Regulation of Type II Collagen Expression by Histone Deacetylase in Articular Chondrocytes*

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Histone deacetylase (HDAC) regulates various cellular processes by modulating gene expression. Here, we investigated the role of HDAC in the expression of type II collagen, a marker of differentiated chondrocytes. We found that HDAC activity in primary articular chondrocytes decreases during dedifferentiation induced by serial monolayer culture and that the activity recovered during redifferentiation induced by three-dimensional culture in a cell pellet. Inhibition of HDAC with trichostatin A or PXD101 was sufficient to block type II collagen expression in primary culture chondrocytes. HDAC inhibition also blocked the redifferentiation of dedifferentiated chondrocytes by suppressing the synthesis and accumulation of type II collagen. HDAC inhibition promoted the expression of Wnt-5a, which is known to inhibit type II collagen expression, and knockdown of Wnt-5a blocked the ability of HDAC inhibitors to suppress type II collagen expression. In addition, the induction of Wnt-5a expression by HDAC inhibitors was associated with acetylation of the Wnt-5a promoter. Taken together, our results suggest that HDAC promotes type II collagen expression by suppressing the transcription of Wnt-5a.

Differentiation of uncommitted mesenchymal cells into chondrocytes is initiated by the proliferation and recruitment of chondrogenic mesenchymal cells into condensations. These precartilage condensations develop into cartilage nodules containing differentiated chondrocytes. Chondrogenesis can be mimicked by micromass culture of mesenchymal cells in vitro. Differentiated chondrocytes are characterized by the ability to synthesize cartilage-specific extracellular matrix molecules including type II collagen and sulfated proteoglycans, which are necessary for normal cartilage development (1, 2). The activity of cartilage-specific matrix molecule synthesis by the differentiated chondrocytes is unstable and rapidly lost in response to certain environmental changes. For instance, proinflammatory cytokines such as interleukin (IL)$^2$-1β cause the loss of differentiated chondrocyte phenotypes (dedifferentiation) by halting the synthesis of cartilage-specific matrix molecules (3, 4). Chondrocyte dedifferentiation is also induced by serial monolayer culture and is accompanied by a gradual shift in the expression of type II collagen and aggrecan to type I and III collagen and versican, respectively (5, 6). Dedifferentiated chondrocytes redifferentiate in three-dimensional culture in an alginate gel or cell pellet (6, 7).

Although maintenance of a differentiated chondrocyte phenotype is important for cartilage homeostasis, the detailed mechanisms of the differentiation and dedifferentiation of chondrocytes remain largely unknown. Because chondrocyte differentiation/dedifferentiation is regulated by various soluble factors including growth factors and cytokines, we hypothesized that histone deacetylase (HDAC) may regulate the differentiation status of chondrocytes. HDAC modulates the growth and differentiation of various cell types by governing chromatin structure and repressing the activity of specific transcription factors (8, 9). The major function of HDAC is to remove acetyl groups from histones, which causes the condensation of the chromatin structure. For example, HDAC can reverse the acetylation of histone H3 on Lys$^2$ and Lys$^14$ and histone H4 on Lys$^8$ and Lys$^{12}$, which are associated with transcriptional activation (10). Chromatin condensation inhibits the access of transcription factors to the promoter regions of specific target genes and ultimately leads to transcriptional repression.

The specific roles of various HDAC family members have been investigated by gene deletion experiments. For instance, knock-out of HDAC4 in mice causes premature ossification of developing bones due to an early onset of chondrocyte hypertrophy (11, 12). Also, HDAC1 is required for the normal formation of the craniofacial cartilage of zebrafish (13). Among the growth factors, leukemia/lymphoma-related factor is known to interact with HDAC1, inhibiting the expression of the cartilage oligomeric matrix protein gene and the differentiation of chondrocytes (14). Furthermore, HDAC4 and 5 are required for the inhibition of Runx2 by transforming growth factor β and are involved in osteoblast differentiation (15). In addition, inhibiting HDAC suppresses the enhancement of chondrogenesis by inhibitors of retinoic acid receptor-mediated signaling in primary cultures of mouse limb mesenchyme (16).
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The above findings suggest that HDAC regulates the differentiated phenotypes of chondrocytes by promoting the expression of cartilage-specific matrix molecules, but the evidence so far is indirect. Therefore, in the current study, we examined the role of HDAC in the expression of type II collagen by primary cultures of chondrocytes. We report that HDAC activity is required for the expression of type II collagen, a marker of differentiated chondrocytes. We also show evidence that HDAC acts by blocking the expression of Wnt-5a.

EXPERIMENTAL PROCEDURES

Cell Culture—Mesenchymal cells were derived from the distal tips of E11.5 mouse embryo limb buds. The cells were suspended at a density of 2.0×10⁵ cells/ml in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) containing 10% (v/v) fetal bovine serum, 50 μg/ml streptomycin, and 50 units/ml penicillin. Chondrogenesis was induced by placing the cells in 15-μl drops in culture dishes and incubating for 5 day (17). Articular chondrocytes were isolated from the knee cartilage of 2–3-week-old New Zealand White rabbits (6). Briefly, cartilage slices were digested enzymatically in 0.2% collagenase type II, and individual cells were grown in complete DMEM. To induce dedifferentiation, passage (P) 0 chondrocytes were serially subcultured up to P4 by plating cells each time at a density of 3×10⁴ cells/cm². Dedifferentiation of dedifferentiated chondrocytes was induced by three-dimensional culture in a cell pellet as described previously (6). Briefly, a suspension (1 ml) of dedifferentiated cells (2×10⁵ cells) in complete DMEM was sedimented by brief centrifugation. Cell pellets were incubated up to 6 day to cause redifferentiation. The differentiation status of chondrocytes was determined by examining the expression of type II collagen. Chondrogenic explants were obtained from rabbit knee joints by cutting them into 32-mm³ pieces and then culturing the explants for 72 h in complete DMEM with or without 0.3 μM trichostatin A (TSA) with daily changes of the culture medium.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)—Total RNA was isolated using RNA STAT-60 (Tel-Test Inc., Friendswoods, TX) and reverse-transcribed with ImProm-II™ reverse transcriptase (Promega, Madison, WI). The resulting cDNA was amplified by PCR using primers 5'-GGG TCT CCT GCC TCC TGC TC-3' (sense) and 5'-CTC CAT CTC TGC TGC CAC GGG GT-3' (antisense), and the PCR product was inserted into the pSPT-18 vector. For Wnt-5a, the full-length cDNA encoding rabbit Wnt-5a was cut with PstI and SalI, and the product was inserted into the pSPT-18 vector. The cloned pSPT-18 plasmids containing type II collagen or the Wnt-5a fragment were linearized with EcoRI or HindIII and transcribed with SP6 and T7 RNA polymerases (Roche Diagnostics), respectively. The resulting transcripts were used to synthesize digoxigenin-conjugated antisense and sense riboprobes. For hybridization, sections of cartilage explants were treated with 0.2 N HCl for 10 min and then permeabilized with 20 μg/ml protease K for 10 min at 37 °C. After acetylation with 0.25% acetic anhydride for 10 min, the sections were incubated with hybridization buffer (40% formamide, 10% dextran sulfate, 1× Denhardt’s solution, 4× SSC, 10 μM dithiothreitol, 1 mg/ml yeast tRNA, and 1 mg/ml salmon sperm DNA) containing denatured sense or antisense digoxigenin-labeled riboprobes. After hybridization overnight at 42 °C, the sections were washed, and nonspecifically bound riboprobes were eliminated by digestion with RNase. The sections were incubated overnight at 4 °C with alkaline phosphatase-conjugated mouse anti-digoxigenin antibody, and hybridization signals were visualized with a solution of 4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-iodolyl-phosphate (Roche Diagnostics).

Western Blot Analysis—Whole-cell lysates were prepared as described previously (6). Proteins were size-fractionated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The nitrocellulose membrane was blocked with 3% nonfat dry milk in Tris-buffered saline and incubated with antibodies against type II collagen, acetylated histone H4 (K8; Upstate Biotechnology) or extracellular signal-regulated protein kinase (Pharmingen). The blots were developed using a peroxidase-conjugated secondary antibody, followed by ECL reagents (Amersham Biosciences, Buckinghamshire, UK).

HDAC Activity Assay—HDAC activity was determined using nuclear extracts, which were prepared by homogenizing chondrocytes in lysis buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1
mm EDTA, 0.1 mm EGTA, 1 mm dithiothreitol) supplemented with protease inhibitors (10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, and 1 mm 4-(2-aminoethyl) benzene-sulfonamide) and phosphatase inhibitors (1 mm NaF and 1 mm Na₂VO₄). Following addition of 0.6% (v/v) Nonidet P-40, the cells were incubated for 15 min on ice and centrifuged at 13,000 × g for 30 s at 4 °C. The nuclear pellets were resuspended in nuclear extraction buffer B (20 mm HEPES, pH 7.9, 0.4 mm NaCl, 1 mm EDTA, 1 mm EGTA, and 1 mm dithiothreitol) containing protease and phosphatase inhibitors and incubated on ice for 15 min. Following centrifugation at 13,000 × g for 5 min at 4 °C, the supernatant (nuclear fraction) was collected and used for the HDAC activity assay. HDAC activity was determined fluorimetrically using a Fluor de Lys HDAC assay system (Biomol, Plymouth Meeting, PA) with a YM-10 membrane (10-kDa molecular mass cutoff) as described by Ryu and Chun (19).

Preparation of Wnt-5a-conditioned Media—Wnt-5a-producing mouse fibroblast L929 cells were obtained from the American Type Culture Collection (Manassas, VA). Conditioned medium was collected from Wnt-5a-producing L929 cells after 36 h in serum-free DMEM. The conditioned medium was clarified by centrifugation at 10,000 × g for 5 min and concentrated 20-fold by ultrafiltration in an Amicon stirred cell (Millipore, Billerica, MA) with a YM-10 membrane (10-kDa molecular mass cutoff) as described by Ryu and Chun (19).

 Knockdown of Wnt-5a by siRNA—A 21-mer siRNA for Wnt-5a was designed based on the coding sequence of rabbit Wnt-5a (GenBank™ accession number DQ388600). The following siRNA oligonucleotides were synthesized by Samchully Pharm (Daejeon, Korea): 5′-UAA UGC CUU UGG CCG UAU

| Gene               | Primer sequences                  | Size/°C | Origin  |
|--------------------|----------------------------------|---------|---------|
| Collagen-II        | 5′-GAC CCC ATG CAQ TAC ATG CG-3′  | 370     | Human   |
| Collagen-II        | 5′-AGG GCC CXT TOA TCG TGC TCT-3′ | 204     | Mouse   |
| Collagen-XI        | 5′-TTTCCCTCAATCAACCCTG-3′        | 356     | Mouse   |
| Chondromodulin-I   | 5′-ACATGACCAAGCAGGAGCCACC-3′     | 504     | Human   |
| HDAC-1             | 5′-TACTGACTACAGGACCAGAT-3′       | 330     | Human   |
| HDAC-3             | 5′-CCGCGCTCCATACAGAATCC-3′       | 390     | Mouse   |
| HDAC-6             | 5′-CGTAAATAGAAGATCAAGACA-3′      | 339     | Human   |
| HDAC-7a            | 5′-GAGGAAAGCAAGCAGGAAGAT-3′      | 438     | Human   |
| HDAC-11            | 5′-TGGAGAATGACAGCAAACTATCA-3′    | 542     | Human   |
| Wnt-5a             | 5′-ACGCTGCAATGGGCTCTACGAGA-3′    | 476     | Human   |
| β-Catenin          | 5′-AAGATACGACACCAATGAAACGA-3′    | 454     | Mouse   |
| Plakoglobin        | 5′-GCCGCCATCGGCGAGTACCCAT-3′     | 380     | Human   |
| c-Jun              | 5′-AGGTGAGCGCATGACGTAGCTTAC-3′   | 251     | Human   |
| p21                | 5′-CCTTCCTAATGATATCCGATTA-3′     | 587     | Mouse   |
| MMP-13             | 5′-TGAGCTACATCTGGTGTTATTCG-3′    | 573     | Human   |
| GAPDH              | 5′-CAGCAGCTGCAAGAAGGCAGA-3′      | 299     | Rabbit  |
| GAPDH              | 5′-CACCTGACAAATCAGAAGCCAG-3′     | 450     | Mouse   |
| Wnt-5a (A)         | 5′-AGGTTGACCAAGGAGGAGGA-3′       | 645     | Mouse   |
| ~ChIP              | 5′-CTCCCTTGGGGAGGAAGGA-3′        | 167     | Mouse   |
| Wnt-5a (B)         | 5′-AGGTTGACCAAGGAGGAGGA-3′       | 449     | Mouse   |
| ~ChIP              | 5′-AGGTTGACCAAGGAGGAGGA-3′       | 131     | Mouse   |
| β-actin            | 5′-GGGAGGTGTCAGGAGGAGGA-3′       | 229     | Mouse   |

TABLE 1 Oligonucleotide primers and PCR conditions

| Gene        | Primer sequences | Size | Origin |
|-------------|------------------|------|--------|
| Collagen-2  | 5′-GAC CCC ATG CAQ TAC ATG CG-3′ | 370 | Human |
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| HDAC-3      | 5′-CCGCGCTCCATACAGAATCC-3′       | 390 | Mouse |
| HDAC-6      | 5′-CGTAAATAGAAGATCAAGACA-3′      | 339 | Human |
| HDAC-7a     | 5′-GAGGAAAGCAAGCAGGAAGAT-3′      | 438 | Human |
| HDAC-11     | 5′-TGGAGAATGACAGCAAACTATCA-3′    | 542 | Human |
| Wnt-5a      | 5′-ACGCTGCAATGGGCTCTACGAGA-3′    | 476 | Human |
| β-Catenin   | 5′-AAGATACGACACCAATGAAACGA-3′    | 454 | Mouse |
| Plakoglobin | 5′-GCCGCCATCGGCGAGTACCCAT-3′     | 380 | Human |
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| Wnt-5a (B)  | 5′-AGGTTGACCAAGGAGGAGGA-3′       | 449 | Mouse |
| ~ChIP       | 5′-AGGTTGACCAAGGAGGAGGA-3′       | 131 | Mouse |
| β-actin     | 5′-GGGAGGTGTCAGGAGGAGGA-3′       | 229 | Mouse |
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A  

| Passage | Coll-II | GAPDH | RT-PCR |
|---------|---------|-------|--------|
| 0       |         |       |        |
| 1       |         |       |        |
| 2       |         |       |        |
| 3       |         |       |        |
| 4       |         |       |        |

B

![Graph A](image1)

C

![Graph B](image2)

D

![Graph C](image3)

FIGURE 1. Differentiation status-dependent HDAC activity in articular chondrocytes. A and B, rabbit articular chondrocytes were serially subcultured up to P4. Type II collagen expression was determined by RT-PCR and Western blotting (A). HDAC activity was determined using the Fluor de Lys HDAC assay system (B). C and D, chondrocytes were maintained as monolayer (lane m) at P0 or P2. Alternatively, dedifferentiated P2 cells were maintained as pellet culture (lane p) for 6 days to induce redifferentiation. Expression of type II collagen was determined by RT-PCR and Western blot analysis (C). HDAC activity was quantified using the Fluor de Lys HDAC assay system (D). The data represent the results of a typical experiment (A and C) or average values with standard deviations (B and D) from more than four independent experiments. Coll-II, type II collagen; ERK, extracellular signal-regulated kinase.

UTT-3’ (sense) and 5’-AAU ACG GCC AAA GCC AUU ATT-3’ (antisense). After heating the siRNA oligonucleotides for 2 min at 90 °C to denature secondary structures within single strands, the sense and antisense strands were allowed to anneal at 30 °C for 1 h. Chondrocytes were then transfected with the siRNA duplex using Lipofectamine 2000 (Invitrogen).

Chromatin Immunoprecipitation (ChIP) Assay—ChIP assays were performed using a commercial ChIP assay kit (Upstate Biotechnology) and chondrocytes differentiated from mesenchymal cells. Mouse mesenchymal cells derived from the distal tips of E11.5 mouse embryo limb buds were maintained as a micromass culture to induce chondrogenic differentiation in the absence or presence of 20 nM TSA or 200 nM PXD101. The cells at day 5 were cross-linked with 1% formaldehyde for 10 min, suspended in 200 μl SDS lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris-HCl, pH 8.0), and incubated for 10 min on ice. The lysates were sonicated at 25% amplitude for five 10-s cycles using a Digital Sonifier 450 (Branson Ultrasonics, Danbury, CT), with cooling on ice for 1 min between each cycle. The samples were centrifuged, and the supernatant was diluted 10-fold with ChIP dilution buffer (Upstate Biotechnology). After preclearing with anti-rabbit IgG and 40 μl of salmon sperm DNA/protein A-agarose for 1 h at 4°C, chromatin was incubated with 3 μg anti-acetylhistone H4 antibody or control IgG in a total volume of 2 ml for 12 h at 4°C. Antibody-protein-DNA complexes were isolated by immunoprecipitation with 60 μl of salmon sperm DNA/protein A-agarose. After extensive washing, the pellets were treated with freshly prepared elution buffer (1% SDS and 0.1 M NaHCO₃), and the eluted fraction was mixed with 20 μl of 5 M NaCl and heated for 4 h at 65 °C. DNA was purified by phenol extraction and used as a template for PCR. The PCR primers for the ChIP assay were designed to correspond to the promoter regions of Wnt-5a, matrix metalloproteinase (MMP)-13, p21, and β-actin (Table 1).

RESULTS

HDAC Activity in Chondrocytes Depends on the Differentiation Status—To investigate the role of HDAC in the maintenance of differentiated chondrocyte phenotypes, we first examined HDAC activities during the dedifferentiation and redifferentiation of articular chondrocytes. Primary chondrocytes were serially subcultured as a monolayer to induce their dedifferentiation. Dedifferentiation was confirmed by a decrease in type II collagen expression (Fig. 1A). HDAC activity was decreased in a similar pattern as type II collagen expression during subculture-induced dedifferentiation (Fig. 1B). The decrease in HDAC activity by dedifferentiation was reversed during the redifferentiation of chondrocytes (Fig. 1D) caused by three-dimensional culture in a cell pellet (Fig. 1C). These results suggest that HDAC activity in chondrocytes is connected to the differentiation status.

Inhibition of HDAC Activity Blocks Type II Collagen Expression in Articular Chondrocyte—To determine whether HDAC activity is associated with maintenance of differentiated phenotypes in chondrocytes, we examined the role of HDAC in type II collagen expression, a marker of differentiated chondrocytes. For this purpose, we used two HDAC inhibitors, TSA (21) and PXD101 (22). Both inhibitors have a broad spectrum of activity against class I, II, and IV HDACs, with little specificity for individual HDAC isoforms (23). Both TSA and PXD101 induced the time- and dose-dependent hyperacetylation of histone H4 in chondrocytes (Fig. 2A and 2C). A fluorimetric HDAC activity assay revealed that these two HDAC inhibitors effectively inhibited HDAC activity (Fig. 2, B and D) without significantly affecting cell viability or proliferation (Fig. 2E).

Inhibition of HDAC by TSA or PXD101 dramatically reduced the number of type II collagen-expressing chondrocytes, as determined by immunofluorescence (Fig. 3C). The inhibitory effects of TSA or PXD101 on type II collagen expression were dose- and time-dependent as determined by RT-PCR, qRT-PCR, and Western blot analysis (Fig. 3, A and B). In contrast to the inhibitory effects of HDAC inhibitors on type II collagen expression, TSA (Fig. 3D) and PXD101 (data not shown) did not affect expression of other chondrogenic molecules such as type XI collagen (24) and chondromodulin-I (25). Expression of plakoglobin, a known HDAC target gene (26), was increased by HDAC inhibition, whereas β-catenin and c-Jun were detected as negative controls.
The results, therefore, indicate that regulation of type II collagen expression by HDAC is specific in chondrocytes. We also examined the effects of the HDAC inhibitors in cartilage explants, which more accurately reflect in vivo conditions. As in primary chondrocytes, TSA and PXD101 suppressed type II collagen expression and induced the hyperacetylation of histone H4 in cartilage explants. Furthermore, inhibition of HDAC by TSA or PXD101 blocked both the expression and accumulation of type II collagen during re-differentiation of dedifferentiated chondrocytes in three-dimensional culture. Collectively, these results indicate that HDAC activity is required for type II collagen expression in chondrocytes.

We next examined which isoform(s) of HDAC is involved in the regulation of type II collagen expression. As shown in Fig. 5A and 5B, the decrease and recovery of HDAC activity during dedifferentiation and re-differentiation, respectively, did not accompany significant changes in the expression of HDAC-1, -6, or -11. We could not detect HDAC-3 and -7a in rabbit articular chondrocytes (data not shown). In addition, overexpression of HDAC-1, -6, or -11 in dedifferentiating P1 chondrocytes did not modulate type II collagen expression (Fig. 5C), suggesting that these HDAC isoforms are not associated with type II collagen expression.

**FIGURE 2. Inhibition of HDAC by TSA or PXD101 in articular chondrocytes.** A and B, chondrocytes were treated with 0.3 μM TSA or 10 μM PXD101 for the indicated times. Acetylation of histone H4 was determined by Western blot analysis (A), and HDAC activity was quantified using the Fluor de Lys HDAC assay system (B). C and D, chondrocytes were treated with the indicated concentrations of TSA or PXD101 for 3 h. Acetylation of histone H4 was determined by Western blot analysis (C), and HDAC activity was quantified using the Fluor de Lys HDAC assay system (D). E, the effect of TSA or PXD101 on cell proliferation and viability was assessed by counting the cell number with a hemocytometer following staining with 0.4% trypan blue. The data represent the results of a typical experiment or average values with standard deviations from four independent experiments. PXD, PXD101; ERK, extracellular signal-regulated kinase.

**FIGURE 3. HDAC inhibition suppresses type II collagen expression.** A and B, chondrocytes were treated with the indicated concentrations of TSA or PXD101 for 24 h (left panels) or with 0.3 μM TSA or 10 μM PXD101 for the indicated times (right panels). Protein and mRNA levels for type II collagen were determined by Western blotting and RT-PCR, respectively (upper panels). The relative expression of type II collagen was determined by qRT-PCR (lower panels). C, chondrocytes were untreated (control) or treated with 0.3 μM TSA or 10 μM PXD101 for 24 h and stained for type II collagen. Nuclei were stained with Hoechst dye. D, chondrocytes were treated with the indicated concentrations of TSA for 24 h. Expression levels of cartilage extracellular matrix molecules (type II collagen, type XI collagen, chondromodulin-I) were determined by RT-PCR. p21 was detected as a target gene of HDAC, whereas β-catenin and c-Jun were detected as negative controls. The data represent the results of a typical experiment or average values with standard deviations from four independent experiments. Coll, collagen; ERK, extracellular signal-regulated kinase; PXD, PXD101.
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FIGURE 4. HDAC inhibition suppresses type II collagen expression in cartilage explants and inhibits the redifferentiation of dedifferentiated chondrocytes. A, cartilage explants were untreated (control) or treated with 0.3 μM TSA for 72 h. The level of the type II collagen transcript was detected by in situ hybridization. Acetylated histone H4 was detected by immunohistochemistry using a Dako LSAB-horseradish peroxidase system. B and C, chondrocytes were subcultured up to P2 as a monolayer (m) or P2 as pellets (p). Differentiated P2 cells were cultured three-dimensionally in cell pellets (p) for 3 or 6 day in the absence or presence of 0.3 μM TSA. The level of type II collagen expression was determined by RT-PCR and Western blotting (B). The accumulation of type II collagen and acetylation of histone H4 in the three-dimensional culture were detected by immunohistochemistry (C). The data represent typical results from four independent experiments. Coll, collagen; ERK, extracellular signal-regulated kinase; WB, Western blot.

FIGURE 5. Expression of HDAC isoforms in articular chondrocytes. A and B, rabbit articular chondrocytes were serially subcultured up to P4 (A). Alternatively, chondrocytes were maintained as monolayer (lane m) at P0 or P2 and dedifferentiated P2 cells were maintained as pellet culture (lane p) for 6 day (B). Expression levels of type II collagen and HDAC isoforms were determined by RT-PCR. C, P1 cells were transfected with HDAC-1, -6, or -11 expression vectors (0.5, 1, and 1.5 μg) and expression levels of type II collagen and HDAC isoforms were determined by RT-PCR. The data represent the results of a typical experiment from more than five independent experiments. Coll, collagen.

transcription, it is unlikely that the collagen gene is a direct target gene of HDAC. Indeed, we previously found that expression of type II collagen in chondrocytes is suppressed by IL-1β via the up-regulation of Wnt-5a (19) and Wnt-7a (27). In contrast, Wnt-11, which is down-regulated by IL-1β, enhances type II collagen expression (19). Therefore, to investigate how type II collagen expression is suppressed by HDAC inhibition, we examined the effect of HDAC inhibitors on the expression of Wnt isoforms. As shown in Fig. 6, TSA and PXD101 up-regulated the expression of Wnt-5a in both primary chondrocytes (Fig. 6, A and B) and cartilage explants (Fig. 6C) as determined by RT-PCR, qRT-PCR, and in situ hybridization. These HDAC inhibitors, however, did not affect the expression of other examined Wnt isoforms including Wnt-7a and -11 (data not shown).

We next examined whether the up-regulation Wnt-5a is involved in the suppression of type II collagen expression by HDAC inhibitors. As reported previously (19), Wnt-5a-conditioned medium inhibited type II collagen expression both at the transcript and protein levels in articular chondrocytes (Fig. 7A). We further examined the role of Wnt-5a up-regulation in type II collagen expression by knocking down Wnt-5a expression using a 21-mer siRNA. The siRNA effectively and dose-dependently blocked the up-regulation of Wnt-5a by TSA or PXD101 and resulted in a concomitant recovery of type II collagen expression as determined by RT-PCR, qRT-PCR, and Western blot analysis (Fig. 7, B and C). These results support the idea that up-regulation of Wnt-5a is linked to the suppression of type II collagen expression by HDAC inhibitors.

Finally, we examined whether Wnt-5a expression is directly regulated by HDAC in chondrocytes to confirm hypothesis that HDAC acts as a bridge between gene expression of Wnt-5a and type II collagen. HDAC inhibition promotes target gene expression by causing the hyperacetylation of promoter sequences, leading to the recruitment of transcriptional complexes. We therefore performed a ChIP assay using an anti-acetyl-histone H4 antibody to determine whether the promoter of Wnt-5a is associated with acetylated histone H4. For these experiments, we used mouse chondrocytes differentiated from mesenchymal cells by micromass culture rather than rabbit articular chondrocytes because the promoter sequence of mouse Wnt-5a is known. Chondrogenesis was induced in the mouse cells by growing them in micromass culture (Fig. 8A, upper panel). HDAC inhibitors blocked type II collagen expression and increased acetylation of histone H4 in differentiated chondrocytes (Fig. 8A, middle and lower panels). HDAC inhibition also caused up-regulation of Wnt-5a as well as a known HDAC target gene p21 (28) (Fig. 8B). Immunoprecipitation of acetylated histone H4 from cells treated with TSA or PXD101 coprecipitated DNA fragment containing the Wnt-5a promoter (Fig. 8C). In these experiments, β-actin was used as a positive control to determine whether same amount of DNA was used in each ChIP assay. In addition, MMP-13 was used as a negative control, and we did not detect it in the absence or presence of TSA (Fig. 8C). Taken together, these results suggest that HDAC directly regulates the transcription of Wnt-5a.

DISCUSSION

HDAC Regulates the Growth and Differentiation of Various Cell Types by Modifying Chromatin Structure and the Activity of Transcription Factors (8, 9). For instance, HDAC3 regulates osteoblast differentiation by repressing osteocalcin expression (29), and the down-regulation of HDAC stimulates adipocyte
differentiation (30). Although HDAC is well known to regulate various cellular processes, the function of HDAC in chondrocytes is largely unknown. Here, we investigated the role of HDAC in the maintenance of chondrocyte differentiation by analyzing the expression of type II collagen. We found that HDAC activity is dependent on the differentiation status: the activity decreases during dedifferentiation and recovers during redifferentiation. The changes in HDAC activity during the de- and re-differentiation of chondrocytes appear to be connected at the post-transcriptional level because the expression of HDAC-1, -6, and -11 do not change during de- and re-differentiation; however, we cannot rule out the possibility that there were changes in the expression of other HDAC isoforms that we were unable to detect due to the use of primers based on human sequences. In addition, our overexpression experiments indicated that HDAC-1, -6, and -11 did not modulate type II collagen expression. Although this indicate that overexpression of individual HDAC-1, -6, or -11 did not affect type II collagen expression, it is possible that unexamined HDAC isoforms or combination of multiple

**FIGURE 6.** Inhibition of HDAC promotes Wnt-5a expression in articular chondrocytes and cartilage explants. A and B, chondrocytes were treated with 0.3 μM TSA or 10 μM PXD101 for the indicated times (A) or with the indicated concentrations for 24 h (B). The level of Wnt-5a transcript was determined by RT-PCR and quantified by qRT-PCR. GAPDH was used as a reference. C, cartilage explants were untreated (control) or treated with 0.3 μM TSA for 72 h. The presence of transcripts for type II collagen and Wnt-5a was examined by in situ hybridization. The data represent typical results from five independent experiments. PXD, PXD101; Coll, collagen.

**FIGURE 7.** Knockdown of Wnt-5a prevents the suppression of type II collagen expression by HDAC inhibitors. A, chondrocytes were treated with 75 μl of Wnt-5a-conditioned medium for the indicated times (upper panel) or with the indicated amounts of control or Wnt-5a-conditioned medium for 36 h (lower panel). Expression of type II collagen was determined by RT-PCR and Western blotting. B and C, cells were transfected with a 21-mer siRNA targeting rabbit Wnt-5a. Transfected cells were treated without or with 0.3 μM TSA (B) or 10 μM PXD101 (C) for an additional 24 h. The expression of Wnt-5a and type II collagen was determined by RT-PCR and Western blot analysis (upper panels). The relative expression of type II collagen was determined by qRT-PCR (lower panels). The data represent typical results from five independent experiments. Coll, collagen; ERK, extracellular signal-regulated kinase; PXD, PXD101; WB, Western blot.
isofoms may regulate type II collagen expression. Therefore, which HDAC isof orm(s) are involved in the expression of type II collagen remains to be further determined.

The inhibitory effects of HDAC inhibition on chondrogenic gene expression appears to specific to type II collagen expression. Among the examined chondrogenic genes, HDAC inhibitors blocked type II collagen expression but did not modulate expressions of type XI collagen and chondromodulin-I (Fig. 3D) and type I and III collagens and aggregan (data not shown). In addition, HDAC inhibitors caused up-regulation of known target genes such as plakoglobin and p21, whereas expressions of other genes such as β-catenin, c-Jun, and GAPDH (Fig. 3D) and Wnt-7a and Wnt-11 (data not shown) are not affected by HDAC inhibitors. This clearly suggests that expressions of type II collagen and Wnt-5a are not nonspecifically regulated by HDAC inhibitors.

A role of HDAC in type II collagen expression was clearly demonstrated by the fact that the HDAC inhibitors TSA and PXD101 suppressed type II collagen expression both in primary cultures and cartilage explants. The involvement of HDAC in cartilage development was originally suggested by reports that a knockdown of HDAC1 disrupts normal cartilage development in zebrafish (13) and that HDAC4 regulates hypertrophic maturation of chondrocytes during skeletal development (11, 12). It has also been shown that HDAC inhibitors block the enhancement of chondrogenesis in mesenchymal cells by inhibition of retinoic acid receptor signaling (16). Our study, however, is the first to provide evidence that HDAC activity regulates type II collagen expression and maintenance of differentiation in articular chondrocytes.

Dedifferentiation of chondrocytes is believed to participate in cartilage destruction by causing an imbalance in the synthesis and degradation of cartilage matrix molecules (31, 32). Therefore, the suppression of type II collagen expression by HDAC inhibitors suggests that HDAC activity is required for the maintenance of cartilage homeostasis. In contrast to our current results, it has been suggested that HDAC is a target for the suppression of cartilage degradation (33, 34). This is based on reports that HDAC inhibitors suppress the ability of IL-1α and oncostatin M to enhance the expression of the matrix metalloproteinase gene in SW1353 chondrosarcoma cells and primary human chondrocytes (34) and that HDAC inhibitors modulate the expression of genes involved in the pathogenesis of adjuvant-induced rheumatoid arthritis (35). Therefore, our finding that HDAC inhibitors block chondrocyte synthesis of type II collagen, a key molecule in cartilage extracellular matrix, raises questions about the use of HDAC inhibitors to suppress cartilage degradation.

Although it is evident that HDAC activity regulates the expression of type II collagen, further studies are needed to more fully elucidate the mechanism. HDAC activity represses gene expression by deacetylating histones, leading to the condensation of chromatin. Therefore, it is likely that HDAC inhibition causes the transcription of target genes by preventing chromatin condensation. This led us to hypothesize that the suppression of type II collagen expression by HDAC inhibitors is not directly mediated by HDAC but rather indirectly by the transcription of target genes that suppress type II collagen expression. We therefore screened various molecules that are known to inhibit type II collagen expression, including Wnt-5a (19), Wnt-7a (27), β-catenin (18), c-Jun (36), and nitric oxide (37). Of these, only the expression of Wnt-5a was enhanced by HDAC inhibitors, which results in the inhibition of type II collagen expression (19). We observed here that HDAC inhibitors caused the up-regulation of Wnt-5a after 3 h (Fig. 6A) and that exogenous Wnt-5a suppresses type II collagen expression within 6 h (Fig. 7A), whereas suppression of type II collagen expression by HDAC inhibitors occurs after 12 h (Fig. 3). This supports our conclusion that the up-regulation of Wnt-5a contributes to the suppression of type II collagen expression. This conclusion was also strongly supported by the fact that a knockdown of Wnt-5a by siRNA abolishes the suppression of type II collagen expression by HDAC inhibitors (Fig. 7). Because...
HDAC inhibition promotes target gene expression by causing the hyperacetylation of promoter sequences, leading to the recruitment of transcriptional complexes, we performed a ChIP assay to determine whether the promoter of Wnt-5a is associated with acetylated histone H4. Immunoprecipitation of acetylated histone H4 from cells treated with TSA or PXD101 coprecipitated DNA containing the Wnt-5a promoter, indicating that the up-regulation of Wnt-5a by HDAC inhibitors was directly linked to the increased acetylation of the Wnt-5a promoter. This clearly supports our hypothesis that HDAC acts as a bridge between gene expressions of Wnt-5a and type II collagen. However, further studies are needed to determine whether Wnt-5a is the only factor mediating the suppression of type II collagen expression by HDAC inhibition.

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