ARTICLE
Journal of Cellular Biochemistry 105:1128–1138 (2008)

Journal of Cellular Biochemistry

Sphingosine-1-Phosphate Acts as a Developmental Stage Specific Inhibitor of Platelet-Derived Growth Factor-Induced Chemotaxis of Osteoblasts

T. Roelofsen,[1,2] R. Akkers,[1,2] W. Beumer,[2,3] M. Apotheker,[2] I. Steeghs,[2] J. van de Ven,[2] C. Gelderblom,[2] A. Garritsen,[2] and K. Dechering[1,2*]

[1]*Department of Applied Biology, Radboud University, Nijmegen, The Netherlands*
[2]*Department of Target Discovery, Schering-Plough Research Institute, Oss, The Netherlands*
[3]*Department of Biotechnology, Delft University of Technology, Delft, The Netherlands*

ABSTRACT

The development and maintenance of a healthy skeleton depends on the migration of cells to areas of new bone formation. Osteoblasts, the bone forming cells of the body, mature from mesenchymal stem cells under the influence of bone morphogenetic protein. It is unclear at what developmental stage the osteoblasts start to migrate to their functional location. We have studied migration of immature pre-osteoblasts and of mature osteoblasts in response to Platelet-derived growth factor (PDGF) and sphingosine-1-phosphate (S1P). PDGF is a growth factor involved in bone remodeling and fracture healing whereas S1P is a circulating sphingolipid known to control cell trafficking. Our data indicate that PDGF acts as a chemotactic cue for pre-osteoblasts. In contrast, S1P is a chemorepellent to these cells. Upon Bone Morphogenetic Protein 2-induced conversion to the osteoblast phenotype, the chemotaxis response to PDGF is retained whereas the sensitivity to S1P is lost. By RNA interference and overexpression experiments we showed that the expression level of the S1P2 receptor is the sole determinant controlling responsiveness to S1P. The combined data indicate that migration of osteoblasts is controlled by the balance between PDGF, S1P and the differentiation state of the cells. We propose that this mechanism preserves the osteoprogenitor pool in the bone marrow, only allowing the more differentiated cell to travel to sites of bone formation. J. Cell. Biochem. 105: 1128–1138, 2008.

KEY WORDS: OSTEOBLASTS; MIGRATION; BONE MORPHOGENETIC PROTEIN; SPHINGOSINE-1-PHOSPHATE; PLATELET-DERIVED GROWTH FACTOR

Adult bone undergoes a process of continuous remodeling, whereby bone tissue is resorbed by osteoclasts followed by generation of new bone by osteoblasts. Following a fracture, a repair cascade is initiated, leading to a complete healing of the fracture. This exceptional regenerative capacity of the skeleton requires a precise coordination of the trafficking of the cells involved in the remodeling process. However, the molecular mechanisms coordinating migration of bone cells have not been well characterized.

Osteoblasts originate from mesenchymal stem cells. The latter undergo self-renewal, to maintain the stem cell pool, and may commit to osteogenic differentiation to generate the specialized osteoblasts [Thomas and Kansara, 2006]. The bone marrow provides a source for mesenchymal stem cells, and changes in the capacity of mesenchymal stem cells to self-renew ultimately lead to changes in bone mass [Hankenson et al., 2000; Bonyadi et al., 2003; Dominici et al., 2004]. It is not clear at what developmental stage the osteoblasts are recruited to areas of new bone formation. Either the cells are recruited as relatively immature stages, and further develop at the site of bone formation, or they mature elsewhere and are recruited as functional osteoblasts. Consistent with the latter notion is the observation that cells that express markers for maturated osteoblasts have been detected in the circulation [Eghbali-Fatourechi et al., 2005; Khosla and Eghbali-Fatourechi, 2006]. These cells are able to form bone in vitro [Eghbali-Fatourechi et al., 2005]. These observations raise the possibility that osteoblasts reach the area of bone formation as a differentiated cell. Alternatively, it has been suggested that bone lining cells that are next to areas of bone formation form a local source of osteoblasts [Dobnig and Turner, 1995]. The bone marrow progenitor pool may than be a source to replenish the lining cell compartment [Parfitt, 2001]. The mechanisms that control the balance between maintenance of the stem cell pool on the one hand, and the generation and recruitment

T. Roelofsen and R. Akkers contributed equally to this work.

*Correspondence to: K. Dechering, Department of Molecular Pharmacology, Room RH2114, Schering-Plough Research Institute, P.O. Box 20, 5340 BH The Netherlands. E-mail: koen.dechering@spcorp.com

Received 6 March 2008; Accepted 7 August 2008 • DOI 10.1002/jcb.21915 • 2008 Wiley-Liss, Inc.
Published online 25 September 2008 in Wiley InterScience (www.interscience.wiley.com).

of specialized osteoblasts to areas of bone formation on the other hand are largely unknown. In the present study we set out to study the migration of osteoblasts as a function of the differentiation state of the cells.

Migration of mesenchymal cells is controlled by a number of growth factors and cytokines. These are either stored in the bone matrix and released by the process of bone resorption, or synthesized locally in response to the resorption process or in response to injury [Fiedler et al., 2002; Fink et al., 2007]. Platelet Derived Growth Factor (PDGF) is synthesized during the very early stages of a bone healing response. Immediately following a bone fracture, PDGF is secreted by platelets that assemble at the injured site [Dimitriou et al., 2005]. PDGF is a polypeptide composed of homodimers or heterodimers of PDGF-A and PDGF-B chains. The predominant form of PDGF in bone tissue is the PDGF-BB form [Fujii et al., 1999]. Orthotopic administration of PDGF-BB enhances fracture healing and increases the number of osteoblasts at the fracture site [Nash et al., 1994]. PDGF-BB is a strong chemotactic factor for osteoblasts and is believed to be involved in the initial recruitment of mesenchymal cells to the fracture site [Godwin and Soltoff, 1997; Fiedler et al., 2002, 2004; Fukuyama et al., 2004].

Chemotaxis of mesenchymal cells to PDGF may be modulated by the sphingolipid S1P. Platelets that assemble at the fracture site and secrete PDGF are also a rich source of S1P [Goparaju et al., 2005; Waters et al., 2006; Alvarez et al., 2007]. S1P signals through a family of G-protein coupled receptors. The expression level of specific receptors determines the response to S1P. The S1P1 receptor has a predominant role in positive regulation of chemotaxis, whereas S1P2 has been shown to inhibit migration [Okamoto et al., 2000; Lepley et al., 2005; Rosen and Goetzl, 2005]. We have show previously that Bone Morphogenetic Protein 2 (BMP2) induces expression of the S1P1 receptor in differentiating osteoblasts, but not in fibroblasts [Vaes et al., 2002]. BMP2 is a strong inducer of osteogenic differentiation of mesenchymal stem cells [ten Dijke, 2006]. The observation that the induction of the osteoblast phenotype coincides with a change in S1P1 expression suggest that undifferentiated and differentiated cells may respond differently to S1P. In the present work we have explored the chemotactic response to PDGF and S1P as a function of the BMP2-induced differentiation state of the cells.

MATERIALS AND METHODS

MATERIALS

Recombinant human PDGF-BB was purchased from Preprotech (Rocky Hill, NJ) and sphingosine 1 phosphate (S1P) from BioMol (Plymouth Meeting, PA). PI3K-inhibitor LY294002, anti-phospho-Akt (Ser 473), anti-phospho-p44/42 MAPK and anti-β-actin antibodies were obtained from Cell Signaling (Danvers, MA). The S1P2 antagonist JTE-013 was obtained from Cayman Chemicals (Ann Arbor, MI). A goat-anti-rabbit horseradish peroxidase-conjugated antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant human Bone Morphogenetic protein 2 (BMP2) was purchased from R&D-systems (Minneapolis, MN). Pertussis Toxin (PTX) was obtained from EMD Biosciences (San Diego, CA). CDP-star alkaline phosphatase ready-to-use substrate was obtained from Roche Diagnostics (Almere, Netherlands). 2-Amino-2-methyl-1,3-propanediol (AMPD), 8-hydroxyquinoline, *o*-Cresolphthalein Complexone and hydroxyapatite were obtained from Sigma–Aldrich (Zwijndrecht, The Netherlands). Lipofectamine 2000 was obtained from Invitrogen (Breda, The Netherlands)

CELL CULTURE AND OSTEOGENIC DIFFERENTIATION

The MC3T3-E1 cell line was obtained from the RIKEN institute (www.riken.jp). Cells were cultured in cell culture medium containing α-minimal essential medium supplemented with 10% bovine calf serum (BCS), L-glutamin (2 mM) and penicillin–streptomycin (2,000 U/ml Penicillin G and 2 mg/ml Streptomycin) at 37°C in a humidified 5% CO_2 atmosphere. Cells were subcultured when they reached 90% confluence. Osteogenic differentiation of MC3T3-E1 cells was induced by placing cells on differentiation medium containing cell culture medium supplemented with 50 μg/ml ascorbic acid, 150 ng/ml BMP2 and 10 mM β-glycerophosphate.

Primary calvaria cells were isolated from 4-day-old C57Bl/6 mice by a sequential trypsin/collagen digestion method described previously [Marzia et al., 2000]. Cells were cultured in α-minimal essential medium supplemented with 10% bovine calf serum (BCS), L-glutamin (2 mM) and penicillin-streptomycin (2,000 U/ml Penicillin G and 2 mg/ml Streptomycin) at 37°C in a humidified 5% CO_2 atmosphere. Confluent monolayers were harvested by trypsinisation and seeded in 24-well plates for experimentation. Osteogenic differentiation was induced as described above for the MC3T3-E1 cells.

Osteogenic differentiation was monitored by measuring the activity of the osteoblast marker alkaline phosphatase and by analyzing mineralization of the extracellular matrix. Alkaline phosphatase was measured by lysing cells in 0.2% Triton X-100 buffered with 0.1 M potassium-phosphate at pH 7.8. Subsequently, 40 μl of CDP-star reagent was added to a 10 μl aliquot of cell lysate and incubated for 30 min. Resulting chemoluminescence was measured in a Victor plate reader (Perkin Elmer, Wellesley, MA). Following cell lysis the matrix-associated calcium was solubilized in 150 μl 0.5 M HCl at 37°C overnight. A 2 μl aliquot of the solubilized calcium was combined with a solution of 0.25 M AMPD, 8.5 mM 8-hydroxyquinoline and 190 μM *o*-Cresolphthalein Complexone. The absorption at 600 nm was measured and compared to that of a standard curve of hydroxy-apatite dissolved in 0.5 M HCl.

GENE EXPRESSION ANALYSIS

Pre-osteoblasts were cultured for 3 days in culture medium or osteogenic differentiation medium containing BMP2 for 3 days. RNA was isolated using the Trizol reagent according to the protocol supplied by the manufacturer (Invitrogen). cRNA labeling, hybridization of Affymetrix Mouse Genome 430A Genechips and data processing was performed as described previously [Vaes et al., 2006].

For real-time PCR, cDNA was synthesized with MMLV reverse-transcriptase and a mixture of random hexamers and oligo dT primers. The mouse S1P2 cDNA was amplified using primers CCCCAACTCCGGGACATAG and GGGACAGCCAGTGGTTGGT. The human S1P2 cDNA was amplified using primers TGACATGGCCGCCCCG and GGCATAGTCCAGAAGG. As a reference, the

cDNA of the housekeeping gene RPL19 was amplified using primers CAGCCCATCCTTGATCAGCT and GGTCTGGTTGGATCCCAATG. PCR was performed using a SYBRgreen nucleotide mix and analyzed in real-time on an ABI PRISM 7900 HT thermocycler following the instruction of the manufacturer (Applied Biosystems, Foster City, CA).

PLASMID CONSTRUCTION

Plasmid EDG050TN00, containing the human S1P2 cDNA sequence fused to an N-terminal 3xHA-tag was obtained from the UMR cDNA resource Center (www.cdna.org). The 3xHA-tagged S1P2 cDNA sequence was excised by HindIII/XbaI restriction digestion and cloned into vector pDNR-1r (Clontech, Mountain View, CA). Subsequently, the insert was transferred to the viral expression vector pLP-LNCX (Clontech) by Cre-mediated recombination. As a negative control gene, the cDNA sequence of the copepod *Pontellina plumata* green fluorescent protein (CopGreen) was used [Shagin et al., 2004]. The CopGreen cDNA sequence was amplified by PCR using primers GAAGTTATCAGTCGACGCCACCATGCCCGCCATG-AAGATC and ATGGTCTAGAAAGCTTTATGTTTCAGGTTCAGGGGG from template plasmid pCop-Green-N (Evrogen, Moscow, Russia) and cloned into the vector pDNR-1r by means of and In-Fusion PCR cloning kit according to the instructions of the manufacturer (Clontech). To generate an HA-tag-CopGreen fusion protein, an HA-tag was inserted into vector pLP-LNCX by PCR with primers CCCAAGCTTGGGCCACCATGTACCCATACGATGTTCCAGATTACG-CTATAACTTCGTATAGCATACATTATACG and CGTCTAGAAAC-TGCTGAGGGCTGG using pLP-LNCX as a template. The PCR product was cloned into vector pLP-LNCX by HindIII/XbaI restriction digestion and ligation to generate vector pLP-LNCX-HA. Subsequently, the CopGreen cDNA sequence was excised from the pDNR-1r vector and inserted into pLP-LNCX-HA by Cre-mediated recombination.

VIRAL TRANSDUCTION

Retroviruses were produced using the Phoenix-ecotropic packaging cell line system as described previously [Swift et al., 1999]. Virus particles were concentrated by centrifugation of 6.5 ml of virus-containing culture medium for 2 h at 14,000 rpm at 4°C. Viral particles were resuspended in 250 μl of culture medium and used to infect MC3T3-E1 cells that were seeded in 24-well plates at 1.5×10^4 cells/well 2 days prior to infection. Following addition of the virus-containing medium, the plates were centrifuged at 1,800 rpm for 45 min at room temperature. The medium was removed and the infection/centrifugation step was repeated. Then, cell culture medium containing 8 μg/ml polybrene was added to the cells and the cells were incubated overnight at 37°C in a humidified 5% CO_2 atmosphere. The next day, cells were put on selection medium containing 0.35 mg/ml geneticine and subcultured for 10 days.

RNA INTERFERENCE

RNA interference was performed by transfecting pools of small-interfering RNAs (Dharmacon, Lafayette, CO) into MC3T3-E1 cells by electroporation. To this end, cells were harvested at 80–90% confluency by trypsinisation and collected in cell culture medium. Electroporation mixtures containing 2×10^6 cells and 1 μM siRNA in 0.4 ml medium were placed in a 0.4 cm cuvette and electroporated at 1,000 V, 50 μF at infinite resistance. Subsequently, 5 ml of culture medium were added and cells were placed in a 25 cm^2 flask and incubated at 37°C in a humidified 5% CO_2 atmosphere overnight. The following day the culture medium was replaced with fresh culture medium and the cells were subcultured for an additional 2 days.

CHEMOTAXIS AND CHEMOKINESIS ASSAYS

Chemotactic responses of MC3T3-E1 and primary calvaria cells were studied using a 96-well chemotaxis system with 8 μm pore size (Neuroprobe Gaithersburg, MD). Membranes were coated with 0.1% gelatin in Phosphate Buffered Salt solution (PBS) for 1 h. Prior to harvesting, cells at 80–90% confluency were switched to low serum medium (α-MEM supplemented with 0.75% BCS, 0.1% bovine serum albumin, 2 mM L-glutamin) and incubated for 2 h. Cells were harvested and resuspended in low serum medium. Subsequently, 2×10^4 cells were loaded in the upper chamber of the chemotaxis system whereas the lower chamber was filled with low serum medium and the chemokines indicated in the figure legends. Chemotaxis was allowed to proceed for 3 h at 37°C in a humidified 5% CO_2 atmosphere. Then, cells at the top of the membrane were removed with a moist cotton swap and the remaining cells on the lower surface were fixed with 1% paraformaldehyde in PBS for 15 min at room temperature. Cell nuclei were visualized by staining with 300 nM DAPI for 15 min at room temperature. Chemotaxis was quantified by counting the number of nuclei in four non-overlapping fields for each well using a Discovery-1 cellular imaging system (Molecular Probes, Sunnyvale, CA).

To study chemokinesis, scratch-wound assays were performed. Briefly, confluent monolayers of MC3T3-E1 cells were pre-incubated in low-serum medium for 2 h. Subsequently, the monolayer was scratched with a pipet tip and the cells were incubated overnight at 37°C in a humidified 5% CO_2 atmosphere in the presence of the chemokines indicated in the figure legends. The next day, cells were washed twice with PBS and fixed with 1% paraformaldehyde in PBS for 15 min. Cells were visualized by staining with a crystal violet solution (0.5% crystal violet, 25% methanol) for 10 min and closure of the scratched area was analyzed by light microscopy.

PHOSPHO-AKT AND PHOSPHO-ERK IMMUNOBLOTTING

Activation of Akt and ERK by PDGF and S1P was studied by analyzing the degree of phosphorylation of these proteins. To this end, MC3T3-E1 cells were switched to low serum medium for 2 h prior to incubation with the compounds indicated in the figure legends. Following compound treatment, cells were lysed in 4% SDS, 100 mM Tris (pH 6.8), 20% glycerol, 0.01% Bromo-Phenol Blue and 200 mM dithiothreitol. The lysates were subjected to SDS–PAGE gel electrophoresis and proteins were transferred to a nitrocellulose membrane by electroblotting. The membranes were blocked in TBS-T (0.05 mM Tris–HCl, 200 mM NaCl, 0.05% Tween-20) supplemented with 5% skim milk for 3 h. For detection of phospho-Akt, the membrane was incubated with a rabbit anti-phospho-Akt (Ser 473) antibody 1:1,000 diluted in TBS-T and 5% skim milk overnight at

4°C. The next day, the membrane was incubated with a conjugated secondary antibody (goat-anti-rabbit HRP) diluted 1:2,000 in TBS-T and 5% skim milk for 1 h at room temperature. HRP activity was detected using the enhanced chemiluminescence method (ECL Plus Western blotting detection system, Amersham Biosciences, Diegem, Belgium). Similarly, phosphorylated forms of ERK1 and 2 were detected using an antibody specific for the phosphorylated forms of these proteins. To demonstrate equal loading of lanes, the blots were probed with a β-actin specific antibody.

STATISTICAL ANALYSIS

Data were analyzed by Student *t*-tests.

RESULTS

PDGF AND S1P AFFECT MIGRATION OF MC3T3-E1 PRE-OSTEOBLASTS

We tested the effect of serum, BMP2, PDGF, and S1P on migration of immature MC3T3-E1 preosteoblasts. The data presented in Figure 1a show that these cells exhibited positive chemotaxis towards PDGF and serum. In contrast, S1P did not induce a chemotactic response. Instead, the low level of spontaneous migration that was observed under low serum conditions was inhibited by S1P. Since BMPs have been described as chemotactic factors for human mesenchymal progenitor cells [Fiedler et al., 2002], we tested the effect of BMP2 on cell migration. The data presented in Figure 1b indicate that under the conditions described here, mouse MC3T3-E1 cells did not show a migratory response to BMP2. In conclusion, our data reveal distinct responses of pre-osteoblasts to the various factors tested here. The pre-osteoblasts show positive chemotaxis towards PDGF and serum, and a negative response towards S1P. BMP2 does not act as a chemotactic factor.

BMP2-INDUCED OSTEOGENIC DIFFERENTIATION ALTERS THE MIGRATORY POTENTIAL OF MC3T3-E1 CELLS

To investigate chemotaxis as a function of the differentiation state of the cells we pretreated the cells with BMP2, which is a potent inducer of osteogenic differentiation of MC3T3-E1 cells [Xiao et al., 2002]. The data presented in Figure 1c indicate that BMP2 converted MC3T3-E1 pre-osteoblasts into alkaline phosphatase positive, mineralizing osteoblasts. The alkaline phosphatase activity was clearly induced at day 3 following the start of the BMP2 treatment, and peaked at day 5. The onset of the mineralization process was at day 3, after which the amount of calcium deposited in the matrix rose almost linearly. In subsequent experiments, we compared the chemotactic response of pre-osteoblast to that of differentiated osteoblasts. The data presented in Figure 1d indicate that both phenotypes showed similar responses to PDGF. In pre-osteoblasts,

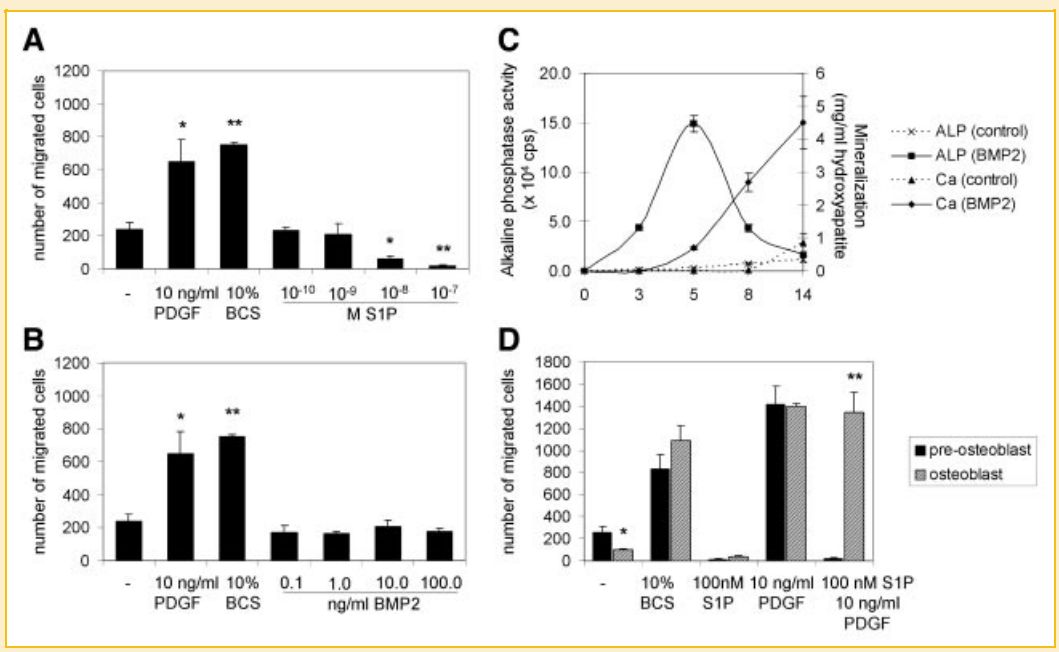

Fig. 1. BMP2-induced osteogenic differentiation alters the migratory potential of MC3T3-E1 cells. Chemotaxis was measured in a trans-well migration assay in response to the factors indicated at the bottom of the figures. A: MC3T3-E1 preosteoblasts show positive chemotaxis towards PDGF and serum whereas cell migration is inhibited by S1P. B: BMP2 does not affect chemotaxis of MC3T3-E1 cells. C: BMP2 induces osteogenic differentiation. MC3T3-E1 cells were stimulated with BMP2 and osteogenic differentiation was monitored by measuring the alkaline phosphatase activity and calcium content of the matrix. As a control, cells were cultured in the absence of BMP2. D: Pre-osteoblasts and osteoblasts show distinct responses to S1P. The chemotactic response to serum, PDGF, S1P and a combination of PDGF and S1P was compared between pre-osteoblasts and cells that were differentiated to osteoblasts by treatment with BMP2 for 3 days. PDGF, platelet-derived growth factor; S1P, sphingosine-1-phosphate; BCS, bovine calf serum. The figure shows the mean values and standard deviations derived from three independent assays. For panels A and B, statistically significant differences between control (–) and stimulated samples are indicated by asterisks ($^{*}P < 0.01$, $^{**}P < 0.001$). For panel D, asterisks indicate statistically significant differences between pre-osteoblasts and osteoblasts.

the response to PDGF was inhibited by S1P. In contrast, the osteoblasts proved to be resistant to S1P inhibition. The combined data indicate that BMP2 is not a chemotactic factor in itself, but that it converts pre-osteoblasts into S1P-insensitive osteoblasts.

S1P IS A CHEMOREPELLENT

As opposed to directional chemotaxis, chemokinesis is chemokine-induced random movement of cells. This process can be studied in scratch wound assays, that monitor cell migration into a scratched area of a monolayer [Masiello et al., 2006]. In this assay, pre-osteoblasts and osteoblasts responded similarly to PDGF, which induces closure of the wounded area (Fig. 2a). Interestingly, osteoblasts showed a higher response to serum than pre-osteoblasts. This was also observed in the chemotaxis experiment, albeit at a much lower magnitude (Fig. 1d). With respect to the response to S1P, the differences between osteoblasts and pre-osteoblasts in chemotaxis were not seen in this chemokinesis experiment. S1P did not inhibit PDGF-induced chemokinesis of either pre-osteoblasts or osteoblasts. These data indicate that S1P is not a general inhibitor of cellular movement, but specifically affects the chemotactic response. To further investigate the actions of S1P we performed a chemotaxis assay in which the S1P and PDGF gradients had similar or opposite directions. The data presented in Figure 2b show that S1P opposed chemotaxis when PDGF and S1P were both added to the lower compartment. When we added S1P to the upper compartment, which reverses the direction of the S1P gradient, it enhanced migration towards PDGF. Lastly, we analyzed the effect of S1P on PDGF-induced chemotaxis in the absence of a specific gradient. To this end, we added S1P to both the upper and lower compartments of a chemotaxis chamber. Under these conditions, chemotaxis to PDGF was similar to that in the absence of S1P (Fig. 2b). From these observations we conclude that S1P is not a simple inhibitor of chemotaxis. Instead, its actions are consistent with those of a chemorepellent as it drives cells down the S1P gradient.

S1P DIFFERENTIALLY AFFECTS MIGRATION OF IMMATURE AND MATURE PRIMARY OSTEOBLASTS

To validate the observed effects of PDGF and S1P on osteoblast migration beyond the MC3T3-E1 cellular model, we studied the

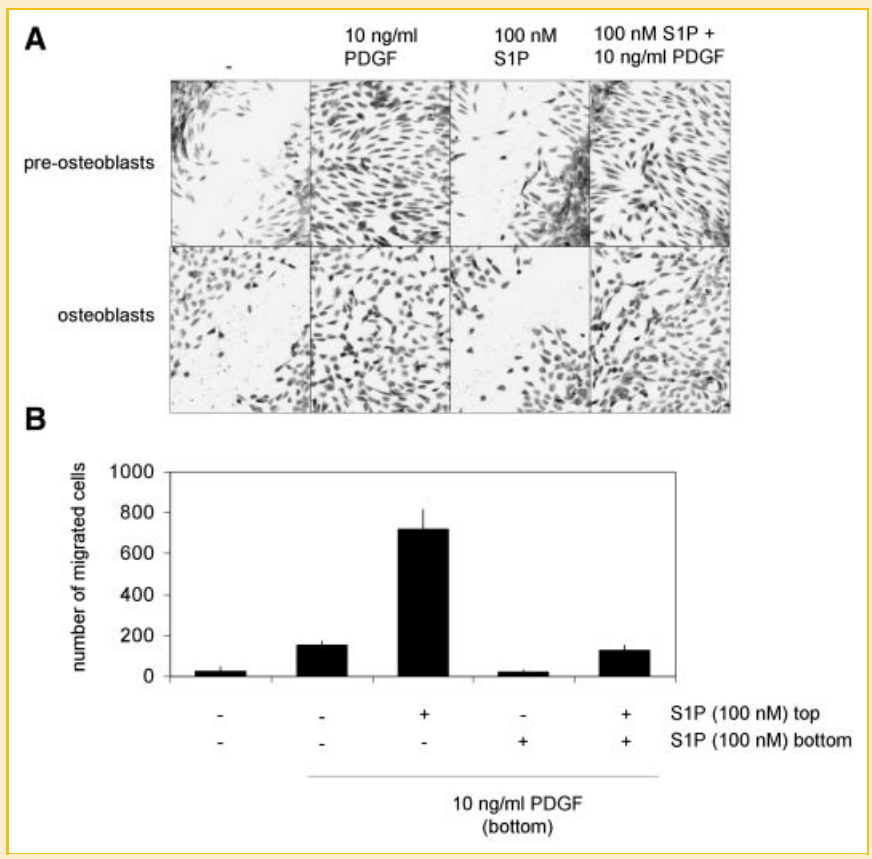

Fig. 2. S1P is a chemorepellent. A: Chemokinesis was studied by scratch-wound assays. Scratched monolayers of MC3T3-E1 cells were incubated overnight with the stimuli indicated at the top of the figure. The assay was performed on MC3T3-E1 pre-osteoblasts and on cells differentiated towards osteoblasts by incubation with BMP2 for 3 days. S1P did not affect PDGF-induced chemokinesis. The assay was performed three times and the figure shows a representative example. B: Checkerboard analysis of the effect of S1P on PDGF-induced chemotaxis. Chemotaxis was measured in a trans-well migration assay. PDGF was added to the lower compartment. S1P was added to the lower compartment, to the cell suspension in the upper compartment, or to both compartments as indicated in the figure. Mean values and standard deviations (indicated by the error bars) were determined from three independent assays.

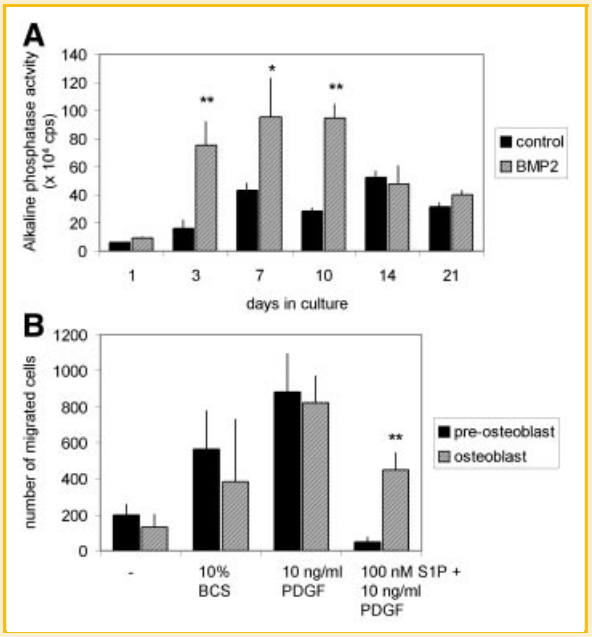

Fig. 3. BMP2-induced osteogenic differentiation alters the migratory potential of primary calvarial cells. Primary pre-osteoblasts were isolated from mouse calvaria and cultured in the absence or presence of BMP2. A: Osteogenic differentiation was monitored as a function of time by measuring the alkaline phosphatase activity in BMP2-treated cells and control cells that were left untreated. B: Pre-osteoblasts and osteoblasts show distinct responses to S1P. Chemotaxis was measured in a trans-well migration assay in response to the factors indicated at the bottom of the figure. The responses were compared between pre-osteoblasts and cells that were differentiated to osteoblasts by treatment with BMP2 for 3 days. The figure shows the mean values and standard deviations derived from three independent assays. Statistically significant differences between control (pre-osteoblasts) and BMP2-treated (osteoblast) cells are indicated by asterisks ($^{*}P < 0.01$, $^{**}P < 0.001$).

chemotactic properties of primary cells isolated from mouse calvaria. Like MC3T3-E1 cells, the calvaria-derived cells develop into mature, alkaline phosphatase positive osteoblasts when exposed to BMP2 (Fig. 3a). We then analyzed the chemotactic responses to serum, PDGF, and a combination of PDGF and S1P of immature cells and of cells differentiated to osteoblasts. The results shown in Figure 3b indicate that the primary cells show modest positive chemotaxis towards serum and a stronger response to PDGF. Like for MC3T3-E1 cells, the chemotactic response of immature pre-osteoblasts could be inhibited by S1P, whereas the osteoblasts were relatively insensitive to S1P. In conclusion, primary calvarial cells and MC3T3-E1 cells show similar chemotactic responses.

S1P DOES NOT AFFECT PDGF SIGNALING AND THE INHIBITORY EFFECT OF S1P IS NOT MEDIATED THROUGH Gi SIGNALING

To provide a molecular explanation for the inhibitory effect of S1P on pre-osteoblast chemotaxis towards PDGF we studied PDGF receptor signaling in MC3T3-E1 cells treated with S1P. Addition of PDGF leads to activation of the PI3kinase-Akt signaling pathway. Indeed, PDGF activated Akt phosphorylation in pre-osteoblasts and osteoblasts (Fig. 4a). This effect could be blocked by the PI3kinase inhibitor LY294002. In contrast, the addition of S1P had no effect on Akt phosphorylation. This indicates that S1P does not interfere with PDGFR-PI3kinase-Akt signaling pathway. S1P1 exclusively couples to Gi, whereas other S1P receptors are more promiscuous in their binding partners. S1P1 activation of Gi leads to the activation of ERK kinase activity [Anliker and Chun, 2004]. In MC3T3-E1 pre-osteoblasts, we observed a rapid induction of ERK phosphorylation by S1P (Fig. 4b). The activation of ERK could be blocked by pertussis toxin, indicating an involvement of Gi. However, blocking of Gi had no effect on chemotaxis (Fig. 4c), showing that neither Gi nor ERK plays a role in the effects of PDGF and S1P on chemotaxis. These results were corroborated by the observation that an inhibitor of MEK (U0126) did not influence pre-osteoblast chemotaxis (data not shown). The observation that the effect on chemotaxis of S1P was insensitive to pertussis toxin suggests that an S1P receptor other than S1P1 is involved in the chemorepellent function of S1P.

S1P2 IS THE PRE-OSTEOBLAST CHEMOREPELLENT RECEPTOR

Since the effects of S1P could not be blocked with pertussis toxin, and are therefore unlikely to be mediated by S1P1-Gi signaling, we studied the expression of other S1P receptors in MC3T3-E1 cells. In agreement with previous data, a microarray experiment showed that MC3T3-E1 pre-osteoblasts express mRNAs for the S1P1 and S1P2 receptors (Fig. 5a) [Lyons and Karin, 2001; Vaes et al., 2002]. Converting the cells to the osteoblast phenotype with BMP2 led to a significant reduction in S1P2 expression (Fig. 5a). The reduction in S1P2 levels detected by the microarray was confirmed in an independent real-time PCR analysis of S1P2 expression (Fig. 5b). We then hypothesized that S1P2 may act as the S1P chemorepellent receptor, and that the resistance of osteoblasts to the inhibitory effect of S1P on chemotaxis is caused by a reduction in S1P2 levels. To test this hypothesis, we analyzed the effect of JTE-013, a highly selective antagonist for S1P2 [Ohmori et al., 2003; Kono et al., 2007]. We tested JTE-013 for its ability to inhibit the S1P chemorepellent effect on pre-osteoblasts. The data presented in Figure 5c indicate that JTE-013 dose-dependently blocked the inhibitory effect of S1P on PDGF-induced chemotaxis. This suggests that S1P2 is responsible for mediating the inhibitory effect of S1P. To substantiate this notion, we decreased S1P2 levels by RNA interference. Transfection of an siRNA directed against the S1P2 mRNA led to a decrease in S1P2 transcript levels, whereas unrelated control siRNA did not affect S1P2 expression (Fig. 6a). Subsequently, cells with downregulated S1P2 expression were subjected to a chemotaxis experiment. The results indicate that cells with low S1P2 levels were resistant to the chemorepellent effect of S1P. Whereas mock-treated cells or cells transfected with a scrambled siRNA sequence showed inhibition of PDGF-induced chemotaxis by S1P, cells transfected with an S1P2-directed siRNA did not react to S1P (Fig. 6b). These data indicate that the S1P2 expression level determines the chemorepellent effect of S1P. Furthermore, it explains that the downregulation of S1P2 expression by BMP2 during osteogenic differentiation leads to a resistance to S1P. To further support this notion, we uncoupled S1P2 expression from the BMP2-induced osteogenic differentiation process by transducing

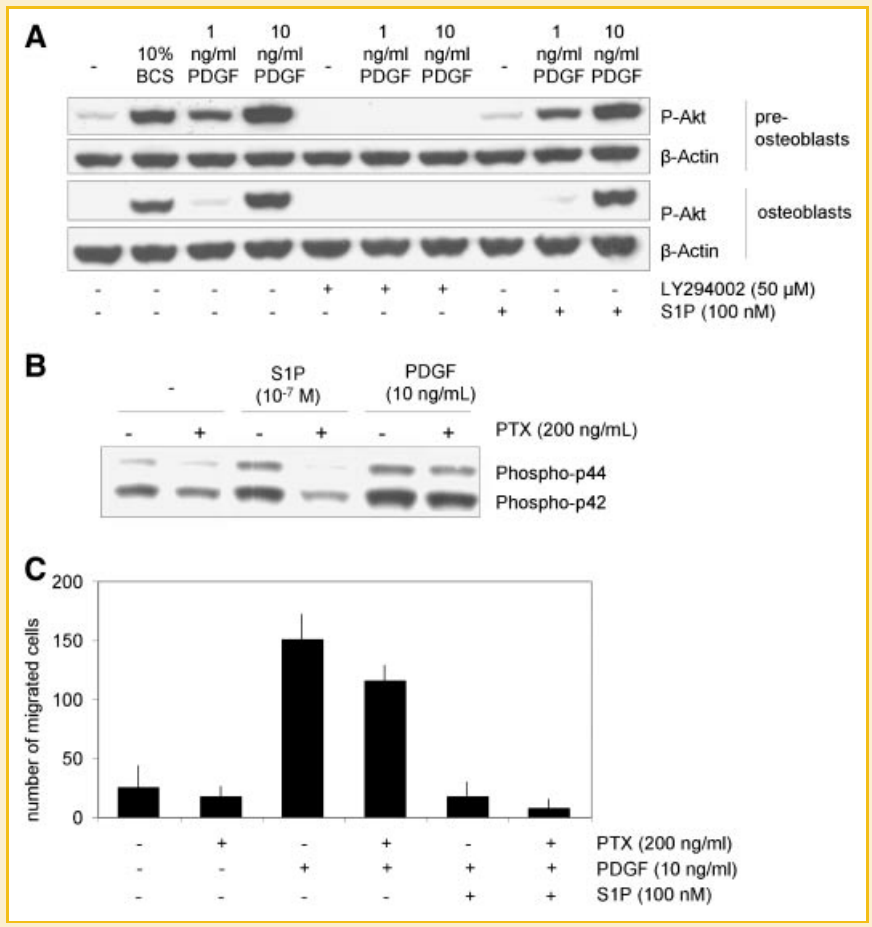

Fig. 4. S1P does not affect PDGFR-PI3K signaling. A: Pre-osteoblasts and osteoblasts were stimulated for 10 min with PDGF or serum (BCS) in the presence and absence of S1P or LY294002, an inhibitor of PI3-kinase. Cellular proteins were extracted and separated by SDS–PAGE. The amount of phosphorylated Akt as a measure the for activity of the PDGFR-PI3K pathway was determined by Western blotting. The blot was reprobed with a β-actin antibody to demonstrate equal loading of samples. B: S1P activation of ERK is mediated by Gi. Cellular extracts were derived from cells stimulated for 10 min with S1P or PDGF in the presence and absence of pertussis toxin (PTX), an inhibitor of Gi signaling. Cellular proteins were separated by SDS–PAGE. The amount of phosphorylated ERK was determined by Western blotting. C: Chemotactic responses to PDGF and S1P are independent of Gi signaling. Chemotaxis was measured in a trans-well migration assay in the presence or absence of pertussis toxin. The figure shows the mean values and standard deviations derived from three independent assays.

MC3T3-E1 cells with an S1P2 gene under control of a constitutively active CMV promoter. As a control, cells were transduced with a gene encoding the fluorescent CopGreen protein which is not expected to affect migration. The transduction of the S1P2 gene was successful as demonstrated by real-time PCR (Fig. 7a). We then measured BMP2-responsiveness and differentiation capacity of the transduced cells. Analysis of the alkaline phosphatase activity showed that the S1P2 and CopGreen transduced cells equally responded to BMP2 and commit to osteogenic differentiation (Fig. 7b). Next, we analyzed the chemotactic response of the transduced cells (Fig. 7c). The data showed that the control cells showed resistance to S1P when converted to the osteoblast phenotype. In contrast, the chemotaxis of S1P2 transduced cells was inhibited both at the pre-osteoblast as well as at the osteoblast stage. These data indicate that at constitutive expression levels of S1P2, BMP2 is unable to convert cells into a S1P-resistant osteoblast. The combined data show that S1P2 expression is the sole determinant of the change in S1P response during the BMP2-induced pre-osteoblast to osteoblast conversion.

DISCUSSION

Bone formation depends on the recruitment of the appropriate cell types. In the present study we show that the chemotactic behavior of osteoblastic cells is altered by a developmental switch. Cells in the pre-osteoblast stage show positive chemotaxis towards PDGF, which is inhibited by S1P. Conversion to the osteoblast phenotype by BMP2 retains the response to PDGF whereas the response to S1P is lost. We show that the response to S1P is controlled by the developmental-stage specific expression of the S1P2 receptor. Gene interference studies showed that knockdown of S1P2 mRNA leads to a loss of the response to S1P. In addition, constitutive overexpression of S1P2 retains sensitivity to S1P during the

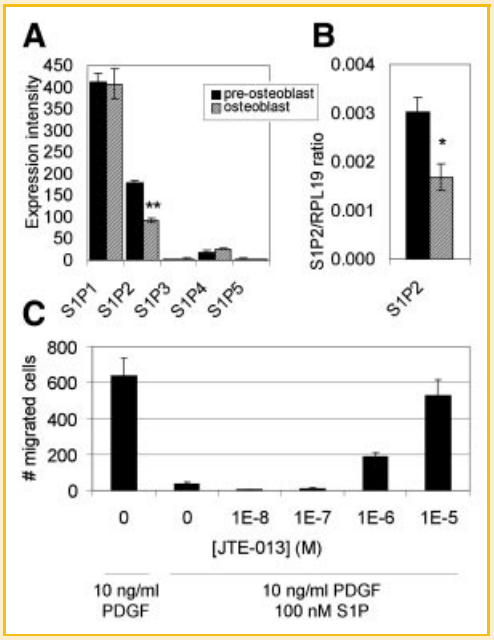

Fig. 5. Osteoblasts downregulate S1P2 expression and antagonizing S1P2 function blocks the chemorepellent action of S1P. A: The mRNA levels of S1P1, S1P2, S1P3, S1P4, and S1P5 mRNAs were measured by microarray hybridization. The figure shows relative expression intensities in pre-osteoblasts and osteoblasts. B: The difference in S1P2 levels between pre-osteoblasts and osteoblasts was confirmed by real-time PCR. The figure shows the S1P2 expression levels relative to that of the house-keeping gene RPL19. C: The S1P2 antagonist JTE-013 blocks the chemorepellent action of S1P. Chemotaxis of MC3T3-E1 was measured in a trans-well migration assay. The figure shows the number of migrated cells in response to PDGF and S1P and varying concentrations of the S1P2 antagonist JTE-013. Error bars indicate standard deviations between three independent assays. Statistically significant differences between pre-osteoblasts and osteoblasts are indicated by asterisks ($^{*}P < 0.01$, $^{**}P < 0.001$).

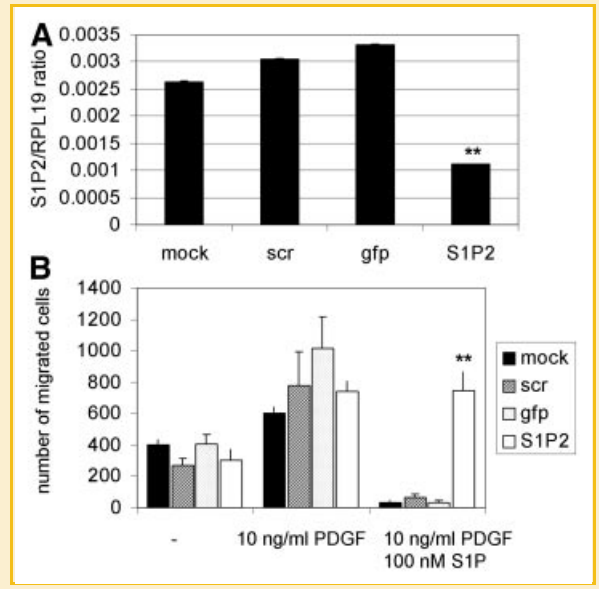

Fig. 6. S1P2 is the S1P chemorepellent receptor. A: RNAinterference downregulates the expression of the S1P2 receptor. MC3T3-E1 pre-osteoblasts were transfected with siRNAs against S1P2. Controls included a scrambled siRNA (scr) and an siRNA against green fluorescent protein (gfp). Mock treated cells were electroporated in the absence of siRNA. Following transfection, cells were cultured for 3 days and the S1P2 expression level was determined by real-time PCR. The figure shows the S1P2 expression levels relative to that of the house-keeping gene RPL19. B: Knockdown of S1P2 leads to a loss of the response to S1P. The siRNA-transfected cells described in (A) were used in a chemotaxis experiment. The figure shows the chemotactic responses to PDGF and S1P. Error bars indicate standard deviations between three independent assays. Statistically significant differences between cells transfected with an S1P2 siRNA and control cells (mock, scr, gfp) are indicated by asterisks ($^{*}P < 0.01$, $^{**}P < 0.001$).

preosteoblast to osteoblast developmental switch. Our data indicate that S1P2 acts as a chemorepellent receptor as the inhibitory effect of S1P requires a gradient of S1P.

The developmental stage specific control of osteoblast differentiation by S1P receptor expression is reminiscent of the way immune cell trafficking is controlled, although the underlying mechanisms differ. In the immune system, S1P controls the trafficking of lymphocytes [Rosen and Goetzl, 2005]. Maturation of lymphocytes leads to an induction of S1P1 expression. As a result, lymphocytes exit from the thymus, and it was shown that the S1P1 expression level is the key determinant in this process [Matloubian et al., 2004]. Our data indicate that maturation of osteoblasts leads to a reduction in S1P2 expression and a consequent enablement of positive chemotaxis. Thus, whereas lymphocyte migration towards their target organ is induced by S1P1 expression, our data suggest that osteoblast migration to areas of bone formation is induced by downregulation of S1P2 expression.

The inhibitory role of S1P2 in pre-osteoblast chemotaxis is in line with its negative role in control of cell migration as reported for other cell types [Yokoo et al., 2004; Goparaju et al., 2005; Lepley et al., 2005]. The checkerboard experiment described here indicated that the direction of the S1P gradient is important in determining the inhibitory effect of S1P. Accordingly, S1P did not inhibit chemokinesis in a scratch wound assay, in which no specific gradient is present. In other cells, for example, mast cells, S1P2 has been shown to inhibit both chemotaxis and chemokinesis [Yokoo et al., 2004]. Since chemokinesis was not inhibited in preosteoblasts, these findings imply that the cellular context ultimately defines the specific response to activation of S1P2. It has been shown that the chemotactic inhibitory action of S1P2 depends on a Rho/PTEN dependent pathway [Sugimoto et al., 2003; Lepley et al., 2005; Sanchez et al., 2005]. What downstream molecules underlie the chemorepellent action of S1P2 in pre-osteoblasts is the subject of our current studies.

Next to the S1P2 receptor, pre-osteoblasts express the S1P1 receptor. It has been shown previously that S1P1 is required for PDGF-induced chemotaxis in fibroblasts and endothelial cells [Hobson et al., 2001; Lee et al., 2001; Goparaju et al., 2005]. Two distinct mechanisms were described. In one mode of action, PDGF activates sphingosine kinase, which synthesizes sphingosine 1-phosphate from sphingosine. The newly synthesized S1P activates S1P1 on the plasma membrane in an autocrine fashion. Subsequently, S1P1 activates Rac, resulting in positive chemotaxis [Hobson et al., 2001; Goparaju et al., 2005]. In a second mode of

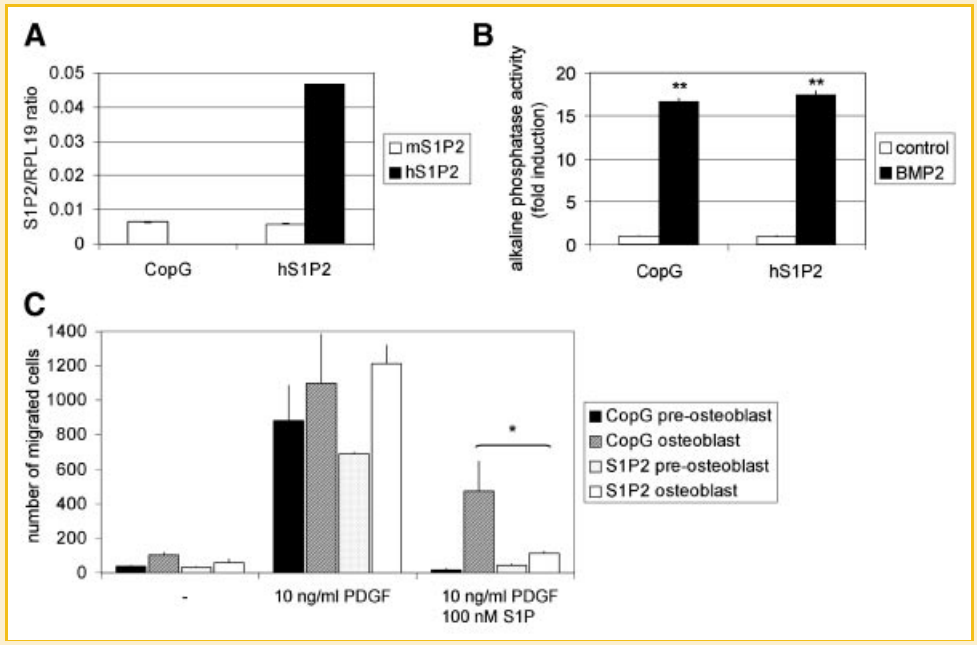

Fig. 7. Overexpression of S1P2 retains S1P sensitivity during osteogenic differentiation. A: Transduced MC3T3-E1-S1P2 cells overexpress hS1P2. The figure shows relative expression levels of mouse (mS1P2) and human (hS1P2) in cells transduced with hS1P2 or the negative control gene CopGreen (CopG). The expression levels of the endogenous mS1P2 transcripts and of the hS1P2 transgene were determined by real-time PCR. Expression values were normalized to those of the house-keeping gene RPL19. B: MC3T3-E1-S1P2 cells differentiate normally into osteoblasts. hS1P2 and CopG transduced cells were cultured in osteogenic differentiation medium or control medium for 3 days. Alkaline phosphatase activity was determined as a measure of osteogenic differentiation. The figure shows the alkaline phosphatase activity relative to the activity in cells cultured in control medium. C: Overexpression of S1P2 alters the sensitivity to S1P in osteoblasts. The figure shows the result of a chemotaxis experiment with cells described in (B). The figure shows the mean values and standard deviations derived from three independent assays. For panel B, statistically significant differences between control (—) and BMP2-treated samples are indicated by asterisks ($^{*}P < 0.01$, $^{**}P < 0.001$). For panel C, asterisks indicate statistically significant differences between hS1P2 and CopG-transduced cells.

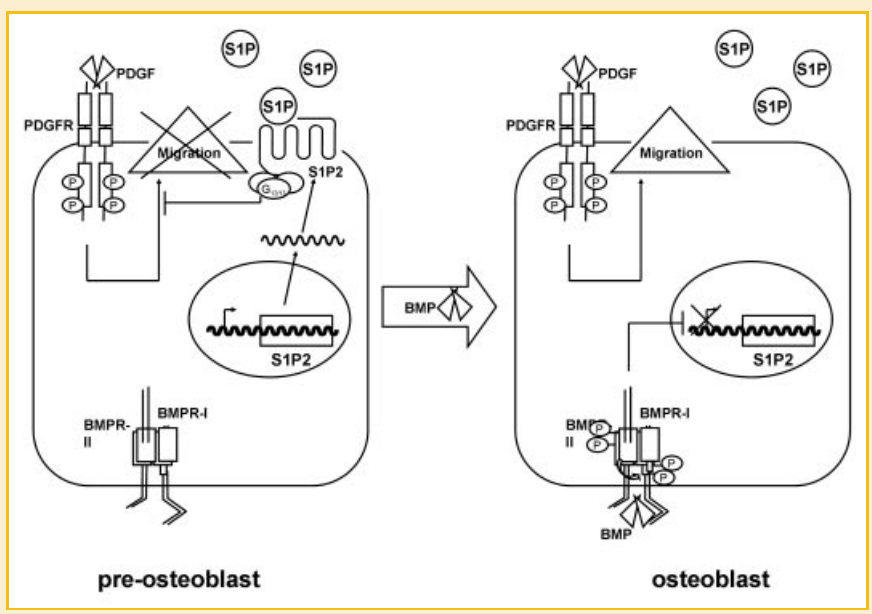

Fig. 8. Cartoon of a working model for the actions of PDGF, BMP and S1P in the control of cell motility. In pre-osteoblasts (left panel), the chemotactic response to PDGF is blocked by high S1P concentrations in the extracellular milieu acting on the S1P2 receptor. When cells differentiate to osteoblasts under the control of BMP (right panel), the activity of the S1P2 gene is repressed. As a consequence, S1P is no longer able to signal and the cells migrate towards PDGF.

action, the PDGF-activated Akt activates the S1P1 receptor by phosphorylation [Lee et al., 2001]. In both modes of action, blocking Gi-dependent S1P1 signaling with pertussis toxin resulted in a block of the chemotactic response to PDGF. We did not observe chemotaxis to S1P in the absence of PDGF under conditions where S1P2 activity was blocked by the antagonist JTE-013 or by small interfering RNAs against the S1P2 mRNA (data not shown). Therefore, the S1P1 receptor seems to play a minor role in this system. Accordingly, pertussis toxin did not affect PDGF-induced chemotaxis of pre-osteoblasts. This indicates that the PDGF-induced chemotactic response in the osteoblast system is distinct from the mechanisms described in fibroblasts.

Previous work has shown that differentiation of osteoblasts may be coupled to their migratory potential [Ozeki et al., 2007]. The transcription factor runx2 is a key driver of osteogenic differentiation. Ablation of the runx2 gene leads to a complete lack of bone formation in mice [Ducy et al., 1997]. In parallel to its actions on the osteogenic differentiation program, runx2 positively affects chemotaxis of osteoblasts towards PDGF [Fujita et al., 2004]. In animals deficient for the runx2 gene, the transcriptional program underlying cell motility is disturbed [Vaes et al., 2006]. These data suggest that differentiation of osteoblasts goes together with the acquisition of a motile phenotype. Our data indicate that the induction of osteogenic differentiation by BMP2 leads to a loss of the sensitivity to the chemorepellent action of S1P. The concentrations of S1P in plasma are as high as 0.2 μM [Yatomi et al., 1997]. Since bone is a well-vascularized tissue, osteoprogenitor cells are in an environment of high S1P concentrations. Our data suggest that this high level of S1P will inhibit pre-osteoblast chemotaxis. Upon conversion to the osteoblast phenotype, S1P2 receptor expression is lowered, permitting cells to respond to a chemotactic cue (Fig. 8). In addition, the simultaneous activation of runx2 contributes to the acquisition of a motile phenotype. We speculate that this mechanism may preserve the osteoprogenitor pool, only allowing the more differentiated cell to travel to sites of bone formation. This notion is supported by the observation that osteogenic cells detected in the circulation express alkaline phosphatase, a marker for the more differentiated osteoblast phenotype [Eghbali-Fatourechi et al., 2005; Khosla and Eghbali-Fatourechi, 2006].

In conclusion, our data indicate that S1P2 acts as a chemorepellent receptor on pre-osteoblasts. Conversion of pre-osteoblasts into osteoblasts by BMP leads to a decrease in S1P2 expression. As a consequence, this allows the cell to respond to chemotactic cues such as provided by PDGF. These data are in line with a model in which osteoblast migrate to areas of new bone formation, whereas migration of the more immature pre-osteoblasts is restricted by S1P. In such a model, S1P2 antagonists like JTE-013 may promote chemotaxis of pre-osteoblasts. This could be of therapeutic benefit in bone conditions that depend on cell trafficking, for example, fracture healing and remodeling of (osteoporotic) bone.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Roland Heerkens and Eugene van Someren for their contributions to this work, and Joop van Zoelen and Guido Zaman for critical reading of the manuscript.

REFERENCES

Alvarez SE, Milstien S, Spiegel S. 2007. Autocrine and paracrine roles of sphingosine-1-phosphate. Trends Endocrinol Metab 18:300–307.

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

Bonyadi M, Waldman SD, Liu D, Aubin JE, Grynpas MD, Stanford WL. 2003. Mesenchymal progenitor self-renewal deficiency leads to age-dependent osteoporosis in Sca-1/Ly-6A null mice. Proc Natl Acad Sci USA 100: 5840–5845.

Dimitriou R, Tsiridis E, Giannoudis PV. 2005. Current concepts of molecular aspects of bone healing. Injury 36:1392–1404.

Dobnig H, Turner RT. 1995. Evidence that intermittent treatment with parathyroid hormone increases bone formation in adult rats by activation of bone lining cells. Endocrinology 136:3632–3638.

Dominici M, Pritchard C, Garlits JE, Hofmann TJ, Persons DA, Horwitz EM. 2004. Hematopoietic cells and osteoblasts are derived from a common marrow progenitor after bone marrow transplantation. Proc Natl Acad Sci USA 101:11761–11766.

Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. 1997. Osf2/Cbfa1: A transcriptional activator of osteoblast differentiation. Cell 89:747–754.

Eghbali-Fatourechi GZ, Lamsam J, Fraser D, Nagel D, Riggs BL, Khosla S. 2005. Circulating osteoblast-lineage cells in humans. N Engl J Med 352: 1959–1966.

Fiedler J, Roderer G, Gunther KP, Brenner RE. 2002. BMP-2, BMP-4, and PDGF-bb stimulate chemotactic migration of primary human mesenchymal progenitor cells. J Cell Biochem 87:305–312.

Fiedler J, Etzel N, Brenner RE. 2004. To go or not to go: Migration of human mesenchymal progenitor cells stimulated by isoforms of PDGF. J Cell Biochem 93:990–998.

Fink EE, Eghbali-Fatourechi GZ, Khosla S. 2007. Remodeling and vascular spaces in bone. J Bone Miner Res 22:1–6.

Fujii H, Kitazawa R, Maeda S, Mizuno K, Kitazawa S. 1999. Expression of platelet-derived growth factor proteins and their receptor alpha and beta mRNAs during fracture healing in the normal mouse. Histochem Cell Biol 112:131–138.

Fujita T, Azuma Y, Fukuyama R, Hattori Y, Yoshida C, Koida M, Ogita K, Komori T. 2004. Runx2 induces osteoblast and chondrocyte differentiation and enhances their migration by coupling with PI3K-Akt signaling. J Cell Biol 166:85–95.

Fukuyama R, Fujita T, Azuma Y, Hirano A, Nakamuta H, Koida M, Komori T. 2004. Statins inhibit osteoblast migration by inhibiting Rac-Akt signaling. Biochem Biophys Res Commun 315:636–642.

Godwin SL, Soltoff SP. 1997. Extracellular calcium and platelet-derived growth factor promote receptor-mediated chemotaxis in osteoblasts through different signaling pathways. J Biol Chem 272:11307–11312.

Goparaju SK, Jolly PS, Watterson KR, Bektas M, Alvarez S, Sarkar S, Mel L, Ishii I, Chun J, Milstien S, Spiegel S. 2005. The S1P2 receptor negatively regulates platelet-derived growth factor-induced motility and proliferation. Mol Cell Biol 25:4237–4249.

Hankenson KD, Bain SD, Kyriakides TR, Smith EA, Goldstein SA, Bornstein P. 2000. Increased marrow-derived osteoprogenitor cells and endosteal bone formation in mice lacking thrombospondin 2. J Bone Miner Res 15:851–862.

Hobson JP, Rosenfeldt HM, Barak LS, Olivera A, Poulton S, Caron MG, Milstien S, Spiegel S. 2001. Role of the sphingosine-1-phosphate receptor EDG-1 in PDGF-induced cell motility. Science 291:1800–1803.

Khosla S, Eghbali-Fatourechi GZ. 2006. Circulating cells with osteogenic potential. Ann NY Acad Sci 1068:489–497.

Kono M, Belyantseva IA, Skoura A, Frolenkov GI, Starost MF, Dreier JL, Lidington D, Bolz SS, Friedman TB, Hla T, Proia RL. 2007. Deafness and stria vascularis defects in S1P2 receptor-null mice. J Biol Chem 282:10690–10696.

Lee MJ, Thangada S, Paik JH, Sapkota GP, Ancellin N, Chae SS, Wu M, Morales-Ruiz M, Sessa WC, Alessi DR, Hla T. 2001. Akt-mediated phosphorylation of the G protein-coupled receptor EDG-1 is required for endothelial cell chemotaxis. Mol Cell 8:693–704.

Lepley D, Paik JH, Hla T, Ferrer F. 2005. The G protein-coupled receptor S1P2 regulates Rho/Rho kinase pathway to inhibit tumor cell migration. Cancer Res 65:3788–3795.

Lyons JM, Karin NJ. 2001. A role for G protein-coupled lysophospholipid receptors in sphingolipid-induced Ca2+ signaling in MC3T3-E1 osteoblastic cells. J Bone Miner Res 16:2035–2042.

Marzia M, Sims NA, Voit S, Migliaccio S, Taranta A, Bernardini S, Faraggiana T, Yoneda T, Mundy GR, Boyce BF, Baron R, Teti A. 2000. Decreased c-Src expression enhances osteoblast differentiation and bone formation. J Cell Biol 151:311–320.

Masiello LM, Fotos JS, Galileo DS, Karin NJ. 2006. Lysophosphatidic acid induces chemotaxis in MC3T3-E1 osteoblastic cells. Bone 39:72–82.

Matloubian M, Lo CG, Cinamon G, Lesneski MJ, Xu Y, Brinkmann V, Allende ML, Proia RL, Cyster JG. 2004. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. Nature 427: 355–360.

Nash TJ, Howlett CR, Martin C, Steele J, Johnson KA, Hicklin DJ. 1994. Effect of platelet-derived growth factor on tibial osteotomies in rabbits. Bone 15:203–208.

Ohmori T, Yatomi Y, Osada M, Kazama F, Takafuta T, Ikeda H, Ozaki Y. 2003. Sphingosine 1-phosphate induces contraction of coronary artery smooth muscle cells via S1P2. Cardiovasc Res 58:170–177.

Okamoto H, Takuwa N, Yokomizo T, Sugimoto N, Sakurada S, Shigematsu H, Takuwa Y. 2000. Inhibitory regulation of Rac activation, membrane ruffling, and cell migration by the G protein-coupled sphingosine-1-phosphate receptor EDG5 but not EDG1 or EDG3. Mol Cell Biol 20:9247–9261.

Ozeki N, Jethanandani P, Nakamura H, Ziober BL, Kramer RH. 2007. Modulation of satellite cell adhesion and motility following BMP2-induced differentiation to osteoblast lineage. Biochem Biophys Res Comm 353:54–59.

Parfitt AM, 2001. The bone remodeling compartment: A circulatory function for bone lining cells. J Bone Miner Res 16:1583–1585.

Rosen H, Goetzl EJ. 2005. Sphingosine 1-phosphate and its receptors: An autocrine and paracrine network. Nature Rev Immunol 7:560–570.

Sanchez T, Thangada S, Wu MT, Kontos CD, Wu D, Wu H, Hla T. 2005. PTEN as an effector in the signaling of antimigratory G protein-coupled receptor. Proc Natl Acad Sci USA 102:4312–4317.

Shagin DA, Barsova EV, Yanushevich YG, Fradkov AF, Lukyanov KA, Labas YA, Semenova TN, Ugalde JA, Meyers A, Nunez JM, Widder EA, Lukyanov SA, Matz MV. 2004. GFP-like proteins as ubiquitous metazoan superfamily: Evolution of functional features and structural complexity. Mol Biol Evol 21:841–850.

Sugimoto N, Takuwa N, Okamoto H, Sakurada S, Takuwa Y. 2003. Inhibitory and stimulatory regulation of Rac and cell motility by the G12/13-Rho and Gi pathways integrated downstream of a single G protein-coupled sphingosine-1-phosphate receptor isoform. Mol Cell Biol 23:1534–1545.

Swift S, Lorens J, Achacoso P, Nolan GP. 1999. Radid production of retroviruses for efficient gene delivery to mammalian cells using 293T cell-based systems. In: Coligan JE, Bierer B, Margulies DH, Shevach EM, Strober W, Coico R, editors. Current protocols in immunology. Wiley Interscience. pp. 14–29.

ten Dijke, P. 2006. Bone morphogenetic protein signal transduction in bone. Curr Med Res Opin 22 (Suppl 1): s7–s11.

Thomas D, Kansara M. 2006. Epigenetic modifications in osteogenic differentiation and transformation. J Cell Biochem 98:757–769.

Vaes BL, Dechering KJ, Feijen A, Hendriks JM, Lefevre C, Mummery CL, Olijve W, van Zoelen EJ, Steegenga WT. 2002. Comprehensive microarray analysis of bone morphogenetic protein 2-induced osteoblast differentiation resulting in the identification of novel markers for bone development. J Bone Miner Res 17:2106–2118.

Vaes BL, Ducy P, Sijbers AM, Hendriks JM, van Someren EP, de Jong NG, van den Heuvel ER, Olijve W, van Zoelen EJ, Dechering KJ. 2006. Microarray analysis on Runx2-deficient mouse embryos reveals novel Runx2 functions and target genes during intramembranous and endochondral bone formation. Bone 39:724–738.

Waters CM, Long J, Gorshkova I, Fujiwara Y, Connell M, Belmonte KE, Tigyi G, Natarajan V, Pyne S, Pyne NJ. 2006. Cell migration activated by platelet-derived growth factor receptor is blocked by an inverse agonist of the sphingosine 1-phosphate receptor-1. FASEB J 20:509–511.

Xiao G, Gopalakrishnan R, Jiang D, Reith E, Benson MD, Franceschi RT. 2002. Bone morphogenetic proteins, extracellular matrix, and mitogen-activated protein kinase signaling pathways are required for osteoblast-specific gene expression and differentiation in MC3T3-E1 cells. J Bone Miner Res 17:101–110.

Yatomi Y, Igarashi Y, Yang L, Hisano N, Qi R, Asazuma N, Satoh K, Ozaki Y, Kume S. 1997. Sphingosine 1-phosphate, a bioactive sphingolipid abundantly stored in platelets, is a normal constituent of human plasma and serum. J Biochem (Tokyo) 121:969–973.

Yokoo E, Yatomi Y, Takafuta T, Osada M, Okamoto Y, Ozaki Y. 2004. Sphingosine 1-phosphate inhibits migration of RBL-2H3 cells via S1 P2: Cross-talk between platelets and mast cells. J Biochem (Tokyo) 135:673–681.