

Histone deacetylase-4 and histone deacetylase-8 regulate interleukin-1 β -induced cartilage catabolic degradation through MAPK/JNK and ERK pathways

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Abstract. Interleukin-1 β (IL-1 β)-induced inflammatory response is associated with osteoarthritis (OA) and its development. Histone deacetylase (HDAC) may be involved in regulating this pathogenesis, but the mechanism has yet to be elucidated. The aim of the present study was to investigate the mechanism underlying the regulation of IL-1 β -stimulated catabolic degradation of cartilage by HDAC. An *in vitro* model of OA was generated using rat articular chondrocytes (rACs) treated with IL-1 β . The role of HDAC in IL-1 β -induced gene expression was investigated using HDAC inhibitors and specific small interfering RNAs (siRNAs). The association of diverse mitogen-activated protein kinase (MAPK) pathways was examined. The IL-1 β -induced expression of a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-4 and ADAMTS-5, and the production of collagen X and cyclooxygenase-2 in rACs was accompanied by the expression of HDAC4 and HDAC8, and were significantly downregulated by HDAC inhibitors and specific siRNAs. IL-1 β -induced activation of extracellular signal-regulated kinase was downregulated by the HDAC inhibitor Trichostatin A, but not significantly by PCI-34051. The activation of c-Jun N-terminal kinase was

observably downregulated by the latter, but only slightly by the former. These results suggest that HDAC4 and HDAC8 may serve as key upstream mediators of MAPK in regulating the IL-1 β -induced cartilage catabolic and degradation. Therefore, inhibiting HDAC4 or HDAC8 or both may be a promising therapeutic strategy in preventing and treating OA.

Introduction

Osteoarthritis (OA) is a joint disease that is commonly reported in the elderly. It is characterized by progressive destruction of articular cartilage, inflammation of synovium, exposure and sclerosis of subchondral bone, and osteophyte formation (1-5). The morbidity of OA is >10% (6,7). The aim of nonsurgical treatment is mainly to alleviate the symptoms including pain and turgescence, but not to prevent the pathogenesis, i.e. to cure cartilage degradation (8-10).

Although the main etiological factor that antagonizes OA remains to be elucidated, it is presumed that an imbalance between anabolic and catabolic metabolism in articular cartilage is an essential event in the progression of OA, which results in pervasive cartilage damage (2).

Previous reports have suggested that a high concentration of proinflammatory cytokine interleukin-1 β (IL-1 β) was detected in the synovial fluid of patients with arthritis and rat models (11), and that neutralizing IL-1 β using antibodies may ameliorate the degeneration and destruction of articular cartilage (12). However, the high cost limits the application of this method. In addition, gene expression of IL-1 β in the peripheral blood mononuclear cells is upregulated in patients with OA (13), implying that IL-1 β may serve a vital role in synovial inflammation and cartilage degradation. The IL-1 β -induced gene expression of matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) in chondrocytes is regulated predominantly at the transcriptional level, particularly by mitogen-activated protein kinase (MAPK) signaling pathways (14). The attenuation of one or more of these signals was considered to reveal a potential target for OA therapy and, therefore, meriting further investigation (15). However, the precise mechanism has yet to be fully elucidated.

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Abbreviations: ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; DMSO, dimethyl sulfoxide; ECM, extracellular matrix; IL-1 β , interleukin-1 β ; MMP, matrix metalloproteinase; NC, negative control; OA, osteoarthritis; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; TSA, Trichostatin A; siRNA, small interfering RNA

Key words: osteoarthritis, a disintegrin and metalloproteinase with thrombospondin motifs, epigenetic, histone deacetylase, mitogen-activated protein kinase

The degradation process of articular cartilage is mainly regulated by the activation of enzymes belonging to the MMP and aggrecanase families (15). A number of previous studies have illuminated the roles of MMPs in collagen catabolism (16,17). For example, MMP-3 and MMP-9 are considered to downregulate the extracellular matrix (ECM) in cartilage (resolving the degenerated collagen) irreversibly (17). Collagen type X (col X) is an indicator of cartilage degradation in this pathogenesis (18,19). Aggrecanase1 was initially identified in 1999 (20), followed by aggrecanase2 (21). They were subsequently termed ADAMTS-4 and ADAMTS-5. It is reported that ADAMTS5 was the primary 'aggrecanase' responsible for aggrecan degradation in a murine model of OA (22); however, ADAMTS4 appeared not to be associated with OA pathogenesis (23). As with other ADAMTS, including ADAMTS-1, ADAMTS-8 and ADAMTS-15, they were of less interest on account of their poor distribution in degradation cartilage tissues in OA (24-26). However, the role of aggrecanase, which belongs to the ADAMTS family, has yet to be elucidated. A previous study suggested that ADAMTS-5 was upregulated in arthritis and served a therapeutic role in OA treatment; silencing ADAMTS-5, alone or combined with ADAMTS-4, which may merit further investigation (27). In addition to ADAMTS, cyclooxygenase (COX)-2 also serves a vital role in cartilage metabolism and inflammation associated with OA. It is widely used in medical reagents, including Celebrex, for its anti-inflammatory action (10,28,29).

Significant interest has emerged in epigenetic disease therapy. For example, inhibition of histone deacetylase (HDAC) activity has been a promising treatment strategy for leukemia and cancer (30). The regulation by HDAC in certain cardiovascular diseases has also been demonstrated in previous studies (31,32). Furthermore, several studies have demonstrated that HDAC inhibitors regulate inflammatory responses (33-36). As they are able to suppress the expression of MMPs to suppress tumor metastasis (37), they are also used in OA treatment (38). Despite extensive investigations, no acknowledged therapy target for HDACs has been reported. Little is known of the effects and underlying mechanism of HDAC on OA pathogenesis (38-41).

As the excessive expression of ADAMTS-4 and ADAMTS-5 and production of col X and COX-2 are implicated in the pathogenesis of OA, their inhibition may be useful for OA treatment. However, inhibiting any one of them may lead to unknown side effects or may not be effective (42). The present study assessed the effect of two types of HDAC inhibitors [Trichostatin A (TSA) and PCI-34051] and specific small interfering RNAs (siRNAs) on the expression of OA-related genes and activation of MAPK pathways in IL-1 β -induced rat articular chondrocytes (rACs) to investigate the underlying mechanisms of OA pathogenesis.

Materials and methods

Isolation and cultivation of rACs. Animal experiments were approved by the Animal Care and Use Committee for Teaching and Research of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China).

Male Sprague-Dawley rats (n=12; weight, 50-60 g; age, 4 weeks; Tongji Medical College) were dissected following

sacrifice and bilateral knee articular cartilage specimens were extracted and cut into 1-mm³ pieces. Cells were isolated using a sequential proteinase and collagenase digestion technique, as described in a previous study (27). The cartilage sections were treated with 0.25% trypsin without EDTA (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 30 min at 37°C and 0.1% collagenase II (Gibco; Thermo Fisher Scientific, Inc.) overnight at 37°C. Subsequently, the cells were resuspended and cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), containing 100 U/ml penicillin G sodium and 100 μ g/ml streptomycin sulfate (Gibco; Thermo Fisher Scientific, Inc.). The cells were expanded in monolayer culture in the medium in an incubator (Thermo Fisher Scientific, Inc.) at 37°C under a humidified atmosphere containing 5% CO₂, with an exchange of the medium every 3 days. Up to ~90% confluence, the chondrocytes were passaged at a ratio of 1:3 using 0.25% trypsin containing 0.05% EDTA. Chondrocytes of the third passage were seeded in 6-well or 60-mm plates at a density of 10⁵ cells/cm² prior to treatment to avoid phenotype loss.

Generation of an *in vitro* OA model and investigation of the expression of HDACs and activation of MAPKs. Cells were incubated in serum-free DMEM/F12 medium overnight at 37°C prior to treatment with different reagents. Recombinant rat IL-1 β (Peprotech, Inc., Rocky Hill, NJ, USA) was resolved in ultrapure water and stored at -20°C prior to use.

IL-1 β -induced rACs were used to generate an *in vitro* OA model, as described in a previous study (43). The chondrocytes were seeded in six-well plates at a density mentioned earlier and treated with 2.5, 5, 10, 20 or 40 ng/ml IL-1 β for 24 h in the serum-free medium once reaching confluence, and untreated cells were used as a control group (44-47).

The chondrocytes were harvested and the gene expression of ADAMTS-4 and ADAMTS-5 for cartilage catabolism was evaluated via reverse transcription-quantitative polymerase chain reaction (RT-qPCR) as detailed below. The expression of collagen type X indicated cartilage degradation and the expression of COX-2 for inflammation were assayed by western blotting as detailed below. Antibodies against col X were purchased from Abcam (Cambridge, UK; cat. no. ab182563) and antibodies against COX-2 were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA; cat. no. 12282).

Two groups, 10 ng/ml IL-1 β and blank control groups, were evaluated for the expression of HDACs by RT-qPCR as detailed below. These groups were observed under an inverted microscope (magnification, x200) directly and following toluidine blue staining (Wuhan Boster Biological Technology, Ltd., Wuhan, China), according to the manufacturer's protocols. Following treatment, the cells were fixed with 4% paraformaldehyde for 30 min at room temperature, then stained with toluidine blue for 5-10 min at room temperature, followed by washing with PBS for 3-5 min several times and finally observed under an inverted microscope (magnification, x200) following air drying.

IL-1 β -treated chondrocytes (10 ng/ml) were used to set sequential time points of 5, 15 and 30 min, and 1, 2, 6 and 24 h

Table I. Sequences of siRNAs.

Gene	Sequence number	Sense (5'-3')	Anti-sense (5'-3')
HDAC4	si1	GAAACUGGACAGUAAGAAA	UUUCUUACUGUCCAGUUUC
	si2	GCAAGAUCUCAUCGUGGA	UCCACGAUGAGGAUCUUGC
	si3	GAAAAGGUUUUACAGCAAA	UUUGCUGUAAAACCUUUUC
HDAC8	si1	UGAUCAUCCGGACUCCAUA	UAUGGAGUCCGGAUGAUA
	si2	GGUCCCGGUUUAUAUCUAU	AUAGAUAAACCGGGACC
	si3	GACGGUACUACAGUGUAA	UUACACUGUAGUACCGUCC

si/siRNA, small interfering RNA; HDAC, histone deacetylase.

to detect the regularity of the OA-related indicators and the activating role of MAPK signaling pathways. The chondrocytes without IL-1 β were set as a blank control.

The OA-related indicators were analyzed using the aforementioned grouping method. The activation of all three signaling pathways was evaluated using western blotting as detailed below. Antibodies against phosphorylated (p)-extra-cellular signal-regulated kinase (ERK)-1/2, total (t)-ERK1/2, p-c-Jun N-terminal kinase (JNK), t-JNK, p-p38 and t-p38 were purchased from Cell Signaling Technology, Inc. (cat. nos. 5726, 9107, 4668, 9258, 9216 and 8690, respectively).

For subsequent experiments, all activators and inhibitors were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, Ontario, Canada) and stored at -80°C prior to use. For the kinase assays, rACs were pretreated with specific inhibitors of ERK (FR180204 at 50 μ M) and JNK (SP600125 at 10 μ M; both from Selleck Chemicals, Houston, TX, USA) and exposed to IL-1 β , or treated with the specific activators of ERK (ceramide C6 at 50 μ M; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) or JNK (anisomycin at 1 μ M; Selleck Chemicals) alone. rACs treated with DMSO were used as a vehicle control and with IL-1 β were used as a positive control. The expression of ADAMTS-4 and ADAMTS-5 was analyzed by RT-qPCR as detailed below.

Investigation of the effect of HDAC inhibitors or specific siRNAs in the in vitro OA model. Subsequently, two HDAC inhibitors (Selleck Chemicals) were selected according to the results of the previous step: TSA, a general HDAC inhibitor (only HDAC8 was not sensitive to it) and PCI-34051, an HDAC8-specific inhibitor. They were dissolved in DMSO and diluted to working concentrations of 10-500 μ M (a further dilution of 1:1,000).

Inhibitors were initially used at various concentrations for 24 h following a 6-h treatment with 10 ng/ml IL-1 β at 37°C to detect the effective concentration for regulating the expression of ADAMTS-4 and ADAMTS-5. Then, 100 nM of both inhibitors were used for 24-h pretreatment and 10 ng/ml IL-1 β treatment at 37°C was performed to detect the regulation of OA-related genes, HDACs at 24 h and MAPK signaling pathway at 15 min.

Three pairs of RNA oligonucleotides specific for HDAC4 and HDAC8 coding regions and non-targeting, scrambled siRNA (siR-Ribo™ Negative Control, Standard; 5 nmol; cat. no. siN05815122147-1-5) were designed and

chemically synthesized (Table I) by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). Cells were seeded on six-wells at a density of 5×10^5 /well prior to transfection, then, 5 μ l Lipofectamine® 3000 (Thermo Fisher Scientific, Inc.) and 100 pmol siRNAs were diffused in Opti-MEM (Thermo Fisher Scientific, Inc.) separately. A non-targeting, scrambled siRNA was used as the negative control. Untreated cells were set as the blank control. Following a 5-min incubation at room temperature, they were mixed for a further 5-min incubation at room temperature. The mixtures were placed into the medium and the medium was replaced with a new one after 6 h at 37°C. Following 24 h of incubation in total, the transfection efficiency was detected using Cy3 under a fluorescence inverted microscope (magnification, x200); the wavelength for the excitation filter was 555-585 nm.

The cells were harvested following 24-h incubation and the expression of HDAC4 and HDAC8 was assessed by western blot analysis. Anti-HDAC4 antibodies were purchased from Cell Signaling Technology, Inc. (cat. no. 5392) and anti-HDAC8 antibodies were purchased from Abcam (cat. no. ab187139) and RT-qPCR, respectively as detailed below.

The sequence of high efficiency was selected for transfection and cells were treated with 10 ng/ml IL-1 β at 37°C for 24 h. Subsequently, the expression of ADAMTS-4, ADAMTS-5 and HDAC was examined by RT-qPCR as detailed below to confirm the effect of HDACs on IL-1 β treatment. The expression of col X and COX-2 was analyzed by western blotting as detailed below separately.

Protein extraction and western blotting. Following the aforementioned treatments, the total protein was extracted from rACs (10^6 cells/well or 2×10^6 cells/plate) using radioimmuno-precipitation assay buffer containing 1% phenylmethylsulfonyl fluoride and 1% phosphatase inhibitor cocktail (optional, when protein was used for phosphorylation detection; Wuhan Boster Biological Technology, Ltd.), according to the manufacturer's protocols. Chondrocytes were initially placed on ice and washed with ice-cold PBS three times. The extract was collected and centrifuged at $12,000 \times g$ for 20 min at 4°C. The concentration of total protein supernatant was determined using a bicinchoninic acid kit (Wuhan Boster Biological Technology, Ltd.). Then, 5X loading buffer (Wuhan Boster Biological Technology, Ltd.) was added in proportion to the protein, mixed and boiled for 5 min. Equal amounts of protein (20 μ g per lane) were resolved on 10% SDS-PAGE and the separated protein was

transferred by electroblotting onto 0.45- μ m polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were preincubated in blocking buffer [5% bovine serum albumin (Wuhan Boster Biological Technology, Ltd.) in Tris-buffered saline with Tween 20 (50 mM Tris, pH 7.6, 150 mM NaCl and 0.1% Tween 20)] for 2 h at room temperature and then incubated at 4°C in blocking buffer with specific primary antibodies at various dilutions [t-p38, p-ERK, p-JNK, t-JNK, col X, COX-2 and HDAC4 at 1:1,000; p-p38 and t-ERK at 1:2,000; GAPDH at 1:10,000; HDAC8 at 1:50,000] overnight or for at least 16 h and subsequently incubated with the secondary antibody (horseradish peroxidase-linked goat anti-rabbit or goat anti-mouse at 1:5,000; Wuhan Boster Biological Technology, Ltd.; cat. nos. BA1054 and BA1050.) for 2 h at room temperature. Immunolabeling was visualized using enhanced chemiluminescence reagent (Wuhan Boster Biological Technology, Ltd.), and recorded and analyzed using the Bio-Rad ImageLab system version 5.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

RNA isolation and RT-qPCR analyses. Following the respective treatments, the total RNA was extracted from rACs (10^6 cells/well or 2×10^6 cells/plate) using AZfresh™ Total RNA Extraction Kit (MiniPrep; Azanno Biotech AB, Gothenburg, Sweden), according to the manufacturer's protocol. The concentration and purity of the total RNA were assayed using a NanoDrop spectrometer (Thermo Fisher Scientific, Inc.). Subsequently, 1 μ g total RNA was used to synthesize cDNA, using a ReverTra Ace qPCR RT Kit (cat. no. FSQ-101; Toyobo Life Science, Osaka, Japan; Code No.FSQ-101) according to the manufacturer's protocol. Briefly, RNA samples were mixed with the kit, incubated at 37°C for 15 min, and 99°C for 5 min. cDNA samples were diluted in diethylpyrocarbonate water and stored at -20°C prior to use. cDNA was amplified and recorded using SYBR® Green Realtime PCR Master Mix (cat. no. QPK-201; Toyobo Life Science) on the iCycler real-time PCR instrument (Bio-Rad Laboratories, Inc.). PCR analysis was performed using gene-specific primers (Table II). All primers were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.). The expression of the gene of interest was normalized to the housekeeping gene GAPDH, with data expressed as relative to the corresponding control group. PCR reactions were performed on a StepOnePlus Real-Time PCR System (Bio-Rad Laboratories, Inc.) according to the manufacturer's instructions. The thermocycling profiles for PCR consisted of 30 cycles of 15 sec at 94°C, 10 sec at 60°C and 40 sec at 72°C, followed by a final 8 min extension at 55°C. Each set of samples included a template-free control. Data were analyzed using the $2^{-\Delta\Delta C_q}$ method (48).

Statistical analysis. All experiments were performed in at least triplicate using independent samples. All data were presented as the mean \pm standard deviation. Comparisons of multiple groups were performed using one-way and two-way analysis of variance, followed by pairwise comparisons with a Bonferroni post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference. All data were analyzed using GraphPad Prism version 6.00 (GraphPad Software, Inc., La Jolla, CA, USA), or SPSS version 20.0 (IBM Corp., Armonk, NY, USA).

Table II. Sequences of primers.

Gene	Sequence
GAPDH	F: 5'-CTCATGACCACAGTCCATGC-3' R: 5'-TTCAGCTCTGGGATGACCTT-3'
ADAMTS-5	F: 5'-TGTTGGTGCGCCAAGGCCAAA-3' R: 5'-CCCTGTGCAGTAGCGGCCAC-3'
ADAMTS-4	F: 5'-ACAATGGCTATGGACACTGCCTCT-3' R: 5'-TGTGGACAATGGCTTGAGTCAGGA-3'
HDAC1	F: 5'-TCCAAGTCCCTTACTTTTCCC-3' R: 5'-CGCTGGTCCCTATCTAGTCC-3'
HDAC2	F: 5'-TAACTGTCAAAGGTCACGCTAA-3' R: 5'-ATACGTCCAACATCGAGCAA-3'
HDAC3	F: 5'-CCAGATCCGCCAGACCATC-3' R: 5'-GGCCTCGTCAGTCCTGTCATA-3'
HDAC4	F: 5'-ACTTTCCCGTGTCACTCCTG-3' R: 5'-TCTTCCTTCGTGGCTTTCC-3'
HDAC5	F: 5'-TCTTTGCATCGCTACGACAAT-3' R: 5'-ATCAAACCCAGCGGAGACTAG-3'
HDAC6	F: 5'-ATGCCGTGACTCCACTATCC-3' R: 5'-TGAACCGATCCACAGGTCCTT-3'
HDAC7	F: 5'-GTGCCAAACCTTCATCTCCC-3' R: 5'-TCCCACCTTCCTATCCCTCA-3'
HDAC8	F: 5'-CTGGAAATCACGCCAAGC-3' R: 5'-TGTCTTCATTATGGGTTCTGC-3'
HDAC9	F: 5'-CTACCGTCAGGGAGGCTAA-3' R: 5'-TCCACCAGTCTGTGAATACGA-3'
HDAC10	F: 5'-GTGTTCTGTTTAGCCGATGTT-3' R: 5'-TCGCCAGTCGCTTGAAAT-3'
HDAC11	F: 5'-GGCAGCATAACCAAAGGC-3' R: 5'-GGAATACCCACATGGTAATGAA-3'

F, forward; R, reverse; ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; HDAC, histone deacetylase.

Results

Successful construction of an in vitro model of OA. rACs were cultured with various concentrations of IL-1 β (2.5-40 ng/ml) for 24 h. The expression levels of OA-related markers were measured via RT-qPCR (Fig. 1A) and western blot analysis (Fig. 1B). The expression of ADAMTS-5 (8.17 ± 1.71) and ADAMTS-4 (3.69 ± 0.31) increased significantly following treatment with 10 ng/ml IL-1 β compared with the control group (1.00 ± 0.16 and 1.00 ± 0.06 ; $P < 0.05$; Fig. 1A-a and -b). The expression of col X and COX-2 also increased following IL-1 β treatment (Fig. 1B-a). The expression of ADAMTS-5 was constant from the beginning of the experiment (1.00 ± 0.21) to 2 h (0.85 ± 0.17 ; $P > 0.05$); then it significantly increased at 4 h (2.50 ± 0.12 ; $P < 0.05$) and reached a peak at 24 h (7.29 ± 0.98 ; $P < 0.05$; Fig. 1A-c). Other indicators, ADAMTS-4, col X and COX-2, demonstrated similar effects (Fig. 1A-d and B-b). The results demonstrated that 10 ng/ml was the appropriate

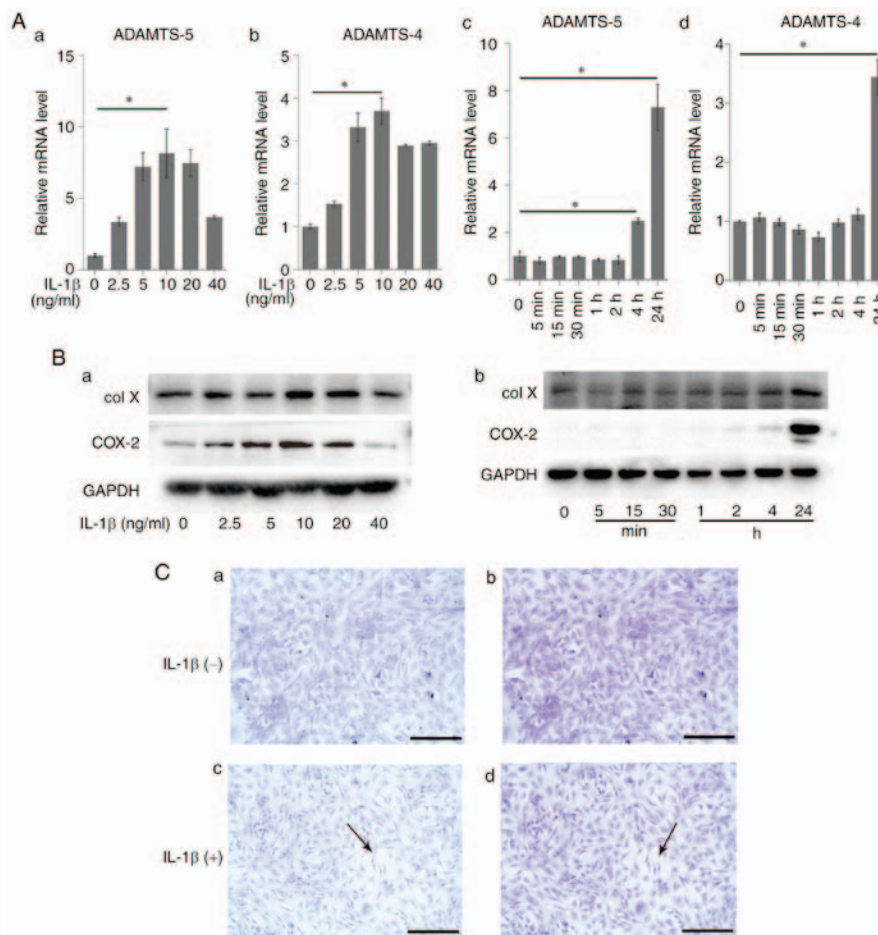


Figure 1. Effect of IL-1 β on osteoarthritis-related gene expression levels and morphological changes in rACs. IL-1 β increased (A) osteoarthritis-related genes and (B) proteins at a various concentrations in a time-dependent manner. Results are expressed with respect to GAPDH expression. Data represent the means \pm standard deviation, n=3. *P<0.05. (C) Representative images of rACs; microscopic observation (left panel) and toluidine blue staining (right panel). (a and b) control; (c and d) IL-1 β 10 ng/ml. Arrows indicate spindle-shaped cells. Magnification, x200; scale bar=200 μ m. IL, interleukin; rACs, rat articular chondrocytes; ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; col X, type X collagen; COX2, cyclooxygenase.

concentration for further steps and the increase in OA-related markers was time-dependent.

Morphological observation of rACs. Untreated rACs were small, exhibiting an identical distribution, and were almost triangular or polygonal. The secretory vesicles and organelles in the cytoplasm exhibited a prolific distribution (Fig. 1C-a). Toluidine blue staining demonstrated that the ECM was rich in proteoglycans and no abnormal cells were observed (Fig. 1C-b). ECM degradation, cell shrinkage and intercellular broadening were observed following IL-1 β treatment. Most cells were elongated and acquired a narrow triangular or spindle shape (Fig. 1C-c). Toluidine blue staining demonstrated morphological changes including degradation of ECM and a decrease in the content of proteoglycans (Fig. 1C-d).

IL-1 β promotes the expression of ADAMTS-4 and ADAMTS-5 through the activation of MAPK signaling. The present study examined the effects of treatment with IL-1 β on the phosphorylation of 3 members of the MAPK family in rACs. The phosphorylation of p38 did not demonstrate notable activation following IL-1 β treatment. However, ERK1/2 demonstrated a rapid increase in the first 5 min, reaching a peak at 15 min and

then decreased gradually. Meanwhile, JNK demonstrated a rapid increase at 15 min, remained constant at 30 min and then decreased rapidly. Levels of the housekeeping gene GAPDH did not change during this period (Fig. 2A).

The expression of ADAMTS-5 (1.00 ± 0.21 vs. 1.06 ± 0.12) and ADAMTS-4 (1.00 ± 0.08 vs. 1.10 ± 0.06) demonstrated no evident change in the negative control (NC) group using DMSO alone to treat the cartilage cells compared with the blank control group (Fig. 2B and C).

The specific activators of JNK (anisomycin, 1 μ M) and ERK (ceramide C6, 50 μ M) significantly upregulated the expression of ADAMTS-5 (1.94 ± 0.32 and 4.96 ± 0.36 vs. 1.00 ± 0.21 ; P<0.05) and ADAMTS-4 (1.46 ± 0.05 and 2.49 ± 0.06 vs. 1.00 ± 0.21 ; P<0.05), respectively, compared with the NC group (Fig. 2B and 2C).

The specific inhibitors of JNK (SP600125, 10 mM; 0.71 ± 0.07 and 1.29 ± 0.05) and ERK (FR180204, 50 μ M; 1.43 ± 0.21 and 1.35 ± 0.06) also significantly blocked the IL-1 β -induced expression compared with the positive control group (5.31 ± 0.35 and 3.12 ± 0.07 ; P<0.05; Fig. 2B and C).

However, the ERK activator-induced expression could not reach the peak in the positive control group even at a higher concentration, suggesting that ERK may serve a subordinate role in the upregulation of ADAMTS-4 and ADAMTS-5.

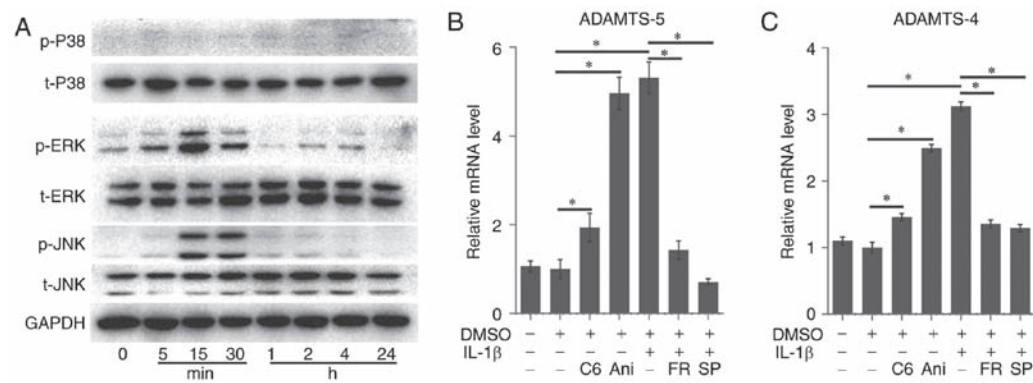


Figure 2. IL-1 β promoted the expression of ADAMTS-4 and ADAMTS-5 through the activation of MAPK signaling. (A) Of the 3 MAPK isoforms, ERK1/2 and JNK were markedly phosphorylated following treatment with IL-1 β . The gene expression of (B) ADAMTS-5 and (C) ADAMTS-4 was facilitated by C6 (ERK activator) or Ani (JNK activator). The IL-1 β -induced gene expression of (B) ADAMTS-5 and (C) ADAMTS-4 was inhibited by FR (ERK inhibitor) or SP (JNK inhibitor). Data present the means \pm standard deviation, n=3. *P<0.05. IL, interleukin; ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; DMSO, dimethyl sulfoxide; C6, Ceramide C6; Ani, Anisomycin; FR, FR180204; SP, SP600125; p, phosphorylated; t, total.

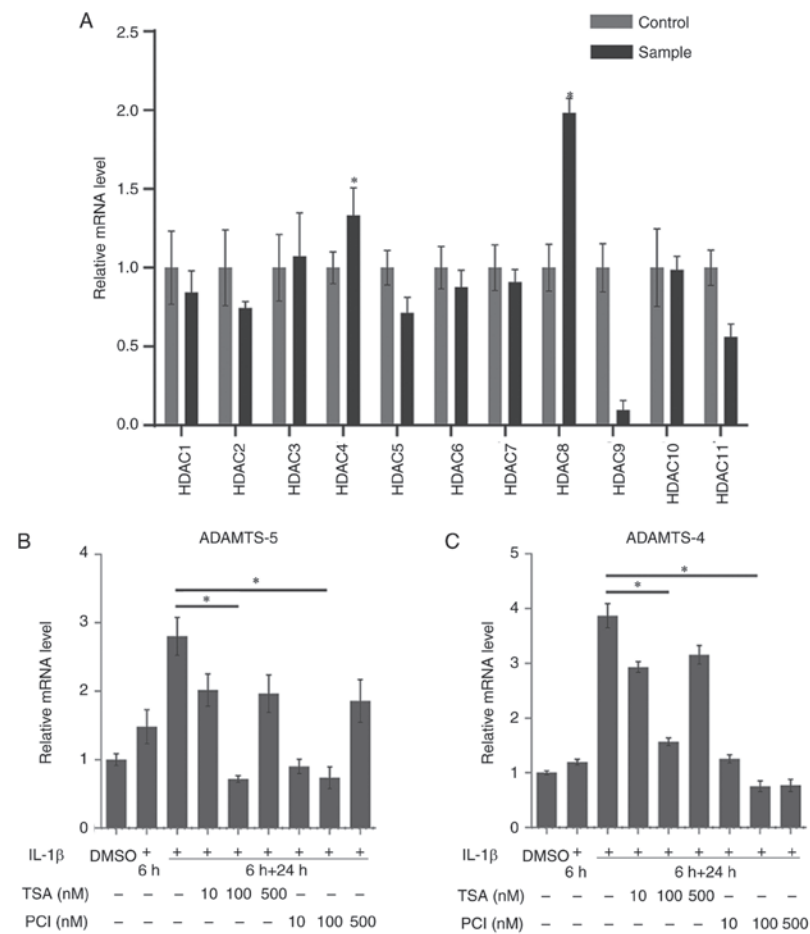


Figure 3. The effect of HDAC inhibitors on ADAMTS-4 and ADAMTS-5 in IL-1 β -treated rACs. (A) HDAC 1-11 mRNA expression in rACs was determined by reverse transcription-quantitative polymerase chain reaction. HDAC 4 and HDAC 8 had significantly higher expression in IL-1 β -treated group compared with the control group. Results are expressed with respect to GAPDH expression. Data represent the means \pm standard deviation, n=3. *P<0.05 vs. control. (B and C) rACs stimulated with IL-1 β (10 ng/ml) 6 h and then treated or not treated with TSA or PCI-34051 for 24 h; ADAMTS-5 and ADAMTS-4 expression was inhibited by TSA (100 nM) and PCI-34051 (100 nM). Results are expressed with respect to GAPDH expression. Data represent the means \pm standard deviation, n=3. *P<0.05. HDAC, histone deacetylase; ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; IL, interleukin; rACs, rat articular chondrocytes; TSA, Trichostatin A; PCI, PCI-34051; DMSO, dimethyl sulfoxide.

Inhibitory effect and mechanism of HDAC in an in vitro OA model. The expression of HDAC1-11 mRNA was evaluated using RT-qPCR. The expression of HDAC4 (1.33 ± 0.17 vs.

1.00 ± 0.10 ; P<0.05) and HDAC8 (1.98 ± 0.09 vs. 1.00 ± 0.15 ; P<0.05) was upregulated following IL-1 β treatment (Fig. 3A). This suggested that HDAC4 and HDAC8 may be associated

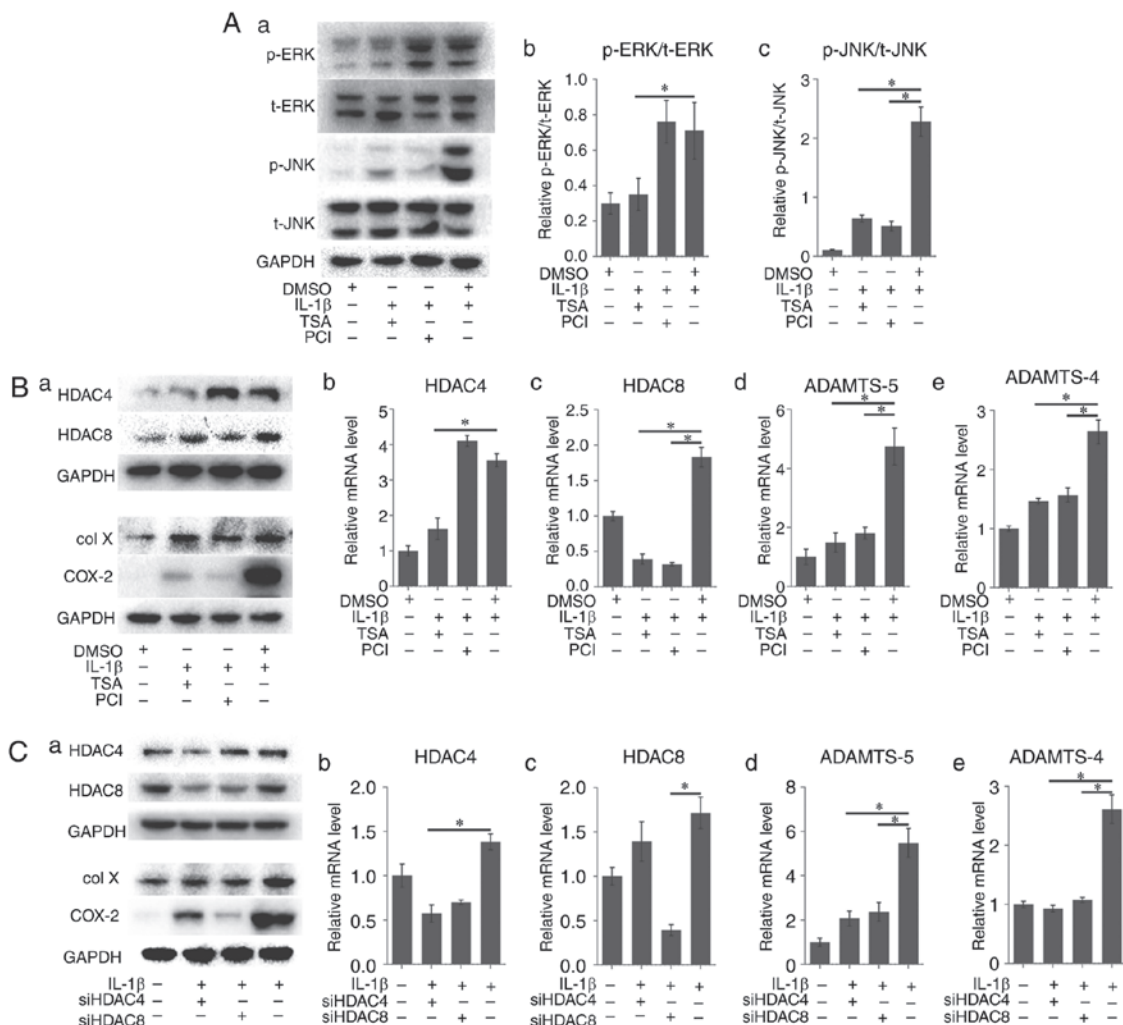


Figure 4. HDAC regulates the osteoarthritis-related genes and protein expression of rACs via ERK1/2 and JNK. (A) IL-1 β -induced ERK1/2 phosphorylation was blocked by TSA, and not blocked by PCI; JNK phosphorylation was blocked by TSA and PCI. (B) HDAC inhibitors (TSA or PCI) suppressed HDAC expression at both mRNA and protein level, and prevented the upregulation of osteoarthritis-related genes (col X, COX2, ADAMTS-5 and ADAMTS-4) expression induced by IL-1 β . Results are presented with respect to GAPDH expression. (C) siRNAs (targeting HDAC4 or HDAC8) suppressed HDAC expression at both mRNA and protein level, and prevented the upregulation of osteoarthritis-related gene expression induced by IL-1 β . Results are expressed with respect to GAPDH expression. Data represent the means \pm standard deviation, n=3. *P<0.05. HDAC, histone deacetylase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; IL, interleukin; TSA, Trichostatin A; DMSO, dimethyl sulfoxide; PCI, PCI-34051; col X, type X collagen; COX2, cyclooxygenase; ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; siRNA, small interfering RNA; p, phosphorylated; t, total. rACs, rat articular chondrocytes.

with the upregulation of the expression of ADAMTS-5 in IL-1 β -treated chondrocytes.

In rACs stimulated with IL-1 β (10 ng/ml) for 6 h and treated or not treated with TSA or PCI-34051 for 24 h, the expression of ADAMTS-5 and ADAMTS-4 were observed over 30 h. TSA at a concentration of 100 nM (0.72 ± 0.25) and PCI-34051 at a concentration of 100 nM demonstrated a significant inhibitory effect on the IL-1 β -induced expression of ADAMTS-5 (2.80 ± 0.28 and 0.74 ± 0.16 ; P<0.05; Fig. 3B). TSA at a concentration of 100 nM (1.57 ± 0.07) and PCI-34051 at 100 nM (0.76 ± 0.10) demonstrated a similar inhibitory effect on the expression of ADAMTS-4 (3.87 ± 0.22 ; P<0.05; Fig. 3C). These results demonstrated that HDAC inhibitors were able to downregulate the expression of ADAMTS in IL-1 β -stimulated rACs and therefore a concentration of 100 nM for TSA and PCI-34051 was selected for subsequent experiments.

TSA- or PCI-pretreated cells were exposed to IL-1 β . ERK phosphorylation demonstrated a marked decrease in the

TSA group compared with the positive control group, but no apparent change in the PCI group was observed. However, JNK phosphorylation demonstrated a marked decrease in the two groups, the latter appearing to be more evident (Fig. 4A).

The expression of col X and COX-2 as analyzed via western blotting demonstrated downregulation compared with the IL-1 β -treated group; the PCI-pretreated group demonstrated a stronger effect (Fig. 4B-a). The expression of HDAC4 in the TSA group (1.62 ± 0.30 vs. 3.56 ± 0.18 ; P<0.05; Fig. 4B-b) and that of HDAC8 in the PCI group decreased significantly (0.32 ± 0.02 vs. 1.83 ± 0.14 ; P<0.05; Fig. 4B-c). The expression of ADAMTS-5 was significantly lower in the pretreated groups than in the positive control group (1.49 ± 0.33 and 1.81 ± 0.20 vs. 4.74 ± 0.62 , P<0.05; Fig. 4B-d), in addition to the expression of ADAMTS-4 (1.46 ± 0.05 and 1.57 ± 0.12 vs. 2.65 ± 0.20 , P<0.05; Fig. 4B-e). These results demonstrated that HDAC inhibitors were able to block MAPK pathways and downregulate OA-related markers at various levels.

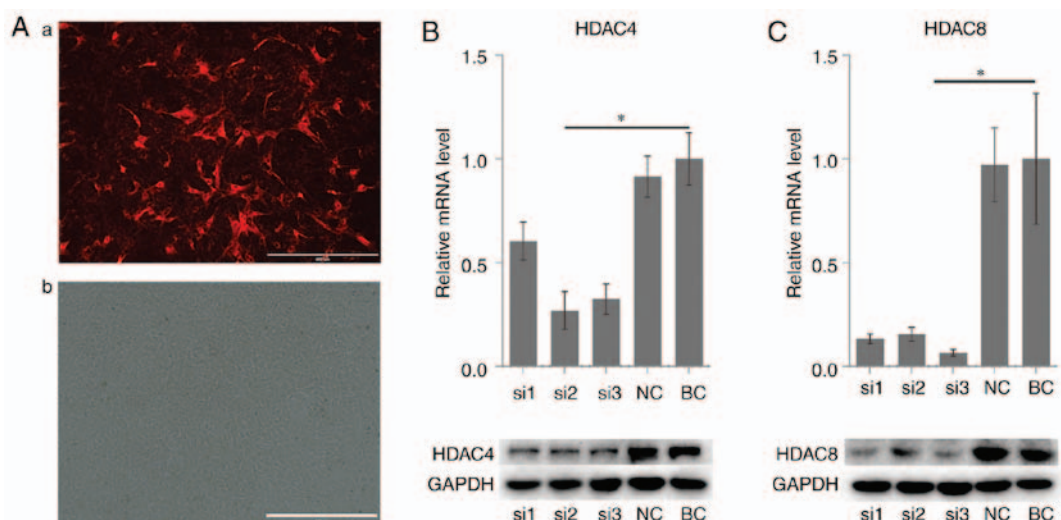


Figure 5. Transfection was successful; mRNA and protein levels of both HDAC4 and HDAC8 were downregulated. (A-a) Red fluorescence image of cells transfected with Cy3 siRNA under a fluorescence microscope and (A-b) the same chondrocytes under a normal optical microscope (magnification, x200; scale bar=400 μ m). (B) Analysis of HDAC4 mRNA expression; western blotting for HDAC4 protein expression. HDAC4 siRNA2 resulted in a significant reduction in the mRNA level and protein expression of HDAC4 in chondrocytes. (C) Analysis of HDAC8 mRNA expression; western blotting for HDAC8 protein expression. HDAC8 siRNA3 resulted in a significant reduction in the mRNA level and protein expression of HDAC8 in chondrocytes. Results are expressed with respect to GAPDH expression. Data represent the means \pm standard deviation, n=3. *P<0.05. HDAC, histone deacetylase; si/siRNA, small interfering RNA; NC, negative control; BC, blank control.

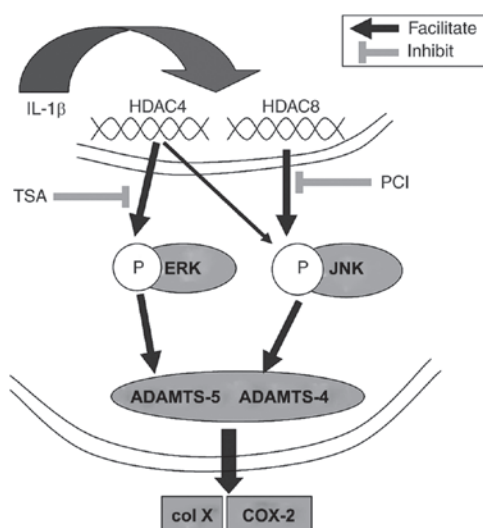


Figure 6. Possible signaling pathways associated with ADAMTS, col X, and COX-2 regulation by IL-1 β in chondrocytes. ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; col X, type X collagen; COX2, cyclooxygenase; IL, interleukin; HDAC, histone deacetylase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; IL, interleukin; TSA, Trichostatin A; PCI, PCI-34051.

The cells pretreated with specific siRNAs targeting HDAC4 or HDAC8 transfection were stimulated with IL-1 β , which demonstrated a similar effect as that of HDAC inhibitors.

The expression of col X and COX-2 as analyzed by western blotting demonstrated similar effects; the HDAC8 siRNA pretreated group demonstrated a stronger inhibitory expression of COX-2 (Fig. 4C-a). The expression of HDAC4 decreased in the siHDAC4 group (0.57 ± 0.10 vs. 1.38 ± 0.09 ; P<0.05; Fig. 4C-b) whereas that of HDAC8 decreased in the siHDAC8 group (0.39 ± 0.06 vs. 1.72 ± 0.18 ; P<0.05; Fig. 4C-c). The expression of ADAMTS-4 and ADAMTS-5 was similar

to that in the inhibitor-pretreated groups (P<0.05; Fig. 4C-d and C-e).

Effect of siRNA on HDAC4 and HDAC8 gene expression in rACs. Subsequently, 3 pairs of siRNA for each gene were used to silence the mRNA expression of HDAC4 and HDAC8 in the rACs. A non-targeting, scrambled siRNA was used as the negative control. The transfection efficiency was observed under a fluorescence microscope at 24 h following transfection. The red fluorescence cells were considered to be transfected successfully (Fig. 5A). All groups demonstrated a downregulation in the mRNA and protein expression of HDAC4 (Fig. 5B) and HDAC8 (Fig. 5C). Appropriate sequences based on the results were selected for subsequent experiments.

Discussion

The most characteristic feature of OA is the large amount of proinflammatory cytokines in articular cartilage produced by synovial cells and progressive damage of cartilage. As current therapies for OA act only on symptoms and do not prevent the pathogenesis of OA (29), researchers have attempted to find effective agents that may inhibit the degeneration and catabolism of articular cartilage. In addition, no reliable indicator exists for the early diagnosis of OA. Therefore, investigating the mechanism for diagnosis and remedy is important. The modulation of synthesis or activity of cartilage degradation-related genes including MMPs and ADAMTS has been one of the major targets in this endeavor.

Previous researchers have focused on tissue engineering to repair the damage of articular cartilage (49-52). However, this type of cartilage may degrade under inflammatory conditions. Although certain stem cell-based therapies may be useful for chondrogenic differentiation under a stable environment (53-56), they may induce osteogenic differentiation under

an OA environment. Therefore, research on anti-inflammatory agents is urgent (57).

Numerous studies have demonstrated that IL-1 β may be an important trigger in the progression of OA (13,58). A previous study also suggested ADAMTS-5 as a downstream indicator for OA therapy and demonstrated that a single dose of ADAMTS-5 siRNA lentivirus injection may prevent cartilage degradation (27). Whether a combination with ADAMTS-4 silencing can achieve an improved effect has been explored (59,60). As a single inhibitor for one type of ADAMTS may be inefficient and possess a number of side effects, the investigation of upstream regulation is crucial. An *in vitro* model of OA was successfully constructed in the present study and the IL-1 β -induced increased expression of ADAMTS-4 and ADAMTS-5, and overproduction of col X and COX-2, were observed in cultured chondrocytes.

MAPK is a group of serine/threonine protein kinases involved in the cellular signal transduction. The members of this signaling pathway group include p38, JNK and ERK1/2 (61). Previous studies have demonstrated the effect of MAPK signals on tumor metastasis (62,63), osteogenesis (64,65), or certain inflammatory diseases (66). The IL-1 β activation of ADAMTS requires the integration of several signaling pathways and activated transcription factors. The present study investigated the regulation and mechanisms of cartilage inflammation and degradation in an *in vitro* model. The effects of IL-1 β -induced MAPK activation in chondrocytes was also explored, and this demonstrated that IL-1 β -induced expression of ADAMTSs was mediated by the MAPK family signal transduction molecules. Following treatment with IL-1 β , the phosphorylation of ERK and JNK occurred in chondrocytes in the initial 30 min; the upregulation of the expression of ADAMTS-4 and ADAMTS-5 occurred at a later time, suggesting a chronological order. Notably, the ERK activator had less ability to upregulate the expression of ADAMTS-4 and ADAMTS-5 compared with IL-1 β or JNK activator, suggesting that ERK may serve a subordinate role in this process or have a synergistic effect with JNK. p38 was not activated in the present study, unlike in the previous findings of Saito *et al* (42). This discrepancy may be due to cell line disparity.

Epigenetic therapy has been widely researched and used in laboratory and clinical trials, mainly in tumor or cardiovascular research and therapy. Several researchers have investigated HDAC inhibitors in OA therapy, owing to their anti-inflammatory properties (67). However, no clear consensus exists as to which type(s) of HDAC serve(s) a decisive role in arthritis pathogenesis and development. For instance, Saito *et al* (42) used two types of HDAC inhibitors to block the expression of ADAMTS-5 in mechanically induced cartilage cells, which was presumed to be via class I HDACs including HDAC1/2/3 or 8. Other researchers have explored the nexus between HDAC4 and HDAC7 in human OA and proposed specific mechanisms (68-70). HDAC inhibitor vorinostat can inhibit the IL-1 β -induced expression of MMPs and this effect is mediated through the phosphorylation of p38 and ERK1/2 (2). These findings demonstrated the therapeutic role and target in the OA treatment of HDACs. The close link between ADAMTS or MMPs and HDAC has promoted the studies of their effects on OA treatment.

The present study selected 2 types of inhibitors. TSA is a broad-spectrum HDAC inhibitor, but HDAC8 was the only one not affected by it. PCI-34051 was a selective HDAC inhibitor

mainly targeting HDAC8. It also had an effect on HDAC2. HDAC4 or HDAC8 demonstrated relatively high expression at an early stage in IL-1 β -treated rACs. Blocking HDAC4 or HDAC8 potentially suppressed ADAMTS-4 and ADAMTS-5 production and expression in IL-1 β -stimulated chondrocytes, suggesting that they may be a promising target for diagnosis and therapy and may be associated with regulation.

As the inhibition of ADAMTS induced by IL-1 β was known to protect from degradation and alleviate the pathology progression, the effect of inhibition of HDAC4 and HDAC8 on IL-1 β -induced inflammation was encouraging.

The effect of HDAC4 and HDAC8 on JNK and ERK1/2 activation in chondrocytes stimulated with IL-1 β was also investigated in the present study. The effect on p38 was not studied as no activation in IL-1 β -stimulated chondrocytes was observed. Notably, TSA was revealed to inhibit the activation of JNK and markedly inhibit the activation of ERK. However, PCI-34051 inhibited the activation of JNK but had almost no effect on the activation of ERK. These results indicated the possible association of JNK and ERK1/2 pathway with the selective inhibition via ADAMTS production. HDAC4 and HDAC8 were associated with this process. Considering the complex effect of inhibitors, specific siRNAs were used to repeat the experiment and similar results were achieved, confirming the regulatory effect of HDAC4 and HDAC8. As other signaling pathways are also associated with the regulation of OA-related gene expression, further studies are required to reveal the precise mechanisms that underlie the regulation.

The inhibitors and siRNAs were revealed to exert a wide range of effects on the expression of genes and proteins associated with OA pathogenesis and correlated with the attenuation of MAPK pathways in rat chondrocytes. The results of the present study enhanced the understanding of the molecular mechanisms of OA and suggested that they may be of potential value in treating and preventing inflammation associated with OA. The lower expression of HDAC9 and HDAC11 may negatively regulate the OA process, which merits further studies. Therefore, the findings of the present study may contribute to the development of anti-OA drugs and diagnostic methods targeting the HDACs.

In conclusion, the present study demonstrated that HDAC inhibitors or siRNAs inhibited the IL-1 β -induced expression of ADAMTS-4, ADAMTS-5, col X and COX2, all of which may serve a pivotal role in the progression of OA, suggesting their inhibitory effect on cartilage degradation. Therefore, HDAC inhibitors merit consideration as therapeutic targets in treating and preventing OA.

Furthermore, the study also demonstrated that this effect was mediated through the regulation of the inhibition of phosphorylation of JNK and ERK1/2 (Fig. 6). This suggested that HDAC4 and HDAC8 may be of diagnostic significance in the early phase of OA.

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Competing interests

The authors declare that they have no competing interests.

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