

STEM CELLS®

Sphingosine 1-Phosphate Mediates Proliferation and Survival of Mesoangioblasts
Chiara Donati, Francesca Cencetti, Paola Nincheri, Caterina Bernacchioni, Silvia Brunelli,
Emilio Clementi, Giulio Cossu and Paola Bruni
Stem Cells published online Apr 26, 2007;
DOI: 10.1634/stemcells.2006-0725

This information is current as of April 30, 2007

The online version of this article, along with updated information and services, is located on
the World Wide Web at:
<http://www.StemCells.com>

STEM CELLS®, an international peer-reviewed journal, covers all aspects of stem cell research: embryonic stem cells; tissue-specific stem cells; cancer stem cells; the stem cell niche; stem cell genetics and genomics; translational and clinical research; technology development.

STEM CELLS® is a monthly publication, it has been published continuously since 1983. The Journal is owned, published, and trademarked by AlphaMed Press, 318 Blackwell Street, Suite 260, Durham, North Carolina, 27701. © 2007 by AlphaMed Press, all rights reserved. Print ISSN: 1066-5099. Online ISSN: 1549-4918.

 **AlphaMed Press**

Sphingosine 1-Phosphate Mediates Proliferation and Survival of Mesoangioblasts

Chiara Donati^{1,2}, Francesca Cencetti^{1,2}, Paola Nincheri¹, Caterina Bernacchioni¹, Silvia Brunelli^{3,4}, Emilio Clementi^{3,5,6}, Giulio Cossu^{2,7}, Paola Bruni^{1,2}

¹Dipartimento di Scienze Biochimiche, Università di Firenze, 50134 Firenze, ²Istituto Interuniversitario di Miologia (IIM), ³Stem Cell Research Institute, Istituto Scientifico H San Raffaele, 20132 Milano, Italy; ⁴Dipartimento di Medicina Sperimentale, Università di Milano-Bicocca, 20052 Monza, Italy; ⁵IRCCS E. Medea, 23842, Bosisio Parini, Italy; ⁶Dipartimento di Scienze Precliniche LITA-Vialba, Università di Milano, 20157 Milano, Italy; ⁷Dipartimento di Biologia, Università di Milano, 20130 Milano.

ABSTRACT

Mesoangioblasts are stem cells capable of differentiating in various mesodermal tissues and are presently regarded as suitable candidates for cell therapy of muscle degenerative diseases as well as myocardial infarction. The enhancement of their proliferation and survival after injection in vivo could greatly improve their ability of repopulating damaged tissues. In this study we show that the bioactive sphingolipid sphingosine 1-phosphate (S1P) regulates critical functions of mesoangioblast cell biology. S1P evoked a full mitogenic response in mesoangioblasts, measured by labeled thymidine incorporation and cell counting. Moreover, S1P strongly counteracted the apoptotic process triggered by stimuli as diverse as serum deprivation, C₂-ceramide or staurosporine, assessed by cell counting as well as histone-associated fragments and

caspase-3 activity determinations. S1P acts both as an intracellular messenger or through specific membrane receptors. Real-time PCR analysis revealed that mesoangioblasts express the S1P-specific receptor S1P₃ and to a minor extent S1P₁ and S1P₂. By employing S1P receptor subtype-specific agonists and antagonists we found that the proliferative response to S1P was mediated mainly by S1P₂. By contrast, the antiapoptotic effect did not implicate S1P receptors. These findings demonstrate an important role of S1P in mesoangioblast proliferation and survival and indicate that targeting modulation of S1P-dependent signaling pathways may be used to improve the efficiency of muscle repair by these cells.

INTRODUCTION

Mesoangioblasts are mesodermal progenitors associated with vessels during the fetal stage of development and persist in post-natal life. They exhibit stem cell features such as pluripotency

and self-renewal ability, and can differentiate, in vivo and in vitro, into different mesoderm cell types such as muscle, bone and adipocytes in response to specific extracellular cues [1,2]. Upon injection into the arterial bloodstream, mesoangioblasts are capable of crossing the

endothelium, migrate in the tissue interstitium and are then incorporated into regenerating skeletal muscle fibers. Such an action opens therapeutic perspectives for degenerative diseases of heart and skeletal muscles. In particular, mesoangioblasts have been successfully employed to correct morphologically and functionally the pathological alterations observed in the alpha-sarcoglycan null mice, a model of severe muscular dystrophy [3]. Moreover, it has been recently shown that mesoangioblasts repair the infarcted heart, being as effective as bone marrow progenitor cells in reducing postinfarction left ventricular dysfunction [4]. Despite these promising results, problems related with in vitro expansion, efficient engrafting and survival of mesoangioblasts in the toxic environment of the damaged muscle still hamper their therapeutic development [5]. The present knowledge of the extracellular agents capable of regulating key biological processes in mesoangioblasts is still limited. The characterization of the physiologically relevant agonists able of regulating proliferation and survival of these stem cells becomes fundamental to enhance their therapeutic efficacy. Studies of the last fifteen years have clearly demonstrated that sphingosine 1-phosphate (S1P), a sphingolipid metabolite physiologically present in the serum, is endowed with powerful biological activities [6,7]. The bioactive sphingolipid is produced from the metabolism of sphingomyelin and although initially regarded as an intracellular mediator of growth factors and cytokines, it is presently recognized to elicit most of its effects as ligand of at least five different specific seven-spanning membrane receptors named S1P₁₋₅ [8]. A number of different studies have demonstrated that S1P regulates key biological processes such as cell proliferation, motility and survival in many different cell types. An important aspect of this regulation is that S1P receptors (S1PR) are differentially coupled to multiple G proteins upstream to distinct signaling pathways; thus, given that their expression pattern is highly cell-specific, S1P can exert different biological

effects depending on the cell type. For example endothelial cells [9] and vascular smooth muscle cells [10] have been shown to proliferate in response to S1P; in contrast, in other cell types such as T lymphocytes [11], hepatocytes [12], and skeletal myoblasts [13], the sphingolipid exerted an anti-proliferative effect. Although in seminal pioneering studies the mitogenic action of S1P had been reported to be independent from S1PR engagement [14], it was subsequently found to implicate in many instances S1P₁ and/or S1P₃ [8], whereas S1P₂ was identified as the receptor responsible for the antiproliferative properties of S1P [12,13]. S1P appears also capable of increasing cell survival and inhibiting the apoptotic process in a number of different cell types [15,16]. Notably, general attention in the dissection of the effects of S1P on cell proliferation and survival has been further increased by recent experimental work showing that a number of growth factors, cytokines and hormones generate S1P and that modulation of S1P metabolism is integral to their mitogenic and/or anti-apoptotic activity [6,17,18]. In this study we report that S1P is a potent mitogenic factor for mesoangioblasts and protects these cells from apoptosis induced by various stimuli such as serum deprivation, C₂-ceramide or staurosporine treatment, supporting the view that S1P availability to these cells can be critical to improve their survival in vivo.

MATERIALS AND METHODS

Cell culture and treatment with agonists, antagonists and inhibitors

Murine mesoangioblasts [2,3] were routinely grown in DMEM supplemented with heat-inactivated 20% fetal calf serum (FCS) (Sigma, St. Louis, MO, USA). Human mesoangioblasts, isolated from adult muscle biopsies [19], were routinely grown in Mega Cell (Sigma St. Louis, MO, USA) supplemented with heat inactivated 5% FCS and 5 ng/ml basic fibroblast growth factor (PeproTech, London, England) [20]. Cells were challenged with 1 μ M D-erythro-S1P (Calbiochem, San Diego, CA, USA), (2 mM stock solution in dimethylsulfoxide). Specific

antagonists of S1PR, VPC23019. [21] and JTE-013 [22] and inhibitors of p38 MAPK or p42/44 MAPK pathway, SB203580 and U0126 (Tocris Cookson Limited, Bristol, United Kingdom), respectively, were administered to the cells 30 min before agonist addition. S1P₁-specific agonist, SEW2871 [23] was a kind gift of Dr. H. Rosen (The Scripps Research Institute, La Jolla, CA, USA) and its efficacy was tested by evaluating its ability to promote p42/44 MAPK phosphorylation (data not shown).

Animals

Alpha-sarcoglycan (-SG) null C57BL/6 mice [24] were a kind gift of Dr. K Campbell (Iowa University, Iowa City, IA, USA). Animals were housed in the pathogen-free facility at our Institution and treated in accordance with the European Community guidelines, and with the approval of the Institutional Ethical Committee.

Cell proliferation determination

To evaluate cell proliferation by [³H]thymidine incorporation, mesoangioblasts, seeded in 12-well plates and utilized when approximately 40% confluent, were serum-starved overnight and then challenged with 1 μM S1P for 24 h. [³H]Thymidine, 1 or 2 μCi/well, (Amersham Pharmacia Biotech, Uppsala, Sweden), was added during the last 2 h of incubation. Cells were washed twice in ice-cold PBS before 500 μl addition of 10% trichloroacetic acid for 5 min at 4 °C. Cells were washed again in ice-cold PBS and 250 μl of ethanol: ether (3:1, v/v) solution was added to the insoluble material. Samples were then lysed in 0.2 N NaOH for 1 h at 37 °C. Incorporation of [³H]thymidine was measured by scintillation counting.

Alternatively, proliferation was evaluated by cell counting. Briefly, mesoangioblasts, seeded in 6-well plates at a density of approximately 1 x 10⁵ cells, were serum-starved overnight and then challenged with 1 μM S1P for the indicated time-intervals before being trypsinized and counted by an hemocytometer.

RT PCR

One μg of total RNA extracted with TriReagent (Sigma, St. Louis, MO, USA) from murine mesoangioblasts was reverse transcribed into DNA as previously described [25] and subjected to PCR using the following software-designed oligonucleotides (Pharmacia Biotech, Uppsala, Sweden):

S1P₁ forward 5'GTGTCCACTAGCATCCCGGAGGTTAAA
GCTCTCCGCAGCTCA 3'; S1P₁ reverse 5'
CCCAACAGGGGTAGCAGGAAGACCCC 3';
S1P₂ forward 5'TCGCGAATG-
CTGATGCTCATCGGG 3'; S1P₂ reverse 5'
TCAGACCACCGTGTGGCCCTCCAG 3';
S1P₃ forward 5'
GCAACCACGCATGCGCAGGGCCAC 3';
S1P₃ reverse 5'
GCGGTTGTGAAATTTATTGTTTTTCCAG
3'; S1P₄ forward 5'
CTGCTGCCCTCTACTCCAA 3'; S1P₄
reverse 5' ATTAATGGCTGAGTTGAACAC
3'; S1P₅ forward
5'GAGCGCCACCTTACCATG 3'; S1P₅
reverse 5'GGAGCAGCTGGTGTCCAT 3' -
actin forward
5'GCGGGAAATCGTGCGTGACATT 3'; -actin
reverse 5'
GATGGAGTTGAAGGTAGTTTCGTG 3'. PCR
amplification products were separated on a 1.2%
agarose gel.

Real time PCR

To quantify mRNA expression of S1P₁, S1P₂ and S1P₃ real time quantitative RT-PCR (TaqMan PCR, Applied Biosystems, Weiterstadt, Germany) using the automated ABI Prism 7700 Sequence Detector System (Applied Biosystems, Foster City, CA) was performed essentially as previously described [26]. All samples were run in triplicate in Micro-Amp optical 96-well plates (Applied Biosystems) with a TaqMan Universal PCR Master Mix (Applied Biosystems). Simultaneous amplification of the target sequence (Assay on demand, S1P₁ Mm00514644_m1, S1P₂ Mm01177794_m1, S1P₃ Mm00515669_m1, Applied Biosystems, Foster City, CA) together with the housekeeping

gene, 18S rRNA, was carried out with the following universal profile: initial denaturation for 10 min at 95 °C was followed by denaturation for 15 s at 95 °C, primer annealing and elongation at 60 °C for 1 min for 40–50 cycles. Results were analysed by ABI Prism Sequence Detection System software (version 1.7) (Applied Biosystems, Foster City, CA) and plotted by Microsoft Excel Software (Microsoft Excel Corp., New York, NY). The 2^{-CT} method was applied as a comparative method of quantification [27].

Western blot analysis

Mesoangioblasts 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 $\text{Na}_4\text{P}_2\text{O}_7$, 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, 14 μM E-64) essentially as described in Donati et al. [13]. To prepare total cell lysates, cell extracts were centrifuged for 15 min at 10,000 *g* at 4 °C. Proteins (30 μg) from lysates were resuspended in Laemmli's sodium dodecylsulfate- (SDS) sample buffer. Samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western analysis as previously described [13]. Bound phospho-p42/p44 MAPK and phospho-p38 MAPK antibodies (Cell Signaling Technology, Inc., Beverly, MA, USA) were detected using ECL reagents (Amersham Pharmacia Biotech, Uppsala, Sweden).

Apoptosis measurement

Mesoangioblasts were seeded at a density of approximately 1×10^5 cells/well and employed for experiments after 24 h. For serum-starvation- and C_2 -ceramide-induced apoptosis, cells were incubated in serum-free medium in the presence or absence of 10 μM C_2 -ceramide (Sigma, St. Louis, MO, USA) for 24 h and when requested 1 μM S1P was administered 30 min and 18 h after serum starvation. For staurosporine-induced apoptosis, 0.5 μM staurosporine (Sigma, St. Louis, MO, USA) was added for the last 4 h of incubation to cells serum-starved for 24 h,

treated or not at 30 min and 18 h incubation with 1 μM S1P. To quantitate DNA fragmentation the Cell Death Detection ELISA^{PLUS} Kit (Roche Applied Science, Mannheim, Germany) was used to quantitatively determine in vitro cytoplasmic histone-associated DNA fragments characteristic of apoptotic cell death. The optical density was determined on an ELISA Reader using 405 and 490 nm (reference) filters. Alternatively, apoptosis was measured by caspase-3 activity assay. Briefly, cells were washed twice with PBS and then lysed for 20 min at 4°C in 20 mM Tris-HCl buffer, pH 7.4, containing 250 mM NaCl, 2 mM EDTA, 0.1% Triton X-100, 5 $\mu\text{g/ml}$ aprotinin, 5 $\mu\text{g/ml}$ leupeptin, 0.5 mM phenylmethylsulfonylfluoride, 4 mM sodium vanadate, and 1 mM DTT [28]. The lysis was completed by sonication, and total protein content was determined in the clarified lysates with the Coomassie Blue reagent (Bio-Rad, Hercules, CA, USA). Aliquots of total proteins (50 μg) were diluted in 50 mM HEPES-KOH buffer, pH 7.0, containing 10% glycerol, 0.1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate, 2 mM EDTA, 10 mM DTT. Caspase-3 activity was determined by incubating the protein sample for 4 h at 37°C in the presence of 30 μM Ac-DEVD-AFC (excitation 400 nm, emission 505 nm) (Biomol Research Laboratories Inc. PA, USA). To determine nonspecific substrate degradation, the assays were also performed by preincubating total protein samples for 15 min at 37°C with or without the specific caspase inhibitor (200 nM Ac-DEVD-CHO) before substrate addition.

The apoptotic response was also evaluated by counting the trypsinized surviving cells by an hemocytometer.

In vivo cell survival assay

D16-GFP mesoangioblasts [3] were pretreated with 1 μM S1P for 12 h, after which cells were suspended in culture medium. Delivery of mesoangioblasts was by injection of 5×10^5 cells directly into tibialis anterior muscles [29]. Muscles recovered from the mesoangioblasts-

injected animals were dissected and frozen in liquid N₂-cooled isopentane. Serial muscle sections were processed for TUNEL assay (Apoptag, Chemicon) to assess the presence of apoptotic nuclei and immunostained as previously described [3] with the anti-GFP antibody (Molecular Probes). The primary antibody was detected using appropriate secondary antibodies conjugated with Alexa 489 (Molecular Probes) and nuclei visualized with the DNA dye 4',6-diamidino-2-phenylindole (DAPI).

Statistical analysis

Data reported are means \pm S.E.M. of triplicates of a representative experiment performed at least 3 times with analogous results. Statistical analysis was performed using Student's *t* test (* *p* < 0.05).

RESULTS

We initially evaluated the pattern of S1PR expression in murine mesoangioblasts. Amplified fragments of ~1000, 600, 225 bp corresponding to mRNA transcripts encoding for S1P₁, S1P₂ and S1P₃, respectively, were detected by RT-PCR analysis (Fig.1A), while S1P₄ and S1P₅ were not (data not shown). Real-time PCR analysis revealed that the relative mRNA abundance was S1P₃ >> S1P₁ > S1P₂ (Fig.1B).

The effect of S1P on mesoangioblast proliferation was evaluated by labeled thymidine incorporation assay experiments. The sphingolipid action was concentration-dependent with a maximal effect at 1 μ M, and a half-maximal effective concentration of 32.6 nM \pm 7.1, *n* = 3 (Fig.2A). The mitogenic action of S1P was also confirmed by cell counting experiments, being the sphingolipid still capable of increasing cell number population up to 96 h of incubation (Fig. 2B). Analogous results were obtained when cell proliferation in response to S1P treatment was evaluated in other independent mesoangioblast clones (A2 and A6) (data not shown). These results demonstrate that S1P behaves in these cells as full mitogen.

Since all the S1PRs that we found expressed by mesoangioblasts have been implicated in the proliferative response elicited by the sphingolipid in different cell types, we investigated the role of individual receptors by using selective agonists and antagonists. The selective S1P₁ agonist SEW2871 in the concentration range 1-30 μ M did not trigger cell proliferation, excluding the involvement of S1P₁ in S1P-stimulated cell proliferation (Fig. 3A). Pharmacological inhibition of S1P₂ using the selective antagonist JTE-013 reduced significantly S1P mitogenic effect, being the compound effective starting from 30 nM (Fig 3B). Instead, the S1P₁/S1P₃ antagonist VPC23019 (100 nM) enhanced basal [³H]thymidine incorporation and slightly reduced the proliferative action of S1P (Fig. 3 C), suggesting a minor role, if any, for S1P₃. On the whole these results indicate that the mitogenic action of S1P in mesoangioblasts occurs mostly via stimulation of S1P₂ receptor. Since the mitogenic effect of S1P can be accompanied by a pro-survival action of the sphingolipid, the potential anti-apoptotic effect of S1P in mesoangioblasts was also investigated. S1P fully prevented the formation of cytoplasmic histone-associated DNA fragments induced by 24 h culture under serum deprivation or in the presence of C₂-ceramide (10 μ M), and significantly reduced the amount of histone associated mono- and oligonucleosomes formed in response to staurosporine (0.5 μ M) administration (Fig 4A). Consistently, S1P significantly diminished the increase in caspase-3 activity and the reduction in total cell number induced by the different apoptogenic stimuli (Fig. 4B and C). S1P exhibited a similar protective effect against apoptosis in mesoangioblasts clones A2 and A6 (data not shown).

To assess the pro-survival effect of S1P in vivo, the D16-GFP mesoangioblasts, previously treated with 1 μ M S1P for 12 h, were injected directly into the right tibialis anterior muscle of -SG-null mice. Twelve hours later, treated and

controlateral (control) muscles were removed and cell death assessed by staining with the TUNEL technique. As shown in Fig. 4D the dystrophic muscle showed signs of apoptosis and provoked cell death of injected GFP-positive mesoangioblasts. Mesoangioblast treatment with S1P before their injection reduced apoptosis by $51 \pm 4.3\%$ ($n = 4$, $P < 0.01$ vs. NT), demonstrating also a clear-cut in vivo pro-survival action of the sphingolipid. The involvement of S1PR in the anti-apoptotic effect of S1P was next examined. As shown in Fig. 5, 1 μM SEW2871 did not prevent the staurosporine-induced formation of histone-associated DNA fragmentation, excluding the involvement of S1P₁. Similarly, the protective effect of S1P was only slightly diminished by JTE-013 (1 μM) or VPC23019 (100 nM), ruling out a role also for S1P₃ or S1P₂. Thus, the anti-apoptotic action of S1P appears to occur through S1PR-independent pathways. The possible molecular mechanisms involved in the biological effects exerted by S1P in mesoangioblasts were then investigated. In particular we studied the involvement of the MAP kinase (MAPK) signaling pathways that have been shown to be activated by S1P and have effect in both mitogenesis and apoptosis [9,30-32]. To this end we used U0126 (10 μM) and SB203580 (5 μM), specific inhibitors of the p42/44 and p38 MAPK pathways, respectively. In control experiments the two inhibitors strongly reduced the S1P-induced activation of p42/44 MAPK and p38 MAPK, respectively (Fig. 6A). In the same Figure it is also shown that cell incubation with 2 μM JTE-013 reduced p38 MAPK and p42/44 MAPK phosphorylation induced by 10 min treatment with 1 μM S1P. Cell treatment with U0126 (10 μM), significantly reduced basal [³H]thymidine incorporation and attenuated the stimulation of DNA synthesis brought about by S1P; conversely, SB203580 (5 μM) did not affect the cell response to S1P (Fig. 6B). Both U0126 and SB203580 did not influence the protective action of S1P during cell death (Fig. 6C). These results indicate that S1P activates the p42/44 as well as the p38 MAPK pathway and that p42/44 MAPK is required for the mitogenic effect of S1P, while

protection from apoptosis is independent from MAPK activation. Finally, in the perspective of a cell therapy in patients, it was evaluated whether the biological effects elicited by S1P in murine mesoangioblasts take place also in the same cell type isolated from human donors. As illustrated in Fig. 7A, treatment with 1 μM S1P significantly stimulated [³H]thymidine incorporation into human mesoangioblasts. The sphingolipid was also found to strongly protect human mesoangioblasts from apoptosis. In particular, 1 μM S1P significantly reduced the formation of cytoplasmic mono- and oligonucleosomes in response to 24 h serum-deprivation or 0.5 μM staurosporine administration (Fig.7B). Notably, the mitogenic and antiapoptotic effect of the sphingolipid was comparable to that induced in murine cells.

DISCUSSION

This study represents the first experimental evidence for a key biological role of S1P in mesoangioblasts. In particular, S1P was found to promote cell proliferation and prevent programmed cell death.

The mitogenic effect exerted by S1P reported here is in agreement with a number of studies performed in different cell types in which the bioactive lipid is capable of stimulating cell proliferation and appears to depend on the action of S1P on its membrane receptors [8-10]. Murine mesoangioblasts were found to express S1P₁, S1P₂, S1P₃, in agreement with the pattern of S1PR expression recently identified in human embryonic stem cells [33]. Although S1P₁ receptor is expressed in mesoangioblasts, it does not play a role in mitogenesis since the specific S1P₁ agonist SEW2871 was unable to reproduce the mitogenic action of the sphingolipid. This is a striking difference with the other cell types so far investigated in which this receptor plays a pivotal role in the sphingolipid-dependent cell proliferation [8-10]. Instead, the observed attenuation of the proliferative response to S1P by S1P₂ antagonist JTE-013 is in favour of a mitogenic role of S1P₂ while the modest

inhibitory effect determined by S1P₁/S1P₃ antagonist VPC23019 rules out a major role for S1P₃ in S1P-induced mesoangioblast proliferation. So far S1P₂ was found to be positively coupled to cell proliferation only in mesangial cells [32], an interesting observation in light of the common perithelial niche of both mesangial cells and mesoangioblasts [19]; in contrast, in other studies the same receptor resulted to be implicated in the anti-proliferative response elicited by the sphingolipid [12,13]. This observation may have important implications in the *in vitro* expansion of these cells for therapeutic purposes, although selective S1P₂ agonists have not been developed yet. The study of the signaling pathways involved in the mitogenic effect of S1P highlighted a key role for p42/44 MAPK, in agreement with a number of other studies performed in different cells [9,34,35].

Another important finding of this study is the powerful pro-survival effect exerted by S1P in mesoangioblast apoptosis induced by serum deprivation or treatment with C₂-ceramide or staurosporine. S1P was found to fully prevent the DNA fragmentation caused by serum deprivation or C₂-ceramide treatment, while chromatin damage induced by the more potent apoptogenic agent staurosporine was appreciably decreased by the sphingolipid. Moreover, S1P was capable of potently attenuating caspase-3 activation and counteracting the reduction in cell number induced by the apoptogenic stimuli. Furthermore, mesoangioblasts pretreated *in vitro* with S1P exhibited an increased survival when injected into tibialis anterior muscles of -SG null mice. On the whole these results demonstrate that S1P plays a key role in the prevention of *in vitro* programmed cell death of mesoangioblasts.

The molecular mechanisms implicated in the anti-apoptotic action of S1P appear complex and need further investigation. The pro-survival effect of the sphingolipid was not mimicked by the S1P₁ agonist SEW2871 and was not significantly influenced by S1PR antagonists VPC23019 or JTE- 013, thus excluding the

involvement of S1PR, and suggesting the possibility of an intracellular action of S1P [14,36,37], since the exogenous sphingolipid may be transported across the plasma membrane, possibly via specific transporters [38]. However, at this stage it cannot be ruled out that the pro-survival effect of S1P in mesoangioblasts is due alternatively to G protein-coupled receptors different from S1PR, as already demonstrated in other cell systems [8,39].

Mesoangioblasts are presently regarded as very promising stem cells in the therapy of muscular dystrophy [3]. However, the reduced survival of these cells after injection *in vivo* is at least in part responsible for their partial ability to repopulate the damaged tissue, representing an obstacle to their employment in cell therapy. The present identification of S1P as powerful mitogenic and anti-apoptotic agent for mesoangioblasts suggests that their efficiency of muscle repair could be increased by the target delivery of exogenous S1P. In view of a future cell therapy it is noteworthy that S1P was here found able to stimulate proliferation of human mesoangioblasts and protect these cells from apoptosis.

Moreover, it has to be considered that S1P is present in the plasma of mammals in a concentration range of 0.1-0.6 μ M [40-41], which is in principle sufficient to fully occupy the majority of S1PR borne by circulating cells. However, since the sphingolipid is largely bound to HDL fraction its real bioavailability remains to be determined [42]. In agreement, it is of note that *in vivo* administration of S1P or S1P agonists is presently regarded as a valid therapeutic approach in a number of different pathologies ranging from renal failure to lung injury [43,44] as well as to prevent transplant rejection and to treat autoimmune disorders [45].

ACKNOWLEDGMENTS

We are indebted with Dr. F. Torricelli and Dr. B. Minuti (Cytogenetics and Genetics Unit, Azienda Ospedaliera Careggi, Florence, Italy) for skilful assistance with real time PCR

REFERENCES

1. De Angelis L, Berghella L, Coletta M, et al. Skeletal myogenic progenitors originating from embryonic dorsal aorta coexpress endothelial and myogenic markers and contribute to postnatal muscle growth and regeneration. *J Cell Biol* 1999;147:869-878.
2. Minasi MG, Riminucci M, De Angelis L et al. The meso-angioblast: a multipotent, self-renewing cell that originates from the dorsal aorta and differentiates into most mesodermal tissues. *Development* 2002;129:2773-2783.
3. Sampaolesi M, Torrente Y, Innocenzi A et al. Cell therapy of alpha-sarcoglycan null dystrophic mice through intra-arterial delivery of mesoangioblasts. *Science* 2003;30:487-492.
4. Galli D, Innocenzi A, Staszewsky L et al. Mesoangioblasts, vessel-associated multipotent stem cells, repair the infarcted heart by multiple cellular mechanisms: a comparison with bone marrow progenitors, fibroblasts, and endothelial cells. *Arterioscler Thromb Vasc Biol* 2005;25:692-697.
5. Cossu G, Sampaolesi M. New therapies for muscular dystrophy: cautious optimism. *Trends Mol Med* 2004;10:516-520.
6. Spiegel S, Milstien S. Sphingosine-1-phosphate: an enigmatic signalling lipid. *Nat Rev Mol Cell Biol*. 2003;4:397-407.
7. Hla T. Physiological and pathological actions of sphingosine 1-phosphate. *Semin Cell Dev Biol* 2004;15:513-520.
8. Ishii I, Fukushima N, Ye X et al. Lysophospholipid receptors: signaling and biology. *Annu Rev Biochem* 2004;73:321-354.
9. Kimura T, Watanabe T, Sato K, et al. Sphingosine 1-phosphate stimulates proliferation and migration of human endothelial cells possibly through the lipid receptors, Edg-1 and Edg-3. *Biochem J* 2000;348:71-76.
10. Tamama K, Kon J, Sato K, et al. Extracellular mechanism through the Edg family of receptors might be responsible for sphingosine-1-phosphate-induced regulation of DNA synthesis and migration of rat aortic smooth-muscle cells. *Biochem J* 2001;353:139-146.
11. Jin Y, Knudsen E, Wang L et al. 2003. Sphingosine 1-phosphate is a novel inhibitor of T-cell proliferation. *Blood* 2003;101:4909-4915
12. Ikeda H, Satoh H, Yanase M, et al. Antiproliferative property of sphingosine 1-phosphate in rat hepatocytes involves activation of Rho via Edg-5. *Gastroenterology* 2003;124:459-469.
13. Donati C, Meacci E, Nuti F et al. Sphingosine 1-phosphate regulates myogenic differentiation: a major role for S1P2 receptor. *FASEB J* 2005;19:449-451.
14. Van Brocklyn JR, Lee MJ, Menzeleev R et al. Dual actions of sphingosine-1-phosphate: extracellular through the Gi-coupled receptor Edg-1 and intracellular to regulate proliferation and survival. *J Cell Biol* 1998;142:229-240.
15. Goetzl EJ, Kong Y, Mei B. Lysophosphatidic acid and sphingosine 1-phosphate protection of T cells from apoptosis in association with suppression of Bax. *J Immunol* 1999;162:2049-2056.
16. Kwon YG, Min JK, Kim KM et al. Sphingosine 1-phosphate protects human umbilical vein endothelial cells from serum-deprived apoptosis by nitric oxide production. *J Biol Chem* 2001;276:10627-10633.

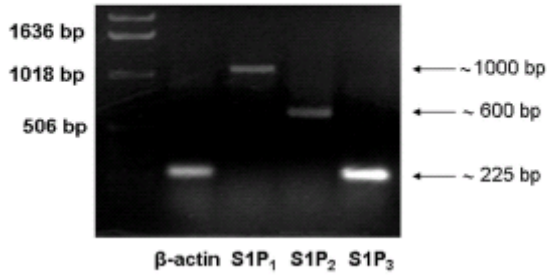
17. Sukocheva O, Wadham C, Holmes A, et al. Estrogen transactivates EGFR via the sphingosine 1-phosphate receptor Edg-3: the role of sphingosine kinase-1. *J Cell Biol* 2006;173:301-310.
18. De Palma C, Meacci E, Perrotta C. et al. Endothelial nitric oxide synthase activation by tumor necrosis factor alpha through neutral sphingomyelinase 2, sphingosine kinase 1, and sphingosine 1 phosphate receptors: a novel pathway relevant to the pathophysiology of endothelium. *Arterioscler Thromb Vasc Biol* 2006;26:99-105.
19. Dellavalle A, Sampaolesi M, Tonlorenzi R, et al., Pericytes of human skeletal muscle are myogenic precursors distinct from satellite cells. *Nat Cell Biol* 2007 doi:10.1038/ncb1542
20. Galvez BG, Sampaolesi M, Brunelli S, et al. Complete repair of dystrophic skeletal muscle by mesoangioblasts with enhanced migration ability. *J Cell Biol* 2006;17:231-243.
21. Davis MD, Clemens JJ, Macdonald TL, et al. Sphingosine 1-phosphate analogs as receptor antagonists. *J Biol Chem* 2005;280:9833-9841.
22. Osada M, Yatomi Y, Ohmori T et al. Enhancement of sphingosine 1-phosphate-induced migration of vascular endothelial cells and smooth muscle cells by an EDG-5 antagonist. *Biochem Biophys Res Commun* 2002;299:483-487.
23. Sanna MG, Liao J, Jo E et al. Sphingosine 1-phosphate (S1P) receptor subtypes S1P1 and S1P3, respectively, regulate lymphocyte recirculation and heart rate. *J Biol Chem* 2004;279:13839-13848.
24. Duclos F, Straub V, Moore SA, et al. Progressive muscular dystrophy in alpha-sarcoglycan-deficient mice. *J Cell Biol* 1998;142:1461-1471.
25. Meacci E, Vasta V, Donati C et al. Receptor-mediated activation of phospholipase D by sphingosine 1-phosphate in skeletal muscle C2C12 cells. A role for protein kinase C. *FEBS Lett* 1999;457:184-188.
26. Becciolini L, Meacci E, Donati C, et al. Sphingosine 1-phosphate inhibits cell migration in C2C12 myoblasts. *Biochim Biophys Acta* 2006;1761:43-51.
27. Livak KJ, Schmittgen TD, Analysis of relative gene expression data using real time quantitative PCR and the 2^{-C_T} . *Methods* 2001;25:402-408.
28. Bucciantini M, Rigacci S, Berti A et al. Patterns of cell death triggered in two different cell lines by HypF-N prefibrillar aggregates. *FASEB J* 2005;19:437-439.
29. Sciorati C, Galvez BG, Brunelli S, et al. E. Ex vivo treatment with nitric oxide increases mesoangioblast therapeutic efficacy in muscular dystrophy. *J Cell Sci* 2006;119:5114-5123.
30. Chihab R, Porn-Ares MI, Alvarado-Kristensson M, et al. Sphingosine 1-phosphate antagonizes human neutrophil apoptosis via p38 mitogen-activated protein kinase. *Cell Mol Life Sci* 2003;60:776-785.
31. Radeff-Huang J, Seasholtz TM, Matteo RG, et al. G protein mediated signaling pathways in lysophospholipid induced cell proliferation and survival. *J Cell Biochem* 2004;92:949-966.
32. Katsuma S, Hada Y, Ueda T et al. Signalling mechanisms in sphingosine 1-phosphate-promoted mesangial cell proliferation. *Genes Cells* 2002;7:1217-1230.
33. Pebay A, Wong RCB, Pitson SM et al. Essential roles of sphingosine-1-phosphate and platelet-derived growth factor in the maintenance of human embryonic stem cells. *Stem Cells* 2005;23:1541-1548.
34. Van Brocklyn J, Letterle C, Snyder P et al. Sphingosine-1-phosphate stimulates human glioma cell proliferation through Gi-coupled receptors: role of ERK MAP kinase and phosphatidylinositol 3-kinase beta. *Cancer Lett* 2002;181:195-204.
35. Pebay A, Toutant M, Premont J, et al. Sphingosine-1-phosphate induces proliferation of astrocytes: regulation by intracellular signalling cascades. *Eur J Neurosci* 2001;13:2067-2076.
36. Olivera A, Rosenfeldt HM, Bektas M, et al. Sphingosine kinase type 1 induces G12/13-mediated stress fiber formation, yet promotes

- growth and survival independent of G protein-coupled receptors. *J Biol Chem* 2003;278:46452-46460.
37. Suomalainen L, Hakala JK, Pentikainen V et al. Sphingosine-1-phosphate in inhibition of male germ cell apoptosis in the human testis. *J Clin Endocrinol Metab* 2003;88:5572-5579.
 38. Boujaoude LC, Bradshaw-Wilder C, Mao C et al. Cystic fibrosis transmembrane regulator regulates uptake of sphingoid base phosphates and lysophosphatidic acid: modulation of cellular activity of sphingosine 1-phosphate. *J Biol Chem* 2001;276:35258-35264.
 39. Im DS Discovery of new G protein-coupled receptors for lipid mediators. *J. Lipid Res* 2004;45:410-418.
 40. Yatomi Y, Igarashi Y, Yang L, et al. Sphingosine 1-phosphate, a bioactive sphingolipid abundantly stored in platelets, is a normal constituent of human plasma and serum. *J Biochem (Tokyo)* 1997;121:969-973.
 41. Berdyshev EV, Gorshkova IA, Garcia JG, et al. Quantitative analysis of sphingoid base-1-phosphates as bisacetylated derivatives by liquid chromatography-tandem mass spectrometry. *Anal Biochem* 2005;339:129-136.
 42. Okajima F. Plasma lipoproteins behave as carriers of extracellular sphingosine 1-phosphate: is this an atherogenic mediator or an anti-atherogenic mediator? *Biochim Biophys Acta* 2002;1582:132-137.
 43. Lien YH, Yong KC, Cho C, et al. S1P(1)-selective agonist, SEW2871, ameliorates ischemic acute renal failure. *Kidney Int* 2006;69:1601-1608
 44. Bischoff A, Czyborra P, Meyer Zu Heringdorf D et al. Sphingosine-1-phosphate reduces rat renal and mesenteric blood flow in vivo in a pertussis toxin-sensitive manner. *Br J Pharmacol* 2000;130:1878-1883.
 45. Gardell SE, Dubin AE, Chun J. Emerging medicinal roles for lysophospholipid signaling. *Trends Mol Med*.2006;12:65-75.

Figure 1. mRNA expression levels of S1PR in D16 mesoangioblasts.

A) RT-PCR analysis. B) Real time PCR analysis. Quantitative mRNA analysis was performed by simultaneous amplification of the target S1P₁, S1P₂, S1P₃ genes together with the housekeeping gene 18S rRNA. Results are expressed as fold changes according to the 2^{-CT} method, utilizing S1P₁ as calibrator.

TOP
Corresponding author: Paola Bruni
Figure 1 A



TOP
Corresponding author: Paola Bruni
Figure 1B

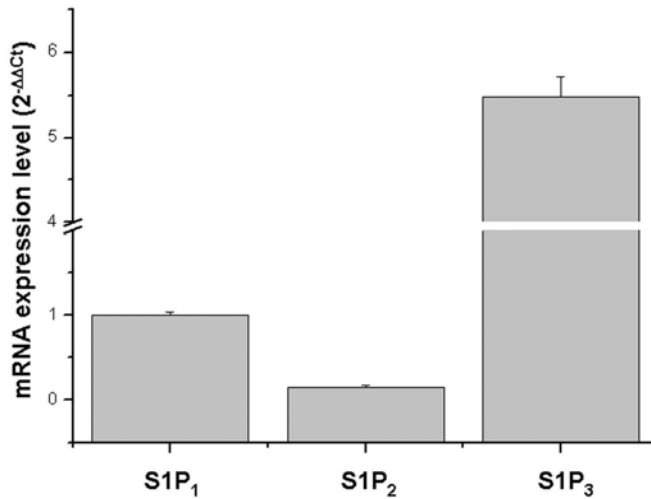
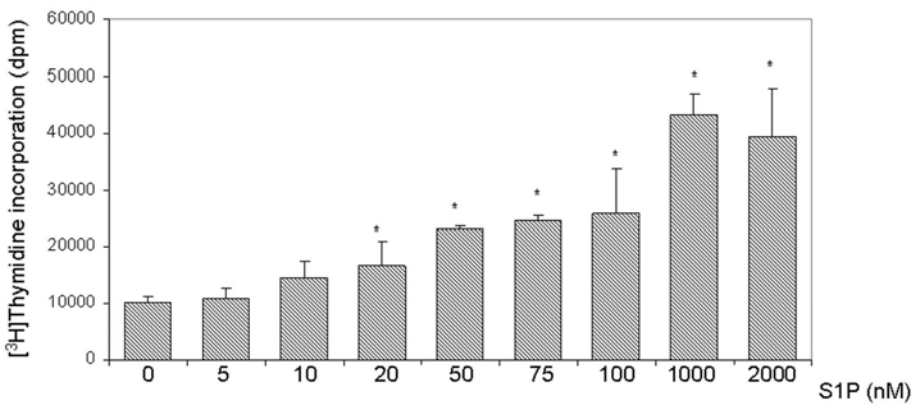


Figure 2. S1P stimulates cell proliferation of D16 mesoangioblasts.

A) Incorporation of [³H]thymidine. Serum-starved D16 mesoangioblasts were stimulated with various concentrations of S1P for 24 h. [³H]thymidine (1 μCi/well) was added in the last 2 h of incubation. B) Cell counting. Serum-starved mesoangioblasts were stimulated with 1 μM S1P for the indicated times before being counted by an hemocytometer.

TOP
Corresponding author: Paola Bruni
Figure 2 A



TOP
Corresponding author: Paola Bruni
Figure 2 B

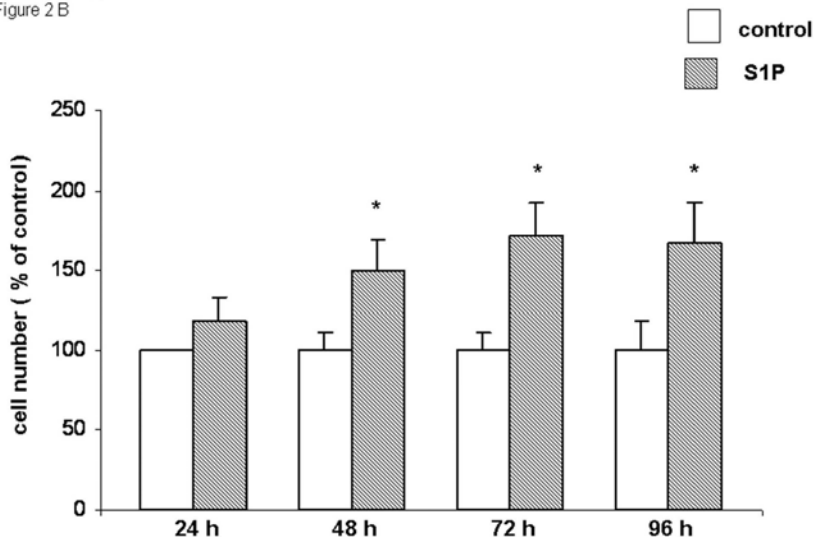
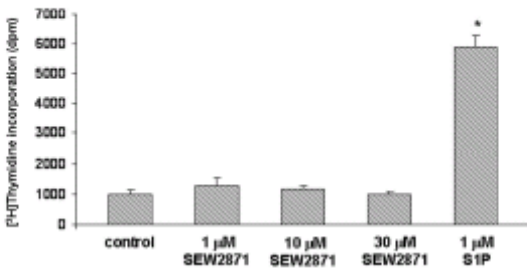


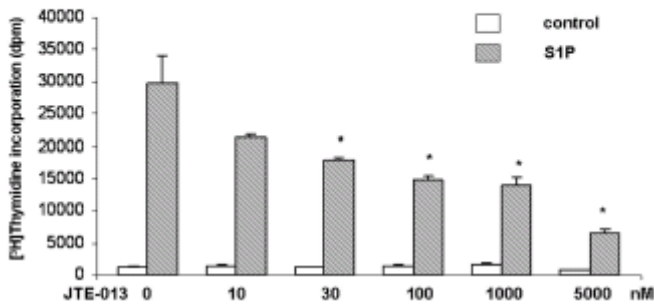
Figure 3. Role of S1PR in the proliferative action of S1P.

Effect of SEW2871 (A), JTE-013 (B) and VPC23019 (C) on mesoangioblast proliferation. The experimental conditions were those described in the legend to Fig.2. Mesoangioblasts were incubated for 24 h in the presence of SEW2871 at the indicated concentrations or pre-incubated for 30 min in the presence of different concentrations of JTE-013 or 100 nM VPC23019 before being stimulated with 1 μ M S1P for 24 h.

TCP
Corresponding author: Paola Bruni
Figure 3 A



TCP
Corresponding author: Paola Bruni
Figure 3 B



TCP
Corresponding author: Paola Bruni
Figure 3 C

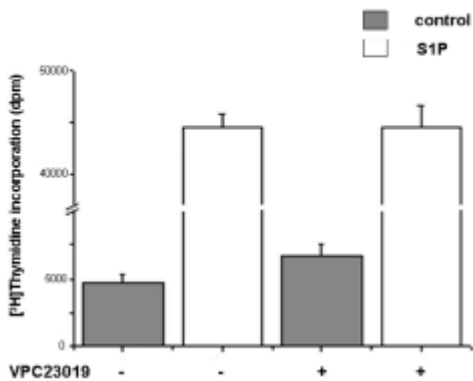
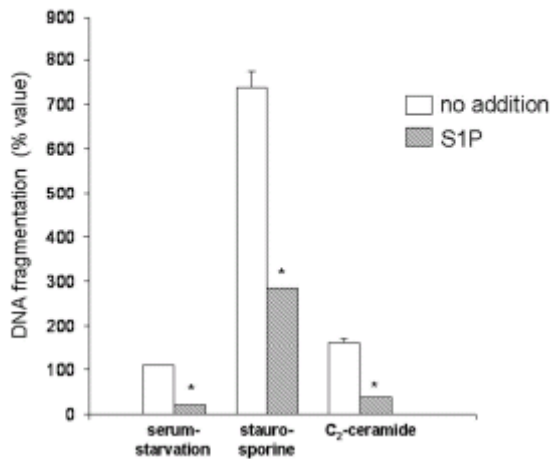


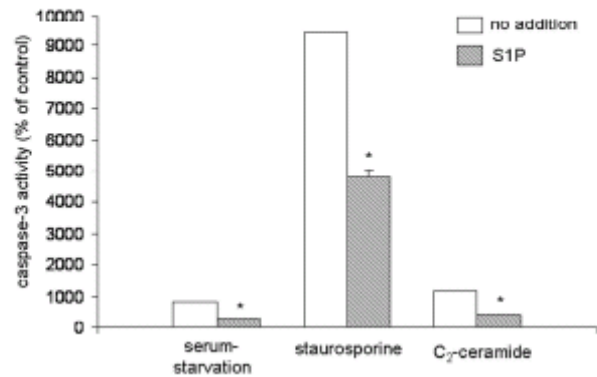
Figure 4. S1P protects D16 mesoangioblasts from apoptosis.

The apoptotic response induced by 24 h serum-starvation, by treating serum-starved cells with 0.5 μ M staurosporine for 4 h or with 10 μ M C₂-ceramide for 24 h in the presence or absence of S1P was evaluated by quantitating DNA fragmentation (A), by measuring caspase-3 activity (B) or by counting the trypsinized surviving cells by an hemocytometer (C). (D) Approximately 5 x 10⁵ GFP- expressing mesoangioblasts pretreated with 1 μ M S1P for 12 h were injected into the tibialis anterior muscles of 4-months-old -SG null mice. After 12 h muscles were recovered. Apoptosis of GFP-expressing mesoangioblasts was assessed by the TUNEL technique (images from one out of 4 reproducible experiments). Also shown is the DAPI staining of the nuclei and its overlay with the GFP and TUNEL staining.

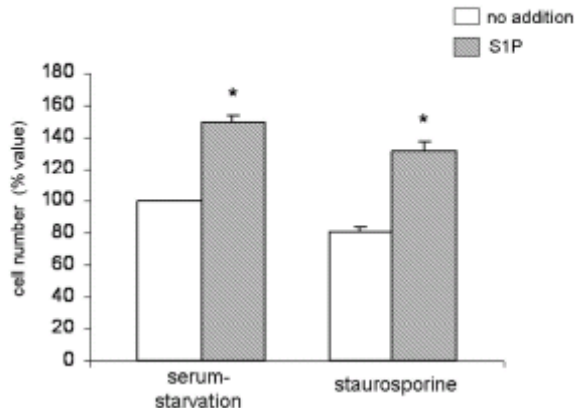
TOP
Corresponding author: Paola Bruni
Figure 4 A



TOP
Corresponding author: Paola Bruni
Figure 4 B



TOP
Corresponding author: Paola Bruni
Figure 4 C



TOP
Corresponding author: Paola Bruni
Figure 4 D

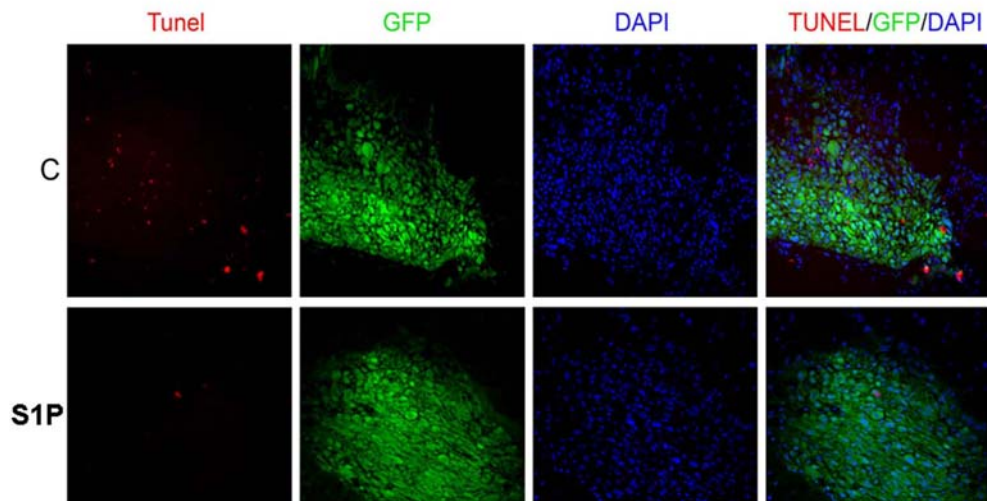


Figure 5. Role of S1PR in the anti-apoptotic action of S1P.

Effect of 1 μ M SEW2871 (grey bar) and 1 μ M JTE-013 or 100 nM VPC23019 in the absence (empty bar) or presence (hatched bar) of 1 μ M S1P on serum-starved mesoangioblast DNA fragmentation induced by 0.5 μ M staurosporine treatment for 4 h.

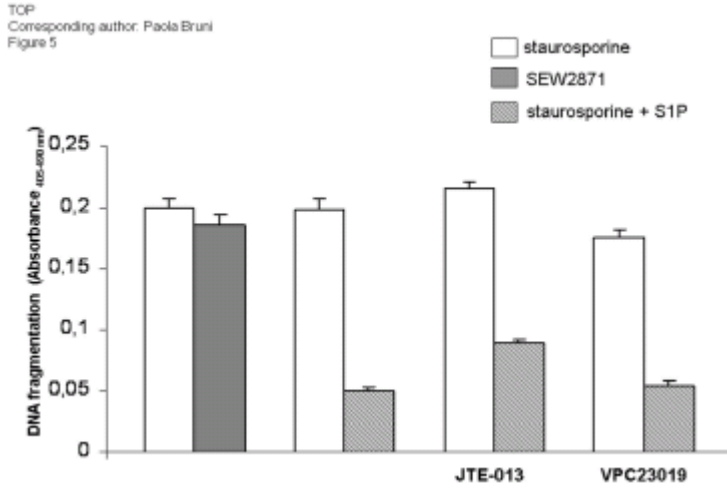
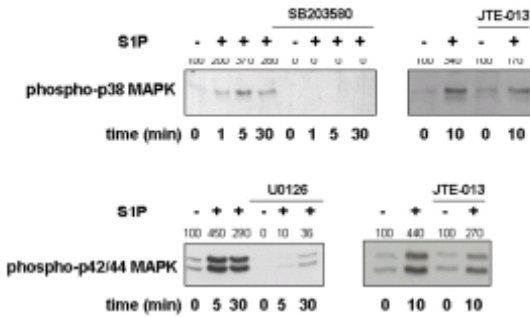


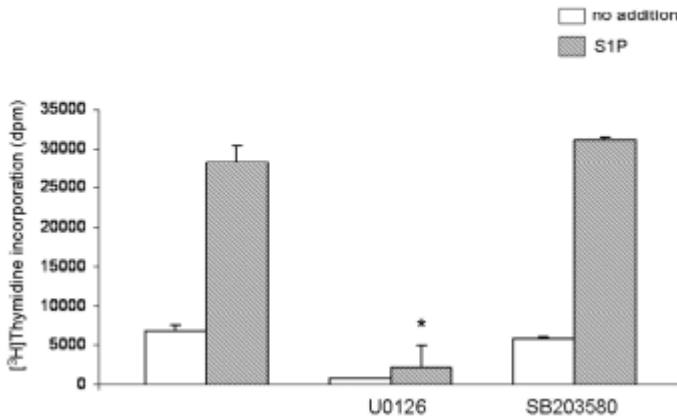
Figure 6. Role of p42/44 MAPK and p38 MAPK pathways in the mitogenic (B) and anti-apoptotic (C) action of S1P in D16 mesoangioblasts.

A) Western blot analysis of lysates (30 μ g) of serum-starved mesoangioblasts incubated with or without 1 μ M S1P for the indicated times in the presence or absence of 10 μ M U0126, 5 μ M SB203580 or 2 μ M JTE-013. B) Effect of 10 μ M U0126 or 5 μ M SB203580 on the proliferative action of S1P in D16 mesoangioblasts. C) Effect of 10 μ M U0126 or 5 μ M SB203580 on the protective effect of S1P on staurosporine-induced DNA fragmentation.

TOP
Corresponding author: Paola Bruni
Figure 6 A



TOP
Corresponding author: Paola Bruni
Figure 6 B



TOP
Corresponding author: Paola Bruni
Figure 6 C

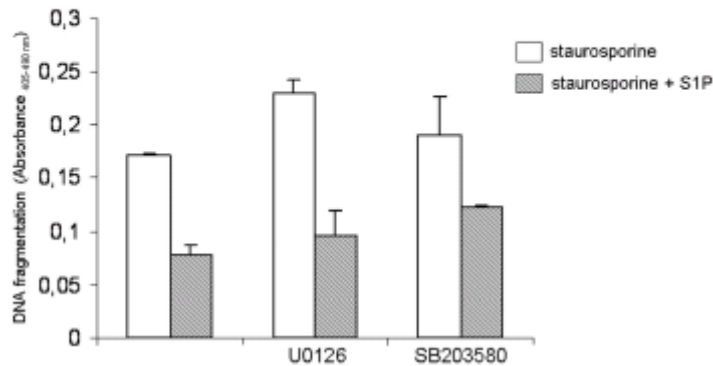
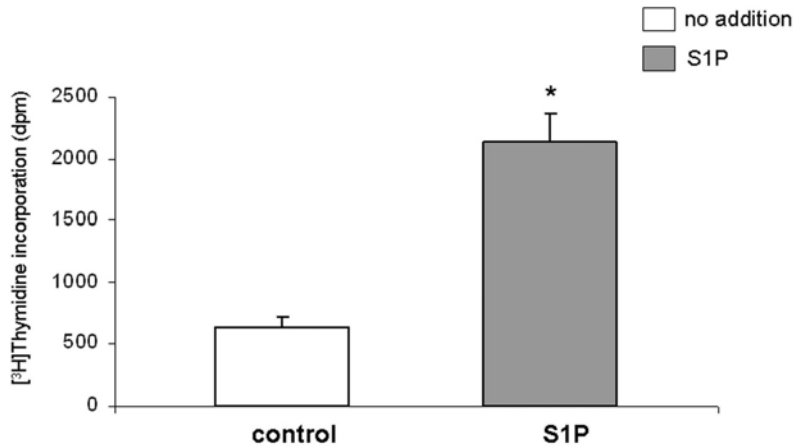


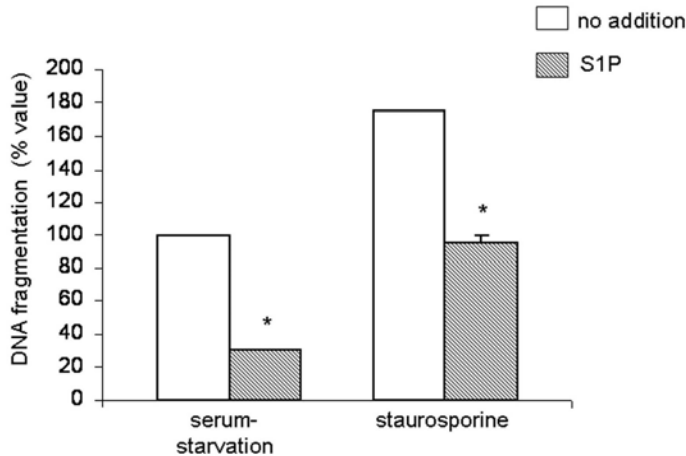
Figure 7. S1P exerts mitogenic (A) and anti-apoptotic effect (B) in human mesoangioblasts.

A) Incorporation of [³H]thymidine. Serum-starved cells were stimulated with 1 μM S1P for 24 h. [³H]thymidine (2 μCi/well) was added to the cells in the last 2 h of incubation. B) The apoptotic response induced by 24 h serum-starvation or by treating serum-starved cells with 0.5 μM staurosporine for 4 h in the absence or presence of S1P was evaluated by quantitating DNA fragmentation.

TOP
Corresponding author: Paola Bruni
Figure 7 A



TOP
Corresponding author: Paola Bruni
Figure 7B



Sphingosine 1-Phosphate Mediates Proliferation and Survival of Mesoangioblasts
Chiara Donati, Francesca Cencetti, Paola Nincheri, Caterina Bernacchioni, Silvia Brunelli,
Emilio Clementi, Giulio Cossu and Paola Bruni
Stem Cells published online Apr 26, 2007;
DOI: 10.1634/stemcells.2006-0725

This information is current as of April 30, 2007

**Updated Information
& Services**

including high-resolution figures, can be found at:
<http://www.StemCells.com>

 **AlphaMed Press**