



Sp7/Osterix induces the mouse pro- α 2(I) collagen gene (*Col1a2*) expression via the proximal promoter in osteoblastic cells



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ABSTRACT

Bone is essentially composed of two components, hydroxyapatite and extracellular matrix proteins. The extracellular matrix of bone is primarily composed of collagen, mostly type I collagen, with lesser amounts of other types of collagen such as type V collagen. Osteoblast differentiation is a multi-step process in which many classes of factors function in a coordinated manner. Sp7/Osterix, which binds to G/C-rich sequences, is a transcription factor that contributes to osteoblast differentiation. The present study aimed to clarify the involvement of Sp7/Osterix with the proximal promoter region of the mouse *Col1a2* gene containing multiple G/C-rich sequences exist. Consequently, a functional analysis of the proximal mouse *Col1a2* promoter showed that a substitution mutation of the second G/C-rich sequence from the transcription site specifically decreased the activity of osteoblastic cells. In addition, the experiments of over-expression of Sp7/Osterix and treatment with its specific siRNA showed that this G/C-rich sequence is responsible for the specific expression in osteoblastic cells. Consistent with these data, Sp7/Osterix bound to the region and increased the expression of the *Col1a2* gene in association with osteoblast differentiation in the culture system.

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1. Introduction

Bone is formed via intramembranous and endochondral ossification. Osteoblasts, which are cells of mesenchymal origin, are responsible for both of these processes. Bone is directly derived from mesenchymal condensation at sites of intramembranous ossification while the intermediate step, cartilage formation, is involved in endochondral ossification. The former process is observed in most craniofacial bones and the lateral region of the clavicles, while the latter process occurs in the long bones of the limbs, vertebrae, basal portion of the skull, medial area of the clavicle and ribs. Recent advancements in molecular biology and mouse genetics have helped to identify various transcription factors that regulate bone formation [1–3]. Runx2 is the α subunit of a heterodimeric transcription factor, and a member of the *Runt* family. In mice, the expression of *Runx2* begins in the notochord on embryonic day 9.5 (E9.5), and later at sites of prechondrogenic

mesenchymal condensation and in chondrocytes [2]. In addition to its essential functions in osteoblast differentiation, Runx2 plays a role in the differentiation of hypertrophic chondrocytes [1]. Sp7/Osterix is a zinc finger-containing transcription factor specific to osteoblasts *in vivo* [3] that acts downstream of Runx2 and strongly binds to GC-rich sequences, including Sp1. The expression of Sp7/Osterix is more specific to osteoblasts than that of Runx2, and the transcripts are not detected before E13 in mice [3].

The extracellular matrix of bone is mostly composed of collagen, primarily type I collagen with other minor types of collagen. Collagen molecules assemble into heterotypic aggregates that subsequently affect the biological and mechanical properties of bone [4]. Collagens are classified according to the fibrillar or non-fibrillar structure [5]. Banded fibrils exhibiting 67-nm periodicity contain heterogeneous molecules of various collagen types. Fibrillar collagen is divided into two groups: major fibrillar collagens (types I, II and III) and minor fibrillar collagens (types V and XI). In bone, both fibrillar collagen (types I, III, V and XXIV) [6] and non-fibrillar collagen (types VI and XII) are observed.

Over 90% of cases of osteogenesis imperfecta (OI) involve autosomal dominant bone disorders caused by mutations in both

Abbreviation: CHIP assay, chromatin immunoprecipitation assay.

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the collagen $\alpha 1(I)$ and $\alpha 2(I)$ chains [7]. Recently, it was reported that 5–10% of recessive cases are caused by non-collagen genes containing *Osterix/Sp7* [8].

It has also been previously demonstrated that *Sp7/Osterix* up-regulates the mouse *Col5a1* and *Col5a3* genes [9,10], which are co-expressed with type I collagen in osteoblasts. The proximal promoter of the human *COL1A2* gene has several GC-rich sequences to which Sp1 binds in order to activate the gene in fibroblasts [11,12]. These GC-rich sequences may also be involved in processes of regulation in association with *Sp7/Osterix* in osteoblasts. In the present study, we examined whether *Sp7/Osterix* binds to and activates the mouse *Col1a2* gene via the proximal promoter region in osteoblasts.

2. Materials and methods

2.1. Cell culture

Mouse MC3T3-E1 and mouse NIH-3T3 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Sanko Junyaku, Tokyo, Japan) at 37 °C in humidified 5% CO₂ and 95% air. After the MC3T3-E1 cells were cultured at confluent, 10 μ M β -glycerophosphate and 50 μ g/mL of ascorbic acid were added in the medium for cell differentiation experiment. The medium was changed every 2 days.

2.2. Real time PCR

Total RNA was extracted from cultured cells using Isogen extraction kit (Nippon Gene, Tokyo, Japan). RNA was stored at –80 °C until used. One micrograms of total RNA was reverse transcribed using ReverTra Ace (Toyobo, Tokyo, Japan) with random primers. For a quantification of mRNA, real-time PCR was performed using a LightCycler TaqMan Master (Roche, IN, USA). The thermal cycling conditions included 1 cycle at 95 °C for 10 min, 40 cycles at 95 °C for 15 s, 60 °C for 1 min. The relative mRNA expression levels were normalized against that of *GAPDH* gene using a comparative threshold cycle method [13]. The primer sets are listed in Table S1.

2.3. Construction of chimeric plasmids

To generate the luciferase constructs, mouse genomic DNA were derived from pBACe3.6RP23 clone (BACPAC Resources Children's Hospital Oakland, CA, USA). Wild type construct was obtained by using Sac site-linked 5' and Xho site-linked 3' specific for the mouse *Col1a2* sequence. Amplified product was inserted into the pGEM-T Easy vector (Promega, WI, USA), digested with Sac and Xho, and subcloned into pGL4.1 basic luciferase vector (Promega, WI, USA). The Xba site, TCTAGA, was introduced into wild type fragment to generate various mutant constructs. The primer sets are listed in Table S1.

2.4. Transient cell transfection and luciferase Assay

Approximately 2×10^5 cells were plated in 35-mm dish for 18 h before transfection. Five μ g of plasmid DNA was transfected into cells using calcium phosphate precipitation methods. Plasmid pRL-TK vector (Promega, WI, USA) was cotransfected as an internal control for transfection efficiency. After 48 h, the transfected cells were harvested, lysed, centrifuged to remove the debris, and subjected to luciferase assay. The luciferase activities were measured with a luminometer (Lumat LB 9507, Perkin-Elmer Life Sciences, Waltham, MA, USA) using the Dual Luciferase Reporter Assay System (Promega, WI, USA) according to the manufacturer's protocol.

The cotransfection experiments were performed using 1.5 μ g of Sp1 or Sp7/*Osterix* expression vectors [10].

2.5. siRNA transfection

The siRNAs targeting mouse Sp1 or Sp7/*Osterix* were purchased (Santa Cruz Biotechnology, CA, USA). The mouse MC3T3-E1 cells were transfected using Lipofectamine2000 (Invitrogen) with a final siRNA concentration of 50 nM. The procedure was described for RT-PCR and luciferase assay using siRNAs previously [10].

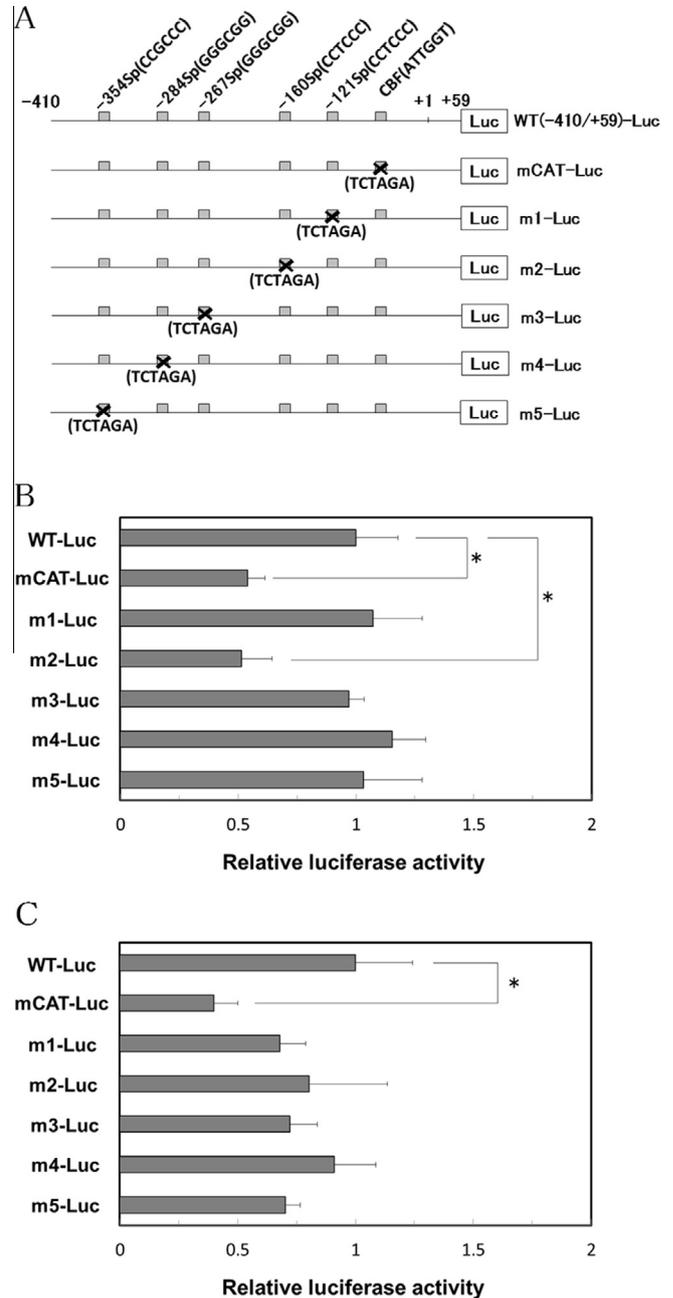


Fig. 1. Luciferase assay of the proximal promoter of the mouse *Col1a2* gene. (A) The luciferase reporter constructs. Transient transfection with the wild-type and six mutant constructs was carried out in the MC3T3-E1 (B) and NIH-3T3 (C) cells. Each value was normalized to the internal control and is shown relative to the activity of the wild-type construct in each cell line. The data represent the mean \pm S.D. of at least three independent experiments. * $p < 0.05$ compared with the wild-type.

2.6. Chromatin immunoprecipitation (CHIP) assay

The CHIP assays were performed using a chromatin immunoprecipitation assay kit (Upstate Biotechnology, NY, USA) according to the manufacturer’s protocol. The anti-Sp1 and anti-Sp7/Osterix antibodies were purchased (Cosmo Bio, Tokyo, Japan). The procedure was described previously [14]. The primer sets are listed in Table S1.

3. Results

3.1. Functional analysis of the proximal promoter region of the Col1a2 gene

The Sp1 binding sites, G/C rich sequences, in the proximal promoter region of the human COL1A2 gene have been previously

reported [11,12]. Sp7/Osterix binds to G/C rich sequence in the proximal promoter region of the Col5a1 and Col5a3 genes in osteoblastic cells [9,10]. Therefore, we performed an experiment in order to examine whether this transcription factor also binds to any Sp1 binding sites in the promoter region of the Col1a2 gene in osteoblastic cells. Consequently a wild-type and five mutant constructs that link to the luciferase gene were generated to assess the promoter activity in MC3T3-E1 cells (Fig. 1A). m2-Luc, which was mutated in the second G/C-rich sequence from the transcription start site, caused an approximately 50% decrease in the promoter activity of the MC3T3-E1 cells (Fig. 1B), but not the NIH-3T3 cells (Fig. 1C). In addition, mCAT-Luc, which was mutated in the CBF/NF-Y binding site, decreased the promoter activity in both the MC3T3-E1 cells and NIH-3T3 cells (Fig. 1B and C). In contrast, m1-Luc, m3-Luc, m4-Luc and m5-Luc had no effect on the promoter activity in the osteoblastic cells (Fig 1B). On the other hand, m1-Luc, m3-Luc and m5-Luc exhibited a tendency to decrease the

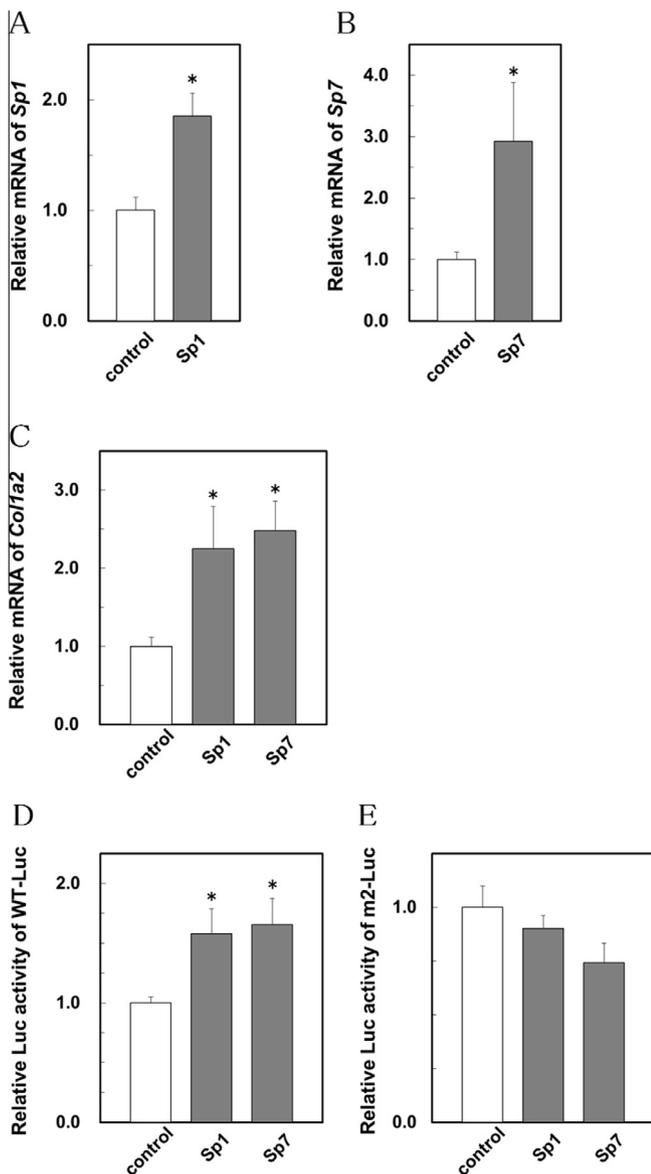


Fig. 2. Overexpression of Sp1 and Sp7/Osterix in the MC3T3-E1 cells. The expression of Sp1 and Sp7/Osterix following the overexpression of Sp1 and Sp7/Osterix expression vector, respectively (A and B). The expression of Col1a2 following the overexpression of Sp1 and Sp7/Osterix (C). The luciferase activity obtained using WT-Luc (D) and m2-Luc (E). An empty expression construct was transfected as a control in each experiment. The data represent the mean ± S.D. of at least three independent experiments. *p < 0.05 compared with the wild-type.

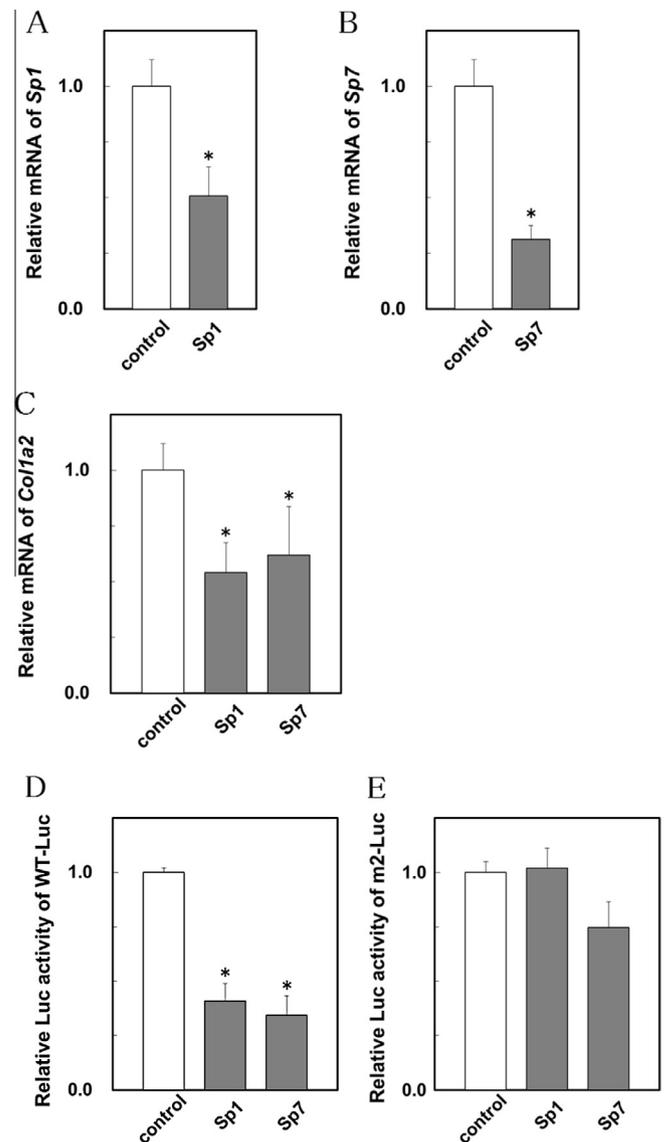


Fig. 3. Inhibition with siRNA against Sp1 and Sp7/Osterix. The expression of Sp1 and Sp7/Osterix after inhibition by specific siRNA of Sp1 and Sp7/Osterix, respectively (A and B). The expression of Col1a2 following the inhibition of Sp1 and Sp7/Osterix (C). The luciferase activity obtained using WT-Luc (D) and m2-Luc (E). Scramble siRNA was transfected as a control in each experiment. The data represent the mean ± S.D. of at least three independent experiments. *p < 0.05 compared with the wild-type.

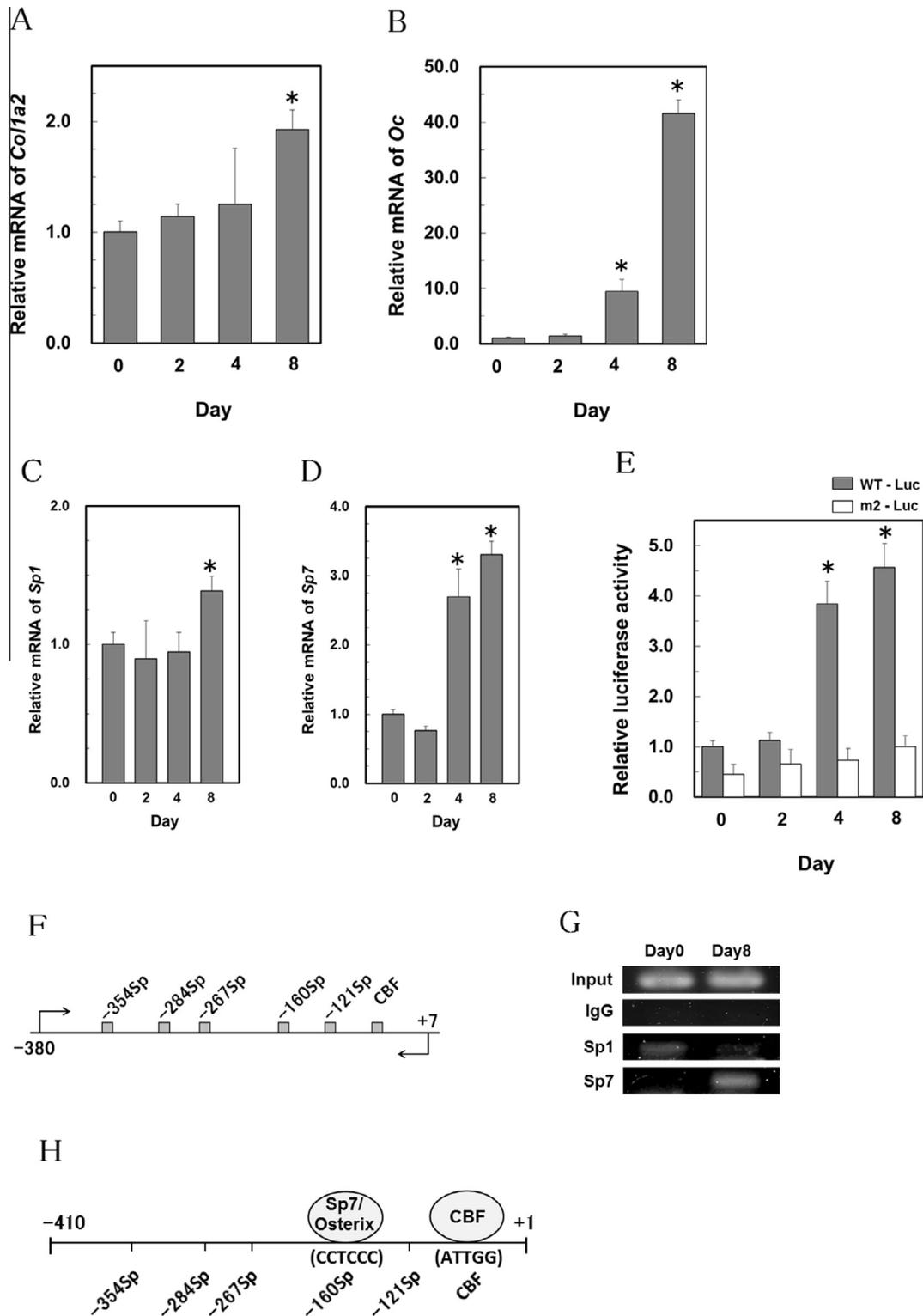


Fig. 4. Gene expression during MC3T3-E1 cell differentiation. The RT-PCR analysis of the *Col1a2* (A), *Osteocalcin* (B), *Sp1* (C) and *Sp7/Osterix* (D) genes. Each value was normalized to the level of *GAPDH*. The value observed on day 0 in each gene is set at 1.0. The data represent the mean \pm S.D. of at least three independent experiments. * $p < 0.05$ compared with the value of each gene on day 0. The luciferase assay of the promoter of the *Col1a2* gene using WT-Luc and m2-Luc constructs during differentiation (E). The value of wild-type on day 0 is set at 1.0. The data represent the mean \pm S.D. of at least three independent experiments. * $p < 0.05$ compared with the WT-Luc on 0 day. Position of primers used for the CHIP assay (F). A CHIP assay was performed to examine the binding of Sp1 and Sp7/Osterix to the *Col1a2* proximal promoter on days 0 and 8. IgG was used as a control. A schematic illustration of the bindings of Sp7/Osterix and CBF to the proximal promoter of the mouse *Col1a2* gene in the osteoblastic cells (H).

promoter activity of the NIH-3T3 cells (Fig. 1C). Therefore Sp1 likely binds to these sites [11,12].

3.2. Endogenous expression of the *Col1a2* gene overexpressed by Sp7/Osterix

The overexpression of Sp1 and Sp7/Osterix was induced in order to examine the effects of Sp1 and Sp7/Osterix in the MC3T3-E1 cells (Fig. 2A and B). Consequently, the mRNA levels of the *Col1a2* gene significantly increased following the overexpression of both factors significantly ($p < 0.05$) (Fig. 2C). The wild-type and m2-Luc luciferase constructs were subsequently cotransfected with Sp1 or Sp7/Osterix in order to examine the effects of these transcription factors on the promoter activity of the *Col1a2* gene in the MC3T3-E1 cells. Both Sp7/Osterix and Sp1 significantly activated the wild-type promoter of the luciferase reporter gene (Fig. 2D). However, neither activity was canceled by the cotransfection of the m2-Luc construct (Fig. 2E).

Conversely, the inhibition of Sp1 and Sp7/Osterix with specific siRNAs was also performed (Fig. 3A and B). As a result the knockdown of Sp1 or Sp7/Osterix decreased the endogenous mRNA levels of the *Col1a2* gene by 50% to 40%, respectively ($p < 0.05$) (Fig. 3C). The promoter activity obtained using the wild-type construct was also decreased by both specific siRNAs (Fig. 3D). Similar to the results of overexpression mentioned above, the knockdown experiments ($p < 0.05$) showed almost no effects on the promoter activity when using the m2-Luc construct (Fig. 3E).

3.3. Activation of the *Col1a2* gene during osteoblast cell differentiation induced by Sp7/Osterix

MC3T3-E1 was differentiated with β -glycerophosphate in order to examine the level of activation of the *Col1a2* gene induced by Sp7/Osterix in the osteoblasts up to day 8. The expression levels of *Col1a2*, *osteocalcin*, *Sp1* and *Sp7/Osterix* were examined using real-time RT-PCR, which showed that the expression of *Col1a2* gradually increased (Fig. 4A), while that of *Osteocalcin* remarkably increased during differentiation (Fig. 4B). Concerning the two transcription factors, the expression of Sp1 was slightly up-regulated (Fig. 4C), while that of Sp7/Osterix increased by approximately 3.5-fold (Fig. 4D) on day 8 after differentiation. Furthermore the activity of the *Col1a2* promoter associated with the wild-type construct increased by approximately 4.5-fold on day 8 after differentiation, although it did not change with m2-Luc, the Sp7/Osterix binding site of which was mutated (Fig. 4E). In order to examine the binding of the transcription factors *in vivo*, a CHIP assay was performed (Fig. 4F). The results subsequently showed that Sp7/Osterix clearly bound to the *Col1a2* proximal promoter region on day 8 after differentiation (Fig. 4G).

4. Discussion

Sp7/Osterix is a zinc finger transcription factor that strongly binds to GC-rich sequence. The DNA-binding domain of Sp7/Osterix is similar to those of Sp1 and Sp3, which are ubiquitously expressed. Our previous studies showed Sp7/Osterix binds to GC-rich sequence in the proximal promoter of the mouse *Col5a1* and *Col5a3* genes and activates these genes in osteoblastic cells [9,10]. Five GC-rich sequences in the proximal domain of the mouse *Col1a2* promoter have been identified. The current study demonstrated that Sp7/Osterix specifically binds to the second GC-rich site from the transcription start site and up-regulates the mouse *Col1a2* gene (Fig. 4H). Recently, it was also demonstrated that Sp7/Osterix induces the mouse *Col1a1* gene expression via the upstream enhancer and proximal promoter regions [15].

Sp7/Osterix forms a complex with NFATc1, resulting in upregulation of the human *COL1A1* gene [16]. Collagen fibrils consist of type I and V collagen molecules, which are cross-linked together in bone [7,17]. Therefore, type I and V collagens are expressed in a coordinate fashion during osteogenesis. This study clearly demonstrated that Sp7/Osterix is responsible for the specific expression of both molecules during fibril formation in bone. Many other types of collagen including type XII, which belongs to the FACIT family localized on the surface of fibrils, is expressed in a small amount in bone. Since mice without type XII collagen have fragile bones with a disorganized collagen fibers [18], Sp7/Osterix may be involved in the expression of the mouse *Col12a1* gene.

Other transcription factors including Runx2 and ATF4 are known to play important roles in osteogenesis. Runx2 is expressed in Sp7/Osterix-deficient mice, indicating that Runx2 works upstream of Sp7/Osterix [3]. Runx2 has also been shown to induce the expression of the mouse *Col1a1* gene by binding at -1347 and -372 bp upstream from the transcription site [19]. In addition, it has been reported that Runx2 binds to the element at the first exon of the *Col1a2* gene and that multimer of this element confer an osteoblast-specific activity. However, this specific activity was not observed in the current study (data not shown). This discrepancy may be due to differences in the constructs, which contain multimer or monomer of the element. ATF4 regulates osteoblast terminal differentiation and is phosphorylated by the kinase RSK2, the inactivation of which result in Coffin-Lowry syndrome, an X-linked disorder associated with mental retardation and skeletal abnormalities [20]. The ATF4 gene is ubiquitously expressed, although its protein is degraded via ubiquitination in many cells except osteoblasts [21]. Type I collagen synthesis is decreased in the osteoblasts of ATF4-deficient mice, which indicates that the activity of ATF4 is regulated post-translationally [20]. In conclusion, osteogenesis is a multi-step process in which mesenchymal cells become osteocytes. Further research is needed to clarify the complex mechanisms underlying the regulation of bone formation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bbrc.2014.08.100>.

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