A cellular atlas of skeletal muscle regeneration and aging

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Summary

An individual skeletal muscle is a complex structure, composed of large contractile myofibers, connective tissue, nerve tissue, immune cells, stem cells and the vasculature. Each of these components contribute to skeletal muscle function, maintenance, regeneration, and if perturbed can potentially contribute to or cause disease that reduces muscle function. To investigate the cellular inventory of skeletal muscle we carried out single cell RNA sequencing on cells isolated from adult uninjured muscle, adult post injury muscle, and from aged uninjured muscle. Our muscle atlas provides the cellular landscape and partial transcriptome of pre-injury, post injury, and aged muscle, identifying dramatic changes in the muscle stem cell, fibroblast and immune cell populations during regeneration. Our data highlight dynamic changes occurring during muscle regeneration, identify potential extrinsic mechanisms that control muscle stem cell behavior, and underscore the inflamed state of aged uninjured muscle.

Introduction

Skeletal muscle is made up of large multi-nucleated, post mitotic fibers, that have a large reserve capacity to regenerate following injury (Baghdadi & Tajbakhsh, 2018; Dumont, Bentzinger, Sincennes, & Rudnicki, 2015; Fukada, 2018; Wosczyna & Rando, 2018). Skeletal muscle regeneration is driven by skeletal muscle stem cells (MuSCs), typically quiescent in uninjured muscle but following injury, leave G0, enter the cell cycle as myoblasts and expand as needed to repair muscle injuries. Post injury MuSC behavior is guided by cell intrinsic and extrinsic mechanisms, where extrinsic factors are produced by a variety of cells including fibroblasts, immune cells, endothelial cells, and fibro-adipogenic progenitors (FAPs). FAPs are a mesenchymal cell population that readily expand following injury and regulate muscle repair by

secreting growth factors (Joe et al., 2010; Lemos et al., 2015; Uezumi, Fukada, Yamamoto, Takeda, & Tsuchida, 2010). Fibroblasts, which produce high levels of extracellular matrix proteins, also expand following injury, and their depletion impairs MuSC expansion and muscle regeneration (Mathew et al., 2011; Murphy, Lawson, Mathew, Hutcheson, & Kardon, 2011). Macrophages influence muscle regeneration by cytokine secretion, including Interferon-γ, TNFα, IL-6, and insulin-like growth factors (IGFs) that in turn regulate FAP, immune cell, and MuSC functions (Arnold et al., 2007; Cheng, Nguyen, Fantuzzi, & Koh, 2008; Langen et al., 2004; Lemos et al., 2015; X. Liu et al., 2017; Perdiguero et al., 2011; Shen, Li, Zhu, Schwendener, & Huard, 2008; Tonkin et al., 2015). T cells, including CD8+ T cells, and regulatory T cells regulate muscle regeneration by secreting MCP-1, Interferon-γ, and amphiregulin (Burzyn et al., 2013; Castiglioni et al., 2015; Panduro, Benoist, & Mathis, 2018; Zhang et al., 2014). Lastly, endothelial cells regulate muscle regeneration via generation of VEGF, HGF, IGF and Ang-1 and by direct cell contact (Bryan et al., 2008; Christov et al., 2007; Mofarrahi et al., 2015; Tatsumi, Anderson, Nevoret, Halevy, & Allen, 1998; Verma et al., 2018). Muscle regeneration is impaired with age, and alterations to extrinsic factors contribute to aged-induced deficiencies. (Blau, Cosgrove, & Ho, 2015; Feige, Brun, Ritso, & Rudnicki, 2018; Hwang & Brack, 2018; Sousa-Victor, García-Prat, Serrano, Perdiguero, & Muñoz-Cánoves, 2015). Given the multitude of cells and extrinsic factors required for skeletal muscle function, generating a cellular inventory and single cell transcriptional profile for muscle regeneration and aging muscle will permit correlation of dynamic changes in cell populations that may influence each other to effectively and rapidly regenerate muscle tissue.

We provide a cellular atlas of muscle regeneration, comparing the single cell transcriptomes and cellular inventories of young uninjured muscle, geriatric uninjured muscle,

adult muscle at 4 d post injury, and adult muscle at 7 d post injury. We found a remarkable diversity in fibroblasts that undergo striking transcriptional changes during regeneration, producing extensive extracellular matrix (ECM), while transiently expressing growth factors and cytokines. Our analysis identifies a heterogeneous macrophage population whose transcriptional profile changes dramatically during regeneration as well as confirms dynamic changes in T cell populations during muscle repair. Unexpectedly, the uninjured, aged muscle shows many similarities to injured adult muscle, revealing an inflamed environment where the fibroblast and immune cell behavior appears to be conferring a partial injury response. Overall, by providing a cellular atlas of muscle repair and aging, we enhance our understanding of the environment during muscle regeneration and provide a valuable resource for further functional studies involving interactions between cell populations during skeletal muscle regeneration.

Results

To investigate the cellular inventory and single cell transcriptome of skeletal muscle, we carried out single cell RNA sequencing on the Tibialis anterior (TA) muscles of adult uninjured mice, aged uninjured mice, 4 d post injury mice, and 7 d post injury mice. We choose 4 d and 7 d post injury time points for analysis based on our discovery that prior to day 5 post injury, MuSCs mostly expand and differentiate producing myonuclei, while after day 5 post injury the majority of MuSC self-renewal occurs, accompanied by generation of peripheral myonuclei but not centrally located myonuclei (See companion article). Thus, single cell sequencing at day 4 and day 7 may identify cells in the muscle interstitium that influence regeneration and provide insight regarding the mechanisms regulating distinct MuSC behavior. Individual TA muscles were dissected, single cell suspensions prepared, with red blood cells and dead cells/debris removed

by Myltenni column selection. The cell numbers were quantified, and single cell RNA sequencing performed with the 10X Genomics platform. A cell clustering analysis with all cells captured (all four different conditions pooled), identified 14 individual cell cohorts, which are displayed in a 2-dimensional TSNE plot (Fig 1A). An interactive 3D TSNE plot is available in supplemental information as an html file (Supplemental Information Fig S1). We identified each of the 14 cell types using a z-score analysis to identify genes with enriched expression as well as established genes for individual cell types (Fig 1B and 1C; Supplemental Information Table S1). The cell cohorts fall into two large groups, immune-related cells and non-immune related cells, with the latter comprising mostly mesodermal cells in skeletal muscle. We identified 7 distinct immune cell populations comprising four macrophage subpopulations (1-aqua, 3-gold, 4-lemonchiffon, 9-orange), a myeloid cell cohort (13-yellow), a T cell cohort (14-green) and a B cell cohort (11-plum) (Fig 1A, 1B). The 7 non-immune cell populations include two cohorts of fibroblasts (2-blue and 5-light blue), MuSCs (7-magenta), endothelial cells (10-pink), differentiated muscle (12-dark red), Schwann cells (6-light brown) and red blood cells (12-red) (Fig 1A, 1B). A cell population identified as differentiated skeletal muscle was unexpected as differentiated skeletal muscle myofibers are large syncytial cells with hundreds of nuclei. The transcripts enriched in this group are either components of the muscle contractile apparatus or other skeletal muscle specific proteins (Supplemental Information Table S1). We suspect that either myofiber fragments or myonuclei were present in our single cell preparations and those were captured in the 10X Genomics microfluidics.

We identified two distinct fibroblast cohorts that segregate by muscle injury. One fibroblast population, (2-blue) is present almost entirely in uninjured muscle, and the second fibroblast population (5-light blue) is exclusively restricted to injured tissue (Fig 2A-2D). The

identity of both fibroblast cohorts was confirmed based on gene enrichment (Supplemental Information Table S1) and on expression of multiple ECM transcripts that include collagen, decorin and fibronectin, whose relative transcript levels were consistently elevated in the post-injury fibroblasts compared to fibroblasts from uninjured muscle (Fig 2E-2F; Supplemental Information Fig S2). Transcripts for gelsolin (Gsn), an actin binding protein, were among few specifically enriched in fibroblasts from uninjured muscle (Supplemental Information Fig S2), while a large number of transcripts were enriched in fibroblasts from injured muscle (5-light blue) including smooth muscle actin (Acta2), periostin (Postn), collagen triple helix repeat-containing protein 1(cthrc1) and TIMP metallopeptidase inhibitor 1(timp1), all reported to be expressed in activated fibroblasts (Fig 2G-2H; Supplemental Information Fig S2) (Bagalad, Mohan Kumar, & Puneeth, 2017; Chapman, Meza, & Lieber, 2016; Gladka et al., 2018). Genes expressed in FAPs including CD34, Ly6a (Sca1), and PDGFra do not segregate with a specific cell subpopulation but instead appear dispersed within the entire fibroblast cohort in either uninjured or injured TA muscle (Fig 2I-2K). Tek and Vcam, whose proteins identify FAP subsets (Malecova et al., 2018), are expressed predominately in uninjured TA muscle fibroblasts or both the uninjured and injured fibroblasts, respectively but do not segregate to a subpopulation (Supplemental Information Fig S2). Cells that express twist2 or Pw1/Peg appear in the general fibroblast population in uninjured and injured muscle tissue (Supplemental Information Fig S2). Twist2 and Peg expressing cells are involved in skeletal muscle regeneration (N. Liu et al., 2017; Mitchell et al., 2010), but the expression pattern for either gene does not segregate into a separate cellular cohort.

The second largest cell cohort were immune cells identified by CD45 (Ptprc) expression (Fig 3A, B). Within the CD45 (Ptprc) expressing cells, we identified seven distinct

subpopulations based on transcriptional profiles (Fig 3A, 3B). Four of the immune cohorts are CD68 expressing macrophages (Tidball, 2017), that express CD74 (Su, Na, Zhang, & Zhao, 2017) (Fig 3C-3D). Among the macrophage subpopulations, cells expressing CD209 (Fig 3G) were identified as dendritic macrophages (9-orange) (Garcia-Vallejo & van Kooyk, 2013; Geijtenbeek et al., 2000; Relloso et al., 2002), while the majority macrophage populations showed enriched expression of F4/80 (Adgre1) (Fig 3I)(3-Gold and 4-Lemonchiffon) (Chazaud, 2014), a minority subset expresses high CCl22 (1-aqua) (Fig 3J) a cytokine associated with regenerative macrophages (Chazaud, 2014). The three non-macrophage, CD45 (Ptprc) expressing cells, include T cells expressing CD2 and CD3 (Fig 3E and Supplemental Information Fig S4) (Ngoenkam, Schamel, & Pongcharoen, 2018; Skånland, Moltu, Berge, Aandahl, & Taskén, 2014; Zhou, Chong, & Littman, 2009), myeloid cells/neutrophils (Fig 3H) that express S100a8 (Pruenster, Vogl, Roth, & Sperandio, 2016; S. Wang et al., 2018) and B cells that express CD79 (Fig 3F) (Chu & Arber, 2001; Naeim, Nagesh Rao, Song, & Grody, 2013).

To identify changes to the cellular cohorts and their transcriptional profiles induced by injury and aging, we next compared the single cell transcriptional profile of cells from uninjured TA muscle with cells from TA muscles at 4 d post injury, 7 d post injury and from aged muscle. Dramatic changes in cell cohort gene expression occurred at 4 d post injury, where in uninjured muscle, fibroblasts (2-blue) were the most abundant cell type captured, followed by immune cells and endothelial cells (Fig 4A and Supplemental Information Table S2). There were dramatic shifts in the fibroblast and T cell populations at 4 d post injury concomitant with large increases in macrophage numbers and MuSC numbers (Fig 4A-4B and Supplemental Information Table 2; Supplemental Information Table Set S3). MuSCs expand and differentiate, with little self-renewal from day 0-4 post injury (See companion paper). Ranked gene GO terms

for metabolic processes, transcription, translation, and cell cycle increase from 0 d to 4 d post injury, consistent with activation, proliferation and differentiation of MuSCs (Supplemental Information Table S4). Examples of genes known to regulate MuSC expansion or differentiation that increase expression at 4 d post injury include Pax7, MyoD, Itga7, FgfR1, Vcam1, and myogenin (Fig 4C and Supplemental Information Table S5).

Remarkable transcriptional changes in the fibroblast, macrophage, and T cell populations occurring by 4 d post injury identify the changes in the cellular environment. For example, smooth muscle actin (Acta2) and periostin (Postn) gene expression increases several hundred fold in fibroblasts indicative of a highly activated state (Fig 4D), (Baum & Duffy, 2011; Chapman et al., 2016; Gladka et al., 2018; Mann et al., 2011), and is accompanied by a more than 10-fold expression increase in several ECM transcripts that influence MuSC behavior (Collagen 1 (Col1a1), Collagen V (Col5a1), Fibronectin (Fn1) and Biglycan (Bgn) (Fig 4D) (Baghdadi et al., 2018; Casar, McKechnie, Fallon, Young, & Brandan, 2004; Lukjanenko et al., 2016). Periostin transcription, which increases nearly 400-fold by 4 d post injury is notable because it regulates muscle regeneration and loss of periostin reduces fibrosis in muscular dystrophy (Hara et al., 2018; Latroche et al., 2017; Lorts, Schwanekamp, Baudino, McNally, & Molkentin, 2012) (Fig 4D). FAPs, a subset of fibroblasts that directly signal to MuSCs are identified by Sca1 (Ly6a) and PDGFRa (Joe et al., 2010; Lemos et al., 2015; Uezumi et al., 2010), whose transcripts are increased at 4 d post injury (Fig 4D). Among the few gene expression changes that decrease in fibroblasts by 4 d post injury are those for decorin (Dcn) and gelsolin (Gsn) (Fig 4D).

Among the four macrophage subpopulations, the most numerous cell type captured at 4 d post injury, transcripts encoding genes for both inflammatory macrophages and regenerative

macrophages were prevalent but neither group was dominant (Fig 4E, Supplemental Information Fig S3, Supplemental Information Table S2). Examples of secreted factors that regulate muscle regeneration whose transcripts increase by 4 d post injury include galectin (LGALS1), complement protein C1qb (C1qb) and osteopontin (SPP1) (Fig 4F) (Naito et al., 2012; Uaesoontrachoon, Wasgewatte Wijesinghe, Mackie, & Pagel, 2013; Uaesoontrachoon et al., 2008; Van Ry, Wuebbles, Key, & Burkin, 2015). Transcripts expressed by regulatory T cells increased at 4 d post injury (Ctla2, Ctal4, Tnfsf18), as did T cell expression of interferon-γ (IFNg), galectin1 (LGALS1), galectin 3 (LGALS3), granzyme B (Gzmb), natural killer cell granule protein (Nkg7) and macrophage migration inhibitory factor (Mif) all of which regulate the inflammatory response or muscle regeneration (Fig 4G) (Boivin et al., 2012; Cheng et al., 2008; Rancourt et al., 2018; Su et al., 2017; Turman, Yabe, McSherry, Bach, & Houchins, 1993; Van Ry et al., 2015).

In contrast to 4 d post injury, few changes in the overall cellular inventory occur between 4 d and 7 d post injury (Fig 5A-5B), yet between 5 d and 7 d post injury MuSC expansion has nearly ceased, accompanied by the peak in MuSC self-renewal (see companion paper) and thus, we chose the 7 d time point to query for accompanying changes in skeletal muscle environment. The 7 d post injury transcriptional profiles for MuSCs are consistent with self-renewal and continued differentiation as evidenced by GO term analysis identifying decreases in gene transcription for genes involved in metabolic processes, translation, and the cell cycle (Supplemental Information Table S7). Although, no significant change in expression of Pax7 occurred between 4 d and 7 d post injury, the Pax7/MyoD ratio of 0.16 increases over 2-fold to 0.38 by 7 d post injury, consistent with MuSC self-renewal and is accompanied by a 2.3-fold increase in myogenin expression (Fig 5C) (Supplemental Information Table S8).

Notable changes in gene expression profiles were observed between 4 d and 7 d post injury despite the overall lack of change in the cellular inventory (Supplemental Information Table Set 6; Fig. 5A, B). In the fibroblast cohort insulin-like growth factors (Igf1 and Igf2) expression increased, accompanied by further increases in periostin (Postn), fibronectin (Fn1), and collagens (Col5a1, Col1a1), with decreases in several cytokine transcripts (Cxcl2, Cxcl12, CxCl10) (Fig 5D), many of which regulate MuSCs (Baghdadi et al., 2018; Brzoska et al., 2012; Clemmons, 2009; Deyhle et al., 2018; Hara et al., 2018; Latroche et al., 2017).

Immune cell cohorts, similar to the fibroblasts changed little from 4 d to 7d post injury. However, we captured more T cells at 7 d post injury than at 4 d post injury (Fig 5A, 5B, Supplemental Information Fig S4 and Supplemental Information Table S2). Within the T cell cohort, expression of IFNg, Lgals1, Gzmb, Nkg7, and Mif decreased by 7 d post injury (Fig 5E), reversing the increase observed during the first 4 d post injury (Fig 4). Expression of genes associated with regulatory T cells (Ctla2, Ctal4, Tnfsf18) did not change significantly from 4 d to 7 d post injury (Fig 5E) similar to the majority of macrophage populations, where expression of inflammatory and regenerative markers did not change significantly from day 4 to day 7 post injury. Within macrophages, expression of galectin-1 (LGALS1), complement protein C1qb (C1qb) and osteopontin (SPP1), all discussed for their notable increases in expression 4 d post injury (Fig 4), decreased by 7 d post injury relative to their expression values at 4 d post injury (Fig 5F).

In aged mice, skeletal muscle function and regenerative capacity are lost arising from cell intrinsic and environmental changes that occur during aging (Blau et al., 2015; Feige et al., 2018; Hwang & Brack, 2018; Sousa-Victor et al., 2015). We queried the skeletal muscle environment in geriatric mice (30 mo old) to determine the extent of transcriptional changes in the cell cohorts

we identified in uninjured and injured adult mouse skeletal muscle (3 mo old). Similar cell populations are present in TA muscles of geriatric mice compared to adult mice (Fig 6A, 6B). However, the transcriptional profile for most cell cohorts differed when compared to uninjured adult TA muscle (Supplemental Information Table Set S9). A hierarchical cluster analysis of gene expression changes in MuSCs for all conditions revealed a striking similarity between aged uninjured and adult injured muscle (Fig. 6C), where GO term analysis of genes ranked by expression change in adult vs aged MuSC revealed increases in metabolic process, translation, and cell cycle, in aged MuSCs (Supplemental Information Table S10). Specific examples of MuSC gene expression in adult uninjured, aged uninjured, and 7 d post injury illustrate the similarities in gene expression values between aged TA muscle and adult 7 d post-injury TA muscle (Fig. 6D). Of the remaining cell cohorts, only T cells clustered similarly, where T cells in aged uninjured muscle are most similar with the gene expression signature of T cells from 7 d post injury adult TA muscle (Fig. 6E). Dramatically upregulated in the aged uninjured T cells compared to adult uninjured T cells are s1000a6 transcripts with more modest increases in expression of natural killer cell granule protein (Nkg7), CD52, regulatory T cell marker Ctla2a, and Ccl5 (Supplemental Information Fig. S5A).

While the gene signatures of fibroblasts and macrophages from aged uninjured TA muscle cluster more closely with adult uninjured TA than from injured TA in hierarchical cluster gene expression analysis (Supplemental Information Fig S6), transcripts from specific genes increased in fibroblasts and macrophages from aged muscle are also increased in the respective cells post injury. For example, expression increased for periostin (Post), biglycan (Bgn), Sca-1 (Ly6a), Interleukin 6 (Il6), complement component C3 (C3), and several cytokines (CxCl12, Cll2, Ccl7) in fibroblasts from aged TA muscle and injured TA muscle compared to adult

uninjured muscle (Fig 6F, 6G). In macrophages, gene expression increased for CxCl2, C1qb, galectin (LGALS1) and osteopontin (SPP1) in aged muscle and injured muscle as compared to adult uninjured muscle (Supplemental Information Fig S5B). These transcripts encode proteins that regulate MuSC behavior during skeletal muscle regeneration (Naito et al., 2012; Rancourt et al., 2018; Uaesoontrachoon et al., 2013; Van Ry et al., 2015). In addition, myeloid cells from aged mice increase expression of transcripts associated with stress and inflammation (Supplemental Information Fig S5C). Overall, the cellular atlas of cells in aged tissue presents an inflamed environment significantly different from adult muscle.

Discussion

We performed single cell RNA sequencing for mononuclear cells in the tibialis anterior muscle at key time points during regeneration to better understand the complexity of transcriptional changes occurring and how those relate to the generation of myonuclei and MuSC self-renewal. We found dramatic transcriptional changes in non-muscle cells for factors that influence MuSCs, further supporting a direct role for their regulation of MuSCs. The cellular atlas in aged muscle identified an inflamed environment with significant gene expression changes in several cell cohorts.

Our findings that prior to day 5 post injury, MuSCs mostly expand and differentiate to produce myonuclei, and after day 5 post injury MuSCs predominately self-renew with a peak at 7 d post injury, was the basis for examining single cell transcription at 4 d and 7 d post injury (See companion article). Consistent with the expansion and production of myonuclei by MuSCs, at 4 d post injury GO term analysis identified major increases in metabolic pathways, translation, and cell cycle, along with increased expression of Pax7, MyoD, and myogenin. Three days later,

at 7 d post injury the MuSC transcriptional signature is dramatically altered where GO term analysis for metabolic pathways, translation, and cell cycle all decreased, along with decreases in MyoD expression. These transcriptional changes are consistent with the decreased expansion, increased self-renewal, and continued differentiation we observed by EdU lineage labeling (see companion paper). MuSCs are influenced by FAPs (Joe et al., 2010; Lemos et al., 2015; Uezumi et al., 2010) and macrophages (Arnold et al., 2007; Cheng et al., 2008; Langen et al., 2004; Lemos et al., 2015; Liu et al., 2017b; Perdiguero et al., 2011; Shen et al., 2008; Tonkin et al., 2015) and thus, the extracellular environment directly alters MuSC behaviors during regeneration. To gain a broader understanding of the cell types involved, we queried changes in gene expression for factors identified to influence MuSCs either in culture or *in vivo.* Fibroblasts and immune cells dramatically upregulate osteopontin, periostin, Igf, collagen V, galectins, and fibronectin upon the first 4 d following injury, all of which can regulate MuSC behavior (Clemmons, 2009; Latroche et al., 2017; Lukjanenko et al., 2016; Pagel, Wasgewatte Wijesinghe, Taghavi Esfandouni, & Mackie, 2014; Rancourt et al., 2018; Rozo, Li, & Fan, 2016; Van Ry et al., 2015). In addition, we observed large changes for expression of cytokines, growth factors, and ECM proteins that are likely to further influence MuSC behavior. Thus, it seems likely that both fibroblasts and immune cells are major regulators of the post injury environment and that the distinct MuSC behaviors seen before and after day 5 post injury are influenced by extrinsic cellular signals as well as potential cell-cell interactions.

The cells our analysis identified as fibroblasts are most likely a group of closely related cells, that include progenitor and differentiated cells such as FAPs, Twist+ cells, PW1/Peg cells, and TCF4+ expressing cells. While our clustering analysis identifies all these cells as fibroblasts, it is clear from the literature that fibroblast subtypes perform distinct functions, thus

identifying better markers and hierarchical relationships among the fibroblast sub-populations will prove important. Fibroblast expression of periostin is worth nothing as it was nearly exclusively expressed by the injured fibroblast, showing a 300-fold transcriptional increase at 4 d post injury and another 4-fold by 7 d post injury. Despite the dramatic increase in periostin expression during regeneration, the effects of periostin are unclear as deletion experiments demonstrate periostin enhances as well as inhibits muscle regeneration (Hara et al., 2018; Latroche et al., 2017; Ozdemir et al., 2014). Moreover, the dystrophic phenotype is reduced in periostin null *mdx* mice (Lorts et al., 2012) and increased periostin expression in fibroblasts correlates with fibrosis in several pathologies (Dobaczewski, Gonzalez-Quesada, & Frangogiannis, 2010; Hamilton, 2008; Merle & Garnero, 2012; Rani, Barbe, Barr, & Litvin, 2009). We identify the predominate cell population producing periostin and the timing of periostin induction during muscle injury providing data for future experiments to investigate how fibroblast produced periostin regulates muscle regeneration.

Post injury macrophages change phenotypes from pro inflammatory to pro regenerative during muscle regeneration to initially aid in removal of necrotic tissue and then to direct tissue repair, respectively (Deng, Wehling-Henricks, Villalta, Wang, & Tidball, 2012; Jin, Warunek, & Wohlfert, 2018; Mounier et al., 2013; Panduro et al., 2018; Ruffell et al., 2009; Tidball, 2017; H. Wang et al., 2014). We confirmed macrophage heterogeneity in uninjured and injured muscle, identifying four distinct cell clusters of post injury macrophages with overlapping expression of genes that identify inflammatory and regenerative cells. The distinct transcriptional changes we observed among post injury macrophages, including large changes to a variety of secreted factors, reflects their dynamic phenotypes and provides mechanistic predictions. For example, transcript levels for osteopontin, a secreted glycoprotein with a role in muscle repair (Pagel et al.,

2014), increases dramatically by 4 d post injury and then declines rapidly from 4 d post injury to 7 d post injury. Osteopontin regulates muscle regeneration by influencing both MuSC and immune cell behavior (Uaesoontrachoon et al., 2013, 2008), while deletion of osteopontin from *mdx* mice reduces pathology in part by shifting macrophages to a regenerative phenotype (Capote et al., 2016; Vetrone et al., 2009). In wild type mice, osteopontin deletion delays the onset of muscle regeneration (Uaesoontrachoon et al., 2013, 2008) and age-dependent increases in osteopontin are linked to impaired muscle regeneration (Paliwal, Pishesha, Wijaya, & Conboy, 2012). Thus, our analysis highlights the diversity of post injury macrophages and provides functional predictions for macrophage control of muscle repair.

We identified a variety of T cells subpopulations and T cells relatives enriched for transcripts encoding CD2, CD3, and CD4. Regulatory T cells, whose numbers are increased in dystrophic tissue and during muscle regeneration dampen immune reactions and regulate muscle regeneration (Josefowicz, Lu, & Rudensky, 2012; Schiaffino, Pereira, Ciciliot, & Rovere-Querini, 2017). Their deletion impairs muscle regeneration, increases fibrosis, and exacerbates the dystrophic phenotype in *mdx* mice (Burzyn et al., 2013; Schiaffino et al., 2017; Villalta et al., 2014), in part by regulating macrophages and MuSCs via secretion of interferon-γ and amphiregulin, respectively (Burzyn et al., 2013; Panduro et al., 2018; Villalta et al., 2014). Unlike fibroblasts and macrophages, whose predominant gene transcription changes occur in the first 4 d post injury, genes in regulatory T cells increased their transcription from day 4 to day 7 post injury suggesting a role in MuSC self-renewal as opposed to MuSC expansion and myonuclear production.

Chronic inflammation is increasingly seen as a major contributor to age-induced muscle wasting (Chung et al., 2009; Dalle, Rossmeislova, & Koppo, 2017; Woods, Wilund, Martin, &

Kistler, 2011) and thus, we predicted that the gene transcription profiles in aged muscle may possess similarities to adult injured muscle. When we queried the single cell transcriptional profile in aged muscle and compared it with adult uninjured and injured muscle we found remarkable similarities in gene expression profiles for MuSCs and T cells in aged muscle and injured muscle, but not for fibroblasts or macrophages whose profiles in aged muscle cluster with that in adult uninjured muscle. Within the immune cell populations from aged muscle, transcripts encoding inflammatory cytokines, Il-6, and complement proteins (C1q C3) were upregulated compared to young adults. In aged muscle, increased IL-6 expression contributes directly to aged-induced MuSC dysfunction (Blau et al., 2015; Price et al., 2014; Sousa-Victor et al., 2014) as well as increases in complement proteins that impair regeneration of aged muscle (Naito et al., 2012). The GO term analysis comparing MuSCs from adult and aged muscle further confirm these data identifying increases in genes involved in translation, cell cycle and metabolism. The aged muscle environment broadly affects MuSCs as their transcriptional profile is altered as are the transcriptional profiles of T cells, suggesting that overall changes in individual cell cohorts may alter the environment sufficiently to affect skeletal muscle regenerative capabilities, which are diminished in aged mice. Although the overall transcriptional profiles for fibroblasts and macrophages were similar to those in young adult muscle, individual genes that regulate MuSC behavior were altered and may contribute to the reduction in MuSC function.

We examined the single cell transcriptomes of the TA muscle during regeneration, following an induced muscle injury and compared those to the transcriptome of uninjured aged skeletal muscle. Muscle injury rapidly and radically alters the transcriptional profiles of the fibroblast and MuSC cohorts corresponding with the primary expansion of MuSCs to produce

myonuclei and at the peak of MuSC self-renewal. In aged mice, MuSC and T cell transcriptional profiles are similar to those in injured muscle. Our data provide transcriptional information from a cellular atlas that we believe will aid in identifying the cell types and signals that direct regeneration and when aberrantly activated contribute to age-induced loss of muscle function and regeneration.

Experimental Procedures

Mice

Mice were bred and housed according to National Institutes of Health (NIH) guidelines for the ethical treatment of animals in a pathogen-free facility at the University of Colorado at Boulder. University of Colorado Institutional Animal Care and Use Committee (IACUC) approved animal protocols and procedures. All experiments were carried out using C57B6 mice (Jackson Labs Stock No. 000664). The young mice were between 3 and 4 months old and the aged mice were between 28 and 32 mo. Only male mice were used in these experiments. For injuries, mice were anesthetized with isofluorane followed by injected with 50μL of 1.2% $BaCl_2$ into the left TA muscle.

TA collections and cell isolations

TA muscles were dissected and placed into 400U/mL collagenase at 37°C for 1h with shaking and then placed into Ham's F-12C supplemented with 15% horse serum to inactivate the collagenase. Cells were passed through three strainers of 100μm, 70μm, and 40μm (BD Falcon) and flow through was centrifuged at 1500×*g* for 5 min and the cell pellets were re-suspended in Ham's F-12C. To remove dead cells and debris, cells were passed over the Miltenyi, dead cell

removal kit columns (Cat# 130-090-101). To remove RBCs, cells were incubated with anti-Ter119 micro magnetic beads and passed over a Miltenyi column (Cat#130-049-901). For the adult and aged uninjured TAs 6 TA muscles (from 3 mice) were pooled together. For the injured TA muscles 2 TA muscles from 2 different mice were pooled together. Cells were then counted using a BioRad TC20 automated cell counter and processed with the 10X genomics single cell sequencing kit.

Single Cell sequencing

To capture, label, and generate transcriptome libraries of individual cells we used the 10X genomics Chromium Single Cell 3' Library and Gel Bead Kit v2 (Cat #PN-120237) following the manufactures protocols. Briefly, the single cell suspension, RT PCR master mix, gel beads, and partitioning oil were loaded into a Single Cell A Chip 10 genomics chip, placed into the chromium controller, and the chromium Single Cell A program was run to generate GEMs (Gel Bead-In-EMulsion) that contain RT-PCR enzymes, cell lysates and primers for illumine sequencing, barcoding, and poly-DT sequences. GEMs are then transferred to PCR tubes and the RT-PCR reaction is run to generate barcoded single cell identified cDNA. Barcoded cDNA is used to make sequencing libraries for analysis with Illuminia sequencing. We captured 1709 cells from young uninjured, 3459 from the aged uninjured, 5077 from the 4 d post injury and 2668 from the 7 d post injury. Sequencing was completed on an Illumina NovaSeq 6000, using paired end 150 cycle 2x150 reads by the genomics and microarray core at the University of Colorado Anschutz Medical Campus.

Bioinformatic methods

Cellranger v2.0.2 (10X Genomics) count module was used for alignment, filtering, barcode counting and UMI counting of the single cell FASTQs, the aggr module used for normalizing samples to same sequencing depth, and the reanalyze module used for final determination of gene expression and t-SNE 2D and 3D coordinates with parameters = [max_clusters=20, tsne_max_dim=3, num_principal_comp=20]. A 3D t-SNE plot was generated using plotly v2.0.15 (Plotly Technologies Inc. Collaborative data science. Montréal, QC, 2015. https://plot.ly) in a custom script and cells clustered manually based on the 3D t-SNE plot. A one way analysis of variance using a linear model of gene expression ~ sample was calculated to determine the p-value for each expressed gene in all cells in a cluster compared to its expression in all other cells, or its expression in cells in specific clusters, or to compare gene expression in cells in a cluster from different conditions. 2D t-SNE cluster plots were generated with CellrangerRkit (2.0.0) visualize clusters module with clustering based on the manual clustering using the 3D t-SNE plots. Gene expression plots were generated with the visualize_gene_markers module [limits=c(0,1.2)] from the log of the normalized gene expressions for each cell.

Heatmaps were generated using Morpheus software from the Broad Institute (https://software.broadinstitute.org/morpheus). Gene Ontology (GO) and gene set enrichment analysis (GSEA) were completed with g:prolifer (https://biit.cs.ut.ee/gprofiler/gost) software and web server (Reimand et al., 2016).

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Author Contributions

BP and BBO conceived the experiments. BP, ND, TA and BO performed experiments, analyzed the data, and made figures. RO and KJ directed and executed the bioinformatic analysis. BP and BBO wrote the manuscript. BBO supervised the research. All authors read and approved the manuscript.

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- Supplemental Table set S6: Lists of gene expression changes and individual gene expression levels between 4 d post injury and 7 d post injury for each cell type.
- Supplemental Table S7: Lists GO term analysis results for changes in MuSC between 4 d post injury and 7 d post injury.
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- Supplemental Table set S9: Lists gene expression changes and individual gene expression levels between adult uninjured and aged uninjured for each cell type.
- Supplemental Table S10: GO term analysis results for changes in MuSC between adult uninjured and aged uninjured

Figure Legends

Figure 1. Cellular atlas of adult skeletal muscle

(A) Fourteen different cell types (color coded and numbered) isolated from all conditions: adult uninjured TA muscle, aged uninjured TA muscle, 4 d post injury TA muscle and 7 d post injury TA muscle displayed as a 2D T-SNE plots shows. (B) Cell identity legend. (C) Heatmap plotting

scaled expression (Z-score) for the 30 most enriched genes for each cell cluster. Supplemental Table S1 contains a list of plotted genes.

Figure 2. Fibroblast single cell transcriptomes in injured and uninjured muscle

Colored coded T-SNE plots, identify (A) cells from all conditions (B) cells from adult uninjured TA muscle, (C) cells from 4 d post injury TA muscle, and (D) cells from 7 d post injury TA muscle. (E-J) T-SNE plots, show cells from all conditions, where scaled individual gene expression is depicted by color.

Figure 3. Immune cell subsets identified by single cell sequencing

(A) T-SNE plots, colored coded to identify cell types, show cells from all conditions. (B-J) T-SNE plots, show cells from all conditions, where scaled individual gene expression is depicted by color.

Figure 4. Comparing single cell transcriptomes between uninjured and 4 d post injury TA muscles.

(A) T-SNE plots of single cell transcriptional profiles from uninjured TA muscle and (B) 4 d post injury TA muscle. (C) Gene expression changes in MuSCs plotted as an expression ratio of 4 d post injury to uninjured. (D) Gene expression changes in fibroblasts population plotted as the expression ratio of 4 d post injury to uninjured (E) Heatmap with scaled gene expression values of common transcripts associated with inflammatory (M1) and regenerative (M2) macrophages. (F) Gene expression changes in macrophages plotted as expression ratio between 4 d post injury

to uninjured. (G) Gene expression changes in T cells plotted as expression ratio of 4 d post injury to uninjured. All changes in expression, reported as expression ratio, have a p-value < 0.05.

Figure 5. Comparing single cell transcriptomes between 4 d post injury and 7 d post injury TA muscles.

(A-B) T-SNE of single cell transcriptional profiles from 4 d post injury and 7 d post injury TA muscle. (C) Gene expression changes in MuSCs plotted as 7 d post injury vs 4 d post injury expression ratio. (D) Gene expression changes in fibroblasts population plotted as the expression ratio between 7 d post injury vs 4 d post injury expression ratio. (E) Gene expression changes in T cells plotted as an expression ratio between 7 d post injury vs 4 d post injury. (F) Gene expression changes in macrophages (gold) plotted as the expression ratio between 7 d post injury vs 4 d post injury expression ratio. All changes in expression, reported as expression ratio, have a p-value < 0.05.

Figure 6. Comparing single cell transcriptomes of uninjured adult and aged muscle.

(A-B) T-SNE of single cell transcriptional profiles from young uninjured and aged uninjured TA muscle. (C) Hierarchical cluster analysis comparing MuSC gene expression signatures. (D) Heatmap comparing scaled gene expression in MuSCs. (E) Hierarchical cluster analysis comparing T cell gene expression signatures. (F) Gene expression changes in fibroblasts population plotted as the expression ratio of aged uninjured to young uninjured. All changes in

expression have a p-value < 0.05. (G) Heatmap with scaled gene expression gene expression changes in fibroblasts from (F).

Supplemental Information Figure S1. 3D T-SNE Plot

3D interactive t-SNE plot in html file format that can be opened with any web browser showing cells from all conditions. Colors for each cell type are the same as shown in Figure 1.

Supplemental Information Figure S2. Fibroblast single cell transcriptomes

(A) T-SNE plots, colored coded to identify cell types, show cells from all conditions. (B-K) T-SNE plots, showing cells from all conditions, where scaled individual gene expression is depicted by color.

Supplemental Information Figure S3. Macrophage single cell transcriptomes at 4 days post injury

T-SNE plots, colored coded to identify cell types, show cells from (A) all conditions or (B) 4 d post injury. (C-H) T-SNE plots, show cells 4 d post injury, where scaled individual gene expression is depicted by color.

Supplemental Information Figure S4. T cell expression at 4 d and 7 d post injury

(A) T-SNE plots, colored coded to identify cell types from 4 d post injury. (B-E) T-SNE plots, identify cell types from 4 d post injury, where scaled individual gene expression is depicted by

color. (F) T-SNE plots, colored coded to identify cell types from 7 d post injury. (G-J) T-SNE plots identify cell types from 7 d post injury, where scaled individual gene expression is depicted by color.

Supplemental Information Figure S5. Gene expression changes between adult and aged uninjured TA muscles

(A) Gene expression changes in T cells plotted as the expression ration of aged uninjured to adult uninjured. (B) Gene expression changes in macrophages plotted as the expression ratio of aged uninjured to adult uninjured. (C) Gene expression changes in myeloid cells plotted as the expression ratio of aged uninjured to adult uninjured. All changes in expression, reported as expression ratio, have a p-value < 0.05.

Supplemental Information Figure S6. Hierarchical cluster analysis of fibroblasts and macrophages

(A) Hierarchical cluster analysis comparing fibroblast gene expression signatures across all conditions. (B) Hierarchical cluster analysis comparing macrophage (gold) gene expression signatures across all conditions.

color. (F) T-SNE plots, colored coded to identify cell types from 7 d post injury. (G-J) T-SNE plots identify cell types from 7 d post injury, where scaled individual gene expression is depicted by color.

Supplemental Information Figure S5. Gene expression changes between adult and aged uninjured TA muscles

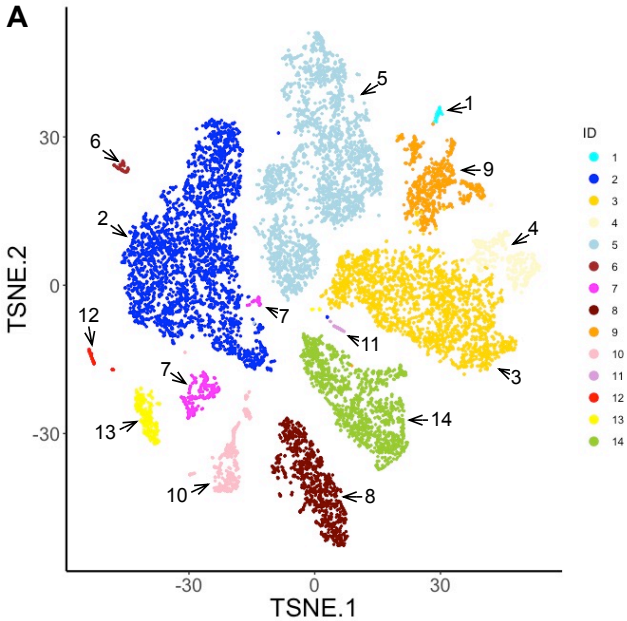
(A) Gene expression changes in T cells plotted as the expression ration of aged uninjured to adult uninjured. (B) Gene expression changes in macrophages plotted as the expression ratio of aged uninjured to adult uninjured. (C) Gene expression changes in myeloid cells plotted as the expression ratio of aged uninjured to adult uninjured. All changes in expression, reported as expression ratio, have a p-value < 0.05.

Supplemental Information Figure S6. Hierarchical cluster analysis of fibroblasts and macrophages

(A) Hierarchical cluster analysis comparing fibroblast gene expression signatures across all conditions. (B) Hierarchical cluster analysis comparing macrophage (gold) gene expression signatures across all conditions.

Figure 1-The cellular atlas of skeletal muscle

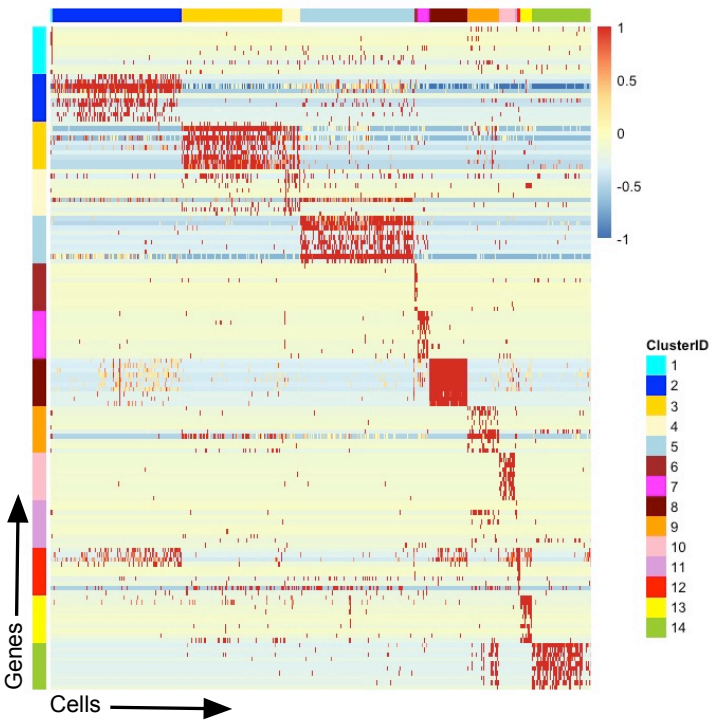
A



B

#	Color		Cell type
1	aqua		Macrophage
2	blue		Fibroblasts
3	gold		Macrophage
4	lemonchiffon		Macrophage
5	lightblue		Fibroblast (activated)
6	lightbrown		Schwann
7	magenta		Muscle stem cell
8	maroon		Skeletal muscle
9	orange		Dendritic macrophage
10	pink		Endothelial
11	plum		B cells and subtypes
12	red		RBC
13	yellow		Innate immune; myeloid
14	green		T cells and subtypes

C



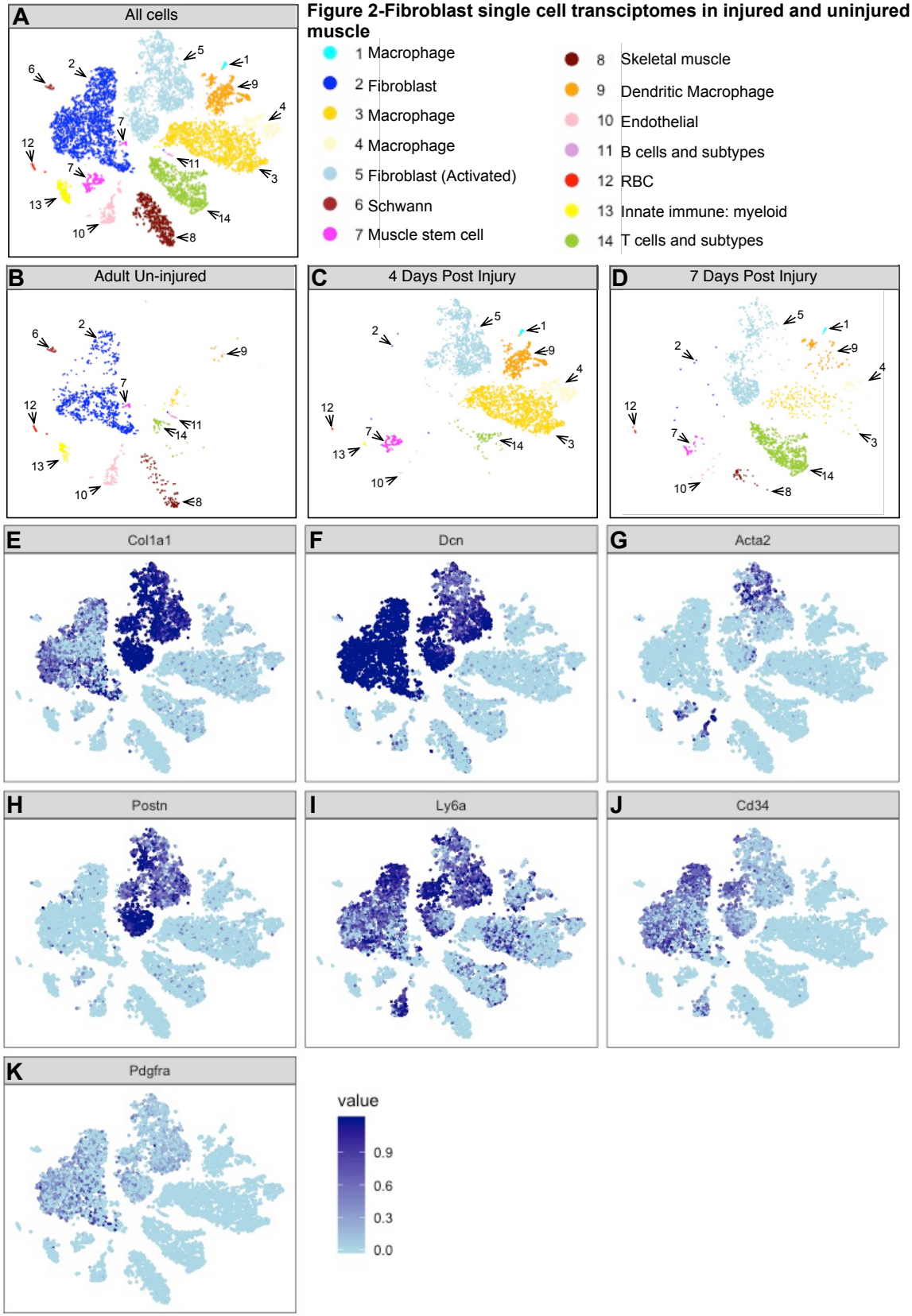
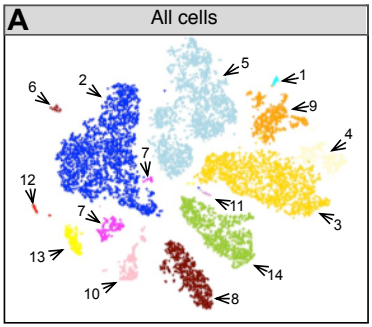


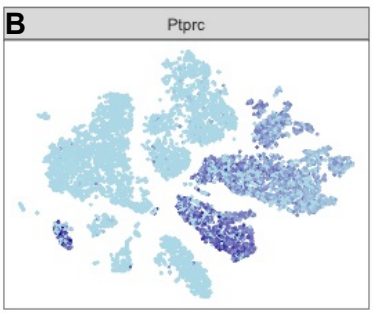
Figure 2-Fibroblast single cell transciptomes in injured and uninjured muscle

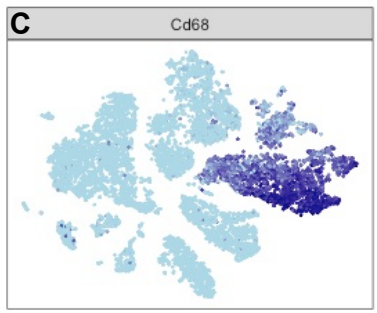
1 Macrophage
2 Fibroblast
3 Macrophage
4 Macrophage
5 Fibroblast (Activated)
6 Schwann
7 Muscle stem cell
8 Skeletal muscle
9 Dendritic Macrophage
10 Endothelial
11 B cells and subtypes
12 RBC
13 Innate immune: myeloid
14 T cells and subtypes

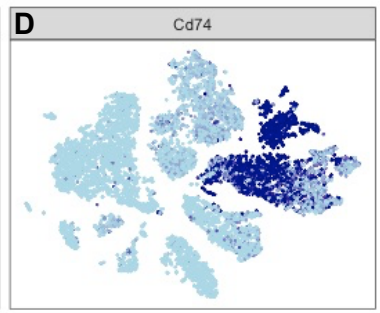
Figure 3-Immune cells subsets identified by single cell sequencing

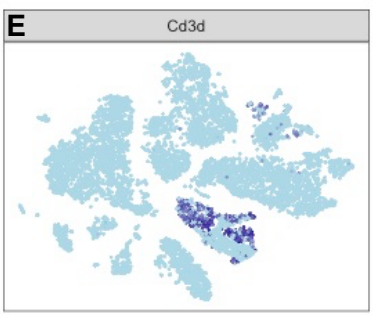


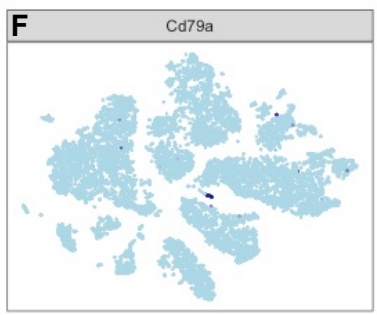
1 Macrophage
2 Fibroblast
3 Macrophage
4 Macrophage
5 Fibroblast (Activated)
6 Schwann
7 Muscle stem cell
8 Skeletal muscle
9 Dendritic Macrophage
10 Endothelial
11 B cells and subtypes
12 RBC
13 Innate immune: myeloid
14 T cells and subtypes

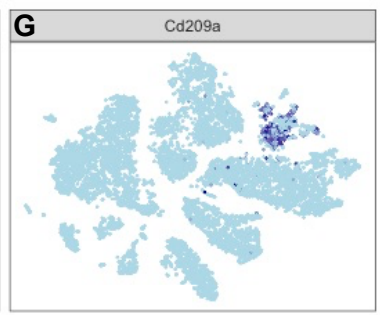


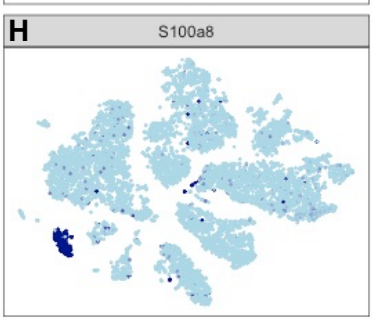


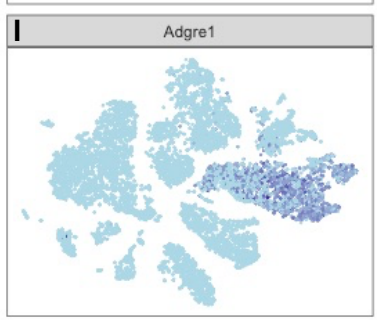


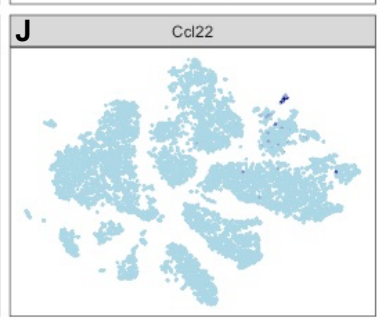












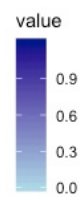


Figure 4 Comparing single cell transcriptomes between un-injured and 4 d post injury muscles

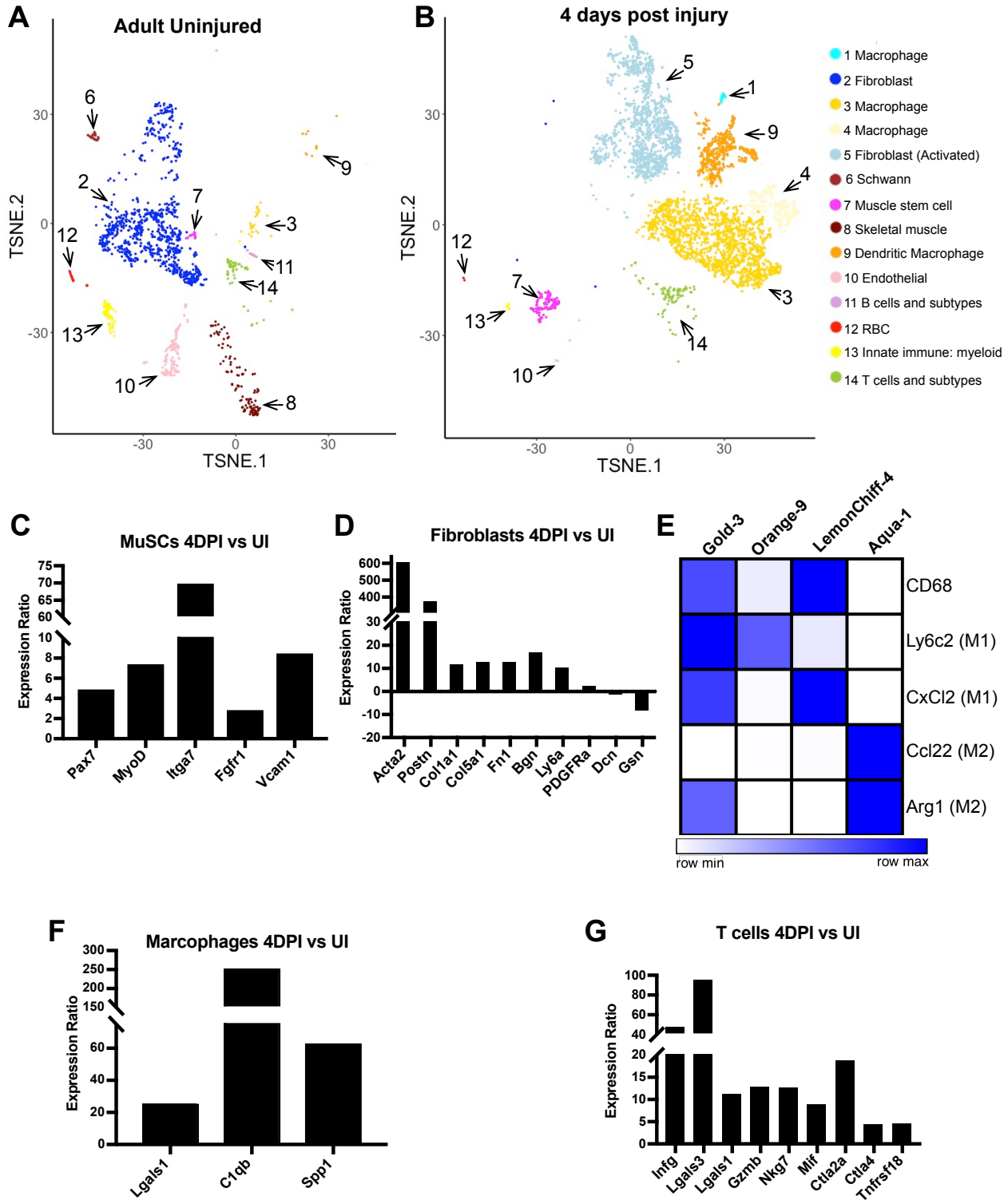
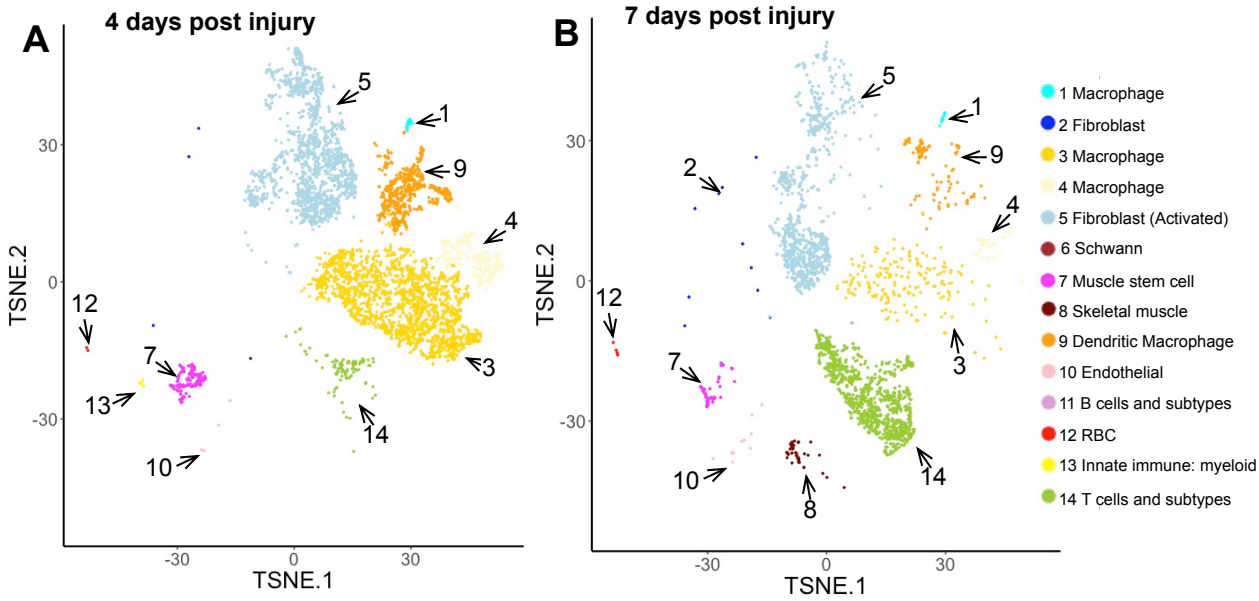
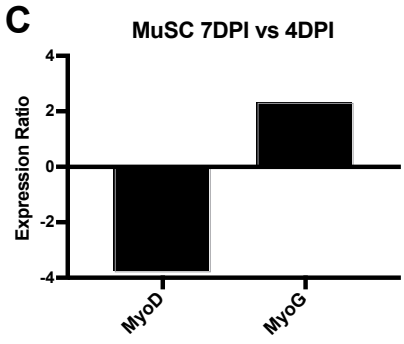
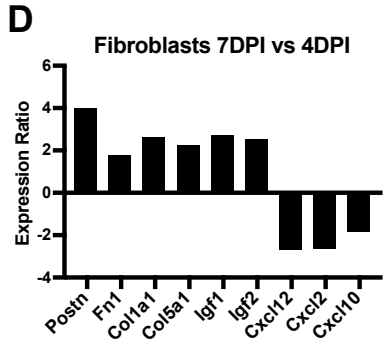
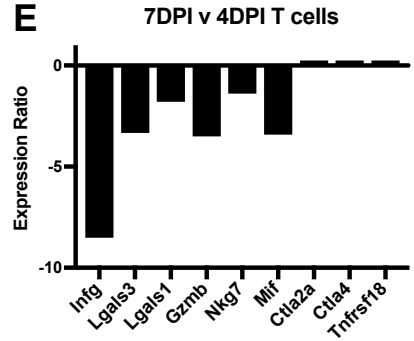


Figure 5 Comparing single cell transcriptomes between 4 d and 7 d post injury muscles









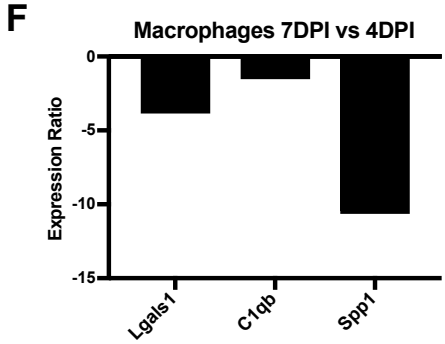


Figure 6 Comparing single cell transciptomes of uninjured adult and aged muscle