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Sp1 upregulates the proximal promoter activity of the mouse collagen α1(XI) gene (*Col11a1*) in chondrocytes

Keijirou Watanabe[1,2] · Mariko Hida[1] · Takako Sasaki[1] · Hiroyuki Yano[3] · Kenji Kawano[2] · Hidekatsu Yoshioka[1] · Noritaka Matsuo[1]

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Abstract Type XI collagen is a cartilage-specific extracellular matrix, and is important for collagen fibril formation and skeletal morphogenesis. We have previously reported that NF-Y regulated the proximal promoter activity of the mouse collagen α1(XI) gene (*Col11a1*) in chondrocytes (Hida *et. al.* In Vitro *Cell. Dev. Biol. Anim.* 2014). However, the mechanism of the *Col11a1* gene regulation in chondrocytes has not been fully elucidated. In this study, we further characterized the proximal promoter activity of the mouse *Col11a1* gene in chondrocytes. Cell transfection experiments with deletion and mutation constructs indicated that the downstream region of the NF-Y binding site (-116 to +1) is also necessary to regulate the proximal promoter activity of the mouse *Col11a1* gene. This minimal promoter region has no TATA box and GC-rich sequence; we therefore examined whether the GC-rich sequence (-96 to -67) is necessary for the transcription regulation of the *Col11a1* gene. Luciferase assays using a series of mutation constructs exhibited that the GC-rich sequence is a critical element of *Col11a1* promoter activity in chondrocytes. Moreover, in silico analysis of this region suggested that one of the most effective candidates was transcription factor Sp1. Consistent with the prediction, overexpression of Sp1 significantly increased the promoter activity. Furthermore, knockdown of Sp1 expression by siRNA transfection suppressed the proximal promoter activity and the expression of endogenous transcript of the mouse *Col11a1* gene. Taken together, these results indicate that the transcription factor Sp1 upregulates the proximal promoter activity of the mouse *Col11a1* gene in chondrocytes.

Keywords Type XI collagen · Chondrocyte · Sp1 · Transcription · Promoter

✉ Noritaka Matsuo
matsuon@oita-u.ac.jp

[1] Department of Matrix medicine, Faculty of medicine, Oita University, 1-1 Idaigaoka, Hasama-machi, Yufu, Oita 879-5593, Japan

[2] Department of Oral and Maxillo-Facial Surgery, Faculty of medicine, Oita University, 1-1, Idaigaoka, Hasama-machi, Yufu, Oita 879-5593, Japan

[3] Division of Radioisotope Research, Department of Research Support, Research Promotion Project, Faculty of medicine, Oita University, 1-1 Idaigaoka, Hasama-machi, Yufu, Oita 879-5593, Japan

Introduction

The collagen superfamily plays an important role, not only in stabilizing the tissues as structural components but also in regulating a variety of biological functions, such as development, differentiation, proliferation, and morphogenesis. There are more than 46 distinct α-chains of collagen genes, which form at least 28 types of collagen proteins (Ricard-Blum 2012). The fibrillar collagens (types I, II, III, V, XI, XXIV, and XXVII) are divided into three subgroups, major (I, II, and III), minor (V and XI), and new (XXIV and XXVII) fibrillar collagens on the basis of their contents in tissues and phylogenetic analyses. Vertebrate fibrillar collagens are either restricted to cartilage (II, XI, and XXVII) or widely distributed in non-cartilage tissues (I, III, V, and XXIV) (Exposito *et al.* 2010; Fang *et al.* 2012). Cartilage is a highly specialized tissue and serves as a template for the growth and development of bones. It contains an extensive extracellular matrix, which included types II, IX, and XI collagen, and provides

mechanical strength to resist compression in joint (Mendler *et al.* 1989).

Type XI collagen is a fibrillar collagen mainly found in the cartilage, and consists of three polypeptide chains, α1(XI), α2(XI), and α3(XI). The α1(XI) and α2(XI) chains are genetically distinct products, whereas the α3(XI) chain is an overglycosylated variant of the α1(II) chain, which is encoded by the type II collagen gene (Ricard-Blum 2012). Type XI collagen co-polymerizes with type II collagen within collagen fibrils and is important for regulation of the fibril diameter and function in connective tissues (Mendler *et al.* 1989). Therefore, the absence and/or mutations of the α1(XI) chain result in abnormally thickened cartilage collagen fibrils. In human disease, mutations in the *COL11A1* gene have been identified in Stickler and Marshall syndromes, which are clinically characterized by altered facial appearance, eye abnormalities, joint alterations, and hearing loss (Annunen *et al.* 1999; Myllyharju and Kivirikko 2001). Furthermore, fibrochondrogenesis, which is short-limbed skeletal dysplasia, is also caused by mutations in the *COL11A1* gene (Tompson *et al.* 2010). These observations suggest that the expression of Col11a1 is critical for maintaining normal structure and function of cartilage.

Among cartilage collagens, type II collagen is well characterized as a model gene of chondrogenic marker. The *COL2A1* gene expression is regulated by the transcriptional activator and/or repressor such as Sox9 (Bell *et al.* 1997; Zhou *et al.* 1998), Sp1/3 (Ghayor *et al.* 2001; Renard *et al.* 2012), Egr-1 (Tan *et al.* 2003), c-Krox (Ghayor *et al.* 2000), and Ap2 (Niebler and Bosserhoff 2013) in both the proximal promoter and the first intron. It has been reported that two chondrocyte-specific enhancer elements were identified in the proximal promoter and the first intron of the *Col11a2* gene and were regulated by Sox9 (Bridgewater *et al.* 1998; Liu *et al.* 2000). In addition, we have first reported that NF-Y regulates the proximal promoter activity of the *Col11a1* gene in chondrocytes (Hida *et al.* 2014). However, the molecular mechanism of transcriptional regulation of the *Col11a1* gene in chondrocytes is not completely characterized yet.

In this study, we have additionally investigated the transcriptional regulation of the mouse *Col11a1* gene in chondrocytes. Transient transfection assays, using deletion and mutation constructs, revealed that the downstream region of the NF-Y binding site, especially in the GC-rich sequence, is necessary for the activation of basal transcription of the mouse *Col11a1* gene. In silico analysis of the GC-rich sequence suggested that a potential candidate was transcription factor Sp1, and it was confirmed by cell transfection experiments with overexpression and/or knockdown of Sp1. Taken together, these results demonstrated that Sp1 upregulates the proximal promoter activity of the mouse *Col11a1* gene in chondrocytes.

Materials and Methods

Cell cultures. Rat chondrosarcoma (RCS) was used in this study. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum at 37°C in humidified 5% CO_2 and 95% air as described previously (Hida *et al.* 2014).

Construction of chimeric plasmids. All mouse Col11a1 promoter-luciferase constructs were derived from the pBACe3.6 RP24-512D13 clone as previously reported (Hida *et al.* 2014). Nucleotide substitutions were generated by site-directed mutagenesis as described previously (Matsuo *et al.* 2003).

For overexpression experiments, mouse full-length Sp1 cDNA was generated by RT-PCR using mouse embryonic cDNA library as a template. A sense primer was designed with KpnI site sequence, and an antisense primer was designed with NotI site sequence, respectively. The PCR product was cloned into the pGEM-T Easy vector (Promega, Fitchburg, WI), followed by digestion with KpnI and NotI, and subcloned into the KpnI/NotI site of pcDNA3 mammalian expression vector (Invitrogen, Carlsbad, CA).

For knockdown experiments, synthetic oligonucleotides were inserted between the mouse U6 promoter and the terminator sequence of the pBAsi-mU6 vector (TAKARA, Tokyo, Japan) to generate a stem-loop type of small interfering RNA (siRNA) in the transfected cells. siDirect2.0 siRNA design software (http://sidirect2.rnai.jp/) was used to select highly effective siRNA sequences of the targeting gene. The sense target sequence for rat Sp1 (5'-GAGTAATGCC TAATATTCAGT-3'), followed by the hairpin loop sequence (5'-CTGTGAAGCCACAGATGGG-3'), and the antisense target sequence were synthesized and cloned into the BamHI/HindIII site of pBAsi-mU6 vector. The sense scramble sequence for negative control (5'-AGATCTCAAG TTCCTCACACC-3') was described previously (Huang *et al.* 2008). All constructs were sequenced with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Waltham, MA).

Cell Transfection and luciferase assay. The cells were plated at a density of 1.5×10^5 cells per 35-mm dish 18 h before transfection. For transient transfection, each of the plasmid DNA was transfected into the cells by using the calcium phosphate-mediated precipitation methods with some modification (Chen and Okayama 1987).

For overexpression and/or knockdown experiments, the cells (1×10^6 cells) were mixed with the expression or knockdown plasmids with luciferase constructs. The cell suspension with plasmid DNAs was added to 2-mm gap cuvettes and was electroporated at 175 V for 5 ms using a NEPA electroporator according to the manufacturer's protocol (Nepa Gene, Chiba, Japan).

Luciferase activity was assayed 24 h later by the Dual-luciferase™ reporter assay system (Promega, WI) using a luminometer (Lumat, 9507, Berthold Technologies, Bad Wildbad, Germany). The pRL-TK *Renilla reniformis* luciferase expression vector was used as an internal control for transfection efficiency. The relative luciferase activity of each construct was compared with the proximal promoter construct, pGL3+1+68 for Figs. 1*b* and 3*b* or pGL3-366+68(wt) for Figs. 2*b* and 4, respectively. Results were expressed as means±SE of five independent experiments.

RNA analysis. Total RNA was isolated from RCS using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instruction. Reverse transcriptions were carried out using Rever Tra Ace reverse transcriptase (TOYOBO, Tokyo, Japan) with Random and Oligo(dT) primers, and the resulting single-stranded cDNA molecules were PCR amplified using gene-specific primers. For a quantification of mRNA, real-time PCR was performed using a LightCycler TaqMan Master (Roche, Indianapolis, IN). 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 using a comparative threshold cycle method (Page and Stromberg 2011).

The primers for Col11a1, Sp1, and GAPDH were as follows: Col11a1 sense, 5'-CCCGACTGTGACTTAACATCC-3', antisense, 5'-GTCCTCTTCTTCTTTGTGTAATTGG-3'; Sp1 sense, 5'-GCTATAGCAAACACCCCAGGT-3', antisense, 5'-CAGGGCTGTTCTCTCCTTCTT-3'; and GAPDH sense, 5'-TGGGAAGCTGGTCATCAAC-3', antisense, 5'-GCATCACCCCATTTGATGTT-3'.

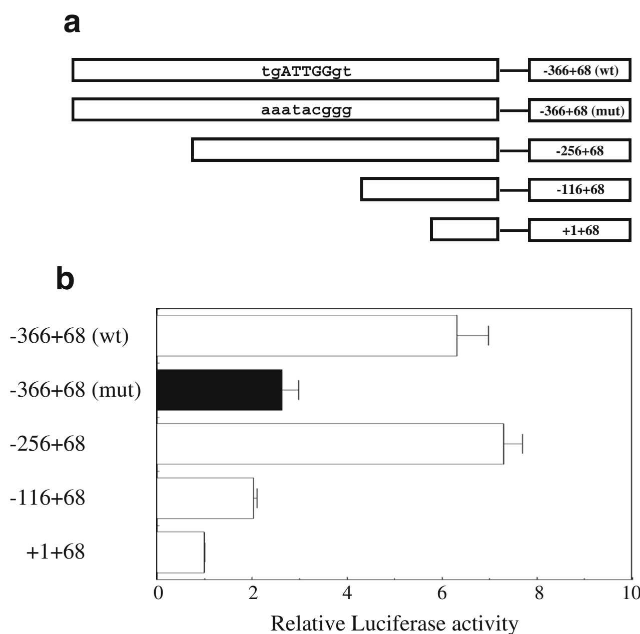

Figure 1. Determination of the minimal promoter region of the mouse *Col11a1* gene. (*a*): Schematic representation of the 5'-deletion and mutation constructs of the mouse Col11a1 promoter. pGL-366+68(mut) was mutated to the NF-Y binding site. (*b*): Deletion and mutation analysis of the mouse Col11a1 proximal promoter. Relative luciferase activities of each construct were assessed in transiently transfected RCS cells. A series of mouse Col11a1 promoter-luciferase constructs were transfected into the cells with the pRL-TK vector as an internal control for transfection efficiency. Relative luciferase activities were normalized against the activity of the pGL+1+68 vector, and results are expressed as mean± SE of five independent experiments.

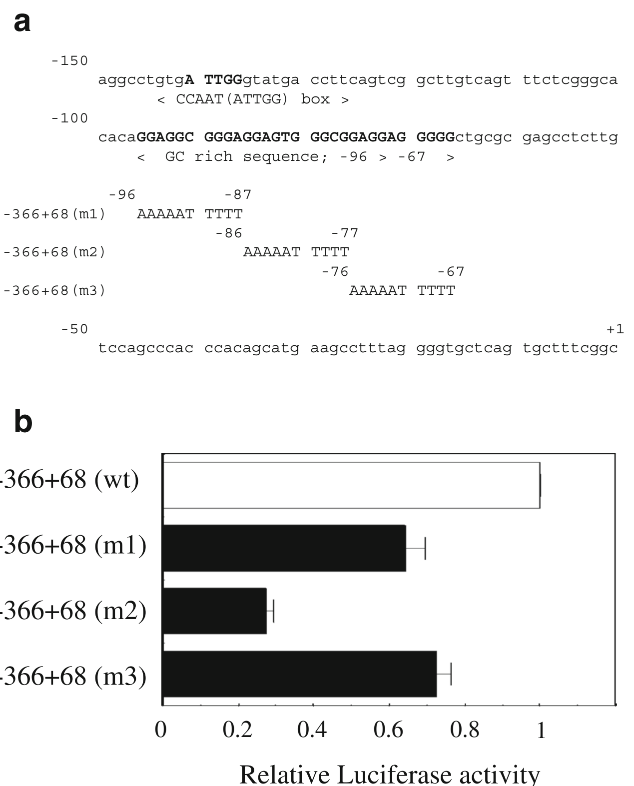

Figure 2. The GC-rich sequence was necessary to regulate the proximal promoter of the mouse *Col11a1* gene. (*a*): Nucleotide sequence of the proximal promoter region of the mouse *Col11a1* gene. The GC-rich sequence (total 30 bp) was divided into three regions (10 bp): m1(-96 to -87), m2(-86 to -77), and m3(-76 to -67), respectively, and these regions were replaced with the AAAAATTTTT sequence. The CCAAT (ATTGG) box and GC-rich sequences are shown by a *capital letter*. (*b*): Mutation analysis of the mouse Col11a1 proximal promoter. A series of mutation luciferase constructs (m1, 2, 3) were transfected into RCS cells with the pRL-TK vector as an internal control for transfection efficiency. Relative luciferase activities were normalized against the Wt (pGL-366+68) vector, and results are expressed as mean±SE of five independent experiments.

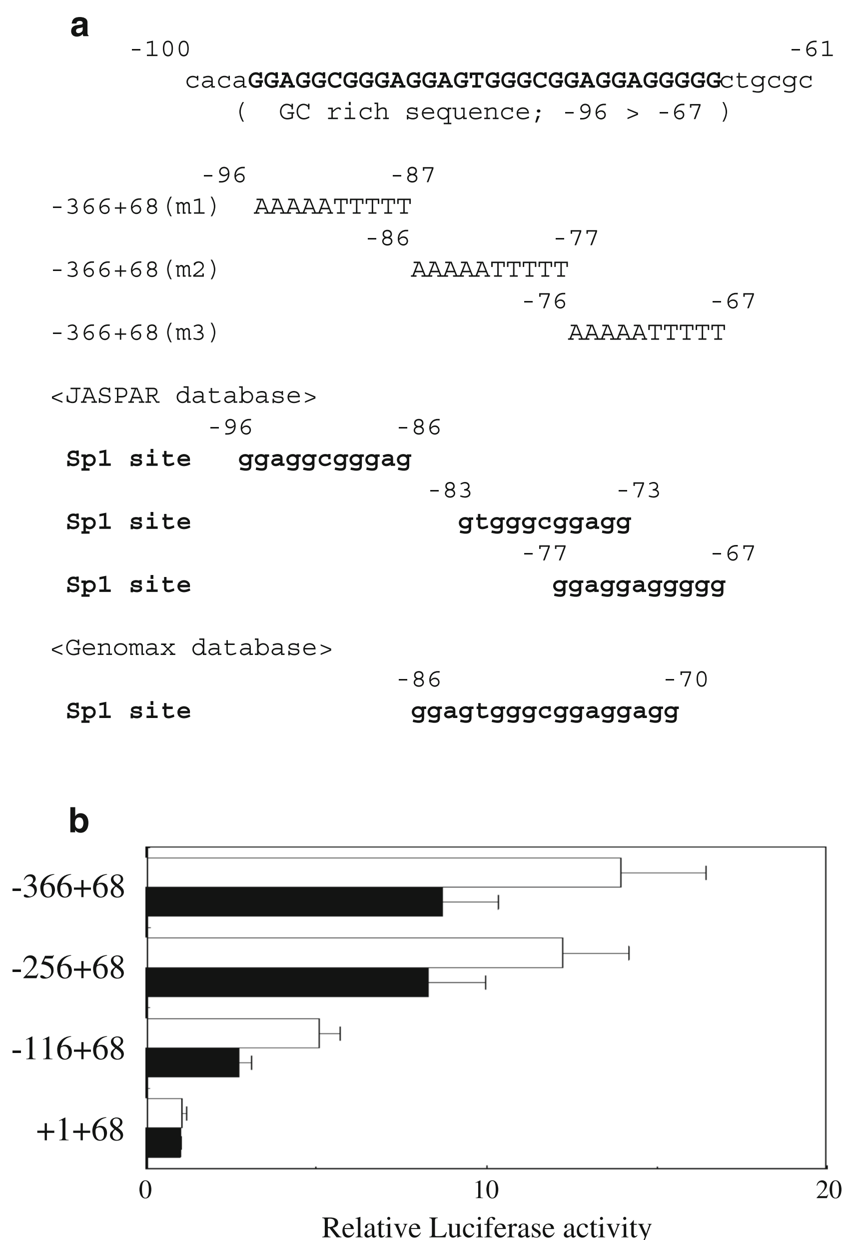

Figure 3. Transcription factor Sp1 upregulated the proximal promoter activity of the mouse *Col11a1* gene. *(a)* Nucleotide sequence of the putative Sp1 binding sites in the proximal promoter region. The JASPAR database indicated three binding sites: -96 to -86, -83 to -73, and -77 to -67, and the Genomax database indicated one binding site: -86 to -70, respectively. The putative Sp1 binding sites are shown by a *bold letter. (b)* Effect of overexpression of Sp1 on the mouse Col11a1 promoter activity. A series of 5'-deletion luciferase constructs were co-transfected with the Sp1 expression vector (pcDNA-Sp1) or pcDNA3 empty vector into RCS cells. Relative luciferase activities were normalized against pGL+1+68 treated with the pcDNA3 empty vector, and results are expressed as mean±SE of five independent experiments. *White*: co-transfection with the pcDNA-Sp1 expression vector, *Black*: co-transfection with the pcDNA3 empty vector.

Results

Downstream region of the NF-Y binding site is necessary to upregulate the proximal promoter activity of the mouse Col11a1 gene. We previously reported that NF-Y regulates the proximal promoter activity of the mouse *Col11a1* gene in chondrocytes (Hida *et al.* 2014). As shown in Fig. 1*b*, however, pGL-116+68 had slightly increased the luciferase activity compared with pGL+1+68, which has no promoter region. Furthermore, pGL-366+68(mut), in which the NF-Y binding site was mutated (Fig. 1*a*), failed to inhibit the proximal promoter activity completely, and the degree of luciferase activity was almost the same as pGL-116+68 (Fig. 1*b*). These results indicated that the additional region between +1 and -116 was also necessary to the proximal transcriptional activity of the mouse *Col11a1* gene.

GC-rich sequence is necessary to upregulate the proximal promoter activity of the mouse Col11a1 gene. In eukaryotes, the TATA box is a highly conserved element, and located approximately -35 to -25 bp upstream of the transcriptional start site. However, the *Col11a1* gene has no TATA sequences in the proximal promoter region as shown in Fig. 2*a*. In this study, we focused on the 30-bp GC-rich sequence (-96 to -67) in the downstream region of the NF-Y binding site, because the GC-rich sequences are also important regulatory elements in the promoter region of many genes, especially the TATA-less genes. A series of mutation constructs (m1, -96 to -87;

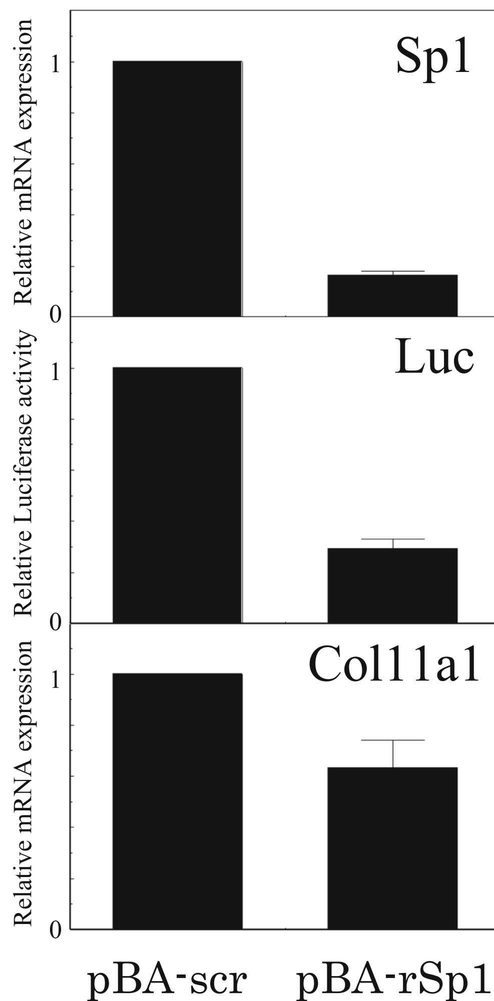

Figure 4. Knockdown of Sp1 expression downregulated the Col11a1 promoter activity and the expression of endogenous Col11a1 transcript in chondrocytes. pGL-366+68(wt) construct was co-transfected with the Sp1 siRNA expression vector (pBA-Sp1) or the scramble siRNA expression vector (pBA-scr) into RCS cells. Relative luciferase activities and the expression level of the endogenous Col11a1 transcript were normalized against pGL-366+68 co-transfected with pBA-scr, and results are expressed as mean±SE of five independent experiments.

m2, -86 to -77; and m3, -76 to -67) were generated as shown in Fig. 2*a*, and then luciferase assays were carried out. Figure 2*b* showed that all mutation constructs have significant reductions in the luciferase activity, and especially, m2 (-86 to -77, mutated in the center of the GC-rich sequence) has a dramatic reduction (70–80%) compared with pGL-366+68(wt). These results suggested that the GC-rich sequence was also important for the promoter activity of the *Col11a1* gene.

Sp1 upregulates the proximal promoter activity of the mouse Col11a1 gene. Next, we performed in silico analysis using JASPAR database (http://jaspardev.genereg.net/) and Genomatix (http://www.genomatix.com/) to identify putative transcriptional factors and their binding sites involved in the GC-rich sequence. As shown in Fig. 3*a*, in silico analysis using both databases indicated that one of the most promising candidate was transcription factor Sp1, which is probably helping recruit the TATA-binding protein in the TATA-less gene.

To confirm whether Sp1 regulated the proximal promoter activity of the mouse *Col11a1* gene in chondrocytes, a series of 5'-deletion luciferase constructs were co-transfected with Sp1 overexpression plasmid into RCS cells. As shown in Fig. 3*b*, all luciferase constructs with the GC-rich sequence had significant increase in the luciferase activity, whereas pGL+1+68 lacking the GC-rich sequence had no effect on the luciferase activity by overexpression of Sp1. The ratio of the relative luciferase activity was also summarized in Table 1. The maximum ratio was exhibited by pGL-116+68, and the ratio was not increased in both pGL-256+68 and pGL-366+68, suggesting that the upstream region of the NF-Y binding site was not affected by overexpression of Sp1.

Consistent with these observations, knockdown of Sp1 expression by siRNA transfection also significantly reduced the proximal promoter activity of the mouse *Col11a1* gene and the expression of the endogenous mouse Col11a1 transcript (Fig. 4). Taken together, our data indicate that the transcription factor Sp1 upregulates the proximal promoter activity of the mouse *Col11a1* gene in chondrocytes.

Discussion

Chondrogenesis is an important biological event in skeletal development and tissue formation. It starts from multipotent mesenchymal cells, and then these cells proliferate, followed by differentiating into chondrocytes. During these processes, the cells secrete chondrogenic matrices such as types II, IX, XI, and XXVII collagens that surround themselves. Therefore, to understand chondrocyte differentiation and cartilage formation, it is necessary to study the regulation of cartilage collagens in chondrocytes (de Crombrugghe *et al.* 2000). Among them, type XI collagen, particularly in Col11a1, play a critical role in chondrocyte differentiation and skeletal organization. That is because the α1(XI) chain-deficient mice (*cho* mice), although α1(II) chain is produced normally, showed

Table 1. Ratio of the relative luciferase activity by overexpression of Sp1

Col11a1 construct	Ratio of relative luciferase activity
pGL+1+68	1.02±0.12
pGL-116+68	1.89±0.06
pGL-256+68	1.60±0.29
pGL-366+68	1.63±0.18

Data represented the ratio of pcDNA-Sp1 to pcDNA3 as referred to in Fig. 3*b*. Results were expressed as means±SE of five independent experiments

irregular collagen fibrils in their cartilage, resulting in a chondrodystrophic phenotype (Li *et al.* 1995).

We previously reported that the transcription factor NF-Y regulates the proximal promoter activity of the *Col11a1* gene in chondrocytes. NF-Y specifically binds to the CCAAT (or ATTGG) sequences, and regulates a wide range of genes, both ubiquitous and/or tissue-specific genes. In most cases, NF-Y cooperatively works together with other transcription factors in the promoters, resulting in the transcriptional activation and/or repression (Dolfini *et al.* 2012). In this study, we provided additional evidence that the transcription factor Sp1 bound to the GC-rich sequence in the proximal promoter, and upregulated the transcription of the *Col11a1* gene in chondrocytes. It has been assumed that the transcription initiation of the eukaryotic gene could be directed by the TATA box or by the CG/GT box in the TATA-less gene (Dikstein 2011). In this context, it is one possibility that Sp1 may recruit TATA-binding proteins and locates them in the correct transcription start site, since the *Col11a1* gene has no TATA box as shown in Fig. 2. In addition to the machinery of recruiting TATA-binding proteins, the Sp1-like family is able to regulate many eukaryotic promoters as the transcriptional activators or repressors. Their regulatory mechanisms of Sp1 transcription factor is as follow: as activators, Sp1 binds to the GC elements in the promoter and/or enhancer regions with other transcription mediators, and as repressors, Sp1-like repressors such as Sp3 or other transcription mediators compete the binding to GC elements with Sp1 (Zhao and Meng 2005). Many transcription factors were shown to regulate the transcription with the Sp1-like family, and the combination between NF-Y and Sp1 has been also reported to activate and/or repress the transcription synergistically (Yamada *et al.* 2000; Ge *et al.* 2002). For other fibrillar collagen genes, the previous reports demonstrated that Sp1 regulates the proximal promoter activity of types I (García-Ruiz *et al.* 2002; Kypriotou *et al.* 2007), II (Dharmavaram *et al.* 1997; Ghayor *et al.* 2001), V (Wu *et al.* 2010a, b), and XI (Goto *et al.* 2006) collagen genes. The proximal promoter of Col2a1 is regulated by the combination of Sp1 and Sp3, which bind to the GC-rich sequence. Sp1 is a potent activator of Col2a1 transcription by binding to the GC-rich element in the promoter and/or enhancer, whereas Sp3 inhibits the promoter activity by competing with Sp1 as described above (Ghayor *et al.* 2001). Sp1, Sp3, and Sp7 upregulate the promoter activity of the *Col11a2* gene in osteoblasts, but not in chondrocytes, and the degree of their transcription activities are almost identical. In this case, Sp3 does not inhibit the promoter activity of the *Col11a2* gene by competing with Sp1 (Goto *et al.* 2006). On the other hand, in non-cartilage collagens, the transcription of type I collagen genes are also regulated by Sp1 and Sp3 in fibroblasts and hepatic stellate cells (García-Ruiz *et al.* 2002; Kypriotou *et al.* 2007). In addition, Sp7/Osterix, one of the Sp1-like transcription factor, upregulates the transcriptional activity of types I (Ortuño *et al.* 2013; Yano *et al.* 2014) and V (Wu *et al.* 2010a, b) genes in osteoblasts. These studies suggested that the Sp1-like family regulates the expression of collagen genes in a gene-specific and/or a tissue-specific manner. Furthermore, the Sp1-like family may regulate, at least in part, in chondrocyte differentiation, cartilage formation, and skeletal development. The interaction between NF-Y and Sp1-like transcription factors of the *Col11a1* gene is not clarified in this study; however, further studies will be required to address these questions.

Another transcriptional regulation is modulated by tissue-specific cis-acting element, such as enhancers and/or silencers. In cartilage, Sox9, one of the HMG-box containing a transcription factor, is essential for chondrocyte differentiation and skeletal development (Akiyama 2008). As described previously, cartilage collagens are composed of types II, IX, and XI collagens. In the *Col2a1* gene, it has been reported that the chondrocyte-specific enhancer element is identified, and Sox9 regulates the *Col2a1* gene expression (Bell *et al.* 1997; Zhou *et al.* 1998). The *Col11a2* gene has also two chondrocyte-specific enhancer elements in the proximal promoter and the first intron and is regulated by Sox9 similar to those of the *Col2a1* gene (Bridgewater *et al.* 1998; Liu *et al.* 2000). The *Col9a1* gene also has Sox9 binding elements in the proximal promoter, resulting in activation of the proximal promoter by Sox9 (Zhang *et al.* 2003; Genzer and Bridgewater 2007). On the other hand, there is no direct evidence that Sox9 regulates the expression of the *Col11a1* gene. However, Lincoln *et al.* have shown that Sox9 is required for heart valve development as well as the *Col11a1* and *Col5a1* genes, suggesting that it is possible that Sox9 may regulate the expression of the *Col11a1* and *Col5a1* genes (Lincoln *et al.* 2006, 2007). In addition, it has been reported that lymphocyte enhancer-binding factor 1 (Lef1) indirectly activates the proximal promoter of the *Col11a1* gene in osteoblasts, and suppresses terminal osteoblast differentiation (Kahler *et al.* 2008). However, the chondrocyte-specific cis-acting elements have not been identified in the *Col11a1* gene yet. Thus, further study on the identification of tissue-specific transcription factor would clarity the mechanism of transcriptional regulation of the *Col11a1* gene, chondrocyte differentiation, cartilage formation, and skeletal development.

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