

Stromal Cell-Derived Factor-1 Induces Matrix Metalloproteinase-13 Expression in Human Chondrocytes

Yung-Cheng Chiu, Rong-Sen Yang, Kuo-Hsien Hsieh, Yi-Chin Fong, Tzong-Der Way, Tu-Sheng Lee, Hsi-Chin Wu, Wen-Mei Fu, and Chih-Hsin Tang

Department of Orthopaedics, Taichung Veterans General Hospital, Taichung, Taiwan (Y.-C.C., T.-S.L.); Departments of Orthopaedics (R.-S.Y.) and Pharmacology (W.-M.F.), College of Medicine, National Taiwan University, Taipei, Taiwan; Tung's Taichung MetroHarbor Hospital, Taichung, Taiwan (K.-H.H.); Department of Orthopaedics, China Medical University Hospital, Taichung, Taiwan (Y.-C.F.); and Departments of Pharmacology (Y.-C.C., C.-H.T.) and Biological Science and Technology (T.-D.W.), School of Medicine (H.-C.W.), China Medical University, Taichung, Taiwan

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ABSTRACT

The production of chemokine stromal cell-derived factor (SDF)-1 is significantly higher in synovial fluid of patients with osteoarthritis and rheumatoid arthritis. Matrix metalloproteinase (MMP)-13 may contribute to the breakdown of articular cartilage during arthritis. Here, we found that SDF-1 α increased the secretion of MMP-13 in cultured human chondrocytes, as shown by reverse transcriptase-polymerase chain reaction, Western blot, and zymographic analysis. SDF-1 α also increased the surface expression of CXCR4 receptor in human chondrocytes. CXCR4-neutralizing antibody, CXCR4-specific inhibitor [1-[[4-(1,4,8,11-tetrazacyclotetradec-1-ylmethyl)phenyl]methyl]-1,4,8,11-tetrazacyclotetradecane (AMD3100)], or small interfering RNA against CXCR4 inhibited the SDF-1 α -induced increase of MMP-13 expression. The transcriptional

regulation of MMP-13 by SDF-1 α was mediated by phosphorylation of extracellular signal-regulated kinases (ERK) and activation of the activator protein (AP)-1 components of c-Fos and c-Jun. The binding of c-Fos and c-Jun to the activator protein (AP-1) element on the MMP-13 promoter and the increase in luciferase activity was enhanced by SDF-1 α . Cotransfection with dominant-negative mutant of ERK2 or c-Fos and c-Jun antisense oligonucleotide inhibited the potentiating action of SDF-1 α on MMP-13 promoter activity. Taken together, our results provide evidence that SDF-1 α acts through CXCR4 to activate ERK and the downstream transcription factors (c-Fos and c-Jun), resulting in the activation of AP-1 on the MMP-13 promoter and contributing cartilage destruction during arthritis.

Chondrocytes are the only cellular components of cartilage. Under normal physiological conditions, chondrocytes maintain an equilibrium between anabolic and catabolic activities that is necessary for preservation of the structural and functional integrity of the tissue. Chondrocytes express various proteolytic enzymes such as aggrecanases and matrix metalloproteinases (MMPs), which, under normal conditions, mediate a very low matrix turnover responsible for cartilage

remodeling (Poole, 2001). However, in pathological conditions such as osteoarthritis (OA) or rheumatoid arthritis (RA), production of these enzymes by chondrocytes increases considerably, resulting in aberrant cartilage destruction (Pelletier et al., 2001; Aigner and McKenna, 2002).

MMPs are a large family of structurally related calcium- and zinc-dependent proteolytic enzymes involved in the degradation of many different components of the extracellular matrix (Nagase and Woessner, 1999; Vincenti, 2001). MMPs are expressed in a number of different cell types, and they play a key role in diverse cellular processes ranging from morphogenesis to tumor invasion and tissue remodeling (Sternlicht and Werb, 2001). Among the MMPs, MMP-13 (collagenase-3) is considered to be of particular interest because of its role in cartilage degradation. MMP-13 actively

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ABBREVIATIONS: MMP, matrix metalloproteinase; OA, osteoarthritis; RA, rheumatoid arthritis; SDF, stromal cell-derived factor; SF, synovial fluid; RT-PCR, reverse transcription-polymerase chain reaction; PCR, polymerase chain reaction; Akt, protein kinase B; ERK, extracellular signal-regulated kinase(s); AP, activator protein; p-, phosphorylated; JNK, c-Jun NH₂-terminal kinase; PD98059, 2'-amino-3'-methoxyflavone; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole; SP600125, anthra(1,9-cd)pyrazol-6(2H)-one; Ly294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; PBS, phosphate-buffered saline; ODN, oligonucleotide; AS, antisense; MS, missense; ChIP, chromatin immunoprecipitation; MAPK, mitogen-activated protein kinase; TNF, tumor necrosis factor; si, small interfering, mut, mutated.

degrades type II collagen, the major type of collagen in cartilage. MMP-13 has been previously shown to be overexpressed in OA (Reboul et al., 1996). Given their important role in cellular functions, the expression and activity of MMPs are tightly regulated at multiple levels of gene transcription, synthesis, and extracellular activity. Complete understanding of the various factors and pathways involved in regulation of MMP expression could be of interest with regard to potential therapies.

Chemokines are a family of small, soluble peptides that regulate cell movement, morphology, and differentiation. They achieve their regulation by signaling through a family of transmembrane G protein-coupled receptors. It has been reported that the concentration of an 8-kDa chemokine, stromal cell-derived factor (SDF)-1, is greatly elevated in the synovial fluid (SF) from patients with OA and RA (Kanbe et al., 2002). Such elevation of SDF-1 concentrations in SF is due, at least partially, to the stimulated synthesis of SDF-1 by synovial fibroblasts under OA and RA conditions (Kanbe et al., 2002). The source of SDF-1 in the joint is from synovium, as demonstrated by immunocytochemistry, protein chemistry, and reverse transcription-polymerase chain reaction (RT-PCR) analysis (Kanbe et al., 2002, 2004). In contrast, the SDF-1 receptor is expressed by chondrocytes in the superficial zone and in the deep zone in articular chondrocytes (Kanbe et al., 2002, 2004). Interaction of SDF-1 with its specific receptor CXCR4 on the surface of chondrocytes induces the release of MMP-3 from chondrocytes (Kanbe et al., 2002). Induction of the release of MMP-3 may contribute to the breakdown of articular cartilage during arthritis (Kanbe et al., 2002).

During OA and RA, synovium may be involved in the induction of catabolic activities in the joint cartilage. Upon stimulation, chondrocytes in the joint cartilage release matrix degradation enzymes, such as MMP-3 and -13, which result in the destruction of cartilage (Pelletier et al., 2001). It has been reported that SDF-1 induced MMP-3 activity in human chondrocytes (Kanbe et al., 2002). However, the effect of SDF-1 on MMP-13 expression in human chondrocytes is mostly unknown. Here, we found that SDF-1 α increased the expression of MMP-13. In addition, ERK, c-Fos/c-Jun, and AP-1 signaling pathways may be involved in the increase of MMP-13 expression by SDF-1 α . The elevated level of SDF-1 in SF from patients with arthritis may contribute to release MMP-13 in cartilage during arthritic pathogenesis.

Materials and Methods

Materials. Protein A/G beads; anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase; and rabbit polyclonal antibodies specific for phosphorylated (p)-ERK, p-p38, p-c-Jun NH₂-terminal kinase (JNK), p-protein kinase B (Akt), Akt, ERK, p38, JNK, c-Fos, c-Jun, and MMP-13 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). PD98059, SB203580, SP600125, and LY294002 were purchased from Calbiochem (San Diego, CA). Rabbit polyclonal antibody specific for CXCR4 was purchased from R&D Systems (Minneapolis, MN). The recombinant human SDF-1 α was purchased from PeproTech (Rocky Hill, NJ). The p38 dominant-negative mutant was provided by Dr. J. Han (Southwestern Medical Center, Dallas, TX). The JNK dominant-negative mutant was provided by Dr. M. Karin (University of California San Diego, La Jolla, CA). The ERK2 dominant-negative mutant was provided by Dr. M. Cobb (Southwestern Medical Center). pSV- β -galactosidase vector

and luciferase assay kit were purchased from Promega (Madison, WI). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Cell Cultures. Primary cultures of human chondrocytes were isolated from articular cartilage as described previously (Lee et al., 2002; Fong et al., 2007). Human articular chondrocytes were isolated from resected cartilage specimens obtained from undergoing primary total knee arthroplasty. Cartilage pieces were minced finely, and chondrocytes were isolated by sequential enzymatic digestion at 37°C with 0.1% hyaluronidase for 30 min and with 0.2% collagenase for 1 h. Isolated chondrocytes were filtered through 70- μ m nylon filters. The cells were grown on plastic cell culture dishes in 95% air, 5% CO₂ with Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA), which was supplemented with 20 mM HEPES and 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin (pH adjusted to 7.6). The cells were used between the second and sixth passages.

Western Blot Analysis of the Cell Lysate and Supernatant. Proteins in the total cell lysate (30 μ g of protein) were separated by 12% SDS-polyacrylamide gel electrophoresis and electrotransferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corporation, Billerica, MA). Blot was blocked in a solution of 4% bovine serum albumin, and membrane-bound proteins were then probed overnight with primary antibodies against SDF-1 α , CXCR4, MMP-13, p-ERK, p-p38, p-JNK, or p-Akt followed by incubation with horseradish peroxidase-conjugated secondary antibodies for 1 h. Antibody-bound protein bands were detected with enhanced chemiluminescence reagents (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK), and they were photographed with Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY). Quantitative data were obtained using a computing densitometer and ImageQuant software (GE Healthcare).

Conditioned medium aliquots were concentrated 100-fold by acetone precipitation and resuspended in 2-fold concentrated reducing Laemmli buffer. Proteins were measured with the detergent-compatible protein assay from Bio-Rad Laboratories (Hercules, CA). Protein samples at 30 μ g were then separated on 10% SDS-polyacrylamide gel, and proteins were analyzed by Western blot analysis as described under Western Blot Analysis of the Cell Lysate and Supernatant.

Zymography Analysis. Conditioned media were collected, centrifuged, and concentrated 100-fold with a Centriprep concentrator (Millipore). Concentrated supernatants were mixed with sample buffer without reducing agent or heating. The sample was loaded into 1 mg/ml gelatin containing SDS-polyacrylamide gel, and then it underwent electrophoresis with constant voltage. Afterward, the gel was washed with 2.5% Triton X-100 to remove SDS, rinsed with 50 mM Tris-HCl, pH 7.5, and then incubated overnight at room temperature with developing buffer (50 mM Tris-HCl, pH 7.5, 5 mM CaCl₂, 1 μ M ZnCl₂, 0.02% thimerosal, and 1% Triton X-100). The zymographic activities were revealed by staining with 1% Coomassie Blue. The sample was also loaded into SDS-polyacrylamide gel and stained with 1% Coomassie Blue as loading control (Chu et al., 2007). For examination of the downstream signaling pathways involved in SDF-1 α treatment, chondrocytes were pretreated with various inhibitors (0.1% dimethyl sulfoxide as vehicle) for 30 min before SDF-1 α administration.

mRNA Analysis by RT-PCR. Total RNA was extracted from chondrocytes using a TRIzol kit (MDBio Inc., Taipei, Taiwan). The reverse transcription reaction was performed using 2 μ g of total RNA that was reverse transcribed into cDNA using oligo(dT) primer and then amplified for 33 cycles using two oligonucleotide primers. The primers used are as follows: MMP-3, sense, AGAGGTGACTCCAC-TCACAT; antisense, GGTCTGTGAGTGAGTGATGATG; MMP-13, sense, TGCTCGCATTCTCCTTCAGGA; antisense, ATGCATCCAG-GGGTCTCTGGC; CXCR4, sense, AATCTTCTGCCACCACCTCT; antisense, GACGCCAACATAGACCACCT; c-Fos, sense, GAATAACA-TGGCTGTGCAGCCAAATGCCGCAA; antisense, CGTCAGATCAA-

GGGAAGCCACAGACATCT; c-Jun, sense, GGAAACGACCTTCTA-TGACGATGCCCTCAA; antisense, GAACCCCTCTGCTCATCTG-TCACGTTCTT; and glyceraldehyde-3-phosphate dehydrogenase, sense, ACCACAGTCCATGCCATCAC; antisense, TCCACCACCCT-GTTGCTGTA. Each PCR cycle was carried out for 30 s at 94°C, 30 s at 55°C, and 1 min at 68°C. PCR products were then separated electrophoretically in a 2% agarose DNA gel and stained with ethidium bromide.

Flow Cytometric Analysis. Human chondrocytes were plated in six-well dishes. The cells were then washed with phosphate-buffered saline (PBS) and detached with trypsin at 37°C. Cells were fixed for 10 min in PBS containing 1% paraformaldehyde. After rinsing in PBS, the cells were incubated with rabbit anti-human antibody against CXCR4 (1:100) for 1 h at 4°C. Cells were then washed again and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit secondary IgG (1:150; Leinco Technologies, Inc., St. Louis, MO) for 45 min and analyzed by flow cytometry using FACSCalibur and CellQuest software (BD Biosciences, San Jose, CA) (Tang et al., 2005).

Oligonucleotide Transfection. Chondrocytes were cultured to confluence; the complete medium was replaced with Opti-MEM (Invitrogen) containing the antisense phosphorothioate oligonucleotides (5 μ g/ml) that had been preincubated with 10 μ g/ml Lipofectamine 2000 (Invitrogen) for 30 min. The cells were washed after 24 h of incubation at 37°C and washed before the addition of medium con-

taining SDF-1 α . All antisense ODNs were synthesized and high-pressure liquid chromatography-purified by MDBio Inc. The sequences used are as follows: c-Fos antisense (AS)-ODN, GCGTTGAGCCCGAGAA and missense (MS)-ODN, GCATTGACGCCAGAGCA; and c-Jun AS-ODN, CGTTTCCATCTTTGCAGT and MS-ODN, ACTGCAAAGATGGAAACG (Naganuma et al., 2000; Zhang et al., 2002).

Generation of DNA Constructs Encoding a Small Interfering RNA against Human CXCR4. Oligonucleotides against human CXCR4 genes were generated and cloned into a pSilencer 3.1-H1 vector (Ambion, Austin, TX) as described previously (Lapteva et al., 2005). We used Lipofectamine 2000 reagent to transfect the chondrocytes with pSilencer 3.1-H1-siCXCR4 or pSilencer 3.1-H1-siCXCR4-mut. Twenty-four hours after transfection, cells were replated in Dulbecco's modified Eagle's medium (Invitrogen) with 10% fetal calf serum.

MMP-13 Promoter Assay. We generated promoter constructs of human MMP-13 genes according to previous reports with some modifications (Pendás et al., 1997; Chu et al., 2007). The primers used for PCR reactions for MMP-13 promoter construct were 5' primer, 5'-CTGAGAGCTCCAACAAGAGATGCTCTCA-3' (forward/SacI; nucleotides -186 to -166); and 3' primer, 5'-GGAAGCTTTCTAGATTGAATGGTGATGCCTGG-3' (reverse/HindIII; nucleotides +10 to +27). The pGL3-Basic vector containing a polyadenylation signal upstream from the luciferase gene was used to construct expression

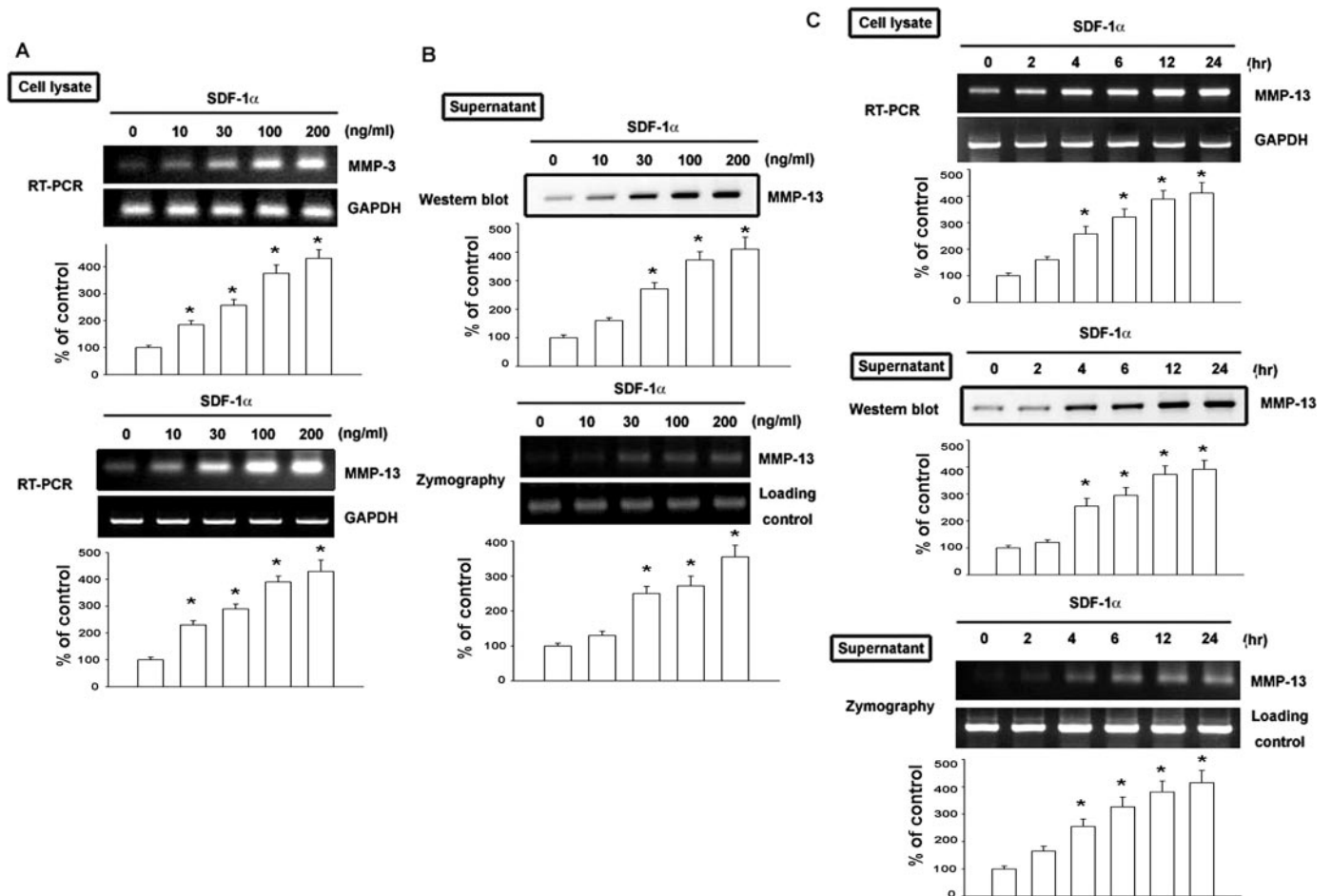


Fig. 1. Concentration- and time-dependent increase in MMP-13 expression by SDF-1 α . Human chondrocytes were incubated with various concentrations of SDF-1 α for 24 h. Then, the cell lysates were collected, and the mRNA levels of MMP-3 and MMP-13 were determined using RT-PCR. A, bottom, quantitative data are shown ($n = 4$). Cells were incubated with various concentrations of SDF-1 α for 24 h (B) or with 100 ng/ml SDF-1 α for 2, 4, 6, 12, or 24 h (C). The cultured medium and cell lysates were then collected, and the mRNA level of MMP-13 in cell lysates was determined using RT-PCR. The protein level of MMP-13 in supernatant was determined using Western blot analysis, and the enzyme activity of MMP-13 in supernatant was determined using zymography. The quantitative data are shown at the bottom ($n = 4$). Data are expressed as means \pm S.E. *, $p \leq 0.05$ compared with control.

vectors by subcloning PCR-amplified DNA to MMP-13 promoter into the SacI/HindIII site of the pGL3-Basic vector. The PCR products were confirmed on the basis of their size as determined by electrophoresis and DNA sequencing. Human chondrocytes were transiently transfected with MMP-13 promoter plasmid using Lipofectamine 2000 reagent. Luciferase activity was measured with the Luciferase reporter assay system (Promega) as described by the manufacturer, using a model TD-70/20 luminometer (Turner Designs, Sunnyvale, CA) (Tang et al., 2006).

DNA Affinity Protein-Binding Assay. Binding of transcription factors to the MMP-13 promoter DNA sequences was assayed as described previously (Pendás et al., 1997). After treatment with SDF-1 α , nuclear extracts were prepared. Biotin-labeled double-stranded oligonucleotides (2 μ g) synthesized based on the human MMP-13 promoter sequence were mixed at room temperature for 1 h with shaking with 200 μ g of nuclear extract proteins and 20 μ l of streptavidin agarose beads in a 70% slurry. Beads were pelleted and washed three times with ice-cold PBS. The bound proteins were then separated by SDS-polyacrylamide gel electrophoresis, followed by Western blot analysis with specific antibodies (Huang and Chen, 2005).

Chromatin Immunoprecipitation Assay. Chromatin immunoprecipitation (ChIP) analysis was performed as described previously (Tang et al., 2007). DNA immunoprecipitated by anti-c-Fos or anti-c-Jun antibody was purified. The DNA was then extracted with phenol-chloroform. The purified DNA pellet was subjected to PCR. PCR products were then resolved by 1.5% agarose gel electrophoresis and visualized by UV. The primers 5'-ACAAGAGATGCTCTCA-3' and 5'-TGAATGGTATGCCTGG-3' were used to amplify across the human MMP-13 promoter region (-182 to +27).

Statistics. The values given are means \pm S.E.M. The significance of difference between the experimental groups and controls was assessed by Student's *t* test. The difference is significant if $p < 0.05$.

Results

SDF-1 α Increased the Expression of MMP-13 in Human Chondrocytes. It has been reported that SDF-1 is greatly elevated in the SF from patients with OA and RA. MMP-3 and -13 have been reported to participate actively in the destruction of cartilage (Pelletier et al., 2004). Therefore, we investigated the effect of SDF-1 on the MMP-13 expression in human chondrocytes. Human chondrocytes were incubated with SDF-1 α (at various concentrations) for 24 h, and the cell lysates and culture medium were then collected. The results from RT-PCR, Western blot, and zymographic analysis indicated that SDF-1 α significantly increased the expression of MMP-13 in both cell lysates and supernatant concentration-dependently (Fig. 1, A and B) (induction of MMP-3 expression was used as positive control; Fig. 1A). The induction of MMP-13 at concentration of 100 ng/ml occurred in a time-dependent manner (Fig. 1C).

SDF-1 α /CXCR4 Interaction Was Responsible for the Expression of MMP-13 in Chondrocytes. Interaction of SDF-1 with its specific receptor CXCR4 on the surface of chondrocytes has been reported to induce the release of MMP-3 from chondrocytes (Kanbe et al., 2002); therefore, we then examined whether SDF-1/CXCR4 interaction is in-

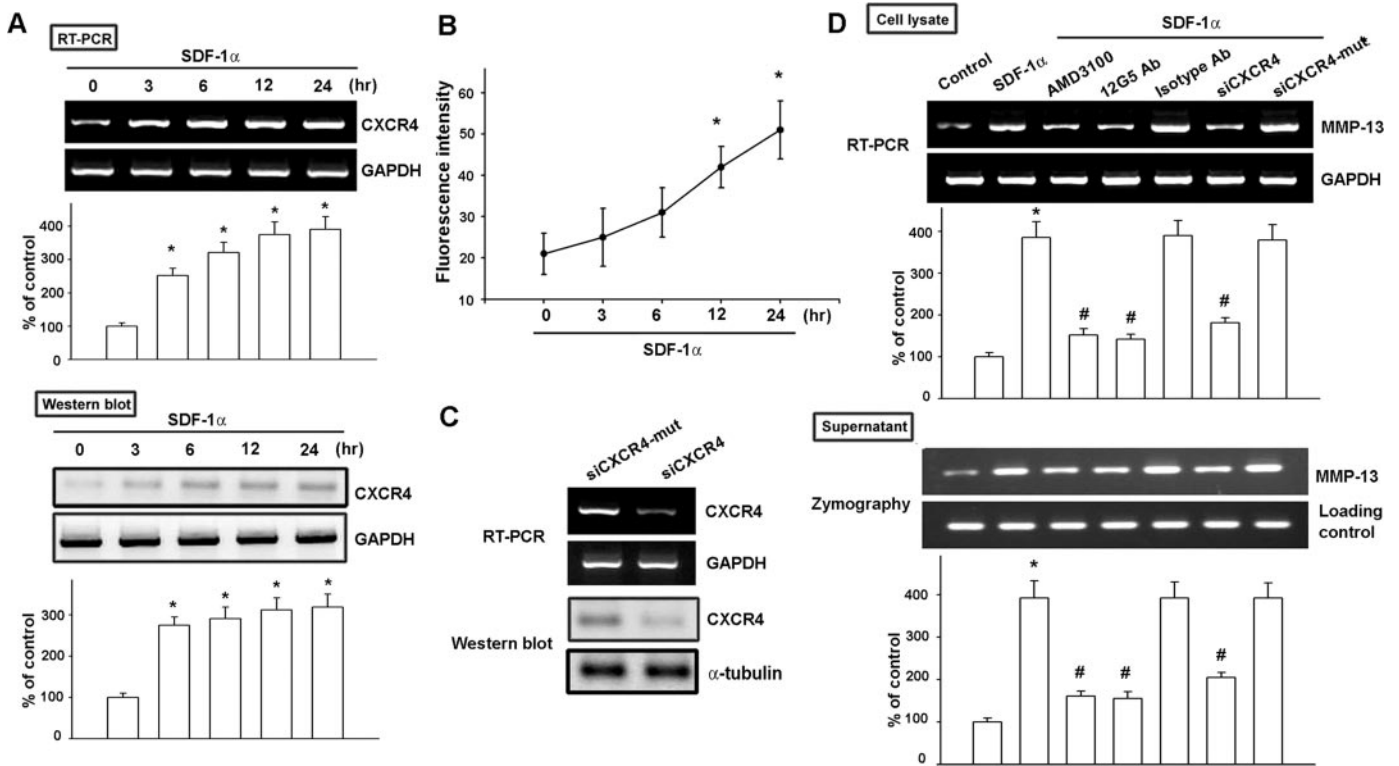


Fig. 2. Involvement of CXCR4 receptor in SDF-1 α -mediated MMP-13 expression in chondrocytes. Chondrocytes were incubated with 100 ng/ml SDF-1 α for indicated times. Then, cell lysates were collected, and the mRNA and protein level of CXCR4 was determined using RT-PCR and Western blot analysis, respectively. A, bottom, quantitative data are shown ($n = 4$). B, cells were incubated with 100 ng/ml SDF-1 α for indicated times, and the cell surface expression of CXCR4 was determined using a flow cytometer. C, cells were transfected with siCXCR4-mut or siCXCR4 for 24 h, and then the mRNA and protein levels of CXCR4 were determined using RT-PCR and Western blot analysis, respectively. Chondrocytes were pretreated with 500 ng/ml AMD3100, 10 μ g/ml 12G5 antibody, and isotype antibody for 30 min or transfected with siCXCR4-mut and siCXCR4 for 24 h followed by stimulation with 100 ng/ml SDF-1 α for 24 h. D, the mRNA level and enzyme activity of MMP-13 was determined by using RT-PCR and zymography analysis, respectively. The quantitative data are shown in the bottom panel ($n = 4$). Data are expressed as means \pm S.E. *, $p \leq 0.05$ compared with control. #, $p < 0.05$ compared with SDF-1 α -treated group.

involved in the signal transduction pathway leading to MMP-13 expression caused by SDF-1 α . Human chondrocytes were treated with SDF-1 α for different times, and the cell lysates were collected. The results from RT-PCR, Western blot, and flow cytometry indicated that SDF-1 α significantly increased both mRNA or protein levels and the cell surface expression of CXCR4 time-dependently (Fig. 2, A and B). Pretreatment of chondrocytes for 30 min with CXCR4-specific chemical inhibitor AMD3100 (500 ng/ml), CXCR4-neutralizing antibody (12G5; 10 μ g/ml), but not mouse monoclonal immunoglobulin isotype control (isotype antibody; 10 μ g/ml) antagonized the SDF-1 α -induced MMP-13 expression (Fig. 2D). Transient transfection of small interfering RNA against CXCR4 (siCXCR4), but not a mutant form of siCXCR4 (siCXCR4-mut), effectively inhibited the expression of MMP-13 caused by SDF-1 α (Fig. 2D). These results sug-

gest that induction of MMP-13 expression by SDF-1 α might occur via the activation of CXCR4 receptor.

ERK Signaling Pathway Was Involved in SDF-1 α -Mediated MMP-13 Up-Regulation. Because the SDF-1 α /CXCR4 interaction has been shown to activate several signaling pathways, including phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase (MAPK), in various cell lines (Kijima et al., 2002; Barbero et al., 2003; Phillips et al., 2003), we performed Western blot analysis to elucidate the signal transduction pathways involved in the SDF-1 α -induced up-regulation of MMP-13. SDF-1 α activated ERK1/2 in chondrocytes, as evidenced by the increase in phosphorylated p42 and p44 (p-ERK) (Fig. 3A). Other signaling pathways, including p38 MAPK, JNK, and Akt were not activated up to 4 h of treatment (Fig. 3A). SDF-1 α -induced mRNA expression and gelatinase activity of MMP-13 were

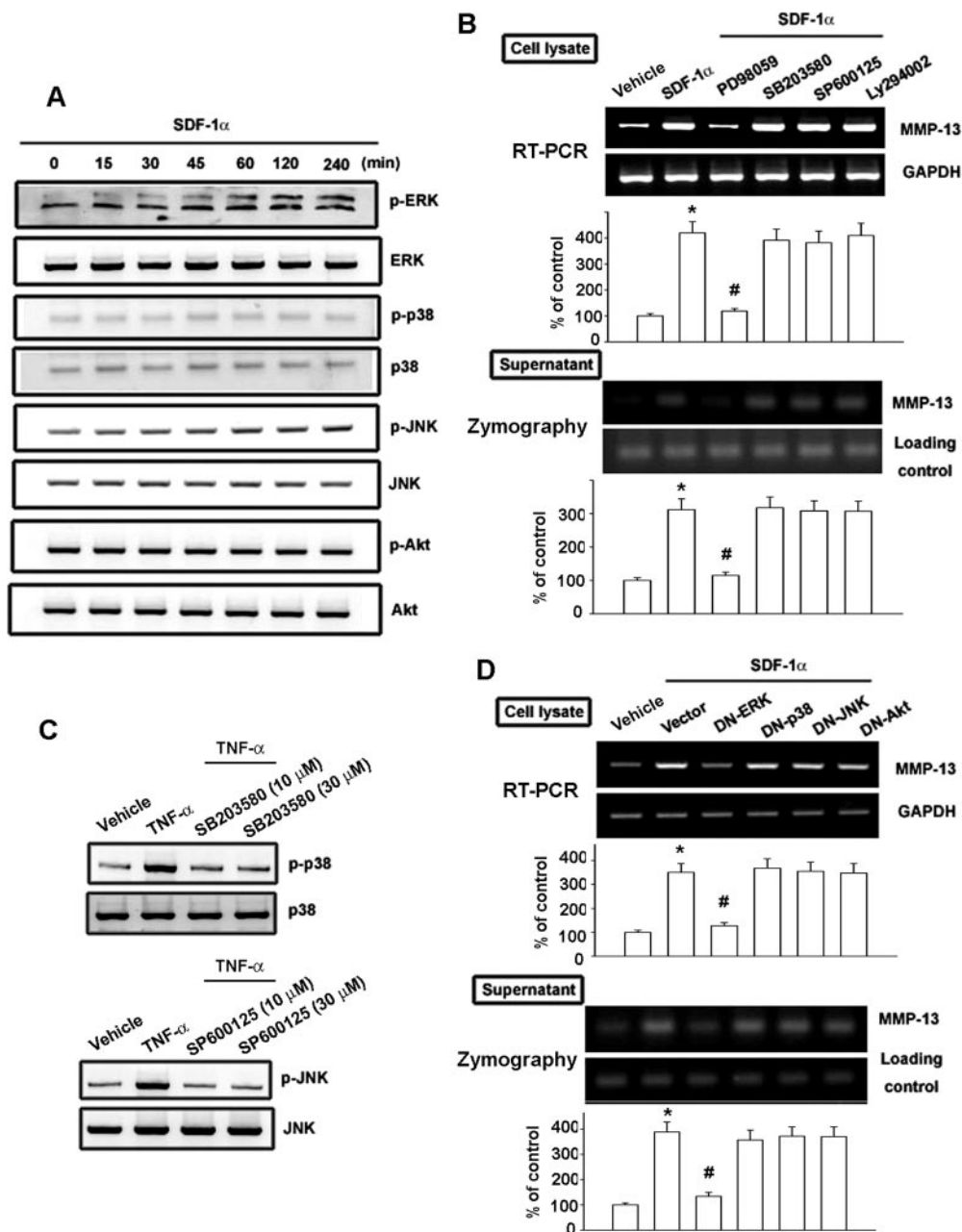


Fig. 3. ERK is involved in the potentiation of MMP-13 expression by SDF-1 α . **A**, chondrocytes were incubated with 100 ng/ml SDF-1 α for the indicated times, and then p-ERK, p-p38, p-JNK, or p-Akt expression was determined by Western blot analysis. Chondrocytes were pretreated for 30 min with 10 and 30 μ M SB203580 or with 10 and 30 μ M SP600125 followed by stimulation with 10 ng/ml TNF- α for 15 min, and p-38 and p-JNK expression was determined by Western blot analysis. Note that 10 and 30 μ M SB203580 or 10 and 30 μ M SP600125 antagonized TNF- α -induced p38 or JNK phosphorylation, respectively. **C**, cells were pretreated for 30 min with 30 μ M PD98059, 10 μ M SB203580, 10 μ M SP600125, and 10 μ M Ly294002 (**B**), or they were transfected with dominant-negative (DN) mutant of ERK, p38, JNK, and Akt (**D**) for 24 h followed by stimulation with 100 ng/ml SDF-1 α for 24 h. The mRNA level and enzyme activity of MMP-13 was determined by using RT-PCR and zymography analysis, respectively. The quantitative data are shown at the bottom ($n = 4$). Data are expressed as means \pm S.E. (percentage of vehicle control). *, $p \leq 0.05$ compared with vehicle. #, $p < 0.05$ compared with SDF-1 α -treated group.

greatly reduced by treatment with ERK inhibitor PD98059 (30 μ M), but these processes were not affected by SB203580 (a p38 MAPK inhibitor; 10 μ M), SP600125 (a JNK inhibitor; 10 μ M), or Ly294002 (a phosphatidylinositol 3-kinase inhibitor; 10 μ M) (Fig. 3B). To confirm that 10 μ M SB203580 and 10 μ M SP600125 are effective on p38 and JNK activity, pretreatment of chondrocytes with 10 and 30 μ M SB203580 or 10 and 30 μ M SP600125 for 30 min completely inhibited 10 ng/ml TNF- α -induced p38 and JNK phosphorylation, respectively (Fig. 3C). In addition, transfection of cells with ERK2 but not p38, JNK, or Akt mutant also antagonized the potentiating effect of SDF-1 α (Fig. 3D). Taken together, these data suggest that the activation of the ERK pathway is required for the SDF-1 α -induced increase of MMP-13 in chondrocytes.

SDF-1 α Increased the Binding of c-Fos and c-Jun to the AP-1 Element on the MMP-13 Promoter. Because the promoter region of human MMP-13 contains an AP-1 binding site and phosphorylation of ERK can lead to AP-1 activation (Eferl and Wagner, 2003; Ala-aho and Kahari, 2005), we further examined the activation of AP-1 components c-Fos and c-Jun after treatment of SDF-1 α . Time-dependent increase in the c-Fos and c-Jun mRNA expression in chondrocytes by SDF-1 α was observed (Fig. 4A). SDF-1 α -activated c-Fos and c-Jun were also evidenced by the accumulation of c-Fos and c-Jun in the nucleus (Fig.

4B). The SDF-1 α -induced c-Fos and c-Jun activation was inhibited by PD98059 but not by SB203580, SP600125, and Ly294002 (Fig. 4C). SDF-1 α -induced mRNA expression and gelatinase activity of MMP-13 were also inhibited by c-Fos and c-Jun AS-ODN but not by MS-ODN (Fig. 4E). It has been reported that human MMP-13 promoter contains an AP-1 binding site between -50 and -44 (Eferl and Wagner, 2003). We next investigated whether c-Fos and c-Jun bind to AP-1 element on the MMP-13 promoter after SDF-1 α stimulation. DNA affinity protein-binding assay experiments showed a time-dependent increase in the binding of c-Fos and c-Jun to the AP-1 element on the human MMP-13 promoter after treatment with SDF-1 α (Fig. 5A). The in vivo recruitment of c-Fos and c-Jun to the MMP-13 promoter (-182 to +27) was assessed by ChIP assays. In vivo binding of c-Fos and c-Jun to the AP-1 element of MMP-13 promoter occurred as early as 30 min, and it was sustained to 240 min after SDF-1 α stimulation (Fig. 5B). The binding of c-Fos and c-Jun to AP-1 element by SDF-1 α was attenuated by PD98059 or ERK mutant but not by SB203580, SP600125, and Ly294002 or p38, JNK, and Akt mutants (Fig. 5, C and D).

Increase of MMP-13 Promoter Activity by SDF-1 α . To further study the pathways involved in the action of SDF-1 α -induced MMP-13 expression, transient transfection was performed using the human MMP-13 promoter-luciferase con-

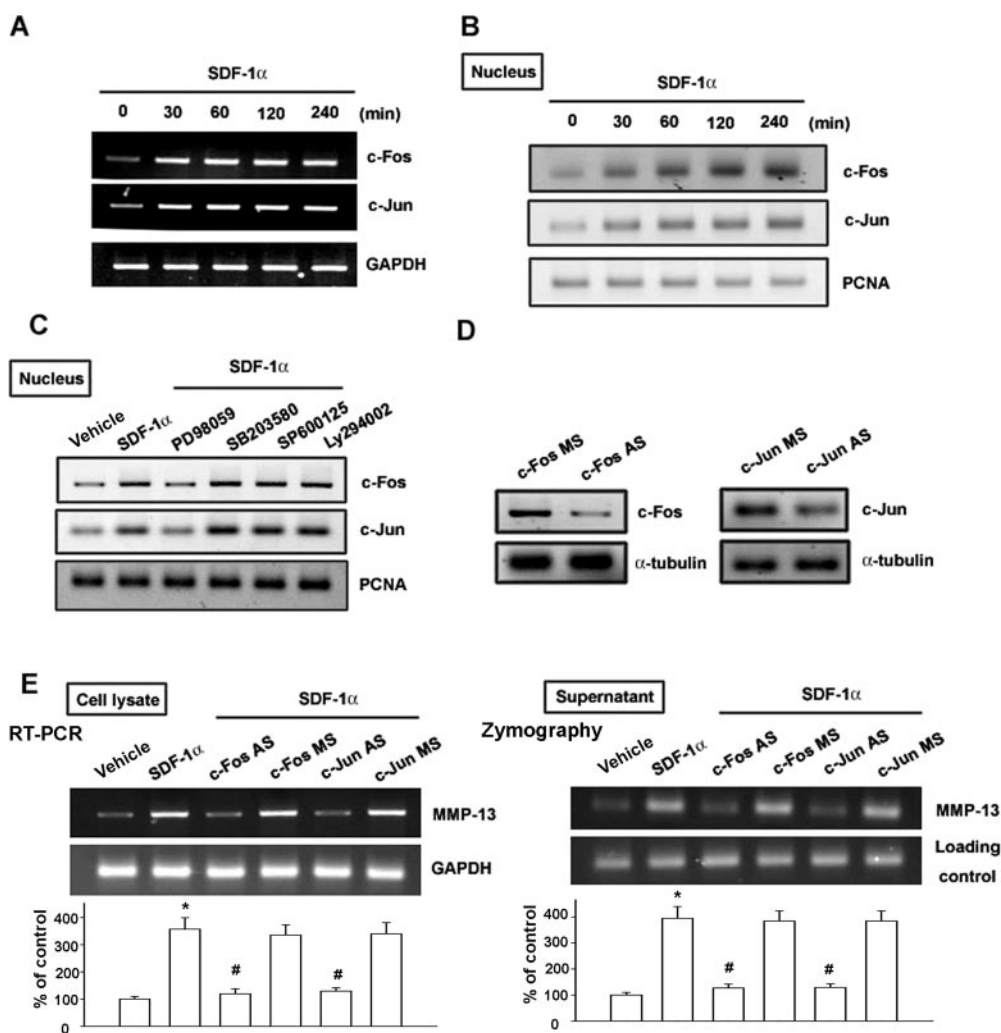


Fig. 4. c-Fos and c-Jun are involved in SDF-1 α -induced MMP-13 expression. A, cells were treated with 100 ng/ml SDF-1 α for the indicated times, and the mRNA levels of c-Fos and c-Jun were determined by using RT-PCR. Cells were treated with 100 ng/ml SDF-1 α for the indicated times (B), or they were pretreated with 30 μ M PD98059, 10 μ M SB203580, 10 μ M SP600125, or 10 μ M Ly294002 for 30 min (C) before stimulation with SDF-1 α for 240 min. The level of nuclear c-Fos and c-Jun was determined by immunoblotting with c-Fos- and c-Jun-specific antibodies, respectively. D, cells were transfected with c-Fos or c-Jun AS-oligonucleotides or MS-oligonucleotides for 24 h, and then the protein level of c-Fos or c-Jun was determined by using Western blot analysis. Cells were transfected with c-Fos or c-Jun (AS) and (MS) for 24 h followed by stimulation with 100 ng/ml SDF-1 α for 24 h, and then the mRNA level and enzyme activity of MMP-13 were determined by using RT-PCR and zymography analysis, respectively (E). The quantitative data are shown at the bottom ($n = 4$). Data are expressed as the means \pm S.E. *, $p \leq 0.05$ compared with vehicle control. #, $p < 0.05$ compared with SDF-1 α -treated group.

struct, which contains the human MMP-13 gene between positions -186 and +27 fused to the luciferase reporter gene. Treatment with SDF-1 α led to a 3.1-fold increase in MMP-13 promoter activity in chondrocytes. The increase of MMP-13 activity by SDF-1 α was antagonized by 30 μ M PD98059 but not by 10 μ M SB203580, 10 μ M SP600125, and 10 μ M Ly294002 (Fig. 6A). Alternatively, a high concentration of SB203580 (30 μ M) or SP600125 (30 μ M) also did not affect SDF-1 α -induced MMP-13 activity (Fig. 6A). In cotransfection experiments, the increase of MMP-13 promoter activity by SDF-1 α was inhibited by the dominant-negative mutant of ERK2 or c-Fos and c-Jun AS-ODN but not by dominant-negative mutants of p38, JNK, and Akt (Fig. 6B). In addition, dominant-negative mutants of ERK2, p38, JNK, and Akt or c-Fos and c-Jun AS-ODN did not affect the basal luciferase activity (Fig. 6B). Taken together, these data suggest that the activation of the ERK, c-Fos/c-Jun, and AP-1 pathway is required for the SDF-1 α -induced increase of MMP-13 in human chondrocytes.

Discussion

SDF-1 is significantly higher in synovial fluid of patients with osteoarthritis and rheumatoid arthritis. MMPs have been demonstrated to contribute to the breakdown of articular cartilage during arthritis (Poole, 2001). In addition, SDF-1 also enhances MMP-3 production in human chondrocytes (Kanbe et al., 2002). Here, we found that MMP-13 is a target protein for the SDF-1 signaling pathway, which required an activation of CXCR4 receptor, ERK, c-Fos/c-Jun, and AP-1.

The synovium of OA and RA patients produces many types of cytokines and chemokines, such as interleukin-1, TNF- α , macrophage inflammatory protein-1, and a variety of MMPs (Yoshihara et al., 2000). MMPs can induce the breakdown of

cartilage. SDF-1 has the additional function to accumulate CD4⁺ memory T cells in the synovium. This indicates that SDF-1 is related to the immune system and the inflammation that attracts lymphocytes to develop RA (Nanki et al., 2000; Blades et al., 2002). It has been reported that SDF-1 is expressed in the synovium but not in cartilage, which can stimulate release of MMP-9 in chondrocytes (Kanbe et al., 2004). MMP-13 expression has been detected in several pathological conditions that are characterized by the destruction of normal collagen tissue architecture (Ala-aho and Kahari, 2005). However, the expression of MMP-13 by SDF-1 α in chondrocytes is mostly unknown. Here, we found that SDF-1 α increased MMP-13 expression by using RT-PCR and zymographic analysis, which plays an important role during arthritis. Previous studies have shown that SDF-1 α /CXCR4 interactions modulate cell migration, invasion, and MMP secretion in several cells (Bartolome et al., 2004, 2006; Fermandis et al., 2004; Ohira et al., 2006). In the present study, we used CXCR4-specific chemical inhibitor AMD3100 and CXCR4-neutralizing antibody to determine the role of CXCR4, and we found that they inhibited SDF-1 α -induced MMP-13 expression, indicating the possible involvement of CXCR4 in SDF-1 α -induced MMP-13 expression in chondrocytes. This was further confirmed by the result that the small interfering RNA against CXCR4 inhibited the enhancement of MMP-13 production by SDF-1 α , indicating the involvement of SDF-1/CXCR4 interaction in SDF-1 α -mediated induction of MMP-13.

A variety of growth factors stimulate the expression of MMP genes via signal transduction pathways that converge to activate AP-1 complex of transcription factors. MAPK pathways, including ERK, JNK, and p38, induce the expression of AP-1 transcription factors (Ala-aho and Kahari, 2005). We found that SDF-1 α enhanced ERK1/2 phosphorylation

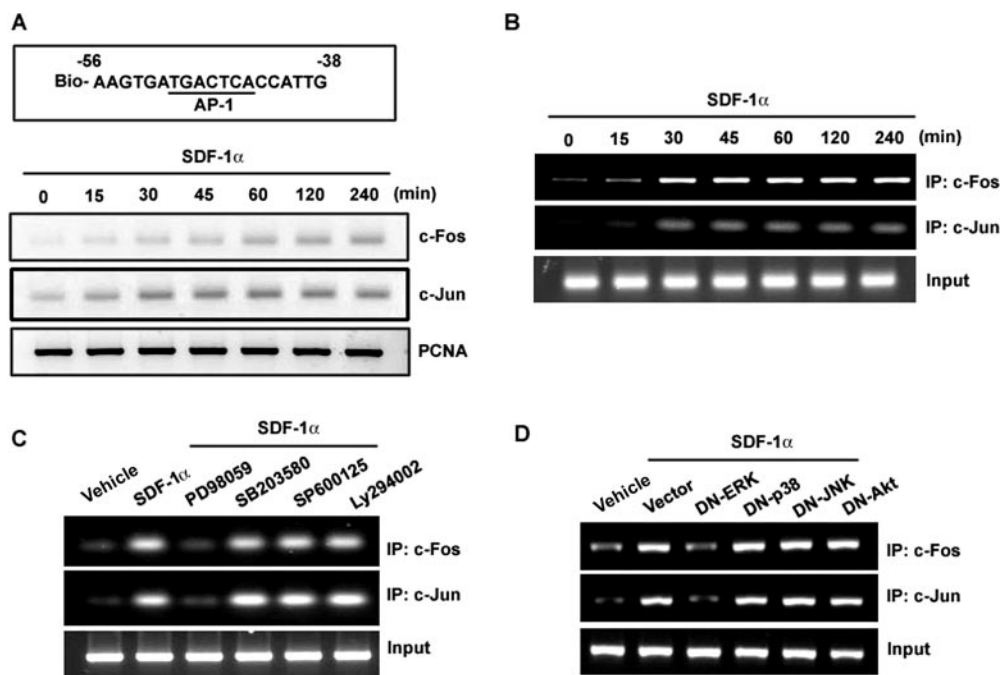


Fig. 5. Time-dependent increase in the binding of c-Fos and c-Jun to the AP-1 site on MMP-13 promoter in chondrocytes. A, top, schematic representing the consensus sequences of AP-1 site on the human MMP-13 promoter labeled with biotin. Chondrocytes were treated with 100 ng/ml SDF-1 α for the indicated times, and nuclear extracts were prepared and incubated with biotinylated AP-1 probe. The complexes were precipitated by streptavidin-agarose beads as described under *Materials and Methods*, and c-Fos or c-Jun in the complexes was detected by Western blot. The equal amount of input nuclear protein was examined by the proliferating cell nuclear antigen protein level. B to D, cells were treated with 100 ng/ml SDF-1 α for the indicated times, or they were pretreated for 30 min with 30 μ M PD98059, 10 μ M SB203580, 10 μ M SP600125, and 10 μ M Ly294002 or transfected with DN mutant of ERK, p38, JNK, and Akt for 24 h followed by stimulation with 100 ng/ml SDF-1 α for 240 min. Then, ChIP assay was performed. Chromatin was immunoprecipitated with anti-c-Fos or anti-c-Jun antibody. One percent of the precipitated chromatin was assayed to verify equal loading (input).

without affecting phosphorylation of Akt and other MAPK pathways (e.g., p38 MAPK and JNK pathways) in human chondrocytes. Previous studies have revealed that SDF-1 α treatment activates ERK1/2 in human lung cancer cells, astrocytes, and glioblastoma and basal cell carcinoma cells (Bajetto et al., 2001; Kijima et al., 2002; Barbero et al., 2003; Phillips et al., 2003; Chu et al., 2007). The SDF-1 α -directed MMP-13 expression was effectively inhibited by ERK inhibitor but not by Akt and other MAPK pathway inhibitors. In addition, dominant-negative mutant of ERK but not p38, JNK, and Akt also inhibited the potentiating action of SDF-1 α . This was further confirmed by the results that the dominant-negative mutant of ERK but not p38, JNK, and Akt inhibited the enhancement of MMP-13 promoter activity by SDF-1 α . A similar signal pathway has also been reported in

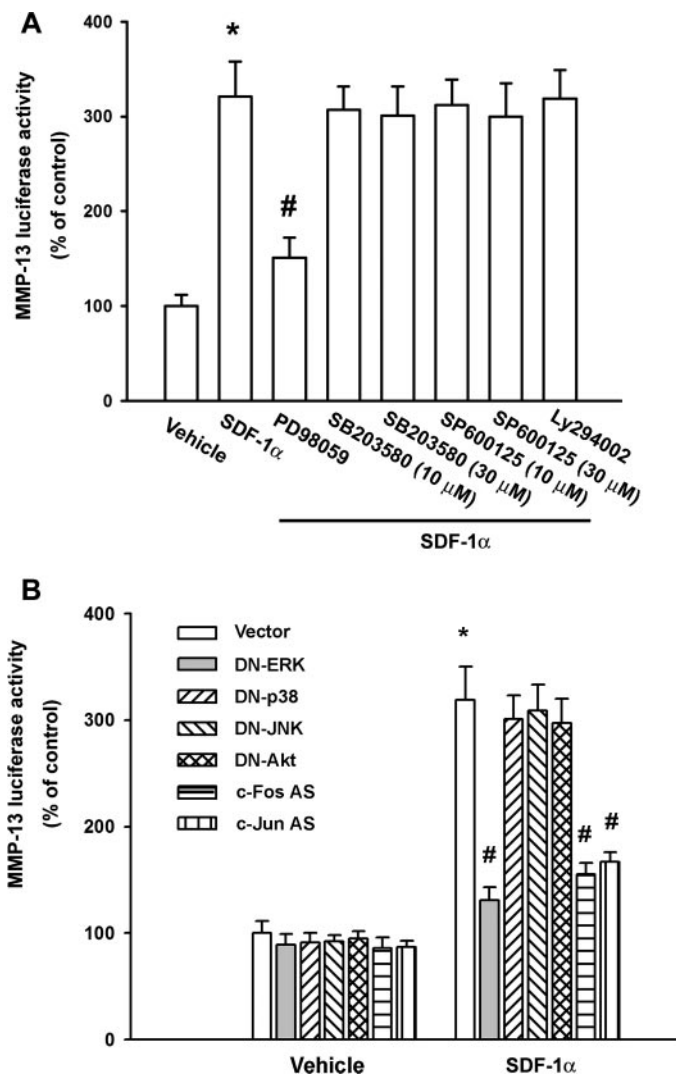


Fig. 6. Signaling pathways involved in the increase of MMP-13 promoter activity by SDF-1 α . **A**, MMP-13 promoter activity was evaluated by transfection with the pMMP-13-Luc luciferase expression vector. Chondrocytes were pretreated with 30 μ M PD98059, 10 and 30 μ M SB203580, 10 and 30 μ M SP600125, or 10 μ M Ly294002 for 30 min before incubation with 100 ng/ml SDF-1 α for 24 h. **B**, cells were cotransfected with pMMP-13-Luc and the DN mutant of ERK, p38, JNK, and Akt or c-Fos and c-Jun AS-oligonucleotides. Then, they were treated for 24 h with SDF-1 α . Luciferase activity was measured, and the results were normalized to β -galactosidase activity. Data are expressed as means \pm S.E. for three independent experiments performed in triplicate. *, $p < 0.05$ compared with vehicle control; #, $p < 0.05$ compared with SDF-1 α -treated group.

the invasion of basal cell carcinoma cells, which involved ERK-dependent MMP-13 expression (Chu et al., 2007). In addition, mechanical strain induced MMP-13 expression also through mitogen-activated protein kinase kinase-ERK signaling pathway to regulate mechanical adaptation (Yang et al., 2004). Taken together, our results provide evidence that SDF-1 α up-regulates MMP-13 in human chondrocytes via the ERK-dependent signaling pathway.

Hormones and growth factors are known to regulate gene expression through AP-1 sites (Angel et al., 1988). It has been reported that SDF-1 α induced MMP-13 secretion through AP-1-dependent pathway in human basal cell carcinoma (Chu et al., 2007). The AP-1 sequence binds to members of the Jun and Fos families of transcription factors. These nuclear proteins interact with the AP-1 site as Jun homodimers or Jun-Fos heterodimers formed by protein dimerization through their leucine zipper motifs. It has been observed that collagenase synthesis is induced in various tissues of transgenic animals overexpressing c-Fos or c-Jun, suggesting that an increase in c-Fos and c-Jun levels can stimulate collagenase expression (Wang et al., 1995). The results of this study show that SDF-1 α induced c-Fos and c-Jun expression and nuclear accumulation. Furthermore, SDF-1 α increased the binding of c-Fos and c-Jun to the AP-1 element on MMP-13 promoter, as shown by DNA affinity protein-binding assay and ChIP assay. Binding of c-Fos and c-Jun to the AP-1 element was attenuated by ERK inhibitor or ERK2 mutant but not by p38, JNK, and Akt inhibitor or p38, JNK, and Akt mutant. These results indicate that SDF-1 α might act through the ERK, c-Fos/c-Jun, and AP-1 pathway to induce MMP-13 activation in human chondrocytes.

In conclusion, the signaling pathway involved in SDF-1 α -induced MMP-13 expression in human chondrocytes has been explored. SDF-1 α increases MMP-13 expression and activity by binding to the CXCR4 receptor and activating ERK and the downstream transcription factors (c-Fos and c-Jun), resulting in the activation of AP-1 on the MMP-13 promoter and MMP-13, may contribute cartilage destruction during arthritis.

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Address correspondence to: Dr. Tang Chih-Hsin, Department of Pharmacology, College of Medicine, China Medical University, 91 Hsueh-Shih Rd., Taichung, Taiwan. E-mail: chtang@mail.cmu.edu.tw