Journal of Steroid Biochemistry & Molecular Biology 130 (2012) 36–44

Contents lists available at SciVerse ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Regulation of SVEP1 gene expression by 17β-estradiol and TNFα in pre-osteoblastic and mammary adenocarcinoma cells

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ARTICLE INFO

Article history:
Received 6 October 2011
Received in revised form
21 December 2011
Accepted 22 December 2011

Keywords:
SVEP1
Cell adhesion molecule
Pre-osteoblastic cells
Mammary adenocarcinoma
Estrogen
TNFα

ABSTRACT

Breast cancer is one of several tumors, including prostate, thyroid and kidney, which display a remarkable predilection for metastasis to bone. The preference to metastasize to bone by tumor cells relies on specific interactions among tumor cells, bone marrow microenvironment and bone cells. Osteomimicry is postulated to enable the survival of tumor cells in the bone tissue. Using gene profiling array and RT-PCR we demonstrated the message expression of few bone matrix proteins in mammary adenocarcinoma cells as well as that of cell adhesion molecules (CAMs). A CAM molecule, named SVEP1, was previously shown to be expressed in osteoblastic cells both *in vivo* and *in vitro* mediating cell adhesion in the bone-marrow niches. Both estradiol ($17\beta E_2$) and TNFα regulate the expression of adhesion molecules and act in bone-cancer-crosstalk. We focused on differential regulation of SVEP1 gene comparing pre-osteoblastic MBA-15 and mammary adenocarcinoma DA3 cells. $17\beta E_2$ and TNFα activated SVEP1 promoter, increased its message and protein levels in both cell types. Using chromatin immunoprecipitation assay, we quantified SVEP1 promoter occupancy by transcription factors; TFIIB, ERα, NF-κB, Sp1 and their binding was also regulated by both factors. By comparing pre-osteoblastic with mammary adenocarcinoma cells, the study expands our understanding of SVEP1 gene expression regulation and it sheds light on its involvement in bone-cancer-microenvironment interactions.

1. Introduction

Cell adhesion molecules (CAMs) mediate cell–cell and cell–extracellular matrix interactions. CAM-mediated interactions initiate the activation of signaling cascades that lead to various cell responses [1,2]. Our group has characterized a CAM molecule named SVEP1, which is a multi-domain protein that possesses multiple complement binding motifs (CCP), an EGF and EGF-like Ca^{2+}-binding domains [3]. CCP domains are present in various proteins that are part of the coagulation and complement cascade, as well as in selectin proteins [4]. 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) [3]. SVEP1 is also expressed by skeletal muscle-activated satellite cells [5]. SVEP1 protein expression was analyzed in a series of human and mouse breast cancer (BC) cell lines [6,52]. SVEP1 messenger RNA was shown to be regulated by estrogen levels *in vivo* and *in vitro* [3,6]. MBA-15 pre-osteoblastic cells are estrogen-receptor (ER) positive cells [7,8] that express SVEP1 gene which is regulated in these cells by estrogen ($17\beta E_2$) [3].

$17\beta E_2$ acts as a stimulator of bone formation *in vivo* and regulates proliferation of osteoblasts [9–11]. This hormone also plays a role in the etiology, progression and proliferation capacity of breast cancer [12–14]. Breast cancer metastases are known to exhibit great affinity to lymph, lung, bone and brain tissues [15]. $17\beta E_2$ modulates the expression of adhesion molecules, as has been elucidated in endothelial cells of the cardiovascular system [16,17]. In these cells, $17\beta E_2$ modulates E-selectin and ICAM-1 expression *in vitro* [18] and P-selectin, ICAM-1 and VCAM-1 expression *in vivo* [19].

Stroma affects cellular activities in various tissues; thus, it is important to study communication between stroma and the tumor cells, specifically in the bone microenvironment [20]. Additionally, inflammatory cytokines are secreted by the stroma and pre-osteoblastic cells, supporting cancer progression in a specific microenvironment [21]. 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*) [22]. Hence, the current research focuses on the possibility that SVEP1 is regulated by TNFα and estrogen. Earlier we have shown that SVEP1 gene is expressed by

Abbreviations: CAM, cell adhesion molecule; ECM, extracellular matrix; TF, tissue factor; ChIP, chromatin immunoprecipitation; qPCR, quantitative PCR.

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0960-0760/$ – see front matter.
doi:10.1016/j.jsbmb.2011.12.015

pre-osteoblastic MBA-15 cells [3] and by human and mouse breast cancer (BC) cell lines [6,52]. In addition, SVEP1 promoter activity is regulated by estrogen and also by its methylation [52].

Breast cancers have the ability to invade and grow as metastases in bone [23], and the microenvironment plays a critical role in regulating the cross talk between breast cancer cells and their metastatic potential. Therefore, we characterized SVEP1 expression levels in a model system composed of pre-osteoblastic cell line, MBA-15 cells and the murine adenocarcinoma cell line, DA3, in order to mimic the potential cross-talk that exists in the bone microenvironment.

The expression of selected osteoblastic- and cell adhesion-related markers was compared between the two cell lines by gene expression array. We found specific adhesion molecules that were highly expressed by the metastatic cell line, while some, including SVEP1, were significantly highly expressed by the pre-osteoblastic cells. SVEP1 gene expression regulation was studied following cell modulation with $17\beta E_2$ and TNFα, both are regulators that act in the bone-cancer-microenvironment. Analysis of SVEP1 mRNA levels in cells grown at basal conditions revealed detectable expression levels of this mRNA in MBA-15 cells but not in DA3 cells. We also demonstrated that $17\beta E_2$ and TNFα augmented the mRNA expression in both of the cell types analyzed. We studied the mechanism of SVEP1 promoter activation by the two modulators, $17\beta E_2$ and TNFα. We used chromatin immunoprecipitation (ChIP) assay to study occupancy of SVEP1-promoter by specific transcription factors (TF), TFIIB, ERα, NF-κB and Sp1 and the alteration in their binding resulted by $17\beta E_2$ and TNFα. The results of the current study expand our understanding of SVEP1 gene expression regulation at the promoter, gene and protein levels. This will shed light on the role of SVEP1 molecule in bone-niche metastatic processes.

2. Materials and methods

2.1. Cell culture

Two cell lines were used in the study: MBA-15, a pre-osteoblastic stromal cell line [24] and DA3, a metastatic murine mammary adenocarcinoma cell line [25]. SVEP1 expression levels were characterized in a model system composed of these specific cell lines in order to mimic the potential cross-talk that exists in the bone microenvironment.

Cells were maintained in growth medium (termed "standard 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 (Beth haEmek, Israel) for 24 h prior to treatment with TNFα (10, 50, 100 ng/ml) (PeproTech Asia) or with 17β-Estradiol ($17\beta E_2$) 1×10^{-8}, 2×10^{-8}, 4×10^{-8} M (Sigma, USA) for 4 or 24 h.

2.2. RNA extraction

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).

2.3. Gene expression analysis

Polymerase chain reactions (PCRs) were performed using specific primers for ERα, Biglycan, Bone-sialo protein (BSP), osteopontin, osteocalcin and SVEP1 (Table 1) 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.

Table 1
Oligonucleotide primers for gene expression analysis by PCR.

Gene	Primer sequence	Expected product size
Biglycan	5′-TGATGAGGAGGCTTCAGGTT 3′-ACTTTGCGGATACGGTTGTC	413 bp
ERα	5′-CCTCCCGCCTTCTACAGGT 3′-CACACGGCACAGTAGCGAG	128 bp
Collα1	5′-TCTCCACTCTTCTAGTTCCT 3′-TTGGGTCATTTCCACATGC	269 bp
Osteoglycin	5′ TGCAACAGGCAATTCTGAAG 3′-TGGTGGTACAGCATCAATGTC	249 bp
GAPDH	5′ TGGAAGGGCTCATGACCAC 3′-ACCTGGTCCTCAGTGTAGC	331 bp
SVEP1	5′ AACCGCCTGTCATAGATTGG 3′-TGTGTACCACACCACCGTTT	178 bp

2.4. RNA hybridization, gene expression array experiment and data analysis

Affymetrix GeneChip® Mouse Gene 1.0 ST arrays were used for gene expression analysis according to the instruction manual, as described in the Affymetrix website (Affymetrix, Santa Clara, CA, USA; 103; http://www.affymetrix.com). We used a total of 4 chips in 2 biological replicates. Microarray analysis was performed on CEL files using Partek® Genomics Suite TM, version 6.5 Copyright© 2010 (http://www.partek.com). Data were normalized and summarized with the robust multi-average method [26], followed by analysis of variance (ANOVA). Cluster analysis of the arrays was obtained by Partek® Genomics Suite TM. Genes of interest that were differentially expressed in the different treatments or cell lines were obtained. Gene expression data were sorted using cutoffs of $p < 0.05$ and fold-difference of 2. We used Gene ontology tools (ToppGene [27]; WebGestalt [28]) for analyses of biological and functional groups. The data is available at: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE34529).

2.5. Bioinformatic analysis

Identification of the putative promoter at the 5′-flanking region of murine SVEP1 gene was applied with 60 kb of genomic sequence upstream the transcription start site (TSS) (GI: 24816888). The *in silico* analysis applied Promoter 2.0 Prediction Server (http://www.cbs.dtu.dk/services/Promoter) for promoter definition. In addition, 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).

2.6. Transcription factors (TF) binding quantified by chromatin immunoprecipitation (ChIP) assay

The ChIP assay is based on formaldehyde fixation of chromatin to DNA–protein complex based on protocol from Upstate biotech [3]. In brief, cells were fixed and chromatin fraction was isolated and sheared by sonication. ChIP was performed with specific antibodies: anti-Estrogen receptor (ERα, SRA-1010, Stressgen, Canada), anti-NF-κB p65 (F-6), anti-TFIIB

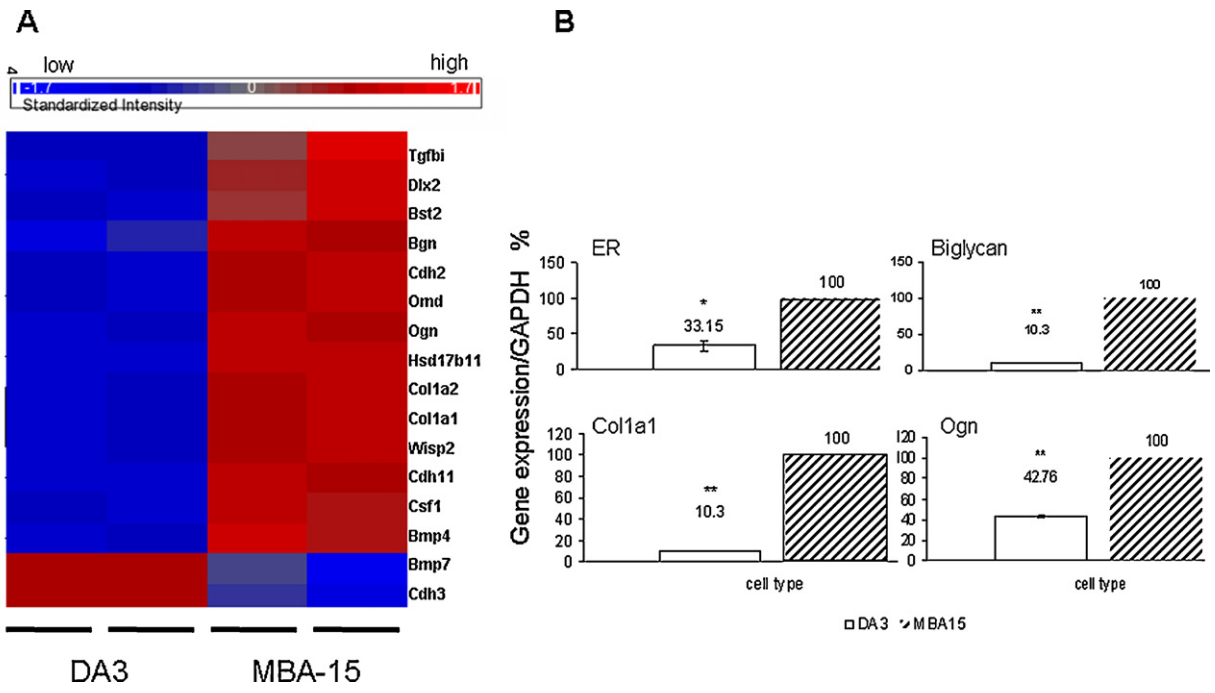


Fig. 1. Osteoblastic genes expression characterization in stromal cell line and mammary adenocarcinoma cells. (A) Unsupervised hierarchical clustering of osteoblastic-related genes. Osteoblastic genes were clustered according to cell type (DA3 and MBA-15, in biological duplicates). Hierarchical clustering analysis was performed (with FDR correction). Clustering was obtained by Partek Genomics Suite, as described earlier. Gene symbols are listed in the right panel. Red colored genes are up-regulated and blue colored genes are down-regulated. Gene symbols are listed in the right panel. Red colored genes are up-regulated and blue colored genes are down-regulated. (B) RT-PCR validation of the expression levels of a few representative osteoblast-related genes. RNA retrieved from DA3 and MBA-15 cells was analyzed by RT-PCR for expression of osteoblastic genes. The expression level for each gene in DA3 cells is calculated as percent of expression compared to the MBA-15 sample (100%) (mean ± SD, $n = 3$, $^{*}p$-value < 0.01, $^{**}p$-value < 0.005); Estrogen-receptor alpha (ERα); Biglycan; Col1a1; Osteoglycin (Ogn). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

(C-18) and-anti Sp1 (PEP2) (Santa Cruz, USA). DNA was isolated upon reversal of the formaldehyde cross-linking and was used as a template for qPCR amplification (see below) with primers for SVEP1 promoter; F-AATTACTGT GGCACTCTCGGT; R-ATTCCATGACACCAGAACCC (GI:24816888) resulted with an amplicon of 140 bp.

2.7. Quantitative PCR (qPCR)

Amplification of SVEP1 promoter was performed as previously described [6]. We used the brilliant SYBR Green QPCR Master Mix kit (Stratagene, USA) in the Stratagene MX 3000 PTM real-time PCR system. Standard curve is based on 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 allow to quantify the initial template based on the Ct value and used for quantification of the gDNA in the ChIP samples harvested by 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.

The expression of selected genes was analyzed using a custom TaqMan low-density array platform (Micro Fluidic Cards; Applied Biosystems, Foster City, CA) in DA3 and MBA-15 cells, previously described in Glait-Santar and Benayahu [52]. The reaction set up consisted of cDNA template made up to 100 μl with deionized water and 95 μl of TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA; Cat no.: 4304437). The mix above was loaded into the array. The samples run as duplicates were only loaded into 4 ports of the array. Thermal cycling conditions were described in Glait-Santar, in preparation. Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) was performed using the ABI Prism7900 HT Sequence Detection System (Applied Biosystems). Three control genes were included in the study: TBP, PPIA and Taf1. Micro Fluidic Cards were analyzed with RQ documents and the RQ Manager Software for automated data analysis. Expression values for target genes were normalized to the concentration of TBP, PPIA and Taf1, that showed the least variation among reference genes in the cell lines. TaqMan® Gene Expression Assays used were: Vcam1: Mm01320970_m1, SVEP1: Mm00465696_m1, Pcdh7: Mm00479579_m1 and L1cam: Mm00493049_m1 (Applied Biosystems, Foster City, CA).

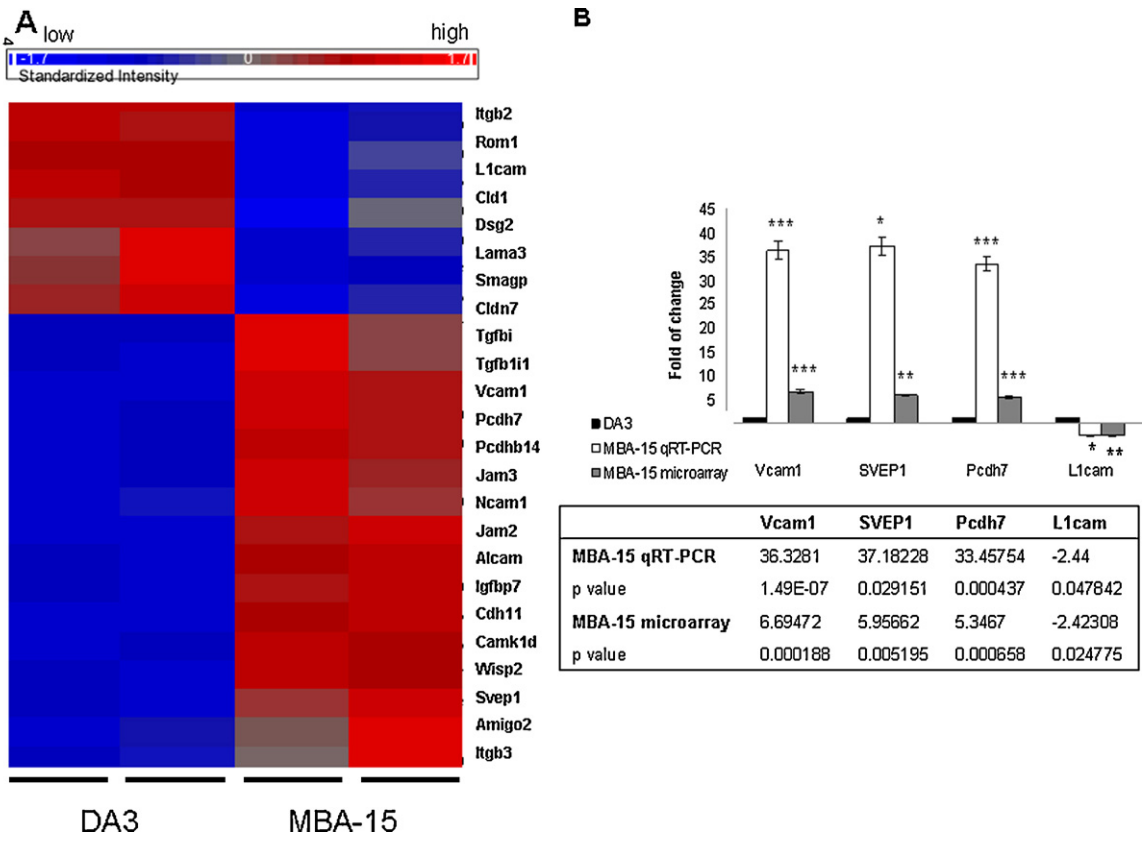
2.8. Protein analysis

2.8.1. 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 primary antibody to SVEP1. The first antibody was incubated for 3 h, followed with 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) [3].

2.8.2. Immunofluorescence (IF) microscopy analysis

Cells were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 20 min, washed with PBS and permeabilized with



	Vcam1	SVEP1	Pcdh7	L1cam
MBA-15 qRT-PCR	36.3281	37.18228	33.45754	-2.44
p value	1.49E-07	0.029151	0.000437	0.047842
MBA-15 microarray	6.69472	5.95662	5.3467	-2.42308
p value	0.000188	0.005195	0.000658	0.024775

Fig. 2. Adhesion related genes expression characterization in stromal cell line and mammary adenocarcinoma cells. (A) Unsupervised hierarchical clustering of cell adhesion molecules. Gene list correlated with either functions of 24 cell adhesion-related genes were clustered according to cell type (DA3 and MBA-15, in biological duplicates). Hierarchical clustering analysis was performed (with FDR correction). Clustering was obtained by Partek Genomics Suite, as described earlier. Gene symbols are listed in the right panel. Red colored genes are up-regulated and blue colored genes are down-regulated. (B) qRT-PCR validation of Vcam1, SVEP1, the expression levels of a few representative genes from the indicated groups, in DA3 (black bars), co-cultured DA3 TLDA (light-gray bars), DA3 microarray (dark-gray bars) cells. Each histogram represents the average of 2 different experiments, each was conducted in triplicates. $^{**}p$-value < 0.01, $^{***}p$-value < 0.001. p-values are supplemented below. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

0.5% Triton in PBS for 5 min. The slides were stained with polyclonal, rabbit anti-SVEP1 (1:100) generated against two synthetic peptides related to 212–219AA, 876–874AA sequence specific to SVEP1 [3]. Slides were stained with 1:500 species-specific secondary antibody, conjugated to Cy-2, designed for simultaneous multiple staining (Jackson Immunoresearch Laboratories, USA) for 1 h at room temperature. Then, slides were washed with PBS and mounted with Vectashield® mounting medium including 4′,6-diamidino-2-phenylindole (DAPI), dimethyl sulfoxide (Vector Laboratories, CA, USA) and observed with a confocal microscope (Leica TCF SP2).

2.9. Statistical analysis

Statistical analyses were carried out by Student's t-test, where values of $p < 0.05$ are statistically significant for $^{*}p$-value ≤ 0.01, $^{**}p$-value ≤ 0.005, $^{***}p$-value ≤ 0.001.

3. Results

The study gains novel insights into the differential expression regulation of SVEP1 gene at the promoter, message and protein levels. In particular, we compared between mammary adenocarcinoma DA3 and pre-osteoblastic MBA-15 cell lines and their response to modulations with TNFα and $17\beta E_2$.

3.1. Comparing the expression of osteoblastic genes in MBA-15- with DA3 cell lines

Gene expression array was performed to compare expression profile of osteoblastic-related genes between MBA-15- and DA3 cells. Fig. 1 illustrates a cluster analysis that is consisted of 16 genes, typically expressed by osteoblastic cells (Fig. 1A, Table S2) that were selected using a fold change >1.5 or <−1.5 and a p-value < 0.01 with FDR correction.

Gene expression array results were validated using RT-PCR analysis. MBA-15- and DA3 cells were studied for the expression of four typical osteoblastic-associated markers: collagen type 1 [alpha 1 (col1a1)], osteoglycin (Ogn), biglycan and estrogen receptor alpha (ERα) (Fig. 1B). Biglycan (C) and col1a1 (D) expression were reduced by 10-fold (p-value <0.0001, <0.0025) in the DA3- than in MBA-15 cells. Osteoglycin (E) expression was approximately 2-fold reduced in DA3- than MBA-15 cell (p-value < 0.003). The osteoblast-related genes that are expressed by MBA-15 and DA3 cells are important in the extracellular matrix formation.

3.2. Characterization of adhesion molecules gene expression in MBA-15- compared with DA3 cell lines

Based on the gene expression array analysis, a second cluster which is consist 24 genes that participate in cell-adhesion pathways (Fig. 2A, Table S1). The genes were selected using a fold change >1.5 or <−1.5 and a p-value < 0.01 with FDR correction.

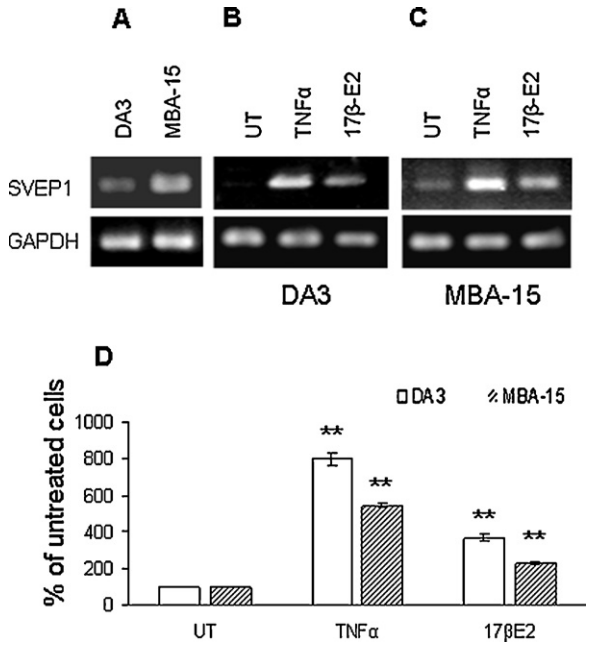


Fig. 3. SVEP1 mRNA expression in DA3 and MBA-15 cells. RT-PCR analysis of SVEP1 mRNA retrieved from (A) DA3 and MBA-15 cells treated with 50 ng/ml TNFα or 10^{-8} M 17βE_2 for 24 h; DA3 (B) and MBA-15 (C). (D) The RNA samples of untreated cells were compared to treated samples and normalized to the GAPDH for each data point. Results are presented as mean ± SD, obtained from three different experiments, each performed in triplicates for each data point. A representative experiment of RT-PCR is shown in the upper part of the figure (**p-value < 0.005).

Interestingly, some of the adhesion-associated genes were highly expressed by DA3 cells, while other, including SVEP1 gene, were highly expressed by the MBA-15 cells (Fig. 2A, Table S1).

Gene expression array results were validated by using qRT-PCR (Fig. 2B). It was found that genes which mediate biological adhesion: Vcam1, SVEP1, Pcdh7, and L1cam are differentially expressed in MBA-15 over the DA3 cells. mRNA expression levels of Vcam1, SVEP1 and Pcdh7 were higher in MBA-15 than those in DA3 cells, respectively (36-fold, p-value = 1.49E−07; 37-fold, p-value = 0.029151 and 33-fold, p-value = 0.000437). While those of L1cam gene were reduced (2.44-fold; p-value = 0.047842) (Fig. 2B).

We focused on SVEP1 molecule that was characterized as a cell surface protein that plays a role in cell adhesion processes. This molecule was previously cloned and its expression was identified *in vivo* in skeletal tissues of bone, periosteum, and bone marrow, and in cultured bone marrow mesenchymal stromal cells (MSC) [3,6,52].

3.3. SVEP1 mRNA expression regulation by TNFα and 17βE_2

The microarray and the qRT-PCR assays demonstrated significant increased SVEP1 gene expression levels (5.9-fold, p-value = 0.005195 and 37-fold; p-value = 0.029151) in MBA-15- compared with DA3 cells (Fig. 2). We studied the SVEP1 expression regulation pattern by RT-PCR assay. SVEP1 expression levels in MBA-15 cells were higher than those that were expressed by DA3 cells (Fig. 3A). Since TNFα and 17βE_2 modulates the expression of adhesion molecules, MBA-15 and DA3 cells were stimulated with 50 ng/ml TNFα or 10^{-8} M 17βE_2. 24 h later, we observed a prominent augmentation of SVEP1 message levels in both cell types (Fig. 3B and C) in response to TNFα or 17βE_2 stimulation, while both factors did not affect significantly the mRNA levels (Fig. S2). Fig. 3D represents quantitation of SVEP1 mRNA levels by a densitometry histogram of at least three independent representative experiments. These results corroborated previous results by us [52].

3.4. Transcription factors (TFs) binding occupancy is regulated by TNFα and 17βE_2

TF occupancy of endogenous SVEP1 promoter was compared between untreated MBA-15 and DA3 cells or when cells were challenged with 50 ng/ml TNFα or 10^{-8} M 17βE_2 for 4 or 24 h (Fig. 4C–J). Antibodies against TFIIB, ERα, NF-κB and Sp1 were used in a ChIP assay and the precipitated DNA was used as a template for quantitative PCR. PCR amplification of the input genomic DNA was performed for the promoter region. Fig. 4A and B are representative amplification and dissociation curve plots of samples that analyzed SVEP1 promoter amplification. The amplified products were analyzed by dissociation curves, and the fluorescence peak corresponding to the PCR product (centered at 86 °C) was distinguishable from the peak that is resulted due to primer-dimer amplified from the negative control sample (centered at 76 °C) (Fig. 4A).

TFIIB is required for transcription initiation by RNA polymerase II. The results illustrate that DA3 cells (Fig. 4D) are highly responsive to TNFα and 17βE_2, as was demonstrated by increased binding of TFIIB to SVEP1 promoter relatively to MBA-15 cells (Fig. 4C). TNFα stimulation of MBA-15 cells caused a 2-fold (p-value = 0.0005) increase in TFIIB-occupancy over untreated cells after 24 h (Fig. 4C). TNFα stimulation of DA3 cells caused a 6-fold (p-value = 0.005) higher binding after 4 h and a 15-fold (p-value = 0.005) increase after 24 h (Fig. 4D). Following 17βE_2 treatment, MBA-15 cells presented a 15-fold (p-value = 0.0005) increase of TFIIB binding to the promoter after 4 h and a 4-fold (p-value = 0.0001) elevated binding after 24 h (Fig. 4C). In DA3 cells, augmentation of TFIIB binding to the promoter was a 6-fold (p-values = 0.0004) higher following 4 h and a 25-fold (p-value = 0.0002) increased binding was measured after 24 h compared to untreated cells (Fig. 4D).

The ChIP assay demonstrated a 2.5-fold (p-value = 0.0002) increased binding of ERα in MBA-15 cells caused by 4 h of TNFα treatment, whereas following 24 h, a 8-fold (p-value = 0.0001) increase was measured (Fig. 4E). In DA3 cells, 4 h of TNFα treatment resulted in a 3-fold (p-value = 0.004) increased ERα binding to SVEP1 promoter and a 2-fold (p-value = 0.005) elevation was measured after 24 h (Fig. 4F). 17βE_2 stimulation of MBA-15 cells increased ERα binding by a 17-fold (p-value = 0.0001) after 4 h and a 3-fold (p-value = 0.0001) after 24 h (Fig. 4E). In DA3 cells, 17βE_2 stimulation for 4 h resulted in a 5.5-fold (p-value = 0.005) increase in ERα binding, while after 24 h no change in binding level was measured (Fig. 4F).

NF-κB is a rapid-acting TF expressed by cells in its inactive state and do not require new protein synthesis to be activated. TNFα-stimulation of MBA-15 cells caused a 9-fold (p-value = 0.0005) increased- and a 2-fold (p-value = 0.00012) increased NF-κB binding after 4 and 24 h, respectively, over untreated cells (Fig. 4G). In DA3 cells, TNFα stimulation caused a 3-fold (p-value = 0.0001) increase and a 26-fold (p-value = 0.0001) increase in NF-κB binding to the promoter after 4 and 24 h, respectively (Fig. 4H). In MBA-15 cells stimulated with 17βE_2, a 4-fold (p-value = 0.0002) increased binding was measured after 4 h, whereas after 24 h the binding was a 1.5-fold (p-value = 0.0005) elevated over untreated cells (Fig. 4G). Interestingly, an inverse effect was noted after 4 h of 17βE_2 treatment in DA3 cells resulted in a 8.8-fold (p-value = 0.0001) reduction of NF-κB binding while no change from control level was noted after 24 h (Fig. 4H).

In MBA-15 cells, Sp1 binding to SVEP1 promoter was a 4-fold (p-value = 0.0005) elevated after 4 h administration of TNFα while no change over untreated cells was noted after 24 h. Following 17βE_2-stimulation of MBA-15 cells, no change in Sp1 binding was noted after 4 h and a slight (1.6-fold) increase was measured after 24 h as compared with untreated cells (Fig. 4I). DA3 cells presented a 2-fold (p-value = 0.0003) elevation of Sp1 binding after 24 h of TNFα administration (Fig. 4J) while these cells treated with 17βE_2

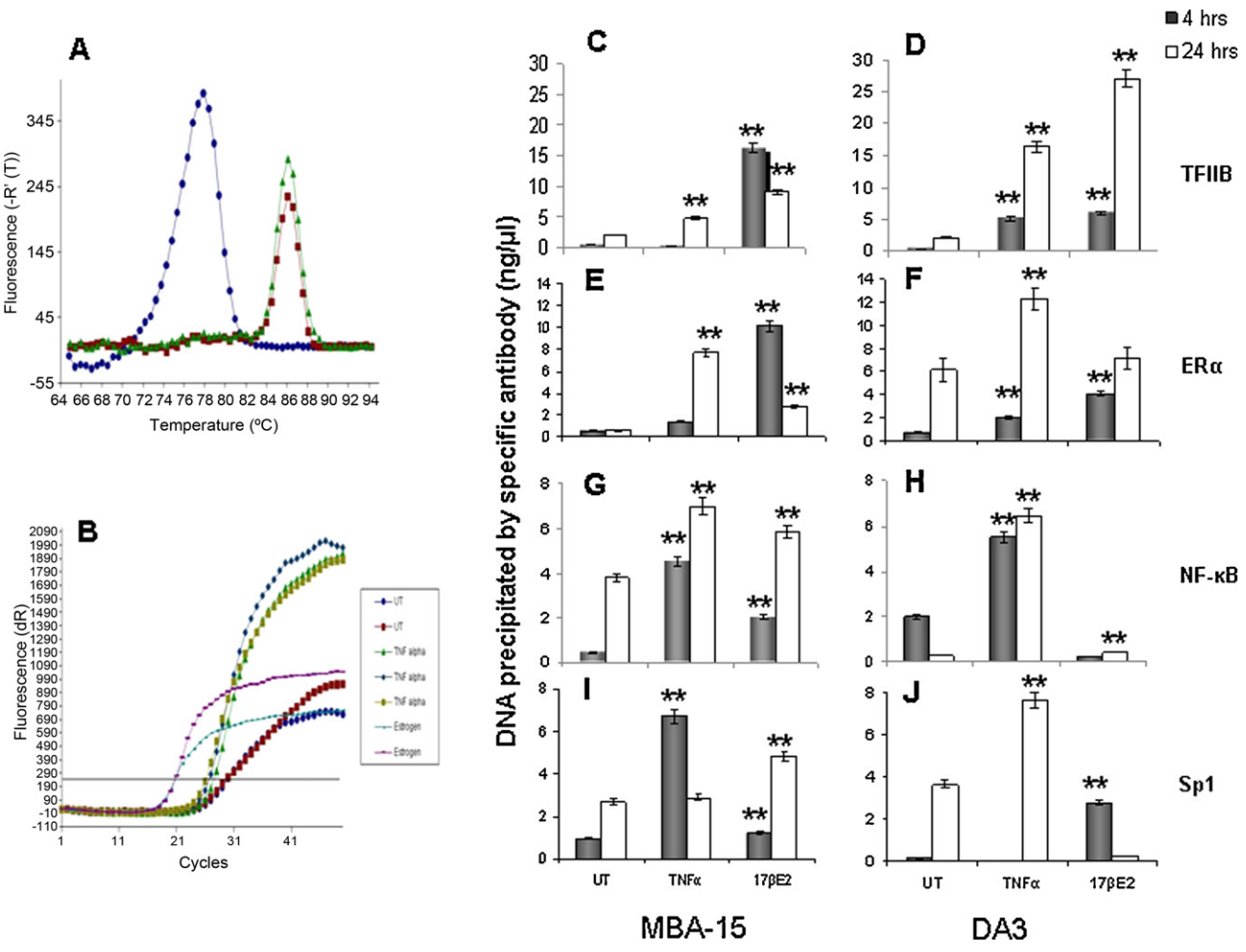


Fig. 4. Quantitative kinetic PCR analysis. Quantitative PCR (A) dissociation and (B) amplification curve plots of PCR of selected analyzed samples. *X* axis represents the number of cycles; *Y* axis represents the fluorescence intensity. (C–J) ChIP analysis followed by Quantitative PCR analysis of specific transcription factors (TFs) binding to endogenous SVEP1 promoter. MBA-15 and DA3 cells were treated with 50 ng/ml TNFα or 10^{-8} M 17βE_2 for 4 (gray bars) or 24 h (white bars). Graphical presentation of kinetic PCR quantitative analysis binding of TFIIB (C, D), ERα (E, F), NF-κB (G, H) and Sp1 (I, J) to amplified SVEP1 promoter DNA are summarized in bar histograms. qPCR results of amplified promoter are summarized as mean ± SD of 3 independent experiments performed in triplicates for each data point (**p-value < 0.005).

for 4 h led to a 19-fold (p-value = 0.0003) increased Sp1 binding. An inverse phenomenon was noted after 24 h of 17βE_2-treatment in DA3 cells, resulted in a 13-fold (p-value = 0.0001) reduction of Sp1 occupancy in the promoter as compared to untreated cells (Fig. 4J).

3.5. SVEP1 protein expression

The regulation of SVEP1 promoter (Fig. 4) and its message (Fig. 3) by TNFα and 17βE_2 was also studied at the protein level (Figs. 5 and 6). Complementary to the mRNA analysis, western blot analysis demonstrated increased SVEP1 protein levels expressed by DA3 and MBA-15 cells following treatment with TNFα or 17βE_2 (Fig. 5A and B). Densitometry of at least two experiments revealed a 8-fold (p-value < 0.001) and a 4-fold (p-value < 0.001) increase of SVEP1 protein levels caused by TNFα and 17βE_2 treatments, respectively, over the untreated cells in DA3 cells. In MBA-15 cells, TNFα and 17βE_2 treatments caused almost 4-fold increase (p-value < 0.001) of SVEP1 protein over untreated cells (Fig. 5C). No changes were noticed in the actin levels.

The expression pattern of SVEP1 protein was analyzed by immunofluoresence assay in MBA-15 and DA3 cells. The immunostaining localized SVEP1 protein to the cytoplasm and cell membranes. The low expression of SVEP1 protein was increased in both cells types; DA3 (Fig. 6A) MBA-15 (Fig. 6B) cells in response to treatment with 50 ng/ml TNFα or 10^{-8} M 17βE_2 for 24 h.

4. Discussion

Breast cancer cells preferentially metastasize to bone, leading to the formation of primarily osteolytic lesions [29,30]. The bone composed mainly from inorganic and organic structures. The inorganic composition of bone is formed from carbonated hydroxyapatite [31] with lower crystallinity. The organic part of matrix is mainly composed of type I collagen and is also composed of various growth factors, the functions of which are not fully known. Factors present include glycosaminoglycans, osteocalcin, osteonectin, bone-sialo protein, osteopontin and cell-induced adhesion molecules. Osteoglycin (Ogn) encodes a protein which induces ectopic bone formation in conjunction with transforming growth factor beta (TGFβ). Ogn and TGFβ are stored within the bone matrix and released when bone is resorbed. Both can serve as natural inhibitors of osteoclast activity by inhibiting osteoclast formation [32]. Collagen, type I, alpha 1 (Col1a1) encodes the major component of type I collagen, the fibrillar collagen found in most connective tissues. Biglycan (Bgn) is a small leucine-rich proteoglycan binds to various extracellular matrix components and has a role in mineralization [33]. It is expressed relatively early during

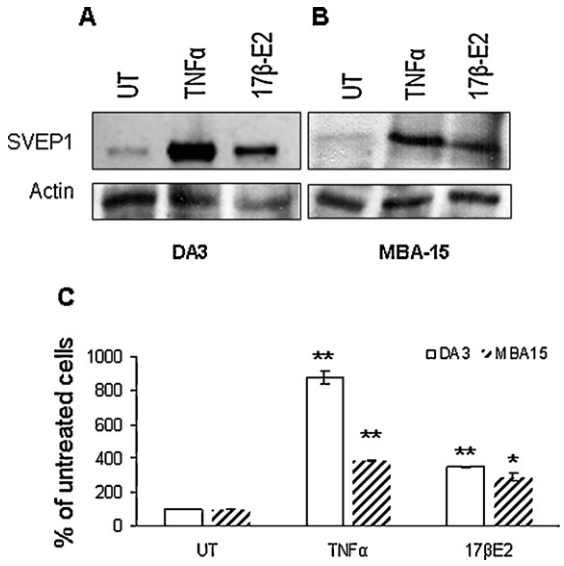


Fig. 5. Western blot analysis of SVEP1 protein immunoprecipitated from DA3 and MBA-15 cells. DA3 (A) and MBA-15 (B) cells treated with 50 ng/ml TNFα or 10^{-8} M $17\beta E_2$ for 24 h; cells were lysed in the presence of protease inhibitors, as indicated in Section 2. Equal amounts of protein (100 μg) were separated by 10% SDS–PAGE, transferred onto nitrocellulose filters, and blotted with anti-SVEP1. (C) The samples of untreated cells were compared to treated samples and normalized to the actin for each data point. Results are presented as mean ± SD, obtained from at least three different experiments. A representative experiment of Western blot is shown in the upper part of the figure (**p-value < 0.002).

matrix synthesis and acts to regulate organization of the extracellular matrix [34].

We compared the expression of osteogenic and cell adhesion-related markers between the two cell lines by DNA microarray analysis. We found specific adhesion molecules that were highly expressed by the metastatic cell line, while some, including SVEP1, were significantly highly expressed by the pre-osteoblastic cells. We evaluated mRNA levels of four genes related to osteogenesis in mammary adenocarcinoma and pre-osteoblastic cell lines.

The osteomimicry hypothesis postulated that cancer cells acquire "bone cell-like" properties that allow them to arrest, survive and grow out as metastases in the bone microenvironment [35]. This hypothesis was in part based on prostate cancer cells, which resemble the breast cancer cells in their affinity to the bone niche [35,36]. Accordingly, we found that DA3 cells express genes that are responsible for maintenance of the bone-organic component.

The bone-marrow microenvironment is composed of the extracellular matrix (ECM), stromal mesenchymal cells and osteogenic cells. The ECM plays a key role in the cells microenvironment, responsible for directing cell fate and maintaining tissue specificity. The ECM acts as a regulator that affects the crosstalk between osteogenic/stroma cells and tumor cells in the bone microenvironment [37,38]. We characterize the differential expression of selected osteogenic genes on the molecular level, focusing on SVEP1 protein. SVEP1, a CAM protein, is expressed by pre-osteoblastic cells and mediates cellular adhesion [3]. SVEP1 expression was shown in a series of human breast cancer (BC) cell lines [6]. This study aimed to gain insight into SVEP1 gene expression and its regulation in cells of the bone microenvironment. Hence, the model system studied relies on pre-osteoblastic and mammary adenocarcinoma cells, which harboring homing ability *in vivo* to bone niches. In addition, there are two factors that act in the bone microenvironment, $17\beta E_2$ and TNFα. Both factors play a role in bone metabolism as well as in carcinogenesis and cancer progression [12–14,39]. Earlier we showed that SVEP1 is regulated by estrogen at the promoter level resulting in up-regulation of both mRNA and protein levels [6]. Other studies have shown the role of both $17\beta E_2$ and TNFα in adhesion molecule expression regulation [18,22].

In the current study, we elaborate on SVEP1 gene expression in $17\beta E_2$- and TNFα-treated cells by comparing pre-osteoblastic MBA-15 to mammary adenocarcinoma DA3 cells. Using bioinformatics analysis, we identified several ERE half-sites interspersed among TFIIB, Sp1, AP1 and NF-κB consensus sequences. A cloned SVEP1 promoter fragment was transiently transfected into cells and activity levels were measured. Treatment of the cells with TNFα and $17\beta E_2$ resulted in increased promoter activity. Activated ER is known by its binding to estrogen response elements (EREs) in promoters (the classical genomic pathway) of estrogen-regulated genes [40,41]. In responsive-genes lacking ERE sites, $17\beta E_2$ acts in an alternate pathway mediated the ER binding to non-palindromic ERE half-sites [42,43]. ERE-half sites contain one inverted repeat

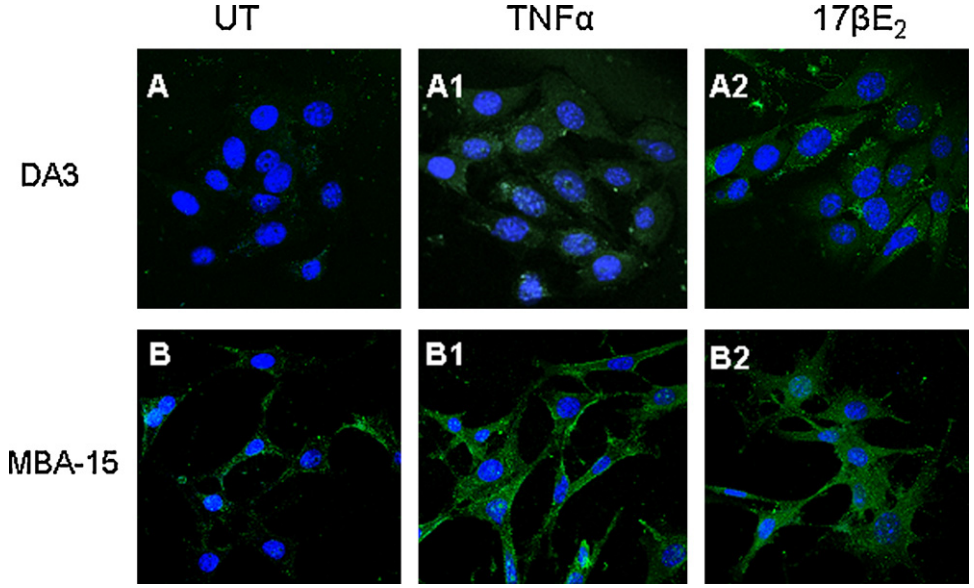


Fig. 6. SVEP1 protein expression in DA3 and MBA-15 cells. Immunofluorecence of SVEP1 protein sub-cellular localization (A, B). DA3 (A) and MBA-15 (B) cells were stained with anti-SVEP1 antibody were visualized by confocal microscopy for SVEP1 protein sub-cellular localization.

that binds ER, generating a heteromeric complex that is often associated with other non-ERE elements such as AP1 and Sp1 [44] or with other proteins such as NF-κB [41,45,46]. One such protein complex, composed of activated ER, co-activator RAC3 and a member of the NF-κB family, affects gene expression at target promoters [47]. ER associates with AP1, forming a complex that plays a role in ERE-independent genomic activities. It was shown that estrogen stimulates such interactions between ER, Fos and Jun, creating a complex that binds at the AP1-site and stimulates ovalbumin [48] or human insulin growth factor 1 expression [49], but represses the choline acetyltransferase gene [50]. Dimeric ER interacts with ERE-half site when stabilized in a heterotrimeric complex with Sp1, which binds to GC-rich promoter sites. ERα has been shown to bind Sp1 consensus sites in close proximity to a single ERE half-site in promoters of several estrogen-regulated genes, such as heat shock protein 27 [51]. Thus, ERα/Sp1 and ERα/AP1 complexes participate in gene activation pathway in which the receptor does not interact directly with DNA, as demonstrated in gene regulation studies in various cell types, including breast carcinoma [46]. We speculate that ER activates SVEP1 gene transcription either directly by binding to ERE sites on promoters or indirectly *via* formation of complex with other transcription factors.

We *in silico* predicted that SVEP1 promoter contains several ERE half-sites in close proximity to Sp1 and AP1 binding sites. Hence, we quantified transcription factor interactions with SVEP1 promoter by comparing promoter occupancy in TNFα- and $17\beta E_2$-treated MBA-15 and DA3 cells using ChIP assay. SVEP1 promoter occupancy by TFIIB in both cell types was significantly increased after $17\beta E_2$- and TNFα treatment, suggesting a mechanism of SVEP1 gene transcription initiation conducted by RNA polymerase II. We speculate that increased binding of ERα to SVEP1 promoter following 4 h of $17\beta E_2$ stimulation in MBA-15 and DA3 cells illustrate a direct activation mechanism of the promoter by ER. $17\beta E_2$ increased NF-κB and Sp1 binding to the promoter in MBA-15 cells, displaying the indirect activation of SVEP1 promoter conducted by ER. The suggested mechanism was based also on quantifying significantly increased mRNA and protein levels following $17\beta E_2$ and TNFα stimulation in both cell lines.

5. Conclusions

Our results strengthen the osteomimicry hypothesis by demonstrating the differential expression of selected osteo-markers genes between the two cells. We conclude that TNFα and $17\beta E_2$ treatments modulate SVEP1 gene expression by affecting the binding of specific transcription factors to SVEP1 promoter. This study presents evidence for hormonal-dependent transcriptional regulation of SVEP1 promoter. The results of the current study expand our understanding of SVEP1 gene expression regulation. Further investigation is required to shed light on SVEP1 expression regulation in the bone-cancer microenvironment. The results of the study may improve our understanding of the SVEP1 gene in mammary derived cells compared to pre-osteoblastic cells that represent the stroma component of the bone marrow.

Conflict of interest

The authors declare that they have no competing interests.

Acknowledgements

The study was partially funded by a grant from EU-FP7, Excell project 214706 and internal funds from Tel Aviv University. This work was performed in partial fulfillment of the requirements for a Ph.D. degree by Chen Glait-Santar in Sackler Faculty of Medicine, Tel Aviv University and is supported in part by the Crown Family Foundation Doctoral Fellowships, USA. The authors thank Dr. Metsada Pasmanik-Chor from the bioinformatics unit of the life sciences faculty of Tel Aviv University for essential and critical assistance.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jsbmb.2011.12.015.

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