Biochimica et Biophysica Acta 1823 (2012) 439–450

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Sphingosine 1-phosphate stimulates proliferation and migration of satellite cells Role of S1P receptors

Serena Calise [a], Sabrina Blescia [a], Francesca Cencetti [a,b], Caterina Bernacchioni [a,b], Chiara Donati [a,b,1], Paola Bruni [a,b,1,*]

[a] Dipartimento di Scienze Biochimiche, Università di Firenze, 50134 Firenze, Italy
[b] Istituto Interuniversitario di Miologia (IIM), Italy

ARTICLE INFO

Article history:
Received 8 August 2011
Received in revised form 14 November 2011
Accepted 16 November 2011
Available online 8 December 2011

Keywords:
Satellite cell
Sphingosine 1-phosphate
S1P receptor
Cell migration
Cell proliferation
Skeletal muscle regeneration

ABSTRACT

Satellite cells are resident stem cells of skeletal muscle; they are normally quiescent but upon post-trauma activation start to proliferate and fuse with damaged fibers contributing to muscle regeneration. In this study the effect of the bioactive sphingolipid sphingosine 1-phosphate (S1P) on the proliferative and migratory response of murine satellite cells has been examined. S1P was found to stimulate labeled thymidine incorporation in a phosphatidylinositol 3-kinase-dependent manner. Moreover, by employing selective S1P receptor agonists and antagonists and silencing individual S1P receptors, the mitogenic action of S1P in satellite cells was shown to depend on $S1P_2$ and $S1P_3$. Notably, by using different experimental approaches S1P was found to positively influence satellite cell migration, necessary for their recruitment at the site of muscle damage. Interestingly, the specific silencing of individual S1P receptor subtypes demonstrated the pivotal role of $S1P_1$ and $S1P_4$ in mediating the S1P migratory effect. This latter result demonstrates for the first time that $S1P_4$ receptor has a role in skeletal muscle cells, supporting the notion that this receptor subtype plays a biological action broader than that so far identified in lymphoid tissue. On the contrary, $S1P_2$ was found to negatively regulate cell migration. Collectively, these results are in favour of an important function of S1P in satellite cell biology that could in principle be exploited as novel pharmacological target for improving skeletal muscle regeneration.

1. Introduction

Sphingosine 1-phosphate (S1P) is a naturally occurring lipid produced by many different cell types, present in circulating blood at low micromolar concentration [1]. A number of studies have clearly demonstrated that S1P can modulate multiple key biological processes including cell proliferation, motility, differentiation and survival by acting either as intracellular mediator or as ligand of at least five distinct G-protein coupled receptors, termed S1P receptors (S1PRs) [2].

This tissue exhibits a remarkable capacity of self-repair upon damage or disuse which relies on the presence of resident stem cells, named satellite cells. These cells are normally quiescent but upon activation start to proliferate and differentiate into myoblasts that in turn fuse with preexisting fibers or generate new myotubes [3]. A large body of literature data is in favour of a physiological role of S1P and its metabolism in skeletal muscle. Pioneering studies performed in murine myoblasts showed that S1P accelerates myogenic differentiation, acting via $S1P_2$ ligation [4]. Subsequently it was demonstrated that endogenous production of S1P, catalyzed by sphingosine kinase-1, followed by $S1P_2$ engagement, was physiologically involved in myoblast differentiation into myotubes [5]. Further studies have shown that inside-out S1P signalling in myoblasts is critically implicated in the molecular mechanisms by which TNFα at low doses enhances myogenesis [6], PDGF modulates chemotactic and mitogenic response [7], TGFβ elicits transdifferentiation of myoblasts into myofibroblasts [8], thus highlighting distinct, even contrasting roles for $S1P_1$, $S1P_2$ and $S1P_3$ [9], that are co-expressed in this cell type.

Notably, recent studies have unveiled a physiological role of S1P in adult skeletal muscle. Zanin et al. showed that S1P robustly protects skeletal muscle fibers from atrophy, being exogenous S1P able to counteract the reduction of muscle mass caused by denervation [10]. Moreover, S1P was found to enhance the regeneration of mouse and rat soleus muscle following myotoxic injury [11] and to protect skeletal muscle fibers from apoptosis, upon eccentric contraction-induced tissue damage [12].

Satellite cells, recognized as crucial players in skeletal muscle self-repair [13], were previously identified as physiological targets of S1P. This sphingolipid was shown to trigger their activation by inducing

Abbreviations: S1P, sphingosine 1-phosphate; S1PR, S1P receptor; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; BSA, bovine serum albumin; PBS, phosphate buffered saline; HS, horse serum

* Corresponding author at: Dipartimento di Scienze Biochimiche, Università di Firenze, Viale G.B. Morgagni 50, 50134 Firenze, Italy. Tel.: +39 0554598328; fax: +39 0554598905.
E-mail address: paola.bruni@unifi.it (P. Bruni).

[1] These authors equally contributed to the work.

0167-4889/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2011.11.016

cell cycle entry; moreover, pharmacological inhibition of sphingolipid metabolism directed towards S1P formation caused perturbation of skeletal muscle repair [14].

Although the results obtained thus far are promising, a better knowledge of the S1P action mechanism in satellite cells is absolutely required in order to understand whether satellite cell responsiveness to S1P could represent a novel target for pharmacological intervention capable of improving skeletal muscle regeneration. For this purpose in this study we have investigated in detail the effect of S1P on satellite cell proliferation and migration, together with the role exerted by S1PRs in these biological actions. Obtained data clearly indicate that S1P robustly affects cell proliferation and motility via $S1P_{2/3}$ and $S1P_{1/4}$ receptors respectively, in favour of a key role of the sphingolipid in satellite cell biology, that could be in principle be exploited to enhance their responsiveness.

2. Materials and methods

2.1. Materials

Biochemicals, TriReagent, cell culture reagents, Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), horse serum (HS), protease inhibitor cocktail, bovine serum albumin (BSA), collagenase, calcein-AM, Blotto, and non-fat dry milk, were purchased from Sigma-Aldrich (St. Louis, MO, USA). MEK inhibitor U0126, $S1P_1$ receptor agonist SEW2871, sphingosine kinase inhibitor, SKI-2 and D-*erythro*-sphingosine 1-phosphate (S1P) were purchased from Calbiochem (San Diego, CA, USA). *(S)*-FTY720-Phosphate (FTY720-P) was purchased from Echelon (Salt Lake City, USA). Short interfering RNA (siRNA) duplexes corresponding to two DNA target sequences of mouse $S1P_1$ (5′-UCACCUACUACUGUUAGAdTdT-3′; 5′-CUUGCUAACUAUUUGGAAAdTdT-3′), mouse $S1P_2$ (5′-CUCUGUACGUCCGAAUGUAdTdT-3′; 5′-GACUAAUCAGAUUGUAGUAdTdT-3′), mouse $S1P_3$ (5′-CCAUAUGAUGCCAACAAGAdTdT-3′; 5′-GUAAGUCAAGCUCCAGUAAdTdT-3′), mouse $S1P_4$ (5′-CUGCUGAACAUCACACUGAdTdT-3′; 5′-CUGACAUCUUUGGUUCUAAdTdT-3′) and scrambled siRNA (5′-UUCUCCGAACGUGUCACGUdTdT-3′) were from Sigma-Proligo (The Woodlands, TX, USA). Lipofectamine RNAiMAX™ (Invitrogen) and all reagents and probes required to perform Real-Time PCR (Applied Biosystems) were purchased from Life Technologies (Carlsbad, CA, USA). Enhanced chemiluminescence (ECL) reagent was obtained from GE Healthcare Europe GmbH (Milan, Italy). Thymidine [methyl-^{3}H] (20.0 Ci/mmol) was from Perkin Elmer (Waltham, MA, USA). Secondary antibodies conjugated to horseradish peroxidase, polyclonal anti-Akt 1/2/3 (H-136) and anti-phospho-Akt 1/2/3 (Ser473) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Polyclonal anti-phospho-ERK1/ERK2 (Thr202/Tyr204), anti-ERK1/ERK2, anti-p38 mitogen-activated protein kinase (MAPK) and monoclonal anti-phospho-p38 MAPK (Thr180/Tyr182) were from Cell Signaling Technology, Inc. (Beverly, MA, USA). The selective $S1P_1$ antagonist W146 and the specific $S1P_{1/3}$ antagonist VPC23019 were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Coomassie Blue reagent was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Chick embryo extract was purchased from Sera Laboratories International Ltd. (Bolney, WS, UK). Collagen type I was purchased from Millipore (Billerica, MA, USA). Matrigel basement membrane matrix was obtained from BD Biosciences (Bedford, MA, USA). Lysophosphatidic acid (LPA) and the selective $S1P_3$ antagonist CAY10444 were purchased from Cayman Chemical (Ann Arbor, MI, USA). Epidermal growth factor (EGF) and insulin-like growth factor 1 (IGF-1) were purchased from PeproTech EC Ltd. (London, UK). The recombinant hepatocyte growth factor (HGF) was purchased from R&D System Inc. (Minneapolis, MN, USA). The p38 MAPK inhibitor SB239063, the phosphatidylinositol 3-kinase inhibitor, LY 294002, the selective $S1P_{2/4}$ receptor antagonist JTE-013 were obtained from Tocris Bioscience (Ellisville, MO, USA). Restore Western Blot stripping buffer was purchased from ThermoFisher Scientific (Rockford, IL, USA). CHEMOTX system, polycarbonate filters (8 μm pores) and modified Boyden chambers were obtained from Neuroprobe (Gaithesburg, MD, USA). Diff-Quick staining solution was purchased from Dade-Behring Holding GmbH (Liederbach, Germany).

2.2. Animals

Male C57BL/6 mice, purchased from Charles River Laboratories Italia S.r.l. (Calco, Lecco, Italy), were housed at controlled temperature (21–24 °C) with a 12–12 h light–dark cycle. Food and water were provided *ad libitum*.

Mice (2–7 month-old) were killed by rapid cervical dislocation, according to the procedure suggested by the Ethical Committee for Animal Experiments of the University of Florence and the EEC guidelines for animal care of the European Community Council (Directive 86/609/EEC). All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.3. Primary satellite cell isolation and culture

Single muscle fibers with associated satellite cells were isolated from *tibialis anterior* (TA) muscles as previously described [15,16]. Briefly the muscles, removed from the hindlimb by microdissection, care being taken to handle them only by their tendons to minimize damage to the fibers, were incubated in 0.2% (wt/vol) collagenase type I/DMEM for 1–2 h at 37 °C. Following enzymatic digestion, the individual myofibers were liberated by repeatedly triturating the muscle with a wide-mouth Pasteur pipette. The disgregated muscles were then pre-plated into 100 mm petri dishes for 1 h at 37 °C to remove fibroblasts which adhere to plastic more avidly than satellite cells [17]. The resulting suspension was then plated into 24-well pre-coated plates with 1 mg/ml Matrigel in plating medium (DMEM supplemented with 10% horse serum, 0.5% chick embryo extract, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml kanamycin, and 2.5 μg/ml amphotericin B) at 37 °C in 5% CO_2. Satellite cells were released from the fibers 12–24 h after plating. After 3–4 days the plating medium was replaced with proliferation medium (DMEM supplemented with 20% foetal bovine serum, 10% horse serum, 1% chick embryo extract, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml kanamycin, and 2.5 μg/ml amphotericin B) for promoting cell division. Isolated satellite cells were routinely tested by western blotting analysis for the expression of Pax7, a paired-box transcription factor expressed by quiescent satellite cells and implicated in the generation of committed myogenic progenitors [18]. All experiments were performed using cultures that had undergone between two and four passages.

2.4. Cell transfection

Cells grown into tissue culture 12-well plates (22,000 cells/well) were transfected with siRNA duplexes using Lipofectamine RNAiMAX, according to the manufacturer's instructions, as previously described [8]. Briefly, 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 cells to a final concentration of 85 nM in DMEM containing serum. After 30 h, cells were shifted to DMEM without serum containing 1 mg/ml BSA and then used for the experiments within 72 h from the beginning of transfection. The specific gene knockdown was evaluated by real-time RT-PCR.

2.5. Cell proliferation

Cell proliferation was determined by measuring [^{3}H]thymidine incorporation as previously described [19]. To evaluate [^{3}H]thymidine

incorporation satellite cells were serum-starved for 24 h and then challenged with or without the indicated concentrations of S1P (2 mM stock solution in dimethyl sulfoxide). [^{3}H]Thymidine (0.5 μCi/well) was added for the last 4 h of incubation. Cells were washed twice in ice-cold PBS before 500 μl addition of 10% trichloroacetic acid (TCA) for 5 min at 4 °C. TCA was removed and 250 μl of ethanol:ether (3:1 v/v) solution was added to the insoluble material and plates were collocated under chemical hood until complete evaporation of solution. Samples were then lysed in 0.25 N NaOH for 1–2 h at 37 °C. Incorporation of [^{3}H]thymidine was measured by scintillation counting. Alternatively, proliferation was evaluated by cell counting. Briefly, satellite cells, seeded in six-well plates at a density of approximately 1×10^5 cells per well, were serum-starved overnight in DMEM containing 0.1% BSA and 2% delipidated FCS and then challenged with 1 μM S1P for 24 h before being trypsinized and counted by a hemocytometer.

2.6. Western blot analysis

Satellite cells were lysed for 30 min at 4 °C in a buffer containing 50 mM Tris, pH 7.5, 120 mM NaCl, 1 mM EDTA, 6 mM EGTA, 15 mM $Na_4P_2O_7$, 20 mM NaF, 1% Nonidet and protease inhibitor cocktail (1.04 mM AEBSF, 0.08 μM aprotinin, 0.02 mM leupeptin, 0.04 mM bestatin, 15 μM pepstatin A, and 14 μM E-64). To prepare total cell lysates, cell extracts were centrifuged for 15 min at 10,000 *g* at 4 °C. Proteins (20 μg) from lysates were resuspended in Laemmli's sodium dodecyl sulphate (SDS) sample buffer. Samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for 90 min at 120 mA, before transfer of proteins to PVDF membranes, which were incubated 1 h at room temperature with 20 mM Tris, pH 7.5, 150 mM NaCl containing 0.1% Tween 20 and 5% non-fat milk. After rinsing, membranes were incubated overnight with the primary antibodies at 4 °C and then with specific secondary antibodies for 1 h at room temperature. Bound antibodies were detected by chemiluminescence.

2.7. Real-Time PCR

Total RNA (1 μg), extracted with TRI Reagent from satellite cells, was reverse transcribed using the High Capacity cDNA Reverse Transcription kit, according to the manufacturer's instructions.

The quantification of S1PR mRNA level 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 [19], by simultaneous amplification of the target sequence ($S1P_1$ Mm00514644_m1, $S1P_2$ Mm01177794_m1, $S1P_3$

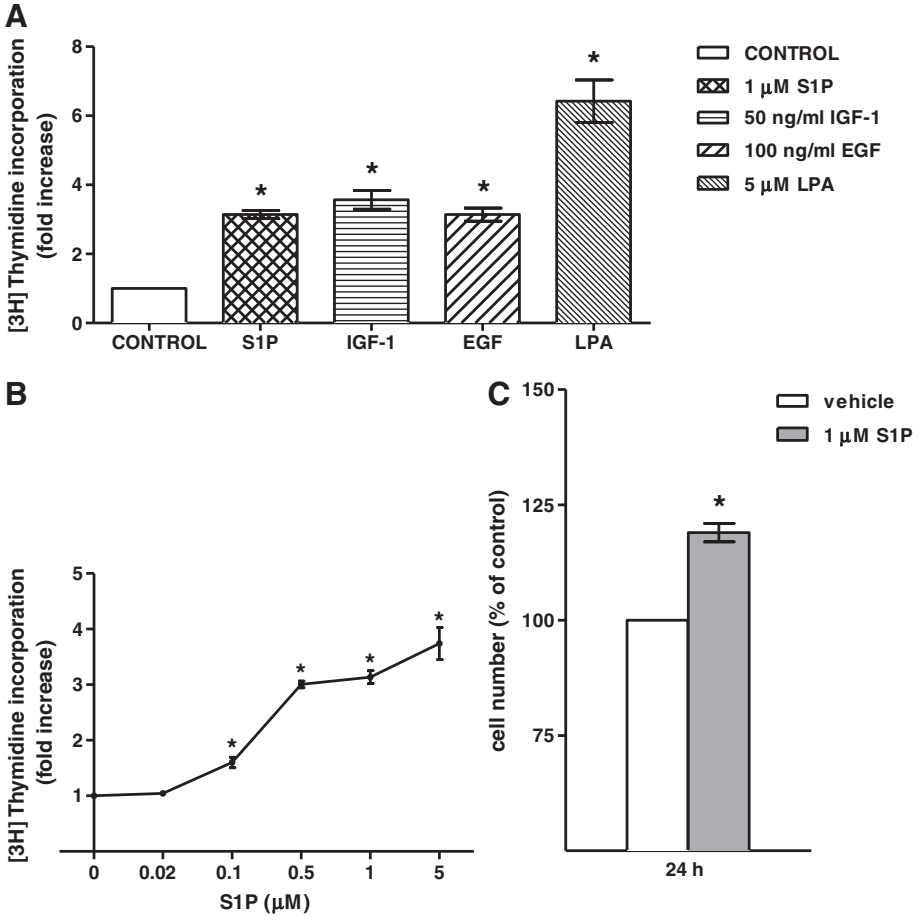


Fig. 1. Effect of S1P on satellite cell proliferation. (A) Labeled thymidine incorporation was measured in serum-starved satellite cells, approximately 60% confluent, treated with the indicated concentrations of S1P, IGF-1, EGF or LPA for 20 h. [^{3}H]Thymidine (0.5 μCi/well) was added during the last 4 h of incubation. [^{3}H]Thymidine incorporation in untreated cells was 9993 ± 842 dpm. The results are reported as fold increase relative to control set as 1. The effect of all the agonists used was statistically significant by Student's *t* test. Data are means ± SEM of at least 3 independent experiments performed in triplicate. (B) Dose dependence of S1P on satellite cell proliferation. Serum-starved cells, approximately 60% confluent, were stimulated with the indicated concentrations of S1P for 20 h. [^{3}H]Thymidine (0.5 μCi/well) was added during the last 4 h of incubation. The results are reported as fold increase relative to control set as 1. Data are means ± SEM of at least 3 independent experiments performed in triplicate. (C) Cell counting. Serum-starved satellite cells were stimulated with 1 μM S1P for 24 h before being counted by a hemocytometer. Asterisk indicates statistical significance: *P<0.05.

Mm00515669_m1, $S1P_4$ Mm00468695_s1 Applied Biosystems, Life Technologies, Carlsbad, CA, USA, $S1P_5$ Prime Time qPCR assay N053190.1.pt.S1PR5, Integrated DNA Technologies, Leuven, Belgium) together with the housekeeping gene 18S rRNA. Results were analyzed by ABI Prism Sequence Detection System software, version 1.7 (Applied Biosystems, Foster City, CA). The $2^{-\Delta\Delta CT}$ method was applied as a comparative method of quantification [20] and data were normalized to ribosomal 18S RNA expression.

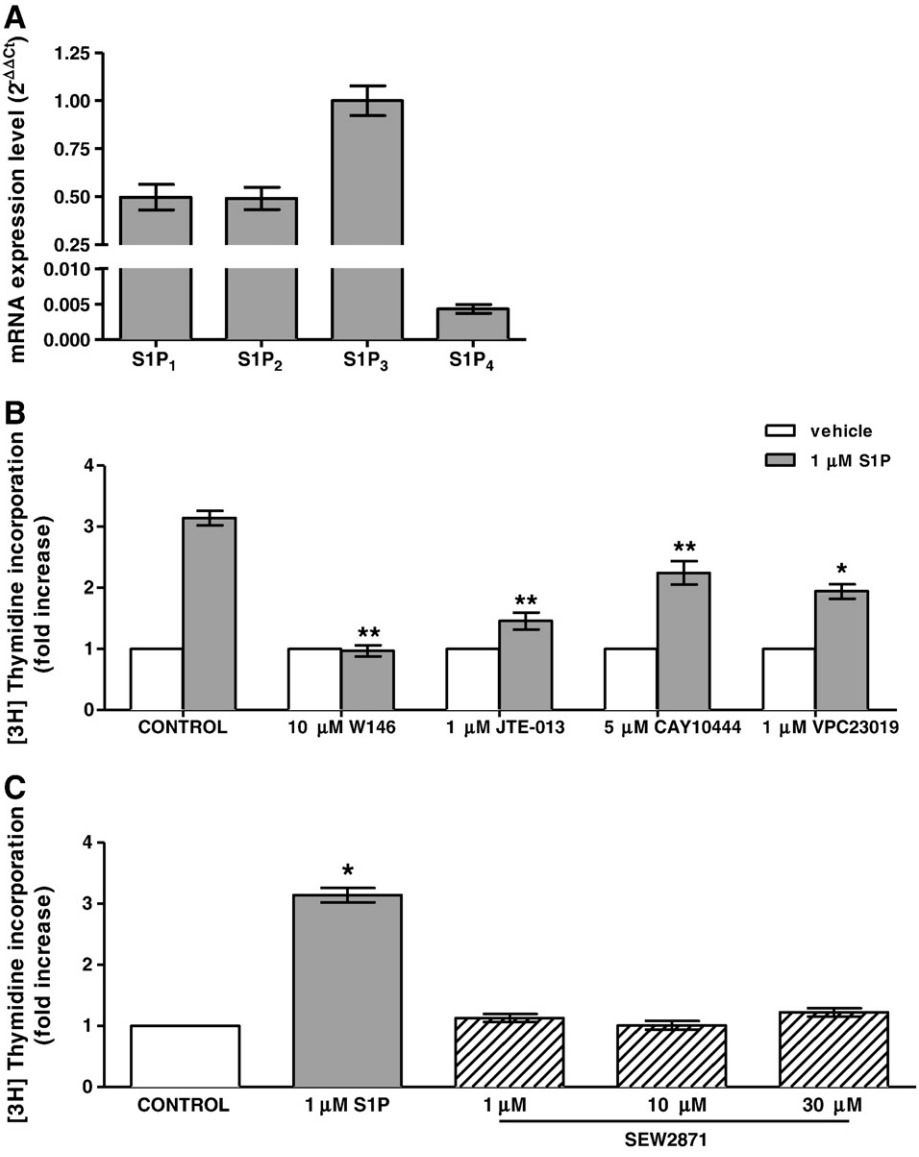


Fig. 2. Role of S1PRs in the mitogenic effect induced by S1P in satellite cells. (A) mRNA expression levels of S1PRs in satellite cells. Quantitative mRNA analysis by real time PCR was performed in satellite cells by simultaneous amplification of the target $S1P_1$, $S1P_2$, $S1P_3$, and $S1P_4$ genes together with the housekeeping gene 18S rRNA. Results are expressed as fold changes according to the $2^{-\Delta\Delta CT}$ method, utilizing $S1P_3$ as calibrator. Data are means ± SEM of 3 independent experiments performed in triplicate. (B) Effect of S1PR antagonists on the proliferative action of S1P. Cells, approximately 60% confluent, were pre-incubated for 30 min in the presence or not of 10 μM W146, 1 μM JTE-013, 5 μM CAY10444 or 1 μM VPC23019 before being stimulated with 1 μM S1P for 20 h. [^{3}H]Thymidine (0.5 μCi/well) was added during the last 4 h of incubation. [^{3}H]Thymidine incorporation in untreated control cells was 9720 ± 608 dpm, in W146 pre-treated cells was 37261 ± 3289 dpm, in JTE-013 pre-treated cells was 12960 ± 1714 dpm, in CAY10444 pre-treated cells was 15553 ± 4892 dpm, and in VPC23019 pre-treated cells was 11340 ± 857 dpm. The results are reported as fold increase relative to control set as 1. Data are means ± SEM of at least 3 independent experiments performed in triplicate. The effect of all the antagonists used was statistically significant by Student's *t* test. (C) Effect of the $S1P_1$ receptor agonist SEW2871 on the proliferative action of S1P. Labeled thymidine incorporation was measured in serum-starved satellite cells, approximately 60% confluent, treated with the indicated concentrations of SEW2871 or with 1 μM S1P for 20 h. [^{3}H]Thymidine (0.5 μCi/well) was added during the last 4 h of incubation. [^{3}H]Thymidine incorporation in untreated cells was 10409 ± 510 dpm. The results are reported as fold increase relative to control set as 1. Data are means ± SEM of at least 3 independent experiments performed in triplicate. The effect of SEW2871 was statistically not significant by Student's *t* test. (D) S1PRs silencing. Real Time PCR was performed in cells transfected with non-targeting siRNA (scrambled) or with siRNA specific for $S1P_1$, $S1P_2$, $S1P_3$ or $S1P_4$; the content of housekeeping gene 18S rRNA was analyzed in parallel. Results are expressed as fold changes according to the $2^{-\Delta\Delta CT}$ method, utilizing as calibrator each receptor subtype in cells transfected with scrambled siRNA. Data are means ± SEM at least of 3 independent experiments performed in triplicate. The effect of siRNA transfection on S1PR mRNA levels was statistically significant by Student's *t* test. (E) Effect of S1PRs silencing on the proliferative action of S1P. Serum-starved satellite cells transfected with non-targeting siRNA (scrambled) or with specific siRNA for individual S1PRs were stimulated with 1 μM S1P for 20 h. [^{3}H]Thymidine (0.5 μCi/well) was added during the last 4 h of incubation. [^{3}H]Thymidine incorporation was 7249 ± 529 dpm in control cells (untreated scrambled-transfected cells), 6175 ± 785 dpm in untreated $S1P_1$- siRNA transfected cells, 4248 ± 1079 dpm in untreated $S1P_2$-siRNA transfected cells, 6856 ± 1208 dpm in untreated $S1P_3$-siRNA transfected cells, 8369 ± 586 dpm in untreated $S1P_4$-siRNA transfected cells. Data are means ± SEM of at least 3 independent experiments performed in triplicate. The effect of $S1P_2$ and $S1P_3$ silencing on S1P-induced proliferation was statistically significant by Student's *t* test. Asterisks indicate statistical significance: *P<0.05; **P<0.01.

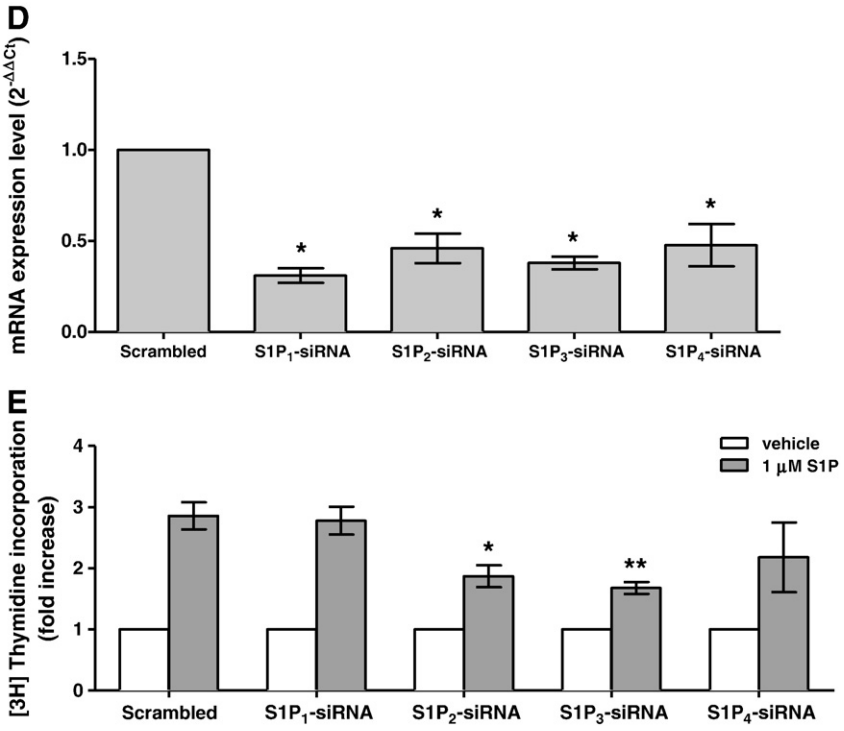


Fig. 2 (*continued*).

2.8. Cell migration

For ChemoTx assay, satellite cells were cultured in proliferating medium until 60–70% confluence and then starved overnight in DMEM containing 0.1% BSA. Cells were washed twice with PBS and then incubated for 30 min at 37 °C in the presence of 1 μM calcein-AM. Stained cells were washed three times with PBS and detached with trypsin. Approximately 8000 cells were placed directly onto chemotaxis filters. The bottom chambers were filled with DMEM containing or not agonists. Cells were incubated for 8 h at 37 °C. The non-migrated cells on the top of the filter were removed by gently wiping the filter with a cotton swab. Fluorescence of migrated cells into the bottom chamber was measured in a multi-well fluorescent plate reader (Fluoroskan Ascent FL, ThermoElectron Corporation, Excitation, 485 nm; Emission, 538 nm). Fluorescence was converted to numbers of cells based on a standard curve generated by seeding known numbers of satellite cells in the bottom of the chamber.

To perform wound healing assay, satellite cells were seeded in 12-well tissue culture plates to a final density of 1×10^5 cells/well and maintained at 37 °C and 5% CO_2 for 24 h until confluence, before being serum-starved overnight. The cell monolayer was wounded by scratching with a 200 μl standard sterile pipette tip. The scratched monolayer was rinsed twice with DMEM containing 0.1% BSA to remove cell debris and incubated with or without 20 nM S1P or 10 ng/ml HGF. At least six scratched areas for each sample were photographed by a Nikon digital camera connected to a Nikon Phase Contrast ELWD 0.3 Microscopy at time 0 and after 8 h of incubation. Satellite cell migration was quantified by calculating the number of cells migrated into the scratched area after 8 h compared to that into the area of the initial wound.

Alternatively, cell migration was evaluated using a modified Boyden chamber system as previously described [21]. Briefly, polycarbonate filters with 8 μm pores were coated with Matrigel (250 μg/ml) for 60 min at 37 °C. S1P (20 nM) was added to the lower chamber while, satellite cells, trypsinized and resuspended in DMEM containing 250 μg/ml heat-inactivated BSA, were placed (2×10^4 cells) into the upper well of the chamber and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 7 h. Polycarbonate filters were fixed overnight with methanol at 4 °C and stained with Diff-Quick staining solution. Migration was assessed by counting the number of migrated cells in six random fields per filter.

2.9. Statistical analysis

Densitometric analysis of the Western Blot bands was performed using ImageJ software and analysis software Quantity One (Bio-Rad Laboratories, Hercules, CA). Graphical representations were performed using Microsoft Excel Software (Microsoft Excel Corp., New York, USA) and Prism 5.0 (GraphPad Software, San Diego, CA). Statistical analysis was performed using Student's *t* test. Asterisks indicate statistical significance: *$P<0.05$; **$P<0.01$.

3. Results

3.1. S1P stimulates proliferation of mouse satellite cells

Satellite cells, although normally quiescent, have the property to proliferate in response to a number of stimuli released upon skeletal muscle damage; however, the panel of extracellular cues endowed with the property of trigger satellite cell proliferation are presently not fully characterized. To investigate the potential mitogenic action of S1P in satellite cells, the effect of 1 μM S1P on labeled thymidine incorporation was evaluated in satellite cells, isolated from TA muscles of C57BL/6 male mice and maintained for 24 h in DMEM medium containing 0.1% BSA, in comparison with the effect elicited by other extracellular cues such as IGF-1 (50 ng/ml), EGF (100 ng/ml), and LPA (5 μM). Concentrations of growth factors and lysophospholipid mediators were chosen as capable of eliciting the maximal biological response on the basis of experiments in which their dose-dependent efficacy had been preliminarily tested. Results illustrated in Fig. 1A clearly show that S1P robustly stimulated DNA duplication in satellite cells, in full agreement with a previous report [14]. Interestingly, the

potency of S1P in eliciting the mitogenic response was comparable to that displayed by IGF-1 and EGF and moderately lower than that induced by LPA, thus far unidentified as mitogen for satellite cells. The effect of the sphingolipid was dose-dependent (EC_{50} = 150 nM) (Fig. 1B). The stimulatory effect of S1P on cell proliferation was also confirmed by cell number determination which was significantly enhanced at 24 h by the bioactive sphingolipid (1 μM) (Fig. 1C).

3.2. S1P-induced satellite cell proliferation depends on ligation to $S1P_{2/3}$

The molecular mechanism by which S1P exerts specific biological effects is quite complex, involving ligation to intracellular targets, presently not fully identified, as well as engagement of specific receptors termed S1PRs. Given that S1PRs were found implicated in the mitogenic effect elicited by S1P in reserve cells isolated from C2C12 cell cultures, that share many biological properties with satellite cells [22], to address their potential role in satellite cells, the pattern of S1PR expression was at first evaluated by quantifying mRNA expression levels of individual S1PRs. As it can be observed in Fig. 2A, four S1PRs were found expressed at mRNA level in satellite cells, with the ranking order of expression $S1P_3 > S1P_1 = S1P_2 >> S1P_4$, whereas $S1P_5$ was not detectable. The role of S1PRs in the mechanism by which S1P stimulated proliferation of satellite cells was then examined, by employing compounds known to act as antagonists of individual S1PRs reported to be selective for $S1P_1$, such as W146 (10 μM), $S1P_{2/4}$, such as JTE-013 (1 μM), $S1P_3$, such as CAY10444 (5 μM) and $S1P_{1/3}$ such as VPC23019 (1 μM). Results illustrated in Fig. 2B clearly show that all the S1PR antagonists impaired the mitogenic response triggered by S1P, being the sphingolipid-dependent [3H]thymidine incorporation abrogated by W146 or JTE-013 treatment and strongly reduced by CAY10444 and VPC23019, suggesting that $S1P_1$, $S1P_2$, $S1P_3$, and $S1P_4$ were all implicated in the observed S1P action. However, in contrast, the selective engagement of $S1P_1$, brought about by SEW2871, did not provoke any increase of DNA duplication above control, even if employed at high concentration (30 μM) (Fig. 2C), suggesting that at least some of these pharmacological compounds at the employed concentrations could exert unspecific effects in this cellular context [23,24]. Thus, to better investigate the role of S1PRs in S1P-stimulated proliferation of satellite cells, individual S1PRs were knocked-down by employing specific siRNA. As illustrated in Fig. 2D, in this adopted experimental condition, RNA interference reduced the expression of each S1PR at least by 50%. Importantly, the down-regulation of $S1P_1$ or $S1P_4$ expression did not alter the ability of S1P to promote satellite cell proliferation, whereas when $S1P_2$ or $S1P_3$ were knocked down, S1P efficacy was diminished approximately by 40%, clearly demonstrating that both S1PRs were involved in transmitting the mitogenic action of S1P in these cells (Fig. 2E).

3.3. S1P stimulates mouse satellite cell migration

Subsequently, it was examined whether S1P was capable of influencing cell migration, which also represents a key biological response necessary for the correct recruitment of satellite cells at the

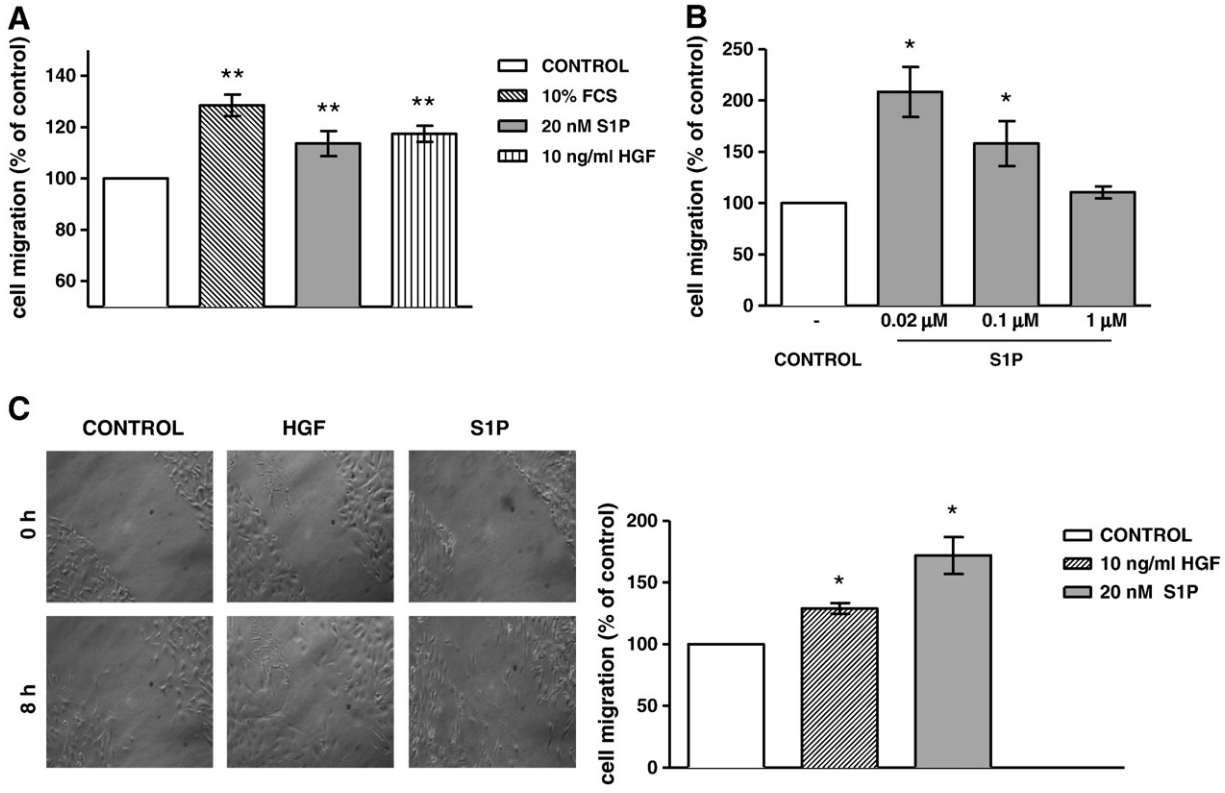


Fig. 3. Effect of S1P on satellite cell migration. (A) Cell migration was measured by 96-well ChemoTx disposable chemotaxis system in satellite cells challenged with 10% FCS, 20 nM S1P and 10 ng/ml HGF for 8 h. Data are means ± SEM of at least 3 independent experiments performed in triplicate. The effect of S1P, HGF and FCS was statistically significant by Student's *t* test. (B) Cell migration across Matrigel-coated porous membranes of Boyden chambers was determined in satellite cells challenged with the indicated concentration of S1P for 7 h. Number of migrated cells in controls was 413 ± 55. Data are means ± SEM of 3 independent experiments performed in triplicate. (C) Scratch wound healing assays were performed in satellite cells challenged with 10 ng/ml HGF and 20 nM S1P for 8 h. Left panel: representative microscopic views at 0 h and 8 h. Right panel: quantitative analysis of cell migration after 8 h. Data represent the means ± SEM of at least 3 independent experiments performed in triplicate and are reported as percentage of control set as 100. The effect of agonists was statistically significant by Student's *t* test. Asterisks indicate statistical significance: *P<0.05; **P<0.01.

site of muscle damage. Results illustrated in Fig. 3A show that 20 nM S1P enhanced cell motility, assessed by employing a cell fluorescence 96-well migration assay kit; moreover, its efficacy was only slightly lower than that of 10% FCS and comparable to the action of HGF, well-known elicitor of satellite cell migration [3,25]. The evaluation of cell motility in response to different amounts of S1P, clearly showed that S1P concentration as low as 20 nM brought about the maximal effect, while the potency of the lipid progressively declined at higher concentrations (Fig. 3B). The high potency of 20 nM S1P as inducer of satellite cell motility was also confirmed by wound healing experiments. Data depicted in Fig. 3C show that 20 nM S1P increased wound repair at a greater extent than HGF.

3.4. S1P-induced satellite cell migration depends on ligation to $S1P_{1/4}$

The role of S1PRs in the pro-migratory action of S1P was then examined. The $S1P_1$ specific agonist SEW2871 (20 nM) potently stimulated cell migration measured using a Boyden chamber assay (Fig. 4A) or by performing wound healing assay (Fig. 4B). Moreover, the immunomodulator FTY720-P (20 nM) that binds to S1PRs except $S1P_2$ stimulated to a similar extent cell migration (Fig. 4A). Cell treatment with S1PR antagonists VPC23019, JTE-013 or W146 in all cases abolished the S1P-induced migration (Fig. 4C and D) suggesting that in principle $S1P_1$, $S1P_2$, $S1P_3$, and $S1P_4$ were all involved in the S1P-induced biological action. However, the specific silencing of

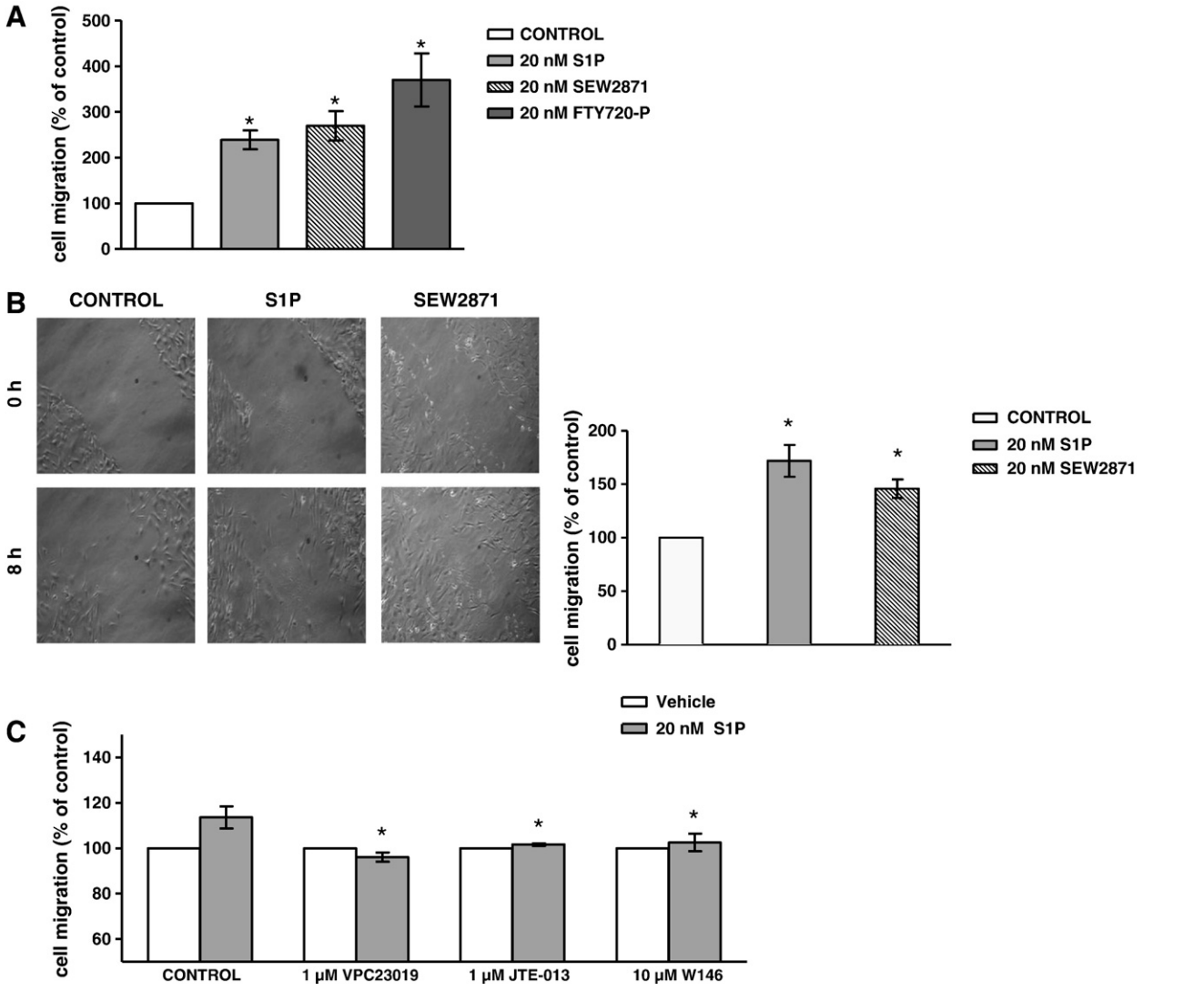


Fig. 4. Role of S1PRs in the migratory action induced by S1P in satellite cells. (A) Cell migration across Matrigel-coated porous membranes of Boyden chambers was determined in satellite cells challenged with 20 nM S1P, 20 nM SEW2871 and 20 nM FTY720-P for 7 h. The results are reported as percentage of control set as 100. Data are means ± SEM of at least 3 independent experiments performed in triplicate. (B) Scratch wound healing assays were performed in satellite cells challenged with 20 nM S1P or 20 nM SEW2871. Left panel: representative microscopic views at 0 h and 8 h. Right panel: quantitative analysis of cell migration after 8 h. The results are reported as percentage of control set as 100. Data are means ± SEM of at least 3 independent experiments performed in triplicate. The effect of SEW2871 and FTY720-P was statistically significant by Student's *t* test. (C-D) Effect of S1PR antagonists on S1P-induced migration. Cell migration was measured in satellite cells pre-incubated for 30 min with 1 μM VPC23019, 1 μM JTE-013, and 10 μM W146 before being challenged with 20 nM S1P for 8 h by (C) a 96-well ChemoTx disposable chemotaxis system and (D) scratch wound healing assays. (D) Upper panel: representative microscopic images at 0 h and 8 h. Lower panel: quantitative analysis of cell migration after 8 h. The results are reported as percentage of control set as 100. (C) Number of migrated cells in untreated control cells was 2123 ± 324, in VPC23019 pre-treated cells was 2215 ± 282, in JTE-013 pre-treated cells was 2133 ± 293, in W146 pre-treated cells was 2164 ± 267. (D) Number of migrated cells in control was 37 ± 9, in VPC23019 pre-treated cells was 38 ± 9, in JTE-013 pre-treated cells was 33 ± 8 and W146 pre-treated cells was 30 ± 7. Data are means ± SEM of at least 3 independent experiments performed in triplicate. The effect of the antagonists was statistically significant by Student's *t* test. (E) Effects of $S1P_{1-4}$ silencing on S1P-dependent cell migration. Satellite cells were transfected with scrambled siRNA, or siRNA targeted to $S1P_1$, $S1P_2$, $S1P_3$ and $S1P_4$ and Boyden chamber assays were performed as described above. Number of migrated cells in control (untreated scrambled-transfected cells) was 186 ± 67, in untreated $S1P_1$-siRNA transfected cells was 158 ± 3, in untreated $S1P_2$-siRNA transfected cells was 112 ± 22, in untreated $S1P_3$-siRNA transfected cells was 176 ± 106 and in untreated $S1P_4$-siRNA transfected cells was 196 ± 80. The down-regulation of $S1P_1$ and $S1P_4$ significantly reduced, while the down-regulation of $S1P_2$ significantly increased, S1P-induced chemotaxis. Asterisk indicates statistical significance: *P<0.05.

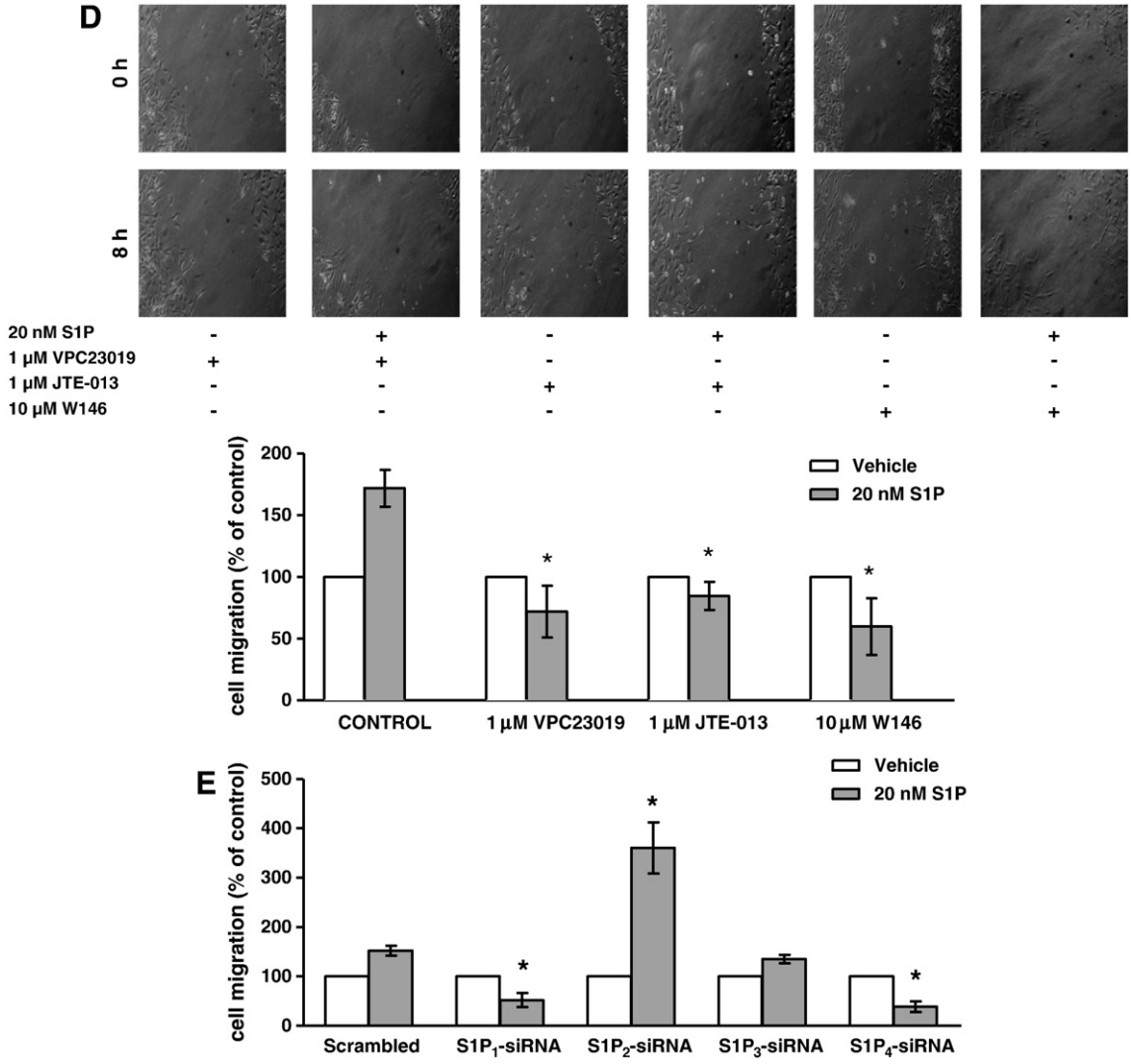


Fig. 4 (*continued*).

individual S1PRs, as illustrated in Fig. 4E, demonstrated the pivotal role exclusively of $S1P_1$ and $S1P_4$. Moreover, RNA interference of S1PRs not only did not confirm the role of $S1P_{2/3}$ contrarily to what shown in Fig. 4C and D by using S1PR antagonists, but also highlighted an anti-migratory role of $S1P_2$ since its specific down-regulation induced a 2-fold increase of S1P-induced cell migration (Fig. 4E).

3.5. S1P-induced satellite cell proliferation and migration depend on distinct signalling pathways

Next, the signalling pathways triggered by S1P treatment of satellite cells were investigated, focusing onto protein kinases known to be involved in the regulation of cell proliferation and migration. Data illustrated in Fig. 5A show that 1 μM S1P provoked a rapid and relevant activation of ERK1/2, whose phosphorylation was maximal at 5 min and still appreciable at 60 min; p38 MAPK phosphorylation was also enhanced by S1P challenged with a time-course similar to that observed for ERK1/2 (Fig. 5B). Akt was also identified as target of S1P, being its phosphorylation moderately enhanced at 5 min challenge (Fig. 5C). To explore the involvement of these signalling pathways in the mechanism by which S1P promoted cell proliferation, specific pharmacological inhibitors were employed. As depicted in Fig. 6A, 5 μM SB239063, 10 μM U0126 and 5 μM LY294002, fully prevented the phosphorylation of p38 MAPK, ERK1/2 and downstream kinase Akt elicited by the sphingolipid respectively. Notably, only the blockade of the PI3K/Akt pathway by LY294002 strongly attenuated the increase of labeled thymidine incorporation elicited by S1P, clearly pointing at a key role of this pathway in the mitogenic response evoked by this sphingolipid (Fig. 6B). Finally, S1P-induced satellite cell migration was completely inhibited when cells were pre-treated with 2.5 μM SB239063, 5 μM LY294002 or 5 μM U0126, highlighting the involvement of all these signalling pathways in this biological action evoked by this sphingolipid (Fig. 6C).

4. Discussion

Satellite cells are skeletal muscle-resident stem cells responsible for the impressive capacity of skeletal muscle to regenerate itself. The peculiar biological properties of satellite cells such as the ability to become activated upon damage, proliferate, migrate at the lesion site, differentiate and fuse with preexisting fibers have been well established, however the underpinning molecular mechanisms have been only in part identified. The full comprehension of extracellular cues enabling the proper molecular events that account for the final biological processes culminating in tissue repair, is necessary in order to individuate novel molecular targets for ameliorating skeletal muscle regeneration in degenerative diseases or in aging.

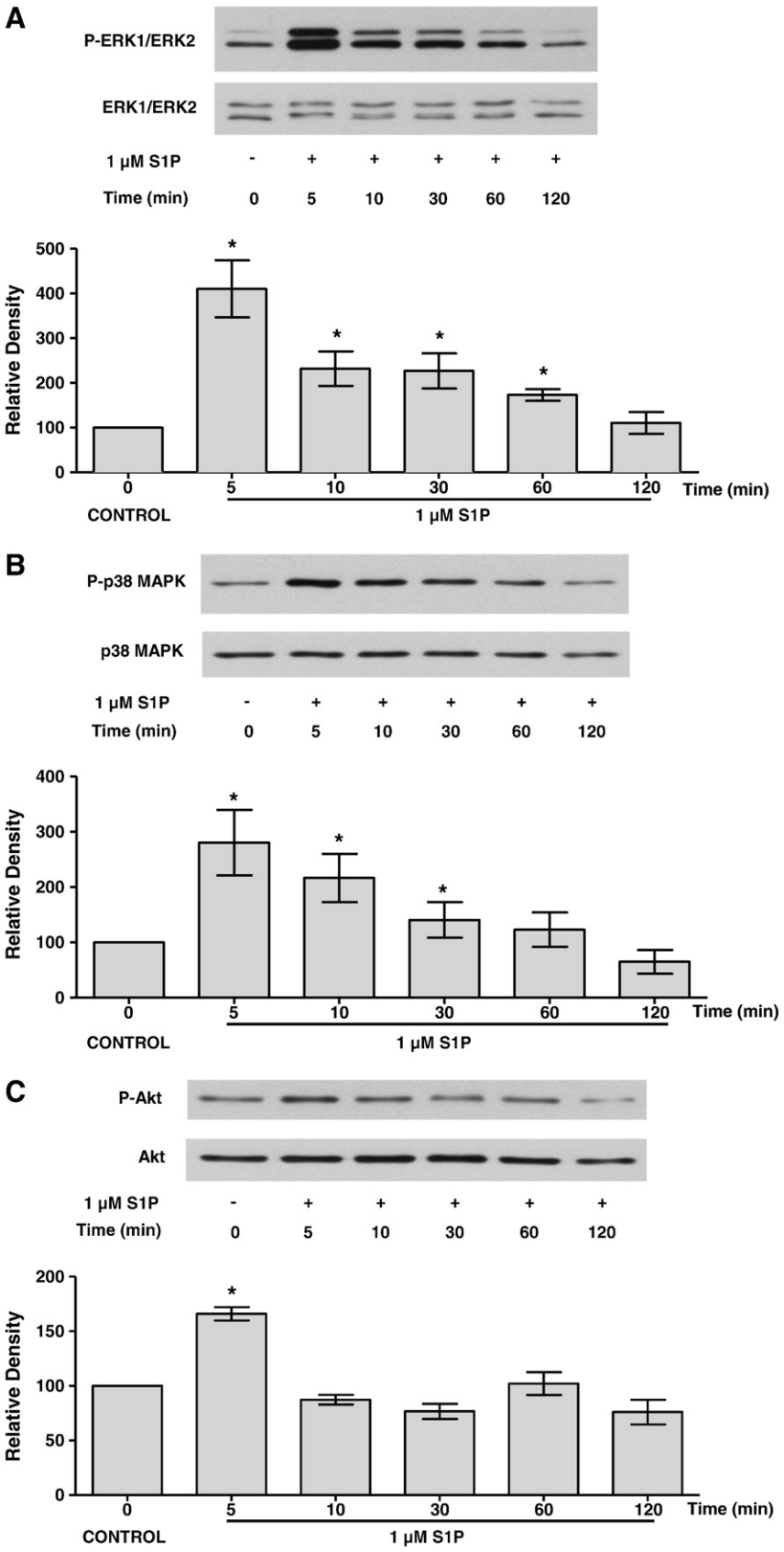
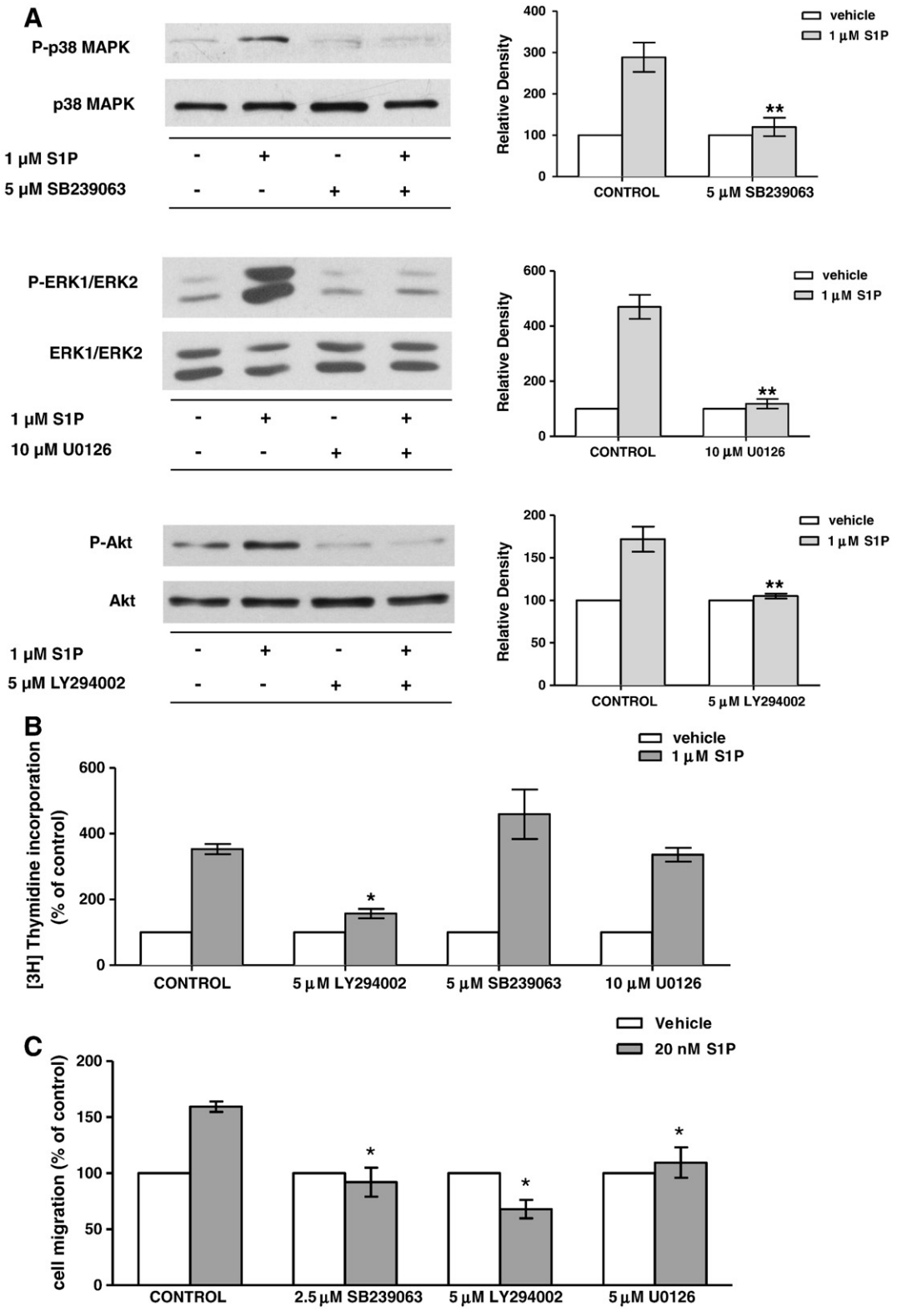


Fig. 5. Effect of S1P on ERK1/2, p38 MAPK and Akt phosphorylation. Serum-starved, ~70% confluent, satellite cells were treated with (+) or without (−) 1 μM S1P for the indicated times. Cell lysates (20 μg) were separated by SDS-PAGE, blotted and immunodetected using specific anti-phospho-ERK1/2 and anti-ERK1/2 (A), anti-phospho-p38 MAPK and anti-p38 MAPK (B) or anti-phospho-Akt and anti-Akt antibodies (C). Densitometric analysis is shown below. A blot representative of 3 independent experiments is presented. Band intensity corresponding to phosphorylated protein was evaluated by densitometric analysis, normalized to its total content and reported as percentage of increase relative to respective control set as 100. Data are means ± SEM of 3 independent experiments. Asterisk indicates statistical significance: *$P<0.05$.

In this study the biological action of S1P in isolated satellite cells has been investigated in detail. The obtained results clearly demonstrate that S1P is a key regulator of satellite cell biology, accounting for promoting not only cell proliferation but also cell migration. Moreover, the pattern of S1PR expression at mRNA level has been determined and the role of individual receptor subtypes in transmitting the biological response has been assessed. The here identified stimulatory effect of S1P on satellite cell proliferation is in line with a previous study in which proliferation of satellite cells associated to myofibers was found to be enhanced by S1P [14]. Moreover, this study highlights a new key biological effect mediated by S1P in satellite cells, such as the stimulation of cell migration, known to be crucial for the proper recruitment of these cells at the site of tissue damage and the successful repair of injured skeletal muscle [3]. Thus, these



latter findings further strengthen the relevance in the control of satellite cell physiology brought about by this sphingolipid.

Here particular attention was paid to the role exerted by S1PRs in the mechanism by which S1P elicited its biological response. Quantitative PCR analysis of satellite cell S1PR mRNA content demonstrated that $S1P_3$ was the receptor subtype expressed at the highest extent.

To investigate the involvement of specific S1PRs in the S1P-induced biological actions, S1PR agonists and antagonists were employed as well as RNA interference technology. However, the findings obtained by using the S1PR agonists and antagonists were in some circumstances opposite to those generated using molecular genetic alterations, due to possible off-target effects of the pharmacological agents, whose specificity is not always well established [23,24]. In this regard, Salomone et al. [26] demonstrated that JTE-013, considered a specific $S1P_2$ ligand, in rodent isolated vessels inhibited vasoconstriction not only to S1P, but also to the prostanoid analog U46619, endothelin-1 and high KCl.

Interestingly, $S1P_2$ together with $S1P_3$ was found to be implicated in the transmission of S1P-induced cell proliferation, similarly to what previously demonstrated in mesenchymal progenitors such as mesoangioblasts [19] and rat hepatocytes [27]. LY294002 was found to strongly attenuate cell proliferation elicited by S1P, clearly pointing at a key role of PI3K/AKT pathway in the mitogenic response evoked by S1P, in contrast to what previously shown in keratinocytes where $S1P/S1P_2$ axis was responsible for an anti-proliferative action exerted via inhibition of AKT phosphorylation [28]. This observation reinforces the notion that the coupling of S1PRs is strictly specific for individual cell types.

These data identify a peculiar coupling of S1PRs to convey the mitogenic signal, distinct from what previously reported in C2C12 reserve cells that share many characteristics with satellite cells, since $S1P_1$, the most represented receptor subtype at mRNA level, was identified in those cells as major transmitter of cell proliferation [22]. In agreement with earlier studies performed in other cell types including mouse myoblasts, embryonic fibroblasts, vascular endothelial cells and lymphocytes [21,29–34], $S1P_1$ was individuated to be crucial in mediating cell migration, whereas $S1P_2$ was shown to negatively regulate this event. Interestingly, a new important finding of this study is that $S1P_4$, even if expressed at low mRNA level, appeared to be involved in the regulation of migration of satellite cells. Although $S1P_4$ expression is commonly considered to be restricted to the lymphoid system where it regulates cell shape and motility [35,36], it has been previously shown to be up-regulated by TGFβ in C2C12 myoblasts [8]. The present results support the notion that this receptor subtype plays a broader role than what expected. In agreement, Long et al. [37] have recently demonstrated that $S1P_4$ may have an important role in breast cancer progression, since it uses HER2 to regulate ERK1/2 in MDA-MB-453 cells.

These results provide increased understanding of the multifaceted roles of S1P in regulating cell fate in normal development, defining specific biological actions exerted by this sphingolipid in skeletal muscle-resident stem cells. Interestingly, in agreement with the present findings, S1P has been previously shown to act as mitogenic cue in various types of stem cells such as human embryonic stem cells [38], mouse and human mesoangioblasts [19] and human bone marrow and adipose tissue-derived stem cells [39]. Nevertheless, exogenous and/or endogenous S1P has been reported to stimulate myogenic differentiation of myoblasts [4,5], act as inducer of adipogenesis in 3T3-L1 cells [40] and elicit differentiation towards smooth muscle of mesoangioblasts and adipose tissue-derived stem cells [41], supporting the notion that S1P is a differentiating cue in cell precursors of mesenchymal origin. These apparent contradictory results could be reconciled by considering that the final biological action of the sphingolipid is strictly dependent on its specific receptor pattern expressed in a given cell type in view of the multiplicity of signals conveyed by S1PRs that can give raise to distinct and even contrasting cellular effects. Thus, it becomes particularly critical the understanding of the extracellular cues responsible for the transcriptional regulation of S1PR expression in stem and progenitor cells and the consequent modification of the expression pattern that could act as decisive time-switch to accomplish tissue repair. Importantly, the bioavailability of S1P as regulator of satellite cells appears to be sufficiently guaranteed by its sub-micromolar concentration in the blood and its conspicuous release by cell types critical for skeletal muscle repair such as macrophages and endothelial cells. It is presently unknown if S1P is endogenously produced by satellite cells in response to challenge with extracellular cues, hence future studies are required to clarify whether endogenous S1P also participates to regulation of satellite cell biological properties.

Acknowledgements

This research was supported by grants from the University of Florence to PB and CD (ex 60%), Ente Cassa di Risparmio di Firenze to PB, Cassa di Risparmio di Lucca to PB and Association Francaise contre les Myopathies to PB.

References

[1] S. Spiegel, S. Milstien, Sphingosine-1-phosphate: an enigmatic signalling lipid, Nat. Rev. Mol. Cell Biol. 4 (2003) 397–407.
[2] I. Ishii, N. Fukushima, X. Ye, J. Chun, Lysophospholipid receptors: signaling and biology, Annu. Rev. Biochem. 73 (2004) 321–354.
[3] F. Le Grand, M. Rudnicki, Satellite and stem cells in muscle growth and repair, Development 134 (2007) 3953–3957.
[4] C. Donati, E. Meacci, F. Nuti, L. Becciolini, M. Farnararo, P. Bruni, Sphingosine 1-phosphate regulates myogenic differentiation: a major role for S1P2 receptor, FASEB J. 19 (2005) 449–451.
[5] E. Meacci, F. Nuti, C. Donati, F. Cencetti, M. Farnararo, P. Bruni, Sphingosine kinase activity is required for myogenic differentiation of C2C12 myoblasts, J. Cell. Physiol. 214 (2008) 210–220.
[6] C. Donati, P. Nincheri, F. Cencetti, E. Rapizzi, M. Farnararo, P. Bruni, Tumor necrosis factor-alpha exerts pro-myogenic action in C2C12 myoblasts via sphingosine kinase/S1P2 signaling, FEBS Lett. 581 (2007) 4384–4388.
[7] P. Nincheri, C. Bernacchioni, F. Cencetti, C. Donati, P. Bruni, Sphingosine kinase-1/S1P1 signalling axis negatively regulates mitogenic response elicited by PDGF in mouse myoblasts, Cell. Signal. 22 (2010) 1688–1699.
[8] F. Cencetti, C. Bernacchioni, P. Nincheri, C. Donati, P. Bruni, Transforming growth factor-beta1 induces transdifferentiation of myoblasts into myofibroblasts via up-regulation of sphingosine kinase-1/S1P3 axis, Mol. Biol. Cell 21 (2010) 1111–1124.

Fig. 6. Role of ERK1/2, p38 MAPK and Akt in the mitogenic and migratory effect of S1P in satellite cells. (A) Effect of U0126, SB239063, LY294002 inhibitors on ERK1/2, p38 MAPK and Akt phosphorylation upon S1P treatment. Serum-starved, ~70% confluent, satellite cells were pre-incubated for 30 min in the presence or not of 5 μM LY294002, 5 μM SB239063, or 10 μM U0126 before being stimulated with 1 μM S1P for 5 min. Cell lysates (20 μg) were separated by SDS-PAGE, blotted and immunodetected using specific anti-phospho-p38 MAPK and anti-p38 MAPK (upper panel), anti-phospho-ERK1/2 and anti-ERK1/2 (middle panel), or anti-phospho-Akt and anti-Akt antibodies (lower panel). A blot representative of 3 independent experiments is presented. Densitometric analyses are shown in the right panels. Band intensity corresponding to phosphorylated protein was evaluated by densitometric analysis, normalized to its total content and reported as percentage of increase relative to respective control set as 100. Data are means ± SEM of 3 independent experiments. (B) Satellite cells, ~60% confluent, were pre-incubated for 30 min in the presence or not of 5 μM LY294002, 5 μM SB239063, or 10 μM U0126 before being stimulated with 1 μM S1P for 20 h. [^{3}H]Thymidine (0.5 μCi/well) was added during the last 4 h of incubation. Results are reported as percentage relative to the respective control set as 100. [^{3}H]Thymidine incorporation was 9966 ± 539 dpm in untreated cells, 5083 ± 471 dpm in LY294002 pre-treated cells, 10046 ± 878 dpm in SB239063 pre-treated cells, and 1840 ± 176 dpm in U0126 pre-treated cells. Data are means ± SEM of 3 independent experiments performed in triplicate. The effect of the LY294002 inhibitor on S1P induced proliferation was statistically significant by Student's *t* test. (C) Cell migration across Matrigel-coated porous membranes of Boyden chambers was determined in satellite cells pre-incubated in the presence or not of 5 μM LY294002, 2.5 μM SB239063, or 5 μM U0126 before being challenged with 20 nM S1P for 7 h. Number of migrated cells in control was 196 ± 16, in SB239063 pre-treated cells was 88 ± 57, in LY294002 pre-treated cells 193 ± 66 and in U0126 pre-treated cells was 239 ± 62. Data are means ± SEM of 3 independent experiments performed in triplicate. Asterisks indicate statistical significance: *P < 0.05; **P < 0.01.

[9] E. Meacci, C. Donati, M. Farnararo, P. Bruni, Sphingosine 1-phosphate signal transduction in muscle cells, Ital. J. Biochem. 52 (2003) 25–27.
[10] M. Zanin, E. Germinario, L. Dalla Libera, D. Sandona, R.A. Sabbadini, R. Betto, D. Danieli-Betto, Trophic action of sphingosine 1-phosphate in denervated rat soleus muscle, Am. J. Physiol. Cell Physiol. 294 (2008) C36–C46.
[11] D. Danieli-Betto, S. Peron, E. Germinario, M. Zanin, G. Sorci, S. Franzoso, D. Sandona, R. Betto, Sphingosine 1-phosphate signaling is involved in skeletal muscle regeneration, Am. J. Physiol. Cell Physiol. 298 (2010) C550–C558.
[12] C. Sassoli, L. Formigli, F. Bini, A. Tani, R. Squecco, C. Battistini, S. Zecchi-Orlandini, F. Francini, E. Meacci, Effects of S1P on skeletal muscle repair/regeneration during eccentric contraction, J. Cell. Mol. Med. 15 (2011) 2498–2511.
[13] J. Dhawan, T.A. Rando, Stem cells in postnatal myogenesis: molecular mechanisms of satellite cell quiescence, activation and replenishment, Trends Cell Biol. 15 (2005) 666–673.
[14] Y. Nagata, T.A. Partridge, R. Matsuda, P.S. Zammit, Entry of muscle satellite cells into the cell cycle requires sphingolipid signaling, J. Cell Biol. 174 (2006) 245–253.
[15] J.D. Rosenblatt, A.I. Lunt, D.J. Parry, T.A. Partridge, Culturing satellite cells from living single muscle fiber explants, In Vitro Cell. Dev. Biol. Anim. 31 (1995) 773–779.
[16] G. Cossu, B. Zani, M. Coletta, M. Bouche, M. Pacifici, M. Molinaro, In vitro differentiation of satellite cells isolated from normal and dystrophic mammalian muscles. A comparison with embryonic myogenic cells, Cell Differ. 9 (1980) 357–368.
[17] A. Musaro, L. Barberi, Isolation and culture of mouse satellite cells, Methods Mol. Biol. 633 (2010) 101–111.
[18] P. Seale, L.A. Sabourin, A. Girgis-Gabardo, A. Mansouri, P. Gruss, M.A. Rudnicki, Pax7 is required for the specification of myogenic satellite cells, Cell 102 (2000) 777–786.
[19] C. Donati, F. Cencetti, P. Nincheri, C. Bernacchioni, S. Brunelli, E. Clementi, G. Cossu, P. Bruni, Sphingosine 1-phosphate mediates proliferation and survival of mesoangioblasts, Stem Cells 25 (2007) 1713–1719.
[20] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(−delta delta C(T)) method, Methods 25 (2001) 402–408.
[21] L. Becciolini, E. Meacci, C. Donati, F. Cencetti, E. Rapizzi, P. Bruni, Sphingosine 1-phosphate inhibits cell migration in C2C12 myoblasts, Biochim. Biophys. Acta 1761 (2006) 43–51.
[22] E. Rapizzi, C. Donati, F. Cencetti, P. Nincheri, P. Bruni, Sphingosine 1-phosphate differentially regulates proliferation of C2C12 reserve cells and myoblasts, Mol. Cell. Biochem. 314 (2008) 193–199.
[23] S. Salomone, C. Waeber, Selectivity and specificity of sphingosine-1-phosphate receptor ligands: caveats and critical thinking in characterizing receptor-mediated effects, Front. Pharmacol. 2 (2011) 9.
[24] N.J. Pyne, S. Pyne, Selectivity and specificity of sphingosine 1-phosphate receptor ligands: "off-targets" or complex pharmacology? Front. Pharmacol. 2 (2011) 26.
[25] R.E. Allen, S.M. Sheehan, R.G. Taylor, T.L. Kendall, G.M. Rice, Hepatocyte growth factor activates quiescent skeletal muscle satellite cells in vitro, J. Cell. Physiol. 165 (1995) 307–312.
[26] S. Salomone, E.M. Potts, S. Tyndall, P.C. Ip, J. Chun, V. Brinkmann, C. Waeber, Analysis of sphingosine 1-phosphate receptors involved in constriction of isolated cerebral arteries with receptor null mice and pharmacological tools, Br. J. Pharmacol. 153 (2008) 140–147.
[27] H. Ikeda, H. Satoh, M. Yanase, Y. Inoue, T. Tomiya, M. Arai, K. Tejima, K. Nagashima, H. Maekawa, N. Yahagi, Y. Yatomi, S. Sakurada, Y. Takuwa, I. Ogata, S. Kimura, K. Fujiwara, Antiproliferative property of sphingosine 1-phosphate in rat hepatocytes involves activation of Rho via Edg-5, Gastroenterology 124 (2003) 459–469.
[28] M. Schüppel, U. Kürschner, U. Kleuser, M. Schäfer-Korting, B. Kleuser, Sphingosine 1-phosphate restrains insulin-mediated keratinocyte proliferation via inhibition of Akt through the S1P2 receptor subtype, J. Invest. Dermatol. 128 (2008) 1747–1756.
[29] A. Kimura, T. Ohmori, R. Ohkawa, S. Madoiwa, J. Mimuro, T. Murakami, E. Kobayashi, Y. Hoshino, Y. Yatomi, Y. Sakata, Essential roles of sphingosine 1-phosphate/S1P1 receptor axis in the migration of neural stem cells toward a site of spinal cord injury, Stem Cells 25 (2007) 115–124.
[30] S.K. Goparaju, P.S. Jolly, K.R. Watterson, M. Bektas, S. Alvarez, S. Sarkar, L. Mel, I. Ishii, J. Chun, S. Milstien, S. Spiegel, The S1P2 receptor negatively regulates platelet-derived growth factor-induced motility and proliferation, Mol. Cell. Biol. 25 (2005) 4237–4249.
[31] T. Ohmori, Y. Yatomi, H. Okamoto, Y. Miura, G. Rile, K. Satoh, Y. Ozaki, G(i)-mediated Cas tyrosine phosphorylation in vascular endothelial cells stimulated with sphingosine 1-phosphate: possible involvement in cell motility enhancement in cooperation with Rho-mediated pathways, J. Biol. Chem. 276 (2001) 5274–5280.
[32] J.H. Paik, S. Chae, M.J. Lee, S. Thangada, T. Hla, Sphingosine 1-phosphate-induced endothelial cell migration requires the expression of EDG-1 and EDG-3 receptors and Rho-dependent activation of alpha vbeta3- and beta1-containing integrins, J. Biol. Chem. 276 (2001) 11830–11837.
[33] M.G. Sanna, J. Liao, E. Jo, C. Alfonso, M.Y. Ahn, M.S. Peterson, B. Webb, S. Lefebvre, J. Chun, N. Gray, H. Rosen, Sphingosine 1-phosphate (S1P) receptor subtypes S1P1 and S1P3, respectively, regulate lymphocyte recirculation and heart rate, J. Biol. Chem. 279 (2004) 13839–13848.
[34] N. Sugimoto, N. Takuwa, H. Okamoto, S. Sakurada, Y. Takuwa, Inhibitory and stimulatory regulation of Rac and cell motility by the G12/13-Rho and Gi pathways integrated downstream of a single G protein-coupled sphingosine-1-phosphate receptor isoform, Mol. Cell. Biol. 23 (2003) 1534–1545.
[35] M.H. Graler, R. Grosse, A. Kusch, E. Kremmer, T. Gudermann, M. Lipp, The sphingosine 1-phosphate receptor S1P4 regulates cell shape and motility via coupling to Gi and G12/13, J. Cell. Biochem. 89 (2003) 507–519.
[36] W. Wang, M.H. Graeler, E.J. Goetzl, Type 4 sphingosine 1-phosphate G protein-coupled receptor (S1P4) transduces S1P effects on T cell proliferation and cytokine secretion without signaling migration, FASEB J. 19 (2005) 1731–1733.
[37] J.S. Long, Y. Fujiwara, J. Edwards, C.L. Tannahill, G. Tigyi, S. Pyne, N.J. Pyne, Sphingosine 1-phosphate receptor 4 uses HER2 (ERBB2) to regulate extracellular signal regulated kinase-1/2 in MDA-MB-453 breast cancer cells, J. Biol. Chem. 285 (2010) 35957–35966.
[38] K. Inniss, H. Moore, Mediation of apoptosis and proliferation of human embryonic stem cells by sphingosine-1-phosphate, Stem Cells Dev. 15 (2006) 789–796.
[39] X. He, S.C. H'Ng, D.T. Leong, D.W. Hutmacher, A.J. Melendez, Sphingosine-1-phosphate mediates proliferation maintaining the multipotency of human adult bone marrow and adipose tissue-derived stem cells, J. Mol. Cell Biol. 2 (2010) 199–208.
[40] T. Hashimoto, J. Igarashi, H. Kosaka, Sphingosine kinase is induced in mouse 3T3-L1 cells and promotes adipogenesis, J. Lipid Res. 50 (2009) 602–610.
[41] P. Nincheri, P. Luciani, R. Squecco, C. Donati, C. Bernacchioni, L. Borgognoni, G. Luciani, S. Benvenuti, F. Francini, P. Bruni, Sphingosine 1-phosphate induces differentiation of adipose tissue-derived mesenchymal stem cells towards smooth muscle cells, Cell. Mol. Life Sci. 66 (2009) 1741–1754.