

Sp7/Osterix is involved in the up-regulation of the mouse pro- α 1(V) collagen gene (*Col5a1*) in osteoblastic cells

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ABSTRACT

Sp7/Osterix, a transcription factor whose expression is restricted in osteoblasts, belongs to the Sp family of transcription factor that bind to G/C-rich sequences. Previous studies have identified a Sp1 binding site in the proximal promoter region of the mouse *Col5a1* gene, but it did not activate or repress this gene in a mouse fibroblast cell line and a human rhabdomyosarcoma cell line. The purpose of the present study was to clarify the involvement of Sp7/Osterix in the mouse *Col5a1* gene. A functional analysis revealed that mutation of the Sp1 binding site specifically decreased the promoter activity in osteoblastic cells. An overexpression of Sp7/Osterix significantly increased the promoter activity and the endogenous mRNA levels of the *Col5a1* gene in osteoblastic cells. Conversely, siRNA-mediated knockdown of Sp7/Osterix decreased the promoter activity and the endogenous mRNA levels of the *Col5a1* gene. These effects on promoter activity were canceled when the mutant construct of Sp1 binding site was introduced. Consistent with these data, the experiments using an osteoblast differentiation model showed increased promoter activity and endogenous mRNA levels, along with increased Sp7/Osterix during differentiation. Therefore, type V collagen appears to be involved in bone formation.

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1. Introduction

Bone formation is a tightly regulated process which occurs when mesenchymal precursor cells differentiate to osteoblasts. Osteoblasts are responsible for two types of bone formation: intramembranous and endochondral ossification. Osteoblasts can directly differentiate from mesenchymal condensations in intramembranous ossification, whereas cartilage formation is an intermediate step in endochondral ossification. In both cases, osteoblasts play a central role in the production of the extracellular matrix and the mineralization of the bone matrix. Recent molecular genetic studies in mice and humans have demonstrated a role for transcription factors that govern bone formation (Karsenty, 2003; Nakashima and de Crombrughe, 2003; Marie, 2008). Osteoblast differentiation from mesenchymal precursors is controlled by a hierarchy of transcription factors. Runx2 and Sp7/Osterix are transcription factors that are expressed in osteoblasts. Runx2 is a member of the Runt family of transcription factors and is expressed in mesenchymal cells at the beginning of skeletal development, and is present in osteoblasts throughout differentiation (Ducy et al., 1997; Komori et al., 1997; Otto et al., 1997). Sp7/Osterix acts downstream of Runx2. Sp7/Osterix, which is a zinc finger-containing transcription factor, belongs to the Sp family of transcrip-

tion factors that bind to G/C-rich sequences and activate mRNA synthesis from genes containing these functional recognition sites (Nakashima et al., 2002).

Collagens are the major constituents of extracellular matrices and are critical for the formation and function of the organs in the body (van der Rest and Garrone, 1991). Type V collagen is a quantitatively minor fibrillar collagen with a broad tissue expression. It is present in tissues where type I collagen is expressed. Type V collagen is incorporated into the fibrils of the more abundant type I collagen, and acts as a regulator of the size and the shape of the fibrils (Birk, 2001). There are several type V isoforms that differ in chain composition. The major isoform is $[\alpha 1(V)]_2\alpha 2(V)$ which is present in many tissues. In addition, the $[\alpha 1(V)]_3$ homotrimer and the $\alpha 1(V)\alpha 2(V)\alpha 3(V)$ heterotrimer have also been identified (Haralson et al., 1980; Rhodes and Miller, 1981). Defects in the human *COL5A1* and *COL5A2* genes have been identified in half of the cases of classic Ehlers–Danlos syndrome (EDS, type I/II) (Toriello et al., 1996; Michalickova et al., 1998).

Characterization of the core promoter of the mouse *Col5a1* gene has revealed that the CBF/NF- κ B factor acts as a transcriptional activator of the *Col5a1* gene in the mouse NIH3T3 cell and the human rhabdomyosarcoma A204 cell (Sakata-Takatani et al., 2004). Two GC-rich domains were also found in the core promoter of the mouse *Col5a1* gene. Sp1 bound to the proximal GC-rich domain, but did not activate or repress the *Col5a1* gene in these cell lines. In the present study, Sp7/Osterix, which binds to GC-rich domains, in the activation of *Col5a1* gene during osteoblast differentiation, was examined.

Abbreviation: CHIP assay, chromatin immunoprecipitation assay.

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2. Results

2.1. Functional analysis of the *Col5a1* gene using the MC3T3-E1 cell line

To examine the effect of the Sp1 binding site in the *Col5a1* in osteoblastic cells, a functional analysis of *Col5a1* gene was performed using MC3T3-E1 cells. We used wild-type and mutant constructs that were previously generated (Fig. 1A) (Sakata-Takatani et al., 2004). The m1-Luc, which has a mutated Sp1 binding site, caused an approximately 50% decrease in promoter activity in MC3T3-E1 cells (Fig. 1B). However, it had no effect in A204 and NIH3T3 cells, as seen in a previous study (Fig. 1C and D) (Sakata-Takatani et al., 2004). The m2-Luc, which was mutated in the CBF/NF-Y site, had decreased promoter activity, and the m3-Luc, which had a mutated downstream Sp1-like binding site, had no effect on the promoter activity in any of the three cell lines (Fig. 1B, C and D).

2.2. The effect of Sp7/Osterix on endogenous expression of the *Col5a1* gene

To examine the effect of Sp1 and Sp7/Osterix, we overexpressed Sp1 and Sp7/Osterix in MC3T3-1 cells. The amounts of overexpressed Sp1 and Sp7/Osterix were examined by real-time PCR, and no

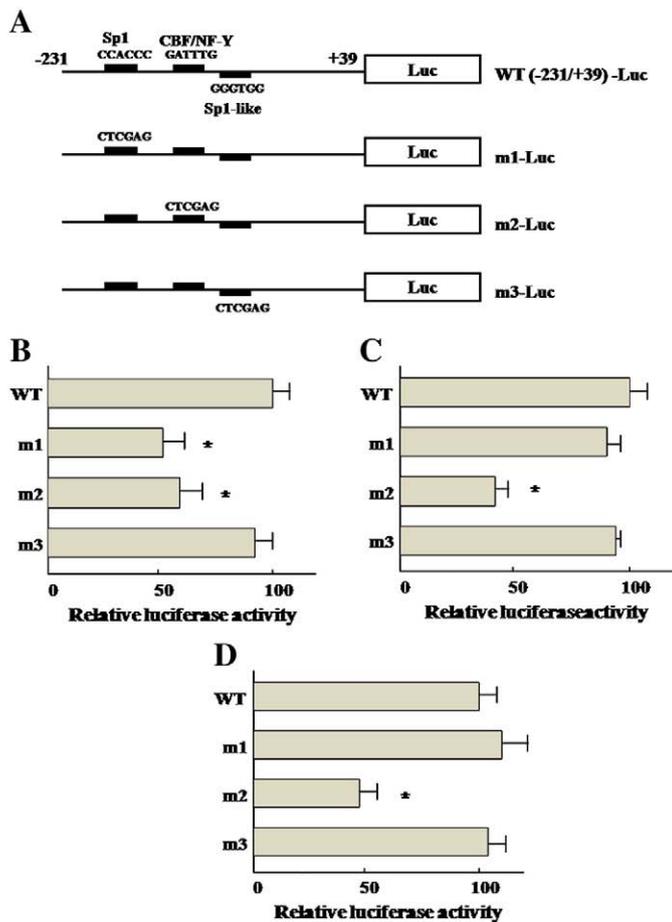


Fig. 1. Functional analysis of the proximal *Col5a1* promoter. (A) A schematic illustration of the luciferase reporter constructs. mut1, mut2 and mut3 are mutated in the Sp1, CBF/NF-Y and Sp1-like binding site of the WT (-231/+39-Luc) construct (Sakata-Takatani et al., 2004). Transient transfections with the wild-type and the mutant constructs were carried out in MC3T3-E1 (B), NIH3T3 (C) and A204 (D) cells. The histograms indicated the percentage of activity normalized to the internal control and are shown relative to the activity of the wild-type construct transfected into each cell line. Data are presented by the mean \pm S.D. of at least three independent experiments. * p <0.05 compared with the wild-type.

significant differences were observed between the expressed amounts (Fig. 2A and B). The overexpression of both Sp1 and Sp7/Osterix increased the endogenous expression of the *Col5a1* gene in MC3T3-E1 cells (Fig. 2C). The effect of Sp7/Osterix overexpression was approximately two times greater than that of Sp1 overexpression.

Conversely, the selective inhibition of Sp1 and Sp7/Osterix by specific siRNAs was performed. No differences were observed between inhibition by siRNA knockdown of Sp1 and Sp7/Osterix (Fig. 3A and B). siRNA knockdown of Sp1 or Sp7/Osterix decreased the endogenous mRNA levels of the *Col5a1* gene by 35 to 40% (p <0.05) (Fig. 3C). In contrast to the overexpression experiments mentioned above (Fig. 2C), the inhibition by siRNA-mediated knockdown of Sp1 and Sp7/Osterix was similar. This may be due to the abundance of siRNA to Sp1 and Sp7/Osterix, comparing to the corresponding mRNAs.

2.3. The effect of Sp7/Osterix on the promoter activity of the *Col5a1* gene

To examine the effect of Sp7/Osterix on the promoter activity of the *Col5a1* gene, Sp7/Osterix was cotransfected with the wild-type and three mutant luciferase constructs into MC3T3-E1 cells. Sp7/Osterix significantly activated the wild-type promoter of the luciferase reporter gene (Fig. 4A). However, transfection had no effect on the cells that were cotransfected with m1-Luc, in which the Sp1 binding site of the promoter of the luciferase reporter gene was mutated (Fig. 4B). However, Sp7/Osterix activated m2-Luc and m3-Luc transcription, in which the CBF/NF-Y and Sp1-like binding sites, respectively, was mutated (Fig. 4C and D). The activity using the m3-Luc construct was similar to that using the wild-type construct, but the luciferase activity using the m2-Luc construct was lower.

Conversely, the selective inhibition of siRNA Sp7/Osterix after Sp7/Osterix overexpression was performed. The *Col5a1* promoter activity using the wild-type construct was decreased by nearly 50% (Fig. 5A). Similarly, the levels using m2-Luc and m3-Luc also decreased

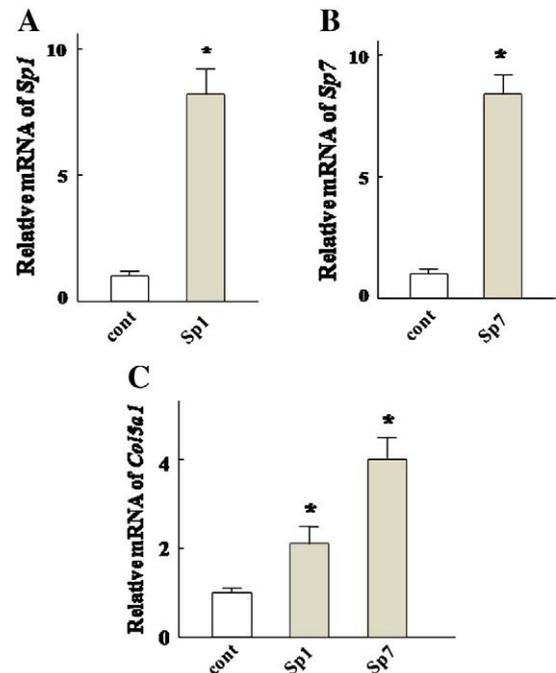


Fig. 2. (A, B) The real-time RT-PCR analysis was performed with total mRNA from MC3T3-E1 cells which were transfected by overexpressing Sp1 (lane 2 in A) and Sp7/Osterix (lane 2 in B). The empty vector was transfected as a control (lane 1 in A and B). (C) Effects of the Sp1 and Sp7/Osterix expression constructs on the endogenous mRNA levels of *Col5a1*. Data are presented by the mean \pm S.D. of at least three independent experiments. * p <0.05 compared with the control.

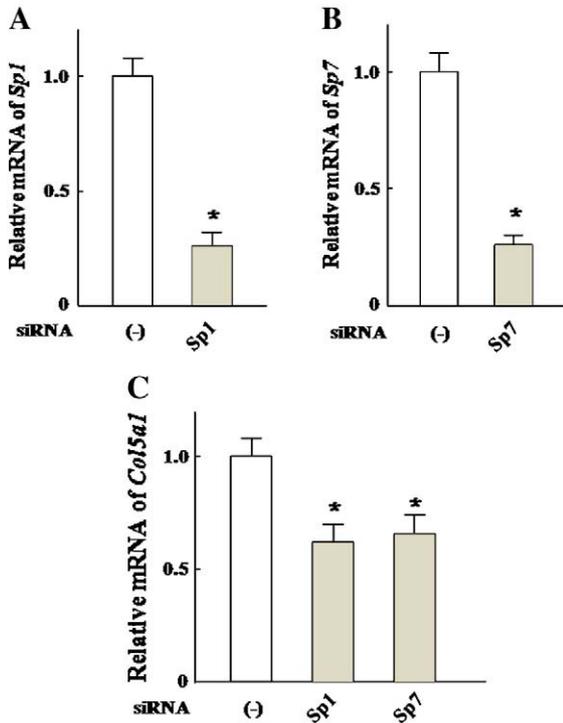


Fig. 3. (A, B) The real-time RT-PCR analysis was performed with total mRNA from the MC3T3-E1 cells, which were transfected with siRNA targeted against Sp1 (lane 2 in A) and Sp7/Osterix (lane 2 in B). (C) The effect of selective inhibition of Sp1 and Sp7/Osterix on the endogenous mRNA levels of *Col5a1*. Data are presented the mean \pm S.D. of at least three independent experiments. * $p < 0.05$ compared with the (-) control.

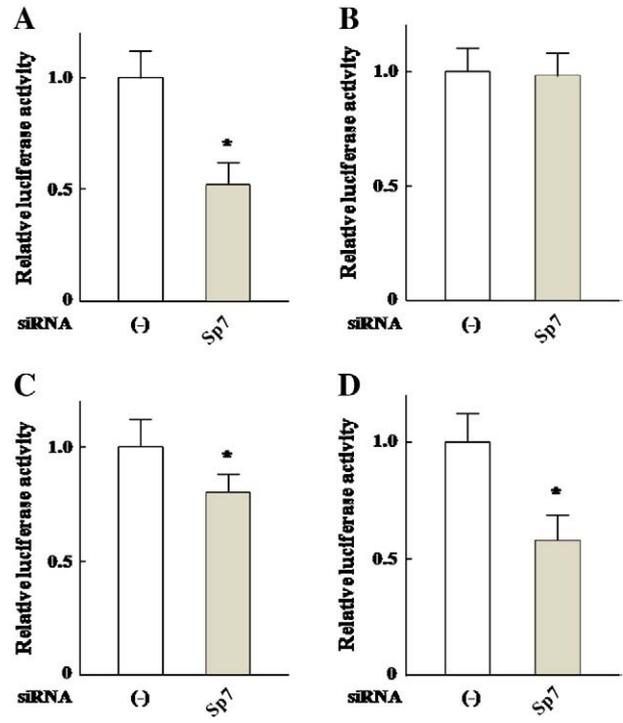


Fig. 5. Effect of inhibition with siRNA against Sp7/Osterix on luciferase activity. The siRNAs were cotransfected with the luciferase reporter constructs, WT (-231/+39-Luc) (A), m1-Luc (B), m2-Luc (C) and m3-Luc (D). The relative luciferase activities were normalized to the non-treated (-) siRNA samples. Note that the inhibition is less effective in the construct that is mutated (C) than is intact (D) in the CBF/NF-Y binding site. Data are presented by the mean \pm S.D. of at least three independent experiments. * $p < 0.05$ compared with the (-) control.

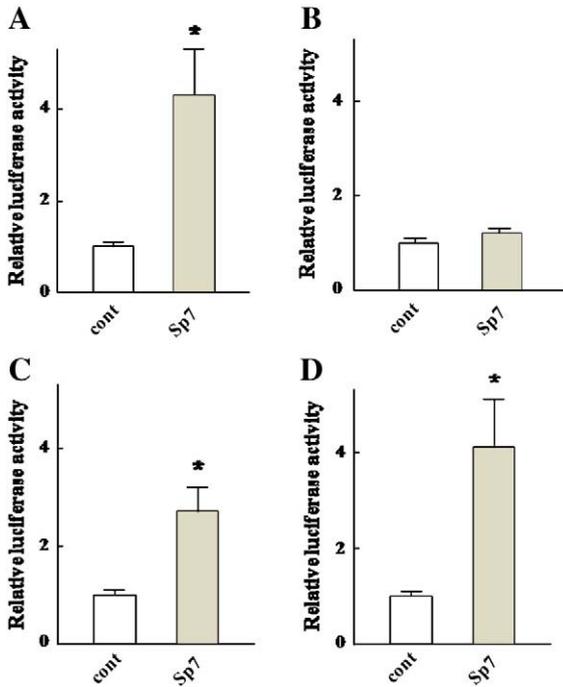


Fig. 4. Transactivation of the *Col5a1* gene by the overexpression of Sp7/Osterix in MC3T3-E1 cells. The Sp7/Osterix expression (Sp7) and empty construct (cont) were cotransfected with the luciferase reporter constructs, WT (-231/+39)-Luc (A), m1-Luc (B), m2-Luc (C) and m3-Luc (D). The relative luciferase activities were normalized against those of the samples transfected with an empty expression vector. Note that the activity is lower in the mutated construct (C) than in the intact (D) in the CBF/NF-Y binding site. Data are presented by the mean \pm S.D. of at least of three independent experiments. * $p < 0.05$ compared with the control.

approximately 20% and 40%, respectively (Fig. 5C and D). However, no changes were seen in the activity of m1-Luc (Fig. 5B).

2.4. Activation of the *Col5a1* gene during the process of MC3T3-E1 cell differentiation by Sp7/Osterix

To further examine the activation of *Col5a1* gene by Sp7/Osterix in osteoblast cells, the cell culture model of osteoblast differentiation was used. MC3T3-E1 was differentiated with β -glycerophosphate for this purpose. The Sp7/Osterix expression was markedly increased, while the expression of Sp1 was consistent with a small level (Fig. 6A). The expression of *Col5a1* gene also increased during osteoblast differentiation (Fig. 6B). The activity of the *Col5a1* promoter with the wild-type construct increased by approximately 3.5-fold on Day 8 after differentiation, but did not change with mut1-Luc (Fig. 6C). Consistent with these data, a CHIP assay revealed that Sp7/Osterix was clearly bound to the *Col5a1* core promoter sequence *in vivo* on 8 day after differentiation (Fig. 6D and E).

3. Discussion

The extracellular matrix of bone is primarily composed of type I collagen, with lesser amounts of other components such as proteoglycans. Type V collagen is cross-linked to type I collagen in the bone (Niyibizi and Eyre, 1989). During the ossification process in the mouse, high pro $\alpha 1(V)$ expression was observed in the osteoblasts of long bones, vertebrae and calvaria, which indicated that type V collagen was involved in the bone formation of both endochondral and intramembranous ossification (Roulet et al., 2007). Our previous study identified the presence of Sp1 and Sp1-like binding sites in the proximal promoter of the *Col5a1* gene (Sakata-Takatani et al., 2004). However, Sp1 did not activate the gene in the NIH 3 T3 cells or in the A204 rhabdomyosarcoma cells. In the present study, we examined

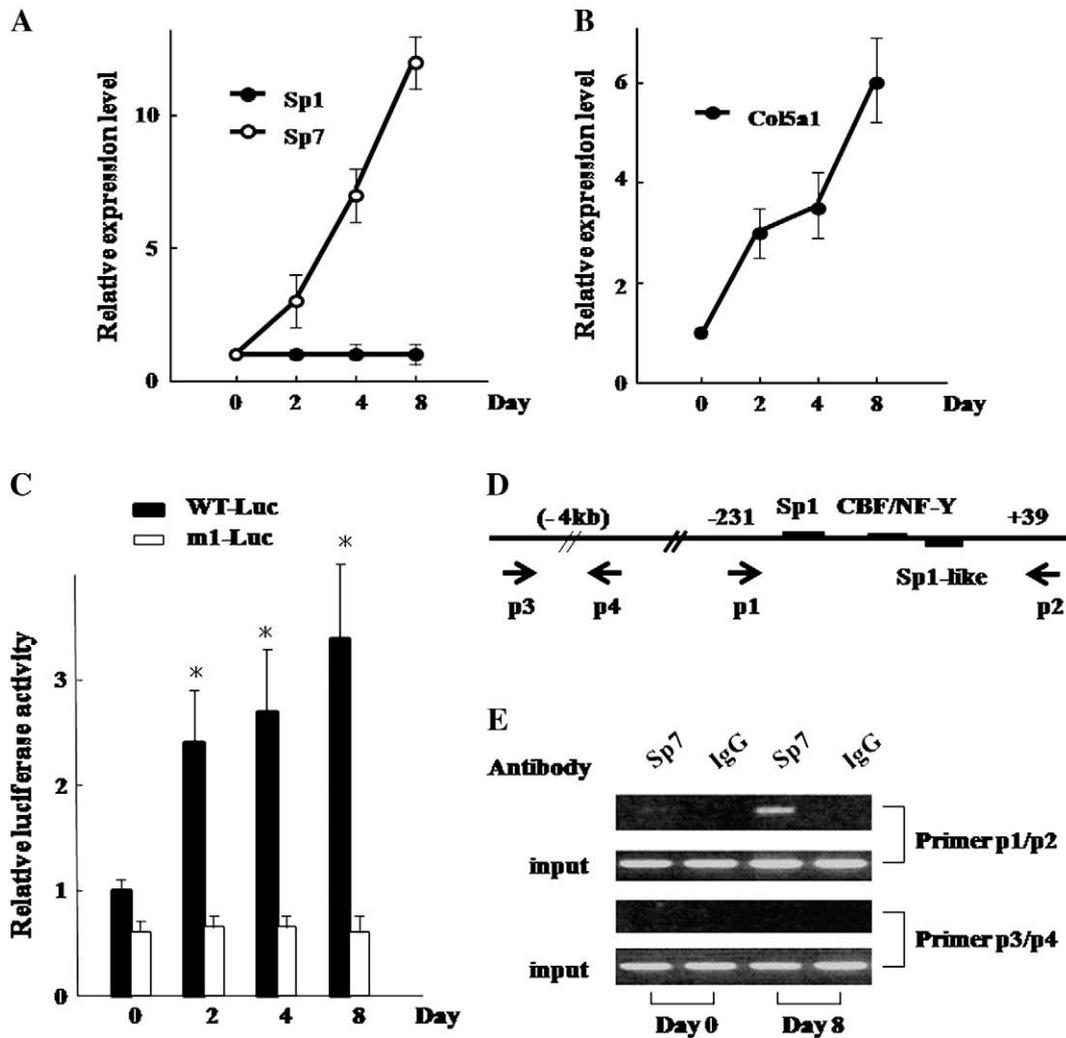


Fig. 6. Expression of Sp1 and Sp7/Osterix during MC3T3-E1 cell differentiation. An RT-PCR analysis for *Sp1* and *Sp7/Osterix* (A) and *Col5a1* (B) gene on 0, 2, 4 and 8 days of differentiation. β -actin was included as a positive control for the PCR. PCR was carried out for 25 cycles. (C) The relative luciferase activity of *Col5a1* promoter using WT (-231/+39-Luc) and m1-Luc. Data are presented by the mean \pm S.D. of three independent experiments. * $p < 0.05$ compared with the WT-Luc on 0 day. (D) Positions of primers used for the CHIP assay. Primer sets, p1 and p2, amplified between the -226 and +70 in the proximal promoter region of the *Col5a1* gene and another set of control primers, p3 and p4, approximately 4 kb upstream. (E) The CHIP assay was performed to examine the binding of Sp7/Osterix to the *Col5a1* promoter at 0 and 8 days. IgG was used as a control.

whether Sp7/Osterix, which belongs to the Sp family, bound to the region and activated the genes in the osteoblastic cells. The results showed that Sp7/Osterix bound to the proximal promoter *Col5a1* gene and activated the gene in osteoblastic cells. However, it did not activate in the NIH 3 T3 cells or in the A204 rhabdomyosarcoma cell as previously shown. Conversely, selective treatment by targeted siRNA knockdown of Sp7/Osterix inhibited the expression of the *Col5a1* gene in MC3T3-E1 cells (Fig. 3C). This experiment also revealed that the activation of the *Col5a1* gene was dependent on increasing amounts of Sp7/Osterix during the differentiation of MC3T3-E1 cells (Fig. 6). These data therefore showed that Sp7/Osterix promotes the expression of the *Col5a1* gene in osteoblastic cells.

Sp7/Osterix is a zinc finger transcription factor and forms a complex with the nuclear factor of activated T cells (NFAT), which activates the Wnt/ β -catenin signaling pathway and regulates bone mass in osteoblasts (Winslow et al., 2006). β -catenin is essential for the differentiation of mature osteoblasts and, consequently, for bone formation (Caetano-Lopes et al., 2007). A complex of Sp7/Osterix and NFAT bound to and activated the human *COL1A1* gene (Koga et al., 2005). We and other investigators have recently demonstrated that Sp7/Osterix binds to an Sp1 binding site in the proximal promoters of the mouse *Col5a3* and the human *COL11A2* gene and up-regulate

those genes, which are expressed in osteoblasts (Wu et al., 2010a; Goto et al., 2006). Many collagen genes are expressed in osteoblastic cells. The complex of Sp7/Osterix and NFAT might be bind to Sp1 binding sites and activate those collagen genes.

Gene transcription is controlled by a series of complex interactions of many transcription factors, which are expressed with ubiquitous and cell specific manners. Previous studies identified that the CBF/NF-Y binding factor had basal activity in the *Col5a1* gene in NIH 3 T3 and A204 cells (Sakata-Takatani et al., 2004). A functional analysis using the mutant of CBF/NF-Y site showed that the promoter activity decreased in osteoblast as well as A204 and NIH 3 T3 cells (m2 in Fig. 1B, C and D). In an experiment of the overexpression with Sp7 and the inhibition with siRNA, the effects were lower in the mutant construct than in the intact construct of CBF/NF-Y site even if Sp1 binding site was normal (Figs. 4 and 5). These data showed the involvement of CBF/NF-Y in the promoter activity of the *Col5a1* gene (Fig. 7). The CBF/NF-Y is ubiquitously expressed, but might be required to coordinate the expression of types I and V collagen in osteoblastic cells as well as in non-osteoblastic cells (Nagato et al., 2004; Wu et al., 2010a). Runx2, which is also identified as a master transcription factor for osteoblast differentiation, acts upstream of Sp7/Osterix and is required for early steps of osteoblast differentiation. Runx2 is a positive regulator of the osteoblast-specific

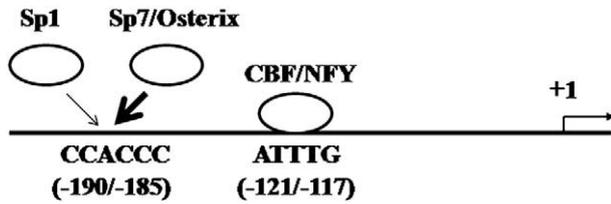


Fig. 7. Schematic illustration of the core promoter of *Col5a* in osteoblastic cells. The bindings of the Sp7(Osterix)/Sp1 and CBF/NFY. The indicated sequences are critical for binding the factors.

expression of both type I collagen genes, and binds to the regions approximately 1.3 kb upstream in the $\alpha 1(I)$ collagen gene and in the first exon of $\alpha 2(I)$ collagen gene (Kern et al., 2001). It should also regulate the *Col5a1* gene, although it could not be identified in the current study. ATF4 is another important transcription factor controlling osteoblasts (Ameri and Harris, 2008). The mRNA of ATF4 is expressed in many cells, but the protein is unstable and degraded in the majority of cell types through ubiquitination except in osteoblasts, where it accumulates (Yang and Karsenty, 2004). It interacts with Runx2 to regulate the transcriptional activity of osteocalcin. It affected the synthesis of type I collagen, which was reduced in the ATF4-deficient mice. These factors may also be involved in the regulation of the *Col5a1* gene. In conclusion, Sp7/Osterix was observed to participate in the activation of the *Col5a1* promoter during osteoblast differentiation. Further study is, however, still required to clarify the complexity of gene regulations regarding bone formation.

4. Experimental procedures

4.1. Cell culture

The cell lines used in this study were mouse preosteoblast MC3T3-E1, human rhabdomyosarcoma A204 and mouse NIH3T3 cells. Cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum (Sanko Junyaku, Tokyo, Japan). For differentiation studies, MC3T3-E1 cells were fed at confluence (time 0) with the above medium additionally containing 10 μ M β -glycerophosphate and 50 μ g/mL of ascorbic acid. The medium for differentiation was changed every 2 days.

4.2. Real-time PCR

Total RNA was isolated from cultured cells using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. RNA was quantified and stored at -80°C until used. Three micrograms of total RNA was reverse transcribed by random hexamer priming using SuperScript II reverse transcriptase (Invitrogen, CA, USA). Real-time PCR was performed using a LightCycler TaqMan Master. The thermal cycling conditions included 1 cycle at 95°C for 10 min, 40 cycles at 95°C for 15 s, and 60°C for 1 min. The relative mRNA expression levels were normalized against that of the *GAPDH* gene from the same RNA preparations, using a comparative threshold cycle method. The primer sets are listed in Table S1.

4.3. Chimeric plasmids

The luciferase constructs, $-231/+39$ -Luc, m1-Luc, m2-Luc and m3-Luc, and the Sp1 and Sp7/Osterix expression plasmid were previously described (Sakata-Takatani et al., 2004; Wu et al., 2010b).

4.4. Transient transfection and luciferase Assays

The cells were plated at a density of 2×10^5 per 35-mm dish for 18 h before transfection. For transient transfection, 0.5 μ g of plasmid

DNA was transfected into these cells by using the LipofectAMINE Plus reagent system. Plasmid pRL-TK vector (Promega) was always cotransfected as an internal control for transfection efficiency. After an additional cultivation for 48 h, the transfected cells were harvested, lysed, centrifuged to pellet the debris, and subjected to luciferase assay. The luciferase activities were measured as chemiluminescence in a luminometer (Lumat LB 9507, PerkinElmer Life Sciences) using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. The cotransfection experiments were performed using 1.5 μ g of Sp1 or Sp7/Osterix expression plasmids (Wu et al., 2010b).

4.5. Chromatin immunoprecipitation (CHIP) assay

The CHIP assays were performed using a chromatin immunoprecipitation assay kit (Upstate Biotechnology, Lake Placid, NY) according to the manufacturer's protocol. The anti-Sp7/Osterix antibodies were purchased (Cosmo Bio, Tokyo, Japan). The primer sets are listed in Table S1. The procedure was described previously (Matsuo et al., 2003).

4.6. siRNA transfection

The siRNA cocktails targeting mouse Sp1 or mouse Sp7/Osterix were purchased (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA). The mouse MC3T3-E1 osteoblastic cells were transfected using Lipofectamine 2000 (Invitrogen) to achieve a final siRNA concentration of 50 nM.

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.matbio.2010.09.002.

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