Biochemical and Biophysical Research Communications 426 (2012) 26–32

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

Global identification of genes regulated by estrogen signaling and demethylation in MCF-7 breast cancer cells

Milica Putnik [a,*], Chunyan Zhao [a], Jan-Åke Gustafsson [a,b], Karin Dahlman-Wright [a]

[a] Department of Biosciences and Nutrition, Novum, Karolinska Institutet, Huddinge S-14183, Sweden
[b] Department of Biology and Biochemistry, Science & Engineering Research Center Bldg, University of Houston, Houston, TX 77204-5056, USA

ARTICLE INFO

Article history:
Received 25 July 2012
Available online 10 August 2012

Keywords:
Estrogen
Estrogen receptor
5-Aza-2′-deoxycytidine
DNA methylation
Breast cancer
Microarray

ABSTRACT

Estrogen signaling and epigenetic modifications, in particular DNA methylation, are involved in regulation of gene expression in breast cancers. Here we investigated a potential regulatory cross-talk between these two pathways by identifying their common target genes and exploring underlying molecular mechanisms in human MCF-7 breast cancer cells. Gene expression profiling revealed that the expression of approximately 140 genes was influenced by both 17β-estradiol (E2) and a demethylating agent 5-aza-2′-deoxycytidine (DAC). Gene ontology (GO) analysis suggests that these genes are involved in intracellular signaling cascades, regulation of cell proliferation and apoptosis. Based on previously reported association with breast cancer, estrogen signaling and/or DNA methylation, CpG island prediction and GO analysis, we selected six genes (BTG3, FHL2, PMAIP1, BTG2, CDKN1A and TGFB2) for further analysis. Tamoxifen reverses the effect of E2 on the expression of all selected genes, suggesting that they are direct targets of estrogen receptor. Furthermore, DAC treatment reactivates the expression of all selected genes in a dose-dependent manner. Promoter CpG island methylation status analysis revealed that only the promoters of BTG3 and FHL2 genes are methylated, with DAC inducing demethylation, suggesting DNA methylation directs repression of these genes in MCF-7 cells. In a further analysis of the potential interplay between estrogen signaling and DNA methylation, E2 treatment showed no effect on the methylation status of these promoters. Additionally, we show that the ERα recruitment occurs at the FHL2 promoter in an E2- and DAC-independent fashion. In conclusion, we identified a set of genes regulated by both estrogen signaling and DNA methylation. However, our data does not support a direct molecular interplay of mediators of estrogen and epigenetic signaling at promoters of regulated genes.

1. Introduction

It is well established that aberrations in both estrogen signaling and epigenetic modifications can lead to alterations of development and cell growth, causing various malignancies, including breast cancer [1,2]. 17β-Estradiol (E2), the predominant endogenous estrogen and ER ligand in humans, regulates proliferation and development of breast epithelial cells that can ultimately lead to development of breast cancer [3]. Many studies have explored the mechanisms by which E2 regulates gene expression in breast cancer cells [4]. More recently the importance of epigenetic modifications for regulation of gene expression has been highlighted in breast cancer [5]. In this study we focus on the potential interplay of the epigenetic mark – DNA methylation and estrogen signaling in breast cancer cells.

Estrogens exert their biological functions via estrogen signaling pathways, primarily mediated by estrogen receptors (ERs), ERα and ERβ. ERs belong to the nuclear receptor superfamily of ligand-regulated transcription factors [6]. ERs, when ligand-activated, form dimers that bind to estrogen responsive elements (EREs) and bring coactivators or corepressors to the ER complex, to regulate gene expression. Estrogen also modulates gene expression through a process referred to as transcription factor cross-talk, in which ERs interact with other transcription factors. By non-genomic mechanisms, estrogens bind to ERs localized in the membrane compartment, which leads to activation of signal transduction pathways in the cytoplasm [7]. In addition to these ligand-induced transcriptional activities of ERs, ligand-independent pathways to activate ERs have been described. Growth factor signaling or stimulation of other signaling pathways leads to activation of kinases that can activate ERs by phosphorylation in the absence of ligand [8].

Breast cancer is the most common cancer in women [9]. E2 production and ERα activity are used as targets in hormonal therapy of breast cancer. ERα-expressing (ER+) cancers can be treated

* Corresponding author. Address: Department of Biosciences and Nutrition, Karolinska Institutet, Hälsovägen 7, S-141 83 Huddinge, Sweden.
E-mail addresses: milica.putnik@ki.se (M. Putnik), chunyan.zhao@ki.se (C. Zhao), jan-ake.gustafsson@ki.se (J.K. Gustafsson), karin.dahlman-wright@ki.se (K. Dahlman-Wright).

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.08.007

with aromatase inhibitors to block estrogen production, or with anti-estrogens, such as tamoxifen, which block ER activity. Tamoxifen is a partial ERα antagonist, competing with E2 for ERα binding and reversing its effect on gene expression [10]. Tamoxifen treatment is suitable for cancers in both pre- and postmenopausal women and has good effects particularly in patients with decreased levels of estrogen. However, 30% of women treated with tamoxifen for 5 years will have recurrent disease within 15 years [11]. As many women eventually develop resistance to current therapies, novel therapeutic alternatives are needed to improve the disease outcome for ER+ breast cancer patients. Regulation of epigenetic modifications might open up new avenues for breast cancer treatment.

DNA methylation is a key epigenetic mechanism for the silencing of many genes, including those involved in cell cycle regulation, DNA repair and apoptosis. Methylation of promoter regions is one mechanism for gene inactivation during cancer development, as demonstrated for several tumor suppressor genes [12]. DNA methylation is a chemical modification of DNA – the addition of a methyl group to the 5 position of the cytosine pyrimidine ring. It can be inherited through cell division, as well as added or removed during lifetime, without changing the original DNA sequence. DNA methylation occurs almost exclusively at cytosines located within CpG dinucleotides. Clusters of CpG dinucleotides, known as CpG islands, are associated with the promoter regions of many genes.

Epigenetic abnormalities are associated with cancer, genetic disorders, autoimmune diseases and aging [13]. Unlike genetic damage, epigenetic changes can sometimes be reversed. This creates a potential for the development of therapeutic strategies based on the regulation of the epigenetic status of cells. Several inhibitors of enzymes controlling epigenetic modifications, specifically DNA methyltransferases and histone deacetylases have shown promising anti-tumorigenic effects for some malignancies [14]. 5-aza-2′-deoxycytidine (DAC), a demethylating agent used in this study, is a cytosine analogue and prevents methylation of CpG islands by incorporating into DNA. DAC is used in treatment of myelodysplastic syndromes and it is undergoing investigation for use in treatment of tumors, together with other drugs targeting DNA methylation [15].

Both estrogen signaling and DNA methylation are shown to be involved in cell cycle control, by regulating proliferative and anti-proliferative genes [16,17]. Imbalance in these signaling pathways could lead to malignant transformation. Hence, the number of studies investigating the cross-talk between these two pathways is increasing. Although there is evidence for overlapping gene regulation by estrogen signaling and DNA methylation [18–23], their common target genes on a genome-wide scale have not yet been reported.

In this study, we for the first time report the comparison of global gene expression profiles regulated by estrogen signaling and demethylation in MCF-7 cells. Furthermore, for a subset of genes identified as regulated by both estrogen signaling and demethylation, we explore potential mechanisms of cross-talk.

2. Materials and methods

2.1. Cell culture and RNA extraction

MCF-7 cells, which express endogenous ERα, but not ERβ, were seeded in plates and cultured in phenol red-free DMEM supplemented with 2.5% dextran-coated charcoal-treated heat-inactivated FBS (Hyclone), either with or without 1 μM DAC (Sigma Aldrich), for 72 h. Culture medium with or without treatment was changed every 24 h. The DAC-treated cells were subsequently treated with vehicle (ethanol), and the DAC non-treated cells were subsequently treated with either vehicle or 10 nM E2 for 6 h. Each treatment was done in four replicates. Total RNA was extracted using RNeasy Mini Kit (Qiagen).

2.2. Microarray analysis

The GeneChip® HT Human Genome U133 Array Plate (Affymetrix) that contains probes for 33,000 well-characterized genes and UniGene clusters was used for microarray analysis. Target synthesis and hybridizations were performed by the Bioinformatics and Expression Analysis core facility at the Karolinska Institute, according to standard protocols. The probe logarithmic intensity error method was employed for statistical analysis of significantly changed genes. We applied a filter of $p < 0.001$ for significantly modulated gene expression and at least a 1.5-fold change in mean differential expression.

Functional annotation and gene ontology analysis was performed using the Database for Annotation, Visualization and Integrated Discovery [24,25]. CpG island analysis was performed using the MethPrimer online software [26].

2.3. Real-time PCR

cDNA synthesis was performed with 2 μg of total RNA, using the TaqMan® Reverse Transcription reagents from Applied Biosystems. mRNA levels were assayed using the SYBRGreen PCR master mix (Applied Biosystems), with specific forward and reverse primers. mRNA levels of the 36B4 (RPLP0) housekeeping gene were used for normalization [27]. The sequences of primer pairs are listed in Supplementary Table 1.

2.4. DNA methylation analysis

The cells were cultured according to the same protocol as used for RNA extraction. Total DNA was extracted using the Invisorb® Spin Cell Mini Kit (Invitek/Westburg). Bisulfite modification was performed with 500 ng of total DNA, using the EZ DNA Methylation Kit™ (Zymo Research) following the manufacturer's instructions. Primers for PCR and genomic sequencing were designed by using the MethPrimer online software, and their sequences are listed in Supplementary Table 2. All PCR reactions were subjected to two rounds of amplifications. The amplification products of the first PCR round were confirmed by electrophoresis and extracted using QIAquick Gel Extraction Kit (Qiagen). 3 μl of the products of the first PCR reaction were subjected to re-amplifications using the same primer pairs. Small aliquots of the final products were confirmed by electrophoresis, and the rest was purified by QIAquick PCR Purification Kit (Qiagen) and sequenced directly by an outside vendor (Macrogen DNA Sequencing Service).

2.5. Chromatin immunoprecipitation (ChIP)

The cells were seeded in 150 mm dishes and grown in the presence or absence of 1 μM DAC for 72 h. Culture medium with or without treatment was changed every 24 h. Cells were then treated with either vehicle or 10 nM E2 for 45 min and ChIP was performed as previously described [28]. The ERα HC-20 and IgG rabbit antibodies (Santa Cruz) were used to perform ChIP for ERα and IgG, respectively. Immunoprecipitated DNA was amplified by real-time PCR using SYBRGreen PCR master mix. The sequences of primer pairs are listed in Supplementary Table 3. All real-time PCR experiments were performed on a 7500 Fast real-time PCR System (Applied Biosystems).

3. Results

3.1. Identification of genes regulated by estrogen signaling and demethylation

To identify genes regulated by estrogen signaling and demethylation, we compared the effects of E2 and DAC on global gene expression profiles in MCF-7 cells. 802 genes were identified as up-regulated by E2, while 851 genes were identified as down-regulated by E2. 1017 genes were identified as up-regulated by DAC, suggesting that demethylation is involved in their regulation. To identify possible common targets, we have compared the DAC up-regulated genes with E2-regulated genes. 88 annotated genes are found to be up-regulated by both E2 and DAC (referred to as E↑D↑ genes in the following text; Fig. 1A), suggesting that E2 has a demethylation-like effect on the regulation of these genes. 49 annotated genes are found to be down-regulated by E2 and up-regulated by DAC (E↓D↑ genes; Fig. 1A), suggesting that E2 has a hypermethylation-like effect on the regulation of these genes. The original microarray data have been deposited in NCBI's Gene Expression Omnibus [29] and are accessible through GEO Series accession number GSE36683 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=bbmvvigqgmaogbq&acc=GSE36683). Genes that were regulated more than 2-fold by either treatment are listed in Table 1. The complete list of regulated genes is provided in Supplementary Table 4.

Gene Ontology (GO) analysis for the 137 genes regulated by both DAC and E2 reveals that the most overrepresented groups were 'regulation of cell motion', 'regulation of cell migration', 'intracellular signaling cascade', 'regulation of cell proliferation' and 'regulation of apoptosis' (Table 2).

3.2. Selection of genes for detailed molecular characterization of the cross-talk between estrogen signaling and DNA methylation

To explore the details of the potential cross-talk between estrogen signaling and DNA methylation at the molecular level, we selected a group a genes for detailed molecular characterization. Based on the following criteria: (1) belonging to the five most overrepresented GO groups, (2) reported association with breast cancer, estrogen signaling and/or DNA methylation, and (3) presence of CpG islands in promoter regions (2000 bp upstream of transcriptional start site), six genes have been selected for further analysis, three from the E↑D↑ group (BTG3, FHL2 and PMAIP1) and three from the E↓D↑ group (BTG2, CDKN1A and TGFB2).

Regulation of gene expression by E2 and DAC for the selected genes was confirmed for the same RNA used for the gene expression profiling analysis using real-time PCR (Fig. 1B), as well as with RNA from two additional independently performed replicate experiments (data not shown). Tamoxifen reversed the effect of E2 after both 6 and 24 h-treatment, confirming that the regulation of mRNA expression of the selected genes by estrogen signaling is mediated via ER (Fig. 2). The dose dependent effect of DAC on mRNA expression of the selected genes is shown in Fig. 3.

3.3. DAC, but not E2, demethylates BTG3 and FHL2 promoters

We assessed the methylation status of the CpG islands in promoters of all selected genes. The promoters of the BTG3 and FHL2 genes were shown to be methylated with DAC inducing demethylation (Fig. 4A). As these genes belong to the E↑D↑ group, we tested the hypothesis that estrogen can have a demethylation-like effect

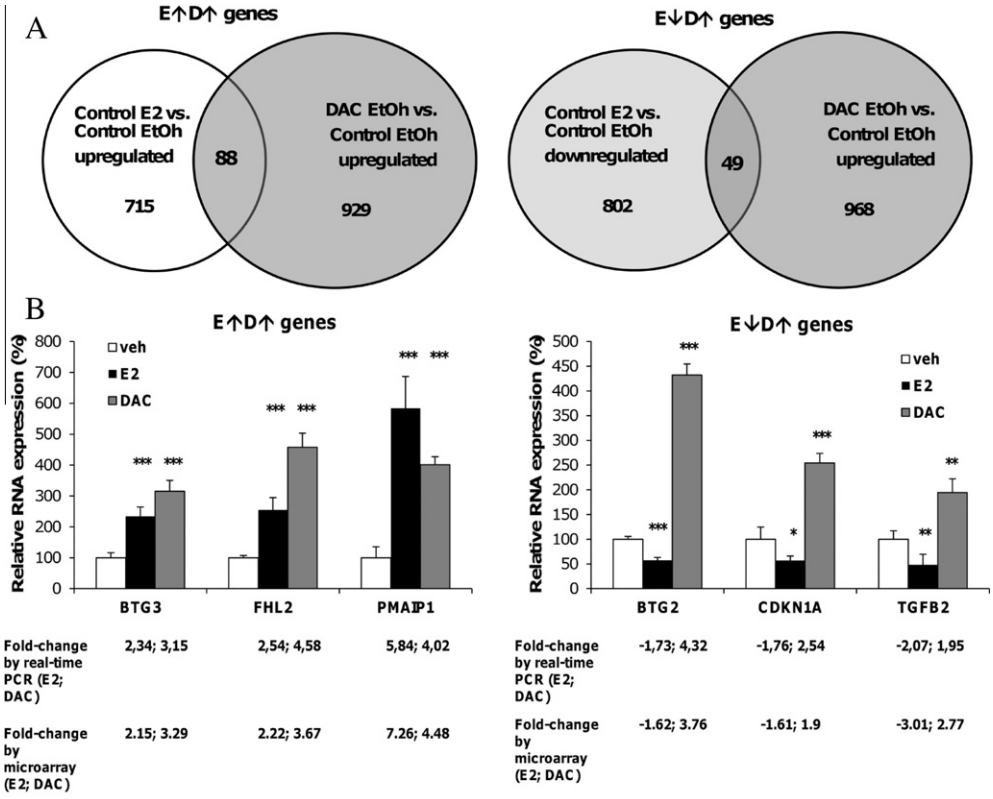

Fig. 1. Identification of genes regulated by estrogen signaling and demethylation. (A) Analysis of microarray results. 88 genes are up-regulated by both E2 and DAC (E↑D↑ genes), and 49 genes are down-regulated by E2, but up-regulated by DAC (E↓D↑ genes). (B) Confirmation of E2- and DAC-regulation of genes, as determined by global gene expression analysis. Real-time PCR analysis of same RNA used for the microarray analysis is presented. The data are the average of four replicates ± SD. Fold change over vehicle from real-time PCR and microarray analysis is shown below the bars. Statistical significance over vehicle is shown using asterisk symbols (*) above the bars. The results are flagged with a single asterisk when $p < 0.05$, with two asterisks when $p < 0.01$, and three asterisks when $p < 0.001$.

Table 1
List of genes regulated more than 2-fold following exposure to either E2 or DAC, compared to control.

	Gene name	Acc. No.	Probe name	Fold change	
				DAC	E2
E↑D↑ genes					
CXCL12	Chemokine (C–X–C motif) ligand 12	NM_000609	209687_PM_at	52.23972664	5.956922044
RASGRP1	RAS guanyl releasing protein 1 (calcium and DAG-regulated)	NM_001128602	205590_PM_at	16.57543329	2.433704715
OLFML3	Olfactomedin-like 3	NM_020190	218162_PM_at	10.79976725	5.476780784
PTGES	Prostaglandin E synthase	NM_004878	210367_PM_s_at	8.750131447	5.05374642
PMAIP1	Phorbol-12-myristate-13-acetate-induced protein 1	NM_021127	204285_PM_s_at	7.264169302	4.475172589
IRS1	Insulin receptor substrate 1	NM_005544	204686_PM_at	5.047926637	2.53205611
SLC16A1	Solute carrier family 16, member 1 (monocarboxylic acid transporter 1)	NM_001166496	202234_PM_s_at	4.414014437	2.219592491
LRRC49	Leucine rich repeat containing 49	NM_017691	219338_PM_s_at	4.36447962	2.384106055
MICAL2	Microtubule associated monoxygenase, calponin and LIM domain containing 2	NM_014632	212473_PM_s_at	4.155880262	5.716021966
RPP25	Ribonuclease P/MRP 25 kDa subunit	NM_017793	219143_PM_s_at	3.854019451	2.030422266
IGF1R	Insulin-like growth factor 1 receptor	NM_000875	203627_PM_at	3.603347621	2.524307238
RGS16	Regulator of G-protein signaling 16	NM_002928	209324_PM_s_at	3.548714615	3.320454941
TUBB2B	Tubulin, beta 2B	NM_178012	214023_PM_x_at	3.547315032	7.181077069
ID4	Inhibitor of DNA binding 4, dominant negative helix-loop-helix protein	NM_001546	209291_PM_at	3.462466065	2.125853014
C4orf19	Chromosome 4 open reading frame 19	NM_001104629	235350_PM_at	3.266550156	2.607615987
CYP26A1	Cytochrome P450, family 26, subfamily A, polypeptide 1	NM_000783	206424_PM_at	3.100268209	2.207504622
DGAT2	Diacylglycerol O-acyltransferase homolog 2 (mouse)	NM_032564	226064_PM_s_at	2.802407567	3.454474967
SDC2	Syndecan 2	NM_002998	212157_PM_at	2.689341872	2.146071278
TSPAN5	Tetraspanin 5	NM_005723	225387_PM_at	2.578505237	2.374815246
UGT8	UDP glycosyltransferase 8	NM_001128174	228956_PM_at	2.467817971	2.145984403
PODXL	Podocalyxin-like	NM_001018111	201578_PM_at	2.324001128	2.566543053
FHL2	Four and a half LIM domains 2	NM_001039492	202949_PM_s_at	2.221543213	3.626033921
ADD3	Adducin 3 (gamma)	NM_001121	201034_PM_at	2.184472295	2.234007659
SLC27A2	Solute carrier family 27 (fatty acid transporter), member 2	NM_001159629	205769_PM_at	2.17714895	2.053090232
LAMA3	Laminin, alpha 3	NM_000227	203726_PM_s_at	2.157791415	2.040753191
BTG3	BTG family, member 3	NM_001130914	213134_PM_x_at	2.149538384	3.294120858
SPATS2L	Spermatogenesis associated, serine-rich 2-like	NM_001100422	222154_PM_s_at	2.123616705	2.08727826
MSI2	Musashi homolog 2 (Drosophila)	NM_138962	243010_PM_at	2.10384776	2.343559823
ADA	Adenosine deaminase	NM_000022	204639_PM_at	2.057867779	3.091598544
RHOU	Ras homolog gene family, member U	NM_021205	223168_PM_at	2.007632136	2.277064702
LRRC50	Leucine rich repeat containing 50	NM_178452	222068_PM_s_at	2.003765588	5.030352966
E↓D↑ genes					
TGFB2	Transforming growth factor, beta 2	NM_001135599	220407_PM_s_at	−3.012428869	2.769296496
ALDH1A3	Aldehyde dehydrogenase 1 family, member A3	NM_000693	203180_PM_at	−2.017140217	6.296403421
BEX4	Brain expressed, X-linked 4	NM_001080425	215440_PM_s_at	−2.503098749	4.208113231
DUSP10	Dual specificity phosphatase 10	NM_007207	221563_PM_at	−2.018636188	2.211673972
KRT17	Keratin 17	NM_000422	212236_PM_x_at	−2.194561935	19.5969634
TMEM40	Transmembrane protein 40	NM_018306	222892_PM_s_at	−2.25714765	4.011490012

Table 2
Gene ontology analysis of E↑D↑ and E↓D↑ genes. Genes selected for further analysis are cited in bold.

GO term	Count	*P*-value	Genes
Regulation of cell motion GO:0051270	10	2.47E-06	B4GALT1, IGF1R, **CDKN1A**, TBC1D8, IL6ST, ADRA2A, ID4, IRS1, ADA, **TGFB2**
Regulation of cell migration GO:0030334	9	6,45E-06	IGF1R, LAMA3, IL6ST, PTP4A1, THBS1, IRS1, CXCL12, ADA, **TGFB2**
Intracellular signaling cascade GO:0007242	25	8.20E-06	ADORA2B, IL6ST, DUSP10, **FHL2**, NFKBIA, RHOU, IGF1R, RASGRP1, AEN, ADRA2A, RHOBTB1, THBS1, RAB8B, NUDT4, PTGER4, SOCS2, CYP26A1, TIFA, IRS1, RASL11B, RAB31, NUDT4P1, JAK1, RASD1, PMEPA1
Regulation of cell proliferation GO:0042127	16	1.10E-04	B4GALT1, TBC1D8, IL6ST, NFKBIA, IRS1, ADA, **TGFB2**, IGF1R, **CDKN1A**, **BTG2**, PTGES, **BTG3**, ADRA2A, ID4, THBS1, KLF4
Regulation of apoptosis GO:0042981	18	1.43E-04	B4GALT1, SOCS2, GPR109B, APH1B, NR4A2, NFKBIA, **PMAIP1**, ADA, **TGFB2**, AMIGO2, IGF1R, **CDKN1A**, CD44, **BTG2**, ALDH1A3, AEN, THBS1, TRAF3

on the promoters of these genes. However, E2 had no effect on the promoter methylation status (Fig. 4A).

3.4. ERα is recruited to the FHL2 CpG island containing promoter

ERα showed significant recruitment to the CpG island containing FHL2 promoter region. DAC did not affect ERα recruitment to the CpG island containing promoter region, suggesting that the FHL2 promoter methylation status has no effect on the ERα recruitment. The recruitment of ERα to the FHL2 promoter was E2-independent (Fig. 4B). ERα showed no significant recruitment to the BTG3 promoter CpG island (data not shown).

4. Discussion

Although there is evidence for interplay between estrogen signaling and DNA methylation, their global overlapping effects on gene regulation have not yet been reported. The aim of this study was to identify the common target genes for estrogen signaling and DNA methylation in human MCF-7 breast cancer cells using a genome-wide gene expression profiling approach. We demonstrate that estrogen signaling and demethylation have the potential to regulate a set of common target genes in MCF-7 cells. However, our data does not support a direct molecular interplay between these pathways at the same regulatory regions.

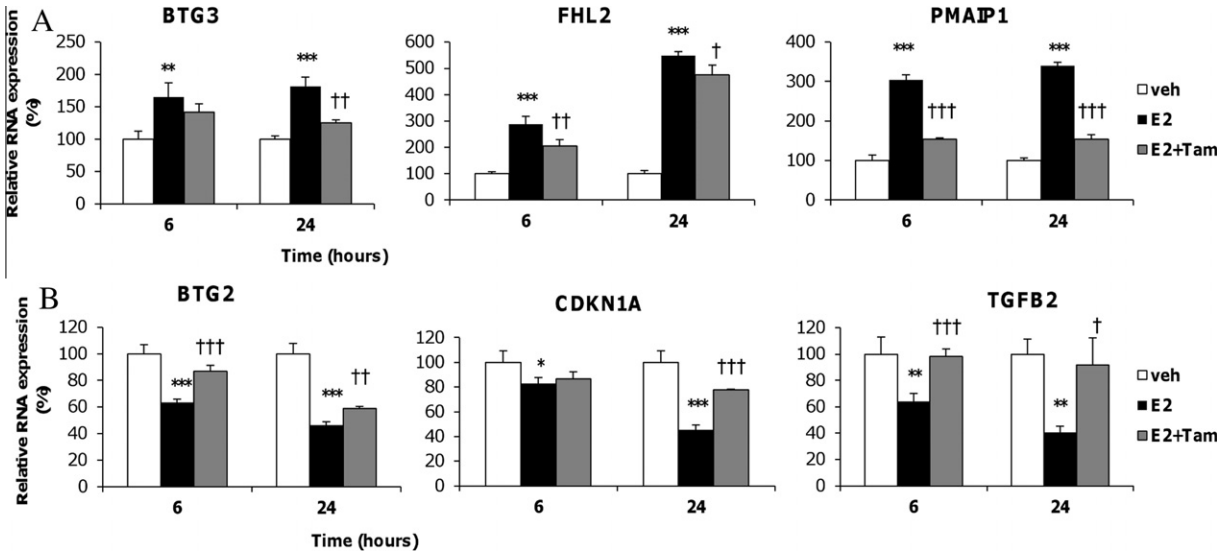

Fig. 2. Real-time PCR analysis of mRNA expression of selected genes after 6 and 24 h treatment with E2 and co-treatment of E2 and 1 μM tamoxifen (Tam). Statistical significance for E2-treated group over vehicle is shown by asterisks (*), and for E2 + Tam group over E2 group using obelisks (†). (A) E↑D↑ genes. (B) E↓D↑ genes.

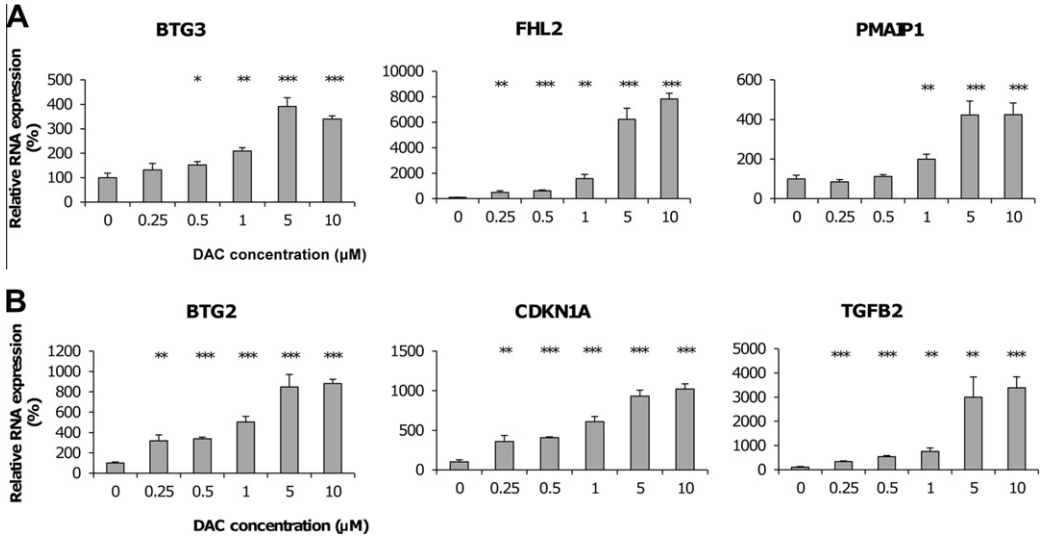

Fig. 3. Real-time PCR analysis of the dose dependent effect of DAC on mRNA expression for the selected genes. Statistical significance is calculated over non-treated group. (A) E↑D↑ genes. (B) E↓D↑ genes.

Using a global gene expression profiling approach, we identified genes regulated by E2 and a demethylating agent DAC. The expression profiles in response to E2 and DAC co-treatment were not further explored, as DAC was found to down-regulate both mRNA and protein levels of ERα in MCF-7 cells (data not shown), in accordance with previous reports [30]. We focused on the up-regulated genes in the DAC-regulated group, since DAC-induced demethylation leads to up-regulation of the genes that are normally directly silenced by DNA methylation.

We compared our data with the study by Fujikane et al. reporting genes regulated by DAC in MCF-7 cells [31]. Of 288 genes found to be up-regulated $\geqslant$5-fold by DAC by Fujikane et al., 38 are confirmed in this study using the same cut-off, and 76 using the $\geqslant$1.5-fold cut-off. Out of nine DAC up-regulated genes confirmed by Fujikane et al. to have methylated promoters, only NTN4 is shown to be up-regulated by DAC in our study. In both studies the cells were exposed to 1 μM DAC for 72 h, but in different cell media (cell media with reduced levels of E2 and growth factors in our study). Other reasons for the limited overlap of DAC-regulated genes identified in the two studies could include the use of different MCF-7 clones as well as the application of different gene expression profiling platforms (Affymetrix U133 Arrays/33,000 genes in our study versus Agilent Whole Genome Microarray/44,000 genes in the study by Fujikane et al.). However, Gene Ontology (GO) analysis in both studies reports that DAC up-regulated genes are involved in apoptosis and that DAC can restore induction of genes silenced by DNA methylation.

We identified a total of 88 genes as up-regulated by both E2 and DAC (E↑D↑ group) and 49 genes as down-regulated by E2 and up-regulated by DAC (E↓D↑ group). Candidate target genes were selected for further analysis based on previously reported association with breast cancer, estrogen signaling and/or DNA methylation, predicted CpG islands and GO analysis. Based on these selection criteria, six genes were selected for a detailed investigation of regulation by E2 and DAC: three from the E↑D↑ group (BTG3, FHL2 and PMAIP1) and three from the E↓D↑ group (BTG2, CDKN1A and TGFB2).

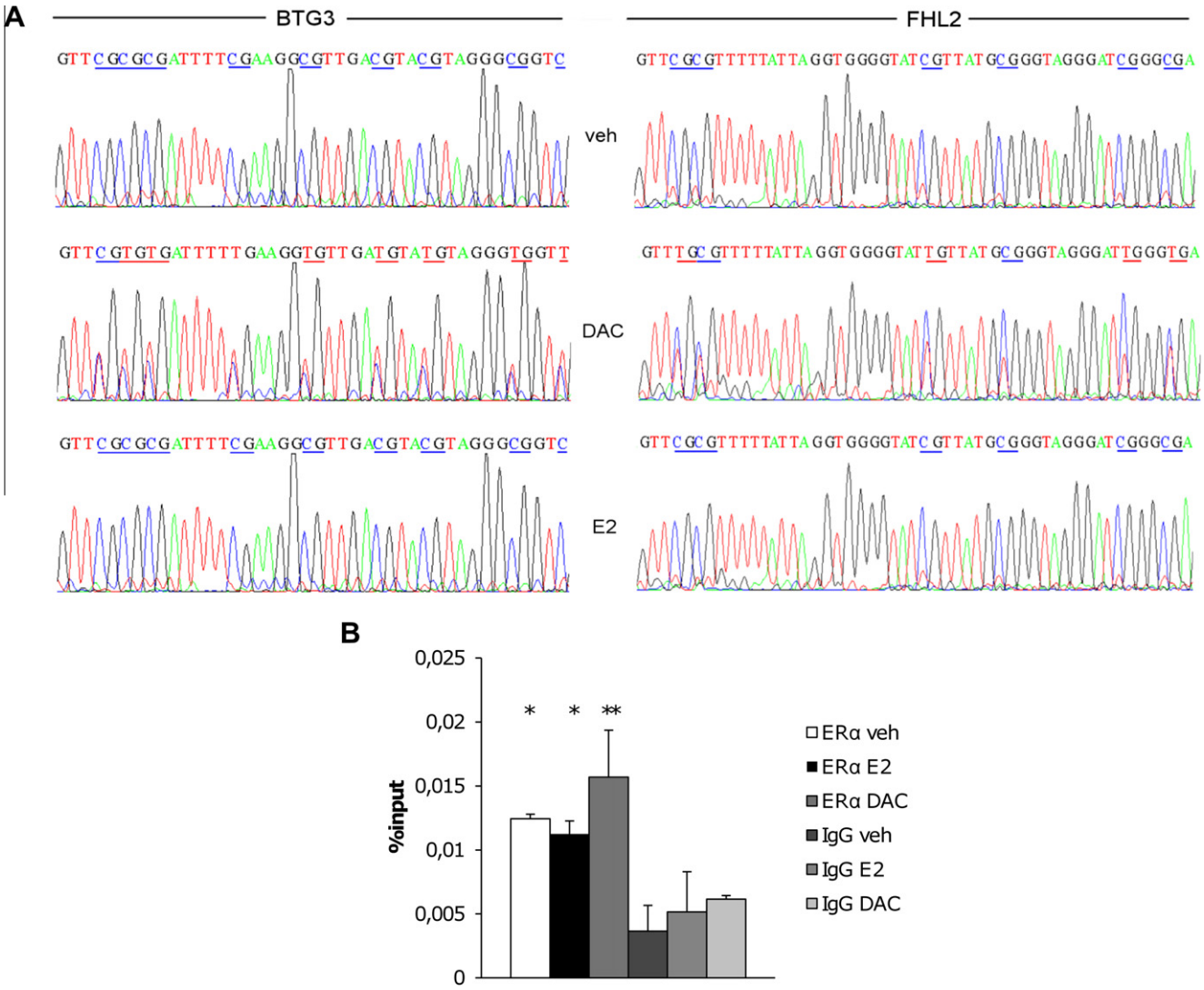

Fig. 4. (A) DAC, but not E2, demethylates BTG3 and FHL2 promoters. After bisulfite treatment, all unmethylated cytosines in the DNA are deaminated and sulfonated, and hence converted to thymines, while methylated cytosines (5-methylcytosines) remain unaltered. Thus, the sequence of the treated DNA will differ depending on whether the DNA is originally methylated or unmethylated. Every treatment was done in four replicates. One representative example is shown. CpG sites are underlined. Blue underlines signify CpG sites that contain methylated cytosines, while red underlines signify CpG sites that contain (partly) demethylated cytosines. (B) ChIP followed by real-time PCR was used for detection of ERα or control IgG recruitment to the CpG island containing promoter of the FHL2 gene after E2- and DAC-treatment. The results are shown as percentage of input and are means ± SD. Statistical significance for ERα recruitment is calculated over corresponding IgG.

We used co-treatment of E2 with tamoxifen to confirm that these genes are targets of estrogen receptor. We also showed DAC dose-dependent expression of the selected genes, suggesting that they are targets of DNA methylation. We next examined the methylation status of the predicted CpG islands in the promoters of the six genes, after DAC and E2 administration using bisulfate sequencing. The promoters of two genes from the E↑D↑ group, BTG3 and FHL2, were found to be methylated, with the methylation being reversed by DAC. E2 showed no demethylating effect on their promoters.

Although PMAIP1, BTG2, CDKN1A and TGFB2 are all found to be up-regulated by DAC, their promoters were not methylated in the absence of DAC. This suggests that DAC regulates the expression of these genes either via demethylation of other methylated DNA regions, such as CpG shores, shelves and open seas [32,33], or indirectly, through demethylation of other genes.

To further explore the hypothesis of a direct molecular interplay between estrogen signaling and DNA methylation at potentially common regulatory elements of the BTG3 and FHL2 genes, we investigated ERα recruitment to the CpG containing promoter regions of these genes. ERα showed significant binding to the FHL2 promoter, however independently of E2 and DAC. This suggests that estrogen signaling does not regulate FHL2 expression via a CpG island containing promoter, but via another regulatory element.

Our results support the previous report that BTG3 is directly regulated by DNA methylation in MCF-7 cells [34]. Furthermore, we show that E2 affects the expression of this gene. However, our data does not support E2-mediated changes in the DNA methylation status of the BTG3 promoter. Genistein, an ER ligand, was shown to have the same demethylating effect as DAC on the BTG3 promoter in prostate and renal cancer cells [35,36], hence estrogen effects on DNA methylation might display cell type- and/or ligand-selective mechanisms.

We confirm the data by Fan et al. that FHL2 is regulated by E2 in MCF-7 cells [18]. Fan et al. also suggested a connection between estrogen signaling and methylation of the FHL2 promoter, as long term disruption of estrogen signaling using estrogen antagonists can lead to hypermethylation of the FHL2 promoter with the

associated loss of E2 responsiveness. Our studies, focusing on regulation of promoter methylation upon short term E2 treatment, do not support a connection between short term E2 treatment and FHL2 promoter methylation.

In conclusion, we have identified genes co-regulated by both estrogen signaling and demethylation in breast cancer cells. However, our data does not support a direct interplay between these signaling pathways at the same regulatory elements.

Acknowledgments

The study was supported by Cancerfonden, Karolinska Institute's faculty funds for partial financing of doctoral students (KID), Karolinska Institute's Center for Biosciences and the EU integrated project CRESCENDO. We are grateful to the Bioinformatic and Expression Analysis core facility at the Karolinska Institute (BEA, www.bea.ki.se) for performing the microarray assay.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.08.007.

References

[1] K. Dahlman-Wright, V. Cavailles, S.A. Fuqua, V.C. Jordan, J.A. Katzenellenbogen, et al., International union of pharmacology. LXIV. Estrogen receptors., Pharmacol. Rev. 58 (2006) 773–781.
[2] S. Sharma, T.K. Kelly, P.A. Jones, Epigenetics in cancer, Carcinogenesis 31 (2010) 27–36.
[3] N. Platet, A.M. Cathiard, M. Gleizes, M. Garcia, Estrogens and their receptors in breast cancer progression: a dual role in cancer proliferation and invasion, Crit. Rev. Oncol. Hematol. 51 (2004) 55–67.
[4] C. Thomas, J.A. Gustafsson, The different roles of ER subtypes in cancer biology and therapy, Nat. Rev. Cancer 11 (2011) 597–608.
[5] J. Jovanovic, J.A. Ronneberg, J. Tost, V. Kristensen, The epigenetics of breast cancer, Mol. Oncol. 4 (2010) 242–254.
[6] S. Nilsson, J.A. Gustafsson, Estrogen receptor action, Crit. Rev. Eukaryot. Gene Expr. 12 (2002) 237–257.
[7] S. Sommer, S.A. Fuqua, Estrogen receptor and breast cancer, Semin. Cancer Biol. 11 (2001) 339–352.
[8] S. Kato, Estrogen receptor-mediated cross-talk with growth factor signaling pathways, Breast Cancer 8 (2001) 3–9.
[9] A. Jemal, F. Bray, M.M. Center, J. Ferlay, E. Ward, et al., Global cancer statistics, CA Cancer J. Clin. 61 (2011) 69–90.
[10] Early Breast Cancer Trialists' Collaborative Group (EBCTCG), Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet 365 (2005) 1687–717.
[11] S. Ali, L. Buluwela, R.C. Coombes, Antiestrogens and their therapeutic applications in breast cancer and other diseases, Annu. Rev. Med. 62 (2011) 217–232.
[12] G.A. Garinis, G.P. Patrinos, N.E. Spanakis, P.G. Menounos, DNA hypermethylation: when tumour suppressor genes go silent, Hum. Genet. 111 (2002) 115–127.
[13] D. Rodenhiser, M. Mann, Epigenetics and human disease: translating basic biology into clinical applications, CMAJ 174 (2006) 341–348.
[14] G. Egger, G. Liang, A. Aparicio, P.A. Jones, Epigenetics in human disease and prospects for epigenetic therapy, Nature 429 (2004) 457–463.
[15] X. Yang, F. Lay, H. Han, P.A. Jones, Targeting DNA methylation for epigenetic therapy, Trends Pharmacol. Sci. 31 (2010) 536–546.
[16] P.M. Das, R. Singal, DNA methylation and cancer, J. Clin. Oncol. 22 (2004) 4632–4642.
[17] J.D. Yager, N.E. Davidson, Estrogen carcinogenesis in breast cancer, N. Engl. J. Med. 354 (2006) 270–282.
[18] M. Fan, P.S. Yan, C. Hartman-Frey, L. Chen, H. Paik, et al., Diverse gene expression and DNA methylation profiles correlate with differential adaptation of breast cancer cells to the antiestrogens tamoxifen and fulvestrant, Cancer Res. 66 (2006) 11954–11966.
[19] P.Y. Hsu, H.K. Hsu, G.A. Singer, P.S. Yan, B.A. Rodriguez, et al., Estrogen-mediated epigenetic repression of large chromosomal regions through DNA looping, Genome Res. 20 (2010) 733–744.
[20] Y.W. Leu, P.S. Yan, M. Fan, V.X. Jin, J.C. Liu, et al., Loss of estrogen receptor signaling triggers epigenetic silencing of downstream targets in breast cancer, Cancer Res. 64 (2004) 8184–8192.
[21] R. Metivier, R. Gallais, C. Tiffoche, C. Le Peron, R.Z. Jurkowska, et al., Cyclical DNA methylation of a transcriptionally active promoter, Nature 452 (2008) 45–50.
[22] B. Ramaswamy, S. Majumder, S. Roy, K. Ghoshal, H. Kutay, et al., Estrogen-mediated suppression of the gene encoding protein tyrosine phosphatase PTPRO in human breast cancer: mechanism and role in tamoxifen sensitivity, Mol. Endocrinol. 23 (2009) 176–187.
[23] M. Widschwendter, K.D. Siegmund, H.M. Muller, H. Fiegl, C. Marth, et al., Association of breast cancer DNA methylation profiles with hormone receptor status and response to tamoxifen, Cancer Res. 64 (2004) 3807–3813.
[24] W. Huang da, B.T. Sherman, R.A. Lempicki, Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists, Nucleic Acids Res. 37 (2009) 1–13.
[25] W. Huang da, B.T. Sherman, R.A. Lempicki, Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources, Nat. Protoc. 4 (2009) 44–57.
[26] L.C. Li, R. Dahiya, MethPrimer: designing primers for methylation PCRs, Bioinformatics 18 (2002) 1427–1431.
[27] R. Akamine, T. Yamamoto, M. Watanabe, N. Yamazaki, M. Kataoka, et al., Usefulness of the 5′ region of the cDNA encoding acidic ribosomal phosphoprotein P0 conserved among rats, mice, and humans as a standard probe for gene expression analysis in different tissues and animal species, J. Biochem. Bioph. Methods 70 (2007) 481–486.
[28] J. Matthews, B. Wihlen, M. Tujague, J. Wan, A. Strom, et al., Estrogen receptor (ER) beta modulates ERalpha-mediated transcriptional activation by altering the recruitment of c-Fos and c-Jun to estrogen-responsive promoters, Mol. Endocrinol. 20 (2006) 534–543.
[29] R. Edgar, M. Domrachev, A.E. Lash, Gene expression omnibus: NCBI gene expression and hybridization array data repository, Nucleic Acids Res. 30 (2002) 207–210.
[30] P. Pryzbylkowski, O. Obajimi, J.C. Keen, Trichostatin A and 5 Aza-2′ deoxycytidine decrease estrogen receptor mRNA stability in ER positive MCF7 cells through modulation of HuR, Breast Cancer Res. Treat. 111 (2008) 15–25.
[31] T. Fujikane, N. Nishikawa, M. Toyota, H. Suzuki, M. Nojima, et al., Genomic screening for genes upregulated by demethylation revealed novel targets of epigenetic silencing in breast cancer, Breast Cancer Res. Treat. 122 (2010) 699–710.
[32] R.A. Irizarry, C. Ladd-Acosta, B. Wen, Z. Wu, C. Montano, et al., The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores, Nat. Genet. 41 (2009) 178–186.
[33] J. Sandoval, H. Heyn, S. Moran, J. Serra-Musach, M.A. Pujana, et al., Validation of a DNA methylation microarray for 450,000 CpG sites in the human genome, Epigenetics 6 (2011) 692–702.
[34] J. Yu, Y. Zhang, Z. Qi, D. Kurtycz, G. Vacano, et al., Methylation-mediated downregulation of the B-cell translocation gene 3 (BTG3) in breast cancer cells, Gene Expr. 14 (2008) 173–182.
[35] S. Majid, A.A. Dar, A.E. Ahmad, H. Hirata, K. Kawakami, et al., BTG3 tumor suppressor gene promoter demethylation, histone modification and cell cycle arrest by genistein in renal cancer, Carcinogenesis 30 (2009) 662–670.
[36] S. Majid, A.A. Dar, V. Shahryari, H. Hirata, A. Ahmad, et al., Genistein reverses hypermethylation and induces active histone modifications in tumor suppressor gene B-Cell translocation gene 3 in prostate cancer, Cancer 116 (2010) 66–76.