB, TB, DEL, PAK, CEC, AVS, DLJ, YW, BN, CSF All authors read and approved the final manuscript.

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Data availability

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Information. The datasets produced in this study are available in the following databases: RNA-seq data: Gene Expression Omnibus GSE314430 [65] and GSE324033 [66]; snRNA-seq data: Gene Expression Omnibus GSE270868 [67].

Declarations

Ethics approval and consent to participate

This study was approved by the Institutional Review Board (#43046) at the University of Kentucky and performed in accordance with the ethical standards of the 1964 Declaration of Helsinki. Informed written consent was obtained from each patient or guardian before participation in this study. Additionally, all minors in this study assented to participation.

Consent for publication

Not applicable.

Competing interests

AVS has received fellowship support from Smith & Nephew and Arthrex in addition to consulting fees from Smith and Nephew, Bioventus, Allosource, and Vericel. DLJ has received travel expenses from Linvatec, in addition to consulting fees from Smith + Nephew and from Xiros Inc. YW is the founder of MyoAnalytics, LLC. The authors declare no other competing interests.

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