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SVEP1 promoter regulation by methylation of CpG sites

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ABSTRACT

SVEP1 gene encodes a cell adhesion molecule (CAM) that was previously shown to be expressed by cells related to skeletal tissues. Here we focus on SVEP1 expression regulation in pre-osteoblastic MBA-15 and mammary adenocarcinoma DA3 cells. We show that SVEP1 message and protein are highly expressed by MBA-15 when compared with DA3 cells. DNA methylation of CpGs sites is an epigenetic mechanism associated with gene silencing. Therefore, we analyzed the methylation status of a region potentially harbors SVEP1 promoter and further activity alterations induced by estrogen (17βE$_2$) and TNFα. We also mapped *in silico* the transcription binding sites namely TFIIB, NF-κB, ERE, AP1 and Sp1 at the putative promoter. Treatments with demethylation reagents, 5′-aza-deoxy-Cytidine (5′-aza-dC), or histone deacetylase inhibitor, Trichostatin A (TSA) resulted with an elevation of SVEP1 mRNA expression in both cell types. Methylation levels of specific CpGs sites located at transcription binding sites were assessed using sodium bisulfite genomic DNA sequencing, methylated DNA immunoprecipitation (meDIP) and Methylation-Specific PCR (MSP). Our results show that the putative promoter of SVEP1 is hypermethylated in DA3— compared with MBA-15 cells, thus regulating SVEP1 expression levels. In addition, by affecting SVEP1 promoter methylation status, 17βE$_2$ and TNFα regulate ectopic SVEP1 promoter and mRNA expression. Our data sheds light on understanding the cell-type specific promoter status for regulation of the SVEP1. Since SVEP1 protein mediates cellular adhesion, this data might be beneficial for the future characterization of SVEP1 expression in the interactions existing in bone.

1. Introduction

Interactions between cells and the extracellular matrix (ECM) and cell–cell interactions are mediated by cell adhesion molecules (CAMs) (Patel et al., 2002; Shur et al., 2002; Witz, 2008). We characterized SVEP1, which is a CAM molecule, possessing complement binding motifs (CCP), an EGF and EGF-like Ca^{+2}-binding domains (Shur et al., 2006). CCP domains are present in various proteins that are part of the coagulation and complement cascade, as well as in selectin proteins (Kansas, 1996). SVEP1 expression was identified in skeletal tissues *in vivo*, mesenchymal stem cells (MSCs) derived from human, mouse and rat bone marrow, and in pre-osteoblastic cells (MBA-15) (Shur et al., 2006). SVEP1 is also expressed by skeletal muscle-activated satellite cells (Shefer and Benayahu, 2010) and was identified in a series of human breast cancer (BC) cell lines (Shur et al., 2007).

It has been recognized that breast cancers have the ability to invade and grow as metastases in the bone (Mundy, 1997). The cross talk between breast cancer cells and their respective microenvironment determine the destiny of the cancer cell and its metastatic potential. Stroma affects cellular activities in various tissues; thus, it is important to study the communication between the stroma and the tumor cells, specifically in the bone microenvironment (Bergfeld and DeClerck, 2010). In addition, inflammatory cytokines are secreted by the stroma and pre-osteoblastic cells, supporting cancer progression in a specific microenvironment (Dvorak, 1986). It was shown that cytokines, such as TNFα and IL-1β, up-regulate expression of receptors and affect adhesion and migration of MSCs *in vivo* (Segers et al., 2006).

Our group demonstrated that SVEP1 mRNA is expressed by cells from the bone marrow and is regulated by estrogen levels *in vivo* and *in vitro* (Shur et al., 2006, 2007). Pre-osteoblastic MBA-15 cells are estrogen-receptor (ER) positive cells (Shamay et al., 1996; Benayahu, 1997) that express the SVEP1 gene which is up-regulated following modulation with 17βE$_2$ (Shur et al., 2006, 2007). SVEP1 protein expression was also detected in human breast cancer cell lines (Shur et al., 2007).

DNA methylation is a major epigenetic alteration affecting gene expression. Methylation, which is catalyzed by the methyltransferases family, is the addition of the methyl group on the 5-carbon of deoxycytosines in CpGs sites (Bird, 2002). Gene expression is affected by DNA methylation and alterations in methylation patterns contribute to cancer development and progression. Epigenetic changes affect the expression of tumor-suppressor genes (Jones and Laird, 1999; Jones and Baylin, 2002; Baylin and Ohm, 2006) and abnormal epigenetic patterns initiate and play a role in the progression of metastatic cancer (Baylin and Ohm, 2006; Feinberg et al., 2006).

Abbreviations: CAM, Cell adhesion molecule; ECM, Extracellular matrix; ChIP, Chromatin immunoprecipitation; TF, Transcription factor; qPCR, quantitative PCR; MSP, Methylation-specific PCR; 5'aza-dC, 5'-aza-deoxycytidine; TSA, Trichostatin A; meDIP, Methylated DNA immunopercipitation.

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The involvement of epigenetic mechanisms in the regulation of SVEP1 promoter activity has not yet been investigated.

Differential expression was also described for the developmental master genes, Oct-4 and Nanong, which are required for normal development (Nichols et al., 1998; Chambers et al., 2003; Mitsui et al., 2003). Oct-4 and Nanong are key molecules in maintaining the pleuripotency of embryonic stem (ES) cells and they are co-expressed in developmental stage- and cell-specific manners (Chambers et al., 2003; Mitsui et al., 2003). A proper formation of cell-type specific DNA methylation profiles is fundamental to cellular differentiation (Shiota and Yanagimachi, 2002). Therefore, it has the capacity to regulate cell type specific expression of a protein. We characterized SVEP1 differential expression in a model system composed of pre-osteoblastic cell line, MBA-15 cells and the murine adenocarcinoma cell line, DA3.

Results obtained demonstrated reduced expression levels of SVEP1 mRNA and protein in the murine mammary adenocarcinoma cell line, DA3, compared with the pre-osteoblastic cell line, MBA-15. Our study was aimed at elucidating the methylation of palindromic dinucleotide CpGs of the SVEP1 promoter constitutes a key event in the regulatory mechanism of its activity. In addition, we gained new data on potential mechanisms responsible for SVEP1 expression regulation by TNFα and 17βE_2.

Using bioinformatic databases, we identified potential transcription factor binding sites at the 5′ region located upstream the SVEP1 gene. The methylation status of CpGs sites was confirmed by the bisulfite genomic DNA sequencing method and Methylation-Specific PCR (MSP). A higher degree of methylation was detected in DNA harvested from DA3 cells relative to MBA-15 cells. The current research focuses on the regulation of the SVEP1 putative promoter by TNFα and 17βE_2 through affecting the methylation content of the promoter. Transcription factor binding sites were differentially methylated between the two cells types with a higher degree of methylation in DNA harvested from DA3 cells relative to MBA-15 cells. These results might explain the low expression levels of SVEP1 mRNA and protein in the DA3 adenocarcinoma cell line. This study presents for the first time, evidence of epigenetic transcriptional regulation of the SVEP1 potential promoter and constitutes the methylation of CpGs sites as a potential mechanism for SVEP1 gene expression regulation. Taken together, we identified that the DNA methylation is probably a part of complex regulatory mechanism affecting SVEP1 promoter activity.

2. Materials and methods

2.1. Cell culture

MBA-15, a pre-osteoblastic stromal cell line (Benayahu et al., 1989) and DA3, a metastatic murine mammary adenocarcinoma cell line (Fu et al., 1990). Cells were maintained in growth medium (termed "basal growth conditions"), Dulbecco's Modified Essential Medium (DMEM) (Gibco, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Beth haEmek, Israel), 1% glutamine and 1% penicillin/streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C. For modulation experiments, cells were cultured in medium supplemented with 3% charcoal-stripped (steroid-depleted) serum serum-stripped (Beth haEmek, Israel) for 24 h prior to treatment with TNFα (10, 50, 100 ng/ml) (PeproTech Asia) or with 17β-Estradiol (17βE_2) 1×10^{-8}, 2×10^{-8}, 4×10^{-8} M (Sigma, USA) for 4 h or 24 h.

2.2. Bioinformatic analysis

Bioinformatics analysis was previously performed to identify putative promoter located upstream the transcription start site (TSS) in the 5′-flanking region of SVEP1 gene (GI: 24816888) (Shur et al., 2007). The analysis applied Promoter 2.0 Prediction Server (http://www.cbs.dtu.dk/services/Promoter) for promoter definition. Transcription factor binding sites were predicted using AliBaba2.1 software (http://www.gene-regulation.com/pub/programs/alibaba2/index.html). Based on these analyses we constructed primers using Primer3 Software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The primers for the promoter region was constructed at 334–194 bp upstream TSS (see methylated DNA immunoprecipitation part).

2.3. SVEP1 promoter cloning and reporter assay activity

Fragment of 798 bp [−1755 bp to −957 bp] from 5′-UTR upstream the transcription start site (TSS) of SVEP1 promoter was amplified from genomic DNA. The primers containing adaptors for Bgl II and EcoR I restriction enzymes (marked in lower case fonts) were F- gagaagatctGTGCACCTTTTCTTTA and R- gggaattccCTCGGAGATTCCATGA. The amplified PCR fragment was cloned into pGLuc vector [pGluc-SVEP1] upstream of a luciferase reporter (New England BioLabs, USA). Cells were transient transfected with 1 μg pGluc-SVEP1 or empty vector [pGLuc-basic] using the jetPEI™ reagent (Polyplus Transfection, France). The cells were co-transfected with 0.2 μg β-galactosidase [β-gal] expression plasmid (pCMVβ, Clontech, USA). Cells were treated for 4 h or 24 h with TNFα (10, 50, 100 ng/ml) or with 17βE_2 (1×10^{-8}, 2×10^{-8}, 4×10^{-8} M). The promoter activity was measured by luciferase assay (Gaussia Luciferase, PJK GmbH; Germany) normalized to β-gal activity (which indicates the transfection efficiency).

2.4. mRNA and gene expression analysis

RNA was extracted from cultured cells using EZ RNA kit (Biological industries, Beth haEmek, Israel) and reverse transcribed to cDNA using avian myeloblastosis virus reverse transcriptase (AMV-RT), oligo-dT and random-hexamers (Takara Shuzo Co.Ltd., Seta, Japan). Polymerase chain reactions (PCR) were performed using specific primers for SVEP1 with PCR mix (Sigma, USA); products were run in 1% agarose gel, detected by ethidium bromide staining and analyzed by "TINA" software. The gel image was captured using a BIS 202D Bio Imaging Densitometer. The integrity of the RNA, the efficiency of the RT reaction and the quality of cDNA subjected to PCR amplification of the transcripts was normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels. SVEP1 mRNA levels were measured using the following primers: 5′-AACCGCCTGTCATAGATTGG; 3′- TGTGTACCACACCACCGTTT. These primers amplify a-178 bp mRNA fragment. GAPDH mRNA levels were measured using the following primers: 5′- TGGAAGGGCTCATGACCAC; 3′- ACCTGGTCCTCAGTGTAGC. These primers amplify a-331 bp mRNA fragment.

2.5. Immunoprecipitation (IP), SDS-PAGE gels and western blot analysis

These procedures and analyses were performed according to the standard protocols (www.protocol-online.net). Briefly, immunoprecipitation was performed with SVEP1 antibody incubated overnight with Protein A immobilized on Sepharose CL-4B (Pharmacia, USA). The immuno-complexes were separated on 7% SDS-PAGE gel for 3 h, then transferred overnight to the nitrocellulose blots and probed with a primary antibody to SVEP1. The first antibody was incubated for 3 h, followed by a secondary antibody, goat anti-rabbit-biotin IgG (Dako, Denmark), and Extravidin-Peroxidase (Sigma, USA), for detection with chemiluminescent substrate (Pierce, USA), exposure to X-OMAT AR film (Kodak) (Shur et al., 2006).

2.6. Message expression of cells treated with 5′-aza-deoxycytidine (5′-aza-dC) and Trichostatin A (TSA)

DA3 and MBA-15 cell lines (6×10^5 cells/100 mm dish) were seeded in 10% FCS in DMEM. After 24 h the cells were treated with 2.5 μM

5′-aza-dC (Sigma, USA), or with 300 nM TSA (Sigma, USA). RNA was extracted from the cells using EZ RNA kit (Beth haEmek, Israel) followed by a reverse transcribed to cDNA using avian myeloblastosis virus reverse transcriptase (AMV-RT) (New England Biolabs) and oligo-dT (Takara Ltd., Japan). The cDNA served as a template for a polymerase chain reaction (PCR) performed with Ready Mix Taq PCR reaction mix (Sigma, USA). The primers for the reaction, designed using Primer3 software (http://frodo.wi.mit.edu/primer3), are 5′- AACCGCCTGTCATAGATTGG, 3′- TGTGTACCACACCACCGTTT localized at 2157–2335 bp (GI: 124783267) resulted with an amplicon of 178 bp. Optical density of the PCR product was measured using Bio-Imaging System, BIS 202D and densitometry analyzed using TINA software.

2.7. Bisulfite-transformation of genomic DNA

Genomic DNA (gDNA) was purified by the Wizard® Genomic DNA Purification Kit (Promega, USA) and was sodium bisulfite modified by the EZ DNA Methylation™ Kit (Zymo Research, CA, USA). Modified DNA was purified and eluted by 1 mM Tris-EDTA buffer (TE) pH-8 and was used for bisulfite sequencing and Methylation Specific PCR (MSP) analyses.

2.8. Bisulfite sequencing of genomic DNA

Genomic DNA was extracted from MBA-15 and DA3 cells and was bisulfite converted. Primers, designed using the MethPrimer software (http://www.urogene.org/methprimer/:), were 5′- GTTTTACGTATGAATATTAAGTTGATG, 3′- AACCTATTCAAATCTCAAACTATTAAA. The analyzed DNA was amplified with Ready Mix Taq PCR reaction mix (Sigma, USA) and PCR amplicon of 246 bp was cloned into pJET1.2 vector (CloneJET, Fermentase). Fifteen separated positive clones were selected for plasmid purification (Promega). Individual clones were picked up and were grown in selective growth medium for about 24 h then clones were sequenced using pJET1.2 forward sequencing primer.

2.9. Methylation-Specific PCR (MSP) assay

Bisulfite modified DNA analyzed by the MSP assay, discriminating between methylated (M) and un-methylated (U) DNA. MSP is based on PCR amplification using two pairs of primers; one pair specific for methylated DNA (M) and the second for unmethylated DNA (U). The amplicon of the analyzed DNA harbored one or more CpG sites. The PCR reaction performed with Ready Mix Taq PCR (Sigma, USA) using the two primer sets (Table 1) that distinguished between the methylated (M)-DNA and un-methylated (UM-DNA) forms. Primers were designed by using the BiSearch web server (http://bisearch.enzim.hu/). Their localization is indicted in Fig. 1.

2.10. Methylated DNA (meDNA) immunopercipitation (meDIP)

Methylated DNA immuno-precipitation (meDIP) assay enables to enrich methylated DNA by chromatin-immunoprecipitation (ChIP) (Weber et al., 2007). meDIP was performed, according to the manufacturer protocol. The ChIP assay was based on formaldehyde fixation of chromatin to DNA–protein complex based on protocol from Upstate biotech (Shur et al., 2006). In brief, cells were fixed and chromatin fraction was isolated and sheared by sonication. ChIP was performed with a specific antibody directed against 5′-methylcytosine modification (Methylated Immunoprecipitation kitP-2019 Epigentek, USA). meDNA was isolated upon reversal of the formaldehyde cross-linking and was used as a template for qPCR amplification (see below). Primers used for the PCR assay were 5′- AATTACTGTGGCACTCTCGGT; 3′- ATTCCATGACACCAGAACCC localized at −334/−194 (GI: 24816888), resulted in an amplicon of 140 bp.

2.11. Quantitative PCR (qPCR)

Amplification of the SVEP1 promoter was performed as previously described (Shur et al., 2007). We used the brilliant SYBR Green QPCR Master Mix kit (Stratagene, USA) in the Stratagene MX 3000 PTM real-time PCR system. Standard curve was based on a diluted known amount of input gDNA used to generate a curve that relates the initial quantity of the specific target in the sample to the threshold cycle (Ct). The standard curve was allowed to quantify the initial template based on the Ct value and used for the quantification of the gDNA in the ChIP samples harvested by the specific transcription factor binding to SVEP1 promoter (see ChIP assay above). A normalizer target (Input) was included in the assay to reduce the effect of sample-to-sample differences. Experiments were performed at least in triplicates for each data point.

2.12. Statistical analysis

Statistical analyses were carried out by a Student's *t*-test, where values of $p<0.05$ are statistically significant for ** p-value ≤ 0.01, *** p-value ≤ 0.001.

3. Results

3.1. In silico analysis of transcription factor binding sites on SVEP1 5′-UTR

The 5′ flanking region of the SVEP1 gene that was predicted as the putative promoter possesses transcription factors (TFs) binding sites and regulatory elements using bioinformatics tools. The *in silico* analysis of the promoter region revealed potential binding sites for transcription factors TFIIB, AP1, NF-κB, ERα and Sp1 (Schematically illustrated in Fig. 1).

3.2. Analysis of SVEP1 mRNA and protein expression

We obtained total RNA from MBA-15 and DA3 cells and we measured SVEP1 and GAPDH mRNA levels by semi-quantitative RT-PCR. As shown in Fig. 2A. B, endogenous SVEP1 mRNA levels were 5-fold lower in DA3 cells than in MBA-15 cells ($p<0.001$).

To investigate whether SVEP1 mRNA levels were correlated with corresponding changes in its protein expression, SVEP1 protein levels were measured in MBA-15 and DA3 cells. Western blotting of whole cell extracts showed a 5-fold ($P<0.001$) reduction of SVEP1 protein levels in DA3 cells compared with the expression in MBA-15 cells (Fig. 2C, D). No changes were noticed in the actin levels.

Table 1
Primers sets SVEP1 promoter methylation analysis by Methylation Specific PCR (MSP). M; Methylated. U; Un-methylated.

Primer set	Forward	Reverse	Location (expected size, bp)
Set #1	M GCGGGAAAGGTGAACGATAA	GGAGAAGAAAACACACACGC	−1371/−1097 (274 bp)
	U GTGGGAAAGGTGAATGATAA	GGAGAAGAAAACACACACAC	
Set #2	M GCGGGAAAGGTGAACGATAA	GATGGATGTGGATACCGAGA	−1371/−1054 (317 bp)
	U GTGGGAAAGGTGAATGATAA	GATGGATGTGGATACCAAGA	

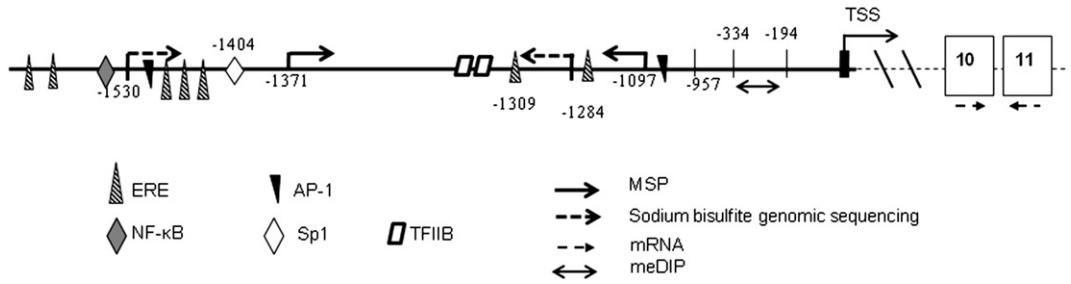

Fig. 1. Schematic diagram of SVEP1 promoter. Bioinformatics analysis of SVEP1 promoter [−1755 bp upstream to TSS]. Primers used for PCR amplification are indicated by arrows, TF binding sites prediction for TFIIB (black trapeze), ERE (light triangles), NF-κB (black diamond), Sp1 (light diamond) and AP1 (inverted triangle).

3.3. Is the expression of SVEP1 message affected by methylation modifications?

We showed that SVEP1 message and protein expression were higher in MBA-15 cells than in DA3 cells (Fig. 2). We assumed that the inhibition of SVEP1 message expression in DA3 cells was associated with acquired promoter methylation on specific CpG sites as was shown in some cases of cancer-related genes (Baylin and Ohm, 2006; Feinberg et al., 2006). To analyze the hypothesis that promoter methylation regulates SVEP1 mRNA expression, cells were treated with 5′aza-dC, an inhibitor of DNA methylation, or TSA, an inhibitor of histone deacetylation. Indeed, after 24 h of treatment, a marked increase ($P<0.01$) in SVEP1 mRNA levels in both cell types was established (Fig. 3A, B). Based on these results, we speculated that DNA methylation involved in the regulation of SVEP1 promoter activity in both cell types.

3.4. Characterization of SVEP1 promoter methylation pattern

Following 5′aza-dC or TSA treatment, an augmentation of SVEP1 message levels was observed in both cell types (Fig. 3). Thus, we further analyzed whether down-regulation of SVEP1 mRNA levels in DA3 cells was associated with changes in promoter methylation status. The promoter methylation content was studied by three assays; sodium-bisulfite genomic DNA sequencing, which enabled direct DNA sequencing (Fig. 4), meDIP (methylated DNA immunoprecipitation) analysis with 5′-methylcytosine antibody (Fig. 5), and amplification of methylated/un-methylated DNA (MSP assay) (Fig. 7).

The bisulfite-modified genomic DNA analyzed a 360 bp fragment that included 7 CpG sites, four ERE-half site (at positions −1472, −1457, −1456, −1309 bp), an AP1 site (at position −1494 bp), and one Sp1 site (at position −1404 bp) located at approximately 500 bp upstream of the TSS (Fig. 4). Series of individual DNA clones retrieved from DA3 cells revealed that specific CpG sites were highly methylated (almost 100% of the analyzed clones) in comparison to MBA-15 cells. Those CpG sites are located in ERE-half binding sites, an AP-1 and Sp1 binding site. In DNA retrieved from MBA-15 cells, a heterogeneous pattern was noted; it was un-methylated for ERE-half site and 50% of the analyzed clones were methylated on AP1 site and Sp1 sites (Fig. 4A). Fig. 4B is a representative direct sequencing of sodium bisulfite-treated DNA, obtained from DA3 and MBA-15 cells. Assessment of methylation is shown in the example, comparing the two sequences of two cytosines (C) was determined and converted to thymines (T) in the DA3 cells, but 5-methylcytosines (shown as C) were unaltered in MBA-15 cells. This resulted in higher methylation in DA3

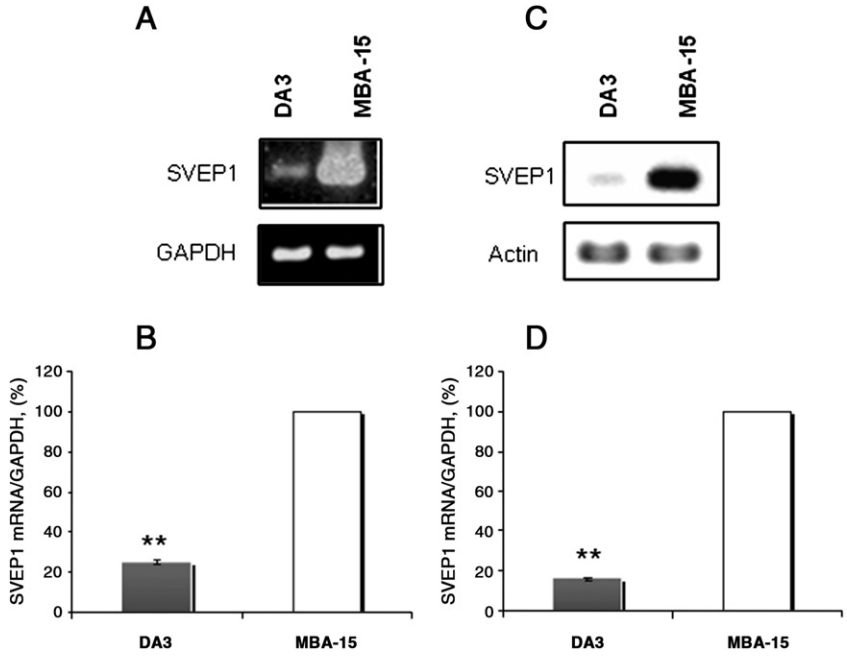

Fig. 2. SVEP1 mRNA and protein expression levels. Cells were harvested, and then total RNA (A) and protein (B) were purified. SVEP1 expression levels were measured by RT-PCR (A, B) and western blot (C, D). Both RT-PCR and western blot densitometry represent data from at least three independent experiments. ** p value<0.001.

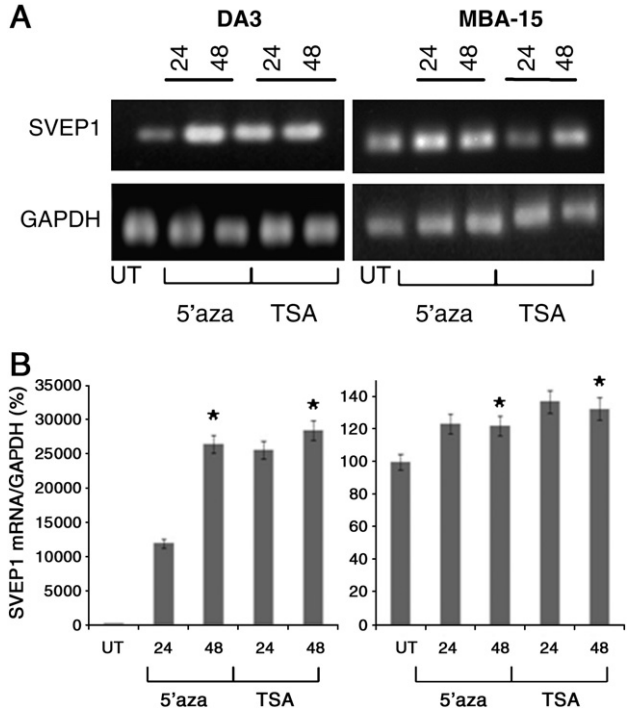

Fig. 3. SVEP1 RNA expression analysis following demethylation treatment. Cells were treated with 2.5 μM 5′-aza-dC or 300 nM TSA for 24 h and 48 h; DA3 and MBA-15 cells (A), Graphical presentation of densitometry analysis of at least 3 independent experiments, * p value<0.023 over untreated cells (B). UT; Untreated.

then in MBA-15 cells (Fig. 4B). The presented sequence illustrated the methylation status of four CpG sites, two of them located in ERE-half site consensus sequence in the SVEP1 promoter. This fragment was methylated in the DA3 cell line but un-methylated in the MBA-15 cell line (Fig. 4B). In summary, the SVEP1 promoter region was highly methylated in DA3 than that in DNA retrieved from MBA-15 cells.

3.5. Quantification of SVEP1 promoter 5′-methylcytosine (5mC) modification

meDIP assay enabled the purification of enriched methylated-DNA by direct immunoprecipitation of the 5′-methylcytosine (5mC) modification related to the CpG sites of the SVEP1 promoter. In DA3 cells, qPCR demonstrated that the SVEP1 promoter had a 10-fold ($P=0.003$) higher methylation levels than in MBA-15 cells (Fig. 5A). It corresponded to the higher DNA-methylation levels identified by the bisulfite-DNA assay (Fig. 4). We quantified meDNA modifications of the promoter in cells after treatment with TNFα and 17βE_2. Following TNFα treatment for 24 h, we detected a 17-fold ($P=0.005$) reduction of 5mC modification levels of SVEP1 promoter DA3 cells, while 17βE_2 treatment caused almost complete elimination of the 5mC modification. In contrast, those treatments caused increased levels of 5mC modification in MBA-15 cells; a 25-fold ($P=0.004$) increase following TNFα- and a 15-fold ($P=0.0045$) up-regulated levels were found following 17βE_2-treatments (Fig. 5B).

3.6. TNFα and 17βE_2 up-regulate ectopic SVEP1 promoter activity

The promoter activity was measured using a cloned construct, pGluc-SVEP1, that was transiently transfected into cells followed by cell activation with TNFα (Fig. 6A) or 17βE_2 (Fig. 6B) for 4 h or 24 h. Luciferase activity served as a read out for the promoter activity normalized to β-gal, which indicates transfection efficiency. The promoter activation measured by luciferase levels in treated cells with 10 ng/ml TNFα for 4 h demonstrated a 1.5-fold ($P<0.005$) increase, compared to untreated cells. In cells treated with 50 ng/ml and 100 ng/ml TNFα, a 2-fold ($P<0.009$) and ($P<0.002$) increase, respectively, were measured, while after 24 h, the elevation in luciferase activity was a 1.5-fold ($P<0.001$) (Fig. 6A). In cells modulated with 1×10^{-8} or 2×10^{-8} M 17βE_2 for 4 h, a 2-fold ($P<0.0008$) elevation was assessed and treated cells with 4×10^{-8} M 17βE_2 demonstrated a 1.5-fold ($P<0.006$) higher luciferase levels. Following 24 h, an elevation of a 1.5-fold ($P<0.001$) was measured for the three concentrations of 17βE_2-treated cells (Fig. 6B). In cells transfected with pGLuc-basic plasmid, no luciferase activity was detected following TNFα or 17βE_2 treatment, indicating the specificity of SVEP1 promoter regulation (data not shown).

3.7. Characterization of the SVEP1 promoter methylation status by MSP

MSP was carried out by using two different sets of primers that were designed to distinguish between methylated (M) and unmethylated (UM) DNA (Fig. 1, Material and methods and Fig. 7). Specifically, a fragment of 274-bp (which is complimentary to the region that was analyzed by the bisulfite genomic DNA sequencing assay) was analyzed. The SVEP1 promoter region had 3-fold (** $P<0.0005$) higher methylation in DA3 cells, as indicated by the ration M/UM. An inverse pattern was noted in MBA-15 cells; 6-fold ($P<0.0005$) higher UM/M (Fig. 7A, B). TNFα or 17βE_2 treatments significantly reduced the M-DNA fraction in DA3 cells (** $P<0.0005$, both) (Fig. 6C). In contrast, a 7-fold ($P=0.0005$) increase of UM-DNA was measured following 17βE_2 treatment but TNFα stimulation did not change the UM-DNA levels (Fig. 7D). An inverse phenomenon was observed for MBA-15 cells. Both TNFα and 17βE_2 treatments increased the M-DNA levels; a 30-fold and a 15-fold ($P=0.0005$) elevation, respectively (Fig. 7E), and a 2.5-fold ($P=0.0005$) decrease of UM-DNA levels (Fig. 7F).

4. Discussion

Epigenetic changes are heritable modifications that are not accompanied by changes in the DNA sequence. Such alterations are responsible for the regulation of biological processes, including cell differentiation, imprinting and silencing of genomic domains (such as inactivation of X chromosome). Epigenetic abnormalities might represent the earliest changes in malignant cell transformation (Lande-Diner and Cedar, 2005; Baylin and Ohm, 2006; Feinberg et al., 2006). DNA methylation is an epigenetic mechanism essential for normal development, influencing cellular events such as transcription, genomic imprinting and genome stability (Jaenisch, 1997). The methylation takes place at the C5 position of cytosine residues and plays an important role in the regulation of gene expression in vertebrates (Eden and Cedar, 1994). It is an essential biological process for mammalian development (Li et al., 1992). DNA methyltransferases (Dnmts) are the group of enzymes responsible for the establishment and maintenance of genomic DNA methylation (Kafri et al., 1992; Kafri et al., 1993).

The study focused on a cell adhesion protein (CAM), SVEP1, that is expressed by pre-osteoblastic (Shur et al., 2006) cells and breast cancer cell lines (Shur et al., 2007) and participates in cellular adhesion processes (Shur et al., 2006). The bone niche is composed of cells and extracellular matrix (ECM), thus creating a crosstalk that regulates interactions of the cells with their ECM. We used a model system composed of mammary adenocarcinoma and pre-osteoblastic cells that physiologically create the stroma in which the metastatic cells are embedded. We previously characterized SVEP1 expression in a series of human breast cancer (BC) cell lines (Shur et al., 2007), and currently, we are presenting its expression regulation in murine

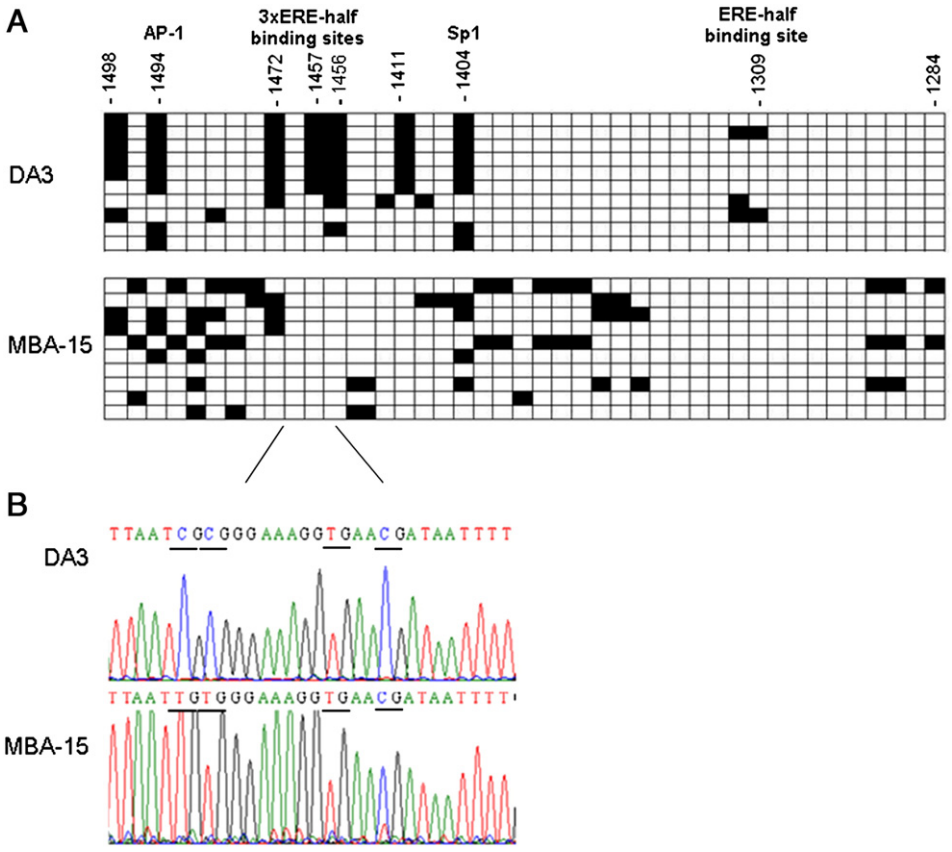

Fig. 4. Assessment of SVEP1 promoter methylation using the sodium-bisulfite sequencing method. Genomic DNA was obtained from MBA-15 and DA3 cells followed by sodium bisulfite-modification as described in Materials and methods. The modified DNA was subjected to direct sequencing. The analyzed promoter region is schematically marked by numbers, which refer the cytosines location in relation to the TSS; methylated CpG sites (black squares), and un-methylated CpG sites (white squares), AP1, Sp1 and ERE-half sites. Results present sequence analysis of 10 independent clones (out of 15 clones) from each cell line (A). Examples of DNA sequencing chromatograms are shown. CpG sites are underlined. Two cytosines (C) are determined and converted to thymines (T) in the DA3 cells, but 5-methylcytosines (shown as C) remained unaltered in MBA-15 cells, meaning that the SVEP1 promoter is more methylated in DA3, but less methylated in MBA-15, cells (B).

mammary adenocarcinoma, DA3 cells compared with pre-osteoblastic MBA-15 cells.

In this study, we identified dynamic changes of methylation at the SVEP1 promoter that may be involved in differential expression of SVEP1 mRNA and protein, and its regulation at the promoter level. 17βE_2 and TNFα are two major factors that act in the bone microenvironment. Both factors play a role in bone metabolism, as well as in carcinogenesis and cancer progression (Barrett-Connor, 1992; Compston, 1992; Oursler et al., 1994; Balkwill and Coussens, 2004). Earlier, we showed that SVEP1 is regulated by 17βE_2 at the promoter level, resulting in up-regulation of its mRNA and protein levels (Shur et al., 2007). Other studies showed the role of both 17βE_2 and TNFα in the regulation of adhesion molecules expression (Aziz and Wakefield, 1996; Segers et al., 2006). The rationale for our study of SVEP1 promoter methylation is based on the fact that expression regulation of selectin ligands that are mediating entry into inflamed sites, was regulated by DNA methylation (Syrbe et al., 2004). In addition, tissue-specific expression of various adhesion molecules is epigenetically regulated. For example, E-selectin is an adhesion molecule transiently and specifically expressed on endothelial cells upon stimulation with cytokines. Its promoter was found to be hypo-methylated in cultured endothelial cells in comparison with non-expressing HeLa cells. Methylation is therefore likely to play a role in blocking E-selectin expression in non-endothelial cells (Smith et al., 1993).

By applying bioinformatics analysis, we identified that SVEP1 putative promoter includes several ERE half-sites interspersed among Sp1, AP1, TFIIB and NF-κB sites. We detected low expression levels of SVEP1 mRNA and protein in DA3 cells compared with MBA-15

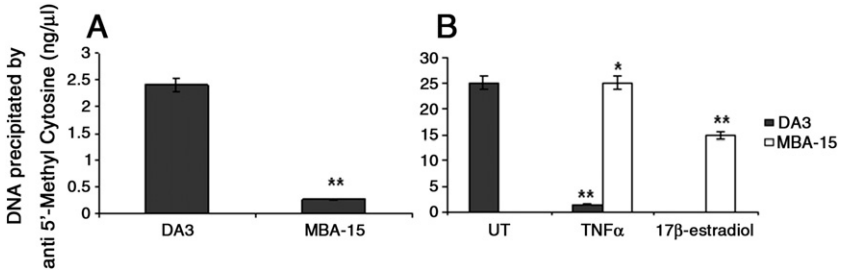

Fig. 5. Quantitative PCR analysis of 5′-methylcytosine modification on SVEP1 promoter analyzed by ChIP assay. Methylated DNA immunoprecipitation (meDIP) analysis of 5′-methylcytosine modification of SVEP1 promoter analyzed by ChIP assay. meDIP analysis of DNA retrieved from DA3 or MBA-15 cells. ** p value < 0.005 *versus* DA3 cells (A), (B) DA3 (gray bars) or MBA-15 (white bars) cells treated with 50 ng/ml TNFα or 10^{-8} M 17βE_2. Bar histograms represent qPCR analysis of amplified promoter DNA normalized by input values (mean ± SD, n = 3 independent experiments performed in triplicates for each data point. ** p value < 0.005 *versus* untreated cells). UT; Untreated.

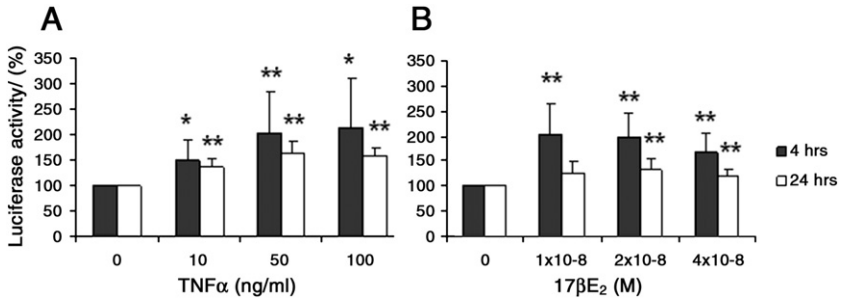

Fig. 6. Luciferase measurements of ectopic SVEP1 promoter activity. Cells were transfected with pGLuc-SVEP1 and were modulated with TNFα for 4 h (black bars) or 24 h (white bars) (A) or with 17βE$_2$ for 4 (black bars) or 24 h (white bars) (B). Promoter activation is expressed as luciferase values normalized for β-galactosidase levels. A value of 100% was given to the basal promoter activity elicited by the pGLuc-SVEP1 construct in the absence of any treatment. Results are mean ± S.D. of 3 independent experiments, performed in duplicates. * P<0.01, ** P<0.005.

cells. We evaluated whether this expression pattern may be caused by methylation alterations at the SVEP1 promoter. The rationale for these analyses was the fact that aberrant promoter methylation is associated with a loss of gene function that can provide a growth advantage to neoplastic cells, as mutations do. For example, BRCA1 gene expression silencing by promoter hyper-methylation occurs in primary breast and ovarian carcinomas (Esteller et al., 2000). Silencing of transcription by DNA methylation can also be achieved by an indirect mechanism at the level of transcription initiation, causing the removal of the transcriptional machinery from active templates (Kass et al., 1997). Therefore, alteration in methylation levels seems to initiate the process that leads ultimately to reduced transcription levels. DNA methylation, leading to transcriptional silencing, is a well-characterized phenomenon in cancer initiation and progression (Struhl, 1998; Cameron et al., 1999; Murzina et al., 1999; Wong et al., 1999). Methylation at specific CpG sites in promoters can determine the transcriptional activity of genes by altering the binding potential of specific transcription factors (Bell and Felsenfeld, 2000; Hark et al., 2000; Bird, 2002). It was shown that such alterations in cancer can lead to the disruption of protein expression and function.

Demethylation reagent, 5′-aza-dC, and the histone deacetylase inhibitor, TSA, have been shown to increase access of TF to promoter sites, resulting in up-regulated gene transcription (Yang et al., 2000; Yang et al., 2001). To study whether methylated CpGs alterations at the SVEP1 promoter are responsible for its reduced expression in DA3 cells, cells were treated with 5′-aza-dC or TSA. In DA3 cells, increased SVEP1 mRNA levels were observed following pharmacological treatments with 5′-aza-dC or TSA, which also resulted in elevated mRNA levels in MBA-15 cells. To confirm that these changes occurred at the SVEP1 putative promoter, we mapped the CpGs sites

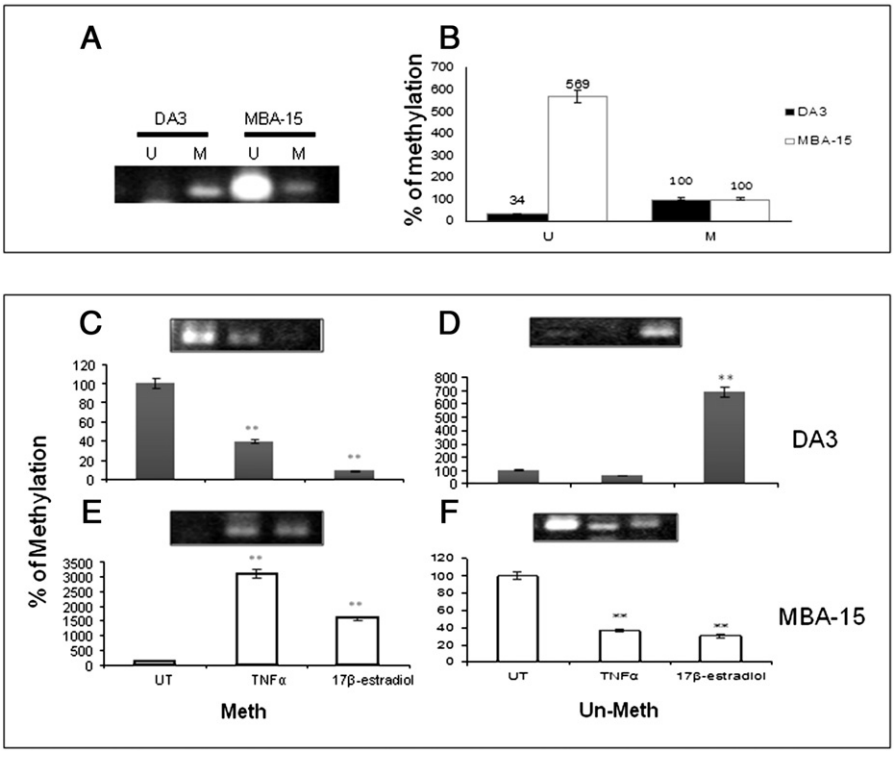

Fig. 7. Methylation of SVEP1 promoter analyzed by methylation specific-PCR (MSP). (A–F) MSP analysis of SVEP1 promoter compared between DA3 and MBA-15 cells. U-un-methylated, M-methylated DNA. (A) SVEP1 promoter methylation in DA3 *versus* MBA-15 cells (B) Graphical presentation of comparative densitometry analysis of SVEP1 promoter amplification in DA3 (gray bars) and MBA-15 (white bars) cells. 100% methylation illustrates quantification of methylated SVEP1 promoter amplification in the untreated cells. (C–F) TNFα and 17βE$_2$ regulate SVEP1 promoter methylation levels. DA3 (C, D gray bars) and MBA-15 (E, F white bars) cells were treated with 50 ng/ml TNFα or 10^{-8} M 17βE$_2$ for 24 h. Bar histograms represent qPCR analysis of amplified promoter DNA normalized by input values (mean ± SD, n = 3 independent experiments performed in triplicates for each data point. ** p value<0.005 *versus* untreated cells). UT; Untreated.

using bisulfite-treated DNA sequencing to detect methylated cytosines on DNA harvested from both cells. The analyzed region includes the ERE-half sites, AP1 and Sp1 site which exhibited higher methylation in DNA obtained from DA3 cells as compared with MBA-15 cells. In addition, the MSP and meDIP assay using anti-5′-methylcytosine antibody showed that the SVEP1 promoter is hypermethylated in DA3 cells as compared to MBA-15 cells. $17\beta E_2$ and TNFα treatments augmented SVEP1 ectopic promoter activity measured by luciferase assay and the mRNA levels in both cells. This observation may be explained by induced demethylation of the promoter in DA3 cells. In MBA-15 cells we noted a partial methylation at these TF binding sites may provide an explanation for increased mRNA levels in response to both regulators. We speculated that a more complex mechanism regulates chromatin conformation and accessibility to SVEP1 promoter through transcription factors binding, DNA methylation and histone acetylation. In the current study, we focused on the analysis of SVEP1 promoter methylation modification. We concluded that SVEP1 gene expression is regulated by specific transcription factors, and that their binding is affected by alterations of CpGs methylation. The relationship between tumor cells and the stroma plays a critical role in cancer growth and metastasis. The stroma contains locally originated or bone-marrow derived cells, which promote the growth and spreading of the cancer cells. Given that SVEP1 protein has been identified to play a role in cellular adhesion processes in the bone niche (Shur et al., 2006), our data sheds light on SVEP1 expression regulation and its potential implications for being a target in the bone-microenvironment.

In summary, the study identifies SVEP1 promoter methylation as part in the transcriptional regulation of the gene. We noted that pharmacological treatments that inhibit DNA methylation resulted with an increased SVEP1 mRNA expression in the DA3 mammary adenocarcinoma cells. In addition, specific transcription factors binding sites were highly methylated in the tumor cells, in comparison to the pre-osteblastic cell line. Using MSP and meDIP assays, we demonstrated that the SVEP1 promoter is highly methylated in the mammary adenocarcinoma cells, in comparison to the pre-osteoblastic cells. These results are complementary and might explain the relatively low expression of SVEP1 mRNA and protein in the DA3 adenocarcinoma cells. The study expands our understanding of SVEP1 gene expression regulation at the promoter level.

Disclosure statement

None of the authors have any actual or potential conflict of interest to disclose

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.gene.2011.09.012.

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