The pathway to muscle fibrosis depends on myostatin stimulating the differentiation of fibro/adipogenic progenitor cells in chronic kidney disease

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Fibrosis in skeletal muscle develops after injury or in response to chronic kidney disease (CKD), but the origin of cells becoming fibrous tissue and the initiating and sustaining mechanisms causing muscle fibrosis are unclear. We identified muscle fibro/adipogenic progenitor cells (FAPs) that potentially differentiate into adipose tissues or fibrosis. We also demonstrated that CKD stimulates myostatin production in muscle. Therefore, we tested whether CKD induces myostatin, which stimulates fibrotic differentiation of FAPs leading to fibrosis in skeletal muscles. We isolated FAPs from mouse muscles and found that myostatin stimulates their proliferation and conversion into fibrocytes. *In vivo*, FAPs isolated from EGFP-transgenic mice (FAPs-EGFP) were transplanted into muscles of mice with CKD or into mouse muscles that were treated with myostatin. CKD or myostatin stimulated FAPs-EGFP proliferation in muscle and increased α-smooth muscle actin expression in FAP-EGFP cells. When myostatin was inhibited with a neutralizing peptibody (a chimeric peptide-Fc fusion protein), the FAP proliferation and muscle fibrosis induced by CKD were both suppressed. Knocking down Smad3 in cultured FAPs interrupted their conversion into fibrocytes, indicating that myostatin directly converts FAPs into fibrocytes. Thus, counteracting myostatin may be a strategy for preventing the development of fibrosis in skeletal muscles of patients with CKD.

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Diseases that injure the kidney, heart, liver, lung, skin, and potentially other organs can initiate a series of complex cellular and molecular events producing fibrosis that leads to cellular dysfunction and ultimately organ failure.[1,2] Despite developments in understanding the pathogenesis of fibrosis in many organs, there are no routinely successful treatments that block the development of fibrosis. The difficulties in designing antifibrotic treatments are related in part to problems with identifying the origin of cells that produce the proteins that contribute to the development of fibrosis and uncovering the pathways that lead to fibrosis.[2] For example, LeBleu *et al.*[3] investigated cells that develop into fibrocytes in damaged kidneys and concluded that different cells contribute to the development of fibrosis in the injured kidneys. There also is evidence that cells initiating fibrosis in injured kidneys or lung tissues arise following epithelial-mesenchymal transition.[4,5] Others report that kidney fibrosis develops following activation of bone marrow–derived cells or perivascular fibrocytes, although the involvement of these cells in the elaboration of collagen type I is controversial.[3,4,6] Responses to injury in other organs such as the liver involve precursors of perivascular mesenchymal cells (e.g., hepatic stellate cells). They contribute to the development of hepatic fibrosis, but the involvement of other cells has not been excluded.[7] In the generation of cardiac fibrosis, there is evidence that transforming growth factor-β1 initiates the epithelial-mesenchymal transition with increased activity of fibrocytes, although fibrosis in the heart could also involve other cells.[8]

Mechanisms that initiate fibrosis in skeletal muscles have received minimal attention and are not even discussed in a popular review.[2] In investigating muscle fibrosis, we found that changes in insulin-like growth factor-1 (IGF-1) in mice with chronic kidney disease (CKD) contributes to fibrosis in injured muscles, but we did not identify specific cells that produce fibrosis nor the pathway that stimulates muscle fibrosis.[1] In injured skeletal muscles, potential precursor of fibrosis are the satellite cells because they possess multilineage capabilities including myogenic, adipogenic, and fibrogenic properties.[9,10] In fact, impaired functions of satellite cells in mice with CKD or in aging mice are associated with skeletal muscle fibrosis.[1,11] Mice bearing satellite cell mutations (e.g., in mice with *Pax7* knockout or with genetic deletions of *Myogenin*, *Myf5*, or *MyoD*) experience accumulation of adipocytes and muscle

fibrosis after muscle injury.[12–15] However, an evaluation of lineage tracing in MyoD-Cre/R26R-EYFP mice concluded that satellite cells do not spontaneously develop into adipocytes or fibrocytes.[16] In addition to satellite cells, we and others have uncovered mesenchymal progenitor cells called fibro/adipogenic progenitor cells (FAPs) in muscle that can develop into adipocytes or fibrocytes.[17–20] These FAPs express platelet-derived growth factor receptor-α (PDGFR-α) and contribute to adipocyte formation in skeletal muscles of mice that are treated with glucocorticoids.[17] FAPs can also stimulate myogenesis.[17,20] Although mice engineered to overexpress PDGFR-α develop systemic fibrosis, the pathway stimulating fibrosis following muscle injury has not been identified.[21] In these experiments, we used *in vitro* and *in vivo* techniques to identify how CKD stimulates the development of fibrosis in skeletal muscles. Our results reveal that CKD-induced fibrosis in skeletal muscles originates from FAPs, and blocking myostatin suppresses muscle fibrosis in mice with CKD.

RESULTS

CKD induces fibrosis in skeletal muscle

CKD in male mice was created by subtotal nephrectomy in 2 stages over 2 weeks. Results from CKD mice were compared with those of sham-operated, pair-fed mice.[22] After recovery from surgery, mice were fed 40% protein yielding blood urea nitrogen values >80 mg/dl versus ~20 mg/dl in control mice.[22,23] After 5 months, Sirius red staining of tibialis anterior (TA) muscles revealed increased collagen deposition in CKD mice versus control mice (Figure 1a). In gastrocnemius muscles, CKD led to higher levels of mRNA of fibrosis markers (Figure 1b). Thus, CKD stimulates fibrosis in muscles.

To evaluate whether CKD also stimulates muscle fibrosis in a model of muscle injury, we injected cardiotoxin into mouse TA muscles, a standard model of muscle injury.[24] After 14 days of muscle injury, mice with CKD exhibited a significant increase ($P < 0.05$) in collagen deposition

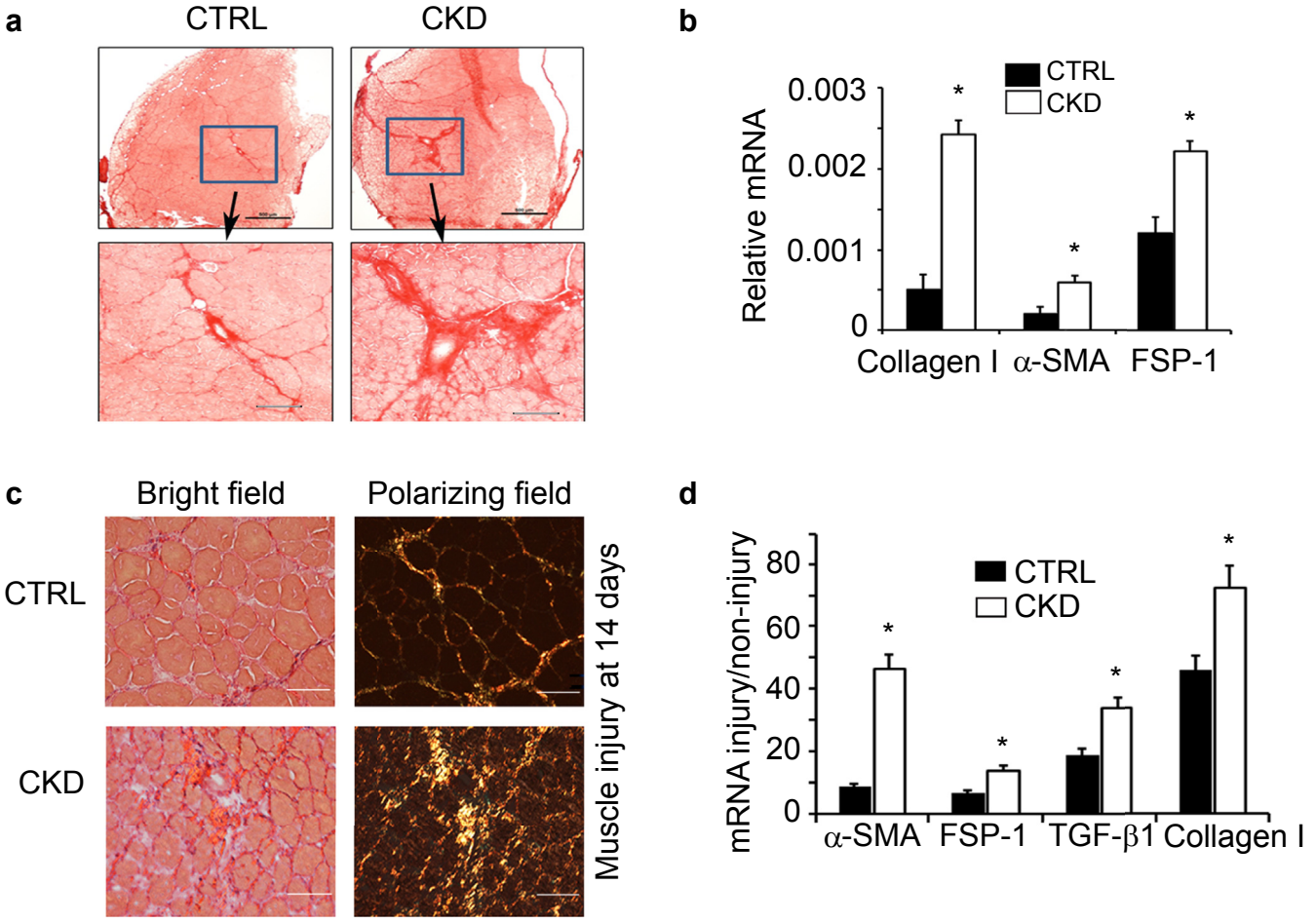

Figure 1 | CKD increases muscle fibrosis. (**a**) Cryo-cross sections of tibialis anterior (TA) muscles were subjected to Sirius red staining (bar = 50 μm); chronic kidney disease (CKD) was associated with increased collagen deposition. (**b**) Fibrotic marker mRNA measured by reverse transcriptase polymerase chain reaction was greater in gastrocnemius muscles of mice with CKD ($n = 4$; *$P < 0.05$ vs. control). (**c**) TA muscles were injured by cardiotoxin injection and compared with the contralateral muscles injected with phosphate-buffered saline. After 14 days, Sirius red–stained cryo-cross sections of muscles of CKD mice (bar = 50 μm) revealed more fibrous tissue. (**d**) mRNA of markers of fibrosis genes was increased in injured muscles of CKD mice ($n = 4$; *$P < 0.05$ vs. control). α-SMA, α-smooth muscle actin; CTRL, control; FSP-1, fibroblast-specific protein 1; TGF-β1, transforming growth factor β1.