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Targeting C1q signaling in fibro-adipogenic progenitors prevents regenerative fibrosis of aged muscle

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Skeletal muscle fibrosis, as occurs with age, in response to injury, or in the setting of degenerative diseases, results in impairments of muscle regeneration and function. Fibro-adipogenic progenitors (FAPs), a distinct population of muscle-resident mesenchymal progenitor cells that reside in the muscle interstitium, play a crucial role in normal muscle regeneration by supporting muscle stem cell proliferation. However, in pathological conditions such as severe or recurrent muscle injury, FAPs can aberrantly differentiate into fibrogenic cells, resulting in excessive deposition of extracellular matrix and fibrosis. In this study, we explore the molecular regulation of FAP differentiation along the fibrogenic lineage to gain insights into the mechanisms of fibrosis in aged muscle in response to injury. Our findings reveal that aging is associated with an increased expression of the complement component 1q (C1q) in muscle-resident macrophages and elevated expression of the complement proteins C1r and C1s in FAPs. Exposure of proliferating FAPs to C1q results in the activation of the Wnt signaling pathway, elevated expression of collagen genes, and FAP fibrogenic differentiation, leading to increased tissue fibrosis. We demonstrate that either pharmacological inhibition of the complement pathway or genetic ablation of C1s in FAPs in aged mice reduces fibrogenic differentiation of FAPs by suppressing Wnt signaling. This reduction in FAP differentiation attenuates the fibrotic response to injury in aged animals as well as in a mouse model of muscular dystrophy. Our study supports the inhibition of complement signaling as a potential therapeutic strategy for mitigating fibrosis in skeletal muscle injury or degeneration.

complement, C1q | aging | fibro-adipogenic progenitors | fibrosis | muscular dystrophy

Fibrosis, defined as the displacement of functional tissue by excessive deposition of extracellular matrix (ECM) proteins, is both a cause and a consequence of tissue dysfunction. In skeletal muscle, fibrosis is apparent in a variety of pathological settings such as severe or recurrent trauma and degenerative diseases such as muscular dystrophies (1–4). With age, mild diffuse fibrosis leads to muscle stiffness, and even minor injuries can result in far greater scarring than in younger individuals. Both the pro-fibrotic milieu and the fibrotic tendencies of resident stem and progenitor cells contribute to the age-related fibrosis of muscle (5). In the muscular dystrophies, recurrent bouts of degeneration lead to the replacement of muscle parenchyma by fibrotic tissue and progressive weakness (4). Fibrosis is also a barrier for successful viral and cell therapy approaches to muscular dystrophies (6–8).

Endogenous fibrogenic cell populations are considered to be the main source of ECM proteins across various tissues (9–11). Historically characterized as fibroblasts, recent studies have revealed considerable heterogeneity within these fibrogenic cell populations not only between different tissues but even within the same tissue (12, 13). Within skeletal muscle, many different markers, including PDGFRα, TCF4, ADAM12, and Dpp4, identify fibrogenic populations (14–18). Among these markers, PDGFRα identifies a distinct population of multipotent mesenchymal progenitors termed "fibro-adipogenic progenitors" (FAPs) (14, 19). While FAPs play an important role in promoting myogenic regeneration under normal conditions, (17) they have also been shown to be a major cellular mediator of fibrotic degenerative changes in a variety of pathological settings (20). In addition, in aged or dystrophic muscle, Pax7-expressing muscle stem cells (MuSCs) can be driven toward a fibrogenic fate, contributing to muscle fibrosis (21, 22). Old fibrogenic cells exert their influence on MuSC fates by altering the surrounding ECM (23). Notably, an increased level of the C1 complement component C1q in aged animals has been implicated in the development of injury-induced fibrosis (24).

In classical activation of the complement cascade, the C1 complex is composed of C1q, C1r, and C1s (25). C1q functions as a molecular scaffold for the two serine proteases,

Significance

Fibrosis in skeletal muscle impairs regeneration and function, especially with age, severe injury, or degenerative disease. In healthy muscle, fibro-adipogenic progenitors (FAPs) support muscle repair. However, under abnormal conditions, FAPs differentiate into cells that deposit excessive extracellular matrix, causing fibrosis. We find that aging elevates the level of the complement component 1q (C1q) protein in muscle macrophages and other complement proteins in FAPs, activating intracellular signaling in FAPs that drives fibrosis. Blocking C1q signaling pharmacologically or genetically reduces fibrosis in aged and dystrophic muscle following injury. This research enhances our understanding of the non-canonical role of the complement pathway in conditions associated with aging and disease, underscoring its potential as a therapeutic target to mitigate muscle fibrosis.

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C1r and C1s. The binding of C1q to its ligands activates C1r and C1s, which then cleave C4 and initiate the downstream complement cascade (26). Aside from its canonical role in these immunological cascades, C1q can activate non-canonical signaling as well (27, 28). In skeletal muscle, C1q activates the Wnt pathway by binding to the canonical Wnt receptor, Frizzled (24). This non-canonical complement signaling has been shown to drive age-related muscle fibrosis as the levels of C1q increase with age in both blood and muscle (24). In dystrophic muscle, the expression of C1q is upregulated in infiltrating macrophages and promotes fibrosis by driving the fibrogenic differentiation of FAPs (29). Using an ex vivo 3D muscle culture, it was recently shown that damaged muscle fibers may also be a source of increased complement proteins (30). This study also demonstrated that inhibiting C4b enhances muscle regeneration in aged animals in vivo (30).

In this report, we show that, in aged muscle, C1q expression is increased in resident macrophages and C1r and C1s expression is increased in FAPs. The increased C1q signaling leads to the activation of the canonical Wnt pathway in FAPs and to their fibrogenic differentiation. We also demonstrate that inhibition of C1 reduces the fibrogenic potential of FAPs and fibrotic changes at the tissue level in aged, injured muscle as well as in a mouse model of muscular dystrophy. These studies suggest that non-canonical C1 signaling may represent a target for therapeutics to reduce or prevent fibrosis that arises from acute or recurrent injury to aged muscle.

Results

C1q Causes Excess Fibrosis Following Injury to Skeletal Muscle. We have previously shown that regeneration of aged muscle is accompanied by an increase in fibrosis compared to young muscle (21). Given that C1q is increased in the serum of aged mice and that inhibition of complement signaling enhances aged muscle regeneration (24), we wanted to determine whether exogenous C1q could promote fibrosis in regenerating muscle. We tested this by treating injured tibialis anterior (TA) muscles of young mice ectopically with C1q embedded in hydrogel, allowing graduate release of C1q during regeneration (*SI Appendix*, Fig. S1*A*). Prolonged exposure of C1q during the regenerative process led to a 2.5-fold increase of interstitial collagen deposition following injury, as measured by Picrosirius red staining (Fig. 1 *A* and *B*).

To determine whether C1r and C1s alone can induce fibrosis during muscle regeneration, we treated injured TA muscles of young mice with either recombinant active C1r or C1s proteins using the same treatment regimen as for C1q (*SI Appendix*, Fig. S1*A*). Contrary to the adverse effect of excessive C1q on muscle regeneration, prolonged exposure to C1r or C1s did not lead to an increase in collagen deposition following acute injury (*SI Appendix*, Fig. S1 *B* and *C*). These results suggest that neither C1r nor C1s can mediate a fibrotic response in regenerating muscle in the absence of elevated C1q.

Complement Gene Expression Is Elevated in Muscle-Resident Cells with Age. To explore the source of the complement proteins in muscle-resident cells, we performed analysis of our previously published single-cell RNA-sequencing (scRNA-seq) data from skeletal muscle (31). This analysis revealed that all 3 genes encoding C1q (C1qa, C1qb, and C1qc) were expressed exclusively in macrophages among all mononucleated cells of uninjured muscle, and the predominant source of C1r and C1s were the FAPs (Fig. 2 *A* and *B*). We also examined the expression of the subcomponents of C1 in different mononucleated cell populations and single myofibers

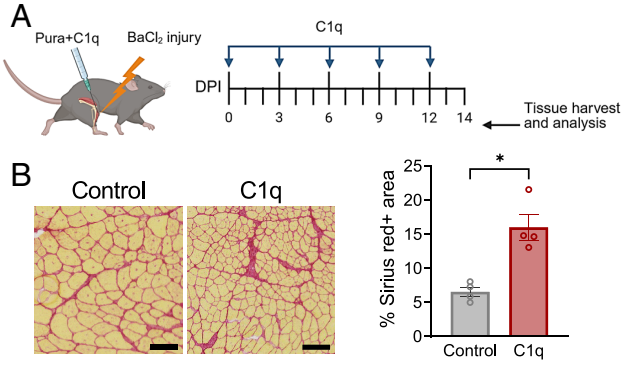

Fig. 1. Increased C1q levels lead to fibrosis following muscle injury. (*A*) Schematic representation of the time course of C1q treatment in vivo. Hydrogel containing C1q or BSA as negative control was administered subcutaneously immediately after $BaCl_2$-mediated injury of TA muscles and every 3 d thereafter. The TA muscles were harvested 14 d postinjury (DPI), cryosectioned, and stained with Picrosirius red to quantify fibrotic areas. (*B*) (*Left*) Representative images of interstitial collagen stained with Picrosirius red in BSA (Control) or C1q-treated TA muscles of young mice. (Scale bars, 100 μm.) (*Right*) Quantification of the fibrotic areas (n = 4). Quantitative data are shown as mean ± SEM, and paired two-tailed Student's *t* tests were used for statistical analysis. *$p < 0.05$.

isolated from uninjured skeletal muscles of young mice using RT-qPCR and confirmed that macrophages were the major source of C1qa, C1qb, and C1qc, whereas FAPs were the predominant source of C1r and C1s (*SI Appendix*, Fig. S2 *A* and *B*). We then determined the expression levels of C1q genes in macrophages freshly isolated from skeletal muscle of young and old mice by RT-qPCR and found that C1qa expression was significantly elevated in macrophages from old mice (Fig. 2*C*), consistent with previous findings (24). Notably, we also found an increase in C1r and C1s transcripts as well as proteins in FAPs freshly isolated from old muscle compared to FAPS from young muscle (Fig. 2 *D*–*F*).

We also examined whether the expression of complement components in relevant cell types changes with age in injured muscle. Using FACS, we isolated MuSCs, FAPs, endothelial cells, as well as monocytes and macrophages ($CD45^+/CD11b^+$) from young and old muscles 3 d after injury, a time point corresponding to peak MuSC proliferation and immune cell infiltration (32, 33). Notably, the infiltrating immune cells in injured muscle are predominantly $CD11b^+$ monocytes and macrophages. We then determined the expression levels of C1 complex subunits in these cells by RT-qPCR analysis and compared their expression between young and old cells. Our analysis revealed that, following injury, although C1s was broadly expressed across multiple cell types, the expression levels of C1r and C1s did not differ significantly between young and old muscle (*SI Appendix*, Fig. S2 *C*–*F*). In contrast, C1qa expression was markedly elevated in monocytes and macrophages isolated from injured old muscle compared to those from young muscle (Fig. 2*G*).

Together, these results indicate that macrophages and FAPs are important sources of C1 complement factors in muscle. Macrophages are the primary source of C1q, and their expression of C1q genes, particularly C1qa, increases significantly with age and is further enhanced following acute muscle injury. In contrast, FAPs are the predominant source of C1r and C1s in both resting and injured muscle. Since C1q forms C1 complexes by associating with C1r and C1s expressed in target cells and acting in an autocrine or paracrine fashion (27), FAPs are therefore likely key targets for C1q-mediated effects in muscle.

C1q Causes Fibrogenic Differentiation of FAPs. To better understand the effect of C1q on FAP gene expression, we performed RNA-seq analysis of FAPs and their progeny treated

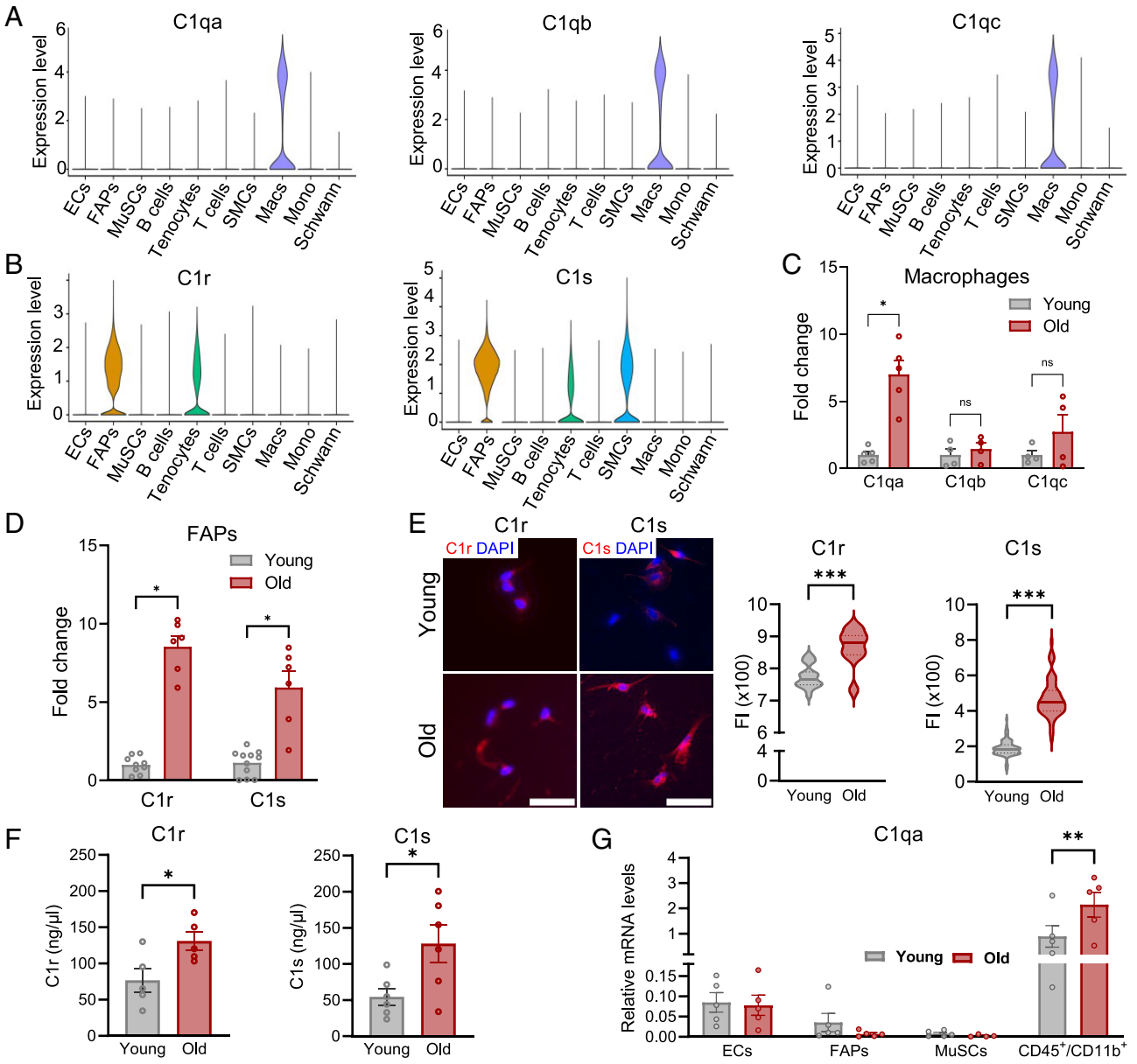

Fig. 2. C1q, C1r, and C1s levels increase with age in skeletal muscle. (*A* and *B*) Violin plots showing expression of C1q components (*A*) and C1r and C1s (*B*) in various cell types of skeletal muscle of young mice as determined by scRNA sequencing. (*C*) Relative expression of C1qa, C1qb, and C1qc in freshly isolated CD45$^+$, F4/80$^+$ macrophages from skeletal muscle of young and old mice determined by RT-qPCR. The expression of each gene is normalized to GAPDH (n ≥ 4). (*D*) Relative expression of C1r and C1s in FAPs from skeletal muscle of young and old mice determined by RT-qPCR. The expression of each gene is normalized to GAPDH (n ≥ 4). (*E*) (*Left* panel) Representative immunofluorescence images of C1r and C1s expression in FAPs from young and old mice. Freshly isolated FAPs were fixed 12 h after plating and stained with antibodies specific to C1r and C1s. DAPI was used to label nuclei. (*Right* panel) Quantification of C1r and C1s expression in FAPs (n = 4). (*F*) Quantification of C1r and C1s in freshly isolated FAPs from young and old mice using ELISA (n ≥ 5). (*G*) Quantification of the relative expression of C1qa in freshly isolated endothelial cells, FAPs, MuSCs, as well as monocytes and macrophages (CD45$^+$/CD11b$^+$) from skeletal muscle of young and old mice 3 DPI, determined by RT- qPCR. The expression of each gene is normalized to GAPDH (n ≥ 4). Quantitative data are shown as mean ± SEM, and unpaired two-tailed Student's *t* tests or two-way Anova were used for statistical analysis. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns: not significant.

with C1q ex vivo. At a false discovery rate of 1%, we identified 4,883 differentially expressed genes (DEGs) in the C1q-treated cells compared to the control cells (Fig. 3*A*). Gene Ontology term analysis of DEGs using the PANTHER pathway database revealed "Extracellular matrix" as the most significantly enriched term (*SI Appendix*, Fig. S3*A*). The DEGs implicated in this term encode several collagen proteins and ECM remodeling factors, including MMPs (matrix metalloproteinases), ADAMTSs (a disintegrin and metalloproteinase with thrombospondin motifs), and TIMPs (tissue inhibitors of MMPs) (Fig. 3 *A* and *B*). Notably, C1q treatment increased the expression of collagens, ADAMTSs, and TIMPs, while decreasing the expression of MMPs (Fig. 3*B*), suggesting that C1q drives transcriptional changes related to ECM accumulation and promotes the fibrogenic conversion of FAP progeny.

To validate these findings, we performed RT-qPCR to study the gene expression changes of Col1a1, Col3a1, Col4a1, TIMP-2, TIMP-3, and MMP-14 in C1q-treated FAPs (Fig. 3*C*). We further confirmed the fibrogenic conversion of FAP progeny by immunofluorescence staining for the fibrogenic marker alpha smooth muscle actin (α-SMA) (Fig. 3*D*). We then examined gene expression in FAPs isolated from TA muscles treated with C1q in vivo. FAPs from C1q-treated muscles showed changes in gene expression consistent with those seen in muscles treated with C1q ex vivo

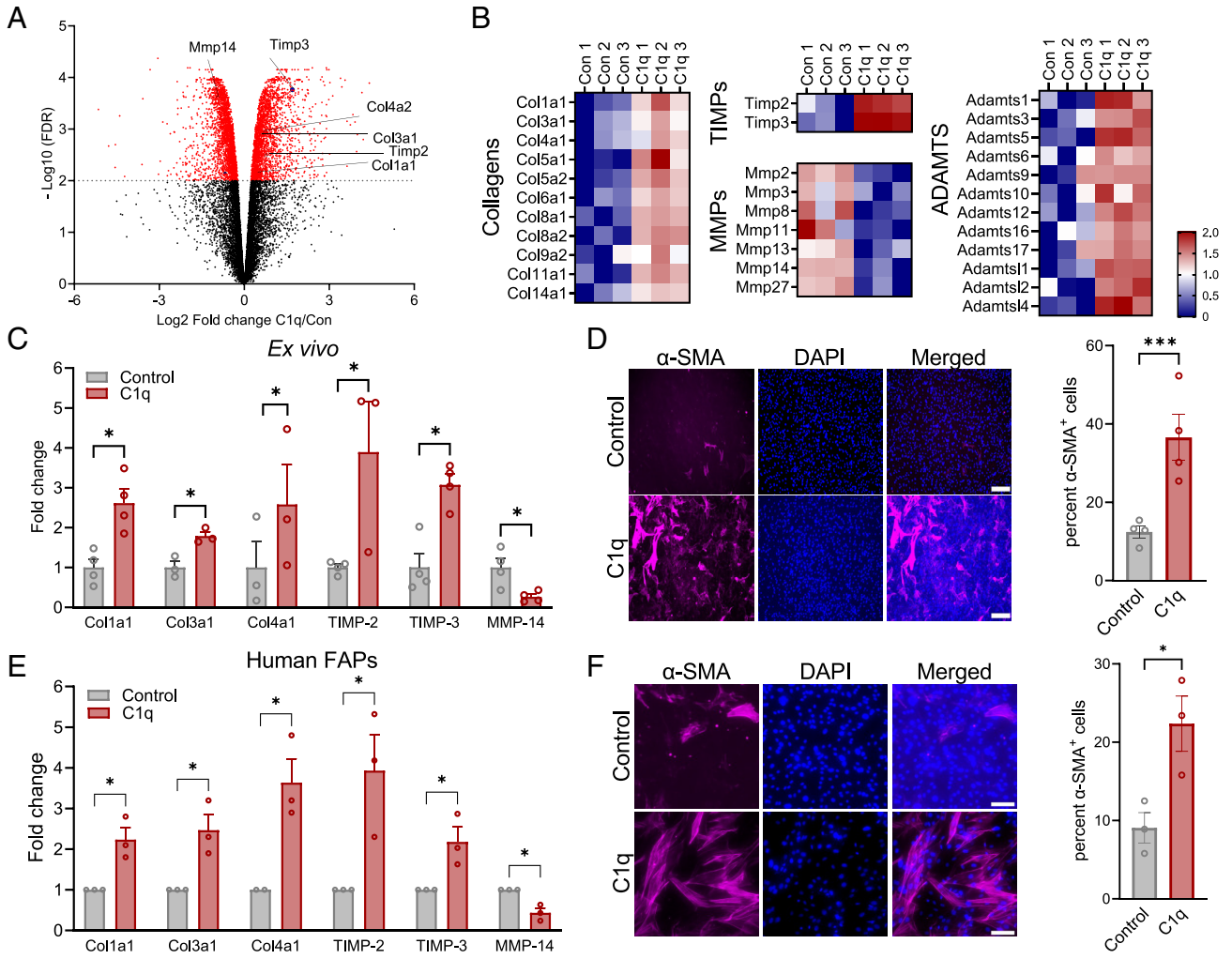

Fig. 3. C1q induces fibrogenic differentiation of FAPs. (*A*) Volcano plot of genes profiled by whole transcriptome RNA-seq from Control and C1q-treated FAPs. (*B*) Heatmaps demonstrating the expression levels of Collagens and ECM remodeling genes in FAPs from Control and C1q-treated groups. (*C*) Quantification of expression of fibrosis-related genes determined by RT-qPCR in FAPs treated with vehicle (Control) or C1q for 5 d. The expression of each gene is normalized to GAPDH (n = 4). (*D*) (*Left* panel) Representative images of FAPs cultured in differentiation medium with vehicle (Control) or C1q for 7 d followed by immunofluorescence with an α-SMA antibody. DAPI was used to label nuclei. (Scale bars, 100 μm.) (*Right* panel) Quantification of the percentage of α-SMA$^+$ cells in control and C1q-treated cells (n = 4). (*E*) Quantification of expression of fibrosis-related genes determined by RT-qPCR in human FAPs treated with vehicle (Control) or C1q for 7 d. The expression of each gene is normalized to GAPDH (n = 3). (*F*) (*Left* panel) Representative images of human FAPs treated with vehicle (Control) or C1q in differentiation medium for 7 d followed by immunofluorescence with an α-SMA antibody. DAPI was used to label nuclei. (Scale bars, 100 μm.) (*Right* panel) Quantification of the percentage of α-SMA$^+$ cells in control and C1q-treated cells (n = 3). Quantitative data are shown as mean ± SEM and unpaired two-tailed Student's *t* tests were used for statistical analysis. *$p < 0.05$, ***$p < 0.001$.

(*SI Appendix*, Fig. S3*B*). To determine whether the effect of C1q treatment on fibrogenic conversion of FAPs is conserved in humans, we treated FAPs isolated from human skeletal muscle with C1q. The transcriptional changes in human FAPs mirrored those seen in mouse FAPs, with a similar increase in the fraction of α-SMA-expressing FAP progeny in response to C1q treatment (Fig. 3 *E* and *F*). Taken together, these results indicate that C1q induces gene expression and cell fate changes consistent with the fibrogenic conversion of FAPs.

C1q Acts to Increase FAP Fibrogenic Differentiation by Activating the Wnt Pathway. C1q has been demonstrated to bind to the Frizzled receptor and activate canonical Wnt pathway (24). We therefore hypothesized that C1q-mediated FAP fibrogenic differentiation might likewise be mediated by the activation of canonical Wnt signaling. We confirmed that freshly isolated FAPs express the Wnt receptor Fzd4, the coreceptor LRP-6, and the β-catenin coactivators, TCF7L1 and TCF7L2 (*SI Appendix*, Fig. S4*A*). Pathway analysis of our RNA-seq dataset comparing C1q-treated to control FAPs indeed revealed enrichment of Wnt-β-catenin signaling in FAPs in response to C1q treatment (*SI Appendix*, Fig. S4*B*). We also confirmed that C1q treatment of FAPs induced accumulation of the active form of β-catenin (*SI Appendix*, Fig. S4*C*). Next, to examine the dependency of C1q-induced Wnt signaling on LRP-6 coreceptor activity, we knocked down LRP-6 in FAPs and then treated the cells with C1q. We observed that the effects of C1q treatment, including induction of expression of Axin-2 (a Wnt target gene) in FAPs, increased fibrosis-related gene expression, and promotion of FAP fibrogenic differentiation, were all LRP-6-dependent (Fig. 4 *A*–*C*). These data suggest that C1q activates canonical Wnt signaling in FAPs to induce fibrogenic differentiation. To further confirm that the canonical Wnt pathway mediates the effect of C1q on FAP fibrogenic differentiation, we treated freshly isolated FAPs with C1q in the presence or absence of the canonical Wnt inhibitor, PNU-74654. We found that PNU-74654 was able to abrogate the C1q-induced expression of Axin-2, collagen, and TIMP genes and the C1q-induced repression of MMP-14 expression (Fig. 4 *D* and *E*).

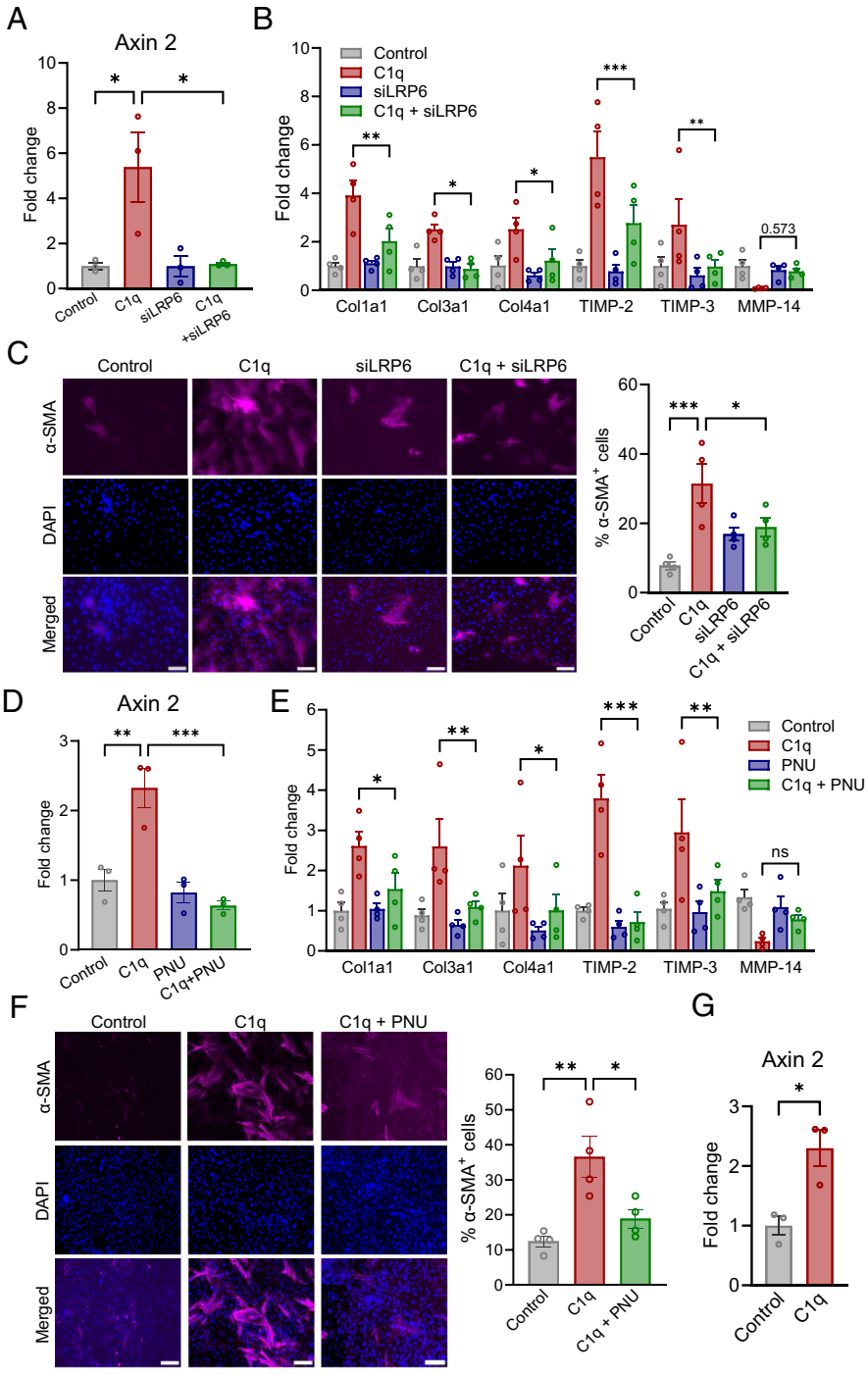

Fig. 4. C1q activates canonical Wnt signaling in FAPs to mediate fibrogenic differentiation. (*A*) Quantification of expression of Axin-2 determined by RT- qPCR in FAPs treated with vehicle (Control), C1q (100 μg/mL), LRP6 siRNA, or C1q plus LRP6 siRNA for 3 d. Axin-2 expression is normalized to GAPDH (n = 4). (*B*) FAPs were treated with vehicle (Control), C1q (100 μg/mL), LRP6 siRNA, or C1q plus LRP6 siRNA for 3 d, and the expression of fibrosis-related genes was determined by RT-qPCR. The expression of each gene is normalized to GAPDH (n = 3). (*C*) (*Left* panel) Representative images of FAPs cultured in differentiation medium treated with vehicle (Control), C1q (100 μg/mL), LRP6 siRNA, or C1q plus LRP6 siRNA for 7 d followed by immunofluorescence analysis with an α-SMA antibody. DAPI was used to label nuclei. (Scale bars, 100 μm.) (*Right* panel) Quantification of percentage of α-SMA$^+$ cells in the 4 conditions (n = 4). (*D*) FAPs were treated with vehicle (control), C1q (100 μg/mL), PNU-74654 (2 μM), or C1q (100 μg/mL) plus PNU-74654 (2 μM) for 48 h, and the expression of Axin-2 was determined by RT-qPCR. Axin-2 expression was normalized to GAPDH (n = 3). (*E*) FAPs were treated with vehicle (control), C1q (100 μg/mL), PNU-74654 (2 μM), or C1q (100 μg/mL) plus PNU-74654 (2 μM) for 48 h, and the expression of fibrosis-related genes was determined by RT-qPCR. The expression of each gene is normalized to GAPDH (n = 4). (*F*) (*Left* panel) Representative images of FAPs cultured in differentiation medium with vehicle (control), C1q (100 μg/mL), or C1q (100 μg/mL) plus PNU-74654 (2 μM) for 7 d followed by immunofluorescence analysis with an α-SMA antibody. DAPI was used to label nuclei. (Scale bars, 100 μm.) (*Right* panel) Quantification of percentage of α-SMA$^+$ cells in the 3 conditions (n = 4). (*G*) Axin-2 expression determined by RT-qPCR in FAPs from muscles treated in vivo with vehicle or C1q. Axin-2 expression is normalized to GAPDH (n = 4). Quantitative data are shown as mean ± SEM, and one- or two-way Anova was used for statistical analysis. *p < 0.05, **p < 0.01, ***p < 0.001, ns: not significant.

PNU-74654 also significantly reduced the percentage of α-SMA+ cells in ex vivo culture of FAPs treated with C1q (Fig. 4*F*). To test whether C1q could activate Wnt signaling in FAPs during muscle regeneration in vivo, we treated injured TA muscles with a C1q-containing hydrogel and isolated FAPs 3 d after injury for RT-qPCR analysis. We found an upregulation of Axin-2 expression

in FAPs from C1q-treated TA muscles compared to FAPs from control muscles (Fig. 4*G*). Taken together, these data indicate that C1q activates the canonical Wnt pathway in FAPs, resulting in their fibrogenic differentiation.

C1q Inhibition Limits the Fibrogenic Differentiation of FAPs in Aged Injured Muscle. C1q inhibition by genetic ablation or neutralizing antibody has been shown to reduce fibrosis during aged muscle regeneration (24). To determine whether this beneficial effect of C1q inhibition can be attributed to changes in FAP differentiation, we treated TA muscles of aged mice with a hydrogel containing C1-INH, an endogenous C1r and C1s inhibitor (34), immediately following injury. The contralateral TA muscles of these mice were treated with a hydrogel containing BSA as control. In comparison to control muscles, C1-INH-treated muscles exhibited a marked reduction in fibrosis (Fig. 5*A*). When we isolated FAPs from these mice, we found that the C1-INH treatment significantly reduced the expression of collagen genes and Axin-2 (Fig. 5*B* and *SI Appendix*, Fig. S5A).

We then tested the effects of C1q deletion on FAP differentiation and fibrosis in aged mice in vivo using a C1qa knockout (C1q KO) strain (35), because C1qa is the only C1 component that is increased in both resting and injured muscle in aged mice (Fig. 2 *C* and *G*). We injured the muscles of mice at 24 mo of age. Consistent with the C1-INH studies, aged C1q KO mice exhibited an almost complete inhibition of the fibrosis seen in aged wild-type (WT) mice in response to injury (Fig. 5*C*). In addition, FAPs from aged C1q cKO mice 3 d after injury express significantly reduced levels of collagen genes compared to those from WT mice (Fig. 5*D*). It should be noted that in the absence of injury, C1q ablation affected neither the mild fibrosis seen in aged muscle nor the expression of fibrogenic genes in FAPs from young or old mice (*SI Appendix*, Fig. S5 *B* and *C*).

Genetic Ablation of C1s in FAPs Reduces Injury-Induced Fibrosis with Age. Given that C1s, a cofactor of C1q, is expressed predominantly in FAPs (Fig. 2*B*), we generated an FAP-specific knockout of C1s to determine whether it is required for the fibrogenic differentiation of FAPs by C1q. To do this, we used a mouse strain with a FAP-specific CreER driver (PDGFRα-CreER) (17). We bred these mice with mice bearing C1s floxed alleles ($C1s^{flox/flox}$) to generate $PDGFR\alpha^{CreER/+}$; $C1s^{flox/flox}$ mice (referred as C1s cKO hereafter), which will have C1s specifically deleted in FAPs in muscle upon tamoxifen administration. We confirmed that the expression of C1s in FAPs isolated from C1s cKO was almost completely abolished following tamoxifen treatment (*SI Appendix*, Fig. S6*A*). As with the C1q KO strain, in the absence of injury we did not observe any effect on age-related muscle fibrosis or on the expression of fibrogenic genes in FAPs from either young animals or old C1s cKO mice (*SI Appendix*, Fig. S6 *B* and *C*). However, the level of fibrosis was reduced in the muscles of aged C1s cKO mice compared to aged WT mice after injury (Fig. 6*A*). FAPs isolated from aged C1s cKO mice expressed reduced levels of fibrogenic genes compared to FAPs isolated from aged WT mice following injury (Fig. 6*B*).

To determine whether C1q-induced fibrosis requires the presence of C1s in FAPs, we treated injured TA muscles of young C1s cKO and WT mice with a hydrogel containing recombinant C1q. The fibrotic response induced by exogenous C1q treatment was significantly blunted in C1s cKO mice (Fig. 6*C*). Likewise, to assess the requirement of C1s to mediate the C1q-induced fibrogenic differentiation of FAPs, we treated FAPs isolated from WT or C1s cKO mice with C1q ex vivo. Whereas C1q treatment resulted in an upregulation of fibrogenic genes and an increased fibrogenic differentiation in WT FAPs, these effects were markedly abrogated in C1s-deficient FAPs (Fig. 6 *D* and *E*). Together, these findings indicate that FAP-derived C1s is essential in mediating the pro-fibrotic effects of excess C1q in muscle regeneration during aging.

C1q Inhibition Results in Reduced Muscle Fibrosis in mdx Mice. It was recently reported that inhibition of complement signaling reduces fibrosis in muscle from mdx mice (29), a mouse model of Duchenne muscular dystrophy. We confirmed that the levels of C1r and C1s are significantly higher in FAPs from mdx mice compared to those from WT mice (*SI Appendix*, Fig. S7*A*).

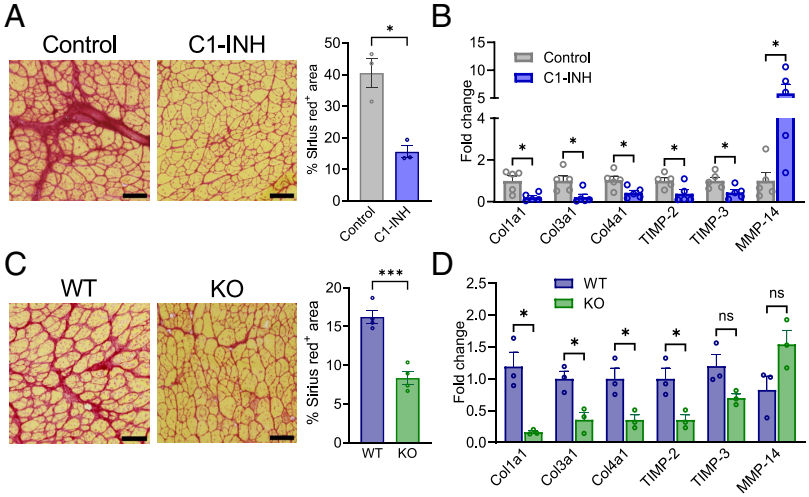

Fig. 5. C1q inhibition/deletion prevents fibrosis in injured aged muscle. (*A*) (*Left* panel) Representative image of collagen matrix in control or C1-INH-treated TA muscles of old mice. (Scale bars, 100 μm.) The TA muscles were isolated 14 DPI and stained with Picrosirius red to assess the fibrotic area. (*Right* panel) Quantification of the fibrotic area measured by Picrosirius red staining (n = 4). (*B*) Quantification of expression of fibrosis-related genes determined by RT-qPCR in FAPs isolated from control and C1-INH-treated TA muscles from WT old mice. The expression of each gene is normalized to GAPDH (n = 4). (*C*) (*Left* panel) Representative images of Picrosirius red staining of collagen matrix in TA muscles of old WT and C1q KO mice. (Scale bars, 100 μm.) The muscles were collected from mice 14 DPI. (*Right* panel) Quantification of the fibrotic area measured by Picrosirius red staining (n = 4). (*D*) Quantification of expression of fibrosis-related genes determined by RT-qPCR in FAPs isolated from old WT and C1q KO mice. The FAPs were collected from injured TA muscles 3 DPI. The expression of each gene is normalized to GAPDH (n ≥ 3). Quantitative data are shown as mean ± SEM and paired two-tailed Student's *t* tests were used for statistical analysis. $*p < 0.05$, $***p < 0.001$, ns: not significant.

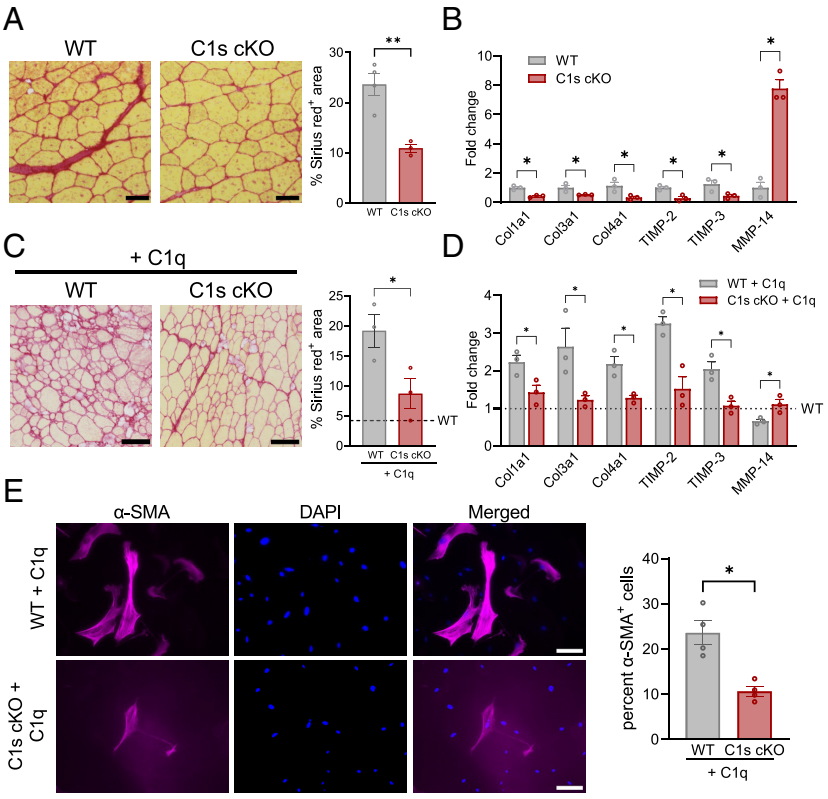

Fig. 6. C1s deletion in FAPs prevents fibrosis in injured aged muscle. (*A*) (*Left* panel) Representative images of Picrosirius red staining of collagen matrix in TA muscles of old WT and C1s cKO mice. (Scale bars, 100 μm.) The muscles were collected from mice 14 DPI. (*Right* panel) Quantification of the fibrotic area measured by Picrosirius red staining (n = 3). (*B*) Quantification of expression of fibrosis-related genes determined by RT-qPCR in FAPs isolated from old WT and C1s cKO mice. The FAPs were collected from TA muscles 3 DPI. The expression of each gene is normalized to GAPDH (n ≥ 3). (*C*) (*Left* panel) Representative images of collagen matrix in C1q-treated TA muscles from young WT and C1s cKO mice. The TA muscles were isolated 14 DPI and stained with Picrosirius red to assess the fibrotic area. (Scale bars, 100 μm.) (*Right* panel) Quantification of the fibrotic area measured by Picrosirius red staining (n = 3). The dotted line represents the fibrotic area in injured TA muscle from WT, untreated mice. (*D*) Fold changes of fibrosis-related gene expression determined by RT-qPCR in FAPs isolated from WT and C1s cKO mice treated with C1q for 7 d. The dotted line represents the expression level in FAPs from untreated WT mice. (*E*) (*Left* panel) Representative images of FAPs from WT and C1s cKO mice treated with C1q in differentiation medium for 7 d followed by immunofluorescence with an α-SMA antibody. DAPI was used to label nuclei. (Scale bars, 100 μm.) (*Right* panel) Quantification of the percentage of α-SMA$^+$ cells in C1q-treated cells from WT and C1s cKO mice (n = 3). Quantitative data are shown as mean ± SEM and unpaired two-tailed Student's *t* tests were used for statistical analysis. $*p < 0.05$, $**p < 0.01$, ns: not significant.

Additionally, we confirmed that pharmacological inhibition of C1 signaling using a hydrogel containing C1-INH reduced fibrosis and the expression of genes encoding collagen and other ECM proteins in FAPs (*SI Appendix*, Fig. S7 *B* and *C*).

As a complementary approach, we examined FAPs and muscle fibrosis in mdx mice in which the expression of C1q had been reduced genetically by crossing the C1q KO with mdx mice. Since mdx mice with complete absence of C1q did not survive beyond 2 wk of age, we studied the effect of C1q reduction on muscle fibrosis in mdx mice hemizygous for C1q (mdx/C1q$^{+/-}$ mice). We confirmed that mdx/C1q$^{+/-}$ mice exhibited approximately a 50% reduction of C1q levels compared to mdx/C1q$^{+/+}$ mice (*SI Appendix*, Fig. S7 *D* and *E*). We compared the diaphragm muscles, which are known to exhibit a high level of fibrosis in the mdx strain (36), from 6-mo-old mdx/C1q$^{+/-}$ mice to age-matched mdx mice. We found a significant decrease in interstitial collagen deposition when C1q expression was reduced (Fig. 7*A*). Contrary to human DMD patients, mdx mice do not exhibit severe fibrosis in lower limb muscles (37). However, it has been shown that repeated microinjuries to the TA muscle lead to a marked increase in fibrosis in mdx limb muscles (38). We thus performed serial microinjuries to TA muscles of mdx/C1q$^{+/+}$ and mdx/C1q$^{+/-}$ mice and compared their extent of fibrosis 1 wk later. We found that the level of fibrotic tissue deposition was reduced by approximately 50% in the TA muscles of mdx/C1q$^{+/-}$ mice compared to control mdx mice (Fig. 7*B*). Together, these data support the findings that reduction in C1q levels is beneficial in terms of limiting fibrosis in both aging and dystrophic muscles.

Discussion

Our studies highlight the role of non-canonical complement signaling, acting via Wnt signaling in FAPs to induce their fibrogenic differentiation, thereby promoting fibrosis in regenerating aged muscle or in dystrophic muscle. We show that inhibiting Wnt signaling abrogates C1q-induced expression of genes encoding ECM proteins in FAPs. This is consistent with previous findings that C1q induces Wnt target genes in FAPs in dystrophic muscle and that inhibition of C1q suppresses fibrosis in aged muscle following injury (24, 29). Importantly, we show that genetic deletion of C1s from FAPs abrogates the propensity of FAPs to undergo fibrogenic differentiation and the ability of C1q to promote fibrosis in aged regenerating muscle. This provides direct evidence that C1s is required for the fibrogenic effects of C1q in FAPs. We also provide evidence that human FAPs are responsive to C1q to undergo fibrogenic differentiation, suggesting that benefits of inhibition of the C1q pathway observed in mice may be applicable to fibrotic muscle disorders in humans.

Since the initial identification of FAPs in muscle (14, 19), there has been much interest in understanding the balance between

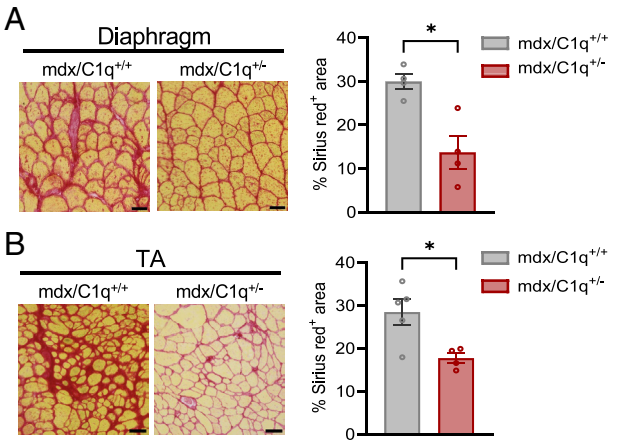

Fig. 7. Inhibition of C1 activity in vivo alleviates fibrosis in mdx mouse muscle. (*A*) (*Left* panel) Representative image of Picrosirius red staining of collagen matrix in the diaphragms of 6-mo-old mdx/C1q$^{+/+}$, and mdx/C1q$^{+/-}$ mice. (Scale bars, 100 μm.) (*Right* panel) Quantification of the fibrotic area measured by Picrosirius red staining (n = 4). (*B*) (*Left* panel) Representative image of Picrosirius red staining of collagen matrix in micro-injured TA muscles of 6-mo-old mdx/C1q$^{+/+}$ and mdx/C1q$^{+/-}$ mice. (Scale bars, 100 μm.) (*Right* panel) Quantification of the fibrotic area measured by Picrosirius red staining (n = 4). Quantitative data are shown as mean ± SEM, and unpaired two-tailed Student's *t* tests were used for statistical analysis. *$P < 0.05$.

their ability to promote muscle homeostasis and repair and their role in muscle degeneration in a variety of pathological conditions (20). It is clear that FAPs play a positive role in muscle physiology, promoting both normal muscle homeostasis as well as muscle regeneration (17, 20). FAPs support the proliferative expansion of MuSCs as well as their differentiation (14, 19, 39). Depletion of FAPs results in muscle atrophy as well as the impairment of the regenerative response (17).

However, excessive FAP proliferation or enhanced FAP differentiation along the fibrogenic, adipogenic, or chondrogenic pathways can contribute to a variety of muscle pathologies including fibrosis, fatty degeneration, and ectopic ossification (20). Suppression of PDGFRα signaling in FAPs reduces FAP proliferation and fibrosis in regenerating, aged muscle and reduces fibrosis in dystrophic muscle (40, 41). Conversely, constitutive activation of PDGFRα signaling in Collagen I-expressing fibroblasts promotes fibrosis during muscle regeneration (41). Clearly, the positive and negative effects of FAP functions are context dependent.

Our findings contribute to the growing body of literature of the role of non-canonical complement signaling in disease pathogenesis, particularly in diseases of aging (42). In addition to contributing to muscle fibrosis, age-related increases in complement components acting through Wnt signaling have been shown to mediate arterial and renal dysfunction (43–45). Consistent with the recent report in dystrophic muscle (29), we found that the activation of complement signaling locally in aged muscle is due to the elevated levels of C1q in tissue-resident macrophages, which only increase with injury, and the high levels of expression of C1r and C1s by FAPs. These results underscore the role of macrophages and FAPs as active mediators of complement activation in muscle associated with aging and injury. Given the fact that C1q increases systemically with age (24, 43), it will be interesting to assess what other tissues express C1r and C1s locally and, as a result, also exhibit age-related tissue dysfunction as a result of non-canonical complement signaling.

The drivers of age-related changes in levels of complement components remain incompletely understood. It has been previously reported that C1q levels rise with age due to elevated production by tissue-resident macrophages (24, 43), and our results are consistent with those findings. In addition, we observed that expression levels of C1r and C1s are significantly increased in FAPs from aged muscle. For each of these complement proteins, key transcription factors regulating their gene expression have been described (46–48). Given the responsiveness of many of these factors to inflammatory stimuli, it is likely that at least some upregulation of expression of these genes in aged tissues is due to the general increase in systemic inflammation with aging (49).

Fibrosis, a major pathophysiological process and pathological outcome in diseases of tissues throughout the body, has been widely studied in the kidney, liver, heart, lung, and skin (50, 51). Fibrosis results from disruption of many signaling pathways (e.g., TGFβ, PDGFRα, VEGF) in many cell types (e.g. fibroblasts, pericytes, immune cells) (16, 52, 53), resulting in organ failure. In that context, our studies could shed light on fibrotic responses more generally in other tissues as FAP-like cells (i.e., PDGFRα-expressing mesenchymal progenitors) have been found throughout the body (54–56). Despite intense study over the past decades, therapeutic interventions to prevent or reduce fibrosis have had limited success (57, 58). As such, there is a compelling need to identify both molecular pathways and cellular mediators for novel therapeutics that target the fibrotic process. C1-INH is currently in clinical use for the treatment of a variety of disorders, including inflammatory/fibrotic diseases (59). Since C1-INH works directly to inhibit C1r and C1s (60), it may be efficacious in any age-related disorders in which the increased levels of C1q lead to pathological consequences. The ability of inhibition of C1 to reduce fibrosis both in aged muscle and in dystrophic muscle suggests that C1 inhibition may be more broadly beneficial in muscle disorders in which fibrosis is a consequence of increased complement signaling.

Methods

Animals. All animals used in this study were housed and maintained in the Veterinary Medical Unit at the Veterans Affairs Palo Alto Health Care System. The Institutional Animal Care and Use Committee (IACUC) approved animal protocols. The young mice used in this study were 2 to 4 mo old, and the old mice were 22 to 24 mo old. Mice were maintained on a 12/12-h light/dark cycle between 20 and 24 °C in individually ventilated cages. Regular health checks were performed weekly on young mice and twice weekly for the aged mice. C1q$^{+/-}$ mice were provided by Dr. Andrea J Tenner, University of California, Irvine, and bred to yield homozygous C1q KO (C1q$^{-/-}$) mice and littermate controls (C1q$^{+/+}$). The specific allele that is deleted in this strain is the C1qa gene, but since the formation of an active C1q complex is dependent on all three C1q genes, the knockout of C1qa effectively eliminates all C1q activity and has subsequently been referred to as a "C1q KO," (61) so we continue to use this designation. The C1s$^{Flox/+}$ mice were provided by Dr. Gareth Howell at Jackson Laboratory. The PDGFRα$^{CreER/+}$ knock-in mice used in this study have been previously described (17). The B6.129 × 1-*GT(ROSA)26Sor*$^{tm1(EYFP)Cos}$/J (Rosa26$^{eYFP/eYFP}$) mice and B6Ros.Cg-*Dmd*$^{mdx\text{-}5Cv}$/J (mdx-5cv) mice were obtained from Jackson Laboratory.

Human Skeletal Muscle Specimens. The human muscle biopsy specimens were used after obtaining informed consent as part of a human studies research protocol approved by the Stanford University Institutional Review Board. Human tissue biopsies were sourced through a collaboration with a not-for-profit organization, Donor Network West (DNW) (San Ramon, CA). Recovery of nontransplantable organs and tissue was considered for research studies only after obtaining records of first-person authorization and/or consent from the family members of the donor. The research protocol was approved by the Institutional Review Board at Stanford University. The donors were a 59-y-old female, a 61-y-old female, and a 51-y-old male. The samples were processed for cell analysis within 1 h of specimen isolation.

$BaCl_2$ Muscle Injury. Before muscle injuries, mice were anesthetized with isoflurane, and the hindlimb skin was disinfected with 70% ethanol. TA muscles were then injected with 50 μL $BaCl_2$ (1.2% w/v in sterile water) (Sigma) with a 31-gauge insulin syringe. Postsurgery buprenorphine analgesia was administered to the injured mice.

Microinjury. Microinjury was performed on mdx-5cv and mdx; $C1q^{+/-}$ mice as previously described (38). Briefly, 6-mo-old mice were anesthetized with 2.5% isoflurane, and hindlimb skin was shaved and disinfected with 70% ethanol. Micropins used for entomology (150 μm diameter, JBP) were sterilized and used. Fifteen micro-punctures were done daily in the TA muscle with micro-pins for 2 wk. Punctures were performed randomly on the whole TA muscle surface.

In Vivo Treatment with C1q, C1r, C1s, and C1-INH. To embed C1q, C1r, C1s, or C1-INH in hydrogel, 10 μg of protein was mixed with Puramatrix or Vitrogel according to the manufacturers' instructions so that the final volume of the mixture is 25 μl. Immediately after $BaCl_2$ injury, the protein-hydrogel mixture was injected subcutaneously between the TA muscles and the skin of one TA of an injured mouse. The contralateral TA were treated with hydrogel containing BSA as controls. Hydrogel treatment was repeated every 3 d until tissue collection. To determine the release kinetics of hydrogel embedded protein, C1q or C1-INH were conjugated with 5 TAMRA-NHS (dissolved in DMSO) in a molar ratio of 2:1 using according to the manufacturer's instructions. Conjugated proteins were then embedded in hydrogel injected subcutaneously at the TA muscles. Fluorescence imaging was then performed using the 594 nm filter every day until the fluorescence signal reached a minimum.

Histology and Histological Analysis. TA muscles were frozen and sectioned into 7 μm sections using a cryostat. The sections were fixed with 4% PFA and stained using the Picrosirius red staining kit (Polysciences). The injured areas showing fibrosis were quantified using FIJI software. For C1q and C1-INH treatment, the muscle region at the site of injection was chosen for quantification. For each muscle sample, three sections were used, and at least three mice were used for each treatment group.

Cell Isolation. Cells were isolated from skeletal muscle based on previously described procedures (17, 62). Briefly, hindlimb muscles isolated from mice before and 3 d after $BaCl_2$ injury were minced with scissors and then enzymatically digested with 760 U/mL Collagenase Type II (Worthington) for 45 min in a 37 °C shaking water bath. Samples were then washed with Ham's F-10 containing 10% Horse serum and 1% Penicillin Streptomycin (PS) (wash medium) and further digested in 1,000 U/mL Collagenase Type II and 11 U/mL Dispase (GIBCO) in wash medium for 30 min in a 37 °C shaking water bath. Samples were then triturated with a 20-gauge needle, washed, and passed through a 40-μm cell strainer to obtain single-cell suspension. Cells were stained with fluorescently conjugated antibodies [dilutions: 1:100 (BioLegend) or 1:500 (eBioscience); *SI Appendix*, Table S1] on a nutating mixer for 30 min at 4 °C, washed in wash medium, and filtered through a 30-μm cell strainer (Falcon). Cell sorting was done with a BD Biosciences FACSAria II with 405, 488, 633 nm lasers or a FACS Aria III with 355, 405, 488, 532, and 633 nm lasers or a Sony Sorter MA900 with 405, 488, 561, and 638 nm lasers. Strict gating schemes were determined and applied based on no antibody or fluorescence minus one control. Sorted cells were collected in the wash medium and reanalyzed for purity.

Myofiber Isolation. Single myofibers were isolated from extensor digitorum longus (EDL) muscles as previously described (63). Briefly, EDL muscles were dissected and digested in Ham's F-10 medium with 500 U/mL Collagenase Type II. The fibers were then triturated, washed extensively, and used for RT- qPCR analysis.

C1q Treatment of FAPs In Vitro. Freshly isolated FAPs were maintained in DMEM supplemented with 20% FBS and 5 ng/mL basic Fibroblast Growth Factor (growth medium) for 2 d. The growth medium was replaced with DMEM supplemented with 10% FBS (differentiation medium) with and without 100 μg/mL of C1q dissolved in high salt buffer (10 mM HEPES and 300 mM NaCl, pH 7.2). C1q was replenished every 24 h when applicable, and the cells were used for immunofluorescence analysis, RT-qPCR, and RNA sequencing after 7 d of treatment with differentiation media.

RT-qPCR. RNA was extracted from 100,000 cells using the RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions and was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Invitrogen). The Roche Light Cycler 480 Real-Time PCR system and custom synthesized primers (*SI Appendix*, Table S1) were used to amplify the cDNA of target genes. Target transcript levels relative to GAPDH transcript levels were quantified using the comparative CT method (64). Measurements were taken in triplicates for each experiment.

RNA Sequencing. Freshly isolated FAPs from individual mice were treated with C1q as described above and RNA was extracted using the Nucleospin RNA XS kit (Macherey-Nagel). cDNA libraries were prepared from RNA by the Stanford Genomics Facility. Libraries underwent single-end 150-bp sequencing at the Stanford Genomics Facility with an Illumina NextSeq 500 to a depth of 20 to 40 million reads. RNA-seq data processing and gene expression analysis were carried out using Galaxy tools. To process raw RNA-seq data, trim_galore was used for adapter and quality trimming (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore) (quality cutoff 20, adapter stringency 1, final length filter 50). STAR (mismatch cutoff 4% of read length, no noncanonical junction alignments) was used to map reads to mm10 (Ensembl release 89, no patches) using transcript annotations from GENCODE. The featureCounts tool of Galaxy package was used to summarize unique mappings over genes. For analysis of gene expression, genes lacking FPKM of at least 1.5 in at least three samples and genes lacking an Entrez ID were filtered out. Raw count data were then normalized for library preparation and sample collection batch effects using RUVs (variation factors $k = 6$). Differential gene-expression analysis was performed for genes with a normalized FPKM value of at least 6 in at least three samples using the Limma software package. The Benjamini–Hochberg FDR control for multiple-hypothesis testing was used to produce q values. Gene ontology (GO) analysis was performed using the Panther database. Heat map of genes belonging to the ECM family was constructed using the Galaxy Heatmap2 tool.

Quantification and Statistical Analysis. Statistical analyses were performed using GraphPad Prism 10 (GraphPad Software).

Data, Materials, and Software Availability. RNA-seq data are deposited in Gene Expression Omnibus with the accession number GSE309478 (65). All study data are included in the article and/or *SI Appendix*.

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The authors declare no competing interest.

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