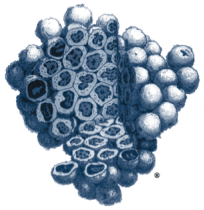

STEM CELLS®

Cooperation of Matrix Metalloproteinases with the RhoA/Rho Kinase and Mitogen-Activated Protein Kinase Kinase-1/Extracellular Signal-Regulated Kinase Signaling Pathways Is Required for the Sphingosine-1-Phosphate-Induced Mobilization of Marrow-Derived Stromal Cells

Mayya Meriane, Stéphanie Duhamel, Laurence Lejeune, Jacques Galipeau and Borhane Annabi

Stem Cells 2006;24;2557-2565; originally published online Aug 24, 2006;
DOI: 10.1634/stemcells.2006-0209

This information is current as of October 30, 2006

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://www.StemCells.com/cgi/content/full/24/11/2557

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. © 2006 by AlphaMed Press, all rights reserved. Print ISSN: 1066-5099. Online ISSN: 1549-4918.

AlphaMed Press

Downloaded from www.StemCells.com at Stanford University Medical Center on October 30, 2006

STEM CELLS®
THE STEM CELL NICHE

Cooperation of Matrix Metalloproteinases with the RhoA/Rho Kinase and Mitogen-Activated Protein Kinase Kinase-1/Extracellular Signal-Regulated Kinase Signaling Pathways Is Required for the Sphingosine-1-Phosphate-Induced Mobilization of Marrow-Derived Stromal Cells

MAYYA MERIANE,[a] STÉPHANIE DUHAMEL,[a] LAURENCE LEJEUNE,[a] JACQUES GALIPEAU,[b] BORHANE ANNABI[a]

[a]Laboratoire d'Oncologie Moléculaire, Département de Chimie, Centre BioMed, Université du Québec à Montréal, Montreal, Quebec, Canada; [b]Department of Medicine, Lady Davis Institute for Medical Research, Montreal, Quebec, Canada

Key Words. Marrow-derived stromal cells • Sphingosine-1-phosphate • Matrix metalloproteinases • Rho GTPases • ROCK MEK1/ERK • Actin cytoskeleton • Cell migration

ABSTRACT

The ease of isolation and ex vivo culture of marrow-derived stromal cells (MSCs) from adult bone marrow renders them a very promising source of adult stem cells for gene transfer and cell therapy. However, little is known about the signaling pathways that control their in vivo mobilization and subsequent biodistribution. Platelet-derived sphingosine-1-phosphate (S1P), a bioactive lipid that acts via G-protein-coupled-receptors, exerts strong chemoattraction upon MSCs through yet-uncharacterized signaling pathways. We show that the S1P-induced migration and morphological changes of MSCs in vitro require the activities of extracellular signal-regulated kinase (ERK), Rho kinase (ROCK), and matrix metalloproteinase (MMP) signaling molecules. Specifically, S1P-induced remodeling of the MSC cytoskeleton led to the rapid (<1 minute) formation of actin stress fibers via activation of the RhoA/ROCK pathway and required the catalytic activity of MMPs. S1P-induced activation of the mitogen-activated protein kinase kinase-1 (MEK1)/ERK pathway also contributed to the induction of the actin stress fibers and to the redistribution of paxillin at the focal adhesions through tyrosine phosphorylation of focal adhesion kinase in an MMP-dependent manner. Moreover, MMP- and ROCK-dependent molecular events are implicated in the regulation of the S1P-induced activation of ERK. Our results demonstrate that MSC mobilization in response to S1P requires cooperation between MMP-mediated signaling events and the RhoA/ROCK and MEK1/ERK intracellular pathways. Therefore, the characterization of the cellular factors and the intracellular signaling pathways underlying MSC mobilization is crucial to achieve high efficacy in therapeutic use. STEM CELLS *2006;24:2557–2565*

INTRODUCTION

Mammalian bone marrow stroma provides a unique microenvironment to a particular pool of somatic stem cells known as marrow-derived stromal cells (MSCs) [1]. The ease of isolation of MSCs from adult bone marrow and the ex vivo culture of MSCs, coupled to their high plasticity potential, renders them a very promising source of adult stem cells for gene transfer and cell therapy [2, 3]. In the adult organism, MSCs traffic across extracellular matrix (ECM)-rich basement membranes and interstitial tissues in response to the appropriate cytokines or growth factors and undergo transdifferentiation into various cell lineages like myoblasts, hepatocytes, and even neuronal cells, thus providing a natural regenerative potential to adult, damaged tissues [2, 4, 5]. However, the mechanisms underlying paracrine regulation of MSC transmigration and differentiation are still poorly understood. Because MSCs are used as drug delivery vehicles such as for targeting invasive and metastatic malignant tumors [3], the characterization of the cellular factors and the intracellular signaling pathways underlying MSC mobilization becomes crucial in order to achieve high efficacy in therapeutic use.

Among the chemokines and inflammatory mediators known to exert potent cellular chemotactic effects, sphingosine-1-phos-

Correspondence: Borhane Annabi, Ph.D., Laboratoire d'Oncologie Moléculaire, Université du Québec à Montréal, C.P. 8888, Succ. Centre-ville, Montréal, Québec, Canada, H3C 3P8. Telephone: 514-987-3000, ext. 7610; Fax: 514-987-0246; e-mail: annabi.borhane@uqam.ca Received April 11, 2006; accepted for publication July 17, 2006; first published online in STEM CELLS *EXPRESS* August 24, 2006. © AlphaMed Press 1066-5099/2006/$20.00/0 doi: 10.1634/stemcells.2006-0209

Downloaded from www.StemCells.com at Stanford University Medical Center on October 30, 2006

phate (S1P) is one of the most important bioactive lysophospholipids secreted in blood plasma upon platelet activation [6] and a very good candidate for the induction of MSC mobilization [7]. S1P acts via G-protein-coupled receptors of the Edg family to induce numerous biological effects on mammalian cells, such as stimulation of cell proliferation, differentiation, apoptosis, angiogenesis, and migration [6, 8]. In particular, S1P1 (Edg1) and S1P2 (Edg5) are the S1P cell surface receptor subtypes that are specifically involved in S1P-induced chemotaxis [9]. Cell migration and chemotaxis require reorganization of the actin cytoskeleton and remodeling of the ECM, which are principally regulated by members of the Rho family of small GTPases (RhoA, Rac1, and Cdc42) and by the family of matrix metalloproteinases (MMPs) [10].

There is growing evidence linking S1P signaling to Rho GTPase activation; it has been shown that some S1P receptor subtypes coupled to Gα12/13 are involved in S1P-mediated RhoA activation [11]. Activation of RhoA leads to the assembly of actin stress fibers and focal adhesions [12, 13], and the activity of RhoA has been correlated to acquisition of a migratory and invasive phenotype [14, 15]. More recently, studies have shown that activation of RhoA induced endothelial cell invasion through the regulation of MMP-9 expression and that RhoA colocalized with the cell surface receptor CD44 at advancing lamellipodia of invasive cells [16]. Accordingly, we have further provided evidence that RhoA also regulated the expression of the transmembrane metalloproteinase MT1-MMP in glioma cells and that the RhoA/Rho kinase (ROCK) signaling pathway regulated MT1-MMP-mediated CD44 functions [17].

To shed light on the paracrine regulation of MSC mobilization, we investigated the early intracellular signaling events involved in platelet-derived S1P-mediated MSC chemotaxis. We show that, in vitro, S1P induces a rapid remodeling of the MSC actin cytoskeleton through the activation of the RhoA/ROCK pathway in an MMP-dependent manner. We also show that S1P-induced MSC migration requires the activity of ROCK. Finally, we demonstrate that the S1P-coordinated activation of the mitogen-activated protein kinase kinase-1 (MEK1)-extracellular signal-regulated kinase (ERK) signaling pathway cooperates with RhoA/ROCK and MMP-mediated molecular events to rapidly induce MSC actin cytoskeleton remodeling and migration. This is the first report that demonstrates cooperation between the RhoA/ROCK and MEK1/ERK intracellular signaling pathways and the role of MMPs in the regulation of S1P-induced chemotaxis of MSCs. Better understanding of the intracellular signaling pathways underlying MSC biology in response to bioactive mediators like S1P will help in optimizing the use of bioengineered MSCs as potent systemic drug delivery vehicles in therapeutic protocols.

Materials and Methods

Cell Culture and Treatment

MSCs were isolated from mouse bone marrow and cultured as previously described [7]. Whole bone marrow was harvested from the femurs and tibias of 18- to 22-g female C57Bl/6 mice (Charles River Laboratories, Laprairie, QC, Canada, http://www.criver.com) sacrificed by CO_2 inhalation as approved by the Université du Québec à Montréal (Montreal, Quebec, Canada) ethical committee (CIPA [Institutional Committee for Animal Protection] protocol number 474). Cells were plated in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) supplemented with 10% inactivated fetal bovine serum (HyClone, Logan, UT, http://www.hyclone.com) and 100 units per ml penicillin/streptomycin. After 5–7 days of incubation in a humidified incubator at 37°C with 5% CO_2, the nonadherent hematopoietic cells were discarded. Adherent MSCs were further grown on noncoated culture dishes and maintained for no more than 14 passages. Analysis by flow cytometry performed at passage 14 revealed that MSCs expressed CD44 yet were negative for CD45, CD31, KDR/flk1 (VEGF-R2), flt-4 (VEGF-R3), and Tie2 (angiopoietin receptor) (data not shown). Short-term (1-hour) pretreatement of MSCs was performed with the following inhibitors: 30 μM Ilomastat (GM6001) (BIOMOL International, L.P., Plymouth Meeting, PA, http://www.biomol.com/1), 10 μM Y27632 (ROCK inhibitor), and 50 μM PD98059 (MEK1 inhibitor) (Calbiochem, San Diego, http://www.emdbiosciences.com). Flow cytometry analysis of annexin-V/propidium iodine staining of the cells showed no effect of the inhibitors used on cell viability (not shown). MSCs were then stimulated for different periods of time with 1 μM S1P (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com).

Chemotactic Cell Migration Assay

Serum-starved MSCs were pretreated for 1 hour in the presence or absence of the above inhibitors. Cells were then trypsinized and seeded at 10^5 cells on 0.15% gelatin/phosphate-buffered saline (PBS) precoated Transwells (8-μm pore size; Corning Life Sciences, Acton, MA, http://www.corning.com/lifesciences) assembled in 24-well Boyden chambers that were filled with 600 μl of serum-free media containing (or not) 30 μM Ilomastat, 10 μM Y27632, or 50 μM PD98059 in the lower compartment. Cells were allowed to adhere for 1 hour and were then stimulated by adding either serum or 1 μM S1P to the lower compartments. Cell migration was allowed to proceed for 6 hours at 37°C in 5% CO_2. Nonmigrating cells that remained on the upper side of the Transwell filter were carefully removed with cotton swabs. Cells that had migrated to the lower side of the filters were fixed with 3.7% formaldehyde, stained with 0.1% crystal violet/20% MeOH, and counted. The migration was quantified by analyzing at least 10 random fields per filter for each independent experiment using Northern Eclipse 6.0 (Empix Imaging, Inc., Mississauga, ON, Canada, http://www.empix.com) computer-assisted imaging software.

Immunofluorescence Analysis

MSCs grown on glass cover slips at approximately 60% confluence were pretreated as described above with or without different inhibitors, then stimulated with 1 μM S1P. Cells were fixed in 3.7% formaldehyde for 10 minutes, permeabilized for 5 minutes with 0.1% Triton X100/PBS, and incubated with 0.1% bovine serum albumin (BSA)/PBS for 20 minutes at room temperature. Cells were incubated with rhodamine-conjugated Phalloidin (Sigma-Aldrich) for 30 minutes at 37°C to stain F-actin. For Paxillin staining, cells were incubated for 1 hour at 37°C with mouse monoclonal anti-Paxillin antibodies (Upstate, Charlottesville, VA, http://www.upstate.com) diluted in 0.1% BSA/PBS. Cells were then gently rinsed with PBS and incubated with fluorescein isothyocyanate-coupled secondary anti-

Downloaded from www.StemCells.com at Stanford University Medical Center on October 30, 2006

mouse antibodies (Sigma-Aldrich) for 1 hour at 37°C. The cover slips were mounted on Mowiol antifading mounting media (Calbiochem) and were left to dry in the dark for 24 hours. The fluorescent cells were analyzed with a microscope (Eclipse TE 2000U; Nikon Corporation, Tokyo, http://www.nikon.com) using an oil immersion ×40 objective lens. Images were recorded with a digital camera and analyzed with the Northern Eclipse 6.0 software.

Western Blot Procedures

Proteins from cell lysates were quantified, and equal protein amounts were separated by SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon, Millipore, Bedford, MA, http://www.millipore.com), which were then blocked for 1 hour at 4°C with 5% nonfat dry milk in TBST (Tris-buffered saline [150 mM NaCl, 20 mM Tris-HCl, pH 7.5], 0.3% Tween 20). Membranes were washed several times with TBST and were probed with the appropriate specific primary antibodies in TBST containing 3% BSA followed by 1 hour of incubation with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (GE Healthcare, Little Chalfont, Buckinghamshire, U.K., http://www.gehealthcare.com) (1:10,000) in TBST containing 5% nonfat dry milk. Immunoreactive bands were revealed with an enhanced chemiluminescence kit (GE Healthcare) followed by exposure to X-OMAT AR Kodak image films (Eastman Kodak, Rochester, NY, http://www.kodak.com). Band intensities were quantified by densitometry using TotalLab TL100 software (Nonlinear USA Inc., Durham, NC, http://www.nonlinear.com).

RhoA Activation Assay

MSCs were grown to approximately 80% confluence on 100-mm dishes and serum-starved in DMEM for 24 hours, then treated with or without the above-described inhibitors before they were stimulated with 1 μM S1P. GTP-RhoA activation was assessed using the RhoA Activation Assay Kit (Upstate) according to the manufacturer's procedure. Immediately after stimulation, cells were rinsed with cold PBS, lysed in 300 μl of MLB buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% NP-40 (Igepal CA-630), 10 mM $MgCl_2$, 1 mM EDTA, 2% glycerol, 25 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride [PMSF] and 10 μg/ml aprotinin/leupeptin), and then briefly centrifuged to remove cell debris. Cell lysates were pulled down by incubation at 4°C with 20 μg of recombinant Rhotekin-ρ-binding domain bound to glutathione-agarose beads for 45 minutes. The beads were then washed three times with MLB and resuspended with MLB containing 5× sample buffer to elute GTP-loaded RhoA from the beads. The samples, as well as 30 μl of whole cell lysates kept for each condition, were separated on a 12% polyacrylamide-SDS gel and then transferred to PVDF membranes. The active GTP-loaded RhoA protein, as well as the total amount of RhoA present in the samples, was detected by probing the membranes with mouse monoclonal anti-RhoA antibodies provided in the same kit.

Focal Adhesion Kinase Immunoprecipitation

MSCs grown on 60-mm dishes and serum-starved overnight were pretreated for 1 hour with or without 30 μM Ilomastat before being stimulated with 1 μM S1P. Cells were harvested with 300 μl of lysis buffer containing 20 mM Tris (pH 7.5), 10% glycerol, 1% Triton X-100, 20 mM NaF, 100 mM NaCl, 1 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml pepstatin. Cell lysates were then centrifuged at 14,000 rpm for 5 minutes at 4°C to remove cell debris, and the supernatants were precleared for 1 hour with 40 μl of protein A-sepharose beads (GE Healthcare). Focal adhesion kinase (FAK) was immunoprecipitated from the precleared lysates by incubation for 2 hours at 4°C with 2 μg of polyclonal anti-FAK antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, http://www.scbt.com) and 25 μl of protein A-sepharose beads. The beads were then washed three times with the lysis buffer before being resuspended and boiled in 5× sample buffer. The immunoprecipitated samples were resolved by 10% polyacrylamide-SDS gel electrophoresis followed by electrotransfer onto PVDF membranes. The phosphorylation level of FAK was assessed by Western blot using mouse monoclonal anti-phosphotyrosine (anti-PY20) antibodies (BD Biosciences, San Jose, CA, http://www.bdbiosciences.com). The amount of FAK present in the whole cell lysates was determined by probing with rabbit polyclonal anti-FAK antibodies (Santa Cruz Biotechnology, Inc.).

ERK Phosphorylation Status

Serum-starved MSCs seeded at approximately 80% confluence in six-well dishes were pretreated for 1 hour with or without inhibitors before being stimulated with 1 μM S1P for different periods of time. Cells were then lysed on ice in a buffer containing 20 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% Nonidet-40, 0.1% SDS, 1% deoxycholate, 5 mM EDTA, 1 mM sodium orthovanadate, 1 mM PMSF, 20 mM NaF, and 10 μg/ml aprotinin and pepstatin. Cell lysates were cleared by centrifugation at 4°C for 20 minutes at 14,000 rpm, and equal amounts of proteins (50 μg/lane) were resuspended with 5× sample buffer before being resolved by SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membranes. The phosphorylation level of ERK in the cell lysates was determined by Western blot using mouse monoclonal anti-phosphospecific anti-ERK (Thr202/Tyr204) antibodies (Cell Signaling Technology, Beverly, MA, http://www.cellsignal.com). Membranes were reprobed with mouse monoclonal anti-ERK antibodies (New England Biolabs Ltd., Pickering, ON, Canada, http://www.neb.com) to show equal protein loading.

Statistical Data Analysis

Data are representative of three or more independent experiments. Statistical significance was assessed using nonparametric one-way analysis of variance with GraphPad Prism Version 4.0 (GraphPad Software, Inc., San Diego, http://www.graphpad.com). Probability values of less than .05 were considered significant, and an asterisk identifies such significance in each figure.

RESULTS

MMPs, ROCK, and ERK Are Required for S1P-Stimulated Migration of MSCs

To characterize the intracellular signaling pathways underlying S1P-mediated MSC chemotaxis, we first examined the effect of S1P on MSC migration under different conditions. For this

www.StemCells.com Downloaded from www.StemCells.com at Stanford University Medical Center on October 30, 2006

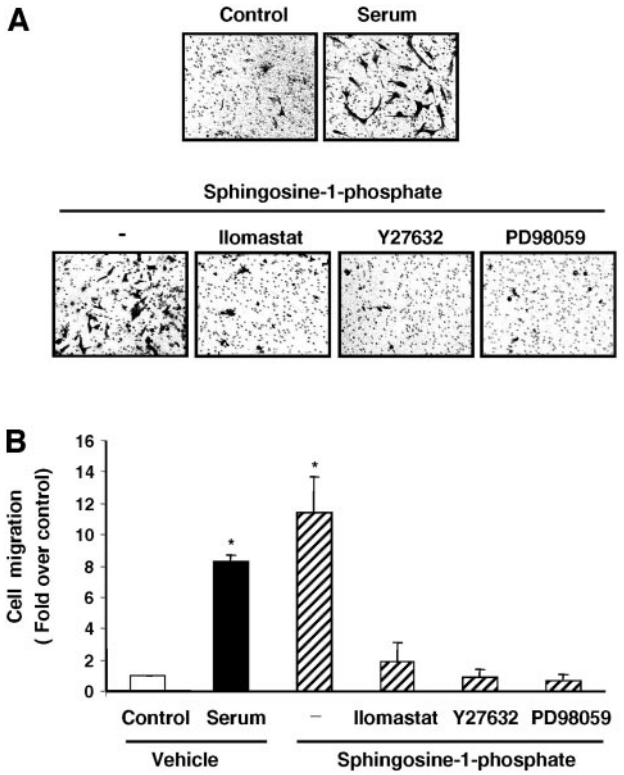

Figure 1. Sphingosine-1-phosphate (S1P)-mediated cell migration of marrow-derived stromal cells (MSCs) requires matrix metalloproteinase, Rho kinase, and extracellular signal-regulated kinase activities. **(A):** Serum-starved MSCs were pretreated for 1 hour with or without 30 μM Ilomastat, 10 μM Y27632, or 50 μM PD98059. Cells were then trypsinized and seeded (10^5 cells) on gelatin-coated filters in modified Boyden chambers. Migration was allowed to proceed for 16 hours at 37°C in the presence of either serum-free media (control) or serum-containing media (serum). Under the same conditions, pretreated MSCs were allowed to migrate in the presence of 30 μM Ilomastat, 10 μM Y27632, or 50 μM PD98059 in serum-free media containing 1 μM S1P. Cells on filters were then fixed and stained; 1 of 10 representative stainings for each condition is shown. **(B):** Values shown represent the means ± SD of three independent experiments in which 10 random fields per filter were counted in each experiment for each condition. Probability values of less than .05 were considered significant compared with control (vehicle); an asterisk identifies such significance.

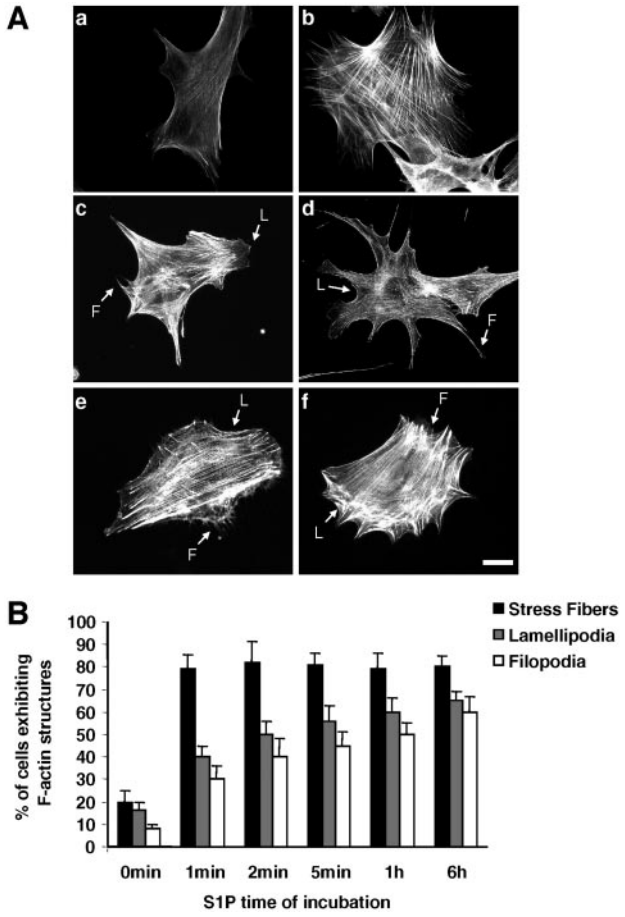

Figure 2. S1P induces a rapid and sustained assembly of actin stress fibers. **(A):** Serum-starved marrow-derived stromal cells were not stimulated **(a)** or were stimulated with 1 μM S1P for 1 minute **(b)**, 2 minutes **(c)**, 5 minutes **(d)**, 1 hour **(e)**, and 6 hours **(f)** and then were fixed and stained with rhodamine-coupled phalloidin to visualize actin filaments. Scale bar = 20 μm. **(B):** Quantification of the percentage of cells exhibiting structures of actin stress fibers lamellipodia (L) and filopodia (F) after S1P treatment. Values shown represent averages for more than 100 cells counted per condition in three independent experiments. Abbreviation: S1P, sphingosine-1-phosphate.

purpose, serum-starved MSCs were pretreated with the following pharmacological inhibitors: Ilomastat, a broad spectrum inhibitor of MMPs; Y27632, a specific inhibitor of ROCK; or PD98059, an inhibitor of the MEK1-ERK1/2 pathway. MSC migration was then analyzed in the presence or absence of S1P using modified Boyden chambers as described in Materials and Methods. (Fig. 1A). As previously documented by us [7], we found that S1P induced a 12-fold increase in MSC migration over the basal level, an effect that was even stronger than serum-derived growth factors, which induced only an eightfold increase in cell migration (Fig. 1B). Pretreatment of MSCs with Ilomastat, as well as with the ROCK inhibitor Y27632, strongly antagonized S1P-induced MSC migration. Inhibition of the MEK1-ERK1/2 signaling pathway with PD98059 also had a powerful antagonizing effect on S1P-induced MSC migration (Fig. 1B). These results implicate molecular events involving MMPs, ROCK, and ERK1/2 as essential for S1P intracellular signaling to induce MSC chemotaxis.

S1P Induces a Rapid Remodeling of the MSC Actin Cytoskeleton

Because cell chemotaxis is regulated by the reorganization of the actin cytoskeleton in response to chemotactic cues [18], we analyzed the effects of S1P treatment on actin organization in MSCs. Serum-starved MSCs were stimulated with 1 μM S1P for different periods of time, and the actin cytoskeleton was visualized by staining with rhodamine-coupled phalloidin. After 1 minute of treatment, S1P induced strong and rapid stress fiber formation compared with the basal level of actin filaments observed in nonstimulated control cells (Fig. 2Aa, 2Ab, 2B). After 2 and 5 minutes, in addition to actin stress fibers, S1P induced lamellipodia and some filopodia structures (Fig. 2Ac, 2Ad, 2B). After 1–6 hours of S1P treatment, the actin stress fibers were maintained while the percentage of cells with la-

Downloaded from www.StemCells.com at Stanford University Medical Center on October 30, 2006

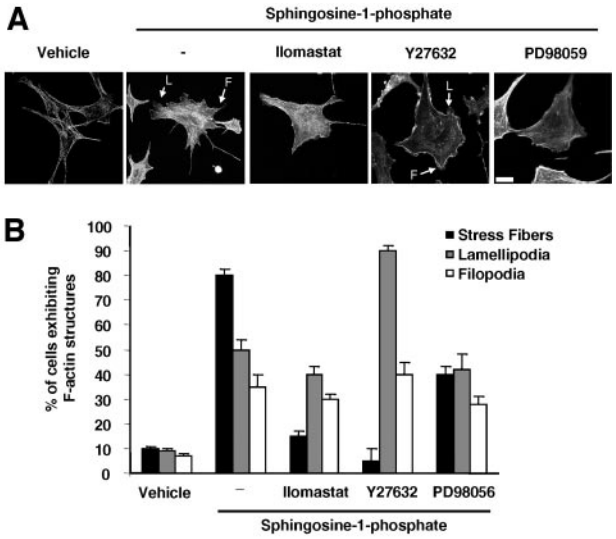

Figure 3. Rho kinase, extracellular signal-regulated kinase, and matrix metalloproteinase activities mediate actin stress fiber assembly after sphingosine-1-phosphate (S1P) stimulation of marrow-derived stromal cells (MSCs). **(A):** Serum-starved MSCs were preincubated for 1 hour with or without 10 μM Y27632, 30 μM Ilomastat, or 50 μM PD98059 prior to 2 minutes of stimulation with 1 μM S1P. Cells were fixed and stained with rhodamine-coupled phalloidin to visualize actin filaments. Scale bar = 20 μm. **(B):** Quantification of the percentage of cells exhibiting actin stress fibers lamellipodia (L) and filopodia (F). Values shown represent averages for more than 100 cells counted per condition in three independent experiments.

mellipodia and filopodia continued to increase (Fig. 2Ae, 2Af, 2B). Although the S1P receptors involved remain to be determined, these results demonstrate that one of the early and immediate events triggered by S1P in MSCs is the strong and sustained induction of actin stress fibers, followed by the slow formation of lamellipodia and filopodia.

ROCK, ERK, and MMPs Are Required for S1P-Induced Actin Stress Fibers in MSCs

We then examined the regulatory mechanisms involved in S1P-mediated actin remodeling in MSCs (Fig. 3A). First, the RhoA/ROCK pathway was investigated by inhibiting ROCK with Y27632 for 1 hour before stimulating the cells with 1 μM S1P for 2 minutes. ROCK inhibition completely antagonized S1P-induced actin stress fibers but increased lamellipodia formation (Fig. 3B). These results suggest that ROCK is the major signaling effector required for S1P-induced actin stress fibers in MSCs. MSCs were then pretreated with Ilomastat for 1 hour to inhibit general MMP activity before cells were stimulated with S1P. Ilomastat also antagonized S1P-induced actin stress fibers but did not affect lamellipodia or filopodia formation, suggesting that MMP-mediated signaling is required only for actin stress fiber formation (Fig. 3B). On the other hand, inhibition of the MEK1-ERK1/2 pathway by PD98059 pretreatment prior to S1P stimulation only partially blocked the induction of the actin stress fibers by S1P, whereas lamellipodia or filopodia formation was unaffected (Fig. 3B). MSCs pretreated with Y27632, Ilomastat, or PD98059 had a basal level of actin filaments similar to those levels seen in untreated control cells (data not shown).

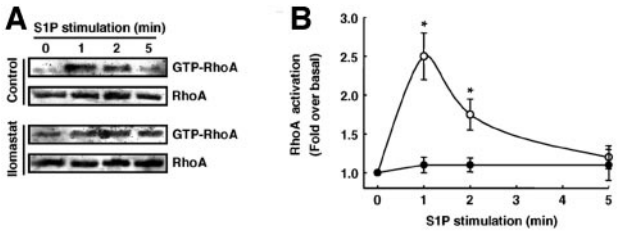

Figure 4. Matrix metalloproteinase catalytic activity is required for the S1P-induced activation of RhoA in marrow-derived stromal cells (MSCs). **(A):** Serum-starved MSCs, preincubated with 30 μM Ilomastat for 1 hour, and control cells were stimulated with 1 μM S1P for 1, 2, and 5 minutes. The cell lysates were subjected to a pull-down assay as described in Materials and Methods using glutathione *S*-transferase-Rhotekin-binding domain fusion protein to measure the level of GTP-loaded RhoA. The proteins from the pull-down as well as from the total cell lysates were analyzed by Western blot using mouse monoclonal anti-RhoA antibodies. **(B):** Quantification of the activation of RhoA by S1P treatment in control (white circles) and Ilomastat-treated cells (black circles). The data are means ± SD of three independent experiments. Probability values of less than .05 were considered significant compared with the respective time 0; an asterisk identifies such significance. Abbreviation: S1P, sphingosine-1-phosphate.

MMP-Mediated Events Regulate RhoA Activation by S1P in MSCs

Because S1P triggered a strong induction of MSC actin stress fibers, a general hallmark of small GTPase RhoA activity, we next investigated whether S1P induced RhoA activation and assessed the level of activated GTPase. Serum-starved MSCs were stimulated with 1 μM S1P, and the levels of GTP-loaded RhoA in the cell lysates were pulled down using recombinant glutathione *S*-transferase-Rhotekin-binding domain as described in Materials and Methods. S1P induced a rapid activation of RhoA after 1 minute of stimulation (Fig. 4A). Interestingly, inhibition of MMP activity by Ilomastat, prior to S1P stimulation, was reflected by an increase in basal activated RhoA which consequently resulted in a lower capacity of S1P to induce further RhoA activation (Fig. 4B), suggesting that potential membrane-bound MMP-mediated events are required for a synergistic RhoA activation by S1P. These data suggest the existence of crosstalk between MMPs, possibly membrane-bound MT1-MMP as we previously suggested [17], and Rho GTPase-mediated signaling pathways.

S1P Induces Remodeling of MSC Focal Adhesions via Paxillin Redistribution and MMPs

We next examined whether S1P affects the organization of the RhoA-mediated focal adhesions by visualizing the subcellular localization of Paxillin, an important protein component of focal adhesions [12, 13]. MSCs were stimulated with or without 1 μM S1P and were fixed and costained for Paxillin and actin filaments using respectively anti-Paxillin antibodies and rhodamine-coupled phalloidin (Fig. 5A). In nonstimulated control cells, a very weak and diffuse Paxillin staining is obtained with a basal level of actin filaments (Fig. 5B). When MSCs were stimulated with S1P, the increase in stress fibers was accompanied by a significant redistribution of Paxillin to the cell periphery and, more specifically, to the tip of the actin filaments (Fig. 5A). Paxillin redistribution all along the stress fibers was visualized by merging the actin and Paxillin stainings showing the colo-

www.StemCells.com Downloaded from www.StemCells.com at Stanford University Medical Center on October 30, 2006

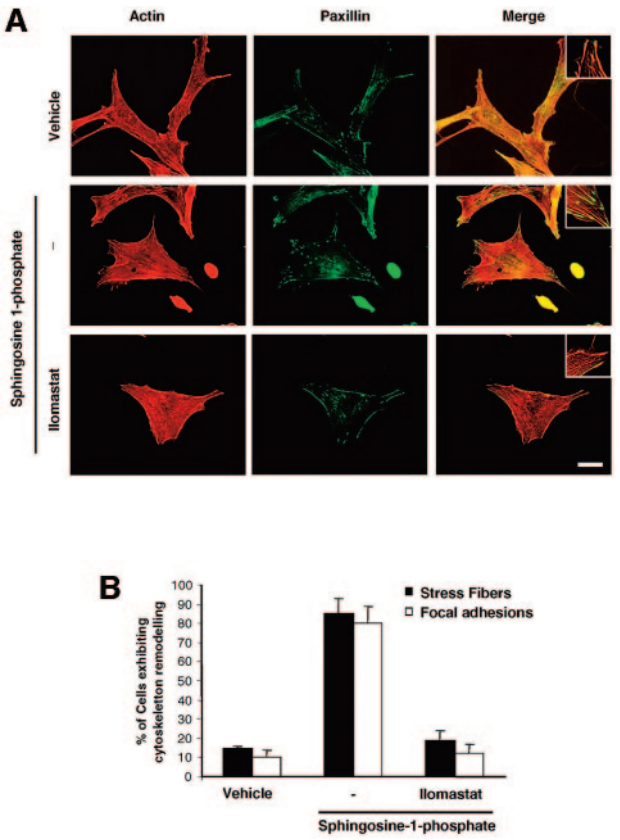

Figure 5. Matrix metalloproteinases are involved in sphingosine-1-phosphate (S1P)-mediated redistribution of Paxillin. **(A):** Marrow-derived stromal cells were serum-starved for 24 hours, preincubated for 1 hour with or without 30 μM Ilomastat, and then treated with 1 μM S1P for 2 minutes. Cells were fixed, and actin filaments were visualized by staining with rhodamine-coupled phalloidin. Paxillin subcellular localization was visualized using anti-paxillin antibodies. Merges between F-actin and Paxillin stainings were performed using Northern Eclipse software. The insets contain a $\times 3$ magnification showing the localization of Paxillin at the tip of the actin stress fibers. Scale bar = 20 μm. **(B):** Quantitative analysis of the percentages of cells exhibiting actin stress fibers and focal adhesions. Values are means $\pm$ SD of three independent experiments.

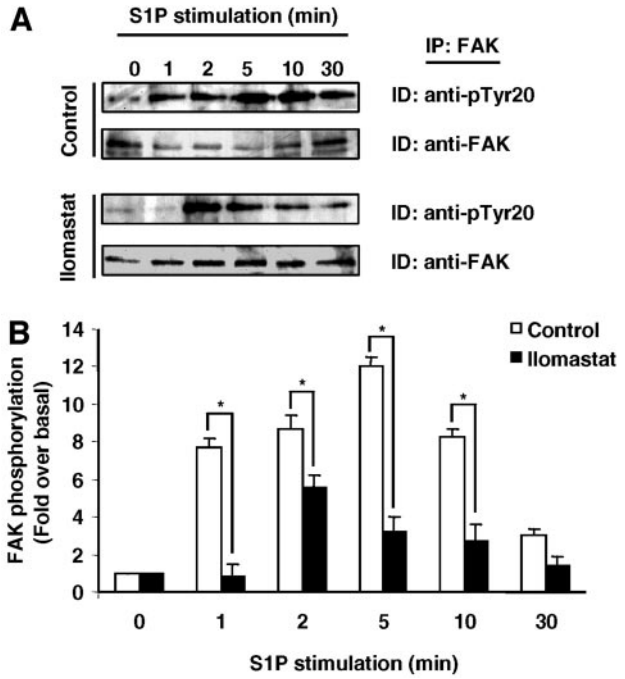

Figure 6. Inhibition of matrix metalloproteinase catalytic activity decreases S1P-induced tyrosine phosphorylation of FAK in marrow-derived stromal cells (MSCs). **(A):** Serum-starved MSCs were preincubated with or without 30 μM Ilomastat for 1 hour and then stimulated with 1 μM S1P for the indicated time. Cells were lysed, and FAK was immunoprecipitated from the lysates using polyclonal anti-FAK antibodies. The immunoprecipitates were analyzed for FAK phosphorylation by Western blot using anti-PY20 anti-phosphotyrosine antibodies. The total amount of FAK in the cell lysates was determined with anti-FAK antibodies. **(B):** Quantitative analysis of FAK phosphorylation. The data are means $\pm$ SD of three independent experiments. Probability values of less than .05 were considered significant when comparison was performed between control and Ilomastat conditions at each time point; an asterisk identifies such significance. Abbreviations: FAK, focal adhesion kinase; ID, immunodetection; IP, immunoprecipitation; S1P, sphingosine-1-phosphate.

calization between actin filaments and Paxillin (Fig. 5A). Paxillin was also redistributed to the tip of short actin filaments located in the center of the cells (Fig. 5A). Finally, we found that S1P-mediated Paxillin redistribution also required MMP activity given that Ilomastat pretreatment strongly antagonized the redistribution of Paxillin by S1P (Fig. 5A, 5B).

MMPs Are Involved in S1P-Induced Tyrosine Phosphorylation of FAK

Phosphorylation of FAK on specific tyrosine residues is known to mediate important signaling pathways linking the ECM to Rho GTPases and to the actin cytoskeleton [19, 20]. To analyze the effects of S1P on phosphorylation levels of FAK, serum-starved MSCs were stimulated with 1 μM S1P for different periods of time, following which FAK was immunoprecipitated from the cell lysates using anti-FAK antibodies (Fig. 6A). Our results show that S1P induced a rapid and strong tyrosine phosphorylation of FAK after 1 minute of stimulation which was sustained for up to 10 minutes of S1P stimulation (Fig. 6B). However, when cells were pretreated with Ilomastat prior to S1P stimulation, FAK tyrosine phosphorylation was transient and significantly diminished as compared with the S1P-treated control cells (Fig. 6B). These results show that MMPs are required for S1P signaling to induce a strong and sustained FAK tyrosine phosphorylation leading to the focal adhesion remodeling needed for cell migration to occur.

MMP- and ROCK-Mediated Signaling Events Are Required for Regulation of ERK Activation by S1P

We have shown that inhibition of the MEK1-ERK1/2 pathway strongly decreased S1P-induced stress fibers in MSCs (Fig. 3). We thus analyzed ERK activation in MSCs that were stimulated (or not) with 1 μM S1P (Fig. 7A). We found that S1P induced a rapid and transient activation of ERK1/2 that decreased after 5 minutes of treatment (Fig. 7B). Inhibition of MMPs by pretreating MSCs with Ilomastat delayed S1P-induced ERK activation until 5 minutes of stimulation (Fig. 7B). This suggests that MMPs are required for the early molecular events that are induced by S1P signaling and that lead to ERK activation. In

Downloaded from www.StemCells.com at Stanford University Medical Center on October 30, 2006

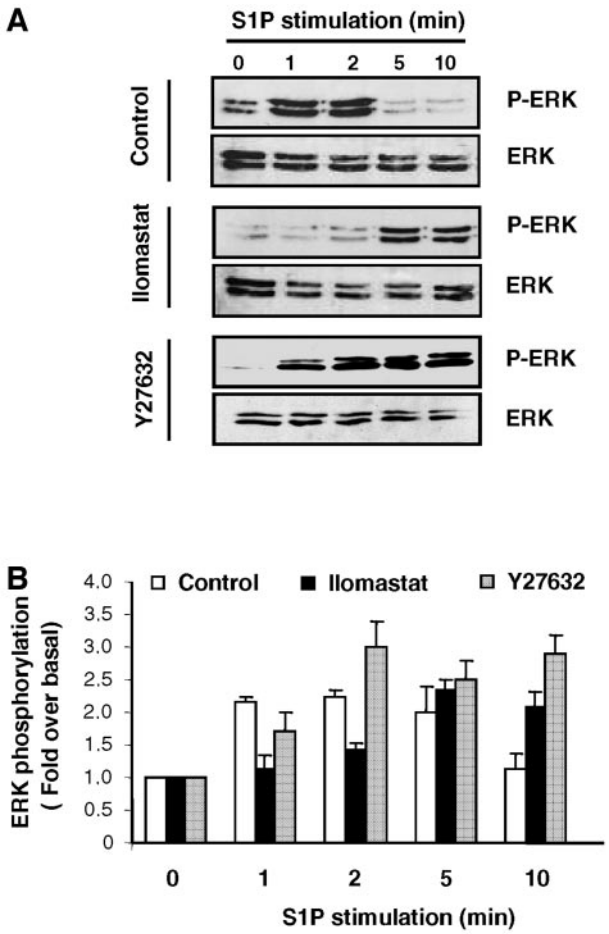

Figure 7. S1P-induced activation of ERK requires matrix metalloproteinase- and Rho kinase-mediated signaling. **(A):** Marrow-derived stromal cells were serum-starved for 24 hours, preincubated with or without 30 μM Ilomastat or 10 μM Y27632 for 1 hour, and then stimulated with 1 μM S1P for the indicated time. Cells were lysed, and the phosphorylation status of ERK was analyzed by Western blot using phospho-specific anti-ERK1/2 antibodies. The total amount of ERK was determined by reprobing with anti-ERK1/2 antibodies. **(B):** Quantitative analysis of ERK activation. The data represent means ± SD of three individual experiments. Abbreviations: ERK, extracellular signal-regulated kinase; P-ERK, phosphorylated extracellular signal-related kinase; S1P, sphingosine-1-phosphate.

contrast, inhibition of ROCK by pretreating MSCs with Y27632 before S1P treatment induced a sustained phosphorylation of ERK, suggesting that ROCK is also involved in the regulation of ERK activation by S1P in MSCs (Fig. 7B). Taken together, our data show that ROCK-mediated signaling pathway and MMPs are required for a fine-tuned regulation of S1P-induced ERK activation in MSCs.

DISCUSSION

The ease of obtaining and expanding MSCs from the adult bone marrow make MSCs a very promising source of adult stem cells, provoking a great deal of interest in many clinical settings, including that of regenerative medicine, immune modulation, and tissue engineering in neurodegenerative pathologies, cardomyopathies, and vasculogenesis [1, 21]. Although recent studies have already demonstrated the feasibility of transplanted MSCs [1], the cellular factors and intracellular signaling pathways underlying the paracrine regulation of their in vivo mobilization remain poorly characterized. Thus, it is now an important challenge to elucidate the intracellular molecular mechanisms governing MSC biology in order to achieve high efficacy in therapeutic use. In this study, we analyzed the early intracellular signaling events triggered by S1P, a bioactive lysophospholipid that is released into serum after platelet activation and that we have found to be the most potent MSC chemotactic factor [7]. Interestingly, S1P is also found in high levels in brain tissue [22] and in brain tumor-derived glioma cells [23], which makes it tempting to suggest that such locally produced S1P by cancer cells could also link S1P-mediated MSC mobilization and recruitment to clinically relevant conditions. In fact, this was recently demonstrated by us as MSCs were significantly recruited in vitro by U-87 glioma-, MCF-7 breast tumor-, and HT-1080 fibrosarcoma-derived cancer cell lines [24].

In particular, we observed that S1P induced a rapid assembly of actin stress fibers that was then followed by focal adhesions, all prerequisite processes required for ECM degradation and cell migration to occur [12, 13, 18]. However, because the roles and regulation of members of the Rho family of small GTPases (in particular, RhoA, Rac1, and Cdc42) are often dictated by the cell type [25], we also investigated their implication in MSC mobilization by S1P. Recent studies have shown that activation of RhoA induced endothelial cell invasion through the regulation of MMP-9 expression [16] and that activated RhoA is required for Rac1-induced lamellipodia and ruffles [26]. Active RhoA recruits and activates its downstream effector ROCK, which then phosphorylates several cytoskeletal proteins like myosin light chain kinase (MLCK) involved in the formation of membrane protrusions at the front of migrating cells [27, 28] and thereby promoting the contraction of actin stress fibers to generate contractile forces [29, 30]. We provide evidence that inhibition of ROCK completely antagonized S1P-induced MSC chemotaxis, likely by the inhibition of actin stress fiber formation which is necessary for the generation of the contractile forces that promote cell locomotion. In light of our data, the RhoA/ROCK pathway thus appears to be an important regulator of S1P-mediated MSC chemotaxis.

RhoA activation is also thought to induce the formation of focal adhesions, which are multiprotein complexes containing structural and signaling proteins [31]. We show that S1P induces a rapid tyrosine phosphorylation of FAK as well as remodeling of the MSC focal adhesions by inducing a rapid redistribution of Paxillin at the cell periphery and, more specifically, at the tips of the actin filaments. These two molecular events are crucial steps required for cell migration. The phosphorylation of FAK is involved in the disassembly of the FAK-Paxillin complex that leads to the remodeling of the focal adhesions observed in motile cells [19]. Although the S1P-induced paxillin redistribution and FAK phosphorylation were reported in endothelial cells [32, 33], our study further establishes that this paxillin redistribution requires the activity of MMPs because Ilomastat, a wide-spectrum inhibitor of the catalytic activity of MMPs [34], abrogated the redistribution of paxillin in S1P-stimulated cells. Moreover, we demonstrate that inhibition of this MMP-dependent process is correlated to a rapid decrease in the tyrosine

www.StemCells.com Downloaded from www.StemCells.com at Stanford University Medical Center on October 30, 2006

phosphorylation of FAK by S1P. Thus, we highlight for the first time the importance of MMPs as potential mediators in S1P-induced MSC mobilization.

It is noteworthy that, whereas the soluble MMPs are involved mostly in the remodeling of the ECM during physiological and pathological processes [10, 35, 36], the membrane-bound MMPs, such as the MT1-MMP, can also function as signaling molecules in MSCs [7]. Recent studies have indeed shown that MT1-MMP cooperates with S1P to induce cell migration of endothelial cells [37]. More importantly, MT1-MMP also triggered ERK activation and cell migration in fibroblasts and glioma cells [38, 39]. In the present study, we show that, in MSCs, S1P induces a rapid and transient activation of ERK that is delayed when MMP catalytic activity was inhibited, proving that MMP activity is required for the early S1P-mediated intracellular signaling that leads to ERK activation. In addition, we show that inhibition of ROCK induces a sustained activation of ERK upon S1P stimulation. Although ERK activation is generally correlated with cell migration induction, our results suggest that the sustained activation of ERK observed upon the ROCK inhibitor Y27632 treatment may not be solely responsible for cell migration induction in MSCs. Altogether, these results show that a time-coordinated activation of ERK involving MMP and ROCK activities is necessary to induce MSC migration in response to S1P stimulation. Accordingly, it has also been shown that the inhibition of the ERK pathway impairs MLCK activation and migration [40, 41] and that the regulation of cell migration occurs by modulating FAK-Paxillin interaction at the focal adhesions. That FAK-Paxillin complex is involved in ERK activation in MSCs suggests the existence of a complex regulatory loop involved in cell migration [40, 42, 43].

Collectively, our data suggest that a finely regulated coordination and cooperation between several intracellular pathways is required for promoting MSC mobilization. Upon S1P stimulation, the coordinated activation of MMPs is required for RhoA/ROCK activation to induce a rapid reorganization of the actin cytokeleton and the remodeling of the focal adhesions. Most importantly, we show that the MEK1/ERK pathway cooperates with these molecular events to further contribute to the chemotactic response of MSCs to S1P. Our results highlight the complex interplay that might exist in vivo between MMP, RhoA/ROCK, and MEK1/ERK intracellular pathways and that leads to the mobilization of MSCs in response to bioactive mediators such as S1P. Whether the immediate morphological changes and early signaling/mobilization effects of S1P that we report may affect MSC differentiation in the long term remains to be determined. The better understanding of the intracellular events underlying MSC biology will, nevertheless, help optimize the use of MSCs as potent therapeutic cytoreagents for gene therapy and other clinical purposes.

ACKNOWLEDGMENTS

B.A. holds a Canada Research Chair in Molecular Oncology from the Canadian Institutes of Health Research (CIHR). M.M. holds a post-Doctoral Fellowship from the CIHR. This study was funded by a grant from the Natural Sciences and Engineering Research Council of Canada (to B.A.). M.M. and S.D. contributed equally to this work.

DISCLOSURES

The authors indicate no potential conflicts of interest.

REFERENCES

1 Short B, Brouard N, Occhiodoro-Scott T et al. Mesenchymal stem cells. Arch Med Res 2003;34:565–571.

2 Gregory CA, Prockop DJ, Spees JL. Non-hematopoietic bone marrow stem cells: Molecular control of expansion and differentiation. Exp Cell Res 2005;306:330–335.

3 Hamada H, Kobune M, Nakamura K et al. Mesenchymal stem cells (MSC) as therapeutic cytoreagents for gene therapy. Cancer Sci 2005; 96:149–156.

4 Pittenger MF, Mackay AM, Beck SC et al. Multilineage potential of adult human mesenchymal stem cells. Science 1999;284:143–147.

5 Iihoshi S, Honmou O, Houkin K et al. A therapeutic window for intravenous administration of autologous bone marrow after cerebral ischemia in adult rats. Brain Res 2004;1007:1–9.

6 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.

7 Annabi B, Thibeault S, Lee YT et al. Matrix metalloproteinase regulation of sphingosine-1-phosphate-induced angiogenic properties of bone marrow stromal cells. Exp Hematol 2003;31:640–649.

8 Saba JD. Lysophospholipids in development: Miles apart and edging in. J Cell Biochem 2004;92:967–992.

9 Spiegel S, English D, Milstien S. Sphingosine 1-phosphate signaling: Providing cells with a sense of direction. Trends Cell Biol 2002;12: 236–242.

10 Sounni NE, Noel A. Membrane type-matrix metalloproteinases and tumor progression. Biochimie 2005;87:329–342.

11 Anliker B, Chun J. Cell surface receptors in lysophospholipid signaling. Semin Cell Dev Biol 2004;15:457–465.

12 Hall A. Rho GTPases and the actin cytoskeleton. Science 1998;279: 509–514.

13 Jaffe AB, Hall A. Rho GTPases: Biochemistry and biology. Annu Rev Cell Dev Biol 2005;21:247–269.

14 Ridley AJ. Rho proteins and cancer. Breast Cancer Res Treat 2004;84: 13–19.

15 Sahai E, Marshall CJ. RHO-GTPases and cancer. Nat Rev Cancer 2002;2:133–142.

16 Abecassis I, Olofsson B, Schmid M et al. RhoA induces MMP-9 expression at CD44 lamellipodial focal complexes and promotes HMEC-1 cell invasion. Exp Cell Res 2003;291:363–376.

17 Annabi B, Bouzeghrane M, Moumdjian R et al. Probing the infiltrating character of brain tumors: Inhibition of RhoA/ROK-mediated CD44 cell surface shedding from glioma cells by the green tea catechin EGCg. J Neurochem 2005;94:906–916.

18 Vicente-Manzanares M, Webb DJ, Horwitz AR. Cell migration at a glance. J Cell Sci 2005;118:4917–4919.

19 Brown MC, Turner CE. Paxillin: Adapting to change. Physiol Rev 2004;84:1315–1339.

20 Mitra SK, Hanson DA, Schlaepfer DD. Focal adhesion kinase: In command and control of cell motility. Nat Rev Mol Cell Biol 2005;6:56–68.

21 Kemp KC, Hows J, Donaldson C. Bone marrow-derived mesenchymal stem cells. Leuk Lymphoma 2005;46:1531–1544.

22 Edsall LC, Spiegel S. Enzymatic measurement of sphingosine 1-phosphate. Anal Biochem 1999;272:80–86.

23 Edsall LC, Cuvillier O, Twitty S et al. Sphingosine kinase expression regulates apoptosis and caspase activation in PC12 cells. J Neurochem 2001;76:1573–1584.

Downloaded from www.StemCells.com at Stanford University Medical Center on October 30, 2006

24 Annabi B, Naud E, Lee YT et al. Vascular progenitors derived from murine bone marrow stromal cells are regulated by fibroblast growth factor and are avidly recruited by vascularizing tumors. J Cell Biochem 2004;91:1146–1158.

25 Yamazaki D, Kurisu S, Takenawa T. Regulation of cancer cell motility through actin reorganization. Cancer Sci 2005;96:379–386.

26 Kurokawa K, Matsuda M. Localized RhoA activation as a requirement for the induction of membrane ruffling. Mol Biol Cell 2005;16: 4294–4303.

27 Totsukawa G, Wu Y, Sasaki Y et al. Distinct roles of MLCK and ROCK in the regulation of membrane protrusions and focal adhesion dynamics during cell migration of fibroblasts. J Cell Biol 2004;164:427–439.

28 Webb DJ, Donais K, Whitmore LA et al. FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. Nat Cell Biol 2004;6:154–161.

29 Amano M, Chihara K, Kimura K et al. Formation of actin stress fibers and focal adhesions enhanced by Rho-kinase. Science 1997;275: 1308–1311.

30 Kimura K, Ito M, Amano M et al. Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). Science 1996;273: 245–248.

31 Schoenwaelder SM, Burridge K. Bidirectional signaling between the cytoskeleton and integrins. Curr Opin Cell Biol 1999;11:274–286.

32 Shikata Y, Birukov KG, Birukova AA et al. Involvement of site-specific FAK phosphorylation in sphingosine-1 phosphate- and thrombin-induced focal adhesion remodeling: Role of Src and GIT. Faseb J 2003; 17:2240–2249.

33 Shikata Y, Birukov KG, Garcia JG. S1P induces FA remodeling in human pulmonary endothelial cells: Role of Rac, GIT1, FAK, and paxillin. J Appl Physiol 2003;94:1193–1203.

34 Grobelny D, Poncz L, Galardy RE. Inhibition of human skin fibroblast collagenase, thermolysin, and Pseudomonas aeruginosa elastase by peptide hydroxamic acids. Biochemistry 1992;31:7152–7154.

35 Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer 2002;2:161–174.

36 Yong VW. Metalloproteinases: Mediators of pathology and regeneration in the CNS. Nat Rev Neurosci 2005;6:931–944.

37 Langlois S, Gingras D, Beliveau R. Membrane type 1-matrix metalloproteinase (MT1-MMP) cooperates with sphingosine 1-phosphate to induce endothelial cell migration and morphogenic differentiation. Blood 2004;103:3020–3028.

38 Annabi B, Thibeault S. Moumdjian R et al. Hyaluronan cell surface binding is induced by type I collagen and regulated by caveolae in glioma cells. J Biol Chem 2004;279:21888–21896.

39 Gingras D, Bousquet-Gagnon N, Langlois S et al. Activation of the extracellular signal-regulated protein kinase (ERK) cascade by membrane-type-1 matrix metalloproteinase (MT1-MMP). FEBS Lett 2001; 507:231–236.

40 Huang C, Jacobson K, Schaller MD. MAP kinases and cell migration. J Cell Sci 2004;117:4619–4628.

41 Klemke RL, Cai S, Giannini AL et al. Regulation of cell motility by mitogen-activated protein kinase. J Cell Biol 1997;137:481–492.

42 Hunger-Glaser I, Salazar EP, Sinnett-Smith J et al. Bombesin, lysophosphatidic acid, and epidermal growth factor rapidly stimulate focal adhesion kinase phosphorylation at Ser-910: Requirement for ERK activation. J Biol Chem 2003;278:22631–22643.

43 Subauste MC, Pertz O, Adamson ED et al. Vinculin modulation of paxillin-FAK interactions regulates ERK to control survival and motility. J Cell Biol 2004;165:371–381.

www.StemCells.com Downloaded from www.StemCells.com at Stanford University Medical Center on October 30, 2006

Cooperation of Matrix Metalloproteinases with the RhoA/Rho Kinase and Mitogen-Activated Protein Kinase Kinase-1/Extracellular Signal-Regulated Kinase Signaling Pathways Is Required for the Sphingosine-1-Phosphate-Induced Mobilization of Marrow-Derived Stromal Cells

Mayya Meriane, Stéphanie Duhamel, Laurence Lejeune, Jacques Galipeau and Borhane Annabi

Stem Cells 2006;24;2557-2565; originally published online Aug 24, 2006;
DOI: 10.1634/stemcells.2006-0209

This information is current as of October 30, 2006

Updated Information & Services	including high-resolution figures, can be found at: http://www.StemCells.com/cgi/content/full/24/11/2557

AlphaMed Press

Downloaded from www.StemCells.com at Stanford University Medical Center on October 30, 2006