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SVEP1 promoter regulation by methylation of CpG sites

C. Glait-Santar, D. Benayahu *

Department of Cell and Developmental Biology, Sackler Faculty of Medicine, Tel-Aviv University, Tel-Aviv 69978, Israel

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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.

* Corresponding author. Tel.: +972 3 6406187; fax: +972 3 6407432.
E-mail addresses: chenglai@post.tau.ac.il (C. Glait-Santar), dafnab@post.tau.ac.il (D. Benayahu).

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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