The inhibition of CTGF/CCN2 activity improves muscle and locomotor function in a murine ALS model

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Abstract

Amyotrophic lateral sclerosis (ALS) is a devastating adult-onset progressive neurodegenerative disease characterized by upper and lower motoneuron degeneration. A total of 20% of familial ALS (fALS) cases are explained by mutations in the superoxide dismutase 1 (SOD1) enzyme. Although more than 20 years have passed since the generation of the first ALS mouse model, the precise molecular mechanisms of ALS pathogenesis remain unknown. CTGF/CCN2 is a matricellular protein with associated fibrotic activity that is up-regulated in several chronic diseases. The inhibition of CTGF/CCN2 with the monoclonal neutralizing antibody FG-3019 reduces fibrosis in several chronic disorders including the mdx mice, a murine model for Duchenne muscular dystrophy (DMD). In this work, we show that there are increased levels of CTGF/CCN2 in skeletal muscle and spinal cord of hSOD1G93A mice. In this scenario, we show evidence that FG-3019 not only reduces fibrosis in skeletal muscle of hSOD1G93A mice, but also improves muscle and locomotor performance. We demonstrate that treatment with FG-3019 reduces muscle atrophy in hSOD1G93A mice. We also found improvement of neuromuscular junction (NMJ) innervation together with a reduction in myelin degeneration in the sciatic nerve, suggesting that alterations in nerve–muscle communication are partially improved in FG-3019-treated hSOD1G93A mice. Moreover, we also found that CTGF/CCN2 is expressed in astrocytes and neurons, predominantly in dorsal areas of spinal cord from symptomatic hSOD1G93A mice. Together, these results reveal that CTGF/CCN2 might be a novel therapeutic target to ameliorate symptoms and improve the quality of life of ALS patients.

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal adult-onset neurodegenerative disease characterized by the degeneration of motoneurons in the motor cortex, brainstem and spinal cord. This loss of motoneurons leads to skeletal muscle wasting, fibrosis, paralysis and eventually death from respiratory failure within 3–5 years of diagnosis (1–3). About 10% of ALS cases are familial (fALS), with an associated genetic component and usually with dominant inheritance. The superoxide dismutase 1 gene (SOD1) was the first one to be linked to ALS and it accounts
for about 20% of fALS cases. Although more than 20 years have passed since generation of the first ALS mouse model (hSOD1G93A), the exact mechanisms by which SOD1 exerts its toxic effects remain unclear. Besides the SOD1 gene, a hexanucleotide repeat expansion GGGGCC in the C9orf72 gene has also been linked to ALS, and accounts for ~50% of fALS cases (4,5). To date, Riluzole and Radicava (edaravone) are the only two FDA-approved drugs for the treatment of ALS; however, Riluzole has limited therapeutic benefits, extending the patients’ life expectancy by only a few months (6). Radicava seems to be more effective in earlier stages of the disease (7,8). Thus, identification of new therapeutic targets is urgently needed.

To date, it has not been well established whether skeletal muscle contributes to degeneration of motoneurons and disease progression. There is evidence showing that restricted expression of SOD1G93A in skeletal muscle is sufficient to induce the characteristic ALS phenotype (9,10), indicating that this tissue is a primary target of SOD1G93A toxicity. However, the molecular mechanisms involved remain obscure.

Fibrosis is a common feature underlying several chronic disorders and involves the replacement of functional tissue by non-functional connective tissue composed of extracellular matrix (ECM) molecules such as proteoglycans, fibronectin and different types of collagens (11-13). The fibrotic process is well described in several chronic disorders including liver disease, idiopathic pulmonary fibrosis, heart failure and skeletal muscle dystrophies (14-17).

Connective tissue growth factor (CTGF/CCN2) is a matricellular protein containing four domains: domain 1, homologous to IGF-1 binding proteins; domain 2, homologous to von Willbrand factor type C repeat; domain 3, homologous to thrombospondin type 1 repeat; and domain 4, which contains a cysteine knot motif that binds heparin sulfate proteoglycans (18). CTGF/CCN2 promotes fibroblast proliferation, ECM synthesis, cell adhesion and migration in several cell types (19). It has been shown that CTGF/CCN2 induces ECM production in skeletal muscle both in vitro (20) and in vivo (21). CTGF/CCN2 was reported to act downstream of transforming growth factor type-β (TGF-β) and can potentiate TGF-β pro-fibrotic effects (22,23). In vivo studies showed that coinjection of TGF-β and CTGF/CCN2 triggers a persistent fibrotic response for at least one week after injection (24). Our laboratory has shown that a human monoclonal antibody against CTGF/CCN2 (FG-3019, FibroGen, Inc., San Francisco, CA, USA) reduces fibrosis and improves muscle strength in mdx mice, a murine model for Duchenne muscular dystrophy (DMD). These results were confirmed using mdx-Ctgf−/− mice (25). FG-3019 also reduces tissue remodeling and fibrosis in liver, cardiovascular system and lungs (18). Post-mortem analyses of the spinal cord from ALS patients showed that CTGF/CCN2 is up-regulated in astrocytes and motoneurons (26). However, there is no evidence about the specific role of CTGF/CCN2 in ALS. We have recently shown that skeletal muscle from hSOD1G93A mice exhibits fibrosis, enhanced TGF-β signaling, induction of markers of fibro/adipogenic progenitors (FAPs), together with CTGF/CCN2 up-regulation during symptomatic stages (27).

In this work, we provide evidence that the inhibition of CTGF/CCN2 activity by the FG-3019 neutralizing antibody not only reduces fibrosis, but also improves muscle and locomotor function in the hSOD1G93A mice. Treatment with FG-3019 also reduces muscle atrophy, improves NMJ innervation and reduces myelin degeneration in the sciatic nerve. We also found that CTGF/CCN2 is expressed in astrocytes and neurons preferably in dorsal areas of spinal cord from symptomatic hSOD1G93A mice. Thus, blocking CTGF/CCN2 activity might be a promising therapeutic approach for improving the quality of life of patients suffering from this neurodegenerative disease.

Results

CTGF/CCN2 is up-regulated in symptomatic hSOD1G93A mice and treatment with the CTGF-blocking antibody FG-3019 improves muscle function and locomotor capacity

We previously reported that skeletal muscle of symptomatic hSOD1G93A mice is fibrotic, with enhanced TGF-β signaling and CTGF/CCN2 up-regulation (27). We first evaluated CTGF/CCN2 protein levels in gastrocnemius muscle from 4-, 8-, 12- and 16-week-old hSOD1G93A mice. We observed that CTGF/CCN2 was significantly up-regulated (4-fold) in symptomatic 16-week-old hSOD1G93A mice (Fig. 1A and B). It was predominantly localized to the endomysium and perimysium of skeletal muscle (Fig. 1C).

We also determined the expression of other CCN family members and found that Ccn1 mRNA was up-regulated in the gastrocnemius from symptomatic hSOD1G93A mice, while Ccn3 mRNA levels were down-regulated (Supplementary Material, Fig. S1A and B). To examine the role of CTGF/CCN2 in ALS progression, hSOD1G93A mice were treated with FG-3019 (FibroGen, Inc.), which inhibits CTGF/CCN2 activity (25 mg/kg). The antibody was administered by intraperitoneal injection three times per week for 2 months, starting with 8-week-old mice (Fig. 2A). As a control, hu IgG (FibroGen, Inc.; 25 mg/kg) was administered on the same schedule. In order to assess whether treatment with FG-3019 delayed disease onset, we measured body weight of wild-type and hSOD1G93A mice treated with the CTGF-blocking antibody or the control hu IgG antibody. FG-3019-treated hSOD1G93A mice exhibited delayed body weight loss compared with hu IgG-treated hSOD1G93A animals, indicating a delay in disease progression (Fig. 2B). To evaluate muscle and locomotor function, hanging and open field tests were performed. In 14-week old animals, hSOD1G93A mice treated with FG-3019 had significantly longer hanging times (82.28 s) than hu IgG-treated hSOD1G93A mice (31.6 s) (Fig. 2C). Moreover, FG-3019-treated mice traveled longer distances (total distance 12.23 m versus 6.60 m) (Fig. 2D) and at faster speeds (average speed of 0.02 m/s versus 0.012 m/s) (Fig. 2E) than hu IgG-treated hSOD1G93A mice.

The isometric forces of diaphragm and tibialis anterior muscles were evaluated ex-vivo to determine the effect of CTGF/CCN2 inhibition on muscle degeneration in hSOD1G93A mice. Muscles from FG-3019-treated hSOD1G93A mice generated greater specific force over a wide range of stimulation frequencies (1–200 Hz) compared with hu IgG-treated hSOD1G93A mice (Fig. 3A and B). Our experiments show that the normalized specific force of diaphragm (89.82 mN/mm2) and tibialis anterior muscles (76.19 mN/mm2) from FG-3019-treated hSOD1G93A mice was increased compared with diaphragm (35.14 mN/mm2) and tibialis anterior (34.73 mM/mm2) from hu IgG-treated hSOD1G93A mice at a frequency of 100Hz (Fig. 3C and D). Considered together, these results indicate that CTGF/CCN2 is up-regulated in symptomatic hSOD1G93A mice and that treatment with CTGF-blocking antibody, FG-3019, improved muscle function and locomotor capacity, thereby slowing ALS progression in hSOD1G93A mice.

In order to confirm that FG-3019 penetrated muscle, immunoblot and immunofluorescence analyses were performed in gastrocnemius from wild-type, hu IgG-treated and...
FG-3019-treated hSOD1\textsuperscript{G93A} mice using anti-human HRP and anti-human Alexa-568 secondary antibodies, respectively. FG-3019 was observed in gastrocnemius muscle (Supplementary Material, Fig. S2A and B) and localized predominantly at the perimysium and endomysium, as shown in Supplementary Material, Figure S2C. We found that CTGF/CCN2 co-localizes with anti-human Alexa-568 staining in consecutive gastrocnemius cross-sections, suggesting that FG-3019 localizes in the same areas as CTGF/CCN2 possibly inhibiting its activity (Supplementary Material, Fig. S2D). We also determined that CTGF/CCN2 levels were similar in hSOD1\textsuperscript{G93A} mice treated with hu IgG or FG-3019 antibody (Supplementary Material, Fig. S2E). These results indicate that FG-3019 antibodies reach the skeletal muscle, but do not significantly alter CTGF/CCN2 levels.

**FG-3019 treatment reduces fibrosis and improves skeletal muscle architecture without affecting TGF-\(\beta\) signaling in hSOD1\textsuperscript{G93A} mice**

Because FG-3019 reduced body weight loss and improved isolated skeletal muscle strength and locomotor function, we further analyzed the skeletal muscle phenotype. At the end of treatment, FG-3019-treated hSOD1\textsuperscript{G93A} mice exhibited decreased fibrosis compared with hu IgG-treated hSOD1\textsuperscript{G93A} mice. Figure 4A–I shows improved muscle structure, evaluated by H&E staining and immunofluorescence. We found a reduction of the interstitial space between myofibers, and of infiltrating mononuclear cells. More importantly, we found that myofibers from FG-3019-treated hSOD1\textsuperscript{G93A} mice were thicker than those from hu IgG-treated hSOD1\textsuperscript{G93A} animals, suggesting that FG-3019 treatment could reduce muscle atrophy (see below). We also found lower levels of ECM deposition, as exemplified by decreased fibronectin and collagen I levels in FG-3019-treated hSOD1\textsuperscript{G93A} mice (Fig. 4D–I). To corroborate the reduction of ECM molecules deposition in FG-3019-treated hSOD1\textsuperscript{G93A} mice that was observed microscopically, we evaluated fibronectin protein levels by immunoblot. We found a significant reduction of fibronectin in FG-3019-treated hSOD1\textsuperscript{G93A} mice (Fig. 4J–K).

All three TGF-\(\beta\) isoforms have been reported to be up-regulated in skeletal muscle of hSOD1\textsuperscript{G93A} mice and ALS patients (28). TGF-\(\beta\)1 and TGF-\(\beta\)2 are also up-regulated in spinal cord of hSOD1\textsuperscript{G93A} mice (29), which activates the Smad-dependent signaling pathway (27). Therefore, we decided to examine whether FG-3019 inhibition of CTGF/CCN2 modulates the TGF-\(\beta\) signaling pathway. We evaluated TGF-\(\beta\)1 transcript levels, total Smad3 levels and the number of p-Smad3-positive cells in FG-3019-treated hSOD1\textsuperscript{G93A} mice. TGF-\(\beta\)1 mRNA levels were similarly elevated in hu IgG-treated and FG-3019-treated hSOD1\textsuperscript{G93A} mice, compared with wild-type mice. Total Smad3 protein levels and p-Smad3-positive cells were similar in FG-3019-treated and hu IgG-treated hSOD1\textsuperscript{G93A} mice (Supplementary Material, Fig. S3A–E). These results show that inhibition of CTGF/CCN2 by FG-3019 is able to reduce fibrosis without affecting canonical TGF-\(\beta\) signaling.

**FG-3019 treatment reduces myofiber atrophy in hSOD1\textsuperscript{G93A} mice**

The results described above (Fig. 4A–C) suggest that CTGF/CCN2 inhibition reduces muscle atrophy in hSOD1\textsuperscript{G93A} mice. Therefore, to quantify myofiber atrophy we measured myofiber diameter in wild-type, hu IgG-treated and FG-3019-treated hSOD1\textsuperscript{G93A} mice using the minimal Feret’s diameter. Figure 5A–D shows that the curve corresponding to the diameter of gastrocnemius myofibers from FG-3019-treated hSOD1\textsuperscript{G93A} mice presents a shift to the right when compared with the curve from hu IgG-treated hSOD1\textsuperscript{G93A} mice. These results indicate that mice treated with FG-3019 have thicker myofibers than hu
IgG-treated mice. We also found that in FG-3019-treated hSOD1\textsuperscript{G93A} mice, 30% of myofibers have a mean diameter of 30–40 μm and 10% of myofibers present a mean diameter of 10–20 μm. In contrast, ~30% of myofibers in hu IgG-treated hSOD1\textsuperscript{G93A} mice have a mean diameter of 20–30 μm and ~25% of myofibers exhibit a 10–20 μm diameter (Fig. 5D). Therefore, the reduction of muscle atrophy observed in FG-3019-treated hSOD1\textsuperscript{G93A} mice could be due to an improvement in nerve–muscle integrity and/or a reduction of fibrosis.

FG-3019 treatment improves NMJ innervation and reduces myelin degeneration in sciatic nerve from hSOD1\textsuperscript{G93A} mice

Denervation of skeletal muscle is one of the hallmarks of ALS that precede muscle atrophy. Since we found increased myofiber diameter in FG-3019-treated hSOD1\textsuperscript{G93A} mice, we wanted to evaluate the integrity of the nerve–muscle communication in these mice. Figure 6A–L shows neuromuscular junctions (NMJs) in diaphragms from wild-type, hu IgG-treated and FG-3019-treated hSOD1\textsuperscript{G93A} mice. Wild-type NMJs were almost totally innervated, as demonstrated by the co-localization of the stains for pre-synaptic terminals (neurofilament H and SV2) and end-plates (α-bungarotoxin). In contrast, only 20% of NMJs from hu IgG-treated hSOD1\textsuperscript{G93A} mice remain innervated during the symptomatic stage (16-weeks old). Interestingly, FG-3019 treatment increases NMJs innervation from 20% to ~50% as shown in Figure 6M.

The reduction in myofiber atrophy and the increased NMJs innervation suggested that the loss of integrity of peripheral nerves in hSOD1\textsuperscript{G93A} mice could be ameliorated by CTGF/CCN2 inhibition. Thus, to further monitor nerve integrity in FG-3019-treated hSOD1\textsuperscript{G93A} mice, we evaluated myelin degeneration in the sciatic nerve by toluidine blue staining and electron microscopy. Figure 7A–F shows that myelin degeneration is reduced in FG-3019-treated hSOD1\textsuperscript{G93A} mice. There was less degenerated myelin structure per total myelin sheaths (Fig. 7G) and per area (Fig. 7H). Figure 7I shows that there is a reduction of axon diameter in hu IgG-treated and FG-3019-treated hSOD1\textsuperscript{G93A} mice compared with wild-type animals. Notably, scatter plots show
Figure 3. FG-3019 treatment improves isometric force of diaphragm and tibialis anterior muscles from hSOD1(G93A) mice in ex-vivo experiments. The graphs show isometric force measurements in diaphragm (A) and tibialis anterior (B) from 16-week-old hu IgG-treated and FG-3019-treated hSOD1(G93A) mice subjected to increasing stimulation frequencies (1–200 Hz). The graphs show isometric-specific force of diaphragm and tibialis anterior from 16-week-old hu IgG-treated and FG-3019-treated hSOD1(G93A) mice at a stimulation frequency of 100 Hz. Values correspond to the mean ± S.E.M. of five animals for each experimental condition. Two-way ANOVA or t-test, *P < 0.05, **P < 0.005, ***P < 0.001.

that g-ratios (ratio of axon diameter to myelin diameter) from hu IgG-treated hSOD1(G93A) mice are decreased compared with g-ratios from wild-type mice. However, we found that there is a slight increase of g-ratios in FG-3019-treated hSOD1(G93A) mice compared with those of hu IgG-treated hSOD1(G93A) mice (Fig. 7), suggesting that myelin thickness could be partially restored. We did not find statistically significant changes in average g-ratios for the three experimental groups (Fig. 7C). Together with the improvement of skeletal muscle structure, our results indicate that sciatic nerve, NMJs and axons are at least partially protected from innervation and degeneration in FG-3019-treated hSOD1(G93A) mice.

CTGF/CCN2 is up-regulated and predominantly located in dorsal area of spinal cord in hSOD1(G93A) mice

Since we found an improvement in skeletal muscle architecture and in peripheral nerves, we asked whether CTGF/CCN2 could have a detrimental role in the spinal cord, which is characterized by neuroinflammation, a process that involved activation of microglia and astrocytes, infiltration of immune cells and high levels of inflammatory cytokines (30, 31). Therefore, we determined CTGF/CCN2 protein levels and tested if the FG-3019 antibody was able to reach the spinal cord in hSOD1(G93A) mice. To address this question, we evaluated CTGF/CCN2 protein levels in spinal cords from 4-, 8-, 12- and 16 weeks old hSOD1(G93A) mice. We found that CTGF/CCN2 levels have a tendency to increase in the early stages, and that this increase becomes statistically significant (3-fold) during the symptomatic stages of 16-week-old hSOD1(G93A) mice (Fig. 8A and B). This result indicates that CTGF/CCN2 is enhanced in spinal cord of hSOD1(G93A) mice.

In order to evaluate if FG-3019 is able to reach the spinal cord, we performed western blots of spinal cord from wild-type, hu IgG-treated and FG-3019-treated hSOD1(G93A) mice using an anti-human HRP. Interestingly, we found that only the spinal cord from hSOD1(G93A) mice that had been treated with FG-3019 showed a positive signal for the anti-human secondary antibody (Fig. 8C and D). This result indicates that FG-3019 is effectively reaching the spinal cord of treated hSOD1(G93A) mice where no signal was found in hu IgG-treated hSOD1(G93A) mice or in wild-type mice. Therefore, FG-3019 can potentially inhibit CTGF/CCN2 activity in this tissue. To gain insight into the cell type that is producing CTGF/CCN2, we performed immunostaining in cross-sections of spinal cord from wild-type and symptomatic (16-week-old) hSOD1(G93A) mice. We determined CTGF/CCN2 protein levels in the spinal cord (Fig. 9K–N). Figure S4 shows a diagram indicating that CTGF/CCN2 activity in this tissue. To gain insight into the cell type that is producing CTGF/CCN2, we performed immunostaining in cross-sections of spinal cord from wild-type and symptomatic (16-week-old) hSOD1(G93A) mice. We found that CTGF/CCN2-positive cells are predominantly in dorsal areas of the spinal cord from hSOD1(G93A) mice (Fig. 9K–N). Figure 9A–J shows a modest CTGF/CCN2 expression in wild-type mice that co-localize with GFAP-positive cells (astrocytes) and neurons (NF-H staining). However, in spinal cord from symptomatic hSOD1(G93A) mice, we found stronger fluorescence intensity for CTGF/CCN2 in both astrocytes and neurons (Fig. 9A–J). Moreover, we found stronger fluorescence intensity for CTGF/CCN2 in neurons (NeuN-positive) preferably in dorsal areas of the spinal cord from hSOD1(G93A) mice (Fig. 9K–N).

CTGF/CCN2 expression is increased in this tissue. To gain insight into the cell type that is producing CTGF/CCN2, we performed immunostaining in cross-sections of spinal cord from wild-type and symptomatic (16-week-old) hSOD1(G93A) mice. We found that CTGF/CCN2-positive cells are predominantly in dorsal areas of the spinal cord, where FG-3019 could be reaching and inhibits CTGF/CCN2 activity.

Discussion

In this study, we show that CTGF/CCN2 is up-regulated not only in the lumbar area, but also in the thoracic area of the spinal cord (Fig. 9B–D). Moreover, we did not find a correlation between ECM molecules and CTGF/CCN2 expression, indicating that CTGF/CCN2 is enhanced in spinal cord of hSOD1(G93A) mice.

Little is known about the role of CTGF/CCN2 in ALS. Post-mortem analyses of spinal cord from ALS patients show that CTGF/CCN2 expression is increased in this tissue (26).
Furthermore, we previously showed that CTGF/CCN2 protein levels are increased in gastrocnemius from symptomatic hSOD1<sup>G93A</sup> mice (27). Spliet et al. found CTGF/CCN2 immunoreactivity in astrocytes and motoneurons in post-mortem spinal cord from sALS and fALS patients (26). On this regard, we found that both astrocytes (GFAP-positive cells) and neurons (NF-H and NeuN-positive cells) are probably the producers of CTGF/CCN2 in spinal cord from symptomatic hSOD1<sup>G93A</sup> mice. Moreover, we observed that CTGF/CCN2 is also up-regulated in thoracic areas of the spinal cord, indicating that its detrimental effect may be not restricted to the lumbar area. We also found an increase in Ccn1 mRNA levels in both hu IgG-treated and FG-3019-treated hSOD1<sup>G93A</sup> mice. CCN1 has also been identified as a pro-fibrotic factor after ischemic kidney injury (32) and it can enhance Smad-dependent TGF-β signaling pathway to promote lung fibrosis (33). In contrast, we found a decrease in Ccn3 mRNA levels in both hu IgG-treated and FG-3019-treated hSOD1<sup>G93A</sup> mice. The evidence shows that CCN3 suppresses ECM deposition acting as an anti-fibrotic factor in several cell types (34–36). Thus, reduced levels of CCN3 in symptomatic hSOD1<sup>G93A</sup> mice could be promoting fibrosis.

The pro-fibrotic role of CTGF/CCN2 is well-described in several tissues such as liver, kidney and skeletal muscle (21,25,37,38). However, the role of CTGF/CCN2 in the nervous system has been only partially explored to date. It has been shown that CTGF/CCN2 acts as a pro-apoptotic factor, induced by glial-derived TGF-β1, which eliminates newborn neurons in an activity-dependent manner in the mouse olfactory bulb and CTGF/CCN2 knock-down results in better odorant detection and discrimination (39). It was shown that CTGF/CCN2 induces astrogenesis and fibronectin expression in neural progenitor cultures (40), suggesting that CTGF/CCN2 could also be acting as an inducer of the expression of ECM molecules in the nervous system. More recently, it has been demonstrated that CTGF/CCN2 is up-regulated in neurons that lack Tsc1 gene, which is known to trigger a hypomyelination phenotype. Even more, the authors show that genetic ablation of CTGF/CCN2 in neurons improves myelination and oligodendrocytes development,
indicating that CTGF/CCN2 is a key actor for myelination in central nervous system (41). In concordance with these results, we found that CTGF/CCN2 inhibition by FG-3019 reduces myelin degeneration in sciatic nerve of hSOD1\(^{G93A}\). However, there is no evidence showing the role of CTGF/CCN2 in myelination of the peripheral nerves. We found a decrease of g-ratios in hSOD1\(^{G93A}\) mice, likely due to the presence of atrophic axons with thicker myelin sheaths as consequence of axon metabolic abnormalities. Similar results have been found in a previous study (42).

We found a slightly restoration of g-ratios in FG-3019-treated hSOD1\(^{G93A}\) mice, suggesting that myelin thickness could be partially restored since we did not find an improvement of axon diameter. The blockade of CTGF/CCN2 activity has been studied in fibrotic chronic diseases such as liver disease (37) and nephropathy (38). Moreover, FG-3019 treatment is able to reduce fibrosis in liver, cardiovascular system and lungs (18). The safety and efficacy of FG-3019 have been evaluated in patients with idiopathic pulmonary fibrosis (43), diabetes and microalbuminuria (44). Our laboratory has shown that FG-3019 treatment is able to reduce fibrosis and improve muscle architecture and strength in mdx mice (25). In this work, we treated hSOD1\(^{G93A}\) mice with FG-3019 and observed several beneficial effects. The treatment with FG-3019 not only reduces fibrosis but also improves muscle and locomotor function, preventing muscle atrophy and improving NMJs innervation in hSOD1\(^{G93A}\) mice. We also show that FG-3019-treated hSOD1\(^{G93A}\) mice have reduced interstitial space between myofibers and better muscle architecture. These results are in agreement with what was previously reported on several tissues including skeletal muscle (18,25,45,46).

It has been shown that CTGF/CCN2 co-localizes with the NMJ (47), suggesting a possible role in this structure. The authors proposed that CTGF/CCN2 could have an important function at the NMJ modulating Wnt signaling. However, the role of CTGF/CCN2 in NMJ maintenance has not been elucidated. Recently, it has been shown that an adeno-associated virus encoding DOK7, a protein that activates muscle-specific kinase (MuSK), is able to suppress NMJs denervation and muscle atrophy in hSOD1\(^{G93A}\) mice (48). Furthermore, the suppression of muscle atrophy observed in FG-3019-treated mice could partially be explained by an unknown role of CTGF/CCN2 at the NMJ.

In this work we also found that FG-3019 reaches the skeletal muscle and spinal cord of hSOD1\(^{G93A}\) mice. It has been reported that blood–brain barrier and blood–spinal cord barrier permeability is compromised at early and late stages of the disease in hSOD1\(^{G93A}\) mice (49). It has also been reported that blood–spinal cord barrier is disrupted in sALS patients, probably leading to degeneration of motoneurons and progression of the disease (50). This evidence explains why the FG-3019 antibody reaches the spinal cord in hSOD1\(^{G93A}\) mice, where it potentially inhibit CTGF/CCN2 activity. The observation of less myelin degeneration in the sciatic nerve suggests that disease-associated alterations in nerve–muscle communication are partially preserved in FG-3019-treated hSOD1\(^{G93A}\) mice. The role of CTGF/CCN2 in spinal cord is largely unexplored; however, it could be involved in the synthesis of ECM molecules and/or may have a pro-apoptotic effect (39,40).

The data presented in this work indicate that CTGF/CCN2 could be a novel therapeutic target for the treatment of ALS and other neurodegenerative diseases characterized by elevated levels of ECM molecules and CTGF/CCN2 up-regulation. This therapeutic approach could ameliorate symptoms and improve the patients’ quality of life.
Materials and Methods

Animal and tissue collection

All protocols were conducted in strict accordance and with the formal approval of the Animal Ethics Committee of the Pontificia Universidad Católica de Chile. The hemizygous transgenic mice carrying the mutant human SOD1<sup>G93A</sup> (B6SJL-Tg (SOD1<sup>G93A</sup>) 1Gur/J) gene were obtained from Jackson Laboratories (Bar Harbor, USA). Wild-type (B6SJL) and hSOD1<sup>G93A</sup> age-matched male mice were used in this study.

Figure 6. FG-3019 treatment improves NMJ innervation in the diaphragm of hSOD1<sup>G93A</sup> mice. (A–L) NF+SV2 (green) and AChR (red) were detected by immunofluorescence in whole diaphragms from 16-week-old wild-type, hu IgG-treated and FG-3019-treated hSOD1<sup>G93A</sup> mice. (M) Quantification of the percentage of innervated NMJs in the diaphragm of wild-type, hu IgG-treated and FG-3019-treated hSOD1<sup>G93A</sup> mice. Values correspond to the mean ± S.E.M. of three animals in each experimental condition. One-way ANOVA, ***P < 0.001. Scale bar corresponds to 100 μm.
Animals were anesthetized with 3.0% isoflurane gas in pure oxygen and sacrificed by cervical dislocation. Gastrocnemius, diaphragm and tibialis anterior muscles, as well as spinal cord were dissected and removed. Muscle samples for cryosectioning were frozen in liquid nitrogen cooled-isopentane (Merck, Darmstadt, GE) and stored at −80°C until processing. For spinal cord cryosections, briefly, animals were perfused with 4% paraformaldehyde and spinal cords were post-fixed with 4% paraformaldehyde overnight at 4°C. Then, spinal cords were dehydrated in 30% sucrose overnight at 4°C. Once dehydrated, spinal cords were sectioned obtaining 20 μm slices.

FG-3019 neutralizing antibody treatment

Human monoclonal IgG antibody against CTGF/CCN2 (FG-3019) and nonspecific human IgG control antibody (hu IgG) were obtained from FibroGen, Inc. Antibodies were administered intraperitoneally to 8-week-old hSOD1G93A male mice at doses of 25 mg/kg each, three times per week for 2 months (25). After treatment, mice were sacrificed as described previously.

Disease progression analysis

Body weight was measured weekly and the onset of disease was determined by the day of maximum body weight. The
experiment stopped when hu IgG-treated hSOD1\textsuperscript{G93A} mice were 16-week-old or reached end-stage, considered as the inability of a mouse to right itself within 30 s after being placed on its side (51). The hanging test was used to evaluate muscle function. Mice were lifted by the tail, allowed to grasp the middle of the wire with their fore limbs, and then were gently lowered to let them put their hind paws on the wire. The tail was released while the mouse was grasping the wire with its four paws, and upon release, time measurement was started. The duration of time each mouse was able to hang from the wire was recorded. Every mouse had three attempts per session, with at least 1 min recovery period between attempts. The open-field test was used to measure spontaneous locomotor activity of mice. Briefly, animals were placed in an empty box (50 \times 50 cm) for 2 min to avoid stress and then their movement was recorded for 10 min. Total distance travelled (m) and average speed (m/s) were calculated using ANY-maze software (ANY-maze, Wood Dale, IL, USA).

Isometric force measurement

The isometric force of isolated tibialis anterior muscle and diaphragm was measured at optimum muscle length (L\textsubscript{o}). Stimulation voltage was determined from micromanipulation of muscle length to produce a maximum isometric twitch force. Maximum isometric tetanic force (P\textsubscript{o}) was determined from the plateau of the frequency-force relationship after successive stimulations at 1–200 Hz for 450 ms, with 2 min rest between the stimuli. After measurements, muscles were removed from the bath, trimmed of their tendons and of any nonmuscle tissue and weighed. Specific force or stress (force normalized per total muscle fiber cross-sectional area; mN/mm\textsuperscript{2}) was calculated from muscle mass and L\textsubscript{o} (25).

Hematoxylin and eosin staining

Gastrocnemius muscle cryosections (7 \textmu m) were placed onto glass slides. Hematoxylin and eosin staining was performed to assess muscle architecture and histology. Briefly, tissue sections were incubated for 10 min in formalin (10% v/v), then washed with water, incubated for 5 min with diluted hematoxylin (Merck, Darmstadt, GE; 25% v/v in H\textsubscript{2}O) and washed with water. Eosin was added for 30 s and then dehydration with ethanol was performed. Finally, Entellan (Merck, Darmstadt, GE) was added to the slices. Sections were imaged using bright field microscopy on a Nikon Eclipse E600.

Immunohistochemistry staining

Gastrocnemius muscle cryosections (7 \textmu m) were fixed in cold ethanol, rinsed in 0.05 M TBS buffer pH 7.6, and incubated overnight with primary antibody against CTGF/CCN2 (Santa Cruz, USA). Sections were washed three times for 5 min in TBS at RT and then incubated with secondary antibody for 30 min followed by three 5 min washes with TBS. Then, sections where incubated with peroxidase-anti-peroxidase (PAP) complex (1:200) (MP Biomedicals, Aurora, OH) for 30 min, then washed in TBS (3x-5 min). The immunoperoxidase reaction was visualized after incubation of sections in 0.1% diaminobenzidine and 0.03% hydrogen peroxide for 2 min. Sections were washed with tap water and counterstained with hematoxylin, then they were dehydrated in an ethanol gradient and cleared with xylene (52).

Analysis of sciatic nerves

Sciatic nerves were extracted and subjected to toluidine blue staining and EM (electron microscopy). For EM, sciatic nerves were fixed overnight with 2.5% glutaraldehyde, 0.01%
picric acid and 0.05 M cacodylate buffer, pH 7.3. Sciatic nerves were incubated in the same buffer with 1% OsO$_4$ and then immersed in 2% uranyl acetate, dehydrated in an ethanol/acetone gradient and infiltrated in Epon (Ted Pella) as described previously (53, 54). Thin sections (80 nm) were obtained and mounted in copper grids and contrasted using 1% uranyl acetate and lead citrate. Observations were made using a Phillips Tecai 12 transmission electron microscope (Eindhoven) at 80 kV.

Figure 9. Localization of CTGF/CCN2 in spinal cord from symptomatic hSOD1$^{G93A}$ mice. (A–J) GFAP (red), NF-H (red) and CTGF (green) were detected by immunostaining in cross-sections of spinal cord from symptomatic (16-week-old) hSOD1$^{G93A}$ mice. Nuclei were stained with Hoechst. (K–M) Digitally reconstructed cross-sections of spinal cord from symptomatic hSOD1$^{G93A}$ mouse immunostained for NeuN (green) and CTGF (red). (N) Inset from M showing co-localization of NeuN and CTGF staining in dorsal area of the spinal cord. (O) Diagram showing the localization of CTGF$^+$ cells within cross-section of spinal cord from symptomatic hSOD1$^{G93A}$ mice. WM: white matter, GM: grey matter. (P) Fibronectin and CTGF/CCN2 were detected by immunoblot in protein extracts of lumbar (L) and thoracic (T) regions of spinal cords from wild-type and symptomatic hSOD1$^{G93A}$ mice. Representative images of two animals in each experimental condition. Scale bar corresponds to 100 μm.
and photographed by a Mega view G2 camera (Olympus). For axon diameter analysis, at least 100 fibers were analyzed in three different areas in all animal using ImageJ software (NIH, USA).

Immunofluorescence microscopy

For gastrocnemius immunofluorescence, cryosections (7 µm) were fixed in 4% paraformaldehyde (Merck, Darmstadt, GE), blocked for 1 h in blocking buffer (1% BSA, BM-0150, Winkler, Santiago, CL; 1% gelatin from cold water fish skin, G7765, Sigma-Aldrich, St. Louis, MO, USA; 0.01% Triton X-100, X100-1L, Sigma-Aldrich) in PBS and incubated overnight at 4°C with the following antibodies: anti-fibronectin (Sigma-Aldrich), anti-collagen-I (Abcam, Cambridge, UK), anti-p-Smad3 (Cell Signaling, Danvers, MA, USA), anti-laminin (Sigma-Aldrich), anti-neurofilament 200 (Sigma-Aldrich), anti-SV2 (SV2 was deposited to the DSHB by Buckley, K.M; DSHB Hybridoma Product SV2), anti-GFAP (DAKO, Santa Clara, CA, USA) or anti-NeuN (Millipore, Billerica, MA, USA). The corresponding Alexa Fluor 568 or 488-conjugated anti-IgGs (Invitrogen, Carlsbad, CA, USA) were used as secondary antibodies or Alexa Fluor 594 conjugated-wheat germ agglutinin (WGA) (Thermo Fisher, Waltham, MA, USA) was used to stain cell surface and ECM (27). For nuclear staining, sections were incubated with 1 µl/ml Hoechst 33258 for 10 min. For spinal cord immunofluorescence, cryosections were washed three times with PBS for 10 min, permeabilized/blocking with 1% BSA. 0.3% Triton X-100 in PBS for 30 min and incubated with the corresponding antibodies as described above. For NMJ staining, briefly, diaphragms were dissected from mice and immediately fixed in 4% PFA for 30 min. Then, they were incubated with α-bungarotoxin (α-BTX; 5µg/mL; Thermo Fisher, Waltham, MA, USA; to stain end-plates) for 30 min at room temperature and blocked 1 h with 1% BSA, 0.4% lysine; 0, 5% Triton X-100 in PBS. Diaphragms were subjected to immunofluorescence using anti-neurofilament H and anti-SV2 (to stain pre-synaptic terminals) as described previously (55). Slices were then washed in water and mounted in fluorescent mounting medium (DAKO). Sections were visualized on a Nikon Eclipse E600 epifluorescence microscope or a Nikon Eclipse C2 si confocal spectral microscope using NIS-Elements software v4.20, 32 bit.

Immunoblot analysis

Skeletal muscles were homogenized in 10 volumes of Tris-EDTA buffer pH 7.4 with 1 µm phenylmethylsulfonyl fluoride (PMSF). Then, one volume of buffer containing 2% glyceral, 4% SDS and 0.125 µ Tris pH 6.8 were added to the homogenates. Spinal cords were homogenized in 10 volumes of RIPA 1× buffer (Cell Signaling) with 1 µm PMSF. Protein concentration was determined in aliquots of muscle extracts using the Bicinchoninic Acid Protein Assay kit (Pierce, Rockford, IL, USA) with BSA as the standard. Aliquots (40–50 µg) were subjected to sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) in 9% polyacrylamide gels, electrophoretically transferred onto PVDF membranes (Millipore) and probed with the following antibodies at 4°C overnight: anti-fibronectin (Sigma-Aldrich), anti-Smad3 (Cell Signaling), anti-CTGF/CCN2 (Santa Cruz, USA), anti-GAPDH (Millipore) or anti-α-tubulin (Sigma-Aldrich). Following incubation for 1 h at room temperature, primary antibodies were detected with horseradish-peroxidase-conjugated secondary antibodies or horseradish-peroxidase-conjugated anti-human secondary antibody for FG-3019 detection. All immunoreactions were visualized by enhanced chemiluminescence (Pierce). Densitometric analysis and quantification were performed using the ImageJ software (NIH).

RNA isolation, reverse transcription and quantitative real-time PCR

Total RNA was isolated from gastrocnemius muscle using TRIzol (Invitrogen) according to the manufacturer’s instructions. Total RNA (2 µg) was reverse transcribed into cDNA using random primers and M-MLV reverse transcriptase (Invitrogen). TaqMan quantitative real-time PCR reactions were performed in duplicate on an Eco Real-Time PCR System (illumina, San Diego, CA, USA) using presynthesized primer sets for mouse-TGFβ1 gene (Mm01178820_m1) and the housekeeping gene GAPDH (Mm9999915_g1; TaqMan Assays-on-Demand, Applied Biosystems, Foster City, CA, USA). For Ccn1 (Fwd: 5’-GAGGAGGATCGGATCTTGTG-3’; Rev.: 5’-ACTGGGACATCCTGGCATATA-G-3’), for Ccn3 (Fwd: 5’-GTCACCAACAGGATGGCACT-3’; Rev.: 5’-GT AGTGGATGGCTTCCAGGGA-3’); and 18 s (Fwd: 5’-TGACGGA GGCCACCAACAG-3’; Rev.: 5’-CACACCCACCACCAGGAAATCG-3’) that was used as a reference gene for Cnn1 and Ccn3. mRNA expression was quantified with the comparative ΔCt method (2−ΔΔCt), using GAPDH as reference gene. The mRNA levels were expressed relative to the mean expression in wild-type mice.

Determination of fiber diameter

Fresh-frozen gastrocnemius muscles were cryosectioned (7 µm) and placed on glass slides, fixed in 4% paraformaldehyde (Merck) and subjected to immunofluorescence using an antilaminin antibody, as described previously. Fiber size was calculated using the ImageJ software (NIH) on reconstructed images of each gastrocnemius muscle. Fibers were manually selected and the minimal Feret diameter of each fiber was computed by the software.

Statistical analyses

The statistical significance of the differences between the means of the experimental groups was evaluated using one-way ANOVA or two-way ANOVA when appropriate. For multiple comparisons, a post-hoc Bonferroni test was performed. A difference was considered statistically significant with a P-value < 0.05. All data analyses and statistical analyses were performed using the Prism 5 software (Graph Pad Software, CA, USA).

Supplementary Material

Supplementary Material is available at HMG online.

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