

Transcriptional regulation of the human DNA methyltransferase 3A and 3B genes by Sp3 and Sp1 zinc finger proteins

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The *DNMT3A* (DNA methyltransferase 3A) and *DNMT3B* genes encode putative *de novo* methyltransferases and show complex transcriptional regulation in the presence of three and two different promoters respectively. All promoters of *DNMT3A* and *DNMT3B* lack typical TATA sequences adjacent to their transcription start sites and contain several Sp1-binding sites. The importance of these Sp1-binding sites was demonstrated by using a GC-rich DNA-binding protein inhibitor, mithramycin A, i.e. on the basis of decrease in the promoter activities and mRNA expression levels of *DNMT3A* and *DNMT3B*. Overexpression of Sp1 and Sp3 up-regulated the promoter activities of these two genes. The physical binding of Sp1 and Sp3 to *DNMT3A* and *DNMT3B* promoters was

confirmed by a gel shift assay. Interestingly, Sp3 overexpression in HEK-293T cells (human embryonic kidney 293T cells) resulted in 3.3- and 4.0-fold increase in *DNMT3A* and *DNMT3B* mRNA expression levels respectively by quantitative reverse transcriptase-PCR, whereas Sp1 overexpression did not. Furthermore, an antisense oligonucleotide to Sp3 significantly decreased the mRNA levels of *DNMT3A* and *DNMT3B*. These results indicate the functional importance of Sp proteins, particularly Sp3, in the regulation of *DNMT3A* and *DNMT3B* gene expression.

Key words: DNA methyltransferase (DNMT), gene regulation, mithramycin A, specificity protein 1 (Sp1), Sp3.

INTRODUCTION

DNMTs (DNA methyltransferases) are the enzymes catalysing DNA methylation [1]. To date, three functional DNMTs have been identified. Dnmt1 has a 5–30-fold preference for hemimethylated substrates; therefore it has been designated as a maintenance methyltransferase [2]. Dnmt3A and Dnmt3B show equal preference for unmethylated and hemimethylated DNA *in vitro* [3]; therefore they are regarded as *de novo* methyltransferases.

The promoters of *DNMT1* have already been characterized [4]. Increased *DNMT1* mRNA expression has been reported for a number of human cancers [5]. In addition, it has been demonstrated that *DNMT1* is the target of *c-fos*-transformed cells [6], shows up-regulated mRNA expression with an inflammatory cytokine interleukin-6 [7] and is controlled by Sp1 (specificity protein 1) and Sp3 [8].

On the other hand, to date, not much data concerning the transcriptional regulation of other methyltransferases have been reported. *Dnmt3A* and *Dnmt3B* are highly expressed in undifferentiated embryonic stem cells and embryonal carcinoma cells, whereas they are barely detectable in differentiated cells and adult tissues, except for some particular organs [3]. *DNMT3A* and *DNMT3B* mRNAs are reported to be overexpressed in tumours and cancer cell lines [5], and inhibition of *DNMT3B* by antisense oligonucleotide induces apoptosis in cancer cells but not in normal cells [9].

We previously identified the promoters of *DNMT3A* and *DNMT3B* [10]. *DNMT3A* has three alternative 1st exons named exons 1A, 1B and 1C, which are controlled by the 1st, 2nd and 3rd promoters respectively. *DNMT3B* has two alternative 1st exons, designated as exons 1A and 1B, from the 1st and 2nd promoters respectively. All promoters of *DNMT3A* and *DNMT3B* lack TATA sequences near their TSPs (transcription start sites).

The 1st and 2nd promoters of *DNMT3A* and the 1st promoter of *DNMT3B* are CpG-rich and contain clusters of Sp1-binding sites in the proximal promoter region, whereas the 3rd promoter of *DNMT3A* and the 2nd promoter of *DNMT3B* are CpG-poor. However, the transcriptional regulation and major transcription factors that regulate these promoters have not yet been reported.

The Sp transcription factor family belongs to the conserved zinc finger DNA-binding domain proteins that recognize the putative DNA-binding motifs GC-box (GGGCGGG) and GT-box (GGTGTGGGG). They are important for the expression of many different housekeeping genes and genes that generally do not contain TATA- or CAAT-boxes in their proximal promoters, as well as tissue-specific genes [11]. Several Sp proteins (Sp1–Sp8) have been identified [11]. Sp1 and Sp3 are ubiquitously expressed [12], whereas the others show tissue-restricted expression patterns [11]. Sp1 is well known as a transcriptional activator, whereas Sp3 can be either a transcriptional activator [13] or repressor of Sp1-mediated transcription [14], depending on the promoter context and cell type.

In the present study, we focused on the transcriptional regulation of *DNMT3A* and *DNMT3B* promoters by the transcription factors Sp1 and Sp3. By means of various experimental approaches, we demonstrated that Sp proteins, particularly Sp3, were essential for the expression of *DNMT3A* and *DNMT3B*. Altogether, our results lead to the first basic understanding of the molecular transcriptional regulation of *DNMT3A* and *DNMT3B*.

MATERIALS AND METHODS

Plasmids

The reporter plasmids containing full-length human *DNMT3A* 1st+2nd promoters pGL3A-P1+2 (–2489/+640), *DNMT3A* 3rd

Abbreviations used: DNMT, DNA methyltransferase; EMSA, electrophoretic mobility-shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HEK-293T cells, human embryonic kidney 293T cells; RT, reverse transcriptase; Sp, specificity protein; TSP, transcription start site.

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promoter pGL3A-P3 (−3007/+1021), *DNMT3B* 1st promoter pGL3B-P1 (−2483/+309) and *DNMT3B* 2nd promoter pGL3B-P2 (−3531/+260) were described previously [10]. All deletion mutants were named according to the nucleotide numbers of their 5′- and 3′-ends relative to the TSPs of each exon (+1). The plasmid pCMV-Sp1 was a gift from S. Smale (University of California, Los Angeles, CA, U.S.A.). The plasmid pCMV4-Sp3/flu was obtained from J.M. Horowitz (North Carolina State University, Raleigh, NC, U.S.A.). Empty mammalian expression vector pRc/CMV (Invitrogen, Groningen, the Netherlands) was used as a negative control.

Site-directed mutagenesis was performed by a PCR-based approach. The Sp1-binding sites at −99/−87 of pGL3A-P3 (−334/+376) and −100/−92 of pGL3B-P2 (−469/+260) were replaced by an *Xba*I restriction site to generate pGL3A-P3 (−334/+376)-M and pGL3B-P2 (−469/+260)-M. As for pGL3B-P1 (−102/+309), the Sp1-binding sites at −99/−92 and −79/−70 were replaced by *Eco*RI and *Xba*I restriction sites respectively to generate pGL3B-P1 (−102/+309)-M. This mutant plasmid was further used as a template to generate pGL3B-P1 (−102/+309)-DM, in which the Sp1-binding site at −62/−49 (5′-GGGCGGGGCGGGG-3′) was also mutated to 5′-GGGC-TTAAACGGGG-3′. All plasmids were sequenced before using them in transfection experiments.

Cell culture, transfection and luciferase assay

HEK-293T cells (human embryonic kidney 293T) cells, adenovirus-transformed HEK cells expressing simian-virus-40 large T antigen, and U-2OS human osteosarcoma cells were grown at 37 °C under a 5% CO₂ and 95% air atmosphere in Dulbecco's modified Eagles's medium containing 10% (v/v) fetal bovine serum.

For the luciferase assay, HEK-293T and U-2OS cells were transfected in 24-well plates in duplicate with 50 ng of reporter plasmid and increasing amounts (100, 150 and 200 ng) of pCMV-Sp1, pCMV4-Sp3/flu or pRc/CMV, using TransIT-LT1 transfection reagent (Mirus, Madison, WI, U.S.A.) for 48 h. For the luciferase assay with a GC-rich DNA-binding protein inhibitor, mithramycin A (Sigma, St. Louis, MO, U.S.A.) was added to a final concentration of 100 nM for 24 h. Cells were lysed with Passive lysis buffer (Promega, Madison, WI, U.S.A.). The luciferase activity of the cell extract was measured with a Luminocounter 700 (Microtech Niti-On, Chiba, Japan) and normalized to the cellular protein concentration. Results were expressed as fold activation, i.e. the ratio of normalized luciferase activity of pCMV-Sp1 and pCMV4-Sp3/flu to that of pRc/CMV.

RNA extraction, cDNA synthesis and semiquantitative PCR analysis

The HEK-293T cells were transfected in 60 mm plates with 2 μg of pCMV-Sp1, pCMV4-Sp3/flu or pRc/CMV using TransIT-LT1 transfection reagent (Mirus) for 48 h. For the GC-rich DNA-binding protein inhibitor experiment, mithramycin A was added to a final concentration of 100 or 200 nM for 24 h. Total RNA was extracted by using an RNeasy Mini kit (Qiagen, Hilden, Germany). The isolated RNA (1 μg) was used for cDNA synthesis as described previously [15].

The synthesized cDNA was then amplified by the PCR method. The locations, sequences and annealing temperatures of primers are shown in Figure 1(A) and Table 1. PCRs (26–35 cycles) were used to cover the linear range of PCR amplification. *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) transcripts were amplified as an internal control for 21 cycles. All RT (reverse transcriptase)-PCR products were ligated into the pGEM-T easy vector (Promega) and confirmed by direct sequencing.

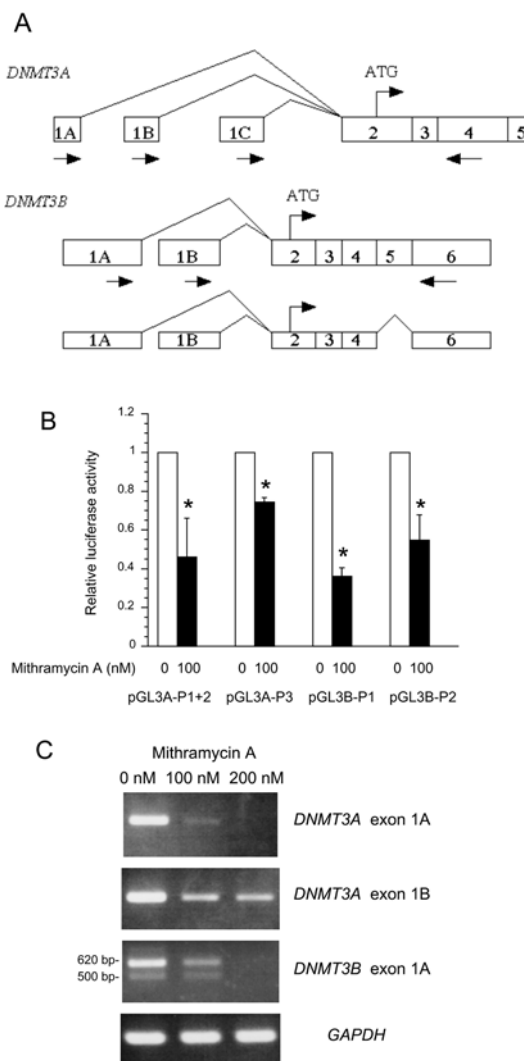


Figure 1 Mithramycin A inhibits *DNMT3A* and *DNMT3B* promoter activities and mRNA expression

(A) Schematic structure of the 5′-region of the human *DNMT3A* and *DNMT3B* mRNAs. Boxed numbers indicate exons, and arrows indicate the positions of sense and antisense primers used for semiquantitative RT-PCR. Three distinct 1st exons of *DNMT3A* (1A, 1B and 1C) are driven by separate promoters (1st, 2nd and 3rd promoters respectively), and spliced to the common exon 2. The 5′-region of *DNMT3B* mRNA contains two alternative 1st exons (1A and 1B), which are spliced to a common exon 2. The structure of the novel alternative spliced variant of *DNMT3B* that lacks exon 5 is shown in the smaller Figure below. (B) The reporter construct containing *DNMT3A* promoters (1st and 2nd promoters, pGL3A-P1 + 2; 3rd promoter, pGL3A-P3) and *DNMT3B* promoters (1st promoter, pGL3B-P1; 2nd promoter, pGL3B-P2) were transfected into HEK-293T cells. Then, luciferase activity was determined in the absence or presence of 100 nM mithramycin A and expressed relative to the activity in the absence of mithramycin A. Results are expressed as the means ± S.D. for three experiments. **P* < 0.05 with the paired *t* test. (C) Semiquantitative RT-PCR analysis of *DNMT3A* and *DNMT3B* 1st exon mRNA variants in HEK-293T cells after incubation with 100 and 200 nM mithramycin A.

LightCycler real-time PCR

The *DNMT3A* and *DNMT3B* mRNA levels in Sp1 or Sp3 transfected HEK-293T cells and Sp3-antisense- or mismatch oligonucleotides-treated cells were quantified with LightCycler™ system using LightCycler DNA Master SYBR Green I (Roche Molecular Biochemicals). As an internal control, *GAPDH* mRNA was also quantified with the same primers used for semiquantitative RT-PCR. The primers for *DNMT3A* were 5′-GAC-TCCATCACGGTGGGCATGG-3′ (sense) and 5′-TGTCCTCT-TGTCCTAACGCC-3′ (antisense). The primers for *DNMT3B*

Table 1 Primer sequences for RT-PCR

Primer	Sequence	<i>T_m</i> (°C)
DNMT3A exon 1A sense	5'-GATCGGGGACCCAGCGCAGA-3'	62
DNMT3A exon 1B sense	5'-GCCCGACCCACCGGCCATAC-3'	62
DNMT3A exon 1C sense	5'-GCCTGCAGCCTGAGCTCAGACC-3'	58
DNMT3A exon 4 antisense	5'-AGATCACCCGAGGGTCTTTGGC-3'	62
DNMT3A exon 17 sense	5'-CATCACGGTGGGCATGGTGC-3'	60
DNMT3A exon 23 antisense	5'-GTCTCTGCCTCGCCAAGC-3'	60
DNMT3B exon 1A sense	5'-AGTGGCCCAAGTAAACCTAGCTC-3'	62
DNMT3B exon 1B sense	5'-GACAGCCTGTCCACATGGAACC-3'	68
DNMT3B exon 6 antisense	5'-CGTTCCTGCCGATGCTGTTC-3'	62
DNMT3B exon 17 sense	5'-CATTGCTTTGGAACCGTAA-3'	55
DNMT3B exon 23 antisense	5'-CTTCTGGCGGGCACCACG-3'	55
GAPDH sense	5'-GACCACAGTCCATGCCATCAC-3'	60
GAPDH antisense	5'-GTCACCACCCTGTGTGTA-3'	60

were 5'-GAGTCCATTGCTGTTGGAACCG-3' (sense) and 5'-ATGTCCTCTGTGTCGCCAACCT-3' (antisense). The amplification programmes were repeated for 40 cycles for *DNMT3B* and *GAPDH* or 45 cycles for *DNMT3A*. The specificity of PCR products was confirmed by melting curve analysis with the presence of single melting curve and reconfirmed by agarose-gel electrophoresis. The standard curve was constructed with 5-fold serial dilutions of HEK-293T cDNA. The Fit Point method was performed for the determination of concentration using LightCycler software version 3.3 (Roche Molecular Biochemicals). The *DNMT3A* and *DNMT3B* mRNA levels were expressed relative to the levels of *GAPDH* mRNA in the same samples.

Western-blot analysis

Western blotting was performed essentially as described in [16]. Total protein (30 µg) from Sp1 or Sp3 transfected HEK-293T cells and Sp3-antisense- or mismatch oligonucleotide-treated cells were immunoblotted with anti-Sp1 (sc-59X; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) at a dilution of 1:2000, anti-Sp3 (sc-644X; Santa Cruz Biotechnology) at 1:6000 and anti-α-tubulin (Bio-Rad) at 1:8000.

EMSA (electrophoretic mobility-shift assay)

The methods for nuclear extract preparation and EMSA were described previously [15]. The sequences of wild-type and mutant-type oligonucleotides are available upon request. For gel supershifts, nuclear extracts were preincubated with 1 µl of the antibody against Sp1 (sc-59X) and/or Sp3 (sc-644X), according to the method described above.

Sp3 knockout with antisense oligonucleotide

The phosphorothioate-modified antisense oligonucleotide (5'-AGTAGCAGCACTTGAATCTGGACT-3') and mismatch oligonucleotide (5'-AGTACCAGGACTTCGAATGTGCACT-3') of the *Sp3* gene were synthesized and HPLC-purified (Qiagen). The HEK-293T cells were transfected with the antisense or mismatch oligonucleotides at a final concentration of 150 nM by using TransIT-Oligo transfection reagent (Mirus). Cells were incubated for 48 h and then harvested for semiquantitative RT-PCR, LightCycler real-time PCR and Western-blot analyses.

RESULTS

Mithramycin A represses *DNMT3A* and *DNMT3B* promoter activities and inhibits mRNA expression

Mithramycin A is an aureolic acid antibiotic that has been shown to inhibit gene expression selectively by displacing transcriptional activators which bind to GC-rich regions of promoters such as Sp1 [17]. Therefore we determined the effects of mithramycin A on the activities of *DNMT3A* and *DNMT3B* promoters. Plasmids containing promoters of *DNMT3A* 1st+2nd promoters pGL3A-P1+2, *DNMT3A* 3rd promoter pGL3A-P3, *DNMT3B* 1st promoter pGL3B-P1 and *DNMT3B* 2nd promoter pGL3B-P2 were transfected into HEK-293T cells and then mithramycin A was added to a concentration of 100 nM. As shown in Figure 1(B), the addition of mithramycin A significantly repressed all promoter activities of *DNMT3A* and *DNMT3B* in HEK-293T cells.

We next analysed the mRNA expression of *DNMT3A* and *DNMT3B* after mithramycin A treatment by the RT-PCR method. As shown in Figure 1(C), the parental HEK-293T cells expressed *DNMT3A/1A* and 1B (from *DNMT3A* 1st and 2nd promoters respectively) and *DNMT3B/1A* (from *DNMT3B* 1st promoter), whereas the expression of *DNMT3A/1C* (from *DNMT3A* 3rd promoter) and *DNMT3B/1B* (from *DNMT3B* 2nd promoter) were undetectable (results not shown). Mithramycin A treatment decreased the *DNMT3A/1A*, 1B and *DNMT3B/1A* mRNA levels in a dose-dependent manner (Figure 1C). With regard to *DNMT3A/1C* and *DNMT3B/1B*, expression levels remained undetectable after mithramycin A treatment (results not shown). Similar results were also obtained with U-2OS cells (results not shown).

Interestingly, although expressed at a much lower level, the RT-PCR product of *DNMT3B/1A* also gave a smaller band (approx. 500 bp) below the major band (620 bp). The direct sequencing result of the smaller product revealed a novel *DNMT3B* isoform that lacks exon 5 (126 bp). The structures of all the alternative spliced variants are shown in Figure 1(A).

Effects of the transcription factors Sp1 and Sp3 on *DNMT3A* and *DNMT3B* promoters

Sp1 and Sp3 are ubiquitously expressed and they are representative GC-rich DNA-binding proteins. Thus, to address the biological effects of Sp1 and Sp3 on *DNMT3A* and *DNMT3B* promoters, reporter plasmids containing the full-length promoters of *DNMT3A* and *DNMT3B* were co-transfected with increasing amounts of an expression vector encoding either Sp1 (pCMV-Sp1) or Sp3 (pCMV4-Sp3/flu) in HEK-293T cells. As shown in Table 2, co-transfection with either Sp1 or Sp3 resulted in the increase of pGL3A-P1+2 luciferase activity in a dose-dependent manner. Similar results were obtained from pGL3A-P3 and pGL3B-P2. With regard to pGL3B-P1, weaker activation by Sp1 and Sp3 was observed. Since transfection with this reporter vector alone has already produced very high basal luciferase activity in many cell lines tested compared with the other reporter vectors ([10]; results not shown), a weaker activation in the co-transfection experiments may result from the high cellular background of endogenous Sp proteins or other transcription factors. The results for other cells (U-2OS) revealed a similar tendency (results not shown).

DNMT3A and *DNMT3B* expression levels in Sp1- and Sp3-overexpressing cells

We next examined whether or not Sp1 and Sp3 overexpression further up-regulates the endogenous mRNA levels of *DNMT3A* and *DNMT3B*. The HEK-293T cells were transiently transfected

Table 2 Effects of Sp1 and Sp3 on *DNMT3A* and *DNMT3B* promoters

The reporter vectors containing full-length promoters of *DNMT3A* and *DNMT3B* were co-transfected with increasing amounts (100, 150 and 200 ng) of Sp1 expression vector pCMV-Sp1, Sp3 expression vector pCMV4-Sp3/flu or empty expression vector pRc/CMV. Luciferase activity was normalized to the protein concentration and expressed relative to the activity in the presence of pRc/CMV in each sample. The results represent the means \pm S.D. for two independent experiments.

Reporter vector	Expression vector ... Amount (ng) ...	pCMV-Sp1			pCMV4-Sp3/flu		
		100	150	200	100	150	200
pGL3A-P1 + 2		1.6 \pm 0.2	2.1 \pm 0.3	6.1 \pm 1.3	1.8 \pm 0.3	2.3 \pm 0.3	3.6 \pm 0.5
pGL3A-P3		2.4 \pm 0.5	4.6 \pm 0.4	13.7 \pm 2.3	3.1 \pm 0.5	4.6 \pm 0.6	6.6 \pm 0.1
pGL3B-P1		1.1 \pm 0.1	1.1 \pm 0.2	1.8 \pm 0.4	0.7 \pm 0.1	0.7 \pm 0.1	1.1 \pm 0.2
pGL3B-P2		1.1 \pm 0.5	1.9 \pm 0.5	10.5 \pm 1.3	0.7 \pm 0.2	0.9 \pm 0.3	2.9 \pm 0.3

with pCMV-Sp1, pCMV4-Sp3/flu or pRc/CMV as a negative control. Semiquantitative RT-PCR analysis with primers recognizing the 3'-portions of *DNMT3A* and *DNMT3B* cDNA showed that *DNMT3A* and *DNMT3B* mRNA expression was increased in HEK-293T cells transfected with the Sp3 vector compared with those transfected with the Sp1 vector and pRc/CMV (Figure 2A, top panels).

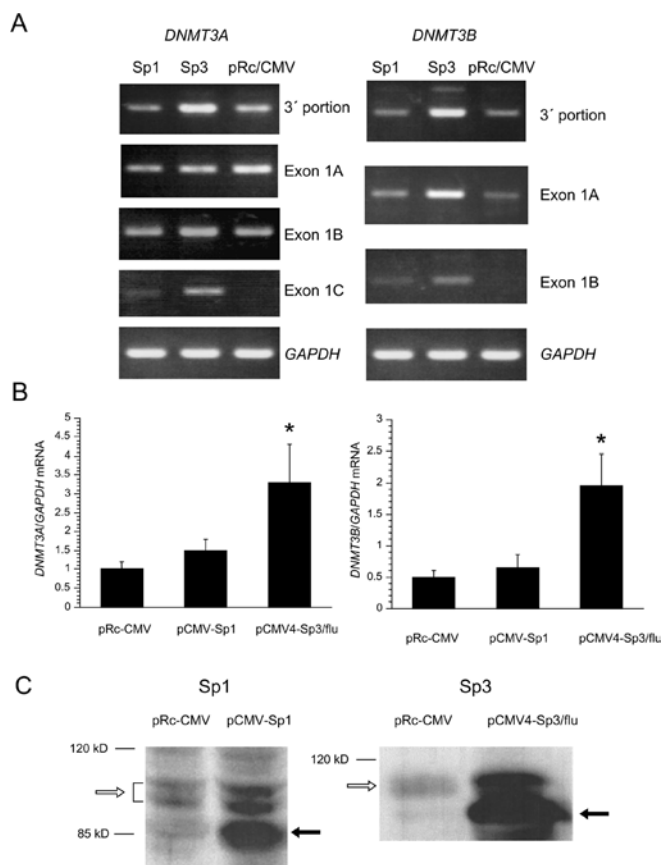
To determine the promoters of *DNMT3A* from which the increase in mRNA expression is derived, we performed RT-PCR with primers specific to all alternative 1st exons of *DNMT3A* or *DNMT3B*. With regard to *DNMT3A*, *DNMT3A/1A* and *1B* were expressed in the parental cells and no significant increase in mRNA expression was observed (Figure 2A). On the other hand, *DNMT3A/1C*, the expression of which was below the detectable level in the parental cells, was significantly up-regulated by Sp3 and to a lesser extent by Sp1. Concerning *DNMT3B*, *DNMT3B/1A* was up-regulated by Sp3. *DNMT3B/1B* was also up-regulated by Sp3 and to a lesser extent by Sp1. The experiments were repeated three times using different cDNAs from three transfection experiments and the same results were obtained.

To confirm the findings of semiquantitative RT-PCR, we performed LightCycler real-time PCR. As shown in Figure 2(B), Sp3 overexpression resulted in 3.3- and 4.0-fold increase in *DNMT3A* and *DNMT3B* levels compared with the levels in cells transfected with the empty vector respectively. As expected, slightly increased *DNMT3A* and *DNMT3B* levels were observed in Sp1-overexpressing cells.

We also confirmed the expression levels of Sp1 and Sp3 proteins in cells transfected with pCMV-Sp1 and pCMV4-Sp3/flu respectively using immunoblot analysis. In Figure 2(C), a significant increase in the exogenous Sp1 protein (approx. 85 kDa), which resulted from the lack of 85 amino acids in the N-terminus of Sp1 [18], was observed. The increase in endogenous Sp1 (upper bands) could be caused by autoregulation [19]. The doublet of Sp1 protein represented a different phosphorylated status. Similarly, the exogenous Sp3 protein, which resulted from the lack of approx. 70 amino acids in the N-terminus of Sp3 [13], was abundantly expressed.

Identification of the Sp1-binding sites responsible for minimal promoter activities in *DNMT3A* and *DNMT3B* promoters

On the basis of the findings for HEK-293T cells that mRNA expression from the *DNMT3A* 3rd promoter and *DNMT3B* 1st and 2nd promoters was up-regulated by Sp3 overexpression and to a lesser extent by Sp1 overexpression, we next examined the Sp1-binding sites responsible for these promoter activities. Previously, we identified the minimal promoters of the *DNMT3A* 3rd promoter pGL3A-P3 (-334/+376), the *DNMT3B* 1st promoter pGL3B-P1 (-604/+309) and the *DNMT3B* 2nd promoter pGL3B-P2

**Figure 2** *DNMT3A* and *DNMT3B* expression levels in Sp1- or Sp3-overexpressing cells

(A) Total RNA from HEK-293T cells transfected with Sp1, Sp3 or pRc/CMV were amplified by semiquantitative RT-PCR method using specific primers for the 3'-portions of *DNMT3A* and *DNMT3B* and all alternative 1st exons of each gene. The experiments were repeated three times using different cDNAs from three transfection experiments. (B) The *DNMT3A* and *DNMT3B* mRNA levels in the same cDNA preparation were quantified by using LightCycler real-time PCR. The *DNMT3A* (left) and *DNMT3B* (right) levels were expressed relative to *GAPDH* mRNA levels in the same samples. Results are expressed as the means \pm S.D. for three experiments. * $P < 0.05$ with the paired t test. (C) Total protein from HEK-293T cells transfected with pCMV-Sp1, pCMV4-Sp3/flu or pRc/CMV was used for Western-blot analyses with anti-Sp1 and anti-Sp3 antibodies respectively. Open arrows indicate the endogenous Sp1 or Sp3 proteins in each Figure. Closed arrows indicate exogenous Sp1 or Sp3 proteins in each Figure.

(-469/+260) in NT2 cells [10]. However, for the *DNMT3B* 1st promoter, pGL3B-P1 showed weak activation by Sp3 (Table 2). We next co-transfected the Sp3 expression vector with a series of 5' deletion mutant reporter vectors of pGL3B-P1. Significant activation (approx. 2-fold) by Sp3 was observed in the deletion

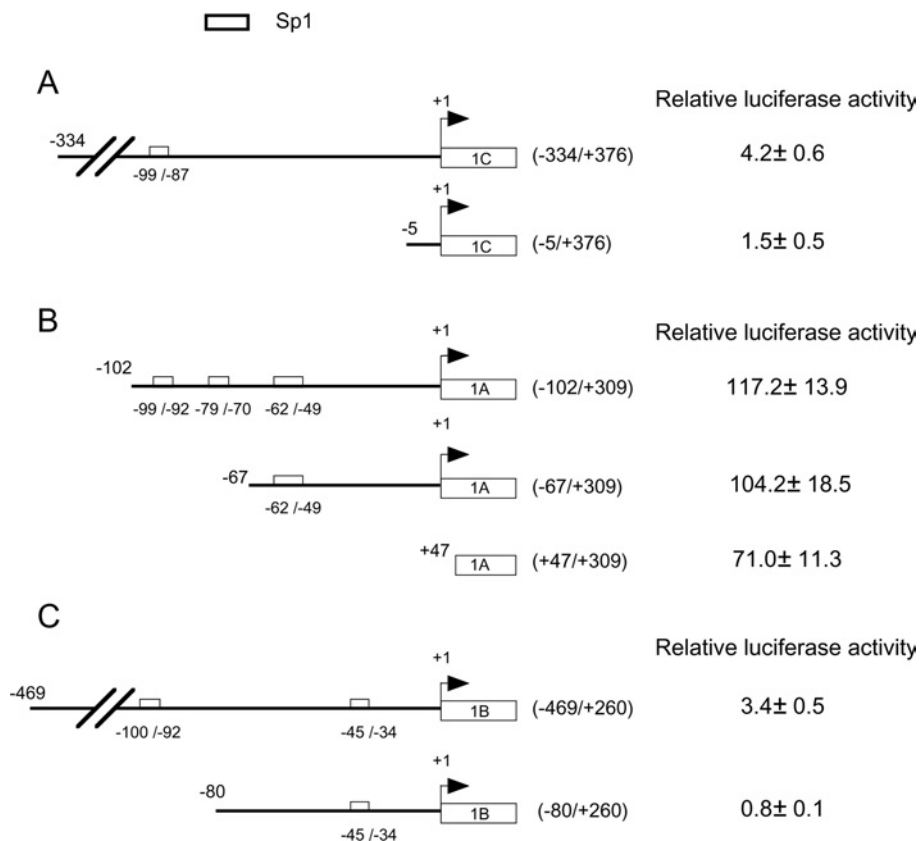


Figure 3 Identification of the Sp1-binding sites responsible for minimal promoter activities of *DNMT3A* and *DNMT3B* in HEK-293T cells

Left panels: schematic structure of the minimal promoter of *DNMT3A* 3rd promoter pGL3A-P3 (–334/+376) (A), *DNMT3B* 1st promoter pGL3B-P1 (–102/+309) (B), *DNMT3B* 2nd promoter pGL3B-P2 (–469/+260) (C) and their deletion constructs are illustrated. The Sp1-binding sites are presented as open boxes and positions are shown relative to the TSP (+1) of each exon. Right panels: the plasmids containing minimal promoter of *DNMT3A* 3rd promoter, *DNMT3B* 1st and 2nd promoters and their deletion mutants were transfected into HEK-293T cells. Luciferase activity was expressed relative to the activity of empty reporter vector pGL3-basic. Results are expressed as the means ± S.D. for three independent experiments.

mutant pGL3B-P1 (–102/+309) (results not shown). Therefore we considered this region to be the minimal promoter of *DNMT3B* 1st promoter in this experiment. Alternatively, there may be suppressive sequences in the 5'-portion of *DNMT3B* 1st promoter, since the shorter reporter sequences exhibited a significant luciferase activity.

The minimal promoters, deletion mutants and the empty reporter vector pGL3-basic were transfected into HEK-293T cells (Figure 3). The results revealed that these minimal promoters still retained high basal activity compared with pGL3-basic. Further 5' deletion of pGL3A-P3 (–334/+376) to (–5/+376) resulted in loss of basal activity (Figure 3A), indicating that crucial sites are located between –334 and –5. Deletion of pGL3B-P1 (–102/+309) to (–67/+309) resulted in a slight loss of the basal activity (Figure 3B). Further 5' deletion to +47/+309 resulted in a 39.4% decrease in basal activity, suggesting that the region –67 to +47 is important. Further 5' deletion of pGL3B-P2 (–469/+260) to (–80/+260) abrogated the basal activity (Figure 3C), which suggests that crucial sites are located between –469 and –80. We then checked the presence of Sp1-binding motifs in these regions with TFSEARCH (<http://www.etl.go.jp/etl/ebrc/research/db/TFSEARCHJ.html>). The results revealed one Sp1-binding site at –99 to –87 of the *DNMT3A* 3rd promoter, three Sp1-binding sites at –99 to –92, –79 to –70 and –62 to –49 of the *DNMT3B* 1st promoter and one Sp1-binding site at –100 to –92 of the *DNMT3B* 2nd promoter.

Effect of site-directed mutagenesis of Sp1-binding sites on the *DNMT3A* and *DNMT3B* promoter activities

To investigate the role of each Sp1-binding site in the *DNMT3A* and *DNMT3B* minimal promoters, site-specific mutagenesis analyses for each Sp1-binding site were performed. As shown in Figure 4, mutations of the Sp1-binding sites at –99 to –87 of the *DNMT3A* 3rd promoter (Figure 4A) and –100 to –92 of *DNMT3B* 2nd promoter (Figure 4C) decreased the promoter activities to 33.3 and 10.3% respectively. Concerning the *DNMT3B* 1st promoter (Figure 4B), mutations of the Sp1-binding sites at –99 to –92 and –79 to –70 resulted in a slight loss of the promoter activity. However, when the binding site at –62 to –49 was also mutated, the promoter activity was reduced to 44.0%. The remaining promoter activity of pGL3B-P1 (–102/+309)-DM could be maintained by the existence of several Sp1-binding sites in the 1st exon [10]. However, these results indicated that these Sp1-binding sites are essential for *DNMT3A* and *DNMT3B* minimal promoter activities.

Sp1 and Sp3 proteins bind to the *DNMT3A* and *DNMT3B* promoters

On the basis of the fact that the Sp1-binding sites at –99 to –87 of the *DNMT3A* 3rd promoter, –62 to –49 of the *DNMT3B* 1st promoter and –100 to –92 of the *DNMT3B* 2nd promoter are essential for the maintenance of the minimal promoter basal activity, we then examined the physical interaction between these

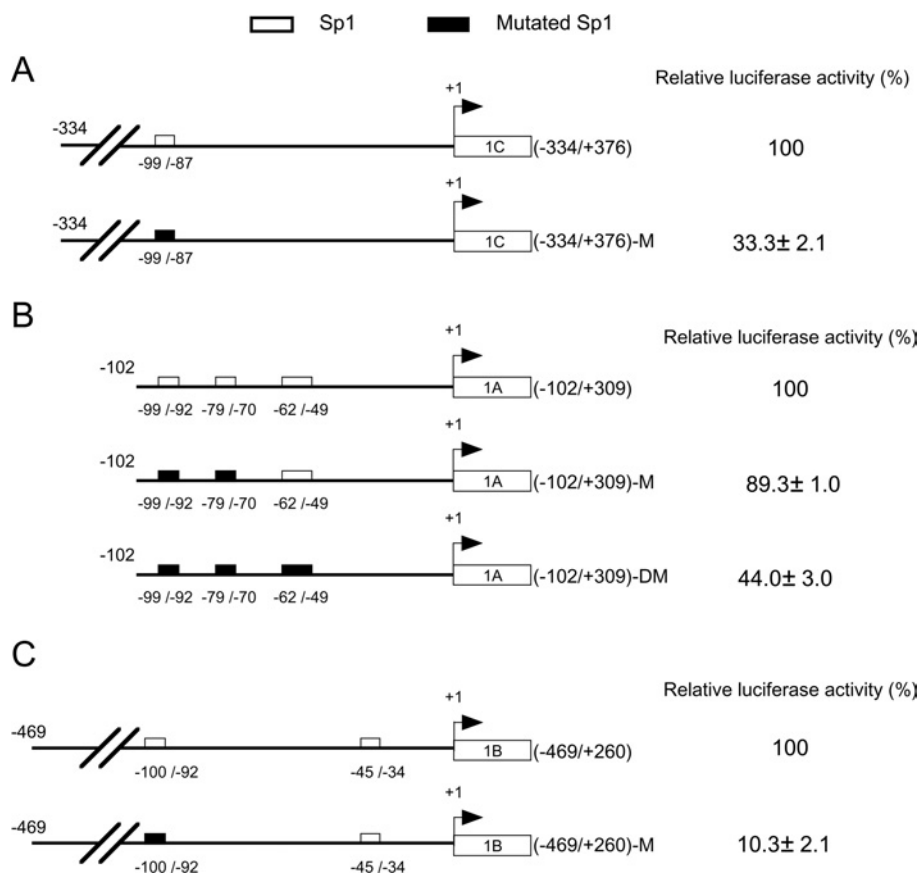


Figure 4 Effect of site-specific mutation of Sp1-binding sites on the *DNMT3A* and *DNMT3B* promoters

Left panels: schematic representation of site-specific mutation of Sp1-binding sites on minimal promoters of *DNMT3A* 3rd promoter (A), *DNMT3B* 1st promoter (B) and *DNMT3B* 2nd promoter (C). The wild-type and mutant-type Sp1-binding sites are shown as open and filled boxes respectively. Right panels: luciferase activities (%) of the mutant reporter vectors were expressed relative to the activities of their wild-type counterparts. Results are expressed as the means \pm S.D. for three independent experiments.

binding sites and transcription factors. Oligonucleotide probes 3A-P3-W1 (from the *DNMT3A* 3rd promoter), 3B-P1-W1 (from the *DNMT3B* 1st promoter) and 3B-P2-W1 (from the *DNMT3B* 2nd promoter) were labelled and incubated with a nuclear extract of HEK-293T cells. The autoradiographs from all three probes were almost comparable. Representative data of the autoradiograph from probe 3B-P2-W1 is shown in Figure 5. The result revealed five major DNA–protein complexes (lane 2). Complexes 1 and 2 were completely competed for by a 500-fold excess of the unlabelled wild-type probe (lane 4), but were not competed for by a 500-fold excess of mutant-type probe (lane 6), indicating that they were specific complexes. To confirm the presence of Sp1 and Sp3 in these complexes, gel supershift was performed by using antibodies against Sp1 and Sp3. Complex 1 considerably decreased in the presence of Sp1 antibody (lane 7) and a combination of Sp1 and Sp3 antibodies further supershifted the remaining bands (lane 9), suggesting that Sp1 and Sp3 were components of complex 1. The addition of Sp3 antibody alone completely abolished complex 2 (lane 8), indicating that it was an Sp3–DNA complex. Since we detected a large Sp3 isoform (110–115 kDa) and small isoforms (70–80 kDa) in HEK-293T cells (Figure 6A), the upper and lower Sp3–DNA complexes may indicate the binding of large and small Sp3 isoforms respectively. No supershift complexes were observed in the presence of preimmune serum (lane 10). However, the last remaining bands of complex 1 (lane 9) could not be supershifted by either Sp1 and/or Sp3 antibodies; therefore their nature remains unknown. Altogether, these results

indicated that Sp1 and Sp3 directly bind to the *DNMT3A* and *DNMT3B* promoters.

Sp3-specific antisense oligonucleotide decreases *DNMT3A* and *DNMT3B* mRNA levels

To determine whether Sp1 and/or Sp3 regulate the *DNMT3A* and *DNMT3B* expression under the physiological conditions in parental HEK-293T cells, we specifically down-regulated Sp1 or Sp3 expression by using the antisense oligonucleotide. In Figure 6(A), Western-blot analysis showed that the Sp3 expression considerably decreased in antisense-treated cells, whereas the Sp1 level was unaffected. We further analysed the expression levels of *DNMT3A* and *DNMT3B* by LightCycler real-time PCR. As shown in Figure 6(B), Sp3 antisense oligonucleotide decreased the mRNA levels of *DNMT3A* and *DNMT3B* to 69.3 and 60.0% compared with the levels in mismatch oligonucleotide-treated cells respectively. Similar results were also obtained from semiquantitative RT–PCR analyses (results not shown). We inhibited Sp1 expression by an Sp1-specific antisense oligonucleotide but no significant decrease in *DNMT3A* and *DNMT3B* mRNA levels were observed (results not shown).

DISCUSSION

DNMTs are believed to play important roles in the development of cancer since they are essential for the maintenance of aberrant

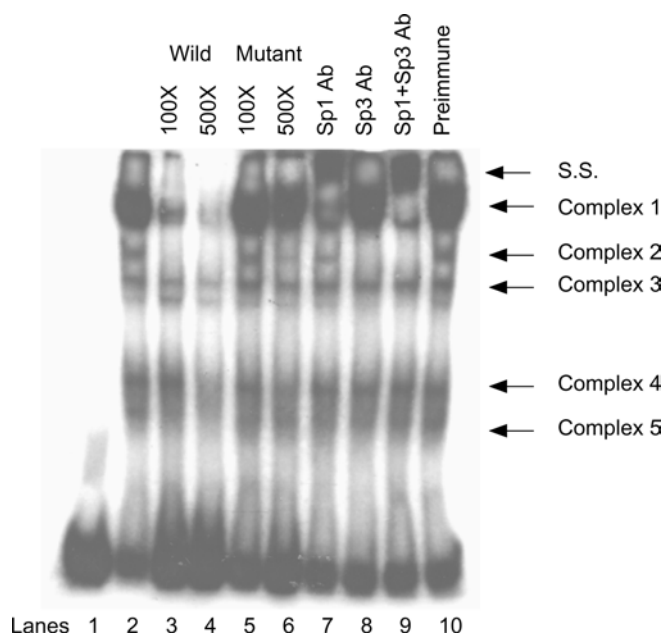


Figure 5 Sp1 and Sp3 bind to the 2nd promoter of *DNMT3B*

The labelled synthetic oligonucleotide spanning nucleotides –119 to –73 relative to the TSP of the *DNMT3B* 2nd promoter (3B-P2-W1) was incubated with nuclear extract of the HEK-293T cells (lane 2) or in the presence of a 100 or 500 molar excess of the unlabelled wild-type probes (lanes 3 and 4) or the mutant-type probes in which the Sp1-binding site at –100/–92 (5'-GAGGCGGGG-3') was mutated to 5'-ATATAGGGG-3' (lanes 5 and 6). For the gel supershift assay, Sp1 and/or Sp3 antibodies were added to the reaction mixture (lanes 7–9). Preimmune serum was used as a control (lane 10). The supershifted bands (S. S.) are indicated.

methylation in cancer cells [20] and for cancer cell survival [9]. However, there are a few studies of the mechanisms involved in the regulation of expression of *DNMT3A* and *DNMT3B*. In the present study, we indicated that Sp1 and Sp3 are essential for the regulation of *DNMT3A* and *DNMT3B* expression. Treatment with mithramycin A, a GC-rich DNA-binding protein inhibitor, repressed the promoter activities and the mRNA levels of *DNMT3A* and *DNMT3B*. The results from site-directed mutagenesis of Sp1-binding sites also suggested that the *DNMT3A* and *DNMT3B* promoter activities are largely dependent on these Sp1-binding sites and Sp proteins that interact with these binding sites. Whereas the results of some previous studies showed that Sp3 is a repressor of Sp1-mediated transcription [14,21], the results from our luciferase assay showed that both Sp1 and Sp3 were positive regulators of the *DNMT3A* and *DNMT3B* promoters not only in HEK-293T cells but also in several other cell lines (A. Jinawath, S. Miyake, Y. Yanagisawa, Y. Akiyama and Y. Yuasa, unpublished work). It has been reported that several promoters are also positively regulated by both Sp1 and Sp3 [8,22,23].

To determine whether the *DNMT3A* and *DNMT3B* genes are the downstream Sp1 and Sp3 target genes, overexpression and knockout experiments were performed. Overexpression of Sp3 in HEK-293T cells significantly increased *DNMT3A* and *DNMT3B* mRNA levels. Although we could not inhibit the Sp3 protein expression by using Sp3 antisense oligonucleotide completely, a significant decrease in *DNMT3A* and *DNMT3B* was found, supporting the view that Sp3 may be required for the expression of *DNMT3A* and *DNMT3B* *in vivo*. By a similar approach, the *BACE1* (beta-amyloid precursor protein cleaving enzyme 1) gene was also shown to be regulated by Sp1, since the *BACE1* mRNA level in Sp1 knockout cells was reduced to 58.6% [24], a reduction

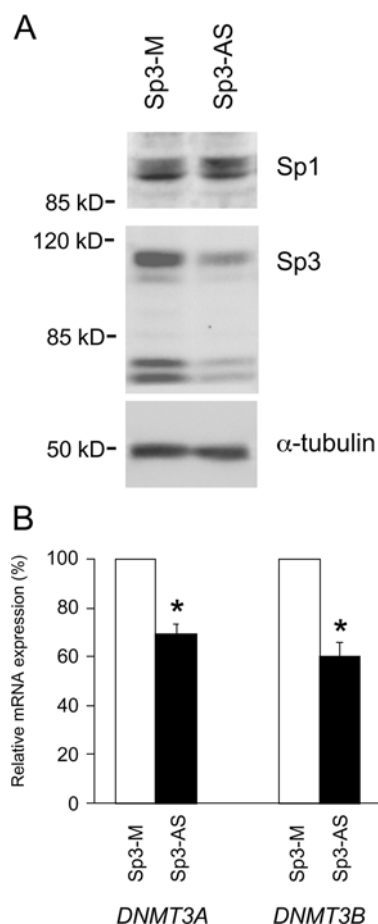


Figure 6 Down-regulation of *DNMT3A* and *DNMT3B* mRNAs with Sp3 antisense oligonucleotide

(A) HEK-293T cells were incubated with either Sp3 antisense oligonucleotide (Sp3-AS) or mismatch oligonucleotide (Sp3-M). Total proteins from both groups were subjected to Western-blot analyses with anti-Sp1 and -Sp3 antibodies. (B) *DNMT3A* and *DNMT3B* mRNA levels from antisense and mismatch oligonucleotide-treated cells were quantified with Light-Cycler real-time PCR, then normalized to the *GAPDH* mRNA levels and expressed relative to the levels in mismatch oligonucleotide-treated cells. * $P < 0.05$ by the *t* test. Results are expressed as the means \pm S.D. for three experiments.

rate similar to ours. On the other hand, Sp1 overexpression did not induce significant up-regulation of *DNMT3A* and *DNMT3B* mRNA levels and treatment with an Sp1 antisense oligonucleotide did not significantly inhibit the expression of these two genes, although physical binding of Sp1 and Sp3 was found in the *DNMT3A* and *DNMT3B* promoters. These results suggest that Sp3 plays a more important role than Sp1 in the regulation of *DNMT3A* and *DNMT3B* in HEK-293T cells. Further experiments will be required to determine the more precise roles of Sp1 and Sp3 in the regulation of these promoters.

Sp3 functions as a repressor or weak activator when it binds to promoters containing multiple binding sites, whereas it functions as an activator when it binds to a promoter containing a single binding site [14]. The *DNMT3A* 3rd and *DNMT3B* 2nd minimal promoters contain one Sp1-binding site at –99 to –87 and –100 to –92 respectively; therefore these promoters may be up-regulated by Sp3 in this model. However, *DNMT3B/1A*, which is the major 5' splicing variant of *DNMT3B* in all the cell lines examined (A. Jinawath, S. Miyake, Y. Yanagisawa, Y. Akiyama and Y. Yuasa, unpublished work), encoded from the *DNMT3B* 1st promoter containing a cluster of Sp1-binding sites, was also

up-regulated by Sp3. Thus the responses of this promoter to Sp3 may depend on the context of a specific promoter. Promoters that contain multiple Sp1-binding sites and are positively regulated by Sp3 have also been reported [23,25].

Several *DNMT3B* isoforms resulting from alternative splicing of exons 10, 21 and/or 22 have been reported [5,26]. Here, we have shown a new splicing variant of the 5'-region of *DNMT3B* for the first time. This variant is associated with *DNMT3B/1A* and lacks exon 5, resulting in the loss of 42 amino acids from the N-terminal portion. We also found this splicing variant lacking exon 5 in association with *DNMT3B/1B* in other cell lines (A. Jinawath, S. Miyake, Y. Yanagisawa, Y. Akiyama and Y. Yuasa, unpublished work). The first 298 amino acids in the N-terminal portion of DNMT3B are essential for the physical binding with DNMT1 and DNMT3A, which may facilitate the rapid establishment of the *de novo* methylation pattern [27]. Therefore the loss of exon 5, which contains 42 amino acids (between amino acids 103 and 144), may impair the binding with other DNMTs. Determination of the functions of these 5' splicing variants of *DNMT3B* and their association with 3' alternative splicing of exons 10, 21 and/or 22 will require further experiments.

Sp1 and Sp3 have been reported to participate in almost all biological aspects of cells including carcinogenesis. For example, overexpression of Sp3 contributes to a repression of TGF- β (transforming growth factor- β) receptor expression in breast cancer cells [21]. Sp1 and Sp3 are required for the induction of vascular endothelial growth factor-A by oxidative stress in gastric cancer cells [22] and constitutive expression of matrix metalloproteinase-2 in astrocytoma cells [28]. Our results indicate that Sp proteins, particularly Sp3, control both *DNMT3A* and *DNMT3B* expressions. Since *DNMT3A* and *DNMT3B* have been reported to be overexpressed in many tumours [5], it is tempting to speculate that the dysregulation of Sp3 and Sp1 contributes to *DNMT3A* and *DNMT3B* overexpression in cancer cells. However, the promoters of *DNMT3A* and *DNMT3B* also contain the binding sites for other transcription factors, including activator protein 1, nuclear factor-Y, E2F, Ets, SRY etc. [10]. To clarify further the mechanisms underlying the regulation of *DNMT3A* and *DNMT3B* expression in a cell- and tissue-specific manner, it is important to analyse other transcription factors as well as Sp proteins.

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