Histone H3K9 methylation is involved in temporomandibular joint osteoarthritis

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Abstract. The morbidity of temporomandibular joint osteoarthritis (TMJOA) increases with age. Condylar articular cartilage degradation, which causes TMJOA, is known to be involved in articular chondrocyte metabolic imbalances in the temporomandibular joint (TMJ) and in other joints of the body. Epigenetic regulation, such as the chemical modification of DNA and histones, is implicated in cartilage homeostasis. However, few studies have been conducted on the epigenetic regulation of condylar articular cartilage degradation. The present study investigated the regulation of histone H3 lysine 9 (H3K9) methylation and its effects on the pathogenesis of degenerative TMJ cartilage disorders. The histone H3K9 methylation level was decreased in degenerated condylar articular cartilage in aged mice. Treatment with chaetocin (a selective H3K9 methylation inhibitor) reduced cell viability and promoted caspase-3/7 activity in ATDC5 mouse chondroprogenitor cells. The inhibition of H3K9 methylation increased matrix metalloproteinase (*Mmp)1* and *Mmp13* mRNA expression in these cells. Furthermore, the expression levels of *Sox9* and collagen α1(II) (*Col2a1*) mRNA, which are anabolic factors for chondrogenic differentiation, were also decreased by treatment with chaetocin, which is an inhibitor of histone methyltransferases. These results indicated that histone H3K9 methylation regulates chondrocyte homeostasis in terms of cell growth, apoptosis and gene expression, and highlighted a possible future therapy option for TMJOA.

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Introduction

Degenerative temporomandibular joint (TMJ) cartilage disorders are one of the most common diseases in dental clinical practice (1). Pathological changes in the TMJ can result in osteoarthritis (OA), which is mainly characterized by condylar cartilage degradation (2). Chondrocytes play a role in the synthesis and degradation of extracellular matrix (ECM) components, including type II collagen and proteoglycans such as aggrecan (3). A decrease in the number of chondrocytes leads to the manifestation of cartilage degradation (4). All components of the condylar cartilage ECM can also be regulated by functionally-balanced matrix metalloproteinases (MMPs), A disintegrin and metalloprotease with thrombospondin motifs (ADAMTS) and tissue inhibitors of matrix metalloprotease that are produced by chondrocytes (5).

Gene expression is regulated by both epigenetic and non-epigenetic mechanisms, leading to the regulation of cell fate, proliferation and differentiation, not only under physiological conditions, but also pathological conditions in humans. Epigenetics is defined as the study of the changes in gene expression without changes in the DNA coding sequence. The major epigenetic mechanisms are DNA methylation and histone modifications, which affect gene expression by changing the chromatin structure (6). The most well-studied histone modifications are methylation and acetylation. One of the crucial histone modifications that affect the chromatin structure is the methylation of histone 3 at lysine 9 (H3K9). There are four known methylated states of H3K9, namely non-methylated, mono-methylated (H3K9Me1), di-methylated (H3K9Me2) and tri-methylated (H3K9Me3) (7), and each modification is associated with the regulation of gene expression and several biological responses (8).

The pathogenesis of OA involves multiple etiologies, including mechanical, biochemical, genetic and epigenetic factors that contribute to the imbalance in the synthesis and destruction of articular cartilage (3,9). Several previous studies have indicated that DNA methylation influences the expression of anabolic and catabolic factors during the pathogenesis of cartilage OA (10-12). Furthermore, histone acetylation mediated by p300/CBP leads to the transcriptional activation

of Sox9, thus indicating that histone acetylation plays a role in sustaining chondrocyte homeostasis (13). However, the biological significance of histone methylation in OA is currently not well understood. The molecular basis of the action of histone methylation on the regulation of chondrocyte homeostasis also remains poorly understood.

The effects of histone methylation on the etiologies of degenerative TMJ cartilage disorders are a subject of growing interest. The present study examined H3K9 to elucidate the role of histone methylation in the pathogenesis of degenerative TMJ cartilage disorders. The histone H3K9 methylation level was investigated in degenerated condylar articular cartilage in mice. The effects of treatment with chaetocin, a selective histone H3K9 inhibitor, were also investigated using chondrogenic ATDC5 cells.

Materials and methods

Ethics and mice. All animal experiments were approved and conducted in accordance with the Animal Care and Use Ethics Committee of Hokkaido University (Sapporo, Japan) and the National Center for Geriatrics and Gerontology (Obu, Japan). Eight-week-old female C57BL/6NCrSlc mice (n=10; weight, ~20 g; Japan SLC, Inc.) and 20-month-old female C57BL/6NCrSlc mice (n=10; weight, ~40 g; Aging Farm, National Center for Geriatrics and Gerontology) were used in the present study. All samples from the mice were collected and analyzed between March 2016 and October 2018 at the Hokkaido University and National Center for Geriatrics and Gerontology. Mice used for experiments were anesthetized by intraperitoneal injection of 0.3 mg/kg medetomidine hydrochloride, 4 mg/kg midazolam and 5 mg/kg butorphanol tartrate. This mixture of three anesthetics [medetomidine, midazolam and butorphanol (MMB)] was developed as an alternative to ketamine for use in rodents (14). The depth of anesthesia in the mice was monitored via the corneal reflex and toe-pinch reflex to verify a lack of response. MMB anesthesia is known to have several adverse effects, including hypothermia (15). No side effects associated with anesthesia were observed in the present study. The mice were euthanized by cervical dislocation under anesthesia. Following this, the skin of the heads of the mice was removed and the heads were dissected into two halves. The TMJs were carefully isolated with all the attached soft tissues removed using small scissors.

Tissue preparation and histological staining. TMJ tissues were fixed in 4% paraformaldehyde for 24 h at 4˚C. The tissues were decalcified in 10% EDTA at pH 7.4 for 7-14 days at room temperature and then embedded in paraffin using conventional methods. By using a microtome (REM-700; Yamato Kohki Industrial Co., Ltd.), serial sagittal sections (5 μm) were cut from the blocks of paraffin with embedded TMJ. The serial sections of each condyle were stained with hematoxylin and eosin (HE) and 0.1% safranin-O/0.02% fast green, according to the following protocols, for analysis under a light microscope (Model Eclipse Ci-S; Nikon Corporation) with a video controller (Digital Sight, DS-L3; Nikon Corporation): For HE staining, the slides ware stained with hematoxylin for 10 min, rinsed and stained with Eosin Y for 5 min at room temperature; for safranin-O fast green staining, the slides were stained with hematoxylin for 7 min, rinsed, stained with 0.05% Fast Green for 5 min with a brief rinse in 1% acetic acid, and stained with 0.1% Safranin-O for 30 min at room temperature. HE staining was used to assess condylar changes, and safranin-O staining and fast green staining were performed to determine the proteoglycan changes.

Histological analysis and application of the modified Mankin scoring system. A modified Mankin scoring system was used to assess the degree of cartilage degradation (16). The scoring of the articular cartilage was based on the pericellular and background staining of safranin-O and fast green, the arrangement of the chondrocytes, and the structural condition of the cartilage. In this system, the score for normal articular cartilage was 0 points, whereas the maximum score for degenerative articular cartilage was 10 points (16).

Antibodies. The following antibodies were used in the present study: Anti-H3K9me1 (cat. no. 07-450; rabbit polyclonal), anti-H3K9me2 (cat. no. 07-441; rabbit polyclonal), and anti-H3K9me3 (cat. no. 07-442; rabbit polyclonal) (all from EMD Millipore).

Immunohistochemistry. Immunohistochemical analysis was performed using primary antibodies against H3K9me1, H3K9me2 and H3K9me3. Paraformaldehyde-fixed, paraffin-embedded 5-μm sagittal sections of the condyles were prepared from 8-week-old and 20-month-old mice. After deparaffinization, antigen retrieval was performed using Liberate Antibody Binding Solution (Polysciences, Inc.) for 35 min and washed in PBS with Tween-20 at room temperature, according to the manufacturer's directions. After endogenous peroxidase was blocked with 1.4% H_2O_2 in methanol for 20 min at room temperature, the sections were incubated overnight at 4˚C with anti-H3K9me1 antibody (1:100), anti-H3K9me2 antibody (1:100) or anti-H3K9me3 antibody (1:100). Thereafter, the sections were incubated with secondary antibodies conjugated to the peroxidase-labeled polymer, Envision+ Dual Link System-horseradish peroxidase (cat. no. K4061; Dako; Agilent Technologies, Inc.) for 2 h at room temperature. Color development was performed using 3,3'-diaminobenzidine tetrahydrochloride (Wako Pure Chemical Industries, Ltd.) for 10 min, and the sections were counterstained with hematoxylin for 5 sec at room temperature. Images were captured using a light microscope (Model Eclipse Ci-S; Nikon Corporation) with a video controller (Digital Sight, DS-L3; Nikon Corporation) at a magnification of x200. When necessary, whole-image adjustments of contrast and brightness were made uniformly to the original data. The total number of cells and immunopositive cells in each section were counted, and the resulting ratio was calculated as the number of immunopositive cells/total cells.

Cell cultures. ATDC5 mouse chondroprogenitor cells were obtained from the RIKEN BioResource Research Center Cell Bank (Tsukuba, Japan) (17). Cells were grown to confluence in DMEM (Sigma-Aldrich; Merck KGaA) with 100 μg/ml kanamycin (Meiji Seika Kaisha, Ltd.) and 10% FBS (SAFC; Merck KGaA) at 37˚C in a humidified atmosphere of 5% CO_2. At 1 day after the cells reached confluence, the medium

was replaced with or without chaetocin (1, 10 or 100 nM; Sigma-Aldrich; Merck KGaA) for 24 h. To investigate whether chaetocin affects *Mmp* mRNA expression, cells were cultured by adding interleukin 1β (R&D Systems, Inc.). Confluent ATDC5 cells were preincubated with DMEM containing 1% FBS for 24 h and then cultured with 10 ng/ml interleukin 1β at the indicated concentrations of chaetocin for 24 h.

Quantification of gene expression by reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from ATDC5 cells with or without exposure to chaetocin by using RNAiso Plus (Takara Bio Inc.), according to the manufacturer's directions. RT was then performed using a PrimeScript RT Master Mix (Perfect Real Time; Takara Bio Inc.). The conditions were as follows: RT reaction at 37˚C for 15 min, RT heat inactivation at 85˚C for 5 sec, and cooling at 4˚C. RT-qPCR was performed using assay-on-demand TaqMan probes [cat. no. Mm00473485_m1 for *Mmp1*, cat. no. Mm00439491_m1 for *Mmp13*, cat. no. Mm00448840_m1 for *Sox9* and cat. no. Mm01309565_m1 for collagen α1(II) (*Col2a1*); Applied Biosystems; Thermo Fisher Scientific, Inc.] and the StepOne® qPCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The qPCR cycling conditions included an initial denaturation at 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. The relative levels of gene expression were quantified using the $\Delta\Delta C_q$ method (18), with *gapdh* (cat. no. Mm99999915_g1; Applied Biosystems; Thermo Fisher Scientific, Inc.) acting as the endogenous control. The fold-change in the mRNA level of chaetocin-treated cells was calculated on the basis of the level in untreated control ATDC5 cells.

Histone isolation and quantification. Total histone proteins were isolated from ATDC5 cells with or without exposure to chaetocin by using an EpiQuik™ Total Histone Extraction kit (Epigentek Group, Inc.), according to the manufacturer's instructions. Global changes in the levels of H3K9 mono-, di- and tri-methylation results were determined using an EpiQuik™ Global Mono-methylation Histone H3K9 Quantification kit (cat. no. P-3030), an EpiQuik™ Global Di-methylation Histone H3K9 Quantification kit (cat. no. P-3032), and an EpiQuik™ Global Tri-methylation Histone H3K9 Quantification kit (cat. no. P-3034) (all Epigentek Group, Inc.), according to the manufacturer's protocol. Briefly, 50 µl C2 buffer was placed in each well, and 200 ng isolated histone proteins was then added to each sample well. Different concentrations of standard histone with H3K9 mono-, di- and tri-methylation (10, 20, 40, 60, 80 or 100 ng/µl) processing were added to standard wells. A blank was maintained and the plate was incubated for 2 h at room temperature. After incubation, the contents of the plate were discarded and the wells were washed with 150 µl C1 buffer. A total of 50 µl C3 buffer containing the secondary antibody was added to each well and then incubated for 60 min at room temperature on an orbital shaker. At the end of this incubation period, the wells were washed with C1 buffer, and 100 µl C4 buffer was added to each well and subsequently incubated for 2 min at room temperature in the dark. The enzymatic reaction was stopped via the addition of 50 µl C5 buffer, and the absorbance was read at 450 nm using a microplate reader (iMark; Bio-Rad Laboratories, Inc.). The fold-change in the histone methylation level of chaetocin-treated cells was calculated on the basis of a comparison with that observed in untreated control ATDC5 cells.

Cell viability assay. The tetrazolium-based colorimetric TetraColor One assay (Seikagaku Corporation) was used to quantitate cell viability. Briefly, ATDC5 cells were seeded into 96-well plates and cultured until confluence, under the aforementioned conditions. At 1 day post-confluence, the medium was replaced with or without chaetocin for 24 h. Thereafter, the substrate WST-8 [composed of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitro phenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] was added to each well. After incubation for an additional 2 h, the absorbance at 450 nm was measured with a microplate reader (iMark; Bio-Rad Laboratories, Inc.). The fold-change in the viability of chaetocin-treated cells was then calculated on the basis of a comparison with that in untreated ATDC5 cells.

Measurement of caspase-3/7 activity. The cellular enzymatic activities of caspase-3/7 were determined using a caspase colorimetric assay (Caspase-Glo 3/7 Assay Systems; Promega, Corporation), as described previously (19). For each reaction, the cells were lysed and incubated with a luminogenic substrate containing the DEVD sequence, which is cleaved by activated caspase-3/7. After incubation at room temperature for 1 h, luminescence was quantified using a Mini Lumat LB 9506 luminometer (Berthold Technologies GmbH & Co. KG). The fold-change in the level of caspase-3/7 activity of chaetocin-treated cells was then calculated on the basis of that present in untreated ATDC5 cells.

Statistical analysis. The quantitative data for modified Mankin score and immunopositive cell ratios of H3K9 methylation are expressed as ranges in box plots, and the differences between two groups were assessed via the Mann-Whitney U test using the statistical program file 'ystat 2008' (Igakutosho Shuppan, Ltd.) developed for Microsoft Excel 2011 (Microsoft Corporation). The quantitative data for RT-qPCR were recorded as the mean ± SD, and comparisons between multiple groups were performed via one-way analysis of variance followed by the application of Dunnett's multiple comparison test (software provided by Osaka University, Japan; http://www.gen-info.osaka-u.ac.jp/MEPHAS/dunnett-e.html). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Histone H3K9 methylation is reduced in the articular cartilage of the degraded TMJ (the resorptive condyle). The condylar cartilage of 8-week-old and 20-month-old C57BL/6NCrSlc female mice was examined to elucidate the differences in site-specific histone modifications associated with TMJ cartilage damage. The surface of the condylar cartilage was intact and smooth in 8-week-old mice (Fig. 1A). The condylar cartilage is composed of fibrous, proliferative and hypertrophic layers, above a layer of subchondral bone. In 8-week-old mice, safranin-O and fast green staining showed that the condylar cartilage exhibited a rich and even distribution of proteoglycans, particularly in the deep layers of the cartilage (Fig. 1B). By contrast, the fibrocartilage layer was lost, and

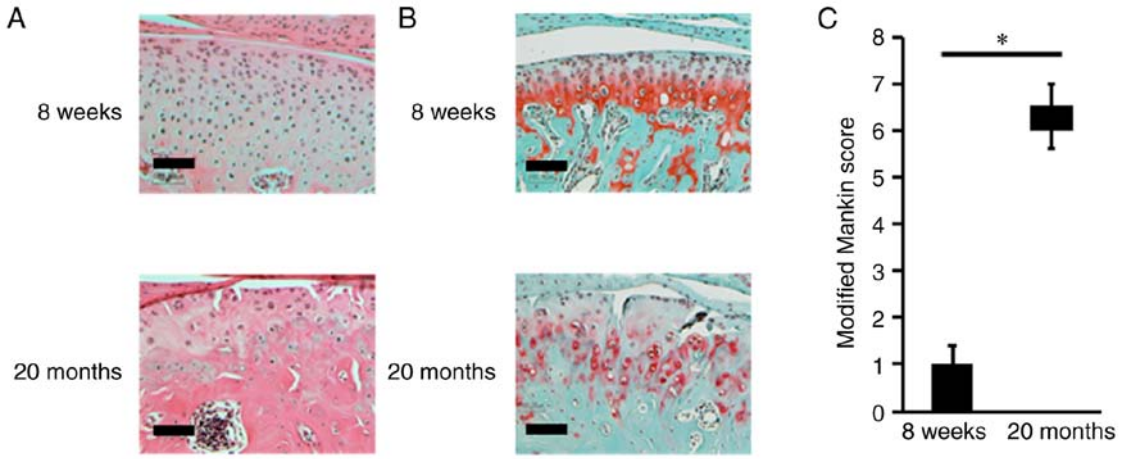

Figure 1. Degraded temporomandibular joint articular cartilage (resorptive condyles). Central sagittal sections of the condyles from 8-week-old (upper) or 20-month-old (lower) C57BL/6NCrSlc female mice were examined. (A) Hematoxylin and eosin staining (magnification, x200). (B) Safranin-O and fast green staining (magnification, x200). The scale bars indicate 100 μm. Representative images from three independent experiments are shown. (C) A comparison of modified Mankin scores is presented. Quantitative data of modified Mankin scores are expressed as ranges in box plots. *P<0.05, Mann-Whitney U test (n=7).

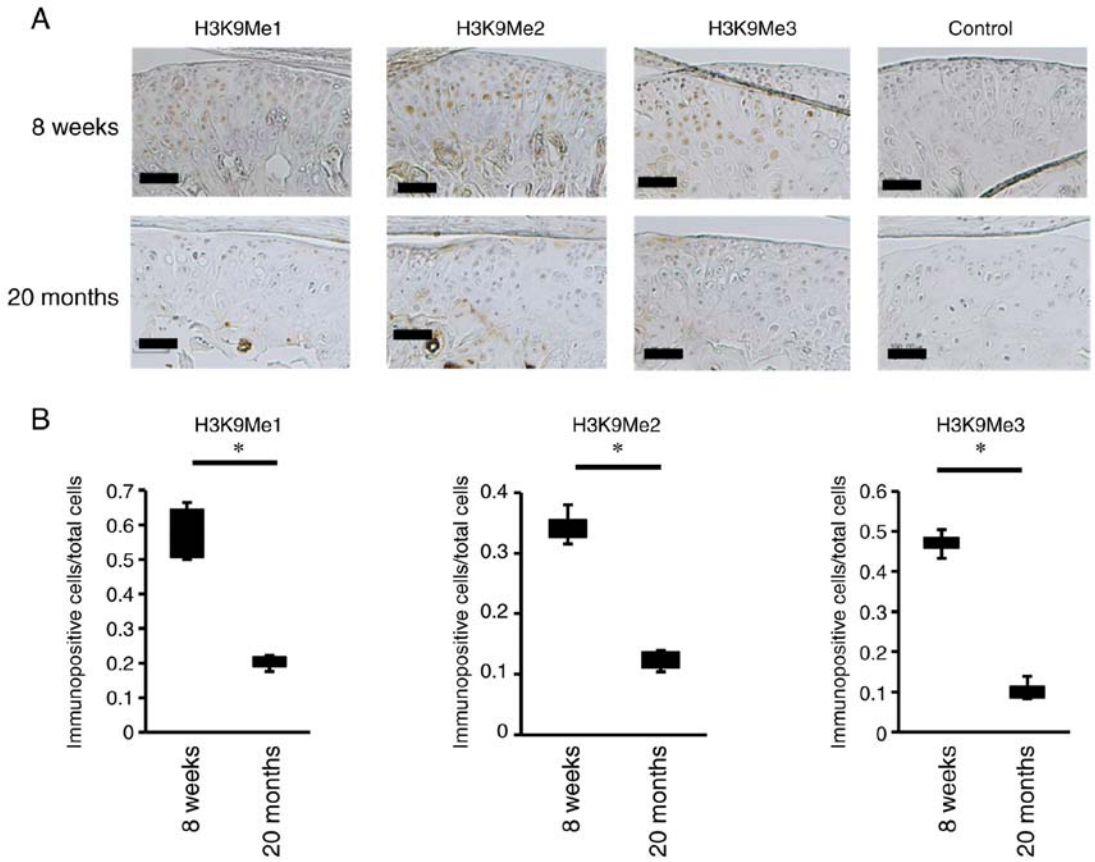

Figure 2. Reduced H3K9 methylation in the degraded temporomandibular joint articular cartilage (resorptive condyles). Central sagittal sections of the condyles from 8-week-old (upper) or 20-month-old (lower) C57BL/6NCrSlc female mice were examined. (A) Immunohistochemical staining with H3K9Me1, H3K9Me2 and H3K9Me3 antibodies (magnification, x200). The secondary antibody for mouse IgG was used as the nonspecific negative control (magnification, x200). The scale bar represents 100 μm. (B) The average numbers of H3K9Me1-, H3K9Me2- and H3K9Me3-positive chondrocytes as proportions of the total cells in all cartilage layers. The stained sections were examined under a light microscope at a magnification of x200. The number of total cells and immunopositive cells in each section were counted, and the ratio of the total cells and immunopositive cells was calculated as the number of immunopositive cells/total cells. The quantitative data of the immunopositive cell ratio of H3K9 methylation derived from three independent experiments are expressed as ranges in box plots. *P<0.05, Mann-Whitney U test (n=3). H3K9Me1, mono-methylated histone H3; H3K9Me2, di-methylated histone H3; H3K9Me3, tri-methylated histone H3.

the proliferative cartilage layer was exposed in 20-month-old mice, thus indicating that the articular cartilage layer was damaged (Fig. 1B). Furthermore, in the aged mice, the articular cartilage appeared thinner, and the number of chondrocytes

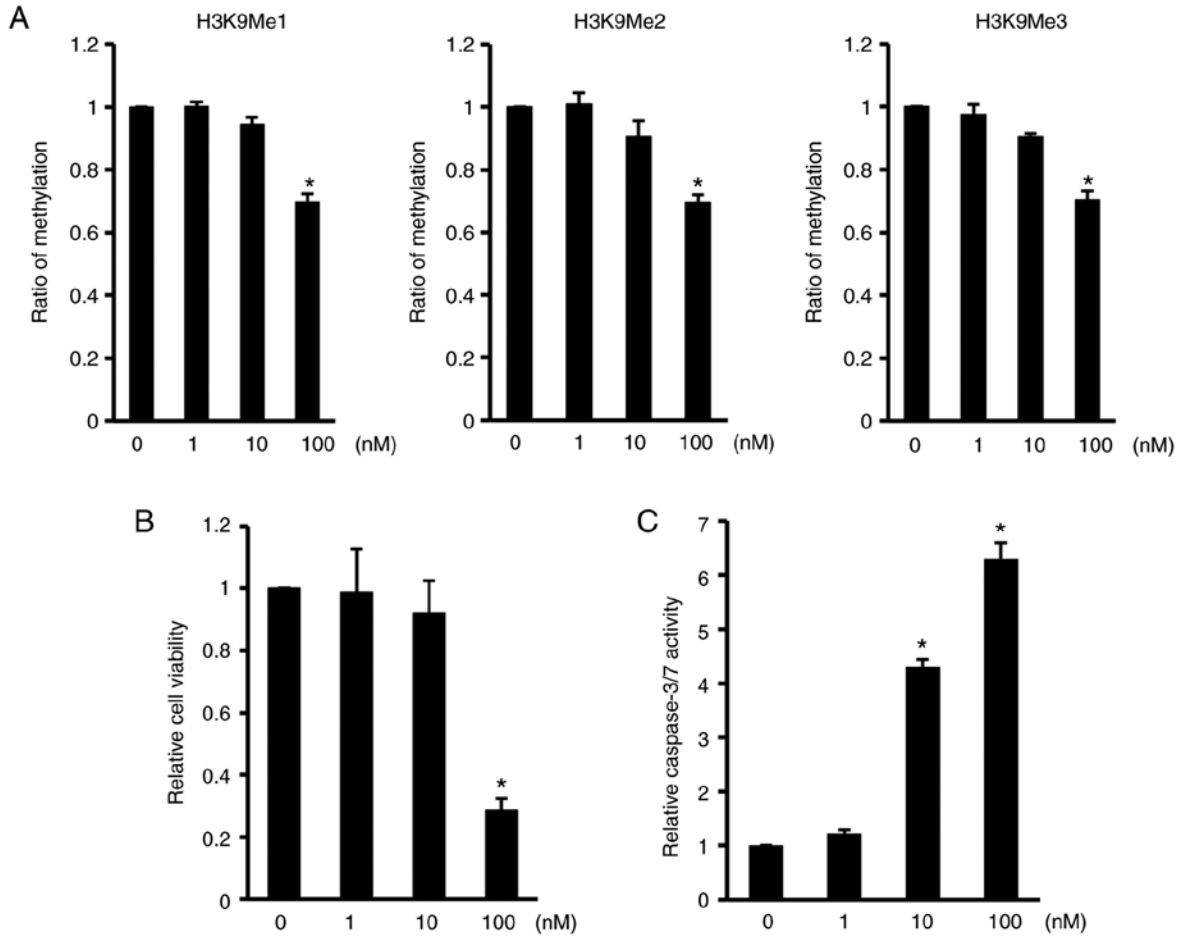

Figure 3. Inhibition of H3K9 methylation regulates cell viability and cellular caspase-3/7 activity in ATDC5 cells. Confluent ATDC5 cells were incubated and treated with the indicated concentration of chaetocin for 24 h. (A) Histone protein was extracted from the cells, and H3 modifications (H3K9Me1, H3K9Me2 and H3K9Me3) were quantified by immunoassay. The fold-change in the histone methylation level of chaetocin-treated cells was calculated on the basis of that seen in untreated ATDC5 cells. (B) Cell viability and (C) cellular caspase-3/7 activities were measured. The fold-changes in viable cells and activities were calculated on the basis of those observed in untreated ATDC5 cells. Data are presented as the mean ± SD; n=3, $^{*}P<0.05$ vs. control, Dunnett's multiple comparison test. H3K9Me1, mono-methylated histone H3; H3K9Me2, di-methylated histone H3; H3K9Me3, tri-methylated histone H3.

appeared decreased. A pronounced loss of proteoglycans was also observed in these mice (Fig. 1B). An analysis of histological changes in the condyles using the modified Mankin scoring system indicated that the degradation of the articular cartilage was significantly higher in 20-month-old mice than in 8-week-old mice (Fig. 1C). Thereafter, H3K9me was examined in sections of the condyles by using specific antibodies for H3K9Me1, H3K9Me2 and H3K9Me3. H3K9Me1-, H3K9Me2- and H3K9Me3-positive chondrocytes were detected in the intact mouse condyles and were localized in the hypertrophic cartilage layer (Fig. 2A). However, in the condyles of cartilage-damaged mice, decreases in H3K9me1-, H3K9Me2- and H3K9Me3-positive cells were observed in the articular cartilage layer (Fig. 2A). Furthermore, the ratio of H3K9Me-positive cells in the condyles of cartilage-damaged mice was lower than that in intact mice (Fig. 2B).

Inhibition of histone H3K9 methylation reduces cell viability and induces apoptosis in ATDC5 cells. To elucidate the effects of histone H3K9 methylation on the proliferation and differentiation of chondrocytes, ATDC5 cells were used for *in vitro* studies. The ATDC5 cell line is derived from mouse teratocarcinoma cells, and characterized as a chondrogenic cell line that exhibits a multistep sequential process analogous to chondrogenic differentiation during endochondral bone formation (20). Chaetocin, a thiodioxopiperazine produced by *Chaetomium spp*., specifically inhibits suppressor of variegation 3-9 homolog 1 (a histone H3K9-specific methyltransferase), thus resulting in a reduction in H3K9 methylation (21). Treatment with chaetocin decreased the methylation levels of H3K9Me1, H3K9Me2 and H3K9Me3 in ATDC5 cells (Fig. 3A). The number of viable ATDC5 cells was also reduced by way of chaetocin treatment (Fig. 3B). Furthermore, caspase-3/7 activity in the cells was promoted by chaetocin (Fig. 3C). These data indicated that the inhibition of H3K9 methylation reduced cell viability and induced apoptosis in ATDC5 cells.

Inhibition of histone H3K9 methylation regulates chondrocyte mRNA expression in ATDC5 cells. To investigate the relationship between H3K9 methylation and cartilage degradation, the expression of MMPs was analyzed by RT-qPCR. The expression of *Mmp1* and *Mmp13* mRNA was significantly induced by chaetocin treatment in ATDC5 cells (Fig. 4), thus suggesting that H3K9 methylation regulates not only chondrocyte proliferation and apoptosis, but also cartilage ECM

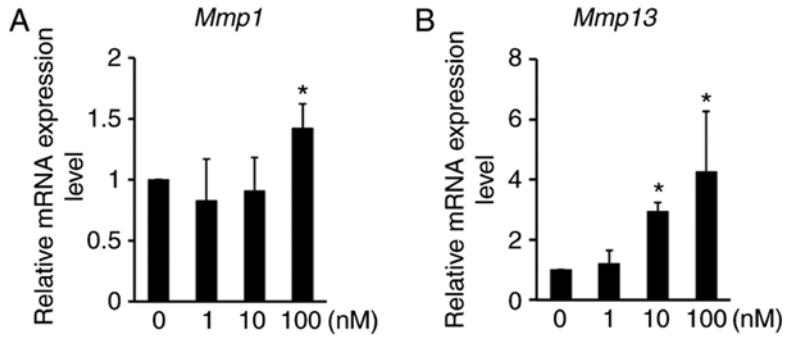

Figure 4. Regulation of *Mmp* mRNA expression via inhibition of histone H3 methylation in ATDC5 cells. Confluent ATDC5 cells were preincubated with DMEM containing 1% FBS for 24 h and then cultured with 10 ng/ml interleukin 1β at the indicated concentrations of chaetocin for 24 h. Total cellular RNA was extracted from the cells and the levels of the (A) *Mmp1* and (B) *Mmp13* mRNA expression levels were measured by reverse transcription-quantitative PCR. mRNA expression levels were normalized against *gapdh* and scaled, with a non-additional control sample set to 1 in each experiment. Data are presented as the mean ± SD; n=3, *P<0.05 vs. control, Dunnett's multiple comparison test. *Mmp*, matrix metalloproteinase.

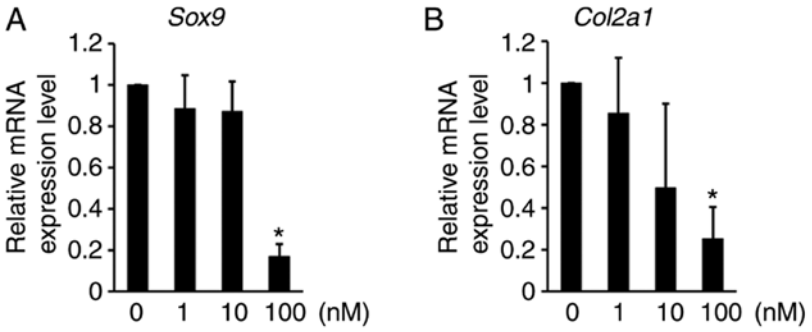

Figure 5. Inhibition of histone H3 methylation regulates *Sox9* and *Col2a1* mRNA expression in ATDC5 cells. Confluent ATDC5 cells were incubated and treated with the indicated concentration of chaetocin for 24 h. Total cellular RNA was extracted from the cells and reverse transcription-quantitative PCR was conducted to estimate the respective (A) *Sox9* and (B) *Col2a1* mRNA expression levels in these cells. mRNA expression levels were normalized against *gapdh* and scaled, with the untreated control sample set to 1 in each experiment. Data are presented as the mean ± SD; n=3, *P<0.05 vs. control, Dunnett's multiple comparison test. *Col12a1*, collagen α1(II).

degradation. Thereafter, RT-qPCR analysis was performed to examine how the inhibition of H3K9 methylation affects anabolic gene expression in ATDC5 cells. The expression levels of *Sox9* and *Col2a1* mRNA, which are well-known to be anabolic factors for chondrogenic differentiation, were decreased by chaetocin treatment in ATDC5 cells (Fig. 5), thus indicating that the inhibition of H3K9 methylation regulates the mRNA expression of anabolic factors that induce chondrocyte differentiation.

Discussion

Histone methylation and demethylation play crucial roles in transcriptional control, thereby affecting gene expression (6). H3K9 methylation is a generally epigenetic mark of heterochromatin formation and transcriptional silencing (22). Previous studies have reported that histone H3K9 methylation at a specific gene promoter is associated with the pathogenesis of OA. A variety of changes in the *Sox9* (the master transcriptional factor that regulates chondrogenesis) gene promoter increase H3K9 methylation in the cartilage in advanced hip OA (23). The induction of microsomal prostaglandin E synthase-1 (*mPges-1*) expression is correlated with decreased levels of H3K9Me1 and H3K9Me2 at the *mPges-1* gene promoter in human OA chondrocytes (24). These observations indicate that H3K9 methylation in specific gene promoters manages the expression of several genes related to OA pathogenesis. The present study demonstrated a decrease in global histone H3K9 methylation in a mouse model of TMJOA. Histone methylation is regulated by histone methyltransferases (HMTs) and histone demethylases (HDMs) (7). An increase in H3K9 methylation appears to be associated with a reduced level of lysine-specific demethylase 3A (25). The conditional knockout of SET domain bifurcated histone lysine methyltransferase 1 (ESET; an ERG-associated protein with a SET domain; also called SETDB1), one of the SET domain-containing lysine-specific HMTs, results in hypertrophy, apoptosis and the terminal differentiation of articular chondrocytes at the knee joint in C57BL/6 mice, thus indicating that HMTs may be implicated in joint diseases, such as OA (26). However, there is a paucity of data available related to the changes in HMTs and HDMs during the pathogenesis of OA. It may be speculated that the regulation of the activity of these HMTs and/or HDMs is associated with the pathogenesis of TMJOA.

OA is associated with structural damage and functional failure in articular cartilage, and cartilage homeostasis is maintained by chondrocytes. OA chondrocytes are characterized by accelerated catabolic processes and the suppression of anabolic processes (27). Matrix-degrading enzymes, including MMPs and ADAMTS, break down the ECM of cartilage (28,29).

The promoters of genes encoding catabolic enzymes such as MMP3, MMP9, MMP13 and ADAMTS4 are demethylated, thus resulting in the increased expression of these enzymes under OA-related pathogenic conditions in chondrocytes (10). The results of the present study indicated that treatment with chaetocin, a selective H3K9 methylation inhibitor, increased the expression of the *Mmp1* and *Mmp13* genes in ATDC5 cells. These observations suggest a link between the epigenetic regulation of DNA methylation and histone modification, including H3K9 methylation, in regulating the gene expression of catabolic enzymes in OA chondrocytes. This relationship may lead to cartilage degradation.

During the pathogenesis of OA, the expression of anabolic factors, including ECM components, is suppressed in OA chondrocytes. DNA methylation and histone modification have been implicated by prior research in the regulation of the expression levels of anabolic factors in OA (11,12), such as the methylation of collagen type IX α1 chain (*Col9a1*) enhancer, which causes the transcriptional repression of *Col9a1* (30). A study involving human chondrocytes found that histone methyltransferase Set7/9 elevated trimethylated H3K4 in the *Col2a1* promoter, thus resulting in increased *Col2a1* expression (31). Under OA-related pathogenic conditions, the promoter of *Sox9* exhibits elevated tri-methylation levels of H3K9 and 27, thus leading to the transcriptional repression of *Sox9* (21). The present results showed that the selective inhibition of H3K9 methylation suppressed anabolic *Sox9* and *Col2a1* expression in ATDC5 cells. It is assumed that the methylation of H3K9 regulates the expression of anabolic and catabolic genes in ATDC5 cells, and may be related to TMJOA pathogenesis. Although the status of histone methylation has been broadly investigated in the development of cancer (32,33), it has not been as widely reported in relation to the pathogenesis of OA, particularly TMJOA.

Many studies have shown that there are very low levels of proliferative activity and apoptotic cell death in chondrocytes in OA cartilage (4,34,35). In the present study, histone H3K9 demethylation by chaetocin decreased the number of viable cells and induced caspase-3/7 activity in ATDC5 cells. The present data are in agreement with those of another study showing that HMT and HDM levels in cancer cells result in regulated proliferation and apoptosis (36). For example, lysine demethylase 4A regulates cell proliferation and apoptosis in colon cancer cells (37). Euchromatic histone lysine methyltransferase 2 is also involved in the proliferation, apoptosis and cell invasion of neuroblastoma cells (38). This is due to the modification of H3K9 methylation, which alters the chromatin structure, cell cycle and apoptosis, and is correlated with the onset of OA and pathological cartilage degradation (39).

The results of the present study demonstrated that H3K9 demethylation occurs in TMJOA mouse cartilage, and that H3K9 demethylation suppresses cell proliferation and negatively regulates chondrocyte homeostasis. TMJ cartilage degradation is a late-onset, complex disease (40). The results of the present study suggested that the epigenetic process of histone methylation may play a crucial role in the pathogenesis of TMJOA. If the molecular mechanisms connecting histone methylation and TMJOA can be clarified by further investigations, histone methylation may be a promising treatment method for TMJOA.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MU conceived the project conducted most of the experiments. KM conducted the animal experiments on 20-month-old C57BL/6NCrSlc female mice. TY and MT provided technical and conceptual support and advice. MU and MT wrote the manuscript. All authors have read and approved the final version of this manuscript for publication.

Ethics approval and consent to participate

Ethical protocols for all animal experiments were approved by the Institutional Animal Care Committee and conducted in accordance with the Animal Care and Use Ethics Committee of Hokkaido University (Sapporo, Japan) and the National Center for Geriatrics and Gerontology (Obu, Japan).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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