Journal of Cellular Biochemistry 95:1135–1145 (2005)

Role of c-Jun N-terminal Kinase in the PDGF-Induced Proliferation and Migration of Human Adipose Tissue-Derived Mesenchymal Stem Cells

Yong Jung Kang, Eun Su Jeon, Hae Young Song, Jae Suk Woo, Jin Sup Jung, Yong Keun Kim, and Jae Ho Kim*

Department of Physiology, Medical Research Institute, College of Medicine, Pusan National University, Busan 602-739, Republic of Korea

Abstract Platelet-derived growth factor (PDGF) is a critical regulator of proliferation and migration for mesenchymal type cells. In this study, we examined the role of mitogen-activated protein (MAP) kinases in the PDGF-BB-induced proliferation and migration of human adipose tissue-derived mesenchymal stem cells (hATSCs). The PDGF-induced proliferation was prevented by a pretreatment with the c-Jun N-terminal kinase (JNK) inhibitor, SP600125. However, it was not prevented by a pretreatment with a p38 MAP kinase inhibitor, SB202190, and a specific inhibitor of the upstream kinase of extracellular signal-regulated kinase (ERK1/2), U0126. Treatment with PDGF induced the activation of JNK and ERK in hATSCs, and pretreatment with SP600125 specifically inhibited the PDGF-induced activation of JNK. Treatment with PDGF induced the cell cycle transition from the G0/G1 phase to the S phase, the elevated expression of cyclin D1, and the phosphorylation of Rb, which were prevented by a pretreatment with SP600125. In addition, the PDGF-induced migration of hATSCs was completely blocked by a pretreatment with SP600125, but not with U0126 and SB202190. These results suggest that JNK protein kinase plays a key role in the PDGF-induced proliferation and migration of mesenchymal stem cells. J. Cell. Biochem. 95: 1135–1145, 2005.

Key words: mesenchymal stem cells; proliferation; migration; PDGF; JNK

Human adipose tissue-derived mesenchymal stem cells (hATSCs) can differentiate toward the osteogenic, adipogenic, myogenic, and chondrogenic lineage [Halvorsen et al., 2001; Erickson et al., 2002; Zuk et al., 2002; Barry and Murphy, 2004]. hATSCs have generated a great deal of interest because of their tantalizing potential use for clinical applications and due to their stem cell properties such as self-renewal, migration, and multi-lineage differentiation capacity. Mesenchymal stem cells (MSCs) are known to be highly proliferative ex vivo, they migrate into injury sites after transplantation, and they differentiate to diverse cell types in local environments [Caplan, 1991; Barry and Murphy, 2004]. To use hATSCs in clinical application, it is necessary to multiply hATSCs in vitro after isolating them from adipose tissues, and they must be induced to efficiently migrate into injury cites. However, the molecular mechanisms involved in the regulation of proliferation, migration, and differentiation of hATSCs are still unknown.

Platelet-derived growth factor (PDGF) is a principal mitogen, survival factor, and chemoattractant for mesenchymal type cells, including MSCs [Kuznetsov et al., 1997; Heldin et al., 1998; Fiedler et al., 2002]. PDGF consists

Abbreviations used: hATSCs, human adipose tissue-derived mesenchymal stem cells; MSCs, mesenchymal stem cells; PDGF, platelet-derived growth factor; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH2-terminal kinase; α-MEM, minimum essential medium alpha; PBS, phosphate-buffered saline; FBS, fetal bovine serum; RT-PCR, reverse transcriptase-polymerase chain reaction; PPARγ2, peroxisome-proliferating activated receptor 2; LPL, lipoprotein lipase; ALP, alkaline phosphatase; DMSO, dimethylsulfoxide.

Grant sponsor: The Korea Science & Engineering Foundation; Grant number: R05-2003-000-12178-0; Grant sponsor: Pusan National University.

*Correspondence to: Jae Ho Kim, Department of Physiology, College of Medicine, Pusan National University, 1-Ga, Ami-Dong, Suh-Gu, Busan 602-739, Republic of Korea.
E-mail: jhkimst@pusan.ac.kr

Received 22 November 2004; Accepted 23 February 2005
DOI 10.1002/jcb.20499

of homodimers or heterodimers of A- and B-polypeptide chains, and they exert their biological effects by binding to two structurally related tyrosine kinase receptors, PDGF-α receptor and PDGF-β receptor [Heldin et al., 1998]. Upon binding to the ligand, the PDGF receptors homo- or hetero-dimerize and then phosphorylate each other on specific tyrosine residues, and thus, they subsequently activate diverse signaling cascades including SHP-2, Src, PLC-γ, Ras, protein kinase A, phosphatidylinositol 3-kinase, and the mitogen-activated protein (MAP) kinases [Heldin et al., 1998; Jones and Kazlauskas, 2000]. Activation of these signaling pathways contributes to the PDGF-induced physiological responses, such as proliferation, actin cytoskeleton rearrangements, and chemotaxis [Heldin and Westermark, 1999]. However, the involvement of the signaling pathways in the PDGF-induced proliferation and migration of MSCs is still unclear.

MAP kinases, including extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), and p38, are the major signal transduction molecules regulated by growth factors, cytokines, and stress [Chang and Karin, 2001]. Each member of the MAP kinase family is activated by phosphorylation and they subsequently translocates into the nucleus to promote diverse physiological responses, including cell proliferation, apoptosis, and a variety of gene expressions. ERK is mainly activated by growth-promoting factors, and it participates in cell proliferation or it contributes to survival in a variety of cells. On the other hand, JNK and p38 are mainly activated by stress signals and both are known to induce apoptosis in many kinds of cells [Davis, 2000; Chang and Karin, 2001; Wada and Penninger, 2004]. Activated JNK phosphorylates serine-63 and serine-73 residues of c-Jun, and it increases the transcription activity of the AP-1 complex, which is responsible for transcription of the apoptosis-related genes [Davis, 2000; Chang and Karin, 2001; Wada and Penninger, 2004]. However, it is still unknown that types of MAP kinases are involved in the PDGF-induced proliferation and migration of MSCs.

In this study, we evaluated the implication of MAP kinases in the PDGF-induced proliferation and migration of MSCs by using specific inhibitors against these MAP kinases. We demonstrated evidences that JNK, but not ERK or p38 protein kinase, plays a pivotal role for the PDGF-induced physiological responses of MSCs, which are derived from adipose tissues.

MATERIALS AND METHODS

Materials

Alpha-minimum essential medium (α-MEM), phosphate-buffered saline (PBS), and trypsin were purchased from Invitrogen. Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). SP600125, propidium iodide, and MTT reagent (3-(4,5-dimethyl-2-thiozol)-2,5-diphenyl-2H-tetrazolium bromide) were purchased from Calbiochem (La Jolla, CA). U0126 and SB202190 were purchased from BIOMOL (Plymouth Meeting, PA). The anti-phospho-ERK (Thr202/204), anti-phospho-JNK (Thr183/Tyr185), anti-phospho-p38 (Thr180/Tyr182), anti-phospho-c-Jun (Ser63), and anti-phospho-Rb (Ser780) antibodies were purchased from Cell Signaling Technology (Beverly, MA). The anti-cyclin D1 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti-actin monoclonal antibody (clone C4) was purchased from MP Biomedicals (Irvine, CA). Human recombinant PDGF-BB was from R&D Systems (Minneapolis, MN). The peroxidase-labeled secondary antibodies were purchased from Amersham Biosciences (Piscataway, NJ). All the other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Cell Culture

Subcutaneous adipose tissue was obtained from elective surgeries with the patient's consent and as was approved by our Institution Review Board and hATSCs were isolated as previously reported [Kang et al., 2003]. Briefly, the liposuction tissues were washed at least three times with sterile phosphate-buffered saline, and then treated with 1 vol of collagenase type I (1g/liter of Hank's balanced salt solution with 1% bovine serum albumin) for 60 min at 37°C with intermittent shaking. The floating adipocytes were separated from the stromal-vascular fraction by centrifugation (300*g*) for 5 min. The cellular pellet was resuspended in α-MEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin, and the cells were plated in tissue culture dishes at 3,500 cells/cm^2. The primary hATSCs were cultured for 4–5 days until they reached confluence, and these cells were defined as passage "0." The passage number of hATSCs

used in these experiments was 3–10, since the self-renewal capacity of hATSCs can be maintained for up to 15 passages without any significant increase of doubling time [Lee et al., 2004], and the PDGF-induced migration ability was not affected up to passage 15. In the current study, hATSCs from four different donors were used for experiments, and these cells showed similar characteristics for the PDGF-induced proliferation and migration. The expression profiles of the hATSCs were highly similar to those expression profiles of human bone marrow-derived mesenchymal stem cells, as was observed by using flow cytometric analysis and microarray analysis [Lee et al., 2004]. The hATSCs were positive for CD29, CD44, CD90, and CD105, all of which have been reported to be marker proteins of mesenchymal stem cells. However, c-kit, CD34, and CD14, which are known as hematopoietic markers, were not expressed in the hATSCs.

Induction of Adipogenic and Osteogenic Differentiation

For induction of adipogenic differentiation, confluent hATSCs were incubated in adipogenic medium (10% FBS, 1 μM dexamethasone, 100 μg/ml 3-isobutyl-1-methylxanthine, 5 μg/ml insulin, and 60 μM indomethacin in α-MEM) for 1 week, and the accumulation of intracellular triglyceride droplets was visualized by using Oil Red O staining as described previously [Novikoff et al., 1980]. Osteogenic differentiation was induced by exposing the cells to osteogenic medium (10% FBS, 0.1 mM dexamethasone, 10 μM β-glycerophosphate, and 50 μg/ml ascorbic acid in α-MEM) over 2–3 weeks, and the extracellular matrix calcification was visualized by using Alizarin red staining as described previously [Keila et al., 1994]. The phase contrast images were photographed by a digital camera that was mounted in an inverted microscope (Olympus IX70).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis

The total RNAs and subsequent messenger RNAs from the hATSCs were purified by using the mRNA isolation system (Novagen, Darmstadt, Germany), as described in the manufacturer's manual. Three microliters of mRNAs was used to make cDNA with oligo dT primers, and then one microliter of the synthesized cDNA was used as a template for the PCR reaction. PCR primers were used to amplify peroxisome-proliferating activated receptor 2 (PPARγ2: sense 5′-GCTGTTATGGGTGAAACTCTG-3′ and antisense 5′-ATAAGGTGGAGATGCAGGTTC-3′, 352 bp), lipoprotein lipase (LPL: sense 5′-ATGGAGAGCAAAGCCCTGCTC-3′ and antisense 5′-TACAGGGCGGCCACAAGTTTT-3′, 298 bp); alkaline phosphatase (ALP: sense 5′-TGGAGCTTCAGAAGCTCAACACCA-3′ and antisense 5′-ATCTCGTTGTCTGAGTACCAGTCC-3′, 453 bp); GAPDH (sense 5′-TCCATGACAACTTTGGTATCG-3′ and antisense 5′-TGTAGCCAAATTCGTTGTCA-3′). PCR was carried out for the initial denaturation of 1 cycle at 95°C for 2 min, followed by 30 cycles of denaturation (95°C for 30 s), annealing for 1 min, and extension (72°C for 2 min) with 2.5 units of Takara *Ex Taq* polymerase (Takara Shuzo Co., Otsu, Shiga, Japan) in a PTC-100 DNA amplification system (MJ Research, Inc., Watertown, MA). This was followed by a final extension step of 72°C for 10 min. At the end of the PCR amplification, the PCR products were analyzed by 1.2% agarose gel electrophoresis and ethidium bromide staining.

Cell Proliferation Assay

Cell proliferation was determined by MTT assay [Alley et al., 1988] and by counting the number of viable cells. For the MTT assay, hATSCs were seeded in a 24-well culture plate at a density of 2×10^4 cells/well, cultured for 48 h in the growth media, serum-starved for 24 h, and then treated with or without various reagents for the indicated times. The cells were washed twice with 500 μl of phosphate-buffered saline and incubated with 100 μl of MTT (0.5 mg/ml) for 2 h at 37°C. The formazan granules generated by the live cells were dissolved in 100 μl dimethylsulfoxide (DMSO), and the absorbance at 562 nm was monitored by using a PowerWave$_x$ microplate spectrophotometer (Bio-Tek Instruments, Inc., Winooski, VT). For the direct counting of cell number, the reagents-treated hATSCs were harvested by trypsinization, suspended in phosphate-buffered saline, and the number of cells was counted by using a hemocytometer.

Western Blotting

The confluent, serum-starved hATSCs were treated with the appropriate conditions, washed with ice-cold phosphate-buffered saline, and then lysed in lysis buffer (20 mM Tris-

HCl, 1 mM EGTA, 1 mM EDTA, 10 mM NaCl, 0.1 mM PMSF, 1 mM Na_3VO_4, 30 mM sodium pyrophosphate, 25 mM β-glycerol phosphate, and 1% Triton X-100, pH 7.4). The lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane, and then stained with 0.1% Ponceau S solution (Sigma-Aldrich). After blocking with 5% nonfat milk, the membranes were immunoblotted with various antibodies, and the bound antibodies were visualized with horseradish peroxidase-conjugated rabbit IgG antibodies and by using the enhanced chemiluminescence Western blotting system (ECL, Amersham Biosciences). In some of the experiments, the Western blotting results were quantified by using a scanning densitometer and NIH Image software.

JNK Activity Assay

The JNK activity was measured by a glutathione based pull-down method using a kit from Cell Signaling Technology (Beverly, MA) according to the manufacturer's directions. Briefly, the cell extracts (0.2 mg of protein) were incubated with 1 μg of immobilized glutathione-*S*-transferase-c-Jun (1–79) fusion protein, centrifuged, washed, and incubated in kinase buffer (25 mM Tris-HCl, pH 7.5, 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$, and 10 μM ATP) for 30 min at 30°C. The phosphorylated substrate was detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and performing immunoblotting using phospho-specific c-Jun antibody.

Measurement of Cell Cycle

The DNA content was measured following the staining of the cells with propidium iodide. The serum-starved hATSCs were treated under the appropriate conditions for 24 h, subsequently trypsinized, washed once in cold PBS, and then fixed in 70% ethanol at −20°C overnight. The fixed cells were pelleted and stained in a propidium iodide solution (50 μg/ml propidium iodide, 50 μg/ml RNase A, 0.1% Triton X-100, and 0.1 mM EDTA) in the dark at 4°C for 1 h prior to flow cytometric quantification of their DNA by a FACScan (Becton Dickinson).

Wounding Assay

The hATSCs were plated onto 24-well culture dishes that had been coated with 10 μg/ml fibronectin, so that they became confluent in the wells following their attachment. The confluent hATSCs were serum-starved for 24 h, wounded with a 200 μl pipette tip, washed with PBS, and the floating cells were removed by PBS washing. Serum-free media containing PDGF-BB and/or SP600125 were added to the wells and the cells were incubated for an additional 20 h. Migration of the wounded cells was evaluated with an inverted microscope (Olympus IX70) and photographed with a digital camera.

Chemotactic Migration Analysis

Migration of the hATSCs was examined according to the procedure described by Law et al. with a slight modification [Law et al., 1996]. Briefly, the hATSCs were suspended at a concentration of 2×10^5 cells/ml in α-MEM and 0.1 ml aliquots of the cell suspension (2×10^4 cells) were added to the top chamber of the gelatin-treated Transwell polycarbonate membranes (having 8-μm pores) in 24-well plates. The lower Transwell compartments contained 0.6 ml of α-MEM, with or without PDGF-BB or the protein kinase inhibitors. For the checkerboard assay, PDGF-BB was added to either the upper or lower chamber at the indicated concentrations. The incubation was continued for 6 h at 37°C, the filters were then disassembled, and the upper surface of each filter was scraped free of cells by wiping it with a cotton swab. The cells that had migrated to the underside of the filter were fixed for 1 min with methanol and stained for 20 min with hematoxylin and eosin. The number of cells that had migrated to the lower surface of each filter was counted in different high power fields at a magnification of 400.

RESULTS

Potential of hATSCs to Differentiate Toward Adipogenic and Osteogenic Lineages

The potential of hATCSs to differentiate toward adipocytes and osteoblasts has been demonstrated in a previous report [Lee et al., 2004]. To further characterize the multi-lineage differentiation of hATSCs, in this present study, we examined the expression levels of the lineage-specific genes by RT-PCR analysis. As shown in Figure 1A, treatment of the hATSCs with adipogenic medium for 7 days resulted in

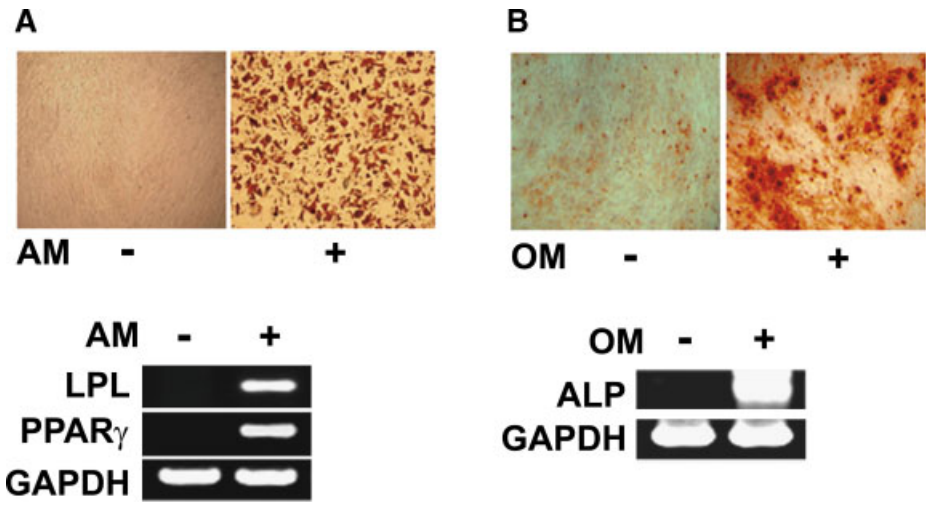

Fig. 1. Differentiation of human adipose tissue-derived mesenchymal stem cells (hATSCs) toward Adipogenic or Osteogenic lineages. **A**: hATSCs were exposed to adipogenic medium (AM) or to control medium for 7 days, and the cells were then fixed and stained with Oil Red O and photographed (upper panels). The expression levels of LPL, PPARγ2, and GAPDH were determined by reverse transcriptase-polymerase chain reaction (RT-PCR) with the indicated primers as described under ''Materials and Methods'' (lower panels). **B**: hATSCs were exposed to osteogenic medium (OM) or to control medium for 21 days, and the cells were then stained with Alizarin Red S and photographed (upper panels). The expression levels of alkaline phosphatase (ALP) and GAPDH were determined by RT-PCR with the indicated primers (lower panels). Representative data from the three independent experiments are shown. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

intracellular staining of lipid droplets with Oil Red O. The expression of LPL and transcription factor PPAR2, which are marker genes for adipogenic differentiation, were appeared in response to the induction of adipogenic differentiation. The exposure of hATSCs to the osteogenic medium for 3 weeks resulted in the calcification of extracellular matrix, as demonstrated by Alizarin red S staining (Fig. 1B). The expression of ALP, which has been shown to be involved in the osteogenic calcification, was induced by the treatment with osteogenic medium. However, in the non-induced hATSCs, the expression of LPL, PPARγ, and ALP were not detected (Fig. 1A,B). Therefore, these results suggest that hATSCs can differentiate into adipogenic or osteogenic lineages under the appropriate condition.

PDGF Induces the Proliferation of hATSCs in a Dose- and Time-Dependent Manner

To determine the effect of PDGF-BB on the proliferation of hATSCs, we measured the cell proliferation by MTT assay. Treatment of hATSCs with 25 ng/ml PDGF-BB induced proliferation that peaked at 24 h (Fig. 2A). Consistent with this PDGF-induced proliferation, the number of hATSCs maximally increased after treatment with PDGF-BB for 24 h (Fig. 2B). In addition, treatment with the indicated concentration of PDGF-BB for 24 h induced proliferation of hATSCs in a concentration-dependent manner as shown in Figure 2C.

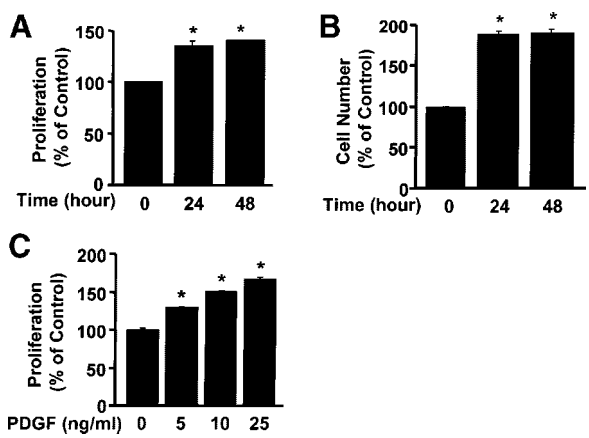

Fig. 2. Time- and dose-dependent effects of platelet-derived growth factor (PDGF) on the proliferation of hATSCs. Serum-starved hATSCs were treated with either 25 ng/ml PDGF-BB or control (1 mg/ml bovine serum albumin) for the indicated time. The effect of PDGF-BB on cell proliferation was determined by using MTT assay (**A**) and number of cells was counted by using a hemocytometer (**B**), respectively. **C:** Dose dependence of PDGF-induced proliferation. The serum-starved hATSC cells were treated with the indicated concentration of PDGF-BB for 24 h, and their proliferation was measured by using MTT assay. The results are expressed as the mean ± S.D. ($n = 9$) and as a percentage of vehicle-treated control. * indicates $P < 0.05$ vs. control (vehicle-treated cells).

JNK is Involved in PDGF-induced Proliferation of hATSCs

To clarify whether MAP kinases are involved in PDGF-induced proliferation, we examined the effect of MAP kinase inhibitors on the proliferation of hATSCs. Pretreatment with SP600125, the JNK inhibitor, completely attenuated the PDGF-induced proliferation (Fig. 3A). In contrast, pretreatment with U0126, which blocks MEK-dependent ERK activation, and SB202190, the p38 inhibitor, had no effect on PDGF-induced proliferation. These results suggest that JNK is specifically involved in PDGF-induced proliferation.

To demonstrate whether JNK can be activated by PDGF-BB treatment, we measured the effect of JNK-dependent phosphorylation of c-Jun. As shown in Figure 3B, the phosphorylation of c-Jun increased with the treatment of PDGF-BB. The PDGF-induced phosphorylation of c-Jun was completely prevented by pretreatment with the JNK inhibitor, SP600125. However, the PDGF-induced phosphorylation of c-Jun was not inhibited by pretreatment with either U0126 or SB202190. To exclude the possibility that the SP600125 nonspecifically inhibits the PDGF-induced phosphorylation of MAP kinases, we next measured the effect of

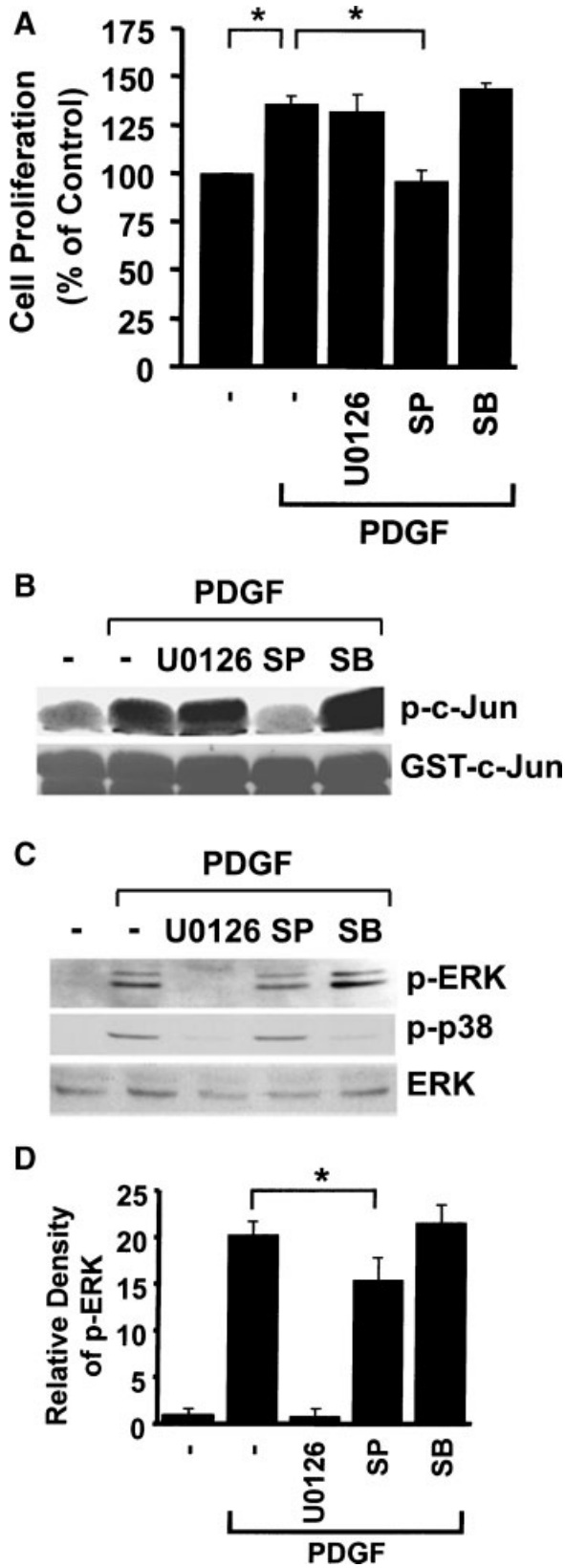

Fig. 3. The c-Jun N-terminal kinase (JNK) is involved in PDGF-induced proliferation in hATSCs. **A**: hATSCs were serum-starved for 24 h, pretreated with vehicle (0.1% DMSO), 10 μM U0126, 10 μM SP600125 (SP), or 10 μM SB202190 (SB) for 15 min at 37°C, and then the cells were exposed to 25 ng/ml PDGF-BB for 24 h. Cell proliferation was probed by MTT assay. Representative data from three independent experiments are shown. Data are expressed as mean ± S.D. ($n = 4$) and as a percentage of the vehicle-treated control. * indicates $P < 0.05$. **B**: JNK activity was measured by using a JNK assay kit (Cell Signaling Technology). Serum-starved hATSCs were pretreated with vehicle (0.1% DMSO), 10 μM U0126, 10 μM SP600125 (SP), or 10 μM SB202190 (SB) for 15 min at 37°C, and then the cells were exposed to 25 ng/ml PDGF-BB for 10 min. An immobilized GST-c-Jun protein was used to pull down JNK enzymes from the cell lysates. Upon addition of a kinase buffer and ATP, the phosphorylation of GST-c-Jun was probed by the Western blotting using anti-phospho-c-Jun (Ser63) antibody (upper panel). The amounts of GST-c-Jun were determined by staining with Ponceau S solution (lower panel). **C:** Phosphorylation of extracellular signal-regulated kinase (ERK) and p38 was probed by the Western blotting of the cell lysates with anti-phospho-ERK1/2 and phospho-p38 antibodies, and the amounts of ERK were probed by anti-ERK antibody to confirm equal loading. Representative data from three independent experiments are shown. **D:** The densities of p-ERK were quantified and normalized to the expression levels of ERK, and mean ± S.D. from three experiments was shown. * indicates $P < 0.05$.

SP600125 on the phosphorylation of ERK and p38. The phosphorylation of ERK was induced by treatment with PDGF-BB, and pretreatment of hATSCs with SP600125 caused a 25% decrease in the phosphorylation of ERK (Fig. 3C,D). However, the PDGF-induced phosphorylation of ERK was completely inhibited by pretreatment with U0126, but not with SB202190 (Fig. 3C). Since the complete inhibition of ERK activation by pretreatment with U0126 had no effects on the cell proliferation, these results suggest that the slight inhibition of the ERK activation by SP600125 pretreatment may not be responsible for the inhibitory effects of SP600125 for the PDGF-induced proliferation. Furthermore, PDGF treatment induced the phosphorylation of p38, and the PDGF-induced phosphorylation of p38 was inhibited by pretreatment with U0126 or SB202190, but it was not inhibited by treatment with SP600125 (Fig. 3C). These results suggest that SP600125 prevented the PDGF-induced proliferation by inhibiting the PDGF-induced activation of JNK.

Role of JNK in the PDGF-induced Cell Cycle Transition From G0/G1 to S Phase

The cell cycle transition from the G0/G1 phase to the S phase has been known to play a key role in the PDGF-induced proliferation [Jones and Kazlauskas, 2000]. The flow cytometry analysis of propidium iodide-stained hATSCs revealed that PDGF treatment caused the number of cells in the G0/G1 phase to decrease from 89% to 67%. In contrast, the percentage of cells in the S phase increased in response to the PDGF treatment from 4% to 25% (Fig. 4A,B). To clarify whether the anti-proliferative effects of the SP600125 inhibitor were due to delayed cell cycle progression, we examined the effect of SP600125 on cell cycle progression. Pretreatment with SP600125 significantly inhibited the PDGF-induced cell cycle progression from the G0/Gl to the S phases (Fig. 4A,B). These results suggest that JNK plays a role in the PDGF-induced proliferation by inducing progression of cell cycle.

Role of JNK for PDGF-induced Cyclin D1 Expression in hATSCs

Cell cycle re-entry and the G1/S transit in proliferative cells commences with the assembly and activation of Cdk4/6-cyclin D and the subsequent activation of Cdk2-cyclin E

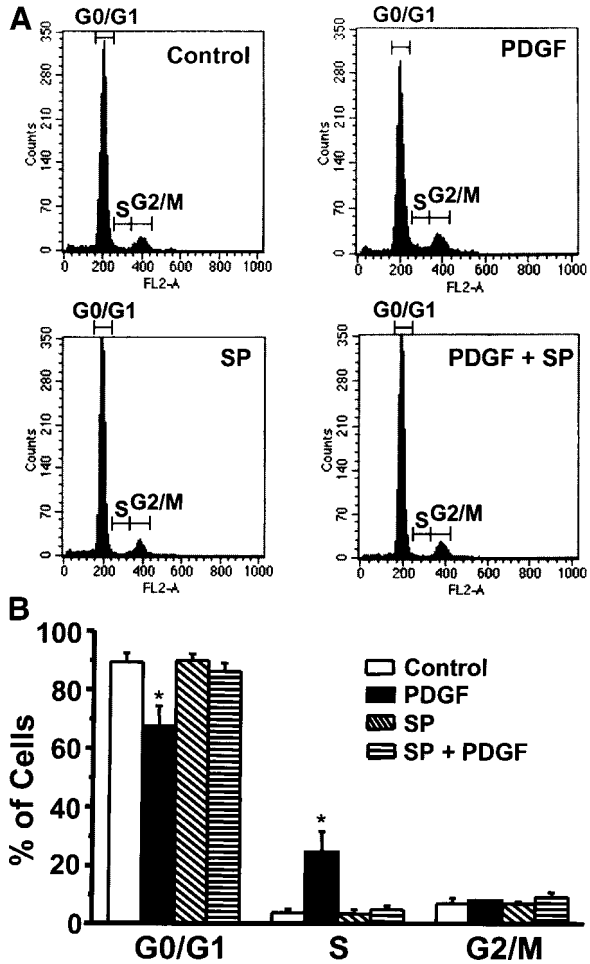

Fig. 4. JNK is involved in the PDGF-induced cell cycle transition from the G0/G1 phase to the S phase. **A**: Serum-starved hATSCs were pretreated with vehicle (0.1% DMSO) or 10 μM SP600125 (SP) for 15 min at 37°C, and then they were treated with 25 ng/ml PDGF-BB for 24 h as indicated. Phases of cell cycle were determined by performing flow cytometry analysis on the propidium iodide-stained cells. Representative data from four independent experiments are shown. **B**: the mean percentages of cells with their DNA content in each of the three phases of the cell cycle over four independent determinations are shown as mean ± S.D. (bars). *, $P < 0.05$ vs. control.

[Morgan, 1997]. To assess the functional links between JNK activation and the cell cycle progression, we analyzed the expression of cyclin D1 in response to PDGF treatment and/or SP600125 treatment. The expression levels of cyclin D1 were elevated at 6 h after treatment with PDGF-BB, and this was sustained for up to 24 h (Fig. 5A). Cyclin D1 associates with Cdk4 and the Cdk4-cyclin D1 complex plays a key role in the cell cycle transition from the G0/G1 phase to the S phases by the phosphorylation of Rb, which is a critical regulator of the G1-S cell cycle transition. Rb exists in hypophosphorylated

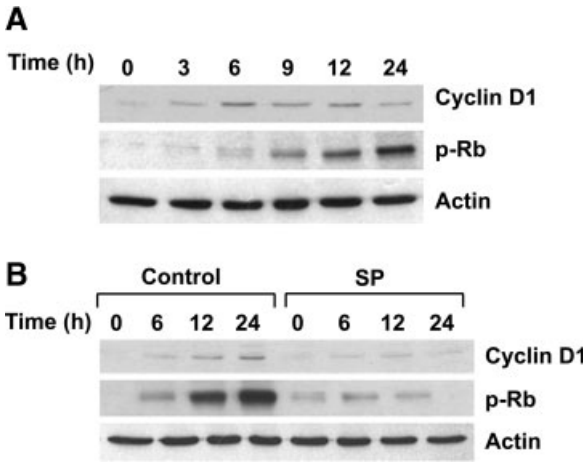

Fig. 5. The role of JNK in the PDGF-induced phosphorylation of Rb by the induction of cyclin D1. **A**: Serum-starved hATSCs were treated with 25 ng/ml PDGF-BB at 37°C for the indicated time. **B**: Serum-starved hATSCs were pretreated with vehicle (0.1% DMSO) or 10 μM SP600125 (SP) for 15 min at 37°C, and then the cells were exposed to 25 ng/ml PDGF-BB for the indicated time. The amounts of cyclin D1 and the phosphorylation of Rb were probed by the Western blotting using anti-cyclin D1 and anti-phospho-Rb antibodies, respectively. Expression of actin was determined by the Western blotting using anti-actin antibody to confirm equal loading. Representative data from three independent experiments are shown.

and hyperphosphorylated forms, and the active and hypophosphorylated form binds to and inhibits the E2F transcription factors that are required for the expression of genes implicated in DNA synthesis [Lundberg and Weinberg, 1998]. However, the inactive and hyperphosphorylated form of Rb releases and activates the transcription factor E2F-1, which allows the expression of the genes that are necessary for DNA replication and mitosis [Ishida et al., 2001]. Therefore, we next explored whether PDGF-BB treatment induces the Cdk4-cyclin D1-dependent phosphorylation of Rb. As shown in Figure 5A, PDGF time-dependently induced the phosphorylation of Rb on Ser 780, which has been previously shown to be preferentially phosphorylated by Cdk4-cyclin D1 [Mittnacht, 1998].

To gain insight into the role of JNK for the PDGF-induced expression of cyclin D1 and the phosphorylation of Rb, we examined the effect of SP600125 on the expression of cyclin D1 and the phosphorylation of Rb. As shown in Figure 5B, the PDGF-induced expression of cyclin D1 was completely prevented by pretreatment with SP600125. In addition, SP600125 pretreatment blocked the PDGF-induced phosphorylation of Rb. These results suggest that JNK plays a pivotal role in the PDGF-induced proliferation and cell cycle transition via the Cdk4-cyclin D1/Rb-dependent pathway.

JNK is Involved in the PDGF-induced Migration of hATSCs

PDGF-BB has been known to induce the chemotactic migration of mesenchymal progenitor cells [Fiedler et al., 2002]. To explore whether PDGF treatment induces migration through the JNK-dependent pathways in hATSCs, we measured migration by performing a wounding assay. Treatment with PDGF-BB induced migration into wounds, and PDGF-induced migration was specifically prevented by SP600125 pretreatment, but migration was not prevented by pretreatment with either U0126 or SB202190 (Fig. 6). By using checkerboard analysis, we next determined whether the PDGF-induced migration of hATSCs was due to directed migration in response to the gradient of chemoattractant (chemotaxis) or if it was due to random motility in the presence of the chemoattractant itself (chemokinesis). Checkerboard analysis was performed with the addition of PDGF-BB in the top, in the bottom, or in both chambers of the Transwell compartments that had 8-μm pores. As shown in Figure 7A, the addition of PDGF-BB in the lower chamber induced migration of hATSCs to the lower chamber. In contrast, addition of PDGF-BB in the upper chamber or in both the chambers abolished the migration of hATSCs. These results suggest that PDGF-BB stimulates the migration of hATSCs in the presence of a positive concentration gradient, and that chemotaxis, but not chemokinesis, is responsible for the PDGF-induced migration. To further confirm whether JNK activation is involved in PDGF-induced migration, we next examined the effect of SP600125 on the PDGF-induced migration. As shown in Figure 7B, SP600125 treatment completely prevented PDGF-induced migration. In contrast, pretreatment with either U0126 or SB202190 did not inhibit the PDGF-induced migration. These results suggest that JNK plays a crucial role in the PDGF-induced migration of hATSCs, and that ERK and p38 are not involved in this migration.

DISCUSSION

In the current study, we demonstrated that JNK is specifically involved in PDGF-induced

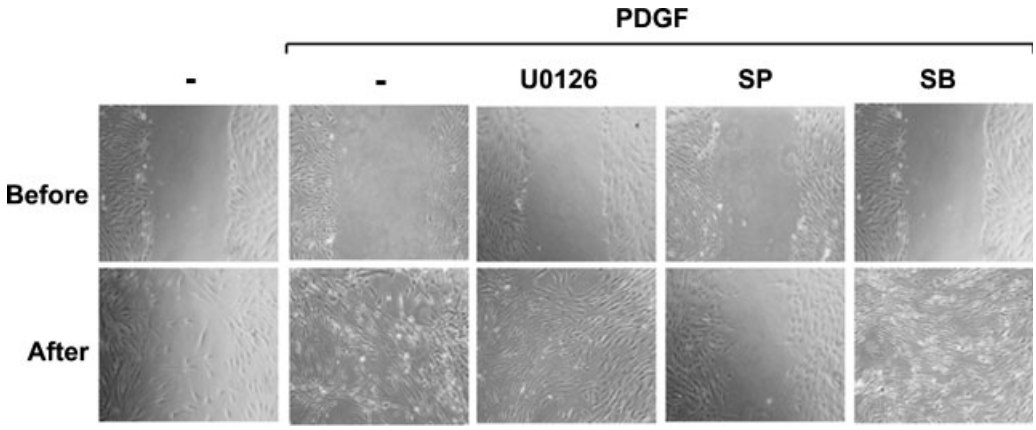

Fig. 6. The role of JNK on PDGF-induced migration of hATSCs. Confluent hATSCs were wounded by manually scraping with a pipette tip. The wounded cells were rinsed with HBBS twice, pretreated with vehicle (0.1% DMSO), 10 μM U0126, 10 μM SP600125 (SP), or 10 μM SB202190 (SB) for 15 min at 37°C, and then they were exposed 25 ng/ml PDGF-BB. Images were taken at the time the wounds were made (before, upper panels) and after incubation for 20 h (after, lower panels).

cell proliferation and in the cell cycle progression from the G0/G1 phase to the S phase. Pretreatment with the JNK-specific inhibitor SP600125 completely attenuated the PDGF-induced cell proliferation and the cell cycle transition. Furthermore, SP600125 treatment blocked the PDGF-induced expression of cyclin D1 and the phosphorylation of Rb. However, the pharmacological inhibition of ERK and p38 had no effect on the PDGF-induced proliferation. In contrast to the observations we made in the present study, it has been well documented that ERK is implicated in proliferation that is stimulated by growth factors, such as PDGF, whereas JNK has been shown to be involved in stress-induced apoptosis [Chang and Karin, 2001]. However, an accumulating body of evidence has recently suggested that JNK plays a key role in cell survival and proliferation for a variety of cell types [Davis, 2000; Manning and Davis, 2003]. Inhibition of basal JNK activity by using SP600125 or anti-sense oligonucleotides attenuated cell proliferation and cell cycle progression in the KB-3 human carcinoma cell line [Du et al., 2004], and treatment with SP600125 inhibited proliferation and cell cycle progression during liver regeneration [Schwabe et al., 2003]. In addition, both the overexpression of a dominant negative JNK mutant and the pharmacological inhibition of JNK both blocked the PDGF-induced proliferation in smooth muscle cells [Kavurma and Khachigian, 2003; Zhan et al., 2003]. These results support our conclusion that JNK plays a crucial role for the PDGF-induced proliferation and the cell cycle progression of hATSCs. However, it has recently been reported that both ERK and JNK are involved in PDGF-induced proliferation for osteoblastic MC3T3-E1 cells [Mehrotra et al.,

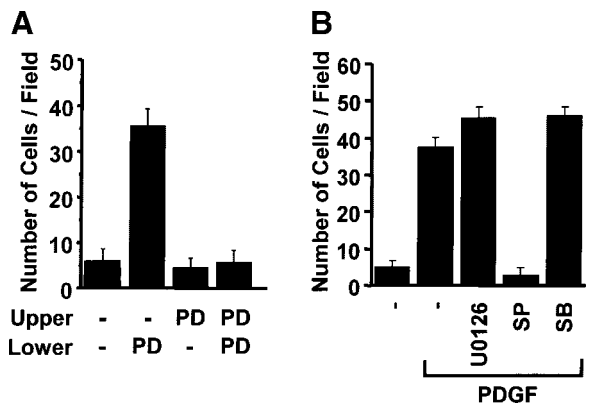

Fig. 7. JNK is involved in the PDGF-induced chemotactic migration in hATSCs. **A**: Checkerboard assays were performed as described under ''Materials and Methods.'' hATSCs were placed on gelatin-coated transwells and allowed to migrate through 8-μm polycarbonate pores. zero or 25 ng/ml PDGF-BB (PD) was added to both the top or/and bottom chambers prior to the motility assay. Migration was measured as the number of cells that migrated through the membrane in 6 h. Data are expressed as the mean ± S.D.; $n = 3$. **B**: Cell suspensions were seeded onto the upper surface of the filters, and then 25 ng/ml PDGF with vehicle (0.1% DMSO), 10 μM U0126, 10 μM SP600125 (SP), or 10 μM SB202190 (SB) were placed in the lower chambers. Cells were allowed to migrate through the membranes. After 6 h, cells on the upper surface of the filter were wiped off with a cotton swab, and the cells that had migrated to the underside of the filter were fixed, stained with Hematoxylin, and counted by brightfield microscopy at ×400 in five in random fields. Representative data from three independent experiments are shown. Data are expressed as mean ± S.D. ($n = 3$) and as a percentage of the vehicle-treated control.

2005]. Although it is still unclear whether the involvement of JNK, but not of ERK, in the PDGF-induced proliferation of hATSCs is specific to different cell types or not, the present study suggests that JNK can be a target molecule to manipulate the proliferation of MSCs.

In the present study, we demonstrated that SP600125 treatment partially attenuated the PDGF-induced activation of ERK, although ERK was not involved in the PDGF-induced proliferation of hATSCs. Consistently, the inhibitory effect of SP600125 for ERK activation has been reported in an experimental model of cerulein-induced pancreatitis [Minutoli et al., 2004]. Unexpectedly, we also observed that the PDGF-induced phosphorylation of p38 was prevented by pretreatment with U0126, the MEK1/2 inhibitor. These results raise a possibility that SP600125 and U0126 may not be specific to JNK and ERK, respectively. Further studies will be needed to demonstrate that the crossover on the specificity of these MAP kinase inhibitors depends on cell types or not.

PDGF is a powerful chemotactic factor for mesechymal lineage cells [Ronnstrand and Heldin, 2001; Fiedler et al., 2002]. In the present study, we revealed that the PDGF-induced migration of hATSCs can be attributed to directed cell migration but not to enhanced chemokinesis (Fig. 7A). Our current study demonstrated that JNK is predominantly involved in PDGF-induced wound healing and the chemotactic migration of MSCs. Since our pharmacological prevention of the PDGF-induced phosphorylation of p38 did not affect the PDGF-induced migration, it is likely that p38 is not involved in the PDGF-induced migration of hATSCs. In contrast, several studies have demonstrated that p38 as well as JNK plays a key role for PDGF-induced migration in smooth muscle cells [Kavurma and Khachigian, 2003; Zhan et al., 2003] and in osteoblastic MC3T3-E1 cells [Mehrotra et al., 2005]. These results suggest that depending on cell types, JNK and p38 are differentially involved in the PDGF-induced migration. The precise molecular mechanism involved in the JNK-mediated migration of hATSCs is not known. Recently, it has been reported that JNK regulates the cell migration of both fish keratocytes and rat bladder tumor epithelial cells through the phosphorylation of Serine 178 on paxillin, which is a key component of the focal adhesion complex [Huang et al., 2003]. Moreover, in vascular smooth muscle cells (VSMCs), which belong to the mesenchymal lineage, the overexpression of dominant negative c-Jun has been reported to block PDGF-induced migration [Ioroi et al., 2003]. Taken together, all these results raise the possibility that JNK may play a role in the PDGF-induced migration of hATSCs through the phosphorylation of c-Jun and paxillin, although further work will be required to determine the exact role of c-Jun and paxillin in the PDGF-induced migration of hATSCs.

The physiological role for the PDGF-induced proliferation and migration of hATSCs is still unclear because we lack precise knowledge regarding the anatomical distribution of hATSCs and the physiological role of PDGF within the adipose tissues. The recruitment of MSCs and their subsequent differentiation to osteoblasts is mandatory for bone development, remodeling, and repair. Since PDGF has been reported to be the major mitogen and chmotactic factor for mesenchymal cells and various osteogenic cell-lines [Pierce et al., 1991], it is likely that PDGF stimulates osteogenesis and bone remodeling by increasing the number of progenitor cells for osteoblasts in bone marrow. It has been demonstrated that PDGF was expressed in fracture hematoma and in osteoblasts during bone fracture healing, suggesting stimulatory effects of PDGF on the formation of bone [Rasubala et al., 2003], and that exogenous PDGF stimulated the fracture healing [Nash et al., 1994]. Therefore, the PDGF-induced proliferation and migration of hATSCs can be useful for in vitro amplification of hATSCs and clinical approaches towards guided tissue regeneration or tissue engineering of bone.

ACKNOWLEDGMENTS

This work was supported in part by the Basic Research Program of the Korea Science & Engineering Foundation grant (R05-2003-000-12178-0) and Pusan National University.

REFERENCES

Alley MC, Scudiero DA, Monks A, Hursey ML, Czerwinski MJ, Fine DL, Abbott BJ, Mayo JG, Shoemaker RH, Boyd MR. 1988. Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. Cancer Res 48:589–601.

Barry FP, Murphy JM. 2004. Mesenchymal stem cells: Clinical applications and biological characterization. Int J Biochem Cell Biol 36:568–584.

Caplan AI. 1991. Mesenchymal stem cells. J Orthop Res 9: 641–650.

Chang L, Karin M. 2001. Mammalian MAP kinase signaling cascades. Nature 410:37–40.

Davis RJ. 2000. Signal transduction by the JNK group of MAP kinases. Cell 103:239–252.

Du L, Lyle CS, Obey TB, Gaarde WA, Muir JA, Bennett BL, Chambers TC. 2004. Inhibition of cell proliferation and cell cycle progression by specific inhibition of basal JNK activity: Evidence that mitotic Bcl-2 phosphorylation is JNK-independent. J Biol Chem 279:11957–11966.

Erickson GR, Gimble JM, Franklin DM, Rice HE, Awad H, Guilak F. 2002. Chondrogenic potential of adipose tissue-derived stromal cells in vitro and in vivo. Biochem Biophys Res Commun 290:763–769.

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.

Halvorsen YD, Franklin D, Bond AL, Hitt DC, Auchter C, Boskey AL, Paschalis EP, Wilkison WO, Gimble JM. 2001. Extracellular matrix mineralization and osteoblast gene expression by human adipose tissue-derived stromal cells. Tissue Eng 7:729–741.

Heldin CH, Ostman A, Ronnstrand L. 1998. Signal transduction via platelet-derived growth factor receptors. Biochim Biophys Acta 1378:F79–F113.

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

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

Ioroi T, Yamamori M, Yagi K, Hirai M, Zhan Y, Kim S, Iwao H. 2003. Dominant negative c-Jun inhibits platelet-derived growth factor-directed migration by vascular smooth muscle cells. J Pharmacol Sci 91:145–148.

Ishida S, Huang E, Zuzan H, Spang R, Leone G, West M, Nevins JR. 2001. Role for E2F in control of both DNA replication and mitotic functions as revealed from DNA microarray analysis. Mol Cell Biol 21:4684–4699.

Jones SM, Kazlauskas A. 2000. Connecting signaling and cell cycle progression in growth factor-stimulated cells. Oncogene 19:5558–5567.

Kang SK, Lee DH, Bae YC, Kim HK, Baik SY, Jung JS. 2003. Improvement of neurological deficits by intracerebral transplantation of human adipose tissue-derived stromal cells after cerebral ischemia in rats. Exp Neurol 183:355–366.

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.

Keila S, Pitaru S, Grosskopf A, Weinreb M. 1994. Bone marrow from mechanically unloaded rat bones expresses reduced osteogenic capacity in vitro. J Bone Miner Res 9: 321–327.

Kuznetsov SA, Friedenstein AJ, Robey PG. 1997. Factors required for bone marrow stromal fibroblast colony formation in vitro. Br J Haematol 97:561–570.

Law RE, Meehan WP, Xi XP, Graf K, Wuthrich DA, Coats W, Faxon D, Hsueh WA. 1996. Troglitazone inhibits vascular smooth muscle cell growth and intimal hyperplasia. J Clin Invest 98:1897–1905.

Lee RH, Kim B, Choi I, Kim H, Choi HS, Suh K, Bae YC, Jung JS. 2004. Characterization and expression analysis of mesenchymal stem cells from human bone marrow and adipose tissue. Cell Physiol Biochem 14:311–324.

Lundberg AS, Weinberg RA. 1998. Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-cdk complexes. Mol Cell Biol 18:753–761.

Manning AM, Davis RJ. 2003. Targeting JNK for therapeutic benefit: From junk to gold? Nat Rev Drug Discov 2:554–565.

Mehrotra M, Krane SM, Walters K, Pilbeam C. 2005. Differential regulation of platelet-derived growth factor stimulated migration and proliferation in osteoblastic cells. J Cell Biochem 93:741–752.

Minutoli L, Altavilla D, Marini H, Passaniti M, Bitto A, Seminara P, Venuti FS, Famulari C, Macri A, Versaci A, Squadrito F. 2004. Protective effects of SP600125 a new inhibitor of c-Jun N-terminal kinase (JNK) and extracellular-regulated kinase (ERK1/2) in an experimental model of cerulein-induced pancreatitis. Life Sci 75:2853–2866.

Mittnacht S. 1998. Control of pRB phosphorylation. Curr Opin Genet Dev 8:21–27.

Morgan DO. 1997. Cyclin-dependent kinases: Engines, clocks, and microprocessors. Annu Rev Cell Dev Biol 13: 261–291.

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.

Novikoff AB, Novikoff PM, Rosen OM, Rubin CS. 1980. Organelle relationships in cultured 3T3-L1 preadipocytes. J Cell Biol 87:180–196.

Pierce GF, Mustoe TA, Altrock BW, Deuel TF, Thomason A. 1991. Role of platelet-derived growth factor in wound healing. J Cell Biochem 45:319–326.

Rasubala L, Yoshikawa H, Nagata K, Iijima T, Ohishi M. 2003. Platelet-derived growth factor and bone morphogenetic protein in the healing of mandibular fractures in rats. Br J Oral Maxillofac Surg 41:173–178.

Ronnstrand L, Heldin CH. 2001. Mechanisms of platelet-derived growth factor-induced chemotaxis. Int J Cancer 91:757–762.

Schwabe RF, Bradham CA, Uehara T, Hatano E, Bennett BL, Schoonhoven R, Brenner DA. 2003. c-Jun-N-terminal kinase drives cyclin D1 expression and proliferation during liver regeneration. Hepatology 37:824–832.

Wada T, Penninger JM. 2004. Mitogen-activated protein kinases in apoptosis regulation. Oncogene 23:2838–2849.

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.

Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH. 2002. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 13:4279–4295.