Distinct human stem cell subpopulations drive adipogenesis and fibrosis in musculoskeletal injury

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Abstract

Fibroadipogenic progenitors (FAPs) maintain healthy skeletal muscle in homeostasis but drive muscle degeneration in chronic injuries by promoting adipogenesis and fibrosis. To uncover how these stem cells switch from a pro-regenerative to pro-degenerative role we perform single-cell mRNA sequencing of human FAPs from healthy and injured muscles across a spectrum of injury. We identify multiple subpopulations with progenitor, adipogenic, or fibrogenic gene signatures. We utilize full spectrum flow cytometry to identify distinct FAP subpopulations based on highly multiplexed protein expression. We uncover that injury severity increases adipogenic commitment of FAP subpopulations and is driven by the downregulation of DLK1. Treatment of FAPs with DLK1 reduces adipogenesis, suggesting that during injury, reduced DLK1 within a subpopulation of FAPs may drive adipogenic degeneration. This work highlights how stem cells perform varied functions depending on tissue context, by dynamically regulating subpopulation fate commitment, which can be targeted improve patient outcomes after injury.

Keywords: Muscle, human, stem cells, fibroadipogenic progenitors, FAP, single cell RNA sequencing, rotator cuff tear, full spectrum flow cytometry, fibrosis, adipogenesis

Introduction

Fibroblasts expressing platelet derived growth factor receptor alpha (PDGFRα) are ubiquitous in adult tissues[1]. PDGFRα-expressing fibroblasts in skeletal muscle are termed fibroadipogenic progenitor cells (FAPs)[2,3]. In healthy tissue, FAPs regulate and promote myogenic regeneration. In chronic injury, however, FAPs impede regeneration by differentiating into adipocytes and fibroblasts and secreting factors that promote adipogenesis and fibrosis, a process known as fibro/fatty degeneration[2–5]. Single cell RNA sequencing (scRNA-seq) studies in both mouse and human tissues provide evidence that subpopulations of PDGFRα+ cells exist in adult tissues, including skeletal muscle[4–11]. A recent investigation of adult mouse skeletal muscle found multiple unique subpopulations, several of which seem to be responsible for *de novo* adipogenesis and tissue mineralization[10]. Heterogeneous FAP subpopulations may explain the multitude of roles these cells play in regenerating tissue.

A comprehensive understanding of human FAP cell heterogeneity and potential functional FAP subpopulations is limited by the challenges of obtaining normal and injured human tissue. Several studies of human skeletal muscle have shown unique FAP populations, but these studies have been limited by cell and patient number[5,8,11]. Furthermore, studies in a clinically relevant chronic injury model are lacking. The human rotator cuff offers a clinically relevant model to study FAP heterogeneity in homeostasis and disease. In rotator cuff tears, poor clinical outcomes and high retear rates are correlated with increased fibro/fatty muscle degeneration and muscle atrophy[12]. Recent studies of rotator cuff muscle degeneration suggest that fibro/fatty degeneration is mediated by FAPs[13]. Understanding the molecular and cellular drivers of fibro/fatty degeneration and muscle atrophy is critical for

improving muscle regeneration after rotator cuff injury. The mechanisms underlying fibro/fatty degeneration in rotator cuff injury may also be translatable to other muscle degenerative states including aging, muscular dystrophy, and other fibro/fatty degenerative diseases[1,14].

In this study, we performed scRNA-seq of over 24,000 human FAPs from healthy tissue and injured rotator cuff muscles across a spectrum of injury. We generated a robust dataset of 14 unique muscle samples, including 6 patients with paired injured rotator cuff muscle and healthy deltoid muscle samples taken together at the time of surgery. We identified FAP heterogeneity through gene and protein expression and discover how specific FAP subpopulations undergo transcriptional changes that correlate with injury severity and promote production of degenerative adipogenic tissue during injury. Utilizing high-dimensional full spectrum flow cytometry we identify distinct progenitor, adipogenic, and fibrogenic FAP subpopulations by multiplexed protein expression. We uncover that injury severity increases adipogenic commitment of FAP subpopulations and that this is driven by downregulation of Delta Like Non-Canonical Notch Ligand 1 (DLK1). Treatment of FAPs with DLK1 reduced adipogenesis, suggesting that during injury, reduced DLK1 within a subpopulation of FAPs may drive adipogenic degeneration. These experiments highlight the significance of stem cell heterogeneity in clinically relevant human samples. Our findings that tissue degeneration is driven by the regulation of specific stem cell populations within the larger stem cell pool presents opportunities for development of targeted therapies to improve patient outcomes for musculoskeletal injuries.

Results

Single cell RNA-sequencing identifies distinct human fibroadipogenic progenitor populations

We evaluated FAP heterogeneity in muscle biopsies from patients undergoing rotator cuff repair. We obtained biopsies from injured supraspinatus muscle with either a partial or full thickness tear and from non-injured deltoid muscle taken from the same patients, as healthy control tissue. As an additional source of healthy muscle, we obtained hamstring (semitendinosus and gracilis) muscle biopsies (**Figure 1a**). Samples were enriched for human FAPs by the surface marker combination CD45-/CD31-/CD56-/PDGFRα+ and single cell RNA sequencing was performed (**Figures 1a and S1a**). We integrated all single cell datasets and identified a total of 7 major cell types by enriched expression of known marker genes including: endothelial cells (*CD31*), pericytes (*RGS5*), fibroblast/myofibroblasts (*ACTA2*), myogenic cells (*PAX7 & MYOD1*), T-cells (*CD3E*), macrophages (*CD68*), and FAPs (*PDGFR*α) (**Figures 1b, S1b, S1c and Supplementary Table S1**). The majority of cells captured (24,929 out of 39,356) expressed high levels of *PDGFRα*. Therefore, we robustly enriched for and profiled human FAPs from injured and non-injured muscle.

We next subclustered the PDGFRα+ FAPs and identified 11 transcriptomically unique FAP subpopulations (**Figures 1c, S1d and Supplementary Table S2**). The FAP subpopulations were named after a top differentially expressed marker in each respective population (**Figures 1d, 1e, and S1e**). We identified a TNMD+ myotendinous progenitor population, two myofibroblast clusters marked by MYL9+ and TNNT1+, and two transitional clusters marked by CCN3+ and LAMA2+ (**Figures S2a and S2b)**. A GLI1+ cluster was notable for high and specific expression of hedgehog signaling genes (*GLI1* and *PTCH1*) and markers of stem/progenitor FAPs (*THY1* and *OSR1*), suggesting that cells in this cluster function as a FAP stem/progenitor cells (**Figures 2a and 2b**)[10,15,16]. A CD74+ population expressed markers of activated progenitor FAPs, including *VCAM1*, *OSR1* and *OSR2*, and *HGF* (**Figure 2c**). VCAM1 marks activated FAPs in adult mice, while OSR1 and OSR2 mark embryologic FAP progenitor populations[6,17]. HGF is a ligand involved in the expansion of progenitor populations during muscle regeneration[18,19]. These findings suggest that the CD74+ population functions as activated progenitors.

We identified a cluster expressing *PIEZO2*, a receptor involved in mechanosensation, suggesting that a subpopulation of FAPs may have mechanosensory function akin to other skeletal muscle stem cell populations (**Figure 2d**)[20–22]. This population also expressed markers of tendon progenitor cells including *SCX, TMND*, and *DPP4 (CD26)*[23]. The PIEZO2+ population may serve as a mechanosensitive population that gives rise to tendon progenitor cells. PIEZO2+ FAPs could be relevant in rotator cuff pathology, given rotator cuff muscle is mechanically unloaded with increasing severity of tear.

We also found two FAP clusters marked by *DLK1* or *CD55*, with upregulation of genes involved in either adipogenesis or fibrosis respectively, the main degenerative FAP phenotypes in skeletal muscle. DLK1 is a known marker of adipose progenitors and has been studied extensively in adipose tissue (**Figure 2e**)[24–26]. MME(CD10), a marker of human

adipogenic FAPs[27], and *ITGA8* and *LRIG1,* genes that increase during adipogenic commitment and differentiation, were all highly expressed in DLK1+ cells[28,29]. The CD55+ population was marked by expression of pro-fibrotic genes, such as *SEMA3C*, *LOXL1*, and *CD248* (**Figure 2f and Supplementary Table S2**)[30–32]. CD55+ cells also express *MSTN* (myostatin), a driver of muscle atrophy, suggesting this population of FAPs may be involved in the regulation of muscle growth and development[33]. Taken together, we hypothesize that DLK+ and CD55+ cells represent subpopulations of FAPs that are primed to become either adipocytes or fibroblasts, respectively.

This dataset demonstrates the heterogeneity of human FAPs and highlights two main FAP subpopulations that express markers associated with adipogenesis or fibrosis. Expression of adipogenic progenitor genes in the DLK1+ population and pro-fibrotic genes in the CD55+ population, suggests that the dual function of FAPs as pro-fibrotic or pro-adipogenesis may derive from discrete subpopulations of FAPs poised to give rise to either fibroblasts or adipocytes[24,27–31].

Transcriptionally heterogenous human FAPs also have heterogenous protein expression of distinct surface markers

Our scRNA-seq human FAP dataset revealed specific expression of genes that encode surface proteins in FAP subpopulations. Co-staining PDGFRα with the surface markers THY1, DLK1, and CD55 confirmed human FAPs have heterogeneous expression of the surface marker proteins identified as population markers in the scRNA-seq analyses (**Figure 2g**). We hypothesized we could label and isolate FAP subpopulations based on the expression of these markers with flow cytometry. To rigorously test whether the transcriptionally-defined FAP subpopulations could be identified at the protein level, we utilized full spectrum flow cytometry. In contrast to conventional flow cytometry, which is limited to a handful of fluorescent markers, full spectrum flow cytometry permits high dimensional profiling of up to 40 fluorescence markers[34]. We used this technique to profile the expression of surface markers in FAPs derived from healthy human muscle (**Figure 3a**). Live (SYTOX-) FAPs were identified by the marker combination CD45-/CD31-/CD56-/CD34+ and further profiled with 6 additional surface markers for FAP subpopulations; CD74, DLK1, CD10 (MME), CD55, CD26 (DPP4), and THY1, which is a highly expressed surface marker in the GLI1+ population (**Figures 3b and S3a**).

To first confirm expression of each protein, we determined the percentage of FAPs expressing each surface protein (**Figure 3c**). We then performed dimensional reduction on the expression levels of all 6 proteins (**Figure 3d**)[35]. Clustering analysis based on the expression of these 6 surface proteins resulted in similar clusters to those observed in the scRNA-seq analysis. THY1+ cells clustered together and were near CD74+ cells. We identified CD55 expressing cells and a subset of CD55+ cells that co-express CD26, which matches the *CD55* and *CD26* transcript expression patterns (**Figure S3b**). Additionally, we found two populations of CD10+ cells, one that did not express DLK1 and a second population that co-expressed DLK1, again in concordance with our scRNA-seq data (**Figures S3b, S3c, and S3d**). As predicted from the scRNA-sequencing, we identified the major distinct subpopulations of FAPs at the protein level utilizing surface markers of progenitor (THY1+), pre-adipogenic (DLK1+), and pre-fibrotic (CD55+) FAPs (**Figures 3e and S3e**). This data confirms that FAP transcriptional heterogeneity is translated into distinct protein expression patterns (**Figure 3f**).

DLK1/CD10 and CD55 are upregulated during FAP differentiation towards adipocyte or fibroblast cell fates

Previous work has suggested that a subpopulation of FAPs expressing *OSR1* function as progenitors for both fibroblasts and adipocytes[10,15,17,36]. We therefore tested our hypothesis that FAP subpopulations include FAP progenitors (marked by *GLI1, THY1*, and *OSR1*) and activated progenitors (marked by *CD74*, *OSR1*, and *OSR2*) which differentiate to FAPs committed to the fibroblast lineage (marked by *CD55*), adipocyte lineage (marked by *DLK1* and *CD10*), or tenogenic lineage (marked by *TNMD*). Pseudotime analysis revealed multiple paths from GLI1+ FAPs to *CD55*, *DLK1*, or *TNMD* expressing FAPs (**Figures 4a and S4a**). Across these cell fate trajectories, we found increasing expression of surface markers for FAP subpopulations (*DLK1*, *CD55*, and *CD26*) and genes associated with adipogenic, fibrogenic, or tenogenic identity (**Figures 4b and S4b**). We then focused on the lineages leading from FAP progenitors to pre-adipogenic and pre-fibrogenic FAPs, as increases in fibrosis and adipogenesis characterize typical muscle degeneration.

We first asked how FAP subpopulations are transcriptomically altered when directed towards a fibrogenic or adipogenic fate. We performed scRNA-seq of FAPs cultured in adipogenic, fibrogenic, or a

standard base expansion media that maintains FAPs in a proliferative state (adipogenic condition reanalyzed from Davies et al.[37]) (**Figures S4c**). Cells from each condition largely grouped together (**Figures S4d and Supplementary Table S3**). We found FAPs maintained in standard base media expressed the stem/progenitor markers *OSR1* and *CD74* (**Figures S4e and S4f**). FAPs cultured in adipogenic media produced a group of adipocytes, marked by expression of the adipocyte marker *PLIN1* and absence of FAP marker *PDGFRα*. Similarly, FAPs cultured in fibrogenic media produced a group of fibroblasts, marked by *ACTA2* and decreased *PDFGRα*. Between the bona fide FAP progenitors, *PLIN1*+ adipocytes, and *ACTA2*+ fibroblasts, we found PDGFRα+ FAPs that co-expressed either *MME*/*CD10* or *CD55* (**Figures S4f and Supplementary Table S4**). FAPs cultured in adipogenic media expressed higher levels of *MME*/*CD10*, while FAPS cultured in fibrogenic media expressed higher levels of CD55, supporting the hypothesis that FAPs marked by *MME*/*CD10* and *CD55* are pre-adipogenic and pre-fibrotic FAPs. Thus, human FAPs could be directed through an intermediate pre-adipogenic or pre-fibrotic FAP state towards *PLIN1*+ adipocyte or *ACTA2*+ fibroblast fate.

We next asked if we could capture FAPs as they transition from progenitors to either adipocytes or fibroblasts and identify their cellular state by protein surface expression. We first confirmed that FAPs increase expression of CD10 and CD55 in adipogenic and fibrogenic media, respectively, after 5 days of treatment (**Figure 4c**). We then analyzed FAPs after 5 days in adipogenic or fibrogenic media with full spectrum flow cytometry for our panel of FAP surface markers (THY1, CD74, DLK1, CD10, CD26, CD55) (**Figures 4d and 4e**). We also measured surface protein expression in FAPs that were not cultured, termed freshly harvested FAPs. FAPs largely clustered together by the media they were grown in, with some overlap, suggesting that the culture media produces distinct expression patterns of these markers (**Figure 4f**). We found cells cultured in the standard expansion media expressed the FAP progenitor markers, CD74 and THY1 (**Figures 4g and 4h**). Unexpectedly, THY1 was also expressed in cells in the adipogenic and fibrogenic media. However, compared to freshly harvested FAPs, the percentage of THY1+ cells was significantly increased in all culture conditions (**Figure S4g**). Expression of the progenitor marker CD26 (DPP4) was high in expansion media and decreased with adipogenic and fibrogenic differentiation (**Figures 4g and 4h**). This data suggests that the progenitor marker THY1 is upregulated by all FAPs following isolation and expansion, while CD26 may serve as a marker of activated proliferating progenitors in culture.

The adipogenic marker CD10 was largely expressed by cells in the adipogenic treatment group, while the fibrogenic marker CD55 was largely expressed by cells in the fibrogenic group (**Figures 4g and 4h**). As in our scRNA-seq dataset of human FAPs in Figures 1 and 2, DLK1+ cells were identified as a subset of CD10+ cells (**Figure 4g**). We found CD10+CD55- cells were near exclusive to adipogenic media conditions while conversely, CD10-CD55+ cells were exclusive to fibrogenic media (**Figure 4i**). Compared to freshly harvested FAPs, these two cell subsets were all also increased in each media respectively (**Figure S4h**). Taken together, the specific expression of CD10 and CD55 suggests that these two surface proteins can been utilized to mark human pre-adipogenic and pre-fibrogenic FAPs. The combination of mRNA and protein data at single cell resolution suggests that two FAP subpopulations contribute to the production of fibrofatty infiltration in human muscle. This data also identifies multiple markers of FAPs as they progress from the progenitor state to distinct lineages (**Figure 4j**).

Severe chronic injury increases adipogenic and fibrotic commitment of FAPs

We next sought to assess FAP subpopulations in a clinically significant model of human muscle disease: rotator cuff injuries, which affect more than 4.5 million people in the United States per year[38,39]. Rotator cuff injuries occur on a spectrum from partial thickness (incomplete) to full thickness (complete) tendon tears (**Figure 5a**)[38]. This spectrum of tendon injury is correlated with the severity of muscle degeneration in chronic injuries. Chronic full thickness tears readily develop fibro/fatty degeneration, while partial tears do not[40]. The degree of degeneration can be quantified clinically with magnetic resonance imaging (MRI) (**Figure 5b**). As degeneration increases, the degree of adipogenic infiltration is classified on MRI from Goutallier 0 (no fat), Goutallier 1-2 (moderate fat), and Goutallier 3-4 (severe fatty degeneration)[41,42]. To investigate the differences in chronic muscle injury, we compared chronically injured rotator cuff muscle to control healthy deltoid muscle taken from the same patients. Patients with full thickness injuries had moderate muscle fatty infiltration while partial tear patients' muscle quality were all without fatty infiltration (patients with severe, Goutallier 3-4, fatty degeneration generally are not candidates for repair) (**Figures 5b and S5a**).

We first compared the proportion of FAP subpopulations between healthy and injury groups in our freshly harvest FAP scRNA-seq dataset (**Figure S5b**). The proportion of each FAP subpopulation did not change in patients with partial thickness tears in comparison to healthy controls (**Figures 5c and S5c**). However, patients with full thickness tears had altered proportions of FAP subpopulations compared to healthy controls. We found an increased proportion of activated CD74+, pre-fibrogenic CD55+, and mechanosensitive PIEZO2+ FAP subpopulations, and a decreased proportion of pre-adipogenic DLK1+ and transitional CCN3+ FAP subpopulation (**Figures 5d and S5d**). Thus, in contrast to partial thickness tears, full thickness tears result in altered FAP lineage dynamics. In full thickness tears, proportions were found altered in favor of an increase in FAP phenotypes that include activated progenitor, fibrogenic, and mechanosensitive cells. Interestingly, the increase in PIEZO2+ cells which express *TPPP3* could function as tendon progenitor cells[43]. Full thickness tendon injuries produce mechanical unloading within the muscle which may push FAPs toward this lineage. Furthermore, the loss of *DLK1* expressing cells could at least in part explain the fatty infiltration seen in the muscle from these patients, through either commitment and differentiation of these cells towards adipocytes or loss in DLK1-mediated suppression of adipogenesis. These findings of FAP transcriptome and relative subpopulation abundance perturbations provide further evidence that injury phenotypes are secondary to specific changes within FAP subpopulations.

We then performed differential gene expression analysis of FAPs from the partial thickness and full thickness injured supraspinatus muscle groups compared to each patient group's corresponding control deltoid muscles. Differentially expressed genes were compared between the two injuries (**Figure 5e and Supplementary Tables S5 and S6**). We found genes that were up- or down-regulated in both injuries (bottom left and top right quadrants), and genes that were altered differentially between the two injuries (top left and bottom right quadrants). Notably *THY1* and *PLA2G2A* expression were significantly upregulated in both partial and full tears. *THY1* has been shown to be upregulated in human FAPs from the muscle of diabetic patients with adipogenic degeneration and *PLA2G2A* expression is increased in obesity[5,44]. Cell activation related genes, *FOS*, *MYC*, and *CDKN1A*, were significantly upregulated in the partial tear group compared to the full tear group. Genes involved in pro-adipogenic and pro-myotendinous regeneration were upregulated as injury progressed from partial to full tear (**Figure 5e**). Genes associated with repression of adipogenesis, such as *DLK1* and *SOX9*, were significantly downregulated in the full thickness tear group but not in the partial tear group (**Figures 5e and 5f**), while genes associated with collagen remodeling and myotendon regeneration, such as *TPPP3*, *FN1*, *FBN1*, and *COL14A1*, were significantly upregulated in the full thickness tear group but not in the partial tear group (**Figures 5e and 5f**)[23,43]. These results correlate with the degree of fatty degeneration seen on MRI, where only full thickness tears show increased adipogenesis and fibrosis[24,45]. These data suggest that FAP populations are altered on a continuum depending on injury severity and may explain the clinical changes in muscle function and structure seen in these patient groups.

We hypothesized that injury conditions may alter signaling between FAPs and other resident or infiltrating cells. To understand how cell signaling changes with injury, we investigated cell-cell interactions between FAPs and the other cell types present in our complete data set (**Figure 5g and S5e**). TGFβ signaling both to and from FAPs and multiple other cell types was elevated in muscle from partial tears and even more so in full thickness tears[4,46,47]. Immunomodulatory signaling such as APP and THY1 signaling from FAPs to macrophages and T-cells increased with injury severity[48–50]. We also found pro-fibrotic (tenascin) signaling from FAPs and pro-adipogenic (visfatin) signaling to FAPs were significantly increased with injury[51,52]. These data suggest that cell-cell signaling to and from FAPs is specific to the underlying degenerative pathology.

FAP subpopulation gene expression in chronic injury leads to a predominantly pro-adipogenic tissue state

We hypothesized that the altered proportions of FAP subpopulations and gene expression changes in injured muscle are reflective of altered FAP lineage commitment and increased production of adipogenic FAPs. To investigate this, we utilized RNA velocity to map cell trajectories in both healthy muscle and chronic full thickness tears (**Figures 6a, 6b, and S6a**)[53]. In healthy muscle, the major cell trajectories arise from the OSR1+/OSR2+/CD74+ progenitor population which splits into 2 pathways towards the pre-adipogenic (DLK1+) and pre-fibrogenic (CD55+) populations (**Figure 6a**). Cells emanating from the mechanosensitive (PIEZO2+) population are directed towards either the pre-fibrogenic or tenocyte (SCX+/TNMD+) progenitor population. We found FAPs from full thickness tear muscle had shifted cell trajectories towards the pre-adipogenic cells (**Figure 6b**). The pathway bifurcation from the CD74+

progenitor population, seen in healthy muscle FAPs, was reduced to a single pro-adipogenic pathway (**Figures 6b**). Taken together, this data suggests the cells in the progenitor subpopulation are pushed towards adipogenesis in chronic injury.

Given the pronounced trajectory changes in the CD74+, CD55+, and DLK1+ populations, we sought to uncover the gene expression differences in these three populations between healthy and full thickness injury muscle (**Figures 6c, S6b and Supplementary Table S7)**. Within the CD74+ population, we found significantly increased expression of *RUNX1*, *HIF1A*, *FGF7*, and *HGF* in FAPs from full thickness injury muscle compared to healthy control muscle. The transcription factors RUNX1 and HIF1A have been shown to attenuate both fibroblast and adipocyte lineage specification[47,54–58]. FGF7 and HGF have known roles in regulating tissue regeneration via several downstream effects including activation of progenitor cells and immunomodulation[18,19,59–61]. These findings suggest that CD74+ cells upregulate genes that control stem/progenitor cell activation and lineage specification during injury. *BMP4* is down regulated in the injured CD74+ population compared to CD74+ cells from healthy muscle. BMP4 regulates adipogenesis by promoting white adipocyte browning[62,63]. Additionally, we found downregulation of *EGFR* in CD74+ cells. Low levels of EGF signaling induce pre-adipocyte differentiation in 3T3-L1 cells[64]. Together, this suggests that upon injury, CD74+ cells activate differentiation programs and secrete factors that may drive FAP cell commitment to an adipogenic lineage.

In the CD55+ pre-fibrogenic population, expression of genes associated with regulation of fibrosis, such as *CD55*, *SEMA3C*, and *CD70*, were increased in injured FAPs (**Figures 6c, S6b, and Supplementary Table S7**)[31,65–68]. *COL3A1*, which is important for fibrillogenesis, was also significantly increased[69,70]. The antifibrotic factors *GDF10* and *CCN5* were significantly decreased in injury[71–74]. GDF10 has also been demonstrated to be a negative regulator of adipogenesis in dystrophic muscles, which may act on FAP progenitor populations[75]. The extracellular matrix gene COL15A1 was also significantly decreased in injury and interestingly is decreased in fibrotic tissue from patients with Dupuytren's contractures, another fibrotic musculoskeletal disease[76]. Furthermore, CD55+ FAPs from injured muscle have significantly less expression of *PDGFRα*, suggesting that these cells are pushed towards terminal differentiation. The altered expression of multiple secreted factors involved in fibrosis and adipogenesis in injury, suggests that CD55+ cells may be important in maintaining a healthy muscle environment. The increased proportion of CD55+ FAPs in chronic injury may lead to a loss of protective anti-fibrotic and anti-adipogenic signaling, which may ultimately lead to the promotion of fibrosis and adipogenesis.

Next, we hypothesized that the gene regulation within pre-adipogenic FAPs (DLK1+) could further explain the increase in fatty infiltration seen in full thickness tear muscle. Akin to the pre-fibrogenic cells, injured DLK1+ FAPs had significantly less expression of *PDGFRα*, suggesting the FAPs differentiate and down regulate *PDGFRα*. Injured DLK1+ FAPs upregulated pro-adipogenic genes, including *ADIRF*, *FOXP1*, *BMP5*, and *TGFBI*, and downregulated negative regulators of adipogenesis, including *NR4A1*, *SOX9*, and *DLK1* (**Figures 6c, S6b and Supplementary Table S7**)[77–84]. IRF1 was also significantly decreased, which may further attenuate lineage specification away from fibroblasts[85,86]. IRF1 has been shown to promote fibrosis through downregulation of a key regulator of adipogenesis, C/EBPβ[85], suggesting that the loss of IRF1 in injured DLK1+ FAPs would promote their differentiation towards adipogenesis rather than fibrosis[87].

Remarkably, *DLK1* expression itself was robustly decreased in injured DLK1+ FAPs with an approximately 5-fold significant difference. A proposed mechanism for DLK1-mediated inhibition of adipogenesis is through upregulation of *SOX9* expression[25,45]. SOX9 inhibits adipogenesis through direct binding to the promoter regions of C/EBPβ and C/EBPδ[45]. We found that *SOX9* was down regulated in injured DLK1+ FAPs. Additionally, injured DLK1+ cells have higher levels of mature spliced *SOX9* mRNA than nascent mRNA, suggesting its transcription is decreased during injury (**Figure S6c**). Similarly, in injured FAPs, nascent *DLK1* mRNA is decreased along the trajectory from progenitor to pre-adipogenic cells (**Figure S6c**). This suggests that upon injury, downregulation of DLK1 leads to increased adipogenesis and fatty infiltration of skeletal muscle. Furthermore, the decrease in *HIF1A* expression in the CD74+ population may further attenuate DLK1-mediated repression of adipogenesis as HIF1A stabilizes the cleavage of membrane bound DLK1 leading to both extracellular and intracellular DLK1 signaling cascades (**Figure 6c**)[88].

Given the expression changes in injured FAP subpopulations, we hypothesized that there would be accompanying FAP-mediated cell-cell interactions driving fibro/fatty degeneration in chronic injury. We found full thickness injury increased signaling pathways associated with degeneration compared to

healthy controls, including cell activation (HGF and THY1), fibrosis (SEMA3), and adipogenesis (BMP) signaling (**Figure 6d**). In full thickness injury, HGF signaling from progenitor and mechanosensitive FAP subpopulations to endothelial and myogenic cells was increased. Interestingly this process appears to be influenced by macrophages (**Figure S6d**). In injury, there was increased THY1 signaling from progenitor FAPs and myogenic cells to macrophages and increased signaling from pericytes and myogenic cells to fibrogenic FAPs (**Figure 6d**). The pro-fibrotic pathway SEMA3 is increased in injury largely through the fibrogenic FAP population as both a sender and influence/mediator of signaling (**Figures 6d and S6d**). Moreover, in the FAP subpopulations we found specific BMP ligands with differential expression changes in injury compared to healthy tissue. BMP receiving and regulation signaling was increased in the FAP progenitor populations after injury, while there was decreased receiving signaling in the adipogenic FAP population (**Figure S6d**). Upregulation of *BMP5* signaling in injury appears to be driving these changes (**Figure S6e**).

These results suggest that both intrinsic FAP subpopulation gene perturbations and FAP extrinsic changes in resident muscle cell gene signaling are driving fibro/fatty degeneration in chronic muscle injury. Taken together, the data from these experiments demonstrate a switch towards adipogenesis in injury conditions. Injured FAP subpopulations upregulate genes that promote adipogenesis and downregulate genes that drive anti-adipogenic pathways.

DLK1 prevents adipogenic differentiation of human fibroadipogenic progenitors

In our scRNA-seq data, we found expression of *DLK1* to mark a subset of CD10+ human pre-adipogenic FAPs. We confirmed that this transcriptionally-defined FAP subpopulation could be identified at the protein level, and found that there is a lineage trajectory of progenitor FAPs to DLK1+ pre-adipogenic FAPs. Furthermore, our data show that *DLK1* expression is significantly decreased in FAPs from chronically injured muscle. Therefore, we hypothesized that DLK1 protein is also decreased in chronic injury, leading to loss of DLK1-mediated inhibition of adipogenesis, which in part results in fatty degeneration of muscle. Given the pronounced decreased expression of *DLK1* mRNA in the pre-adipogenic population within the chronic full thickness tear injury muscle, we first sought to confirm that DLK1 protein expression was also decreased. We compared FAPs isolated from healthy deltoid muscle to FAPs from injured full thickness tears with full spectrum flow cytometry for DLK1 protein expression (**Figures 6e**). Akin to the scRNA-seq data, FAP specific DLK1 protein expression was significantly decreased in injured compared to healthy muscle (**Figure 6f**). We hypothesized that DLK1 loss results in increased adipogenic differentiation of FAPs and that this could be reversed through treatment with DLK1 protein. We tested this *in vitro* by culturing FAPs in adipogenic media for 1 week with recombinant DLK1 protein compared to PBS control (**Figure 6g**). Addition of DLK1 reduced adipogenic differentiation by greater than 50% (**Figure 6h**). Thus, we propose that loss of DLK1 protein expression in chronic full thickness rotator cuff injuries contributes to fatty degeneration seen in these patients (**Figure 7**).

Discussion

Musculoskeletal injuries and specifically rotator cuff injuries, impact millions of people in the United States each year, are an enormous burden on the health care system, and cause significant morbidity in these patient populations[39]. In this study, we sought to investigate stem cell functional heterogeneity in a clinically relevant model of human muscle disease that is also directly applicable to many other muscle degenerative pathologies. Our dataset provides a valuable resource of over 24,000 fibroadipogenic progenitor cells, and over 14,000 cells of other major resident cell types of healthy and injured skeletal muscle. We utilized scRNA-seq to identify human FAP subpopulations and propose that these are functionally heterogeneous. We highlight five clusters which likely serve functionally distinct roles in homeostasis and regeneration: progenitor (GLI1+ and CD74+), pre-adipogenic (DLK1+), pre-fibrogenic (CD55+), and transitional mechanosensitive (PIEZO2+) subpopulations. Utilizing full spectrum flow cytometry, we were able to resolve these unique stem and progenitor cell populations through surface markers and provide a roadmap for prospective isolation as full spectrum flow sorting machines become more widely available[89]. We then show that FAP subpopulations are perturbed in injury and these changes are highly sensitive to injury severity. In severe chronic injury, subpopulation gene expression and cell proportions are both affected with the net result of increased FAP-specific adipogenesis, mirroring the clinical phenotype. Finally, we propose that DLK1 is both a marker of pre-adipogenic cells and is a crucial regulator of FAP adipogenesis. DLK1 protein is sufficient to significantly inhibit FAP commitment to adipogenic differentiation possibly through upregulation of SOX9.

OSR1 is thought to mark true FAP progenitor populations in mice[15,17,36]. Here we identified two distinct human FAP OSR1+ populations which express high amounts of either *GLI1* or *CD74*. The GLI1+ population expressed high levels of hedgehog signaling markers and *THY1*. Both *GLI1* and *THY1* expressing cells are highly proliferative and responsible for *de novo* adipogenesis in skeletal muscle[5,90]. The supply of hedgehog ligand has yet to be confirmed but is thought to come from Schwann or endothelial cells[90]. Interestingly we found high expression of the hedgehog signaling protein desert hedgehog (*DHH*) in a subset of endothelial cells which may be the cellular origin of this ligand in human skeletal muscle (**Figure S7a**). In the intestine, PDGFRα+ subepithelial mesenchymal cells that express GLI1 maintain the epithelial stem cell population through secretion of Wnt2b or Wnt3a[91]. The human GLI1+ FAP population identified here also has specific and high expression of *WNT2b* (**Figure S7b**). It is likely that FAP-specific control of the stem cell niche occurs through the regulation of WNT ligand expression in hedgehog responsive cells.

In muscle injuries, progenitor cells give rise to new populations that prevent proper regeneration. Treatments that can prevent progenitors from giving rise to degenerative cell populations could improve patient outcomes after injury. For example, treatment of FAPs with a beta-agonist induces FAP beigeing and improves muscle regeneration[37]. *CXCL12* was upregulated in beigeing FAPs and is also a marker of activated CD74+ FAPs in our dataset (**Figure S7c**). We hypothesize CXCL12 could be critical for directing FAP progenitor fate and could possibly function through CXCL12 – CXCR4 signaling, as expression of *CXCR4* is present on both muscle stem cell and immune cell populations (**Figures S7a**)[92,93]. By uncovering novel markers of FAP subpopulations, we can begin to unravel the mechanisms by which either pro-regenerative or degenerative FAP states are produced. It will be critical for future studies to investigate the "tunability" of this population to influence FAP differentiation towards pro-regenerative subpopulations and away from degenerative adipogenic and fibrogenic states.

We further demonstrate that utilization of full spectrum flow cytometry enables highly specific characterization of stem cell populations in skeletal muscle. Identification of surface markers of pre-fibrogenic and pre-adipogenic FAP populations provides a method for the direct study of FAP heterogeneity both *in vivo* and *in vitro*. CD55 marks fibrogenic FAPs and this subpopulation is inducible through prolonged TGFβ stimulation. DLK1 and CD10 mark pre-adipogenic FAPs and CD10 expression is increased along adipogenic differentiation. It is also interesting that DLK1 expression *in vitro* was decreased. DLK1 expression is decreased by dexamethasone, a common component in adipogenesis treatment media[26]. We propose that DLK1 marks pre-adipogenic FAPs but is lost upon adipogenic differentiation. The identification of these surface markers offers an attractive way to prospectively study and isolate subpopulations including fibrogenic and adipogenic FAPs.

We next applied this knowledge of FAP subpopulations to a chronic injury model of rotator cuff injuries. We found underlying gene and protein expression are altered depending on incomplete versus complete tendon injuries, suggesting that FAPs respond uniquely to the severity of the rotator cuff tendon tear. As injury severity increases (partial to full tear) FAP lineage specification switches to a predominantly adipogenic cellular program. This correlates to the clinical phenotype, with increased severe fatty infiltration on MRIs of patients with larger, chronic rotator cuff tears[40]. Indeed, there appears to be FAP subpopulation specific changes that while unique to each population, are all leading to a net increase of adipogenesis in response to injury severity.

There are currently no accepted pharmacologic strategies to prevent muscle degeneration. FAP subpopulation-specific changes in injury offer novel avenues for therapeutics development. For example, in rotator cuff injuries the degree of muscular fatty degeneration is negatively correlated with clinical outcomes even after successful tendon repair. When degeneration becomes severe, repair is overwhelmingly likely to fail, thus these patients must undergo a more extreme treatment consisting of reverse total shoulder replacement to restore shoulder function[42,94,95]. Even mild levels of fatty degeneration persist after repair, highlighting the need for adjunctive medical treatments[95]. As such, intervening before degeneration becomes advanced is of paramount importance. We propose the CD74+/OSR1+/OSR2+ population is the human progenitor population that can be targeted in injury to ameliorate fatty degeneration. Even in chronic tears, there appear to be an abundant number of these cells in supply, despite the overall switch to a pro-adipogenic program, which could be reprogrammed to promote muscle regeneration. In chronically injured muscle, we find increased expression of transcription factors *HIF1A* and *RUNX1* in CD74+ progenitors which may explain adipogenic cell trajectories following rotator cuff injury. We see more evidence for this pro-adipogenic program switch within the pre-adipogenic

DLK1+ population itself. We found DLK1 expression is greatly decreased in pre-adipogenic FAPs in injured muscle. One proposed mechanism for the anti-adipogenic effects of DLK1 is through upregulation of *SOX9*, which was significantly decreased in injured cells within this population[25,45]. Moreover, we were able to confirm that DLK1 protein expression is decreased in FAPs from chronic full thickness tears. We then found that DLK1 treatment of human FAPs can inhibit FAP adipogenesis even in a pro-adipogenic environment. Thus, in this model of muscle degeneration there is a critical loss of a negative feedback loop which normally prevents commitment of pre-adipogenic FAPs to adipogenesis. These findings demonstrate the importance of understanding stem and progenitor cell heterogeneity within musculoskeletal injuries. This dataset can provide a valuable resource for the identification of molecular targets to direct progenitors towards pro-regenerative fates and suppress the production of degenerative phenotypes. It will be critical for future translative studies to utilize this knowledge of human FAP heterogeneity to target specific cell states and populations in degenerative diseases.

Materials and Methods

Muscle Procurement and Digestion

This study was conducted under the approval of the Institutional Review Board (IRB # 18-26000 and 18-26760) at The University of California San Francisco (UCSF). Biopsies were obtained from individuals undergoing surgery at UCSF. Written informed consent was obtained from all subjects. Deltoid and supraspinatus muscle samples were obtained during arthroscopic surgery with a biting grasper tool. Hamstring muscle was obtained from harvested hamstring tendons utilized for anterior cruciate ligament reconstruction. All samples were transferred from the operating room on ice and stored in 30% FBS (fetal bovine serum, Gibco, #10437028) in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, #11965084) until processing. Muscle was trimmed of excess fat, tendon, and connective tissue. Muscle was then mechanically and enzymatically digested in 1 mg/ml collagenase III (Worthington Biochemical Corporation, #NC0021929) in DMEM with 10% FBS and 1% Penicillin/Streptomycin (Gibco, #15140122) at 37°C for 70 minutes with intermittent manual needle trituration with an 18-gauge needle[96]. Digests were washed with PBS (Gibco, #10010023) and further digested with 0.25% trypsin-EDTA (Gibco, #25200056) at 37°C for 12–15 min. Suspensions were passed through 40 µm nylon mesh (Corning, #431750), erythrocytes were lysed with ACK lysing buffer (Quality Biological, #188-156-101) for 5–7 min on ice and washed in PBS with 2% FBS. Cells were washed and resuspended in 100 µl flow cytometry buffer (PBS with 2% FBS, 5 mM EDTA (Corning, #MT-46034CI), 2 mM HEPES (Quality Biological, #118-089-721)) per 1 gram of starting tissue weight. Antibody cocktail consisting of: anti-CD31 (eBioscience, #48-0319-42, eFluor 450) (2 µl per gram of starting muscle weight), anti-CD45 (eBioscience, #48-0459-42, eFluor 450) (2 µl), anti-CD56 (Miltenyi Biotec, #130-114-548, APC-Vio770) (8 µl), anti-CD34 (BD, #555823, PE-Cy5) (6 µl) and anti-PDGFRα (BioLegend, 323506, PE) (8 µl) was then added to the cells for 30 minutes on ice. Cells were washed and resuspended in flow cytometry buffer with 1:1000 SYTOX Blue (ThermoFisher, #S34857). Flow cytometry analysis and cell sorting were performed at the UCSF Flow Cytometry Core with BD FACSAria2 machines operated using FACSDiva software. Flow cytometry isolations were analyzed with FACSDiva and FlowJo software.

Single Cell mRNA Sequencing

Viable CD31-/CD45-/CD56-/CD34+/PDGFRa+ FAPs were collected and spiked into a collection of all live (SYTOX-)cells to obtain samples with >50% FAPs. Cells were resuspended in 0.04% BSA (EMD Millipore, #12659-500gm) in PBS at 1000 cells per µl to prepare for scRNA-seq. For each sample 5,000-10,000 cells were loaded onto one lane of a 10X Chromium chip per sample, using the Chromium Single Cell 3' Reagent kit (10X Genomics, version 3.1, #PN-1000123). Manufacturer's instructions were followed for Gel Beads-in-emulsion (GEM) generation, cDNA production and library construction. Libraries were sequenced on Illumina NovaSeq. CellRanger (version 3.0) was used with default settings to de-multiplex, align reads to the human genome (10X Genomics pre-built GRCh38 reference genome) and count unique molecular identifiers (UMIs). Doublets were removed from individual datasets with DoubletFinder based on loading concentrations and the 10X Genomics estimates for doublet rates[97]. Seurat (version 4.1) was used for downstream analysis of individual datasets, including filtering, dimension reduction, clustering, UMAP, and differential gene expression analysis[98]. Samples were integrated according to the Seurat Integration Tutorial. Scaling, dimension reduction, and clustering were performed in Seurat to integrate data for visualization. Differential gene expression analysis and gene expression visualization were performed in Seurat on the original, non-integrated values. For pseudotime analyses, UMAP dimensions from Seurat were imported into Monocle3 (version 1.0)[99].

Pseudotime ordering and branch analysis was then performed according to the Monocle3 tutorial. RNA velocity analyses performed with scVelo according to the Differential Kinetics tutorial (version 0.2.5)[53,100]. CellChat (version 1.6.0) was utilized to predict cell-cell communication of ligand receptor pair interactions with standard parameters following the author's tutorial[101].

Full spectrum flow cytometry

For analysis of freshly harvested samples, muscle was digested, and cell suspensions were prepared as above. Cells were stained with anti-CD31 (eBioscience, #63-0319-42, Super Bright 600) (2 µl per gram of starting muscle weight), anti-CD45 (eBioscience, #48-0459-42, eFluor 450) (2 µl), anti-CD56 (Miltenyi Biotec, #130-114-548, APC-Vio770) (8 µl), anti-CD34 (BD, #555823, PE-Cy5) (6 µl), anti-THY1 (eBioscience, #11-0909-42, FITC) (2 µl), anti-CD74 (BioLegend, #326808, PE) (2 µl), anti-DLK1 (R&D, #FAB1144N, Alexa Fluor 700) (4 µl), anti-CD10 (BioLegend, #312226, BV711) (2 µl), anti-CD26 (BioLegend, #302710, APC) (1 µl), and anti-CD55 (BioLegend, #311314, PE-Cy7) (2 µl). We utilized single color controls both with both beads (ThermoFisher, #01-1111-42) and cells. For analysis of cultured FAPs, cells were gently lifted with 0.05% trypsin, washed, resuspended in 100 µl flow cytometry buffer (PBS with 2% FBS, 5 mM EDTA, 2 mM HEPES) per $1x10^6$ cells, and stained with anti-THY1 (2 µl per $1x10^6$ cells), anti-CD74 (2 µl), anti-DLK1 (4 µl), anti-CD10 (2 µl), anti-CD26 (1 µl), and anti-CD55 (2 µl). Fluorescence minus one (FMO) controls were made with one marker left out of the antibody cocktail for each respectively. In both fresh digests and cultured cell preparations, cells were washed and resuspended in flow cytometry buffer with 1:2000 SYTOX Blue viability dye (ThermoFisher, #S34857) added just prior to analysis. Flow cytometry analysis and cell sorting were performed at the UCSF Flow Cytometry Core with Cytek Aurora machines operated using SpectroFlo software. Flow cytometry data were analyzed with SpectroFlo and FlowJo software. Dimension reduction was performed in FlowJo with UMAP[35]. Data was then visualized in R with ggplot2.

FAP culture

FAPs were isolated from fresh tissue digests with the surface marker combination CD31-/CD45-/CD56-/CD34+. Primary cells were cultured in expansion media: Ham's F-10 Nutrient Mix (Gibco, #11550043) containing 1% Penicillin/Streptomycin, 10% FBS, and 10ng/mL bFGF (Gibco, #PHG0264). Cell media was changed every 2-3 days and cells were passaged at 70-80% confluency. For adipogenic differentiation, FAPs were grown to 80% confluency in expansion media and then switched to adipogenic media (StemPro Adipogenesis Differentiation Kit, Gibco, # A1007001). Media was changed every 2 days. For fibrogenic differentiation, FAPs were grown to 80% confluency in expansion media and then switched to expansion media containing, 10 ng/ml of TGFβ1 (Invitrogen, #PHG9204). Media was changed every 2 days.

DLK1 treatment

FAPs were seeded on polystyrene plates at a density of 60,000 cells/cm^2. At 80% confluence cells were treated with adipogenic media (Gibco, #A1007001) containing 5 µg/ml rDLK1 (R&D Systems, #1144-PR-025) or PBS vehicle. Media was changed every 2 days.

Immunofluorescence and imaging

Human muscle biopsies were obtained from the operating room and transferred on ice. Tissues were flash frozen within O.C.T. Compound (Tissue-Tek, #4583) in liquid nitrogen cooled 2-methylbutane (Millipore Sigma, #MX0760) and stored at -80 degrees. Transverse muscle sections were cut at 8 µm and stored at -80 degrees. Slides were warmed at room temperature prior to initiating staining protocols. Slides were fixed in 4% PFA (Electron Microscopy Sciences, #50-980-487) at room temperature for 10 min, washed in PBST (PBS with 0.1% Tween-20 (Millipore Sigma, #P1379)), then blocked with protein-free serum block (Agilent, #X090930-2) for 1 hour and incubated at 4 degrees overnight with the corresponding primary antibodies. Primary antibodies and dilutions for human muscle cross section imaging: anti-human PDGFR alpha antibody 1:100 (R&D Systems, #AF-307-NA), anti-Laminin 2 alpha 1:250 (Abcam, #ab11576), anti-CD55 1:100 (Invitrogen, #MA1-911610), anti-CD90 (Thy-1) 1:100 (Invitrogen, #14-0909-82), and anti-DLK-1 1:100 (Abcam, #ab89908). After a PBST wash the corresponding secondary antibodies were applied for 1 hr at room temperature. Secondary antibodies and dilutions for human muscle cross section imaging: Donkey anti-mouse (Alexa Fluor® 488) 1:500 (Abcam, #ab150105), donkey anti-goat (Alexa Fluor® 594) (Invitrogen, #A-11058) 1:500, and donkey anti-rat (Alexa Fluor® 647) 1:500 (Abcam, #ab150155). After a PBST wash, slides were mounted with VECTASHIELD mounting medium with DAPI (Vector

Laboratories, #H-2000-2). For immunofluorescence imaging of cultured cells, cells were washed with PBS and fixed with 4% PFA at room temperature for 10 min, and then washed, blocked, and stained in the same manner as with slides described above. Primary antibodies and dilutions used for stains of cultured cells: anti-CD10 1:100 (Santa Cruz Biotechnology, INC., # sc-19993) and anti-CD55 1:100 (Invitrogen, #MA1-911610). Secondary antibodies and dilutions used for stains of cultured cells: Donkey anti-mouse (Alexa Fluor® 488) 1:500 (Abcam, #ab150105). Cells were counter-stained with DAPI 1:10,000 (Millipore Sigma, #D9542-10mg) for 5 minutes at room temperature, then washed with PBST, and kept in PBS for imaging. For quantification of lipid production in cell cultures, cells were fixed with 4% PFA at room temperature for 10 minutes, rinsed three times for 30 seconds in DI water, and then stained with 0.36% Oil Red O solution (EMD Millipore, #90358) for 30 minutes. Cells were once again rinsed three times for 30 seconds in DI water and then kept in 10% glycerol in PBS for imaging. All images were taken on a Zeiss Axio Observer D1 fluorescence microscope and were analyzed using ImageJ.

MRI imaging and analysis:

T2-relaxtion magnetic resonance imaging studies were utilized to grade muscle fatty infiltration in the supraspinatus and deltoid muscles as originally described by Goutallier et al[41]. Classification as follows: Goutallier 0: no appreciable fat content, Goutallier 1: some fat streaking, Goutallier 2: fat is appreciable but less fat than muscle, Goutallier 3: fat content equal to muscle, Goutallier 4: fat content is greater than muscle.

Statistical Testing

All statistical testing was performed using Prism 9.4 software. Statistical tests and p-value cut-offs used for each experiment are listed under the relevant heading.

Resource Availability

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Brian Feeley (brian.feeley@ucsf.edu).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

Raw and processed scRNA-seq data will be available on NIH. scRNA-seq data will be deposited on CellxGene.

Code and scripts utilized in this study will be made available on GitHub.

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Author Contributions

Conceptualization, S.M.G. and B.T.F.; Methodology, S.M.G. and B.T.F.; Software, S.M.G. and J.L.; Validation, S.M.G. and B.T.F.; Formal Analysis, S.M.G., A.D., and J.L.; Investigation, S.M.G., A.D., J.L., H.C., and M.L.; Data Curation, S.M.G.; Writing – Original Draft, S.M.G.; Writing – Review & Editing, S.M.G., A.D., X.L., M.R.D., and B.T.F.; Visualization, S.M.G. and J.L.; Supervision, X.L. and B.T.F.; Project Administration, X.L. and B.T.F.; Funding Acquisition, S.M.G., X.L., and B.T.F.

Declaration of interests

The authors declare no competing interests.

Inclusion and diversity

We support inclusive, diverse, and equitable conduct of research.

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Figure Titles and Legends:

Figure 1: Single cell sequencing identifies human fibroadipogenic progenitor populations. **a)** Experimental design for single cell mRNA sequencing (scRNA-seq). **b)** UMAP of all cells sequenced (39,356) labeled by cell type. Inset: *PDGFRα* expression. **c)** UMAP of the PDGFRα+ FAPs (24,929 cells). Upregulated gene markers of each population are shown in the same color and outlined with dashed lines. **d)** Dot plot demonstrating expression of each subpopulation marker. **e)** Expression plots demonstrating expression of each subpopulation marker. See also Supplementary Figure 1.

Figure 2: Distinct human fibroadipogenic progenitor populations are marked by *GLI1*, *CD74*, *DLK1*, *PIEZO2*, and *CD55*. **a)** Cartoon FAP subpopulation UMAP with highlighted populations. **b-f** Expression plots of upregulated genes in the **b)** GLI1+, **c)** CD74+, **d)** DLK1+, **e)** PIEZO2+, and **f)** CD55+ populations. **g)** Left: cartoon UMAP plots. Right: immunofluorescence of human muscle cross sections. Large yellow arrows: PDGFRα+ cells positive for the population marker. Small white arrows: PDGFRα+ cells negative for the population marker. Scale bar = 20 µm. n = 3 biological replicates. See also Supplementary Figure 2.

Figure 3: Transcriptionally heterogenous human fibroadipogenic progenitors also have heterogenous protein expression of distinct surface markers. **a)** Experimental design of full spectrum flow cytometry analysis of human FAPs. **b)** Representative full spectrum flow cytometry plots showing FAP surface protein expression. **c)** Bar plot of total expression of each surface marker on all FAPs. **d)** Representative UMAP of full spectrum flow cytometry data. **e)** Bar plot of the percentage of FAPs which express one marker only, all markers, or none of 4 surface markers. **f)** Cartoon of human FAP subpopulations with the surface marker profiles of each population. n = 6 biological replicates for all data. All plots represent mean ± SEM. See also Supplementary Figure 3.

Figure 4: DLK1/CD10 and CD55 are upregulated during FAP differentiation towards adipocyte or fibroblast cell fates. **a)** Pseudotime analysis demonstrates distinct human FAP lineages that progress from progenitor cells to adipogenic, fibrogenic, and tenogenic FAPs. **b)** Gene expression plots along pseudotime for each lineage from progenitor cells down adipogenic, fibrogenic, and tenogenic pathways. Cells placed along pseudotime for each path depicted above the plots. Pseudotime split into 20 equal sized bins. **c)** Immunofluorescence images of day five FAPs grown in media conditions: standard expansion media (std), adipogenic media (adipo), and fibrogenic media (fibro). Scale bar = 20 µm. **d)** Experimental design of human FAP full spectrum analysis of cultured cells. **e)** Representative flow cytometry plots of FAP surface protein expression. **f)** UMAP of all cultured cells labeled by condition. **g)** Surface protein expression plots on UMAP. **h)** Bar plot of total surface marker expression on FAPs cultured in each media. **i)** Bar plot of CD10+CD55- and CD10-CD55+ expressing FAPs. **j)** Cartoon schematic of proposed human FAP lineages and accompanying gene/protein markers. n = 6 biological replicates for all data. All plots represent mean ± SEM. Statistical test for all plots: two-way ANOVA with Tukey's multiple comparison correction. *p < 0.05 **p < 0.01 ***p < 0.001. See also Supplementary Figure 4.

Figure 5: Severe chronic injury increases adipogenic and fibrotic commitment of FAPs. **a)** Cartoon cross-section of the human shoulder. Rotator cuff tenon tears are classified as either partial (incomplete thickness) or full (complete thickness) tears. **b)** Representative cross-section, T2-weighted, MRI images of human rotator cuff muscles. Supra (supraspinatus), infra (infraspinatus), subscap (subscapularis) muscles. Fatty infiltration classification: G0-4. **c-d** Paired sample plots of cell proportion within each subpopulation in **c)** partial thickness tendon tears compared to healthy deltoid muscle from the same patients, and **d)** full thickness tendon tears compared to healthy deltoid muscle from the same patients. Paired samples linked with lines. n = 3 partial and n = 3 full thickness tear unique biological replicates. Statistical test for all plots: paired two-way ANOVA. *p < 0.05 **p < 0.01. **e)** Scatter plot of differential gene expression comparing changes in partial and full thickness tear muscle compared to healthy deltoid muscle control muscle. Significant expression changes were $Log_2FC > 0.32$ and $p < 0.05$ (blue dotted lines). **f)** Volcano plot demonstrating significant gene differential expression in full thickness tear muscle samples compared to healthy control muscle. Significant genes were $Log_2FC > 0.32$ and $p < 0.05$ (red circles). **g)** Cell-cell interaction plots of significant pathways upregulated in both partial and full thickness tears. Width of lines indicate strength of signaling between populations. Arrows depict signaling directionality and signal lines are colored by cell population of origin. See also Supplementary Figure 5.

Figure 6: FAP subpopulation gene expression in chronic injury leads to a predominantly proadipogenic tissue state. **a)** RNA velocity trajectory analysis of healthy deltoid control muscle from patients with full thickness tears. FAP subset UMAP is overlayed with arrows demonstrating predicted cell trajectories. Cells colored by FAP subpopulations. **b)** RNA velocity trajectory analysis of FAPs from full thickness tear samples. **c)** Violin plots of gene expression in the progenitor CD74+, pre-fibrogenic CD55+, and pre-adipogenic DLK1+ populations. Significant genes were $Log_2FC > 0.32$ and *p < 0.05 **p < 0.01 ***p < 0.001. **d)** Cell-cell interaction plots. Significant pathways upregulated in full thickness tears compared to healthy control muscle. Width of lines indicate strength of signaling between populations. Arrows depict signaling directionality and signal lines are colored by cell population of origin. **e)** Representative flow cytometry plots of human FAPs from either healthy or full thickness tear muscle, analyzed for DLK1 expression. **f)** Bar plot of FAP specific DLK1 expression in healthy versus full thickness tear muscle. Paired patient samples are connected by lines. *p < 0.05. n = 4 biological replicates in e and f. **g)** Representative oil-red-o (ORO) images of FAPs grown in adipogenic media

with PBS control vs. recombinant DLK1. Lipid is stained deep red. Scale bar = 40 µm. **h)** Quantification of adipogenesis of FAPs grown in adipogenic media with PBS control vs. recombinant DLK1. n = 3 biological replicates in g and h. All plots are mean ± SEM. Statistical test for all plots: paired t test. *$p < 0.05$. See also Supplementary Figure 6.

Figure 7: DLK1 prevents adipogenic differentiation of human fibroadipogenic progenitors. Cartoon schematic of proposed human FAP lineages and accompanying gene markers in healthy muscle and chronic injury muscle. CD55 is increased along fibrogenic differentiation while CD10 increased along adipogenic differentiation. DLK1 both marks preadipogenic FAPs and prevents adipogenic differentiation possibly through increased expression of SOX9.

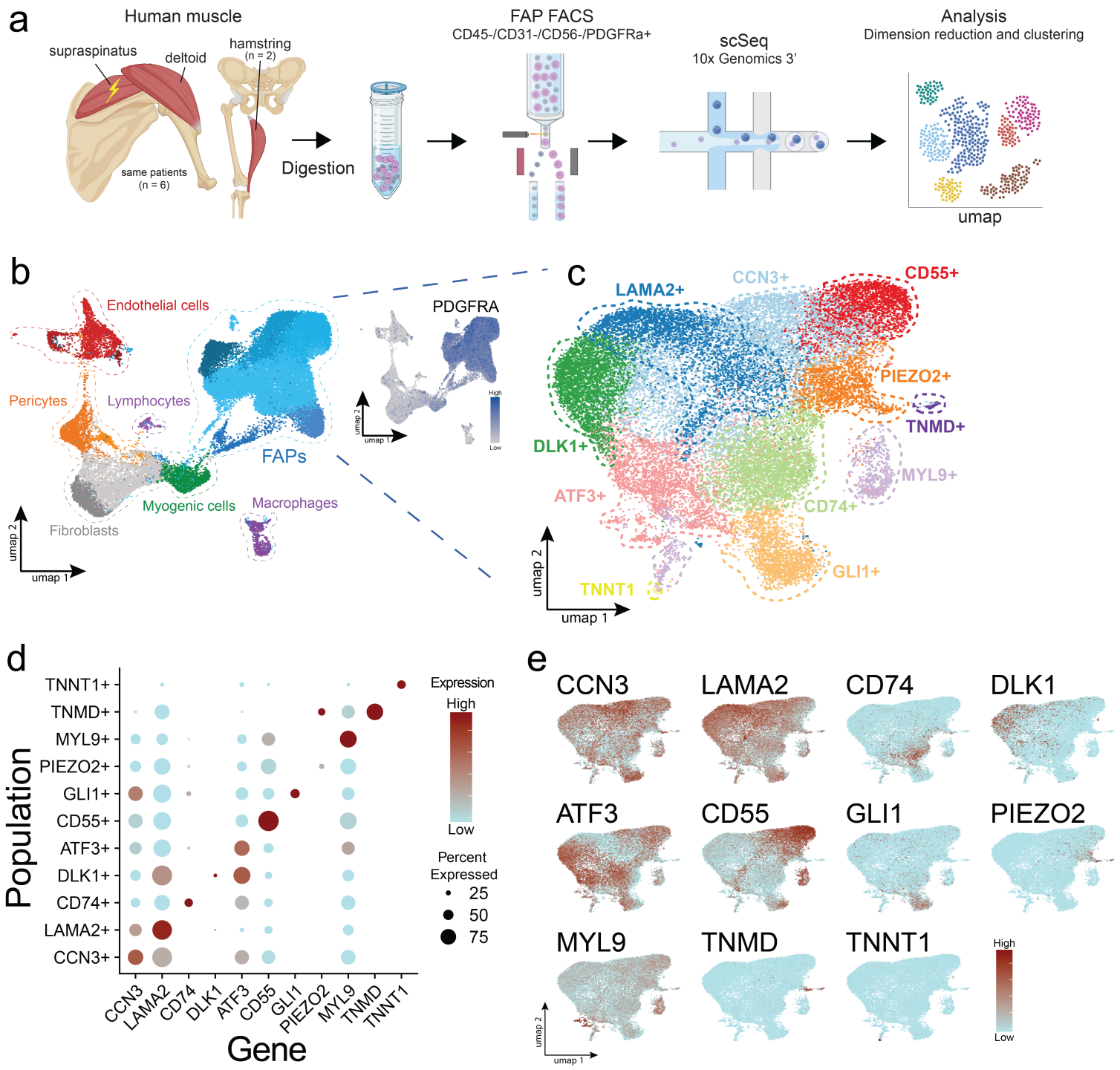

a
Human muscle
supraspinatus
deltoid
hamstring
(n = 2)
same patients
(n = 6)
Digestion
FAP FACS
CD45-/CD31-/CD56-/PDGFRa+
scSeq
10x Genomics 3'
Analysis
Dimension reduction and clustering
umap
b
Endothelial cells
Pericytes
Lymphocytes
FAPs
Myogenic cells
Macrophages
Fibroblasts
umap 2
umap 1
PDGFRA
High
Low
c
LAMA2+
CCN3+
CD55+
PIEZO2+
TNMD+
DLK1+
MYL9+
ATF3+
CD74+
GLI1+
TNNT1
umap 2
umap 1
d
Population
TNNT1+
TNMD+
MYL9+
PIEZO2+
GLI1+
CD55+
ATF3+
DLK1+
CD74+
LAMA2+
CCN3+
CCN3
LAMA2
CD74
DLK1
ATF3
CD55
GLI1
PIEZO2
MYL9
TNMD
TNNT1
Gene
Expression
High
Low
Percent
Expressed
25
50
75
e
CCN3
LAMA2
CD74
DLK1
ATF3
CD55
GLI1
PIEZO2
MYL9
TNMD
TNNT1
High
Low
umap 2
umap 1

Figure 1

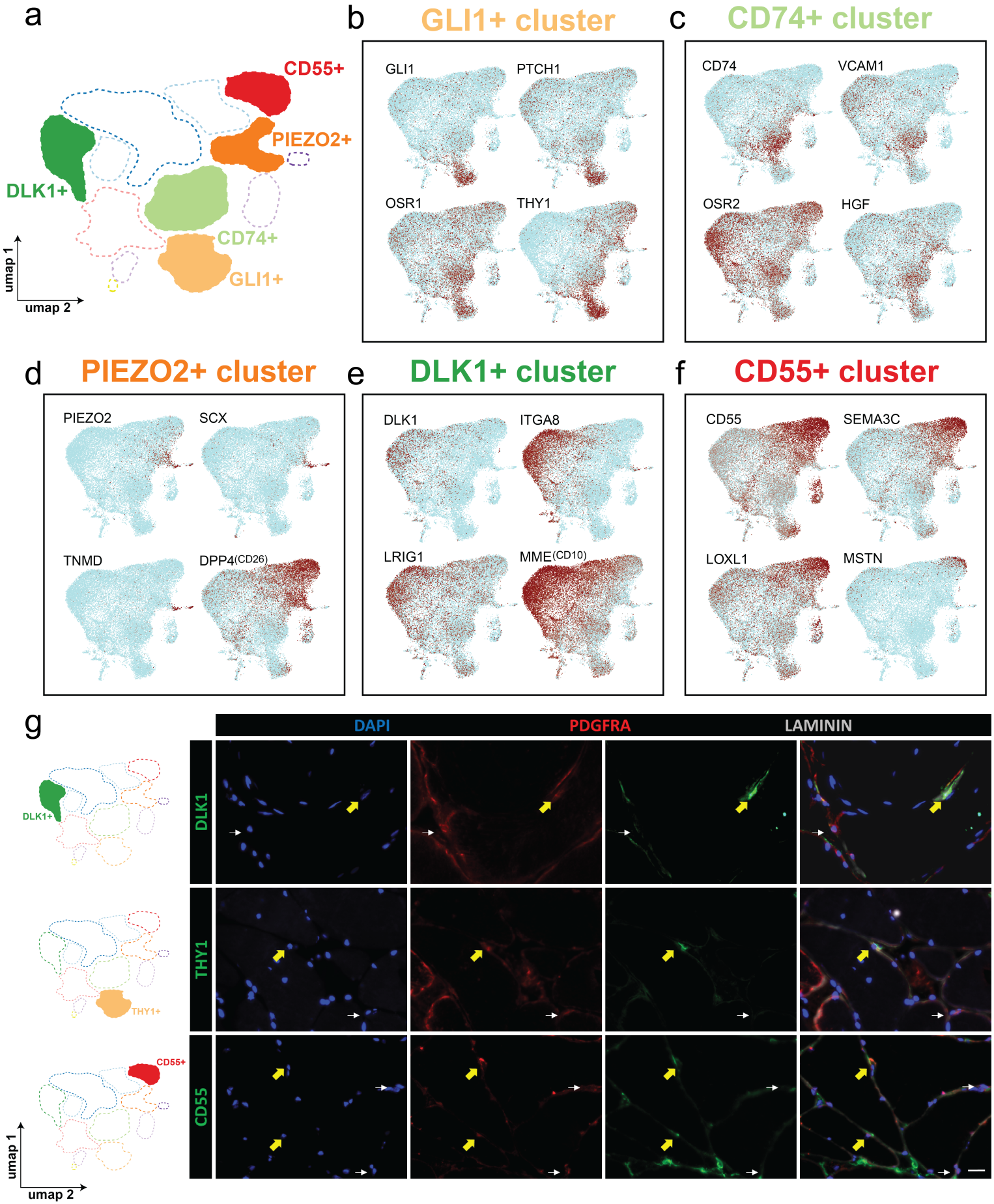
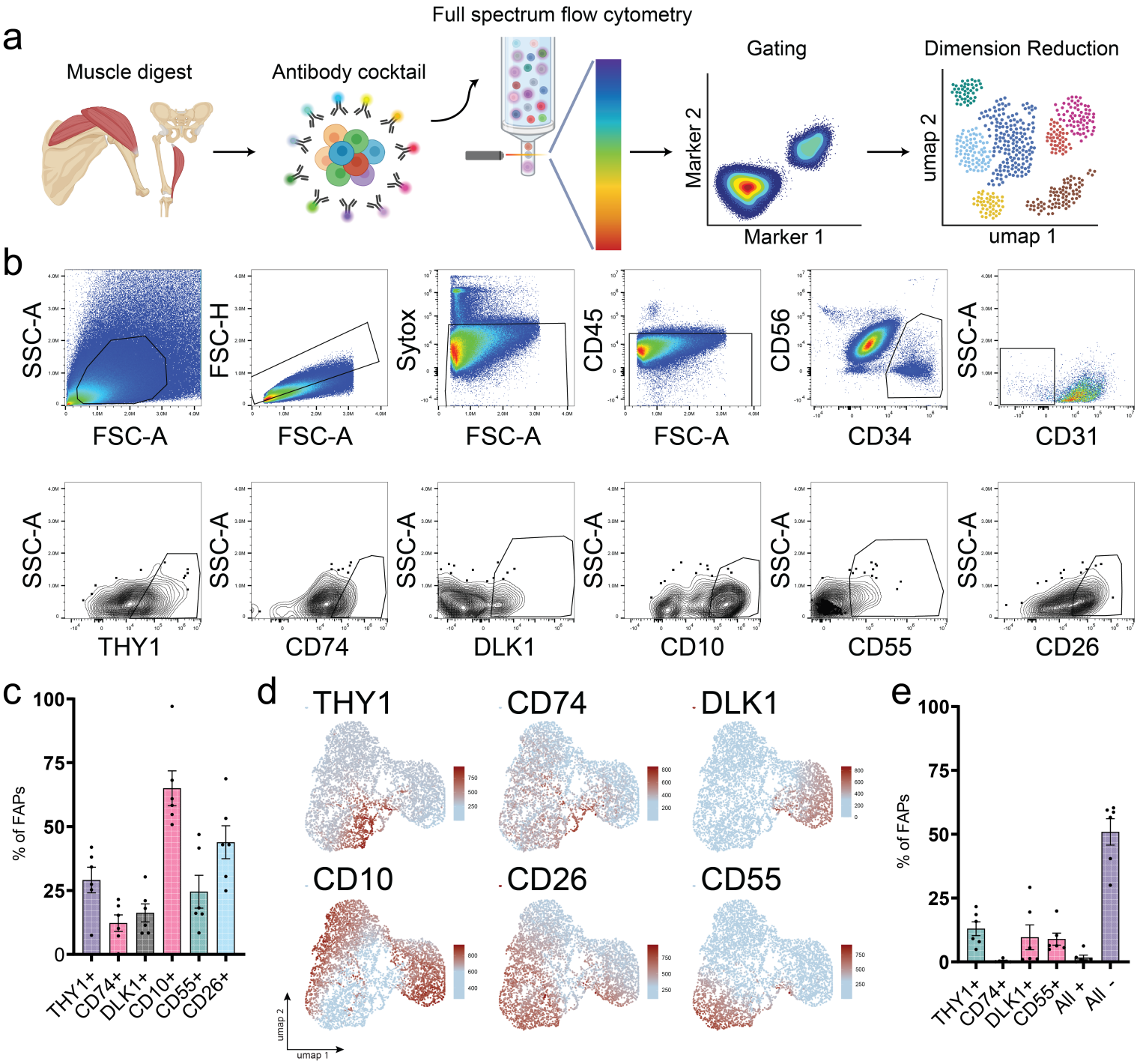

a
CD55+
PIEZO2+
DLK1+
CD74+
GLI1+
umap 1
umap 2
b
GLI1+ cluster
GLI1
PTCH1
OSR1
THY1
c
CD74+ cluster
CD74
VCAM1
OSR2
HGF
d
PIEZO2+ cluster
PIEZO2
SCX
TNMD
DPP4(CD26)
e
DLK1+ cluster
DLK1
ITGA8
LRIG1
MME(CD10)
f
CD55+ cluster
CD55
SEMA3C
LOXL1
MSTN
g
DAPI
PDGFRA
LAMININ
DLK1
THY1
CD55
DLK1+
THY1+
CD55+
umap 1
umap 2

Figure 2


a
Full spectrum flow cytometry
Muscle digest
Antibody cocktail
Gating
Dimension Reduction
Marker 2
Marker 1
umap 2
umap 1
b
SSC-A
FSC-A
FSC-H
Sytox
CD45
CD56
CD34
CD31
THY1
CD74
DLK1
CD10
CD55
CD26
c
% of FAPs
THY1+
CD74+
DLK1+
CD10+
CD55+
CD26+
d
THY1
CD74
DLK1
CD10
CD26
CD55
umap 2
umap 1
e
% of FAPs
THY1+
CD74+
DLK1+
CD55+
All +
All -

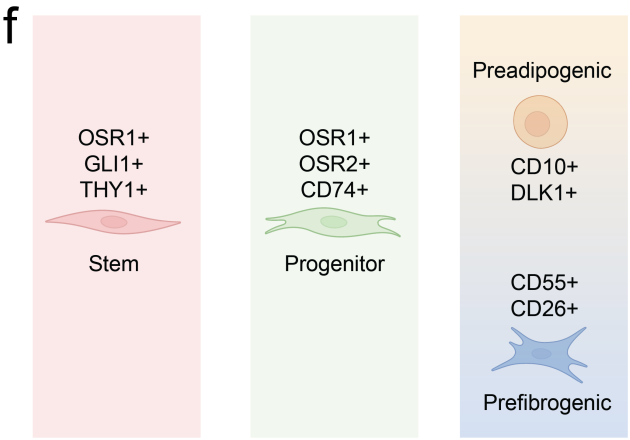

f
OSR1+
GLI1+
THY1+
Stem
OSR1+
OSR2+
CD74+
Progenitor
Preadipogenic
CD10+
DLK1+
CD55+
CD26+
Prefibrogenic

Figure 3

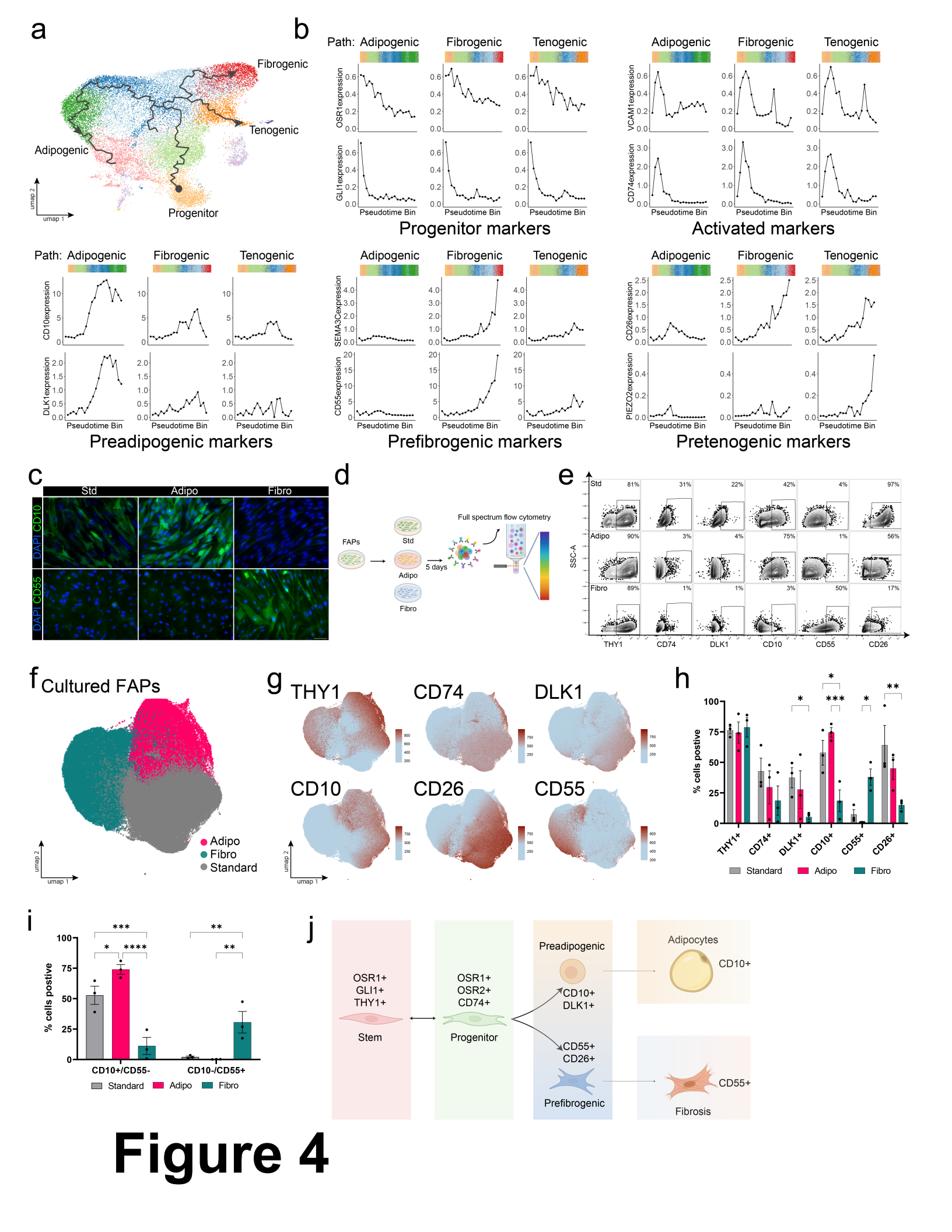

a
Fibrogenic
Tenogenic
Adipogenic
Progenitor
umap 2
umap 1
b
Path: Adipogenic Fibrogenic Tenogenic
OSR1expression
GLI1expression
Pseudotime Bin
Progenitor markers
VCAM1expression
CD74expression
Activated markers
CD10expression
DLK1expression
Preadipogenic markers
SEMA3Cexpression
CD55expression
Prefibrogenic markers
CD26expression
PIEZO2expression
Pretenogenic markers
c
Std
Adipo
Fibro
DAPI CD10
DAPI CD55
d
FAPs
Std
Adipo
Fibro
5 days
Full spectrum flow cytometry
e
Std
Adipo
Fibro
SSC-A
THY1
CD74
DLK1
CD10
CD55
CD26
f
Cultured FAPs
Adipo
Fibro
Standard
umap 2
umap 1
g
THY1
CD74
DLK1
CD10
CD26
CD55
umap 2
umap 1
h
% cells postive
THY1+
CD74+
DLK1+
CD10+
CD55+
CD26+
Standard
Adipo
Fibro
i
% cells postive
CD10+/CD55-
CD10-/CD55+
Standard
Adipo
Fibro
j
OSR1+
GLI1+
THY1+
Stem
OSR1+
OSR2+
CD74+
Progenitor
Preadipogenic
CD10+
DLK1+
CD55+
CD26+
Prefibrogenic
Adipocytes
CD10+
CD55+
Fibrosis

Figure 4

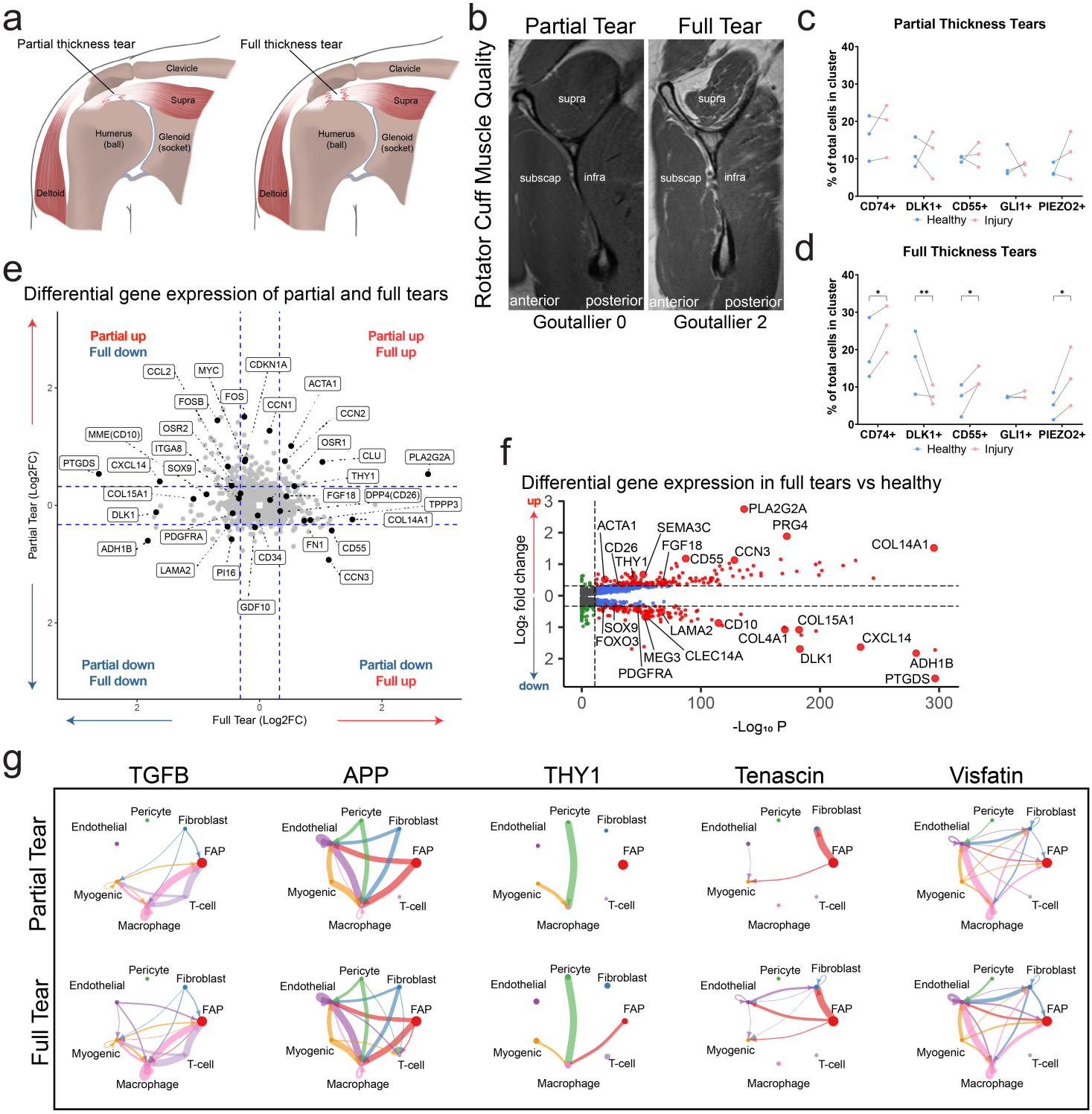

a
Partial thickness tear
Full thickness tear
Clavicle
Supra
Humerus (ball)
Glenoid (socket)
Deltoid
b
Partial Tear
Full Tear
Rotator Cuff Muscle Quality
supra
subscap
infra
anterior
posterior
Goutallier 0
Goutallier 2
c
Partial Thickness Tears
% of total cells in cluster
CD74+
DLK1+
CD55+
GLI1+
PIEZO2+
Healthy
Injury
d
Full Thickness Tears
e
Differential gene expression of partial and full tears
Partial up
Full down
Partial up
Full up
Partial down
Full down
Partial down
Full up
Partial Tear (Log2FC)
Full Tear (Log2FC)
CCL2
MYC
CDKN1A
ACTA1
FOSB
FOS
CCN1
CCN2
OSR2
MME(CD10)
ITGA8
OSR1
CLU
PLA2G2A
PTGDS
CXCL14
SOX9
THY1
COL15A1
FGF18
DPP4(CD26)
TPPP3
DLK1
COL14A1
PDGFRA
ADH1B
FN1
CD55
CD34
LAMA2
PI16
CCN3
GDF10
f
Differential gene expression in full tears vs healthy
up
down
Log₂ fold change
-Log₁₀ P
PLA2G2A
PRG4
ACTA1
SEMA3C
CD26
FGF18
THY1
CD55
CCN3
COL14A1
SOX9
FOXO3
LAMA2
CD10
COL15A1
COL4A1
CXCL14
MEG3
CLEC14A
DLK1
ADH1B
PDGFRA
PTGDS
g
TGFB
APP
THY1
Tenascin
Visfatin
Partial Tear
Full Tear
Pericyte
Fibroblast
Endothelial
FAP
Myogenic
T-cell
Macrophage

Figure 5

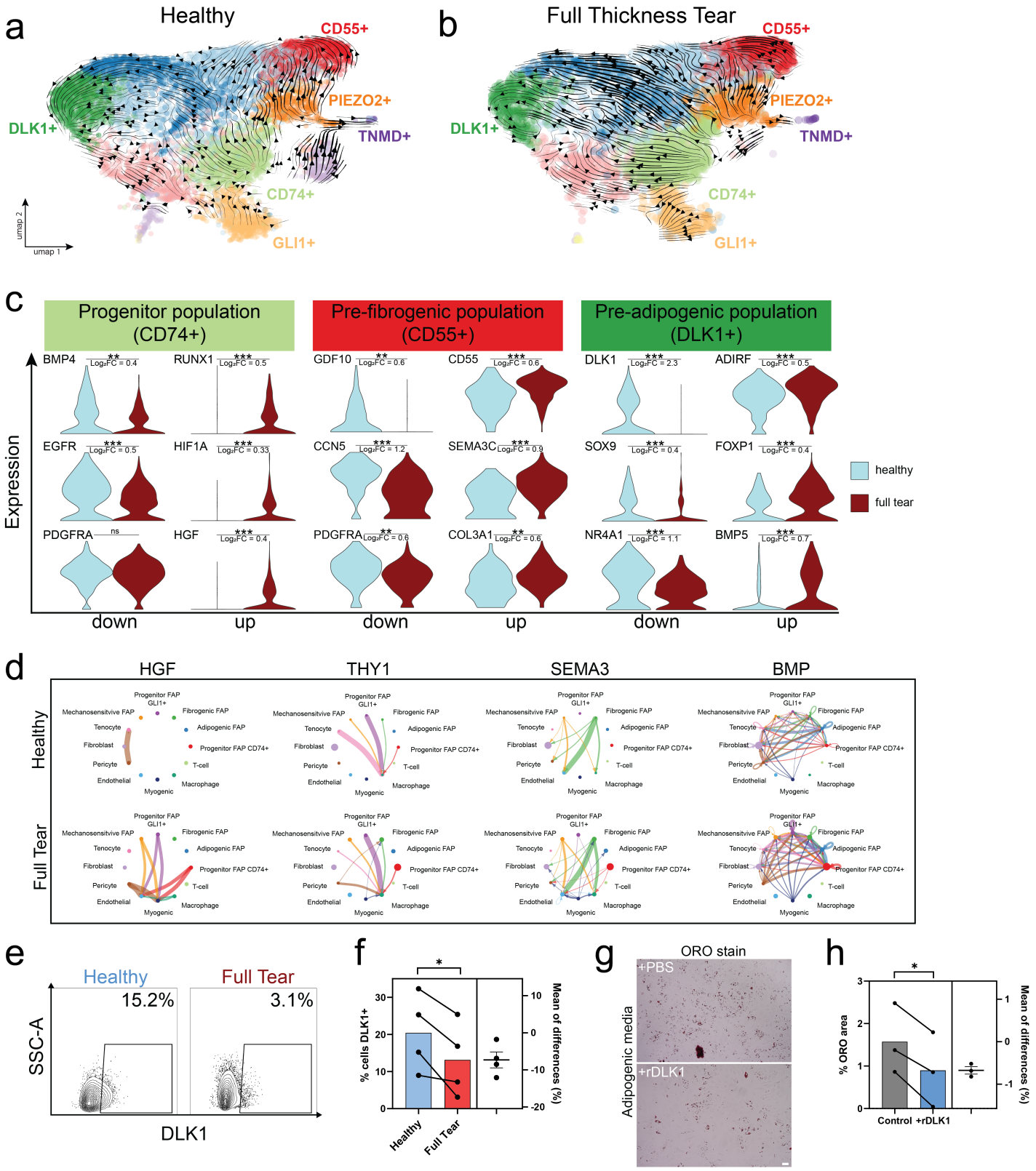

a
Healthy
CD55+
PIEZO2+
TNMD+
DLK1+
CD74+
GLI1+
umap 2
umap 1
b
Full Thickness Tear
CD55+
PIEZO2+
TNMD+
DLK1+
CD74+
GLI1+
c
Progenitor population (CD74+)
Pre-fibrogenic population (CD55+)
Pre-adipogenic population (DLK1+)
Expression
BMP4
RUNX1
GDF10
CD55
DLK1
ADIRF
EGFR
HIF1A
CCN5
SEMA3C
SOX9
FOXP1
PDGFRA
HGF
PDGFRA
COL3A1
NR4A1
BMP5
healthy
full tear
down
up
down
up
down
up
d
HGF
THY1
SEMA3
BMP
Healthy
Full Tear
Progenitor FAP GLI1+
Fibrogenic FAP
Adipogenic FAP
Progenitor FAP CD74+
T-cell
Macrophage
Myogenic
Endothelial
Pericyte
Fibroblast
Tenocyte
Mechanosensitvive FAP
e
Healthy
Full Tear
15.2%
3.1%
SSC-A
DLK1
f
% cells DLK1+
Mean of differences (%)
Healthy
Full Tear
g
ORO stain
+PBS
+rDLK1
Adipogenic media
h
% ORO area
Mean of differences (%)
Control
+rDLK1

Figure 6

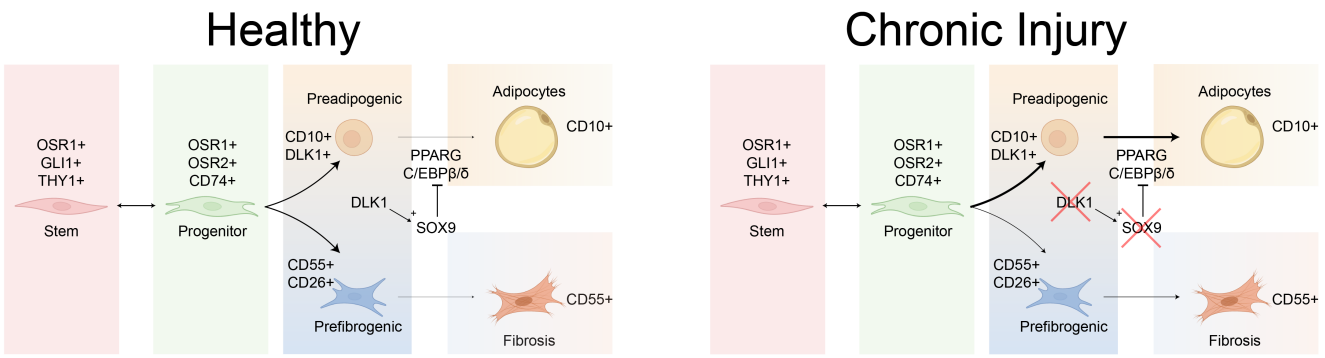

Healthy
OSR1+
GLI1+
THY1+
Stem
OSR1+
OSR2+
CD74+
Progenitor
Preadipogenic
CD10+
DLK1+
PPARG
C/EBPβ/δ
DLK1
SOX9
CD55+
CD26+
Prefibrogenic
Adipocytes
CD10+
CD55+
Fibrosis
Chronic Injury
OSR1+
GLI1+
THY1+
Stem
OSR1+
OSR2+
CD74+
Progenitor
Preadipogenic
CD10+
DLK1+
PPARG
C/EBPβ/δ
DLK1
SOX9
CD55+
CD26+
Prefibrogenic
Adipocytes
CD10+
CD55+
Fibrosis

Figure 7