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SMAD3 and SP1/SP3 Transcription Factors Collaborate to Regulate Connective Tissue Growth Factor Gene Expression in Myoblasts in Response to Transforming Growth Factor β

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ABSTRACT

Fibrotic disorders are characterized by an increase in extracellular matrix protein expression and deposition, Duchene Muscular Dystrophy being one of them. Among the factors that induce fibrosis are Transforming Growth Factor type β (TGF-β) and the matricellular protein Connective Tissue Growth Factor (CTGF/CCN2), the latter being a target of the TGF-β/SMAD signaling pathway and is the responsible for the profibrotic effects of TGF-β. Both CTGF and TGF 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. By using luciferase reporter assays, site directed mutagenesis and specific inhibitors in C2C12 cells; 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 and that SP1/SP3 factors are involved in TGF-β-mediated CTGF expression. J. Cell. Biochem. 116: 1880–1887, 2015.

KEY WORDS: FIBROSIS; CTGF/CCN2; SKELETAL MUSCLE; DUCHENNE MUSCULAR DYSTROPHY; TGF-BETA; SMAD

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 liver [Paradis et al., 2002] and heart [Lang et al., 2008], among others. 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 depletion of the muscle's satellite cells [Charge and Rudnicki, 2004]. Fibrosis is also observed in DMD animal models including the *mdx* mice [Bulfield et al., 1984].

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., 1999]. 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 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 is should be present once

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every 1024 bp in the genome), the low specifity (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]. It has been found that TGF-β induces the expression of the matricellular protein connective tissue growth factor (CTGF/CCN2) in fibroblasts [Igarashi et al., 1993] and, more important, the profibrotic effects of TGF-β are CTGF dependent [Grotendorst, 1997].

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 and Abraham, 2006]). In pathological conditions, CTGF has been proposed to have a central role in fibrosis in several tissues (reviewed in [Leask, 2013]) and, importantly, to be required for the onset of fibrosis in vivo [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., 2012a; 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] and AP-1 in keloid fibroblasts [Xia et al., 2007].

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, which involves SMAD3 and SP1/SP3 transcription factors.

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% CO_2 and controlled humidity. These myoblasts have the capability of differentiate and fuse, forming contractile myotubes in differentiation conditions.

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 h 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 h. At that point, FBS was added to reach a final concentration of 10% and cells were cultured for 3 h. The cells were then serum-starved for 12–14 h and 10 ng/mL TGF-β (R&D systems) or vehicle was then added to the culture and incubated for further 24 h and cells were lysed and assayed with Dual-Luciferase Reporter Assay System according to manufacturer instructions (Promega). When 3 μM SIS3 (Calbiochem) and 200 μM Mythramicine A (Abcam) were used, they were added 1 h before TGF-β treatment. 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). All the experiments were done at least three times in triplicate.

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.

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.

REAL-TIME PCR

C2C12 cells were seeded in 6-well plates to reach 60–70% confluence, starved overnight and treated with 10 ng/mL TGF-β (R&D Systems) or vehicle for 8 h. Total RNA were extracted with TRIzol® reagent (Life Technologies), 1 μg or RNA was reverse transcribed using M-MLV reverse transcriptase (Life Technologies) and then used for CTGF mRNA quantification using the comparative ΔCt method (2-ΔΔCT) with 18S as the reference gene. When 3 μM SIS3 (Calbiochem) and 200 μM Mythramicine A (Abcam) were used, they were added 1 h before TGF-β treatment.

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 5 mm 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 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 120 s and was initiated 3 min after injection of the substrate.

IN SILICO ANALYSIS OF THE CTGF PROMOTER

The promoter sequence of CTGF was analyzed using the MatInspector tool 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 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-FL) shows an increased response to TGF-β than the vector carrying the shorter fragment (pCTGF-0.9) (Fig. 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.

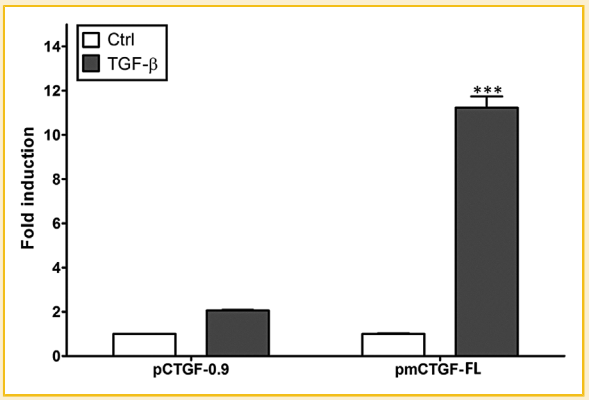

Fig. 1. Induction of pmCTGF-FL vector in myoblasts. C2C12 cells were transfected with pCTGF-0.9 and pmCTGF-FL vectors in 24-well plates in triplicate. The graph shows fold induction of TGF-β versus control (Ctrl, vehicle), mean with SEM (n = 3 in triplicate). $^{***}P < 0.001$ Two-way ANOVA Bonferroni posttests.

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 TGFβ. The analysis showed 1250 putative TFBS, including several sites related to TGF-β (Suppl. Table I). The TFBS of importance for TGF-β/SMAD are summarized in Table I: 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 (Suppl. Table I).

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 and we conducted reporter assays on myoblasts. As shown in Figure 2, 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 I) because it has been shown that AP-1 transcription factors could act synergically with SMAD3 to promote gene expression [Sundqvist et al., 2013; Bai et al., 2014]. The mutation of the AP-1 site showed no decrease of the reporter gene expression (Suppl. Figs. 1A and B) suggesting that the AP-1 site is not involved in the TGF-β-mediated expression of

TABLE I. Putative Transcription Factor Binding Sites related to TGF-β in the CTGF promoter. 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.

Detailed family information	Position		Strand	Sequence
	from	to		
AP1, Activating protein 1	-4834	-4824	(+)	tctgAATCatg
GC-Box factors SP1/GC	-4469	-4455	(+)	ctGGGGtgtgttcat
GC-Box factors SP1/GC	-4362	-4348	(-)	ctttggagGGACtaa
GC-Box factors SP1/GC	-3735	-3721	(-)	gcgGGGCaggggcg
AP1, Activating protein 1	-3532	-3522	(+)	ttgAGTCacg
Vertebrate SMAD family of transcription factors	-3380	-3372	(+)	aGTCTggtc
AP1, Activating protein 1	-2998	-2988	(+)	gctGAGTcatt
GC-Box factors SP1/GC	-2847	-2833	(-)	gaAGGGtgtgtgaca
Vertebrate SMAD family of transcription factors	-2816	-2808	(+)	tGTCTgtat
AP1, Activating protein 1	-2292	-2282	(-)	attGAGTaact
GC-Box factors SP1/GC	-2251	-2237	(-)	aggGGGCaggctcag
AP1, Activating protein 1	-2080	-2070	(-)	aatGAGTgagg
GC-Box factors SP1/GC	-1665	-1651	(-)	ggtgGGAGgggtaa
Vertebrate SMAD family of transcription factors	-1307	-1299	(-)	tGTCTgtct
GC-Box factors SP1/GC	-954	-940	(-)	ttGGGGtttgttctg
GC-Box factors SP1/GC	-27	-13	(-)	ggcGGGCggcgctgg
Vertebrate SMAD family of transcription factors	121	129	(-)	tGTCTggac
GC-Box factors SP1/GC	131	145	(-)	cagGGGCggaggttg

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 I). Therefore, we first decided to construct a deletion mutant of pmCTGF-FL that lacks most of the 5′ UTR region of the *CTGF* gene (Fig. 3A). The deletion of the 5′ UTR region of the *CTGF* gene showed a significant reduction on the TGF-β-mediated CTGF expression (Fig. 3B), 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 I, 121–129) 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

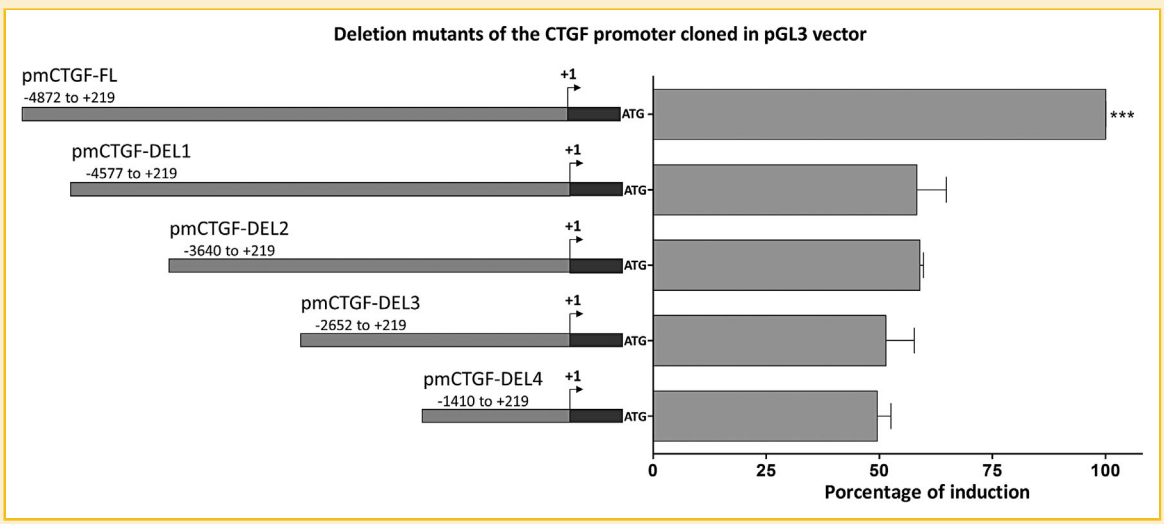

Fig. 2. Reporter assay of the deletion mutants of CTGF promoter region. LEFT. Schematic representation of the deletion mutants cloned in pGL3 vector to conduct reporter assays. The promoter region is shown in light gray, whereas the 5′ UTR of the *CTGF* gene dark gray. The numbers indicate the position of the bases related to transcription start, indicated by an arrow. RIGHT. The level of expression of each reporter plasmid is related to the level of expression of the full length plasmid (pmCTGF-FL). The graph shows mean with SEM (n = 3, in triplicate). $^{***}P < 0.0001$ Mann–Whitney *t*-test.

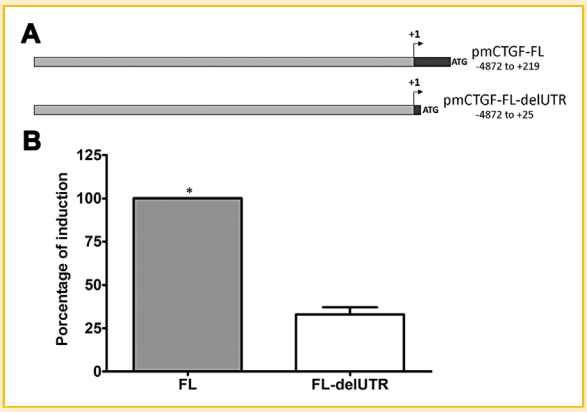

Fig. 3. Deletion mutant of the 5′ UTR of CTGF promoter. (A) Schematic representation of the deletion mutants cloned in pGL3 vector to conduct reporter assays. The promoter region is shown in light gray, whereas the 5′ UTR of the *CTGF* gene is shown dark grey. The numbers indicate the position of the 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-FL). The graph shows mean with SEM (n = 4, in triplicate). $^{*}P < 0.05$ Mann–Whitney *t*-test.

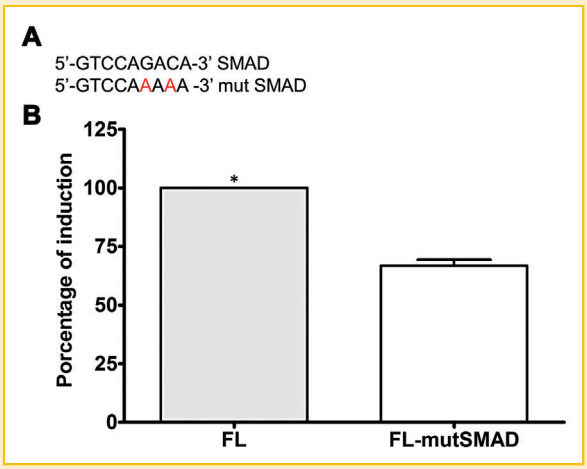

Fig. 4. Mutation on the SBE of the 5′ UTR of pmCTGF-FL. (A) The sequence of the wild-type (SMAD) and mutated SMAD element (mut SMAD). Mutated nucleotides are shown in red. (B) The level of expression of the mutated reporter plasmid is related to the level of expression of the full length plasmid (pmCTGF-FL). The graph shows mean with SEM (n = 4, in triplicate). $^{*}P < 0.05$ Mann–Whitney *t*-test.

nucleotides in the sequence (Fig. 4A) and performed reporter assays of the constructs. As seen in Figure 4B, 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. Next, we tried to immunoprecipitate SMAD3 in this SBE, but results were not informative (Data not shown). Using the SMAD3 specific inhibitor SIS3 [Jinnin et al., 2006], we found that the expression of CTGF mRNA was abolished with SIS3 (Fig. 5A) and the induction of the pmCTGF-FL plasmid was also inhibited (Fig. 5B). Also, the inhibition of the induction of pmCTGF-FL by SIS3 was bigger than that of pmCTGF-FL-mutSMAD (Fig. 5B), suggesting that SMAD3 might be enhancing the transcription of CTGF expression mediated by TGF-β through this site. It is also important to notice that the mutation on the SBE reduced the expression of the reporter gene expression by 32% (Fig. 4B), whereas the deletion of the 5′ UTR reduced the expression by 67% (Fig. 3B). This suggests that there are additional TFBS that are important in the TGF-β-mediated expression of CTGF.

SMAD3 AND SP1/SP3 FACTORS ARE INVOLVED IN CTGF EXPRESSION MEDIATED BY TGF-β, THROUGH THE 5′ UTR OF THE *CTGF* GENE

In order to further understand how the 5′ UTR region of the *CTGF* gene control it's expression in response to TGF-β, we focused on an SP1 site that is located in very close proximity to the SBE (131–145), 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]. C2C12 myoblasts treated with Mithramycin A, a selective inhibitor of SP1/SP3 transcription factors [Fernandez-Guizan et al., 2014; Sleiman et al., 2011], prior to TGF-β treatment, shows a decreased expression of CTGF mRNA but to a much lesser extent than when these cells are treated with SIS3 (Fig. 5A), suggesting that SP1/SP3 factors are partially involved in TGF-β-mediated CTGF expression. When Mithramycin A is applied to C2C12 cells transfected with the deletion mutants treated with TGF-β, its inhibitory effect is not present when the entire 5′ UTR region is deleted, suggesting that the SP1 site present in this region is responsible for the effect.

DISCUSSION

CTGF plays a central role in the onset and maintenance of fibrosis in the skeletal muscle. There are compiling evidence showing that the muscle fiber might be an important source for CTGF production in the dystrophic context. As proof of concept, we show that electroporating the pmCTGF-FL plasmid in *tibialis anterior* muscle of C57BL/6JRj shows an increased expression in the muscle than the empty vector (pGL3) from day 1 to day 7 postelectrotransfer (ET) (Suppl. Figs. 2A and B). 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 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]. However, the induction of CTGF mediated by TGF-β in muscle cells has not been extensively

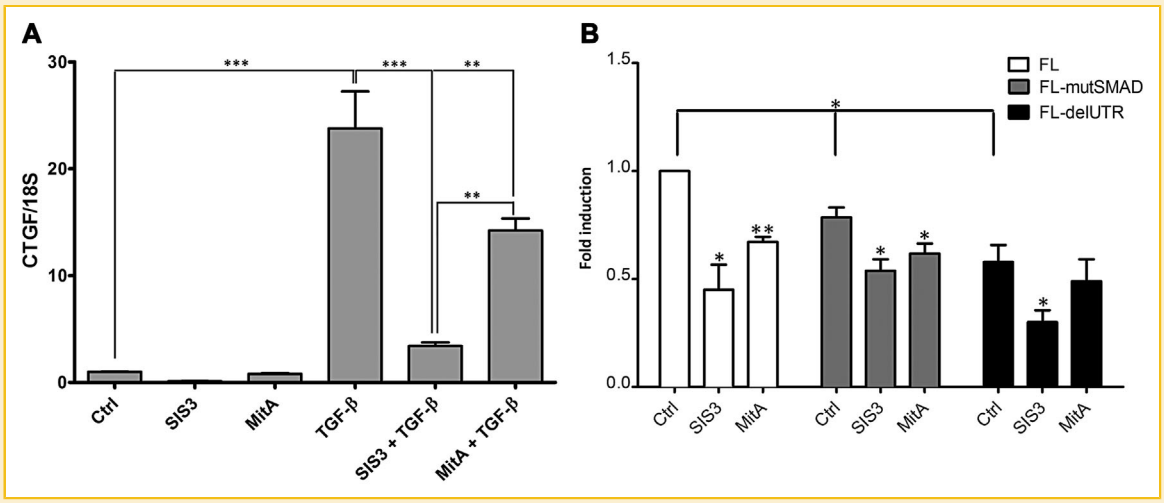

Fig. 5. Effect of SMAD3 and SP1/SP3 inhibitors in CTGF expression. (A) mRNA quantification of CTGF mRNA in C2C12 treated with vehicle (Ctrl) or 10 ng/mL TGF-β, with or without 3 μM SIS3 and 200 nm of Mithramycin A (MitA). The results are presented as fold induction of Ctrl and graph shows mean with SEM (n = 4, in duplicate). $^{***}P < 0.0001$, $^{**}P < 0.001$, One-way ANOVA Bonferroni multiple comparison test. (B) The level of expression of the reporter gene is related to the level of expression of the full length plasmid (pmCTGF-FL). The graph shows mean with SEM (n = 3, in triplicate). $^{**}P < 0.01$, $^{*}P < 0.05$ Two-way ANOVA test Bonferroni posttests.

studied. We have previously shown that TGF-β can induce the expression of CTGF in myoblast and C2C12-derived myotubes [Vial et al., 2008], so it is of particular interest to characterize the regulation of the expression of CTGF in myoblasts, myotubes, and skeletal muscle.

In this paper, we found a novel SMAD-Binding Element in the 5′ UTR of the *CTGF* gene that can act as an enhancer on the TGF-β-mediated expression of CTGF in myoblasts, similar to what it has been described for the TGFBRE before [Leask et al., 2001]. In addition, we also found evidence that suggests that SP1/SP3 transcriptions factor are acting together with SMAD3 in order to activate CTGF transcription. Although the presence of active TFBS in the 5′ UTR of genes is unusual, there have been examples of active SBEs [Pommier et al., 2012] and SP1/SP3 sites [Bianchi et al., 2009] present in 5′ UTR regions. 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]. 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). Our data suggest an interaction of SMAD3 with SP1/SP3 factors and the formation of a bigger transcriptional complex and, therefore, the SMAD3 protein might have not been exposed within the complex to allow the recognition by the antibody during the ChIP procedure. It will be very interesting to further characterize the complex formed in the 5′ UTR region of the *CTGF* gene and analyze the different interactions that drive the expression of CTGF mediated by TGF-β in myoblasts.

Our data also indicate 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]).

We are also aware that our bioinformatical analysis didn′t pick up some SBEs already described in the literature or deliver results somehow different. For example, the SBE, described in the human CTGF promoter by Fujii et al. [2012] together with and adjacent TEAD site, was recognized in our analysis as an ETS-1 factor binding site (−178 to −158, Suppl. Table I). Similarly, 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 part of a RXR heterodimer and Nuclear receptor subfamily 2 factors binding site in our bioinformatical analysis (−160 to −148, Suppl. Table I) and it doesn′t corresponds to any of the SBEs we found. This site was shown to be 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]. Although the literature shows that the effect of retinoic acid in fibrosis is not yet clear (reviewed in [Zhou et al., 2012]), it could be an interesting approach to explore its effect in CTGF and TGF-β expression and signaling in muscular fibrosis.

All these evidences taken together show a vast, complicated, and interlaced scenario of several factors and pathways that are playing a role in the onset and maintenance of fibrosis in different tissues and dissimilar pathological states. A deeper understanding of how the most important fibrotic factors are expressed is paramount for the development of better therapies against fibrosis.

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