Effects of HDAC4 on IL-1β-induced matrix metalloproteinase expression regulated partially through the WNT3A/β-catenin pathway

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Abstract
Background: Histone deacetylase 4 (HDAC4) regulates chondrocyte hypertrophy and bone formation. The aim of the present study was to explore the effects of HDAC4 on Interleukin 1 beta (IL-1β)-induced chondrocyte extracellular matrix degradation and whether it is regulated through the WNT family member 3A (WNT3A)/β-catenin signaling pathway.

Methods: Primary chondrocytes (CC) and human chondrosarcoma cells (SW1353 cells) were treated with IL-1β and the level of HDAC4 was assayed using Western blotting. Then, HDAC4 expression in the SW1353 cells was silenced using small interfering RNA to detect the effect of HDAC4 knockdown on the levels of matrix metalloproteinase 3 (MMP3) and MMP13 induced by IL-1β. After transfection with HDAC4 plasmids, the overexpression efficiency was examined using Real-time quantitative polymerase chain reaction (qRT-PCR) and the levels of MMP3 and MMP13 were assayed using Western blotting. After incubation with IL-1β, the translocation of β-catenin into the nucleus was observed using immunofluorescence staining in SW1353 cells to investigate the activation of the WNT3A/β-catenin signaling pathway. Finally, treatment with WNT3A and transfection with glycogen synthase kinase 3 beta (GSK3β) plasmids were assessed for their effects on HDAC4 levels using Western blotting.

Results: IL-1β downregulated HDAC4 levels in chondrocytes and SW1353 cells. Furthermore, HDAC4 knockdown increased the levels of MMP3 and MMP13, which contributed to the degradation of the extracellular matrix. Overexpression of HDAC4 inhibited IL-1β-induced increases in MMP3 and MMP13. IL-1β upregulated the levels of WNT3A, and WNT3A reduced HDAC4 levels in SW1353 cells. GSK-3β rescued IL-1β-induced downregulation of HDAC4 in SW1353 cells.

Conclusion: HDAC4 exerted an inhibitory effect on IL-1β-induced extracellular matrix degradation and was regulated partially by the WNT3A/β-catenin signaling pathway.

Keywords: Histone deacetylase 4; Matrix metalloproteinase 13; Matrix metalloproteinase 3; Osteoarthritis; WNT3A

Introduction
Osteoarthritis (OA) is one of the most common degenerative joint disorders in human,¹² which is characterized by hyperplasia of subchondral bone and the degeneration of articular cartilage, resulting in the loss of joint function.¹³,¹⁴ The temporomandibular joint (TMJ) is a synovial joint that plays an important role in craniofacial growth and function, and can be affected by OA.¹⁵ The maintenance of homeostasis in articular cartilage is crucial for its structural integrity and function. The balance between matrix destruction and repair is regulated by the catabolic and anabolic activities of chondrocytes.¹⁶

Although the specific mechanisms of OA are unclear, growing evidence suggests that catabolic alterations and inflammation play critical roles in the development of OA.¹⁷ Proinflammatory cytokines such as interleukin (IL)-1β are the critical mediators of the disturbed processes implicated in OA pathophysiology,⁸ which lead to the apoptosis of chondrocytes and degradation of the extracellular matrix (ECM), eventually resulting in cartilage degeneration.⁸,¹⁰,¹² Specifically, upregulation of cytokines increases the expression of matrix-degrading proteins such as matrix metalloproteinases (MMPs) significantly, especially matrix metalloproteinase 1 (MMP1), matrix metalloproteinase 3 (MMP3), and matrix metalloproteinase 13 (MMP13).¹⁶,¹⁷ These MMPs contribute to the degradation of type II collagen and aggrecan, which are the major components of the cartilage matrix.¹⁸,¹⁹ Therefore, inhibition of IL-1β–induced catabolic metabolism and inflammatory responses could delay the progression of OA.

The initiation and progression of OA is a complex process that involves many cell types, signaling pathways, and molecular changes in the ECM. Epigenetic alterations...
occurred in chondrocytes during the initiation and progression of OA, 
and histone deacetylases (HDACs) play crucial roles in all stages of chondrocyte maturation, likely contributing to disease phenotypes. Class II HDACs initiate transcriptional changes in response to cytokines and growth factors, playing key roles in chondrocyte maintenance and maturation. HDAC4 is the most extensively studied class II HDAC. HDAC4 has a crucial role in the regulation of chondrocyte hypertrophy during skeletogenesis in mice; HDAC4-null mice displayed aberrant chondrocyte hypertrophy and subsequent premature ossification in the chondrocostal cartilage, and HDAC4 could repress chondrocyte hypertrophy in vitro. Hypertrophic chondrocytes express high levels of MMP-3 and MMP-13. We, therefore, hypothesized that HDAC4 would affect IL-1\( \beta \)-induced MMP expression.

Several critical signaling pathways act as key regulators and activators of cellular and molecular processes during OA development. WNT signaling is one such pathway and it was reported to stimulate matrix catabolic genes and the activity of catabolic proteins in articular chondrocytes, suggesting its possible role in OA. WNT is a family of extracellularly secreted glycoproteins whose various receptors regulate canonical \( \beta \)-catenin-dependent and non-canonical \( \beta \)-catenin-independent signaling pathways. The protein level of WNT3A, a major activator of canonical WNT/\( \beta \)-catenin signaling, and that of \( \beta \)-catenin were increased in a model of OA. \( \beta \)-catenin is a central molecule in the canonical WNT signaling pathway, which controls multiple developmental processes in skeletal and joint development. However, the relationship between the WNT signaling and HDAC4 in the pathogenesis of TMJ osteoarthritis remains unclear. In the present study, we explored the role of HDAC4 in the development of OA and whether it was regulated through the WNT signaling pathway.

Methods

Primary chondrocyte isolation and culture

Chondrocytes were isolated from the TMJ condyles of 4-week-old Wistar rats (River Experimental Animal Technique Company, Beijing, China) as described in previous study. Cartilage was digested with 0.25% trypsin (Sigma, St. Louis, MO, USA) for 15 min, followed by digestion with 0.2% type II collagenase (Invitrogen, San Diego, CA, USA) for 4 to 6 h. Chondrocytes were then suspended in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA), supplemented with 50 units of penicillin/streptomycin (Gibco) and 2 mmol/L glutamine. Cells were plated in 60-mm plates at a density of 1.5 \( \times \) 10\(^6\) cells/plate. After primary culture for 5 days, the chondrocytes were harvested. All animal procedures were approved by the Animal Use and Care Committee of Peking University, China.

SW1353 cell culture

SW1353 human chondrosarcoma cells were purchased from ATCC (Manassas, VA, USA). The cells were cultured in DMEM supplemented with 10% v/v FBS, 2 mmol/L glutamine, and 50 units of penicillin/streptomycin. Cells were grown at 37°C in an atmosphere containing 5% CO\(_2\) and 95% humidity.

Cell treatment and transfection

HDAC4 expression in SW1353 cells was silenced using small interfering RNA (siRNA; GenePharma, Shanghai, China) transfection. The sequences of siRNAs are listed in Table 1. Chondrocytes were seeded at a density of 2.0 \( \times \) 10\(^3\) cells/well in 6-well plates and transfected with siRNAs specific to HDAC4 (si-HDAC4; 50 nmol/L) or with a negative control siRNA (si-NC; 50 nmol/L) as a negative control, using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The sequences of the siRNAs used are listed in Table 1. The cells were then incubated with IL-1\( \beta \) (10 ng/mL) for 24 h. The HDAC4 overexpression vector and empty vector (Genechem, Shanghai, China) were transfected into cells using Lipofectamine 3000 reagent. Cells were plated in a 6-well tissue culture plate 48 to 72 h before transfection. Cells at 80% to 90% confluence were transfected with 2.5 \( \mu \)g of plasmid DNA. Transfected cells were maintained in complete DMEM for 48 h. Cells were then incubated with IL-1\( \beta \) (10 ng/mL) for 24 h and used for further analyses. For cell treatment, cells at 80–90% confluence were washed with phosphate buffered saline (PBS) and were further cultured in serum free media overnight. IL-1\( \beta \) (Sigma, St. Louis, MO, USA) or WNT3A (R&D Systems, Minneapolis, MN) was added into the media.

Immunofluorescence microscopy

For immunofluorescence microscopy, cells were grown on glass coverslips and fixed with 4% paraformaldehyde at 4°C for 15 min. The cells were permeabilized and blocked with 0.5% Triton X-100 and 5% bovine serum albumin in...
phosphate-buffered saline (PBS) for 30 min. The coverslips were then exposed to primary antibodies at 4°C overnight, followed by fluorescein isothiocyanate–conjugated secondary antibodies for 1 h. The nuclei were counterstained using 4',6-diamidino-2-phenylindole (DAPI) for 5 min. The slides were mounted with 90% glycerol in PBS, and fluorescence was examined under a fluorescence microscope.

RNA isolation and qRT-PCR

Total RNA was isolated from cells using TRIzol (Invitrogen, Carlsbad, CA, USA). Total RNA (1 μg) was reverse-transcribed using a PrimeScript-RT reagent kit (Takara Biotechnology, Kusatsu, Japan), and resultant cDNA was analyzed using quantitative real-time polymerase chain reaction (qPCR) using the 7500 Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA) and SYBR Premix Ex Taq™ (Takara Biotechnology), according to the manufacturer’s guidelines. The primers used are listed in Table 2. All experiments were performed in triplicate and mRNA levels were standardized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The relative mRNA expression levels were calculated using the 2(−ΔΔCT) method.

Western blotting

Cells were lysed using 2% sodium dodecyl sulfate (SDS) lysis buffer (0.05 mol/L Tris-HCl, pH 7.4, 2 mmol/L EDTA, 2% SDS) supplemented with protease inhibitor cocktail (Calbiochem, La Jolla, CA, USA) and phenylmethylsulfonyl fluoride (PMSF, Sigma). The protein concentration was determined by the bicinchoninic acid method by using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Proteins (50 μg) were fractionated by SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in TRIS-buffered saline containing 0.1% Tween 20. Rabbit anti-HDAC4 (1:1000 dilution, Cell Signaling Technology, Danvers, MA, USA), mouse anti-MMP3 (1:2000 dilution, ProteinTech), and rabbit anti-MMP13 (1:500 dilution, Protein Tech) antibodies were used to detect the proteins. The blots were developed using a horseradish peroxidase-conjugated secondary antibody and analyzed using enhanced chemiluminescence detection.

Statistical analysis

All data were expressed as mean ± standard deviation. Statistically significant differences were calculated using the Student’s t test in GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, USA). P < 0.05 was considered statistically significant.

Results

HDAC4 was downregulated in chondrocytes and SW1353 cells treated with IL-1β

To study the function of HDAC4 during the progression of OA, a cellular OA model was established by treating primary chondrocytes and SW1353 cells with IL-1β. The levels of HDAC4 and MMP isoforms were analyzed using Western blotting in rat primary TMJ condylar chondrocytes and SW1353 cells treated with IL-1β at different concentrations. As shown in Figure 1, treatment of condylar chondrocytes and SW1353 cells with IL-1β caused a time and dose-dependent downregulation of HDAC4 levels, with a corresponding increase in MMP3 and MMP13 levels.
Knockdown of HDAC4 enhanced IL-1β-induced increases in MMP levels in SW1353 cells

The effects of HDAC4 siRNA were detected using qRT-PCR and Western blotting [Figure 2A]. As shown in Figure 2B, MMP-3 and MMP-13 levels were higher in the si-HDAC4 group than those in the control group. The effect of HDAC4 on IL-1β-induced extracellular matrix degradation in SW1353 cells was then detected. As shown in Figure 2F, MMP-3 and MMP-13 levels were higher in the IL-1β treated group than those in the control group. The protein levels of MMP-3 and MMP-13 significantly increased in the si-HDAC4 group after incubation with IL-1β (10 ng/mL) for 24 h.

Overexpression of HDAC4 inhibited IL-1β-induced increases in MMP levels in SW1353 cells

To further clarify the hypothesis that HDAC4 participates in IL-1β-mediated induction of MMP3 and MMP13, SW1353 cells were transfected with the HDAC4 overexpression plasmid. As shown in Figure 2D, the levels of MMP3 and MMP13 were downregulated after transfection of the HDAC4 overexpression plasmid. Then HDAC4 was overexpressed in SW1353 cells and IL-1β was added to explore the role of this gene in OA progression. IL-1β significantly upregulated the protein levels of MMP3 and MMP13, whereas pretreatment with HDAC4 overexpression resulted in significant decreases in MMP3 and MMP13 levels [Figure 2E]. Therefore, overexpression of HDAC4 inhibited IL-1β-mediated induction of MMP3 and MMP13, at levels corresponding to transfection efficiency [Figure 2C].

IL-1β activated the WNT3A/β-catenin pathway

We investigated the WNT3A/β-catenin signaling pathway to explore the underlying mechanism of HDAC4. As shown in Figure 3A, treatment of SW1353 cells with IL-1β caused time-dependent upregulation of WNT3A. β-catenin protein levels and their distribution in cells were determined using Western blotting and immunofluorescence staining. Western blotting revealed decreased levels of phosphorylated β-catenin in IL-1β treated cells [Figure 3B]. Immunofluorescence staining revealed that the translocation of β-catenin into the nucleus increased, which was induced by IL-1β stimulation [Figure 3C].

**Figure 2:** Effects of HDAC4 in IL-1β stimulated SW1353 cells. (A) qRT-PCR and Western blotting analyses were used to evaluate the efficiency of HDAC4 knockdown. *P < 0.01 compared with si-NC group. (B) SW1353 cells were transfected with si-HDAC4 or si-NC, and the protein levels of MMP3 and MMP-13 were assayed using Western blotting. (C) Relative mRNA expression of HDAC4 after overexpression plasmid transfection. *P < 0.01 compared with si-NC group. (D) Protein levels of MMP3 and MMP13 after transfection of the HDAC4 overexpression plasmid were assayed using Western blotting. (E) HDAC4 was overexpressed in SW1353 cells and IL-1β was added and incubated for 24 h. Protein levels of MMP3 and MMP-13 were assayed using Western blotting. (F) SW1353 cells transfected with si-HDAC4 or si-NC were treated with IL-1β for 24 h and the cells were collected for Western blotting analysis of MMP3 and MMP-13 levels. qRT-PCR: Real-time quantitative polymerase chain reaction; si/siRNA: Small interfering RNA; NC: Negative control.
**WNT3A downregulated HDAC4 expression in SW1353 cells**

To determine the expression patterns of HDAC4 induced by WNT3A, SW1353 cells were treated with purified WNT3A protein for different time periods and at different concentrations. As shown in Figure 4A and 4B, HDAC4 levels were downregulated in a time- and dose-dependent manner; however, the induction of MMP-3 and MMP-13 by WNT3A was significantly upregulated, according to the results of the RT-PCR and Western blotting.

**Glycogen synthase kinase 3 beta (GSK3β) rescued IL-1β-induced downregulation of HDAC4 in SW1353 cells**

To investigate and validate the role of WNT/β-catenin signaling in the expression of HDAC4, we transfected

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**Figure 3:** The WNT3A/β-catenin pathway was activated by IL-1β. (A) Levels of WNT3A in the SW1353 cells treated with IL-1β (10 ng/mL) at different times. (B) SW1353 cells were treated with IL-1β (10 ng/mL), and protein levels of β-catenin and phosphorylated β-catenin were assayed using Western blotting. (C) SW1353 cells were treated with IL-1β (10 ng/mL), and the localization of β-catenin was observed using immunofluorescence. WNT3A: WNT family member 3A.

**Figure 4:** The regulatory mechanism of WNT3A/β-catenin on HDAC4. (A) Levels of HDAC4 in SW1353 cells treated with WNT3A at different concentrations. (B) Levels of HDAC4 in SW1353 cells treated with WNT3A (200 ng/mL) for the indicated time periods. HDAC4 levels were determined using Western blotting. (C) qRT-PCR analyses were used to evaluate the efficiency of GSK3β overexpression. *P* < 0.01 compared with si-NC group. (D) Protein levels of HDAC4 were assayed using Western blotting after transfection of the GSK3β overexpression plasmid. GSK3β: Glycogen synthase kinase 3 beta.
SW1353 cells with a GSK3β overexpression vector; GSK3β is a major inhibitory factor involved in the WNT/β-catenin signal pathway. The transfection efficiency was confirmed using qRT-PCR [Figure 4C]. The Western blotting results showed that the levels of HDAC4 were markedly reduced after treatment with IL-1β, but were restored after GSK3β overexpression in SW1353 cells [Figure 4D].

**Discussion**

A clear understanding of the etiology and molecular pathogenesis of OA is necessary to develop optimal treatments for this disease. Epigenetics has attracted significant research attention in the study of OA pathogenesis and treatment. Several HDACs were identified as important regulators of cartilage development and degradation. In a previous study, HDAC4 was shown to repress chondrocyte hypertrophy and endochondral bone formation. Decreased HDAC4 contributed to the pathogenesis of cartilage degeneration. However, high expression of HDAC4 has also been reported in OA chondrocytes, which implicated HDAC4 in promoting the catabolic activity of chondrocytes. Conflicting expression patterns of HDAC4 observed in OA chondrocytes could be due to changes in HDAC expression at different stages of OA. However, the exact role of HDAC4 in OA was unclear. In the present study, we aimed to determine the role of HDAC4, a histone deacetylase involved in IL-1β-induced MMPs expression, in OA, and its regulatory mechanism. IL-1β is a critical mediator of OA progression; therefore, an in vitro OA model was constructed by stimulating rat condylar chondrocytes and SW1353 cells with IL-1β, which caused upregulation of MMP3 and MMP-13 levels. The same expression patterns are observed during OA. We found that the HDAC4 level was gradually downregulated after IL-1β treatment in a dose and time-dependent manner, indicating that HDAC4 is associated with cartilage degradation during OA.

MMPs, a family of proteolytic enzymes, are prominently involved in the breakdown of the ECM in OA. Increased MMP expression is closely related to OA progression. MMP-3 and MMP-13 play a crucial role in the progression of OA by degrading components of the ECM, such as type II and type IV collagen. Inhibition of IL-1β-mediated inflammation could be an effective therapeutic strategy for symptomatic relief and structural repair in OA. Inhibition of MMP-3 and MMP-13 expression might be related with chondro-protective effects. Clinical trials of the use of MMP inhibitors as a disease-modifying treatment have been unsuccessful because of severe side effects and inefficient MMP inhibition. In the present study, we examined the effects of HDAC4 knockdown and overexpression in IL-1β-induced cells. Knockdown of HDAC4 exerted an enhanced catabolic effect by upregulating MMP-3 and MMP-13 levels. Furthermore, HDAC4 overexpression exerted an anti-catabolic effect by downregulating the levels of MMP-3 and MMP-13 in IL-1β-stimulated cells. Thus, silencing of HDAC4 could cause metabolic imbalance in the ECM and overexpression of HDAC4 could have anti-inflammatory effect in IL-1β-induced cells.

The WNT/β-catenin signaling pathway participates in a series of cellular events, including cell differentiation, proliferation, migration, and cartilage homeostasis. Many studies have shown the important role of WNT/β-catenin signaling in the pathogenesis of OA. In the present study, the relationship between HDAC4 and WNT/β-catenin signaling was investigated. The WNT/β-catenin signaling pathway was activated by stimulation WNT3A, a well-known activator of canonical WNT/β-catenin signaling, which significantly decreased the level of HDAC4. At the same time, cells treated with the WNT3A showed significantly higher levels of MMP-3 and MMP-13, indicating that HDAC4 was associated with the WNT/β-catenin pathway. In addition, the WNT/β-catenin signaling was inhibited by overexpression of GSK3β, which rescued IL-1β-induced downregulation of HDAC4 in SW1353 cells. These data suggested that the effects of HDAC4 in IL-1β-induced MMPs expression were associated with the WNT/β-catenin signaling pathway, indicating that inhibiting the WNT/β-catenin pathway might have an anti-inflammatory effect partially by regulating HDAC4.

In the growth plate, HDAC4 is a negative regulator of chondrocyte hypertrophy. However, the role of HDAC4 during OA cartilage degeneration and its mechanism is unclear. Our results provided an insight into the role of HDAC4 in an in vitro OA model, and revealed an association of HDAC4 with the WNT/β-catenin signaling pathway. Future studies are necessary to investigate the mechanism of action of HDAC4 on OA-related genes. In addition, the relationship between HDAC4 and other signaling pathways also merits further investigation.

In summary, our results suggest that decreased HDAC4 is at least partially responsible for the upregulation of OA-related genes, such as MMP3 and MMP13, in vitro, and HDAC4 upregulation might downregulate MMP3 and MMP13, indicating that HDAC4 exerts a protective effect on IL-1β-induced ECM degradation and is regulated partially by the WNT3A/β-catenin signaling pathway [Figure 5]. The exact role of HDAC4 in the progression of OA will be confirmed in future animal experiments.

**Figure 5**: A schematic of involved molecular connections in the TMJOA development. TMJOA: Temporomandibular joint osteoarthritis; IL-1β: Interleukin 1 beta; WNT3A: WNT family member 3A; HDAC4: Histone deacetylase 4; GSK3β: Glycogen synthase kinase 3 beta; MMP: Matrix metalloproteinase.
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