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Cell-specific epigenetic regulation of ChM-I gene expression: Crosstalk between DNA methylation and histone acetylation

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Abstract

The expression of the chondromodulin-I (ChM-I) gene, a cartilage-specific gene, is regulated by the binding of Sp3 to the core promoter region, which is inhibited by the methylation of CpG in the target genome in the osteogenic lineage, osteosarcoma (OS) cells. The histone tails associated with the hypermethylated promoter region of the ChM-I gene were deacetylated by histone deacetylase 2 (HDAC2) in three ChM-I-negative OS cell lines. Treatment with an HDAC inhibitor induced the binding of Sp3 in one cell line, which became ChM-I-positive. This process was associated with acetylation instead of the dimethylation of histone H3 at lysine 9 (H3-K9) and, surprisingly, the demethylation of the core promoter region. The demethylation was transient, and gradually replaced by methylation after a rapid recovery of histone deacetylaion. These results represent an example of the plasticity of differentiation being regulated by the cell-specific plasticity of epigenetic regulation.

Keywords: Chondromodulin-I; Histone modification; Acetylation; Differentiation; Epigenetic; Chondrogenic; Methylation; H3-K9; HDAC2

The expression of cell type-specific genes is tightly regulated by a hierarchical mechanism composed of genetic and epigenetic factors. In some cases, a single transcription factor may determine cell fate. In other instances, however, the expression of such a master gene is found in cells in other tissue-lineages. For example, the expression of the SOX9 gene, which is the master gene for chondrogenic cell lineage, is also found of in cells of the ostogenic lineage [1]. Transcription factors regulating the expression positively or negatively must bind to specific DNA elements to exert their function, and the factors regulating such binding significantly affect their function and therefore the expression of each gene.

Chondromodulin-I (ChM-I) is a potent vascular endothelial cell growth inhibitor purified from bovine epiphyseal cartilage [2], the expression of which in matured limbs is limited to cells in the resting, proliferating, and early hypertrophic zone of the growth plate [2], and not in other mesenchymal tissues including bone. Therefore, it is a suitable material for analyzing the molecular mechanism regulating cell-specific genes, and we have shown that the Sp3 transcription factor is a major driver of binding to a specific portion in the core promoter region [1]. Because Sp3 is a universal transcription factor ubiquitously expressed in many tissues, the cell-specific expression of the ChM-I gene requires additional factors. Using osteosarcoma (OS) cells, which are malignant mesenchymal

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tumor cells with features of osteogenic lineage cells, we have shown that DNA methylation of the Sp3-binding sequence is one such mechanism to shut down the expression in cells of the osteogenic lineage [1]. Treatment with a demethylating reagent, however, failed to restore the expression of the ChM-I gene in some OS cells, suggesting the presence of other regulatory mechanisms [1].

Modification of the histone tail is another epigenetic regulatory mechanism [3]. Histone proteins assemble into nucleosomes, which function as DNA packaging. The amino-terminal tails of histone protrude from the nucleosome and are subject to chemical modifications including phosphorylation, ubiquitination, acetylation, and methylation [4]. The acetylation of lysine residues on histone H3 and H4 leads to the formation of an open chromatin structure and allows regulatory factors to easily access the chromatin [4], which is regulated by the stochastic balance of histone acetyltrasferases (HAT) and histone deacetylases (HDAC). Notably, dimethylation of histone H3 at lysine 9 (H3-K9) tightly inhibits the gene expression, correlated with the methylation of CpG [5,6]. These modifications of the histone tail and CpG methylation status determine the differentiation [7].

Here, we investigated the epigenetic regulation of the ChM-I gene in a variety of OS cell lines and found that modifications of the histone tail determine the expression. We found evidence for crosstalk between the modification of histones and methylation of CpG, in which histone acetylation can inhibit the maintenance of CpG methylation by substituting H3-K9 dimethylation with H3-K9 acetylation.

Materials and methods

Cell lines and culture conditions. The human cell lines Saos2, HuO, HOS, MG63, and U2OS were obtained from ATCC or Japanese Cancer Research Resources Bank. The human osteosarcoma cell lines TAKAO and ANOS were established in our laboratory. All the cell lines used in this study were maintained in DMEM (Sigma) with 10% fetal bovine serum (Hyclone, South Logan, UT), 100 U/ml penicillin, and 100 mg/ml streptomycin, in 5% CO_2 at 37 °C.

SDS–PAGE. Western blotting was performed as previously mentioned [1]. The primary antibodies used were as follows: acetylated H3, acetylated H4, dimethylated H3-K9, acetylated H3-K9, and H3 from Upstate Biotechnology (Lake Placid, NY), HDAC2, HDAC3, and HDAC6 from Zymed Laboratory (San Francisco, CA), and Sp1 and Sp3 from Santa Cruz Biotechnology (Santa Cruz, CA).

Reverse transcription (RT)-PCR and quantitative RT-PCR. RNA was isolated using Trizol Reagent (Life Technologies, Rockville, MD) following the manufacturer's directions from frozen tumor tissues and the cultured cell lines. All RT reactions were performed using 1 μg of total RNA with a Super Script First Strand Synthesis System for RT-PCR kit (Life Technologies) according to the instructions supplied. RT-PCR amplification was performed as previously mentioned [1].

Demethylation drug treatment. The cells (1×10^5) were seeded on 60-mm dishes in DMEM with 10% FBS. After cells attaching to the dishes, the cells were treated with 1 μM of 5-Aza-2′-deoxycytidine (5-Aza-dC) (Sigma–Aldrich) for 96 h.

Histone deacetylase inhibitor (HDACi) treatment. The cells (1×10^5) were seeded on 60-mm dishes in DMEM with 10% FBS. After attaching to the dishes, they cells were treated with histone deacetylase inhibitors; MS-275 provided by Nihon Scherring (Chiba, Japan), The cells were harvested after 24 h.

Bisulfite genomic sequencing. The bisulfite modification of DNA samples was performed using the EpiTect bisulfite kit (Qiagen, Tokyo, Japan). Bisulfite-modified DNA spanning residues −297 to −104 relative to the transcription start point [1] was amplified, cloned into the TA-vector (Invitrogen), and sequenced using an ABI 377 semiautomatic sequencer (PE Applied Biosystems).

Chromatin immunoprecipitation (ChIP). The suitability of each antibody for the ChIP assay was confirmed by immunoprecipitation–Western blotting (data not shown). Tissue samples were treated using an EpiQuik tissue ChIP kit according to the manufacturer's directions (Epigentek Group Inc. Brooklyn, NY). Cells were harvested and mixed with formaldehyde at a final concentration of 1.0% for 10 min at 37 °C to cross-link protein to DNA. Cells then were suspended in 0.2 ml of SDS lysis buffer and settled on ice for 10 min. DNA cross-linked with protein was sonicated into fragments of 200–1000 bp. One-tenth of the sample was set aside as an input control, and the rest was precleared with salmon sperm DNA protein A–Sepharose beads (Upstate Biotechnology) for 30 min with agitation. The soluble chromatin fraction was collected with each antibody at 4 °C overnight with rotation. Immune complexes were collected with salmon sperm DNA protein A–Sepharose beads and washed with the manufacturer's low salt, high salt, and LiCl buffers and then washed twice with TE buffer (10 mM Tris–HCl and 1 mM EDTA). The chromatin-antibody complexes were eluted with elution buffer (1% SDS and 0.1 M $NaHCO_3$). Protein–DNA cross-links were reversed with 5 M NaCl at 65 °C for 4 h, proteinase K treatment and phenol–chloroform extraction were carried out, and then the DNA was precipitated in ethanol. PCR amplification was performed using primers specific for the *ChM-I* promoter (sense, 5-GAATGCAGGCCAGTGAGAAGGT-3; antisense, 5-GCACCCTGGGATCTGTCCCGCT-3). The reaction was performed with an initial denaturation of 5 min at 94 °C followed by 30 cycles of 1 min at 94 °C, 1 min at 63 °C, and 1 min at 72 °C with a final extension at 72 °C for 7 min.

Results

The promoter lesion of ChM-I was deacetylated by binding of HDAC2 in ChM-I-negative OS cell lines

Three of seven OS cell lines (TAKAO, Saos2, and MG63) showed no expression of the ChM-I gene even in the RT-PCR analyses, among which two (TAKAO and Saos2) turned out to be positive on treatment with 5-Aza-dC (Fig. 1 A). The state of the histone tails of these seven OS cell lines was analyzed by Western blotting (Fig. 1B) and ChIP assay (Fig. 1C). The acetylation of global lesions of the histone H3 and H4 tails did not differ between the ChM-I-positive and negative cell lines (Fig. 1B). But the histone H3 and H4 tails associated with the lesion in the promoter of ChM-I was locally acetylated in ChM-I-positive cells, and deacetylated in the negative cells (Fig. 1C), indicating that the status of histone acetylation correlated with the expression of the ChM-I gene. To find the HDAC responsible for the deacetylation, the expression of HDAC2, HDAC3, and HDAC6 was analyzed by Western blotting, which showed no clear correlation with the expression of ChM-I gene (Fig. 1D). The ChIP assay, however, showed that HDAC2, but not HDA3 or HDAC6, bound to the promoter region in the ChM-I-negative cell lines (Fig. 1E). These results suggested that HDAC2 was

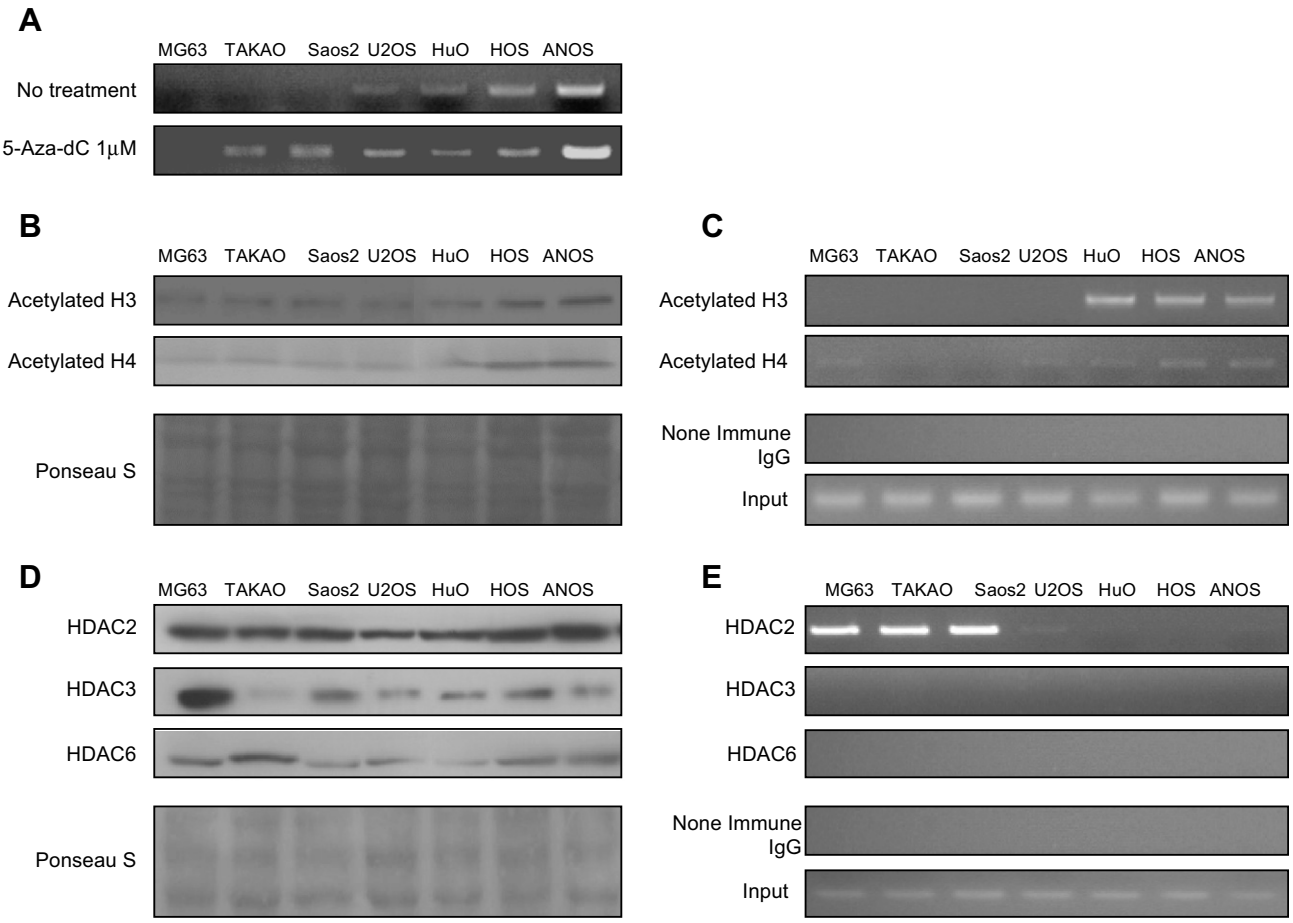

Fig. 1. Histone H3 and H4 tails surrounding the promoter of ChM-I were deacetylated by the binding of HDAC2 in ChM-I-negative OS cells. (A) mRNA expression of ChM-I before and after the treatment with 5-aza-dC (1 μM). (B) Protein expression of acetylated H3 and H4 tail in OS cells. (C) ChIP assay demonstrating the association of acetylated H3 and H4 with the core promoter region of the ChM-I gene in OS cells. (D) Protein expression of HDAC2, HDAC3, and HDAC6. (E) ChIP assay demonstrating the binding of HDACs to the core promoter region of the ChM-I gene in OS cells.

responsible for the deacetylation of histone associated with the promoter region of the ChM-I gene in ChM-I-negative OS cells.

Inhibition of HDAC2 restored the binding of Sp3 and the expression of ChM-I gene

MS-275 is an inhibitor for class I HDACs including HDAC2. The global histone acetylation was promoted by MS-275 in both ChM-I-positive (ANOS) and negative (MG63 and TAKAO) cells (Fig. 2A), whereas the local histone acetylation status caused by the MS-275 treatment differed among the cell lines. Histone H4 was further acetylated in ANOS, and the acetylation of both histone H3 and H4 was induced in MG63. No such induction of histone acetylation was observed in TAKAO (Fig. 2B).

Then we analyzed the binding of Sp3 and HDAC2 to the promoter region of the ChM-I gene after the treatment with MS-275 and/or 5-Aza-dC (Fig. 2C). In ANOS, the binding status of Sp3 or HDAC2 was not changed by any treatment (Fig. 2C). In MG63, the binding of Sp3 was induced in association with the elimination of the binding of HDAC2 by MS-275, but not by 5-Aza-dC treatment (Fig. 2C). In TAKAO, the binding of Sp3 was induced by 5-Aza-dC treatment with no change in the binding of HDAC2, and treatment with MS-275 caused a reduction in the binding of HDAC2, but failed to induce the binding of Sp3 (Fig. 2C). Simultaneous treatment with 5-Aza-dC and MS-275 resulted in a reduction in the binding HDAC2. The expression of the ChM-I gene in three cell lines correlated with the status of Sp3 binding; MS-275 treatment and 5-Aza-dC treatment induced the expression in MG63 and TAKAO, respectively. The binding of Sp1, HDAC3, and HDAC6 was not changed by MS-275 and/or 5-aza-dC treatment in these three cell lines (Fig. 2C). We performed identical experiments using Saos2, another ChM-I-negative OS cell line, and obtained essentially the same results as in TAKAO (data not shown).

CpG methylation of the promoter lesion was demethylated by MS-275 in a DNA replication-dependent manner

The induction of ChM-I gene expression in MG63 by HDACi was an unexpected result because the promoter

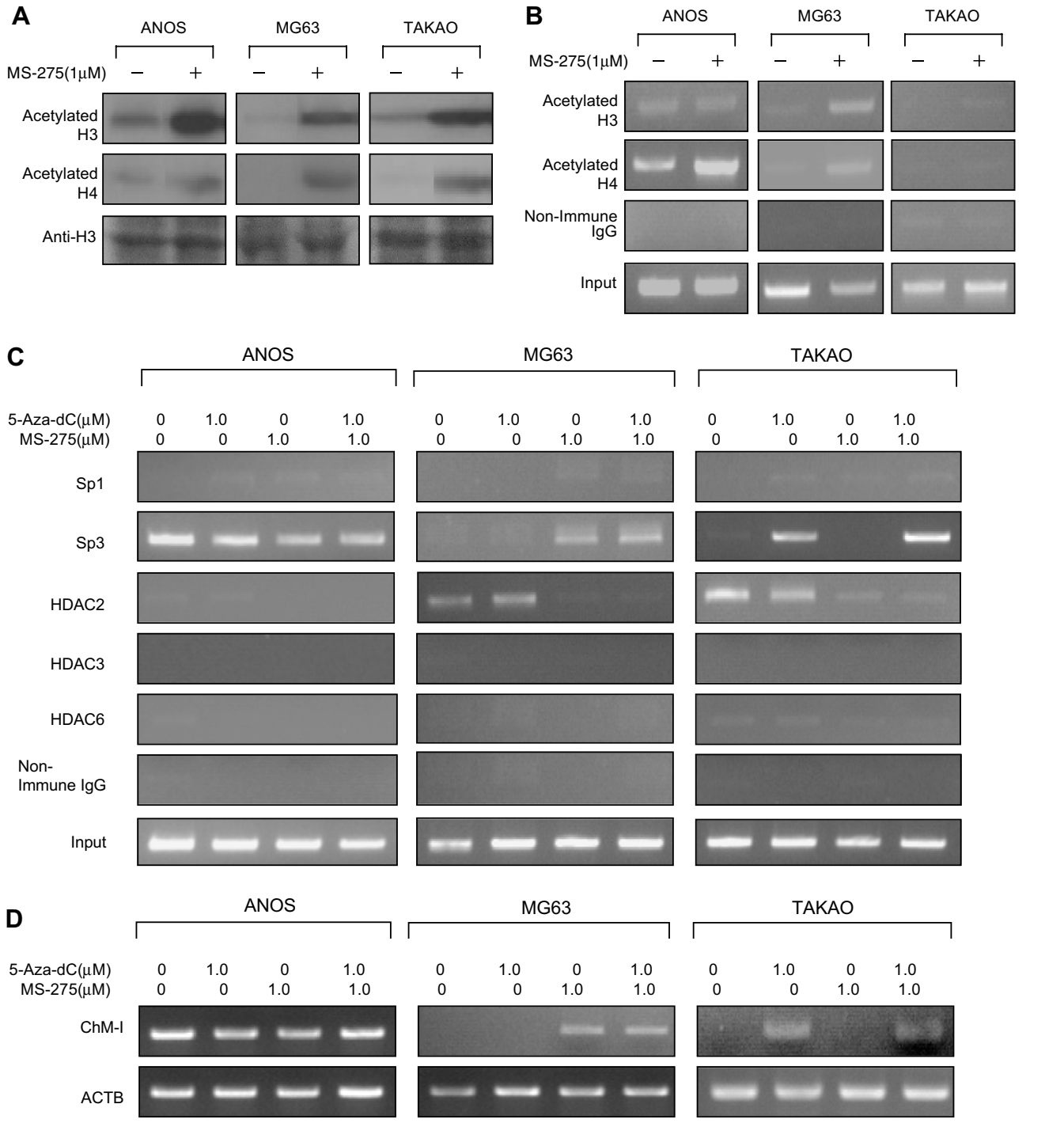

Fig. 2. Histone H3 and H4 tails were acetylated by MS-275, eliminating HDAC2. (A) Protein expression of acetylated H3 and H4 in OS cells with or without treatment with MS-275 (1 μM). (B) ChIP assay demonstrating the association of acetylated H3 and H4 with the core promoter region of the ChM-I gene in OS cells with or without treatment with MS-275 (1 μM). (C) ChIP assay demonstrating the binding of Sp1, Sp3, HDAC2, HDAC3, and HDAC6 with the core promoter region of the ChM-I gene in OS cells. Cells were treated with 5-Aza-dC and MS-275 at the indicated concentration. (D) mRNA expression of the ChM-I gene in OS cells treated with 5-Aza-dC and MS-275 at the indicated concentration.

region was heavily methylated in this cell line [1]. Bisulfite genomic sequencing of the promoter region revealed that the methylation of MG63 was significantly reduced and almost equivalent with that of ANOS (Fig. 3A). Methylation in the promoter region of TAKAO was also reduced but to much less of an extent (Fig. 3A). Conversion between acetylation and methylation of H3-K9 is known to regulate the methylation status of CpG [5]. Notably, the dimethylation of H3-K9 is known to be correlated with methylation of CpG [6]. Therefore, the modification of H3-

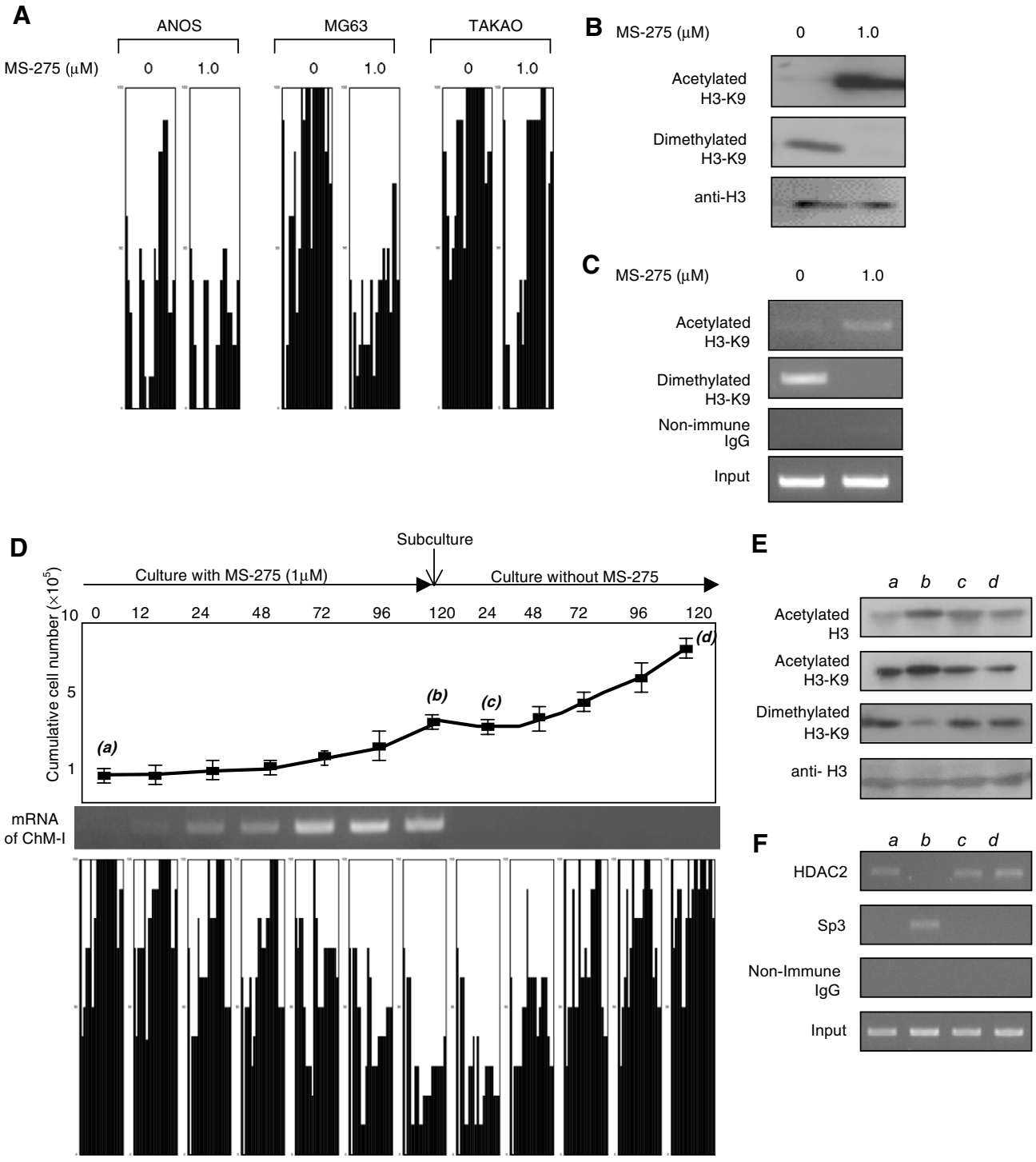

Fig. 3. Modification of the histone tail was associated with the methylation of CpG in the promoter region of the ChM-I gene. (A) Methylation status of the core promoter region of OS cells analyzed by bisulfite sequencing. Cells were treated with the indicated concentration of MS-275 for 96 h. *Y*-axis indicates the number of methylated alleles and *X*-axis indicates the position of each CpG site relative to the transcription start site. (B) Protein expression of acetylated H3-K9 and dimethylated H3-K9 in MG63 treated with the indicated concentration of MS-275. (C) ChIP assay demonstrating the association of acetylated H3-K9 and dimethylated H3-K9 with the core promoter region of ChM-I gene in MG63 cells treated with the indicated concentration of MS-275. (D) Temporal association of the mRNA expression and the methylation status of the core promoter region of the ChM-I gene in MG63 treated with MS275 (1 μM). Cumulative cell number (upper column), mRNA expression (middle column), and methylation status of the core promoter region of the ChM-I gene (lower column) were sequentially analyzed at the indicated time points. (E) Protein expression of acetylated H3, acetylated H3-K9, and dimethylated H3-K9 of MG63 at the time point indicated in (D). (F) ChIP assay demonstrating the binding of HDAC2 and Sp3 to the core promoter region of the ChM-I gene in MG63 at the time points indicated in (D).

K9 was analyzed by Western blotting (Fig. 3B) and ChIP assay (Fig. 3C). The dimethylation was converted to acetylation by MS-275 treatment in both global (Fig. 3B) and local promoter areas (Fig. 3C) in MG63.

To investigate the temporal relationship between the demethylation process and the acetylation process, the methylation status of the promoter region of MG63 was sequentially analyzed after exposure to MS-275 (Fig. 3D). The expression of ChM-I was gradually up-regulated in parallel with the demethylation of the promoter region in a DNA replication-dependent manner (Fig. 3D, a and b). At 120 h after the exposure to MS-275, the modification of H3-K9 was converted from dimethylation to acetylation (Fig. 3E, b), and Sp3 replaced HDAC2 (Fig. 3F, b). The expression of the ChM-I gene completely disappeared 24s hr after the withdrawal of MS-275 from the culture medium (Fig. 3D, c). At this time point, the binding of HDAC2 was restored eliminating the binding of Sp3 (Fig. 3F, c), although the promoter region was still hypomethylated (Fig. 3D, c). The methylation of CpG was gradually increased in a DNA replication-dependent manner and returned to the original status at 120 h after the withdrawal of MS-275 (Fig. 3D, d). These results suggested that MS-275 inhibited the maintenance of CpG methylation after DNA replication by replacing methylated H3-K9 with acetylated H3-K9.

Discussion

Both histone deacetylation and DNA methylation are important mechanisms to silence the transcription of genes unrelated to the biological phenotype of each cell. Acetylation of the histone tail is catalyzed by histone acetyltransferase (HAT) and analyzed using HDAC, each of which consists of a large family [4]. The acetylation status of histone associated with each genomic locus will be determined by the balance of two factors, HAT and HDAC, in each case, and therefore the deacetylation is regarded as a reversible silencing mechanism [4]. On the other hand, because there are no intrinsic factors with demethylase activity, DNA methylation catalyzed by DNA methyltransferase has been considered an irreversible silencing mechanism [3]. Recent reports have shown that these two mechanisms were not independent and moreover, closely related to each other [8–10].

One of the most intriguing results of this study is that the methylation of CpG was reduced by HDACi without further treatment with 5-Aza-dC. The reduction in methylation by HDACi seems to be dependent on DNA-replication (Fig. 3C). Several reports have emphasized the interplay between DNA methylation and histone methylation [11,12], notably, that H3-K9 dimethylation directry and H3-K9 acetylation inversely correlates with DNA methylation [5]. Loss-of-function mutations in H3-K9 methyltransferases of *Neurospora* and *Arabidopsis* were found to reduce overall levels of DNA methylation in vivo [8]. Consistent with these findings in fungi and plants, we here showed that the reduction in level of dimethylated histone H3-K9 was associated a reduction of DNA methylation at a specific residue in the regulatory region of a human gene, presumably due to the increase in acetylated histone H3-K9 caused by HDACi. The demethylating effect of another HDACi has been reported [9,10]. Further analysis of this intriguing matter may provide new insight into the regulation of gene expression by chromatin remodeling factors.

Hypermethylation of CpG dinucleotides in the core promoter region was a common feature of the three ChM-I-negative OS cell lines, which also shared deacetylation of the histone tail associated with the promoter region. The response to DNA-demethylating or histone-acetylating treatment was, however, quite different. In the case of TAKAO and Saos2, HDACi treatment failed to induce histone acetylation and therefore Sp3 binding, whereas 5-Aza-dC treatment induced Sp3 binding without modifying of histone acetylation, suggesting that DNA methylation may be a dominant factor in these cells. In the case of MG63, however, HDACi effectively induced histone acetylation and Sp3 binding in association with DNA demethylation. We have no clear explanation for the difference between these cell lines. MG63 is a unique cell line, in which the expression of osteogenic markers is not remarkable under normal culture conditions [1]. The transcription of the alkaline phosphates (ALP) gene was suppressed by CpG methylation and induced by 5-Aza-dC [13]. In contrast, 5-Aza-dC treatment failed to induce the expression of the osteocalcin and ChM-I genes, even when CpG methylation was eliminated [1,13]. In contrast, TAKAO and Saos2 retained features of osteogenic cells. Saos2 has strong ALP activity without any induction, and produces abundant immature bone tissue, osteoid, in the subcutaneous environment of athymic mice (data not shown). It is intriguing whether the difference in differentiation stage relates to the difference in the plasticity of epigenetic regulation, and the ChM-I gene in OS cells will be a suitable material with which to investigate this important issue.

Acknowledgments

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