Methylation in the Core-promoter Region of the Chondromodulin-I Gene Determines the Cell-specific Expression by Regulating the Binding of Transcriptional Activator Sp3*

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Transcriptional regulation of cell- and stage-specific genes is a crucial process in the development of mesenchymal tissues. Here we have investigated the regulatory mechanism of the expression of the chondromodulin-I (ChM-I) gene, one of the chondrocyte-specific genes, in osteogenic cells using osteosarcoma (OS) cells as a model. Methylation-specific sequence analyses revealed that the extent of methylation in the core-promoter region of the ChM-I gene was correlated inversely with the expression of the ChM-I gene in OS primary tumors and cell lines. 5-Aza-deoxycytidine treatment induced the expression of the ChM-I gene in ChM-I-negative OS cell lines, and the induction of expression was associated tightly with the demethylation of cytosine at −52 (C(−52)) in the middle of an Sp1/3 binding site to which the Sp3, but not Sp1, bound. The replacement of C(−52) with methyl-cytosine or thymine abrogated Sp3 binding and also the transcription activity of the genomic fragment including C(−52). The inhibition of Sp3 expression by small interfering RNA reduced the expression of the ChM-I gene in ChM-I-positive normal chondrocytes, indicating Sp3 as a physiological transcriptional activator of the ChM-I gene. These results suggest that the methylation status of the core-promoter region is one of the mechanisms to determine the cell-specific expression of the ChM-I gene through the regulation of the binding of Sp3.

Chondromodulin-I (ChM-I) is a 25-kDa glycoprotein originally purified from bovine epiphyseal cartilage on the basis of growth-promoting activity for chondrocytes (1) and subsequently revealed to be a potent vascular endothelial cell growth inhibitor (2). During embryonal development, the expression of the ChM-I gene is first observed in all of the cartilaginous tissues, which are composed of prehypertrophic chondrocytes (3). As the development proceeds, hypertrophic chondrocytes develop in the center of cartilaginous bone rudiments where the expression of the ChM-I gene shows a marked decrease (3). No expression of the ChM-I gene is observed in bone tissues developing after vascular invasion in the area adjacent to hypertrophic chondrocytes (3). In matured limbs, the expression of the ChM-I gene is limited to cells in the resting, proliferating, and early hypertrophic zone of the growth plate (2–4). These results suggest that the expression of the ChM-I gene is regulated strictly in a cell- and stage-related manner, although the molecular mechanisms leading to this spatiotemporal expression have not been elucidated.

Osteosarcoma (OS) is defined as a sarcoma that produces a bone matrix called osteoid, suggesting that the precursor cells of OS are cells of the osteogenic lineage (5). The degree of differentiation as osteoblasts, however, differs considerably among OS ranging from tumors with a large amount of osteoid and expressing a number of bone-related genes such as alkaline phosphatase (ALP) and osteocalcin (OCN) genes, namely osteoblastic OS (OBOS), to tumors in which an osteoid is hardly seen, fibroblastic OS (FBOS) (6–8). A particular intriguing subtype of OS is chordroblastic OS (CBOS) in which tumor cells directly produce immature cartilage in addition to osteoid (5, 9), suggesting that tumor cells in this subtype have the potential to differentiate into both osteogenic and chondrogenic cells. These clinical findings suggest that the precursor cells of OS range from mesenchymal stem cells to mature osteoblasts and that OS cells can be used as materials to investigate the regulatory mechanisms of cell- and stage-specific genes such as the ChM-I gene.

Here we first analyzed the expression of the ChM-I gene in primary OS tumors and cell lines and found that the gene was expressed strongly in CBOS but not in tumors of other subtypes. This result prompted us to investigate the involvement in regulation of the expression of the ChM-I gene of an epigenetic mechanism, which has been studied extensively as the mechanism controlling the expression of cell- and stage-specific genes (10). We found that the expression of the ChM-I gene was regulated positively by a transcription factor, Sp3, and that the binding of Sp3 was regulated by the methylation status in the core-promoter region of the ChM-I gene, especially at one Sp3 binding site.

EXPERIMENTAL PROCEDURES

Tissue Samples—Primary tumor tissues from 24 OS cases were obtained at either biopsy or resection. All were conventional high grade tumors, and histological subtypes were OBOS in 13 cases, CBOS in 6 cases, and FBOS in 5 cases. Tumor specimens were frozen quickly and kept at −80 °C until nucleic acid extraction. As a control of the expres-
sion of the ChM-I gene, total RNA was extracted from the articular cartilage of the ankle joint of an 11-year-old male who underwent an above-knee amputation because of osteosarcoma of the femur.

**Cell Culture and Reagents**—The human osteosarcoma cell lines Saso2, HuO, HOS, MG63, and U2OS were obtained from either ATCC or the Japanese Cancer Research Resources Bank. The human osteosarcoma cell line TAKAO is a clonal cell line derived from SU cells (11). ANOS was established in our laboratory using material from a 12-year-old female with CBOS. These cell lines were maintained in Dulbecco’s modified Eagle’s medium with 10% heat-inactivated fetal bovine serum. Chondrocytes were isolated from articular cartilage tissue from the ankle joint of an 11-year-old male who underwent an above-knee amputation because of a malignant bone tumor of the femur. *Drosophila* SL2 cells were kindly provided by T. Ishihara and maintained in Drosophila’s insect medium supplemented with 10% heat-inactivated fetal bovine serum.

**Western Blotting**—Proteins were extracted from each cell line by SDS lysis buffer and settled on ice for 10 min. DNA cross-linked with 45 volts for 3 h, and the gel then was dried and autoradiographed. For the competition assay, the DNA-protein complex was digested with 1 µg of anti-Spi or anti-Sp3 antibody for 1 h before being mixed with labeled DNA. Luciferase Assay—The 533-bp fragment from −446 to +87 relative to the transcription initiation site of the ChM-I gene was amplified by PCR, digested by SacI and XhoI, and cloned into a luciferase reporter plasmid, PGV-B (Toyokyo Ink, Tokyo, Japan), which was designated PGV-B-IF. The fragment having a thymine residue at position −52 was synthesized by PCR, cloned into pSP64K (Stratagene), and designated PGV-B-mtf1. SL2 cells (5×10⁵) were seeded in a 35-mm dish, and 1 µg of each reporter plasmid was co-transfected with 1 µg of the Sp1 (pPAcSpi) or Sp3 (pPAcSp3) containing a truncated form of Sp3 and pPAcUsP3 containing a full-length form of Sp3 expression vector (16) into Schneider cells using EFFECTEN (Qiagen) according to the manufacturer’s instructions. Transfection efficiency was standardized by the co-transfection of 1 ng of pRL-TK control vector (Toyokyo Ink). Cells were harvested 24 h after transfection, and luciferase assays were performed with the PicaGene Dual SeaPansy system (Toyokyo Ink). Firefly-luciferase activity and SeaPansy-luciferase activity were measured as relative light units with a luminometer (Lumino, STRATEC Biomedical Systems). The firefly-luciferase activity was normalized for transfection efficiency based on the SeaPansy-luciferase activity. Each experiment was performed in triplicate. Transfection experiments also were performed using ANOS, TAKAO, and primary chondrocytes instead of Schneider cells and the human Sp3 expression vector pRC/CMV/Sp3 (for review see Ref. 16) instead of pPAcUsP3.

**RNA Interference—**RNA interference was achieved using small interfering RNA (siRNA) for the Sp1 and Sp3 genes basically as recommended by the manufacturer (Dharmacon). The 21-nucleotide duplexes containing the pattern AA/N19/UU were selected to obtain symmetric 2-nt 3’-overhangs of an identical sequence. Luciferase siRNA duplex (GL2R1N, Dharmacon) was used as a negative control. Transient transfections of siRNAs (1 µg) were performed using Lipofectamine 2000 (Invitrogen). RNAs and proteins were prepared 48 h after transfection and used for the RT-PCR and Western blotting.

**Chromatin Immunoprecipitation (ChIP)—**The suitability of each antibody for the ChIP assay was confirmed by immunoprecipitation-Western blotting assay (data not shown). 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 200–1,000 bp. One-tenth of the sample was set aside as an input control, and the rest was preclared with salmon sperm DNA protein A-Sepharose beads (Upstate Biotech- nology). 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-Cl and 1 mM EDTA). The chromatin-antibody complexes were eluted with elution buffer (1% SDS and 0.1 M NaHCO₃). Protein DNA cross-links were reversed with 5 mM 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’-GATTGACCCGCAATGAGATG-3’; antisense, 5’-GCCAGGCTGGATCTCCTCGGTG-3’; respectively) and filling in by DNA polymerase I (TOYOBO, Osaka, Japan). The reaction was performed in 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. We confirmed the exponential increase of PCR product at this number of cycles in several preliminary experiments (data not shown).

**RESULTS**

**CBOS Expresses the Cartilage-related Genes—**The expression of the ChM-I gene along with a number of bone- and cartilage-related genes was analyzed by RT-PCR in 24 OS, and the representative data of 12 cases were presented in Fig. 1A. No substantial difference was observed in the expression levels of bone-related genes among three types of OS. As for the cartilage-related genes, however, three subtypes showed a considerable difference. CBOS expressed all of the analyzed carti-
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Fig. 1. Expression of bone- and cartilage-related genes in OS. mRNA expression of bone-related genes (OSF2, OCN, ALP, and COL1A1) and cartilage-related genes (SOX9, ChM-I, COL2A1, COL9A1, COL11A2, and AGC) in primary OS tumors (A) and OS cell lines (B) were analyzed by RT-PCR.

Fig. 2. Expression of the ChM-I in OS. A, quantitative analyses of mRNA expression of the ChM-I gene in primary tumors and cell lines. Expression level of the ChM-I gene in each sample was demonstrated as a value relative to that in normal cartilage tissue as described under “Experimental Procedures.” B, Western blot analysis of the ChM-I protein in OS cell lines.

The mRNA expression of bone-related genes (OSF2, OCN, ALP, and COL1A1) and cartilage-related genes (SOX9, ChM-I, COL2A1, COL9A1, COL11A2, and AGC) varied significantly among other cell lines. The expression of cartilage-related genes were expressed in ANOS that was established between CBOS and other types of OS was the expression of the downstream genes such as COL2A1 and AGC genes showed clear difference among subtypes. The most notable difference was the expression level of the SOX9 gene in CBOSs showed hypomethylation of CpGs in this region, which showed a clear association with the histological subtype (Fig. 2A). Most CBOS samples showed a value comparable with that in normal cartilage (0.33–8.15; mean value, 2.58), whereas the expression level in FBOS samples was extremely low (<0.0017). OBOS showed intermediate values with variances among samples (0.8–0.0004) (Fig. 2A). The expression level of the ChM-I gene in OS cell lines also varied considerably (0.98–0.02) with one cell line, ANOS, having a value (0.98) equivalent to that of the articular cartilage (Fig. 2A). ChM-I protein was detected only in ANOS by Western blotting (Fig. 2B), suggesting that mRNA expression level similar to that in articular cartilage was required to be detected by Western blotting.

Regulatory Regions of the ChM-I Gene Are Methylated in ChM-I-negative Cells—To investigate the role of epigenetic regulation for the expression of the ChM-I gene, the methylation status of the transcription regulatory region of the ChM-I gene was analyzed. Yanagihara et al. (15) report that the 533-bp region from −487 to +46 relative to the transcription start site contained the major transcription activity of the ChM-I gene. Twenty-one CpG sites were found in this region, and the methylation profile of each site was analyzed by bisulfite genomic sequencing (Fig. 3A). In normal cartilage cells, the CpG sites in this region were hypomethylated. ANOS in which the expression of the ChM-I gene was equivalent to that in normal cartilage cells also showed hypomethylation at the CpG sites from −136 to +15, whereas the CpG sites located further downstream were methylated. In the other six cell lines, the mRNA expression level was correlated with the extent of methylation. The promoter region of Saos2 with no expression of the ChM-I gene was methylated extensively, whereas that of HOS with a relatively high level of expression of the ChM-I gene was hypomethylated. To investigate whether the methylation in the core-regulatory region was a product of cell culture, the methylation profile in this region was analyzed in the primary tumors of four cases of each subtype (Fig. 3B). All of the four CBOSs showed hypomethylation of CpGs in this region, which
were methylated heavily in all of the FBOSs and OBOSs. These results confirmed that the methylation of the core-regulatory region was not a product of cell culture and was closely associated with the reduced expression of the ChM-I gene in primary OS tumors.

**Methylation of C(-52) Is Correlated with Reduced Expression of the ChM-I Gene**—To further investigate the significance of the methylation as the regulatory mechanism, three cell lines (TAKAO, Saos2, and MG63) showing no expression of the ChM-I gene by standard RT-PCR (Fig. 1B) were treated with a

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**Fig. 3. Methylation profile of CpG sites in the core-regulatory region of the ChM-I gene in OS.** Methylation profiles of OS cell lines (A) and primary OS tumors (B). Bisulfite genomic sequencing data for 10 alleles in each sample are presented, and closed and open squares indicate the methylated and non-methylated alleles, respectively. Numbers in boxes on the left indicate the position of each CpG site relative to the transcription start site. The expression of the ChM-I gene in each sample detected by standard RT-PCR was demonstrated above the bisulfite genomic sequencing data.
demethylating agent, 5-Aza-dC. The expression of the ChM-I gene was induced in all three cell lines, although MG63 required a much higher concentration of 5-Aza-dC (10 μM) than TAKAO and Saos2 (Fig. 4A). Bisulfite genomic sequencing before and after the treatment with 5-Aza-dC showed extensive demethylation in the core-regulatory region in TAKAO and, to a lesser extent, in Saos2 but no demethylation in MG63 (Fig. 4B), which was consistent with the level of the ChM-I gene expression at this concentration of 5-Aza-dC (Fig. 4A). Comparing the methylation profiles at each site before and after the treatment, we found that the methylation of cytosine at −52 (C(−52)) showed a close correlation with the expression of the ChM-I gene. Almost all of the alleles of C(−52) in the three cell lines (9/10 in TAKAO, 10/10 in Saos2, and 10/10 in MG63) were methylated before the 5-Aza-dC treatment. After the treatment, 7 and 6 of 10 alleles were free from methylation in TAKAO and Saos2, respectively, whereas only one of ten alleles was demethylated in MG63. In the other OS cell lines and primary OS tumors, the methylation profile of C(−52) was correlated well with the level of expression (Fig. 2, A and B), suggesting that non-methylated C(−52) is critical for the expression of the ChM-I gene in OS cells.

**Sp3, but Not Sp1, Binds to the Sp1/3 Binding Site Containing C(−52) Using TESS and TFSEARCH**

We found that C(−52) was in the middle of the consensus sequence of the Sp1/3 binding site (CGCGG). To investigate whether this site is a target for the protein binding, a 21-bp oligonucleotide corresponding to −72 to −45 was synthesized (GR2) and used for electrophoresis mobility shift assay with the nuclear extract of ANOS. Electrophoresis mobility shift assay showed that GR2 bound to nuclear protein and that the binding was inhibited by unlabeled GR2 but not by unlabeled GR1 corresponding to −96 to −69, which indicates specific binding (Fig. 5A). Both C(−52)tmC-GR2 and C(−52)/T-GR2 failed to bind the nuclear extract, indicating that the preservation of a non-methylated C(−52) was critical for the protein binding (Fig. 5B). The addition of anti-Sp1 antibody to the GR2-protein mixture had no effect on the GR2-protein binding (Fig. 5C). On the other hand, a supershifted band was observed when anti-Sp3 antibody was added to the GR2-protein mixture (Fig. 5C), indicating that Sp3 bound to the Sp1/3 site including C(−52) in ANOS. The binding of Sp3 to the core-promoter region in vivo was confirmed by ChIP analysis (Fig. 5D). Sp3, but not Sp1, bound
to the core-promoter region in ANOS. Neither Sp1 nor Sp3 binding was detected in MG63 before or after the treatment with 5-Aza-dC (1.0 μM), whereas Sp3 binding was induced in TAKAO after the treatment with 5-Aza-dC, which was consistent with the results on ChM-I gene expression.

Sp3 Up-regulates ChM-I Promoter Activity through the Sp1/3 Sites Containing C(−52)—The Sp1 (pPacSp1) or Sp3 (pPacSp3 or pPacUSp3) expression vector was introduced into SL2 cells together with the reporter plasmid containing the wild-type (PGV-B-f1) or mutant (PGV-B-mtf1) fragment (Fig. 6). Co-transfection of PGV-B-f1 with pPacSp1 (Fig. 6b) and pPacSp3 (Fig. 6c) showed transcriptional activity at a level similar to that obtained with the empty expression vector (pPac) (Fig. 6a), whereas pPacUSp3 greatly enhanced the activity (Fig. 6d). This induction was abolished completely when PGV-B-mtf1 was used instead of PGV-B-f1 (Fig. 6h). These results suggested a positive regulatory role for Sp3 in the ChM-I gene expression, which was mediated by the Sp1/3 site containing C(−52). The induction of the transcription activity of the ChM-I gene by Sp3 was analyzed further in OS cell lines and primary chondrocytes (Fig. 7). Transfection of human Sp3 expression vector (pRC/CMV/Sp3) into ANOS (Fig. 7A) or primary chondrocytes (Fig. 7B), which were positive for the expression of endogenous ChM-I gene, induced the luciferase activity when the reporter plasmids containing the wild-type fragment with C(−52) was co-transfected. The induction level in primary chondrocytes was much lower than that in ANOS, probably because of low transfection efficiency determined by the activity of the control luciferase plasmid, pRL-TK (data not shown). No induction was observed when the reporter plasmids containing the mutant fragment with T(−52) were used. Similar results were observed also in TAKAO (Fig. 7C), which was negative for the expression of the endogenous ChM-I gene, further suggesting that the expression of endogenous ChM-I gene in TAKAO was repressed by methylation at the C(−52) site.

Reduction of the Sp3 Gene Expression Results in the Reduction of the ChM-I Gene Expression—Both Sp1 and Sp3 were expressed in all of the OS cell lines (Fig. 8A). To further investigate the role of Sp3 in the expression of the ChM-I gene, double-stranded siRNA for Sp1 or Sp3 was transfected into ChM-I-expressing cells. siRNA for Sp1 and Sp3 effectively reduced the mRNA expression of corresponding genes in ANOS, whereas the expression of the ChM-I gene was reduced only when the expression of the Sp3 was inhibited (Fig. 8B). The expression level of the ChM-I gene was confirmed by quantitative RT-PCR, which showed the clear association of siRNA for the Sp3 with the reduction of the ChM-I gene expression (Fig. 8D). The effects of siRNA for Sp1 and Sp3 were analyzed further at protein level, and again, the reduction of ChM-I protein expression was observed only when the expression of Sp3 was inhibited by siRNA (Fig. 8F). Identical results were obtained when human primary chondrocytes were used instead of ANOS. The reduction of Sp3 correlated with the reduction of the ChM-I gene at mRNA (Fig. 8, C and E) and protein level (Fig. 8G), suggesting that Sp3 is a positive regulator of the ChM-I gene in normal chondrocytes.

**DISCUSSION**

Cells of the chondrogenic and osteogenic lineages are considered to share mesenchymal stem cells as a common ancestor.
(17, 18), and we and others (19, 20) provide the evidence for the presence of bi-directional precursors, which can differentiate into either chondrogenic or osteogenic cells. In addition to the ChM-I gene, CBOS expressed a number of cartilage-related genes such as the COL2A1, COL9A1, and AGC genes (Fig. 1, A and B), suggesting that CBOS may stem from bi-directional precursors. The expression of "the master gene has been proposed to regulate the expression of the entire set of genes required for differentiation to specific direction, and in the case of cells of chondrogenic lineage, the SOX9 has been regarded as such (21). However, all seven OS cell lines expressed the SOX9 gene irrespective to the expression of downstream genes such as the COL2A1 gene (Fig. 1B) (21). These results suggested the presence of mechanisms to turn off the expression of cartilage-specific genes in OS cells. The results in this study demonstrated an example in which the epigenetic mechanism is involved in such a tissue-specific expression of cartilage-related genes.

Little is known regarding the transcriptional regulation of the ChM-I gene. As for extrinsic signals, several growth factors have been shown to reduce the expression of the ChM-I gene including fibroblast growth factor-2, transforming growth factor-β, parathyroid hormone, and parathyroid hormone-related peptide (3, 4), but no definite positive regulators for the expression of the ChM-I gene are known at present. As for the genomic structures responsible for the transcriptional regulation, Yanagihara et al. (15) determine the major regulatory region for the transcription of the ChM-I gene and found that the transcription factor YY-1 down-regulated the transcription (15). However, the role of YY-1 in vivo was not demonstrated.
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![Diagram of experimental results]

FIG. 8. Expression of Sp3 associates with the expression of ChM-I. A, Western blot analyses of Sp1 and Sp3 in cell lines. B–G, inhibition of the Sp3 expression reduced the expression of ChM-I gene. siRNA (1 µg) for Sp1, Sp3, or luciferase (GL2) was transfected into ANOS (B, D, and F) or primary chondrocytes (C, E, and G), and total RNA and protein were extracted at 48 h after the transfection. mRNA expression of Sp1, Sp3, and ChM-I genes was analyzed by the standard RT-PCR (B and C). The expression of the ChM-I gene was evaluated further by quantitative RT-PCR as described under “Experimental Procedures” (D and E). *p < 0.05. The expression of Sp1, Sp3, and Ch-M-I protein was analyzed by Western blotting (F and G).

and the relationship of YY-1 with the down-regulators mentioned above has yet to be proved. In this report, we have shown for the first time that Sp3 is a potent positive regulator of the ChM-I gene expression, and the data from the siRNA experiments clearly indicated that Sp3 is a critical factor also in normal chondrocytes. Sp3 is a transcription factor belonging to the Sp1 family, which binds to the consensus GC or GT box (22). Sp3 was isolated as a homologue of Sp1 (23) and reported as the factor that inhibits the function of Sp1 (16). However, evidence is accumulating to suggest that Sp3 also has the ability to stimulate transcription involving the promoters of various genes (24–26), suggesting that Sp3 is a bi-functional transcriptional regulator (24, 27). The studies of Sp3-deficient mice indicate that Sp3 is essential for postnatal survival, late tooth development, and late bone development (28, 29), suggesting its involvement in bone and cartilage metabolism.

Because Sp3 is a transcription factor expressed ubiquitously and indeed all of the OS cell lines expressed Sp3 (Fig. 8A), it is reasonable to assume that the binding of Sp3 to the core-promoter region is regulated by some mechanisms that may determine the cell- and stage-specific expression of the ChM-I gene. The regulation of higher order chromatin structures by DNA methylation is crucial to tissue-specific gene expression and global gene silencing (30, 31), and we found that the binding of Sp3 was regulated by methylation in the core-regulatory region of the ChM-I gene and that treatment with 5-Aza-dC induced the expression of the ChM-I gene in association with the binding of Sp3. We have no clear explanation as to why MG63 required a high concentration of 5-Aza-dC to reduce the methylation (Fig. 3A). There was no significant difference in the expression of the Dnmt1 gene among the cell lines (data not shown). Because the growth of MG63 showed no significant change following treatment with 10 µM 5-Aza-dC, which caused severe growth inhibition in all of the other cell lines, there seemed to be a mechanism specific to MG63. Hypomethylation at CpG islands is a common feature of cancer cells (31), and treatments with inhibitors for methyltransferase such as 5-Aza-dC leads to the reactivation of methylation-silenced genes in many cancers (32). Considering these findings, the methylation-associated silencing of the ChM-I gene in OBOS and FBOS might be a transformation-related phenomenon. However, it is also likely that the silencing of the ChM-I gene is a physiological phenomenon occurring during the differentiation of mesenchymal cells as demonstrated for other genes (33–35). We showed that the removal of methylation restored the binding of Sp3 and induced the expression of the ChM-I gene in osteoblastic cells (Fig. 4B). However, quantitative RT-PCR revealed that the level of expression was lower than normal level (data not shown), suggesting that the binding of Sp3 is necessary but not enough to gain the full transcriptional activity. The investigation of negative regulators such as YY-1 or other epigenetic regulatory mechanisms such as histone acetylation is required to understand the entire picture of the transcriptional regulation of the ChM-I gene.

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