Transforming Growth Factor-β1 Induces Transdifferentiation of Myoblasts into Myofibroblasts via Up-Regulation of Sphingosine Kinase-1/S1P₃ Axis

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The pleiotropic cytokine transforming growth factor (TGF)-β1 is a key player in the onset of skeletal muscle fibrosis, which hampers tissue repair. However, the molecular mechanisms implicated in TGFβ1-dependent transdifferentiation of myoblasts into myofibroblasts are presently unknown. Here, we show that TGFβ1 up-regulates sphingosine kinase (SK)-1 in C2C12 myoblasts in a Smad-dependent manner, and concomitantly modifies the expression of sphingosine 1-phosphate (S1P) receptors (S1PRs). Notably, pharmacological or short interfering RNA-mediated inhibition of SK1 prevented the induction of fibrotic markers by TGFβ1. Moreover, inhibition of S1P₁, which became the highest expressed S1PR after TGFβ1 challenge, strongly attenuated the profibrotic response to TGFβ1. Furthermore, downstream of S1P₁, Rho/Rho kinase signaling was found critically implicated in the profibrotic action of TGFβ1. Importantly, we demonstrate that SK/S1P axis, known to play a key role in myogenesis via S1P₂, consequently to TGFβ1-dependent S1PR pattern remodeling, becomes responsible for transmitting a profibrotic, antIdifferentiating action. This study provides new compelling information on the mechanism by which TGFβ1 gives rise to fibrosis in skeletal muscle, opening new perspectives for its pharmacological treatment. Moreover, it highlights the pleiotropic role of SK/S1P axis in skeletal myoblasts that, depending on the expressed S1PR pattern, seems capable of eliciting multiple, even contrasting biological responses.

INTRODUCTION

Repair and maintenance of skeletal muscle depends on satellite cells, tissue resident stem cells that become activated after tissue injury and rapidly generate myoblasts that proliferate, migrate at the lesion site, fuse or differentiate into new myofibers (Hawke and Garry, 2001). However, the recovery of the injured skeletal muscle often is hindered by the development of fibrosis. Skeletal muscle fibrosis is a major pathological hallmark of chronic myopathies in which myoblasts are replaced by progressive deposition of extracellular matrix proteins (Huard et al., 2002; Sato et al., 2003). Moreover, skeletal muscle traumas provoked by repetitive muscle contractions, laceration or denervation lead also to muscular fibrosis (Best and Hunter, 2000). A major role in the onset of fibrosis is exerted by myofibroblasts that result from transdifferentiation of muscle progenitor cells during the regenerative process (Li and Huard, 2002). Among the environmental stimuli released at the injured area, transforming growth factor (TGF)-β1 has been individuated as the major inducer of differentiation of myogenic cells into fibrotic cells (Sato et al., 2003; Li et al., 2004; Gosselin et al., 2004); however, the molecular mechanisms responsible for the elicited biological response have not been so far investigated.

TGFβ1, the prototypic member of a large family of multifunctional growth factors, regulates crucial events of development, disease, and repair (Rahimi and Leof, 2007). The mechanism by which TGFβ-related molecules elicit their multiple biological effects is quite complex and presently not fully elucidated. The cytokine signal is transduced via a heteromeric complex of two types of transmembrane serine/threonine kinase receptors that phosphorylate receptor-activated Smad proteins. Subsequently, the translocation into the nucleus of Smad complex and its association with additional transcriptional factors determines the transcriptional regulation of target genes (Massague and Wotton, 2000; Derynck and Zhang, 2003). The long-lasting action exerted by TGFβ1 implies the triggering of a complex cascade of transcriptional events not fully characterized yet. Several recent studies have demonstrated that some of the effects elicited by TGFβ1 are transmitted by the pathway initiated by enhancement of sphingosine kinase (SK), followed by intracellular generation of the bioactive lipid sphingosine 1-phosphate (SIP) (Watterson et al., 2007). Once formed, SIP can in turn act as extracellular ligand of a panel of five distinct membrane receptors or as intracellular mediator (Alvarez et al., 2007). In particular, it has been shown that the SK/SIP pathway is critically implicated in the mechanism by which TGFβ elicits invasive behavior of esophageal cancer cells (Miller et al., 2008), antiapoptotic action in mesoangioblasts (Donati et al., 2009), and profi-
brotic effect in various types of fibroblasts (Yamanaka et al., 2004; Kono et al., 2007; Gellings Lowe et al., 2009). In this regard, up-regulation of SK1 has been found to be critical for the TGFβ1-induced transcriptional regulation of the tissue inhibitor of metalloproteinase-1, which inhibits degradation of extracellular matrix in fibroblasts (Yamanaka et al., 2004). Moreover, recent reports have shown that SK1 plays a role in TGFβ1-dependent extracellular matrix remodeling and myofibroblast differentiation of lung fibroblasts (Kono et al., 2007), and collagen production by cardiac fibroblasts (Gellings Lowe et al., 2009).

Previously, we have established that SK1/SIP$_3$ axis is required for serum withdrawal-induced myogenic differentiation of myoblasts (Meacci et al., 2008). Moreover, the prodifferentiation effect exerted by low doses of tumor necrosis factor-α in mouse myoblasts was found to rely on the triggering of SK1/SIP$_3$ pathway (Donati et al., 2007b). However, at present is not known whether SK is regulated by TGFβ1 in myoblasts and eventually participates to its biological action. The well-established profibrotic action of TGFβ1 together with the emerging role of SK1/SIP$_3$ axis as profibrotic signaling pathway spurred us to investigate whether TGFβ1 regulates SK in myoblasts and whether it has any role in its biological response.

Data reported here show for the first time that TGFβ1 simultaneously up-regulates SK1 and profoundly modifies SIP receptor (SIPR) expression in mouse myoblasts. Importantly, SIP$_3$, which becomes the dominant signaling receptor upon TGFβ1 challenge, seems to be critically implicated in the transdifferentiation of myoblasts into myofibroblasts. These results represent the first experimental evidence that induction of SK1/SIP$_3$ axis, subsequent to SIPR expression pattern remodelling, is exploited by TGFβ1 to carry on cell transdifferentiation. Importantly, they open new perspectives for pharmacological treatment of skeletal muscle fibrosis.

MATERIALS AND METHODS

Materials

Biochemicals, TRI Reagent, cell culture reagents, DMEM, fetal calf serum (FCS), protease inhibitor cocktail, bovine serum albumin (BSA), monoclonal anti-serum, smooth muscle actin (SMA) antibodies, monoclonal anti-skeletal fast myosin heavy chain (MHC) (clone MY-23), and tetracycline-hydrodramine B isothiocyanate (TRITC)-phalloidin conjugate were purchased from Sigma-Aldrich (St. Louis, MO). Mouse skeletal muscle C2C12 cells were obtained from the European Collection of Cell Cultures (Salisbury, UK). TGFβ1 was purchased from PeproTech (London, United Kingdom). SK-I2 [2-(p-hydroxyanilino)-(4'-chlorophenyl)diethanolamine], U0126, Y27632, and D-enylphospho-SIP were from Calbiochem, (San Diego, CA). Short interfering RNA (siRNA) duplexes corresponding to two DNA target sequences of mouse SK1 (5'-UGAGAACUGGGCCUCUUAAdTdT3', 5'-GGUUAUAGCUAGUCUCUA-AdTdT3'; mouse SIP1, 5'-CCUCUAGUGGVGGCGUAdTdT3', 5'-GCAGUAAUCGAGUGUAdTdT3'; mouse SIP2, 5'-CCGUAAGCUGCCCAGUATGTT3'; mouse SIP3, 5'-GCGUAACACGCAGCUGATT3'; mouse SIP4, 5'-ACGCAGGCACACACAGUATGTT3'; mouse SIP5, 5'-CGACUAGCAACACAGUATGTT3'; mouse SIP6, 5'-CAACAGCAACACACAGUATGTT3'; mouse SIP7, 5'-CCUGAAGAAAGCCCGTCTCCA3'). Oligofectamine Reagent and Lipofectamine RNAiMAX were obtained from Invitrogen (Carlsbad, CA). Enhanced chemiluminescence (ECL) reagents and [32P]ATP (300 Ci/ml) were obtained from GE Healthcare Europe (Milan, Italy). Anti-Smad4, D-eruvinol (19.8 Ci/mmol), was from PerkinElmer Life and Analytical Sciences (Boston, MA). Secondary antibodies conjugated to horseradish peroxidase, monoclonal anti-RhoA, polyclonal anti-laminin, and polyclonal anti-Smad2/3 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-transgelin antibodies were from Everst Biotech (Upper Heyford, Oxfordshire, United Kingdom). Monoclonal antibodies against β-actin were from Sigma-Aldrich (Denver, CO). Monoclonal anti-caveolin-3 and anti-hemo-agglutinin (HA) antibodies were from BD Biosciences Transduction Laboratories (Lexington, KY). Polyclonal antibodies against Smad4 were purchased from Cell Signaling Technology (Danvers, MA). Fluorescein-conjugated horse-anti-mouse secondary antibodies were obtained from Vector Laboratories (Burlingame, CA). The specific anti-SK1, antagonist ViscTextUtils SIP1, antagonist W146 were from Avanti Polar Lipids (Alabaster, AL). All reagents and probes required to perform real-time PCR were from Applied Biosystems (Foster City, CA). puSiRNA-SIP$_3$ vector was a kind gift of Prof. Y. Ishikawa (Hokkaido University, Sapporo, Japan).

Cell Culture

Murine C2C12 myoblasts were routinely grown in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO$_2$. For myofibroblast transdifferentiation and myogenic differentiation experiments, cells were seeded in p35 or in p60 plates and when 90% confluent they were shifted to DMEM without serum containing 1 mg/ml BSA.

Sphingosine Kinase Activity Assay

SK activity was measured as described in Olivera et al. (1994) with few modifications, as described previously (Donati et al., 2007b). Specific activity of SK was expressed as picomoles of SIP formed per minute per milligram of protein.

Western Blot Analysis

Cell lysates, cytosolic and membrane fractions, were prepared from confluent serum-starved C2C12 myoblasts as described previously (Meacci et al., 2000) and then subjected to SDS-polyacrylamide gel electrophoresis electrophoresis and Western analysis (Donati et al., 2005). Specific anti-SK1 polyclonal antibodies (directed against the 16 carboxy-terminal amino acids of the mouse SK1, kindly provided by Dr. Y. Banno, Gifu University School of Medicine, Gifu, Japan; Murate et al., 2001) were used to immunodeplete endogenous SK1. Rabbit polyclonal antibodies generated against SK2 (Igarashi et al., 2003) were a kind gift of Dr. S. Nakamura (Department of Molecular and Cellular Biology, Kobe University Graduate School of Medicine, Kobe, Japan). The expression of fibrosis markers and myogenic differentiation markers was evaluated on total cell lysates (20 μg) after 24 and 48 h agonist challenge, respectively. Equally loaded protein was checked by expression of the nonmuscle-specific β-actin isoform of actin. The evaluation of RhoA subcellular localization was performed by Western blotting analysis of membrane and cytosolic fractions (10 μg) with a specific monoclonal anti-RhoA antibody.

Reverse Transcription-Polymerase Chain Reaction

Total RNA (2 μg), extracted with TRI Reagent from C2C12 myoblasts was reverse transcribed using SuperScript II reverse transcriptase (Invitrogen) and subjected to PCR using SIP1 (1090 base pairs), SIP2 (336 base pairs), SIP3 (225 base pairs), SIP4 (324 base pairs), and β-actin (250 base pairs) PCR primers as described previously (Donati et al., 2007a). β-Actin was used as an internal reference control to normalize relative levels of gene expression.

Quantitative Real-Time Reverse Transcription (RT)-PCR

The quantification of SIP1 mRNA was performed by Real-Time PCR employing TaqMan Gene Expression Assays. Each measurement was carried out in triplicate, using the automated ABI Prism 7700 Sequence Detector System (Applied Biosystems, Foster City, CA), as described previously (Donati et al., 2007a), by simultaneous amplification of the target sequence (SIP1, Mm00514644_m1, SIP2, Mm01177794_m1, SIP3, Mm00515669_m1, and SIP4, Mm00486995_s1; Applied Biosystems) together with the housekeeping gene 18S rRNA. Results were analyzed by ABI Prism Sequence Detection System software, version 1.7 (Applied Biosystems). The 2$^{-ΔΔCε}$ method was applied as a comparative method of quantification (Livak and Schmittgen, 2001), and data were normalized to ribosomal 18S RNA expression.

Lipid Analysis

Cell sphingolipids were labeled with [3-3H]sphingosine (0.1 μCi/ml for 48 h at 37°C), extracted, and separated on high performance-thin layer chromatography (HPTLC) essentially as described in Khbaza et al., (2003). Radioactive lipid fraction, identified by comparison with standards and quantified by a Beta-Imager 2000 instrument (BioSpace, Paris, France), with an acquisition time of 48 h, essentially as described in Pinetti et al. (2000). The radioactivity associated with individual lipids was determined with the specific β-Vision software (Biospace).

Immunostaining and Fluorescence Microscopy

C2C12 cells were seeded on microscope slides, precoated with 2% gelatin, and then treated or not with 10 nM TGFβ1, 60 min before 5 ng/ml TGFβ1 treat-

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ment. After 24 h cells were fixed in 2% paraformaldehyde in PBS for 20 min and permeabilized in 0.1% Triton X-100-PBS for 30 min. Cells were then blocked in 3% BSA for 1 h and incubated with anti-α-SMA antibody for 2 h and fluorescein-conjugated anti-mouse secondary antibody for 1 h. To stain F-actin filaments, the specimen was incubated with TRITC-phalloidin for 40 min. Images were obtained using an SP5 laser scanning confocal microscope (Leica, Wetzlar, Germany) with a 63× objective.

Cell Transfection

Cells grown into six-well dishes (60,000 cells/well), were transfected with siRNA duplexes using Oligofectamine Reagent transfection system, as described previously (Donati et al., 2007b). In some experiments, cells were transfected using Lipofectamine RNAiMAX, according to manufacturer’s instructions. In brief, Lipofectamine RNAiMAX was incubated with siRNA in DMEM without serum and antibiotics at room temperature for 20 min, and afterward the lipid/RNA complexes were added with gentle agitation to C2C12 cells to a final concentration of 50 nM in serum containing DMEM. After 24 h, cells were shifted to DMEM without serum containing 1 mg/ml BSA and then used for the experiments within 48 h from the beginning of transfection. The specific gene knockdown was evaluated by Western blot analysis or alternatively by real-time RT-PCR.

Statistical Analysis

Statistical analysis was performed using Student’s t test. Graphical representations were performed using Excel software (Microsoft, Redmond, WA) and Prism 4.0 (GraphPad Software, San Diego, CA). Densitometric analysis of the Western blot bands was performed using imaging and analysis software Quantity One (Bio-Rad Laboratories, Hercules, CA). Asterisks indicate statistical significance (*p < 0.05 and **p < 0.001).

RESULTS

TGFβ1 Regulates SK1 and Sphingolipid Metabolite Levels in C2C12 Myoblasts

The SK/S1P pathway is important in the regulation of cell fate of multiple cell types and has been already found im-

![Figure 1](image_url)

Figure 1. TGFβ1 affects SK activity and induces SK1 expression. Confluent C2C12 myoblasts were incubated with DMEM containing 1 mg/ml BSA for the indicated time intervals in the presence of 5 ng/ml TGFβ1. (A) Aliquots of cell extracts (50 μg) were used to determine SK activity. Data represent the mean ± SEM of three independent experiments each performed at least in duplicate. The effect of TGFβ1 was statistically significant by Student’s t test (*p < 0.05, **p < 0.001). (B) Top, aliquots of total cell lysates (20 μg) were used to perform Western analysis, using specific anti-SK1 and anti-SK2 antibodies. A representative blot is shown. Bottom, densitometric analysis of at least three independent experiments. Data are the mean ± SEM and are reported as protein expression normalized to β-actin, -fold change over control. (C) Two micrograms of total RNA was subjected to semiquantitative RT-PCR analysis, using specific primers for SK1, SK2, and β-actin. (D) Left, Western analysis of SK1 was performed in membrane and cytosolic fractions prepared form myoblasts treated (+) or not (−) with 5 ng/ml TGFβ1 for the indicated time intervals. A blot representative of three independent experiments with analogous results is shown. Right, densitometric analysis of three independent experiments. Data reported are the mean ± SEM of membrane/cytosol ratio to respective control set as 1.
plicated in myogenic differentiation of mouse myoblasts (Donati et al., 2005; Meacci et al., 2008); however, its involvement in TGF-β1 action in these cells has not been so far investigated. To explore this issue, at first we examined whether the cytokine was capable of regulating SK activity in C2C12 cells. Experimental data illustrated in Figure 1A show that cell treatment with 5 ng/ml TGF-β1 exerted a biphasic effect on SK activity. The enzymatic activity was moderately reduced at early time points (1 min – 4 h range), whereas it was significantly enhanced at 18 h and still clearly appreciable at 48 h. These data are in agreement with a previous report in which the cytokine was found to reduce fibroblast SK activity and S1P cellular content within the first hour of incubation, whereas it enhanced SK activity at more prolonged times of incubation (Yamanaka et al., 2004). Given that the time course of TGF-β1-dependent increase of SK activity was compatible with transcriptional regulation of SK1 or SK2, to verify this possibility Western analysis of both proteins was performed. As shown in Figure 1B, TGF-β1 treatment for 24–72 h resulted in a sustained marked increase of SK1 protein content. Instead, SK2 protein content, which was time-dependently increased by serum starvation, was not changed by the cytokine possibly due to its proapoptotic role (Igarashi et al., 2003).

In agreement with the augmented protein content, SK1 mRNA levels, measured by semiquantitative PCR assay, were enhanced already at 4 h after TGF-β1 challenge, and the stimulatory effect of the cytokine was maintained at 18 h (Figure 1C). Figure 1C also shows that mRNA content of SK2 was unaffected. Given that stimulation of SK activity by multiple agonists resides on enzyme translocation to membrane, eventual TGF-β1-induced intracellular redistribution of SK1 also was explored. Western analysis of SK1 performed in cytosolic and membrane fractions of myoblasts incubated with 5 ng/ml TGF-β1 for various time intervals revealed that the cytokine increased at similar extent SK1 protein content of cytosolic and membrane fractions (Figure 1D), thus not specifically affecting an intracellular pool of SK1. These results demonstrate that the sustained increase of SK activity elicited by TGF-β1 is exclusively attributable to the up-regulation of SK1 expression.

To further examine the effect of TGF-β1 on sphingolipid metabolism, myoblasts, previously steady-state metabolically labeled with [3H]sphingosine, were administered with the cytokine (5 ng/ml) for various time intervals and then subjected to lipid extraction and HPTLC separation followed by digital autoradiography to quantify the various labeled sphingolipid metabolites. Data presented in Figure 2 show that [3H]ceramide levels were enhanced by TGF-β1 within 6 h, whereas at more prolonged times (18–24 h) they were significantly reduced. In parallel, [3H]sphingosine levels where not appreciably modified throughout the incubation. Interestingly, treatment with TGF-β1 at 18 and 24 h raised [3H]S1P levels by ~60%. Thus, consistently with the complex regulation of SK activity, TGF-β1 elicited changes of cellular bioactive sphingolipid metabolite levels.

**Involvement of SK1 in the Profibrotic Effect of TGF-β1**

To assess the ability of TGF-β1 to promote the transdifferentiation of myoblasts into myofibroblasts, the expression of fibrosis marker proteins such as α-SMA, laminin, and transgelin in C2C12 myoblasts was evaluated. Western analysis data presented in Figure 3A show that the expression of α-SMA and laminin in untreated cells underwent to a time-dependent increase, in agreement with their transient expression in skeletal myoblasts during differentiation (Olwin and Hall, 1985; Woodcock-Mitchell et al., 1988); moreover, the expression levels of all the chosen markers were appreciably enhanced by 5 ng/ml TGF-β1 at all the examined time intervals. In particular, TGF-β1 exerted a remarkable effect on transgelin whose levels were barely detectable in controls, whereas they were highly augmented in the cytokine-treated myoblasts. These data were in full agreement with a previous report (Li et al., 2004), in which the profibrotic action of TGF-β1 in C2C12 cells was described for the first time.

Next, the potential role of TGF-β1-dependent up-regulation of SK1 in the profibrotic action of the cytokine was examined. For this purpose, C2C12 myoblasts were treated with SKI-2, a specific pharmacological inhibitor of SK, already successfully used to block the enzymatic activity in these cells (Donati et al., 2007b). As shown in Figure 3B, enhanced expression of α-SMA, laminin, and transgelin induced by 24-h treatment with TGF-β1 was completely reversed by preincubation with 10 μM SKI-2. Confocal immu-
nolour fluorescent analysis revealed the marked enhanced expression of α-SMA in C2C12 cells treated with 5 ng/ml TGFβ1 for 24 h, which resulted mainly localized with F-actin filaments (Figure 3C). In agreement, SKI-2 treatment (10 μM) completely prevented the cytokine-induced expression of α-SMA. To further assess the involvement of SK1 in the biological response evoked by TGFβ1 in C2C12 myoblasts, SK1 was knocked down by employing siRNA technology. In
TGF-β reduced basal expression levels of SK1 (Figure 3D); moreover, cytokine affected S1PR expression pattern. Notably, semi-myoblasts, we examined whether cell treatment with the can exploit SK/S1P axis to exert its profibrotic effect in role of SK1 in the transdifferentiation of myoblasts into robustly decreased (Figure 3E), further supporting the key implicated mechanism could rely on additional molecular proteins was then investigated. Treatment of myoblasts specifically silenced prior cell challenge with 5 ng/ml TGF-β, lamina and laminin, or transgelin (Tagln) was analyzed by Western blotting in cell lysates, using specific primary antibodies. Bottom, densitometric analysis is reported as described in the legend to Figure 1B.

accordance with previous reports (Donati et al., 2007b; Meacci et al., 2008), specific siRNA treatment efficaciously reduced basal expression levels of SK1 (Figure 3D); moreover, it fully inhibited the TGFβ1-induced SK1 up-regulation, without affecting SK2 protein content. In agreement, the TGFβ1-dependent expression increase of laminin and α-SMA was totally abrogated when SK1 was down-regulated by RNA interference, whereas transgelin content was robustly decreased (Figure 3E), further supporting the key role of SK1 in the transdifferentiation of myoblasts into myofibroblasts brought about by TGFβ1. Because SIP acts as profibrotic mediator in various cell types, such as cultured fibroblasts and retinal pigmented epithelial cells (Urata et al., 2005; Keller et al., 2007; Swaney et al., 2008), the effect of exogenous SIP on fibrosis marker proteins was then investigated. Treatment of myoblasts with 1 μM SIP for various time intervals did not influence the expression of α-SMA, laminin, or transgelin (Figure 4). Analogous results were obtained employing SIP in the concentration range 1–100 nM (data not shown). Thus, TGFβ1-directed transdifferentiation of myoblasts into myofibroblasts required up-regulation of SK1 but was not mimicked by the addition with exogenous SIP, suggesting that the implicated mechanism could rely on additional molecular effects exerted by TGFβ1 on SK/SIP signaling pathway.

**Profibrotic Effect of TGFβ1 Is Dependent on S1PR Expression Change**

To gain further insight into the mechanism by which TGFβ1 can exploit SK/SIP axis to exert its profibrotic effect in myoblasts, we examined whether cell treatment with the cytokine affected S1PR expression pattern. Notably, semi-quantitative PCR analysis depicted in Figure 5A shows that incubation of myoblasts for 6 or 18 h in the presence of 5 ng/ml TGFβ1, apparently reduced the band corresponding to S1P1; increased that of S1P3; and rendered clearly detectable a band of 324 base pairs, corresponding to S1P4. More importantly, quantitative analysis of S1PR expression performed by real-time PCR confirmed that TGFβ1 strongly modified S1PR mRNA expression levels. In particular, it markedly reduced S1P1 and at a lesser extent S1P3 at 6 and 18 h of incubation, whereas it highly increased S1P3 at both time intervals and very potently up-regulated S1P4 mRNA levels at 18 h (Figure 5B). As a consequence, when the expression of each S1PR was referred to that of S1P1 to calculate the ranking order, the obtained profile in untreated myoblasts was S1P1 > S1P3 > S1P1 >> S1P4 whereas it became S1P3 > S1P1 > S1P3 > S1P1 in TGFβ1-treated cells at 6 h and S1P3 > S1P1 > S1P2 > S1P4 at 18 h (Figure 5C).

Because the profound alteration of S1PR pattern provoked by TGFβ1 could be implicated in the cross-talk between the cytokine and the SIP signaling pathway, next we examined whether the profibrotic action exerted by TGFβ1 was S1PR-mediated. For this purpose, the expression of fibrosis marker proteins was evaluated in C2C12 cells challenged with TGFβ1 in the presence or absence of 1 μM VPC23019, selective S1P1, antagonist or 10 μM W146, selective antagonist of S1P3. Results illustrated in Figure 6, A and B, show that the increased content of α-SMA, laminin or transgelin after TGFβ1 treatment was abrogated when SIP1 and S1P3 were blocked but was not affected by inhibition of S1P1, suggesting a role of S1P3 but not SIP1 in TGFβ1-induced response. To further support this hypothesis, individual S1PRs were knocked down by specific siRNA. Notably, as shown in Figure 6C, siRNA directed against SIP3 which significantly reduced the receptor expression, strongly attenuated the enhancement of laminin and transgelin levels elicited by TGFβ1 and abrogated the increase of α-SMA. Conversely, the down-regulation of SIP1 or SIP3 by specific siRNA did not alter the profibrotic effect of the cytokine (Figure 6, D and E). Altogether, these data support the view that transactivation of S1P3 by TGFβ1 is critical for the induction of myofibroblastic phenotype.

To confirm the importance of S1P3 in TGFβ1-induced myofibroblast transdifferentiation, we studied whether exogenous SIP rescued the effect of TGFβ1 in myoblasts where the production of endogenous SIP was blocked. As it can be observed from Figure 7, A and B, in cells in which SK was inhibited by 10 μM SKI-2 administration or SKI was specifically silenced prior cell challenge with 5 ng/ml TGFβ1, treatment with 1 μM SIP 6 h after the addition of the cytokine partially restored the stimulatory effect of TGFβ1 on transgelin expression. Importantly, SIP could not rescue the up-regulation of transgelin brought about by TGFβ1 in cells where SK was pharmacologically inhibited and SIP3 specifically silenced by siRNA administration (Figure 7C). Moreover, in contrast to what observed in native cells (Figure 4), SIP challenge of S1P3-overexpressing myoblasts provoked an appreciable increase of transgelin expression, whereas the profibrotic response elicited by TGFβ1 was significantly enhanced (Figure 7D). These data provide further experimental evidence that up-regulation of SIP3 by TGFβ1 is required to readress SIP signaling toward the onset of myofibroblastic phenotype.

**Up-Regulation of SK1/SIP3 Induced by TGFβ1 Is Smad Dependent**

Given that Smad proteins transmit most of the transcriptional effect exerted by TGFβ1, subsequently their implication in the up-regulation of SK1 and/or SIP3 by TGFβ1 was
examined. For this purpose, myoblasts transfected with specific siRNAs, capable of significantly attenuating the expression of Smad2, Smad3, or Smad4 (Figure 8A), were tested for the responsiveness to TGFβ1 in regard to up-regulation of SK1/S1P3 axis. As shown in Figure 8B, silencing of Smad2, Smad3, or Smad4 blunted the TGFβ1-dependent increase of SK1 protein content at 24 h; moreover, it also abolished the enhancement of S1P3 mRNA content elicited at the same time of incubation (Figure 8C). In agreement with these results, silencing of Smad2 or Smad3 strongly attenuated the expression of transgelin, which was fully abolished by Smad4 knockdown (Figure 8B).

**Figure 5.** TGFβ1 modulates the expression of S1PRs. (A) Two micrograms of total RNA extracted from C2C12 myoblasts stimulated (+) or not (−) with 5 ng/ml TGFβ1 for the indicated time intervals was subjected to semiquantitative RT-PCR analysis, using specific primers for S1P1, S1P2, S1P3, S1P4, and β-actin. (B) Quantitative mRNA analysis was performed by real-time PCR in total RNA extracted from C2C12 myoblasts stimulated (+) or not (−) with 5 ng/ml TGFβ1 for the indicated time intervals. S1PR mRNA quantification was based on the 2^−ΔΔCt method, using individual S1PR subtype of the unchallenged specimen as calibrator. (C) Quantitative mRNA analysis was performed as described in B. Results are expressed as -fold changes according to the 2^−ΔΔCt method, using S1P1 as calibrator. Data are the mean ± SD of one representative experiment performed in triplicate and repeated three times with analogous results.

**Profibrotic Response Elicited by TGFβ1 Is Downstream of Rho/Rho Kinase Pathway**

Next, to investigate the signaling pathways downstream of SK1/S1P3 axis implicated in the TGFβ1-directed myoblast transdifferentiation into myofibroblasts, cells were incubated with compounds such as U0126 (10 μM) or Y27632 (25 μM), which specifically inhibit extracellular signal-regulated kinase (ERK)1/2 and Rho kinase pathway, respectively. As shown in Figure 9A, prevention of ERK1/2 or Rho kinase activation did not impair TGFβ1-induced SK1, but Rho kinase inhibition strongly reduced the positive effect of the cytokine on transgelin protein content. Thus, these results suggest that Rho kinase is downstream of SK1 induction and upstream of fibrosis marker expression triggered by TGFβ1.

Because Rho kinase activity relies on stimulation by RhoA, it was then examined the effect of TGFβ1 on RhoA intracellular distribution, a validated procedure to assess the entity of active GTP-bound RhoA in C2C12 myoblasts (Mecacci et al., 2000). As illustrated in Figure 9B, 5 ng/ml TGFβ1 provoked an early increase of membrane-bound RhoA, evident already at 1 h of incubation, and a more delayed effect clearly appreciable at 8 h, still detectable at 16 h. Interestingly, preincubation with 10 μM SKI-2 did not affect the
early RhoA activation but abrogated the delayed activation, indicating that SK activation was upstream of this TGFβ1-driven molecular event. Moreover, the enhancement of membrane-bound RhoA induced by 8-h TGFβ1 treatment was prevented by previous cell incubation with 1 μM VPC23019 (Figure 9C). These findings were corroborated by results obtained in myoblasts where SK1 or S1P3 were knocked down by transfection with specific siRNA. Both experimental conditions prevented TGFβ1-dependent RhoA association to membrane at 8 h of incubation (Figure 9D). These data strongly support the view that up-regulation of SK1 as well as S1P3 are critically required for RhoA activa-

Figure 6. Role of S1PRs in TGFβ1-induced transdifferentiation of C2C12 myoblasts. (A) Left, confluent serum-starved C2C12 myoblasts were treated with or without 1 μM VPC23019 30 min before 5 ng/ml TGFβ1 challenge for 24 h. The content of laminin, α-SMA, and transgelin (Tagln) was analyzed by Western blotting of whole cell lysates (20 μg). Right, densitometric analysis. Data are reported as described in the legend to Figure 1B. (B) Left, confluent serum-starved C2C12 myoblasts were treated with or without 10 μM W146, 30 min before 5 ng/ml TGFβ1 challenge for 24 h. Fibrosis marker proteins were detected as described in A. Right, data are reported as described in the legend to Figure 1B. (C) C2C12 myoblasts, transfected with scrambled (SCR) or S1P3 siRNA, incubated in the absence (−) or in the presence (+) of 5 ng/ml TGFβ1 for the last 24 h of transfection, were checked for down-regulation by real-time PCR (left). Middle, fibrosis marker proteins were analyzed as described in A. Right, densitometric analysis. Data are reported as described in the legend to Figure 1B. Myoblasts transfected with SCR or S1P3 siRNA (D) and with SCR or S1P3 siRNA (E) were treated and used as described in C.
tion by TGFβ1, necessary in turn for the induction of myo-


tifibroblastic phenotype via Rho kinase stimulation.

**SK1/S1P₃** Signaling Pathway Is Implicated in the

Antimyogenic Action of TGFβ1

The antidifferentiating property of TGFβ1 is well established (Olson et al., 1986; Leask and Abraham, 2004). In agreement, treatment of myoblasts with the cytokine (5 ng/ml) caused a strong reduction of expression of known myogenic markers such as myosin heavy chain (MHC), and caveolin-3 at all the chosen times of incubation (Figure 10). Figure 10 also shows that the inhibition of SK signaling pathway by pre-incubation with 10 μM SKI-2 attenuated the inhibitory effect of TGFβ1 on myogenesis at 48 h of incubation. In accordance, a similar reduction of the antimyogenic effect of TGFβ1 was detected in myoblasts where SK1 was specifically down-regulated by siRNA treatment. Moreover, Figure 10 shows that the inhibitory effect of TGFβ1 on myogenesis was likewise diminished in cells where S1P₃ was knocked down. These results support the view that transactivation of S1P₃ by TGFβ1 plays also a role in its antimyogenic action.

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**Figure 7.** Dominant S1P₃ signaling is re-

quired for the induction of profibrotic re-

sponse by S1P. (A) Left, confluent serum-

starved C2C12 myoblasts were pretreated or

not with 10 μM SKI-2 for 60 min and then

incubated in the presence (+) or absence (−) of

5 ng/ml TGFβ1, 6 h before the challenge with

1 μM S1P for 24 h. Transgelin (Tagln) was
detected in aliquots (20 μg) of total cell lysates
by Western blot analysis. Right, densitometric
analysis. Data are reported as described in
the legend to Figure 1B. (B) C2C12 myoblasts,
transfected with scrambled (SCR) or SK1
siRNA, were incubated for 24 h in the presence
(+) or absence (−) of 5 ng/ml TGFβ1, 6 h
before 1 μM S1P challenge. Tagln was detected
as described in A. Right, densitometric analy-

sis. Data are reported as described in the leg-

end to Figure 1B. (C) Left, C2C12 myoblasts,
transfected with SCR or S1P₃ siRNA, were

treated with 10 μM SKI-2 for 60 min and then

incubated in the presence (+) or absence (−) of

5 ng/ml TGFβ1, 6 h before 1 μM S1P challenge.

Tagln was detected as described in A. Right,
densitometric analysis. Data are re-

ported as described in the legend to Figure 1B.

(D) Left, C2C12 cells were transiently trans-
fected with pcDNA3-S1P₃ or empty vector.

Cells were treated with 1 μM S1P or 5 ng/ml
TGFβ1 for 24 h and cell lysates used to detect
Tagln as described in A. Inset, S1P₃ expression
levels was evaluated in transfected C2C12
myoblasts by Western blot analysis using spe-

fic monoclonal anti-HA antibodies. Right,
densitometric analysis. Data are re-

ported as described in the legend to Figure 1B.
DISCUSSION

In this study, we describe a novel signaling pathway triggered by TGFβ1 in C2C12 myoblasts via Smad-dependent up-regulation of SK1 and S1P3, that is critically implicated in the transdifferentiation of myoblasts into myofibroblasts through Rho/Rho kinase signaling pathway.

Recent studies have highlighted a pivotal role of TGFβ1-directed SK1 up-regulation in mediating the profibrotic action in different types of fibroblasts (Yamanaka et al., 2004; Kono et al., 2007; Gellings Lowe et al., 2009), in full accordance with the profibrotic effect exerted by S1P in various cellular contexts, including human lung and dermal fibroblasts and retinal pigmented epithelial cells (Urata et al., 2005; Keller et al., 2007; Swaney et al., 2008). This study for the first time demonstrates that the TGFβ1-directed transcriptional regulation of SK1 and S1P3, upstream of the up-regulation of myofibroblast marker proteins is a Smad-mediated event, where the role of Smad2, Smad3, and Smad4 seems to be critical.

Previously, we demonstrated that in myoblasts SK/S1P axis, via S1P2 engagement, is physiologically required for the achievement of myogenic differentiation of C2C12 myoblasts triggered in vitro by serum withdrawal (Meacci et al., 2008) or by challenge with low doses of tumor necrosis factor-α (Donati et al., 2007b). In agreement, exogenous S1P was recognized previously to exert promyogenic activity in C2C12 cells, via engagement of S1P2 (Donati et al., 2005). The present finding that S1P does not influence the expression of fibrosis marker proteins in mouse myoblasts is consistent with the past results and with the notion that skeletal muscle fibrosis is associated with impairment of skeletal muscle differentiation (Best and Hunter, 2000; Sato et al., 2003). Nonetheless, herein SK1 up-regulation was found to be critical for transmitting the profibrotic effect of TGFβ1 in myoblasts. Strikingly, the different biological outcome mediated by SK/S1P axis after TGFβ1 challenge seems to depend on the effect exerted by the cytokine on S1PR expression pattern. Thus, the combined action on S1PR expression remodeling and SK1 up-regulation convey the TGFβ1-directed S1P inside-out signaling to act primarily via S1P3, which becomes the prevailing expressed receptor, rather than S1P2. Interestingly, the stimulation of the SK/S1P3 axis by TGFβ1 was also implicated in its antidifferentiating activity, supporting the view that in these cells S1P-dependent pathways

Figure 8. Role of Smad proteins in TGFβ1-dependent SK1 and S1P3 up-regulation and in TGFβ1-induced transdifferentiation of C2C12 myoblasts. C2C12 myoblasts were transfected with Smad2 or Smad3 or Smad4 siRNA, using scrambled (SCR) siRNA as a control. (A) Top, C2C12 myoblasts were checked for expression of Smad2, Smad3, or Smad4 by Western blot analysis, using specific anti-Smad2/3 or anti-Smad4 antibodies. Densitometric analysis is showed in the bottom panels. Data are reported as described in the legend to Figure 1B. (B) Left, C2C12 myoblasts, transfected with SCR, Smad2, Smad3, or Smad4 siRNA, were incubated in the presence (+) or absence (−) of 5 ng/ml TGFβ1 for 24 h. Total cell lysates (20 µg) were subjected to Western blot analysis to detect SK1 and Transgelin (Tagln) expression. Right, densitometric analysis is reported as described in the legend to Figure 1B. (C) Quantitative analysis of S1P3 mRNA was performed by real-time PCR in total RNA extracted from C2C12 myoblasts transfected with SCR, Smad2, Smad3, or Smad4 siRNA, stimulated (+) or not (−) with 5 ng/ml TGFβ1 for 24 h. Results are expressed as -fold changes according to the 2−ΔΔCT method, using S1P3 subtype of the unchallenged specimen as calibrator. Data are the mean ± SD of one representative experiment performed in triplicate and repeated three times with analogous results.
are exploited by TGFβ1 to elicit multiple biological effects. It is also interesting to note that the here identified Smad-dependent up-regulation of SK1/S1P3 brought about by TGFβ1 contributes to better define at mechanistic level the

Figure 9. TGFβ1-dependent transdifferentiation of C2C12 myoblasts is dependent on RhoA/Rho kinase activation, downstream of SK1 and S1P3. (A) Left, C2C12 myoblasts were treated with or without 10 μM U0126 or 25 μM Y27632, 30 min before 5 ng/ml TGFβ1 challenge for 24 h. The content of SK1 and transgelin (Tagln) was analyzed by Western analysis of whole cell lysates (20 μg). Right, densitometric analysis. A blot representative of three independent experiments is shown. Data are reported as described in the legend to Figure 1B. (B) Left, C2C12 myoblasts were incubated for the indicated time intervals with or without 5 ng/ml TGFβ1 in the presence or absence of 10 μM SKI-2. Western analysis of RhoA was performed in membrane and cytosolic fractions (10 μg). Right, densitometric analysis of three independent experiments. Data are reported as described in the legend to Figure 1D. (C) Left, C2C12 myoblasts were treated with or without 1 μM VPC23019. Western analysis was performed as described in B. Right, densitometric analysis is reported as described in the legend to Figure 1D.

are exploited by TGFβ1 to elicit multiple biological effects. It is also interesting to note that the here identified Smad-dependent up-regulation of SK1/S1P3 brought about by TGFβ1 contributes to better define at mechanistic level the
well-known antimyogenic action elicited by TGFβ1 via Smad proteins (Liu et al., 2001; Zhu et al., 2004).

It should be noted that our study on S1PR expression relies exclusively on expression levels of mRNA, due to the lack of suitable S1P3 and S1P2 antibodies. However, even though the actual increase of S1P3 and decrease of S1P2 protein content could not be measured, the altered S1PR profile at myoblast plasma membrane could be inferred from the finding that in myoblasts exogenous S1P is myogenic and not profibrotic, but after TGFβ1 treatment it becomes able to enhance fibrosis and reduce myogenic marker expression, similarly to what observed in S1P3-overexpressing myoblasts.

Several lines of experimental evidence are provided here in support of a key role of S1P3 in transmitting the profibrotic action of TGFβ1 in myoblasts. In agreement, S1P3 was previously individuated downstream of TGFβ1 regulation of α-SMA expression in lung fibroblasts (Kono et al., 2007), although in these cells it shared with S1P2 the profibrotic signaling. Moreover, the structural analogue of S1P, FTY720-phosphate, has been recently reported to elicit myofibroblast differentiation of fibroblasts via S1P3, because its effect was abrogated in S1P3-null mice (Keller et al., 2007). Intriguingly, in the same study Smad3 activation was identified downstream of S1P3 signaling, whereas here Smad3, together with Smad2 and Smad4, was found upstream of S1P3 transcriptional regulation, suggesting the occurrence of positive loop in the regulation of these two proteins worth of a more careful investigation.

The Rho/Rho kinase pathway is known to be involved in the fibrotic process that affects multiple different tissues (Dolman et al., 2008; Soon and Yee, 2008; Haudek et al., 2009). Interestingly, in our study Rho/Rho kinase was identified upstream of S1P3-dependent up-regulation of fibrosis marker expression brought about by TGFβ1. These data are in agreement with a previous study in which Rho kinase was found implicated in the mechanism by which SK1 regulates differentiation of lung fibroblasts mediated by TGFβ1 (Kono et al., 2007).

Another original finding of this study entails the up-regulation of S1P4 by TGFβ1, supporting the notion that TGFβ1 elicits myoblast transdifferentiation into myofibroblasts.
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S1P, in addition to acting in lymphoid tissue (Wang et al., 2005; Matsuyuki et al., 2006) is probably involved in biological responses in other cellular settings. The delayed enhancement of S1P2 expression in comparison with the more rapid induction of SK1 and S1P3 suggests that its up-regulation requires the synthesis of secondary factors down-stream of TGFβ1. Although in this study S1P2 was found disengaged from the TGFβ1-induced profibrotic response its transcriptional regulation by TGFβ1 probably participates to some other molecular event that concur to achieve the full biological response to the cytokine.

In summary, the present results for the first time demonstrate the key role of SK1/S1P3 axis in the TGFβ1-induced transdifferentiation of myoblasts into myofibroblasts and more importantly demonstrate that TGFβ1 by remodelling S1PR expression redirects the promyogenic effect of endogenous S1P into a profibrotic, antidifferentiating cue. A model of TGFβ1 action is illustrated in Figure 11.

The concept that the peculiar flexibility of S1P inside-out signaling is strictly dependent on a given S1PR profile is also supported by a previous study in which platelet-derived growth factor-BB-induced up-regulation of SK1 and SK2, together with that of S1P2, was found as part of a regulatory loop finalized to trigger hepatic wound repair by hepatic myofibroblasts (Serriere-Lanneau et al., 2007). In the same vein, up-regulation of S1P2 triggered by cross-linking of the high-affinity receptor for immunoglobulin E, contributed to the regulatory balance between mast cell migration and degranulation (Jolly et al., 2004).

TGFβ1 is the main elictor of overproduction of the extra-cellular matrix proteins that cause muscle fibrosis, a biological process that negatively interferes with repair and functional recovery of injured skeletal muscle (Best and Hunter, 2000; Sato et al., 2003; Li et al., 2004). Thus, neutralization of TGFβ1 action seems to be a promising approach to improve muscle regeneration (Huard et al., 2002; Cohn et al., 2007). Finally, it is worth mentioning that the adverse effects exerted by TGFβ1 on muscle regeneration in multiple myopathic states imply not only the stimulation of fibrotic response but also a decline of performance of satellite cells, the muscle-resident stem cells that differentiate into myoblasts (Cohn et al., 2007). In this regard, because S1P has been individuated among the most powerful regulators of satellite cell proliferation (Nagata et al., 2006), it seems to be of great importance to understand whether cross-talk with S1P pathway plays any role in the deleterious action of TGFβ1 in these cells.

In conclusion, this study has identified the unique ability of TGFβ1 to exploit S1P signaling to transdifferentiate myoblasts into myofibroblasts, by simultaneously affecting SK1 and S1PR expression pattern, providing new clues on the molecular mechanisms underlying the profibrotic and antidifferentiating response elicited by the cytokine that could be pharmacologically addressed to ameliorate skeletal muscle regeneration.

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