

Activation of the P38/CREB/MMP13 axis is associated with osteoarthritis

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Purposes: Osteoarthritis (OA) is a common joint disease characterized by the degradation of articular cartilage and joint inflammation. Interleukin-1 β induces P38/cAMP response element binding protein (CREB) pathway activation, resulting in increased expression of matrix metalloproteinase-13 (MMP13) in chondrocytes. However, the role of the P38/CREB/MMP13 axis is unclear in the progression of OA. In this study, we aimed to answer the following questions: (1) how does the P38/CREB/MMP13 axis in cartilage from patients with OA compare with control specimens? (2) Can the P38 agonist anisomycin (ANS) induce mouse OA?

Materials and methods: Surgical specimens of human cartilage were divided into OA and control groups. Surgical specimens of mouse cartilage were divided into control and ANS-induced groups. Safranin O staining of the cartilage tissues was performed to evaluate the extracellular matrix. Reverse transcription-polymerase chain reaction was performed using these tissues to investigate messenger RNA expressions of type II collagen, aggrecan, MMP13, and ADAM metalloproteinase with thrombospondin type 1 motif 5. Phosphorylated (p)-P38, p-CREB, and MMP13 were evaluated by Western blot analysis. Anisomycin was used to activate P38, and p-P38, p-CREB, and MMP13 were evaluated by immunofluorescence and Western blot analysis.

Results: Safranin O staining showed that the extracellular matrix degraded in humans with OA and ANS-induced mouse cartilage samples. The expressions of p-P38, p-CREB, and MMP13 were all upregulated in osteoarthritic cartilage or anisomycin-induced chondrocytes, suggesting that the P38/CREB/MMP13 axis may play a role in the progression of OA.

Conclusions: The P38/CREB/MMP13 axis is active in osteoarthritic chondrocytes and may cause the degeneration of cartilage. Effective new therapy directed against this pathway could be developed.

Keywords: degenerative joint disease, CREB, anisomycin, mice

Introduction

Osteoarthritis (OA) is a degenerative joint disease characterized by degradation of articular cartilage and joint inflammation. A third of the worldwide population aged more than 50 years have OA.¹ Oral or topical nonsteroidal anti-inflammatory drugs can relieve joint pain and stiffness.² However, joint replacement surgery is still the only effective treatment for terminal OA. Thus, it is urgent to research the underlying molecular pathogenesis mechanisms and explore new strategies for treating OA.

Aging, genetic predisposition, and mechanical injury are the primary causes of OA.^{3,4} As the only cell type in cartilage, chondrocytes regulate the balance between synthesis and degradation of extracellular matrix remodeling.⁵ Matrix metalloproteinases (MMPs)

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are considered the most important catabolic enzymes associated with digestion of collagen fibers and proteoglycans.⁶ In addition, pro-inflammatory cytokines, including interleukin (IL)-1 β , tumor necrosis factor (TNF)- α , and IL-6, are able to stimulate the expression of MMPs during the development of OA.^{7,8} Among the MMPs, the expression of MMP13 is more restricted to connective tissue.⁹ It targets type II collagen in cartilage for degradation and degrades proteoglycan, types IV and IX collagen, osteonectin, and perlecan in cartilage.¹⁰ Additionally, the expression of MMP13 was upregulated in cartilage obtained from the femoral heads of patients with OA.¹¹ Studies have reported that IL-1 β induced the P38/cAMP response element binding protein (CREB) pathway activation, resulting in increased expression of MMP13 in chondrocytes.¹² CREB is a transcription factor that binds to certain DNA sequences called cAMP response elements and thereby increases or decreases the transcription, and thus the expression of certain genes. CREB recruitment following a specific CpG demethylation leads to the increased expression of MMP13 in chondrocytes.¹³ However, the role of the P38/CREB pathway is unclear in the progression of OA.

Therefore, we aimed to answer the following questions: (1) how does the P38/CREB/MMP13 axis in cartilage from patients with OA compare with control specimens? (2) As a p38 agonist, can anisomycin (ANS) stimulate p38 phosphorylation in various cells and induce mouse OA?

Materials and methods

Human cartilage and mouse model

Human cartilage samples were collected according to protocols approved by the Ethics Committee of Sir Run Run Shaw Hospital, and the methods were performed in accordance with the approved guidelines. Written informed consent was obtained from all subjects. Control cartilage was harvested from patients of the fractured femoral neck without a history of OA at the time of surgery for total hip replacement (n=20), and the pathological cartilage was acquired from patients with end-stage symptomatic hip OA at the time of surgery for total hip replacement (n=10). All the samples came from patients without autoimmune, gouty, and infectious arthritis. Human cartilage samples were obtained from the weight-bearing area of the head of the femur. According to the Outerbridge classification, control samples were grade 1, and OA samples were grade 3 or 4. Clinical characteristics of the patients were collected, as shown in Table 1.

Table 1 Clinical characteristics of the patients

Control				
10 paired clinical OA and control tissues				
Gender	Age (year)	Height (cm)	Weight (kg)	BMI
Female	66	155	55	22.8928
Male	67	160	60	23.4375
Female	50	166	56.5	20.5037
Female	67	155	70	29.1363
Male	66	155	53	22.0603
Female	69	150	41	18.2222
Female	68	155	59	24.5577
Female	68	158	54	21.6311
Female	69	153	65	27.7670
Female	70	162	62.5	23.8149
10 control tissues for primary chondrocytes isolation				
Female	72	160	72	28.125
Female	71	170	63	21.7993
Female	69	159	48	18.9865
Male	60	169	78	27.3099
Female	74	164	65	24.1672
Female	68	154	50	21.0828
Female	60	164	55	20.4491
Female	70	160	35	13.6719
Male	69	168	52	18.4240
Female	60	163	60	22.5827
OA				
10 paired clinical OA and control tissues				
Gender	Age (year)	Height (cm)	Weight (kg)	BMI
Male	61	174	82	27.0842
Male	73	165	60	22.0386
Female	62	155	67	27.8876
Female	60	153	68	29.0487
Female	64	160	53	20.7031
Male	70	178	65	20.5151
Female	67	162	67	25.5296
Female	73	158	73	29.2421
Male	70	175	81	26.4489
Female	72	160	60	23.4375

Animal handling and experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals and following approval from the Institute of Health Sciences Institutional Animal Care and Use Committee. Briefly, 30 healthy, 8-week-old male C57BL/J6 mouse were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China), and mice were divided into the control (CON) and ANS groups (n=15 per group). Mice in the ANS group received an

intra-articular injection of 10 μ l (100 μ M) of ANS (Sigma-Aldrich, St. Louis, MO, USA) twice per week for 6 weeks. Mice in the CON group received an injection of phosphate-buffered saline (PBS) twice per week for 6 weeks. They were housed in an animal facility under controlled temperature (22–24 °C) and humidity (50–60%) conditions and a 12 hr light/dark cycle with free access to food and water. Once weekly, a professional animal keeper cleaned and equipped the home cages with new bedding. Mice were euthanized with carbon dioxide if they reached the humane end point, which was based on 20% reduction in body weight.

Histological analysis and immunohistochemistry

Human cartilage samples were obtained from the weight-bearing area of the head of the femur. Mice cartilage samples were obtained from the entire knee. Cartilage tissue specimens were fixed in 4% paraformaldehyde for paraffin embedding. Each paraffin-embedded cartilage sample was sectioned at 5 μ m, and every tenth section was stained with 0.1% safranin O solution and 0.001% Fast Green solution (Sigma-Aldrich). Histological images were acquired by using the Nikon Biological Microscope Ni-U (Nikon Instruments, Inc., Melville, NY, USA) and analyzed by Image J software (National Institute of Health, Bethesda, MD, USA). Cartilage destruction of mice knee joints was scored by 2 observers who were blinded to group-identifying information using the Osteoarthritis Research Society International (OARSI) grading system.¹⁴ For immunohistochemistry, the sections were incubated at 4 °C with antibodies for HOXA1 (Abcam, Cambridge, MA, USA) overnight and for 2 hrs at room temperature with secondary antibodies (Beyotime Institute of Biotechnology, Inc., Jiangsu, China). The number of positively stained cells on the entire articular surface (including the femoral condyle and tibial plateau area) per specimen was counted, and the percentage of positive cells was calculated.¹⁵

Culturing of chondrocytes

Chondrocytes were isolated from human and mouse articular cartilage tissues as described previously.¹⁶ Chondrocytes were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Gibco, Grand Island, NY, USA) for 24 hrs at 37 °C. The cells were filtered through a 0.075-mm cell strainer

and washed before culturing or microRNA/messenger (m)-RNA isolation with sterile PBS. Primary chondrocytes at 80% confluence were used for the experiments. During the culture period, cells were incubated at 37 °C in a humidified atmosphere of 5% carbon dioxide and 95% air. Human and mice chondrocytes were grown on a 20-mm glass-bottom cell culture dish (801001; Nest Biotechnology Co., Ltd., Shanghai, China) with or without being treated with ANS (10 μ M) for 24 hrs for immunofluorescence (5×10^5 cells/dish). Additionally, they were grown on 20-mm cell culture dish with or without being treated with ANS (10 μ M) for 24 hrs for protein collection (1×10^6 cells/dish).

RNA extraction and quantitative real-time polymerase chain reaction analysis

OA and control cartilage tissues were grinded with liquid nitrogen, and total RNA was extracted using TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA), according to the manufacturer's instructions. RNA was stored at –80 °C. Reverse transcription was performed using 1.0 μ g of the total RNA and HiFiScript complementary (c)-DNA Kit (CW BIO, Beijing, China). Amplification reactions were set using 20- μ l reaction volumes containing amplification primers and UltraSYBR Mixture with ROX (CW BIO) and detected by the ABI 7500 Sequencing Detection System (Applied Biosystems, Foster City, CA, USA). A 1- μ l volume of cDNA and 1- μ l volume of primer (Sangon Biotech, Shanghai, China) were used in each amplification reaction. The following cycling conditions were used: 40 cycles of denaturation at 95 °C for 5 seconds and amplification at 60 °C for 24 seconds. All reactions were run in triplicate and normalized to the mRNA house-keeping gene β -actin. The primer sequences are shown in Table 2.

Western blotting

Protein samples were isolated from cartilage tissues or chondrocytes with or without being treated with ANS (10 μ M). Cartilage tissues were pulverized into powder in liquid nitrogen, and then the lysis buffer (50 mM Tris HCl [pH 7.4], 150 mM NaCl, 20 mM ethylenediaminetetraacetic acid, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], and protease inhibitors) was added and centrifuged at 160,609 g for 10 mins at 4 °C. Cell samples were washed with cold PBS and lysed in a lysis buffer. Proteins were resolved on a 10%

Table 2 Primer sequences

Human β -actin	Forward 5'-TCATGCGTTCTCCTCAGATG-3' Reverse 5'-AATCCACTGGTGAACCAAGC-3'
Human MMP13	Forward 5'-TGGCATTGCTGACATCATGA-3' Reverse 5'-GCCAGAGGGCCCATCAA-3'
Human collagen 2A1	Forward 5'-ATGACAATCTGGCTCCCAACTGC-3' Reverse 5'-GACCGGCCCTATGTCCACACCGAAT-3'
Human aggrecan	Forward 5'-TGAGGAGGGCTGGAACAAGTACC-3' Reverse 5'-GGAGGTGTAATTGCAGGGAACA-3'
Human ADAMTS-5	Forward 5'-TACTTGGCCTCTCCCATGAC-3' Reverse 5'-TTTGGACCAGGGCTTAGATG-3'
Mouse β -actin	Forward 5'-CCTCTATGCCAACACAGT-3' Reverse 5'-AGCCACCAATCCACACAG-3'
Mouse MMP13	Forward 5'-GACAGTGGAGGTGGCCTTAC-3' Reverse 5'-ACAAGTGGGTAGATAACAAGGT-3'

Abbreviations: MMP13, matrix metalloproteinase-13; ADAMTS-5, ADAM metalloproteinase with thrombospondin type 1 motif 5.

SDS-polyacrylamide electrophoresis gel and transferred to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA, USA). After blocking with 5% nonfat milk in Tris-buffered saline plus 0.1% Tween 20, the membranes were incubated with primary antibodies against phosphorylated (p)-P38, p-CREB, MMP13, or β -actin (Abcam). After washing, primary antibodies were detected using horseradish peroxidase-conjugated secondary anti-mouse or anti-rabbit antibodies (Beyotime Institute of Biotechnology Inc.) and visualized using an enhanced chemiluminescence kit (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Immunofluorescence

Chondrocytes were grown on a 20-mm glass bottom cell culture dish (801001; Nest Biotechnology Co., Ltd., Shanghai, China) with or without being treated with ANS (10 μ M) for 24 hrs. Then the cells were fixed with 4% paraformaldehyde for 15 mins, washed two times with PBS containing 0.05% Tween-20, permeabilized with 0.3% Triton X-100 for 5 mins, and then blocked with 1% bovine serum albumin for 30 mins. Next, the cells were incubated with the p-P38, p-CREB, and MMP13 antibodies (1:200 dilutions, Abcam) at 4 °C overnight. After three washes with PBS, the cells were incubated with goat anti-rabbit immunoglobulin G conjugated to fluorescent Cy5 dye (1:100 dilutions, Abcam) in PBS. The 4',6-diamidino-2-phenylindole (Life Technologies, Carlsbad, CA, USA) was used for nuclear staining. Immunofluorescent images

were obtained using the Nikon Eclipse TI microscope (Nikon Instruments Inc.), Zeiss LSM780 confocal microscope (Zeiss, Jena, Germany), or Zeiss Colibri epifluorescence microscope and processed with Image J (National Institute of Health).

Statistical analysis

Data are expressed as a mean \pm standard deviation. The significance of differences between the 2 groups was determined using an independent-samples *t*-test. Statistical analyses were performed using SPSS 22.0 (IBM Corp., Armonk, NY, USA). $P < 0.05$ or $P < 0.01$ was considered statistically significant.

Results

P38/CREB/MMP13 is active in human osteoarthritic cartilage

Figure 1A shows an x-ray of the hip preoperatively. The result of safranin O and fast green staining showed that cartilage suffered more severe destruction and loss of integrity in osteoarthritic samples (Figure 1B). Quantitative polymerase chain reaction (qPCR) of the RNA of tissues showed that ADAM metalloproteinase with thrombospondin type 1 motif 5 (ADAMTS5) and MMP13 were upregulated in osteoarthritic cartilage, whereas Col2 and aggrecan were downregulated in osteoarthritic cartilage tissues (Figure 1C–F). Protein from osteoarthritic and control cartilage was used to evaluate the expression levels of p-P38, p-CREB, and MMP13 by

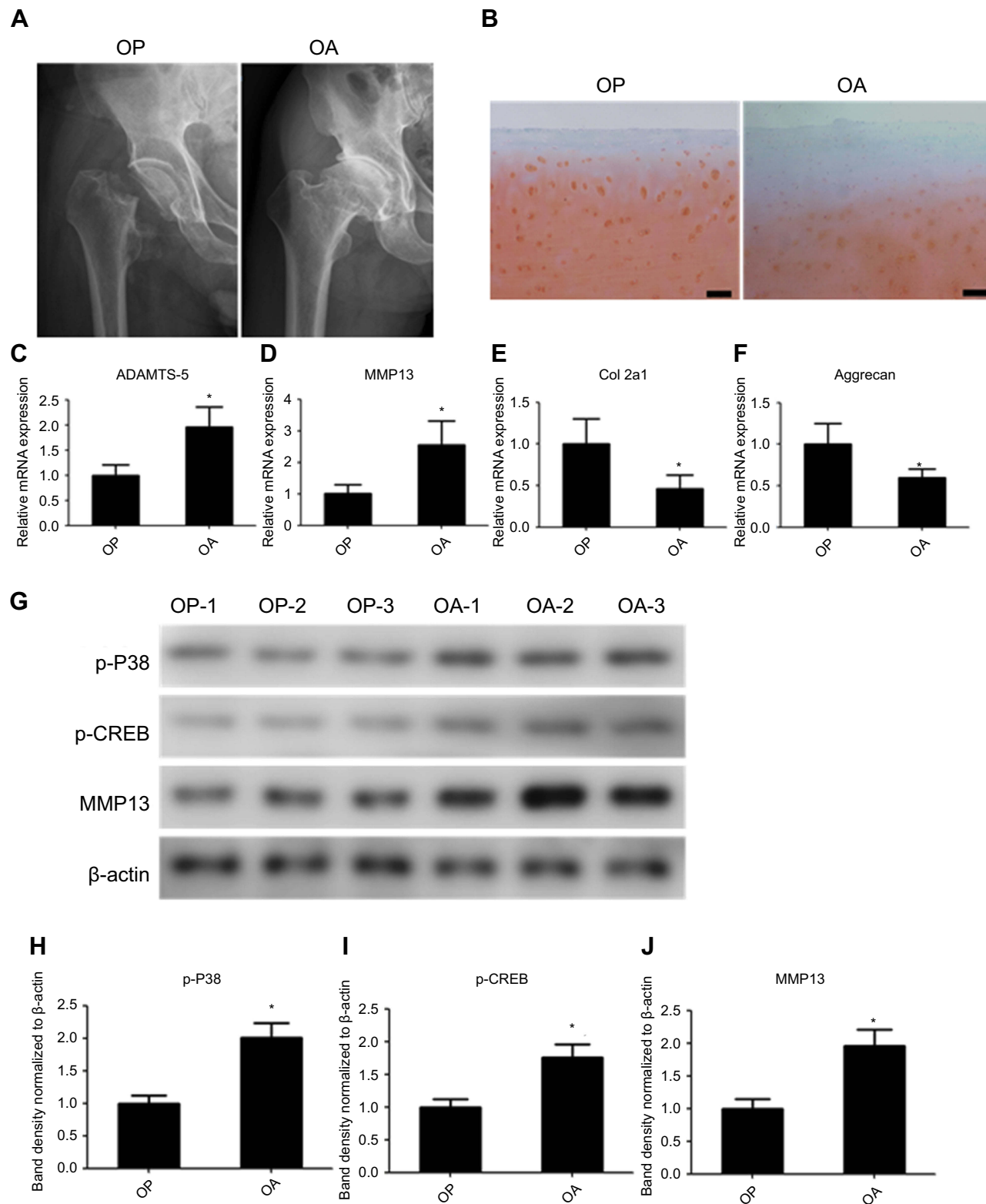


Figure 1 P38/CREB/MMP13 is active in human osteoarthritic chondrocytes. **(A)** X-rays of the hip from patients with OA and the control. **(B)** Safranin O and fast green staining of the cartilage tissues from patients with OA and the control. Relative messenger RNA expression of **(C)** ADAM metalloproteinase with thrombospondin type I motif 5, **(D)** MMP13, **(E)** Col2, and **(F)** aggrecan in human osteoarthritic and control cartilage samples. **(G)** Western blot analysis of p-P38, p-CREB, and MMP13 in human chondrocytes from osteoarthritic and the control cartilage. The band density analysis of **(H)** p-P38, **(I)** p-CREB, and **(J)** MMP13 were analyzed by Image J software and normalized to β-actin. n=10, data represent the mean±standard error of the mean. *P<0.05.

Abbreviations: CREB, cAMP response element binding protein; MMP13, matrix metalloproteinase-13; OA, osteoarthritis; p, phosphorylated.

Western blot analysis. The result showed that p-P38, p-CREB, and MMP13 had higher expression in osteoarthritic cartilage (Figure 1G), and the band density is calculated in Figure 1H–J.

P38/CREB/MMP13 is active in ANS-induced human and mouse chondrocytes

Primary chondrocytes in the CON group were isolated from control cartilage and normal mice. ANS was used to activate the P38 signal in the chondrocytes. Then p-P38, p-CREB, and MMP13 were evaluated by immunofluorescence and Western blot analysis. The result of immunofluorescence showed that p-P38 was active as expected, and p-CREB and MMP13 were upregulated (Figure 2A). The result of Western blot analysis was in line with that of immunofluorescence (Figure 2B). The band density is calculated in Figure 2C–D.

ANS-induced mouse OA is involved with activation of the P38/CREB/MMP13 axis

After mice received an intra-articular injection of ANS (10 μ l [100 μ m]) or PBS twice per week for 6 weeks, radiography was used to evaluate degeneration of the knee joint (Figure 3A). The result of safranin O and fast green staining suggested severe destruction and loss of integrity in mouse cartilage with ANS (Figure 3B), and the mice with ANS had a higher OARSI score (Figure 3D). Additionally, the effect size (r) was 0.825 between the two groups (Table 3). The result of immunohistochemistry showed a higher MMP13 positive cell rate in mouse cartilage with ANS (Figure 3C and E). The qPCR of the RNA of tissues showed that MMP13 was upregulated in mouse cartilage tissues with ANS (Figure 3F). Protein from mouse cartilage tissues in the ANS or CON group was used to evaluate the expression levels of p-P38, p-CREB, and MMP13 by Western blot analysis. The result showed that p-P38, p-CREB, and MMP13 had higher expressions in mice with ANS (Figure 3G), and the band density is calculated in Figure 3H–J.

Discussion

OA is a complex degenerative joint disease affected by many factors. Imbalance between synthesis and decomposition of the cartilage matrix is the primary cause of OA, which leads to cartilage destruction. MMPs are catabolic proteases capable of degrading all types of extracellular matrix proteins. In particular, MMP13 is the main protease

involved in the degradation of Col2. MMP13 is crucial for OA progression, and the pharmacologic inhibition of MMP13 was an effective strategy to decelerate articular cartilage loss in a murine model of injury-induced knee OA.^{16,17} In the present study, we found that the P38/CREB/MMP13 axis is active in osteoarthritic chondrocytes, and P38 agonist ANS may induce mouse OA. Therefore, effective new therapy directed against this pathway could be developed.

Additionally, previous studies reported that activation of the p38-mitogen-activated protein kinase (MAPK) signaling pathway may lead to the expression of proinflammatory cytokines, which in turn promote the activation of P38, and this plays a key role in the development of OA.^{18,19} Proinflammatory agents such as IL-1 β , TNF- α , and reactive oxygen species induce the synthesis of MMPs, which lead to the degradation of cartilage.^{20–22} Blockage of p38 MAPK could inhibit the downstream inflammatory cytokine production and MMP expression in chondrocytes.^{23,24} Local treatment with a p38 MAPK inhibitor improved inflammation and joint degradation in mouse models, indicating treatment potential for human OA.²⁵ Recent studies showed that the activation of CREB resulted in an increased expression of MMP13 in chondrocytes.^{12,13} Moreover, IL-1 β -induced CREB activation by phosphorylation of Ser133 is mainly dependent on p38 kinases.^{26,27} Herein, p-P38 and p-CREB expressions were increased in human osteoarthritic cartilage. Thus, we first drew the conclusion that the P38/CREB/MMP13 axis is active in human OA.

As a p38 agonist, ANS could stimulate p38 phosphorylation in various cells.^{28–32} Our results suggested that the P38/CREB/MMP13 axis was activated by ANS in human and mouse chondrocytes. In addition, p-P38, p-CREB, and MMP13 expressions were increased in ANS-induced mouse cartilage. Therefore, we concluded that the intra-articular injection of ANS could induce mouse OA, which might involve activation of the P38/CREB/MMP13 axis.

There are several limitations in our study. First, the c-Jun N-terminal kinase (JNK) pathway was also activated by ANS.^{30,33} Thus, ANS-stimulated chondrocytes are not limited to activation of the P38 pathway. The JNK pathway also might be involved in ANS-induced OA. All these assumptions need further research with the specific p38 or JNK agonist. Second, activation of the P38/CREB/MMP13 axis was evaluated by Western blot and immunofluorescence in our study. Thus, other methods need to be used to verify the P38/CREB/MMP13 axis, such as

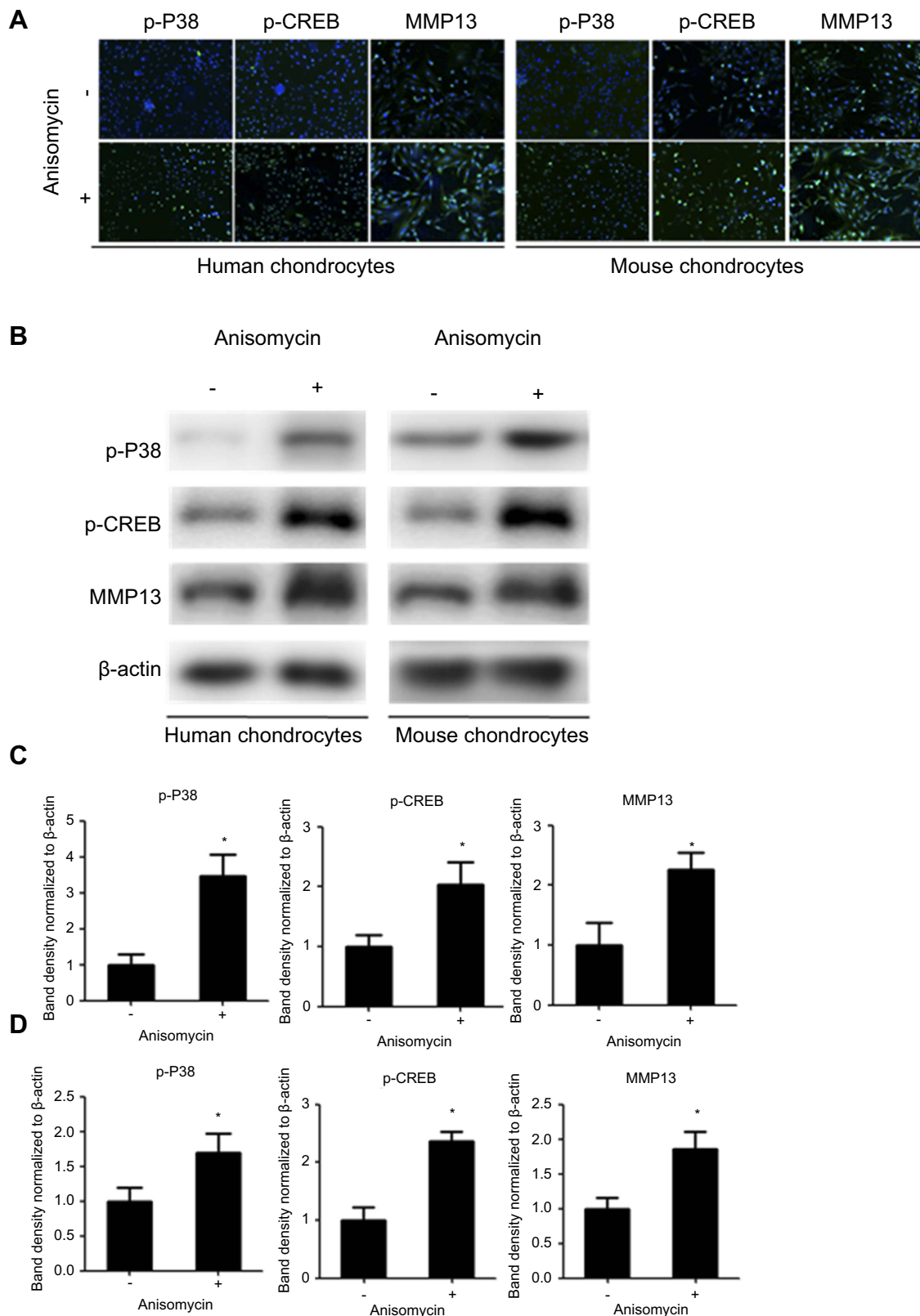


Figure 2 P38/CREB/MMP13 is active in ANS-induced human and mouse chondrocytes. **(A)** Immunofluorescence analysis of p-P38, p-CREB, and MMP13 expression in chondrocytes treated with or without ANS (10 μ M) for 24 hrs. **(B)** Western blot analysis of p-P38, p-CREB, and MMP13 in chondrocytes treated with or without ANS (10 μ M) for 24 hrs. The band density analysis of p-P38, p-CREB, and MMP13 from **(C)** human chondrocytes and **(D)** mouse chondrocytes were analyzed by Image J software and normalized to β -actin. n=10, data are expressed as the mean \pm standard error of the mean. * P <0.05.

Abbreviations: CREB, cAMP response element binding protein; MMP13, matrix metalloproteinase-13; p, phosphorylated; ANS, anisomycin.

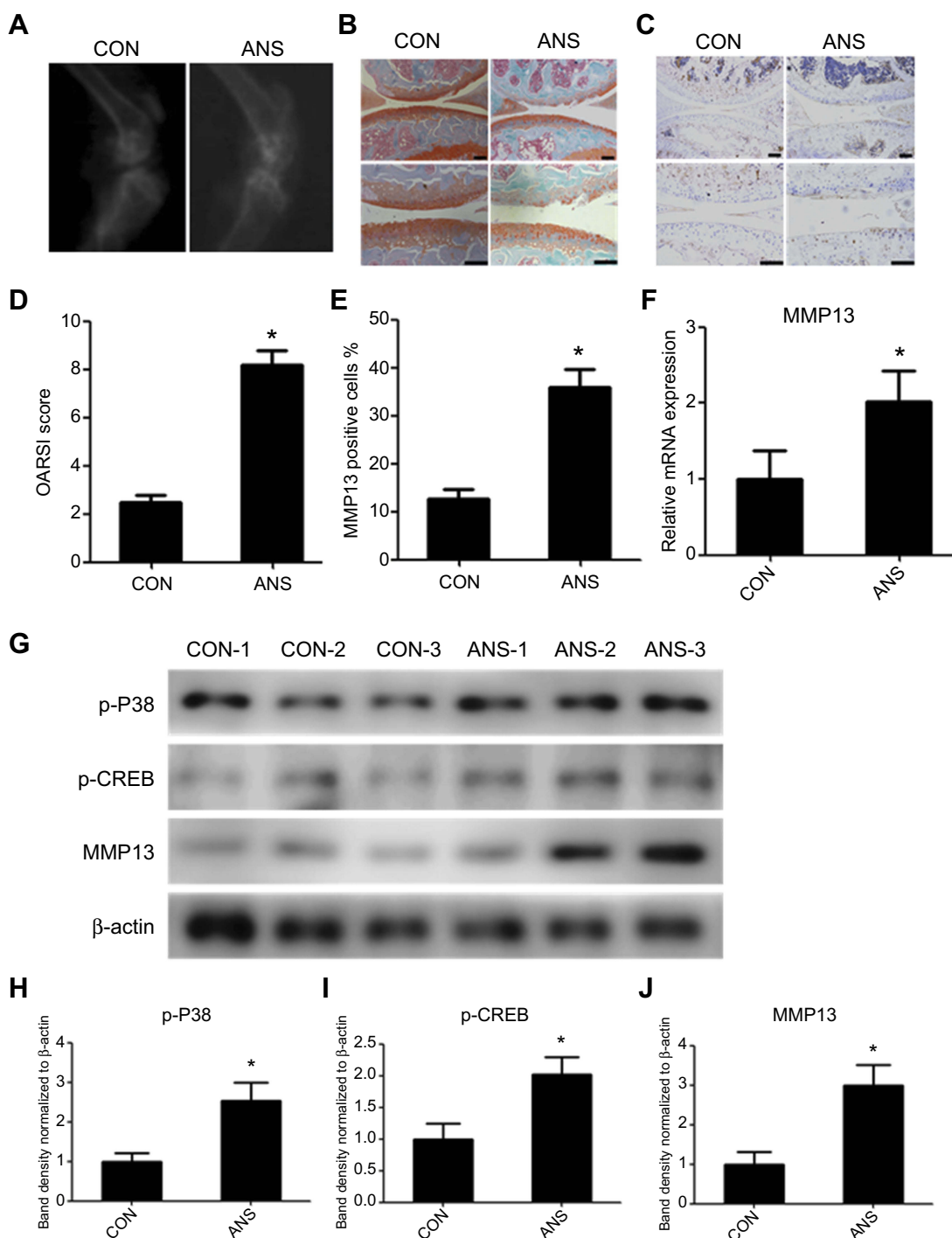


Figure 3 ANS-induced mouse osteoarthritis. **(A)** X-ray of the knee from the control ANS-induced mice. **(B)** Safranin O and fast green staining of cartilage tissues from mice in the CON and ANS groups. **(C)** Immunohistochemistry analysis of MMP13 expression in the CON group and ANS-induced mouse cartilage. **(D)** Osteoarthritis Research Society International scores were determined according to the safranin O and fast green staining results. **(E)** The percentage of MMP13-positive cells was calculated according to immunohistochemistry. **(F)** Relative messenger RNA expression of MMP13 in the CON group and ANS-induced mouse cartilage samples. **(G)** Western blot analysis of p-P38, p-CREB, and MMP13 in the CON group and ANS-induced mouse cartilage. The band density analysis of **(H)** p-P38, **(I)** p-CREB, and **(J)** MMP13 was performed using Image J software and normalized to β-actin. n=15, Data are expressed as the mean±standard error of the mean. *P<0.05. **Abbreviations:** CREB, cAMP response element binding protein; MMP13, matrix metalloproteinase-13; p, phosphorylated; ANS, anisomycin.

hybridization in situ in an in vivo study. In addition, the p38 inhibitor should be used to verify the effect of P38/CREB/MMP13 axis in reverse.

This study is the first to examine the effect of the P38/CREB/MMP13 axis in OA. We concluded that the P38/CREB/MMP13 axis is active in osteoarthritic chondrocytes,

Table 3 Details about type of OARSI scoring (mean/max/sum)

	Control	ANS
Mean	2.6	8.2
Max	5	12
Min	1	5
Sum	41.6	131.2
Stdev	1.17	2.44

Note: The Effect Size (R) between Control and ANS is 0.825.

and it may cause the degeneration of cartilage. These results provide a basis for targeting the P38/CREB/MMP13 axis in the prevention and treatment of OA.

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Disclosure

The authors report no conflicts of interest in this work.

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