PONTIFICIA UNIVERSIDAD CATÓLICA DE CHILE
Facultad de Ciencias Biológicas
Programa de Doctorado en Ciencias Biológicas
Mención Biología Celular y Molecular.

EFFECT OF HYPOXIA IN SKELETAL MUSCLE FIBROSIS; REGULATION OF CTGF/CCN2 EXPRESSION BY HIF-1α AND TGF-β

Tesis presentada a la Pontificia Universidad Católica de Chile en cumplimiento parcial para optar al grado de Doctor en Ciencias Biológicas con mención en Biología Celular y Molecular.

Por

ROGER CHRISTIAN VALLE TENNEY

DIRECTOR DE TESIS: DR. ENRIQUE BRANDAN

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ACTA APROBACIÓN COMITÉ DE LA TESIS

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ABBREVIATIONS

ALS: Amyotrophic Lateral Sclerosis
AP-1: Activator Protein 1
CTGF/CCN2: Connective tissue growth factor
DMD: Duchenne Muscular Dystrophy
DMOG: Dimethyloxaloylglycine
ECM: Extracellular matrix
EGF: Epidermal growth factor
EPO: Erythropoietin
FAP: Fibro adipogenic progenitor
H&E: Hematoxylin and eosin
HIF-1α: Hypoxia-Inducible Factor 1α
HRE: Hypoxia Response Elements
hSOD1^{G93A}: Mouse model for ALS
IGF: Insulin growth factor
IHC: Immunohistochemistry
iNOS: Inducible nitric oxide synthase
mdx: Dystrophin deficient mouse model for DMD
mRNA: Messenger ribonucleic acid
NF-κB: Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
nNOSμ: Neuronal nitric oxide synthase
NO: Nitric oxide
PDGF: Platelet-derived growth factor
PHD: HIF-prolyl hydroxylases domain-containing enzymes
qPCR: Quantitative polymerase chain reaction
SBE: Smad Binding Elements
Sgcd-/-: Sarcoglycan null mice
siRNA: Short interference ribonucleic acid
SP-1: Specific protein 1
TGF-β: Transforming growth factor β
VEGF: Vascular endothelial growth factor

RESUMEN

La fibrosis es una condición recurrente en varias patologías asociadas al músculo esquelético entre ellas: la Distrofia Muscular de Duchenne (DMD), diferentes patologías neuromusculares como la Esclerosis Lateral Amiotrófica (ELA) o aquellas asociadas a denervación, y en daño crónico. La fibrosis muscular se caracteriza por la acumulación excesiva de proteínas de matriz extracelular (ECM), inflamación persistente, daño muscular y sobreexpresión de factores profibróticos. Entre estos últimos se encuentran; el factor de crecimiento transformante tipo β (TGF-β) y el factor de crecimiento de tejido conectivo (CTGF/CCN2).

Varios antecedentes muestran que músculos fibróticos también están asociados a un daño vascular. Resultados preliminares de nuestro laboratorio en modelos murinos que desarrollan fibrosis muestran una disminución en la cantidad de capilares que rodean las fibras musculares. En consecuencia, esto se asocia a una disminución en la perfusión sanguínea y por lo tanto a una disminución en la tensión de oxígeno en el tejido. Este fenómeno se define como hipoxia y lleva a la activación de mecanismos moleculares de respuesta que promueven la supervivencia celular y adaptación a bajas concentraciones de oxígeno. Sin embargo, la relación entre hipoxia y fibrosis no ha sido estudiada en el músculo esquelético.

El factor transcripción inducible por hipoxia 1α (HIF-1α) es el regulador central de la respuesta molecular a hipoxia, mientras que CTGF/CCN2 es una proteína matricelular clave en la inducción de la fibrosis. Existe evidencia previa que vincula la regulación de la expresión de CTGF/CCN2 en respuesta a hipoxia y específicamente HIF-1α, de manera opuesta en distintos tejidos. Sin embargo, no está descrito como hipoxia regula la expresión de CTGF/CCN2 en el músculo esquelético.

El objetivo de esta tesis es estudiar el mecanismo molecular y las vías de señalización involucradas en la regulación de la expresión de CTGF/CCN2 en respuesta a hipoxia y TGF-β en distintos tipos celulares que componen el músculo esquelético *in vitro* e *in vivo.*

Para este análisis sometimos sistemáticamente fibroblastos, mioblastos y miotubos, de líneas celulares y cultivo primario, a condiciones de hipoxia en una cámara de cultivo con atmosfera controlada junto con estimulación con TGF-β.

En primer lugar, encontramos que los tratamientos a distintos tiempos en atmosfera hipóxica no indujeron la expresión de CTGF/CCN2 en ninguno de los tipos celulares mencionados anteriormente. Interesantemente, encontramos que específicamente los miotubos sobreexpresan CTGF/CCN2 tras la activación conjunta de la ruta de señalización de hipoxia mediada por HIF-1α y TGF-β. Describimos que el efecto de ambas rutas sobre la expresión de CTGF/CCN2 es de tipo sinérgico, depende del factor de transcripción HIF-1α y requiere de las rutas de señalización no canónicas de TGF-β. Finalmente, probamos estos resultados *in vivo* utilizando 2 metodologías para activar la ruta señalización de hipoxia mediada por HIF-1α: mediante estabilizadores farmacológicos de HIF-1α y a través de isquemia por escisión de la arteria femoral. Combinamos estas metodologías con inyecciones intramusculares de TGF-β y logramos recapitular el efecto observado *in vitro.* Tras la activación farmacológica de HIF-1α y la inyección intramuscular de TGF-β observamos un efecto sinérgico en la expresión de CTGF/CCN2 y dicha expresión se encontró localizada en las fibras musculares *in vivo.*

En base a esto postulamos que las fibras musculares son la principal fuente de CTGF/CCN2 en el músculo esquelético en respuesta a hipoxia y TGF-β; y contribuyen a la progresión de la patología fibrótica.

ABSTRACT

Fibrosis is a recurrent condition of several pathologies associated to the skeletal muscle, among them; Duchenne Muscular Dystrophy (DMD), motor-neuron diseases including Amyotrophic Lateral Sclerosis (ALS) or denervation, and chronic damage. Muscle fibrosis is characterized by the excessive accumulation of extracellular matrix (ECM) proteins, persistent inflammation, muscle waste, and over-expression of profibrotic factors. Such as transforming growth factor type β (TGF-β) and connective tissue growth factor (CTGF/CCN2).

Several reports show that fibrotic muscles are also associated with vascular damage. Preliminary results from our laboratory show that murine models that develop fibrosis are associated with a decrease in the number of capillaries that surrounds each muscle fiber. In consequence, this is linked to a decrease in blood perfusion; and therefore, to a drop in the oxygen tension in the tissue. This phenomenon is called hypoxia and triggers the activation of molecular mechanisms of hypoxic response that allows the survival and adaptation under low oxygen concentrations. However, the relationship between hypoxia and fibrosis in skeletal muscle has not been studied.

The transcription factor Hypoxia-Inducible Factor 1α (HIF-1α) is the central regulator of hypoxic response, while CTGF/CCN2 is a key matricellular protein in fibrosis induction. There is evidence that links CTGF/CCN2 expression regulation by hypoxia, and specifically by HIF-1α in opposite ways in different tissues. However, the regulation of CTGF/CCN2 expression by hypoxia in skeletal muscle has not been described.

The aim of this thesis is to study the molecular mechanism and the signaling pathways involved in the regulation of expression of CTGF/CCN2 in the different cell types that compose skeletal muscle *in vitro* and *in vivo.*

For this analysis, we systematically exposed fibroblasts, myoblasts and myotubes to hypoxic conditions in a controlled atmosphere cell culture chamber together with TGF-β stimulation.

First, we found that hypoxic treatment at different times under hypoxic atmosphere did not induce the expression of CTGF/CCN2 in none of the cell types mentioned above. Interestingly, we found that specifically myotubes over-express CTGF/CCN2 after combined activation of hypoxic and TGF-β signaling pathways. We classify this phenotype as a synergistic effect and requires the HIF-1α transcription factor and non-canonical TGF-β signaling pathways.

Finally, we demonstrate these results *in vivo* using two different methodologies to activate hypoxic signaling mediated by HIF-1α; using pharmacological HIF-1α stabilizers and by ischemia through femoral artery scission. We combined these methodologies with intramuscular injections of TGF-β, and we were able to resemble the effect observed *in vitro.* HIF-1α activation, together with an intramuscular injection of TGF-β synergistically induce CTGF/CCN2 expression in skeletal muscle. Moreover, CTGF/CCN2 was localized in muscle fibers *in vivo.*

Based on these results, we hypothesize that muscle fibers are the main source of CTGF/CCN2 in the skeletal muscle in response to hypoxia and TGF-β, contributing to the fibrotic pathology progression.

INTRODUCTION

SKELETAL MUSCLE

Skeletal muscle is the most abundant tissue in the human body, and it is dedicated to perform body movement. The basic cellular unit of skeletal muscle is the myofiber. Myofibers are elongated, multinucleate cells formed by the fusion of mononuclear myoblasts and contain sarcomere specialized structures that enable contraction (Alberts et al., 2002). Myofibers are organized by different layers of connective tissue composed by extracellular matrix (ECM) proteins mainly collagens, proteoglycans, and glycoproteins (Gillies et al., 2012).

In physiological conditions and after an injury during the healing process, controlled deposition of ECM proteins allows regeneration serving as a scaffolding network for new-forming muscle fibers (Brandan and Gutierrez, 2013b) However, under many pathological conditions, this process is altered.

SKELETAL MUSCLE FIBROSIS

Skeletal muscle fibrosis is a pathological condition where functional and contractile fibers are replaced by rigid scar-like tissue. Besides, there is a persistent infiltration of mononuclear cells mainly inflammatory, overexpression of profibrotic factors, myofibroblasts activation, and ECM deposition (Mann et al., 2011; WynnThomas A. and Ramalingam, 2012; Mahdy et al., 2018). Among the ECM proteins increased in muscle fibrosis are fibronectin, collagens, and proteoglycans (Cáceres et al., 2000; Alvarez et al., 2002; WynnT a et al., 2013; Pessina et al., 2014).

Duchenne Muscular Dystrophy (DMD) is a genetic disorder that results in the absence of the protein dystrophin, the pathology is characterized to develop fibrosis in the skeletal muscle, and the mouse model for the study of this pathology is the *mdx* mice. The absence of

dystrophin leads to the loss of the anchoring of the myofiber to the basal lamina, inducing fiber degeneration, loss of muscle mass, weakness and increased ECM accumulation (Figure 1) (Kharraz et al., 2014; Mahdy et al., 2018; Smith and Barton, 2018).

Fibrosis is also present in motor-neuron diseases. Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disorder characterized by progressive degeneration of upper and lower motoneurons, the loss of motoneuron innervation leads to skeletal muscle wasting, paralysis and eventually death from respiratory failure (Pansarasa et al., 2014; Mancuso and Navarro, 2015). Similarly to symptomatic stages of ALS disease, animals subjected to surgical denervation where synaptic transmission from motoneurons to the skeletal muscle is interrupted, displays muscle fibrotic-like phenotype overexpressing ECM proteins and profibrotic factors (Pessina et al., 2014; Gonzalez et al., 2017; Rebolledo et al., 2019).

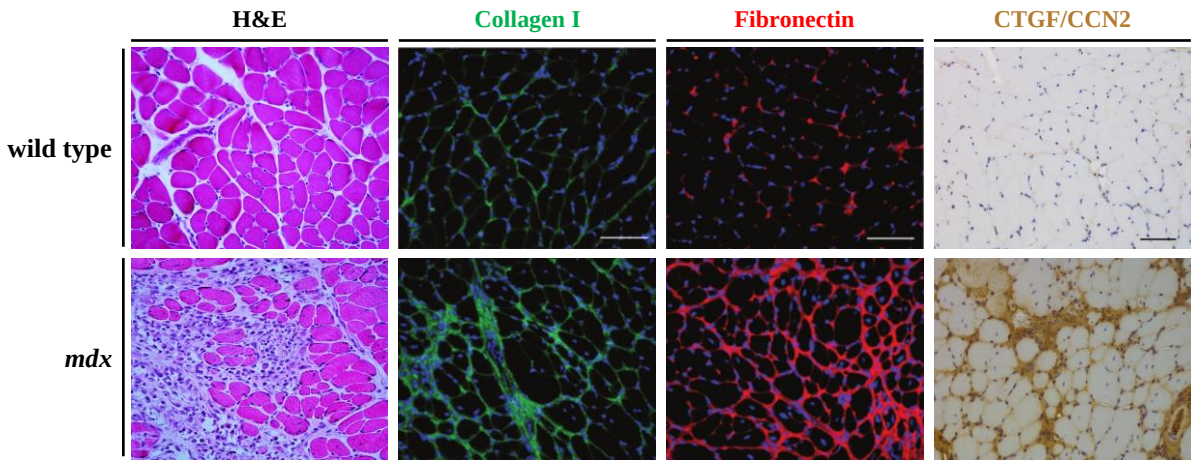

Figure 1. Fibrosis in skeletal muscles from *mdx* dystrophic mice. Microscopic images of muscles from wild type and *mdx* show the fibrotic phenotype. Hematoxylin and eosin (H&E) evidence damage, increased infiltration of mononuclear cells, and loss of muscle mass. Immunolabeling of collagen I, fibronectin and CTGF/CCN2 showed augmented expression of ECM and profibrotic factors. Images extracted from Morales et al. 2013 and Acuña et al. 2017.

PROFIBROTIC FACTORS

Several cytokines have emerged as key drivers of fibrosis in different models including skeletal muscle; transforming growth factor β (TGF-β) (Pohlers et al., 2009; Narola et al., 2013; Accornero et al., 2014), connective tissue growth factor (CTGF/CCN2) (Morales et al., 2013), platelet-derived growth factor (PDGF) (Mueller et al., 2016), epidermal growth factor (EGF) and insulin growth factor (IGF) among others (Borthwick et al., 2013; Mahdy et al., 2018).

Transforming Growth Factor β (TGF-β)

The transforming growth factor beta (TGF-β) cytokines are a sub-family composed of 3 ligands; TGF-β1, 2, and 3. These ligands are part of a 30-member TGF-β super-family ligands (Massagué, 1998; Weiss and Attisano, 2013). TGF-β signals through the canonical Smad-dependent pathway or Smad-independent pathways (Derynck and Zhang, 2003). Canonical TGF-β signaling begins with the activation of the TGF-β receptor I kinase followed by heterodimerization with TGF-β receptor II after ligand binding. Subsequent phosphorylation of Smad2/3 allows binding to Smad4 and translocation into the nucleus, where this protein complex drives the expression of target genes such as collagen, fibronectin, and CTGF/CCN2 among others (Shi and Massagué, 2003; Leask and Abraham, 2004; Vial et al., 2008). Smad independent pathways involved activation of AKT, Rho, ERK, JNK, and p38 MAPK kinase pathways differentially among cell types (Zhang, 2017).

TGF-β signaling participates in several physiological processes such as development, migration, and ECM production (Massagué, 1998, 2012). However, TGF-β overexpression has also been implied in pathologies such as cancer, autoimmune, cardiovascular and fibrotic

diseases, including muscle pathologies (Bernasconi et al., 1999; Gordon and Blobe, 2008; Acuña et al., 2014; Pessina et al., 2014; Gonzalez et al., 2017; Song et al., 2017).

In skeletal muscle, TGF-β is secreted mainly by macrophages during early stages of regeneration but remains persistent in fibrotic muscles (Gosselin et al., 2004; Zhou et al., 2006; Novak et al., 2014; Lemos et al., 2015). Functional experiments have demonstrated that TGF-β plays a key role in muscle fibrosis: overexpression of TGF-β1 specifically in skeletal muscle cells by a transgenic approach induces muscle waste and endomysial fibrosis (Narola et al., 2013). Oppositely, TGF-β signaling inhibition in myofibers by expression of a dominant negative form of the TGF-β receptor II, mitigated the dystrophic phenotype observed in sarcoglycan-null (Sgcd-/-) mice (Accornero et al., 2014). **In this context, TGF-β is an important constituent of the skeletal muscle profibrotic environment.**

Connective Tissue Growth Factor (CTGF/CCN2)

Another crucial pro-fibrotic factor in skeletal muscle is CTGF/CCN2; it is considered one of the downstream effectors of fibrotic TGF-β signaling (Duncan et al., 1999; Biernacka et al., 2011). CTGF/CCN2 is a member of the CCN family of matricellular proteins, it promotes fibroblast proliferation, ECM production, cell adhesion and migration of a variety of cell types (Grotendorst and Duncan, 2005; Brandan and Gutierrez, 2013a; Kubota and Takigawa, 2015) including skeletal muscle cells (Mezzano et al., 2007; Vial et al., 2008; Córdova et al., 2015). CTGF/CCN2 exerts its functions interacting with various molecules such as integrins, proteoglycans, and receptor low-density lipoprotein-1 (Leask and Abraham, 2006; Shi-Wen et al., 2008).

CTGF/CCN2 is vital during development (Ivkovic, 2003), barely expressed in adult normal-physiological muscles (Morales et al., 2013) and restricted to early steps of wound healing (Alfaro et al., 2013). In contrast, CTGF/CCN2 is increased in challenged skeletal muscle, e.g. under pathological conditions such as: the *mdx* mouse, the rodent model for DMD (Figure 1) (Sun et al., 2008; Morales et al., 2013), after total sciatic nerve denervation (Rebolledo et al., 2019), symptomatic stages of hSOD1^{G93A} ALS mouse model (Gonzalez et al., 2017) and older mice as models of sarcopenia (Ibebunjo et al., 2012; Du et al., 2013). Accordingly, CTGF/CCN2 reduction or blockage attenuates skeletal muscle fibrosis in these models (Morales et al., 2013; Gonzalez et al., 2018; Rebolledo et al., 2019) demonstrating its critical role in fibrosis progression. **Considering these pieces of evidence, it is very important to study how CTGF/CCN2 is regulated under skeletal muscle fibrotic conditions.**

HYPOXIA

Exercise or local damage decreases oxygen availability in human skeletal muscles. The drop of oxygen tension in the tissue is named hypoxia, in this scenario cells respond to restore oxygen supply in the tissue, and most of the hypoxic response occurs through the induction of the transcription factor Hypoxia-Inducible Factor 1α (HIF-1α) (Koh et al., 2008).

In normoxic conditions, HIF-1α is constitutively expressed and continuously degraded via the ubiquitin-proteasome pathway after hydroxylation by HIF-prolyl hydroxylases domain-containing enzymes (PHD) (Ivan et al., 2001). Low oxygen pressure inhibits PHD, allowing HIF-1α stabilization and translocation to the nucleus where promotes the expression of target genes, e. g. Vascular endothelial growth factor (VEGF) and erythropoietin (EPO), necessaries for cellular adaptation to oxygen deficiency and survival under hypoxic conditions (Ameln,

2005; Ke and Costa, 2006). Hypoxic signaling activation and vasculature remodeling physiologically occur in response to acute events (Koh et al., 2008; Gustafsson, 2011; Hotta et al., 2018).

RELATION BETWEEN HYPOXIA AND SKELETAL MUSCLE FIBROSIS

Several studies suggest a relationship between hypoxia and fibrotic diseases. Nonetheless, this concept has not been fully addressed in the skeletal muscle; despite this, several reports have shown vasculature impairments in muscle disuse and dystrophic pathologies, that support this relationship.

On the one hand, muscle contraction by itself induces vasodilation in skeletal muscle microvasculature, promoting blood perfusion (HongKwang-Seok and Kim, 2017). Oppositely, after denervation of hind limb muscles by sciatic nerve transection, where contraction cannot occur, results in a loss and necrosis of vascular bed followed by muscle atrophy (Carpenter and Karpati, 1982; Borisov et al., 2000). Preliminary data from our laboratory show that the ALS mouse model hSOD1^{G93A}, that present motorneuron degeneration, also exhibits a decrease in the number of capillaries surrounding each muscle fiber. These antecedents indicate that the loss of muscular activity contributes to impair blood perfusion and thereby might activate a hypoxic response.

By the other hand, it has been described that dystrophic *mdx* or Sgcd-/- mice present muscle vasculature damage (Figure 2) (Gargioli et al., 2008; Matsakas et al., 2013), and the damage extent correlates with aging and the pathological progression of the disease (Latroche et al., 2015). The vascular hypothesis of muscular dystrophy explains part of the pathological state of the disease, describing muscle fiber apoptosis via functional ischemia due to reduced vasculature and mislocalization of the muscle-specific isoform of neuronal nitric oxide synthase

(nNOSμ) (Thomas et al., 2003; Thomas, 2013). Nitric oxide (NO) produced by sarcolemmal nNOSμ acts as a local paracrine signal that inhibits vasoconstriction (Chavoshan et al., 2002; Jendzjowsky and DeLorey, 2013). Therefore, functional ischemia through blood flow impairment is a prevalent feature in skeletal muscle dystrophy. **This evidence suggests that hypoxic signaling could be active in fibrotic skeletal muscles.**

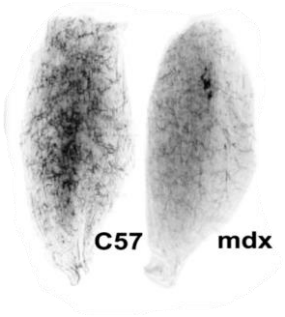

Figure 2. Vasculature damage in the dystrophic muscle. Vascular mapping images of tibialis anterior muscles from *wild type* (C57Bl/10J) and *mdx* mice, perfused with gouache pigment solution. Image extracted from Matsakas et al. 2013.

Studies carried out on tissues different from skeletal muscle have pointed out a relationship between hypoxia and fibrosis (Norman et al., 2000; Darby and Hewitson, 2016). It has been described that hypoxia by itself is able to induce ECM proteins in epidermal fibroblasts from systemic sclerosis patients (Distler et al., 2007), human renal fibroblasts (Norman et al., 2000), proximal tube cells (Orphanides et al., 1997), tubular epithelial cells (Kimura et al., 2008) and epithelial kidney cells (Rana et al., 2015).

The other axis of the relationship between hypoxia and fibrosis is through profibrotic factors. Hypoxia is able to induce CTGF/CCN2 profibrotic factor in mouse tubular cells (Higgins et al., 2004; Lee et al., 2009), scleroderma skin fibroblasts (HongK. H. et al., 2006), chondrosarcoma cell line (Kondo et al., 2006) and human lung fibroblasts (Cheng et al., 2016). However, there are some studies with opposed results, where hypoxia inhibits the expression of CTGF/CCN2 in human renal tubular cells, even blocking TGF-β induced expression of CTGF/CCN2 (KroeningSven et al., 2009; KroeningS. et al., 2010).

These differences between cell type response indicate that the mechanisms involved in fibrosis formation by hypoxia are not conserved among cells and tissues. Moreover, the complexity of the response could be paradoxical in some cases; given that are keloids scars with high content of ECM that are vastly vascularized (Lokmic et al., 2012), or in chondrocytes where hypoxia repress the ECM production and the expression of CTGF/CNN2 (Tran et al., 2013). **For these reasons, it is important to study how hypoxia contributes to the regulation of CTGF/CCN2 profibrotic factor in skeletal muscle cells.**

CROSSTALK BETWEEN HYPOXIA AND TGF-β

It hat has been previously described a crosstalk between hypoxia and TGF-β signaling, e. g. dermal fibroblasts transition into myofibroblasts is potentiated by both signaling pathways (Zhao et al., 2017), and the expression of some genes such as VEGF and EPO can be up-regulated by hypoxia and TGF-β (Sanchez-Elsner et al., 2001; Sánchez-Elsner et al., 2004).

In summary, hypoxic signaling mediated by HIF-1α together with TGF-β could regulate CTGF/CCN2 expression in different cellular models. **The role of hypoxia in skeletal muscle fibrosis and the possible contribution of both, HIF-1α and TGF-β, signaling pathways in CTGF/CCN2 expression and fibrotic response remains unexplored**.

Considering that TGF-β signaling is augmented in skeletal muscle fibrosis and the associated vasculature damage suggests hypoxic signaling activation. Together with that CTGF/CCN2 promoter region possess Smad Binding Elements (SBE) (Córdova et al., 2015) as well as Hypoxia Response Elements (HRE) (Higgins et al., 2004), **we aim to study how CTGF/CCN2 might be regulated by hypoxia and TGF-β signaling in skeletal muscle cells.**

HYPOTHESIS

Hypoxic signaling through HIF-1α, together with TGF-β contributes to CTGF/CCN2 increased expression in skeletal muscle fibrosis.

GENERAL OBJECTIVE

To determine the molecular mechanism and the signaling pathway involved in the induction of CTGF/CCN2 in response to hypoxia and TGF-β, in the muscular tissue.

SPECIFIC OBJECTIVES

1. To study the hypoxic effect and the expression of HIF-1α in the regulation of CTGF/CCN2 and its contribution to the fibrotic effect *in vitro*. As a first approach, we will use cell lines of fibroblasts (NIH/3T3), myoblasts, and myotubes (C2C12). Then, primary cell culture of fibroblasts and differentiated myotubes derived from myoblasts.

2. To determine the contribution of TGF-β dependent signaling pathways involved in the expression of CTGF/CCN2 under hypoxia and TGF-β treatments, we will use pharmacological inhibitors of TGF-β canonical and non-canonical signaling pathways mediators. Moreover, we will determine HIF-1α dependence by using siRNA silencing.

3. To study the contribution of hypoxic signaling over CTGF/CCN2 expression *in vivo, w*e will use pharmacological stabilizers of HIF-1α and hind limb ischemia surgery to activate hypoxic signaling, together with intramuscular injections of TGF-β.

MATERIALS AND METHODS

All the Material and Methods are described properly in the Material and Methods sections of the attached paper.

RESULTS

SPECIFIC OBJECTIVE 1

To study the hypoxic effect and the expression of HIF-1α in the regulation of CTGF/CCN2 and its contribution to the fibrotic effect *in vitro, w*e evaluated cell lines of fibroblasts (NIH/3T3), myoblasts and myotubes (C2C12) and then, primary cell culture of fibroblasts and differentiated myotubes derived from myoblasts.

Summary of the results of the specific objective 1

We exposed fibroblasts (NIH/3T3), myoblasts and myotubes (C2C12) to a hypoxic atmosphere with/without TGF-β1 during different time periods and we analyzed the CTGF/CCN2 expression/secretion by Western-blot and qPCR. We found that hypoxia by itself was not able to induce CTGF/CCN2 in any of the cell types analyzed. By the other side, TGF-β alone induced CTGF/CCN2, and the temporal expression exhibits a peak at 8 hours. Notably, we found that specifically in myotubes, the co-treatment with hypoxia and TGF-β1 induced CTGF/CCN2 over-expression. CTGF/CCN2 over-expression was maintained up to 16-24 hours in the cells and secreted to the medium (Western-blot). Moreover, this was a transcriptional effect since the mRNA follows the same kinetics (qPCR).

We confirmed that myotubes were responsible for CTGF/CCN2 expression by immunofluorescence microscopy and cell disaggregation analyses. We also corroborated this phenomenon using a pharmacological HIF-1α stabilizer (DMOG) under normoxic conditions. Additionally, we classified the effect of hypoxia and TGF-β over CTGF/CCN2 expression in myotubes as a synergistic effect. This phenomenon was also reproducible in primary cell culture of myotubes from E19 rat fetus.

SPECIFIC OBJECTIVE 2

To determine the contribution of TGF-β dependent signaling pathways involved in the expression of CTGF/CCN2 under hypoxia and TGF-β treatments.

Summary of the results of the specific objective 2

We use several pharmacological inhibitors of the canonical and non-canonical TGF-β signaling pathway to analyze which one was involved in the expression of CTGF/CCN2 in myotubes in response to hypoxia and TGF-β. We found that the inhibition of TGF-β receptor type I kinase activity (SB-525334), SP1/SP3 transcription factors (Mithramycin-A), MEK1/2 (UO126) and JNK activity (SP600125) abolished the synergistic effect of hypoxia and TGF-β on CTGF/CCN2 expression in myotubes. In contrast, Smad3 phosphorylation (SIS3) and P38 MAPK activity (SB203580) was not required for this effect. On the other hand, we used siRNA to analyze the requirement of the HIF-1α transcription factor in this phenotype. We found that the synergistic effect of hypoxia and TGF-β over CTGF/CCN2 expression in myotubes is HIF-1α dependent.

SPECIFIC OBJECTIVE 3

To study the contribution of hypoxic signaling over CTGF/CCN2 expression *in vivo*.

Summary of the results of the specific objective 3

To evaluate *in vivo,* the mechanism found *in vitro* in myotubes, we performed skeletal muscle hypoxia by two different approaches. First, we use a pharmacological stabilizer of HIF-1α DMOG (HIF prolyl-hydroxylase inhibitor) injected intraperitoneally. Secondly, we performed femoral artery scission to induce hind limb ischemia. Both methodologies allow us to activate hypoxic signaling in skeletal muscle. After that, we injected TGF-β1 via

intramuscular in the muscle to resemble the conditions that activate CTGF/CCN2 overexpression in myotubes *in vitro*.

We observed CTGF/CCN2 induction in skeletal muscle *in vivo* only when both, TGF-β and hypoxic signaling pathways are active together, similar to the phenotype found *in vitro*. Additionally, immunohistochemistry analysis reveals that under this condition, muscle fibers appear positive for CTGF/CCN2 staining confirming cell type specificity *in vivo*.

All the above results are included in the attached manuscript.

MANUSCRIPT

Role of hypoxia in skeletal muscle fibrosis: Synergism between hypoxia and TGF-β signaling upregulates CCN2/CTGF expression specifically in muscle fibers

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RUNNING TITLE

Hypoxia and TGF-β are involved in CCN2/CTGF mediated skeletal muscle fibrosis

LIST OF ABBREVIATIONS

Amyotrophic lateral sclerosis, (ALS)

Connective Tissue Growth Factor, (CCN2/CTGF)

Duchenne Muscular Dystrophy, (DMD)

Extracellular matrix, (ECM)

Hypoxia-inducible factor-1α, (HIF-1α)

Immunofluorescence staining, (IF)

HIF Prolyl Hydroxylase domain containing enzyme, (PHD)

Tibialis anterior, TA

Transforming growth factor type-β, (TGF-β)

ABSTRACT (not structured and no more than 200 words)

Several skeletal muscle diseases are characterized by fibrosis, the excessive accumulation of extracellular matrix. Transforming growth factor-β (TGF-β) and connective tissue growth factor (CCN2/CTGF) are two profibrotic factors augmented in fibrotic skeletal muscle, together with signs of reduced vasculature that implies a decrease in oxygen supply. We observed that fibrotic muscles are characterized by the presence of positive nuclei for hypoxia-inducible factor-1α (HIF-1α), a key mediator of the hypoxia response. However, it is not clear how a hypoxic environment could contribute to the fibrotic phenotype in skeletal muscle.

We evaluated the role of hypoxia and TGF-β on CCN2 expression in vitro. Fibroblasts, myoblasts and differentiated myotubes were incubated with TGF-β1 under hypoxic conditions. Hypoxia and TGF- β1 induced CCN2 expression synergistically in myotubes but not in fibroblasts or undifferentiated muscle progenitors. This induction requires HIF-1α and the Smad-independent TGF-β signaling pathway. We performed in vivo experiments using pharmacological stabilization of HIF-1α or hypoxia-induced via hindlimb ischemia together with intramuscular injections of TGF-β1, and we found increased CCN2 expression. These observations suggest that hypoxic signaling together with TGF-β signaling, which are both characteristics of a fibrotic skeletal muscle environment, induce the expression of CCN2 in skeletal muscle fibers and myotubes.

KEYWORDS

Skeletal Muscle, Fibrosis, Hypoxia, HIF-1α, CCN2/CTGF, TGF-β.

CONFLICT OF INTERESTS STATEMENT:

KEL is an employee and shareholder of FibroGen, Inc. The other authors have no conflicts of interest.

INTRODUCTION

Under conditions of skeletal muscle injury and healing, there are controlled deposition of extracellular matrix (ECM) proteins that allows regeneration by serving as a scaffolding network for the formation of new muscle fibers [1]. Nevertheless, persistent or chronic damage of skeletal muscle leads to inflammation, overexpression of profibrotic factors, myofibroblast activation and exacerbated ECM deposition [2-5]. These features define fibrosis, which is characteristic of several pathological conditions such as skeletal muscle dystrophies (MD), motoneuron diseases, denervation and different models of myotrauma [6-9]. In these pathologies, functional and contractile fibers are replaced by rigid scar-like tissue containing ECM proteins such as fibronectin, collagens, and proteoglycans [4, 5, 10-14].

Profibrotic factors reside mainly in the interstitial space and are increased in fibrotic skeletal muscle [6, 15, 16]. It has been reported that transforming growth factor β (TGF-β), connective tissue growth factor (CCN2/CTGF) [17], platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and insulin-like growth factor (IGF), among others [2, 18], can interact directly with ECM proteins. In this manner, they create a profibrotic environment that modulates locally different cell types residing in skeletal muscle tissue to induce ECM accumulation.

TGF-β signaling is involved in several physiological processes such as development, migration and ECM production [19]. Moreover, TGF-β overexpression has also been implied in pathologies such as cancer, autoimmune, cardiovascular and fibrotic diseases including muscle pathologies. TGF-β is secreted mainly by macrophages in the skeletal muscle during early stages of regeneration but remains persistent in fibrotic muscles [6-8, 16, 20-25]. TGF-β signals through the canonical Smad-dependent pathway or Smad-independent pathways [26, 27]. Canonical TGF-β signaling begins with the activation of the TGF-β receptor I kinase followed by heterodimerization with TGF-β receptor II after ligand binding. Subsequent phosphorylation of Smad2/3 allows binding to Smad4 and translocation into the nucleus, where this protein complex drives the expression of target genes such as collagen, fibronectin, and CCN2 among others [27-30]. TGF-β also signals through several non-canonical pathways including MAPKs (ERK, p38, JNK), AKT, JAK1, Smad1 and others, differentially among cell types [31, 32].

CCN2 is a crucial profibrotic factor that is a member of the CCN family of matricellular proteins. It promotes fibroblast proliferation, ECM production, cell adhesion and migration of a variety of cell types including skeletal muscle cells [2, 30, 33-36]. CCN2 is vital during development [37], barely expressed in adult normal healthy muscles [15] and restricted to early steps of wound healing [38]. In contrast, its expression is increased in challenged skeletal muscle, e.g. in pathological conditions such as dystrophic skeletal muscle from the mdx mouse model for Duchenne muscular dystrophy (DMD) [15,39, 40], under conditions of repetitive damage [8], in a transgenic mouse model for Amyotrophic lateral sclerosis (ALS, tg hSOD1G93A) [7, 41], and after denervation by sciatic nerve transection [9]. Accordingly, CCN2 reduction or blockage attenuates skeletal muscle fibrosis in these models [9, 15, 41,42], demonstrating its critical role in fibrosis progression.

Hypoxic signaling activation and vasculature remodeling physiologically occur in response to acute events. In human skeletal muscle, a decrease in oxygen availability during exercise or due to local damage leads to the activation of the hypoxia response pathway [43]. Normally, the

hypoxia-inducible factor-1α (HIF-1α) is constitutively expressed but rapidly degraded via the ubiquitin-proteasome pathway after hydroxylation-labeling by HIF-prolyl hydroxylases domain-containing enzymes (PHD). Low oxygen pressure inhibits PHD, allowing HIF-1α stabilization and translocation into the nucleus where it promotes the expression of target genes, e.g. Vascular endothelial growth factor (VEGF) and erythropoietin (EPO), which are necessary for cellular adaptation to oxygen deficiency and survival under hypoxic conditions [44, 45]. Nevertheless, this necessary adaptation strategy when chronic, might be accompanied by a detrimental response. The stable expression of HIF-1α in tubular epithelial cells promotes interstitial renal fibrosis [46]; in models of skin scleroderma, systemic sclerosis and renal fibrosis, the activation of HIF-1α signaling can up-regulate the expression of profibrotic CCN2 and consequently increase the accumulation of ECM proteins [47-52]. Moreover, crosstalk between HIF-1α with TGF-β signaling and with other profibrotic pathways has been reported [53-56]. Thereby, studies carried out on tissues different from skeletal muscle have pointed out a relationship between hypoxia and fibrosis in a cell type-specific manner [57, 58]. A decrease in oxygen availability also occurs in chronic skeletal muscle damage such as muscular dystrophies, evidenced by a decrease in muscle vasculature, besides the described fibrosis [59, 60]. Furthermore, the fibrosis itself impairs gas exchange by increasing the distance and providing a physical barrier between capillaries and myofibers [61-63]. Therefore, vasculature, blood flow, and gas exchange impairments are present under pathological conditions in the skeletal muscle, suggesting that hypoxic signaling is active and might be contributing to the establishment and maintenance of the fibrotic phenotype.

In this work, we analyze the relationship between HIF-1α and the hypoxic response in the establishment of skeletal muscle fibrosis. We observed that the HIF-1α dependent hypoxic response pathway is active in different models of skeletal muscle pathology where fibrosis is a hallmark. Also, pharmacological stabilization of HIF-1α or hypoxia-induced via ischemia, together with increased TGF- β, are able to induce CCN2 in vivo. In vitro studies indicate that induction of CCN2 by hypoxia requires TGF-β signaling in a Smad-independent way, and both pathways work synergistically to induce CCN2 specifically in myotubes.

RESULTS

Fibrotic skeletal muscle shows activation of HIF-1α signaling and decreased capillary density.

Reduction of the skeletal muscle vascular network has been previously reported in dystrophic mice exhibiting insufficient blood supply [59, 60, 64]. Here, we evaluated the capillary density in skeletal muscles from three different mouse models where fibrosis is present. Also, in these models, CCN2 has been shown to have an important role in the development of skeletal muscle fibrosis. We evaluated the mdx mouse model for DMD, denervation by sciatic nerve transection, and the transgenic hSOD1G93A mouse model for ALS [6, 7, 9, 11, 15, 41]. We performed immunofluorescence staining (IF) against CD31 to mark endothelial cells in transversal cryo-sections from gastrocnemius muscles and quantified the capillary density as the number of capillaries surrounding every single fiber. We observed that wt healthy muscle has a frequency curve with a peak of five capillaries per fiber. However, all the fibrotic models display a frequency curve displaced to the left, indicating decreased capillary density and suggesting decreased perfusion of the skeletal muscle (Figure 1A).

To evaluate if hypoxic signaling is active in skeletal muscles from our fibrotic models, we tested HIF-1α stabilization and nuclear localization. IF staining against HIF-1α showed a significant increase in the number of positive nuclei, localized in muscle fibers and interstitial cells, only in fibrotic skeletal muscle compared to wt or control tissue (Figure 1B, C). Also, increased levels of HIF-1α was detected by western blot analysis of total homogenates from denervated, mdx and ALS muscles, that also overexpress ECM proteins such as fibronectin and profibrotic factor CCN2 (Figure 1D, E). These results indicate that fibrotic skeletal muscles from different etiologies have reduced vasculature and that hypoxic signaling through HIF-1α is active in these models. In our denervation model, HIF-1α accumulation can be detected as soon as two days after sciatic nerve transection (Supplementary Figure 1A, B). Moreover, HIF-1α protein accumulation was increased in muscles of mdx mice from the age of 2 months throughout the aging process, correlated with the progression of the pathology (Supplementary Figure 1C). These results suggest that hypoxia is an early event in the establishment of skeletal muscle fibrosis.

Hypoxia plus TGFβ1 induce CCN2 overexpression in myotubes.

Several research groups and our laboratory have reported extensively that the TGF-β signaling pathway is upregulated in skeletal muscle fibrosis from different etiologies. These observations, summarized in Table 1, include increased TGF-β1 ligand/mRNA, TGF-β receptors (TβR) and Smad- dependent signaling, that have been analyzed in several models of skeletal muscle fibrosis such as mdx mice, myotrauma (BaCl2 and laceration), ALS models and denervation, and also in DMD patients.

We observed activated hypoxic response in skeletal muscle pathological conditions, all of them associated with fibrosis and increased TGF-β signaling and up-regulation of CCN2. The Ccn2 promoter region has binding elements for SMAD and SP1 transcription factors, which are effectors of TGF-β signaling [34]. Also, hypoxia response elements for HIF-1α can be found in the Ccn2 promoter region (Higgins et al., 2004). Therefore, we hypothesize that both, HIF-1α

and TGF-β signaling pathways, could collaborate in the skeletal muscle to induce CCN2 expression during the development of skeletal muscle fibrosis. In order to test our hypothesis, we evaluated possible crosstalk between hypoxic and TGF-β signaling pathways on CCN2 expression in skeletal muscle.

To evaluate CCN2 expression under hypoxia and TGF-β treatment, we used different cell types as in vitro models for resident cells of skeletal muscle tissue: NIH/3T3 fibroblasts, C2C12 myoblasts and C2C12 myotubes (7 days post-differentiation). We treated cultured cells with TGF-β1 (5 ng/mL) under normoxic (atmospheric O2 ~21%) and hypoxic (0.5% O2, 5% CO2, ~95% N2) conditions for 4 to 48 h using a controlled atmosphere cell culture chamber. Using this hypoxic protocol, we were able to stabilize HIF-1α (Figure 2A) and induce HIF-1α target genes PDH3 and Glut1, shown as a control of hypoxia conditions (Figure 2D). We found that hypoxia per se was not able to strongly induce CCN2 expression in fibroblasts, myoblasts (supplementary figure 2A, B) or myotubes (Figure 2A). On the other hand, TGF-β1 treatment shows a time course of CCN2 expression with a peak at 4-8 h that declines at 24 – 48 h in myotubes (Figure 2A), fibroblasts and myoblasts (supplementary figure 2A, B). Interestingly, when we performed TGF-β1 treatments combined with hypoxia conditions, we observed a robust CCN2 protein overexpression in myotubes that was larger than TGF-β1 treatment alone. This increased expression was maintained for up to 24 h in the myotubes extracts and in the secreted medium (Figure2A, B), and is specific for myotubes, because it does not occur in fibroblast and myoblast cell lines. We also evaluated CCN2 mRNA levels, which increased in response to hypoxia plus TGF-β1 treatment in myotubes and were maintained high for up to 24 h, indicating that the up-regulation of CCN2 occurs at the transcriptional level (Figure 2C). To confirm that the effect observed in C2C12 myotubes was not a response dependent on the cell line, we evaluate the effects of hypoxic conditions plus TGF-β1 treatment in primary culture cells from E19 rat fetus. Once again, we observed an increased CCN2 expression, larger than the one in response to TGF-β1 alone, specifically in primary myotubes (Figure 2E, F) but not primary muscle fibroblasts (Supplementary Figure 2C).

Next, we aimed to determine if the larger increment in CCN2 expression in response to combined hypoxia and TGF-β1 treatment, compared to TGF-β1 alone, corresponds to a synergistic or additive effect on CCN2 expression in myotubes. We treated myotubes with sub-optimal concentrations of TGF-β1 that were not able to trigger CCN2 expression. We found that treatment with 0.001 ng/mL TGF-β1 for 8 h did not induce CCN2 expression in myotubes. However, when myotubes were also exposed to hypoxia the overexpression of CCN2 was triggered (Figure 3A, B). The same effect was also observed at 0.01 ng/mL TGF-β1 (Figure 3A, B). These experiments suggest that hypoxia conditions and TGF-β-1 treatment together potentiate on the expression of CCN2 in a synergistic manner.

The effect of hypoxia and TGF-β1 on CCN2 expression is specific for myotubes.

Since C2C12 cell differentiation into myotubes is not complete, there is always a remaining population of cells that do not differentiate, stays quiescent and is maintained as “reserve cells” [65, 66]. We aimed to determine which cell population in the culture, reserve cells or differentiated myotubes, was responsible for the synergistic effect between hypoxia and TGF-β1 on the up-regulation of CCN2. First, we differentiated C2C12 cell cultures for seven days and then exposed them for 8 h to hypoxic conditions, TGF-β or both. Then, we evaluated CCN2

expression by IF (Figure 4A). In control normoxic and hypoxic conditions we observed a few mononuclear cells expressing CCN2. The same is observed when we pharmacollogicaly stabilize HIF-1α using DMOG, an inhibitor of PHD. TGF-β1 treatment alone induced CCN2 expression exclusively in mononuclear cells. Remarkably, hypoxia or DMOG plus TGF-β1 induced CCN2 expression almost exclusively in myotubes. This response is shown as the colocalization of CCN2 signal and myosin heavy chain skeletal fast (MyHC) used as myotube marker (Figure 4A). Also, CCN2 signal in myotubes is in a perinuclear pattern suggesting localization in the secretory pathway (Figure 4B, Supplementary Figure 3). As a control for hypoxia, we tested for HIF-1α positive nuclei, which appear in both myotubes and mononuclear cells maintained at low oxygen pressure (Figure4C). Over 60% of total myotubes in the culture are involved in upregulating CCN2. For quantification purposes, each myotube was considered as one branch of the differentiated cell culture (Figure 4E, Supplementary Figure 3). Moreover, to validate these results we performed primary cell cultures from hind limb muscles of rat fetuses E19. The primary myoblasts were differentiated into myotubes and then treated under hypoxic conditions and/or TGF-β1. We observed the same synergistic phenotype as in C2C12 myotubes in vitro model (Figure 4D, Supplementary Figure 3).

We used a different approach to assess which cell population in the culture was responsible for the synergistic cooperation between hypoxia and TGF-β1 on CCN2 expression. We exposed C2C12 differentiated cell cultures under hypoxic conditions and TGF-β1 for 8 h and then separated myotubes from reserve cell using a differential trypsin disaggregation methodology [66] (Figure 5A). We obtained myotubes from the mild trypsinization supernatant and reserve cells as mononuclear cells attached to the plate. Myotubes were identified by MyHC expression and reserve cells by its absence (Figure 5B). As acontrol, we analyzed secreted CCN2 in the culture medium before the separation and we observe the synergistic up-regulation effect under hypoxia and TGF-β1 treatment together. Using this methodology, we again observed that hypoxia plus TGF-β1 increases CCN2 levels in a synergistic manner only in myotubes but not in reserve cells (Figure 5B, C). These results suggest that myotubes are the cell type that responds synergistically to hypoxia plus TGF-β inducing CCN2 expression and secretion.

Synergistic cooperation between hypoxia and TGF-β1 on CCN2 expression in myotubes requires HIF-1α and the TGFβ1 non-canonical signaling pathway.

We analyzed the HIF-1α dependence on the synergistic upregulation of CCN2. Differentiated C2C12 myotubes were transfected with a custom Stealth siRNA against HIF-1α 16 h prior to hypoxia and TGF-β1 treatments. We found that HIF-1α silencing prevents the synergistic effect of hypoxia and TGF-β on CCN2 expression (Figure 6A). Then, we evaluated part of the TGF-β signaling pathway on the effect of hypoxia plus TGF-β1 on CCN2 expression in myotubes. For this purpose, we treated differentiated C2C12 myotubes with different inhibitors of the TGF-β signaling pathway for 30 min before hypoxia and TGF-β1 treatments. We found that the inhibition of TGF-β receptor type I kinase activity (SB-525334), SP1/SP3 transcription factors (Mithramycin-A), MEK1/2 (UO126) and JNK activity (SP600125) abolished the synergistic effect of hypoxia and TGF-β on CCN2 expression in myotubes. In contrast, Smad3 phosphorylation (SIS3) and P38 MAPK activity (SB203580) was not required for this effect (Figure 6B). Moreover, differential phosphorylation in ERK, SMAD3 and P38 proteins was observed 1 h after myotubes were exposed to hypoxia and TGF-β1 (Supplementary Figure 4). Controls for each pharmacological inhibitor are shown in Supplementary Figure 4. These results

suggest that the synergistic cooperation between hypoxia and TGF-β1 on CCN2 expression in myotubes is mediated by HIF-1α and requires a Smad3-independent TGF-β signaling pathway.

HIF-1α stabilization in combination with intramuscular administration of TGF-β1 induces CCN2 expression in skeletal muscle fibers in vivo.

We aimed to evaluate the myotube-specific synergistic mechanism of hypoxia and TGF-β1 on CCN2 expression in vivo. For this purpose, two approaches were performed to evaluate hypoxia: first, we mimic hypoxia using a HIF-PHD inhibitor that allows HIF-1α stabilization in vivo (DMOG) and second, we induced short term hind limb ischemia by femoral artery scission during 4h to stop the blood flow through the leg and consequently to decrease the oxygen levels. Subsequently, we injected a small amount of TGF-β1 intramuscularly into the Tibialis Anterior (TA) muscle and analyzed the muscles 4 h post-injection (Figure 7A). 5 ng of intramuscular TGF-β1 injection in TA muscle did not induce CCN2 expression 4 h post-injection (Figure 7B-D). However, longer treatments of 8 h were capable of inducing CCN2 (Supplementary Figure 5 A, B). Nevertheless, when TGF-β1 was injected into muscles, either in DMOG treated mice or in a condition of hind limb ischemia, we observed a higher amount of CCN2 expression by western blot analysis (Figure 7 B-D) and qPCR (Figure 7 C-E). Additionally, we performed immunofluorescence against CCN2 on TA muscles cryo-sections from mice treated with DMOG and/or TGF-β1. Interestingly, we observed a remarkable increase in CTGF/CCN2 immunostaining around the injection site only when both signaling pathways were active together (Figure 7 F). The same effect was observed with ischemia plus TGF- β1 (Supplementary Figure 5C,D). More detailed analysis of CCN2 expression in cryosections across the whole injected muscle was performed, showing that combined DMOG + TGF-β treatment increases the longitudinal area expressing CCN2 (Supplementary Figure 5E, F). Furthermore, activation of both HIF-1α and TGF-β signaling, but not each treatment by itself, allowed us to visualize muscle fibers expressing CCN2 (Figure 7G). This observation resembles the synergistic phenotype observed in vitro in differentiated myotubes. Altogether, these results show that the activation of hypoxic signaling, either pharmacologically or via ischemia, in combination with TGF-β signaling activation, triggers CCN2 expression in skeletal muscle cells.

DISCUSSION

Our laboratory and other research groups have extensively demonstrated that CCN2 is a strong profibrotic factor and that its inhibition is enough to significantly improve the fibrotic muscle phenotype [9, 15, 41]. Also, several publications have focused on evaluating the role of hypoxia in skeletal muscle regeneration or metabolism [67-69]. However, the possible fibrotic effect of hypoxia in skeletal muscle has not been addressed until now. In this work, we show that hypoxia signaling is active in fibrotic skeletal muscle and we evaluated the contribution of HIF-1α on the regulation of profibrotic factor CCN2 and fibrosis. We showed that hypoxia crosstalk with TGF-β signaling, which is also up-regulated in fibrotic skeletal muscle, synergistically increases CCN2 expression specifically in myotubes.

The extent of vasculature damage correlates with aging and the pathological progression of skeletal muscle [70]. The vascular hypothesis of MD explains part of the pathological stage of the disease, describing muscle fiber apoptosis via ischemia due to reduced vasculature and mislocalization of nNOSμ [71, 72]. NO produced by sarcolemmal nNOSμ acts as a local paracrine signal that promotes blood flow by inhibiting vasoconstriction [73, 74], and the reestablishment of nNOSμ to the plasma membrane improves muscle function in mdx mice [75]. Therefore, vasoconstriction and decreased blood flow that probably decreases oxygen availability, are tightly related to muscle pathologies, including fibrosis. In human skeletal muscles, physiologic activation of HIF-1α occurs during exercise promoting VEGF and EPO expression for adaptation to oxygen deprivation [45]. Acute skeletal muscle damage increases microvasculature permeability and allows leukocyte infiltration during a time window that is resolved in about seven days [76]. Thus, hypoxic signaling activation and vasculature remodeling occur physiologically in response to acute events. Notwithstanding, the role of HIF-1α in skeletal muscle cells is very complex. In vitro experiments in myoblasts have shown that myogenesis is impaired under hypoxic conditions [67] and, in the absence of HIF-1α, myogenesis is also inhibited [77]. In vivo experiments have shown that muscle regeneration is delayed under hypoxic conditions [78] and myogenesis decreases in ischemia experiments [67]. Furthermore, in mice with HIF-1α knocked out in myeloid cells that were challenged with injury, experiments have demonstrated an essential role of hypoxic signaling in these cells during skeletal muscle regeneration [68]. In agreement with these observations, the opposite state, hyperbaric oxygen treatment promotes myogenesis via satellite cell activation and reduces inflammation [69]. Therefore, the physiological role of HIF-1α in the skeletal muscle is restricted to a specific threshold and in a very cell type-specific manner. However, little is known about its role under pathological conditions.

The regulation of CCN2 expression is also highly cell-type specific. Several reports have shown that hypoxia induces CCN2 expression in fibrosis and tumor progression [48, 79, 80]. Among them, hypoxia induces CCN2 in mouse tubular cells regulated by the hypoxia response elements present in the CCN2 promoter region [47], and the induction depends on p38 signaling [51]. However, CCN2 regulation by hypoxia is highly cell-type specific and can be inhibited in other cell types such as chondrocytes [81] or human renal tubular cells, where HIF-1α activation downregulates CCN2 expression, with differential activation of Smad and YAP/STAT signaling pathways [82]. HIF-1α was also reported to reduce TGF-β-mediated upregulation of CCN2 expression in human renal cells [83]. Thus, it is appropriate to emphasize that CCN2 regulation

by hypoxia or HIF-1α stabilization is tightly dependent on cell identity. In effect, in the present study, we show that CCN2 expression is upregulated specifically in myotubes subjected to both hypoxia and TGF-β1 stimulation. Although not explored here, the explanation for the specificity of the phenotype could rely on CCN2 promoter region, specifically expressed transcription factors and epigenetic regulation in myotubes versus myoblasts. Further studies are needed to analyze the transcription factor profile in myotubes submitted to hypoxia and TGF-β, based on signaling pathways required for CCN2 synergistic expression (Figure 6). We hypothesize that this pronounced effect might require a multiprotein complex that includes at least HIF-1α, Smad, SP1 and AP-1 transcription factors. It has been described that HIF-1α can directly interact with Smads proteins [84], which in turn can interact with several transcription factors, in some cases serving as a scaffolding complex that regulates a variety of genes [85]. Furthermore, other transcription factors or co-factors specific for myotubes, not present in other cell types, might stabilize and potentiate the complex facilitating this synergism.

It is thought that fibroblasts are the source of CCN2 secreted into the interstitial space and that CCN2 works as an autocrine signal for activation, proliferation, and differentiation into myofibroblasts [86, 87]. Fibroblasts are considered the major producers of ECM proteins in skeletal muscle mainly by synthesizing collagens, which could explain fibrosis formation by the increased number of these cells in fibrotic muscles [88]. However, considering the results presented here, we proposed that skeletal muscle fibers could be the major source of CCN2 in skeletal muscle tissue due to their representative mass in the tissue and that CCN2 could signal to regulate fibroblast activation (Figure 8). These results are in agreement with previous results showing that only myofiber-selective inhibition of CCN2 improved muscle regeneration and function in a dystrophic model [89].

Our results and previous observations by IHC/IF analysis of CCN2 expression in skeletal muscle of mdx mice and DMD patients suggest that CCN2 localizes in both, the interstitial space and the muscle fibers [16, 21, 39, 90-92]. Moreover, we showed that the overexpression of CCN2 specifically in myotubes is triggered by molecular mechanisms that involve synergy between HIF-1α and the TGF-β signaling pathways. In this way, fibroblasts resident in the interstitial space can receive CCN2 as a myofibroblast activation paracrine signal from muscle fibers (Figure 8). Consistent with this, independent experiments attempting to treat fibrosis-inhibiting fibroblast activation with tyrosine kinase inhibitors have shown an improvement in the pathology without diminishing CCN2 expression [93].

It is probable that both fibrosis and altered micro vascularization/hypoxia are part of a two-way dialog. ECM accumulation can increase distance and create a physical barrier between capillaries and muscle fibers (or other muscle residing cells), impairing gas and soluble factors exchange in both directions, as well as opposing neovascularization, reinnervation and hindering efficient cell therapy [60, 62, 63]. In other words, chronic damage can continuously activate the hypoxia response, beyond its positive effects, causing increased ECM accumulation which, in turn, can decrease oxygen availability in muscle fibers that once more turns on hypoxia signaling, creating a “vicious oxygen cycle” that contributes to the establishment of muscle fibrosis (Figure 8).

In conclusion, we have demonstrated that muscle fibers are the primary source of CCN2 in fibrotic skeletal muscles. Our data also demonstrate that hypoxia synergizes with TGF-β to induce CCN2 expression and muscle fibrosis. The fibrosis associated with many neuromuscular diseases increases muscle hypoxia, leading to a positive feedback loop with CCCN2 to promote progressive muscle fibrosis. These observations suggest that inhibition of CCN2 by hypoxic signaling could attenuate the progressive fibrosis that leads to the decline of muscle function in a variety of diseases.

EXPERIMENTAL PROCEDURES

Animals and tissue harvest.

All protocols were conducted with the approval of the Scientific Ethics Committee for Animal Care and Environment of the Pontificia Universidad Católica de Chile. C57BL/10ScSn-Dmd (mdx mice), C57BL/10 and C57BL/6j (wild-type mice), and B6SJL-Tg (SOD1G93A) 1Gur/J and its wt control (B6SJL) were purchased from The Jackson Laboratory (Bar Harbor, ME). The Ctgf/Ccn2 hemizygous (Ctgf+/-) mouse was kindly donated by Professor Roel Goldschmeding (UMC Utrecht, The Netherlands).

For tissue harvesting, animals were anesthetized with isoflurane and euthanized by cervical dislocation. Muscles were quickly dissected for cryo-sectioning, frozen in isopentane cooled in liquid nitrogen and stored at −80 °C until processing.

In vivo treatments with HIF-1α stabilizer.

Dimethyloxaloylglycine (DMOG) Sigma Aldrich, was diluted in sterile PBS solution and the dose used was 100 mg/kg given by intraperitoneal (I.P.) injection.

TGF-β1 intramuscular injection.

5 ng of hrTGF-β1 (Biolegend) were diluted in 50 µL of NaCl 0.9% saline solution in siliconized tubes (Sigmacote Sigma-Aldrich). The solution was injected in the Tibialis Anterior muscle using siliconized syringes by slowly pressing the plunger while the needle was removed to spread the solution through the muscle.

Hind limb ischemia.

The hind limb ischemia protocol was based on Niiyama et al., 2009 [94]. Animals were placed in a 37°C pre-heated plaque mounted in a stereoscopic microscope. Surgery area was shaved, and a 0.5 cm incision was performed in the skin. Fat and connective tissue was carefully removed, and the femoral artery was separated from the femoral vein and sciatic nerve. The femoral artery was ligated with two double knots of surgical silk 7-0 and then excised between them. Finally, the area was sutured with 7-0 silk.

Sciatic nerve transection

A small incision (≤ 0.5 mm) was made in the skin muscle packages from gluteal and biceps femoris muscles were carefully separated by cutting the facia. The sciatic nerve was exposed and a small (2-5 mm) section was removed to prevent reinnervation. Denervation was performed unilaterally, using the contralateral hindlimb as control.

Cell culture and hypoxic conditions.

C2C12 myoblasts and NIH3T3 fibroblasts (American Type Culture Collection) were grown in DMEM (Gibco) with 10% FBS (HyClone) at 37°C with 8% or 5% CO2. Myotube differentiation was initiated by plating C2C12 myoblasts at 7,000 cells/cm2 and allowing them

to proliferate for 48 hr at 8% CO2. Differentiation was then induced by changing the medium to DMEM containing 2% equine serum (Hyclone) and incubation for 7 days, in the presence or absence of Ara-C (100 µM, Sigma-Aldrich) from days 3-5.

Cell culture hypoxia was performed in a 2.2 L polycarbonate controlled atmosphere cell culture chamber based on the design of Wang et al., 2014 [95]. Air was replaced with a mixture of 95% nitrogen and 5% CO2, and pure oxygen was directly injected to the desired final concentration. The pressure was maintained at 2 psi to control sealing.

The following inhibitors were used in the cell culture assays 30 min before treatment: TGF-β receptor type I kinase activity SB-525334 2 µM (Sigma-Aldrich), Smad3 phosphorylation inhibitor SIS3 3 µM (Merck Millipore), SP1/SP3 inhibitor Mithramycin-A 2 µM (Abcam AB142723), P38 MAPK inhibitor SB203580 10µM (Tocris), MEK1/2 inhibitor UO126 10µM (Sigma-Aldrich), JNK inhibitor SP600125 10µM (BioMol).

Reserve cells and myotube isolation.

The differentiated C2C12 culture was treated for 8h and cell types were isolated using a mild trypsinization method modified from Stuelsatz et al., 2010 . Briefly, floating myotubes were collected with PBS trypsin 0.005% solution with gentle agitation for approximately 15 min. Reserve cells attached to the dish were collected using PBS 0.05% trypsin solution.

Western-blot analysis.

Skeletal muscle tissue samples were processed as described in Rebolledo et al., 2019 9, using the following primary antibodies: anti-fibronectin (Sigma-Aldrich F3648), anti-HIF-1α (Novus Biologicals NB100-479 lot. AB-2), anti-HIF-1α for muscle extracts (BD Biosciences 610959), anti-CTGF/CCN2 (Santa Cruz sc-14939), anti-CTGF/CCN2 for muscle extracts (Abcam ab6992), anti-GAPDH (Biolegend

631402), anti-α-tubulin (Sigma-Aldrich T5168), anti-phospho-Smad3 (Cell Signaling 9520S), anti- Smad3 (Cell Signaling 9523S), anti-phospho-ERK 1/2 (Cell Signaling 9101S), anti-ERK 1/2 (Cell Signaling 9102), anti-phospho- JNK (Cell Signaling 92515), anti-JNK (Cell Signaling 9252), anti- phospho-P38 (Cell Signaling 9211-S), anti-P38 (Cell Signaling 9212). Primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies incubated for 1 h at room temperature.

Immunoblots were visualized with enhanced chemiluminescence (Pierce) using a ChemiDoc-It HR 410 imaging system (Upland).

HIF-1α siRNA silencing.

HIF-1α expression was knocked down with mouse siRNA (Stealth, Thermoscientific): AAGCAUUUCUCUCAUUUCCUCAUGG [77]. Myotubes were transfected using Transit-X2 (Mirrus) 16 h prior to placing the cells under hypoxia conditions, according to the manufacturer’s instructions.

RNA isolation, reverse transcription, and quantitative real-time PCR.

Total RNA was isolated from muscle tissues or cell samples using Trizol (Invitrogen, Calif., USA), according to the manufacturer's instructions. Total RNA (2 μg) was reverse-transcribed to cDNA using random primers and M-MLV reverse transcriptase (Invitrogen, CA, USA). Quantitative real-time polymerase chain reactions (qPCR) were performed in duplicate on an Eco Real-Time PCR System (Illumina, CA, USA). mRNA expression was quantified using the comparative dCt method (2-ddCT), using 18S or D-glyceraldehyde- 3-phosphate dehydrogenase (GAPDH) as the reference gene. The primer sequences used for mouse genes were: CTGF/CCN2 (Fwd: CAGGCTGGAGAAGCAGAGTCGT, Rev: CTGGTGCAGCCAGAAAGCTCAA), 18 s (Fwd: TGACGGA AGGGCACCACCAG, Rev: CACCACCACCCACGGAATCG), GAPDH (Fwd: AGGTCGGTGTGAACGGATTTG, Rev: TGTAGACCATGTAGTTGAGGTCA), EPO (Fwd: CATCTGCGACAGTCGAGTTCTG, Rev: CACAACCCATCGTGACATTTTC), Glut1 (Fwd: GCTGTGCTTATGGGCTTCTC, Rev: CACATACATGGGCACAAAGC), PHD3 (Fwd: TTGGGACGCCAAGTTACACG, Rev: TGGCATAGGAGGGCTGGACTT), RER1 (Fwd: GCCTTGGGAATTTACCACCT, Rev: CTTCGAATGAAGGGACGAAA).

Indirect immunofluorescence.

Cells or skeletal muscle cryosections (7μm) were fixed for 15 min in 4% paraformaldehyde, washed with PBS and permeabilized with PBS–Triton X-100 0.1% for 5 min. Then samples were and blocked in PBS 1% BSA for 30 min. Primary antibodies were diluted in PBS 1% BSA and incubated overnight at 4°C. Primary antibodies are listed below: anti-fibronectin (Sigma-Aldrich F3648), anti-CD31 (Abcam ab28364), anti-Collagen type I (Abcam ab34710), anti-HIF-1α (Santa Cruz sc-10790), anti-CTGF (Cell Signaling D8Z8U), anti-laminin (Sigma, L0663), anti-phospho-Smad3 (Cell Signaling 9520S). The samples were washed with PBS and incubated with Alexa-fluor conjugated secondary antibodies (1:1,000; Life Technologies, Carlsbad, CA) for 1 h at room temperature; Hoechst 33258 (2 mg/ml; 1:5,000) was also added to stain nuclei. The samples were washed with PBS 1% and mounted with Dako medium (Carpinteria, CA). Images were taken in a Nikon Eclipse E600 epifluorescence microscope or in an Olympus BX51 microscope for field reconstructions.

Statistical analyses.

The statistical significance of the experimental groups was evaluated using T-test, one-way ANOVA or two-way ANOVA, as appropriate. For multiple comparisons, a post-hoc Bonferroni test was performed. Significance was considered when the P-value was < 0.05. Statistical analyses were performed using the Prism 5 software (Graph Pad Software, CA, USA).

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AUTHOR CONTRIBUTIONS

R. Valle-Tenney, D. Rebolledo and E. Brandan designed research, R. Valle-Tenney and D. Rebolledo performed research, R. Valle-Tenney, D. Rebolledo and E. Brandan analyzed data, R. Valle-Tenney, D. Rebolledo and K. E. Lipson and E. Brandan wrote the paper.

Table 1. TGF-β signaling is increased in skeletal muscle fibrosis.

Summary of published evidence that shows TGF-β signaling in different models of skeletal muscle fibrosis. References are classified by TGF-β signaling component and the methodology used in each model of muscle fibrosis.

LEGENDS TO FIGURES

Figure 1. Fibrotic skeletal muscle shows lower capillary density and activation of HIF-1α signaling

Fibrotic skeletal muscle was obtained from 6 month-old mdx mice model of DMD, unilateral denervated C57BL/10 mice and a post-symptomatic transgenic hSOD1G93A mouse model for ALS. A. Gastronecmius cryosections (7μm) were immunostained against CD31 and laminin, and the number of CD31 puncta surrounding each fiber was counted (capillary density). The fibrotic models show a decrease in capillary density with respect to control muscle. B-C: Immunostaining against HIF-1α and laminin in GT cryosections (C). Fibrotic skeletal muscle has a higher number of positive nuclei for HIF-1α; quantification is shown in B. D. Western blot from whole homogenates shows increased levels of HIF-1α in fibrotic muscles, together with increased ECM-protein fibronectin and profibrotic factor CCN2. E. Densitometric analysis from western blot in D. T-test comparing each model with its respective control. mdx N=3, denervation N= 7, tg hSOD1G93A N=3. *** P<0.001, * P<0.05.

Figure 2. Hypoxia plus TGF-β1 induce CCN2 overexpression in C2C12 myotubes.

A. C2C12 cells were differentiated during 7 days in DMEM with 2% equine serum and treated under hypoxic conditions (0.5% O2) with/without TGF-β1 (5 ng/mL) during 4, 8, 16 and 24 h. Control normoxic conditions set as 21% O2. Secreted CCN2 and VEGF were analyzed by precipitation of cell culture media with heparin-agarose beads. B. Western blot statistical analysis, significance relative to control, two way ANOVA N=3, *** P<0.001, * P<0.05. C. mRNA levels of CCN2 in myotubes under hypoxia and/or TGF-β1 during 4, 8, 16, 24, 48 h D. qPCR analysis of HIF-1α downstream genes as hypoxia controls in myotubes treated during 24 h. Significance between hypoxia + TGF-β1 and TGF- β1, two way ANOVA N=3, *P<0.05. E. E19 rat fetuses were used to obtain primary myoblasts from hind limb muscles, then myoblasts were differentiated into myotubes during 4 days in MEM 10% equine serum and exposed under hypoxic conditions and treated with/without TGF-β1 (5 ng/mL) during 8 h. F. Western blot analysis of CCN2 under hypoxia and TGF-β1 treatment shows a synergistic effect of both stimuli 1way ANOVA N=3, * P<0.05

Figure 3. Hypoxia plus TGF-β1 induce CCN2 overexpression in a synergistic manner in C2C12 myotubes.

CCN2 expression is triggered by sub-optimal concentrations of TGF-β1 only when C2C12 myotubes are exposed to hypoxic conditions. A. C2C12 cells were differentiated for 7 days in DMEM 2% equine serum, myotubes were treated with TGF-β1 (0.001 to 1 ng/mL) during 8 h

under normoxic (21% O2) or hypoxic conditions (0.5% O2). CCN2 expression was analyzed by western blot from cell extracts and from the cell culture media (secreted CCN2) by precipitation with heparin-agarose beads. B. Western blot statistical analysis, significance relative to control, one way ANOVA N=3, * P<0.05, *** P<0.001.

Figure 4. Hypoxia plus TGF-β1 induce CCN2 overexpression specifically in myotubes.

A. Immunofluorescence staining of myosin heavy chain skeletal fast (MyHC) and CCN2 in C2C12 myotubes treated during 8 h with DMOG or under hypoxic conditions (0.5% O2) with/without TGF-β1 (5 ng/mL). Image reconstruction from 49 fields taken with 20X objective. Nuclei are shown in blue (Hoechst) and colocalization between CCN2 (red) and MyHC (green), in magenta (pseudocolor, colocalization threshold analysis ImageJ 1.52p software). Scale bar 100 µm. B. Representative IF confocal image of a myotube expressing CCN2 under hypoxia and TGF-β1 for 8 h. Scale bar 10 µm. C. HIF-1α nuclear localization in C2C12 myotubes during hypoxic treatments. Scale bar 100 µm. D. Percentage of C2C12 myotubes expressing CCN2 under hypoxia plus TGF-β1. Each myotube branch was considered as a unit. Statistical analysis relative to control one way ANOVA N=3, *** P<0.0001. E. Representative images of primary E19 rat fetal myotube cell culture expressing CCN2 under hypoxia and TGF-β1 treatments.

Figure 5. Hypoxia plus TGF-β1 induce CCN2 synergistic overexpression specifically in isolated myotubes.

A. Schematic methodology for the isolation of myotubes and reserve cells (modified from Stuelsatz et al. 2010). 7 day C2C12 differentiated cell culture was treated during 8 h under hypoxia with/without TGF-β1. Then, trypsin (0.005%) was added for 20 min, and myotubes were obtained from the supernatant while reserve cells were retained attached to the plate. B. Analysis of secreted CCN2 of the cell culture media using heparin-agarose beads previous trypsin disaggregation. Western blot analysis of reserve cells and myotubes under hypoxia and TGF-β1. DNA was used as a loading control. Myotube extracts were characterized by the expression of MyHC. C. Statistical analysis of CCN2 expression in myotubes treated with HYP + TGF-β1 v/s TGF-β1. N=3 one-way ANOVA N=3, *** P<0.001.

Figure 6. Synergistic cooperation between hypoxia and TGF-β1 on CCN2 expression in myotubes requires HIF-1α and the TGF-β1 non-canonical signaling pathway.

A. C2C12 myotubes were transfected with 200 nM of Stealth siRNA against HIF-1α during 16 h and exposed to hypoxia and TGF-β1 for 8 h. Significance relative to HYP + TGF-β1 paired T-test N=5,## relative to control, ** relative to HYP + TGF-β1, P<0.01. B. C2C12 myotubes differentiated during 7 days were pre-treated for 30 min with the following inhibitors: TGF-β receptor type I kinase activity SB-525334 2 µM, Smad3 phosphorylation inhibitor SIS3 3 µM, SP1/SP3 inhibitor Mithramycin-A 2 µM, MEK1/2 inhibitor UO126 10 µM, JNK inhibitor SP600125 10 µM, and P38 MAPK inhibitor SB203580 10 µM. After that, hypoxia plus TGF-β1 treatment were performed for 8 h. CCN2 levels were measured by Western blot. One-way ANOVA N=3, *** P<0.001 (# significance relative to control, * significance relative to HYP + TGF-β1)

Figure 7. HIF-1α signaling and TGF-β1 induce CCN2 synergistically in skeletal muscle fibers in vivo.

A. Scheme of animal treatment. C57BL/6J mice were pre-treated with an I.P. injection of DMOG (150 mg/kg in PBS solution) or ischemia via femoral artery excision. 4 h later, mice were unilaterally injected with intramuscular (I.M.) TGF-β1 (5 ng in saline solution 0.9% NaCl) in the tibialis anterior (TA). Mice were euthanized 4 h after TGF-β1 injection, and both TA were collected for analysis. B, D. Western blot analysis of CCN2 expression in TA muscle extracts from mice treated with DMOG or ischemia, and TGF-β1. Statistical analysis: B; Paired T-test N=3, * P<0.1, C; one way ANOVA N=2, *** P<0.001. C, E. qPCR analysis of CCN2 expression in TA muscles of mice treated with DMOG or ischemia, and TGF-β1. Statistical analysis D; one way ANOVA N=3, *** P<0.001, E; one way ANOVA N=2, ** P<0.01. F. Immunofluorescence staining of CCN2 in transversal cryo-sections of TA muscles treated with DMOG and/or TGF-β1. Reconstruction images of whole TA muscle. Laminin is showed in green and nuclei in blue. G. Representative image of a region near of injection site of TA muscle from mice treated with DMOG and TGF-β. CCN2 is shown in red, laminin in green and nuclei in blue. Arrows indicate muscle fibers positive for CCN2 staining.

Figure 8. Proposed cellular model: "Vicious oxygen cycle".

Initially, muscle disuse or damage triggers HIF-1α accumulation and activation of hypoxic signaling concomitantly with TGF-β secreted by macrophages or other sources, leading to the over-expression of CCN2 profibrotic factor by muscle fibers. CCN2 acts as a local paracrine signal over fibroblasts and stimulate its transition into myofibroblast resulting in exacerbates ECM production. The ECM itself creates a physical barrier that difficult oxygen perfusion into cells by increasing the distances between blood vessels and muscle fibers. We hypothesize that the resulting fibrotic state in skeletal muscle is a sum of factors that are enhanced between them, where different cell types interplay creating a vicious cycle that continuously worsens the skeletal muscle physiology, resulting in chronic ECM deposition, vascular damage, muscle waste, and persistent inflammation.

Supplementary Figure 1. Time course of HIF-1α expression after denervation and during mdx mice aging.

A. Denervation induces accumulation and nuclear localization of HIF-1α as soon as 2 days after denervation. IF staining against HIF-1α in cryosections of gastrocnemius muscles from unilaterally sciatic nerve transected mice at 2, 4 days, 1 week and 2 weeks post denervation. Laminin was used to identify muscle fibers. Below, quantification and statistical analysis of HIF-1α percentage of positive nuclei relative to total nuclei number. The t-test between control and denervated pair for each time point. N=3 for each time point. B. Western blot analysis of HIF-1α expression in denervated gastrocnemius muscles at 2, 4 days, 1 week and 2 weeks post denervation. Densitometric analysis of HIF-1α protein levels relative to the contralateral control for each day. N=3 for each time point. C. Western blot analysis of HIF-1α expression in total gastrocnemius homogenates from 6 month-old C57BL/10 wt mice and mdx mice of different ages ranging from 1 to 9-month-old (2 individuals per age; M1 and M2).

Supplementary Figure 2. Hypoxia plus TGF-β1 do not exhibit synergism on CCN2 expression in NIH/3T3 fibroblasts, C2C12 myoblasts or primary muscle fibroblasts.

NIH/3T3 fibroblasts (A), C2C12 myoblasts (B) and primary muscle fibroblasts from E19 rat fetus (C) were treated under hypoxic conditions (0.5% O2) with/without TGF-β1 (5 ng/mL) during different time periods up to 48 h. Control normoxic conditions were set as 21% O2. Western blot analysis of HIF-1α, CCN2, and GAPDH.

Supplementary Figure 3. Hypoxic signaling induce CCN2 specifically in myotubes in vitro.

A. Representative immunofluorescence microscopy of myosin heavy chain skeletal fast (MyHC) and CCN2 in C2C12 myotubes treated during 8 h with DMOG and/or TGF-β1. Nuclei in blue (Hoechst). Scale bar 100 µm. B. Quantification of positive myotubes for CCN2 treated during 8 h with DMOG and/or TGF-β1. C. Quantification of primary E19 rat fetus myotubes positive for CCN2 treated during 8 h with hypoxia and/or TGF-β1. Analysis of myotubes positive for CCN2 was made by colocalization analysis between MyHC and CCN2. For quantification purposes each myotube was considered as 1 single branch.

Supplementary Figure 4. Signaling pathways activated in C2C12 myotubes immediately after exposure to hypoxia and/or TGF-β1 treatment.

Western blot analysis of phosphorylation of SMAD3, ERK, JNK, and P38 in C2C12 myotubes after 1h of exposure to hypoxia (0.5% O2) and/or TGF-β1 treatment (5 ng/mL). Additionally, pretreatments were performed 30 min before with the following specific inhibitors: TGF-β receptor type I kinase activity SB-525334 2 µM, Smad3 phosphorylation inhibitor SIS3 3 µM, SP1/SP3 inhibitor Mithramycin-A 2 µM, P38 MAPK inhibitor SB203580 10 µM, MEK1/2 inhibitor UO126 10 µM, JNK inhibitor SP600125 10 µM.

Supplementary Figure 5. Histological analysis of TA muscles treated with ischemia or DMOG and TGF-β.

A. Control of intramuscular injection of TGF-β1 (5 ng/50 µL). Analysis was performed 8h after injection. Three independent experiments are shown M1, M2 and M3. B. Quantification of CCN2 protein expression normalized to GAPDH, T-Test N=4 *P<0.05. C. Immunofluorescence staining of CCN2 (red), MyHC (green) and Nuclei (blue) in transversal cryo-sections of TA muscles treated with ischemia and/or TGF-β1. D. quantification of total CCN2 signaling in ROI. For quantification purposes ROI was considered adjacent to injection site. E. Scheme of histological analysis of TA muscles from mice treated with DMOG and intramuscular injection of TGF-β. Muscle cryo-section samples were taken every 200 µm to cover most of the muscle tissue, reconstruction of immunofluorescence microscopy images was analyzed quantifying CCN2 intensity in total muscle area. F. Quantification of CCN2 intensity in muscle sections of mice treated with TGF-β and/or DMOG.

TGF-β signaling is augmented in skeletal muscle fibrosis

Component	Method	Model	References
TGF-β1 ligand	IHC TGF-β1	DMD	[8], [20], [21]
	Elisa of biopsies	DMD	[8]
	WB TGF-β 1, 2, 3.	ALS	[7]
TGF-β1 mRNA.	In situ hybridization.	*mdx*	[22]
	qPCR of the damaged area.	*mdx*	[16]
	qPCR	Denervation, laceration.	[8], [9]
	qPCR	ALS	[7].
	qPCR	Denervation, *mdx,* $BaCL_2$ damage.	[6], [8]
TβR I, II, III	qPCR, In situ hybridization	*mdx*	[25]
Smad signaling in skeletal muscle.	pSmad3 IF positive nuclei/cm2 (Young and old animals).	*mdx*	[23]
	pSMad2/3 IHC of human muscle.	DMD	[8]
	pSMad2 IF nuclear accumulation.	*mdx*	[24]
	pSmad3 IF % positive nuclei (Symptomatic animals).	ALS	[7]

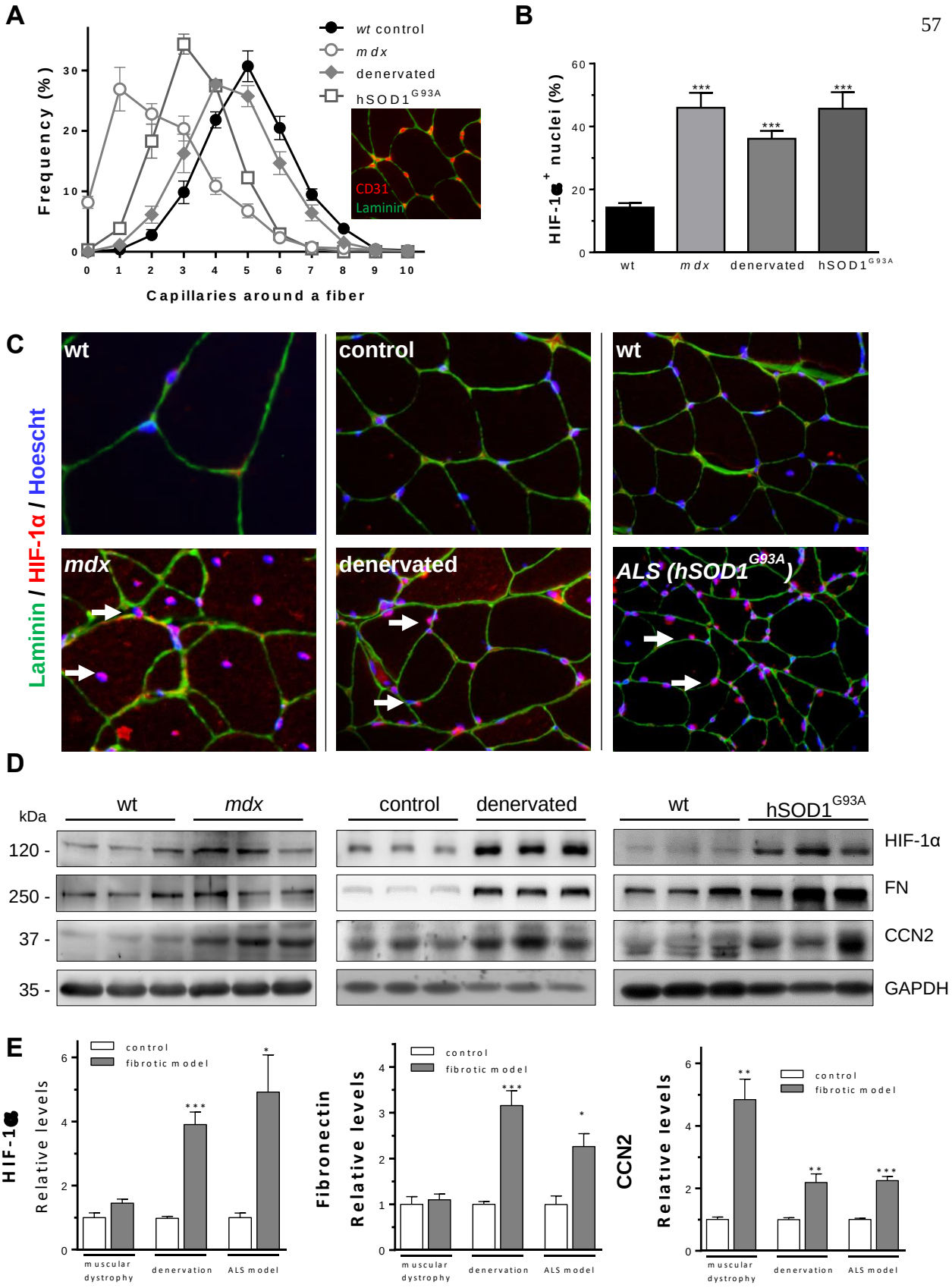

A
wt control
mdx
denervated
hSOD1G93A
Frequency (%)
Capillaries around a fiber
CD31
Laminin
B
HIF-1α+ nuclei (%)
wt
mdx
denervated
hSOD1G93A
C
wt
control
wt
mdx
denervated
ALS (hSOD1G93A)
Laminin / HIF-1α / Hoescht
D
kDa
wt
mdx
control
denervated
wt
hSOD1G93A
120 -
250 -
37 -
35 -
HIF-1α
FN
CCN2
GAPDH
E
HIF-1α
Relative levels
Fibronectin
CCN2
control
fibrotic model
muscular dystrophy
denervation
ALS model

Figure 1

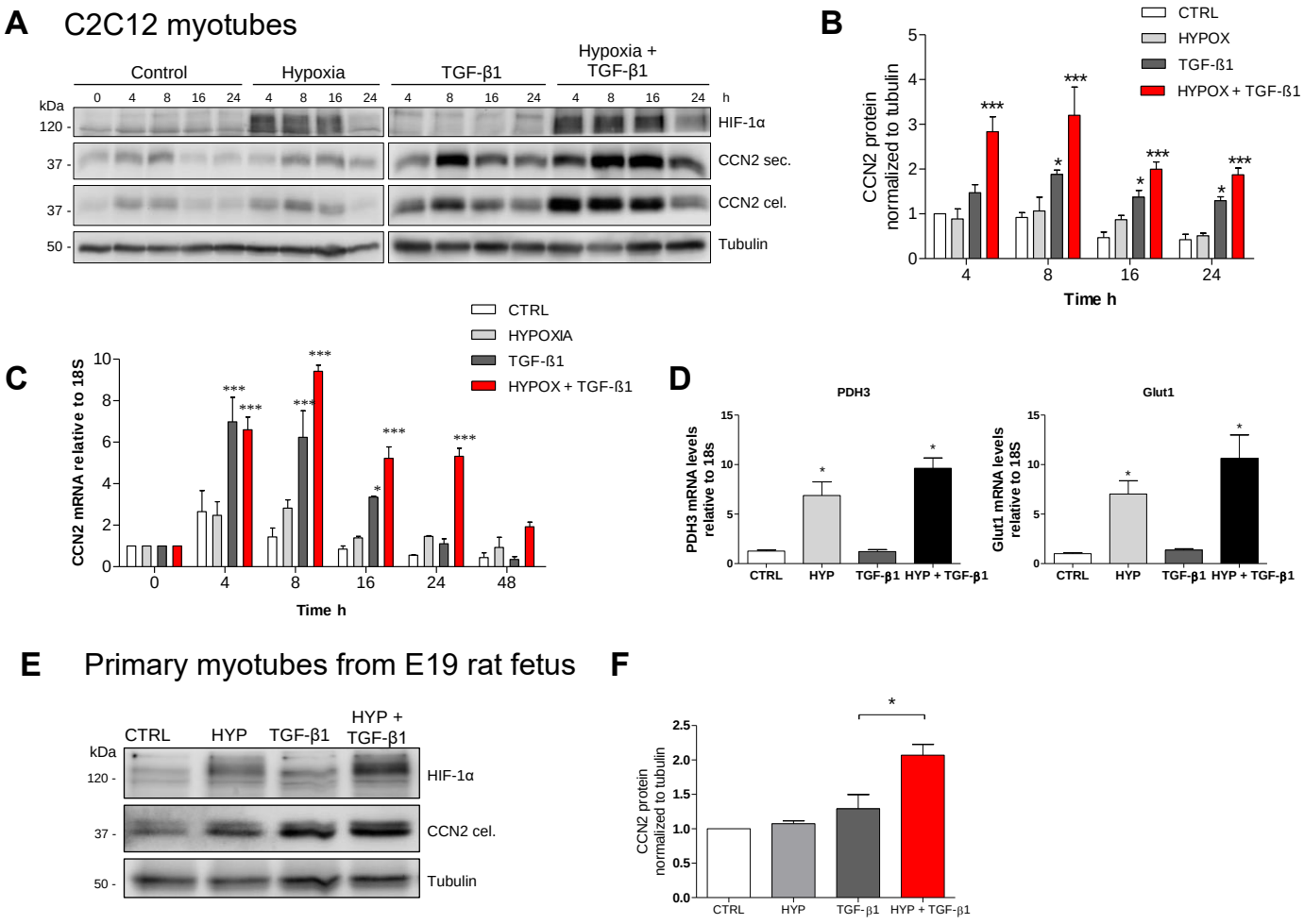

A
C2C12 myotubes
Control
Hypoxia
TGF-β1
Hypoxia + TGF-β1
kDa
0 4 8 16 24 4 8 16 24 4 8 16 24 4 8 16 24 h
120 -
37 -
37 -
50 -
HIF-1α
CCN2 sec.
CCN2 cel.
Tubulin
B
CTRL
HYPOX
TGF-ß1
HYPOX + TGF-ß1
CCN2 protein normalized to tubulin
Time h
C
CTRL
HYPOXIA
TGF-ß1
HYPOX + TGF-ß1
CCN2 mRNA relative to 18S
Time h
D
PDH3
PDH3 mRNA levels relative to 18s
Glut1
Glut1 mRNA levels relative to 18S
CTRL
HYP
TGF-β1
HYP + TGF-β1
E
Primary myotubes from E19 rat fetus
CTRL
HYP
TGF-β1
HYP + TGF-β1
kDa
120 -
37 -
50 -
HIF-1α
CCN2 cel.
Tubulin
F
CCN2 protein normalized to tubulin
CTRL
HYP
TGF-β1
HYP + TGF-β1

Figure 2

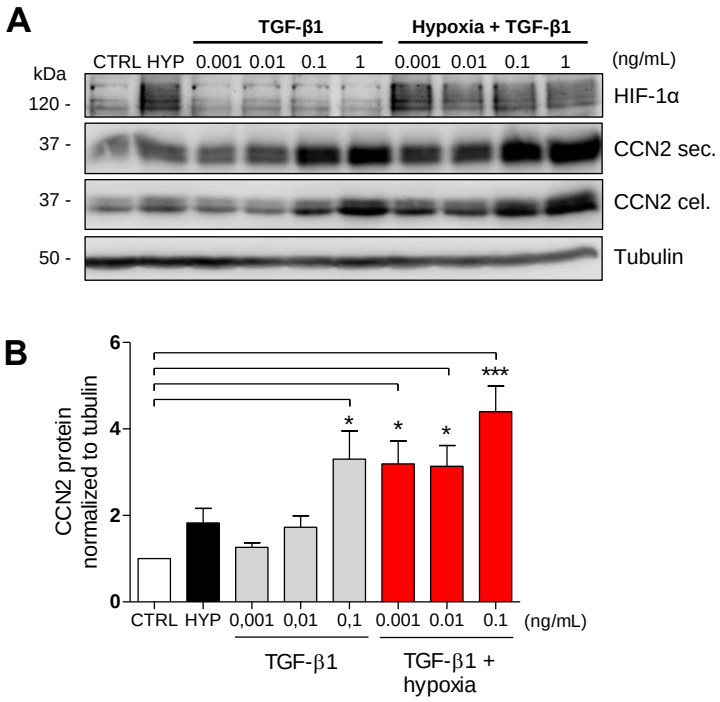

A
TGF-β1
Hypoxia + TGF-β1
CTRL HYP 0.001 0.01 0.1 1 0.001 0.01 0.1 1 (ng/mL)
kDa
120 -
37 -
37 -
50 -
HIF-1α
CCN2 sec.
CCN2 cel.
Tubulin
B
CCN2 protein normalized to tubulin
6
4
2
0
*
*
*
***
CTRL HYP 0,001 0,01 0,1 0.001 0.01 0.1 (ng/mL)
TGF-β1
TGF-β1 + hypoxia

Figure 3

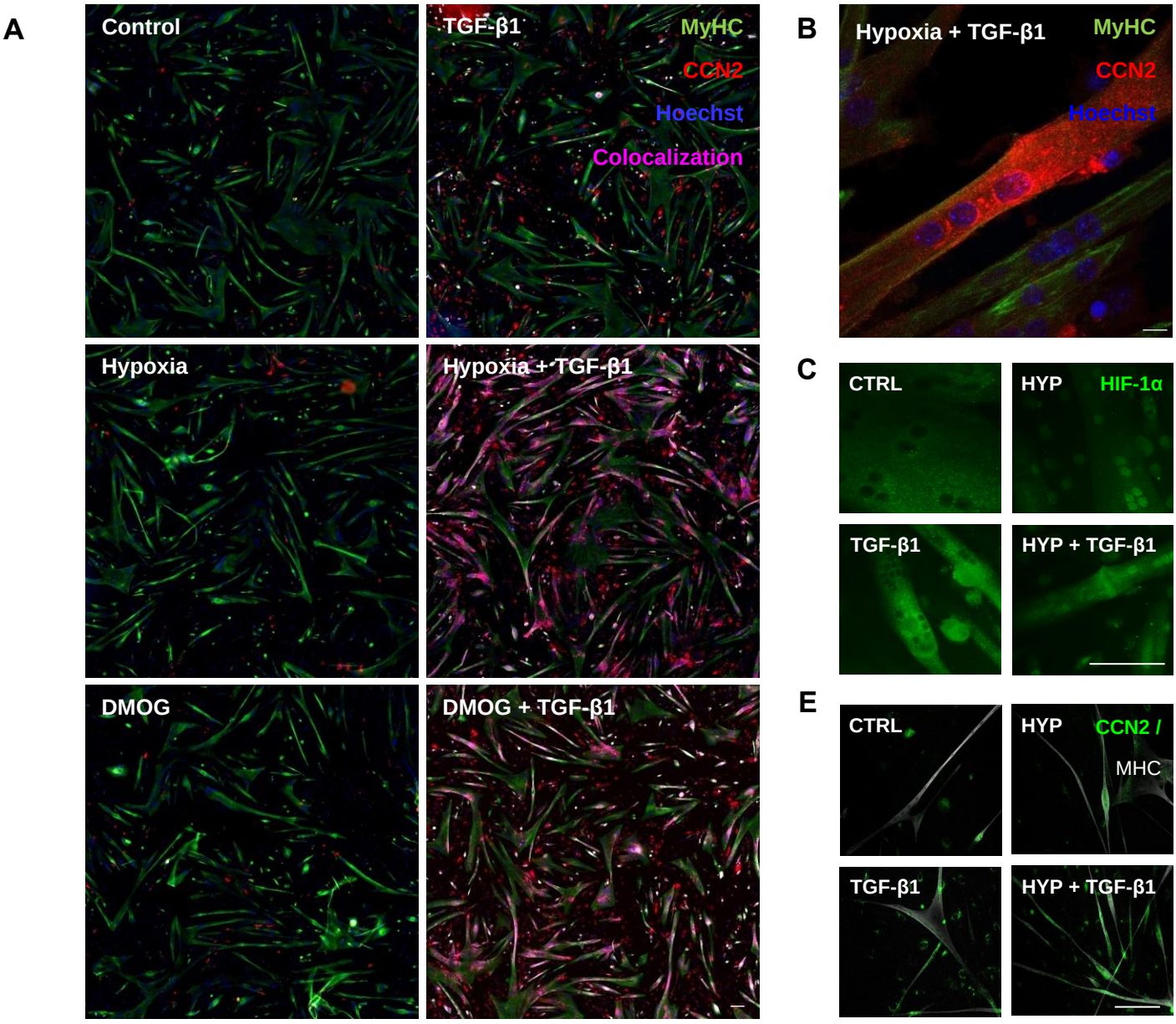

A
Control
TGF-β1
MyHC
CCN2
Hoechst
Colocalization
Hypoxia
Hypoxia + TGF-β1
DMOG
DMOG + TGF-β1
B
Hypoxia + TGF-β1
MyHC
CCN2
Hoechst
C
CTRL
HYP
HIF-1α
TGF-β1
HYP + TGF-β1
E
CTRL
HYP
CCN2 /
MHC
TGF-β1
HYP + TGF-β1
D
% of CCN2 (+) C2C12 myotubes
80
60
40
20
0
***
***
CTRL
HYP
TGF-ß1
HYP +
TGF-ß1

Figure 4

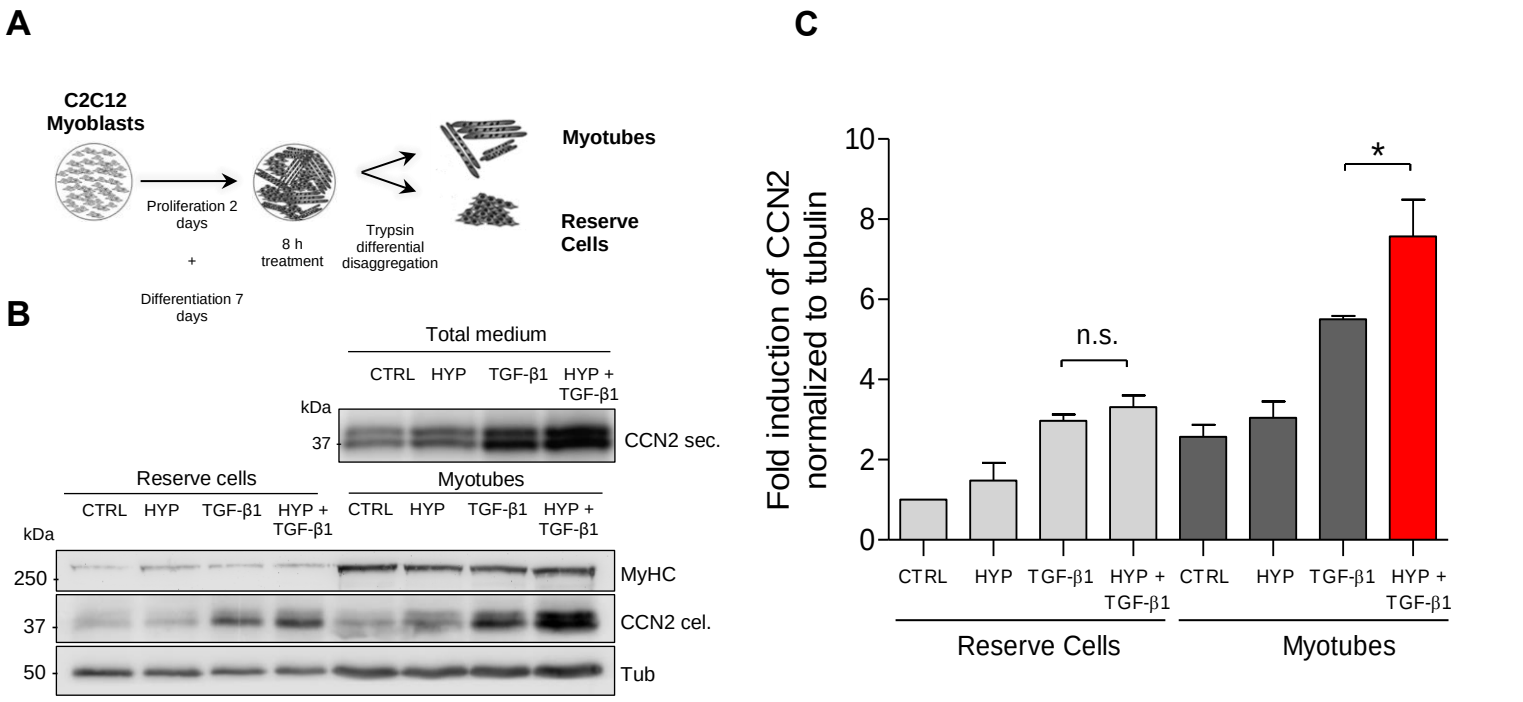

A
C2C12
Myoblasts
Proliferation 2 days
+
Differentiation 7 days
8 h treatment
Trypsin differential disaggregation
Myotubes
Reserve Cells
B
Total medium
CTRL HYP TGF-β1 HYP + TGF-β1
kDa
37
CCN2 sec.
Reserve cells
Myotubes
CTRL HYP TGF-β1 HYP + TGF-β1 CTRL HYP TGF-β1 HYP + TGF-β1
kDa
250
MyHC
37
CCN2 cel.
50
Tub
C
Fold induction of CCN2 normalized to tubulin
10
8
6
4
2
0
n.s.
*
CTRL HYP TGF-β1 HYP + TGF-β1 CTRL HYP TGF-β1 HYP + TGF-β1
Reserve Cells
Myotubes

Figure 5

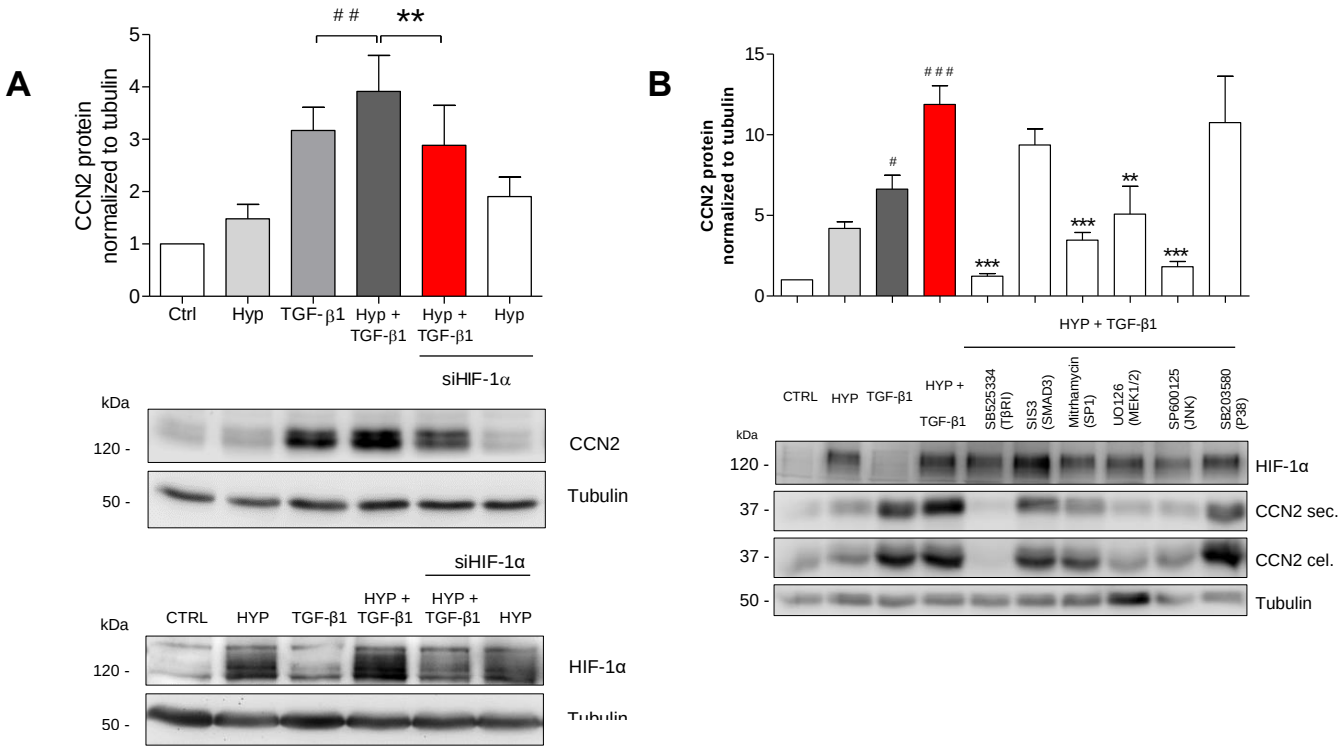

A
CCN2 protein normalized to tubulin
# #
**
Ctrl
Hyp
TGF-β1
Hyp + TGF-β1
Hyp + TGF-β1
Hyp
siHIF-1α
kDa
120 -
50 -
CCN2
Tubulin
siHIF-1α
CTRL
HYP
TGF-β1
HYP + TGF-β1
HYP + TGF-β1
HYP
HIF-1α
Tubulin
B
CCN2 protein normalized to tubulin
##
#
***
**
HYP + TGF-β1
CTRL
HYP
TGF-β1
HYP + TGF-β1
SB525334 (TβRI)
SIS3 (SMAD3)
Mitrhamycin (SP1)
UO126 (MEK1/2)
SP600125 (JNK)
SB203580 (P38)
kDa
120 -
37 -
37 -
50 -
HIF-1α
CCN2 sec.
CCN2 cel.
Tubulin

Figure 6

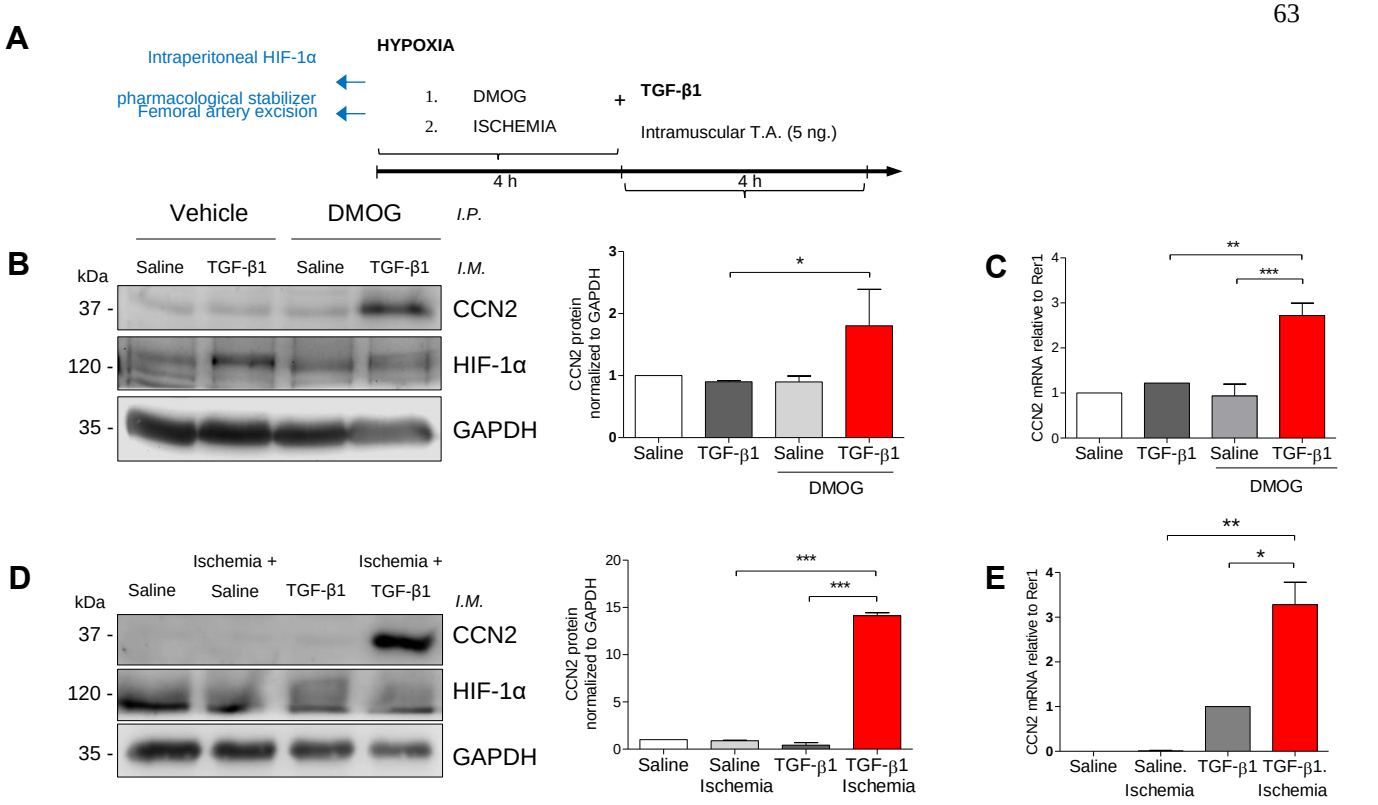

A
Intraperitoneal HIF-1α
pharmacological stabilizer
Femoral artery excision
HYPOXIA
1. DMOG
2. ISCHEMIA
+
TGF-β1
Intramuscular T.A. (5 ng.)
4 h
4 h
Vehicle
DMOG
I.P.
B
kDa
Saline
TGF-β1
Saline
TGF-β1
I.M.
37 -
120 -
35 -
CCN2
HIF-1α
GAPDH
CCN2 protein normalized to GAPDH
Saline
TGF-β1
Saline
TGF-β1
DMOG
C
CCN2 mRNA relative to Rer1
Saline
TGF-β1
Saline
TGF-β1
DMOG
D
Ischemia +
Ischemia +
Saline
Saline
TGF-β1
TGF-β1
kDa
I.M.
37 -
120 -
35 -
CCN2
HIF-1α
GAPDH
CCN2 protein normalized to GAPDH
Saline
Saline Ischemia
TGF-β1
TGF-β1 Ischemia
E
CCN2 mRNA relative to Rer1
Saline
Saline. Ischemia
TGF-β1
TGF-β1. Ischemia
F
CTRL
DMOG
TGF-β1
DMOG + TGF-β1
Laminin
Nuclei
CCN2
G
DMOG + TGF-β1
Laminin / Nuclei / CCN2

Figure 7

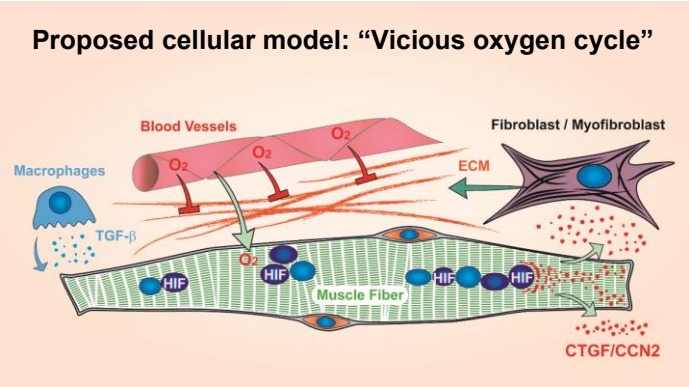
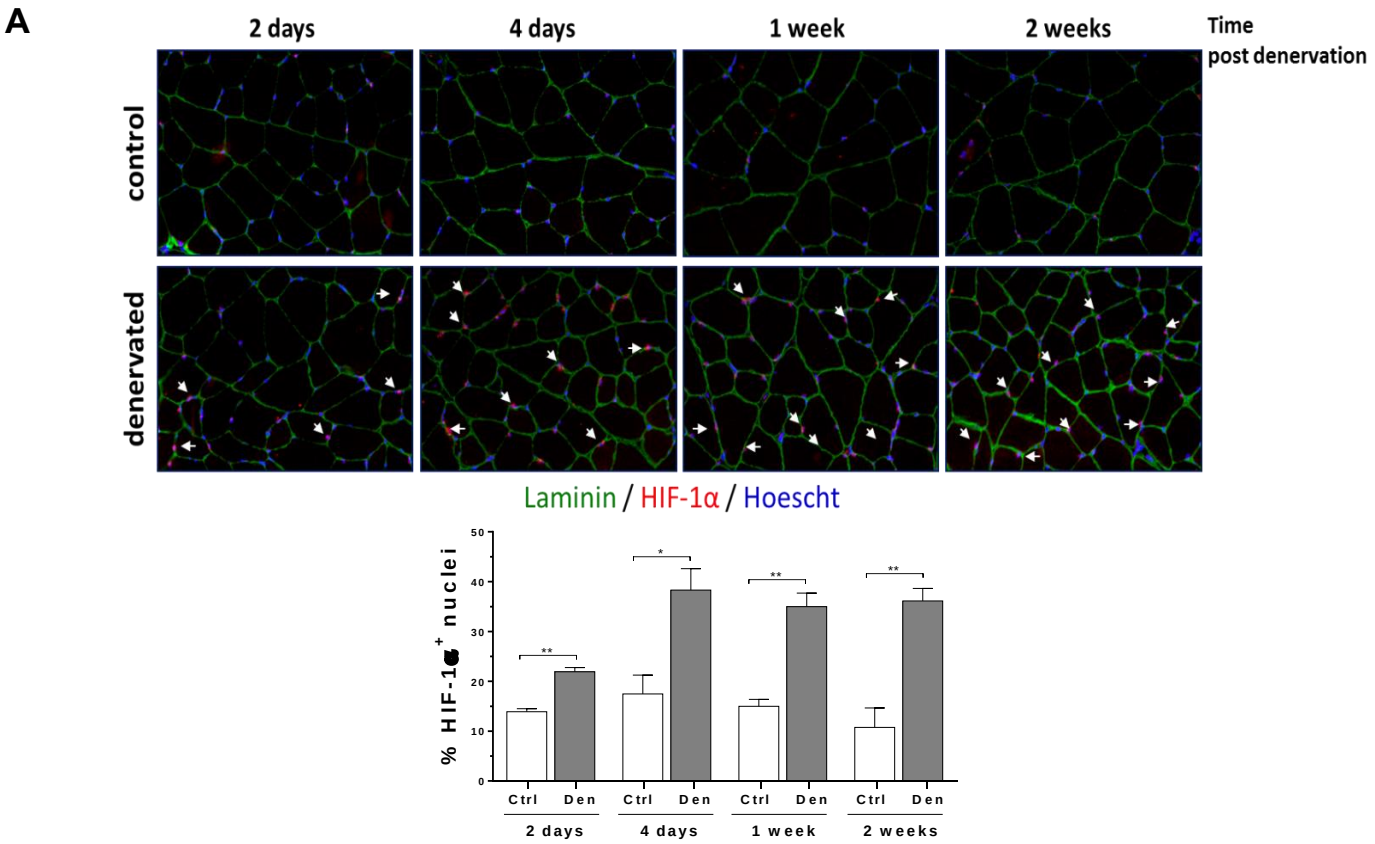
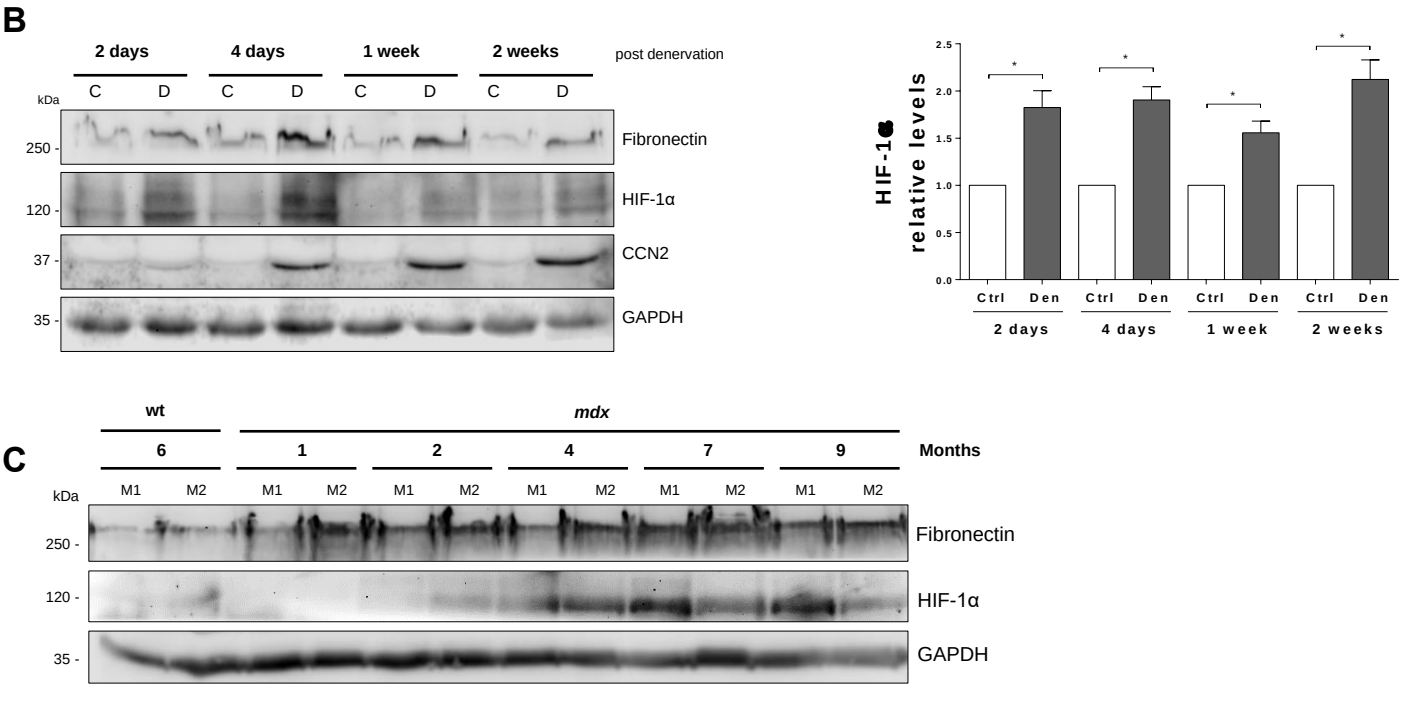
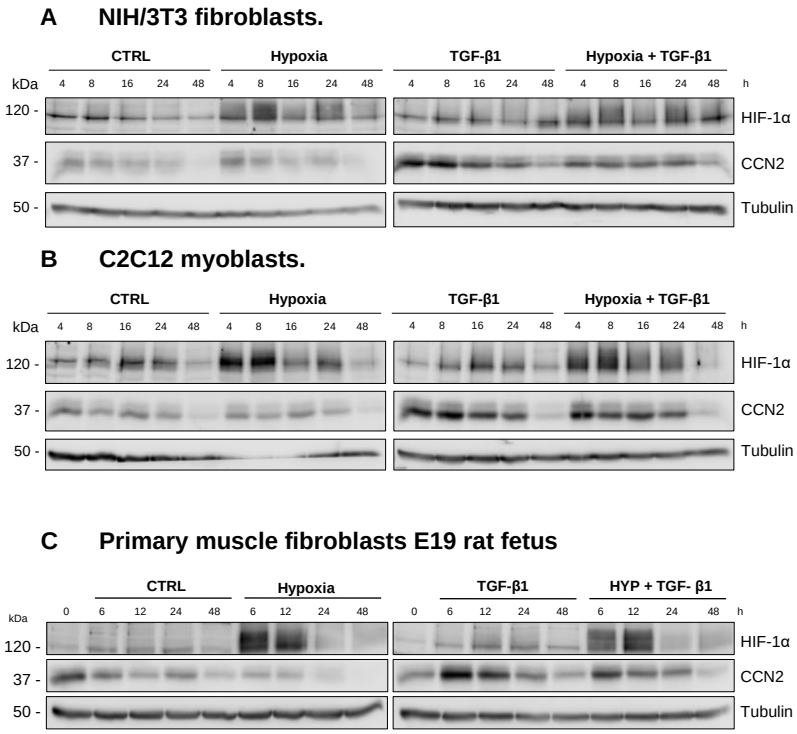

Proposed cellular model: “Vicious oxygen cycle”
Blood Vessels
O2
O2
O2
Fibroblast / Myofibroblast
ECM
Macrophages
TGF-β
O2
HIF
HIF
HIF
HIF
Muscle Fiber
CTGF/CCN2

Figure 8

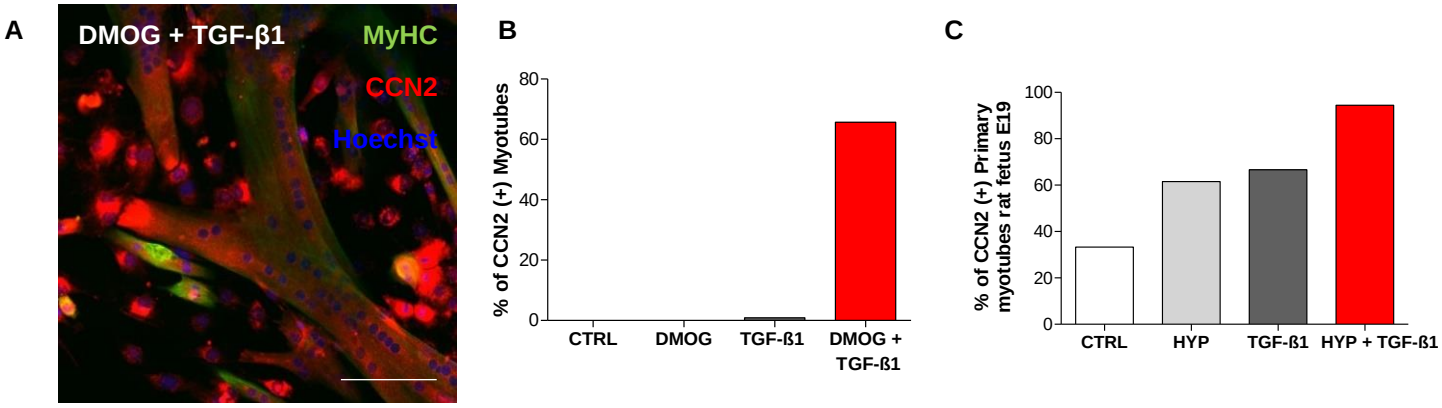




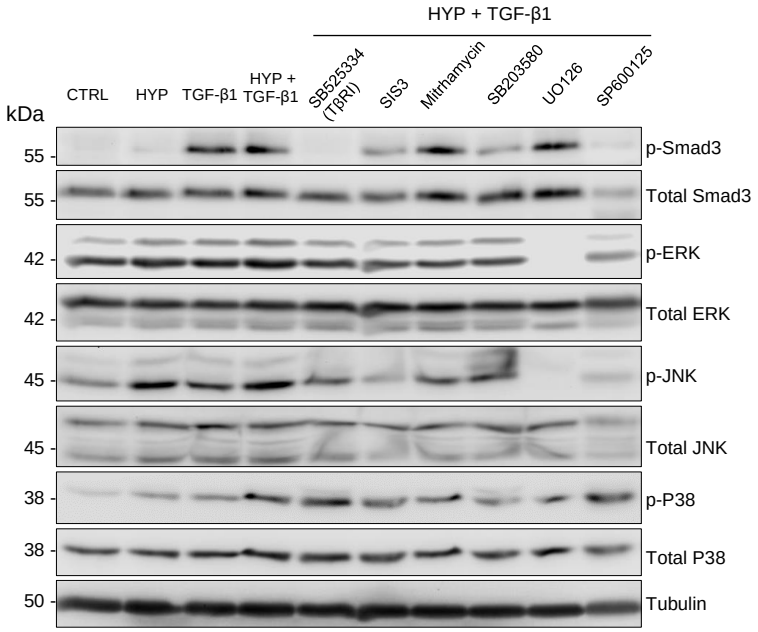
Supplementary Figure 1



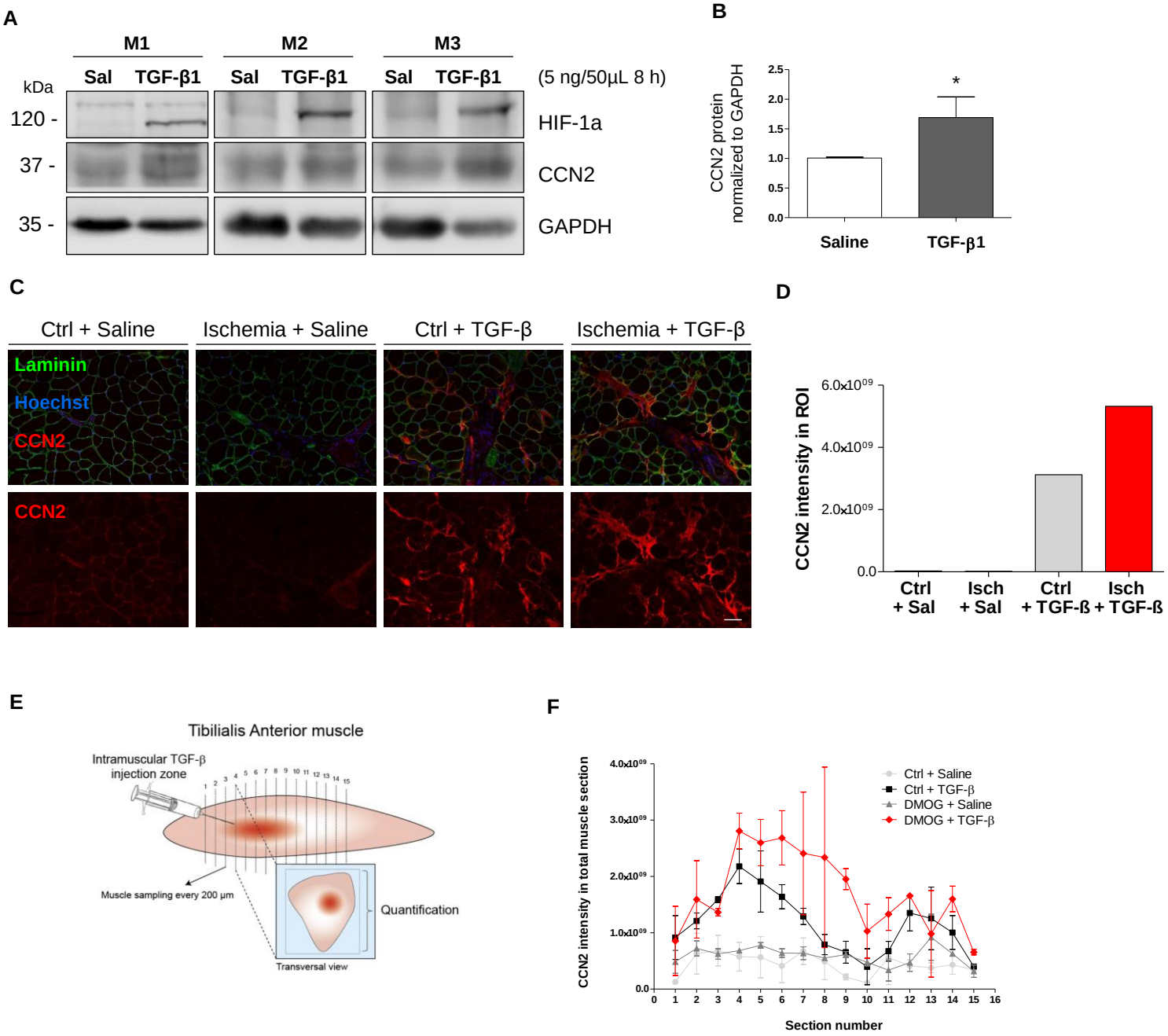
Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5

DISCUSSION

Muscle fibrosis cellular mechanisms

Several muscle pathologies including dystrophies, motor-neuron diseases and pathological states associated to chronic myotrauma are related to fibrosis development in the skeletal muscle tissue (Pessina et al., 2014; Gonzalez et al., 2017; Smith and Barton, 2018).

The fibrotic environment in skeletal muscle is overcrowded of mononuclear cells, ECM proteins, and several profibrotic cytokines. Among the mononuclear cells that infiltrate fibrotic skeletal muscle are fibroblasts, immune, myogenic, and vascular cells. These different cell populations proliferate transiently during regeneration in a synchronized and coordinated fashion (Bentzinger et al., 2013). However, the overlapping degeneration and regeneration cycles that occur in muscular dystrophies lead to an asynchronous remodeling of the microenvironment that finally develop the fibrotic state (Dadgar et al., 2014).

During early steps of muscle physiological regeneration process after injury, the first cell population involved in regenerative response are myeloid cells, including neutrophils and macrophages. Macrophages are subclassified in two main phenotypes: M1 macrophages (CD68+); these cells are considered pro-inflammatory and plays an important role in regeneration due they express inducible nitric oxide synthase (iNOS), promoting NO production that contributes to satellite cells activation (Wozniak and Anderson, 2007; Rigamonti et al., 2013). By the other side, M2 macrophages (CD163+/CD206+) plays an anti-inflammatory role during the healing process (Tidball and Villalta, 2010; Bentzinger et al., 2013). Importantly, M2 macrophages are characterized to secrete TGF-β during skeletal muscle regeneration (Hsieh et al., 2018). Therefore, in the fibrotic context where inflammatory response persists in the skeletal

muscle tissue. Immune cells, specifically M2 macrophages contribute to the over-production of the TGF-β profibrotic cytokine.

Another cell population important in muscle regeneration are the fibroblasts. In physiological conditions fibro adipogenic progenitor (FAP) subpopulation present in a steady state in the muscle, rapidly expand in response to muscle injury and promotes regeneration enhancing the proliferation of myogenic progenitors (Joe et al., 2010; Murphy et al., 2011; Contreras et al., 2016). In the fibrotic context, fibroblasts population proliferates and persists over time by their activation and differentiation into a myofibroblast phenotype (Uezumi et al., 2011). Fibroblasts are considered the main effectors of fibrotic phenotype since they express ECM matrix proteins such as fibronectin and collagens (Mezzano et al., 2007; Uezumi et al., 2011; Chapman et al., 2017) in response to several cytokines and ECM signals. Among them, PDGF (Mueller et al., 2016), TGF-β (Uezumi et al., 2011) and CTGF/CCN2 (Frazier et al., 1996; Grotendorst and Duncan, 2005).

Taking into account the previous evidence that demonstrate that CTGF/CCN2 is crucial for skeletal muscle fibrosis formation and progression in several animal models (Morales et al., 2013; Gonzalez et al., 2018; Rebolledo et al., 2019), it is proper to hypothesize that CTGF/CCN2 profibrotic effect in muscle is exerted over fibroblasts through activation and differentiation into myofibroblasts phenotype, to subsequent overproduce ECM proteins. In parallel, CTGF/CCN2 has also been implied in myogenic dysregulation (Vial et al., 2008; Nishida et al., 2015). Henceforth, we focused on the study of CTGF/CCN2 regulation of expression as a profibrotic effector in skeletal muscle. Specifically, in this thesis we addressed the role of hypoxic signaling over CTGF/CCN2 expression.

Vasculature in skeletal muscle fibrosis

The vascular hypothesis of muscular dystrophy has been studied for several years (Boysen and Engel, 1975); some attempts have failed to probe the DMD vascular hypothesis (Bradley et al., 1975). Nevertheless, further research has proved that the vascular component is part of the fibrotic disease and can be manipulated to improve muscle condition. Vascular therapies including vaso-relaxation drugs and proangiogenic treatments have been successful in improving dystrophic phenotype in DMD patients or *mdx* mice (Ennen et al., 2013; Matsakas et al., 2013; Podkalicka et al., 2019). Although there is considerable evidence that link vascular damage in skeletal muscle pathologies, such as in dystrophies; DMD, *mdx* and sgcd -/- (Gargioli et al., 2008; Matsakas et al., 2013), and denervation (Borisov et al., 2000; Hudlicka, 2007). The role hypoxic signaling in skeletal muscle had not been addressed until now.

The role of HIF-1α in skeletal muscle cells is very complex, *in vitro* experiments in myoblasts have shown that myogenesis is impaired under hypoxia conditions where HIF-1α is active (Majmundar et al., 2012) and conversely, in the absence of HIF-1α myogenesis is also inhibited (Ono et al., 2006). *In vivo* experiments have shown that muscle regeneration is delayed under hypoxic conditions (Chaillou et al., 2014) and myogenesis decreases in ischemia experiments (Majmundar et al., 2015). On the other side, knockout mice for HIF-1α in myeloid cells challenged with injury experiments, demonstrate an essential role of hypoxic signaling in these cells during skeletal muscle regeneration (Scheerer et al., 2013). Therefore, HIF-1α physiological role in skeletal muscle is restricted to a very-bounded threshold and in a cell type-specific manner.

Experimental approach

In this work, we attempted to mimic the skeletal muscle profibrotic environment, putting in context two main axes; the presence of TGF-β profibrotic cytokine and hypoxia signaling pathway through the activation of HIF-1α transcription factor. Thus, we analyzed skeletal muscle cells lines and primary cell culture, in a screening-like approach, under treatment with both stimuli, to analyze the profibrotic response evaluating the expression and secretion of the profibrotic factor CTGF/CCN2.

We did not evaluate all the cell types residing in skeletal muscle, being excluded from this analysis immune and endothelial cells. Though immune cells largely contribute to secrete TGF-β by macrophage activation (Novak et al., 2014), they are not able to express/secrete CTGF/CCN2 (Daniel et al., 2005). By the other side, although some endothelial cells are able to express CTGF/CCN2 (Samarin et al., 2009), they respond to CTGF/CCN2 promoting angiogenesis during development (Ivkovic, 2003; Hall-Glenn et al., 2012). We focused on fibroblasts because of their ability to act as fibrotic effectors (Chapman et al., 2017) and myogenic/muscle cells due to the mass and their potential to control the overall muscle response in regeneration and fibrosis (Li et al., 2004; Frontera and Ochala, 2015).

As a result of systematically expose fibroblasts, myoblasts, and myotubes to the hypoxic controlled atmosphere, we found that hypoxia by itself does not affect CTGF/CCN2 expression in any of the cellular types mentioned above. Strikingly, we found that co-treatment with hypoxia and TGF-β induce a robust overexpression of CTGF/CCN2 only in myotubes (Figure 3 of the manuscript).

Even though it has been described that muscle fibroblasts are able to express CTGF/CCN2 in response to TGF-β (Uezumi et al., 2011), we did not find an additive or

repressive effect of hypoxia over CTGF/CCN2 by TGF-β stimulation. **Considering previous works, it is proper to affirm that the CTGF/CCN2 regulation by hypoxia or HIF-1α stabilization is tightly dependent on cell identity.**

Several reports of CTGF/CCN2 staining in skeletal muscle show positive signal in muscle fibers (Sun et al., 2008; Morales et al., 2013, 2018; Liu et al., 2016; Song et al., 2017; Acuña et al., 2018). However, the fact that muscle fibers express CTGF/CCN2 had never been addressed at a molecular mechanism level.

Thereby, in the present study, we show that CTGF/CCN2 expression is upregulated specifically in myotubes submitted to hypoxia and TGF-β1 stimulation. The explanation for the specificity of the phenotype could rely on CTGF/CCN2 promoter region and epigenetic regulation by differential transcription factor profile in myotubes versus fibroblasts and myoblasts.

Regulation of CTGF/CCN2 expression

CTGF/CCN2 promoter region possess several transcription factor response elements. Including; Smad Binding Elements (SBE), Hypoxia Response Elements (HRE), Activator Protein 1 (AP-1), Specific protein 1 (SP-1), Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB), SOX9, among others. Summarized in Figure 3.

CTGF/CCN2 can be directly regulated by Smad proteins through SBE in the promoter region. In this work, we found that CTGF/CCN2 induction by hypoxia and TGF-β was not abolished by Smad3 inhibitor SIS3. However, SIS3 treatment allows only partial inhibition of Smad3 phosphorylation. In consequence, we cannot fully discard Smad3 participation on the synergistic effect of hypoxia and TGF-β. Moreover, it has been described that HIF-1α can

directly interact with Smad proteins (Sanchez-Elsner et al., 2001) and therefore both transcription factor could be involved in CTGF/CCN2 regulation of expression in myotubes.

It has been reported that Smad can interact with several transcription factors, in some cases serving as a scaffolding to form a large transcription complex that regulates a variety of genes (Euler-Taimor and Heger, 2006). Among them, SP1/SMAD3/HIF-1α multiprotein complex on the regulation of endoglin (Sánchez-Elsner et al., 2002), and YAP-TEAD4–Smad3-p300 complex on CTGF/CCN2 in cancer cells (Fujii et al., 2012). In the context of CTGF/CCN2 expression, we postulate that the transcriptional profile in myotubes allows the formation of a multipart transcription factor complex that facilitates the synergistic effect of hypoxia and TGF-β signaling.

Related to non-canonical TGF-β signaling pathways, it has been described that the promoter region of CTGF/CCN2 gene has an AP-1 site that is responsive to GLI proteins which are downstream of MEK/ERK pathway (Cheng et al., 2016). By the other side, HIF-1α has also been implied in transcriptional regulation of several genes by interacting with other transcription factors, forming complexes. Cyr61 (CCN1) gene expression is induced by hypoxia in melanoma cells by HIF-1 interaction with c-Jun/activator protein-1 (AP-1) (Kunz and Ibrahim, 2003). The role of HIF-1α in CTGF/CCN2 regulation of expression is intriguing. This regulation is highly cell-type specific; several reports show opposite effects among different cell types, e. g. Hypoxia induces CTGF/CCN2 expression in skin fibroblasts (HongK. H. et al., 2006; Mingyuan et al., 2018) or mouse tubular cells (Higgins et al., 2004). Oppositely, HIF-1α activation downregulates CTGF/CCN2 expression in chondrocytes (Tran et al., 2010) or human renal tubular cells (Preisser et al., 2016).

Although we did not analyze the transcription factor profile in myotubes submitted to hypoxia and TGF-β, based on signaling pathway requirements for CTGF/CCN2 synergistic expression (Figure 6 of the manuscript), we hypothesize that this pronounced expression might require a multiprotein complex that includes at least: HIF-1α, Smad, SP1 and AP-1 transcription factors (Figure 3). Taking these results together, it would be interesting to study which specific transcription factors o coactivators participate in the expression of CTGF/CCN2, specifically in myotubes in response to hypoxia and TGF-β signaling. These analyses could allow the identification of new epigenetic therapeutic targets to stop or decrease the profibrotic signaling, specifically in skeletal muscle and no other cells/genes where hypoxic signaling is beneficial, to ameliorate fibrotic diseases.

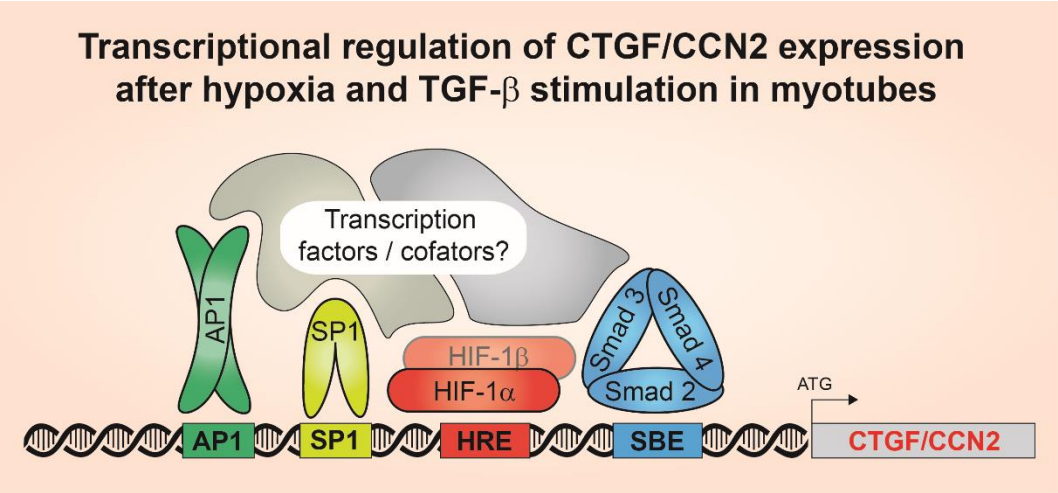


Table 1. Transcription factor binding sites in CTGF/CCN2 mouse promoter

Site	Location	Cellular model	References
HRE	-1558 to -1554 -3745 to -3741	Primary kidney tubular epithelial cells	(Higgins et al., 2004)
AP-1	-345 to -334	Smooth muscle cells (SMCs)	(Chaqour et al., 2006)
SP-1	-4469 to -4455 -4362 to -4348 -3735 to -3721 -2847 to -2833 -2251 to -2237 -1665 to -1651 -954 to -940 -27 to -13 131 to 145	Putative in silico analysis	(Córdova et al., 2015)
NF-kB	-315 to -305	Smooth muscle cells (SMCs)	(Chaqour et al., 2006)
SBE	-399 to -387 -3380 to -3372 -2816 to -2808 -1307 to -1299 121 to 129	Scleroderma fibroblasts Putative in silico analysis C2C12 myoblasts	(Holmes et al., 2001) (Córdova et al., 2015)
SOX9	-289 to -282	Nucleus pulposus cells of the intervertebral disc	(Oh et al., 2016)
BCE-1 (TGFβRE)	-380 to -367	Fibroblasts	(Grotendorst et al., 1996; Leask et al., 2001)

Figure 3. Transcriptional regulation model of CTGF/CCN2 after hypoxia and TGF-β stimulation in myotubes. We hypothesize that myotubes present a specific transcriptional factor profile that allows CTGF/CCN2 synergistic overexpression after hypoxic and TGF-β signaling activation. To elicit CTGF/CCN2 overexpression, we hypothesize that there would be specific factors or cofactors in myotubes/muscle fibers that stabilize a multiprotein complex in the promoter region of CTGF/CCN2 gene, that includes at least: HIF-1α, Smad, SP1 and AP-1 transcription factors. **Table 1.** Transcription factor binding sites in CTGF/CCN2 mouse promoter

***In vivo* observations**

Recently, our laboratory has described that skeletal muscles from the ALS mouse model hSOD^{G93A} develop fibrosis only in the symptomatic stage of the disease, this is related to motor neuron degeneration, and therefore to muscular activity. In this work, fibrotic markers such as collagen, fibronectin, TGF-β, and CTGF/CCN2 are increased in 6-month-old symptomatic mice (Gonzalez et al., 2017). In agreement with these results, here we found that hypoxic signaling through HIF-1α is also augmented at this age, supporting the relationship between hypoxia and fibrosis in a motor-neuron related pathology. We do not evaluate HIF-1α stabilization in pre-symptomatic stages of the pathology progression from hSOD^{G93} mice. However, it would be interesting to analyze if hypoxic signaling is activated previous fibrosis development in this ALS mouse model, these results would give us clues about the temporality role of hypoxic signaling in fibrosis progression.

Regarding the temporality, we took advantage of the sciatic nerve denervation model to address this question. This model quickly develops fibrosis in the skeletal muscle in a two-week time window (Pessina et al., 2014). Our laboratory recently reported that fibrotic markers start to increase slightly since day two after denervation. Fibronectin and collagen follow different kinetics; fibronectin increased up to four-fold by day 7 post denervation, while collagen I slowly double its expression by day 14 after denervation. Additionally, CTGF/CCN2 is also augmented in denervated muscles and remarkably mediates muscle fibrosis development after denervation. Notably, the increase of CTGF/CCN2 and TGF-β1 expression start from day 4 and it is maintained up to 14 days after denervation. (Rebolledo et al., 2019).

Interestingly, in the present work, we found that hypoxic signaling is active starting from day 2 after denervation; we observed positive nuclei for HIF-1α in muscle and interstitial cells,

and HIF-1α protein stabilization by Western blot (Supplementary figure 1 of the manuscript). These findings show that hypoxic signaling precedes CTGF/CCN2 expression after skeletal muscle denervation. Therefore, it suggests that CTGF/CCN2 expression requires HIF-1α and TGF-β signaling; conditions that are only meet from day 4 after denervation. The temporality of these events supports and resemble the results found *in vitro*, where hypoxia and TGF-β has a synergistic effect over CTGF/CCN2 expression in fibers only when both signaling pathways are active together.

We also analyzed the expression of HIF-1α in the *mdx* dystrophic mouse at different ages. We found that HIF-1α appears elevated since the first month of life and continue increasing through time (Supplementary figure 1 of the manuscript). This finding is in line with the temporality hypothesis, where the hypoxic signaling activation would be an early event in the pathology progression of the *mdx* mice, preceding fibrosis formation. Early HIF-1α accumulation at the first month in the *mdx* mice could be explained by mild injury and muscle disuse, at this point the overall damage has not overpassed the uncontrolled point of no return in the fibrosis development, and HIF-1α could still be playing a beneficial pro-angiogenic role. Eventually, at later ages sustained hypoxic signaling activation will trigger profibrotic factors expression and ECM deposition, resulting in fibrosis establishment, which is also correlated with vasculature damage appearance (Latroche et al., 2015). Supporting this idea, our data show that chronic/sustained HIF-1α stabilization using a pharmacological inhibitor of the PHD increases fibronectin in skeletal muscle through the induction of CTGF/CCN2 (Figure 2 of the manuscript).

Proposed model

Putting in context all the features of muscle fibrosis analyzed in this work: including ECM, profibrotic factors, and vascularization impairment, we hypothesize that the resulting fibrotic state is a sum of factors that are enhanced between them, where different cell types interplay creating a vicious cycle that continuously worsens the skeletal muscle physiology (Figure 4).

By the one side, the capillary dysfunction presented in different pathological states associated to fibrosis, including; denervation (Carpenter and Karpati, 1982; Borisov et al., 2000), injury damage (Kang et al., 2013; Scheerer et al., 2013) and dystrophies (Gargioli et al., 2008; Matsakas et al., 2013), contributes to promote hypoxic signaling activation. Then, hypoxic signaling activation can induce the overproduction of CTGF/CCN2 in muscle fibers when they are co-stimulated with TGF-β coming from macrophages. CTGF/CCN2 profibrotic factor paracrinal over-expression can increase fibroblasts activation and differentiation into myofibroblasts phenotype. Moreover, macrophages can also be responsive to CTGF/CNN2 (Daniel et al., 2005). Thus CTGF/CCN2 could contribute to attracting more mononuclear cells to the damaged region, maintaining elevated the expression/secretion of TGF-β by these cells. In a simplified view of the process, the fibroblast over-activation can directly increase the ECM proteins production eliciting its accumulation. At the same time, ECM accumulation can create a physical barrier that increases the distance between capillaries and muscle fibers, impairing gas exchange in both directions (Chilibeck et al., 1997). In conjunction, all these features contribute to the formation fibrotic-like phenotype, over-expressing pro fibrotic factors which in turn continuously decrease the oxygen availability in muscle fibers, and therefore repeating the cycle to finally establish the fibrosis in the skeletal muscle.

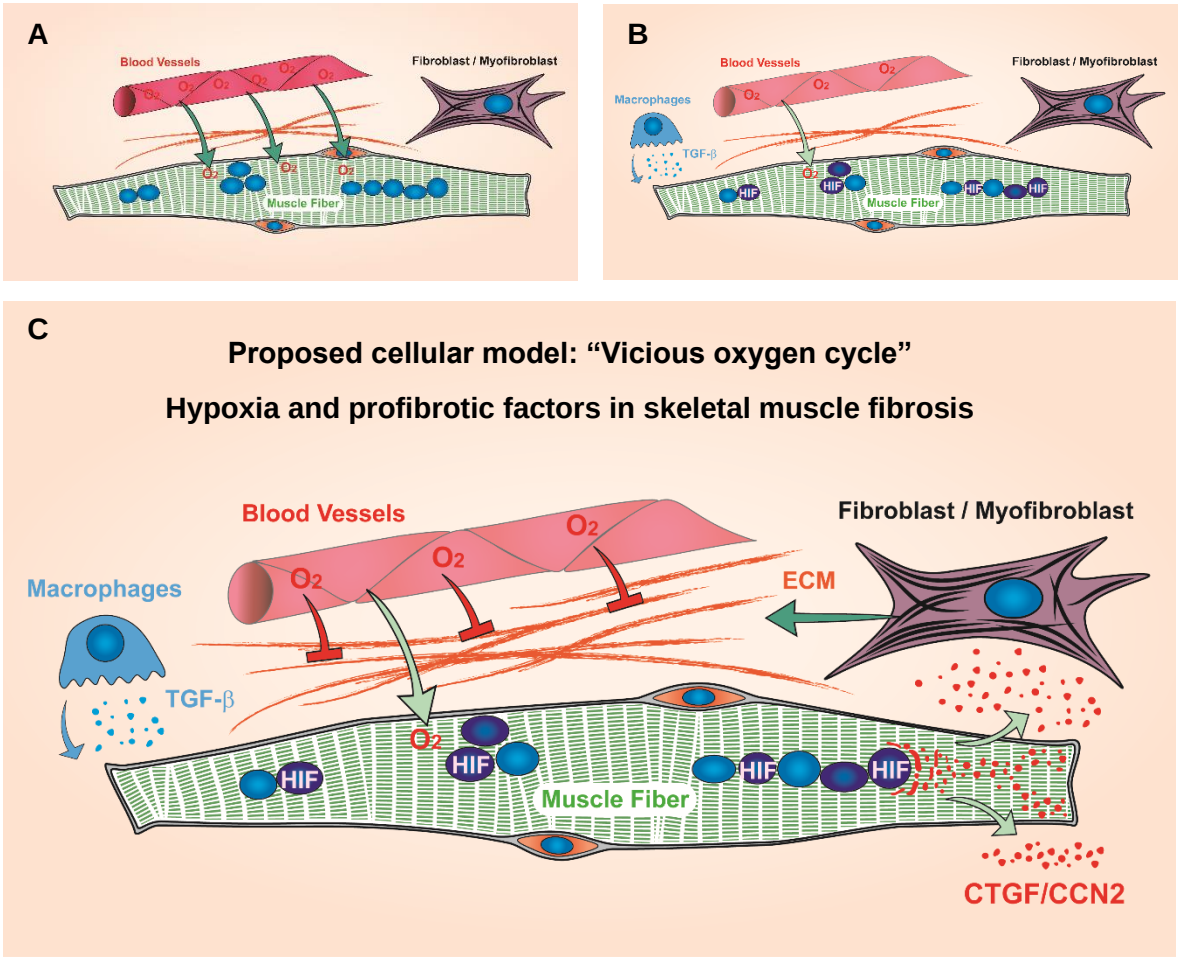


Figure 4. Proposed cellular model: "Vicious oxygen cycle". A. Initially, in physiological conditions the oxygen flow freely through blood vessels to muscle cells. B. Vasculature damage by muscle disuse or damage decreses oxygen perfusion in the tissue leading to HIF-1α-mediated hypoxic signaling activation, accompanied with TGF-b secretion by activated macrophages. C. The sum of hypoxic and TGF-β signaling activation contributes to the over-expression of CTGF/CCN2 profibrotic factor by muscle fibers. CTGF/CCN2 acts as a local paracrine signal over fibroblasts and stimulate its transition into myofibroblast resulting in ECM production. The ECM itself creates a physical barrier that blocks oxygen perfusion into cells by increasing the distances between blood vessels and muscle fibers. We hypothesize that the resulting fibrotic state in skeletal muscle is a sum of factors that are enhanced between them, where different cell types interplay creating a vicious cycle that continuously worsens the skeletal muscle physiology, resulting in chronic ECM deposition, vascular damage, reduced oxygen blood flow, muscle waste, and persistent inflammation.

CONCLUSION

In this thesis, we found that hypoxia is a key feature of skeletal muscle fibrosis. Hypoxic signaling activation through HIF-1α is a recurrent characteristic in several muscle pathologies related to fibrosis. Moreover, here we described a mechanism where HIF-1α together with TGF-β1 synergistically induces the expression of CTGF/CCN2 profibrotic factor specifically in muscle fibers *in vivo* and myotubes *in vitro.* These results contribute to the understanding of the cellular and molecular basis of fibrosis formation in skeletal muscle pathologies, putting in context the hypoxic signaling.

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