中 國 醫 藥 大 學
臨床醫學研究所

碩士學位論文

# 探討 SDF-1 刺激對軟骨細胞分泌細胞間質水解酵素之作用

Stromal cell-derived factor-1 induces matrix metalloprotease-13 expression in human chondrocytes

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# Contents

# 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-1α increased the secretion of MMP-13 in cultured human chondrocytes, as shown by reverse transcriptase-polymerase chain reaction, Western blot, and zymographic analysis. SDF-1α 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)], orsmall interfering RNA against CXCR4 inhibited the SDF-1α-induced increase of MMP-13 expression.

The transcriptional regulation of MMP-13 by SDF-1α 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-1α. Cotransfection with dominant-negative mutant of ERK2 or c-Fos and c-Jun antisense oligonucleotide inhibited the potentiating action of SDF-1α on MMP-13 promoter activity.

Taken together, our results provide evidence that SDF-1α 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.

# 中文摘要

Stromal cell-derived factor-1(SDF-1)在退化性關節炎及風濕性關節炎病患的關節液中皆可以發現其含量顯著的提高。細胞間質水解酵素(Matrix metalloproteinases; MMP)則早已被證實會破壞關節軟骨導致關節炎的發生。但是到底經由何種訊息傳導路徑來調控人類軟骨細胞分泌MMP-13，則尚未有定論。在本論文中，我們經由RT-PCR，Western blot及Zymography發現SDF-1會誘導人類軟骨細胞分泌MMP-13。SDF-1也會促使它在人類軟骨細胞上的接受器CXCR4提高表現量。相對的，CXCR4抗體(neutralizing antibody)，CXCR4化學抑制劑(AMD3100)及CXCR4 si-RNA則會抑制SDF-1誘導人類軟骨細胞分泌MMP-13的表現。此外，SDF-1誘導人類軟骨細胞分泌MMP-13的表現是經由磷酸化ERK訊息傳導路徑及增加轉錄因子c-Fos及c-Jun的表現來啟動MMP-13驅動子(promotor)的活力。轉錄因子c-Fos及c-Jun結合到MMP-13驅動子(promotor)上AP-1序列從而增加MMP-13的表現可以由將人類軟骨細胞轉殖帶有MMP-13驅動子的質體(luciferase plasmid)再給予SDF-1的刺激來證明。相對的，將人類軟骨細胞轉殖dominant-negative mutant of ERK(DN-ERK)或antisense c-Fos(AS c-Fos)及antisense c-Jun(AS c-Jun)則會抑制SDF-1誘導人類軟骨細胞分泌MMP-13的表現。此抑制效果也可由冷光偵測儀上的表現得知。綜合以上實驗結果，我們發現SDF-1可以經由人類軟骨細胞接受器CXCR4，接著磷酸化ERK訊息傳導路徑並活化轉錄因子c-Fos及c-Jun結合到MMP-13驅動子(promotor)上AP-1序列，最後誘導人類軟骨細胞分泌MMP-13破壞軟骨導致關節炎的產生。

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身為公立醫學中心的臨床醫師，平時要應付醫療與行政上的煩瑣事務，已經令我有左支右絀分身乏術之感。但是現今醫療環境的激烈競爭，卻又驅使我不得不投入這場學位大戰中。所幸過去兩年來，我犧牲有限的與家人相處及運動休閒的時光，全心投入基礎研究的領域中。如今看來，實在是一項值得的投資。它帶給我的絕非只是一張碩士文憑，而是未來會影響我一生的真正的寶物!發現臨床工作無法解決的問題，卻在基礎研究中透露出解決問題的曙光。真是令人欣喜若狂。這讓我期許有朝一日能在某個骨科疾病的治療上為人類提供些微貢獻。回想初入學時，臨醫所標榜的，要指導我們成為醫師科學家。經過這些時間的薰陶,我不敢自居已經是一位醫師科學家，但毫無疑問的師父已經把我帶入門，其它就看日後的修行了。

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# Figure contents

# Abbreviation

| | |
|---|---|
| ChIP | Chromatin Immunoprecipitation |
| DAPA | DNA Affinity Protein-Binding Assay |
| DNA | Doxyribonucleic acid |
| ECM | Extracellular matrix |
| ERK | Extracellular signal regulated kinase |
| FACS | Flow cytometry analysis |
| MAPK | Mitogen-activated protein kinase |
| MMP-13 | Matrix metalloproteinases |
| OA | Osteoarthritis |
| PBS | Phosphate buffer saline |
| PCR | Polymerase chain reaction |
| RA | Rheumatic arthritis |
| Si-RNA | Small interfering RNA |
| SDF-1 | Stromal cell derived factor-1 |
| SF | Synovium fluid |

# Introduction

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 calciumand 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 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-1α concentrations in SF is due, at least partially, to the stimulated synthesis of SDF-1α by synovial fibroblasts under OA and RA conditions (Kanbe et al., 2002). The source of SDF-1α 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-1α 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-1α 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).

STROMA-DERIVED FACTOR 1 (SDF-1, or CXCL12) was initially cloned by Tashiro et al. (Tashiro et al., 1993) and later identified as a growth factor for B cell progenitor cells, a chemotactic factor for T cells and monocytes, and in B cell lymphopoiesis and bone marrow myelopoiesis. CXCL12 is a 68-amino acid small (8kDa) cytokine that belongs to the CXC chemokine family. CXCL12 is expressed in two isoforms, SDF-1α and SDF-1β, from a single gene that encodes two splice variants. The two encoded proteins are almost identical, except for the last four amino acids of SDF-1β, which are absent in SDF-1α. Biological and functional differences between the CXCL12 isoforms have not been described. The CXCL12 gene is mapped in chromosome 10, whereas most of the other genes encoding CXC chemokines reside on

chromosome 4 (Shirozu et al., 1995).

It was long thought that CXCL12 bound exclusively to CXCR4 and that CXCR4 was its sole receptor. However, CXCR7 was identified as another receptor for CXCL12 at the end of 2005 (Balabanian et al., 2005). The immunological activities of CXCL12/CXCR4 have been largely studied in the context of immune cell trafficking. Interestingly, both CXCL12 and CXCR4 knockout mice are embryonic lethal, with any surviving pups dying within an hour of birth, suggesting that the CXCL12/ CXCR4 pathway mediates multiple biological activities (Nagasawa et al., 1996, Tachibana et al., 1998). The lethal effect of CXCL12 and CXCR4 knockout is related to the pleiotropic activity of CXCL12 and CXCR4, which are critical for hematopoietic, neural, vascular, and craniofacial organogenesis (Ma et al., 1998, Nagasawa et al., 1996, Tachibana et al., 1998).

CXCL12 was initially cloned from bone marrow stromal cells (Tashiro et al., 1993). Strikingly, CXCL12 is widely expressed in various organs including heart, liver, brain, kidney, skeletal muscle, and lymphoid organs. Vascular endothelial cells, stromal fibroblasts, and osteoblasts are the major cellular source for CXCL12 in these organs (Gupta et al., 1998, Katayama et al., 2006, Petit et al., 2002, Ponomaryov et al., 2000, Zou et al., 1998). Interestingly, high levels of functional CXCL12 were first reported in human ovarian cancer in 2001 (Kryczek et al., Scotton et al., 2001, Zou et al., 2001). Subsequent studies documented a strong correlation between CXCL12 expression and bone marrow and lymph node metastasis of breast (Muller et al., 2001) and prostate cancer (Taichman et al., 2002). Interest in the role of CXCL12/ CXCR4 in tumor pathology was provoked by these studies. In addition to ovarian cancer, CXCL12 expression is reported in breast cancer (Bachelder et al., 2002, Kang et al., 2005), glioblastoma (Barbero et al., 2003, Porcile et al.,

2005), pancreatic cancer (Koshiba et al., 2000, Marchesi et al., 2004), prostate cancer (Darash-Yahana et al., 2004, Sun et al., 2003), thyroid cancer (Hwang et al., 2003), and many other human tumors.

The matrix metalloproteinase (MMP) family members are the major enzymes that degrade the components of the extracellular matrix (Vincenti MP, 2001, Nagase H and Woessner JF, 1999). At the time of writing this article, 20 members of this family have been identified (Stetler-Stevenson WG and Yu AE, 2001). All are active at neutral pH, require Ca2+ for activity and contain a central zinc atom as part of their structure. Most MMPs are secreted into the extracellular space in a latent proform, and require proteolytic cleavage for enzymatic activity. A few MMPs, however, are activated intracellularly by a furin-like mechanism and therefore, these enzymes are fully active when they reach the extracellular space (Nagase H and Woessner JF, 1999). Most cells in the body express MMPs, even though some enzymes are often associated with a particular cell type. For example, the principle substrate of MMP-2 (also known as gelatinase A) and MMP-9 (also known as gelatinase B) is the type IV collagen in basement membrane and thus, these enzymes are usually expressed by endothelial cells, although other cells (e.g. stromal fibroblasts, macrophages, tumor cells) also express them (Vincenti MP, 2001, Borden P and Heller RA, 1997).

MMP-3 (also known as stromelysin) activates MMP-1 (also known as collagenase-1) and cleaves a broad range of matrix proteins (Vincenti MP et al., 1996); MMP-1, which is an interstitial collagenase, and MMP-3 are among the most ubiquitously expressed MMPs. In contrast, MMP-13 (also known as collagenase-3) has a more restricted pattern of expression within connective tissue, and is usually produced only by cartilage and bone during development, and by chondrocytes in osteoarthritis (OA) (Borden P et al., 1996, Mengshol JA, 2000).

Expression of MMPs is low in normal cells, and these low levels allow for healthy connective tissue remodeling. In pathologic conditions, however, the level of MMP expression increases considerably, resulting in aberrant connective tissue destruction. Excess MMP production is associated with the pathology of many diseases, including periodontitis, atherosclerosis, tumor invasion/metastasis and arthritic disease (Vincenti MP, 2001, Borden P and Heller RA, 1997). In rheumatoid arthritis (RA) and OA, connective tissue destruction is mediated primarily by chondrocytes, by synovial fibroblasts and on occasion, by osteoclasts (Mengshol JA et al., 2000, Goldring MB, 2000).

The interstitial collagens (types I, II and III), are the principle targets of destruction, and the secreted collagenases (MMP-1 and MMP-13) have the major role in this process. These MMPs are induced in response to the cytokines and growth factors usually found in arthritic joints. MMP-9 is also an inducible MMP, but its role in connective tissue destruction in arthritis appears to be secondary, since it contributes to the degradation of collagen only after the chains of the triple helix have been cleaved by the interstitial collagenases (Nagase H and Woessner JF, 1999). In contrast, MMP-2 and MMP-14 (membrane type 1-MMP), are constitutively expressed, with minimal regulation, and they may have a relatively minor role in the pathophysiology of arthritis. Thus, the collagenases (MMP-1, MMP-8 [also known as neutrophil collagenase] and MMP-13) have the unique ability to cleave the triple helix of collagen, thereby allowing the chains to unwind; the chains then become susceptible to further degradation by other MMPs. Recently, MMP-8 (traditionally termed neutrophil collagenase) has been found in arthritic lesions, even in the absence of neutrophils, indicating that chondrocytes, and perhaps synovial cells, can produce this enzyme (Tetlow LC et al., 2001, Shlopov BV et al., 1997). MMP-13 may have a particular role in cartilage

degradation because it is expressed by chondrocytes, and because it hydrolyzes type-II collagen more efficiently than the other collagenases (Mitchell PG et al., 1996). However, MMP-1 is more abundant and it also degrades interstitial collagens effectively (Vincenti MP, 2001, Nagase H and Woessner JF, 1999). We will, therefore, focus this discussion on the mechanisms controlling transcription of MMP-1 and MMP-13 in arthritic disease, although the concepts may be applicable to other members of this gene family and to other pathologic conditions.

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-1α induced MMP-3 activity in human chondrocytes (Kanbe et al., 2002). However, the effect of SDF-1α on MMP-13 expression in human chondrocytes is mostly unknown. Here, we found that SDF-1α 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-1α. The elevated level of SDF-1α 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 | Santa Cruz Biotechnology |
| Anti-mouse IgG conjugated horseradish peroxidase | Santa Cruz Biotechnology |
| Anti-rabbit IgG conjugated horseradish peroxidase | Santa Cruz Biotechnology |
| Phosphorylated (p)-ERK | Santa Cruz Biotechnology |
| P-p38 | Santa Cruz Biotechnology |
| P-c-Jun | Santa Cruz Biotechnology |
| NH2-terminal kinase (JNK) | Santa Cruz Biotechnology |
| P-protein kinase B (Akt) | Santa Cruz Biotechnology |
| Akt | Santa Cruz Biotechnology |
| ERK | Santa Cruz Biotechnology |
| p38 | Santa Cruz Biotechnology |
| JNK | Santa Cruz Biotechnology |
| c-Fos | Santa Cruz Biotechnology |
| c-Jun | Santa Cruz Biotechnology |
| MMP-13 | Santa Cruz Biotechnology |
| PD98059 | Calbiochem |
| SB203580 | Calbiochem |
| SP600125 | Calbiochem |
| Ly294002 | Calbiochem |
| Rabbit polyclonal antibody specific for CXCR4 | R&D Systems |
| Recombinant human SDF-1α | PeproTech |

| | |
|---|---|
| p38 dominant-negative mutant | Provided by Dr. J. Han |
| JNK dominant-negative mutant | Provided by Dr. M. Karin |
| ERK2 dominant-negative mutant | Provided by Dr. M. Cobb |
| pSV-β-galactosidase vector | Promega |
| Luciferase assay kit | Promega |
| All other chemicals | Sigma-Aldrich |

***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-μm 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 μg/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 (30μg 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-1α, 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 30μg 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, 1μM 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-1α treatment, chondrocytes were pretreated with various inhibitors (0.1% dimethyl sulfoxide as vehicle) for 30 min before SDF-1α 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 2μg 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, AGAGGTGACTCCACTCACAT; antisense, GGTCTGTGAGTGAGTGATAG; MMP-13, sense, TGCTCGCATTCTCCTTCAGGA; antisense, ATGCATCCAGGGGTCCTGGC; CXCR4, sense, AATCTTCCTGCCCACCATCT; antisense, GACGCCAACATAGACCAC -CT; c-Fos, sense, GAATAACATGGCTGTGCAGCCAAATGCCGCAA; antisense, CGTCAGATCAAGGGAAGCCACAGACATCT; c-Jun, sense, GGAAACGACCTTCTATG -ACGATGCCCTCAA; antisense, GAACCCCTCCTGCTCATCTGTCACGTTCTT; and glyceraldehyde-3-phosphate dehydrogenase, sense, ACCACAGTCCATGCCATCAC; antisense, TCCACCACCCTGTTGCTGTA. 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 antirabbit 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(5μg/ml) that had been preincubated with 10μg/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 containing SDF-1α. All antisense ODNs were synthesized and highpressure liquid chromatography-purified by MDBio Inc. The sequences used are as follows: c-Fos antisense (AS)-ODN, GCGTTGAAGCCCGAGAA 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 (Penda´s et al., 1997; Chu et al., 2007). The primers used for PCR reactions for MMP-13 promoter construct were 5′primer, 5′-CTGAGAGCTCCAACAAGAGAT GCTCTCA-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 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 (Penda´s et al., 1997). After treatment with SDF-1α, nuclear extracts were prepared. Biotin-labeled doublestranded oligonucleotides (2μg) synthesized based on the human MMP-13 promoter sequence were mixed at room temperature for 1 h with shaking with 200μg of nuclear extract proteins and 20 μl 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 antic- 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′-AACAAGAGAT GCTCTCA-3′

and 5′-TGAATGGTGATGCCTGG-3′ were used to amplify across the human MMP-13 promoter region (－182 to＋27).

***Statistics.***

The values given are means ± 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-1α 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-1α(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-1αsignificantly 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-1α/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- volved in the signal transduction pathway leading to MMP-13 expression caused by SDF-1α. Human chondrocytes were treated with SDF-1α for different times, and the cell lysates were collected. The results from RT-PCR, Western blot, and flow cytometry indicated that SDF-1α 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; 10μg/ml), but not mouse monoclonal immunoglobulin isotype control (isotype antibody; 10μg/ml) antagonized the SDF-1α-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-1α(Fig. 2D). These results suggest that induction of MMP-13 expression by SDF-1α might occur via the activation of CXCR4 receptor.

***ERK Signaling Pathway Was Involved in SDF-1α-Mediated MMP-13 Up-Regulation.***

Because the SDF-1α/CXCR4 interaction has been shown to activate several signalingpathways, 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-1α-induced up-regulation of MMP-13. SDF-1αactivated 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-1α-induced mRNA expression and gelatinase activity of MMP-13 were greatly reduced by treatment with ERK inhibitor PD98059 (30μM), but these processes were not affected by SB203580 (a p38 MAPK inhibitor; 10μM), SP600125 (a JNK inhibitor; 10μM), or Ly294002 (a phosphatidylinositol 3-kinase inhibitor; 10μM)

(Fig. 3B). To confirm that 10 _M SB203580 and 10μM SP600125 are effective on p38 and JNK activity, pretreatment of chondrocytes with 10 and 30μM SB203580 or 10 and 30μM 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-1α(Fig. 3D). Taken together, these data suggest that the activation of the ERK pathway is required for the SDF-1α-induced increase of MMP-13 in chondrocytes.

***SDF-1αIncreased 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-1α. Time-dependent increase in the c-Fos and c-Jun mRNA expression in chondrocytes by SDF-1αwas observed (Fig.4A). SDF-1α-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-1α-induced c-Fos and c-Jun activation was inhibited by PD98059 but not by SB203580, SP600125, and Ly294002 (Fig. 4C). SDF-1α-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-1α 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-1α(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-1αstimulation(Fig. 5B). The binding of c-Fos and c-Jun to AP-1 element by SDF-1αwas 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-1α.***

To further study the pathways involved in the action of SDF-1α-induced MMP-13 expression, transient transfection was performed using the human MMP-13 promoter-luciferase con- struct, which contains the human MMP-13 gene between positions -186 and +27 fused to the luciferase reporter gene. Treatment with SDF-1αled to a 3.1-fold increase in MMP-13 promoter activity in chondrocytes. The increase of MMP-13 activity by SDF-1α was antagonized by 30μM PD98059 but not by 10μM SB203580, 10μM SP600125, and 10μM Ly294002 (Fig. 6A). Alternatively, a high concentration of SB203580 (30μM) or SP600125 (30μM) also did not affect SDF-1α-induced MMP-13 activity (Fig. 6A). In cotransfection experiments, the increase of MMP-13 promoter activity by SDF-1α was inhibited by the dominant-negative mutant of ERK2 or c-Fos and c-Jun AS-ODN but not by dominantnegative 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-1α-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 $CD^{4+}$ 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-1α in chondrocytes is mostly unknown. Here, we found that SDF-1αincreased MMP-13 expression by using RT-PCR and zymographic analysis, which plays an important role during arthritis. Previous studies have shown that SDF-1α/CXCR4 interactions modulate cell migration, invasion, and MMP secretion in several cells (Bartolome et al., 2004, 2006; Fernandis 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-1α- induced MMP-13 expression, indicating the possible involvement of CXCR4 in SDF-1α-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-1α, indicating the involvement of SDF-1/CXCR4 interaction in SDF-1α-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-1α enhanced ERK1/2 phosphorylation 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-1α 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-1α- 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-1α. 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-1α. A similar signal pathway has also been reported in 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-1αup-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-1α 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-1αinduced c-Fos and c-Jun expression and nuclear accumulation. Furthermore, SDF-1αincreased 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-1αmight 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-1α-induced MMP-13 expression in human chondrocytes has been explored. SDF-1αincreases 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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# Figure

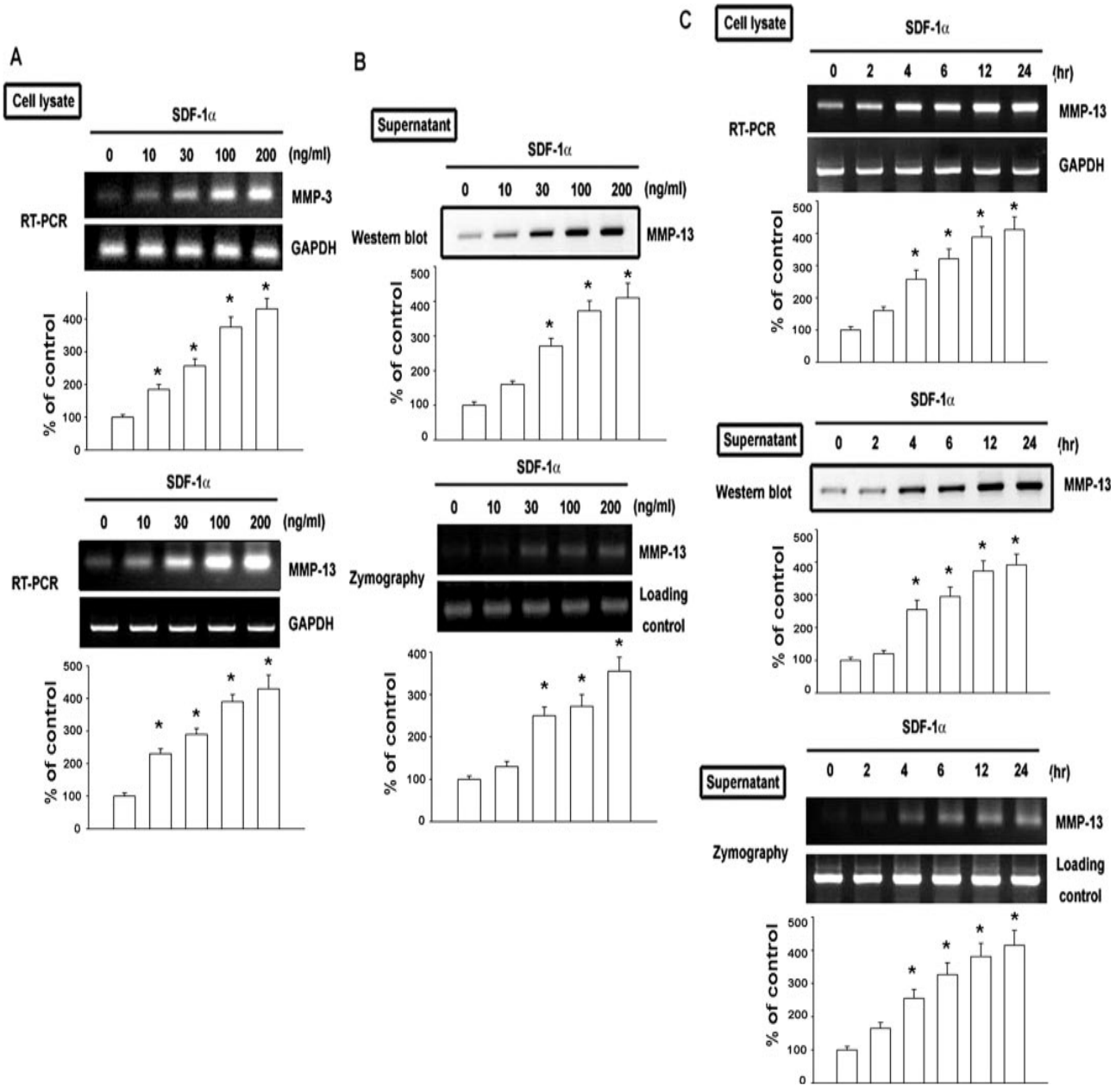

A
Cell lysate
SDF-1α
0 10 30 100 200 (ng/ml)
RT-PCR
MMP-3
GAPDH
% of control
SDF-1α
0 10 30 100 200 (ng/ml)
RT-PCR
MMP-13
GAPDH
% of control
B
Supernatant
SDF-1α
0 10 30 100 200 (ng/ml)
Western blot
MMP-13
% of control
SDF-1α
0 10 30 100 200 (ng/ml)
Zymography
MMP-13
Loading control
% of control
C
Cell lysate
SDF-1α
0 2 4 6 12 24 (hr)
RT-PCR
MMP-13
GAPDH
% of control
SDF-1α
Supernatant
0 2 4 6 12 24 (hr)
Western blot
MMP-13
% of control
SDF-1α
Supernatant
0 2 4 6 12 24 (hr)
Zymography
MMP-13
Loading control
% of control

**Fig. 1. Concentration- and time-dependent increase in MMP-13 expression by SDF-1α.**

Human chondrocytes were incubated with various concentrations of SDF-1α 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-1α for 24 h (B) or with 100 ng/ml SDF-1αfor 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 ± S.E. *, $p \leqq 0.05$ compared with control.

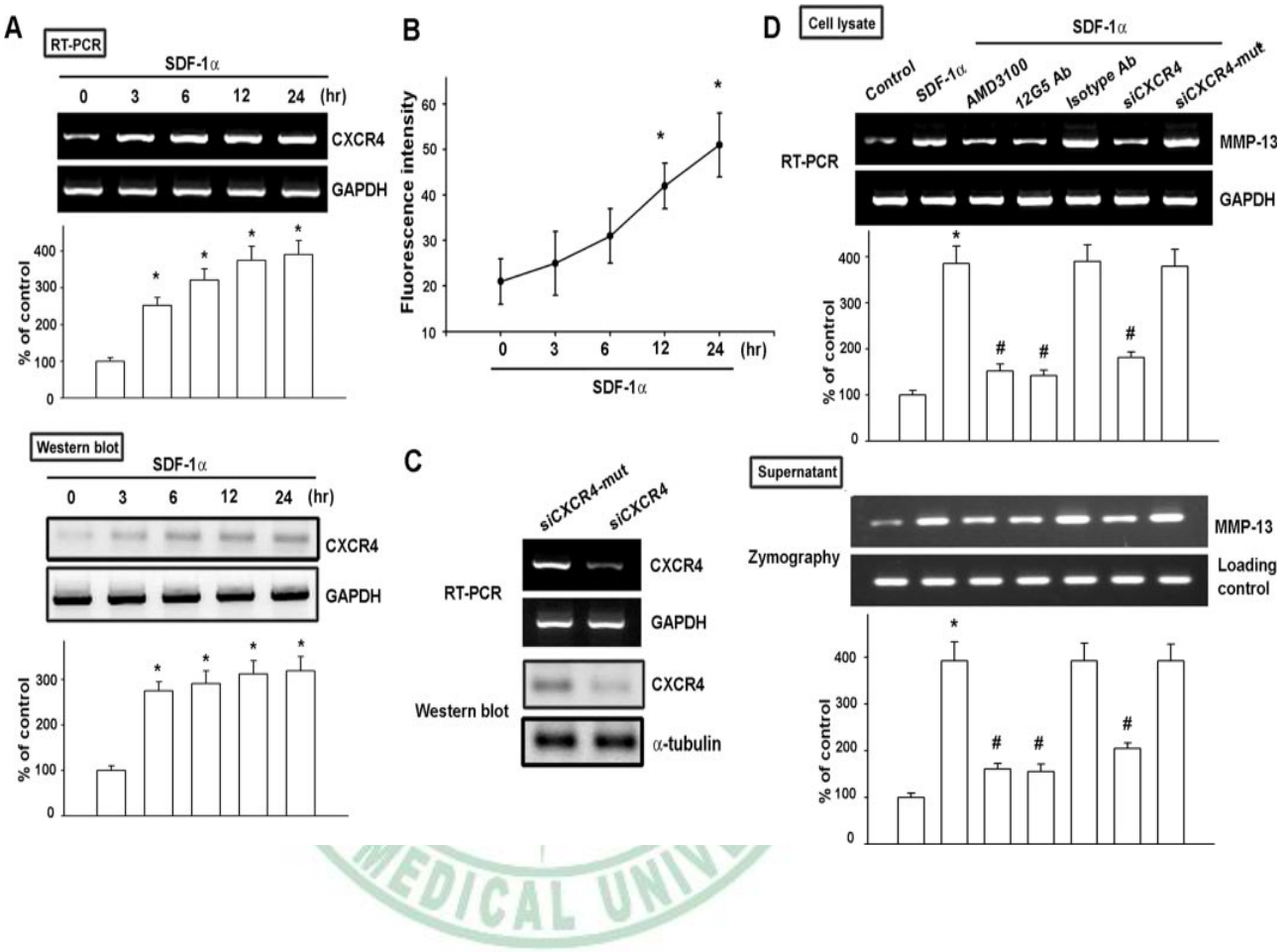

A
RT-PCR
SDF-1α
0 3 6 12 24 (hr)
CXCR4
GAPDH
% of control
Western blot
SDF-1α
0 3 6 12 24 (hr)
CXCR4
GAPDH
% of control
B
Fluorescence intensity
0 3 6 12 24 (hr)
SDF-1α
C
siCXCR4-mut
siCXCR4
RT-PCR
CXCR4
GAPDH
Western blot
CXCR4
α-tubulin
D
Cell lysate
SDF-1α
Control
SDF-1α
AMD3100
12G5 Ab
Isotype Ab
siCXCR4
siCXCR4-mut
RT-PCR
MMP-13
GAPDH
% of control
Supernatant
Zymography
MMP-13
Loading control
% of control

**Fig. 2. Involvement of CXCR4 receptor in SDF-1α-mediated MMP-13 expression in chondrocytes.**

Chondrocytes were incubated with 100 ng/ml SDF-1α 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-1α 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, 10μg/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-1α 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 ± S.E. *, $p \leqq 0.05$ compared with control. #, $p < 0.05$ compared with SDF-1α-treated group.

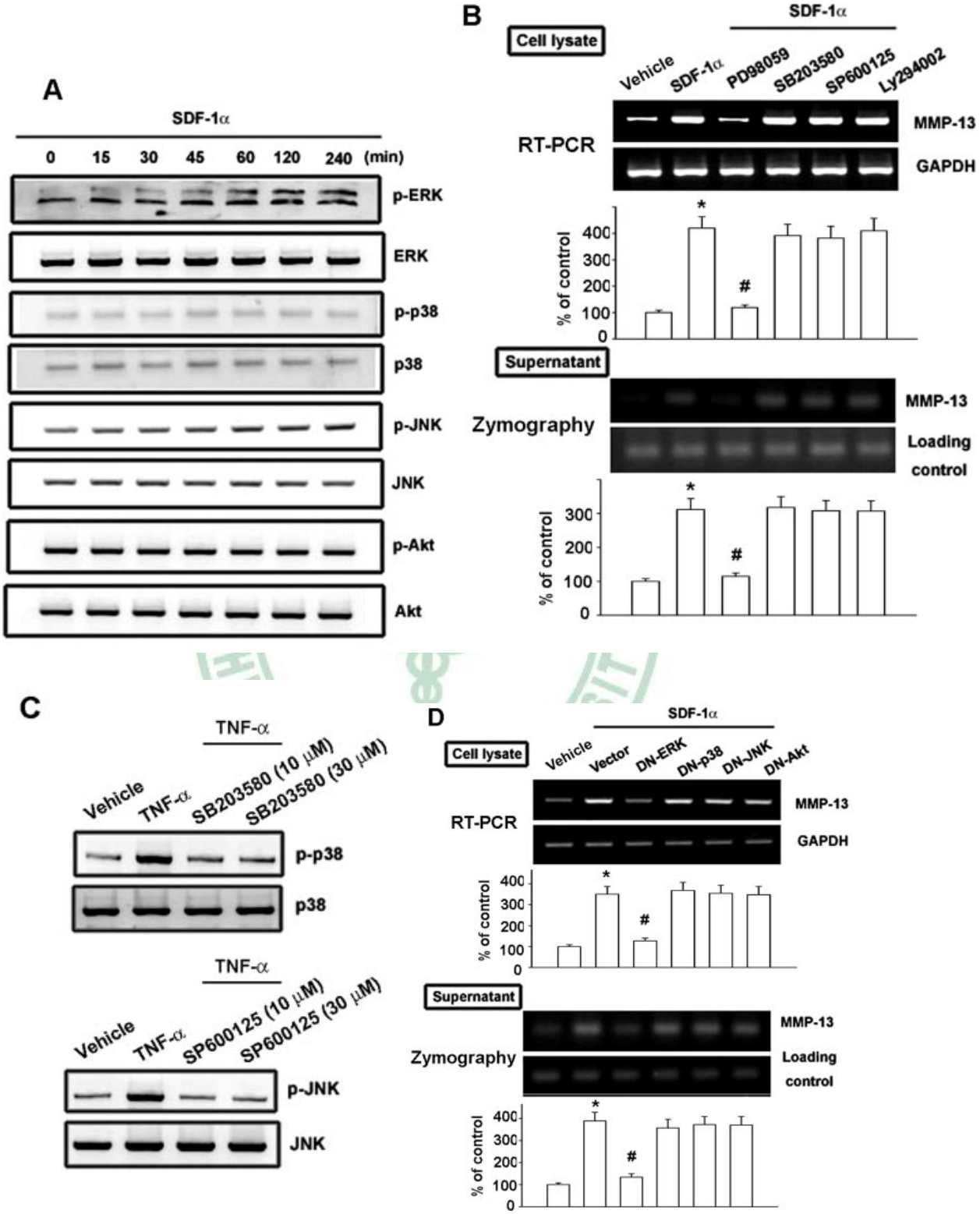

A
SDF-1α
0 15 30 45 60 120 240 (min)
p-ERK
ERK
p-p38
p38
p-JNK
JNK
p-Akt
Akt
B
SDF-1α
Cell lysate
Vehicle
SDF-1α
PD98059
SB203580
SP600125
Ly294002
RT-PCR
MMP-13
GAPDH
% of control
Supernatant
Zymography
MMP-13
Loading control
C
TNF-α
Vehicle
TNF-α
SB203580 (10 μM)
SB203580 (30 μM)
p-p38
p38
TNF-α
Vehicle
TNF-α
SP600125 (10 μM)
SP600125 (30 μM)
p-JNK
JNK
D
SDF-1α
Cell lysate
Vehicle
Vector
DN-ERK
DN-p38
DN-JNK
DN-Akt
RT-PCR
MMP-13
GAPDH
% of control
Supernatant
Zymography
MMP-13
Loading control

**Fig. 3. ERK is involved in the potentiation of MMP-13 expression by SDF-1α.**

A, chondrocytes were incubated with 100 ng/ml SDF-1α 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 30μM SB203580 or with 10 and 30μM 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 30μM SB203580 or 10 and 30μM SP600125 antagonized TNF-α-induced p38 or JNK phosphorylation, respectively. C, cells were pretreated for 30 min with 30μM PD98059, 10μM SB203580, 10μM SP600125, and 10μM 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-1αfor 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 ± S.E. (percentage of vehicle control). *, $p \leq 0.05$ compared with vehicle. #, $p < 0.05$ compared with SDF-1α-treated group.

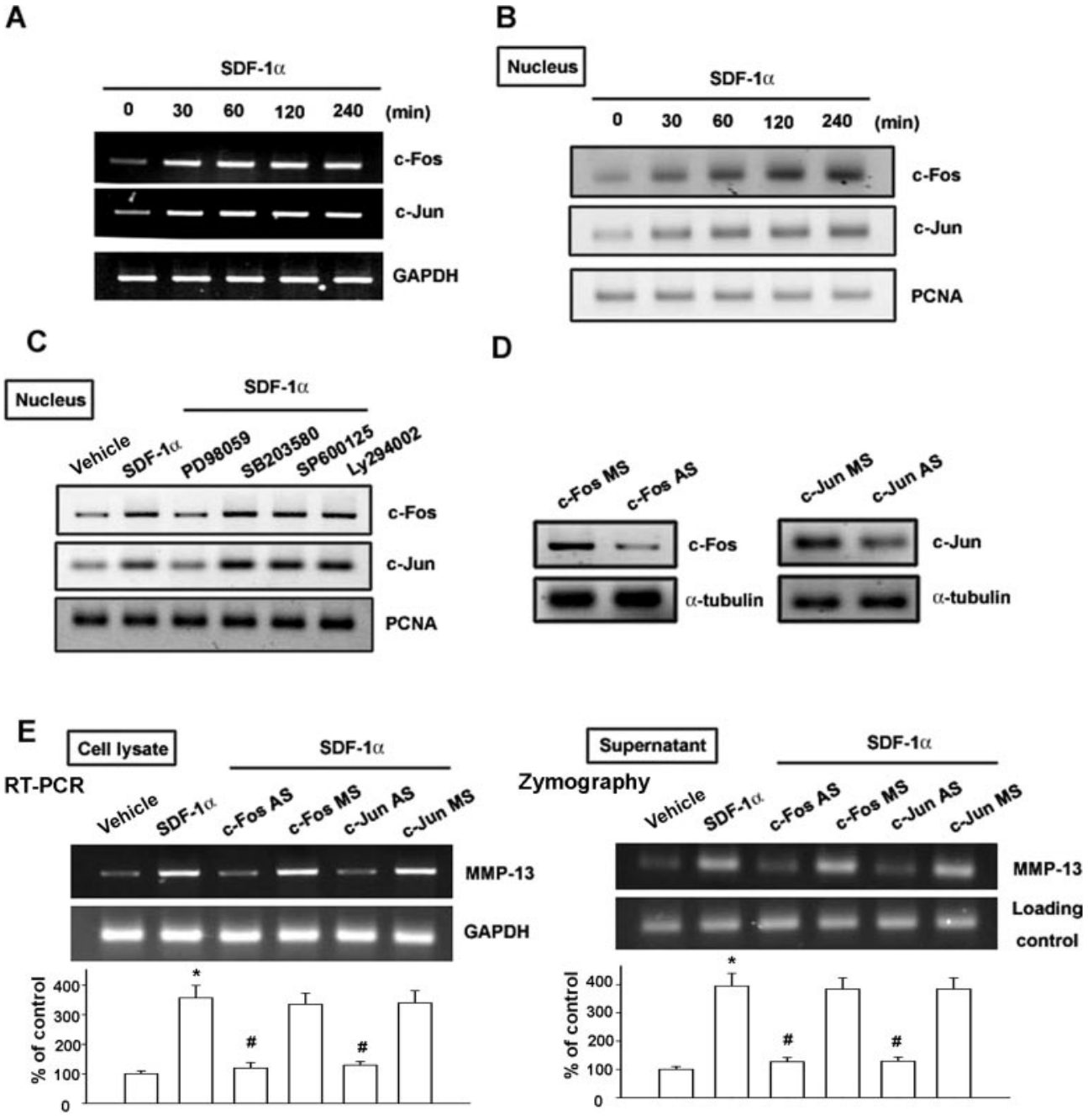

A
SDF-1α
0 30 60 120 240 (min)
c-Fos
c-Jun
GAPDH
B
Nucleus
SDF-1α
0 30 60 120 240 (min)
c-Fos
c-Jun
PCNA
C
Nucleus
SDF-1α
Vehicle
SDF-1α
PD98059
SB203580
SP600125
Ly294002
c-Fos
c-Jun
PCNA
D
c-Fos MS
c-Fos AS
c-Fos
α-tubulin
c-Jun MS
c-Jun AS
c-Jun
α-tubulin
E
Cell lysate
SDF-1α
RT-PCR
Vehicle
SDF-1α
c-Fos AS
c-Fos MS
c-Jun AS
c-Jun MS
MMP-13
GAPDH
% of control
400
300
200
100
0
*
#
#
Supernatant
SDF-1α
Zymography
Vehicle
SDF-1α
c-Fos AS
c-Fos MS
c-Jun AS
c-Jun MS
MMP-13
Loading control
% of control
400
300
200
100
0
*
#
#

**Fig. 4. c-Fos and c-Jun are involved in SDF-1α-induced MMP-13 expression**.

A, cells were treated with 100 ng/ml SDF-1α 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-1α for the indicated times (B), or they were pretreated with 30μM PD98059, 10μM SB203580, 10μM SP600125, or 10μM Ly294002 for 30 min (C) before stimulation with SDF-1α for 240 min. The level of nuclear c-Fos and c-Jun was determined by immuno- blotting 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-For 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-1α 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 ± S.E. *, $p \leqq 0.05$ compared with vehicle control. #, $p < 0.05$ compared with SDF-1α-treated group.

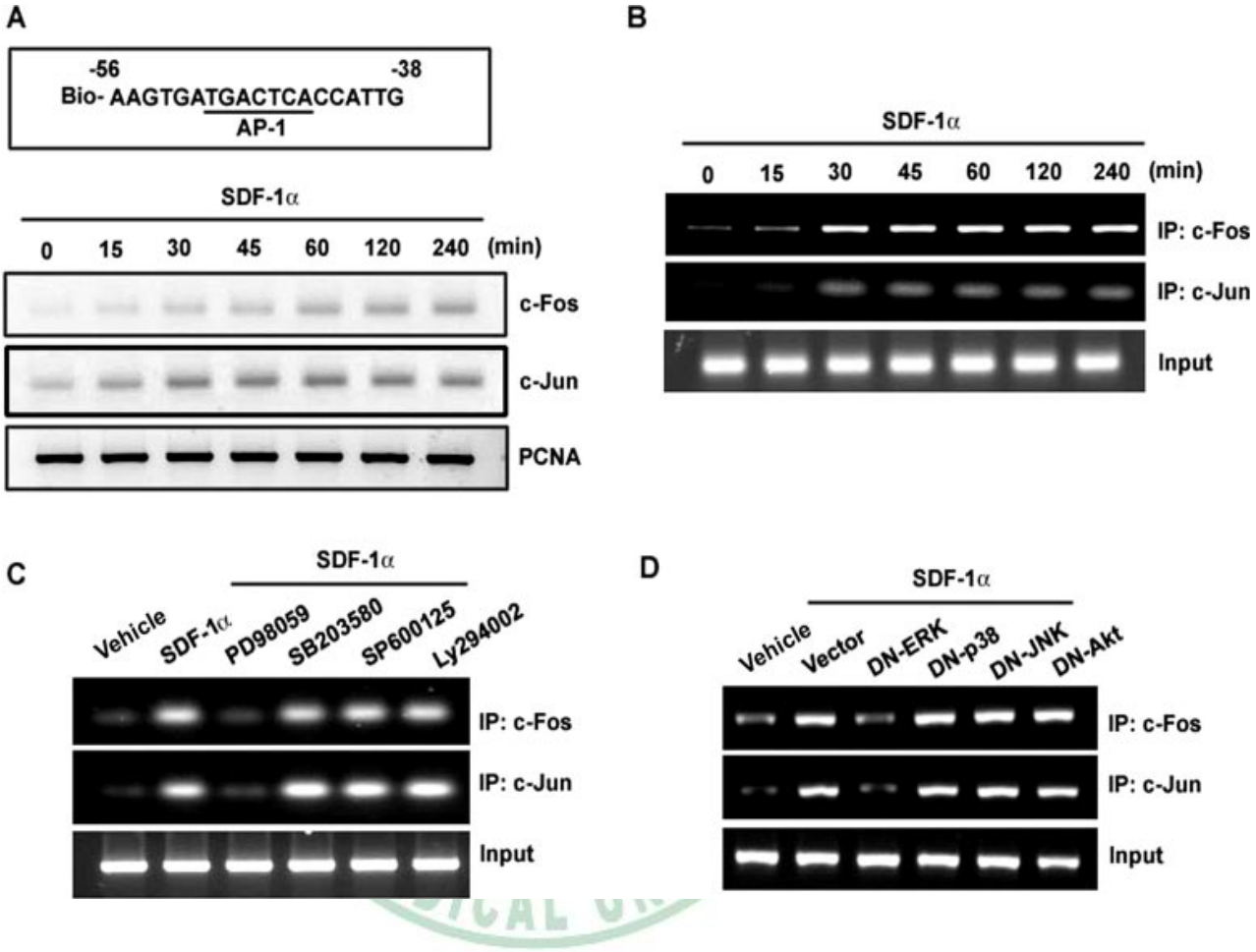
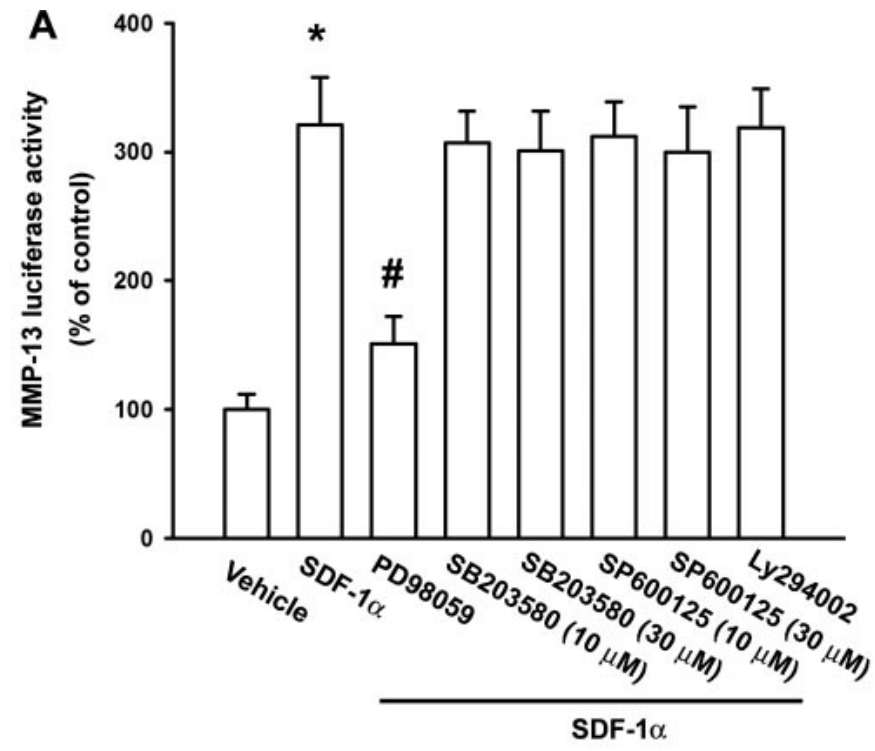

A
-56
-38
Bio- AAGTGATGACTCACCATTG
AP-1
SDF-1α
0 15 30 45 60 120 240 (min)
c-Fos
c-Jun
PCNA
B
SDF-1α
0 15 30 45 60 120 240 (min)
IP: c-Fos
IP: c-Jun
Input
C
SDF-1α
Vehicle
SDF-1α
PD98059
SB203580
SP600125
Ly294002
IP: c-Fos
IP: c-Jun
Input
D
SDF-1α
Vehicle
Vector
DN-ERK
DN-p38
DN-JNK
DN-Akt
IP: c-Fos
IP: c-Jun
Input

**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-1α 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-1α for the indicated times, or they were pretreated for 30 min with 30μM PD98059, 10μM SB203580, 10μM SP600125, and 10μM Ly294002 or transfected with DN mutant of ERK, p38, JNK, and Akt for 24 h followed by stimulation with 100 ng/ml SDF-1α 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).


A
MMP-13 luciferase activity
(% of control)
400
300
200
100
0
*
#
Vehicle
SDF-1α
PD98059
SB203580 (10 μM)
SB203580 (30 μM)
SP600125 (10 μM)
SP600125 (30 μM)
Ly294002
SDF-1α

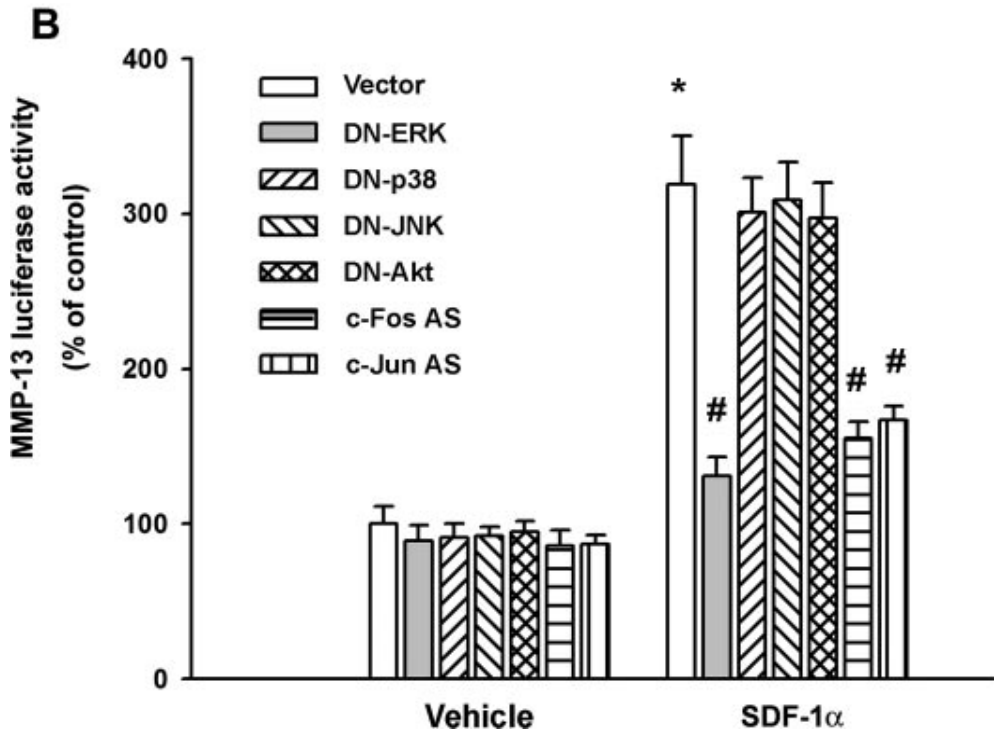

B
MMP-13 luciferase activity
(% of control)
400
300
200
100
0
Vector
DN-ERK
DN-p38
DN-JNK
DN-Akt
c-Fos AS
c-Jun AS
*
#
#
#
Vehicle
SDF-1α

**Fig. 6. Signaling pathways involved in the increase of MMP-13 promoter activity by SDF-1α.**

A, MMP-13 promoter activity was evaluated by transfection with the pMMP-13-Luc luciferase expression vector. Chondrocytes were pretreated with 30μM PD98059, 10 and 30μM SB203580, 10 and 30μMSP600125, or 10μMLy294002 for 30 min before incubation with 100 ng/ml SDF-1α 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-1α. Luciferase activity was measured, and the results were normalized to β-galactosidase activity. Data are expressed as means ± S.E. for three independent experiments performed in triplicate. *, $p \leqq 0.05$ compared with vehicle control; #, $p < 0.05$ compared with SDF-1α-treated group.

# 台中榮民總醫院人體試驗委員會

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## 人體試驗研究計畫許可書

中華民國 96 年 10 月 25 日

計畫名稱：探討adiponectin在滑液纖維母細胞產生IL-6的訊息傳遞路徑

(計畫書版本：Version 1, 20071008；受試者同意書版本：Version 2, 20071025;

本會編號：C07134)

計畫主持人：骨科部邱詠証醫師

協同主持人：骨科部李土生及中國醫藥大學湯智昕助理教授

上述計畫已於中華民國 96 年 10 月 8 日經本院人體試驗委員會第 78 次會議審查通過，本證明有效期限至 97 年 10 月 7 日止。

(每屆滿一年人體試驗委員會必須進行審查，請於有效期限到期二個月前繳交期中報告)

人體試驗委員會主任委員

副院長　陳穎從

陳穎從

## Clinical Trial Authorization

Date: 25 October 2007

The project entitled, " Role of adiponectin on AMPK activation and IL-6 production in human synovial fibroblasts " ( Protocol: Version 1, 20071008, ICF: Version 2, 20071025; IRB TCVGH No: C07134 ) submitted by the investigator Yung-Cheng Chiu, the sub- investigator Tu-Sheng Lee , and Chih-Hsin Tang, has been approved by the Institutional Review Board of Taichung Veterans General Hospital at the 78th full committee meeting on 8 October 2007. This permission is valid to 7 October 2008.

Ying-Tsung Chen

Ying-Tsung Chen, M.D.

Chairman, Institutional Review Board, TCVGH

本會組織與執行皆符合國際醫藥法規協合會之藥品優良臨床試驗規範

The committee is organized and operates according to ICH-GCP and the applicable laws and regulations

# Publication

The Journal of Immunology

# Adiponectin Enhances IL-6 Production in Human Synovial Fibroblast via an AdipoR1 Receptor, AMPK, p38, and NF-κB Pathway[1]

Chih-Hsin Tang,[2*] Yung-Cheng Chiu,[†] Tzu-Wei Tan,[*] Rong-Sen Yang,[2‡] and Wen-Mei Fu[2§]

**Articular adipose tissue is a ubiquitous component of human joints, and adiponectin is a protein hormone secreted predominantly by differentiated adipocytes and involved in energy homeostasis. We investigated the signaling pathway involved in IL-6 production caused by adiponectin in both rheumatoid arthritis synovial fibroblasts and osteoarthritis synovial fibroblasts. Rheumatoid arthritis synovial fibroblasts and osteoarthritis synovial fibroblasts expressed the AdipoR1 and AdipoR2 isoforms of the adiponectin receptor. Adiponectin caused concentration- and time-dependent increases in IL-6 production. Adiponectin-mediated IL-6 production was attenuated by AdipoR1 and 5′-AMP-activated protein kinase (AMPK)α1 small interference RNA. Pretreatment with AMPK inhibitor (araA and compound C), p38 inhibitor (SB203580), NF-κB inhibitor, IκB protease inhibitor, and NF-κB inhibitor peptide also inhibited the potentiating action of adiponectin. Adiponectin increased the kinase activity and phosphorylation of AMPK and p38. Stimulation of synovial fibroblasts with adiponectin activated IκB kinase α/β (IKK α/β), IκBα phosphorylation, IκBα degradation, p65 phosphorylation at Ser (276), p65 and p50 translocation from the cytosol to the nucleus, and κB-luciferase activity. Adiponectin-mediated an increase of IKK α/β activity, κB-luciferase activity, and p65 and p50 binding to the NF-κB element and was inhibited by compound C, SB203580 and AdipoR1 small interference RNA. Our results suggest that adiponectin increased IL-6 production in synovial fibroblasts via the AdipoR1 receptor/AMPK/p38/IKKαβ and NF-κB signaling pathway.** *The Journal of Immunology,* **2007, 179: 5483–5492.**

Adipose tissue is a ubiquitous tissue, which can be found as a structural component of many organs of the human body, including the skin, gut, heart, and joints, and frequently serves the purpose of smoothing out gaps or incongruities between different tissues. Adipocyte has the ability to synthesize and release proinflammatory molecules, complement factors, signaling molecules, growth factors, and adhesion molecules (1, 2), suggesting an integrated function of adipocytes in tissue inflammation. Among these molecules are IL-6, macrophage migration inhibitory factor, M-CSF, TNF-α, complement factor 3a, complement factor B, leptin, resistin, and adiponectin (3–8). For these molecules, the term "adipocytokines" was introduced (1), which reflects the novel function of adipose tissue as an immunological, endocrine, and paracrine organ.

Adiponectin (also known as Acrp30, AdipoQ, and GBP28), an adipocytokine secreted by adipocytes, has been receiving a great deal of attention due to its insulin-sensitizing effects and possible therapeutic use for metabolic disorders (9, 10). Accumulating evidence has suggested a novel link between adipose tissue, adipocytokines, and inflammatory joint disease (11–13). It has been described in the synthesis of proinflammatory cytokines and growth factors in the infrapatellar fat pad from patients with osteoarthritis (OA)[3] (4), and Yamasaki et al. (14) demonstrated that fibroblasts have the potential to transform into adipocytes under the influence of cytokines. Moreover, it has been found that adipocytokine levels (resistin and adiponectin) are greatly elevated in the synovial fluid from patients with OA and rheumatoid arthritis (RA) (15).

IL-6 is a multifunctional cytokine that plays a central role in both innate and acquired immune responses. IL-6 is the predominant mediator of the acute phase response, an innate immune mechanism that is triggered by infection and inflammation (16, 17). IL-6 also plays multiple roles during the subsequent development of acquired immunity against incoming pathogens, including regulation of the expressions of cytokine and chemokine, stimulation of Ab production by B cells, regulation of macrophage and dendritic cell differentiation, and the response of regulatory T cells to microbial infection (16, 17). In addition to these roles in pathogen-specific inflammation and immunity, IL-6 levels are elevated in chronic inflammatory conditions, such as RA (18, 19). Several consensus sequences, including those for NF-κB, CREB, NF-IL-6, and AP-1 in the 5′

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[2] Address correspondence and reprint requests to Dr. Tang Chih-Hsin, Department of Pharmacology, College of Medicine, China Medical University, No. 91, Hsueh-Shih Road, Taichung, Taiwan; Yang Rong-Sen, Department of Orthopaedics, National Taiwan University and Hospital, Taiwan; or Fu Wen-Mei, Department of Pharmacology, College of Medicine, National Taiwan University, Taiwan. E-mail addresses: chtang@mail.cmu.edu.tw, rsyang@ntuh.gov.tw, or wenmei@ntu.edu.tw

[3] Abbreviations used in this paper: OA, osteoarthritis; siRNA, small interference RNA; IKK, IκB kinase; LPS, Lipopolysaccharide; IL, interleukin; TNF, tumor necrosis factor; RT-PCR, reverse transcriptase-polymerase chain reaction; DAPA, DNA affinity protin-binding assay; ChIP, chromatin immunoprecipitation assay; RA, rheumatoid arthritis; AMPK, 5′-AMP-activated protein kinase; RASF, rheumatoid arthritis synovial fibroblasts; OASF, osteoarthritis synovial fibroblasts.

promoter region of the IL-6 gene, have been identified as regulatory sequences that induce IL-6 in response to various stimuli (20, 21). NF-κB, a key transcription factor that regulates IL-6 expression, is a dimer of either transcription factor p65 or transcription factor p50 (22). In a resting state, this dimer is associated with IκBs to retain NF-κB in the cytosol (23). IκB kinase (IKK), which is activated through stimulation by cytokines and bacterial products, phosphorylates IκBα at Ser (32) and Ser (36) and IκBβ at Ser (19) and Ser (23) (24, 25), to produce ubiquitination of IκBα/β at lysine residues and degradation by the 26S proteasome (26).

Adiponectin was originally described as an adipocytokine exclusively expressed by adipose tissue (1). Interestingly, adiponectin shares strong homologies with the complement factor C1q and the proinflammatory cytokine TNF-α. Thus, it belongs to the C1q-TNF-superfamily, the members of which are thought to be derived from a common progenitor molecule and to share common (proinflammatory) functions (27). Adiponectin activates intracellular signaling pathways by activation of 5′-AMP-activated protein kinase (AMPK). Treatment with adiponectin or ectopic expression of its receptors has been shown to increase AMPK phosphorylation and fatty acid oxidation in muscles, and this effect was abolished by the use of dominant-negative AMPK (28, 29). However, the signaling pathway for adiponectin on IL-6 production in synovial fibroblasts is mostly unknown. In the present study, we explored the intracellular signaling pathway involved in adiponectin-induced IL-6 production in synovial fibroblast cells. The results show that adiponectin activates AdipoR1 receptor and results in the activation of AMPK/p38/IKKαβ and NF-κB, leading to up-regulation of IL-6 expression.

## Materials and Methods

### *Materials*

Protein A/G beads, anti-mouse and anti-rabbit IgG-conjugated HRP, rabbit polyclonal Abs specific for IκBα, p-IκBα, IKKα/β, p65, p50, p-p38, p38, and GST-IκBα fusion protein were purchased from Santa Cruz Biotechnology. Rabbit polyclonal Ab specific for AMPKα phosphorylated at Thr (172), IKKα/β phosphorylated at $Ser^{180/181}$, p65 phosphorylated at Ser (276), AMPKα, AMPKα1, and AMPKα2 were purchased from Cell Signaling and Neuroscience. NF-κB inhibitor (PDTC), IκB protease inhibitor (TPCK), SB203580, 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), compound C, and adenosine-9-β-D-arabino-furanoside (AraA) were obtained from Calbiochem. The NF-κB inhibitor peptide (in a cell-permeable form) was purchased from Biomol. IL-6 enzyme immunoassay kit was purchased from Cayman Chemical. [γ-$^{32}$P]ATP was purchased from Amersham Biosciences. The NF-κB luciferase plasmid was purchased from Stratagene. The IKKα (KM) and IKKβ (KM) mutants were gifts from Dr. H. Nakano (Juntendo University, Tokyo, Japan). The p38 dominant negative mutant was provided by Dr. J. Han (Southwestern Medical Center, Dallas, TX). pSV-β-galactosidase vector, luciferase assay kit was purchased from Promega. All other chemicals were obtained from Sigma-Aldrich.

### *Cell cultures*

Synovial tissues were obtained from ten patients with RA and ten patients with OA undergoing knee replacement surgeries (Taichung Veterans General Hospital, Taichung, Taiwan). Patients with RA and OA are fulfilled with diagnostic criteria of American College of Rheumatology (ACR), respectively (46, 47). All of rheumatic arthritis received at least 3 years of DMARD, steroid, or anti-inflammatory drugs therapy. Fresh synovial tissues were minced and digested in a solution of collagenase, and DNase. Isolated fibroblasts were filtered through 70 μM nylon filters. The cells were grown on the plastic cell culture dishes in 95% air-5% $CO_2$ with RPMI 1640 (Invitrogen Life Technologies) which was supplemented with 20 mM HEPES and 10% heat-inactivated FBS, 2 mM-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) (pH adjusted to 7.6). Fibroblasts from passages four to nine were used for the experiments.

### *Measurements of IL-6 production*

Human synovial fibroblasts were cultured in 24-well culture plates. After reaching confluence, cells were treated with 0.1 μg/ml, 0.3 μg/ml, 1 μg/ml, 3 μg/ml, 10 μg/ml, 30 μg/ml human full-length adiponectin (Catalog no. 1065-AP; R&D Systems), and then incubated in a humidified incubator at 37°C for 24 h. For examination of the downstream signaling pathways involved in adiponectin treatment, cells were pretreated with various inhibitors (ara A (0.5 mM); compound C (10 μM); SB203580 (10 μM); PDTC (60 μM); TPCK (3 μM); NF-κB inhibitor peptide (10 μg/ml)) for 30 min before adiponectin (3 μg/ml) administration. After incubation, the medium was removed and stored at −80°C until assay. IL-6 in the medium was assayed using the IL-6 enzyme immunoassay kits, according to the procedure described by the manufacturer.

### *siRNA transfection*

Two pairs of small-interfering RNAs (siRNAs) were synthesized by MDBio. The sequences of human AdipoR1 and AdipoR2 siRNAs were used as previously described (29). The siRNA against human AMPKα1 and AMPKα2 were purchased from Santa Cruz Biotechnology. Cells were transfected with siRNAs (0.4 nmol) using Lipofectamine 2000 (Invitrogen Life Technology) according to the manufacturer's instructions.

### *mRNA analysis by reverse transcriptase-PCR (RT-PCR)*

Total RNA was extracted from synovial fibroblasts using a TRIzol kit (MDBio). The reverse transcription reaction was performed using 2 μg of total RNA that was reverse transcribed into cDNA using oligo(dT) primer, then amplified for 33 cycles using two oligonucleotide primers: IL-6: AAATGCCAGCCTGCTGACGAAG and AACAACAATCTGAGGTGC CCATGCTAC; AdipoR1: CCTTTCCCCAAGCTGAAGCTGC and CCTT GACAAAGCCCTCAGCGAT; AdipoR2: AACGAGCCAACAGAAAAC CGATTG and ATACACACAGAAACAGGCAACATTTG; GAPDH: AAGCCCATCACCATCTTCCAG and AGGGGCCATCCACAGTCTT CT (30).

Each PCR cycle was conducted 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.

### *Western blot analysis*

The cellular lysates were prepared as described previously (31). Proteins were resolved on SDS-PAGE and transferred to Immobilon polyvinyldifluoride (PVDF) membranes. The blots were blocked with 4% BSA for 1 h at room temperature and then probed with rabbit anti-human Abs against IκBα, IKKαβ, p65, p50 or p-AMPK (1/1000) for 1 h at room temperature. After three washes, the blots were subsequently incubated with a donkey anti-rabbit peroxidase-conjugated secondary Ab (1/1000) for 1 h at room temperature. The blots were visualized by ECL using Kodak X-OMAT LS film (Eastman Kodak). Quantitative data were obtained using a computing densitometer and ImageQuant software (Molecular Dynamics).

### *Transfection and reporter gene assay*

Human synovial fibroblasts were cotransfected with 0.8 μg κB-luciferase plasmid, 0.4 μg β-galactosidase expression vector. Fibroblasts were grown to 80% confluent in 12 well plates and were transfected on the following day by Lipofectamine 2000 (LF2000; Invitrogen Life Technologies). DNA and LF2000 were premixed for 20 min and then applied to the cells. After 24 h transfection, the cells were then incubated with the indicated agents. After further 24 h incubation, the medium were removed, and cells were washed once with cold PBS. To prepare lysates, 100 μl reporter lysis buffer (Promega) was added to each well, and cells were scraped from dishes. The supernatant was collected after centrifugation at 13,000 rpm for 2 min. Aliquots of cell lysates (20 μl) containing equal amounts of protein (20–30 μg) were placed into wells of an opaque black 96-well microplate. An equal volume of luciferase substrate was added to all samples, and luminescence was measured in a microplate luminometer. The value of luciferase activity was normalized to transfection efficiency monitored by the cotransfected β-galactosidase expression vector.

### *Preparation of nuclear extracts*

The nuclear extracts were prepared as described previously (32). Cells were harvested and suspended in hypotonic buffer A (10 mM HEPES (pH 7.6), 10 mM KCl, 1 mM DTT, 0.1 mM EDTA, and 0.5 mM PMSF) for 10 min on ice and vortexed for 10 s. Nuclei were pelleted by centrifugation at 12,000 × *g* for 20 s. The supernatants containing cytosolic proteins were

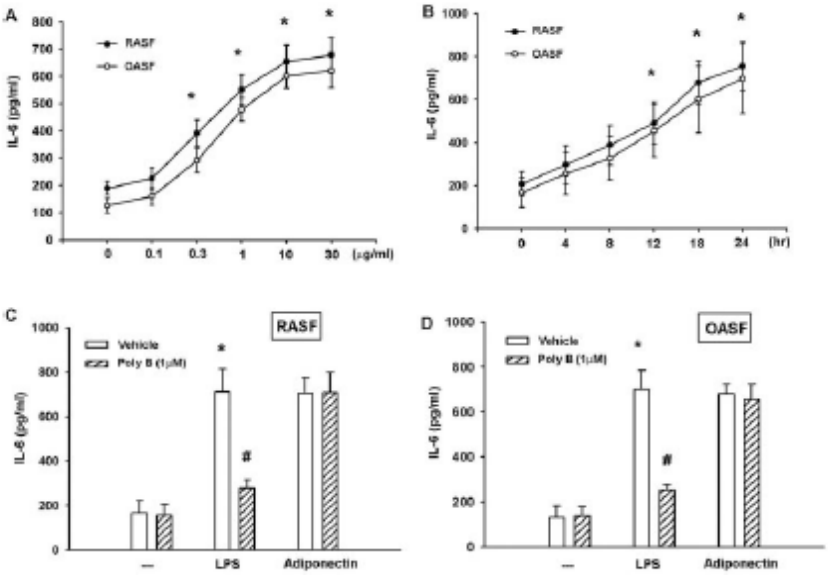


FIGURE 1. Concentration- and time-dependent increases in IL-6 production by adiponectin. Human synovial fibroblasts were incubated with various concentrations of adiponectin for 24 h (*A*) or with adiponectin (3 μg/ml) for 4, 8, 12, 18, or 24 h (*B*). Media were collected to measure IL-6. Results are expressed of four independent experiments performed in triplicate. *, $p \leq 0.05$ as compared with basal level. *C* and *D*, RASF or OASF cells were pretreated with polymyxin B (poly B, 1 μM) for 30 min followed by stimulation with LPS (1 μM) or adiponectin (3 μg/ml) for 24 h. Media were collected to measure IL-6. Results are expressed of four independent experiments performed in triplicate. *, $p \leq 0.05$ as compared with basal level. #, $p < 0.05$ as compared with LPS or adiponectin-treated group.

collected. A pellet containing nuclei was suspended in buffer C (20 mM HEPES (pH 7.6), 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 25% glycerol, and 0.4 M NaCl) for 30 min on ice. The supernatants containing nuclei proteins were collected by centrifugation at 12,000 × *g* for 20 min and stored at −70°C.

### *AMPK in vitro kinase assay*

AMPK activity assays were conducted as previously described (33). In brief, total cell extracts were prepared from synovial fibroblasts in 1× radioimmunoprecipitation assay buffer and precipitated with saturated ammonium sulfate solution (final concentration 35%). Assays were performed at 30°C for 10 min in 25 μl reaction mixtures containing 5 μg protein extracts in a reaction buffer (40 mmol/l HEPES (pH 7.0), 80 mmol/l NaCl, 5 mmol/l magnesium acetate, 1 mmol/l DTT, 200 μmol/l each of AMP and ATP, and 2 μCi [$\gamma$-$^{32}$P] ATP) with or without 200 μmol/l SAMS peptide (Upstate). For immunoprecipitated kinase assays, cell lysates were immunoprecipitated with anti-AMPK-α1 or AMPKα2 Abs (Upstate), washed with 40 mmol/l HEPES (pH 7.0), and suspended in 20 μl reaction buffer. The reaction mixtures were spotted onto P81 cation exchange papers, washed three times with 1% phosphoric acid, and measured using a scintillation counter. AMPK activity was expressed as [$^{32}$P] incorporated per microgram of protein.

### *Protein kinase assays*

The cellular lysates were prepared as described previously (34). Equal amounts of protein were incubated with specific Abs against p38 or IKKα/β in the presences of protein A/G-agarose beads for 12 h at 4°C with gentle rotation. The beads were washed three times with lysis buffer and

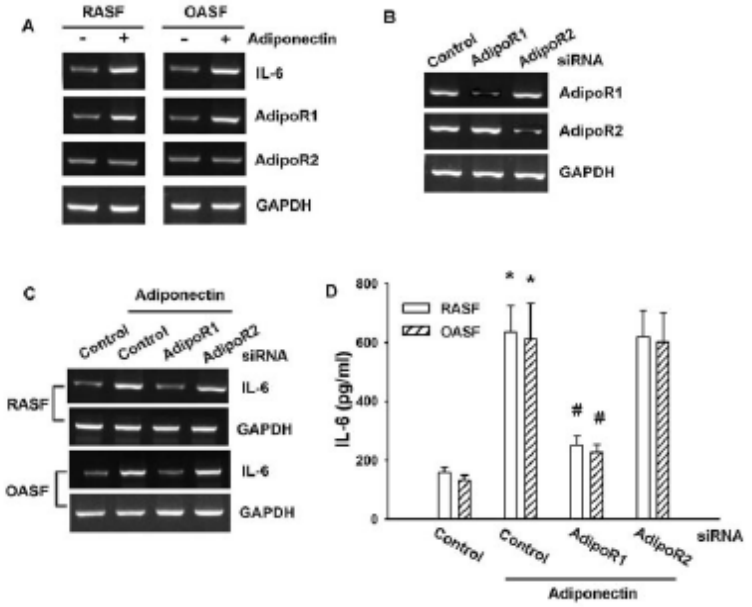


FIGURE 2. Involvement of AdipoR1 receptor in adiponectin-mediated IL-6 production in synovial fibroblasts. Total RNA was extracted from RASF or OASF cells, and subjected to RT-PCR for IL-6, AdipoR1, and AdipoR2 mRNAs using the respective primers. Note that both RASF and OASF cells express IL-6, AdipoR1, and AdipoR2 receptor mRNA, and IL-6 and AdipoR1 mRNA increased in response to adiponectin (3 μg/ml) application for 12 h (*A*). RASF cells were transfected with AdipoR1, AdipoR2, or control siRNA for 24 h, the mRNA levels of AdipoR1 or AdipoR2 was determined by using RT-PCR analysis (*B*). RASF or OASF cells were transfected with AdipoR1, AdipoR2, or control siRNA for 24 h followed by incubation with adiponectin (3 μg/ml) for 24 h to analyze the mRNA and protein expression, respectively. Total RNA and medium were collected, and the expressions of IL-6 were analyzed by RT-PCR and ELISA (*C* and *D*). Results are representative of at least three independent experiments. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase. *, $p \leq 0.05$ as compared with control. #, $p < 0.05$ as compared with adiponectin-treated group.

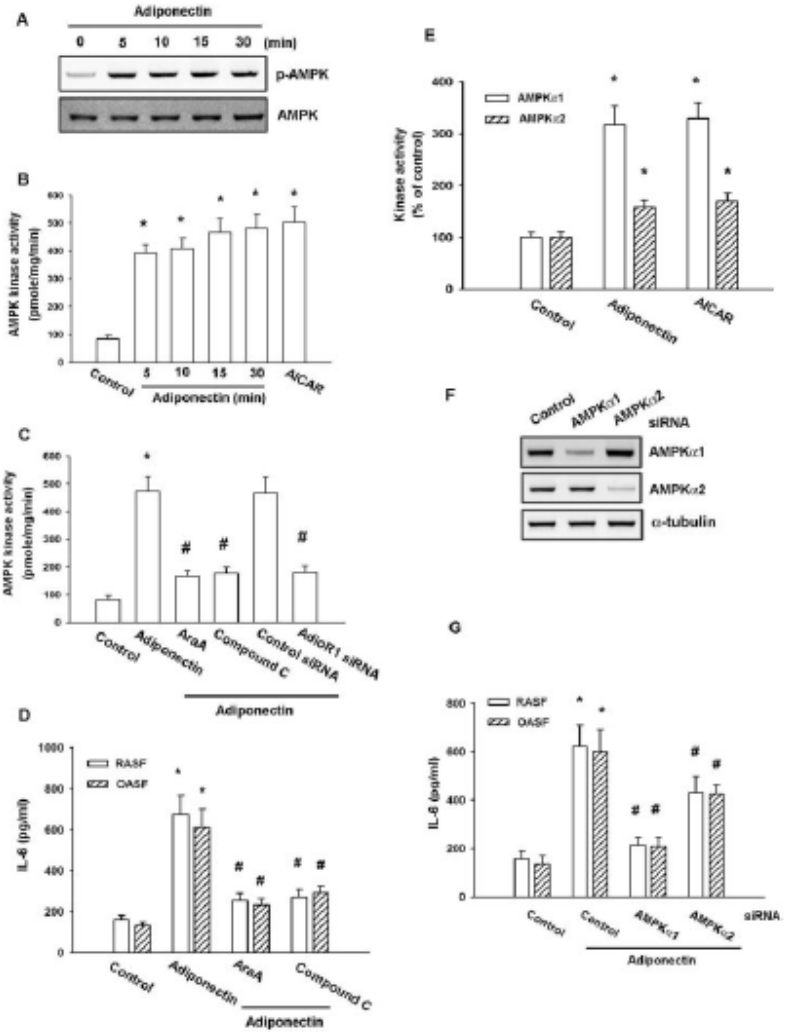


**FIGURE 3.** AMPK is involved in adiponectin-induced IL-6 production. *A*, Cells were incubated with adiponectin (3 μg/ml) for indicated time intervals. Cell lysates were prepared, and then immunoblotted with Ab for phosphor-AMPKα Thr (172) (*upper panel*) or AMPKα (*lower panel*), respectively. *B* and *C*, Cells were incubated with adiponectin (3 μg/ml) for indicated time intervals, or pretreated with araA (0.5 mM) and compound C (10 μM) for 30 min or transfected with AdipoR1 and control siRNA followed by stimulation with adiponectin for 30 min, and AMPK kinase activity was performed as described in *Materials and Methods*. *D*, RASF or OASF cells were pretreated for 30 min with araA (0.5 mM) or compound C (10 μM), and then stimulated with adiponectin (3 μg/ml) for 24 h. Media were collected to measure IL-6. *E*, Cells were incubated with adiponectin (3 μg/ml) or AICAR (1 mM) for 30 min, and cell lysates were then immunoblotted with an Abs specific for AMPKα1 or AMPKα2. The AMPKα1 or AMPKα2 kinase activity was performed as described in *Materials and Methods*. *F*, RASF cells were transfected with AMPKα1, AMPKα2, or control siRNA for 24 h, the protein levels of AMPKα1 or AMPKα2 was determined by using Western blot analysis. *G*, RASF or OASF cells were transfected with AMPKα1, AMPKα2, or control siRNA for 24 h, and then stimulated with adiponectin (3 μg/ml) for 24 h. Media were collected to measure IL-6. Results are representative of at least three independent experiments. *, $p \leq 0.05$ as compared with control. #, $p < 0.05$ as compared with adiponectin-treated group.

two times with kinase buffer (20 mM HEPES, pH 7.4, 20 mM $MgCl_2$, and 2 mM DTT). The kinase reactions were performed by incubating immunoprecipitated beads with 20 μl of kinase buffer (20 mM ATP and 3 μCi of [$\gamma$-$^{32}$P]ATP) at 30°C for 30 min. To assess p38 and IKKα/β activities, 2 μg of MBP and 0.5 μg of GST-IκBα were added as the substrate. The reaction mixtures were analyzed by SDS-PAGE followed by autoradiography.

*DNA affinity protein-binding assay (DAPA)*

Binding of transcription factors to the IL-6 promoter DNA sequences was assayed, as described (21). Following treatment with adiponectin, nuclear extracts were prepared. Biotin-labeled double-stranded oligonucleotides (2 μg) synthesized based on the IL-6 promoter sequence, were mixed at room temperature for 1 h with shaking with 200 μg nuclear extract proteins, and 20 μl streptavidin agarose beads in a 70% slurry. Beads were pelleted and washed three times with cold PBS, then the bound proteins separated by SDS-PAGE, followed by Western blot analysis with specific Abs.

*Chromatin immunoprecipitation assay*

Chromatin immunoprecipitation (ChIP) analysis was performed as described previously (32). DNA immunoprecipitated by anti-p65 or anti-p50 Ab 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′-CAAGACATGCCAAAGTGCTG-3′ and 5′-TTGAGA CTCATGGGAAAATCC-3′ were used to amplify across the human IL-6 promoter region (−288 to −39).

*Statistics*

For statistical evaluation, Mann-Whitney *U* test for non-Gaussian parameters and Student's *t* test for Gaussian parameters (including Bonferroni correction). The difference is significant if the *p* value is <0.05.

## Results

*Adiponectin induces IL-6 production in synovial fibroblasts*

Adiponectin is significantly higher in synovial fluid of patients with osteoarthritis and rheumatoid arthritis (15). Human synovial fibroblast was chosen to investigate the signal pathways of adiponectin in the production of IL-6, an inflammatory response gene. Treatment of rheumatoid arthritis synovial fibroblast (RASF) or osteoarthritis synovial fibroblast (OASF) with adiponectin (0.1–30

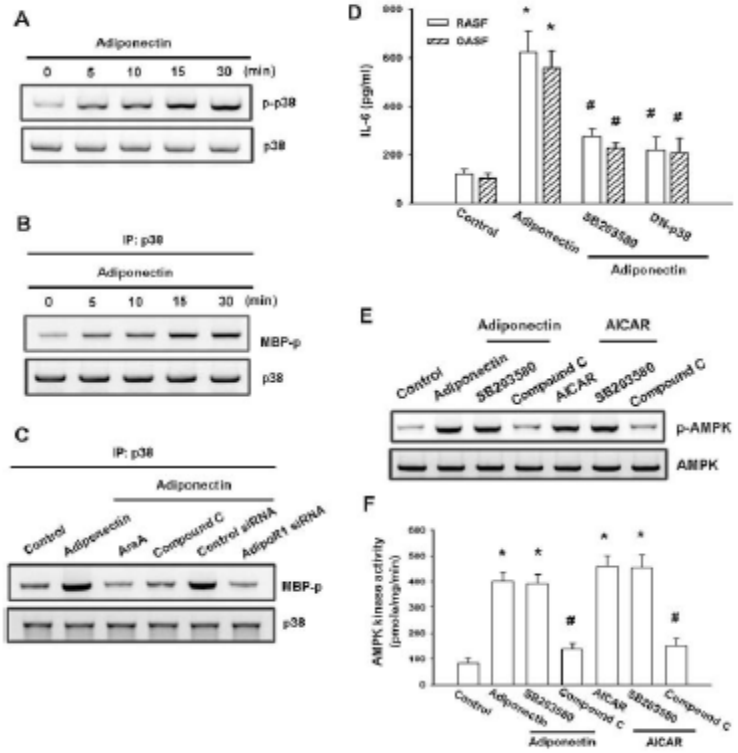


**FIGURE 4.** p38 is involved in adiponectin-mediated IL-6 production in synovial fibroblasts. *A*, Synovial fibroblasts were incubated with adiponectin (3 $\mu$g/ml) for indicated time intervals, cell lysates were then immunoblotted with an Ab specific for phosphor-p38. *B* and *C*, Cells were incubated with adiponectin (3 $\mu$g/ml) for indicated time intervals, or pretreated with araA (0.5 mM) and compound C (10 $\mu$M) for 30 min or transfected with AdipoR1 and control siRNA followed by stimulation with adiponectin for 30 min, and cell lysates were then immunoprecipitated with an Ab specific for p38. One set of immunoprecipitated was subjected to the kinase assay (KA) described in *Materials and Methods* using MBP as a substrate (*upper panel*). The other set of immunoprecipitates was subjected to 10% SDS-PAGE and analyzed by immunoblotting (WB) with the anti-p38 Ab (*lower panel*). Equal amounts of the immunoprecipitated kinase complex present in each kinase assay were confirmed by immunoblotting for p38. *D*, RASF or OASF cells were pretreated with SB203580 (10 $\mu$M) or transfected with dominant negative mutant (DN) of p38 for 24 h, and then stimulated with adiponectin (3 $\mu$g/ml) for 24 h. Media were collected to measure IL-6. *E* and *F*, RASF cells were pretreated for 30 min with SB203580 (10 $\mu$M) or compound C (10 $\mu$M), and then stimulated with adiponectin (3 $\mu$g/ml) or AICAR (1 mM) for 30 min, and cell lysates were then immunoblotted with an Abs specific for phosphor-AMPK$\alpha$ Thr (172). The AMPK kinase activity was also performed as described in *Materials and Methods*. Results are representative of at least three independent experiments. *, $p \leq 0.05$ as compared with control. #, $p < 0.05$ as compared with adiponectin-treated group.

$\mu$g/ml) for 24 h induced IL-6 production in a concentration-dependent manner (Fig. 1*A*), this induction occurred in a time-dependent manner (Fig. 1*B*). After adiponectin (10 $\mu$g/ml) treatment for 24 h, the amount of IL-6 released had increased in both RASF and OASF cells (Fig. 1*B*). To further confirm this stimulation-specific mediation by adiponectin without LPS contamination, polymyxin B, an LPS inhibitor, was used. We found that polymyxin B (1 $\mu$M) completely inhibited LPS (1 $\mu$M)-induced IL-6 release. However, it had no effect on adiponectin (10 $\mu$g/ml)-induced IL-6 release in both RASF and OASF (Fig. 1, *C* and *D*).

### *Involvement of AdipoR1 receptor in adiponectin-mediated increase of IL-6 production*

AdipoR1 is expressed abundantly in skeletal muscle, and AdipoR2 is predominantly expressed in liver (29). However, little is known about the expression of AdipoR1 and AdipoR2 in synovial fibroblasts. To investigate the role of AdipoR1 and AdipoR2 subtype receptors in adiponectin-mediated increase of IL-6 production, we assessed the distribution of these adiponectin receptor subtype receptors in human synovial fibroblasts by RT-PCR analysis. The mRNAs of AdipoR1 and AdipoR2 subtype receptors could be detected in RASF and OASF (Fig. 2*A*). Upon adiponectin treatment for 12 h, the mRNA levels of IL-6 and AdipoR1subtype receptor were evidently increased, whereas other subtypes AdipoR2 receptor mRNA remained unchanged (Fig. 2*A*). We next examined which adiponectin subtype receptors are involved in the adiponectin-mediated increase of IL-6 release, specific inhibition of AdipoR1 receptor expression was accomplished with siRNA (Fig. 2*B*). It was found that AdipoR1 receptor-specific siRNA but not AdipoR2 siRNA or control siRNA significantly blocked adiponectin-mediated increase of IL-6 production in human RASF and OASF by using RT-PCR and ELISA (Fig. 2, *C* and *D*). These results suggest that AdipoR1 may be involved in adiponectin-induced IL-6 expression and release in human synovial fibroblasts.

### *The signaling pathways of AMPK and p38 are involved in the potentiating action of adiponectin*

Adiponectin has been shown to increase fatty acid oxidation via activation of AMPK (35). Fig. 3, *A* and *B* show that adiponectin enhanced AMPK$\alpha$ phosphorylation at the Thr (172) and activity in

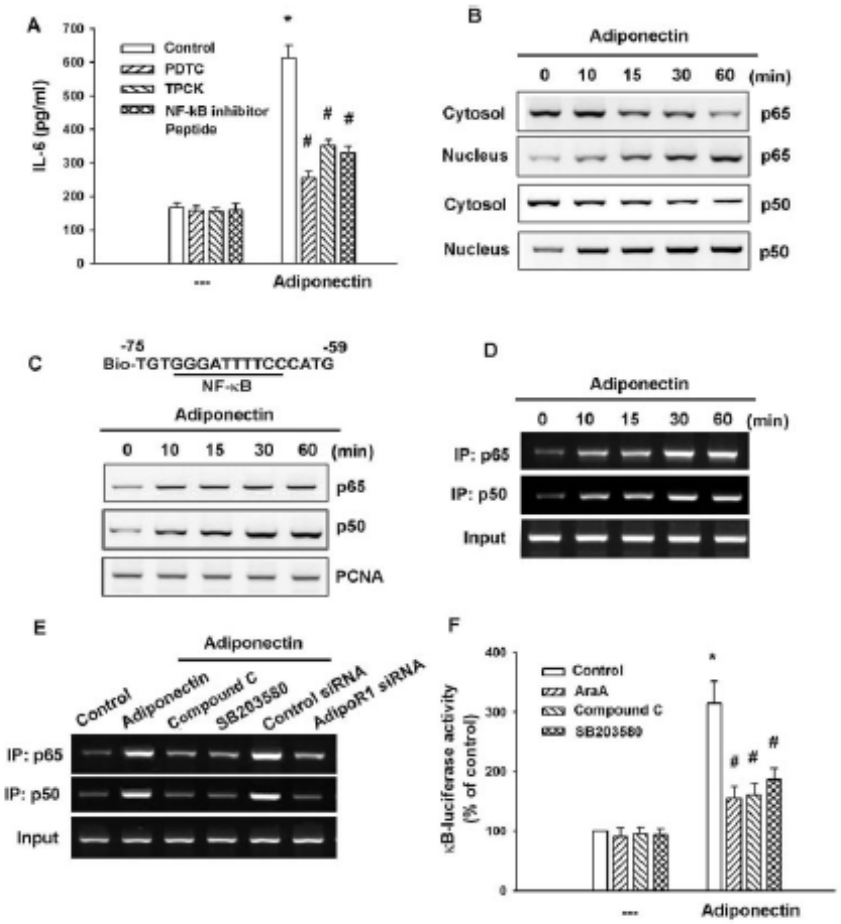


FIGURE 5. NF-κB is involved in the potentiation of IL-6 production by adiponectin. *A*, RASF cells were pretreated for 30 min with PDTC (60 μM), TPCK (3 μM), and NF-κB inhibitor peptide (10 μg/ml) followed by stimulation with adiponectin (3 μg/ml) for 24 h. Media were collected to measure IL-6. Results are expressed of four independent experiments performed in triplicate. *, $p \leq 0.05$ as compared with control. #, $p < 0.05$ as compared with adiponectin-treated group. *B*, Cells were treated with adiponectin (3 μg/ml) for indicated time intervals, and the levels of cytosolic and nuclear p65 or p50 were determined by immunoblotting with p65 or p50 specific Abs, respectively. *C*, The upper schematic illustration represents the consensus sequences of NF-κB site on the IL-6 promoter labeled with biotin. Cells were treated with adiponectin (3 μg/ml) for indicated time intervals, and nuclear extracts were prepared and incubated with biotinylated NF-κB probe. The complexes were precipitated by streptavidin-agarose beads as described under *Materials and Methods* and p65 or p50 in the complexes was detected by Western blot. The equal amount of input nuclear protein was examined by the PCNA protein level. *D* and *E*, Cells were treated with adiponectin (3 μg/ml) for the indicated time intervals, or pretreated with SB203580 (10 μM) and compound C (10 μM) or transfected with AdipoR1 siRNA, and then stimulated with adiponectin (3 μg/ml) for 60 min, and ChIP assay was then performed. Chromatin was immunoprecipitated with anti-p65 or anti-p50 Ab. One percent of the precipitated chromatin was assayed to verify equal loading (Input). *F*, Cells were transfected with κB-luciferase expression vector and then pretreated with araA (0.5 mM), compound C (10 μM) and SB203580 (10 μM) for 30 min before incubation with adiponectin (3 μg/ml) for 24 h. Luciferase activity was then assayed. Results are representative of at least three independent experiments. *, $p \leq 0.05$ as compared with control. #, $p < 0.05$ as compared with adiponectin-treated group.

a time-dependent manner. Pretreatment of cells for 30 min with AMPK inhibitors (araA (0.5 mM) or compound C (10 μM)) and transfection with AdipoR1 siRNA markedly attenuated the adiponectin-induced AMPK kinase activity (Fig. 3*C*). Fig. 3*D* also shows that adiponectin-induced IL-6 production was inhibited by araA or compound C in human RASF and OASF. In addition, treatment of cells with araA (0.5 mM) or compound C (10 μM) did not affect cell viability, which was assessed by the MTT assay (data not shown). In an attempt to determine which catalytic subunit of AMPKα1 or AMPKα2 mediated adiponectin signaling in human synovial fibroblasts, we performed in vitro kinase assay using Abs specific for each isoform. The kinase activity of both α1 and α2 isoforms was increased by adiponectin treatment, the α1 isoform by 3-fold and α2 isoform by ~50% (Fig. 3*E*). In addition, treatment of cells with 5-Aminoimidazole-4-caroxamide-1-β-D-ribofuranoside (AICAR; 1 mM), which activates AMPK after being metabolized to 5-aminoimidazole-4-caroxamide-1-β-D-ribofuranoside-5-monophosphate in the cells, also increased the kinase activity of AMPKα1 and AMPKα2 (Fig. 3*E*). To further examine whether AMPKα1 activation is involved in the signal transduction pathway leading to IL-6 production by adiponectin, the AMPKα1 siRNA was used. AMPKα1 siRNA specifically inhibited the expression of AMPKα1 but not AMPKα2 (Fig. 3*F*). Fig. 3*G* also shows that adiponectin-induced IL-6 production was inhibited by

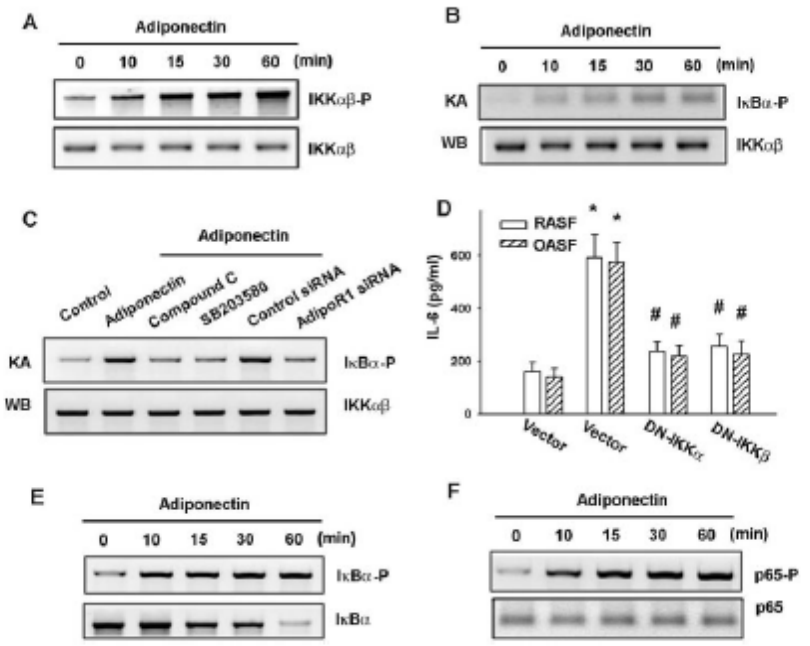


FIGURE 6. Adiponectin induces IKK α/β activation, IκBα phosphorylation, IκBα degradation, and p65 Ser (276) phosphorylation in synovial fibroblasts. Synovial fibroblasts were incubated with adiponectin (3 μg/ml) for indicated time intervals, cell lysates were then immunoblotted with an Ab specific for phosphor-IKKα/β (*A*). Cells were incubated with adiponectin (3 μg/ml) for indicated time intervals, or pretreated with SB203580 (10 μM) and compound C (10 μM) or transfected with AdipoR1 siRNA followed by stimulation with adiponectin for 60 min, and cell lysates were then immunoblotted with Ab specific for IKKα/β. One set of immunoprecipitates was subjected to the kinase assay (KA) described in *Materials and Methods* using the GST-IκBα fusion protein as a substrate (*upper panel*). The other set of immunoprecipitates was subjected to 10% SDS-PAGE and analyzed by immunoblotting (WB) with the anti-IKKα/β Ab (*lower panel*). Equal amounts of the immunoprecipitated kinase complex present in each kinase assay were confirmed by immunoblotting for IKK α/β (*B* and *C*). Cells were transfected with IKKα, IKKβ mutant, or vector for 24 h followed by stimulation with adiponectin for 24 h. Media were collected to measure IL-6 (*D*). Results are representative of at least three independent experiments. *, $p \leq 0.05$ as compared with control. #, $p < 0.05$ as compared with adiponectin-treated group. Synovial fibroblasts were incubated with adiponectin for indicated time intervals, and cytosolic levels of IκBα phosphorylation, IκBα degradation, and p65 Ser (276) phosphorylation were determined by immunoblotting using phospho-IκBα, IκBα-specific and p65 phosphorylated at Ser (276) Abs, respectively (*E* and *F*).

AMPKα1 siRNA. In addition, AMPKα2 siRNA also slightly attenuated adiponectin-induced IL-6 production. Therefore, AMPKα1 is much more important in adiponectin-induced IL-6 release, although the role of AMPKα2 cannot be ruled out. It was recently reported that adiponectin activates p38 MAPK in addition to AMPK in C2C12 cells (29), we tested whether p38 might also be involved in adiponectin-induced IL-6 production. As shown in Fig. 4*A*, treatment of fibroblasts with adiponectin resulted in a time-dependent phosphorylation of p38. Next, we directly examined p38 kinase activity in response to adiponectin. In vitro p38 kinase activity was measured using MBP as a p38 exogenous substrate. Fig. 4*B* shows that treatment of fibroblasts with adiponectin induced an increase in p38 activity began 5 min, peaked at 10–30 min. We also found that pretreatment of cells for 30 min with araA, compound C or transfection with AdipoR1 siRNA markedly inhibited the adiponectin-induced p38 activity (Fig. 4*C*). We then investigated the role of p38 in mediating adiponectin-induced IL-6 expression using the specific p38 inhibitor SB203580. Pretreatment of cell with SB203580 (10 μM) or transfection with dominant negative mutant of p38 attenuated adiponectin-induced IL-6 production (Fig. 4*D*). In addition, treatment of cells with SB203580 (10 μM) did not affect cell viability, which was assessed by the MTT assay (data not shown). Next, we examine the relationship of AMPK and p38, when AMPK was chemically inhibited with compound C, the stimulation of AMPK phosphorylation and activity was attenuated. In contrast, inhibition of p38 with SB203580 did not affect adiponectin-induced phosphorylation and activity of AMPK. Similar results were obtained when AMPK was directly activated using AICAR (Fig. 4, *E* and *F*). AICAR increase the phosphorylation and kinase activity of AMPK and these effects were inhibited by compound C but not SB203580 (Fig. 5, *E* and *F*). Therefore, these results indicated that p38 may function as a downstream signaling molecule of AMPK in the adiponectin signaling pathway.

### *Involvement of NF-κB in adiponectin-induced IL-6 production*

NF-κB activation has been reported to be necessary for IL-6 induction in macrophages (36). To examine whether NF-κB activation is involved in the signal transduction pathway leading to IL-6 expression caused by adiponectin, the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) was used. Fig. 5*A* shows that PDTC (30 μM) inhibited the enhancement of IL-6 production induced by adiponectin. Furthermore, pretreatment of synovial fibroblasts with an IκB protease inhibitor (L-1-tosylamido-2-phenylenylethyl chloromethyl ketone (TPCK, 3 μM)) and NF-κB inhibitor peptide (10 μg/ml) (36) also antagonized the potentiating action of IL-6 (Fig. 5*A*). In addition, treatment of cells with PDTC (30 μM), TPCK (3 μM) or NF-κB inhibitor peptide (10 μg/ml) did not affect cell viability, which was assessed by the MTT assay (data not shown). It has been report that the NF-κB binding site between

−72 and −63 was important for the activation of the IL-6 gene (21). NF-κB activation was further evaluated by analyzing the translocation of NF-κB from cytosol to the nucleus, as well as by DNA affinity protein-binding assay (DAPA) and chromatin immunoprecipitation (ChIP) assay. Treatment of cells with adiponectin resulted in a marked translocation of p65 and p50 NF-κB from the cytosol to the nucleus (Fig. 5*B*). DAPA experiments showed a time-dependent increase in the binding of p65 and p50 to the NF-κB element on the IL-6 promoter after adiponectin treatment (Fig. 5*C*). The in vivo recruitment of p65 and p50 to the IL-6 promoter (−288 to −39) was assessed by ChIP assay. In vivo binding of p65 and p50 to the NF-κB element of IL-6 promoter occurred as early as 10 min and sustained to 60 min after adiponectin stimulation (Fig. 5*D*). The binding of p65 and p50 to NF-κB element by adiponectin stimulation was attenuated by compound C, SB203580 and AdipoR1 siRNA (Fig. 5*E*). To further confirm the NF-κB element involved in the action of adiponectin-induced IL-6 expression, transient transfection was performed using the κB promoter-luciferase constructs. Synovial fibroblasts incubated with adiponectin (3 μg/ml) led to a 3.1-fold increase in κB promoter activity. The increase of κB activity by adiponectin was antagonized by araA, compound C and SB203580 (Fig. 5*F*). These results suggest that NF-κB activation is necessary for adiponectin-induced IL-6 production in human synovial fibroblasts.

*Adiponectin causes an increase in IKK α/β phosphorylation, IκBα phosphorylation and IκBα degradation*

We further examined the upstream molecules involved in adiponectin-induced NF-κB activation. Stimulation of cells with adiponectin induced IKKα/β phosphorylation and activity in a time-dependent manner (Fig. 6, *A* and *B*). Pretreatment of cells with compound C and SB203580 or transfection with AdipoR1 siRNA attenuated adiponectin-induced IKKα/β activity (Fig. 6*C*). Furthermore, transfection with IKKα or IKKβ mutant markedly inhibited the adiponectin-induced IL-6 production (Fig. 6*D*). These data suggest that IKKα/β activation is involved in adiponectin-induced IL-6 production in human synovial fibroblasts. Treatment with synovial fibroblasts with adiponectin also caused IκBα phosphorylation and IκBα degradation in a time-dependent manner (Fig. 6*E*). Next, we further examined p65 phosphorylation at Ser (276) by adiponectin in synovial fibroblasts. Treatment of cells with adiponectin induced p65 phosphorylation at Ser (276) in a time-dependent manner (Fig. 6*F*).

## Discussion

In contrast to the ample data in the field of endocrinology and cardiovascular disease, little is known about the role of adipose tissue and adipocytokines, especially of adiponectin, in immunological and inflammatory disease, such as arthritis (6, 37, 38). It has been reported that adiponectin is significantly higher in synovial fluid of patients with osteoarthritis and rheumatoid arthritis (15). Here we further identify IL-6 as a target protein for the adiponectin signaling pathway that regulates cell inflammatory response in both RASF and OASF. Using pharmacological and genetic inhibitors show that these inhibitors all attenuated adiponectin-induced IL-6 release, indicating adiponectin through the same signaling pathway to induce IL-6 production in RASF and OASF. Ehling et al., (30) demonstrated that the adiponectin time-dependent and concentration-dependent induce IL-6 production in human synovial fibroblasts, and the adiponectin receptor expressed in both RA and OA fibroblast. In addition, using p38, protein kinase A, protein kinase C and cAMP-dependent PKA inhibitor to block signaling pathways, only incubation with p38 inhibitor SB203580 and transfection with p38 siRNA significantly inhibited the adiponectin-induced IL-6 production (30). There are several novel findings in our current research. First, we demonstrated that the AdipoR1 but not AdipoR2 was involved in the adiponecin-induced IL-6 production. Second, AMPKα1 was more important than AMPKα2 in the adiponectin-induced IL-6 production. Third, IKKαβ/NF-κB pathway was involved in adiponecitn-induced IL-6 release. Fourth, the potentiation of IL-6 by adiponectin required an activation of the AdipoR1 receptor, AMPKα1, p38, IKKαβ and NF-κB signaling pathway. Fifth, the same signaling pathway was required for adiponectin-induced IL-6 production in both RASF and OASF. Our findings suggest that adiponectin acts as an inducer of inflammatory cytokines such as IL-6 and as an enhancer of the inflammatory response in RA and OA.

Two adiponectin receptors, AdipoR1 and AdipoR2, that mediated the biological effects of adiponectin was identified recently (29). AdipoR1 is a high-affinity receptor for globular adiponectin and a low-affinity receptor for the full-length ligand, whereas AdipoR2 is an intermediate-affinity receptor for both forms of adiponectin (29). AdipoR1 is abundantly expressed in skeletal muscle, whereas AdipoR2 is predominantly expressed in the liver. However, the express of AdipoR1 or AdipoR2 receptors in synovial fibroblast are large unknown. We found that RASF and OASF cells express both AdipoR1 and AdipoR2 receptor isoforms by RT-PCR analysis. In addition, adiponectin increases the expression of IL-6 and AdipoR1 but not AdipoR2. Furthermore, transfection with AdipoR1 but not AdipoR2 siRNA antagonized the adiponectin-induced IL-6 production. These results suggest that AdipoR1 is an upstream receptor in adiponectin-induced IL-6 release.

AMPK is a heterotrimeric serine/threonine kinase composed of α catalytic a subunit and regulatory β and γ subunit (39). It has been previously shown that AMPK is involved in the signaling pathway for the metabolic effects of adiponectin (39). We demonstrated that the AMPK inhibitors ara A and compounds C antagonized the adiponectin-mediated potentiation of IL-6 expression, suggesting that AMPK activation is an obligatory event in adiponectin-induced IL-6 expression in these cells. In an attempt to determine which catalytic subunit of AMPK α1 or α2 mediated adiponectin signaling in human synovial fibroblasts. We found that adiponectin and AICAR (AMPK activator) increased kinase activity of AMPKα1 but only slightly increased the kinase activity of AMPKα2. This was further confirmed by the results that the siRNA of AMPKα1 inhibited the enhancement of IL-6 production by adiponectin. However, only slightly effect of AMPKα2 siRNA in adiponectin-induced IL-6 expression. Therefore, AMPKα1 is much more important in adiponectin-induced IL-6 release. Although we cannot ruled out the effect of AMPKα2 in adiponectin-induced IL-6 production in synovial fibroblasts. It has been reported that AMPK interacts with p38 to regulated glucose metabolism (40). We examined the potential role of p38 in the signaling pathway adiponectin-induced IL-6 expression. Pretreatment of synovial fibroblasts for 30 min with SB203580 or transfection with p38 mutant for 24 h markedly attenuated the adiponectin-induced IL-6 production. In addition, we also found that treatment of synovial fibroblasts with adiponectin induced increases in p38 phosphorylation and kinase activity. These effects were inhibited by araA, compound C and AdipoR1 siRNA, indicating the involvement of AdipoR1-AMPK-dependent p38 activation in adiponectin-mediated IL-6 induction. It has also reported that AMPK is downstream molecule of p38 in the control of myocardial glucose metabolism (41). In this study, we found that p38 inhibitor SB203580 cannot antagonized the adiponectin or AICAR-increased AMPK phosphorylation and kinase activity.

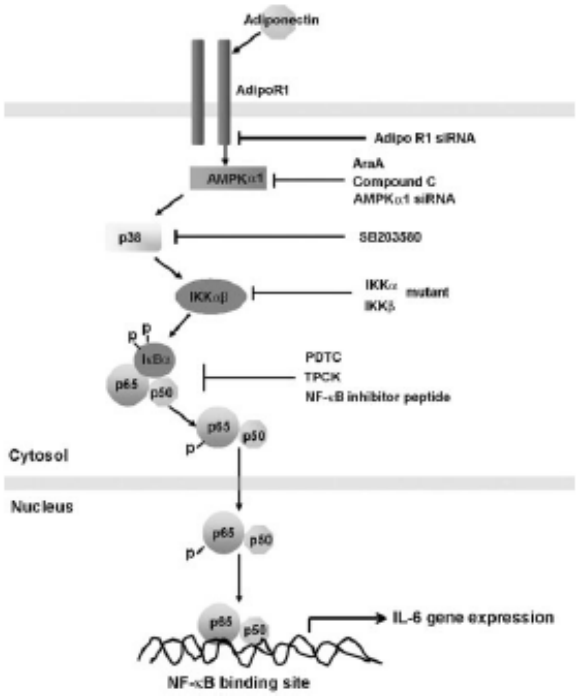


FIGURE 7. Schematic diagram of the signaling pathways involved in adiponectin-induced IL-6 production in synovial fibroblasts. Adiponectin increases IL-6 expression by binding to the AdipoR1 receptor and activation of AMPKα1, p38, IKKαβ, which enhances binding of p65 and p50 to the NF-κB site, resulting in the transactivation of IL-6 expression.

Therefore, these results indicated that p38 may function as a downstream signaling molecule of AMPK in the adiponectin signaling pathway.

There are several binding sites for a number of transcription factors including NF-κB, CREB, NF-IL-6, and AP-1 box in the 5′ region of the IL-6 gene (20, 21). Recent studies on the IL-6 promoter have demonstrated that IL-6 induction by several transcription factors occurs in a highly stimulus-specific or cell-specific manner. For example, NF-κB has been shown to control the induced transcription of IL-6 in mouse macrophages (36). In osteoblasts, vasoactive intestinal peptide-induced IL-6 expression is mediated by AP-1 and CREB (42). The results of this study show that NF-κB activation contributes to adiponectin-induced IL-6 production in synovial fibroblasts, and that the inhibitors of the NF-κB-dependent signaling pathway, including PDTC, TPCK or NF-κB inhibitor peptide inhibited adiponectin-induced IL-6 expression. In an inactivated state, NF-κB is normally held in the cytoplasm by the inhibitor protein IκB. Upon stimulation, such as by TNF-α, IκB proteins become phosphorylated by the multisubunit IKK complex, which subsequently targets IκB for ubiquitination, and then are degraded by the 26S proteasome. Finally, the free NF-κB translocates to the nucleus, where it activates the responsive gene (43). In the present study, we found that treatment of synovial fibroblasts with adiponectin resulted in increases in IKKα/β phosphorylation and activity, p65 and p50 translocation from the cytosol to the nucleus, and the binding of p65 and p50 to NF-κB element on IL-6 promoter. Using transient transfection with κB-luciferase as an indicator of NF-κB activity, we also found that adiponectin-induced an increase in NF-κB activity. The IKKs can be stimulated by various proinflammatory stimuli, including IL-1β, peptidoglycan and thrombin (43, 44). These extracellular signals activate the IKK complex, which is comprised of catalytic subunits (IKKα and IKKβ) and a linker subunit (IKKγ/NEMO). This kinase complex, in turn, phosphrylates IκBα at Ser (32) and Ser (36) and signals for ubiquitinrelated degradation. The released NF-κB is then translocated into the nucleus where it promotes NF-κB-dependent transcription (45). The findings of our experiments showed that pretreatment of synovial fibroblasts with compound c, SB203580 or transfection with AdipoR1 siRNA antagonized the increase of IKKα/β activity by adiponectin. Based on these findings, we suggest that the AdipoR1/AMPK/p38 pathway is involved in adiponectin-induced IKKα/β activation. Here, we also found that treatment with adiponectin (3 μg/ml) for 24 h caused TNF-α and IL-1β release in human synovial fibroblasts from 52 ± 8 to 105 ± 16 pg/ml ($n = 3$, $p < 0.05$) and 196 ± 13 to 363 ± 18 pg/ml ($n = 3$, $p < 0.05$), respectively. In addition, pretreatment of cells with araA, compound C, SB203580, PDTC, TPCK or NF-κB inhibitor peptide also antagonized adiponectin-induced TNF-α and IL-1β release (data not shown). These results suggest that the similar signaling pathways are involved in adiponectin-induced cytokines/chemokines release.

In conclusion, the signaling pathway involved in adiponectin-induced IL-6 production in human synovial fibroblasts has been explored. Adiponectin increases IL-6 production by binding to the AdipoR1 receptor and activation of AMPK, p38 and IKKαβ, which enhances binding of p65 and p50 to the NF-κB site, resulting in the transactivation of IL-6 production (Fig. 7).

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## Disclosures

The authors have no financial conflict of interest.

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