ORIGINAL

Sp6 regulation of *Rock1* promoter activity in dental epithelial cells

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Abstract : Sp6 is a transcription factor of the SP/KLF family and an indispensable regulator of the morphological dynamics of ameloblast differentiation during tooth development. However, the underlying molecular mechanisms remain unclear. We have previously identified one of the Sp6 downstream genes, *Rock1*, which is involved in ameloblast polarization. In this study, we investigated the transcriptional regulatory mechanisms of *Rock1* by Sp6. First, we identified the transcription start sites (TSS) and cloned the 5′-flanking region of *Rock1*. Serial deletion analyses identified a critical region for *Rock1* promoter activity within the 249-bp upstream region of TSS, and chromatin immunoprecipitation assays revealed Sp6-binding to this region. Subsequent transient transfection experiments showed that *Rock1* promoter activity is enhanced by Sp6, but reduced by Sp1. Treatment of dental epithelial cells with the GC-selective DNA binding inhibitor, mithramycin A, affected *Rock1* promoter activity in loss of enhancement by Sp6, but not repression by Sp1. Further site-directed mutagenesis indicated that the region from -206 to -150 contains responsive elements for Sp6. Taken together, we conclude that Sp6 positively regulates *Rock1* transcription by direct binding to the *Rock1* promoter region from -206 to -150, which functionally distinct from Sp1. J. Med. Invest. 61 : 306-317, August, 2014

Keywords **:** *amelogenesis, dental epithelial cells, Rock1, Sp6, transcription start site*

INTRODUCTION

Sp6, a transcription factor of the SP/KLF family, is one of the key regulators of tooth development. Loss-of-function studies demonstrated that Sp6 is required for ameloblast differentiation in tooth development ; the deficiency of Sp6 causes tooth anomalies, including supernumerary teeth, enamel deficiency, cusp defects, root formation, and abnormal dentin structure (1, 2). However, the molecular basis for Sp6 function remains unclear. To clarify the role of Sp6, we established a gain-of-function system *in vitro* using the rat dental epithelial cells G5, and found that Sp6 regulates amelogenesis by down-regulation of the BMP antagonist *follistatin* (3). Subsequently, we generated *Sp6* transgenic rats as another gain-of-function system *in vivo* and revealed perturbed morphological and metabolic differentiation of ameloblasts following ectopic Sp6 expression (4). These data suggested the indispensable role of Sp6 in tooth development.

Recently, we also demonstrated that a frameshift mutation in *Sp6*, which disrupts the third zinc finger domain, is responsible for the autosomal recessive

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The nucleotide sequence of promoter and 5′ end of rat *Rock1* has been deposited into GenBank under the accession number AB861944.

amelogenesis imperfecta phenotype in AMI rats. We observed morphological changes of ameloblasts in *Ami/Ami* and *Wt/Ami* incisors (5). However, the underlying mechanism through which *Sp6* mutations lead to abnormal morphology remains unclear. Concurrently, we investigated the downstream targets of Sp6 using comparative microarray analyses and found that *Rock1* is upregulated in the presence of the Sp6 protein (6).

ROCK1 (Rho-associated coiled-coil-forming kinase 1) is a serine/threonine kinase which is activated by the small GTP-binding protein RhoA. There are two isoforms, ROCK1 and ROCK2, which share 65% identity in their complete amino acid sequences and 92% identity in their kinase domains (7). However, the previous study showed distinct roles of ROCK isoforms in the same cells. ROCK1 activity is essential for formation of stress fibers and focal adhesion, on the other hand, ROCK2 acts as a counterbalancing regulator of microfilament bundle and focal adhesion sites (8).

During tooth development, ROCK contributes to the polarity, proliferation, and differentiation of ameloblasts by regulating actin cytoskeleton organization and cell-cell adhesion (9). RhoA also regulates the expression of *amelogenin* and *DSPP* during cytodifferentiation of ameloblasts and odontoblasts, respectively (10). However, the regulation of *Rock1* expression has not been investigated, although the activators of Rock1 have been widely discussed.

In the present study, we demonstrated that Sp6 positively regulates *Rock1* expression by direct binding to the *Rock1* promoter. We also observed the critical elements to affect both Sp6-enhanced *Rock1* promoter activity and Sp6 binding to the *Rock1* promoter. In addition, we found that Sp1 downregulated *Rock1* transcription. The present data suggest a unique role of Sp6 in tooth development and morphogenesis that may be essential for future studies of tooth regeneration.

MATERIALS AND METHODS

Cell Culture

Rat dental epithelia-derived cells, G5 (11) and its *Sp6*-stable transformant, C9 (3), were cultured in a Dulbecco's modified Eagle's medium and Ham's F12 medium (D/F12, Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS ; JRH Biosciences, Lenexa, KS, USA) incubated at 37°C in 5% CO_2.

RNA Ligase-Mediated 5′-Rapid Amplification of cDNA Ends (RLM-RACE)

Total RNA was extracted from molars of 7-day-old stroke-prone spontaneously hypertensive rats (SHRSP) (5) using the TRI reagent (MRC, Cincinnati, OH, USA). RNA was then used in RLM-RACE assays with a GeneRacer kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. In brief, total RNA was dephosphorylated with calf intestinal alkaline phosphatase followed by tobacco acid pyrophosphatase treatment to remove cap structures from the 5′ ends of full-length mRNAs. Oligo RNAs were then added to the 5′ ends of the RNA using RNA ligase. Ligated RNAs were reverse transcribed to cDNAs using oligo dT-primers, and these cDNAs were then used as templates in PCR amplification. Initial and nested PCRs were performed using the antisense primers 5′-GGGATCC-CGCAGCAGGTTGTCGA-3′ and 5′-CAAGTTCAA-CCAACTTCCTCCGCGG-3′, respectively. Amplified products were subcloned into pGEM-T easy plasmid vectors (Promega, Madison, WI, USA), and eleven clones were sequenced.

In Silico Promoter Analysis

Potential transcription factor binding sites were identified in the 2500-bp region upstream to the newly identified transcription start site of *Rock1* (accession number : AB861944) using the TFBIND algorithm (http : //tfbind.hgc.jp/). These sites were confirmed using the GENETYX software program, version 10 (Genetyx), and overlapping sites that were identified in the "A" region were used in further analyses.

Chromatin Immunoprecipitation (ChIP)-PCR assay

Sp6 binding to the *Rock1* promoter in C9 cells were determined using a ChIP-IT Express Enzymatic kit (Active Motif, Carlsbad, CA, USA) with some modifications. In brief, cells were cross-linked with 1% formaldehyde for 10 min, harvested on ice, and were Dounce-homogenized 40 times in ice bath. DNA-protein complexes were enzymatically digested to the lengths of 200-1000 bp, as determined by gel electrophoresis. Rabbit IgG antiserum directed against anti-HA high affinity clone 3F10 (Roche, Mannheim, Germany) was used (3 μg of antisera per sample) to direct chromatin antibody pull-down at 4°C overnight in a rotary mixer (NRC-20D ; Nissin, Tokyo, Japan). Immunoprecipitated chromatin was treated sequentially with elution

buffer, reverse cross-linking buffer, and proteinase K to recover DNA. The primer sequences and PCR conditions used to amplify chromatin DNA are presented in Table 1. ChIP assays were performed in G5 cells cotransfected with pCI-neo mammalian expression vector (Promega) harboring HA-tagged rat *Sp6* (WT) and pGL3-mutant reporter constructs. DNA was amplified using forward primers containing the mutant sequences (in bold) 1 (5′-CCTGTCCTCCACT**AAA**TTT**AAT**-3′), 2 (5′-TCCGACTTCTCTT**AAA**CC**GAA**-3′), 3 (5′-CGCCCTCCGCCT**ATA**AG**AAT**-3′), and 4 (5′-GCCTGCCAGGCCC**AATAAT**-3′) and the reverse primer 5′-TCCTGTTCAAACAAACGGGG-3′. PCR reactions were performed with 33 cycles of denaturing at 95°C for 45 s, annealing at 57°C for 45 s, and extension at 72°C for 20 s.

Plasmid Constructs

Rat genomic DNA template was amplified by PCR using a PrimeSTAR Max Premix (Takara, Shiga, Japan) with the forward primer 5′-TGGGCACATGTTTTCCCATCTGTGGT-3′ and the reverse primer 5′-TCTAAGGCGCTAGCGGCTGTCTAGC-3′, which flanked the *Rock1* region (from -2709 to +383). PCR reactions were performed with 20 cycles of denaturing at 98°C for 10 s, annealing at 55°C for 5 s, and extension at 72°C for 15 s. Nested PCRs were performed to generate *Rock1* promoter 5′ deletion constructs using the reverse primer 5′-CCGAAGCTTTTATCCGGTCGCTGCTGGGGTTACG-3′ and the forward primers Luc#1 5′-CTAGAGCTCGTCTGAAGAGTTGTGCCTAGCCCTTG-3′, Luc#2 5′-CTAGAGCTCTGACCTATGATGAAACCCAAGGGTTGAT-3′, Luc#3 5′-CTAGAGCTCCCAGCTCAGCCACCAGAGGCTGG-3′, and Luc#4 5′-CTAGAGCTCAGGCGGACATATTAGTCCCTCTGAGC-3′, which yielded varying PCR product lengths. PCR reactions were performed with 30 cycles of denaturing at 98°C for 10 s, annealing at 55°C for 5 s, and extension at 72°C for 15 s. For further 5′ deletion constructs in region "A", we used the forward primers Luc#3.1 5′-CTAGAGCTCTCCTCCACTGGGTTTCCGACTTCTC-3′, Luc#3.2 5′-CTAGAGCTCTCCGGCTCCGCCTCTCTCCCCTCC-3′, Luc#3.3 5′-CTAGAGCTCTCGCGACCCGGCCAGCCCCGC-3′, Luc#3.4 5′-CTAGAGCTCCTGCGTGCGCGTGCGCGAGCGCG-3′, and Luc#3.5 5′-CTAGAGCTCGCGCGCCGGCGGTCCCCGTTTG-3′, which yielded varying amplicon lengths. PCR reactions were performed with 30 cycles of denaturing at 98°C for 10 s, annealing at 55°C for 5 s, and extension at 72°C for 5 s. Additional *Sac*I and *Hin*dIII restriction sites were incorporated into forward and reverse primers, respectively, to facilitate subcloning of amplified fragments. The PCR fragments and the luciferase reporter vector pGL3-basic were separately digested with *Sac*I and *Hin*dIII before ligation, and reporter constructs were sequenced. *Sp6* expression vector was described previously (3). *Sp1* expression vector was constructed using pCIneo mammalian expression vector (Promega). In brief, the entire rat *Sp1* coding region was generated by RT-PCR using PrimeSTAR GXL DNA Polymerase (Takara) from total RNA of C9 cells (3). FLAG-tag was added at the N-terminus of *Sp1* cDNA in the forward primer. The primer sequences are the follows ; Forward, 5′-CCACCATGGACTACAAAGACGATGACGACAAGAGCGACCAAGATCACTCAATGGATG-3′ (the underlined region indicates the position of the FLAG-tag sequence) ; Reverse, 5′-ATCTCAGAAACATTGCCACTGATATTAATG-3′.

Site-Directed Mutagenesis

Constructs containing mutant SP1 and AP2 sites from the *Rock1* promoter region ranging from -206 to -150 were prepared using an AMAP Multi Site-Directed Mutagenesis kit (MBL, Woburn, MA, USA), according to the manufacturer's protocol. Luc#3 reporter plasmids were used as PCR templates, and the products were ligated using phosphorylated

Table 1. ChIP-PCR primers

Rock1 Promoter	Primer Sequence (5′ → 3′)	Annealing (°C)	Extension (s)	Cycles
-249 to +17 ("A" region)	F : CCAGCTCAGCCACCAGAG	58	30	38
	R : TCCTGTTCAAACAAACGGGG			
-206 to -150	F : GATTATAAAGATGACGATGATAAA TCCTCCACTGGGTTTCCGACTTCTC	67	15	35
	R : TTTATCATCGTCATCTTTATAATCA CACGGGCGGGGCCTGGCAGG			

primers containing the following mutant sequences (in bold) : mutation 1, 5′-TCCCTGTCCTCCACT**AAA**TTT**AAT**ACTTCTCTTCGCCCTCC-3′ ; mutation 2, 5′-GTTTCCGACTTCTCTT**AAA**CC**GAA**GCCTGCCAGGCCCCG-3′ ; mutation 3, 5′-CTTCGCCCTCCGCCT**ATA**AG**AAT**CCGCCCGTGTCCGGC-3′ ; and mutation 4, 5′-CCGCCTGCCAGGCCC**AATAAT**TGTCCGGCTCCGCCT-3′. Mutant promoter plasmids were transformed into competent *E. coli* JM109 cells after treatment with *Dpn*I and then sequenced.

Transient Transfection and Dual Luciferase Reporter Assay

G5 cells were plated at 6.0×10^4 cells/well in 24-well plates one day before transfection. Cells were cotransfected with 400 ng of the pGL3-reporters p*Rock1*-Luc or p*Rock1*-Luc-Mut, 40 ng of pSP6 or pSP1, and 4 ng of the renilla luciferase reporter plasmid pRL-TK using the X-tremeGENE HP DNA Transfection Reagent (Roche), according to the manufacturer's instructions. After 24 h, cell lysates were prepared, according to the manufacturer's protocol, for the Dual Luciferase Reporter Assay System (Promega), and both firefly and renilla luciferase activities were measured using a Lumat LB 9507 (Berthold, Bad Wildbad, Germany). For the DNA binding inhibitory analysis by mithramycin A, the cells were cultured with a vehicle (DMSO) or varying doses of mithramycin A (Sigma, Tokyo, Japan) 5 h after transfection. The transient transfection and dual luciferase reporter assay were repeated at least two times in triplicates. The promoter activity of each *Rock1* promoter construct was measured by luciferase activity and was normalized by that of the longest promoter construct without any mutation. For the analysis of the effect of Sp6 on *Rock1* promoter activity, the activity was analyzed by comparing the luciferase activity of each promoter construct in *Sp6*-cotransfected sample with that of the same construct in pCIneo-cotransfected sample. For mithramycin A treatment, DMSO was used as control. The average of control samples is indicated as 100.

Reverse Transcription-PCR (RT-PCR)

G5 cells were plated at 2.88×10^5 cells/well in six-well plates. The cells were transiently transfected with the empty pCI-neo, Sp6, or Sp1 expression vectors and were harvested after 24 h. Total RNA was extracted using the TRI reagent (MRC), according to the manufacturer's instructions. RNA was reversed transcribed using an RNA PCR kit AMV Ver. 3.0 (Takara), according to the manufacturer's instructions. Synthesized cDNA was analyzed by PCR using GoTaq DNA polymerase (Promega) with the forward primer 5′-GGATGCTACCTGATCACCAG-3′ and the reverse primer 5′-CCGTAGGCAAACCCGCAAG-3′. PCR reactions were performed with 33 cycles of denaturing at 95°C for 45 s, annealing at 57°C for 45 s, and extension at 72°C for 30 s. RT-PCR analysis was performed by three separate experiments under the same experimental conditions. Expression level of *Rock1* mRNA was normalized to that of *18S* rRNA.

Statistics

For the luciferase assay, data are mean ± SD from at least two separate experiments with triplicate samples. For RT-PCR analysis, data are means ± SD from three separate experiments (n = 3). Statistical analysis was performed by Student's *t*-test using Microsoft Excel 2010 software. A p value less than 0.05 was considered to be statistically significant.

RESULTS

Determination of Rock1 Transcription Start Sites (TSS)

Homology searches of human, mouse, and rat *Rock1* cDNA in GenBank identified 5′ end structures with 84% and 96% homology to human and mouse sequences, respectively, compared with rat sequence (Fig. 1A). However, TSS of rat *Rock1* has not been identified yet. Therefore, we first performed RLM-RACE to define TSS after removing the 5′-cap structures from intact full-length mRNA using tobacco acid pyrophosphatase. RLM-RACE products were then subcloned into the pGEM-T easy vectors. Based on sequence analyses of 11 randomly picked clones, we identified multiple potential TSS and defined the most 5′ end position as +1 (Fig. 1B). Sequencing data from this study have been deposited into GenBank sequence database (accession number : AB861944).

Detailed inspections of sequence data revealed that the rat *Rock1* promoter does not contain TATA or CCAAT boxes, which is in agreement with a report stating that TATA-less promoters often show multiple TSS (12). Recent bioinformatic analyses indicated that approximately 76% of human core promoters lack TATA-like elements, have high GC contents, and are enriched in SP1-binding sites.

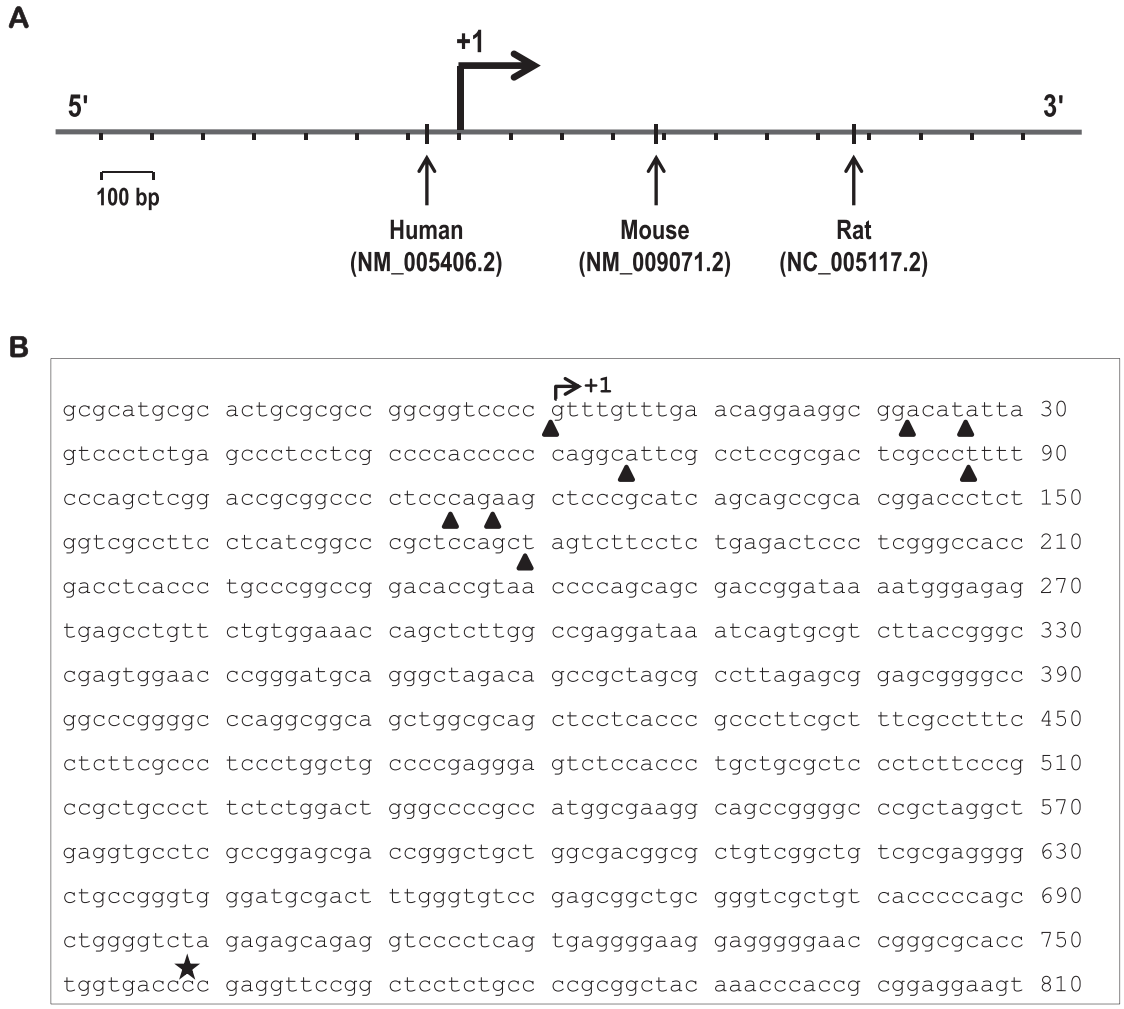

Figure 1. Determination of *Rock1* transcription start sites (TSS)
A : The newly identified TSS ; the most 5′ end of the TSS is indicated with a black arrow at the position +1. Relative positions of the first nucleotide in human, mouse, and rat *Rock1* genes reported in GenBank are shown by vertical lines with their accession numbers. B : Multiple TSS were determined by RLM-RACE ; denoted as black triangle. TSS is indicated with a black arrow. Asterisk indicates the position of reported cDNA from rat *Rock1* (accession number : NC_005117.2).

Moreover, TATA-less genes are frequently involved in basic "housekeeping" processes, such as protein biosynthesis, cell growth and/or maintenance, and intracellular transport (13).

Sp6 Binds to the "A" Region and Positively Regulates Rock1 Promoter Activity

Searches for potential Sp6-binding sites using TFBIND algorithm on the *Rock1* promoter revealed multiple potential SP1 sites and GC-boxes at around 2,500-bp upstream of TSS. Subsequent *in silico* analyses using GENETYX software also identified five SP1-binding sites and one GC-boxes within 249-bp upstream region of TSS, which overlapped with TFBIND algorithm finding. Since we did not find any other sites within 2,500 bp of promoter region by GENETYX software, we focused on this 249-bp region, named as "A" (Fig. 2A). To define regulatory regions of the *Rock1* promoter, a series of reporter vectors were constructed with serial deletion of *Rock1* 5′-flanking promoter sequences by inserting at the upstream of the firefly luciferase coding sequence in the promoter-less plasmid vector, pGL3-basic (Fig. 2B). Luciferase reporter assays were then performed to determine the effects of Sp6 on promoter activity. The Luc#4 reporter construct, which lacked the "A" region, exhibited no significant promoter activity or enhancement. In contrast, Luc#1, Luc#2, and Luc#3 constructs exhibited higher luciferase activities and were enhanced 1.5-fold by co-transfection with *Sp6* expression plasmids compared with the control vector (Fig. 2B) indicating that the "A" region is required for basal activity and enhancement by Sp6.

To confirm Sp6 binding to the *Rock1* promoter region, we performed ChIP assays using *Sp6*-stable

transformant, C9 cells, and anti-HA antibody to precipitate HA-tagged Sp6 protein followed by PCR using specific primers for the "A" region. The predicted 266 bp-band of PCR products was detected, suggesting the specific binding of Sp6 to the "A" region (Fig. 2C). Subsequent analyses of endogenous *Rock1* mRNA expression in G5 cells with transient transfection of *Sp6* expression vector were correlated significantly with Sp6-mediated promoter activity ($p < 0.001$, Fig. 2D).

Sp6 is Functionally Distinct from Sp1

To determine Sp6-binding sites within the "A" region, we dissected the overlapped SP1 sites and GC-boxes as shown in Fig. 2A, into several reporter constructs (Fig. 3A, Luc#3.1-Luc#3.5), and transiently cotransfected those reporter constructs and *Sp6* or *Sp1* expression vector into G5 cells. Luc #3 and Luc#3.1 demonstrated the enhancement of promoter activity by Sp6 at the similar level, however, deletion of the promoter region from -206 to -150 in Luc#3.2

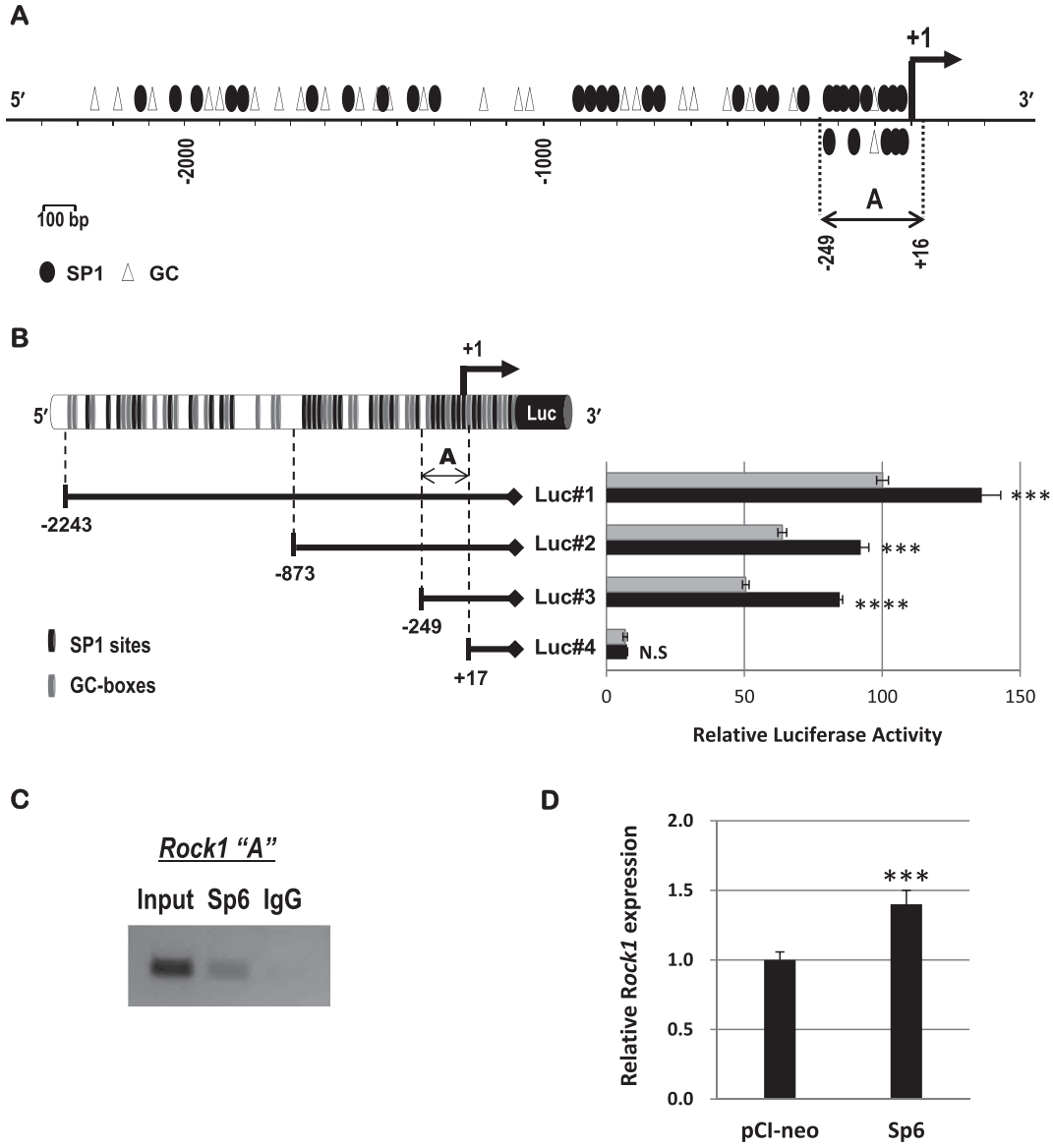

Figure 2. Sp6 binds to the *Rock1* promoter "A" region and enhances its activity
A : Potential Sp6-binding sites identified by *in silico* analysis. Two results were obtained using TFBIND algorithm and GENETYX software, and indicated with symbols above and below the line, respectively, within the 2,500 bp upstream region of TSS. B : Sp6 effect on *Rock1* promoter activity. Left, schematic structure of the serial 5′ deletion constructs of the *Rock1* promoter. Right, luciferase activity of the desired deletion constructs with empty pCIneo (gray bar) or *Sp6* expression vectors (black bar). Figures are representative of three independent experiments. *** $p < 0.001$, **** $p < 0.0001$; N. S, not significant. C : Detection of Sp6-binding on region "A" by ChIP-PCR. Chromatin from C9 cells was immunoprecipitated with antibody against HA-tagged Sp6 (Sp6) or non-immune (IgG). PCR products of flanking the "A" region were shown. Input, whole chromatin DNA. D : Sp6 effect on *Rock1* mRNA level in G5 cells. *Rock1* mRNA level was normalized by *18S* level. pCIneo, transfected with empty vector ; Sp6, transfected with *Sp6* expression vectors. *** $p < 0.001$.

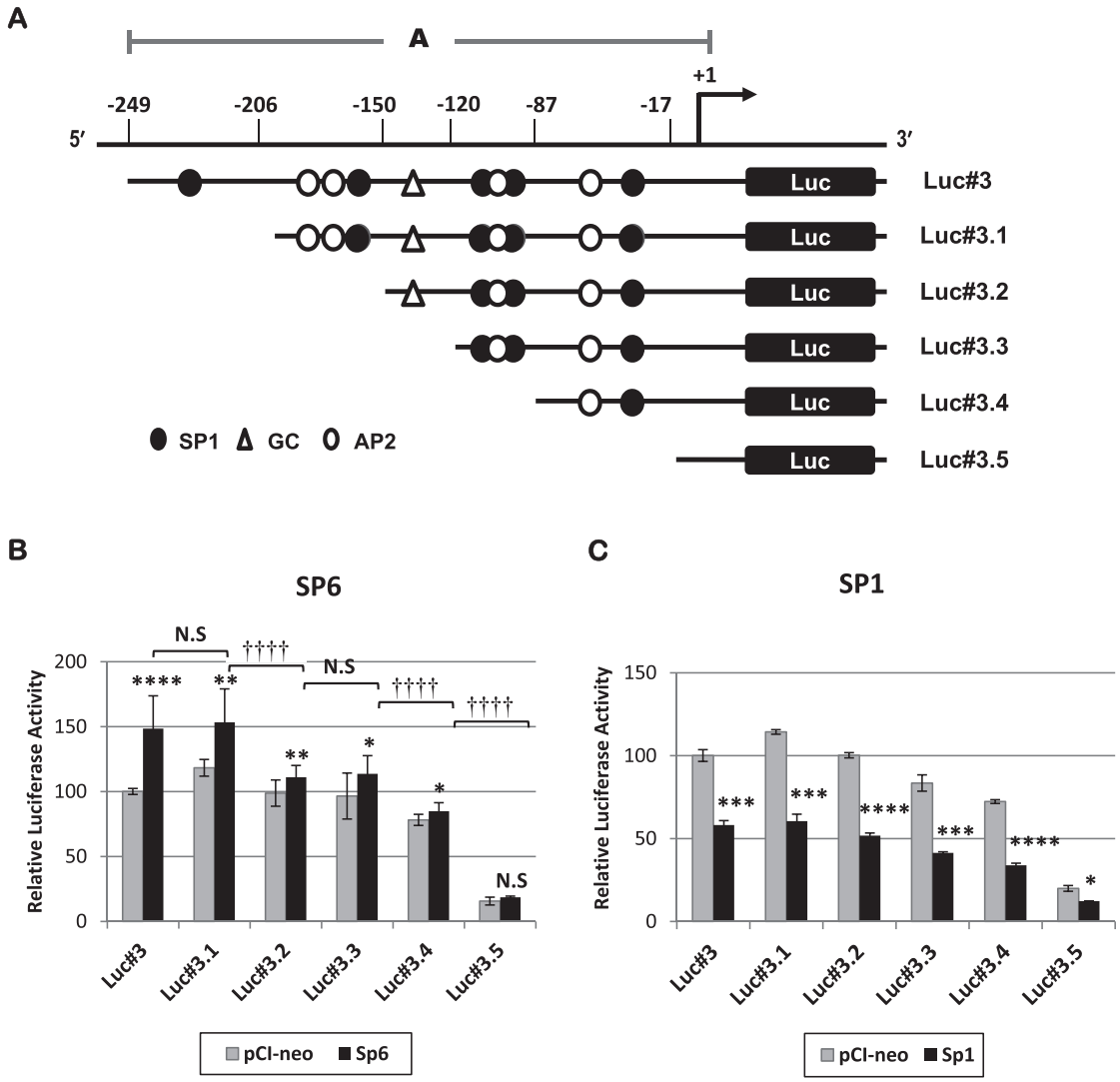

Figure 3. Differential regulation of *Rock1* promoter activity by Sp1 and Sp6
A : Serial 5′ deletion constructs of "A" region with indicated consensus binding sites. B and C : *Rock1* promoter activity by dual luciferase reporter assay. G5 cells were transiently cotransfected with the serial 5′ deletion constructs and either *Sp6* (B) or *Sp1* (C) expression vectors using an empty pCIneo vector as a control. Results are expressed as mean±SD from three independent experiments (n=9) or representative experiments (n=3) in B and C, respectively. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ when compared with control (pCIneo) ; †††† $p<0.0001$ when compared Sp6 effect between each construct as indicated by bar ; N. S, not significant.

resulted in a significant reduction (†††† $p<0.0001$) of enhancing activity by Sp6 (Fig. 3B). Since Sp1 binding site is located in this region, it is possible that Sp6 may compete with Sp1 for binding this region. Indeed, both *Sp1* and *Sp6* mRNA are endogenously expressed in G5 cells (data not shown). To assess the effects of Sp1 on these promoter activities, we co-transfected with *Sp1* expression vector and reporter plasmids. As shown in Fig. 3C, Sp1 suppressed *Rock1* promoter activity with any length of promoter, suggesting that Sp6 is functionally distinct from Sp1. These results indicated that Sp6 and Sp1 may positively and negatively regulate *Rock1* promoter activity, respectively. In addition, we observed the basal activity of *Rock1* promoter in Luc#3.5.

Furthermore, we determined whether mithramycin A, a compound that interferes with Sp1 binding to GC-rich DNA sequences, affects *Rock1* promoter activity directed by Sp1 or Sp6. Mithramycin A is frequently used to explore sequence specificities of DNA-binding factors, and its binding to GC-rich sequences prevents binding of regulatory proteins such as Sp1, resulting in inhibition of promoter-dependent transcription *in vitro* (14). In our study, treatment with mithramycin A reduced the Sp6-mediated enhancement of *Rock1* promoter activity in dose-dependent manner, especially up to 200 nM (Fig. 4A). However, *Rock1* promoter repression by Sp1 was not affected with mithramycin A (Fig. 4B).

As shown in Fig. 4C, Sp6-mediated enhancement of *Rock1* expression was observed at the mRNA level and the enhancement was abolished in the presence of mithramycin A. However, in contrast, reduction of *Rock1* expression by Sp1 was not observed at mRNA level and the *Rock1* expression was not affected by mithramycin A treatment.

Determination of Sp6 Responsive Elements

The finding that mithramycin A inhibited the effects of Sp6 enhancement on the *Rock1* promoter activity with the reporter constructs containing the region from -206 to -150, prompted us to further confirm Sp6 responsive elements within this region.

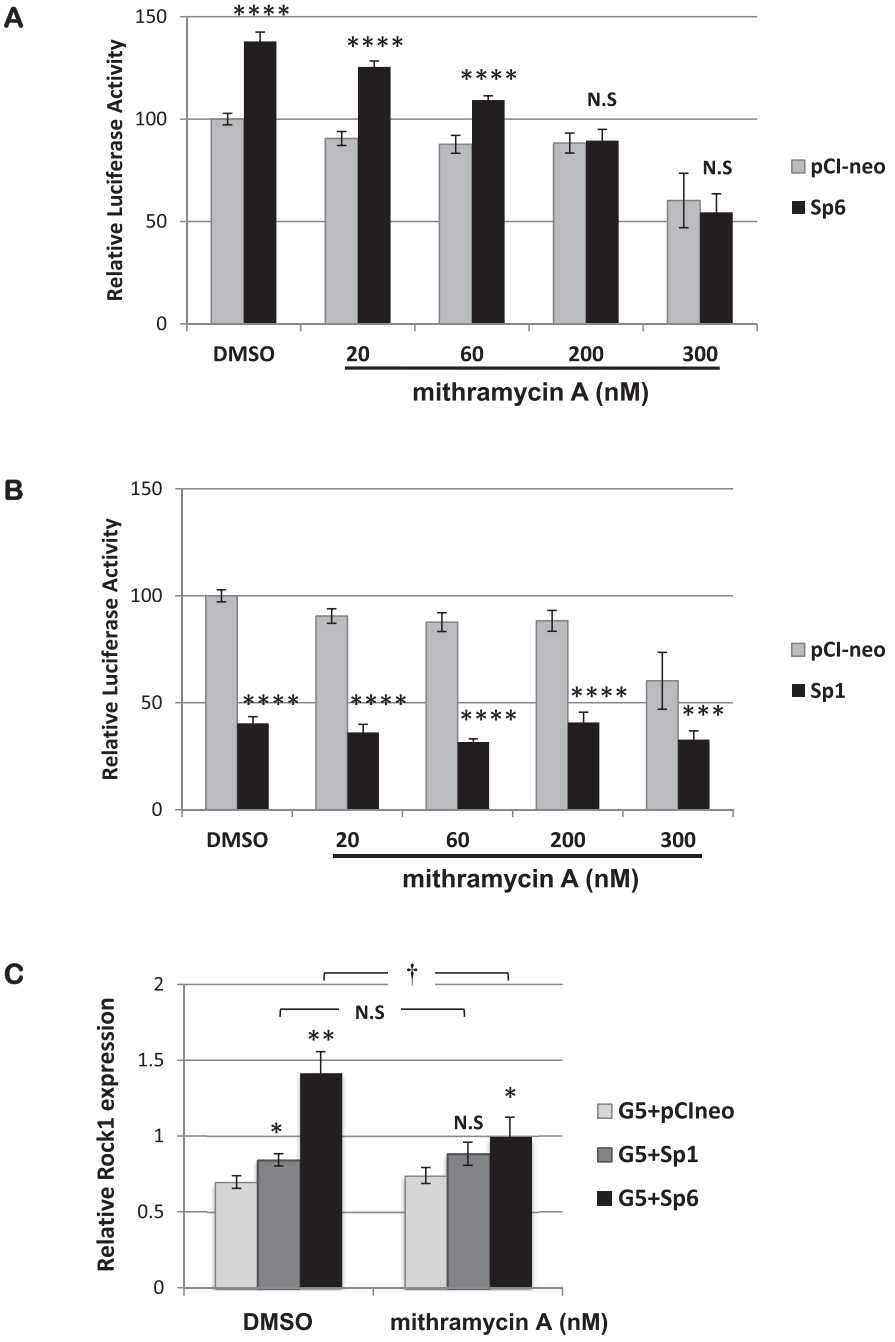

Figure 4. Mithramycin A inhibits Sp6 but not Sp1 function in *Rock1* promoter activity
A and B : Effect of mithramycin A on *Rock1* promoter activity. G5 cells were transiently cotransfected with Luc#3 reporter construct and either *Sp6* (A) or *Sp1* (B) expression vectors using an empty pCIneo vector as a control. Five hours of post-transfection ; cells were treated with either a vehicle (DMSO) or with varying concentrations of mithramycin A. C : Effect of mithramycin A on *Rock1* mRNA expression. *Rock1* mRNA levels were analyzed in G5 cells cotransfected with *Sp6*, *Sp1* or empty vector, pCIneo with and without mithramycin A treatment. Results (A and B) are expressed as mean ± SD from two independent experiments, each in triplicate samples (n=6). * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$, **** $p< 0.0001$ when compared with control (pCIneo) ; † $p< 0.05$ when compared with control (DMSO) treatment ; N. S, not significant.

Therefore, we performed ChIP assays in G5 cells using anti-HA antibody to precipitate HA-tagged Sp6 protein and followed by PCR using specific primers for the region -206 to -150. The specific binding of Sp6 to the *Rock1* promoter region -206 to -150 was observed as the predicted PCR products (Fig. 5A). To ensure this result, we mutated the potential SP1 sites that were identified using GENETYX software for *in silico* analyses (Fig. 5B). We also mutated AP2 sites, because AP2 site is GC-rich and the region from -206 to -150 contains two potential AP2 sites, which may be occupied by Sp6. In addition

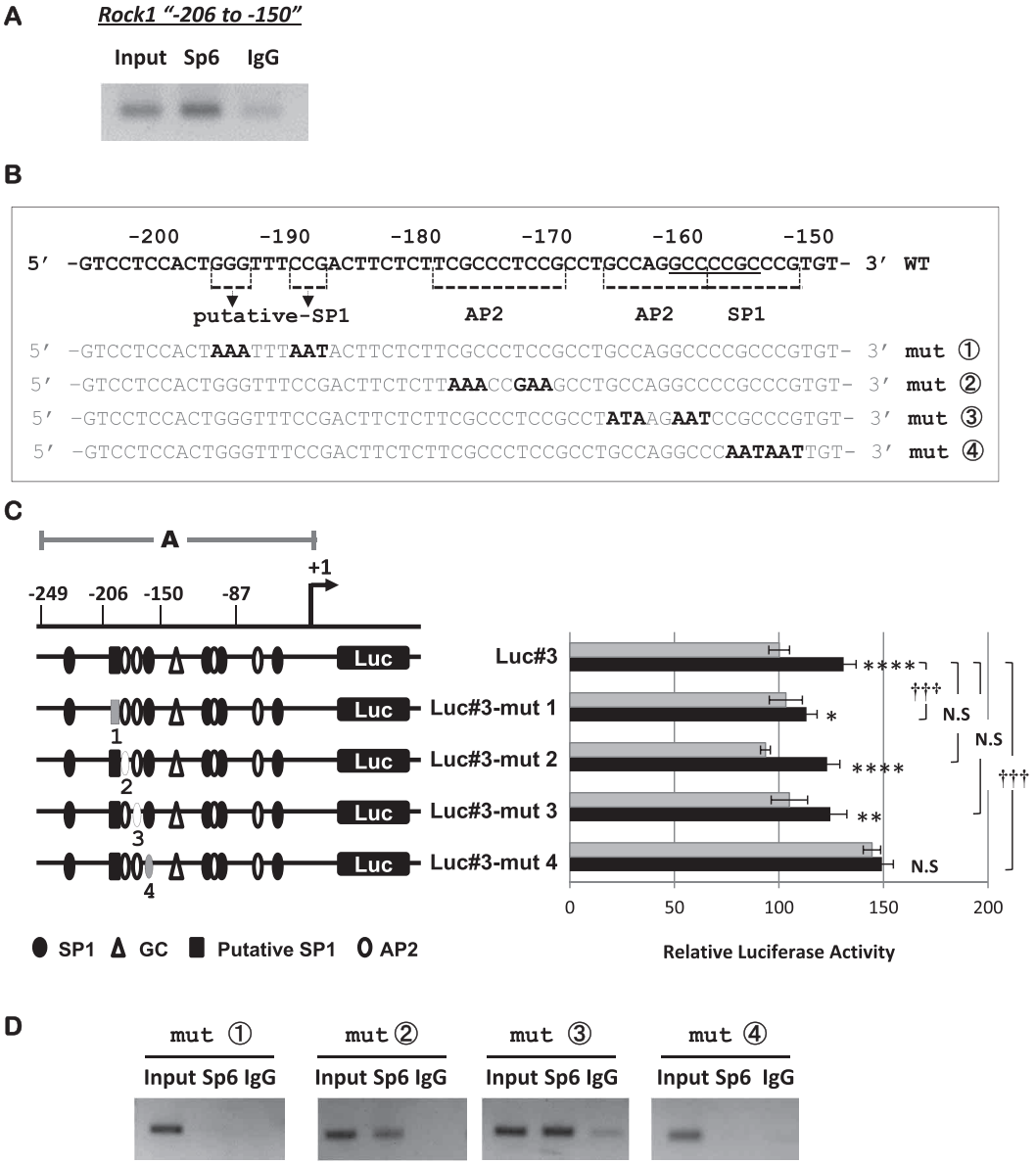

Figure 5. Determination of Sp6 responsive elements on *Rock1* promoter region
A : Detection of Sp6-binding on region -206 to -150 by ChIP-PCR. G5 cells were transiently cotransfected with the Luc#3.1 reporter and the *Sp6* expression vector. Twenty-four hours of post-transfection ; chromatin from G5 cells was immunoprecipitated using an antibody against HA-tagged Sp6 (Sp6) or with non-immune (IgG). PCR products with primers flanking the region from -206 to -150 are shown. Input, whole chromatin DNA. B : Wild-type (WT) and mutant *Rock1* promoter sequences (-207 to -150). Top sequence represents the region from -206 to -150 with indicated TF-binding sites. Mut 1-4 indicated the mutated nucleotides (bold) in this region. Underline, GCF-binding site. C : Sp6 effect on the luciferase activity of *Rock1* mutant promoter constructs. Left, schematic structure of the mutant constructs of the *Rock1* promoter ranging from -206 to -150 ; mut 1-4 indicate mutants 1-4, respectively. Right, luciferase activity of each mutant construct cotransfected with empty pCIneo (gray bar) or *Sp6* expression vector (black bar). Results are expressed as mean ± SD from two independent experiments, each in triplicate samples (n=6). * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$, **** $p< 0.0001$ when compared with control (pCIneo) ; ††† $p< 0.001$ when compared with the wild type Luc#3 construct ; N. S, not significant. D : Detection of Sp6-bindings on *Rock1* mutant promoter by ChIP-PCR. G5 cells were transiently cotransfected with mutant constructs and *Sp6* expression vectors. Twenty-four hours of post-transfection, chromatin from G5 cells was immunoprecipitated using an antibody against HA-tagged Sp6 (Sp6) or non-immune (IgG). PCR products are shown. Specific primers for each mutation were synthesized with mutant nucleotides at the 3′ end. Input, whole chromatin DNA.

to the mutations in SP1 and AP2 sites identified by *in silico* analyses, we mutated 5′-GGGTTTCCG-3′ in position number 1 (Fig. 5C) because GGG is a putative DNA-binding site for Sp1 zinc finger 3 (15). CCG is the complement sequence of CGG, which is possibly recognized by Sp1, because mutation of the 5′-GCGGGAGG-3′ motif in the *IER3* promoter markedly reduces basal and abrogated vorinostat-induced promoter activity (16). Hence, we have referred to 5′-GGGTTTCCG-3′ as a putative SP1-binding site.

As shown in Fig. 5C, mutation of putative SP1-binding site (Luc#3-mut 1) significantly reduced Sp6-mediated enhancement of *Rock1* promoter activity(††† $p<0.001$), whereas mutation of two AP2 sites, Luc#3-mut-2 and Luc#3-mut-3 constructs, had no effect on the luciferase activity. Of interest, while mutation of SP1 site significantly increased basal activity ($p<0.0001$), Sp6 overexpression had no effect on promoter activity (Fig. 5C, Luc#3-mut 4).

Next, we found the functional linkage between Sp6-enhancing effect on *Rock1* promoter activity and Sp6 binding to each mutant construct by ChIP-PCR analyses (Fig. 5D). We observed that Sp6 could bind to the Luc#3 reporter constructs with mutated AP2 sites (mut 2 and mut 3) but not with mutated putative SP1 and SP1 sites (mut 1 and mut 4). These data suggested that two sites containing GGGtttCCG and CGCCCG motifs in *Rock1* promoter region -206 to -150 are critical for the Sp6-enhanced expression of *Rock1*. Therefore, Sp6 may have binding selectivity to GC-rich sequences. In addition, there is another possibility of Sp6 effect to *Rock1* promoter activity ; the unknown molecule(s) could bind to AT-rich sequence of mut1, and recruit Sp6 through protein-protein interaction, resulting in enhancement of the promoter activity. However, further investigation is necessary to explain the underlying mechanism.

DISCUSSION

The present study explored the regulatory mechanisms of *Rock1* promoter activity by Sp6, and demonstrated that Sp6 could bind directly to the proximal 249-bp region of the *Rock1* promoter and enhanced basal promoter activity. This is the first report to characterize the effect of Sp6 on *Rock1* promoter regulation with its different role from another Sp family member, Sp1.

Distinct regulation by Sp family members is a quite interesting issue in terms of cell- and tissue-specificity (17). Nine members in Sp family have been identified with conserved three zinc finger DNA binding domains at C-terminus. In addition, Sp1-4 proteins contain long N-terminal regulatory domains, but Sp5-9 proteins have shorter N-terminus domains (17, 18). These structural differences may explain a part of the distinct regulatory function.

Functional promoter analysis using luciferase reporter assay indicated that over-expression of Sp6 in G5 cells enhanced *Rock1* promoter activity and endogenous *Rock1* mRNA expression (Fig. 2D and Fig. 4C). However, we observed suppression of *Rock1* promoter activity in the presence of Sp1 (Fig. 3B), and this suppressive effect did not correlate with endogenous *Rock1* mRNA expression (Fig. 4C). Indeed, we only examined the effect of Sp1 on the "A" region of the *Rock1* promoter by *in vitro* system. It remains unclear whether other regions in *Rock1* promoter are involved in Sp1-mediated endogenous promoter activity. Furthermore, we can measure the steady-state levels of mRNA by RT-PCR analysis, which are regulated at the transcriptional level and stability in a post-transcriptional manner (19). The mRNA stability is regulated by the interaction between *cis*-acting, *i.e.* sequence and structures, and *trans*-acting factors, *i.e.* alternative splicing regulatory factors, turnover and translation regulator RNA-binding proteins, and non-coding RNAs (20). It is possible that the post-transcriptional regulator(s) could maintain endogenous *Rock1* mRNA level against Sp1-mediated suppression.

Furthermore, we observed that mithramycin A treatment interfered Sp6-, but not Sp1-mediated *Rock1* promoter activity. These findings did not show the direct evidence of Sp1- or Sp6- binding to GC-rich DNA elements on *Rock1* promoter region, but they clearly demonstrated that Sp6-mediated regulation of *Rock1* transcription relies on GC-rich DNA sequence. It suggested that Sp1 has different regulatory mechanisms from Sp6, possibly through the protein-protein interaction with other transcription factor(s). This hypothesis may explain that mutated-putative SP1 site did not show any elevation of basal activity in Figure 5C.

Mutation analysis of *Rock1* promoter elements revealed additional interesting findings ; mutation of known SP1 and putative SP1 sites abolished Sp6-mediated enhancement of *Rock1* promoter activity and increased basal activity in Luc#3-mut4 construct, respectively (Fig. 5C). The latter finding raised a question for the underlying mechanisms.

Since we found Sp1 has suppressive effect in Rock1 promoter, the increased basal activity may simply occur by abolished endogenous Sp1 binding. However, we also have to consider the involvement of other molecules. We observed transient Ap2-over-expression suppressed *Rock1* promoter activity (data not shown). Ap2 has been shown to bind to not only the authentic sequence motif 5′-GCCN$_3$GGC-3′, but also to the different motifs such as 5′-CCCCAGGC-3′ in the *SV40* enhancer element and E-box site 5′-CACGTG-3′ in the Myc-response element (21, 22). Therefore, Ap2 might bind to the region containing Sp1 site 5′-CGCCCG-3′ to suppress promoter activity.

In addition, mutation of SP1 sites might abolish the suppressive effects of transcriptional repressor GC-factor (GCF), because the GCF-binding motif 5′-GCCCCGC-3′ was indicated in the *Rock1* promoter region from the region of -162 to -156 overlaps with AP2- and SP1-binding sites by *in silico* analysis (Fig. 5B). GCF was originally isolated as a factor that binds to GC-rich sequences (GCGGGGC) and represses transcription of *epidermal growth factor receptor* (*EGFR*), *β-actin*, and *calcium-dependent protease* promoters (23).

The biological role of Sp6-*Rock1* axis could be suggested by their expression patterns and functions. Immunohistochemical analyses demonstrated that Sp6 and ROCK1 express sequentially (3, 9). Cytosolic ROCK1 were detected at low level in basal epithelial cells, inner enamel epithelium cells, and presecretory ameloblasts (9). Moreover, ROCK1 was strongly expressed in secretory and mature ameloblasts (9), following the Sp6 expression in the nuclei of presecretory and secretory ameloblasts (3). On the other hand, the Rho family inhibitor RhoGDI was downregulated during ameloblast secretory stage (24). Interestingly, expression of RhoA, the direct upstream activator of ROCK, and ROCK1 were also detected strongly in highly polarized ameloblasts, but weakly in non-polarized cells of inner epithelium and dental papilla during tooth development (9, 10).

Rock1 plays pivotal roles in epithelial cell polarity by regulating PAR-1b activity and restricting basement membrane position (25), supporting that ROCK1 contributes to the polarity, proliferation, and differentiation of ameloblasts (9). In agreement with this, we found that Sp6 regulates ameloblast morphology and polarity by restoring polarization, alignment, and height of ameloblasts in *Ami/Ami Tg* rats with an *Sp6* transgene (5). Taken together, our findings suggested that Sp6 plays an important role in amelogenesis by regulating *Rock1* expression, which involved in ameloblasts morphology and polarity. Further analyses are required to investigate the role of Sp6 as a transcription factor during tooth development and develop the *in vivo* system for tooth regeneration.

CONFLICT OF INTERESTS

The authors declare no conflict of interest

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