



Role of CTGF and TNF on fibrosis in muscular dystrophy

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Jaime Gonzalo Cordova. Role of CTGF and TNF on fibrosis in muscular dystrophy. Genomics [q-bio.GN]. Université Pierre et Marie Curie - Paris VI; Pontificia universidad católica de Chile (Santiago de Chile), 2014. English. NNT: 2014PA066546 . tel-01137592

HAL Id: tel-01137592

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Pontificia Universidad Católica de Chile

Université Pierre et Marie Curie

ROLE OF CTGF AND TNF ON FIBROSIS IN MUSCULAR DYSTROPHY

By:

JAIME GONZALO CÓRDOVA STEGER

August 2014.



Pontificia Universidad Católica de Chile

Université Pierre et Marie Curie

ROLE OF CTGF AND TNF ON FIBROSIS IN MUSCULAR DYSTROPHY

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

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Agosto 2014.



Pontificia Universidad Católica de Chile

Université Pierre et Marie Curie

ED 387: iViv: Interdisciplinaire pour le vivant

ROLE OF CTGF AND TNF ON FIBROSIS IN MUSCULAR DYSTROPHY

Thèse de doctorat de Biologie Cellulaire et Moléculaire

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Août 2014.

To my beloved parents, Marta y Jaime,
and to my dear Pachi.

ACKNOWLEDGEMENTS

I am sincerely grateful to:

My advisors, Dr. Enrique Brandan and Dr. Pascal Bigey, for the continuous support and guidance throughout my research project, for the stimulating discussions and their valuable suggestions that helped me to design and perform this work. Also, their effort in proof reading the drafts, are greatly appreciated.

Members of the evaluation committee: Dr. Germain Trugnan, Dr. Antoine Kirchler, Dr. Martín Montecino and Dr. Claudio Cabello; who reviewed this thesis and contributed to improve the manuscript with their insightful comments and corrections.

I also thanks to for the pre-doctoral fellowships from: Beca VRI de doctorado 2007, PUC; Beca de doctorado 2008 and Beca de apoyo a la realización de tesis doctorado 2009-2010, CONICYT; Beca de pasantía MECESUP 2009 and 2011; Beca Chile cotutela de doctorado 2010.

This work was supported by grants from the Basal Center of Excellence in Science in Technology (CONICYT-PFB12/2007) FONDECYT 1110426 and Fundación Chilena para Biología Celular Proyecto MF-100 to Dr. Enrique Brandan.

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ABBREVIATIONS

ATRA:	All-trans retinoic acid
CTGF:	Connective Tissue Growth Factor
DISC:	Death Inducing Signaling Complex
DD:	Death Domain
DMD:	Duchenne Muscular Dystrophy
DGAC:	Dystrophin Glycoproteins-Associated Complex
ECM:	Extracellular Matrix
ET:	Electrotransfer
GRMD:	Golden Retriever Muscular Dystrophy
HFMD:	Hypertrophic Feline Muscular Dystrophy
HGF:	Hepatocyte Growth Factor
LRP:	Low Density Lipoproteins-Related Receptor
MAPK:	Mitogen-Activated Protein Kinase
RAR:	Retinoic Acid Receptor
RXR:	Retinoic X Receptor
SBE:	SMAD Binding Element

SMA: Smooth Muscle Actin

SMAD: Small Mothers Against Decapentaplegic

TA: *tibialis anterior*

TFBS: Transcription Factor Binding Sites

TGF: Transforming Growth Factor type β

TGFBR: TGF- β Receptor

TNF: Tumor Necrosis Factor

TNFR: TNF Receptor

UTR: Untranslated Region

VEGF: Vascular Endothelial Growth Factor

wt: wild-type

ABSTRACT

The Duchenne Muscular Dystrophy (DMD) is an X-linked disease characterized by progressive and accumulative damage in the muscle due to the absence of the dystrophin protein. Fibrosis, the excessive accumulation of extracellular matrix (ECM) proteins, is also present in the muscle of DMD patients and several animal models (such as the mdx mice), due to continuous inflammation in the tissue produced by contraction-relaxation cycles. Among the factors that induce fibrosis are Transforming Growth Factor type β (TGF- β) and Connective Tissue Growth Factor (CTGF), the latter being a target of the TGF- β /Small Mothers Against Decapentaplegic (SMAD) signaling pathway and is the responsible for the profibrotic effects of TGF- β and are augmented in fibrosis tissues. Little is known about the regulation of the expression of CTGF mediated by TGF- β in muscle cells. In here, we described a novel SMAD Binding Element (SBE) located in the 5' UTR region of the CTGF gene important for the TGF- β mediated expression of CTGF in myoblasts. In addition, our results suggest that additional transcription factor binding sites (TFBS) present in the 5' UTR of the CTGF gene are important for this expression.

On the other hand, the Tumor Necrosis Factor (TNF) is a potent inflammatory cytokine that is present in DMD muscles and is responsible for muscle necrosis and inflammatory cell

infiltration in several tissues. In this study, we show that the increased expression of the soluble TNF Receptor I (smTNFRI) by electrotransfer (ET) in the *tibialis anterior* (TA) muscle attenuates inflammation, damage and fibrosis in the skeletal muscle of the mdx mice. In addition, we found an increase in isolated muscle strength in the mdx mice. Therefore, we propose that ET could be used as an efficient anti-TNF therapy for treating muscle dystrophies.

RESUMEN

La Distrofia Muscular de Duchenne (DMD) es una enfermedad ligada al X, que se caracteriza por daño progresivo y acumulativo en el músculo debido a la ausencia de la proteína distrofina. La fibrosis, que es la excesiva acumulación de proteínas de la matriz extracelular (ECM), también está presente en el músculo de pacientes de DMD y varios modelos animales (como el ratón mdx), debido a la inflamación continua en el tejido producida por repetidos ciclos de contracción y relajación. Entre los factores que indican fibrosis se encuentran el Factor de Crecimiento Transformante tipo β (TGF- β) y el Factor de Crecimiento de Tejido Conectivo (CTGF), este último es blanco de la señalización mediada por TGF- β /Small Mothers Against Decapentaplegic (SMAD) y es responsable de los efectos profibróticos de TGF- β y está aumentado en tejidos fibróticos. Poco se sabe de la regulación de la expresión de CTGF mediada por TGF- β en células musculares. En esta tesis, describimos un nuevo elemento de unión a SMAD (SBE) localizado en la región 5' UTR del gen de CTGF, que es importante para la expresión de CTGF mediada por TGF- β en mioblastos. Adicionalmente, nuestros resultados sugieren que sitios de unión de factores de transcripción (TFBS) adicionales, presentes en la región 5' UTR del gen de CTGF son importantes para su expresión.

Por otra parte, el Factor de Necrosis Tumoral (TNF) es una potente citoquina inflamatoria que está presente en músculos de pacientes con DMD y que es responsable de la necrosis en el músculo y de la infiltración de células inflamatorias en distintos tejidos. En esta tesis, encontramos que el aumento en la expresión del receptor soluble de TNFR I (smTNFRI) mediante la técnica de electrotransferencia (ET) en el músculo *tibialis anterior* (TA) atenúa la inflamación, el daño y la fibrosis en el músculo esquelético del ratón mdx. Adicionalmente, encontramos un aumento significativo en la fuerza isométrica en músculo aislado del ratón mdx. Por este motivo, proponemos que la ET, podría ser usada como una terapia eficiente contra TNF para el tratamiento de distrofias musculares.

RÉSUMÉ

La dystrophie musculaire de Duchenne (DMD) est une maladie liée à l'X caractérisée par la détérioration progressive et cumulative des muscles en raison de l'absence de la protéine dystrophine. Le muscle des patients atteints de DMD ainsi que plusieurs modèles animaux de la maladie (comme le souris mdx), présentent également une fibrose, accumulation excessive de protéines de la matrice extracellulaire, à cause de l'inflammation continue dans le tissu produit par des cycles de contraction et de relaxation. Parmi les facteurs qui induisent la fibrose, il y a le Facteur de Croissance de Transformation de type β (TGF- β) et le Facteur de Croissance du Tissu Conjonctif (CTGF). Ce dernier est une cible de la voie de signalisation médiée par TGF- β /Small Mothers Against Decapentaplegic (SMAD) et est responsable des effets profibrotiques de TGF- β , d'où son augmentation dans les tissus de la fibrose. La régulation de l'expression de CTGF médiée par TGF- β dans les cellules musculaires est peu connue. Dans ce travail, nous avons décrit un nouvel élément de liaison SMAD (SBE) situé dans la région 5'UTR du gène de CTGF, important pour l'expression de CTGF médiée par le TGF- β dans des myoblastes. De plus, nos résultats suggèrent que les sites de liaison supplémentaires du facteur de transcription (TFBS) présents dans le 5'UTR du gène de CTGF sont importants pour cette expression.

Par ailleurs, le Facteur de Nécrose Tumorale (TNF) est une cytokine inflammatoire puissante qui est présente dans les muscles atteints de DMD et est responsable de la nécrose du muscle et de l'infiltration de cellules inflammatoires dans plusieurs tissus. Dans cette étude, nous montrons que l'augmentation de l'expression du récepteur soluble de TNF I (smTNFRI) par électrotransfert (ET) dans le muscle *tibialis anterior* (TA) de la souris atténue l'inflammation, les dommages et la fibrose dans le muscle squelettique des souris mdx. En outre, nous avons constaté une augmentation de la force musculaire dans le muscle isolé chez la souris mdx. Par conséquent, nous proposons l'ET comme thérapie efficace anti-TNF pour le traitement de dystrophies musculaires.

1. INTRODUCTION

The pathophysiological fibrosis is characterized by an excessive accumulation of extracellular matrix components (ECM), produced by a cascade of events occurring after a tissue injury and resulting in permanent scars formation.

Fibrosis could alter tissue function and causes chronic diseases in several organs and tissues such as kidney, liver, lung, muscle, etc (Wynn, 2008). Despite the wide range of tissues susceptible to fibrosis, fibrotic tissues share common features, such as cell degeneration, inflammatory cell infiltration, chronic inflammation and proliferation of fibroblast-like cells (Serrano et al., 2011). This imbalance is also supported by the production of growth factors, proteolytic enzymes, angiogenic factors and fibrogenic cytokines, which together disturb the microenvironment of the damaged tissue and stimulate the deposition of connective tissue elements that progressively reshape, destroy and replace the normal tissue architecture. However, the identity of some cellular factors involved in the fibrogenic pathways is still unknown. Therefore, the improvement of our understanding of those factors as well as the mechanisms involved in the fibrogenic process is crucial for the development of new and more powerful strategies to the treatment of fibrosis-related diseases.

1.1 Duchenne muscular dystrophy

The skeletal muscle fibrosis is frequently associated with a clinical and molecular heterogeneous group of diseases known as muscular dystrophies. Phenotypically, these diseases are characterized by inflammation and weakening of the muscle tissue, which compromises patient mobility (Durbeej and Campbell, 2002, Amato and Griggs, 2011).

Muscular dystrophies are a diverse group of genetic muscular diseases, being Duchenne Muscular Dystrophy (DMD) the most severe (Shieh, 2013). DMD is an X-linked disease that affects between one in 3600 to 6000 live male births (Bushby et al., 2010). Patients with this condition gradually show muscle weakness, they require the use of wheelchair in their teens and they present orthopedic and respiratory complications that lead to death between the second and third decade of life (Bushby et al., 2010). Cardiac involvement is also common, 25% of DMD patients show evidence of preclinical cardiomyopathies before their sixth year of age and by the time they reach age of 30, almost 100% of the patients have developed some kind of heart disease (McNally, 2007).

At the molecular level, DMD is characterized by a severe reduction or the absence of the protein dystrophin (Koenig et al., 1987, Kunkel et al., 1987). The dystrophin gene is about 2500 Mb, conformed by 86 exons and located in Xp21 (Muntoni et al., 2003). The dystrophin protein is 427 kDa in its complete form (there are also shorter isoforms) is expressed in skeletal, cardiac muscle and brain, predominantly. Specifically in the muscle, dystrophin is expressed under the plasma membrane of muscle fibers (sarcolemma) and it is anchored to the actin cytoskeleton and to several transmembrane glycoproteins (dystrophin glycoproteins-associated complex or DGAC) through β -dystroglycan (Blake et al., 2002). Since DGAC also binds to ECM proteins,

dystrophin act as a bridge that anchors the muscle fiber cytoskeleton to the extracellular space, which brings stability to the muscles fiber during cycles of contraction and relaxation (Ervasti and Sonnemann, 2008). The absence of dystrophin, might cause the rupture of the sarcolemma of the muscle fiber during contraction (Allen and Whitehead, 2011). Even though dystrophin act as a scaffolding protein, it seems that this is not its only function, because it could play a role in intracellular signaling through its association with DGAC (Batchelor and Winder, 2006).

At a cellular level, muscle tissue of DMD patients show evidence of degeneration, regeneration, myofiber atrophy, fatty accumulation and necrosis of muscle fibers, inflammation and fibrosis (Spencer and Tidball, 2001, Alvarez et al., 2002, Desguerre et al., 2009a, Desguerre et al., 2009b, Serrano and Munoz-Canoves, 2010, Zhou and Lu, 2010, Villalta et al., 2011). Even in the first biopsies performed by Duchenne and reported in 1868 was described a “hyperplasia of the interstitial connective tissue with production of fibrotic tissue, as the main anatomical lesion of the muscles in the pseudohypertrophic paralysis” (Tyler, 2003). At early stages of the disease, necrotic muscle fibers rise and therefore areas of muscle regeneration emerge. Later, after repeated cycles of degeneration a decreased ability of muscle regeneration take place together with a chronic inflammatory process and a significant increase of fibrosis (Emery, 2002). Excessive ECM proteins in fibrotic conditions also affects the interaction between the sarcolemma and the basal lamina (Ervasti, 2007). Several ECM molecules that are augmented in the dystrophic muscles of mice and humans have been identified as collagen I and III, and fibronectin (Morrison et al., 2000, Wynn, 2008).

There are several animal models for DMD as the dog, GRMD (golden retriever muscular dystrophy), the cat, HFMD (hypertrophic feline muscular dystrophy), and the mouse, mdx (x-linked muscular dystrophy). Neither of the aforementioned models express dystrophin, but the

dog is the model that resembles most to the disease in humans (Blake et al., 2002). However, the most studied animal model for DMD is the mdx mouse. This mouse is considered a valid genetic model of the disease, since it has a point mutation in exon 23 of dystrophin gene that produces a stop codon which cause the absence of the protein in the sarcolemma of muscle fibers (Sicinski et al., 1989). Although mdx mice are normal at birth, skeletal muscles undergo an extensive process of degeneration between 3-5 postnatal weeks. This acute degeneration phase results in an extensive regeneration process with a progressive fibrosis. However, for unknown reasons in older animals the muscle regeneration process fails and the mice become extremely weak and die earlier than the wild-type (wt) animals (Tanabe et al., 1986, Pastoret and Sebillé, 1995, Caceres et al., 2000).

Interestingly, mdx mouse histopathology is similar to that observed in DMD patients. Moreover, these animals show a progressive atrophy and loss of muscle mass. Due to continuous cycles of muscle degeneration and regeneration, mdx mice exhibit an increased variability of muscle fiber size, nuclei located in a central position and an increased presence of myofibroblasts and inflammatory type of cells (Bulfield et al., 1984, Briguet et al., 2004).

The fibrotic phenotype in mdx mice is less severe than in DMD patients, mainly in the muscles of lower extremities as the *tibialis anterior*, an effect caused by captivity. However, pathological features of mdx diaphragm are more similar to that of DMD limb muscles due to the constant movement of this muscle in the breathing process (Stedman et al., 1991, Connolly et al., 2001). The muscle damage in the limbs of mdx mice can be accelerated through exercise protocols to emulate the DMD phenotype in humans (De Luca et al., 2005, Morales et al., 2013b, Cabrera et al., 2014).

The excess of ECM components produces muscle dysfunction and contributes to the lethal phenotype of this disease (Desguerre et al., 2009b). Although the use of cellular and gene therapies that restore dystrophin expression might deliver a cure for DMD, so far there are no effective therapies against this disease. Recent studies have shown that decreasing muscle fibrosis could represent an effective therapy for DMD (Morales et al., 2013b, Acuna et al., 2014, Cabrera et al., 2014). Reducing fibrosis not only improves muscle function but increases the process of muscle regeneration (Cohn et al., 2007, Turgeman et al., 2008) and improves cell transplant to restore potential dystrophin in the muscle fiber (Cordier et al., 2000, Gargioli et al., 2008, Morales et al., 2013b, Cabrera et al., 2014) suggesting that antifibrotic therapies combined with cell therapy would have a significant potential in the treating of this disease.

Therefore understanding the cellular and molecular mechanisms involved in the development fibrosis associated with dystrophin deficiency is critical to the development of therapies antifibrotic for DMD.

1.2 Muscle damage and repair

Regenerative capacity of skeletal muscle depends on a population of cells located under the basal lamina of muscle fibers, called satellite cells. In normal conditions, satellite cells are mitotically inactive, but in response to stimuli such as stress induced by trauma, they become activated and start to proliferate and differentiate to form new fibers or merging to pre-existing fibers (Seale and Rudnicki, 2000, Hawke and Garry, 2001, Chen and Goldhamer, 2003).

After acute injury, normal muscle repair starts by removing damaged or dead fibers through inflammatory cells such as macrophages, and then replacing or repairing the injured tissue through satellite cells (Mauro, 1961). In chronic fibrosis cases, including DMD, newly produced muscle fibers are susceptible to degeneration because they carry a molecular defect that leads to repeated cycles of degeneration and regeneration of the muscle fibers and allows the establishment of a chronic inflammatory process (Porter et al., 2002). In the muscle of DMD patients, this chronic injury leads not only to ECM deposition, but also to a decreased nutrition of the muscle fibers (Klingler et al., 2012) and a depletion of the muscle's satellite cells (Charge and Rudnicki, 2004). Yet, cellular and molecular mechanisms that underlie the inflammatory process and lead to fibrosis onset and development are still unknown. Therefore, the identification of those factors that link both processes is key to the development of new therapeutic strategies.

Macrophages are the main type of inflammatory cells found after muscle injury. In the dystrophic muscle, macrophages remove dead cells and also modulate the regeneration process (Tidball, 2005). Current evidence suggests that nature, duration and intensity of the inflammatory response in damaged muscle critically influences normal muscle repair, while in the dystrophy muscle, promotes the formation of fibrotic tissue, particularly during disease progression (Tidball, 2005). Interfering the transient inflammatory response induced by an acute injury can affect removal of cell debris and therefore the formation of new muscle fibers. However, interfering chronic inflammation in muscular dystrophies has a beneficial effect since it decreases degeneration and blocks fibrosis progression, thus improving regeneration (Tidball, 2005). Indeed, several studies have demonstrated that anti-inflammatory agents, acting on cytokines (such as TNF and its cellular receptors) and proteins of the pro-inflammatory

pathways (such as NF- κ B), slow the progression of dystrophy in mdx mice (Pelosi et al., 2007, Peterson and Guttridge, 2008, Radley et al., 2008, Cabrera et al., 2014).

After muscle injury, a heterogeneous population of macrophages playing opposite roles have been identified (Arnold et al., 2007). At early stages after a muscle injury, a population of macrophages (M1, positive ED-1 (CD68)), producing high levels of pro-inflammatory cytokines such as TNF and IL-1 β , are found in association to monocytes recruitment and removal of necrotic material (Arnold et al., 2007). Later, during advanced stages of the regeneration process, a population of anti-inflammatory macrophages (M2C, positive ED-2 (CD163)) are found in abundance when tissue repair is carrying on (Arnold et al., 2007). Thus, pro-inflammatory macrophages might increase myogenic cell proliferation, whereas anti-inflammatory macrophages could stimulate differentiation, *in vitro* (Arnold et al., 2007). There is an interesting work that uses a co-injection of macrophages and human myoblasts in immunodeficient mice. They show that injecting pro-inflammatory macrophages together with human myoblasts, enhances the proliferation state of the myoblasts as well as their migration and, more importantly, these pro-inflammatory macrophages can switch to an anti-inflammatory state *in vivo*, and then start to stimulate differentiation and muscle regeneration (Bencze et al., 2012).

1.3 Pro-fibrotic and pro-inflammatory cytokines in DMD

Most pro-fibrogenic factors are produced by inflammatory cells infiltrated into the tissue, mesenchymal cells, fibroblasts and also by specific cells in the tissue, facilitating the fibrogenic paracrine effects and, therefore, perpetuating an inflammation-driven fibrosis. The activation of fibroblasts and the expression of ECM components are stimulated by pro-fibrotic cytokines like Transforming Growth Factor type β (TGF- β) (Wynn, 2008).

TGF- β is a potent pro-fibrotic cytokine that contributes to the pathogenesis of several fibrotic disorders (Branton and Kopp, 1999), including muscular dystrophies (Bernasconi et al., 1999). To date, there are three isoforms described for TGF- β (TGF- β 1, TGF- β 2 and TGF- β 3) and all of them are synthesized as precursor proteins (Zhou et al., 2006). The canonical TGF- β signaling pathway is the following: TGF- β binds to the TGF- β receptor type II (TGFBRII), which forms a complex with TGF- β receptor type I (TGFBRI) and causes the phosphorylation and activation of TGFBRI, this complex phosphorylates SMAD2/3, which, in turn binds SMAD4 (Massague, 1998). In the nucleus, the SMAD proteins recognize the sequence called SMAD Binding Element (SBE), first described as 5'-GTCTAGAC-3' (Zawel et al., 1998). Later, it was shown that SMAD complex recognizes the sequence 5'-GTCT-3' or its complement 5'-AGAC-3', although the optimal binding sequence is thought to be 5-CAGAC-3' and, more importantly, the affinity observed of SMAD for this sequence was shown too low to be effective alone *in vivo* (Shi et al., 1998). The short length of the SBE (calculations show that it should be present once every 1024 bp in the genome), the low specificity (SMAD1, SMAD3 and SMAD4 can bind to the SBE) and the low affinity binding of SMAD proteins, suggest that additional components should be required for a specific, high-affinity binding of SMAD-

containing complexes to target genes (Massague and Wotton, 2000, Massague et al., 2005). On the other hand, it also has been shown that TGF- β can signal through several additional pathways, like the p38 mitogen-activated protein kinase (MAPK), activated ERK, c-abl, JNK, among others (Shi and Massague, 2003). These signaling pathways appear to be modifying the expression of genes in a selective manner. For example, FAK, JNK and TAK1 are required for the differentiation of fibroblasts to a fibrotic phenotype known as myofibroblasts, whose main characteristic is its high capacity to synthesize ECM proteins and have contractile activity due to expression of alpha smooth muscle actin (α -SMA) (Vaughan et al., 2000, Hinz, 2007).

TGF- β is present in the muscles of patients with several congenital dystrophies, including DMD, and in the *mdx* diaphragm (Bernasconi et al., 1995, Bernasconi et al., 1999, Zhou et al., 2006). Moreover, the direct *in vivo* injection of recombinant TGF- β in the muscle stimulates the expression of TGF- β in muscle cells in an autocrine fashion and induces the formation of connective tissue in the area of injection (Zhu et al., 2007, Brandan et al., 2008). Interestingly, it has been found that TGF- β induces the expression of Connective Tissue Growth Factor (CTGF/CCN2) in fibroblasts (Igarashi et al., 1993) and, more importantly, it has been shown that the pro-fibrotic effects of TGF- β are CTGF-dependent (Grotendorst, 1997, Leask and Abraham, 2004).

CTGF is a cysteine-rich modular protein belonging to the Cyr61/CTGF/NOV (CCN) family of growth factors (Perbal, 2004). CTGF is involved in a number of biological processes including differentiation, proliferation (Yosimichi et al., 2001, Grotendorst et al., 2004), adhesion (Ball et al., 2003), migration (Gao and Brigstock, 2006), apoptosis (Hishikawa et al., 1999a, Hishikawa et al., 1999b), ECM production (Frazier et al., 1996), chondrogenesis (Ivkovic et al., 2003) and angiogenesis (Babic et al., 1999). One of the main features of CTGF

is to be the main inducer of ECM production and therefore of the fibrotic process in a large variety of fibrotic diseases (Brigstock, 1999, Leask and Abraham, 2006). Based on studies of expression of CTGF during development, it has been suggested that this factor plays a role in the formation of cartilage, bone, teeth and maturation of nerve cells (Leask and Abraham, 2003). Consistent with this, mice deficient in CTGF die at birth due to alterations in specific production of bone matrix, chondrocyte proliferation and ossification of the ribs (Ivkovic et al., 2003). Although CTGF is not expressed under normal conditions in most adult tissues, expression of this protein can be induced by TGF- β , hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), angiotensin II, glucocorticoid, endothelin-1 and hypoxia, among other stimuli (Leask and Abraham, 2006, Leask, 2009).

To date, a specific high-affinity receptor for CTGF has not been identified, but it is known that some of its functions, such as adhesion and migration, requires the presence of integrins and heparan sulfate proteoglycans (Babic et al., 1999, Ball et al., 2003, Gao et al., 2004). CTGF also interacts with low density lipoproteins-related receptor (LRP-1) through which it would be internalized and degraded via endosomes (Segarini et al., 2001). We have also shown that Decorin interacts with CTGF and inhibits its pro-fibrotic activity (Vial et al., 2011, Brandan and Gutierrez, 2013).

CTGF levels correlate with the degree and severity of fibrosis in many fibrotic tissues. Some of them include skin disorders such as systemic sclerosis and keloids (Igarashi et al., 1993), atherosclerotic lesions (Oemar et al., 1997), pulmonary fibrosis (Lasky et al., 1998), renal fibrosis (Ito et al., 1998), chronic pancreatitis (di Mola et al., 1999) and liver fibrosis (Paradis et al., 1999). In addition, CTGF is increased in the muscle tissue of patients with different dystrophies, including DMD (Sun et al., 2008), and in the mdx mice (Cabello-Verrugio et al.,

2012a, Morales et al., 2013b). Additionally, we found that the exogenous increase of CTGF in the muscle of wild type mice led to a decrease in muscle strength and an increase in the expression of ECM proteins (Morales et al., 2011). Also, we have previously shown that TGF- β induces CTGF mRNA and protein expression, and also that CTGF itself reduces differentiation markers in myoblasts, like desmin and MyoD along with an increase in FN accumulation (Vial et al., 2008). Furthermore, in another previous work, we showed that reducing CTGF expression or blocking CTGF function in *mdx* mice, slowed down the progression of the dystrophic phenotype, seen as an increase in muscle strength, a reduction in the deposition of ECM proteins and, more important, led to a better response to muscle stem cell therapy in treated *mdx* mice (Morales et al., 2013b). These findings confirm that CTGF is an attractive target for antifibrotic therapy, so it is essential to understand how its expression is regulated, particularly in muscle cells.

The first attempt to study the regulation of CTGF by TGF- β , was done by Grotendorst et al. where they identified a TGF- β response element, using a 900 bp fragment of the CTGF promoter controlling the expression of the luciferine gene in human skin fibroblasts (Grotendorst et al., 1996). When we tested this promoter in myoblasts cells in response to TGF- β , we found a weak induction of luciferase, but that it was surprisingly low compared to our observation of the mRNA induction by Northern blot analysis (Vial et al., 2008). One explanation for this difference is that in myoblasts, there are additional transcription factor binding sites (TFBS) that are required for the induction of CTGF by TGF- β . A TGF- β response element was described to control the TGF- β mediated expression of CTGF in fibroblasts (Grotendorst et al., 1996) and a SBE (Holmes et al., 2001), however, the full 5' UTR region was not included in these studies. Also, several other transcription factors have been described to

contribute to the TGF- β mediated expression of CTGF: SP1 in scleroderma fibroblasts (Holmes et al., 2003), MAPKs and PKC in mesangial cells (Chen et al., 2002) and fibroblasts (Leask et al., 2003); and AP-1 in keloid fibroblasts (Xia et al., 2007) and nucleus pulposus (Tran et al., 2010).

Regarding inflammation, Tumor necrosis factor (TNF) is a potent inflammatory cytokine that increases when myofibers are damaged, it is expressed in myoblasts and myotubes (Collins and Grounds, 2001) and it is also increased in the plasma levels of DMD patients (Porreca et al., 1999). TNF is mainly produced by macrophages, and also by a variety of other tissues including lymphoid cells, mast cells, endothelial cells, fibroblasts and neuronal tissue (Wajant et al., 2003). TNF is produced mainly as a transmembrane proteins which is cleaved by the metalloprotease TNF-converting enzyme, then, TNF acts as a homotrimer that binds to the TNF Receptors to exert its effects (Grell, 1995, Wajant et al., 2003). The TNF receptor I (TNFRI), which is ubiquitously expressed, or the TNF receptor II, which is mostly inducible and present in endothelia and hematopoietic cells (Tracey et al., 2008). More importantly, TNFRI has been recognized as the main TNFR responsible for the initiation of the inflammatory response (Loetscher et al., 1993, van der Poll et al., 1996). In addition, soluble versions of the TNF receptors (sTNFR) occurs naturally and might have a role in the modulation of the TNF inflammatory response (Engelmann et al., 1990, Nophar et al., 1990, Seckinger et al., 1990). Also, the levels of sTNFRs in serum increase with several pathological conditions (Aderka et al., 1991, Cope et al., 1992, Diez-Ruiz et al., 1995, Torre-Amione et al., 1996, Thevenon et al., 2010).

We have previously shown that by expressing a chimeric protein composed of the sTNFRI receptor coupled with the Fc fragment of IgG1 by electrotransfer (ET) in skeletal muscle, is

effective for the treatment of rheumatoid arthritis in a mouse model (Bloquel et al., 2004) and uveitis in a rat model (Bloquel et al., 2006). Therefore, these data suggests that the expression of sTNFRI by ET might be used as an efficient anti-TNF therapy for treating muscle dystrophies.

1.4 Muscle dystrophy therapies

A study performed on twenty five DMD patients, showed that among several pathological features, including myofibrillar atrophy, necrosis and replacement by fatty tissue, only fibrosis observed in biopsies from early stages of disease correlates to poor muscle strength and results in age-related progressive muscle weakening (Emery, 2002). This finding supports the idea that fibrosis directly contributes to progressive muscle dysfunction that leads to death in DMD patients.

Treatment of DMD patients involves corticosteroids (prednisone) administration, which partially improves muscular strength and extends the ability to walk in the early years, but eventually produces undesirable side effects (Angelini, 2007). Until now, there are not effective treatments to combat fibrosis in DMD.

Among the therapies assayed for DMD treatment, there are gene therapies including to reverse gene mutations that prevent the proper expression of dystrophin, the use of overexpression vectors, as well as attempts at exon-skipping (Lu et al., 2011, Adkin et al., 2012) to generate functional dystrophin protein isoforms. Furthermore, there have been cellular therapies using different cells with myogenic potential, several reports shows that cells derived from bone marrow have been used (Mafi et al., 2011), satellite cells (Cerletti et al., 2008, Sacco

et al., 2008), pericytes (Peault et al., 2007), muscle-derived stem cells (Qu-Petersen et al., 2002) and mesangioblasts (Sampaolesi et al., 2006). However, the effectiveness of these approaches is still a problem. Furthermore, the amount of target tissue for these interventions and an excess in connective tissue and ECM impose a physical barrier, which could impair the efficacies of these therapies (Muir and Chamberlain, 2009). In this context, several issues have been observed in cell therapies for DMD. First, the injected cells are distributed locally, which means that a patient must perform multiple injections to treat a complete muscle (Huard et al., 1992). Second, an immune response against injected satellite cells has been found even in the case of coincidence between the major histocompatibility complexes, therefore new therapies should consider an inhibition of the inflammatory response that eventually caused the death of most satellite cells during the first 72 hours after the injection (Fan et al., 1996, Guerette et al., 1997). On the other hand, the efficiency of transplanted cells can be increased by using cells with myogenic potential overexpressing metalloproteinase MMP-9 (Gargioli et al., 2008), belonging to a proteolytic family of enzymes that have multiple ECM components as substrates (Woessner, 1991). This suggests that cells that have the capacity to degrade the ECM become more migratory and have better distribution in the dystrophic muscle.

Antagonism to TGF- β signaling by a variety of strategies have been shown to inhibit fibrosis and enhance muscle regeneration in several experimental models. However, no agent has shown the ability to reduce fibrosis once formed. For example, direct immunomodulation of TGF- β inhibited the accumulation of connective tissue (Isaka et al., 2000, Denton et al., 2007) and progression of fibrosis in the diaphragm of mdx mice, but increased significantly inflammation (Andreetta et al., 2006). These data suggest a strong relationship between fibrosis and inflammation, so therapies that inhibits inflammation and decrease fibrosis could improve

cell-based therapies in DMD. We have shown before that decreasing fibrosis has a beneficial effect in cell based therapies (Morales et al., 2013b, Cabrera et al., 2014).

Several anti-TNF therapies are in use for the treatment of rheumatoid arthritis using a recombinant versions of sTNFRII or blocking antibodies (Thalayasingam and Isaacs, 2011) and also, a pegylated form of sTNFRI has been used with good results reducing renal fibrosis (Therrien et al., 2012). Two of those therapies have been used in relation to muscle dystrophy. Enbrel® (etanercept), a chimeric protein of the sTNFRII with the Fc fragment of human IgG, has been used to successfully protect dystrophic muscle from inflammatory damage in the mdx mice (Nemoto et al., 2011) and Remicade® (infliximab), an anti-TNF antibody, has also been used and shown to reduce muscle fiber necrosis in dystrophic mice (Grounds and Torrisi, 2004). In addition, a modification of the infliximab antibody, cV1q, has been used to reduce damage and necrosis in muscles of wt and mdx mice (Radley et al., 2008, Piers et al., 2011). All these therapies rely on the production of purified proteins, which can be expensive and the successful treatment require repetitive injections. Gene therapy offers many advantages compared to recombinant protein therapies, such as low cost, good quantity and long-term production of the required protein with a single procedure and little secondary effects. We have previously shown that by expressing a chimeric protein composed of the sTNFRI receptor coupled with the Fc fragment of IgG1 by electrotransfer (ET) in skeletal muscle, has been proven effective for the treatment of rheumatoid arthritis in a mouse model (Bloquel et al., 2004) and uveitis in a rat model (Bloquel et al., 2006). Therefore, these data shows that the expression of sTNFRI by ET might be used as an efficient anti-TNF therapy for treating muscle dystrophies.

At this point, we have described a very complex scenario showing that pro-fibrotic and pro-inflammatory cytokines are present in the muscles of DMD patients and in the mdx mice, which

can be inducing fibrosis. As CTGF has been shown as the growth factor responsible for many of the pro-fibrotic effects of TGF- β , it appear as an attractive target for therapy design in DMD and to study its expression in the skeletal muscle. On the other hand, by using the TA muscle as a biofactory for producing a blocking agent for TNF, might be a novel and efficient way to reduce inflammation and fibrosis in the skeletal muscle of the mdx mice and inflammation-related pathologies.

1.5 Hypothesis and Objectives.

Hypothesis 1:

Novel transcription factor binding sites control TGF- β mediated CTGF expression in C2C12 myoblasts.

General Objective 1:

To study the transcriptional regulation of CTGF in C2C12 myoblasts.

Specific Objectives 1:

- To identify the regions of the CTGF promoter that are relevant for the TGF- β mediated expression of CTGF in C2C12 myoblasts by the use of deletion mutants of a vector carrying 5kb of the murine CTGF promoter.
- To analyze TFBS that participate in the transcriptional regulation of CTGF mediated by TGF- β in C2C12 myoblasts by site-directed mutagenesis.

Hypothesis 2:

Using the TA muscle for producing sTNFRI will block inflammation and reduce damage and fibrosis in the dystrophic muscle.

General Objective 2:

To evaluate the role of TNF in the onset and progression of fibrosis in the dystrophic muscle of the mdx mice.

Specific Objective 2:

- To evaluate the blockage of TNF by the expression of soluble TNF receptor I in the TA muscle of the mdx mice and its relationship with the onset and progression of fibrosis.

2. CHAPTER I

The results presented in the next section were obtained to accomplish the Objective 1, previously described.

The manuscript presented below, was submitted in August 2014 to Journal of Cellular Biochemistry (Manuscript ID: JCB-14-0445).

A novel SMAD binding element in the 5' UTR of Connective Tissue Growth Factor Gene controls its expression in myoblasts in response to Transforming Growth Factor β

Running Title: Transcriptional regulation of CTGF in myoblasts

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Abstract

Fibrotic disorders are characterized by an increase in extracellular matrix protein expression and deposition, being Duchenne Muscular Dystrophy one of them. Among the factors that induce fibrosis are Transforming Growth Factor type β (TGF- β) and Connective Tissue Growth Factor (CTGF), the latter being a target of the TGF- β /SMAD signaling pathway and is the responsible for the profibrotic effects of TGF- β . CTGF and TGF-both cytokines are increased in tissues affected by fibrosis but little is known about the regulation of the expression of CTGF mediated by TGF- β in muscle cells. In here, we described a novel SMAD Binding Element (SBE) located in the 5' UTR region of the CTGF gene important for the TGF- β mediated expression of CTGF in myoblasts. In addition, our results suggest that additional transcription factor binding sites (TFBS) present in the 5' UTR of the CTGF gene are important for this expression.

Keywords: Fibrosis, CTGF/CCN2, skeletal muscle, Duchenne Muscular Dystrophy, TGF-beta, SMAD.

Introduction

The main feature of fibrotic disorders is the increased expression and accumulation of extracellular matrix (ECM) proteins, like fibronectin and collagen. These disorders are found in several tissues, like the kidney (Ito et al. 1998), liver (Paradis et al. 2002), lung (Lasky et al. 1998) and heart (Lang et al. 2008). In Duchenne Muscular Dystrophy (DMD), an X-linked recessive disease, characterized by a severe and progressive muscle loss, fibrosis is also observed (Blake et al. 2002). Fibrosis is the result of chronic inflammatory reactions induced by tissue injury, among other factors (Wynn 2008). In the muscle of DMD patients, this chronic

injury leads not only to ECM deposition, but also to a decreased nutrition of the muscle fibers (Klingler et al. 2012) and a depletion of the muscle's satellite cells (Charge et al. 2004). Fibrosis is also observed in DMD animal models including the *mdx* mice (Bulfield et al. 1984, Stedman et al. 1991, Caceres et al. 2000, Passerini et al. 2002).

Among the factors that contribute to fibrosis, one of the most important is transforming growth factor type β (TGF- β), which augmented expression has been described in the muscles of patients with several congenital dystrophies, including DMD, and in the *mdx* diaphragm (Bernasconi et al. 1995, Bernasconi et al. 1999, Zhou et al. 2006). The canonical TGF- β signaling pathway is the following: TGF- β binds to the TGF- β receptor type II (TGFBRII), which forms a complex with TGF- β receptor type I (TGFBRI) and causes the phosphorylation and activation of TGFBRI, this complex phosphorylates SMAD2/3, which, in turn binds SMAD4 (Massague 1998). In the nucleus, the SMAD proteins recognize the sequence called SMAD Binding Element (SBE), first described as 5'-GTCTAGAC-3' (Zawel et al. 1998). Later, it was shown that SMAD complex recognize the sequence 5'-GTCT-3' or its complement 5'-AGAC-3', although the optimal binding sequence is thought to be 5-CAGAC-3' and, more importantly, the affinity observed of SMAD for this sequence was shown too low to be effective *in vivo* (Shi et al. 1998). The short length of the SBE (calculations show that it should be present once every 1024 bp in the genome), the low specificity (SMAD1, SMAD3 and SMAD4 can bind to the SBE) and the low affinity binding of SMAD proteins, suggest that additional components should be required for a specific, high-affinity binding of SMAD-containing complexes to target genes (Massague et al. 2000, Massague et al. 2005). It has been found that TGF- β induces the expression of connective tissue growth factor (CTGF/CCN2) in fibroblasts (Igarashi et al. 1993)

and, more important, the pro-fibrotic effects of TGF- β are CTGF-dependent (Grotendorst 1997, Leask et al. 2004).

CTGF is a member of the CCN family of proteins. CTGF is a secreted protein involved in many physiological processes, including adhesion, angiogenesis, migration, tissue repair and bone formation (reviewed in (Leask et al. 2006)). In pathological conditions, it has been described to participate in cancer progression (reviewed in (Chu et al. 2008, Dhar et al. 2010)), it's been proposed to have a central role in fibrosis in several tissues (reviewed in (Lipson et al. 2012, Leask 2013)) and, importantly, to be required for the onset of fibrosis *in vivo* (Li et al. 2006, Liu et al. 2011).

CTGF is increased in the muscle tissue of patients with different dystrophies, including DMD (Sun et al. 2008), and *mdx* (Cabello-Verrugio et al. 2012, Morales et al. 2013). Additionally, we found that the exogenous increase of CTGF in the muscle of wild type mice led to a decrease in muscle strength and an increase in the expression of ECM proteins (Morales et al. 2011). We have previously shown that TGF- β induces CTGF mRNA and protein expression, and also that CTGF itself reduces differentiation markers in myoblasts, like desmin and MyoD along with an increase in fibronectin accumulation (Vial et al. 2008). Furthermore, in another previous work we showed that reducing CTGF expression or blocking CTGF function in *mdx* mice, slowed down the progression of the dystrophic phenotype, seen as an increase on muscle strength, a reduction in the deposition of ECM proteins and, more important, led to a better response to muscle stem cell therapy in treated *mdx* mice (Morales et al. 2013). These findings confirm that CTGF is a very interesting target for antifibrotic therapy, so it is essential to understand how its expression is regulated, particularly in muscle cells.

A TGF- β response element was described to control the TGF- β mediated expression of CTGF in fibroblasts (Grotendorst et al. 1996) and a SBE (Holmes et al. 2001), however, the full 5' UTR region was not included in these studies. Also, several other transcription factors have been described to contribute to the TGF- β mediated expression of CTGF: SP1 in scleroderma fibroblasts (Holmes et al. 2003), MAPKs and PKC in mesangial cells (Chen et al. 2002) and fibroblasts (Leask et al. 2003); and AP-1 in keloid fibroblasts (Xia et al. 2007) and nucleus pulposus (Tran et al. 2010).

In this work, we describe a novel SBE located in the 5' UTR region of the murine CTGF gene that regulates the expression of CTGF induced by TGF- β in C2C12 myoblast cell line.

Materials and Methods

Cell culture

C2C12 mouse myoblast cells were acquired from the American Type Culture Collection and were grown in DMEM culture medium (Life Technologies) with 10% Fetal Bovine Serum (FBS, HyClone) and Penicillin-Streptomycin (Life technologies) in a culture chamber at 37°C, 5% CO₂ and controlled humidity. This cell line correspond to a subcloning made by Blau et al (Blau et al. 1985) from the myoblast cell line produced by Yaffe et al (Yaffe et al. 1977). These myoblasts have the capability of differentiate and fuse, forming contractile myotubes in differentiation conditions as described (Larrain et al. 1997).

Animals

C57BL/6JRj animals (Charles River) were kept in temperature and humidity controlled facility, and had free access to water and food until they were used for study at 8 weeks of age. All protocols were conducted in strict accordance and with the formal approval of the Animal Ethics

Committee of the Pontificia Universidad Católica de Chile and following the Paris Descartes Ethics Committee recommendations.

Transfections and luciferase reporter assay

C2C12 cells were plated on 24-well plates 24 hours prior to the transfection procedure, until 60-70% confluence was reached. Plates were rinsed with PBS and medium was replaced with Opti-MEM (Life Technologies). Later, cells were incubated with the different plasmid constructions, Lipofectamine and PLUS Reagent in Opti-MEM according to the manufacturer protocol (Life Technologies) for 4 hours. At that point, FBS was added to reach a final concentration of 10% and cells were cultured for 3 hours. The cells were then serum-starved for 12-14 hours and 10 ng/mL TGF- β (R&D systems) or vehicle was then added to the culture and incubated for further 24 hours and cells were lysed and assayed with Dual-Luciferase Reporter Assay System according to manufacturer instructions (Promega). pRL-SV40 (Promega) plasmid was used as internal transfection control and pBluescript II (Agilent) plasmid was used to normalized the amount of DNA transfected in each well. Light emission of luciferase and renilla was measured with Mithras LB 940 Multimode Microplate Reader (Berthold).

CTGF promoter cloning and plasmid construction

To clone the promoter of CTGF, we used was the BAC RP24-346F6 (Access number BH044826, from Children's Hospital Oakland Research Institute, CHORI) as template, which is part of a genomic library constructed from the spleen and brain of C57BL6/J mice. Using *Pfu* polymerase (Fermentas) and a standard PCR protocol, we cloned a 5091 bp fragment (ranging from -4872 to +219 of the CTGF gene) into the pGL3 vector (Promega) that includes the full 5' UTR region of the CTGF gene and it was fully sequenced in both strands with primer walking procedure, Genbank accession number KF905227. In addition, all the deletion mutants were

constructed using PCR in the same way as the full plasmid and sequenced. The pCTGF-0.9 vector was kindly donated by A. Leask (Abraham et al. 2000).

Site-directed mutagenesis

Site-directed mutagenesis was performed with QuikChange II Site-Directed Mutagenesis Kit (Stratagene) according to manufacturer protocol. Primer used for mutations were designed with QuikChange Primer Design online software (Stratagene). For SBE mutation, 5'-CCG CCT GGA GCG TCC AAA AAC CAA CCT CCG C-3' and 5'-GCG GAG GTT GGT TTT TGG ACG CTC CAG GCG G-3' primers were used, bases used for mutations are underlined and correct mutations were confirmed by sequencing.

Electrotransfer procedure and *in vivo* luciferase activity

C57BL6/J animals of 8 weeks of age were anesthetized by intraperitoneal injection of 0.3 ml of a mix of ketamine (100 mg/kg, Chlorkétam, Vétoquinol, Paris, France) and xylazine (10 mg/kg, Rompun, Bayer Santé, Puteaux, France) in 0.9% NaCl sterile solution. Hind legs were shaved and 30 µg of plasmid diluted in 40 µL of saline, or saline alone, were injected into *tibialis anterior* muscle of both legs. Then, the muscle was coated with conductive gel (Eko-gel, Eurocamina, Italy) to ensure electrical contact and two homemade stainless steel external plate electrodes were placed about 5mm apart at each side of the leg. Eight transcutaneous pulses of 200 V/cm and 20 ms were then applied at a frequency of 4 Hz with a square pulse electroporator (Sphergen, Evry, France). To measure luciferase activity *in vivo* after electrotransfer, mice were anesthetized and 10 mg/mL luciferine solution (Synchem) in sterile saline was injected intraperitoneally. Optical imaging was performed as described elsewhere (Bloquel et al. 2006). Briefly, luminescence was detected using a cooled GaAs intensified charge-coupled device (ICCD) camera (Photon-Imager; Biospace, Paris, France). Distance from the lens to the mouse

was of 30 cm. Operating temperature was set at -25°C. Duration of luminescence acquisition was 120s and was initiated 3 minutes after injection of the substrate.

In silico analysis of the CTGF promoter

The promoter sequence of CTGF was analyzed using the MatInspector tool (Quandt et al. 1995, Cartharius et al. 2005) and Transfac® vertebrate database version 7.0.

Results

CTGF promoter expression and analysis in myoblasts and skeletal muscle

The first attempt to study the regulation of CTGF by TGF- β , was done by Grotendorst et al. where they identified a TGF- β response element, using a 900 bp fragment of the CTGF promoter controlling the expression of the luciferase gene in human skin fibroblasts (Grotendorst et al. 1996). When we tested this promoter (in here called pCTGF-0.9) in myoblasts cells in response to TGF- β , we found a weak induction of luciferase, this induction was surprisingly low compared to our observation of the important TGF- β mediated induction of CTGF mRNA seen in the same cell line by Northern blot analysis (Vial et al. 2008). One explanation for this difference is that in myoblasts, there are additional transcription factor binding sites (TFBS) that are required for the induction of CTGF by TGF- β present in more distal regions of the CTGF promoter or in the 5' UTR of the CTGF gene. To test this hypothesis, we cloned a 5091 bp fragment of the murine CTGF promoter, ranging from -4972 to +219 of the CTGF gene, in the pGL3 vector and we conducted luciferase assays in myoblasts to test its response to TGF- β . We found that the vector carrying the larger fragment of the CTGF promoter (pmCTGF-5.1) shows an increased response to TGF- β than the vector carrying the shorter fragment (pCTGF-0.9)

(Figure 1). This result suggests that there are other TFBS that might have a role in the TGF β -mediated expression of CTGF in myoblast cell line.

Next, we wanted to know if the pmCTGF-5.1 construct could be expressed in skeletal muscle. For that purpose, we electroporated the pmCTGF-5.1 plasmid in *tibialis anterior* muscle of C57BL/6JRj mice and we measured the expression of luciferase *in vivo*. We found that the pmCTGF-5.1 plasmid shows an increased expression in the muscle than the empty vector (pGL3) from day 1 to day 7 post electrotransfer (ET) (Figure 2A and 2B). The control situations, saline injection and injection of plasmids without electrical pulses, showed no detectable luminescence (data not shown). These results suggest that the CTGF gene could be transcriptionally activated in mature muscle fibers.

CTGF promoter *in silico* analysis

In order to elucidate which TFBS could be responsible for the TGF- β mediated expression of CTGF in myoblasts, we used the MatInspector tool in order to identify the TFBS present in the CTGF promoter with special focus on those related to TGF β . The analysis, showed 1250 putative TFBS, including several sites related to TGF- β . The TFBS of importance for TGF- β /SMAD are summarized in Table 1: eight AP-1 sites were found, together with ten SP1 sites and four SBEs. A TATA-box (-38 to -32) was also recognized in the CTGF gene.

The 5' UTR region of the CTGF gene bears elements of transcriptional regulation in response to TGF- β

To analyze which TFBS are responsible for the expression of CTGF in response to TGF- β , we constructed deletion mutants by PCR as shown in Figure 3 and we conducted reporter assays on

myoblasts. As shown in Figure 4, all the deletion mutants showed a significant decrease in the activation mediated by TGF- β and no additional decrease was found between the deletion mutants, suggesting that the region comprised between -4872 and -4578 of the CTGF promoter region carries regulatory elements that could be controlling the TGF- β mediated expression of CTGF in myoblasts. This region has several putative TFBS that could account for the decrease in transcriptional activation of the CTGF promoter when deleted and we chose to test the AP-1 site (tctgAATCatg) located in -4834 to -4824 (Table 1), because it has been shown that AP-1 transcription factors could act synergically with SMAD3 to promote gene expression (Verrecchia et al. 2001, Sundqvist et al. 2013, Bai et al. 2014). The mutation of the AP-1 site showed no decrease of the reporter gene expression (Supplementary Figure 1A and 1B) suggesting that the AP-1 site is not involved in the TGF- β mediated expression of CTGF. Further analyses are required in order to comprehend how this region regulates the expression of the CTGF gene in myoblasts.

The bioinformatical analysis also showed the presence of a SBE in the 5' UTR region of the CTGF promoter (Table 1). Therefore, we first decided to construct a deletion mutant of pmCTGF-5.1 that lacks most of the 5' UTR region of the CTGF gene (Figure 5A). The deletion of the 5' UTR region of the CTGF gene showed a significant reduction on the TGF- β mediated CTGF expression (Figure 5B), suggesting that there are relevant TFBS that are responsible for the induction of the CTGF gene expression by TGF- β .

A SBE in the 5'UTR of the CTGF gene is important for the induction of CTGF by TGF- β

In the 5' UTR of the CTGF gene, there is a SBE (Table 1, 121 to 129) that could be important for the induction of CTGF by TGF- β in myoblasts. To test this hypothesis, we mutated the SBE

changing the important nucleotides in the sequence (Figure 6A) and performed reporter assays of the constructs. As seen in Figure 6B, there is a significant reduction in the expression of the reporter gene when the SBE is mutated, suggesting that this sequence is important for the transcriptional control of CTGF expression by TGF- β in myoblasts. It is important to notice that the mutation on the SBE reduced the expression of the reporter gene expression by 32% (Figure 6B), whereas the deletion of the 5' UTR reduced the expression by 67% (Figure 5B). This suggests that there are additional TFBS that are important in the TGF- β mediated expression of CTGF. Further experiments will be required in order to fully understand the mechanisms involved in this process in myoblasts.

Discussion

CTGF plays a central role in the onset and maintenance of fibrosis in the skeletal muscle. Exogenous increase of CTGF in the muscle of wild type mice can induce augmented extracellular matrix components and decrease isometric force in the muscle, all features of dystrophic pathologies (Morales et al. 2011). Interestingly, returning of CTGF expression to normal levels, led to a reversion of the fibrotic phenotype (Morales et al. 2011), showing that therapies against fibrotic pathologies could be successful even when the disease is already present. Also, reducing CTGF levels slowed down the progression of muscular dystrophy in the mdx mice and led to an increase in cell therapy (Morales et al. 2013).

There are compelling evidence showing that the muscle fiber might be an important source for CTGF production in the dystrophic context. Usually, CTGF is not expressed in the normal state of the muscle but CTGF levels increase importantly when damage and inflammation are present, and under pathological conditions (Cabello-Verrugio et al. 2012). TGF- β is increased in the

muscles of DMD patients (Bernasconi et al. 1995) and those of several dystrophic mice (Onofre-Oliveira et al. 2012). Moreover, CTGF also contributes to an increase in TGF- β binding to its receptors and an increase in TGF- β signaling (Abreu et al. 2002). It has also been shown that CTGF and TGF- β act cooperatively to elicit a fibrotic tissue response (Wang et al. 2011). In the mdx mice, TGF- β expression seems to be originated in areas populated by inflammatory cells and regenerating fibers (Zhou et al. 2006). This correlates with the fact that CTGF is expressed in the endomysium and regenerating fibers of human dystrophic patients (Sun et al. 2008) and, as we showed in here, that CTGF promoter can be activated in the skeletal muscle of mice. However, the induction of CTGF mediated by TGF- β in muscle cells has not been extensively studied. We have previously shown that TGF- β can induce the expression of CTGF in myoblast and C2C12-derived myotubes (Vial et al. 2008), this has been also found in rat L6-derived myotubes (Maeda et al. 2005), so it is of particular interest to characterize the regulation of the expression of CTGF in myoblasts, myotubes and skeletal muscle. Further analyses will be required in order to fully comprehend how CTGF expression is regulated in muscle cells.

In this paper, we found a novel SMAD Binding Element in the 5' UTR of the CTGF gene that is important for the TGF- β mediated expression of CTGF in myoblasts. In addition, we showed that the 5' UTR might have additional TFBS important for CTGF expression in myoblasts. The full 5' UTR region was not included in previous studies regarding TGF- β mediated CTGF expression (Chen et al. 2002, Holmes et al. 2003, Leask et al. 2003, Xia et al. 2007, Tran et al. 2010) and our bioinformatical analysis shows the presence of several TFBS that could be acting together with the SBE described in this paper for the TGF- β mediated CTGF expression in myoblasts. It is known that SBEs alone are not strong enough to confer TGF- β inducibility, due to SMADs low binding affinity to this site (Massague et al. 2005), this might be the reason why

we couldn't immunoprecipitate SMAD3 in the CTGF promoter when we analyzed the novel SBE located in the 5' UTR (data not shown). Due to the possible interaction of SMAD3 with other factors and the formation of a bigger transcriptional complex, the SMAD3 protein might have not been exposed within the complex to allow the recognition by the antibody during the ChIP procedure. Within the 5' UTR region that confers TGF- β induction to the reporter gene, we found several TFBSs that could be acting together with the SBE in the 5' UTR of CTGF gene. Among these TFBSs, a SP1 site which is located in very close proximity to the SBE (131 to 145) and could be evaluated for the TGF- β induction of CTGF, as the SP1 factors are reported to be acting together with SMADs proteins to enhance transcription (Botella et al. 2009, Lu et al. 2010, Fausther et al. 2012).

Our data also indicates that an upstream region (-4872 and -4578) of the CTGF promoter is involved in TGF- β mediated expression of CTGF and that the AP-1 site located in this region would not be involved with this induction. Between the TFBSs found in this region, there are two TCF/LEF (-4810 to -4794 and -4721 to -4705) sites that could be implicated in the expression of CTGF. Interestingly, several experimental evidences show a cross-talk between Wnt and TGF- β signaling, and Wnt pathway has been proposed as a novel therapeutical target for fibrotic disorders (reviewed in (Cisternas et al. 2014)).

The GTGTCAAGGGGTC element described first as a TGF- β response element (Grotendorst et al. 1996) and later named BCE-1 (Chen et al. 2002), was recognized as a RXR heterodimer and Nuclear receptor subfamily 2 factors binding site in our bioinformatical analysis (-160 to -148). This site was recognized by Retinoic Acid Receptor/Retinoid X Receptor (RAR/RXR) heterodimers and was important for All-trans retinoic acid (ATRA) mediated expression of CTGF in fibroblasts (Fadloun et al. 2008). In addition, ATRA therapy Induces myositis in

leukemia patients (van Der Vliet et al. 2000, Pecker et al. 2014), suggesting that ATRA might have a role in inflammation in the muscle. On the other hand, in activated and hence, fibrogenic hepatic stellate cells that participates in hepatic fibrosis, the levels of RAR and RXR were diminished, together with lower concentration of RXR/RAR activators (Ohata et al. 1997) and, in another study, several agonists of RAR and RXR produced a decrease in the expression of fibrotic proteins (Hellemans et al. 2004). ATRA was also found to reduce the expression of TGF- β and CTGF in scleroderma fibroblasts (Xiao et al. 2011). Moreover, ATRA has been reported to reduce TGF- β expression and signaling in lung fibrosis (Song et al. 2013) and mesangial cells (Han et al. 2014). Taken together, this evidence shows that the effect of retinoic acid in fibrosis is not yet clear (reviewed in (Zhou et al. 2012)) but it could be an interesting approach to explore its effect in CTGF and TGF- β expression and signaling in muscular fibrosis.

Recently, it has been shown a fibrotic effect of CTGF in the absence of TGF- β signaling in liver fibrosis (Sakai et al. 2014). This evidence shows the relevance of CTGF in fibrotic disorders and that, other signaling pathways, besides TGF- β /SMAD signaling, are involved in CTGF expression. Therefore, the study of the precise regulation of CTGF expression can be helpful to understand the mechanisms of the onset and progression of fibrosis in different tissues.

Acknowledgments

This study was supported by research grants from CARE PFB12/2007, FONDECYT 1110426, Fundación Chilena para Biología Celular Proyecto MF-100. Beca VRI de doctorado 2007, PUC. Beca de doctorado 2008 and Beca de apoyo a la realización de tesis doctorado 2009-2010,

CONICYT. Beca de pasantía MECESUP 2009 and 2011. Beca Chile cotutela de doctorado 2010.

We are grateful to the Animal Housing Facility (*in vivo* experiments) of the Centre de Recherche Pharmaceutique de Paris (Paris Descartes University).

Disclosure

The authors declare they have no competing interests as defined by molecular medicine, or other interests that might be perceived to influence the results and discussion reported in this paper.

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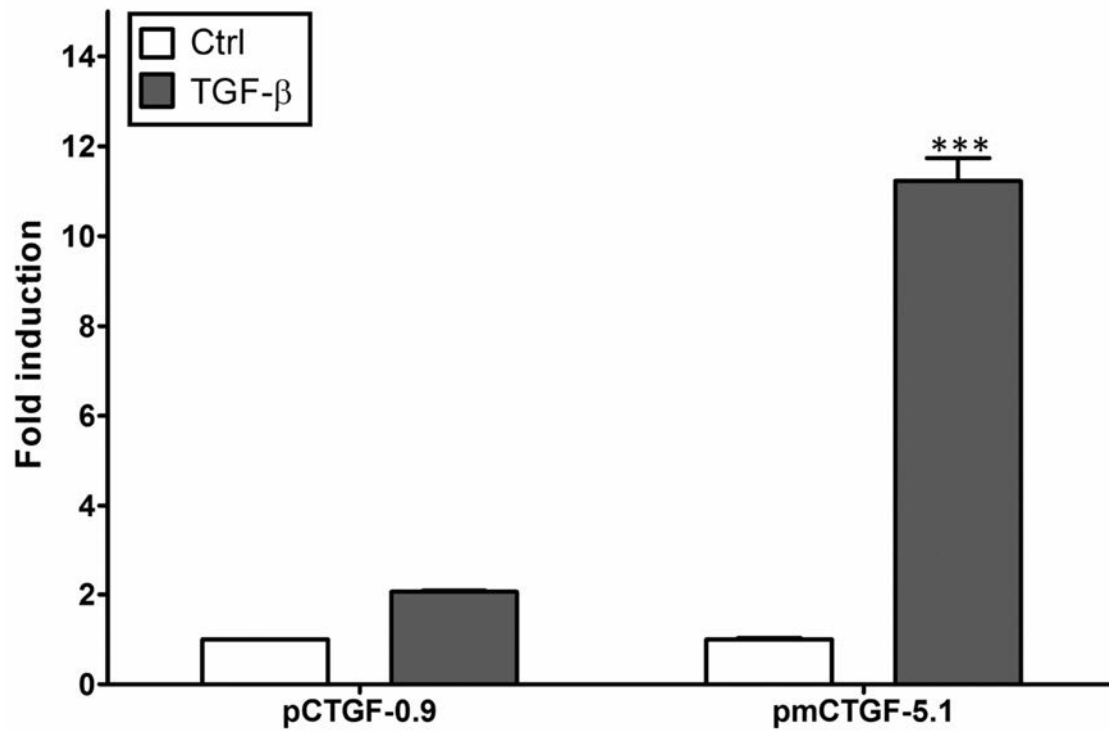
Figure 1

Figure 1: Induction of pmCTGF-5.1 vector in myoblasts. C2C12 cells were transfected with pCTGF-0.9 and pmCTGF-5.1 vectors in 24-well plates in triplicate. The graph shows fold induction of TGF-β versus control (Ctrl, vehicle), mean with SEM (n=3). ***P<0.0001 Mann-Whitney t test.

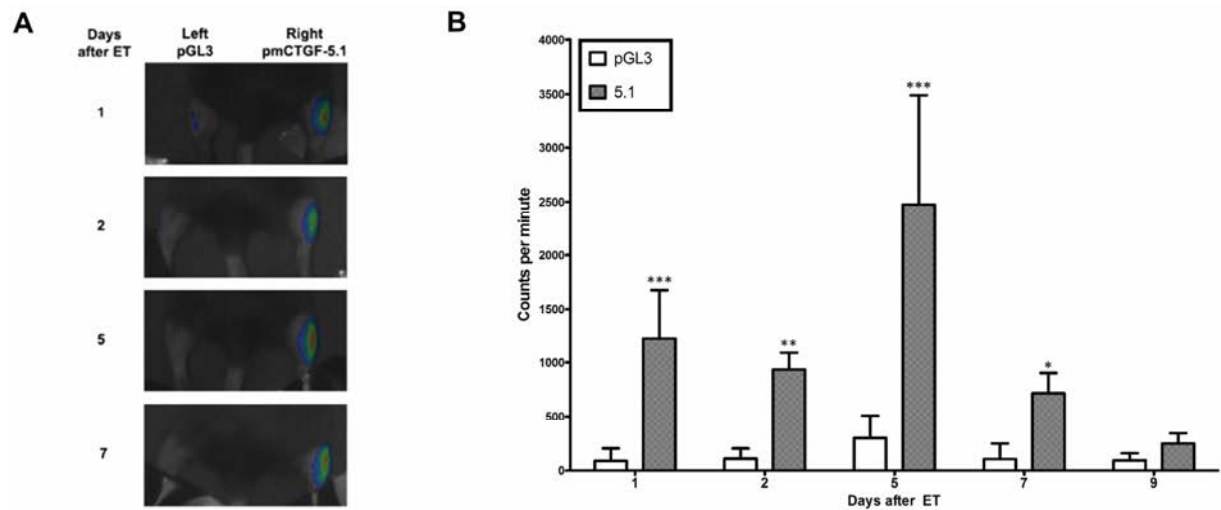
Figure 2

Figure 2: Induction of pmCTGF-5.1 in *Tibialis anterior* muscle in mice. **A.** Representative photograph of luciferine luminescence in mice muscle electroporated with pGL3 and pmCTGF-5.1. **B.** Quantification of luminescence in the muscle of mice electroporated with pGL3 and pmCTGF-5.1, each muscle electroporated with the respective plasmids was measured for 120 s. The graph shows mean with SEM (n=6). *P<0.05, **P<0.01 and ***P<0.001 2way ANOVA test.

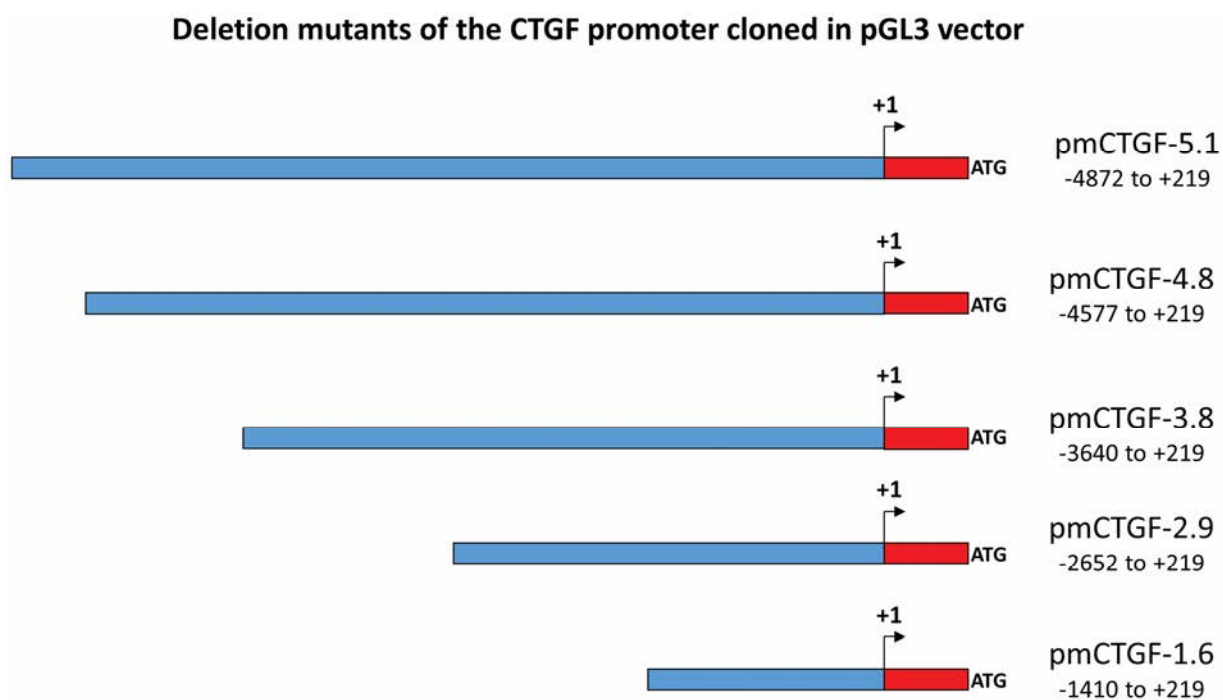
Figure 3

Figure 3: Deletion mutants of CTGF promoter region. Schematic representation of the deletion mutants cloned in pGL3 vector to conduct reporter assays. The promoter region is shown in blue whereas the 5'UTR of the CTGF gene is shown in red. The numbers indicate the position of the bases related to transcription start.

Figure 4

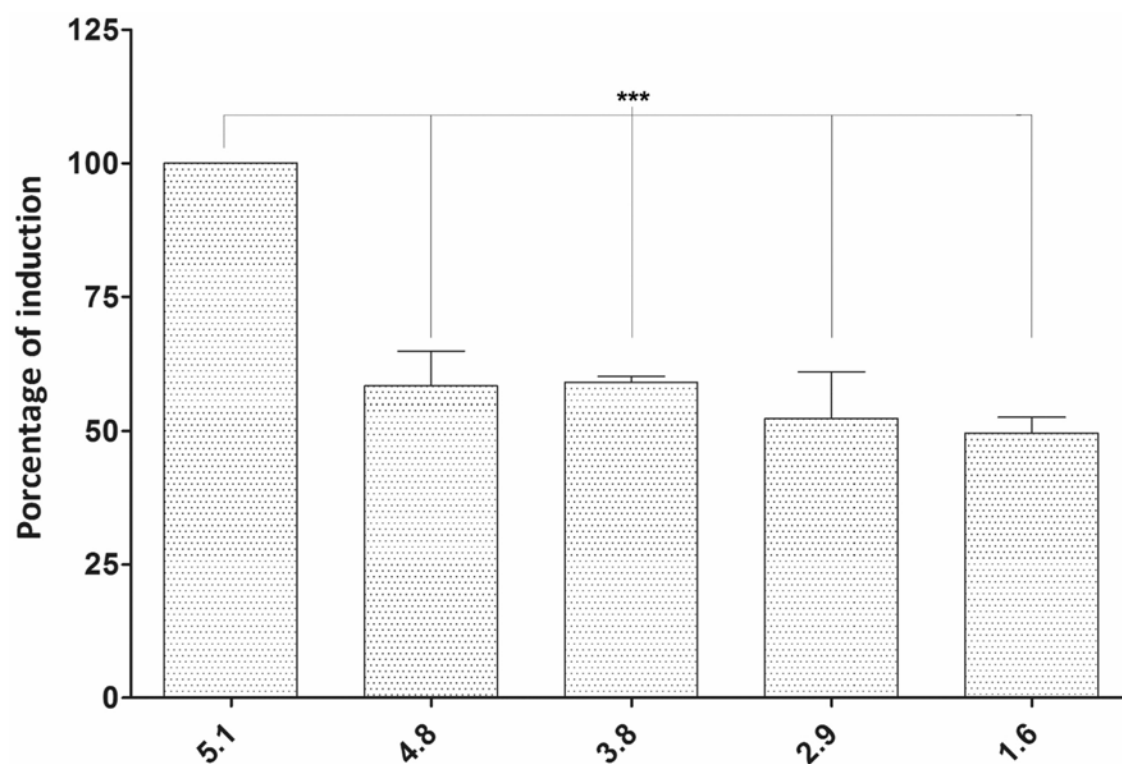


Figure 4: Reporter assay of the deletion mutants of CTGF promoter region. The level of expression of each reporter plasmid is related to the level of expression of the full length plasmid (pmCTGF-5.1). The graph shows mean with SEM (n=3). ***P<0.0001 Mann-Whitney t test.

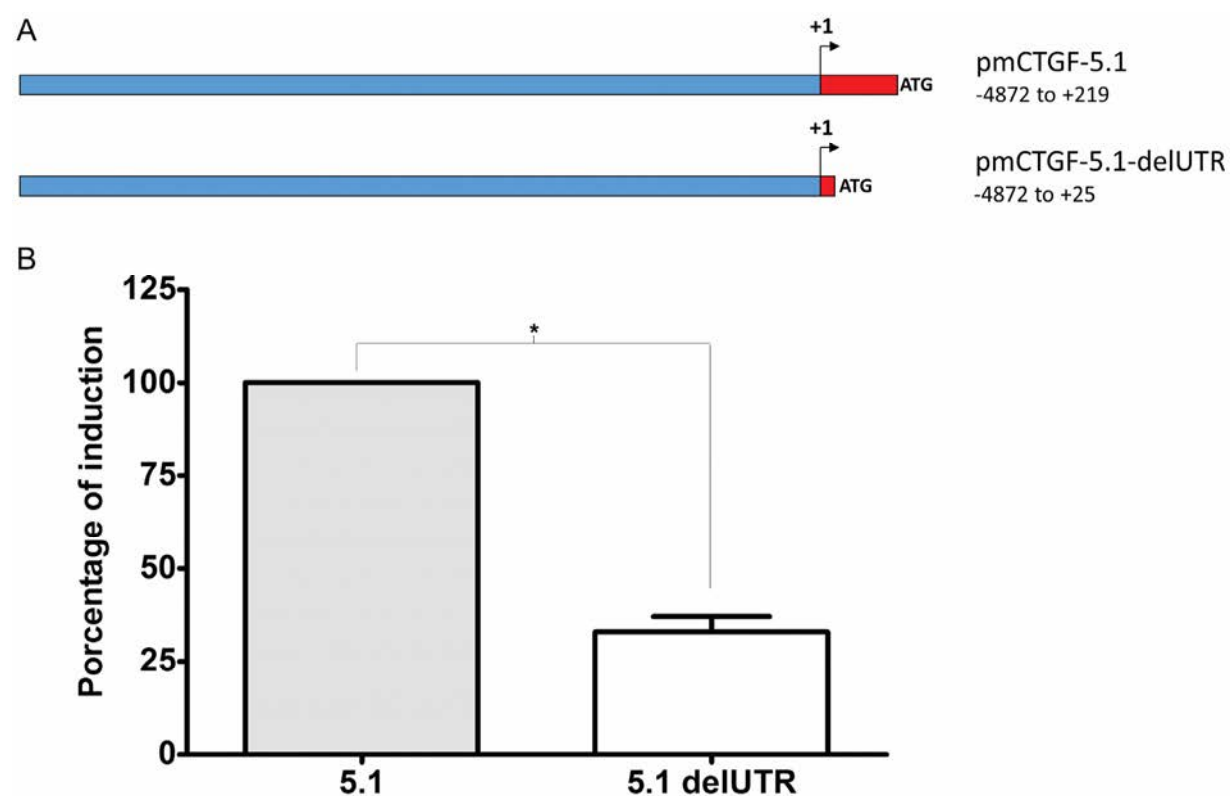
Figure 5

Figure 5: Deletion mutant of the 5' UTR of CTGF promoter. **A.** Schematic representation of the deletion mutants cloned in pGL3 vector to conduct reporter assays. The promoter region is shown in blue whereas the 5'UTR of the CTGF gene is shown in red. The numbers indicate the position of the bases related to transcription start. **B.** The level of expression of the deletion mutant is related to the level of expression of the full length plasmid (pmCTGF-5.1). The graph shows mean with SEM (n=4). *P<0.05 Mann-Whitney t test.

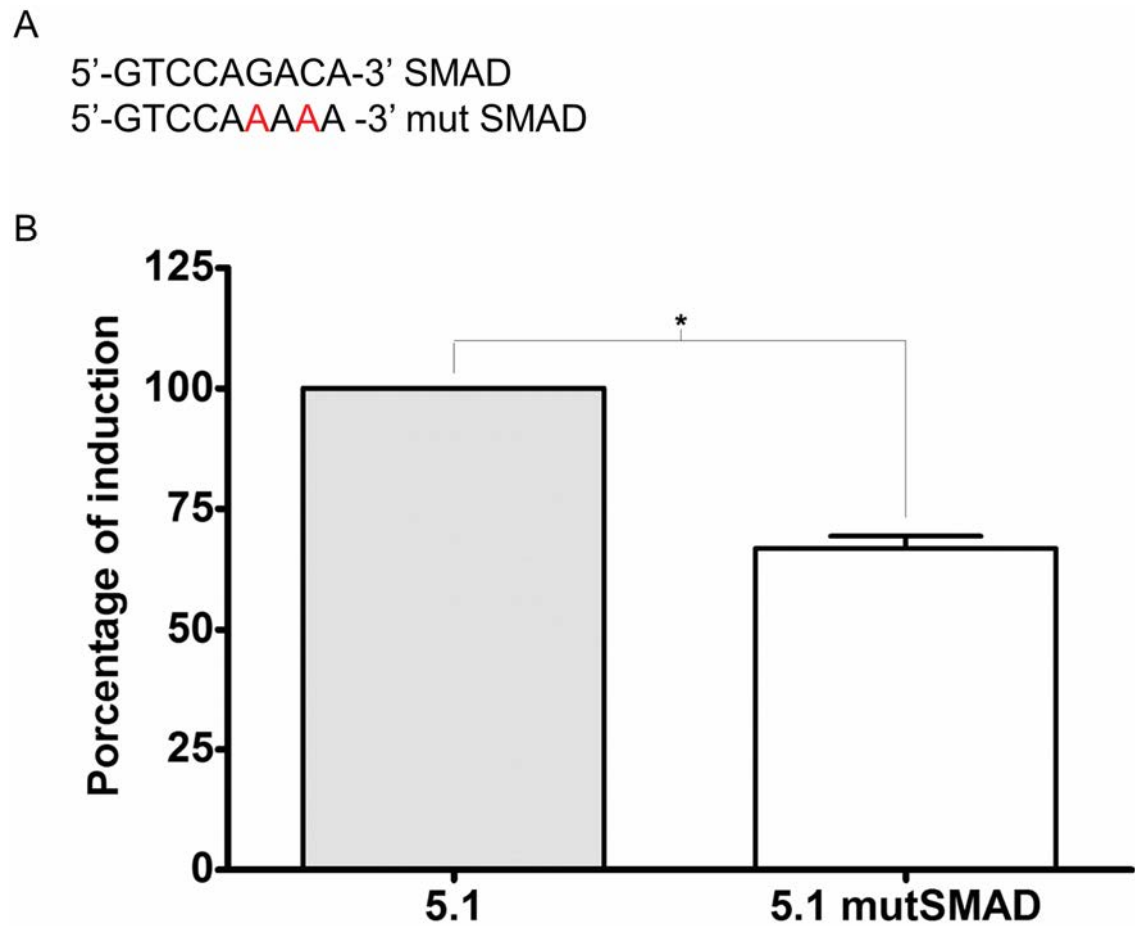
Figure 6

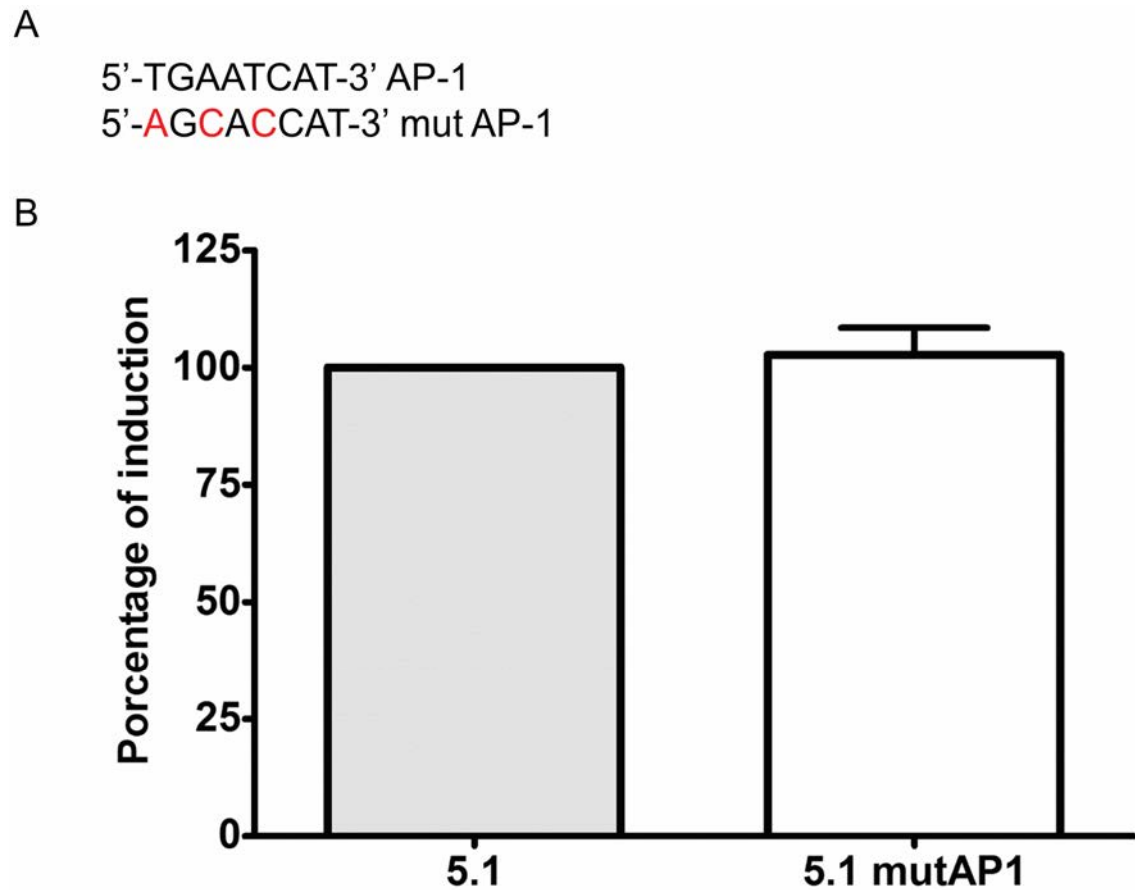
Figure 6: Mutation on the SBE of the 5'UTR of pmCTGF-5.1. **A.** The sequence of the wild type (AP-1) and mutated AP-1 element (mut AP-1). Mutated nucleotides are shown in red. **B.** The level of expression of the mutated reporter plasmid is related to the level of expression of the full length plasmid (pmCTGF-5.1). The graph shows mean with SEM (n=4). *P<0.05 Mann-Whitney t test.

Table 1: Putative Transcription Factor Binding Sites related to TGF- β in the CTGF promoter.

Detailed Family Information	Position		Strand	Sequence
	from	to		
AP1, Activating protein 1	-4834	-4824	(+)	tctgAA ^T Catg
GC-Box factors SP1/GC	-4469	-4455	(+)	ctGGG ^G tgtgtcat
GC-Box factors SP1/GC	-4362	-4348	(-)	ctttggagGGAC ^T taa
GC-Box factors SP1/GC	-3735	-3721	(-)	gcgGGGCagggggcg
AP1, Activating protein 1	-3532	-3522	(+)	tttgAG ^T Cacg
Vertebrate SMAD family of transcription factors	-3380	-3372	(+)	aGTCT ^T ggtc
AP1, Activating protein 1	-2998	-2988	(+)	gctGAG ^T catt
GC-Box factors SP1/GC	-2847	-2833	(-)	gaAGGG ^T tgtgtgaca
Vertebrate SMAD family of transcription factors	-2816	-2808	(+)	tGTCT ^T gtat
AP1, Activating protein 1	-2292	-2282	(-)	attGAG ^T taact
GC-Box factors SP1/GC	-2251	-2237	(-)	aggGGGCaggctcag
AP1, Activating protein 1	-2080	-2070	(-)	aatGAG ^T gagg
GC-Box factors SP1/GC	-1665	-1651	(-)	ggtgGGA ^G ggggtaa
Vertebrate SMAD family of transcription factors	-1307	-1299	(-)	tGTCT ^T gtct
GC-Box factors SP1/GC	-954	-940	(-)	ttGGGG ^T ttgttctg
GC-Box factors SP1/GC	-27	-13	(-)	ggcGGGCggcgctgg
Vertebrate SMAD family of transcription factors	121	129	(-)	tGTCT ^T ggac
GC-Box factors SP1/GC	131	145	(-)	cagGGGCggagggtg

5091 pb of the CTGF promoter was analyzed using MatInspector tool. The TFBS present in this region related to TGF- β are shown. Capital letters corresponds to the core sequence and red letters have a conservation value (ci-Value) in the matrix higher than 60. Position is relative to the initiation of transcription.

Supplementary Figure 1



Supplementary Figure 1: Mutation on the AP-1 site of pmCTGF-5.1. **A.** The sequence of the wild type (AP-1) and mutated AP-1 element (mut AP-1). Mutated nucleotides are shown in red. **B.** The level of expression of the mutated reporter plasmid is related to the level of expression of the full length plasmid (pmCTGF-5.1). The graph shows mean with SEM (n=3). Mann-Whitney t test was used. For AP-1 mutation, 5'-GAG TAT TCT AGT TAC GTT GAT CAA ATA AAA TCA GCA CCA TGT GTT TGA ATA ACA AAA GAA ACA AAG CAC-3' and 5'-GTG CTT TGT TTC TTT TGT TAT TCA AAC ACA TGG TGC TGGA TTT TAT TTG ATC AAC GTA ACT AGA ATA CTC-3' primers were used, bases used for mutations are underlined. Mutations were confirmed by sequencing.

3. CHAPTER II

The results presented in the next section were obtained to accomplish the Objective 2, previously described.

The manuscript presented below, will be submitted to Clinical Science.

**Electrotransfer of Soluble Tumor Necrosis Factor Receptor I improves muscle strength
and reduces fibrosis in dystrophic mice**

Running Title: TNF blockage improves muscle dystrophy in mdx mice

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Abstract

The Duchenne Muscular Dystrophy (DMD) is an X-linked disease characterized by progressive and accumulative damage in the muscle due to the absence of the dystrophin protein. Fibrosis is also present in the muscle of DMD patients and several animal models, due to continuous inflammation in the tissue produced by contraction-relaxation cycles. TNF is a potent inflammatory cytokine that is present in DMD muscles and is responsible for muscle necrosis and inflammatory cell infiltration in several tissues. In this study, we show that the increased expression of sTNFR1 in dystrophic mice by electrotransfer (ET) in mouse tibialis anterior (TA) attenuates inflammation, damage and fibrosis in the skeletal muscle of the mdx mice. In addition, we found an increase in isolated muscle strength in the mdx mice. Therefore, we propose that ET could be used as an efficient anti-TNF therapy for treating muscle dystrophies.

Keywords: TNF, TNF receptor, Muscular Dystrophy, Fibrosis, Muscle Strength, Muscle Therapy

Introduction

Muscular dystrophies are a diverse group of genetic muscular diseases, being Duchenne Muscular Dystrophy (DMD) the most severe (Shieh, 2013). DMD is an X-linked disease that affects between one in 3600 to 6000 live male births (Bushby et al., 2010). It is characterized by the absence of the protein dystrophin (Koenig et al., 1987; Kunkel et al., 1987), that acts as an anchor between the actin cytoskeleton and the basal lamina of the muscle fiber (Ervasti and Sonnemann, 2008). This absence might cause the rupture of the sarcolemma of the muscle fiber during contraction (Allen and Whitehead, 2011). Thus, children with this condition will

gradually show muscle weakness, they will require the use of wheelchair in their teens and they will present cardiac, orthopedic and respiratory complications that will lead to death between the second and third decade of life (Bushby et al., 2010). At the moment, there is no cure or efficient therapy for this disease.

Pathological features of DMD includes myofiber atrophy, fatty accumulation, degeneration and necrosis of muscle fibers, elevated creatine kinase levels, inflammation and fibrosis (Alvarez et al., 2002; Desguerre et al., 2009a; Desguerre et al., 2009b; Serrano and Munoz-Canoves, 2010; Spencer and Tidball, 2001; Villalta et al., 2011; Zhou and Lu, 2010). Fibrosis is the increased expression and accumulation of Extracellular Matrix (ECM) proteins, like collagen and fibronectin; and it is the results of chronic inflammation induced by repetitive injury in a tissue, among other factors (Wynn, 2008). Fibrosis is also observed in DMD animal models including the *mdx* mice (Bulfield et al., 1984; Caceres et al., 2000; Passerini et al., 2002; Stedman et al., 1991). In addition, we have previously showed that decreasing fibrosis leads to a better response to muscle stem cell therapy in treated *mdx* mice, a murine model for DMD (Morales et al., 2013b). These data suggests that interfering with fibrosis onset by reducing inflammation, might be useful for stem cell based therapies in DMD.

Tumor necrosis factor (TNF) is a potent inflammatory cytokine that increases when myofibers are damaged, it is expressed in myoblasts and myotubes (Collins and Grounds, 2001) and it is increased in the plasma levels of DMD patients (Porreca et al., 1999). TNF acts as a homotrimer that binds to the TNF Receptors to exert its effects. The TNF receptor I (TNFRI), which is ubiquitously expressed, or the TNF receptor II, which is mostly inducible and present in endothelia and hematopoietic cells (Tracey et al., 2008). More importantly, TNFRI has been recognized as the main TNFR responsible for the initiation of the inflammatory response

(Loetscher et al., 1993; van der Poll et al., 1996). Unlike TNFRII, TNFRI bears an intracellular Death Domain (DD) close to the C-terminal end of the protein (Tartaglia et al., 1993). The binding of TNF to TNFRI can trigger the formation of two different TNFRI signaling complexes, while Complex I leads to anti-apoptotic effects and pro-inflammatory events, the formation of Complex II leads to apoptosis signaling (Ihnatko and Kubes, 2007; Micheau and Tschopp, 2003). After TNFRI activation, the TRADD adaptor protein interacts with the TNFRI DD and, subsequently, allows the interactions with several adaptor proteins such as TRAF2, cIAP1, cIAP2 and RIP1 forming the Complex I, which, in turn, activates several signaling pathways that will lead to NF- κ B and AP-1 transcription factors activation (Aggarwal et al., 1996; Chen et al., 2008; Hsu et al., 1996; Mahoney et al., 2008; Micheau and Tschopp, 2003). On the other hand, the formation of complex II or death inducing signaling complex (DISC), requires the internalization of the TNFRI after complex I is formed transiently and then TNFRI interacts with FADD and caspase 8/10 to initiate apoptosis (Micheau and Tschopp, 2003). In addition, soluble versions of the TNF receptors (sTNFR) occurs naturally and might have a role in the modulation of the TNF response (Engelmann et al., 1990; Nophar et al., 1990; Seckinger et al., 1990). Also, the levels of sTNFRs in serum increase with several pathological conditions (Aderka et al., 1991; Cope et al., 1992; Diez-Ruiz et al., 1995; Thevenon et al., 2010; Torre-Amione et al., 1996).

There are reports that shows that TNF might have an anti-fibrotic effect: reduces the expression of collagen I (Verrecchia and Mauviel, 2004), suppresses transforming growth factor type β (TGF- β) signaling, and important pro-fibrotic cytokine (Yu et al., 2009) and reduces the expression of connective tissue growth factor (CTGF), a fibrotic effector of TGF- β (Laug et al., 2012). But, on the other hand, there are also numerous reports that support the notion that

reducing inflammation by the inhibition of TNF signaling, has a largely beneficial effect that results in less damage and, therefore, less fibrosis. TNF and its receptors are required for fibrosis onset in lung (Liu et al., 1998; Piguet et al., 1990a), TNF increases fibroblast proliferation necrosis in the dermis (Piguet et al., 1990b), also enhances the activation of pro-fibrotic fibroblasts (Camara and Jarai, 2010) and can lead to an induction of TGF- β (Sullivan et al., 2005).

Several anti-TNF therapies are in use for the treatment of rheumatoid arthritis using a recombinant version of sTNFRII or blocking antibodies (Thalayasingam and Isaacs, 2011) and also, a pegylated form of sTNFRI has been used with good results reducing renal fibrosis (Therrien et al., 2012), but they all rely on the production of purified proteins, which can be expensive and require repetitive injections. Gene therapy offers many advantages compared to recombinant protein therapies, such as low cost, good quantity and long-term production of the required protein with a single procedure and little secondary effects. We have previously shown that by expressing a chimeric protein composed of the sTNFRI receptor coupled with the Fc fragment of IgG1 by electrotransfer (ET) in skeletal muscle, has been proven effective for the treatment of rheumatoid arthritis in a mouse model (Bloquel et al., 2004) and uveitis in a rat model (Bloquel et al., 2006).

In this study, we show that the increased expression of sTNFRI in the mdx mice, the murine model of DMD by the ET in mouse tibialis anterior (TA) of mouse sTNFRI-IgG1 chimera encoding plasmid attenuates inflammation, damage and fibrosis in the skeletal muscle of the mdx mice. In addition, we found an increase in isolated muscle strength in the mdx mice. Therefore, showing that ET might be used as an efficient anti-TNF therapy for treating muscle dystrophies and other inflammation related pathologies.

Materials and Methods

Animal handling, electrotransfer and experimental exercise

C57BL/10ScSnJ and C57BL/10ScSn-Dmd^{mdx}/J strain mice (The Jackson Laboratory, USA) were kept in a facility with controlled temperature and humidity and had free access to water and food until they were used for study at 8 weeks of age. For ET, animals were anesthetized using isoflurane gas, hind legs were shaved, and 30 µg of empty plasmid or soluble TNF Receptor I plasmid diluted in 40 µL of saline, were injected into TA muscle of the left leg. Then, the muscle was coated with conductive gel to ensure electrical contact and two platinum external plate electrodes (7mm Tweezertrodes, BTX, Harvard Apparatus, USA) were placed about 5mm apart at each side of the leg. Eight transcutaneous pulses of 180 V/cm and 20 ms were then applied at a frequency of 4 Hz with an ECM 830 square pulse electroporator (BTX Harvard Apparatus, USA). Serum was collected by retro orbital blood extraction in anesthetized mice and used for measuring soluble TNF Receptor I expression with Mouse TNF RI/TNFRSF1A DuoSet ELISA kit (R&D Systems, USA). Ten session of forced experimental exercise was made by using a treadmill three times per week for 30 minutes; each session was carried at a velocity of 12 m/min and started at day 7 post-ET. The animals were euthanatized and contralateral TA or diaphragm muscles were removed for analysis. All protocols were conducted in strict accordance and with the formal approval of the Animal Ethics Committee of the Pontificia Universidad Católica de Chile and following the Paris Descartes Ethics Committee recommendations.

Skeletal muscle histology

Muscle architecture and histology were analyzed by hematoxylin-eosin staining (H&E) of transverse sections of muscle as described elsewhere (Cabello-Verrugio et al., 2012).

Immunoblot analysis

Muscles were homogenized in 10 volumes of Tris-EDTA buffer with 1 mM PMSF as described previously (Morales et al., 2011). Briefly, protein concentration of muscle extract was determined using the BCA protein assay kit (Pierce, USA) and 50µg of the samples were subjected to SDS-PAGE 10% polyacrylamide gels, transferred onto PVDF membranes (Schleicher & Schuell, USA), and probed with specific antibodies against fibronectin (Sigma, USA) and tubulin (Sigma, USA) as described previously (Morales et al., 2013b). All immunoreactions were visualized using an enhanced chemiluminescence kit (Pierce, USA). Densitometric analysis and quantification were performed using ImageJ software (NIH, USA).

Immunofluorescence microscopy

Cryosections of 7 µm were fixed in 4% paraformaldehyde and blocked for 1 hour in 10% BSA and incubated for 1 hour at room temperature with specific antibodies against fibronectin (Sigma, USA) and F4/80 (Abcam, USA). FITC-conjugated goat anti rabbit IgG and rabbit anti mouse IgG (Invitrogen, USA) were used as secondary antibodies. Nuclear staining was achieved by incubating the muscle sections with 1 µg/ml Hoechst 33258 in PBS for 10 min (Morales et al., 2013b). The coverslips were mounted using Fluoromount (Dako, USA) and observed under a Nikon Diaphot inverted microscope equipped for epifluorescence. Area of fluorescence was measured in the photographs of randomly found loci of degeneration/regeneration in the cryosections of muscles treated with anti-F4/80 antibodies and then measuring the area corresponding to positive fluorescence using ImageJ software (NIH, USA).

Contractile Properties of Isolated Muscle

The isometric force of isolated muscles was measured as described previously (Morales et al., 2013b). Briefly, optimum muscle length (Lo) and stimulation voltage were determined from

micromanipulation of muscle length to produce maximum isometric twitch force. Maximum isometric tetanic force (P_o) was determined from the plateau of the frequency-force relationship after successive stimulations at 1 to 200 Hz for 450 ms, with 2-minute rests between stimuli. After functional testing, muscles were removed from the bath, trimmed of their tendons and any adhering non-muscle tissue, blotted once on filter paper and weighed. Muscle mass and L_o were used to calculate specific net force (force normalized per total muscle fiber cross-sectional area (CSA), mN/mm^2) (Morales et al., 2013b).

Results

Expression of smTNFRI improves histological features of tibialis anterior muscle in the mdx mice

We wanted to use the TA muscle as a biofactory to produce the chimeric protein made from the murine soluble TNF receptor I (smTNFRI) and the Fc fragment of the murine IgG1 in order to get a systemic anti-TNF therapy. Our first goal was to assay the expression of the smTNFRI chimeric protein in sedentary mdx mice or in mdx mice subjected to forced exercise using a treadmill to induce muscle damage and inflammation. For this, we ET the plasmids containing the smTNFRI-IgG1 chimera and an empty vector as a control in the *tibialis anterior* (TA) muscles of mdx mice and we determined the concentration of smTNFRI in the serum at different days post-ET. We found a strong expression of the smTNFRI in animals electroporated with the coding plasmid (smTNFRI) as compared with the animals electrotransferred with empty vector (Ctrl) which show a basal expression of the endogenous soluble receptor (Figure 1).

Next we wanted to know whether the expression of smTNFRI improved the histological features of the mdx muscle. We wanted to evaluate the systemic effects of smTNFRI, for that reason,

we ET the left muscle of mice and used the contralateral muscle for the analyses. The mdx muscle shows the typical features of the dystrophy phenotype (Figure 2): the heterogeneous size of muscle fibers, the central nuclei and the foci of degeneration and regeneration with the infiltration of inflammatory cells (arrows). The number and size of these foci are increased by the damage induced by exercise (Sedentary vs Exercised, Figure 2) and are greatly reduced by the expression of smTNFRI (Control vs smTNFRI). These could be caused by a decreased in the necrosis of muscle fibers and the inhibition of inflammatory cell infiltration caused by the blockage of TNF by smTNFRI.

Blockage of TNF with smTNFRI expression changes muscle architecture and reduces inflammatory cell infiltration in the tibialis anterior muscle of exercised mdx mice

Given that we found the largest histological differences between exercised mdx mice treated with smTNFRI vs Control mice, we wanted to evaluate the difference in the accumulation of fibronectin (FN) and the presence of macrophages in muscle cryosections of these groups. For that purpose, we performed an indirect immunofluorescence to visualize FN as a marker of fibrosis and F4/80 as a marker of macrophages. We found that the production of smTNFRI reduces the fluorescence of fibronectin in the TA muscle of mdx mice (Figure 3A), but it does not decrease the amount of FN protein (Figure 3B and 3C). On the other hand, we found lesser amounts of macrophage infiltration in the muscle sections (Figure 4A and 4B). These results shows that blocking TNF action by ET improves muscle architecture and reduces infiltration of inflammatory cells in the TA muscle of the mdx mice.

Expression of smTNFRI increased muscle strength in tibialis anterior muscle of the mdx mice

Next, we wanted to know if the amelioration of the histological features of the mdx muscle has an effect in muscle strength. For this, we measured isometric force in isolated TA muscles of mdx mice. We found that muscles from mice treated with smTNFRI showed a significant increase in isometric force compared to control mice (Figure 5A), reaching the strength level of the wt mice (Figure 5A and 5B). These results show that the amelioration of the histological features of the TA muscle is associated with an increase in muscle performance.

Electrotransfer of smTNFRI also reduces fibrotic features in diaphragm muscle of the mdx mice

We were also interested in studying the effect of the expression of the smTNFRI in the TA has an effect on other muscles. The diaphragm muscle is in constant cycles of contraction and relaxation and the contractile force continuously damages it during respiration. For this reason, the histology of the diaphragm muscle shows more damage than any other muscle in the body (Figure 6, control) but it still shows a slightly better histology when the mice has been electrotransferred with smTNFRI (Figure 6, smTNFRI) and shows a decrease in the deposition of fibronectin (Figure 7). This improvement in the histology and reductions of fibronectin in the muscle is accompanied with a minor, non-significative, increase in strength (Figure 8). These data suggest that the treatment with smTNFRI improves diaphragm histology and reduces fibronectin, a marker of fibrosis.

Discussion

There are many different approaches directed to develop effective therapies to treat DMD. We have worked mainly in aiming our efforts in diminishing fibrosis, because this is one of the main sources of the failure of cell based therapies (Morales et al., 2013b). We have demonstrated that diverse targets can reduce fibrosis and ameliorate muscle function, such as reducing the amount of CTGF (Morales et al., 2013b), targeting some members of the Renin-Angiotensin system (Acuna et al., 2014; Morales et al., 2013a) and by using andrographolide, a botanic drug, to inhibit NF- κ B (Cabrera et al., 2014) with very good results in mice. In here, we have shown that electroporation of the soluble TNF receptor I Improves several features of the dystrophic muscles. Another group used a similar approach with very good results preventing injury in radiation-Induced lung fibrosis, using a two injections protocol of a plasmid coding for smTNFRI and transfected skeletal muscle of mice with polyetylenimine (Przybyszewska et al., 2011). Comparing both methods, ET seems to be more effective, since ET can achieve 50 times higher serum levels of smTNFRI using only one injection, probably due to a higher efficiency of plasmid transfer to muscle fibers.

First, we showed that the systemic production of smTNFRI chimera produces a beneficial effect in the TA muscle, improving muscle architecture and reducing inflammation and, more importantly, increasing the strength of the TA mdx muscle to levels indistinguishable from wt TA muscle. In the TA muscle, the treatment with smTNFRI was able to prevent the onset of fibrosis, since, at the time of the beginning of the treatment, the TA muscle only shows minor damage. This might be the reason why we did not find a decrease in FN levels. As the muscle is not fibrotic yet, we were able to improve muscle architecture, seen as a decrease in the space between the muscle fibers.

A different scenario occurs in the diaphragm muscle, as this muscle shows an advance degree of damage and fibrosis. Even though we are able to ameliorate muscle histology and fibrosis in the diaphragm of mdx muscle, we only found a small increase tendency in muscle strength but it was significantly lower than that of wt diaphragm. This could be explained by the constant contraction/relaxation cycles that the diaphragm muscle has to endure during the mice life. We believe that better results could be achieved by starting the treatment in younger animals or with longer periods of treatment, in order to be able to rescue the damage induced by the continuous use of the diaphragm muscle for respiration. At least, we were able to stop the progression of fibrosis and muscle damage in the diaphragm muscle of mdx mice.

There are five anti-TNF therapies approved for the treatment of rheumatoid arthritis (Thalayasingam and Isaacs, 2011), two of them has been used to protect inflammatory damage in the muscle. Enbrel® (etanercept), a chimeric protein of the soluble TNF receptor II with the Fc fragment of human IgG, has been used to successfully to protect dystrophic muscle from inflammatory damage in the mdx mice (Nemoto et al., 2011) and Remicade® (infliximab), an anti-TNF antibody, has also been used and shown to reduce muscle fiber necrosis in dystrophic mice (Grounds and Torrisi, 2004). In addition, a modification of the infliximab antibody, cV1q, has been used to reduce damage and necrosis in muscles of wt and mdx mice (Piers et al., 2011; Radley et al., 2008). Although successful, these systemic anti-TNF therapies have risen some concerns regarding the apparition of side effects in human patients (Desai and Furst, 2006; Jain and Singh, 2013; Rosenblum and Amital, 2011), like malignancies (Cohen and Dittrich, 2001), lupus (Williams et al., 2009), dermatological adverse reactions (Mocci et al., 2013), interstitial lung disease (Perez-Alvarez et al., 2011) and infections (Besada, 2011; Komano et al., 2011). Also, there is a recent study showing that infliximab (cV1q) therapy on mdx mice shows

negative effects on cardiac function (Ermolova et al., 2014). Taken together, this evidence suggests that anti-TNF systemic treatments should be taken with extreme caution in order to avoid secondary effects.

Even though the ET therapy we use in this study is systemic, titration of the amount of plasmid used for ET could be used in order to minimize the quantity of smTNFRI released to the serum while maintaining a higher local dose in the muscle. In a model of uveitis, we have previously shown that a therapeutic effect can be achieved locally without any detection of sTNFRI in serum, reducing the possibility of systemic secondary effects (Bloquel et al., 2006). Therefore, more studies should be carried out in order to achieve the local and systemic effect of sTNFRI without the dangers of secondary effect due to high sTNFRI levels in the serum. Another advantage of ET therapy is that it avoids repeated injections needed with the purified protein or antibody treatments.

There are several studies that show that ET therapies could be used to treat numerous pathologies with low risk of secondary effects (Rochard et al., 2011; Trollet et al., 2006). Also, inducible promoters can be used in order to achieve the desired concentration of the protein produced, locally and/or systemically. In addition, there are more than 80 clinical trials in different stages using ET, which shows an increased interest in using ET to treat different diseases like keloids, cancer, HIV, among others (clinicaltrials.org).

In conclusion, in this study we show that ET of smTNFRI in the muscle could be used as an effective therapy for the treatment of DMD and other inflammatory diseases. Although more studies are needed in order to evaluate the desired levels of smTNFRI in the serum, in order to avoid secondary effects that could be produced by systemic anti-TNF therapy.

Acknowledgments

This study was supported by research grants from CARE PFB12/2007, FONDECYT 1110426, Fundación Chilena para Biología Celular Proyecto MF-100. FONDECYT 3140396. Beca VRI de doctorado 2007, PUC. Beca de doctorado 2008 and Beca de apoyo a la realización de tesis doctorado 2009-2010, CONICYT. Beca de pasantía MECESUP 2009 and 2011. Beca Chile cotutela de doctorado 2010.

We are grateful to the Animal Housing Facility (in vivo experiments) of the Centre de Recherche Pharmaceutique de Paris (Paris Descartes University).

Disclosure

The authors declare they have no competing interests or other interests that might be perceived to influence the results and discussion reported in this paper.

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Figure 1

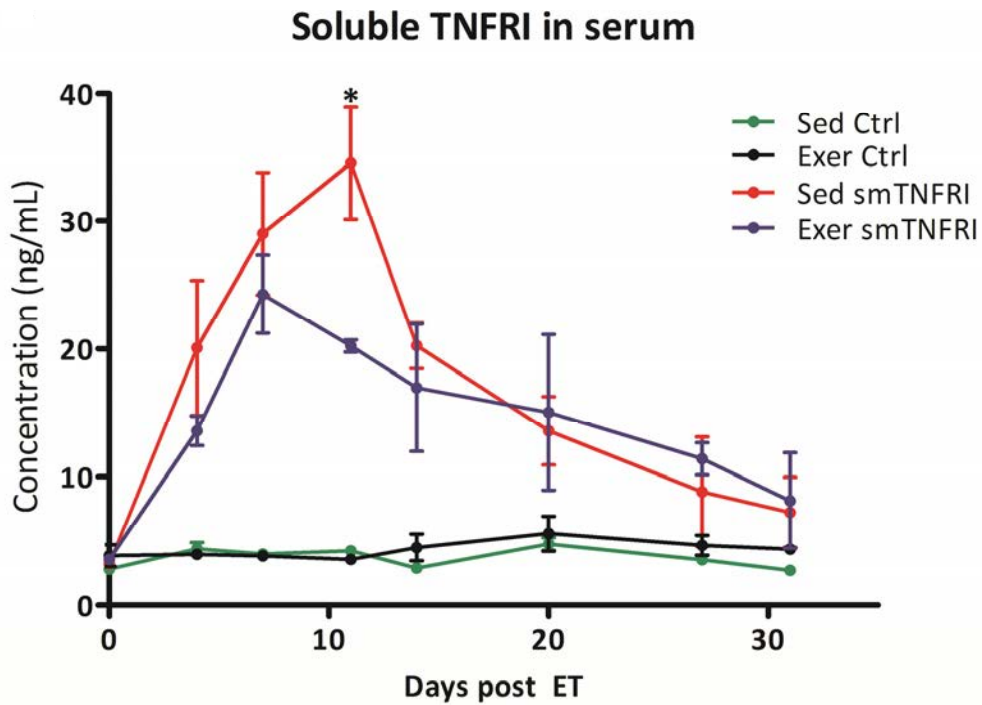


Figure 1: Expression of soluble TNF Receptor I in the serum of mdx mice. Tibialis anterior muscle of mdx mice was electrotransferred with empty vector (ctrl) of one coding for smTNFRI-IgG1 (smTNFRI). Each group was separated in two groups, one group was subjected to voluntary exercise (Exer) and one was sedentary (Sed). Every few days serum was collected and TNFRI concentration was measured, for a minimum of 3 animals for each day to a maximum of 5 animals. Graph shows mean plus SEM. 2way ANOVA with Bonferroni post test was used (*, $p < 0.05$).

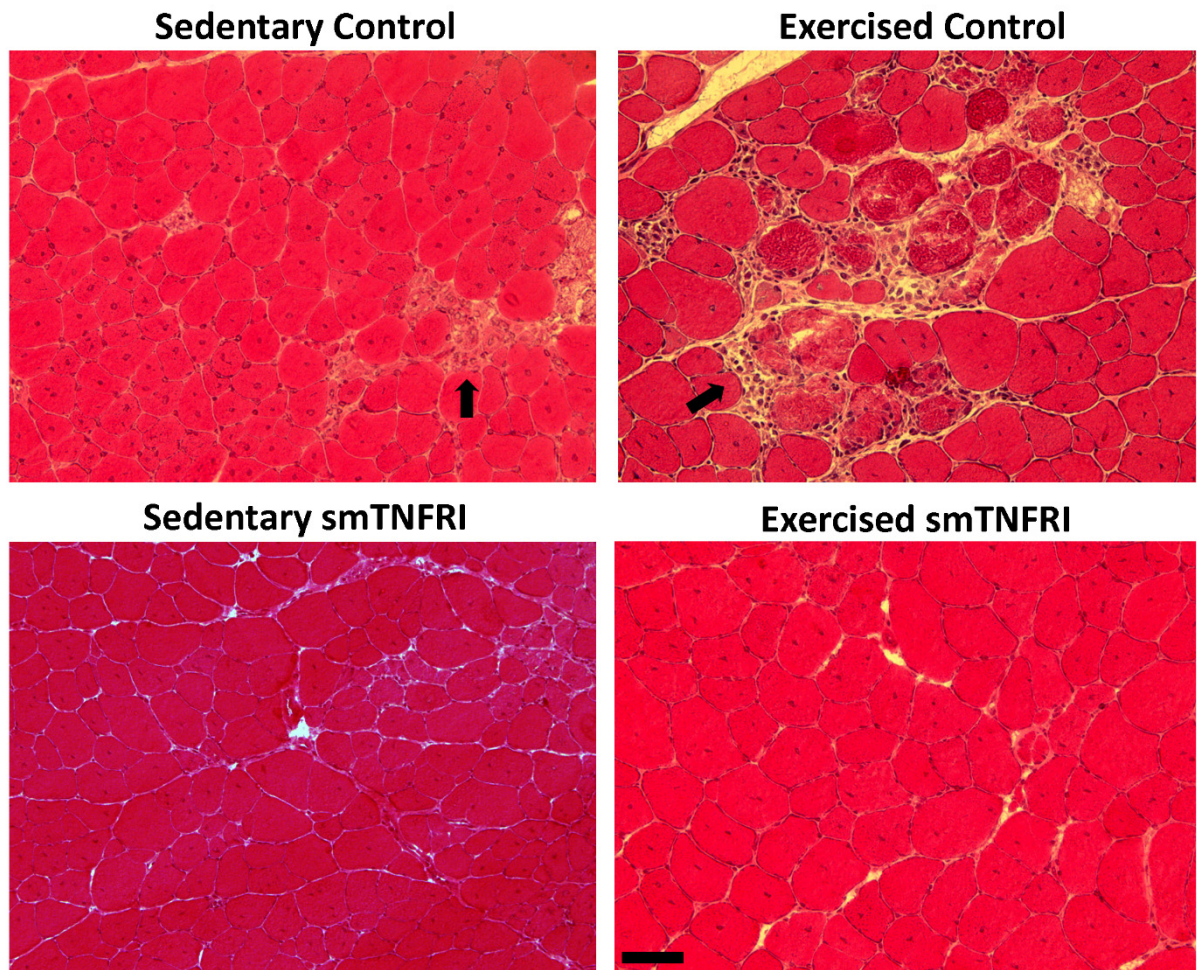
Figure 2

Figure 2: Expression of smTNFRI improves histological features in tibialis anterior muscles of mdx mice. Cryosections of contralateral tibialis anterior muscles of each group of mdx mice were treated for H&E staining and photographed (Bar=50 μ m). A representative photograph for each condition are shown (n=6).

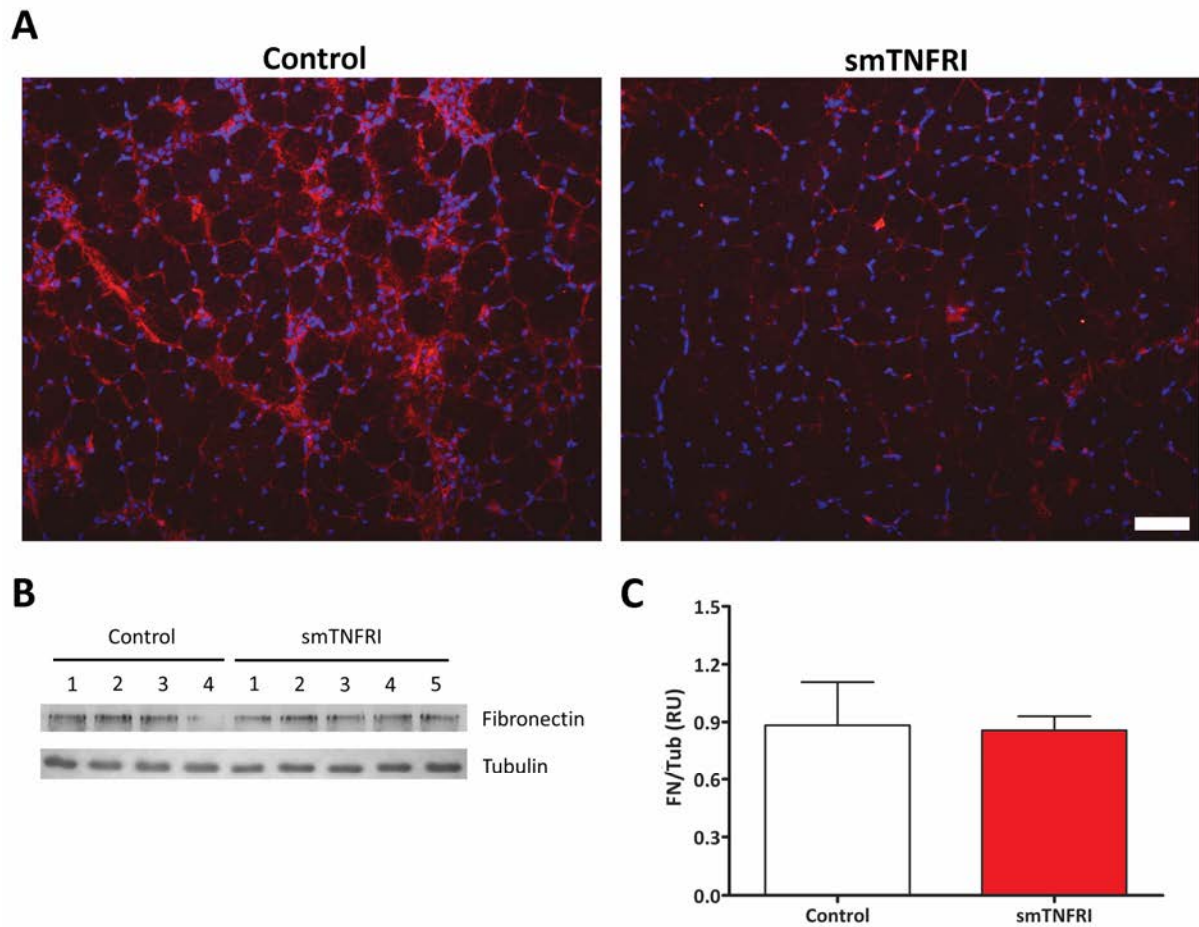
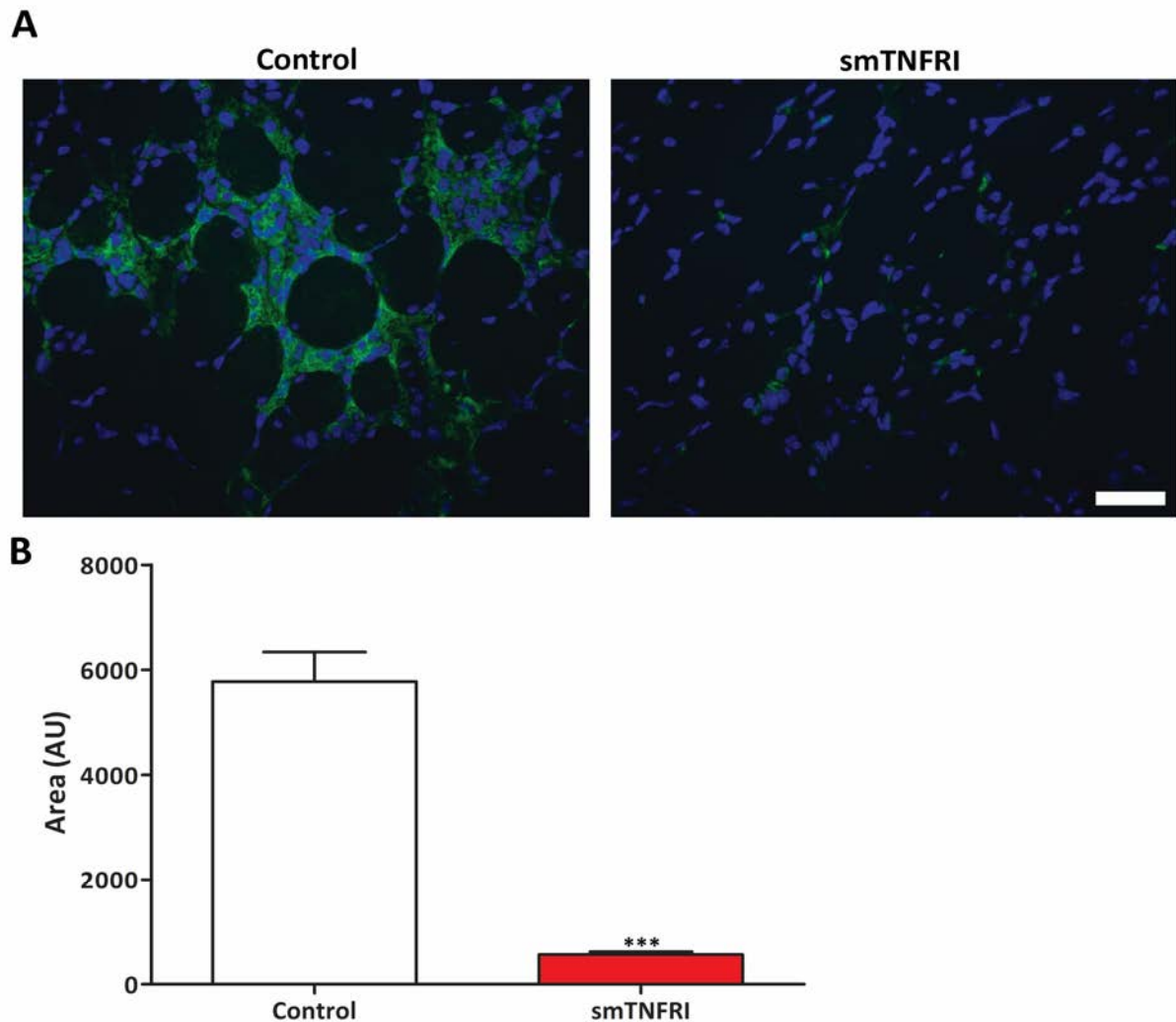
Figure 3

Figure 3: Expression of smTNFRI ameliorates TA muscle architecture. A. Cryosections of muscles from animals exercised and electrotransferred with empty vector (Ctrl) or smTNFRI-IgG1 encoding plasmid were stained for fibronectin and Hoechst 33258 and photographed. Photographs are representative of n=6 animals. Bar 100 μ m. B. Control (lanes 1-4) and smTNFRI treated (lanes 5-9) muscle extracts were analyzed by western blotting to detect fibronectin (FN) and tubulin (loading control). B. Relative expression of fibronectin in muscle extracts. C. Graph shown mean plus SEM, t test analysis was used.

Figure 4**Figure 4: Expression of smTNFRI decreases macrophage infiltration in TA mdx muscle.**

A. Cryosections of muscles from animals exercised and electrotransferred with empty vector (Ctrl) or smTNFRI-IgG1 encoding plasmid were stained for F4/80 and Hoechst 33258 and photographed. Photograph are representative of n=6 animals. Bar 50µm. B. Two or three photographs were taken randomly in the muscle cryosections of control and treated animals, and area (AU=Arbitrary Units) positive for immunofluorescence was measured with ImageJ software. Graph shows mean plus SEM, t test was used (***) $p < 0.0001$.

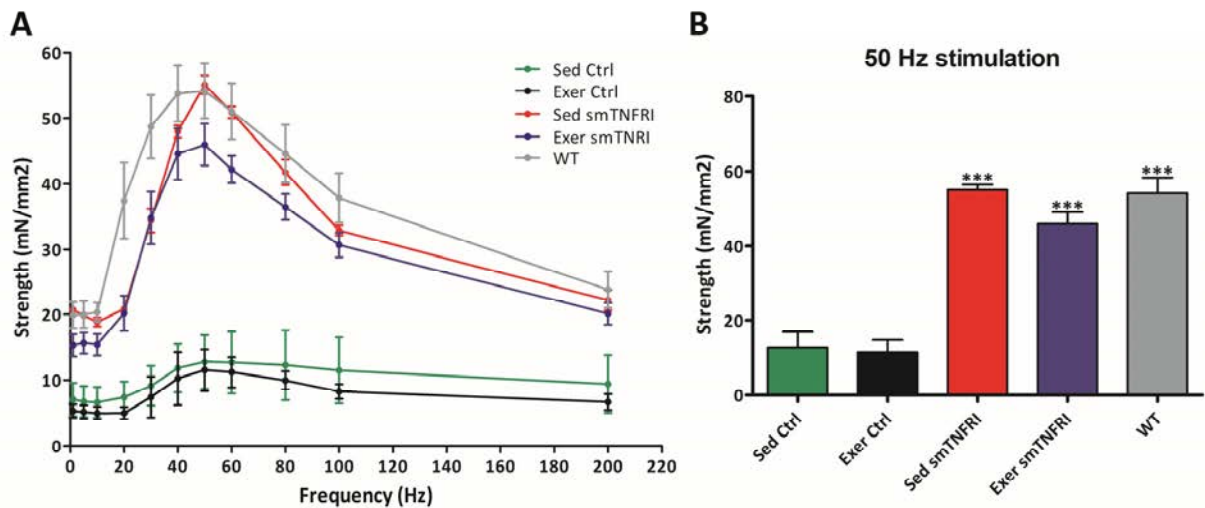
Figure 5

Figure 5: TNF blockage by the expression of smTNFRI augments the strength in the tibialis anterior muscle of the mdx mice. A. Isolated tibialis anterior muscles isolated from wild type mice (WT), sedentary mdx mice (Sed), exercised mdx mice (Exer), electrotransferred with empty vector (Ctrl) and electrotransferred with smTNFRI-IgG1 coding plasmid (smTNFRI) were subjected to different frequency stimuli to assay isometric force. Graphic shows mean plus SEM (WT, n=3; Sed Ctrl, n=4; Sed smTNFRI, n=3; Exer Ctrl, n=3 and Exer smTNFRI, n=5). B. An example of the strength of the different groups of mdx mice stimulated at 50 Hz. Graph shows mean plus SEM, 2way ANOVA with Bonferroni post test (***) $p < 0.001$.

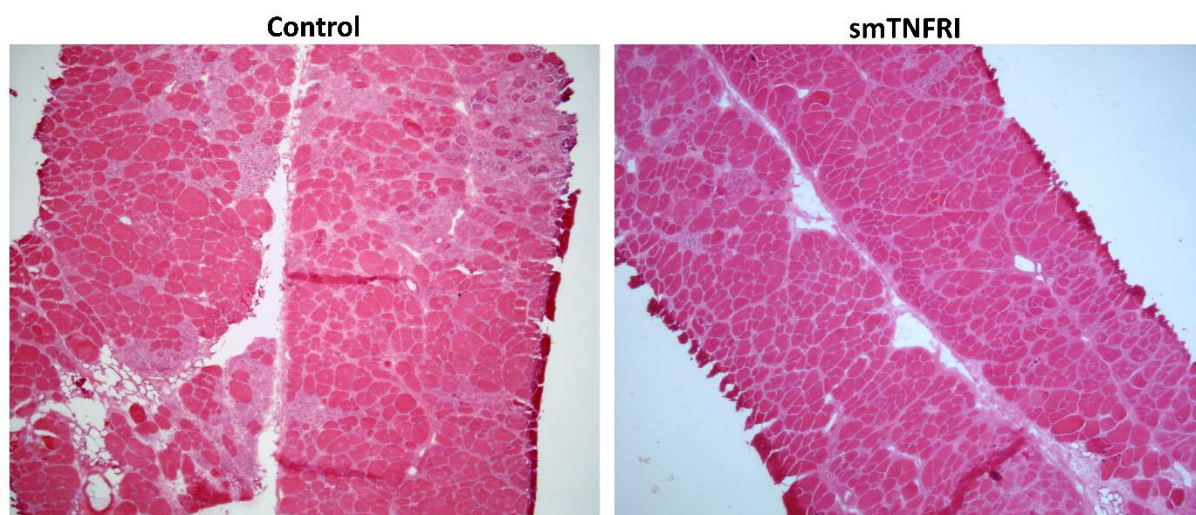
Figure 6

Figure 6: Amelioration of diaphragm histology in the mdx mice. Cryosections of Diaphragm muscles of each group of mdx mice were treated for H&E staining and photographed (Bar 100 μ m). A representative photograph for each condition are shown (control, n=4; smTNFRI, n=5).

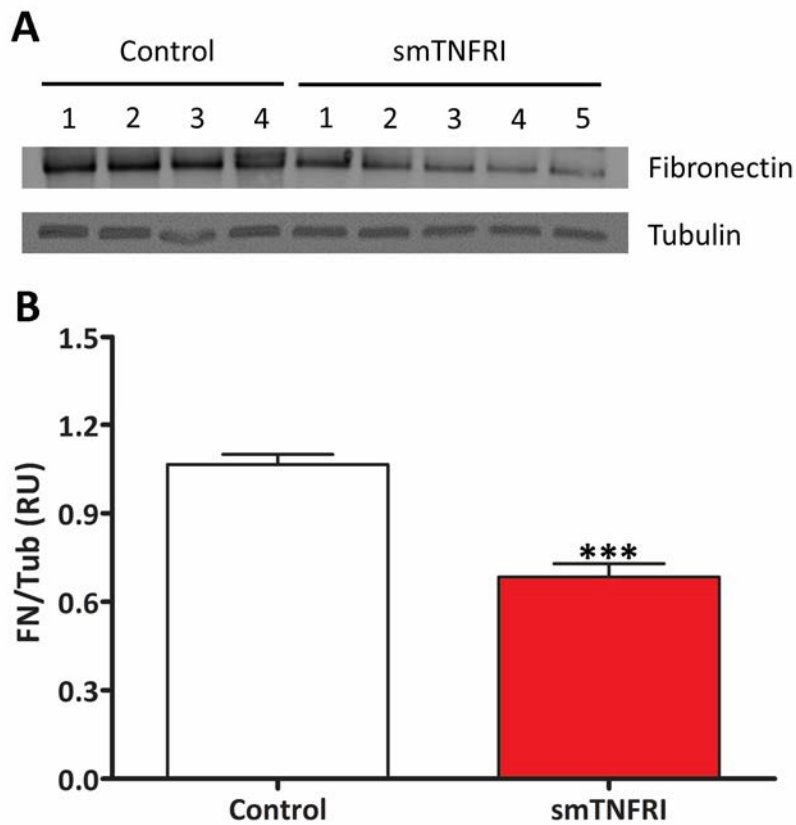
Figure 7

Figure 7: Reduction of fibronectin accumulation in diaphragm of mdx mice. A. Control (lanes 1-4) and smTNFRI treated (lanes 5-9) muscle extracts were analyzed by western blotting to detect fibronectin (FN) and tubulin (loading control). B. Relative expression of fibronectin in muscle extracts. Graph shown mean plus SEM, t test analysis was used (***) $p < 0.001$.

Figure 8

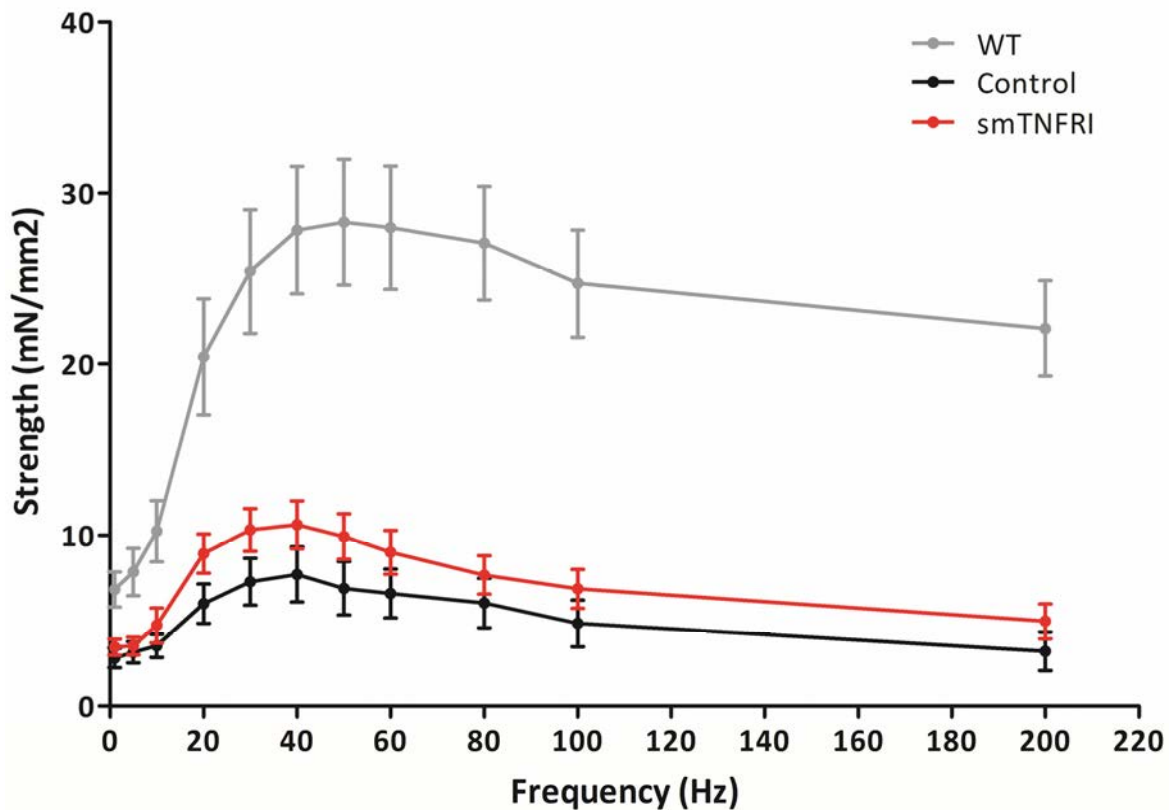


Figure 8: Diaphragm strength of wt, control and smTNFRI treated mdx mice. Isolated diaphragm muscles isolated from wild type mice (WT), sedentary mdx mice (Sed), exercised mdx mice (Exer), electrotransferred with empty vector (Ctrl) and electrotransferred with smTNFRI-IgG1 coding plasmid (smTNFRI) were subjected to different frequency stimuli to assay isometric force. Graphic shows mean plus SEM (WT, n=6, Ctrl, n=4 and smTNFRI, n=4).

4. DISCUSSION

In this thesis, we have shown a vast, complex and interlaced scenario between several important pro-fibrotic and pro-inflammatory cytokines that play major roles in the onset and progression of fibrosis in DMD and that could participate in many other fibrotic or inflammatory-related pathologies.

There are many different approaches directed to develop effective therapies to treat DMD. Despite the strong relationship between TGF- β and the development of fibrosis, therapies targeted to block the activity of this factor are ineffective due to the adverse effects of the inhibition of TGF- β . Mice lacking TGF- β show a severe autoimmune and inflammatory phenotype (Crowe et al., 2000) and an increase in tumorigenesis (Tang et al., 1999).

Our lab has worked mainly in directing our efforts to diminishing fibrosis, because this is one of the main sources for failure of cell based therapies (Morales et al., 2013b). We have demonstrated that diverse targets can reduce fibrosis and ameliorate muscle function, such as reducing the amount of connective tissue growth factor (CTGF/CCN2) (Morales et al., 2013b), targeting some members of the Renin-Angiotensin system (Morales et al., 2013a, Acuna et al., 2014) and by using andrographolide, a botanical drug, to inhibit NF- κ B (Cabrera et al., 2014) with very good results in mdx mice.

4.1 CTGF expression and transcriptional regulation

CTGF plays a central role in the onset and maintenance of fibrosis in the skeletal muscle. Exogenous increase of CTGF in the muscle of wild type mice can induce augmented extracellular matrix components and decreased isometric force in the muscle, all features of dystrophic pathologies (Morales et al., 2011). Interestingly, returning of CTGF expression to normal levels, led to a reversion of the fibrotic phenotype (Morales et al., 2011), showing that therapies against fibrotic pathologies could be successful even when fibrotic features are already present. Also, reducing CTGF levels slowed down the progression of muscular dystrophy in the mdx mice and led to an increase in cell therapy (Morales et al., 2013b).

There are compelling evidence showing that the muscle fiber might be an important source for CTGF production in the dystrophic context. Usually, CTGF is not expressed in the normal state of the muscle but CTGF levels increase importantly when damage and inflammation are present, and under pathological conditions (Cabello-Verrugio et al., 2012b). TGF- β is increased in the muscles of DMD patients (Bernasconi et al., 1995) and those of several dystrophic mice (Onofre-Oliveira et al., 2012). Moreover, CTGF contributes to an increase in TGF- β binding to its receptors and to increase TGF- β signaling (Abreu et al., 2002). It has also been shown that CTGF and TGF- β act cooperatively to elicit a fibrotic tissue response (Wang et al., 2011). In the mdx mice, TGF- β expression seems to be originated in areas populated by inflammatory cells and regenerating fibers (Zhou et al., 2006). This correlates with the fact that CTGF is expressed in the endomysium and regenerating fibers of human dystrophic patients (Sun et al., 2008) and, as we showed in this thesis, the CTGF promoter can be activated in the skeletal muscle of mice. However, the induction of CTGF mediated by TGF- β in muscle cells has not

been extensively studied. We have previously shown that TGF- β can induce the expression of CTGF in myoblasts and C2C12-derived myotubes (Vial et al., 2008). This has been also found in rat L6-derived myotubes (Maeda et al., 2005), so it is of particular interest to characterize the regulation of CTGF expression in myoblasts, myotubes and skeletal muscle. In addition, in muscle derived stem cells TGF- β increased the expression of CTGF and collagen I and III, and, interestingly, this increase in collagen by TGF- β was partially inhibited by the use of a CTGF blocking antibody (Chen et al., 2013). Also, in a mouse model of HIV infection, TGF- β , CTGF and collagen I levels were increased in the muscle (Kusko et al., 2012). Exercise also has been shown to induce CTGF expression in the muscle (Kivela et al., 2007) and Lysophosphatidic Acid (LPA) increases CTGF expression in C2C12 myoblasts, requiring the activation of JNK signaling (Cabello-Verrugio et al., 2011). Also, the inhibition of Angiotensin-converting enzyme, decreases fibrosis and CTGF expression in the mdx mice (Acuna et al., 2014). Further studies are required in order to understand how CTGF expression is regulated in muscle cells.

In this thesis, we found a novel SMAD Binding Element in the 5' UTR of the CTGF gene that is important for the TGF- β mediated expression of CTGF in myoblasts. In addition, we showed that the 5' UTR might have additional TFBS important for CTGF expression in myoblasts. The full 5' UTR region was not included in previous studies regarding TGF- β mediated CTGF expression (Chen et al., 2002, Holmes et al., 2003, Leask et al., 2003, Xia et al., 2007, Tran et al., 2010) and our bioinformatical analysis shows the presence of several TFBS that could be acting together with the SBE described in this paper for the TGF- β mediated CTGF expression in myoblasts. It is known that SBEs alone are not strong enough to confer TGF- β inducibility, due to SMADs low binding affinity to this site (Massague et al., 2005). This might be the reason why we could not immunoprecipitate SMAD3 in the CTGF promoter when we

analyzed the novel SBE located in the 5' UTR (data not shown). Due to the possible interaction of SMAD3 with other factors and the formation of a bigger transcriptional complex, the SMAD3 protein might have not been exposed within the complex to allow the recognition by the anti-SMAD3 antibody during the ChIP procedure. Within the 5' UTR region that confers TGF- β induction to the reporter gene, we found several TFBS that could be acting together with the SBE in the 5' UTR of CTGF gene. Among these TFBSs, a SP1 site which is located in very close proximity to the SBE (131 to 145) and which could be evaluated for the TGF- β induction of CTGF, as the SP1 factors are reported to be acting together with SMADs proteins to enhance transcription (Botella et al., 2009, Lu et al., 2010, Fausther et al., 2012).

Our data also indicates that an upstream region (-4872 to -4578) of the CTGF promoter is involved in TGF- β mediated expression of CTGF and that the AP-1 site located in this region would not be involved with this induction. Between the TFBSs found in this region, there are two TCF/LEF (-4810 to -4794 and -4721 to -4705) sites that could be implicated in the expression of CTGF. Interestingly, several experimental evidences show a cross-talk between Wnt and TGF- β signaling, and Wnt pathway has been proposed as a novel therapeutical target for fibrotic disorders (reviewed in (Cisternas et al., 2014)).

Besides TGF- β , several other factors have been shown to induce CTGF expression in various tissues/cell lines. CTGF expression can be induced VEGF in endothelial cells (Suzuma et al., 2000), BMP-2 in an osteoblastic cell line (Nishida et al., 2000), high glucose in human mesangial cells (Murphy et al., 1999), LPA in human mesangial cells (Goppelt-Strube et al., 2001) and H₂O₂ in Human lens epithelial cells (Park et al., 2001).

The GTGTCAAGGGGTC element described first as a TGF- β response element (Grotendorst et al., 1996) and later named BCE-1 (Chen et al., 2002), was recognized as a RXR

heterodimer and Nuclear receptor subfamily 2 factors binding site in our bioinformatical analysis (-160 to -148). This site was recognized by Retinoic Acid Receptor/Retinoid X Receptor (RAR/RXR) heterodimers and was important for All-trans retinoic acid (ATRA) mediated expression of CTGF in fibroblasts (Fadloun et al., 2008). In addition, ATRA therapy induces myositis in leukemia patients (van Der Vliet et al., 2000, Pecker et al., 2014), suggesting that ATRA might have a role in inflammation in the muscle. On the other hand, in activated and hence, fibrogenic hepatic stellate cells that participate in hepatic fibrosis, the levels of RAR and RXR were diminished, together with lower concentration of RXR/RAR activators (Ohata et al., 1997) and, in another study, several agonists of RAR and RXR produced a decrease in the expression of fibrotic proteins (Hellemans et al., 2004). ATRA was also found to reduce the expression of TGF- β and CTGF in scleroderma fibroblasts (Xiao et al., 2011). Moreover, ATRA has been reported to reduce TGF- β expression and signaling in lung fibrosis (Song et al., 2013) and mesangial cells (Han et al., 2014). Taken together, this evidence shows that the effect of retinoic acid in fibrosis is not yet understood (reviewed in (Zhou et al., 2012)), but it could be an interesting approach to explore its effect in CTGF and TGF- β expression and signaling in muscular fibrosis.

Recently, it has been shown a fibrotic effect of CTGF in the absence of TGF- β signaling in liver fibrosis (Sakai et al., 2014). This evidence shows the relevance of CTGF in fibrotic disorders and that, other signaling pathways, besides TGF- β /SMAD signaling, are involved in CTGF expression. Therefore, the study of the precise regulation of CTGF expression can be helpful to further understand the mechanisms of the onset and progression of fibrosis in different tissues.

4.2 Tumor Necrosis Factor

Here, we have shown that electroporation of the soluble murine TNF receptor I improves several features of the dystrophic muscles.

First, we showed that the systemic production of smTNFRI chimera produces a beneficial effect in the TA muscle, improving muscle architecture and reducing inflammation and, more importantly, increasing the strength of the TA mdx muscle to levels indistinguishable from wt TA muscle. In the TA muscle, the treatment with smTNFRI was able to prevent the onset of fibrosis, since, at the time of the beginning of the treatment, the TA muscle only shows minor damage and low level of inflammation. This might be the reason why we did not find a decrease in FN levels. As the muscle is not fibrotic yet, we were able to improve muscle architecture, seen as a decrease in the space between the muscle fibers, showing that the TA muscle of the mdx mice is a good model for studying the initial steps of fibrosis.

A different scenario occurs in the diaphragm muscle, as this muscle shows an advance degree of damage and fibrosis. Even though we are able to ameliorate muscle histology and fibrosis in the diaphragm mdx muscle, we only found a small increase tendency in muscle strength but it was significantly lower than that of wt diaphragm. This could be explained by the constant contraction/relaxation cycles that the diaphragm muscle has to endure during the mice life, producing degeneration and regeneration of muscle fibers. We believe that better results could be achieved by starting the treatment in younger animals or with longer periods of treatment, in order to be able to rescue the damage induced by the continuous use of the diaphragm muscle for respiration. At least, we were able to stop the progression of fibrosis and

muscle damage in the diaphragm muscle of mdx mice. It will be interesting also, to study if longer periods of treatment with smTNFRI will decrease CTGF expression in the dystrophic muscles of mdx mice.

There are five anti-TNF therapies approved for the treatment of rheumatoid arthritis (Thalayasingam and Isaacs, 2011), two of them have been used to protect inflammatory damage in the muscle. Enbrel® (etanercept), a chimeric protein of the soluble TNF receptor II with the Fc fragment of human IgG, has been used to successfully protect dystrophic muscle from inflammatory damage in the mdx mice (Nemoto et al., 2011) and Remicade® (infliximab), an anti-TNF antibody, has also been used and shown to reduce muscle fiber necrosis in dystrophic mice (Grounds and Torrisi, 2004). In addition, a modification of the infliximab antibody, cV1q, has been used to reduce damage and necrosis in muscles of wt and mdx mice (Piers et al., 2011). Although successful, these systemic anti-TNF therapies have risen some concerns regarding the apparition of side effects in human patients (Desai and Furst, 2006, Rosenblum and Amital, 2011, Jain and Singh, 2013), like malignancies (Cohen and Dittrich, 2001), lupus (Williams et al., 2009), dermatological adverse reactions (Mocci et al., 2013), interstitial lung disease (Perez-Alvarez et al., 2011) and infections (Besada, 2011, Komano et al., 2011). Also, there is a recent study showing that infliximab (cV1q) therapy on mdx mice shows negative effects on cardiac function (Ermolova et al., 2014). Taken together, this evidence suggests that anti-TNF systemic treatments should be taken with extreme caution in order to avoid secondary effects.

Even though the ET therapy we use in this study is systemic, titration of the amount of plasmid could be considered in order to minimize the quantity of smTNFRI released to the serum. In a model of uveitis, we have previously shown that a therapeutic effect can be achieved locally without any detection of sTNFRI in serum, reducing the possibility of systemic

secondary effects (Bloquel et al., 2006). Therefore, more studies should be carried out in order to achieve the local and systemic effect of sTNFRI without the dangers of secondary effect due to high sTNFRI levels in the serum. Another advantage of ET therapy is that it avoids repeated injections needed with the purified protein or antibody treatments.

There are several studies showing that ET therapies could be used to treat numerous pathologies with low risk of secondary effects (Trollet et al., 2006, Rochard et al., 2011). Also, inducible promoters can be used in order to achieve the desired concentration of the protein produced, locally and/or systemically. In addition, there are more than 80 clinical trials in different stages using ET, which shows an increased interest in using ET to treat different diseases like keloids, cancer, HIV, among others (clinicaltrials.org).

In conclusion, in this thesis we show that ET of smTNFRI in the muscle could be used as an effective therapy for the treatment of DMD and other inflammatory diseases. However, more studies are needed in order to evaluate the desired levels of sTNFRI in the serum, avoiding secondary effects that could be produced by an anti-TNF therapy.

4.3 Combined therapies for the treatment of DMD

The main goal for a successful DMD therapy will be to set-up a proper environment for an effective stem cell therapy. This proper environment should have the following status: First, it should promote muscle stem cell migration and proliferation; and second, it has to be capable to induce stem cell differentiation and muscle fiber formation.

Our lab has shown that reducing fibrosis is an important step for successful stem cell therapies. Migration can be achieved by blocking CTGF (Morales et al., 2013b) and by blocking NF- κ B (Cabrera et al., 2014) and, other group, by using stem cells expressing MMP-9 (Gargioli et al., 2008). In addition, other therapies focused in reducing fibrosis, such as angiotensin-1-7 treatment (Acuna et al., 2014) and smTNFRI expression, could be tested for the success of stem cells-based therapies. Regarding the proliferation step, Sonic hedgehog treatment have shown good results increasing the proliferation of stem cells and muscle regeneration in the mdx mice (Piccioni et al., 2014).

Together, this data shows that several promising lines of investigation are in course to develop better strategies to treat DMD and combined therapies between the factors discussed above could be used in order to achieve better understanding on fibrosis onset and development, and to restore normal function of the dystrophic muscle with stem cells therapies.

5. CONCLUSIONS

- In here, we describe a novel SBE that controls the expression of CTGF mediated by TGF- β in C2C12 myoblasts, located in the 5'UTR region of the CTGF gene.
- We find that additional TFBS are present in the 5'UTR region of the CTGF that may be implicated in TGF- β mediated CTGF expression.
- We describe that blocking TNF actions by the expression of soluble TNF Receptor I using the TA muscle as a bioreactor in the mdx mice, ameliorates the architecture and strength in the dystrophic muscle as well as it decreases fibrosis.
- The electrotransfer of sTNFRI in the muscle could be used as an effective therapy for the treatment of DMD.

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