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Sp7/Osterix induces the mouse pro-α2(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 primary 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 overexpression 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.

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 occur 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, Ranx2 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, primary 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 case 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 α1(I) and α2(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* upregulates 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% CO_2 and 95% air. After the MC3T3-E1 cells were cultured at confluent, 10 μM β-glycerophosphate and 50 μg/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 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 μg 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 μg 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].

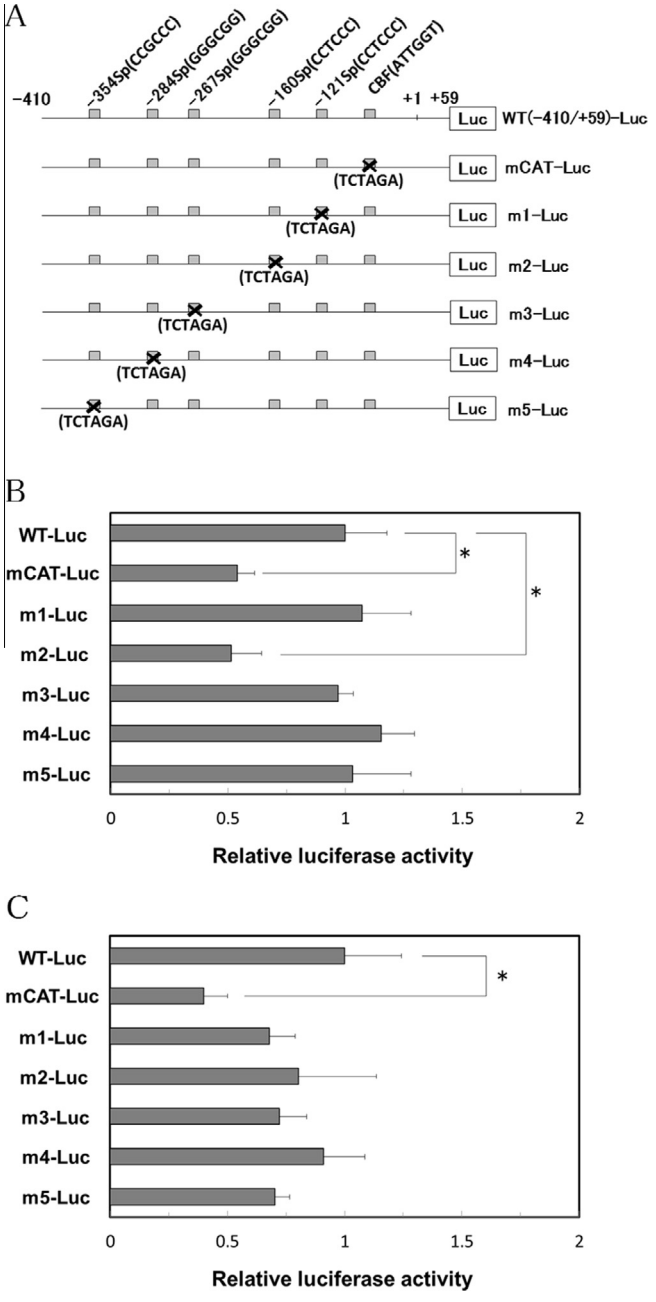

Fig. 1. Luciferase assay of the proximal promoter of the mouse *Col1a2* gene. (A) The luciferase reporter constructs. Transient transfection with the wild-type and six mutant constructs was carried out in the MC3T3-E1 (B) and NIH-3T3 (C) cells. Each value was normalized to the internal control and is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