Matrix Biology 29 (2010) 701–706

Contents lists available at ScienceDirect

Matrix Biology

journal homepage: www.elsevier.com/locate/matbio

Sp7/Osterix is involved in the up-regulation of the mouse pro-α1(V) collagen gene (*Col5a1*) in osteoblastic cells

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ARTICLE INFO

Article history:
Received 12 July 2010
Received in revised form 21 September 2010
Accepted 23 September 2010

Keywords:
Type V collagen
Sp7/Osterix
Gene regulation
Osteoblastic cell
Bone formation

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 Sp1binding 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.

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 Cromrrugghe, 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 transcription 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 $[\alpha1(V)]_2\alpha2(V)$ which is present in many tissues. In addition, the $[\alpha1(V)]_3$ homotrimer and the $\alpha1(V)\alpha2(V)\alpha3(V)$ 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-Y 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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doi:10.1016/j.matbio.2010.09.002

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 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

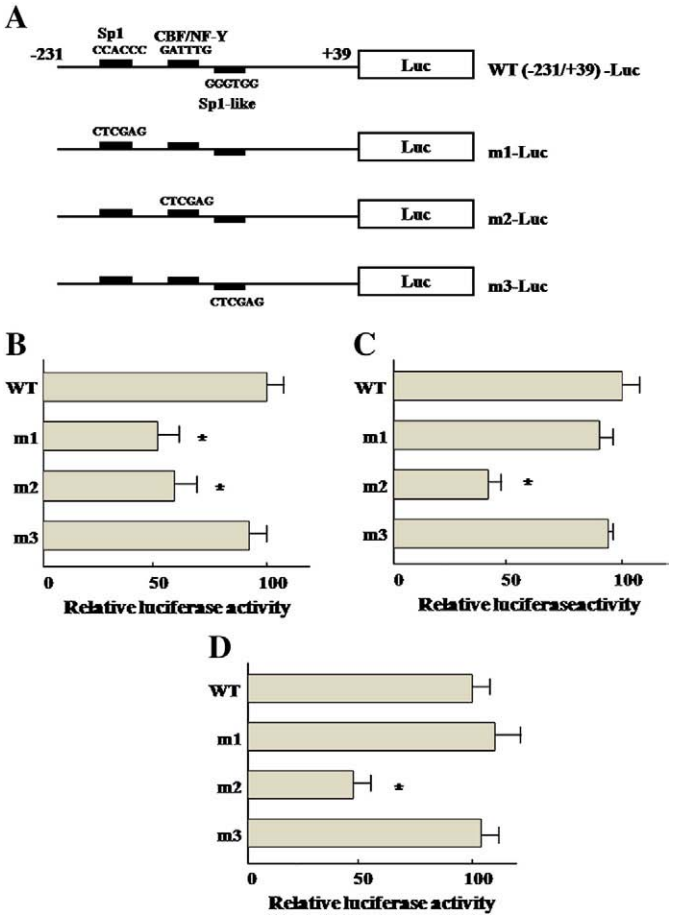

Fig. 1. Functional analysis of the proximal *Col5a1* promoter. (A) A schematic illustration of the luciferase reporter constructs. mut1, mut2 and mut3 are mutated in the Sp1, CBF/NF-Y and Sp1-like binding site of the WT(−231/+39-Luc) construct (Sakata-Takatani et al., 2004). Transient transfections with 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 ± S.D. of at least three independent experiments. *$p<0.05$ compared with the wild-type.

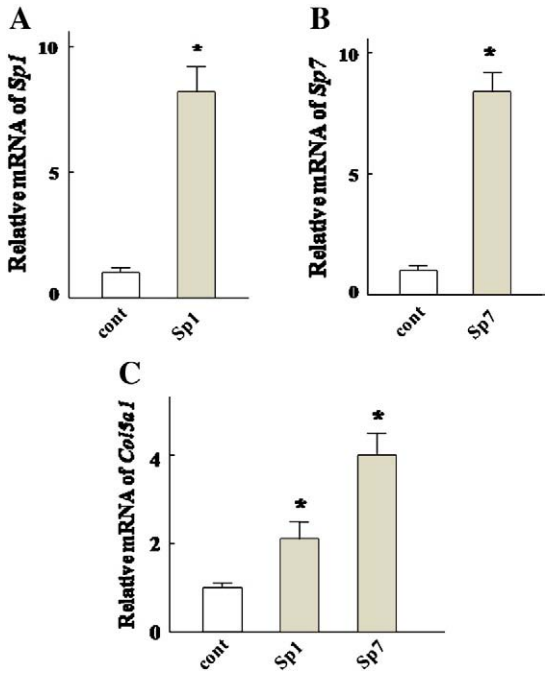

Fig. 2. (A, B) The real-time RT-PCR analysis was performed with total mRNA from MC3T3-E1 cells which were transfected by overexpressing Sp1 (lane 2 in A) and Sp7/Osterix (lane 2 in B). The empty vector was transfected as a control (lane 1 in A and B). (C) Effects of the Sp1 and Sp7/Osterix expression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