

ORIGINAL**Ctip2-mediated *Sp6* transcriptional regulation in dental epithelium-derived cells**

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Abstract : Tooth development relies on the interaction between the oral ectoderm and underlying mesenchyme, and is regulated by a complex genetic cascade. This transcriptional cascade is regulated by the spatiotemporal activation and deactivation of transcription factors. The specificity proteins 6 (*Sp6*) and chicken ovalbumin upstream promoter transcription factor-interacting protein 2 (*Ctip2*) were identified in loss-of-function studies as key transcription factors required for tooth development. *Ctip2* binds to the *Sp6* promoter *in vivo* ; however, its role in *Sp6* expression remains unclear. In this study, we investigated *Sp6* transcriptional regulation by *Ctip2*. Immunohistochemical analysis revealed that *Sp6* and *Ctip2* colocalize in the rat incisor during tooth development. We examined whether *Ctip2* regulates *Sp6* promoter activity in dental epithelial cells. Cotransfection experiments using serial *Sp6* promoter-luciferase constructs and *Ctip2* expression plasmids showed that *Ctip2* significantly suppressed the *Sp6* second promoter activity, although the *Sp6* first promoter activity was unaffected. *Ctip2* was able to bind to the proximal region of the *Sp6* first promoter, as previously demonstrated, and also to the novel distal region of the first, and second promoter regions. Our findings indicate that *Ctip2* regulates *Sp6* gene expression through direct binding to the *Sp6* second promoter region. *J. Med. Invest.* 61 : 126-136, February, 2014

Keywords : *ameloblast differentiation, Ctip2, Sp6, tooth development, transcriptional regulation*

INTRODUCTION

Tooth development is a good model of both the spatiotemporal regulation of genetic cascades and interaction between ectoderm and underlying neural crest-derived mesenchyme, and has the advantage of easy operation for organ culture (1-3). Tooth patterning and shaping are modulated by signaling pathways that mediate epithelial-mesenchyme interaction in developing teeth. These regulatory signals

function in complex networks characterized by abundant activators and inhibitors. The inhibition or stimulation of any of the major conserved signaling pathways in knockout mice can lead to arrested tooth formation or abundant *de novo* tooth formation (3).

The specificity protein (Sp) family of transcription factors comprises nine proteins that share a well-conserved, C₂H₂-type DNA-binding domain composed of three tandem zinc finger motifs in their C-terminal region (4, 5). Sp family proteins positively or negatively regulate the transcription of various target genes, depending on the cellular context. Although all Sp transcription factors exhibit a high degree of structural conservation of the three C-terminal zinc finger domains, each Sp protein has

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The nucleotide sequence of rat *Ctip2* cDNA has been deposited at GenBank under the following accession numbers ; JF520429 for *Ctip2*-long and JF520428 for *Ctip2*-short.

a distinctive role in the target gene regulation. Sp6 (also known as epiprofin) is a member of the Sp family expressed and implicated in the development of several epithelium-containing tissues, such as the epidermis, teeth, hair follicles, lungs, and limbs (6-8).

In particular, Sp6 functions in cell fate determination in the dental epithelium by regulating proliferation and differentiation. Thus far, two loss-of-function studies have attempted to understand Sp6 function *in vivo* (9, 10). In one study, Sp6-deficient model mice exhibited delayed tooth eruption followed by the formation of supernumerary teeth. These mice sometimes exhibited defects of tooth structure such as fragile, fused, and reduced in size. Another study featured a rat spontaneous disease model with mutated Sp6, known as AMI, exhibiting enamel defects reminiscent of the amelogenesis imperfecta phenotype (11). We also recently established an Sp6 transgenic rat as a gain-of-function model (12). In these rats, ectopic Sp6 expression caused morphologic and metabolic changes in ameloblast differentiation. These findings indicate the importance of the spatiotemporal regulation of Sp6 expression during normal tooth development. However, the mechanisms by which Sp6 gene expression is regulated remain unclear.

Ctip2 (also known as Bcl11b or Rit1) was recently reported to bind to the Sp6 promoter in mice (13, 14). Ctip2 is also a C₂H₂-type zinc finger transcription factor involved in the development of several tissues, including neurons, T cells, skin, and teeth (13, 15-19). Mice lacking both Ctip2 alleles [Ctip2-knockout (KO) mice] die shortly after birth from unknown causes, but exhibit defects in the immune system, central nervous system, skin, teeth, and hair cells of the cochlea (13, 15-20). Ctip2 regulates gene expression by directly binding to a GC-rich consensus sequence in its target genes or by indirectly binding to promoter regions through other transcription factors, such as Sp1 and COUP-TF (21, 22). Ctip2 functions as either a transcriptional repressor or activator, depending on context (20). These observations suggest that Ctip2 plays a critical role in the development and tissues homeostasis.

A recent study revealed the disruption of several aspects of enamel formation in Ctip2-KO mice, including enamel knot, stellate reticulum, and cell polarity (13). Both mRNA and protein expression levels of ameloblast differentiation markers, such as amelogenin, ameloblastin, and enamelin, were decreased in Ctip2-KO mice. Furthermore, Msx2

and Sp6 mRNA levels, important transcription factors in tooth development whose promoters Ctip2 is known to bind to, were downregulated in Ctip2-KO mice. Based on these findings, we hypothesized that Ctip2 is an upstream regulator of Sp6 expression during tooth development.

In this study, we investigated the role of Ctip2 in the regulation of Sp6 expression. To explore the potential interaction between Ctip2 and Sp6, we first analyzed their localization *in vivo* using postnatal day 1 (P1) rat mandibular incisors. Subsequently, we examined the effect of Ctip2 on Sp6 promoter activity using promoter-luciferase reporter constructs. Finally, we assessed whether Ctip2 can directly bind to the Sp6 promoter region. We demonstrated that Ctip2 represses Sp6 gene expression through direct binding.

MATERIALS AND METHODS

Immunohistochemistry

Stroke-prone spontaneous hypertensive (SHRSP) rats (11) were maintained and treated in accordance with the guidelines for animal experimentation of the University of Tokushima, Tokushima, Japan, and the experimental protocol was approved by the Ethics Committee for Animal Experimentation of the University of Tokushima (No. 11114). Surgery was performed under ether anesthesia, and all efforts were made to minimize suffering. Mandibular incisors were excised from SHRSP rats at P1. Mandibular incisors were fixed with 4% paraformaldehyde and blocked in paraffin. Sections of paraffin-embedded incisors 4 μm thick were used for immunohistochemistry. Endogenous peroxidase activity was prevented in each section using 3% H₂O₂ in methanol. Antigens were retrieved by microwave using Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA, USA). The sections were subsequently blocked with 3% normal horse serum in phosphate-buffered saline [PBS(-)], and incubated with anti-Sp6 (1 : 1000) as previously described (8) or anti-Ctip2 antibody [1 : 300 (ab28448) ; Abcam, Cambridge, UK]. For negative controls, normal rabbit serum (1 : 1000) or immunoglobulin G (IgG) (1 : 300) were used. The sections were incubated with the primary antibodies overnight at 4°C. After washing with 1× PBS(-) twice for 5 min, tissue sections were incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody (Histofine ; Nichirei Biosciences, Tokyo, Japan), at the same dilution as

the primary antibody, for 30 min at room temperature. Immune complexes were detected using 3,3'-diaminobenzidine-catechol substrate concentrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). After hematoxylin counterstaining, the stained sections were observed under a light microscope (BX51-34 FL-1-K-O ; Olympus Corporation, Tokyo, Japan).

Isolation of rat Ctip2 isoforms and construction of Ctip2 expression vectors

The entire rat *Ctip2* coding region, with additional nucleotides for the *Sal*I site at the 5'-end and FLAG-tag plus *Not*I site at the 3'-end was generated by reverse transcription polymerase chain reaction (RT-PCR) using PrimeSTAR GXL DNA Polymerase (Takara Bio, Shiga, Japan) from rat molar RNA (P7). FLAG-tagging was designed at the C-terminus of *Ctip2* cDNA in the reverse primer. DNA fragments were amplified using the primers 5'-CTAGTCGACGCCATGTCCCGCCGCAAACAGGGCAACCCGCAG-3' and 5'-CTAGCGGCCGCTTACTTATCGTCGTCATCCTTGTAATCGCTCCTCTCAGCCTGCTCGATTTTGAC-3' (the underlined region indicates the FLAG-tag sequence), and cloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA). The sequences of these fragments were confirmed using an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). After double digestion with the *Sal*I and *Not*I restriction enzymes, the DNA fragments *Ctip2*-long and *Ctip2*-short plus their FLAG-tag sequences were recloned into the mammalian expression vector, pCI-neo (Promega).

In silico analysis

Comparison of *Ctip2* amino acid (aa) sequences among species and search for DNA binding motif of transcription factors were performed using GENETYX software (GENETYX, Tokyo, Japan).

Ctip2 expression in rat dental epithelial cells

The mRNA expression of *Ctip2* isoforms in rat dental epithelial G5 cells was confirmed by RT-PCR. Total RNA was isolated using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) and treated with DNase I (Invitrogen Japan, Tokyo, Japan). cDNA was synthesized with random primers using the TaKaRa RNA PCR Kit (AMV) version 3.0 (Takara Bio), according to the manufacturer's protocols. RT-PCR analysis was performed using GoTaq DNA Polymerase (Promega). Gene-specific primers

(5'-ACCATGTGGAGGCTGCTATC-3' and 5'-GCTGTTGAAGGGCTGCTTAC-3') were synthesized (Hokkaido System Science, Hokkaido, Japan).

Cell culture

Rat dental epithelial-derived G5 cells were previously established in our laboratory (23). COS-7 cells as described previously (24) were purchased from the RIKEN BioResource Center through the National BioResource Project of the Ministry of Education, Culture, Sports, Science and Technology, Japan. G5 and COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/Nutrient Mixture F-12 and DMEM medium, containing 10% fetal bovine serum at 37°C in a 5% CO₂ atmosphere. G5 and COS-7 cells were passaged at 80% and 75% confluence, respectively.

Dual-luciferase reporter assay

Sp6 promoter constructs (A: -4688/+76; B: +165/+5283; C: +4319/+7890; D: +4319/+5435; E: +5427/+7890) has been prepared and reported previously (25). When G5 cells reached up to 75% confluence in 24-well plates, the cells were cotransfected with 4 ng of pRL-TK, 400 ng of *Sp6* promoter constructs, and 40 ng of expression vectors [either *Ctip2*-long, *Ctip2*-short, or pCIneo empty vector (Promega)] using 0.8 µl of X-tremeGENE HP DNA Transfection Reagent (Roche Applied Science, Tokyo, Japan), according to the manufacturer's instructions. pGL3-Control and pGL3-Basic vectors (Promega) were used instead of *Sp6* promoter constructs as positive and negative controls, respectively. The DNA mixture was incubated for 15 min at room temperature. Untreated G5 cells were not cotransfected with any plasmids. All samples were incubated at 37°C in a 5% CO₂ atmosphere for 24 h. Cotransfected cells were harvested with 1× Passive Lysis Buffer (Promega). Luciferase activity in the extracted lysates was measured using a Lumat LB 9507 Luminometer (Berthold Japan, Tokyo, Japan) and the Dual-Luciferase Reporter Assay System (Promega), according to the manufacturer's instructions. The ratio of firefly to *Renilla* luciferase activities was calculated and normalized to the background value of the pGL3-control Vector. Assays were performed in triplicate.

Chromatin immunoprecipitation analysis

The chromatin immunoprecipitation (ChIP) assay was performed using the ChIP-IT Express Enzymatic Kit (Active Motif, Tokyo, Japan), according

to the manufacturer's instructions. In brief, when COS-7 cells were grown to 70% confluence in 100-mm dishes, cells were cotransfected with the *Sp6* promoter-luciferase constructs (A or D) and *Ctip2* expression vectors (*Ctip2*-long or *Ctip2*-short) using 10 µl of X-tremeGENE HP DNA Transfection Reagent (Roche Applied Science) according to the manufacturer's protocols. After incubation for 24 h, cotransfected cells were fixed with 37% formaldehyde in serum-free culture medium for 7 min at room temperature on a shaker. Fixation was stopped using glycine stop solution, and nuclei were extracted using a 1 ml Dounce homogenizer (Wheaton Science Products, Millville, NJ, USA). For immunoprecipitation, 1 µg of anti-Ctip2 antibody (Abcam) was used. PCR was performed using the following ChIP-PCR primers: D-region forward, 5'-AAGGATCTCTGGAAACCAGG-3'; reverse, 5'-TGGCTACCAGTGCTTTTGAC-3'; proximal forward, 5'-AGCTGCCTCTTCCAGATGAA-3'; reverse, 5'-CCAGGAAGTAGTGGCCTGAT-3'; distal forward, 5'-GCCCCACTGTTTAGCTCTGA-3'; reverse, 5'-AGACCCCTCCTCCTTAACCA-3'; using the ChIP-IT Express Enzymatic Kit (Active Motif Japan).

RESULTS

Ctip2 and *Sp6* colocalization in the rat incisor

To examine *Ctip2* and *Sp6* expression patterns during ameloblast differentiation in teeth, we performed an immunohistochemical analysis using mandibular rat incisors at P1, in which all stages of ameloblast differentiation can be observed. *Sp6* was particularly detected in the ameloblast and odontoblast layers. *Ctip2* was also detected in both layers parallel to the *Sp6* signal indicated by white arrows as shown in Figure 1B. Among the differentiation stages of ameloblasts, both *Ctip2* and *Sp6* signals were clearly detected at the pre-ameloblast stage to the secretory stage, and localized in the nuclei of ameloblasts and odontoblasts. However, at the maturation stage, both signals were detected only in ameloblasts, not odontoblasts. These results showed that *Ctip2* and *Sp6* colocalize in the nuclei of cells in the ameloblast layer undergoing ameloblast differentiation, suggesting that *Ctip2* and *Sp6* have a functional association.

Polymerase chain reaction-cloning of rat Ctip2 isoforms

Two *Ctip2* isoforms have been reported in humans

and three in mice (14). On the other hand, the genetic information regarding rat *Ctip2* was only able to retrieve from GenBank under the accession number NM_001108057. At first, we compared both the aa and mRNA sequences between human (h), mouse (m), and rat (r). The accession numbers of the aa sequences were: NP_612808 for hCTIP2-1, NP_075049 for hCTIP2-2, NP_001073352 for mCtip2a, NP_067374 for mCtip2b, and NP_001101527 for rCtip2. The accession numbers of the mRNA sequences were: NM_138576.2 for hCTIP2-1, NM_022898.1 for hCTIP2-2, NM_001079883 for mCtip2a, NM_021399 for mCtip2b, and NM_001108057 for rCtip2. As shown in Figure 2A, aa sequences were highly conserved among the three species, although we observed that reported rat *Ctip2* does not contain 22 amino acid residues at N-terminus. mRNA sequences were also highly conserved among the three species, and we found that the surrounding sequences of the start codon were identical at the rates of 62 out of 70 and 53 out of 55 nucleotides upstream and downstream of the first ATG codon, respectively, between human and mouse *Ctip2* (Figure 2B). Therefore, next we searched the rat homologous sequences corresponding to 500 nucleotides at the 5'-end region of mouse *Ctip2* (taken from GenBank sequence database) in rat genome using Basic Local Alignment Search Tool (BLAST: <http://blast.ncbi.nlm.nih.gov/Blast.cgi>). As shown in Figure 2C, we found the identical sequences in the upstream region of second ATG. Based on these results, we isolated the entire *Ctip2* coding region by PCR-cloning using rat molar RNA as a template.

Before *Ctip2* cDNA cloning, we first examined the presence of *Ctip2* isoforms in rat molar RNA by RT-PCR and found two transcripts of the *Ctip2* (Figure 2D). Next, we designed the cloning primers combined with FLAG tag sequence, and performed PCR-cloning. Two isoforms of *Ctip2* coding sequence (cfs) were cloned and confirmed by sequencing (data not shown). We named them *Ctip2*-long and *Ctip2*-short and their sequences were deposited in GenBank with the accession numbers JF520429 and JF520428, respectively. *Ctip2*-long comprised four exons with a total of 2643 base pairs (bp), and *Ctip2*-short comprised three exons with a total of 2439 bp (Figure 2E). To examine the functional role of *Ctip2* in dental epithelial cells, we re-cloned both isoforms plus a C-terminal FLAG-tag into the mammalian expression vector, pCIneo. To confirm the expression levels of exogenous *Ctip2* mRNA transcribed from the constructed expression

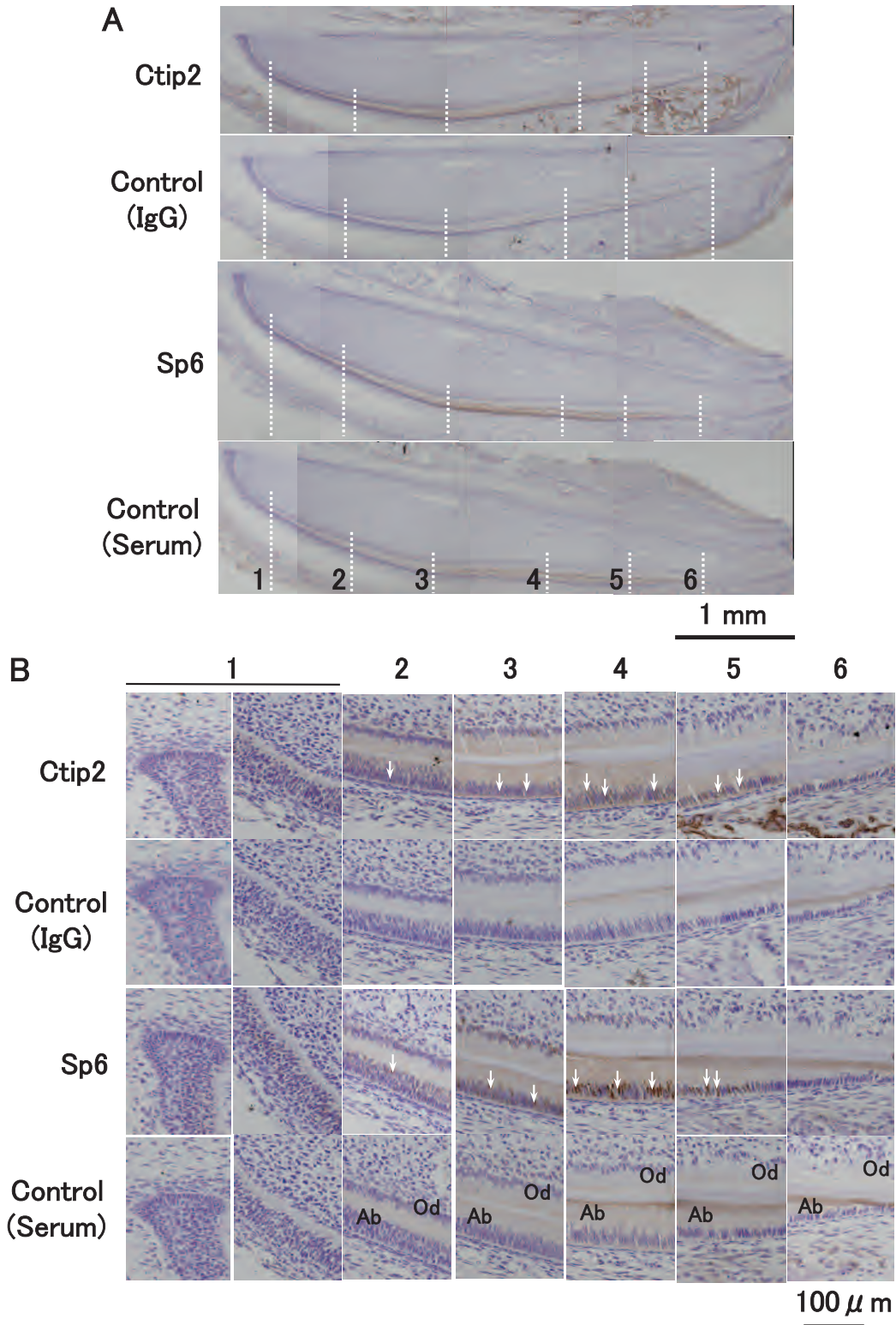


Figure 1 : Colocalization of *Ctip2* and *Sp6* during ameloblast differentiation.

Immunohistochemical analyses were performed using anti-*Ctip2* and anti-*Sp6* antibodies on serial sections of rat mandibular incisors (P1). (A) Whole structure of the incisor at low magnification. Numbers 1-6 indicate the stages of ameloblast differentiation : 1, undifferentiated ; 2, pre-ameloblast ; 3, presecretory ; 4, secretory ; 5, maturation ; 6, reduced. Scale bar indicates 1 mm. (B) High-magnification images of the incisor. White arrows show the positive signals indicating protein expression. Scale bar indicates 100 μ m. Numbers correspond to the same stages of differentiation as in (A). Abbreviations : Ab, ameloblasts ; Od, odontoblasts.

vectors, we transiently transfected either *Ctip2*-long in pCIneo (C-L) or *Ctip2*-short in pCIneo (C-S) into G5 cells. As expected, both exogenous mRNA of

Ctip2 isoforms were strongly detected compared to endogenous ones by RT-PCR analysis (Figure 2F)

promoter. Instead of that, we found another motif “AGCCAG” within the proximal region, but not in distal region. Then we decided to focus on both motifs GGCCGG and AGCCAG as the potential binding motifs of Ctip2. To test the functional association between Ctip2 and *Sp6* *in vitro*, we performed a luciferase reporter assay using several *Sp6* promoter constructs harboring the GGCCGG and AGCCAG motifs (Figure 3A). We consistently detected a suppressive effect of both Ctip2-long and Ctip2-short on *Sp6* promoter activity in the B- and C-regions, but almost no promoter activity in the A-region. We observed more significant repression and higher basal activity in the C-region than in the B-region, suggesting the presence of critical Ctip2 binding sites or Ctip2-mediated regulation in the C-region.

To further refine the Ctip2 target site(s), we divided the C-region into two parts, D- and E-regions and repeated the luciferase promoter analysis. As shown in Figure 3B, we repeatedly observed a suppressive effect of both Ctip2-long and Ctip2-short on the *Sp6* promoter at a similar ratio in the D-region but not in the E-region. Moreover, we also observed no significant effect of Ctip2 on luciferase activity via the E-region. Taken together, these results suggest that both Ctip2-long and Ctip2-short negatively regulate *Sp6* promoter activity through the *Sp6* second promoter, but not the first and potential third

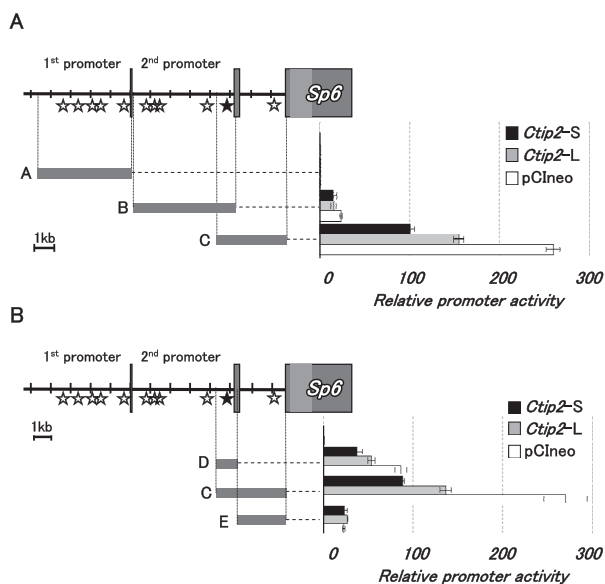


Figure 3 : Ctip2-mediated suppression of *Sp6* promoter activity in G5 cells.

(A, B) G5 cells were cotransfected with the indicated luciferase reporter construct containing *Sp6* promoter and either *Ctip2*-long (*Ctip2*-L), *Ctip2*-short(*Ctip2*-S), or an empty vector, and the cells were incubated for 24 h. A-E indicate the truncated *Sp6* promoter region used in reporter constructs. White star, AGCCAG ; black star, GGCCGG.

promoters in G5 cells.

Ctip2 binding to the *Sp6* promoter

To confirm whether Ctip2 directly binds to the *Sp6* promoter regions, we examined specific target regions within A-region of the *Sp6* first promoter into the proximal and distal parts, according to a previous study (13). In addition, we selected the D-region, within the *Sp6* second promoter that contains a GGCCGG motif, as a candidate region for a Ctip2 binding site based on the results of the luciferase promoter analysis (Figure 4A). We performed ChIP analysis with Ctip2 antibodies and subsequent PCR-targeting of both the proximal and distal regions within A-region of the *Sp6* first promoter and also D-region of the *Sp6* second promoter. We observed that both Ctip2-long and Ctip2-short were able to bind to the both proximal and distal region of the *Sp6* first promoter and also D-region of the *Sp6* second promoter (Figure 4B). These results indicated that both the Ctip2-long and Ctip2-short forms directly bind to both *Sp6* first and second promoters.

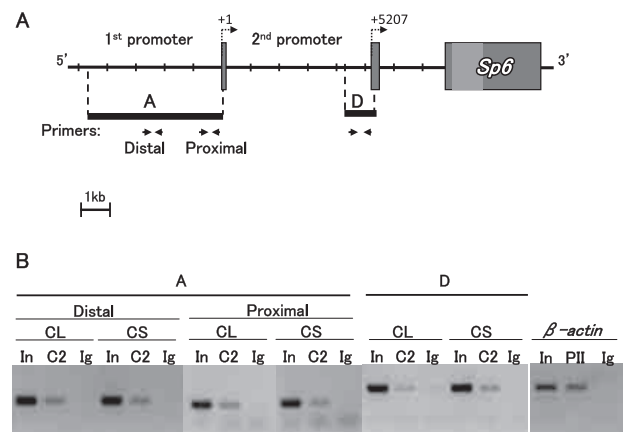


Figure 4 : Ctip2 binding to *Sp6* promoter.

COS-7 cells were cotransfected with the indicated reporter construct containing the *Sp6* promoter and either *Ctip2*-long, *Ctip2*-short, or an empty vector and incubated for 24 h. (A) Primer designs for chromatin immunoprecipitation (ChIP)-polymerase chain reaction (PCR). Black arrows indicate primers. Dotted arrows indicate transcription start sites from exon 1a (marked as +1) and exon 1b (marked as +5207). (B) ChIP-PCR binding. A and D indicate the *Sp6* promoter regions as in (A). Abbreviations : CL, Ctip2-long ; CS, Ctip2-short ; In, 1 : 10 input of chromatin sample ; C2, Ctip2 ; Ig, Immunoglobulin G, used as a control.

DISCUSSION

Golonzhka *et al.* (13) demonstrated that *Sp6* expression was downregulated in *Ctip2*-KO mice and that Ctip2 was able to bind to the *Sp6* proximal promoter *in vivo*. *Sp6* was originally demonstrated to

exert growth-promoting activity (6). In our previous study, we demonstrated the Sp6 expression and functions in ameloblast differentiation (27), which is believed to play a role in the late stages of amelogenesis (28). However, the functional relationship and molecular basis of Sp6 regulation by Ctip2 remain unelucidated.

In this study, we confirmed the concomitant expression profile of both Ctip2 and Sp6 during ameloblast differentiation in the rat incisor (Figure 1). We observed that both Ctip2 and Sp6 colocalized from the pre-ameloblast to the maturation stages, but primarily during differentiation rather than proliferation. These results supported our expectation of a functional association between Ctip2 and Sp6 during ameloblast differentiation.

By *in silico* analysis, we observed the Ctip2 structure to be highly homologous among human, mouse, and rat. Wakabayashi *et al.* discovered three different Ctip2 transcripts by RT-PCR using RNA isolated from the mouse thymus (14). However, we were unable to detect the third Ctip2 isoform, comprising exons 1 and 4, in P7 rat molar RNA. Wakabayashi *et al.* also demonstrated a high frequency of Ctip2 loss of heterozygosity and the Ctip2-mediated restoration of growth suppression in Ctip2 deficient cells, implying that Ctip2 functions in growth regulation (14). Although Sp6 has also been shown to enhance cells growth (6), direct evidence for a functional association between Ctip2 and Sp6 promoter regulation remains unclear. To further explore the role of Ctip2 in dental epithelial differentiation through Sp6, we isolated Ctip2 cDNA to examine the effect of its expression on Sp6. To investigate the functional association between Ctip2 and Sp6 regulation, we analyzed the effect of Ctip2 expression on Sp6 promoter activity. Ctip2 expression had no effect on the activity of the first Sp6 promoter, which was demonstrated to contain Ctip2 binding sites by a previous study (13). In contrast, Ctip2 exerted a suppressive effect on the activity of the Sp6 second promoter in dental epithelium-derived G5 cells (Figure 3A and 3B). These findings clearly demonstrate that Ctip2 negatively regulates Sp6 expression in our *in vitro* system, which is different from that reported in previous studies (13).

There are four possible reasons to explain the apparent discrepancy of Ctip2-Sp6 regulation between our findings and the previous study (13). First, we observed Ctip2-regulation at the level of Sp6 promoter activity by luciferase assay, however, Golonzhka *et al.* demonstrated Sp6 mRNA level by qRT-PCR.

The mRNA level is regulated by promoter activity, transcriptional efficiency, and mRNA stability. Therefore, it is difficult to compare directly the promoter activity and the mRNA level. Second, Sp6 promoter activity was evaluated by luciferase reporter assay, thereby DNA fragment of Sp6 promoter region was inserted into the luciferase reporter vector. This condition is different from *in vivo* situation, in which genes are usually regulated by epigenetic modifications depending on the developmental cues (29). Third, we solely examined the effects of Ctip2 on the Sp6 promoter *in vitro*. However, *in vivo* system, Ctip2 may promote a complex formation with other transcriptional cofactors to regulate Sp6 transcription as previously shown (20). In addition, signals of some cytokines from the environment during tooth development through epithelial and mesenchymal interactions may also affect the Ctip2-mediated transcriptional regulation. Fourth, Sp6 is known to be expressed in ameloblasts and odontoblasts in teeth, skin and hair follicles (6). We used dental epithelial cells as the indicator host cells of the promoter activity, while Golonzhka *et al.* examined Sp6 expression using mandibular tissue of Ctip2-KO mouse. Therefore, we cannot directly compare the results because their samples are the mixture of several cell types, suggesting that Sp6 expression might be the results of multiple effects.

Next, we used ChIP-PCR analysis to determine whether Ctip2 binds directly to the Sp6 promoter regions. Our results demonstrated specific binding of Ctip2-long and Ctip2-short to the both Sp6 first and second promoter (Figure 4B). Taken together, these results suggest that the Sp6 second promoter, but not the first, could be functionally responsible for the downregulation.

We repeatedly observed that Ctip2 exerted a repressive effect through the B-, C- and D-regions, which was most clearly evident via the C- and D-regions, but Ctip2 had no effect via the E-region. These findings suggested that the D-regions of the second Sp6 promoter harbored Ctip2 binding site(s) for Sp6 regulation. Further *in silico* analysis of the promoter sequences identified the presence of a potential Ctip2 binding motif (GGCCGG) in the D-region, shown in Figure 3A (asterisks), which was previously reported as a putative Ctip2 binding site in the p57KIP2 promoter in SK-N-MC cells (21, 26).

Ctip2 was originally identified as a transcriptional repressor that binds directly to GC-rich consensus sequences in its target genes and interacts with the nucleosome remodeling deacetylase (NuRD)

complex (20, 21, 26, 30), co-operating with COUP-TF, Sp1, LSD1, HDAC or SIRT1 (22, 31-34) or associate with cyclin-dependent kinase inhibitors through 7SK snRNA in a complex with negative regulator (35). Conversely, Ctip2 was shown to activate *nuclear factor- κ B* transcription (20). Together, these observations suggest that Ctip2 can act as either a transcriptional repressor or activator in a context dependent manner. Another previous study reported a dynamic, mitogen-activated protein kinase (MAPK)-regulated pathway involving sequential, linked, and reversible post-translational modifications of Ctip2 (36). MAPK-mediated Ctip2 phosphorylation causes rapid Ctip2 desumoylation and initiates a cycle of dephosphorylation and resumoylation. Ctip2 sumoylation results in recruitment of the coactivator p300 to the promoters of Ctip2-repressed targets, consequently inducing transcription. These findings suggest two possible molecular mechanisms by which Ctip2 can regulate its target genes. First, Ctip2 can mediate target gene repression by recruiting negative regulators or chromatin modifying enzymes as a co-repressor in a complex, such as LSD1, HDAC1/2, SUV39H1, HP1 and SIRT1 or NuRD and P-TEFb (20-22, 26, 30-35). Second, Ctip2 could mediate the switching of target gene transcription from negative to positive, abrogating repression by Ctip2 post-translational modification (36).

Based on our findings, we summarized our data in Figure 5. Ctip2 binds directly to both *Sp6* first and second promoter regions (small lower bar) and functionally regulates the repression of *Sp6* through only the second promoter in the context of G5 cells *in vitro*. One mechanism by which Ctip2 exerts its suppressive effects in G5 cells may involve its participation with other co-repressor(s) in a complex with NuRD or P-TEFb (20-22, 26, 30-35). In the physiological context (*in vivo*), developmental cues from genetic cascades or biological signaling molecules surrounding tissues during tooth development may also affect the regulation of *Sp6* expression. One possible explanation for this is that these developmental cues function in a mechanism that, through modification of Ctip2, switches its activity from negative to positive and *vice versa*. Of note, the post-transcriptional modification of Ctip2 reportedly allows it to recruit the coactivator acetyltransferase p300 into complexes, consequently reducing repression activity (36).

In conclusion, we demonstrated a molecular linkage between Ctip2 and *Sp6* expression in our *in vitro*

system. However, there are some discrepancies between our data and previous report (13). Further investigations are required to better understand the molecular cascades underlying tooth development.

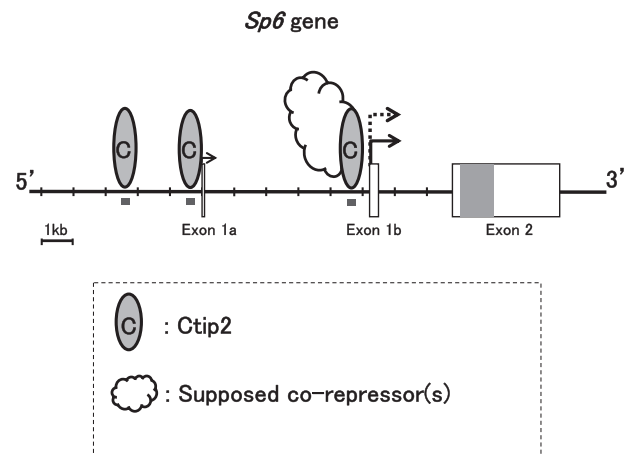


Figure 5 : Summary of Ctip2-mediated *Sp6* regulation. In the absence or presence of Ctip2, *Sp6* second promoter activity is indicated by dotted or solid arrows, respectively. *Sp6* first promoter activity is indicated by solid arrow because of no different activities in both conditions. A supposed Ctip2 co-repressor(s) is shown as a cloud. Small lower bars indicate the Ctip2 binding site. Gray box indicates the *Sp6* coding region.

CONFLICT OF INTEREST

None of the authors have any conflicts of interest to declare.

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