Regulation of estrogen receptor β1 expression in breast cancer by epigenetic modification of the 5' regulatory region

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Abstract. ERβ1 is often down-regulated in breast cancer compared to normal breast but mechanisms surrounding this are unclear. We examined whether loss of heterozygosity (LOH) or methylation at ERβ promoters (0N, 0K) and/or untranslated exon 0N were involved in ERβ down-regulation in breast cancer tissues and cell lines and if treatment with the de-methylating agent 5-aza-deoxycytidine and/or the histone deacetylase inhibitor Trichostatin A could influence expression *in vitro*. We found no evidence of correlation between LOH at 14q22-24 (genomic locus containing *ERβ/ESR2*), and ERβ1 expression in primary breast cancers. A negative correlation between ERβ1 mRNA expression and methylation status was observed for promoter 0N in BT-20, MDA-MB-453 and T47D cells. Promoter 0K was consistently unmethylated. In primary breast tumours, methylation of the untranslated exon 0N, downstream of promoter 0N, but not of promoter 0N itself, correlated with down-regulation of ERβ. In MDA-MB-453 cells, treatment with 5-aza-deoxycytidine was sufficient to induce ERβ1 expression from the 0N promoter while in BT-20 both agents were required. Examination of various sites on *ESR2* highlighted epigenetic but not genetic regulation of ERβ1. In particular methylation adjacent to promoter 0N was a key regulatory event for ERβ1 silencing. A combination of de-methylating agents and histone deacetylase inhibitors fully restored ERβ1 expression which may offer a novel therapeutic angle for breast cancer management.

Introduction

Despite its discovery in 1996, the mechanistic role of estrogen receptor (ER) β in breast cancer remains incompletely under-

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stood. Five ERβ isoforms exist, formed by alternative splicing of exon 8 (1). Of these, ERβ1 is regarded as the wild-type isoform regulating gene transcription in response to estradiol (2). ERβ1 is constitutively expressed in the normal mammary gland but frequently down-regulated in breast cancer (3,4) where it may function as a tumour-suppressor (5,6). Loss of ERβ1 might be the result of genetic modifications, such as the homozygous deletion of both copies of the ERβ gene (*ESR2*) or from loss of heterozygosity (LOH) together with mutation (7). Alternatively, down-regulation of ERβ1 expression in the absence of genetic mutations might be the result of epigenetic modifications.

Epigenetic regulation of total ERβ expression has previously been reported in various cancers (8-11). The ERβ promoter region contains several CpG islands, depicted schematically in Fig. 1. Transcription from two different ERβ promoters, termed promoters 0N and 0K, generates ERβ mRNA isoforms that diverge in their 5'-untranslated regions (UTRs) by including the alternative untranslated exons 0N or 0K. Evidence suggests that total ERβ expression and, more specifically, the expression of ERβ1, may be regulated by hypermethylation of CpG islands located within promoter 0N or exon 0N in various primary tumours and tumour cell lines (4,10-12). Interestingly, we have reported that ERβ untranslated exons are differentially associated with mRNAs for each ERβ isoform (13), adding weight to the current evidence suggesting epigenetic events at specific sites might influence the expression of individual ERβ isoforms.

Unlike genetic alterations, changes in DNA methylation are potentially reversible and the transcriptional reactivation of tumour suppressor genes through promoter de-methylation represents an attractive strategy for anticancer treatment currently being evaluated in clinical trials (14). ERβ1, -β2 and -β transcripts derived from promoter 0N can be re-expressed in breast cancer cell lines following treatment with DNA methyltransferase (DNMT) inhibitors (10). DNA de-methylation and histone deacetylase (HDAC) inhibition have been associated with re-expression of total ERβ in prostate and ovarian cancer cell lines (9,15). However, the effects of combination therapy on the re-expression of ERβ1 in breast cancer cells have yet to be explored.

Here, we aimed to determine the underlying mechanisms of ERβ1 de-regulation, performing LOH analysis to examine the influence of genetic modifications in the silencing of ERβ1
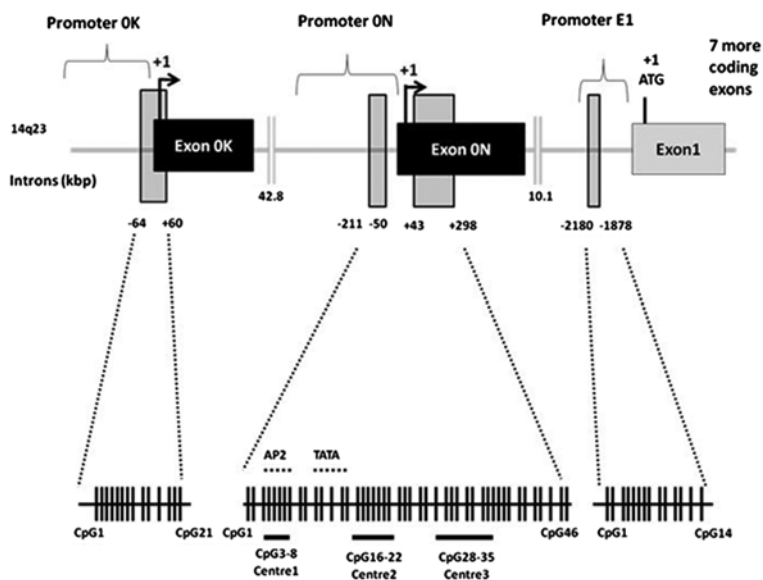
Figure 1. Schematic diagram depicting the location of ERβ promoters, untranslated exons, exon 1 and CpG islands. The ERβ gene has three promoters, distal (P0K), central (P0N) and proximal (PE1). 5'-untranslated exons (0K and 0N; black boxes), transcriptional (black arrows), translational (ATG) start site and intron sizes are shown. CpG islands are shown (grey boxes). Map of CpG sites is shown as vertical lines. Three methylation centres (1-3) are depicted by the solid underline. Centre 1 (CpG 3-8) is located in P0N and Centre 2 (CpG 16-22) and Centre 3 (CpG 28-35) are in the exon 0N. The TATA box and AP2 are depicted by the dotted lines and located in P0N CpG island. The numbers above indicate position of CpG islands respect to transcriptional start sites (+1). Sequence position for PE1 CpG island relative to translational start site (+1).

expression in breast cancer and examining whether aberrant methylation of the CpG islands, located in ERβ promoters (0K and 0N) and a 5'-untranslated region (exon 0N) was involved in the regulation of ERβ1 expression in breast cancer cell lines and in primary breast cancer. We also examined the effects of a combination of DNA methylation and HDAC inhibition on the re-expression of ERβ1 mRNA and ERβ mRNAs containing untranslated exons (0N or 0K) in ERβ1-negative breast cancer cell lines.

Materials and methods

Case selection. Following ethical approval from the Leeds (East) Research Ethics Committee (06/Q1206/180), 51 snap frozen tumour and adjacent matched normal tissues were selected from the Leeds Breast Tissue Bank. The cohort comprised 10 grade 1, 17 grade 2 and 24 grade 3 tumours; 25 were lymph node positive and 26 were node negative. Samples were harvested prior to freezing by specialised breast histopathologists (AMH/SL) who ensured that tumour samples contained at least 80% of tumour cells. Immunohistochemical analysis of ERβ1 in matched formalin-fixed paraffin-embedded cases and gene expression of ERβ1 in frozen tissue was conducted as previously described (16,17).

Tissue culture. BT-20, MDA-MB-453 and T47D breast cancer cell lines were maintained in RPMI-1640 medium, supplemented with 5% heat-inactivated fetal bovine serum (FBS; both Invitrogen), in a 5% CO_2 humidified incubator at 37˚C. Bimonthly *Mycoplasma* checks (MycoAlert Mycoplasma detection assay, Lonza) were consistently negative. Short tandem repeat profiles confirmed cell identity.

DNA extraction and LOH analysis. DNA was extracted using standard phenol/chloroform methods. Multiplex PCR was performed in a reaction volume of 10 µl containing 10 ng sample DNA, 1 pmol/µl of each primer pair (fluorescently labelled forward primer), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, PCR buffer, 1.25 U Taq polymerase (Promega) and molecular grade water. LOH was determined using four microsatellite markers D14S1026, AL359235, D14S63 and AL122035, which span the chromosome 14q22-24 region. Primer sequences were obtained from the Genome Database (http://gdbwww.gdb.org/) or Ensembl (http://www.ensembl.org). Cycle conditions were: 95˚C for 5 min, 95˚C for 30 sec, 58˚C for 30 sec, 72˚C for 30 sec for 35 cycles and a final extension of 72˚C for 10 min. Resulting products were sequenced (ABI 377 Perkin-Elmer). Allele ratios of tumour and normal samples were calculated from the peak heights obtained from the electrophoretograms and a tumour/normal ratio calculated. A value of <0.5 indicated LOH (18). LOH was correlated to immunohistochemical expression of ERβ1.

DNA extraction, bisulphite modification and methylation analysis. All extraction kits were from Qiagen and the manufacturer's instructions were followed. Primers for bisulphite PCR (BSP) and methylation-specific PCR (MSP) are shown in Table I. DNA (1 µg) was extracted from frozen breast cancer tissues or breast cell lines (DNeasy Blood and Tissue Kit). This was bisulphite modified (EpiTect Bisulphite Kit). BSP was performed (Multiplex PCR Kit). Following initial melting at 95˚C for 10 min, cycle conditions were: i) 45 cycles of 95˚C, 30 sec, 56˚C for 45 sec, 72˚C for 45 sec (promoter 0N and exon 0N), ii) 40 cycles of 95˚C, 1 min, 63˚C for 1 min, 72˚C for 1 min (promoter 0K), both followed by a final extension at 72˚C for 10 min. PCR products were analysed on a 1.5% agarose gel containing 0.5 µg/ml ethidium bromide and visualized under UV illumination. Bands were excised from the gel and purified (QIAquick Gel Extraction Kit). Purified DNA was directly sequenced. MSP was performed using the EpiTect MSP Kit.

Table I. Primer sequences for BSP, MSP and QRT-PCR.

Primer sets	Forward (5'→3')	Reverse (5'→3')	Size (bp)
BSP			
Promoter 0K	GTTGGGGTTATTTCGGGGTTGTT	CCTCCAACAAAACAAACACATTCA	295
Promoter 0N and exon 0N	GTTATTATTTTTGTGGGTGGATTGG	ACCTTACCTTCTCTAAAATAC	500
MSP			
Promoter 0N-W	CCCAGACTGGCTGTATCAGTGTCGG	TGACCTCTAAGTGGGAGCACCCTCG	178
Promoter 0N-M	TTTAGATTGGTTGTATTAGTGTCGG	TAACCTCTAAATAAAAACACCCTCG	178
Promoter 0N-U	TTAGATTGGTTGTATTAGTGTTGG	CCTCTAAATAAAAACACCCTCAAA	174
Exon 0N-W	GGAGGGACCACCCGAGCTGC	CCACCTGTTGAGGAAAGCGAGCG	102
Exon 0N-M	GGAGGGATTATTCGAGTTGC	CCACCTATTAAAAAAAACGAACG	102
Exon 0N-U	GGGAGGGATTATTTGAGTTGTG	CCACCTATTAAAAAAAACAAACAC	101
QRT-PCR			
ERβ1	TGGGCACCTTTCTCCTTTAGTGG	GCTTCACACCAGGGACTCTTTTGAG	87
ERβ (exon 0N)	CGGGAGACCCCCCCTAATGC	CTCAAAGATTCGTGGGCAAGTATAATG	105
ERβ (exon 0K)	AGTTACTGAGTCCGATGAATGTGCTTG	CTCAAAGATTCGTGGGCAAGTATAATG	108

W, wild-type primer set; M, methylation-specific primer set; U, unmethylation-specific primer set.

Cycle conditions were: 95˚C for 10 min, 45 cycles of 95˚C for 30 sec, 56-62˚C for 45 sec, 72˚C for 45 sec and a final extension at 72˚C for 10 min. PCR products were run and analysed on a 1.5% ethidium bromide-stained agarose gels as described above. MSP primers could equally bind both unmethylated and methylated DNA (19). To limit PCR bias that could occur during amplification, MSP primers were optimized using touchdown PCR with gradient annealing temperature. As positive controls, a human control DNA set containing bisulphite methylated-converted, unmethylated DNA and unmethylated-unconverted DNA were used (EpiTect PCR Control DNA Set). The optimum annealing temperature for methylated specific primer was chosen at an annealing temperature which favoured the methylated specific primer to bind specifically to the methylated template control but not to unmethylated template control and unconverted unmethylated DNA control and vice versa.

Pharmacological restoration of ERβ1 mRNA and ERβ 5'-UTR expression using DNA methyl transferase (DNMT) and histone deacetylase (HDAC) inhibitors. BT-20 and MDA-MB-453 cells were seeded in 6-well plates at $3x10^4$ cells/cm². After overnight attachment, the DNMT inhibitor, 5-aza-2'-deoxycytidine (5-aza-dC) was added at final concentration of 5 μM (BT-20) and 1 μM (MDA-MB-453) for 7 days. Fresh medium containing 5-aza-dC was added every two days. Trichostatin A (TSA) was added when required at 300 nM for the last 24 h. Cells were harvested on day 8 for RNA and DNA extraction. Controls cells received DMSO vehicle. QRT-PCR was performed for ERβ1 and ERβ mRNAs containing untranslated exons (0K or 0N). Primer sequences are in Table I.

Results

LOH analysis. To determine whether genetic modifications influence ERβ1 expression, we performed LOH on 27 breast tumours from our cohort of 51 (Fig. 2). LOH was identified in 2/12 (17%) cases at the AL359235 locus. No LOH was observed in 23 cases that were informative for the marker D14S63, directly adjacent to the ERβ gene. Similarly only 1/20 (5%) cases showed LOH at the D14S1026 marker located within the ERβ gene. However, LOH was more frequently observed at the AL122035 locus, in 5 of 24 informative cases (21%) screened. Comparison of LOH data with ERβ1 immunohistochemistry showed no correlation between LOH and ERβ1-negative tumours. Since LOH did not appear to be associated with loss of ERβ1 expression, we performed MSP to determine the methylation status of promoter 0N and untranslated exon 0N in a subset of cases. Methylation was seen in 5/12 samples, all from ERβ1-negative tumours suggesting epigenetic rather than genetic events are important in the regulation of ERβ1 expression.

Correlation of ERβ1 mRNA expression with methylation status of its promoters using BSP and MSP. The location of ERβ promoters, untranslated exons, exon 1 and CpG islands is depicted schematically in Fig. 1. BSP (Fig. 3A) was performed on promoter 0N, exon 0N (3 CpG islands), and promoter 0K. ERβ1-positive T47D cells were predominantly unmethylated at both promoter 0N and exon 0N, whereas BT-20 (ERβ1-negative) and MDA-MB-453 (low ERβ1 expression) were mainly methylated (Fig. 3B). Promoter 0K was unmethylated in all cell lines. Parallel MSP analysis showed BT-20 and MDA-MB-453 cells were methylated at promoter 0N and exon 0N, whereas T47D was unmethylated at promoter 0N with partial methylation at exon 0N (Fig. 3C). Methylation at ERβ promoter 0N and exon 0N was negatively associated with ERβ1 expression in breast cancer cell lines (Fig. 3D). As promoter 0K was unmethylated in our cell line panel we did not undertake MSP for promoter 0K. These data suggest that epigenetic modification at promoter 0N and exon 0N is a key regulator of ERβ1 expression in breast cancer cells.
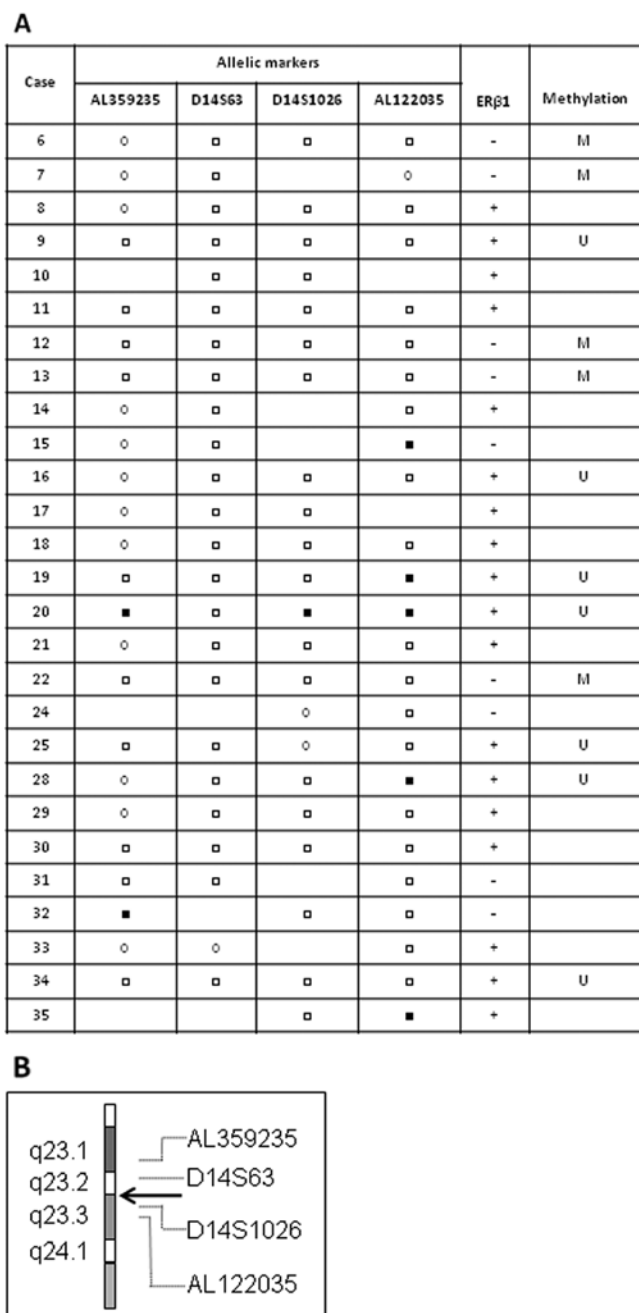
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Case	Allelic markers				ERβ1	Methylation
	AL359235	D14S63	D14S1026	AL122035		
6	○	□	□	□	-	M
7	○	□		○	-	M
8	○	□	□	□	+	
9	□	□	□	□	+	U
10		□	□		+	
11	□	□	□	□	+	
12	□	□	□	□	-	M
13	□	□	□	□	-	M
14	○	□		□	+	
15	○	□		■	-	
16	○	□	□	□	+	U
17	○	□	□		+	
18	○	□	□	□	+	
19	□	□	□	■	+	U
20	■	□	■	■	+	U
21	○	□	□	□	+	
22	□	□	□	□	-	M
24		□	○	□	-	
25	□	□	○	□	+	U
28	○	□	□	■	+	U
29	○	□	□	□	+	
30	□	□	□	□	+	
31	□	□		□	-	
32	■		□	□	-	
33	○	○		□	+	
34	□	□	□	□	+	U
35			□	■	+	

B

Figure 2. Analysis of LOH in 27 breast tumours. LOH and methylation profiles with respect to ERβ1 status in breast cancer (A). □, retained; ■, LOH; ○, non-informative (homozygous); no symbol, insufficient DNA for analysis; M, methylated; U, unmethylated. Positions of allelic markers on chromosome 14q (B). Arrow refers to genomic location of ERβ at 63.8 Mb on chromosome 14 and dotted lines to the relative position of each allelic marker.

Relationship between ERβ1 mRNA expression and promoter methylation patterns in primary human breast tumours and matched normal material. Having detected aberrant methylation of ERβ promoter 0N and exon 0N in human breast cancer cell lines, MSP analysis was subsequently conducted on primary breast tumours and tissue from 3 matched normal tumour pairs to determine the methylation pattern. MSP was used as whole tissue extracts were analyzed containing a mixture of both cancerous and non-cancerous cells, making the detection of changes specific to cancerous cells challenging. The sensitivity of MSP allows for detection of aberrantly methylated alleles even if they contribute relatively little to the overall DNA in a sample (21). As shown by a representative MSP analysis (Fig. 4), promoter 0N (A) and exon 0N (B) were differentially methylated. As promoter 0K was unmethylated in our cell line panel we did not study this in clinical samples. Hypermethylation of ERβ promoter 0N was observed in 7/24 (29%) of breast tumour samples, whereas hypermethylation of ERβ exon 0N was found in 16/24 cases (66%). No evidence of promoter 0N and exon 0N methylation was observed in 17/24 (71%) and 8/24 (33%) breast cancer specimens, respectively. Promoter 0N or exon 0N methylation was either undetectable (n=4) or weakly detectable (n=2) in normal breast tissue adjacent to tumours from the same patients (data not shown). These results suggested that promoter 0N and exon 0N methylation in the ERβ gene is a common feature of breast carcinoma and may account for the frequent down-regulation of ERβ1. The relationship between ERβ promoter 0N and exon 0N methylation, and ERβ1 mRNA expression in breast tumours is shown in Fig. 5. ERβ1 mRNA expression was significantly reduced in tumour versus normal samples, with no significant difference in ERβ1 expression between the promoter 0N methylated and unmethylated groups. In contrast, breast cancers with methylated exon 0N sequences exhibited a significant down-regulation of ERβ1 mRNA expression, compared to the unmethylated exon 0N samples (P=0.03; Mann-Whitney). This suggests methylation at exon 0N, rather than promoter 0N, may be an important regulatory event leading to ERβ1 silencing in primary breast cancers.

Pharmacological restoration of ERβ1 expression after in vitro DNA demethylation and histone deacetylation. To determine if ERβ promoter methylation was functionally correlated with ERβ1 silencing, ERβ1-negative or -low breast cancer cell lines, BT-20 and MDA-MB-453, were treated with either 5-aza-dC, TSA or both. As shown in Fig. 6A, both cell lines expressed low levels of ERβ1 mRNA, and ERβ mRNAs containing untranslated exons (0N or 0K). MDA-MB-453 cells had partial methylation of promoter 0N and exon 0N and treatment with 5-aza-dC was sufficient to induce ERβ1 and mRNAs containing untranslated exons (0N or 0K) with no additional re-expression seen following combination treatment with TSA (Fig. 6B). When BT-20 cells (complete methylation of both ERβ promoter 0N and exon 0N), were exposed to 5-aza-dC and TSA, ERβ1 mRNA expression was restored (Fig. 6B). Interestingly, our results are the first to show that the combination of 5-aza-dC and TSA had a synergistic effect in these breast cells and greatly enhanced expression of ERβ1 and transcripts containing the untranslated exon 0N with negligible effects on mRNAs containing exon 0K.

Discussion

It is well recognized that ERβ1 is frequently down-regulated in breast cancer compared with normal tissue (3,4), however little is known about the mechanisms responsible for this down-regulation. Here, we present evidence that ERβ1 expression is down-regulated in breast cancer cells epigenetically but not by LOH. We have shown that aberrant methylation of CpG islands, located in promoter 0N and exon 0N, is involved in the regulation of ERβ1 expression in epithelial breast cancer cell lines and primary breast cancers. We have also demonstrated that a combination of de-methylating agents and HDAC inhibi-
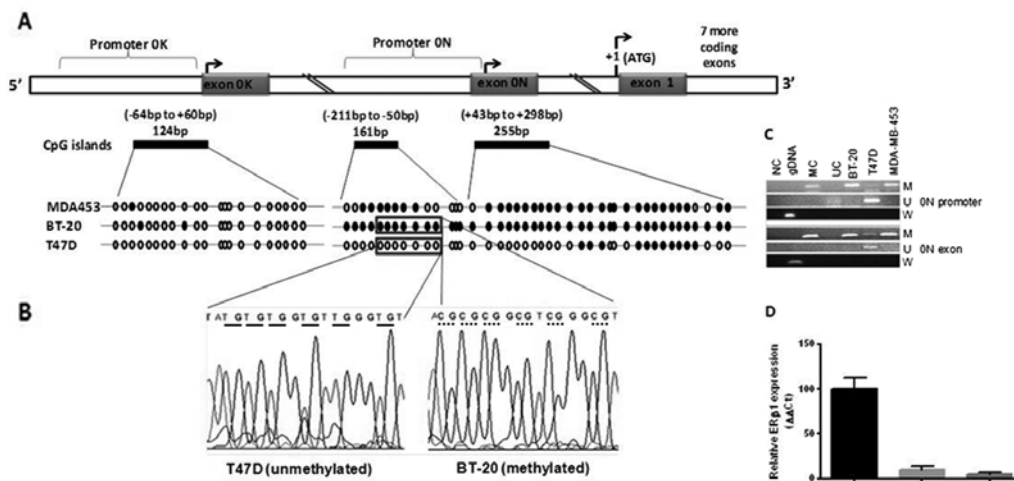
Figure 3. ERβ1 mRNA expression and methylation status of its promoters in breast cell lines. Example of BSP (A), in 3 breast cancer cell lines. Circles represent CpG sites (solid, methylated; open, unmethylated). Complete methylation of promoter 0N was seen in BT-20, partial methylation in MDA-MB-453 and mostly unmethylated in T47D cells. Promoter 0K was mainly unmethylated in all cell lines. (B), Representative DNA sequencing of promoter 0N from methylated (BT20, dotted underline) and unmethylated (T47D, solid underline) cells are shown. (C), MSP shows methylation of promoter 0N in BT20 and MDA-MB-453 cells but not in T47D. Exon 0N was methylated in all 3 cell lines. U, PCR products amplified with unmethylated primers. M, amplified products with methylated primers. Wild-type (W) primer set served as a control for annealing to methylated or unmethylated DNA without bisulphite modification. NG, negative control; GDNA, genomic DNA; MC, methylated converted DNA control; UC, unmethylated converted DNA control. (D), ERβ1 mRNA expression in ERβ1⁺ T47D and ERβ1⁻ BT-20 and ERβ1-low MDA-MB-453 breast cancer cells is shown.
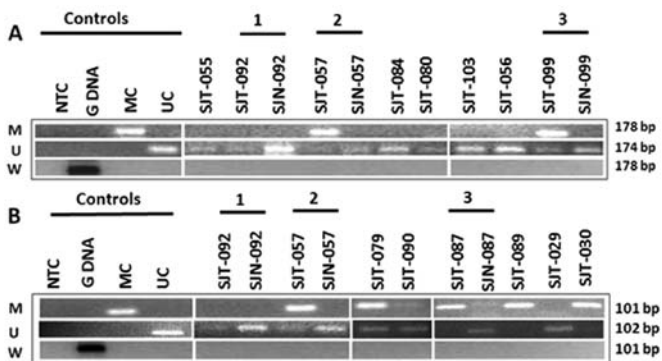

Figure 4. Promoter methylation patterns in primary human breast tumours and matched normal material. MSP analysis of (A) promoter 0N, and (B) exon 0N in normal breast (SJN) and primary breast invasive carcinomas (SJT). Promoters 0N and exon 0N were differentially methylated. M, methylation-specific primers. U, unmethylation-specific primers. W (wild-type) primer set served as a control for annealing to methylated or unmethylated DNA without bisulphite modification. NG, negative control; GDNA, genomic DNA; MC, methylated converted DNA control; UC, unmethylated converted DNA control.
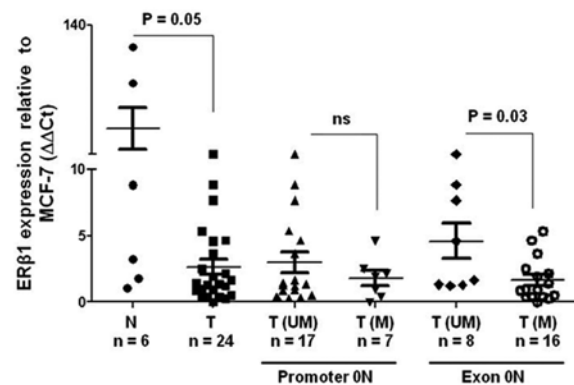

Figure 5. Relationship between ERβ1 mRNA expression and promoter 0N and exon 0N methylation in primary human breast tumours. Scatter plot illustrating significant down-regulation of ERβ1 expression assessed by QRT-PCR between normal breast and breast tumours (P=0.05, Mann-Whitney U-test) and the significant association between ERβ1 expression and exon 0N but not promoter 0N methylation in these tissues. Exon 0N methylated tumours showed significant down-regulation of ERβ1 expression as compared with exon 0N unmethylated tumours (P=0.03, Mann-Whitney U-test). Horizontal lines represent group medians and vertical lines: range with ± SE.

tors can have a synergistic effect, greatly enhancing expression of ERβ1 mRNAs derived from promoter 0N. Importantly, our data suggest that this combination might offer a therapeutic approach for the treatment and/or chemoprevention of some ERβ1-negative breast tumours.

BSP and MSP were used to examine the methylation status of ERβ promoters (0N and 0K) and the downstream adjacent untranslated exon 0N in breast cancer cell lines. Our results showed that CpG islands located in promoter 0N and exon 0N were differentially methylated in breast cells with differing ERβ1 statuses. Promoter 0K was unmethylated in our cell line panel. ERβ1 mRNA expression was inversely associated with the methylation status of both promoter 0N and exon 0N, but not promoter 0K in these breast cell lines, suggesting that CpG islands located in promoter 0N and exon 0N are important regu-

latory sites for the regulation of ERβ1 expression. Zhao *et al* showed a similar negative association between the expression of ERβ1 and -β2 mRNA and the methylation status of exon 0N in breast cells (10). Likewise, others have shown a negative correlation between total ERβ expression and methylation of promoter 0N and exon 0N in various human cancers (8,11,22). Interestingly, we have shown that ERβ1 mRNA transcripts in some breast cells predominantly contain the untranslated exon 0N rather than exon 0K (13), indicating that promoter 0N and exon 0N may be critical regulatory regions for the control of this specific ERβ isoform.

Next, we examined whether a correlation exists between the methylation status of either ERβ promoter 0N and/or
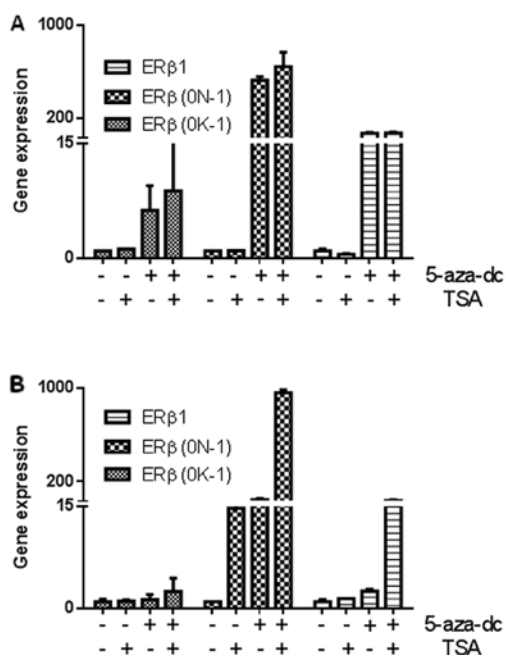
Figure 6. Pharmacological restoration of ERβ1 mRNA and ERβ 5'-UTR expression. MDA-MB-453 (A) and BT20 (B) cells were treated with either 5-aza-dC, TSA or both. In BT20 cells which had complete methylation of 0N, both agents were required for induction of ERβ1 mRNA and ERβ mRNAs containing exon 0N [ERβ (0N-1)] but not ERβ mRNAs containing exon 0K [ERβ (0K-1)]. In MDA-MB-453 cells treatment with 5-aza-dC was sufficient to induce ERβ1 and ERβ (0N-1) expression with no additional re-expression seen with TSA and little effects on ERβ (0K-1).

untranslated exon 0N and ERβ1 expression in primary breast cancers. ERβ1 expression was significantly down-regulated in breast tumours compared with normal breast tissue, therefore we examined whether this might have been caused by the hypermethylation of ERβ promoter 0N and/or the untranslated exon 0N. MSP analysis of promoter 0N and exon 0N revealed that CpG islands within these two regions were differentially methylated. Our data showed a total of 7/24 (29%) cases had detectable promoter 0N methylation, whereas 16/24 (67%) cases had detectable exon 0N methylation. No methylation of promoter 0N or exon 0N was found in four cases of normal breast tissue, whereas two normal samples showed a weak methylation signal (data not shown). Data for the methylation status in normal breast tissue remains inconclusive due to the small cohort size used in our study. We also found that ERβ1 expression was significantly down-regulated in methylated-exon 0N breast tumours compared with unmethylated breast tumours, confirming that DNA hypermethylation might have caused epigenetic silencing of ERβ1 expression in these primary breast cancers. This compliments a recent study that used MSP to measure ERβ methylation in DNA extracted from primary invasive ductal breast tumours and circulating DNA in a cohort of Indian patients (23). In late stage cancers they showed a significant correlation between methylation status and loss of expression of total ERβ protein. Our data are also consistent with previous reports, which have estimated the methylation of exon 0N by direct BSP in breast clinical samples (10) and various other cancers (8,11,12,22,24, 25). Hierarchical clustering previously identified three methylation 'hotspots' within two ERβ CpG islands located within promoter 0N and

exon 0N in prostate cancer cells (11). This study suggested two mechanisms responsible for methylation at promoter 0N and exon 0N. The first involves methylation seeding to first establish methylation at promoter 0N and exon 0N as a stochastic phenomenon at low levels in normal and tumour cells (11). This may provide an explanation for the weak methylation signal detected in two cases of normal breast tissue in our study. The second mechanism involves methylation spreading, which first occurs at the CpG island located within exon 0N and then extends to promoter 0N (11). Thus, it has been suggested that DNA methyltransferases (DNMTs) have a lower opportunity to access the CpG islands at promoter 0N due to steric hindrance from transcription-initiation complexes and upstream enhancer sequences, which are occupied with the binding of transcription factors. In contrast, the CpG sites within exon 0N are more accessible to DNMTs and therefore have a greater opportunity to be methylated first (11). These suggestions are in agreement with our findings in clinical samples, where exon 0N had a higher frequency of methylation compared with promoter 0N and suggest that aberrant methylation at exon 0N is the key regulatory site for ERβ1 expression.

Next, we examined whether expression of ERβ1 mRNA could be pharmacologically restored following *in vitro* DNA de-methylation and histone acetylation. We used cell lines with zero or minimal ERβ1 expression (BT-20, MDA-MB-453, respectively), which showed high frequencies of methylation at promoter 0N and exon 0N, to examine whether 5-aza-dC and TSA have a synergistic effect on ERβ1 re-expression. In MDA-MB-453 cells, 5-aza-dC alone was sufficient to reactivate expression of ERβ1 mRNA derived from promoter 0N, but not from promoter 0K, with no additional significant restoration observed following TSA treatment. Importantly, in BT-20 cells TSA alone had little effect on re-expression of ERβ1 mRNAs derived from promoter 0N. In contrast, 5-aza-dC greatly enhanced re-expression of ERβ1 mRNA derived from promoter 0N only. Strikingly, a combination of de-methylating agents and HDAC inhibitors had a synergistic effect on the restoration of ERβ1 mRNA derived from promoter 0N, re-activating ERβ1 more than with either agent alone. To our knowledge, we are the first to show that this combination can enhance expression of the ERβ1 isoform in breast cancer. It is worth noting that it remains unknown if re-expressed ERβ1 mRNA is translated into functional protein. In particular, we have shown that untranslated exons have potent and differential influences on expression acting at the level of translation in a cell-specific manner (13). We were unable to address this issue in the present study due to technical difficulties with ERβ1-specific antibodies preventing us from assessing ERβ1 protein expression in parallel by western blot analysis.

In conclusion, our results add to the growing body of evidence showing that ERβ1 is regulated at multiple levels in breast cancer (13,20,26,27). Our data indicate that epigenetic mechanisms involving DNA hypermethylation and/or histone acetylation, at sites adjacent to promoter 0N, play key roles in the regulation of ERβ1 expression. Importantly, our data indicate that a combination of de-methylating agents and HDAC inhibitors might provide an epigenetic approach for the treatment and/or chemoprevention of some ERβ1-negative breast cancers. While the prospect of introducing epigenetic therapy to the clinic presents several clinical and translational

challenges, our results warrant further preclinical investigation; in particular to define the precise mechanism of action of these agents and to consider their potential development for future clinical trials. This is already ongoing for some types of haematological (28) and solid (14,29) malignancies.

Acknowledgements

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