Adiponectin Increases MMP-3 Expression in Human Chondrocytes Through AdipoR1 Signaling Pathway

Kwok-Man Tong,[1,2] Chao-Ping Chen,[1,3] Kui-Chou Huang,[1] Dong-Chen Shieh,[2] Hsu-Chen Cheng,[4] Chung-Yuh Tzeng,[1] Kun-Hui Chen,[1] Yung-Cheng Chiu,[1,2,5] and Chih-Hsin Tang[6,7]*

[1]Department of Orthopaedics, Taichung Veterans General Hospital, Taichung, Taiwan

[2]Department of Nursing, Hungkuang University, Taichung, Taiwan

[3]Department of Exercise and Health Science, National Taiwan College of Physical Education, Taichung, Taiwan

[4]Department of Life Sciences, National Chung Hsing University, Taichung, Taiwan

[5]Graduate Institute of Clinical Medical Science, China Medical University, Taichung, Taiwan

[6]Department of Pharmacology, School of Medicine, China Medical University and Hospital, Taichung, Taiwan

[7]Graduate Institute of Basic Medical Science, China Medical University and Hospital, Taichung, Taiwan

ABSTRACT

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. The adiponectin is significantly higher in synovial fluid of patients with osteoarthritis and rheumatoid arthritis. Matrix metalloproteinases (MMP)-3 may contribute to the breakdown of articular cartilage during arthritis. We investigated the signaling pathway involved in MMP-3 caused by adiponectin in human chondrocytes. Adiponectin increased the secretion of MMP-3 in cultured human chondrocytes, as shown by qPCR, Western blot, and ELISA analysis. Adiponectin-mediated MMP-3 expression was attenuated by AdipoR1 but not AdipoR2 siRNA. Pretreatment with 5′-AMP-activated protein kinase (AMPK) inhibitor (araA and compound C), p38 inhibitor (SB203580), and NF-κB inhibitor (PDTC and TPCK) also inhibited the potentiating action of adiponectin. Activations of p38, AMPK, and NF-κB pathways after adiponectin treatment were demonstrated. Taken together, our results provide evidence that adiponectin acts through AdipoR1 to activate p38 and AMPK, resulting in the activations of NF-κB on the MMP-3 promoter and contribute cartilage destruction during arthritis. J. Cell. Biochem. 112: 1431–1440, 2011. © 2011 Wiley-Liss, Inc.

KEY WORDS: ADIPONECTIN; CHONDROCYTES; MMP-3; AMPK; p38

Chondrocytes are the only cellular components of cartilage. Under normal physiologic 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 [van Osch et al., 2009]. However, in pathologic conditions such as osteoarthritis (OA) or rheumatoid arthritis (RA), chondrocytes production of these enzymes increases considerably, resulting in aberrant cartilage destruction [Pelletier et al., 2001; Aigner and McKenna, 2002].

MMPs are a large family of structurally related calcium- and zinc-dependent proteolytic enzymes involved in the degradation of many different components of the extracellular matrix [Vincenti, 2001]. MMPs are expressed in a number of different cell types and play a key role in diverse cellular processes [Sternlicht and Werb, 2001]. MMP-3 (stromelysin-1) is secreted as an inactive soluble pro-form which can be activated by a variety of proteases [Carmeliet et al., 1997]. Like most MMPs, MMP-3 is not expressed in normal tissue,

but is rapidly induced in cases of tissue repair or during remodeling processes [Page-McCaw et al., 2007]. MMP-3 has been previously shown to be overexpressed in OA [Burrage et al., 2006]. 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.

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 [Scherer et al., 1995; Hu et al., 1996]. Accumulating evidence has suggested that a novel links between adipose tissue, adipocytokines, and inflammatory joint disease [Bokarewa et al., 2003; Dumond et al., 2003]. It has been described that the synthesis of proinflammatory cytokines and growth factors in the infrapatellar fat pad from patients with OA [Mohamed-Ali et al., 1997]. Moreover, it has been found that adipocytokine levels (resistin and adiponectin) are greatly elevated in the synovial fluid from patients with OA and RA [Schaffler et al., 2003].

Adiponectin was originally described as an adipocytokine exclusively expressed by adipose tissue [Scherer et al., 1995]. 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 functions [Shapiro and Scherer, 1998]. 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 [Yamauchi et al., 2002, 2003]. It has been reported that adiponectin induced MMP-3 expression in human chondrocytes [Lago et al., 2008]. However, the detail signaling pathways in adiponectin-induced MMP-3 expression in human chondrocytes are mostly unknown. Here we found that adiponectin increased the expression of MMP-3. In addition, AdipoR1 receptor, p38, AMPK, and NF-κB signaling pathways may be involved in increasing of MMP-3 expression by adiponectin. The elevated level of adiponectin in synovial fluid from patients with arthritis may contribute to release MMP-3 in cartilage during arthritic pathogenesis.

MATERIALS AND METHODS

MATERIALS

Anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase, rabbit polyclonal antibodies specific for β-actin, AdipoR1, AdipoR2, AMPKα1, AMPKα2, p-p38, p38, IKK, p-IκBα, IκBα, and p65 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibody specific for AMPKα phosphorylated at Thr^{172}, IKKαβ phosphorylated at Ser$^{180/181}$ and p65 phosphorylated at Ser^{536}were purchased from Cell Signaling and Neuroscience (Danvers, MA). PDTC, TPCK, SB203580, 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), compound C, and adeno-sine-9-β-D-arabino-furanoside (AraA) were obtained from Calbiochem (San Diego, CA). Human full-length adiponectin was obtained form R&D Systems (Minneapolis, MN). The NF-κB luciferase plasmid was purchased from Stratagene (La Jolla, CA). 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 (South-western Medical Center, Dallas, TX). pSV-β-galactosidase vector, luciferase assay kit was purchased from Promega (Madison, WI). All other chemicals were obtained from Sigma–Aldrich (St. Louis, MO).

CELL CULTURE

Primary cultures of human chondrocytes were isolated from articular cartilage as previously described [Chiu et al., 2007; Hsu et al., 2007]. After approval by the local ethics committee, human articular chondrocytes were isolated from knee replacement surgeries of patients with OA. Cartilage pieces were minced finely, and chondrocytes were isolated by sequential enzymatic digestion at 37°C with 0.1% hyaluronidase for 30 min and 0.2% collagenase for 1 h. Isolated chondrocytes were filtered through 70 μM nylon filters. The cells were grown on the plastic cell culture dishes in 95% air–5% CO_2 with Dulbecco's modified Eagle's medium (DMED; Gibco, Grand Island, NY) 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). To verify the chondrocytic phenotype of the isolated cells, the type II collagen (marker gene of chondrocytes) has been examined. We found that the isolated cells expressed the type II collagen mRNA and protein (Supplementary Figure S1).

siRNA TRANSFECTION

Two pairs of small-interfering RNAs (siRNAs) were synthesized by MDBio, Inc. (Taipei, Taiwan). The sequences of human AdipoR1 and AdipoR2 siRNAs were used as previously described [Tang et al., 2007]. 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) according to the manufacturer's instructions.

FLOW CYTOMETRIC ANALYSIS

Human chondrocytes were plated in six-well dishes. The cells were then washed with PBS and detached with trypsin at 37°C. Cells were fixed for 10 min in PBS containing 1% paraformaldehyde. After rinsed in PBS, the cells were incubated with mouse anti-human antibody against AdipoR1 or AdipoR2 (1:100) for 1 h at 4°C. Cells were then washed again and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit secondary IgG (1:100; Leinco Tec., Inc., St. Louis, MO) for 45 min and analyzed by flow cytometry using FACS Calibur and CellQuest software (BD Biosciences).

WESTERN BLOT ANALYSIS

The cellular lysates were prepared and proteins were then 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 antibodies against p-p38, p38, p-IKK, IKK, p-p65, p65, or β-actin

(1:1,000) for 1 h at room temperature. After three washes, the blots were subsequently incubated with a donkey anti-rabbit peroxidase-conjugated secondary antibody (1:1,000) for 1 h at room temperature. The blots were visualized with enhanced chemiluminescence using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY). Quantitative data were obtained using a computing densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

ELISA ASSAY

Human chondrocytes (2×10^4) were cultured in 24-well culture plates. Cells were incubated with adiponectin for 24 h at 37°C. After incubation, the medium was removed and stored at −80°C until assay. MMP-3 in the medium was assayed using the MMP-3 enzyme immunoassay kits, according to the procedure described by the manufacturer (Biocompare, San Jose, CA).

QUANTITATIVE REAL–TIME PCR

Total RNA was extracted from chondrocytes with a TRIzol kit (MDBio, Inc.). The reverse transcription reaction was performed using 2 μg of total RNA (in 2 μl RNase-free water) that was reverse transcribed into cDNA with an MMLV RT kit (Promega) and following the manufacturer's recommended procedures. Quantitative real-time PCR (qPCR) analysis was carried out with TaqMan® one-step PCR Master Mix (Applied Biosystems, Foster City, CA). cDNA template (2 μl) was added to each 25-μl reaction with sequence-specific primers and TaqMan® probes. All target gene primers and probes were purchased commercially (β-actin was used an internal control) (Applied Biosystems). qPCR assays were carried out in triplicate on a StepOnePlus sequence detection system. The cycling conditions were: 10-min polymerase activation at 95°C followed by 40 cycles at 95°C for 15 s and 60°C for 60 s. The threshold was set above the non-template control background and within the linear phase of target gene amplification to calculate the cycle number at which the transcript was detected (denoted C_T).

ELECTROPHORETIC MOBILITY SHIFT ASSAY

Electrophoretic mobility shift assay was performed by using EMSA "gel shift" kit (Panomics, Redwood City, CA) according to the manufacturer's protocol. Oligonucleotide corresponding to NF-κB-binding sequence (5'-AGTTGAGGGGACTTTCCCAGGC-3') was used [Liu et al., 2006]. Nuclear extract (3 μg) of cells was incubated with poly d(I-C) at room temperature for 5 min. The nuclear extract was then incubated with biotin-labeled probes at room temperature for 30 min. After electrophoresis on a 6% polyacrylamide gel, the samples on gel were transferred onto a presoaked Immobilon-Nyt membrane (Millipore, Billerica, MA). The membrane was baked at 80°C for 1 h, cross-linked in an oven for 3 min then developed by adding the blocking buffer and streptavidin–horseradish peroxidase conjugate and subjected to Western blot analysis.

REPORTER ASSAY

The chondrocytes were transfected with reporter plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. Twenty-Four hours after transfection, the cells
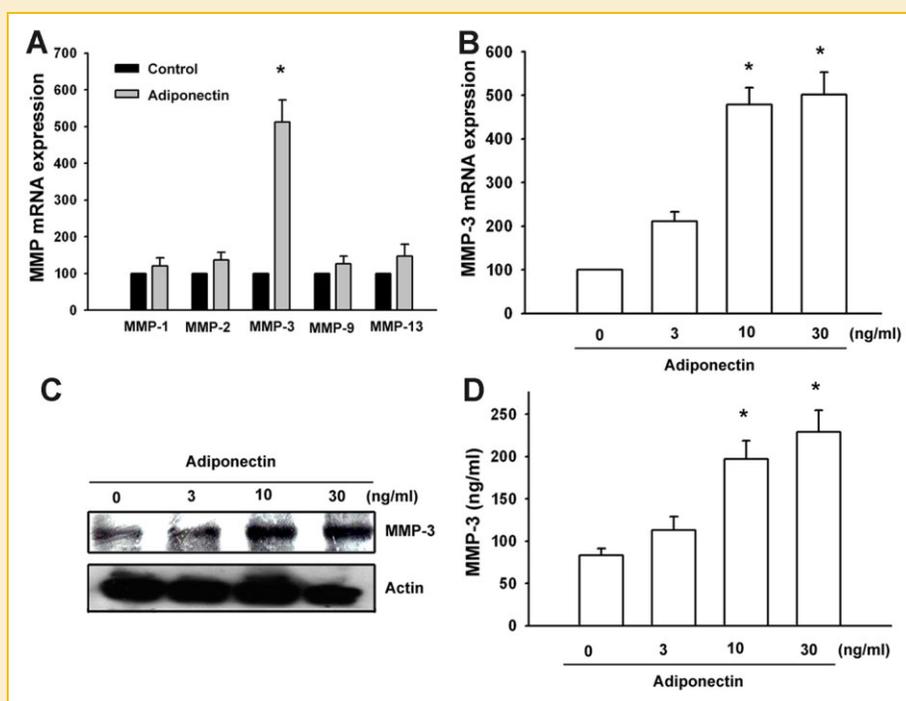
Fig. 1. Adiponectin induced MMP-3 expression in human chondrocytes. A: Cells were incubated with adiponectin (30 ng/ml) for 24 h, and the mRNA level of MMPs was determined using qPCR. B: Cells were incubated with adiponectin (3–30 ng/ml) for 24 h, and MMP-3 mRNA expression was determined using qPCR. Cells were incubated with various concentrations of adiponectin, and protein expression of MMP-3 was examined by Western blot (C) and ELISA (D) assay. Results are expressed as the mean ± SEM. *$P < 0.05$ compared with control; #$P < 0.05$ compared with adiponectin–treated group.

were treated with inhibitors for 30 min and then adiponectin or vehicle was added for 24 h. Cell extracts were then prepared, and luciferase and β-galactosidase activities were measured [Tang et al., 2007; Lai et al., 2008].

STATISTICS

The values given are means ± SEM. The significance of difference between the experimental groups and controls was assessed by Student's t-test. The difference is significant if the P-value is <0.05.

RESULTS

ADIPONECTIN INCREASED THE EXPRESSION OF MMP-3 IN HUMAN CHONDROCYTES

It has been reported that adiponectin is greatly elevated in the synovial fluid from patients with OA and RA [Schaffler et al., 2003]. MMPs have been reported participate actively in the destruction of cartilage [van Osch et al., 2009]. Therefore, we investigated the effect of adiponectin on the MMPs expression in human chondrocytes. qPCR analysis showed that adiponectin significantly increased the expression of MMP-3 mRNA but not MMP-1, -2, -9, and -13 in human chondrocytes (Fig. 1A). Stimulation of cells

with adiponectin also increased MMP-13 mRNA expression dose-dependently (Fig. 1B). Furthermore, adiponectin further increased protein expression of MMP-3 in chondrocytes in a dose-dependent manner by using Western blot and ELISA assay (Fig. 1C,D). These data suggest that adiponectin induced MMP-3 expression in human chondrocytes.

INVOLVEMENT OF ADIPOR1 RECEPTOR IN ADIPONECTIN-MEDIATED MMP-3 EXPRESSION IN HUMAN CHONDROCYTES

AdipoR1 is expressed abundantly in skeletal muscle, and AdipoR2 is predominantly expressed in liver [Yamauchi et al., 2003]. Next, we examine whether AdipoR receptor is involved in adiponectin-mediated MMP-3 expression in human chondrocytes. Stimulation of cells with adiponectin increased the cell surface expression of AdipoR1 but not AdipoR2 by flow cytometry (Fig. 2A). However, adiponectin did not affect the mRNA expression of AdipoR1 and AdipoR2 (Fig. 2B). Therefore, adiponectin increases AdipoR1 localization but not expression. Transfection of cells with AdipoR1 and AdipoR2 siRNA reduced the protein expression of AdipoR1 and AdipoR2, respectively (Fig. 2C; upper panel). Transient transfection of siRNA against AdipoR1 but not AdipoR2 effectively inhibited the
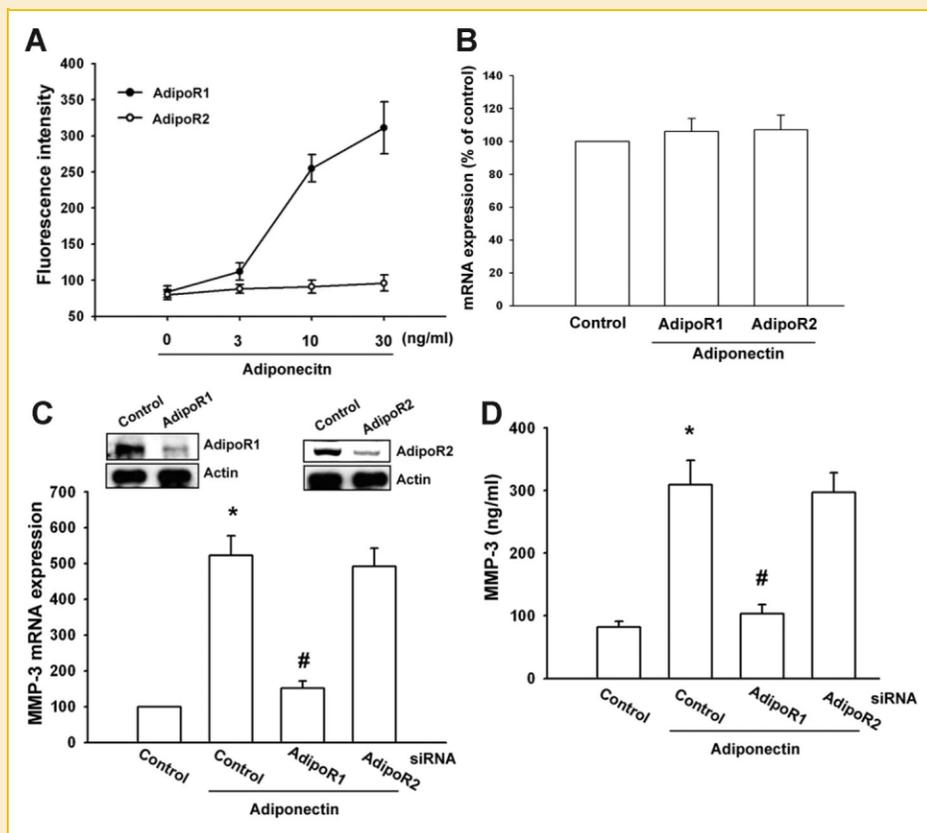
Fig. 2. Involvement of AdipoR1 in adiponectin-increased MMP-3 expression in human chondrocytes. A: Cells were incubated with adiponectin (3–30 ng/ml) for 24 h, and the cell surface AdipoR expression was determined using flow cytometry. B: Cells were incubated with adiponectin for 24 h, and the mRNA expression of AdipoR was determined using qPCR. C; upper panel: Cells were transfected with AdipoR1, AdipoR2, or control siRNA for 24 h, the AdipoR1 and AdipoR2 expression was examined by Western blot. Cells were transfected with AdipoR1, AdipoR2, or control siRNA for 24 h followed by stimulation with adiponectin, the MMP-3 expression was examined by qPCR (C) and ELISA (D). Results are expressed as the mean ± SEM. *$P < 0.05$ compared with control; #$P < 0.05$ compared with adiponectin-treated group.

MMP-3 expression (Fig. 2C,D). These data suggest that adiponectin/AdipoR1 receptor interaction plays a key role in MMP-3 expression in human chondrocytes.

THE SIGNALING PATHWAYS OF p38 AND AMPK ARE INVOLVED IN THE POTENTIATING ACTION OF ADIPONECTIN

Adiponectin has been shown to increase IL-6 production via activation of p38 pathway [Tang et al., 2007]. We tested whether p38 might also be involved in adiponectin-induced MMP-3 expression. As shown in Figure 3A, treatment of cells with adiponectin resulted in a time-dependent phosphorylation of p38. We then investigated the role of p38 in mediating adiponectin-induced MMP-3 expression using the specific p38 inhibitor SB203580. Pretreatment of cells with SB203580 (10 μM) or transfection with dominant negative mutant of p38 attenuated adiponectin-induced MMP-3 mRNA and protein expression (Fig. 3B,C). Next, we examine the effect of p38 mutant on p38 phosphorylation. Transfection of cells with p38 mutant increased p38 expression and reduced p38 phosphorylation (Fig. 3B; upper panel). Therefore, p38 is involved in adiponectin-mediated MMP-3 expression in human chondrocytes. It has reported that AMPK is downstream molecule of p38 in the control of myocardial glucose metabolism [Jaswal et al., 2007]. Figure 4A shows that adiponectin enhanced AMPKα phosphorylation at the Thr^{172} in a time-dependent manner. Pretreatment of cells for 30 min with AMPK inhibitors [araA (0.5 mM) or compound C (10 μM)] markedly attenuated the adiponectin-induced MMP-3 production (Fig. 4B,C). 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-ribo-furanoside-5-monophosphate in the cells, also increased the MMP-3 expression of chondrocytes (Fig. 4B,C). In an attempt to determine which catalytic subunit of AMPKα1 or AMPKα2 mediated adiponectin signaling in human chondrocytes, we performed qPCR and ELISA assay using siRNA specific for each isoform. Transfection of cells with AMPKα1 or AMPKα2 siRNA reduced AMPKα1 or AMPKα2 protein expression, respectively (Fig. 4D). Furthermore, AMPKα1 and AMPKα2 siRNA antagonized adiponectin-induced MMP-3 expression (Fig. 4E,F). Therefore, AMPKα1 and AMPKα2 involved in adiponectin-mediated MMP-3 expression in chondrocytes. Next, we examine the relationship of p38 and AMPK, pretreatment of cells for 30 min with Ara A, compound C, and SB203580 reduced AMPK phosphorylation (Fig. 4G). AMPK was directly activated using AICAR. AICAR increase the phosphorylation of AMPK did not inhibited by SB203580 (Fig. 4H). In addition, pretreatment of cells with SB203580 but not compound C and Ara A reduced adiponecitn-enhanced p38 phosphorylation (Fig. 4I). Therefore, these results indicated that AMPK may function as a downstream signaling molecule of p38 in the adiponectin signaling pathway.

NF-κB SIGNALING PATHWAY IS INVOLVED IN THE ADIPONECTIN-MEDIATED MMP-3 UP-REGULATION

As previously mentioned, NF-κB activation is necessary for the MMP-3 expression [Tang et al. 2010]. To examine whether NF-κB activation is involved in adiponectin-induced MMP-3 expression,
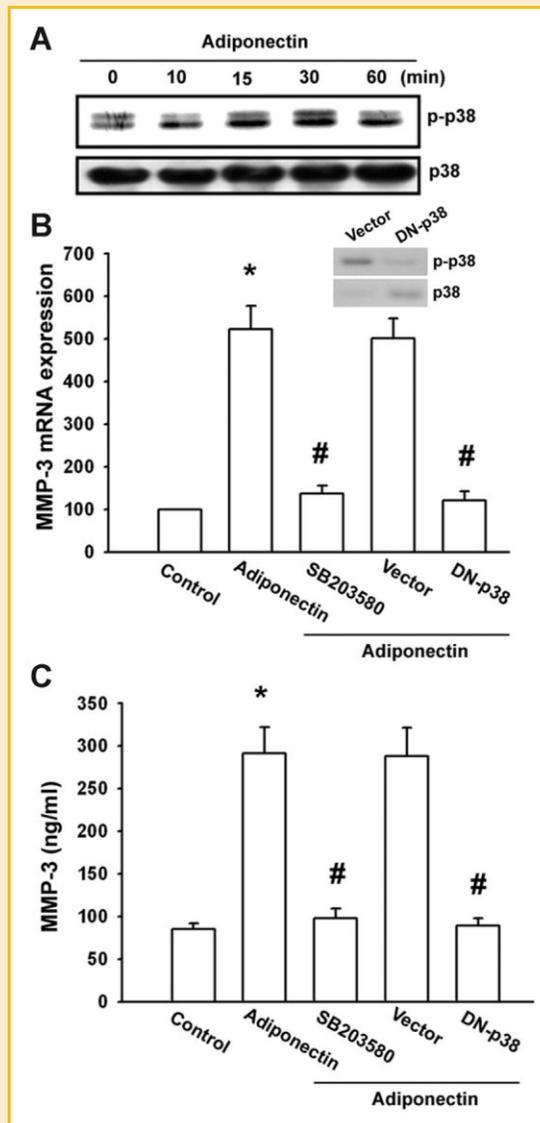
Fig. 3. p38 is involved in adiponectin-mediated MMP-3 expression in human chondrocytes. A: Cells were incubated with adiponectin for indicated time intervals, and p-38 expression was determined by Western blot. B,C: Cells were pretreated for 30 min with SB203580 (10 μM) or transfected with dominant negative (DN) mutant of p38 for 24 h followed by stimulation with adiponectin, the MMP-3 expression was examined by qPCR and ELISA. B; upper panel: Cells were transfected with dominant negative (DN) mutant of p38 for 24 h, the p38 and p-p38 expression was examined by Western blot. Results are expressed as the mean ± SEM. *$P < 0.05$ compared with control; #$P < 0.05$ compared with adiponectin-treated group.

an NF-κB inhibitor, PDTC, was used. Figure 5A,B shows that cells pretreated with PDTC (10 μM) and inhibited the adiponectin-induced MMP-3 expression of human chondrocytes. Furthermore, cells pretreated with TPCK (3 μM), an IκB protease inhibitor, also reduced adiponectin-induced MMP-3 expression of human chondrocytes (Fig. 5A,B). Treatment of cells with PDTC or PTCK reduced basal p65 phosphorylation (Fig. 5A; upper panel). We further examined the upstream molecules involved in adiponectin-induced NF-κB activation. Stimulation of cells with adiponectin-induced
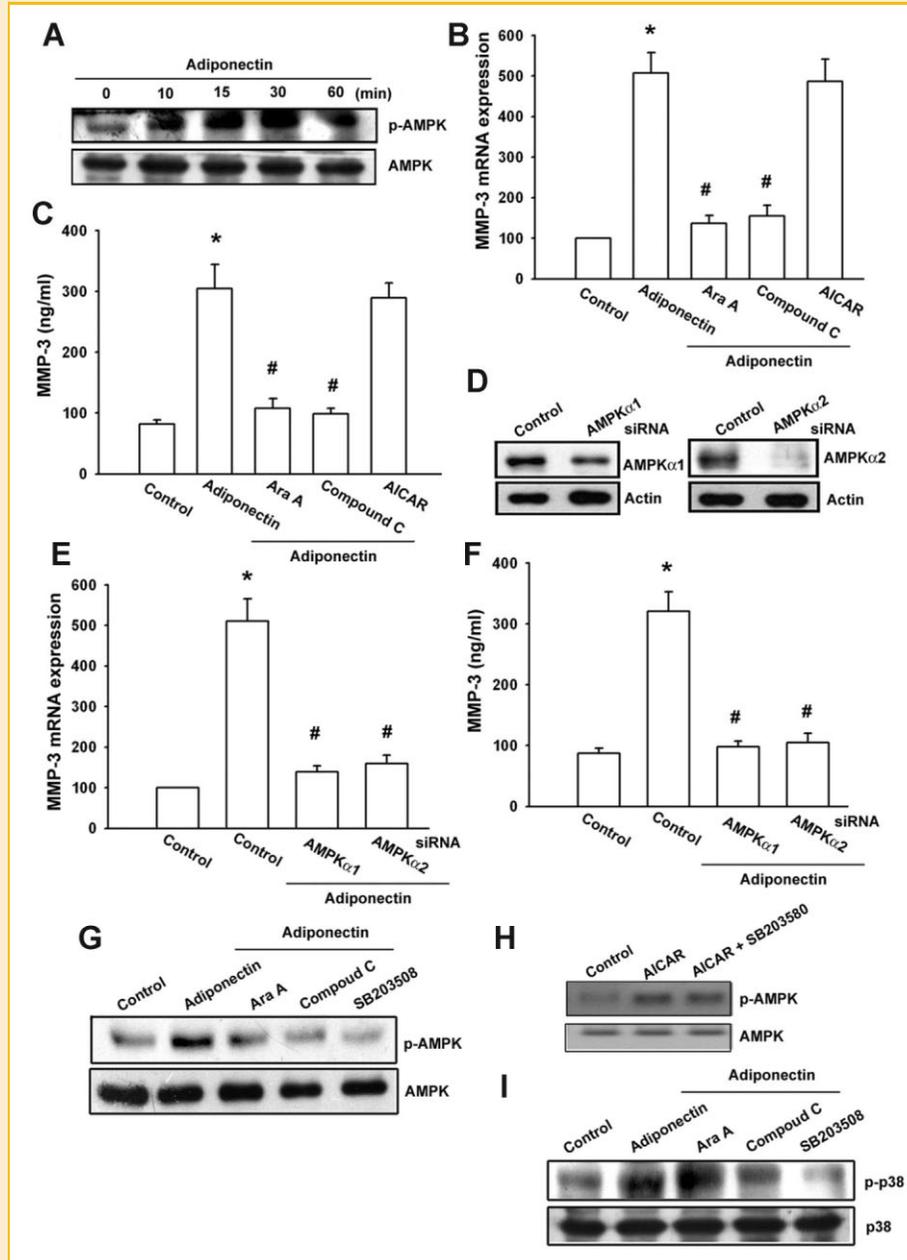
Fig. 4. AMPK pathway is involved in adiponectin-mediated MMP-3 expression in human chondrocytes. A: Cells were incubated with adiponectin for indicated time intervals, and p-AMPK expression was determined by Western blot. B,C: Cells were pretreated with AraA and compound C for 30 min followed by stimulation with adiponectin or treated with AICAR (1 mM) for 24 h. The MMP-3 expression was examined by qPCR and ELISA. D: Cells were transfected with AMPKα1, AMPKα2, or control siRNA for 24 h, the AMPKα1 and AMPKα2 expression was examined by Western blot. E,F: Cells were transfected with AMPKα1, AMPKα2, or control siRNA for 24 h followed by stimulation with adiponectin, the MMP-3 expression was examined by qPCR and ELISA. G,I: Cells were pretreated with compound C, Ara A, or SB203580 for 30 min followed by stimulation with adiponectin for 60 min, and p-AMPK and p-p38 was examined by Western blot. H: Cells were pretreated with SB203580 for 30 min followed by stimulation with AICAR for 60 min, and p-AMPK was examined by Western blot. Results are expressed as the mean ± SE. *$P < 0.05$ compared with control; #$P < 0.05$ compared with adiponectin-treated group.

IKKα/β phosphorylation in a time-dependent manner (Fig. 5C). Furthermore, transfection with IKKα or IKKβ mutant markedly inhibited the adiponectin-induced MMP-3 expression (Fig. 5D,E). These data suggest that IKKα/β activation is involved in adiponectin-induced MMP-3 expression of human chondrocytes. Transfection of cells with IKKα or IKKβ mutant reduced IKKα/β phosphorylation (Fig. 5D; upper panel). Treatment with chondro-

cytes with adiponectin also caused IκBα phosphorylation in a time-dependent manner (Fig. 5C). Previous studies showed that p65 Ser[536] phosphorylation increases NF-κB transactivation [Madrid et al., 2001], and the antibody specific against phosphorylated p65 Ser[536] was used to examine p65 phosphorylation. Treatment of cells with adiponectin for various time intervals resulted in p65 Ser[536] phosphorylation (Fig. 5C). These results indicated that NF-κB
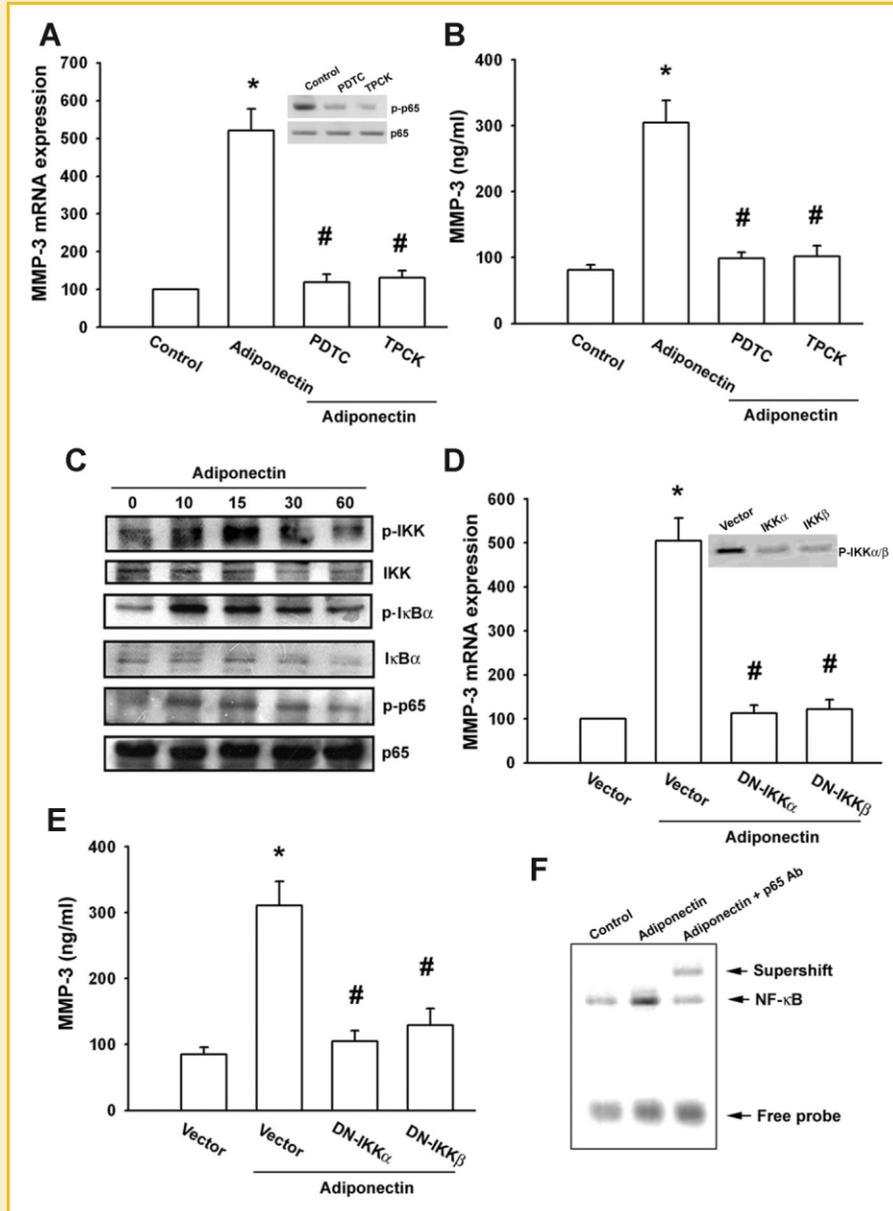
Fig. 5. Adiponectin induces MMP-3 expression through NF-κB. A,B: Cells were pretreated for 30 min with PDTC (10 μM) or TPCK (3 μM) followed by stimulation with adiponectin, the MMP-3 expression was examined by qPCR and ELISA. A; upper panel: Cells were pretreated with PDTC or TPCK, p65 phosphorylation was examined by Western blot. C: Cells were incubated with adiponectin for indicated time intervals, and p-IKK, p-IκBα, and p-p65 expression was determined by Western blot. D,E: Cells were transfected with DN-IKKα, DN-IKKβ, or vector for 24 h followed by stimulation with adiponectin for 24 h, the MMP-3 expression was examined by qPCR and ELISA. D; upper panel: Cells were transfected with DN-IKKα, DN-IKKβ, or vector for 24 h, the p-IKKαβ expression was examined by Western blot. F: Nuclear extracts from cells exposed to adiponectin for 60 min, and p65 binding to NF-κB element was examined by EMSA analysis. Preincubation of p65 antibody (Ab, 5 μg) with nuclear extracts isolated from cells exposed to adiponectin induced the supershift of NF-κB DNA complex. Results are expressed as the mean ± SE. *$P < 0.05$ compared with control; #$P < 0.05$ compared with adiponectin-treated group.

activation is important for adiponectin-induced MMP-3 expression of human chondrocytes. NF-κB activation was further evaluated by analyzing the electrophoretic mobility shift assay. Stimulation of cells with adiponectin resulted activation of NF-κB-specific DNA–protein complex formation (Fig. 5F). To identify the specific subunit involved in the formation of the NF-κB complex, supershift assay was performed using antibody specific for anti-p65. Incubation of nuclear extracts with anti-p65 antibody increased supershift of NF-κB DNA–protein complex (Fig. 5F). To directly determine NF-κB activation after adiponectin treatment, human chondrocytes were transiently transfected with κB-luciferase as an indicator of NF-κB activation. As shown in Figure 6A, adiponectin treatment of chondrocytes for 24 h caused increase in κB-luciferase activity. In addition, Ara A, compound C, SB203580, PDTC, and TPCK reduced adiponectin-mediated NF-κB activity (Fig. 6A). Cotransfection of cells with AdipoR1, AMPKα1 and AMPKα2 siRNA or p38, IKKα and
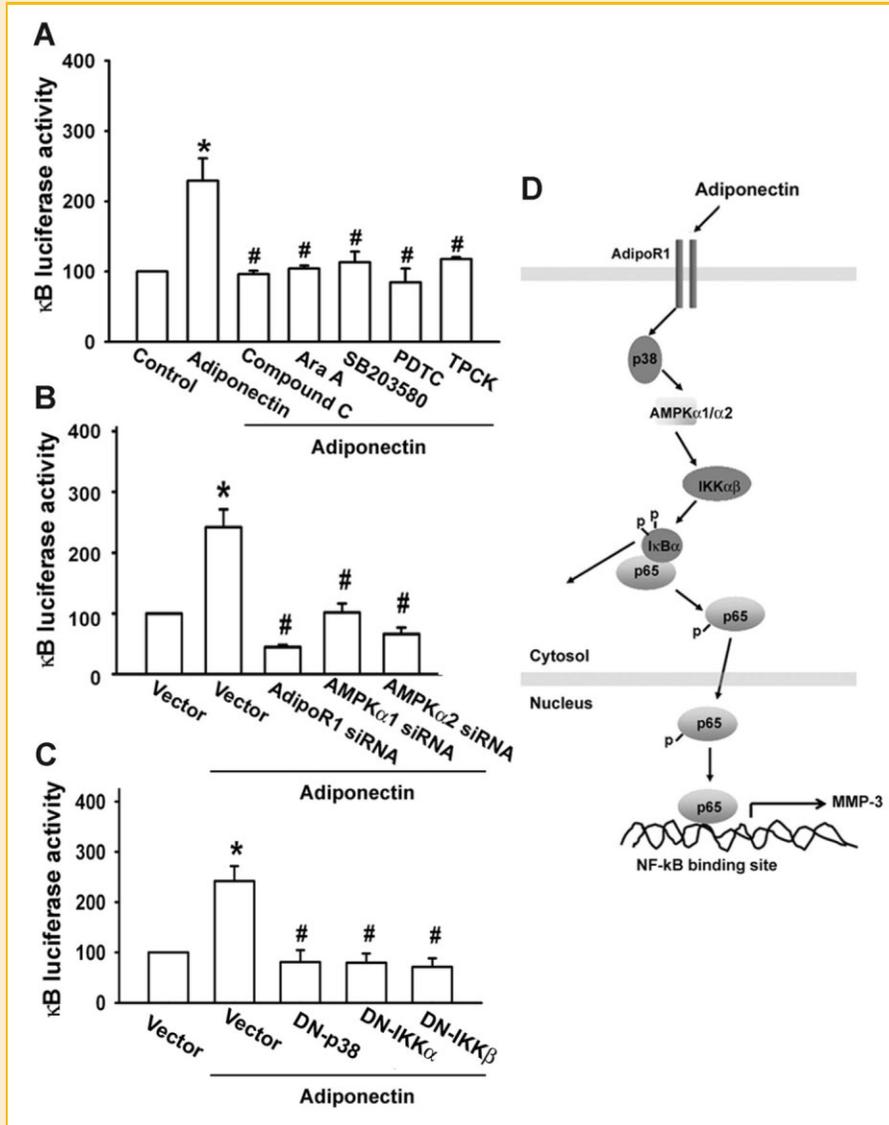
Fig. 6. AdipoR1, p38, and AMPK pathway is mediated adiponectin-mediated NF-κB activation. Cells transiently transfected with κB-luciferase plasmid for 24 h and then pretreated with Ara A, compound C, SB203580, PDTC, and TPCK (A) for 30 min or cotransfected with AdipoR1, AMPKα1, and AMPKα2 siRNA (B) or cotransfected with p38, IKKα, and IKKβ mutant (C) before incubation with adiponectin for 24 h. Luciferase activity was measured, and the results were normalized to the β-galactosidase activity. D: Schematic presentation of the signaling pathways involved in adiponectin-induced MMP-3 expression of human chondrocytes. Adiponectin and AdipoR1 interaction activates p38 and AMPK pathways, which in turn induces NF-κB activation, which leads to MMP-3 expression of human chondrocytes.

IKKβ mutant also reduced adiponectin-induced NF-κB activity (Fig. 6B,C). Taken together, these data suggest that activation of AdipoR1 receptor, p38, AMPK pathways are required for adiponectin-induced NF-κB activation in human chondrocytes.

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 [Choy et al., 1992; Hotamisligil et al., 1995]. It has been reported that adiponectin is significantly higher in synovial fluid of patients with OA and RA [Schaffler et al., 2003]. MMPs have been demonstrated may contribute to the breakdown of articular cartilage during arthritis. It has been reported that adiponectin down-regulates MMP-13 and induces TIMP-2 (an inhibitor of MMPs) in chondrocytes [Chen et al., 2006]. Lago et al. [2008] also reported that adiponectin induces IL-6, MCP-1, MMP-3, and MMP-9 expression in human chondrocytes. Here we found that MMP-3 is a target protein for adiponectin signaling pathway, which is required an activation AdipoR1 receptor, p38, AMPK, IKKαβ, and NF-κB signaling pathway. Therefore, adiponectin plays a pleiotropic role in chondrocytes. In this study, we do not have normal human chondrocytes to examine the role of adiponectin in normal chondrocytes. We found

that high levels of basal p-p38 and p-IKK in this study. Therefore, OA patients constitutively exposed to higher levels of adiponectin and increased basal p38 and IKK phosphorylation.

Two adiponectin receptors, AdipoR1 and AdipoR2, that mediated the biological effects of adiponectin was identified recently [Yamauchi et al., 2003]. 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 [Yamauchi et al., 2003]. AdipoR1 is abundantly expressed in skeletal muscle, whereas AdipoR2 is predominantly expressed in the liver. However, the effects of AdipoR1 and AdipoR2 receptor in MMP-3 expression of human chondrocytes are large unknown. We found that human primary chondrocytes express both AdipoR1 and AdipoR2 receptor isoforms by Western blot and flow cytometry analysis. Stimulation of adiponectin increased AdipoR1 but not AdipoR2 expression. In addition, AdipoR1 but not AdipoR2 siRNA reduced adiponectin-increased MMP-3 expression. Therefore, AdipoR1 is more important than AdipoR2 in adiponectin-increased MMP-3 expression.

AMPK is a heterotrimeric serine/threonine kinase composed of α catalytic a subunit and regulatory β and γ subunit [Carling, 2004]. It has been previously shown that AMPK is involved in the signaling pathway for the metabolic effects of adiponectin [Carling, 2004]. We demonstrated that the AMPK inhibitors Ara A and compounds C antagonized the adiponectin-mediated MMP-3 expression, suggesting that AMPK activation is an obligatory event in adiponectin-induced MMP-3 production in these cells. In an attempt to determine which catalytic subunit of AMPK α1 or Aα2 mediated adiponectin signaling in human chondrocytes. We found that AMPKα1 and AMPKα2 siRNA reduced adiponectin-mediated MMP-3 expression. Therefore, AMPKα1 and AMPKα2 are involved in adiponectin-induced MMP-3 expression. It has reported that AMPK is downstream molecule of p38 in the control of myocardial glucose metabolism [Jaswal et al., 2007; Tang and Lu, 2009]. We examined the potential role of p38 in the signaling pathway of adiponectin-induced MMP-3 expression. Pretreatment of human chondrocytes for 30 min with SB203580 or transfection with p38 mutant for 24 h markedly attenuated the adiponectin-induced MMP-3 expression. In addition, we also found that treatment of cells with adiponectin induced increases in p38 phosphorylation. In addition, compound C and Ara A did not affect adiponectin-enhanced p38 phosphorylation, indicating the involvement of AdipoR-p38-dependent AMPK activation in adiponectin-mediated MMP-3 expression.

NF-κB has been shown to control the induced transcription of MMP-3 in human chondrosarcoma [Tang et al. 2010]. The results of this study show that NF-κB activation contributes to adiponectin-induced MMP-3 production in human chondrocytes, and that the inhibitors of the NF-κB-dependent signaling pathway, including PDTC or TPCK inhibited adiponectin-induced MMP-3 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 [Hatada et al., 2000]. In the present study, we found that treatment of chondrocytes with adiponectin resulted in increases in IKKαβ phosphorylation and NF-κB-specific DNA–protein complex formation. 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. 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 phosphorylates IκBα at Ser^{32} and Ser^{36} and signals for ubiquitin-related degradation [Chen et al., 1995]. The released NF-κB is then translocated into the nucleus where it promotes NF-κB-dependent transcription. There is also a strong evidence that IKKα and IKKβ are themselves phosphorylated and activated by one or more upstream activating kinases [Hatada et al., 2000]. p65 is phosphorylated at Ser^{536} by a variety of kinases through various signaling pathways, and this enhances the p65 transactivation potential. The results of this study showed that adiponectin increased the phosphorylation of IKK, IκBα, and p65. On the other hand, Ara A, compound C, SB203580, PDTC, and TPCK reduced adiponectin-mediated NF-κB promoter activity. Our data indicated that AdipoR1/p38/AMPK and NF-κB pathways might play important role in the expression of MMP-3 of human chondrocytes.

In conclusion, the signaling pathway involved in adiponectin-induced MMP-3 expression in human chondrocyte cells has been explored. Adiponectin increases MMP-3 expression and activity by binding to the AdipoR1 receptor and activation of p38 and AMPK, resulting in the activations of NF-κB on the MMP-3 promoter and contribute cartilage destruction during arthritis (Fig. 6D). Whether the other adipokines (leptin) involved in tissue destruction during OA are needs further examination.

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