Journal of Cellular Biochemistry 93:741–752 (2004)

Differential Regulation of Platelet-Derived Growth Factor Stimulated Migration and Proliferation in Osteoblastic Cells

Meenal Mehrotra,[1] Stephen M. Krane,[2] Kristen Walters,[1] and Carol Pilbeam[1]*

[1]Department of Medicine, University of Connecticut Health Center, Farmington, Connecticut 06030
[2]Department of Medicine, Harvard Medical School and Massachusetts General Hospital, Boston, Massachusetts 02129

Abstract Osteoblastic migration and proliferation in response to growth factors are essential for skeletal development, bone remodeling, and fracture repair, as well as pathologic processes, such as metastasis. We studied migration in response to platelet-derived growth factor (PDGF, 10 ng/ml) in a wounding model. PDGF stimulated a twofold increase in migration of osteoblastic MC3T3-E1 cells and murine calvarial osteoblasts over 24–48 h. PDGF also stimulated a tenfold increase in ^{3}H-thymidine (^{3}H-TdR) incorporation in MC3T3-E1 cells. Migration and DNA replication, as measured by BrdU incorporation, could be stimulated in the same cell. Blocking DNA replication with aphidicolin did not reduce the distance migrated. To examine the role of mitogen-activated protein (MAP) kinases in migration and proliferation, we used specific inhibitors of p38 MAP kinase, extracellular signal regulated kinase (ERK), and c-Jun N-terminal kinase (JNK). For these signaling studies, proliferation was measured by carboxyfluorescein diacetate succinimidyl ester (CFSE) using flow cytometry. Inhibition of the p38 MAP kinase pathway by SB203580 and SB202190 blocked PDGF-stimulated migration but had no effect on proliferation. Inhibition of the ERK pathway by PD98059 and U0126 inhibited proliferation but did not inhibit migration. Inhibition of JNK activity by SP600125 inhibited both migration and proliferation. Hence, the stimulation of migration and proliferation by PDGF occurred by both overlapping and independent pathways. The JNK pathway was involved in both migration and proliferation, whereas the p38 pathway was predominantly involved in migration and the ERK pathway predominantly involved in proliferation. J. Cell. Biochem. 93: 741–752, 2004.

Key words: signaling pathways; MAP kinase; ERK; p38 kinase; JNK; CFSE

Migration of the bone-forming osteoblastic cells is postulated to be involved in many processes essential to bone physiology, including embryonic skeletal development and subsequent bone remodeling and responses to mechanical loading. Migration is also thought to be important in pathologic processes, such as wound healing, fracture repair, fibrosis, and tumor metastasis. Many growth factors, including fibroblast growth factors, platelet-derived growth factor (PDGF), insulin-like growth factor-I, bone morphogenetic proteins, vascular endothelial growth factor, and transforming growth factor-β, can promote migration in a variety of cell types and have been proposed as therapeutic agents in wound healing or fracture and targets to inhibit in fibrosis or metastasis [Khan et al., 2000; Lieberman et al., 2002; Reddi et al., 2003].

PDGF has been shown to stimulate migration or chemotaxis in multiple types of cells, including vascular smooth muscle cells [Jacob et al., 2002; Kingsley et al., 2002; Zhan et al., 2003], fibroblasts [Anand-Apte et al., 1997], monocytes [Siegbahn et al., 1990], endothelial

This work was presented in part at the 25th annual meeting of the American Society for Bone and Mineral Research, Minneapolis, MN, September 2003.

Grant sponsor: American Association of Orthodontics Foundation (Biomedical Research Award); Grant sponsor: National Institute of Health; Grant numbers: ROI-AR47673, AR-44815.

*Correspondence to: Carol Pilbeam, Room AM047, University of Connecticut Health Center, 263 Farmington Avenue, Farmington, CT 06030.
E-mail: pilbeam@nso.uchc.edu

Received 6 January 2004; Accepted 22 March 2004
DOI 10.1002/jcb.20138

cells [Matsumoto et al., 1999], pancreatic cells [Masamune et al., 2003], hepatic myofibroblasts [Tangkijvanich et al., 2002], mesenchymal precursor cells [Fiedler et al., 2002], and osteoblasts [Tsukamoto et al., 1991; Lind et al., 1995; Godwin and Soltoff, 1997]. PDGF is a dimer, consisting of disulfide-bonded A- and B-polypeptide chains (PDGF-AA, PDFG-AB, and PDGF-BB), which binds and activates two tyrosine kinase receptors, α- and β [Heldin and Westermark, 1999]. PDGF-BB binds to both α- and β-receptors while PDGF-AA binds only to α-receptors. Although platelets are a major storage site for PDGF, many tissues have been shown to synthesize PDGF [Heldin and Westermark, 1999]. It was originally thought that osteoblasts produced primarily PDGF-AA [Zhang et al., 1991; Centrella et al., 1992] although more recent studies have shown that osteoblasts also produce PDGF-BB [Rydziel and Canalis, 1996]. In vivo, PDGF may enhance bone repair [Bolander, 1992]. Both PDGF-A and -B chains are expressed in multiple cells involved in healing fractures, with PDGF-B chains being most highly expressed in osteoblasts during bone formation [Andrew et al., 1995; Fujii et al., 1999]. Activation of either the α- or β-receptor can stimulate migration, although PDGF-BB is generally a stronger inducer than PDGF-AA [Gilardetti et al., 1991; Bornfeldt et al., 1995; Heldin and Westermark, 1999; Yu et al., 2001].

Most growth factors that are potent stimulators of migration are also potent stimulators of cell proliferation. PDGF has been shown to be a potent mitogen for osteoblasts in rat calvarial organ cultures [Canalis et al., 1989; Hock and Canalis, 1994] and in human [Zhang et al., 1991] and rat primary osteoblastic cultures [Centrella et al., 1992]. Signaling pathways for both proliferation and migration often involve the mitogen-activated protein (MAP) kinases. MAP kinases are a family of serine/threonine kinases, including extracellular signal regulated kinase (ERK1 and ERK2), the stress-activated p38 MAP kinase (α, β, γ, δ), and c-jun N-terminal kinase (JNK1, JNK2, and JNK3), which regulate multiple cellular functions [Johnson and Lapadat, 2002]. All three of these MAP kinase pathways have been shown to be involved in PDGF-stimulated proliferation and/or migration in various cell types [Bornfeldt et al., 1995; Hinton et al., 1998; Cospedal et al., 1999; Taylor, 2000; Pearson et al., 2001; Tangkijvanich et al., 2002; Huang et al., 2003; Javelaud et al., 2003; Kavurma and Khachigian, 2003; Zhan et al., 2003].

In this study, we examined the dependence of PDGF (PDGF-BB)-stimulated migration on proliferation in murine osteoblastic MC3T3-E1 cells and compared the effects of specific inhibitors of the ERK, p38 kinase, and JNK pathways on migration and proliferation. We found that PDGF could stimulate both migration and proliferation in the same cell and that migration was independent of proliferation. Proliferation and migration were differentially regulated by the MAP kinases. The JNK pathway was involved in both migration and proliferation whereas the p38 kinase pathway was involved only in migration and the ERK pathway only in proliferation.

MATERIALS AND METHODS

Materials

Rat PDGF (PDGF-BB) was purchased from R&D Systems, Inc. (Westerville, OH). Rat-tail collagen Type I was purchased from Collaborative Biomedical Products, Corporation. Aphidicolin was purchased from Calbiochem-Novabiochem Laboratories (La Jolla, CA). SB203580, SB202190, PD98059, U0126, and SP600125 were purchased from Biomol (Plymouth Meeting, PA). Crystal violet and other chemicals were purchased from Sigma Chemicals Co. (St. Louis, MO).

Collagen-Coated Petri Dishes

Petri dishes (100 mm) were incubated for 1 h at 37°C on a rocker with 6 ml of 50 μg/ml of rat-tail collagen in 0.02 N acetic acid to provide a collagen concentration of 5 $\mu g/cm^2$. Dishes were stored at 4°C, and the collagen solution aspirated off and the dish washed with phosphate buffered saline (PBS) prior to cell plating. Cells plated on collagen-coated Petri dishes grew normally, while cells plated on non-coated dishes did not adhere.

Cell Culture

MC3T3-E1 cells were plated at 5,000 cells/cm^2 in 100-mm tissue culture dishes, cultured in a humidified atmosphere of 5% CO_2 in air at 37°C. Cells were fed every 3 days with Dulbecco's modified Eagle's medium (DMEM) without phenol red (Sigma) supplemented with 10%

heat inactivated fetal calf serum (FCS) (Gibco BRL, Grand Island, NY), 100 μg/ml penicillin (Sigma), and 50 μg/ml streptomycin (Sigma), and passaged on day 7.

Primary calvarial osteoblastic cells were obtained from neonatal CD-1 mice, maintained in colonies according to protocols approved by the Animal Care and Use Committee of the University of Connecticut Health Center. Calvariae were dissected, washed with PBS, and digested with crude collagenase P (Roche Molecular Biomedicals, Inc., Indianapolis, IN) in a solution of 1 ml trypsin/EDTA and 4 ml PBS for 10 min at 37°C with gentle rocking. The digestion procedure was repeated to provide five populations of cells (fraction 5 was digested for 20 min). Cells from populations 2–5 were pooled and cultured to confluence under the same conditions as MC3T3-E1 cells before being replated for experiments.

Wounding Model

Cells were plated at 100,000 /cm^2 in collagen-coated 100-mm Petri dishes and cultured for 24 h prior to wounding in DMEM with 10% FCS. At this plating density, cells reached confluence in about 24 h and were uniformly distributed near the wound line. For wounding, a linear line was created down the center of the Petri dish using a sterilized, taped razor blade. One half of the plate was gently scraped to remove cells, and plates were washed twice with sterile PBS. Dishes were then incubated with or without PDGF (10 ng/ml) in DMEM with 0.1% bovine serum albumin (BSA) for 24–48 h. If inhibitors were employed, cells were incubated with the inhibitors 30 min prior to wounding.

For measurement of migration, cells were stained with crystal violet, and photographs of the migration front were taken under the microscope. Photographs were imported into the NIH Image 1.61 program (free software from NIH). For each region, a straight line of known length (L) was drawn along the wound line and the area (A) perpendicular from this line to the migration front was measured. The average distance of migration (D) of the cell front from the wound line was calculated as $D = A/L$ in arbitrary units of pixels. In each experiment (except for the signaling pathway experiments), there were three Petri dishes per treatment group. Five to seven areas were analyzed in each dish, and the average migration distance for each plate was calculated. The average for each dish was then used to calculate the mean ± SEM for that experiment.

^{3}H-Thymidine Incorporation

DNA synthesis in control and PDGF-treated cells was assessed by the incorporation of ^{3}H-thymidine into newly replicated DNA. Cultures were pulsed with 5 μCi/ml ^{3}H-thymidine (NEN Life Science Products, Inc., Boston, MA) 24 h after wounding and incubated for another 2 h. Cells were washed with PBS and then extracted twice with trichloroacetic acid (TCA). The TCA insoluble fraction was assayed by scraping the cells in 0.5 M NaOH and counting by liquid scintillation. DNA content was determined fluorometrically with diaminopheylindole (DAPI) using a neutralized (with acetic acid) aliquot of the NaOH fraction.

BrdU Staining

Staining by BrdU, which incorporates into the nuclei of cells undergoing DNA synthesis, was used as another measure of cell proliferation. Twenty-four hours after wounding, cells were incubated with 10 μM BrdU (Roche Molecular, Indianapolis, IN) for 2 h. Cells were then fixed in 95% and 100% ethanol and rehydrated in decreasing ethanol series. Cellular DNA was denatured by incubation with 2N HCl for 1 h at 37°C. The cells were then incubated with anti-BrdU antibody (6 μg/ml; Roche Molecular, Indianapolis, IN) for 1 h and stained with biotinylated secondary anti-mouse antibody (Vectastain Elite ABC kit, Vector Labs, Inc., Burlingame, CA) for 1 h. Cells were then incubated with avidin/biotin-HRP reagent (Vectastain Elite ABC kit) for 1 h. The color was developed by using the DAB peroxidase substrate kit (Vector Labs). Cells were counterstained with Harris haematoxylin (Sigma Chemicals Co., St. Louis, MO) and visualized under the microscope.

Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE) Staining and Flow Cytometry

As another measure of proliferation, we stained the cells with 5-(and -6)-carboxyfluorescein diacetate succinimidyl ester (CFDA, SE; Molecular Probes, Eugene, OR). The fluorescence of this dye is stable for several months and can be analyzed by flow cytometry using excitation at 488 nm and the FL-1 detection channel. MC3T3-E1 cells were washed twice with sterile PBS at room temperature and then incubated

with CFDA, SE at a concentration of 50 μM in PBS (made from a 5 mM stock of CFDASE) for 10 min at 37°C. After 10 min, the reaction was terminated by adding FCS and incubating for a further 10 min. The cells were finally washed twice with sterile PBS and 1% FCS. The labeled cells were plated at 50,000 cells/cm^2 and grown for 24 h before treating with the inhibitors and PDGF for 24 h. Mean fluorescence intensity was measured by flow cytometry using a FACSCalibur and CellQuest software (Becton Dickinson Biosciences, Mountain View, CA).

Statistical Analysis

Each experiment was performed three times, unless specified otherwise, and the mean and standard error of the mean (SEM) were calculated. Statistical significance of differences was determined by analysis of variance (ANOVA), with post-hoc comparison of more than two groups by Bonferroni or *t*-test as appropriate (SigmaStat software, Jandel Scientific, San Rafael, CA).

RESULTS

Effects of PDGF on Cell Migration

MC3T3-E1 cells were plated on collagen-coated Petri dishes, and 24 h later the cell layer was wounded and the remaining cells treated with vehicle or PDGF (10 ng/ml) in serum free media. Migration of the cell front for 48 h is shown in Figure 1A. Measurement of the migration of the cell front from the wound line is shown in Figure 1B. Data from three independent experiments are shown in Figure 1C. PDGF significantly increased the migration of cells from the wound line relative to control cultures in all experiments, with the mean increase (±SEM) being 2.4 ± 0.4-fold. Following the same protocol, two experiments were performed on primary calvarial cells from neonatal mice, and PDGF stimulated a 1.9 ± 0.3-fold increase in the movement of cells out from the wound line (data not shown), similar to the results seen with the MC3T3-E1 cells.

Effects on Cell Migration of Inhibiting Cell Proliferation

PDGF is a potent mitogen and the increased migration of the cell front in PDGF-treated cultures compared to control cultures might be coupled to increased proliferation. Hence, we measured migration in the presence of aphidicolin, an inhibitor of DNA replication. To confirm that proliferation was inhibited, we examined the incorporation of ^{3}H-thymidine into newly replicated DNA, normalized to the total DNA content, during the last 2 h of 24 h culture of MC3T3-E1 cells treated with PDGF (10 ng/ml) or vehicle, in the presence or absence of aphidicolin (Fig. 2). PDGF increased ^{3}H-thymidine incorporation tenfold in cells

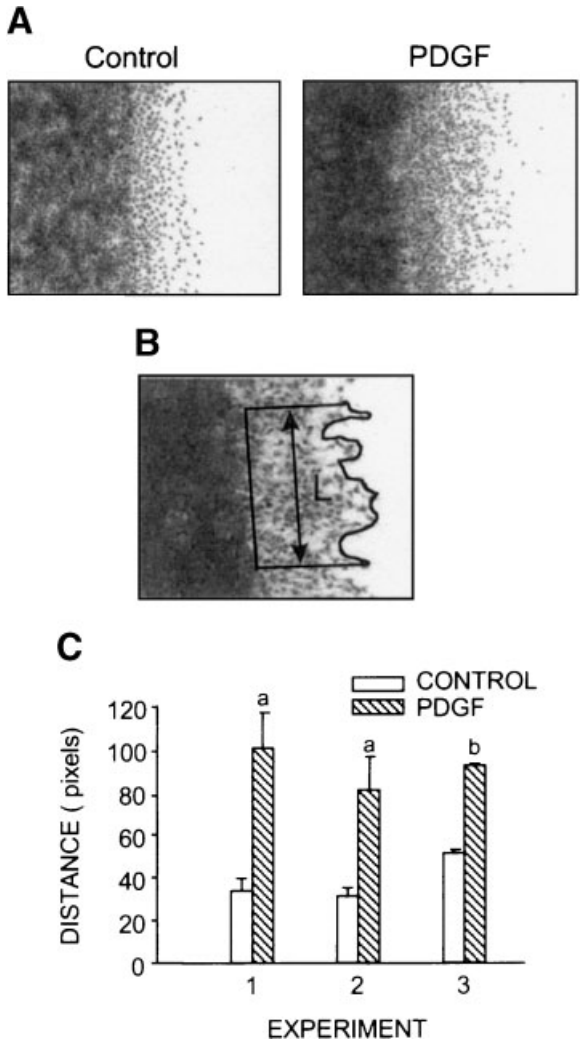

Fig. 1. Effect of platelet-derived growth factor (PDGF) on migration of MC3T3-E1 cells. **A**: Photomicrographs of MC3T3-E1 cells treated with vehicle (control) and PDGF (10 ng/ml) for 48 h after wounding. **B**: Method used for quantifying migration of cell front. Cultures were stained with crystal violet and non-overlapping photographs of the migration front were taken. For each area, a straight line of known length, L, was drawn along the wound line, and the area, A, of the cell front perpendicular to L outlined and calculated by NIH Image. The average distance of migration, D, of the cell front from the wound line was calculated as D = A/L in arbitrary units of pixels. **C**: Effect of PDGF (10 ng/ml) on migration in three different experiments. Each bar represents the mean ± SEM. [a]Significant effect of PDGF, $P < 0.01$; [b]$P < 0.05$.

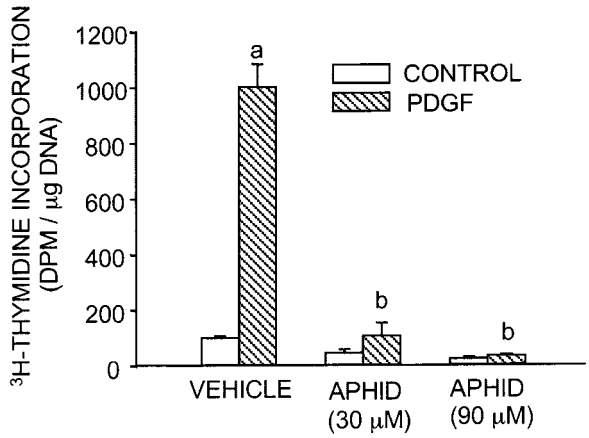

Fig. 2. Effect of aphidicolin on PDGF-stimulated cell proliferation. MC3T3-E1 cells were treated with PDGF (10 ng/ml), with or without aphidicolin (30 or 90 μM). Incorporation of ^{3}H-thymidine into newly replicated DNA, normalized to the total DNA content, was measured during the last 2 h of a 24 h culture. Bars are means ± SEM. [a]Significant effect of PDGF, $P < 0.01$. [b]Significant effect of aphidicolin, $P < 0.01$.

without aphidicolin. However, in cells treated with either 30 or 90 μM aphidicolin, PDGF had no significant effect on ^{3}H-thymidine incorporation.

To examine the effects of aphidicolin on migration of MC3T3-E1 cells, treatment with aphidicolin was begun 1 h before wounding. After wounding, cells were treated with PDGF (10 ng/ml) or vehicle, in the presence or absence of aphidicolin, for 24 h. In three independent experiments, PDGF increased migration on average by 2.3 ± 0.3-fold in the presence of aphidicolin 30 μM, compared to 1.8 ± 0.3-fold in the absence of aphidicolin (Fig. 3A). In the presence of aphidicolin 90 μM, PDGF stimulated an increase in migration of 2.2-fold, compared to 2.1-fold without aphidicolin (Fig. 3B). Migration in cultures without PDGF was not significantly affected by aphidicolin. This same protocol was repeated with neonatal mouse calvarial cells. In two experiments, PDGF (10 ng/ml) increased the distance of cell migration by 1.7-fold in the presence of aphidicolin (90 μM), compared to 1.8-fold without aphidicolin (data not shown). Migration in control cultures was not effected by aphidicolin.

To visualize both proliferating and migrating cells, some cultures of MC3T3-E1 cells were stained with BrdU during the last 2 h of culture (Fig. 3C). Many cells that migrated out from the wound line were stained for BrdU, indicating that the same cell could both replicate and migrate. Treatment with aphidicolin (30 μM) decreased the number of BrdU stained cells in the migration front but not the distance migrated outward from the wound line.

Effects on Migration of Inhibiting the MAP Kinase Pathways

To study the roles of p38 kinase and ERK signaling pathways in osteoblastic migration, MC3T3-E1 cells were treated with specific inhibitors of p38 MAP kinase activity, SB203580 and SB202190, and specific inhibitors of ERK activity, PD98059 and U0126. In each experiment, the average distance of migration was calculated from seven non-overlapping areas of equal length along the wound line in one dish. The mean values (±SEM) for three independent experiments are shown in Figure 4A. The inhibitors, SB203580 and SB202190, are reported to selectively inhibit p38 MAP kinase with IC_{50} of 50–70 nM [Clerk and Sugden, 1998; Fox et al., 1998]. Both of these inhibitors, used at a dose of 10 μM, completely blocked the PDGF stimulated migration of osteoblastic cells (Fig. 4A). The inhibitors, PD98059 and U0126, selectively inhibit MAP kinase kinase (MEK) activity and ERK phosphorylation. The maximal effects of PD98059 are reported to occur at 10–100 μM [Pang et al., 1995], while the IC_{50} for U0126 is 60–70 nM [Favata et al., 1998]. PD98059 and U0126, used at 40 and 20 μM, respectively, had no effect on PDGF-stimulated migration (Fig. 4A). There was no significant change in the basal migration of the cells when treated with any of these inhibitors alone.

To examine the role of the JNK signaling pathway in PDGF-stimulated migration, cells were treated with the selective inhibitor of JNK activity, SP600125. This inhibitor was shown to block the phosphorylation of c-Jun with an IC_{50} of 5–10 μM in Jurkat T-cells [Bennett et al., 2001]. SP600125 at 1 μM had no effect on migration of MC3T3-E1 cells (data not shown) but SP600125 at 10 μM decreased migration in control cultures by 30% and in PDGF-stimulated cultures by 50% (Fig. 4B). In the presence of SP600125, PDGF stimulated a 1.6-fold increase in migration, compared to a 2.2-fold increase in the absence of SP600125. The same results were obtained in a second experiment.

Effects on Cell Proliferation of Inhibiting the MAP Kinase Pathways

To study the effects of the MAP kinase inhibitors on cell replication in response to PDGF,

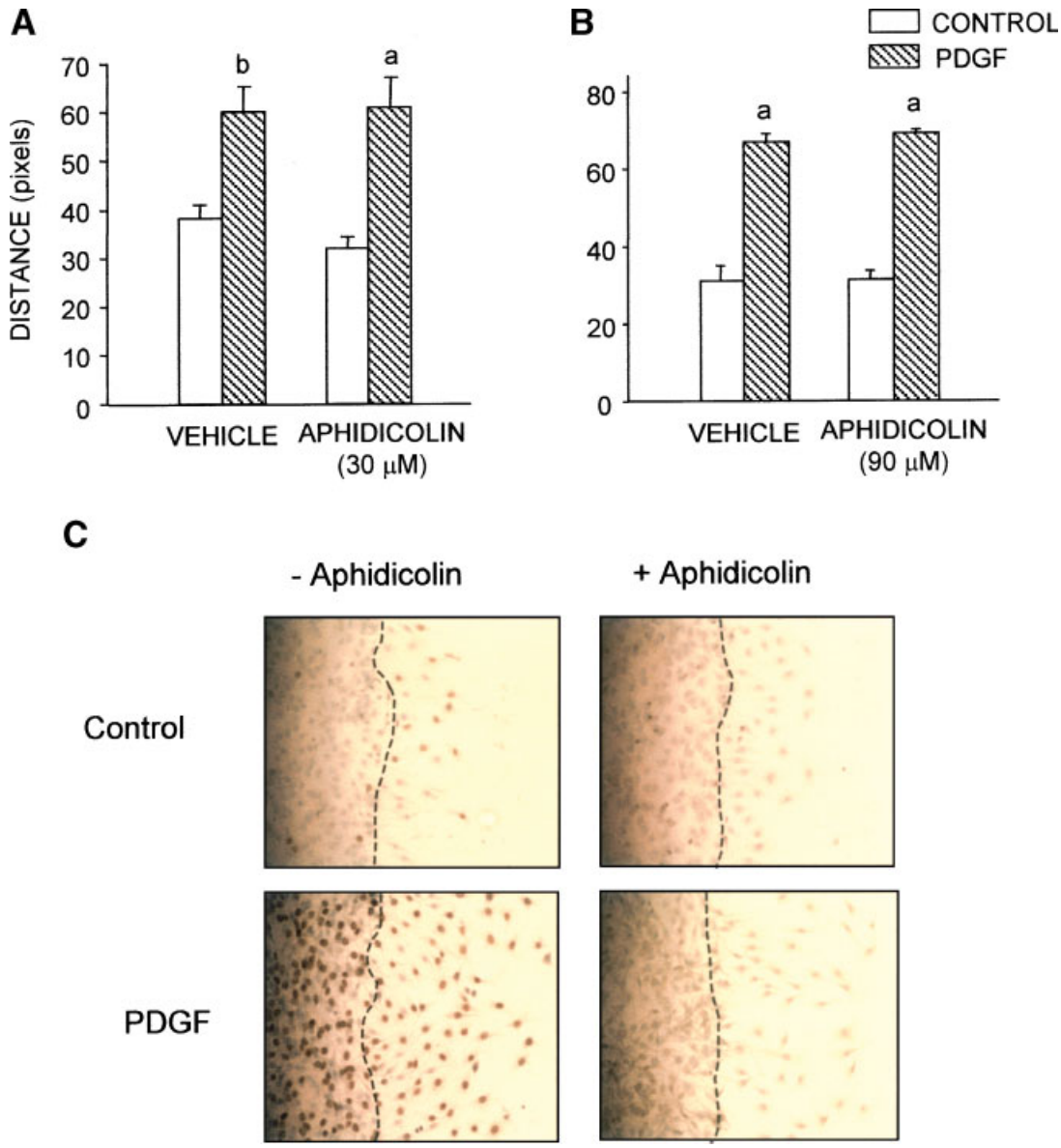

Fig. 3. Effect of aphidicolin on migration. MC3T3-E1 cells were treated with vehicle (control) or PDGF (10 ng/ml) after wounding, in the presence or absence of aphidicolin (**A**) 30 μM or (**B**) 90 μM. Results are from three independent experiments. Bars are means ± SEM. [a]Significant effect of PDGF, $P < 0.01$; [b]$P < 0.05$. (**C**) Photomicrographs of BrdU staining of MC3T3-E1 cells, treated with PDGF (10 ng/ml) in the presence and absence of aphidicolin (30 μM), and allowed to migrate for 24 h after wounding. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

MC3T3-E1 cells were stained with CFDA, SE, which spontaneously penetrates cell membranes, is converted to anionic carboxyfluorescein succinimidyl ester (CFSE) by intracellular esterases and couples to other proteins in cells resulting in long-term retention. When cells divide, the CFSE labeling is distributed equally between the daughter cells, the mean fluorescence intensity per cell is halved, and the peak of fluorescence intensity moves towards the left (Fig. 5). Cells were stained with CFSE at the time of plating and cultured for 24 h before addition of PDGF or vehicle. Selective inhibitors and aphidicolin were added 30 min before PDGF. The peak intensity of cells treated with aphidicolin (30 μM) was taken to reflect the cell population that had not undergone division after treatment with PDGF. For each experiment, the percentage of the cell population that had undergone ≥2 cell divisions was calculated

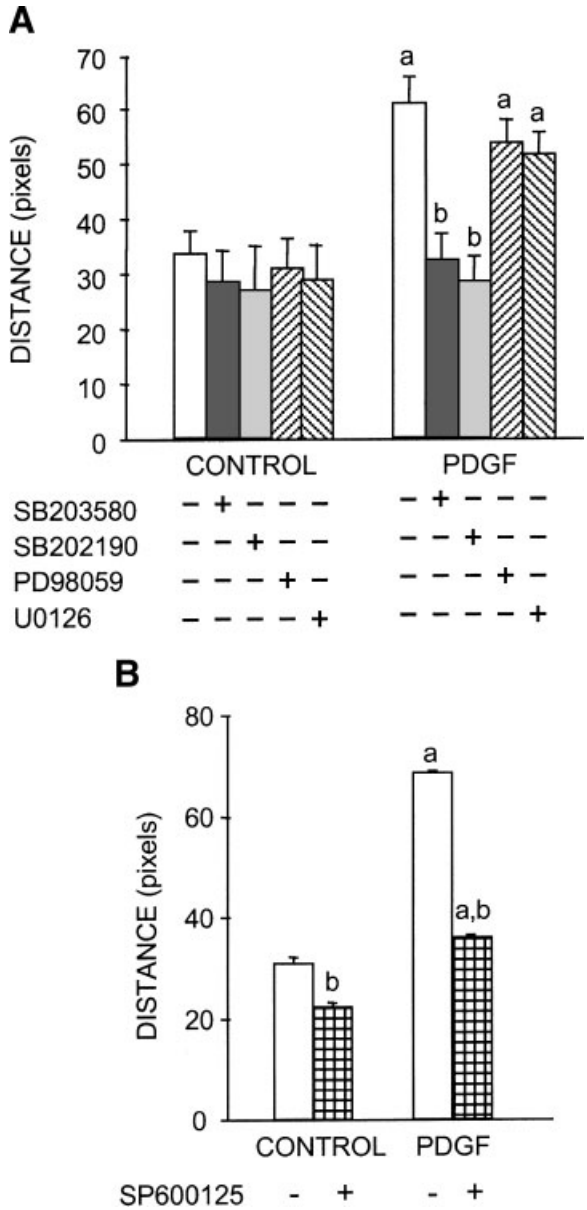

Fig. 4. Role of MAP kinase signaling pathways in PDGF stimulated migration. MC3T3-E1 cells were treated with PDGF (10 ng/ml) or vehicle, with or without inhibitor, and allowed to migrate for 24 h. Inhibitors were added 30 min prior to wounding. **A**: Effects of inhibiting the p38 kinase pathway (SB203580, 10 μM; SB202190, 10 μM) and the ERK pathway (PD98059, 40 μM; U0126, 20 μM) on migration. **B**: Effects of the specific JNK inhibitor (SP600125, 10 μM) on migration. [a]Significant effect of PDGF, $P < 0.01$. [b]Significant effect of inhibitor, $P < 0.01$.

by setting the right hand gate at 2 cell divisions to the left of the aphidicolin peak fluorescence intensity.

Using this methodology, PDGF (10 ng/ml) caused a twofold increase in the percentage of cells that underwent $\geq$2 cell divisions over 24 h (Fig. 6A–C). The p38 kinase inhibitor SB203580

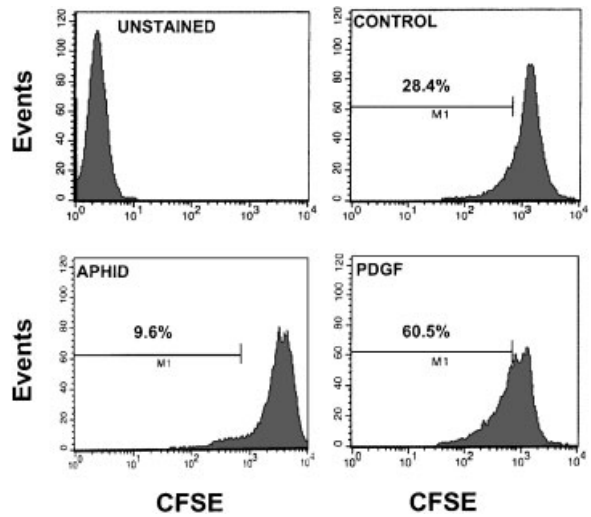

Fig. 5. Measurement of cell replication using carboxyfluorescein succinimidyl ester (CFSE) staining and flow cytometry. Peak intensity of cells treated with aphidicolin (30 μM) was taken to reflect the cell population that had not undergone division. The gate was then set at 2 cell divisions to the left of the aphidicolin peak intensity for each experiment. The percentage of the population of cells with $\geq$2 divisions was calculated for each experiment.

used at 10 μM, the same dose that blocked PDGF-stimulated migration, had no inhibitory effect on either control or PDGF-stimulated proliferation (Fig. 6A). There was a trend for the p38 kinase inhibitor SB202190 to decrease both control and PDGF-stimulated proliferation, but it was not statistically significant (Fig. 6B). Both of the ERK inhibitors, used at the same doses that had no effect on PDGF-stimulated migration, decreased cell division in control and PDGF treated cultures by about 50% (Fig. 6A,B). A dose response for the JNK inhibitor, SP600125, showed no effect on PDGF-stimulated cell division at 1 μM, while SP600125 at 20 and 40 μM had similar inhibitory effects to SP600125 at 10 μM (data not shown). Results of treatment with SP600125 (10 μM) are shown in Figure 6C. SP600125 decreased proliferation in control and PDGF treated cultures by 75–80%. Despite the marked inhibition of proliferation in general, there was still a stimulatory effect on proliferation by PDGF in the presence of these inhibitors (Fig. 6A–C).

DISCUSSION

Local growth factors induce migration in osteoblastic cells during skeletal development,

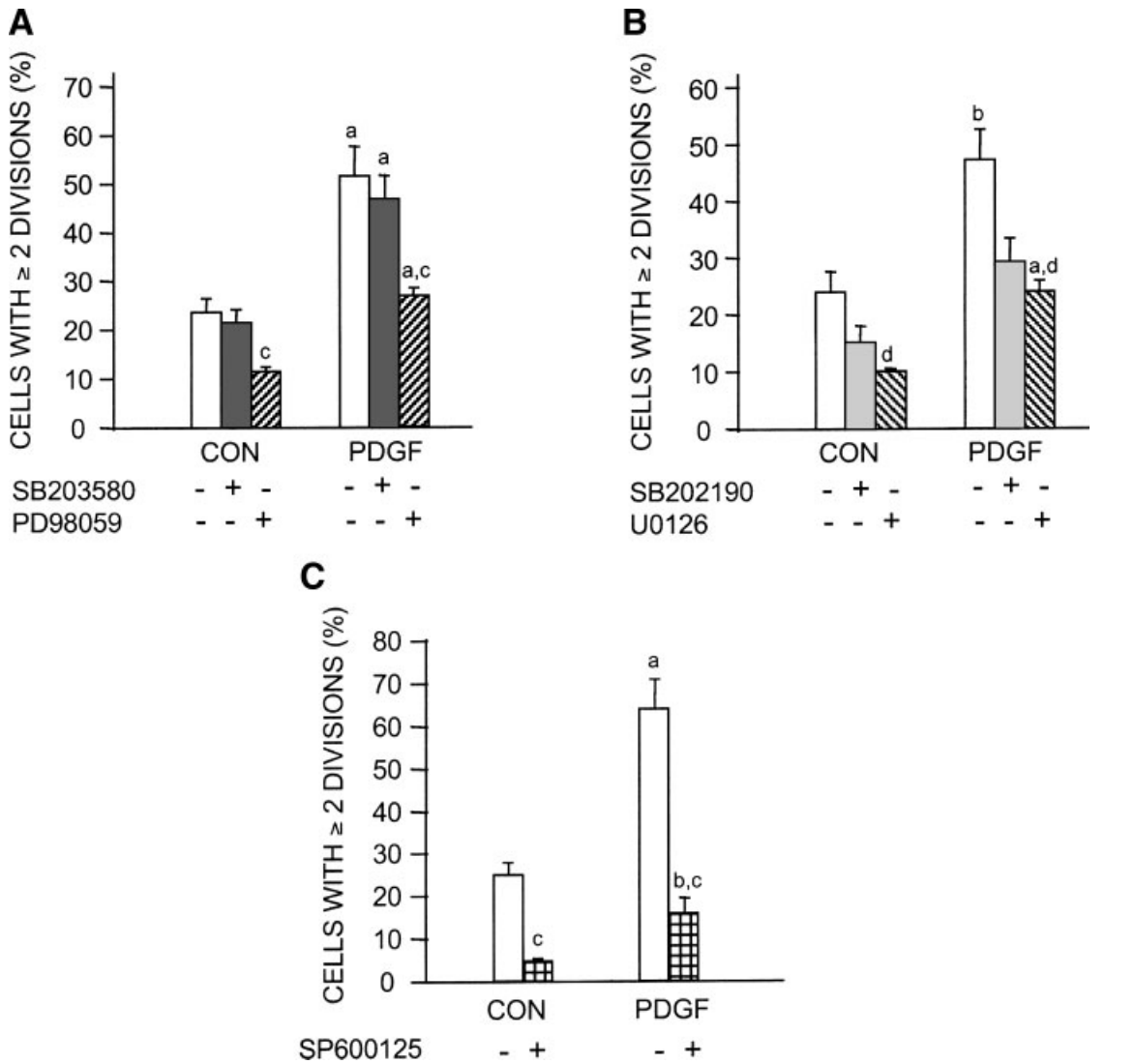

Fig. 6. Role of MAP kinase signaling pathways in PDGF stimulated proliferation. MC3T3-E1 cells were labeled with CFSE, plated at 50,000 cells/cm^2 and grown 24 h before treating with PDGF (10 ng/ml) or vehicle for another 24 h. Inhibitors and aphidicolin were added 30 min prior to treating with vehicle or PDGF. Bars are the means ± SEM, calculated from three to four independent experiments, for the percentage of cells that underwent two or more cell divisions when compared to aphidicolin-treated cells, as described in the text. **A**: Comparison of inhibitors of the p38 kinase (SB203580, 10 μM) and ERK pathways (PD98059, 40 μM). **B**: Comparison of two other inhibitors of the p38 kinase (SB202190, 10 μM) and ERK pathways (U0126, 20 μM). **C**: Effects of the specific JNK inhibitor (SP600125, 10 μM). [a]Significant effect of PDGF, $P < 0.01$; [b]$P < 0.05$. [c]Significant effect of inhibitor, $P < 0.01$; [d]$P < 0.05$.

bone growth and remodeling, and fracture repair. PDGF has been shown to be a stimulator of migration in osteoblasts [Tsukamoto et al., 1991; Lind et al., 1995; Godwin and Soltoff, 1997]. Using a wounding model where movement of the cell front out from the wound line could be directly visualized and measured, we found that PDGF reproducibly stimulated a twofold increase in migration in MC3T3-E1 and calvarial osteoblastic cells. Migration and proliferation are often linked, both being induced by similar factors. The question arises of whether or not migration of a cell is in part a function of the movement apart of daughter cells after replication. In this regard, PDGF has been shown to stimulate proliferation in osteoblasts [Canalis et al., 1989; Zhang et al., 1991; Centrella et al., 1992; Hock and Canalis, 1994], and, using several methods to measure replication, we confirmed that PDGF is a potent mitogen for MC3T3-E1 cells. Visualization of cells undergoing DNA replication by BrdU

staining showed that both migration and replication could occur in the same cells. Treatment with an inhibitor of DNA replication, aphidicolin, inhibited cell replication by 90% and reduced the density of cells in the migrating front but had no effect on the distance that the cell front moved in either control or PDGF treated cultures. Hence, the number of cells migrating, but not the rate of migration, depended on cell proliferation. Tangkijvanich et al. [2002] have reported similar results in hepatic myofibroblasts. They observed that pretreatment with the anti-mitotic agent mitomycin C did not alter the rate of migration in the absence or presence of PDGF.

In non-osteoblastic cell models, multiple MAP kinase signaling pathways have been implicated in PDGF-stimulated migration. The p38 kinase pathway has been found to mediate PDGF-stimulated migration in smooth muscle cells [Hedges et al., 1999; Iijima et al., 2002], aortic endothelial cells [Matsumoto et al., 1999], and pancreatic stellate cells [Masamune et al., 2003]. ERK activation was shown to be necessary for PDGF-stimulated chemotaxis or migration in vascular smooth muscle cells and retinal epithelial cells [Hinton et al., 1998; Cospedal et al., 1999; Yamboliev and Gerthoffer, 2001; Kingsley et al., 2002], whereas in similar cell models, others found no effect on migration by inhibiting ERK activation [Bornfeldt et al., 1995]. Effects of inhibition of the p38 kinase and ERK pathways on cell migration have also been compared. In a study of vascular smooth muscle cells, similar contributions of the p38 and ERK pathways to PDGF-stimulated migration were found [Zhan et al., 2003]. On the other hand, in three other reports of migration of vascular smooth muscle cells [Kavurma and Khachigian, 2003], hepatic myofibroblasts [Tangkijvanich et al., 2002], and corneal epithelial cells [Sharma et al., 2003], the p38 kinase pathway but not the ERK pathway appeared to mediate PDGF-induced cell migration. In the current report, PDGF-stimulated migration of MC3T3-E1 cells out from the wound line was blocked by both of the selective p38 MAP kinase inhibitors, SB203580 and SB202190, but was unaffected by either of two selective ERK inhibitors, PD98059 and U0126.

Cells also migrated out from the wound line in serum free medium in the absence of PDGF. This migration might be stimulated by growth factors or cytokines released on wounding of the cell monolayer. In an immortalized rat calvarial cell line, wounding or conditioned media from the wounded monolayer was shown to phosphorylate ERK in the unperturbed cell layer [Katz et al., 2002]. The selective ERK inhibitors, however, had no effect on basal MC3T3-E1 cell migration in our study. The absence of any effect of the p38 kinase inhibitors on basal migration suggests that the autocrine/paracrine factor is not PDGF.

Disruption or inhibition of the JNK pathway has been shown to inhibit basal migration in smooth muscle cells [Cospedal et al., 1999] and dermal fibroblasts [Javelaud et al., 2003]. Retinoic acid has been shown to inhibit cardiac neural crest migration by blocking JNK activity [Li et al., 2001]. A recent study has demonstrated that phosphorylation of paxillin, a focal adhesion adaptor protein, by JNK, is required for the rapid movement of fish keratocytes and rat bladder epithelial cells [Huang et al., 2003]. In several studies, JNK signaling was also found to play a role in PDGF-stimulated migration in smooth muscle cells [Kavurma and Khachigian, 2003; Zhan et al., 2003]. We found that the selective JNK inhibitor SP600125 decreased basal and PDGF-stimulated migration in MC3T3-E1 cells by 30–50%. The effects of this inhibitor were not specific for PDGF since the inhibitor did not significantly reduce the PDGF-stimulated increase in migration (treated/control).

Our observation that inhibition of proliferation did not block migration suggests that some of the signaling pathways in migration and proliferation might be independent. ERK is the MAP kinase thought to be predominantly involved in the regulation of mitogenesis in multiple cell types [Pearson et al., 2001]. ERK kinase has been shown to be essential for PDGF-stimulated mitogenesis in smooth muscle cells, fibroblasts, and other non-osteoblastic cells [Bornfeldt et al., 1995; Taylor, 2000; Tangkijvanich et al., 2002; Kavurma and Khachigian, 2003; Zhan et al., 2003]. Two studies, in which effects of ERK and p38 inhibitors in hepatic myofibroblasts [Tangkijvanich et al., 2002] and corneal epithelial cells [Sharma et al., 2003] were compared, found that the ERK pathway and not the p38 pathway mediated PDGF-stimulated proliferation.

Our results in osteoblastic MC3T3-E1 cells are similar to those of Tangkijvanich et al. [2002] and Sharma et al. [2003]. Both of the

ERK inhibitors, used at doses that had no effect on PDGF-stimulated migration, decreased cell division in control and PDGF treated cultures by about 50%. Hence, the effect of ERK inhibitors on proliferation was not specific for PDGF-stimulated proliferation. As discussed above, it is possible that the wounding itself stimulated the activation of signaling pathways leading to proliferation. Both of the p38 MAP kinase inhibitors SB203580 and SB202190 blocked PDGF-induced migration, and although there was a non-significant trend for SB202190 to decrease proliferation, SB203580 had no effect on proliferation. The reason for the difference in the effects on proliferation is unclear. Both of the p38 inhibitors are pyridinylimidazole compounds that inhibit p38 MAP kinase α and β isoforms but not γ and δ isoforms [Singh et al., 1999; Davies et al., 2000]. They are both considered to be reasonably specific inhibitors and their degree of specificity is similar, although SB203580 may be about five times as effective in inhibiting the β isoform as SB202190 [Davies et al., 2000]. SB202190 has been shown to negatively regulate the p42/44 MAP kinase (ERK) pathway [Singh et al., 1999], and it is possible that this crossover could be more important for SB202190 than for SB203580.

All three kinases, ERK, p38, and JNK, have been examined in smooth muscle cells and it was considered that all contributed to PDGF-stimulated proliferation in smooth muscle cells [Kavurma and Khachigian, 2003; Zhan et al., 2003]. In our study, the selective JNK inhibitor, SP600125, used at the lowest dose that inhibited migration or proliferation, inhibited proliferation in both control and PDGF-treated cultures by 75–80% but did not prevent a PDGF-stimulated increase in proliferation. The inhibition of cell division was similar to that seen with aphidicolin in the CFSE flow cytometry studies (data not shown) and could reflect a generalized toxicity. However, aphidicolin itself did not inhibit migration. Nevertheless, it should be noted that some studies have found SP600125 to be very non-specific in its effects [Bain et al., 2003].

In summary, we have shown that PDGF-stimulated osteoblastic migration and proliferation can be differentially regulated. The dissection of the signaling pathways for these processes might lead to novel therapeutic interventions in wound healing and fracture repair.

ACKNOWLEDGMENTS

The authors thank Shikhar Mehrotra for helping with the FACS analysis. The study was supported by Biomedical Research Award from the American Association of Orthodontics Foundation to K.W. and C.P. and by National Institute of Health ROI-AR47673 to C.P. and AR-44815 to S.M.K.

REFERENCES

Anand-Apte B, Zetter BR, Viswanathan A, Qiu RG, Chen J, Ruggieri R, Symons M. 1997. Platelet-derived growth factor and fibronectin-stimulated migration are differentially regulated by the Rac and extracellular signal-regulated kinase pathways. J Biol Chem 272:30688–30692.

Andrew JG, Hoyland JA, Freemont AJ, Marsh DR. 1995. Platelet-derived growth factor expression in normally healing human fractures. Bone 16:455–460.

Bain J, McLauchlan H, Elliott M, Cohen P. 2003. The specificities of protein kinase inhibitors: An update. Biochem J 371:199–204.

Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu W, Leisten JC, Motiwala A, Pierce S, Satoh Y, Bhagwat SS, Manning AM, Anderson DW. 2001. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. Proc Natl Acad Sci USA 98:13681–13686.

Bolander ME. 1992. Regulation of fracture repair by growth factors. Proc Soc Exp Biol Med 200:165–170.

Bornfeldt KE, Raines EW, Graves LM, Skinner MP, Krebs EG, Ross R. 1995. Platelet-derived growth factor. Distinct signal transduction pathways associated with migration versus proliferation. Ann NY Acad Sci 766: 416–430.

Canalis E, McCarthy TL, Centrella M. 1989. Effects of platelet-derived growth factor on bone formation in vitro. J Cell Physiol 140:530–537.

Centrella M, McCarthy TL, Kusmik WF, Canalis E. 1992. Isoform-specific regulation of platelet-derived growth factor activity and binding in osteoblast-enriched cultures from fetal rat bone. J Clin Invest 89:1076–1084.

Clerk A, Sugden PH. 1998. The p38-MAPK inhibitor, SB203580, inhibits cardiac stress-activated protein kinases/c-Jun N-terminal kinases (SAPKs/JNKs). FEBS Lett 426:93–96.

Cospedal R, Lobo M, Zachary I. 1999. Differential regulation of extracellular signal-regulated protein kinases (ERKs) 1 and 2 by cAMP and dissociation of ERK inhibition from anti-mitogenic effects in rabbit vascular smooth muscle cells. Biochem J 342:407–414.

Davies SP, Reddy H, Caivano M, Cohen P. 2000. Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J 351:95–105.

Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, Van Dyk DE, Pitts WJ, Earl RA, Hobbs F, Copeland RA, Magolda RL, Scherle PA, Trzaskos JM. 1998. Identification of a novel inhibitor of mitogen-activated protein kinase kinase. J Biol Chem 273: 18623–18632.

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.

Fox T, Coll JT, Xie X, Ford PJ, Germann UA, Porter MD, Pazhanisamy S, Fleming MA, Galullo V, Su MS, Wilson KP. 1998. A single amino acid substitution makes ERK2 susceptible to pyridinyl imidazole inhibitors of p38 MAP kinase. Protein Sci 7:2249–2255.

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.

Gilardetti RS, Chaibi MS, Stroumza J, Williams SR, Antoniades HN, Carnes DC, Graves DT. 1991. High-affinity binding of PDGF-AA and PDGF-BB to normal human osteoblastic cells and modulation by interleukin-1. Am J Physiol 261:C980–C985.

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.

Hedges JC, Dechert MA, Yamboliev IA, Martin JL, Hickey E, Weber LA, Gerthoffer WT. 1999. A role for p38(MAPK)/HSP27 pathway in smooth muscle cell migration. J Biol Chem 274:24211–24219.

Heldin CH, Westermark B. 1999. Mechanism of action and in vivo role of platelet-derived growth factor. Physiol Rev 79:1283–1316.

Hinton DR, He S, Graf K, Yang D, Hsueh WA, Ryan SJ, Law RE. 1998. Mitogen-activated protein kinase activation mediates PDGF-directed migration of RPE cells. Exp Cell Res 239:11–15.

Hock JM, Canalis E. 1994. Platelet-derived growth factor enhances bone cell replication, but not differentiated function of osteoblasts. Endocrinology 134:1423–1428.

Huang C, Rajfur Z, Borchers C, Schaller MD, Jacobson K. 2003. JNK phosphorylates paxillin and regulates cell migration. Nature 424:219–223.

Iijima K, Yoshizumi M, Hashimoto M, Akishita M, Kozaki K, Ako J, Watanabe T, Ohike Y, Son B, Yu J, Nakahara K, Ouchi Y. 2002. Red wine polyphenols inhibit vascular smooth muscle cell migration through two distinct signaling pathways. Circulation 105:2404–2410.

Jacob A, Molkentin JD, Smolenski A, Lohmann SM, Begum N. 2002. Insulin inhibits PDGF-directed VSMC migration via NO/ cGMP increase of MKP-1 and its inactivation of MAPKs. Am J Physiol Cell Physiol 283:C704–C713.

Javelaud D, Laboureau J, Gabison E, Verrecchia F, Mauviel A. 2003. Disruption of basal JNK activity differentially affects key fibroblast functions important for wound healing. J Biol Chem 278:24624–24628.

Johnson GL, Lapadat R. 2002. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science 298:1911–1912.

Katz RW, Teng SY, Thomas S, Landesberg R. 2002. Paracrine activation of extracellular signal-regulated kinase in a simple in vitro model of wounded osteoblasts. Bone 31:288–295.

Kavurma MM, Khachigian LM. 2003. ERK, JNK, and p38 MAP kinases differentially regulate proliferation and migration of phenotypically distinct smooth muscle cell subtypes. J Cell Biochem 89:289–300.

Khan SN, Bostrom MP, Lane JM. 2000. Bone growth factors. Orthop Clin North Am 31:375–388.

Kingsley K, Huff JL, Rust WL, Carroll K, Martinez AM, Fitchmun M, Plopper GE. 2002. ERK1/2 mediates PDGF-BB stimulated vascular smooth muscle cell proliferation and migration on laminin-5. Biochem Biophys Res Commun 293:1000–1006.

Li J, Molkentin JD, Colbert MC. 2001. Retinoic acid inhibits cardiac neural crest migration by blocking c-Jun N-terminal kinase activation. Dev Biol 232:351–361.

Lieberman JR, Daluiski A, Einhorn TA. 2002. The role of growth factors in the repair of bone. Biology and clinical applications. J Bone Joint Surg Am 84-A:1032–1044.

Lind M, Deleuran B, Thestrup-Pedersen K, Soballe K, Eriksen EF, Bunger C. 1995. Chemotaxis of human osteoblasts. Effects of osteotropic growth factors. Apmis 103:140–146.

Masamune A, Satoh M, Kikuta K, Sakai Y, Satoh A, Shimosegawa T. 2003. Inhibition of p38 mitogen-activated protein kinase blocks activation of rat pancreatic stellate cells. J Pharmacol Exp Ther 304:8–14.

Matsumoto T, Yokote K, Tamura K, Takemoto M, Ueno H, Saito Y, Mori S. 1999. Platelet-derived growth factor activates p38 mitogen-activated protein kinase through a Ras-dependent pathway that is important for actin reorganization and cell migration. J Biol Chem 274: 13954–13960.

Pang L, Sawada T, Decker SJ, Saltiel AR. 1995. Inhibition of MAP kinase kinase blocks the differentiation of PC-12 cells induced by nerve growth factor. J Biol Chem 270: 13585–13588.

Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K, Cobb MH. 2001. Mitogen-activated protein (MAP) kinase pathways: Regulation and physiological functions. Endocr Rev 22:153–183.

Reddi AH, Roodman D, Freeman C, Mohla S. 2003. Mechanisms of tumor metastasis to the bone: Challenges and opportunities. J Bone Miner Res 18:190–194.

Rydziel S, Canalis E. 1996. Expression and growth factor regulation of platelet-derived growth factor B transcripts in primary osteoblast cell cultures. Endocrinology 137: 4115–4119.

Sharma GD, He J, Bazan HE. 2003. p38 and ERK1/2 coordinate cellular migration and proliferation in epithelial wound healing: Evidence of cross-talk activation between MAP kinase cascades. J Biol Chem 278:21989–21997.

Siegbahn A, Hammacher A, Westermark B, Heldin CH. 1990. Differential effects of the various isoforms of platelet-derived growth factor on chemotaxis of fibroblasts, monocytes, and granulocytes. J Clin Invest 85: 916–920.

Singh RP, Dhawan P, Golden C, Kapoor GS, Mehta KD. 1999. One-way cross-talk between p38(MAPK) and p42/44(MAPK). Inhibition of p38(MAPK) induces low density lipoprotein receptor expression through activation of the p42/44(MAPK) cascade. J Biol Chem 274: 19593–19600.

Tangkijvanich P, Santiskulvong C, Melton AC, Rozengurt E, Yee HF, Jr. 2002. p38 MAP kinase mediates platelet-

derived growth factor-stimulated migration of hepatic myofibroblasts. J Cell Physiol 191:351–361.

Taylor CC. 2000. Platelet-derived growth factor activates porcine thecal cell phosphatidylinositol-3-kinase-Akt/PKB and ras-extracellular signal-regulated kinase-1/2 kinase signaling pathways via the platelet-derived growth factor-beta receptor. Endocrinology 141:1545–1553.

Tsukamoto T, Matsui T, Fukase M, Fujita T. 1991. Platelet-derived growth factor B chain homodimer enhances chemotaxis and DNA synthesis in normal osteoblast-like cells (MC3T3-E1). Biochem Biophys Res Commun 175: 745–751.

Yamboliev IA, Gerthoffer WT. 2001. Modulatory role of ERK MAPK-caldesmon pathway in PDGF-stimulated migration of cultured pulmonary artery SMCs. Am J Physiol Cell Physiol 280:C1680–C1688.

Yu J, Moon A, Kim HR. 2001. Both platelet-derived growth factor receptor (PDGFR)-alpha and PDGFR-beta promote murine fibroblast cell migration. Biochem Biophys Res Commun 282:697–700.

Zhan Y, Kim S, Izumi Y, Izumiya Y, Nakao T, Miyazaki H, Iwao H. 2003. Role of JNK, p38, and ERK in platelet-derived growth factor-induced vascular proliferation, migration, and gene expression. Arterioscler Thromb Vasc Biol 23:795–801.

Zhang L, Leeman E, Carnes DC, Graves DT. 1991. Human osteoblasts synthesize and respond to platelet-derived growth factor. Am J Physiol 261:C348–C354.