Anti-proliferative effect of estrogen in breast cancer cells that re-express ER α is mediated by aberrant regulation of cell cycle genes

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Abstract

Estrogen receptor (ER)-negative breast carcinomas do not respond to hormone therapy, making their effective treatment very difficult. The re-expression of ER α in ER-negative MDA-MB-231 breast cancer cells has been used as a model system, in which hormone-dependent responses can be restored. Paradoxically, in contrast to the mitogenic activity of 17 β -estradiol (E2) in ER-positive breast cancer cells, E2 suppresses proliferation in ER-negative breast cancer cells in which ER α has been re-expressed. We have used global gene expression profiling to investigate the mechanism by which E2 suppresses proliferation in MDA-MB-231 cells that express ER α through adenoviral infection. We show that a number of genes known to promote cell proliferation and survival are repressed by E2 in these cells. These include genes encoding the anti-apoptosis factor *SURVIVIN*, positive cell cycle regulators (*CDC2*, *CYCLIN B1*, *CYCLIN B2*, *CYCLIN G1*, *CHK1*, *BUB3*, *STK6*, *SKB1*, *CSE1 L*) and chromosome replication proteins (*MCM2*, *MCM3*, *FEN1*, *RRM2*, *TOP2A*, *RFC1*). In parallel, E2-induced the expression of the negative cell cycle regulators *KIP2* and *QUIESCIN Q6*, and the tumour-suppressor genes *E-CADHERIN* and *NBL1*. Strikingly, the expression of several of these genes is regulated in the opposite direction by E2 compared with their regulation in ER-positive MCF-7 cells. Together, these data suggest a mechanism for the E2-dependent suppression of proliferation in ER-negative breast cancer cells into which ER α has been reintroduced.

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Introduction

Estrogens are important regulators of growth and differentiation in the normal mammary gland and participate in the development and progression of breast cancer (Pike *et al.* 1993). The mitogenic effects of estrogens on breast epithelial cells are mediated, at least in part, via the altered expression of genes involved in cell cycle regulation (Prall *et al.* 1997). Transcriptional regulation of estrogen-responsive genes is mediated by two members of the nuclear receptor superfamily, estrogen receptor (ER) α and ER β . These ERs function as ligand-activated transcription factors that recruit a variety of coregulator proteins to activate or repress the expression of estrogen-responsive genes (Moggs & Orphanides 2001, Hall *et al.* 2001, McKenna and O'Malley 2002, Tremblay and Giguere 2002).

ER antagonists are used widely as therapeutic agents in the treatment of ER-positive breast cancers (Vogel 2003). In contrast, ER-negative breast cancers cannot be controlled by hormone therapy, making their effective treatment very difficult. This led to the suggestion that re-introducing ER into these cells would allow them to

be controlled using anti-estrogen therapies. However, paradoxically, the reintroduction of ER α into ER-negative breast cancer cells results in the suppression of proliferation by 17 β -estradiol (E2) (Garcia *et al.* 1992, Levenson and Jordan 1994). The mechanism underlying this anti-proliferative effect of E2 in these cells is not known.

We have used global gene expression profiling to identify the molecular pathways through which estrogens suppress proliferation in ER-negative MDA-MB-231 breast cancer cells that re-express ER α . Our data reveal that, in these cells, E2 regulates the expression of a number of genes involved in cell proliferation and survival that have been previously associated with mitogenic stimulation by estrogens. However, strikingly, many of these genes are regulated in the opposite direction compared with their response in ER-positive MCF-7 breast cancer cells exposed to estrogens. Identification of the molecular networks associated with the suppression of proliferation in ER-negative breast cancer cells may allow the development of new strategies to control the growth of ER-negative breast tumours.

Materials and methods

Cell culture

MDA-MB-231 cells were cultured routinely at 37 °C in humidified chambers at 5% CO₂ in Minimal Essential Media (MEM) supplemented with non-essential amino acids, 2 mM glutamine, Penicillin, Streptomycin and 5% charcoal-dextran-treated fetal calf serum. HEK293 cells were cultured as described in He *et al.* (1998). MCF-7 cells were maintained at 37 °C in humidified chambers at 5% CO₂ in RPMI 1640 media containing phenol red, 2 mM glutamine, Penicillin, Streptomycin and 10% heat-inactivated fetal bovine serum. Prior to dosing with vehicle control (ethanol) or E2 (Sigma), MCF-7 cells were incubated for 4 days in RPMI 1640 media without phenol red and containing 2 mM glutamine, Penicillin, Streptomycin and 5% charcoal-dextran-treated fetal bovine serum.

Adenoviral system used to express ER α in human MDA-MB-231 cells

Full-length human ER α (1–595; Green *et al.* 1986) cDNA was cloned into the shuttle vector pAdTrack-cytomegalovirus (CMV). The resulting construct was linearised and cotransformed into *E. coli* BJ5183 cells, together with an adenoviral backbone plasmid, pAdEasy-1 (He *et al.* 1998, see also Murphy & Orphanides 2002). Selected recombinants were analysed by restriction endonuclease digestion. Finally, recombinant plasmids encoding ER α were linearised and transfected into an adenovirus packaging cell line, HEK 293, in order to generate recombinant adenovirus that expresses ER α (Ad-ER α). A control recombinant adenovirus construct containing the *E. coli* β -galactosidase gene (Ad-LacZ) was constructed in a similar manner. Recombinant adenovirus was harvested from HEK293 cells using Arklone extraction, purified by ultracentrifugation through a caesium chloride gradient and dialysed in a Slide-a-lyser cassette (Perbio Science, Cramlington, Northumbria, UK). The purified adenovirus was aliquoted and stored at –80 °C. Each virus stock was titered in MDA-MB-231 cells, to determine the multiplicity of infection (MOI). For analysis of E2-dependent transcriptional responses, MDA-MB-231 cells were transfected with either Ad-LacZ or Ad-ER α at a MOI of 2500. Since both Ad-LacZ and Ad-ER α were engineered to co-express the green fluorescent protein (GFP), infection levels could be quantified by monitoring the expression of the GFP using fluorescent microscopy (% Infectivity=GFP cells/total cells $\times$ 100). Twelve hours after the initial infection, transfected MDA-MB-231 cells were photographed using both light and fluorescent microscopy, to determine the % of GFP-expressing cells. Both Ad-LacZ and Ad-ER α reproducibly gave between 90 and 100% infectivity of

MDA-MB-231 cells under these conditions. Expression of ER α in cells transfected with Ad-ER α was confirmed by Northern blot analysis (Sambrook *et al.* 1989) using 1% denaturing agarose gels containing 10 μ g total RNA per lane and a 417 bp ³²P-labelled probe generated by PCR of the ER α cDNA (forward: 5'-ATACGAAAAGACCGAAGAGGAG-3'; reverse: 5'-CCAGACGA GACCAATCATCA-3').

Reporter assay for ER-mediated transcription in MDA-MB-231 cells infected with adenovirus encoding ER α

MDA-MB-231 cells infected for 24 h with adenovirus (MOI=2500) encoding either β -galactosidase (control; Ad-LacZ) or ER α (Ad-ER α) were co-transfected with a luciferase reporter construct that contained two copies of the vitellogenin estrogen response element (ERE) and also with a CMV-phRenilla plasmid (Promega), to measure transfection efficiency. After 24 h, cells were treated with 0.01% ethanol, as a control, or E2 in fresh medium at the concentrations indicated. Cells were incubated for a further 24 h before harvesting for lysis and luciferase assays using the Dual-luciferase assay system (Promega). Results are expressed in terms of relative luciferase activity after normalisation for renilla luciferase activity $\pm$ S.D.

RT-PCR analysis of the endogenous estrogen-responsive gene pS2 in MDA-MB-231 cells infected with adenovirus encoding ER α

Cells infected for 24 h with adenovirus (MOI=2500) encoding β -galactosidase (control; Ad-LacZ) or ER α (Ad-ER α) were treated for 24, 30 and 50 h with 0.01% ethanol, as a control, or 10^{–8} M E2. Total RNA was isolated using Trizol reagent (Life Technologies) and purified according to the manufacturer's instructions. DNA-free RNA was prepared using a DNA-free kit (Ambion, Huntingdon, Cambs, UK) according to the manufacturer's instructions. DNase-treated RNA (1 μ g) was reverse transcribed with oligo-dT using the Superscript II kit (Invitrogen) according to the manufacturer's instructions. PCR analysis of pS2 gene expression was performed using the oligonucleotide primers 5'-TGACTCGGGGTGCCT TTGAG-3' and 5'-GTGAGCCGAGGCACAGCTG CAG-3'. The β -actin gene (5'-ACCATGGATGATG ATATCGC-3' and 5'-ACATGGCTGGGGTGTTG AAG-3') was used as a control.

Cell proliferation assay

Cells were maintained in MEM containing 5% CDFCS and were seeded at 5000 cells/well in 24-well dishes in the same media. After overnight infection with Ad-LacZ

Table 1 Taqman Gene expression assays used for quantitative real-time PCR

Gene symbol	Affymetrix probe set	Taqman gene expression assay	GenBank accession number	Exon location	Central nucleotide location
MCM2	202107_s_at	Hs00170472_m1	NM_004526	2/3	299
MCM3	201555_at	Hs00172459_m1	NM_002388	12/13	1938
MCM7	201112_s_at	Hs00428518_m1	NM_182776	9/10	1794
CSE1L	202870_s_at	Hs00169158_m1	NM_001316	14/15	1614
CDC2	210559_s_at	Hs00176469_m1	NM_001786	12	107
CDC20	203213_at	Hs00415851_g1	NM_001255	12	59
BUB1	209642_at	Hs00177821_m1	NM_004336	1/2	73
BIRC5	202095_at	Hs00153353_m1	NM_001168	3/4	393
FEN1	204768_s_at	Hs00748727_s1	NM_004111	2	643
FOSL1	204420_at	Hs00759776_s1	NM_005438	4	1103
RPLP0/36B4	201033_x_at	Hs99999902_m1	NM_001002	—	267

or Ad-ER α (MOI=2500), the medium was removed and replaced with fresh medium containing either 0.01% ethanol, as a control, or 10^{-8} M E2 for 24 h. Cells were then incubated with 1 μ Ci [methyl- 3 H]thymidine at 37 °C for 4 h. Plates were sequentially washed and fixed with ice cold PBS, 10% TCA, MeOH and the incorporated label was recovered by incubation of the wells in 0.5 M NaOH for 30 min at 37 °C. Lysates were transferred to vials containing Optiphase 'hi-safe' 3 scintillation cocktail (PerkinElmer Life Sciences, Beaconsfield, Bucks, UK) and [3 H]thymidine incorporation (c.p.m.) was determined in a scintillation counter.

Affymetrix GeneChip transcript profiling and data analysis for MDA-MB-231 cells infected with adenovirus encoding ER α

Cells infected for 24 h with adenovirus (MOI=2500) encoding β -galactosidase (control; Ad-LacZ) or ER α (Ad-ER α) were treated for 48 h with 0.01% ethanol, as a control, or 10^{-8} M E2. Total RNA was isolated using Trizol reagent (Life Technologies) and purified according to the manufacturer's instructions. Biotin-Labeled complementary RNAs were synthesized using the Bioarray HighYield RNA Transcript Labeling Kit (Affymetrix, High Wycombe, Bucks, UK) from 5 μ g total RNA and hybridised to Affymetrix human U133A GeneChips as described in the Affymetrix GeneChip Technical Manual. Microarrays were then scanned and the intensities were averaged using Microarray Analysis Suite 5.0 (Affymetrix). The mean signal intensity of each array was globally normalized to 500. Affymetrix pivot files were imported into GeneSpring 6.0 (Silicon Genetics, Redwood City, CA, USA) and normalised to the 50th percentile of each GeneChip and to the median of each gene. Normalised data was filtered to exclude genes that lack a present flag or a raw signal strength >500 in any of the treatment groups. The three

independent biological replicates data sets for MDA-MB-231 cells infected Ad-LacZ ($\pm$ E2) or Ad-ER α ($\pm$ E2) were initially filtered using a one-sample Student's *t*-test ($P < 0.05$) to identify statistically differentially expressed genes within each treatment group. The resulting 574 genes were subsequently analysed using a one-way ANOVA test with the following conditions: parametric test assuming equal variance, Benjamini and Hochberg false discovery rate < 0.01 (Benjamini & Hochberg 1995), Tukey post-hoc testing (see <http://www.silicongenetics.com> for further details). Using these criteria, less than 1% of the 88 genes selected by ANOVA can be expected to be significant by chance. Genes with similar expression profiles were grouped together using hierarchical clustering (Pearson correlation). Gene names used in this manuscript were derived by homology searching of nucleotide sequence databases (BLASTn) using Affymetrix probe target sequences and the NetAffx (Liu *et al.* 2003) database. All genes described in the figures and text showed statistically significant alterations in expression in all three replicate studies. MIAME (Minimum Information About a Microarray Experiment)-compliant microarray data for the three independent replicate studies were submitted to the Gene Expression Omnibus (GEO) database (GEO 2004).

Quantitative real-time PCR analysis of gene expression

DNA-free RNA was prepared using 'DNA-free' (Ambion) according to the manufacturer's instructions. DNase-treated RNA (0.7 μ g) was reverse transcribed with random hexamers using Superscript III kit (Invitrogen) according to the manufacturer's instructions. All quantitative real-time PCR reactions were carried out using an ABI Prism 7700 sequence detection system (Applied Biosystems, Warrington, Chester, UK).

The thermal cycler conditions were, 2 min at 50 °C and 10 min at 95 °C followed by 15 seconds at 95 °C (denaturation) and 1 min at 60 °C (anneal-extension) for 40 cycles. The total volume for each reaction was 20 µl comprising 9 µl diluted cDNA (0.5 ng/µl), 10 µl Taqman Universal Master mix and 1 µl Taqman gene expression assay (Applied Biosystems). Each Taqman gene expression assay contains forward primer, reverse primer and Taqman MGB probe (primer locations and corresponding gene accession numbers are shown in Table 1). Each RNA sample was assayed in triplicate and the mean Ct value was calculated. The fold change was determined using the $\Delta\Delta C_t$ method. All genes were normalised to the control gene RPLP0/36B4 (Accession number: NM_001002; Laborda 1991).

Results

Reintroduction of functional ER α into ER-negative MDA-MB-231 breast cancer cells by adenoviral transfection

We used a recombinant adenoviral delivery system to examine the molecular mechanisms through which the reintroduction of ER α into ER-negative MDA-MB-231 breast cancer cells confers E2-dependent suppression of proliferation. (He *et al.* 1998, Fig. 1A). Recombinant adenoviruses were engineered to co-express the full-length human ER α cDNA (amino acids 1–595; Green *et al.* 1986) and GFP, as described in the Materials and methods (Ad-ER α). Recombinant adenoviruses containing the *E. coli* LACZ gene in place of the human ER α gene were used as a control (Ad-LacZ). Quantification of infection levels in MDA-MB-231 cells by fluorescent microscopy revealed that GFP was expressed in >90% of cells after infection (Fig. 1B). The expression of a transcript corresponding to the transfected human ER α cDNA was confirmed by Northern blotting (Fig. 1C). Quantitative real-time PCR analysis of ER α gene expression levels (data not shown) revealed that the reintroduction of ER α into ER-negative MDA-MB-231 breast cancer cells by adenoviral transfection results in higher levels (~ 5 -fold) of ER α expression than those found in MCF-7 breast cancer cells, consistent with previous studies (Lazennec & Katzenellenbogen 1999).

To confirm that ER α re-expression in MDA-MB-231 cells was functional, we used a reporter-based transfection assay that measures the ability of ligand-activated ERs to regulate transcription via a consensus multimerised ERE present on a transiently transfected plasmid. In cells infected with Ad-ER α , but not in cells infected with Ad-LacZ, the addition of E2 (10^{-8} M and 10^{-7} M) increased reporter gene expression (4.8- and 3.3-fold respectively; Fig. 2A), demonstrating that adenoviral infection resulted in the expression of transcriptionally active ER α . We next examined the ability of

Figure 1 Reintroduction of ER α into ER-negative MDA-MB-231 breast cancer cells. (A) Recombinant adenovirus engineered to co-express both GFP and human ER α (Ad-ER α) was used to infect MDA-MB-231 cells. Recombinant adenovirus containing the *E. coli* LACZ gene in place of ER α (Ad-LacZ) was used as a control. (B) MDA-MB-231 cell infection efficiencies of greater than 90% were measured routinely using the adenovirus constructs at a MOI of 2500. Left panel: light microscopy of MDA-MB-231 cells 24 hr after infection with Ad-ER α . The efficiency of viral infection was determined by measuring the proportion of cells that exhibit GFP fluorescence (right panel). (C) Northern blot analysis of ER α expression 24 h after Ad-LacZ (lane 1) or Ad-ER α (lane 2) infection of MDA-MB-231 cells.

adenoviral-encoded ER α to regulate endogenous (i.e. chromosomal) genes. For this purpose, we selected the classical estrogen responsive gene, pS2 (also known as TFF1; Davidson *et al.* 1986), which contains a consensus ERE in its promoter region and is regulated directly by ERs (Berry *et al.* 1989). Cells infected with Ad-LacZ or Ad-ER α were treated with 10^{-8} M E2 or vehicle (ethanol) for 24, 30 or 50 h prior to RT-PCR analysis of pS2 gene expression. As expected, pS2 gene expression was induced by E2 in cells expressing ER α (Fig. 2B, lanes 7 to 12), but not in control cells (Fig. 2B, lanes 1 to 6). Therefore, the adenovirus-encoded ER α is capable of activating an endogenous chromosomal gene in the presence of E2.

Figure 3 E2 inhibits proliferation in MDA-MB-231 breast cancer cells that re-express ER α after transfection with Ad-ER α . MDA-MB-231 cells were infected with either Ad-LacZ or Ad-ER α . The cells were then treated with control vehicle (0.01% ethanol) or E2 at the concentrations indicated for 24 h. Proliferation was measured by [methyl- 3 H]thymidine incorporation. Values are the mean $\pm$ s.d. of three determinations. Similar results were obtained in two independent experiments.

We next examined the effect of adenoviral transfection of ER α on cell proliferation. The re-expression of human ER α in MDA-MB-231 breast cancer cells restores hormone responsiveness, but leads to the inhibition of proliferation by E2 (Garcia *et al.*, 1992, Levenson and Jordan 1994, Lazennec and Katzenellenbogen 1999). Consistent with these observations, E2 caused a 3-fold decrease in proliferation in MDA-MB-231 cells that re-express ER α (Fig. 3).

We conclude that adenoviral transfection of ER α into the ER-negative MDA-MB-231 breast cancer cell line confers both transcriptional and anti-proliferative responses to E2. Therefore, this model system is suitable for investigating the mechanism by which E2 suppresses proliferation in ER-negative cells that re-express ER α .

Figure 2 MDA-MB-231 cells infected with adenovirus encoding ER α contain transcriptionally active ERs. (A) Cells were infected with adenovirus encoding β -galactosidase (Ad-LacZ control) or ER α (Ad-ER α) and were co-transfected with a luciferase reporter construct, that contained two copies of the vitellogenin ERE, and with the CMV-phRenilla plasmid (to measure transfection efficiency). After 24 h, cells were treated with 0.01% ethanol, as a control, or estradiol at the concentrations indicated. Results are expressed as relative luciferase activities after normalisation for Renilla luciferase activity $\pm$ s.d. ($n=6$). (B) MDA-MB-231 cells infected with Ad-LacZ or Ad-ER α were treated with vehicle (ethanol) or E2 (10^{-8} M) for the times indicated and the expression of the endogenous pS2 gene was analysed by RT-PCR. The β -actin gene was used as a control.

Figure 4 Microarray analysis of estrogen-responsive genes in MDA-MD-231 breast cancer cells transfected with Ad-LacZ or Ad-ERα. Statistical analysis of Affymetrix HG-U133A GeneChip data was performed on three independent biological replicate studies of MDA-MB-231 cells infected with either Ad-LacZ or Ad-ERα prior to 48 h incubation with either vehicle control (0.01% ethanol) or 10⁻⁸M E2. Differentially expressed genes within each treatment group were identified using a one sample Student's *t*-test ($P < 0.05$). The resulting 547 genes were subsequently filtered using a stringent one-way ANOVA test combined with Benjamini and Hochberg multiple testing correction (false discovery rate < 0.01 ; Benjamini & Hochberg 1995). Using these criteria, less than 1% of the 88 genes shown can be expected to be significant by chance. Genes with similar expression profiles were grouped together using hierarchical clustering (Pearson correlation) and the resulting gene tree is shown. The magnitude of fold-induction or -repression for each gene (relative to the median of its expression across all experimental samples) is indicated by the colour bar. Data shown are based on three replicate studies. Quantitative data for the magnitude of each gene expression change, together with gene descriptions and Affymetrix probe set IDs are shown in Table 2.

Changes in gene expression associated with estrogen-induced suppression of proliferation in ER-negative MDA-MB-231 breast cancer cells that re-express ERα

Statistical analysis of genes regulated by E2

We used microarray gene expression profiling to obtain a holistic view of the endogenous transcriptional targets of ERα in our model system. The expression of 22 483

genes in each of four treatment groups (Ad-LacZ, Ad-LacZ+E2, Ad-ERα and Ad-ERα+E2) was measured using the Affymetrix human GeneChip U133A, and the resulting data were subjected to rigorous statistical analyses (Materials and methods). 574 gene probe sets were selected as being significantly ($P < 0.05$) under- or over-expressed in one or more of the 4 treatment groups using a Student's *t*-test, based on data from three independent biological replicates. A stringent ANOVA

Table 2 Genes regulated by E2 in MDA-MB-231 cells that re-express ER α

Affymetrix probe set	Gene name		Biological pathway/ process	Ratio of gene expression E2:AO (mean $\pm$ S.D.)
201531_at	zinc finger protein 36	ZFP36	mRNA catabolism	3.8 $\pm$ 1.9
212442_s_at	LAG1 longevity assurance homolog 6	LASS6		2.2 $\pm$ 0.6
201339_s_at	sterol carrier protein 2	SCP2	steroid biosynthesis	1.9 $\pm$ 0.5
218002_s_at	chemokine ligand 14	CXCL14		30.3 $\pm$ 10.7
210517_s_at	A kinase anchor protein 12	AKAP12		3.2 $\pm$ 0.6
209304_x_at	growth arrest and DNA damage-inducible, beta	GADD45B	cell cycle regulation/apoptosis	4.1 $\pm$ 1.5
210059_s_at	mitogen-activated protein kinase 13	MAPK13	regulation of translation/signaling	3.1 $\pm$ 1.3
211168_s_at	regulator of nonsense transcripts 1	RENT1	mRNA catabolism	3.2 $\pm$ 1.6
219480_at	snail homolog 1	SNAI1	transcription	12.6 $\pm$ 6.3
205016_at	transforming growth factor, alpha	TGFA	regulation of cell cycle/signaling	4.3 $\pm$ 1.0
203058_s_at	3'-phosphoadenosine 5'-phosphosulfate synthase 2	PAPSS2	nucleic acid metabolism	8.4 $\pm$ 3.8
205206_at	Kallmann syndrome 1 sequence	KAL1	cell adhesion	2.6 $\pm$ 0.7
40829_at	WD and tetratricopeptide repeats 1	WDTIC1		1.6 $\pm$ 0.3
218322_s_at	acyl-CoA synthetase long-chain family member 5	ACSL5	fatty acid metabolism	2.9 $\pm$ 0.8
204158_s_at	T-cell, immune regulator 1, ATPase, H ⁺ transporting	TCIRG1	defense response/proliferation	5.9 $\pm$ 1.8
200884_at	creatine kinase	CKB	amino acid metabolism	5.4 $\pm$ 1.7
218532_s_at	hypothetical protein FLJ20152	FLJ20152		10.7 $\pm$ 5.5
205105_at	mannosidase, alpha 2A1	MAN2A1	carbohydrate metabolism	1.9 $\pm$ 0.4
201720_s_at	Lysosomal-associated multispanning membrane protein-5	LAPTM5		3.9 $\pm$ 0.8
205899_at	cyclin A1	CCNA1	regulation of cell cycle	5.9 $\pm$ 3.1
31637_s_at	nuclear receptor subfamily 1, group D, member 1	NR1D1	transcription/circadian rhythm regulator	2.2 $\pm$ 0.4
203060_s_at	3'-phosphoadenosine 5'-phosphosulfate synthase 2	PAPSS2	nucleic acid metabolism	6.9 $\pm$ 1.9
201721_s_at	Lysosomal-associated multispanning membrane protein-5	LAPTM5		4.3 $\pm$ 1.5
218692_at	hypothetical protein FLJ20366	FLJ20366		3.0 $\pm$ 0.6
205009_at	trefoil factor 1	TFF1	cell growth/defense response	22.3 $\pm$ 4.9
210357_s_at	spermine oxidase	SMOX	electron transport	4.4 $\pm$ 1.2
211429_s_at	serine protease inhibitor, clade A, member 1	SERPINA1	acute-phase response	8.5 $\pm$ 1.2
220486_x_at	hypothetical protein FLJ22679	FLJ22679		1.9 $\pm$ 0.2
204326_x_at	metallothionein 1X	MT1X	response to metal ion	2.3 $\pm$ 0.3
202833_s_at	serine protease inhibitor, clade A, member 1	SERPINA1	acute-phase response	10.9 $\pm$ 0.3
218749_s_at	solute carrier family 24, member 6	SLC24A6		4.2 $\pm$ 1.6
203059_s_at	3'-phosphoadenosine 5'-phosphosulfate synthase 2	PAPSS2	nucleic acid metabolism	8.4 $\pm$ 1.7
213004_at	angiotensin-like 2	ANGPTL2	development	6.9 $\pm$ 2.3
217744_s_at	TP53 apoptosis effector	PERP	apoptosis	2.9 $\pm$ 1.2
207935_s_at	keratin 13	KRT13	cytoskeleton	16.8 $\pm$ 0.4
212216_at	putative amino acid transporter	KIAA0436		1.7 $\pm$ 0.3
203071_at	semaphorin 3B	SEMA3B	signaling	7.7 $\pm$ 1.9
202267_at	laminin, gamma 2	LAMC2	cell adhesion	4.2 $\pm$ 1.3
201131_s_at	E-cadherin	CDH1	cell adhesion	35.2 $\pm$ 35.4

Table 2 Continued

	Gene name		Biological pathway/ process	Ratio of gene expression E2:AO (mean±S.D.)
Affymetrix probe set				
202950_at	crystallin, zeta	CRYZ		2.4±0.5
216323_x_at	tubulin, alpha 2	TUBA2	microtubule	4.0±1.8
203661_s_at	tropomodulin 1	TMOD1	cytoskeleton	7.5±2.9
202053_s_at	aldehyde dehydrogenase 3 family, member A2	ALDH3A2	glycolysis/ascorbate, aldarate and fatty acid metabolism	2.0±0.2
204368_at	solute carrier organic anion transporter family, member 2A1	SLCO2A1	lipid transport	8.9±0.8
209035_at	midkine	MDK	regulation of cell cycle/signaling	7.9±4.2
204664_at	alkaline phosphatase	ALPP	glycerolipid metabolism/folate biosynthesis	129.1±86.8
213308_at	SH3 and multiple ankyrin repeat domains 2	SHANK2	signaling	4.1±1.7
214476_at	trefoil factor 2	TFF2	defense response	35.1±20.8
213001_at	angiopoietin-like 2	ANGPTL2	development	3.9±0.8
217165_x_at	metallothionein 2A	MT2A	copper ion homeostasis	2.8±0.5
203585_at	zinc finger protein 185	ZNF185		3.0±0.6
210740_s_at	inositol 1,3,4-triphosphate 5/6 kinase	ITPK1	signal transduction	2.7±0.8
206461_x_at	metallothionein 1H	MT1H	response to metal ion	2.8±1.2
205068_s_at	Rho GTPase activating protein 26	ARHGAP26	growth/cytoskeleton	2.6±0.2
212057_at	KIAA0182 protein	KIAA0182		2.6±1.1
202458_at	serine protease 23	SPUVE	proteolysis	2.6±0.1
201858_s_at	proteoglycan 1	PRG1		2.5±0.5
219369_s_at	OUT domain, ubiquitin aldehyde binding 2	OTUB2		4.2±1.5
211474_s_at	serine protease inhibitor, clade B, member 6	SERPINB6	acute-phase response	2.2±0.4
213909_at	leucine rich repeat containing 15	LRRC15		3.1±1.0
202756_s_at	glypican 1	GPC1	development	4.7±2.2
219045_at	ras homolog gene family, member 5	RHOF	signaling	4.0±2.1
205148_s_at	chloride channel 4	CLCN4	ion transport	3.1±1.3
202976_s_at	Rho-related BTB domain containing 3	RHOBTB3		2.8±1.3
201349_at	solute carrier family 9, isoform 3 regulator 1	SLC9A3R1	signaling/cytoskeleton	7.5±5.0
221667_s_at	heat shock protein 8	HSPB8		7.0±2.5
215189_at	keratin 6	KRTHB6		11.8±8.5
210827_s_at	E74-like factor 3	ELF3	transcription	2.1±0.4
212135_s_at	ATPase, Ca ²⁺ transporting	ATP2B4	ion transport	2.2±0.4
210609_s_at	tumor protein p53 inducible protein 3	TP53I3	apoptosis	3.2±0.8
217190_x_at	estrogen receptor alpha	ESR1	transcription/signaling	1.5±0.4
215552_s_at	estrogen receptor alpha	ESR1	transcription/signaling	1.3±0.4
211233_x_at	estrogen receptor alpha	ESR1	transcription/signaling	1.2±0.5
211234_x_at	estrogen receptor alpha	ESR1	transcription/signaling	1.2±0.5
211235_s_at	estrogen receptor alpha	ESR1	transcription/signaling	1.2±0.4
218399_s_at	cell division cycle associated 4	CDCA4	regulation of cell cycle	0.6±0.09
202870_s_at	cell division cycle 20 homolog	CDC20	regulation of cell cycle	0.5±0.1
211519_s_at	kinesin family member 2C	KIF2C	mitosis/proliferation	0.5±0.1
201897_s_at	CDC28 protein kinase subunit 1B	CKS1B	cell proliferation	0.6±0.1
210983_s_at	minichromosome maintenance deficient 7	MCM7	DNA replication	0.5±0.1

Table 2 Continued

	Gene name		Biological pathway/ process	Ratio of gene expression E2:AO (mean±S.D.)
Affymetrix probe set				
202503_s_at	KIAA0101 gene product	KIAA0101		0.5±0.07
204420_at	FOS-like antigen 1	FOSL1	transcription/proliferation	0.4±0.1
208002_s_at	acyl-CoA hydrolase	BACH	lipid metabolism	0.6±0.07
202095_s_at	survivin	BIRC5	cell cycle	0.5±0.06
			regulation/apoptosis	
204521_at	predicted protein clone 23733	HSU79724		0.4±0.1
220155_s_at	bromodomain containing 9	BRD9		0.7±0.08
208994_s_at	peptidyl-prolyl isomerase G	PPIG	protein folding/RNA splicing	1.0±0.06
210006_at	DKFZP564O243 protein	DKFZP564O243	aromatic compound metabolism	0.9±0.05

Eighty-three genes demonstrated significantly induced or repressed expression after E2 stimulation of MDA-MB-231 cells that re-express ER α (based on ANOVA using Benjamini and Hochberg multiple testing correction (false positive discovery rate <0.01; Benjamini & Hochberg 1995)). Mean gene expression ratios $\pm$ S.D. (E2-treated relative to vehicle control; based on three independent biological replicate experiments) are shown together with Affymetrix GeneChip probe set, gene name, biological pathway/process (as of the NetAffx update on 23 June 2004). Two or more Affymetrix GeneChip probe sets for the same gene indicates that probe sets specific for independent regions of the same gene demonstrated estrogen-regulated expression.

test (Benjamini and Hochberg multi-testing correction, false positive rate <0.01; Benjamini & Hochberg 1995) was then applied, resulting in the identification of 88 gene probe sets showing differential expression between one or more of the 4 treatment groups (Fig. 4). Five of these gene probe sets represented the ER α gene, confirming that this gene was re-expressed in MDA-MB-231 cells infected with the ER α adenoviral construct. None of the 88 gene probe sets were E2-responsive in MDA-MB-231 cells transfected with the control adenovirus construct (Ad-LacZ), consistent with the ER-negative status of this cell line. In contrast, 83 probe sets showed a transcriptional response to E2 in MDA-MB-231 cells transfected with Ad-ER α . The magnitude of E2-dependent alterations in gene expression for the 83 gene probe sets, together with their gene ontology descriptions and functional classifications, are shown in Table 2. These genes include the classical E2-responsive gene pS2/TFF1 (Davidson *et al.* 1986, Berry *et al.* 1989) and TGFA, both of which have previously been observed to be up-regulated by E2 after adenoviral transfection of ER α into MDA-MB-231 cells (Lazennec & Katzenellenbogen 1999).

Gene ontology and promoter analysis of ER α -dependent estrogen-responsive genes.

The molecular functions of the 83 E2-responsive genes we identified fall into a broad range of gene ontology classifications (Liu *et al.* 2003, Bard & Rhee 2004), including cell cycle control, signalling, growth factors, transporters, defense responses and cell adhesion (Table 2), highlighting the diverse gene networks and metabolic and cell regulatory pathways through which E2 exerts its

effects on breast cancer cells. Many of these genes have not previously been identified as being E2-responsive in breast cancer cells, and include genes whose function is unknown (e.g. *FLJ20152*, *FLJ20366*, *FLJ22679*; Table 1).

In order to gain more evidence that these genes were regulated directly by ER α , we searched their regulatory regions for EREs, based on similarity to the consensus ERE sequence (Klinge 2001). *In silico* analysis of the sequences found 3000 bp immediately upstream of the transcriptional start site of each of the 83 genes and revealed the presence of one or more candidate EREs (Table 3). This provides further evidence that the genes we have identified are regulated by ER α directly. A number of these ERE motifs have also been identified independently in a recent genome-wide screen for ERE motifs in the human and mouse genomes (Bourdeau *et al.* 2004).

Estrogen represses the expression of genes that promote cell proliferation and survival

Several of the E2-responsive genes we have identified (e.g. *MCM7*, *CDC20*, *CKSB1*, *SURVIVIN*; Table 2) have been shown previously to be involved in the regulation of cell proliferation and survival. Among these genes is: (1) the *MCM7* (minichromosome maintenance deficient 7) gene, encoding a DNA replication licensing factor that functions to limit a cell to a single round of replication per cell cycle (Blow & Hodgson 2002); (2) the WD-repeat protein *CDC20*, essential for progression through mitosis (Vodermaier 2001); (3) the *CKS1B* gene, encoding a substrate-targeting subunit of the SCF ubiquitin ligase complex that regulates the entry into S

Table 3 Identification of putative estrogen-response elements (EREs) in E2-responsive genes identified by microarray analysis of MDA-MD-231 cells that re-express ER α

	Gene name		No. of EREs	Closest to consensus ERE sequence
Affymetrix probe set				
201531_at	zinc finger protein 36	ZFP36	3	GACCAnnnTGACT
212442_s_at	LAG1 longevity assurance homolog 6	LASS6	no sequence information available	
201339_s_at	sterol carrier protein 2	SCP2	1	GGACAnnnTGACT
218002_s_at	chemokine ligand 14	CXCL14	2	AGGCAnnnTGACT
210517_s_at	A kinase anchor protein 12	AKAP12	3	TCTCAnnnTGTC
209304_x_at	growth arrest and DNA damage-inducible, beta	GADD45B	6	GGTCAnnnTGGTC (3)
210059_s_at	mitogen-activated protein kinase 13	MAPK13	3	CGCCAnnnTGACC
211168_s_at	regulator of nonsense transcripts 1	RENT1	2	GATCAnnnTGAAC
219480_at	snail homolog 1	SNAI1	3	TGGCAnnnTGAGC
205016_at	transforming growth factor, alpha	TGFA	4	AGCCAnnnTGAGC
203058_s_at	3'-phosphoadenosine 5'-phosphosulfate synthase 2	PAPSS2	4	AGTCAnnnTGACC
205206_at	Kallmann syndrome 1 sequence	KAL1	2	TGTCAnnnTGAAG
40829_at	WD and tetratricopeptide repeats 1	WDTC1	1	GTTCAnnnTGACA
218322_s_at	acyl-CoA synthetase long-chain family member 5	ACSL5	4	GATCAnnnTGAAC
204158_s_at	T-cell, immune regulator 1, ATPase, H ⁺ -transporting	TCIRG1	6	GGTCAnnnTGACA
200884_at	creatine kinase	CKB	3	GGGCAnnnTGAGG
218532_s_at	hypothetical protein FLJ20152	FLJ20152	3	TGTCAnnnTGCCC
205105_at	mannosidase, alpha 2A1	MAN2A1	2	AATCAnnnTGACC
201720_s_at	Lysosomal-associated multispanning membrane protein-5	LAPTM5	3	GGGCAnnnTGACC
205899_at	cyclin A1	CCNA1	1	TTTCAnnnTGAAC
31637_s_at	nuclear receptor subfamily 1, group D, member 1	NR1D1	4	AGTCAnnnTGACT
203060_s_at	3'-phosphoadenosine 5'-phosphosulfate synthase 2	PAPSS2	4	AGTCAnnnTGACC
201721_s_at	Lysosomal-associated multispanning membrane protein-5	LAPTM5	3	GGGCAnnnTGACC
218692_at	hypothetical protein FLJ20366	FLJ20366	4	GTTCAnnnTGAAG
205009_at	trefoil factor 1	TFF1	2	GGTCAnnnTGGCC
210357_s_at	spermine oxidase	SMOX	4	GACCAnnnTGACC
211429_s_at	serine protease inhibitor, clade A, member 1	SERPINA1	2	GGGCAnnnTGACT
220486_x_at	hypothetical protein FLJ22679	FLJ22679	1	ACTCAnnnTGAGT
204326_x_at	metallothionein 1X	MT1X	4	CGACAnnnTGACA
202833_s_at	serine protease inhibitor, clade A, member 1	SERPINA1	2	GGGCAnnnTGACT
218749_s_at	solute carrier family 24, member 6	SLC24A6	6	TGTCAnnnTGCCC
203059_s_at	3'-phosphoadenosine 5'-phosphosulfate synthase 2	PAPSS2	4	AGTCAnnnTGACC
213004_at	angiopoietin-like 2	ANGPTL2	1	GATGAnnnTGAGG
217744_s_at	TP53 apoptosis effector	PERP	4	GGTCAnnnTGGTC
207935_s_at	keratin 13	KRT13	4	TGTCAnnnTGACT
212216_at	putative amino acid transporter	KIAA0436	no sequence information available	
203071_at	semaphorin 3B	SEMA3B	3	GTGCAnnnTGACC
202267_at	laminin, gamma 2	LAMC2	2	GGTCAnnnTGCCA
201131_s_at	E-cadherin	CDH1	1	GGCCAnnnTGATG
202950_at	crystallin, zeta	CRYZ	1	AGCCAnnnTGAAG
216323_x_at	tubulin, alpha 2	TUBA2	?	?
203661_s_at	tropomodulin 1	TMOD1	3	CATCAnnnTGATC
202053_s_at	aldehyde dehydrogenase 3 family, member A2	ALDH3A2	3	TGCCAnnnTGACC
204368_at	solute carrier organic anion transporter family, member 2A1	SLCO2A1	6	GATCAnnnTGAGG
209035_at	midkine	MDK	2	TCTCAnnnTGACA
204664_at	alkaline phosphatase	ALPP	4	GGTCAnnnTGGCA

Table 3 Continued

	Gene name		No. of EREs	Closest to consensus ERE sequence
Affymetrix probe set				
213308_at	SH3 and multiple ankyrin repeat domains 2	SHANK2	3	GGTCAnnnTCAGC
214476_at	trefoil factor 2	TFF2	3	AGTCAnnnTGGCC
213001_at	angiopoietin-like 2	ANGPTL2	1	GATGAnnnTGAGG
217165_x_at	metallothionein 2A	MT2A	2	TTTCAnnnTGAAA
203585_at	zinc finger protein 185	ZNF185	4	GGTCAnnnTGACT
210740_s_at	inositol 1,3,4-triphosphate 5/6 kinase	ITPK1	5	GGTCAnnnTGGCC
206461_x_at	metallothionein 1H	MT1H	2	GTTCAnnnTGGCA
205068_s_at	Rho GTPase activating protein 26	ARHGAP26	1	GCTCAnnnTGGGC
212057_at	KIAA0182 protein	KIAA0182	no sequence information available	
202458_at	serine protease 23	SPUVE	1	AATCAnnnTGTTT
201858_s_at	proteoglycan 1	PRG1	6	AGTCAnnnTGAGC
219369_s_at	OUT domain, ubiquitin aldehyde binding 2	OTUB2	4	AGTCAnnnTGCCT
211474_s_at	serine protease inhibitor, clade B, member 6	SERPINB6	2	AGTGAnnnTGAGC
213909_at	leucine rich repeat containing 15	LRRC15	no sequence information available	
202756_s_at	glypican 1	GPC1	1	GGTCAnnnTGAGG
219045_at	ras homolog gene family, member 5	RHOF	1	TGACAnnnTGAGC
205148_s_at	chloride channel 4	CLCN4	2	AGACAnnnTGAGA
202976_s_at	Rho-related BTB domain containing 3	RHOBTB3	2	GGTCAnnnTCACT
201349_at	solute carrier family 9, isoform 3 regulator 1	SLC9A3R1	1	GCCCAnnnTGAGG
221667_s_at	heat shock protein 8	HSPB8	2	GGACAnnnTGAGA
215189_at	keratin 6	KRTHB6	3	GGCCAnnnTGACC
210827_s_at	E74-like factor 3	ELF3	6	GACCAAnnnTGAGC
212135_s_at	ATPase, Ca++ transporting	ATP2B4	3	ACTCAAnnnTGTCT
210609_s_at	tumor protein p53 inducible protein 3	TP53I3	6	TGTCAnnnTGAGA
217190_x_at	estrogen receptor alpha	ESR1	2	AGTCAnnnTGAGA
215552_s_at	estrogen receptor alpha	ESR1	2	AGTCAnnnTGAGA
211233_x_at	estrogen receptor alpha	ESR1	2	AGTCAnnnTGAGA
211234_x_at	estrogen receptor alpha	ESR1	2	AGTCAnnnTGAGA
211235_s_at	estrogen receptor alpha	ESR1	2	AGTCAnnnTGAGA
218399_s_at	cell division cycle associated 4	CDC44	5	AATCAAnnnTGACC
202870_s_at	cell division cycle 20 homolog	CDC20	3	GTTCAnnnTGATT
211519_s_at	kinesin family member 2C	KIF2C	6	GTACAnnnTGACC
201897_s_at	CDC28 protein kinase subunit 1B	CKS1B	1	TGTCAnnnTGCCA
210983_s_at	minichromosome maintenance deficient 7	MCM7	6	AGGCAAnnnTGACT
202503_s_at	KIAA0101 gene product	KIAA0101	3	GGTCAnnnTGGTC
204420_at	FOS-like antigen 1	FOSL1	4	GATCAAnnnTGCCT
208002_s_at	acyl-CoA hydrolase	BACH	4	TTTCAnnnTGAGC
202095_s_at	survivin	BIRC5	5	GGACAnnnTGATT
204521_at	predicted protein clone 23733	HSU79724	3	GCCCAnnnTGACC
220155_s_at	bromodomain containing 9	BRD9	2	GAGCAAnnnTGACA
208994_s_at	peptidyl-prolyl isomerase G	PPIG	4	GACCAAnnnTGACC
210006_at	DKFZP564O243 protein	DKFZP564O243	4	CGTCAnnnTGCCT

Putative EREs within 3000 bp upstream from the transcriptional start site of each gene were identified by comparison to the consensus ERE (GGTCAnnnTGACC) allowing for a maximum of 2 base changes in either half-site. The total number of EREs found within the 3000 bp region is also indicated. Promoter sequences for genes associated with each Affymetrix probe set were extracted from the UCSC mouse genome browser via the NetAffx database (Liu *et al.* 2003).

phase (Reed 2003) and (4) *SURVIVIN*, is a member of the inhibitor of apoptosis (IAP) family that is involved in the regulation of cell division (Kobayashi *et al.* 1999). Importantly, the repression of these genes by E2 is consistent with the suppression of proliferation observed in E2-treated MDA-MB-231 cells that re-express ER α (Fig. 3). We speculate, therefore, that these transcriptional responses are associated directly

with the anti-proliferative effects of E2 observed in these cells.

In order to identify additional E2-responsive regulators of cell proliferation and survival that may have been missed by our initial stringent ANOVA analysis, in which multi-testing correction was employed to minimise the false discovery rate (Fig. 4), we re-interrogated our transcript profiling data. We found 34 additional

Table 4 Transcriptional responses associated with cell proliferation and survival in E2 stimulated MDA-MB-231 cells that re-express ER α

Affymetrix probe set	Gene name		Biological pathway/ process	Ratio of gene expression E2:AO (mean $\pm$ S.D.)
205899_at	cyclin A1	CCNA1	cell cycle	6.0 $\pm$ 3.0
201621_at	neuroblastoma suppression of tumorigenicity 1	NBL1	cell cycle	5.1 $\pm$ 2.8
219534_x_at	cyclin-dependent kinase inhibitor 1C (p57, Kip2)	CDKN1C	cell cycle	3.9 $\pm$ 3.1
217744_s_at	TP53 apoptosis effector	PERP	apoptosis	2.9 $\pm$ 1.2
213348_at	cyclin-dependent kinase inhibitor 1C (p57, Kip2)	CDKN1C	cell cycle	2.3 $\pm$ 1.0
210538_s_at	baculoviral IAP repeat-containing 3	BIRC3	apoptosis	3.1 $\pm$ 1.9
209304_x_at	growth arrest and DNA damage-inducible, beta	GADD45B	cell cycle/apoptosis	4.1 $\pm$ 1.5
201482_at	quiescin Q6	QSCN6	cell cycle/cell proliferation	2.2 $\pm$ 0.9
222036_s_at	minichromosome maintenance deficient 4	MCM4	DNA replication	0.7 $\pm$ 0.4
210559_s_at	cell division cycle 2 (CDK1)	CDC2	cell cycle	0.5 $\pm$ 0.08
204092_s_at	serine/threonine kinase 6	STK6	cell cycle	0.7 $\pm$ 0.2
213677_s_at	postmeiotic segregation increased 1	PMS1	cell cycle	0.8 $\pm$ 0.02
209642_at	budding inhibited by benzimidazoles 1 homolog	BUB1	cell cycle	0.6 $\pm$ 0.07
205394_at	CHK1 checkpoint homolog	CHEK1	cell cycle/cell proliferation	0.7 $\pm$ 0.07
204768_s_at	flap structure-specific endonuclease 1	FEN1	DNA replication	0.8 $\pm$ 0.1
202107_s_at	minichromosome maintenance deficient 2	MCM2	DNA replication/cell cycle	0.7 $\pm$ 0.05
218399_s_at	cell division cycle associated 4	CDCA4	cell cycle	0.6 $\pm$ 0.09
218355_at	kinesin family member 4A	KIF4A	cell cycle	0.5 $\pm$ 0.09
202705_at	cyclin B2	CCNB2	cell cycle	0.6 $\pm$ 0.05
211519_s_at	kinesin family member 2C	KIF2C	cell cycle	0.5 $\pm$ 0.1
203213_at	cell division cycle 2 (CDK1)	CDC2	cell cycle	0.5 $\pm$ 0.2
202870_s_at	cell division cycle 20 homolog	CDC20	cell cycle	0.5 $\pm$ 0.1
218009_s_at	protein regulator of cytokinesis	PRC1	cell cycle	0.5 $\pm$ 0.1
202095_s_at	baculoviral IAP repeat-containing 5 (survivin)	BIRC5	cell cycle/apoptosis	0.5 $\pm$ 0.06
201112_s_at	chromosome segregation 1-like	CSE1L	cell proliferation/apoptosis	0.7 $\pm$ 0.1
214710_s_at	cyclin B1	CCNB1	cell cycle	0.5 $\pm$ 0.07
201897_s_at	CDC28 protein kinase regulatory subunit 1B	CKS1B	cell proliferation	0.6 $\pm$ 0.1
209773_s_at	ribonucleotide reductase M2 polypeptide	RRM2	DNA replication	0.5 $\pm$ 0.07
217786_at	SKB1 homolog	SKB1	cell cycle/cell proliferation	0.7 $\pm$ 0.1
201291_s_at	DNA topoisomerase II alpha	TOP2A	DNA replication	0.5 $\pm$ 0.2
212563_at	block of proliferation 1	BOP1	cell proliferation	0.6 $\pm$ 0.03
210983_s_at	minichromosome maintenance deficient 7	MCM7	DNA replication/cell cycle	0.5 $\pm$ 0.1
208795_s_at	minichromosome maintenance deficient 7	MCM7	DNA replication/cell cycle	0.5 $\pm$ 0.1
208796_s_at	cyclin G1	CCNG1	cell cycle	0.7 $\pm$ 0.2

Table 4 Continued

Affymetrix probe set	Gene name		Biological pathway/ process	Ratio of gene expression E2:AO (mean±S.D.)
200920_s_at	B-cell translocation gene 1	BTG1	cell cycle/cell proliferation/apoptosis	0.7±0.09
204240_s_at	structural maintenance of chromosomes 2-like 1	SMC2L1	cell cycle	0.6±0.05
210766_s_at	chromosome segregation 1-like	CSE1L	cell proliferation/apoptosis	0.9±0.3
201555_at	minichromosome maintenance deficient 3	MCM3	DNA replication/cell cycle	0.8±0.2
219350_s_at	diablo homolog	DIABLO	apoptosis	0.7±0.06
211036_x_at	anaphase promoting complex subunit 5	ANAPC5	cell cycle	0.8±0.08
201457_x_at	budding inhibited by benzimidazoles 3 homolog	BUB3	cell cycle	0.8±0.07
208021_s_at	replication factor C 1	RFC1	DNA replication	0.6±0.04

Gene ontology classifications for DNA replication, cell cycle, cell proliferation and cell survival were used to interrogate 574 genes exhibiting a significant (students *t*-test $P < 0.05$) alteration in their expression due to estrogen stimulation. Mean gene expression ratios $\pm$ S.D. (E2-treated relative to vehicle control; based on 3 independent biological replicate experiments) are shown together with Affymetrix GeneChip probe set, gene name, biological pathway/process (as of the NetAffx update on 23 June 2004). Two or more Affymetrix GeneChip probe sets for the same gene indicates that probe sets specific for independent regions of the same gene demonstrated estrogen-regulated expression.

E2-responsive probe sets (Student's *t*-test $P < 0.05$) whose gene ontology classifications were consistent with a role in the regulation of cell cycle progression, proliferation or survival (Table 4). This analysis revealed that E2 down-regulated the expression of many additional genes involved in cell cycle progression (*CDC2*, *CYCLIN B1*, *CYCLIN B2*, *CYCLIN G1*, *CHK1*, *BUB3*, *STK6*, *SKB1*, *CSE1*) and chromosome replication (*MCM2*, *MCM3*, *FEN1*, *RRM2*, *TOPII*, *RFC1*). A number of negative regulators of the cell cycle were also induced by E2, including *KIP2*, *NBL1* (neuroblastoma suppressor of tumorigenicity 1, also known as DAN; Ozaki *et al.* 1995) and *QUIESCIN Q6* (Coppock *et al.* 1998). The functional relationships between the numerous estrogen-responsive cell cycle regulators identified in this study (Table 4) are summarised in the cell cycle pathway map shown in Fig. 5. The overall effect of changes in the expression of these cell cycle genes is consistent with the observed suppression of proliferation (Fig. 3).

Opposing estrogen-dependent transcriptional regulation of cell cycle genes in MDA-MB-231 cells that re-express ER α and ER α -positive MCF-7 breast cancer cells

Further evidence for the involvement of the genes described above in the suppression of proliferation in our model system is provided by previous reports showing that the expression of many of the same genes is regulated in the opposite direction in ER-positive MCF-7 breast cancer cells treated with E2 (Table 5). Transcript profiling previously revealed that *FEN1*, *MCM2*, *MCM3*, *MCM7*, *CDC2*, *CDC20*, *BUB1*, *STK6*,

CSE1L and *SURVIVIN* are up-regulated during E2-induced proliferation of MCF-7 cells (Lobenhofer *et al.* 2002, Frasor *et al.* 2003). This is in contrast to the repression of these genes by E2 in MDA-MB-231 cells that re-express ER α in the microarray analysis reported here (Table 5). Quantitative real-time PCR analysis of the E2-responsiveness of these genes in both MCF-7 cells and MDA-MB-231 cells that re-express ER α confirms and extends our observations of opposing transcriptional responses to E2 in these two cell types (Fig. 6). We conclude that the paradoxical anti-proliferative effects of E2 in MDA-MB-231 cells that overexpress ER α may be due to the aberrant regulation of key cell cycle regulators.

Opposing estrogen-dependent transcriptional regulation of growth-related genes in MDA-MB-231 cells that re-express ER α and ER α -positive MCF-7 breast cancer cells

In addition to the aberrant regulation of cell cycle genes, we also found that a number of growth-related genes were regulated by E2 in the opposite direction in ER-negative MDA-MB-231 cells that re-express ER α compared with ER-positive MCF7 cells. These include E-CADHERIN (*CDH1*), an important mediator of cell-cell interactions that acts as a tumour suppressor gene and whose loss of expression is associated with invasive growth (Thiery 2002). *CDH1* is down-regulated by E2 in ER-containing breast cancer cells (Oesterreich *et al.* 2003), but is up-regulated in MDA-MB-231 cells transfected with ER α (Table 5). This suggests that the negative growth response to E2 in these cells may

Figure 5 E2-responsive cell cycle genes in MDA-MB-231 cells that re-express ER α . The cell cycle pathway map was originally adapted from KEGG and was obtained from www.GenMAPP.org (Dahlquist *et al.* 2002). Red and green boxes indicate up- and down-regulation of gene expression by estrogen, respectively.

involve alteration of epithelial cell architecture. Surprisingly, the SNAIL gene, a known negative transcriptional regulator of CDH1, was also up-regulated by E2 in these cells (Table 2), an event that is normally associated with the loss of expression of CDH1 (Fujita *et al.* 2003). Nevertheless, our data reveal the altered expression in these cells by E2 of two genes associated with the invasive growth pathway in breast cancer.

Components of the c-myc and AP-1 transcription factors also show opposing transcriptional responses in breast cancer cells containing endogenous versus transfected ER α . The gene encoding the AP-1 transcription factor, Fos-like antigen 1 (FOSL1; also known as FRA-1), is repressed by E2 in MDA-MB-231 cells transfected with ER α (Table 2, Fig. 6), consistent with previous observations that AP-1 activity is inhibited by E2 in MDA-MB-231 cells stably transfected with ER α (Philips *et al.* 1998). Furthermore, c-myc has previously been reported to be repressed by E2 in MDA-MB-231 cells transfected with adenovirally encoded ER α (Lazennec & Katzenellenbogen 1999). The repression of these genes by E2 in cells that re-express ER α is in marked contrast to their induction by E2 in MCF-7 cells (Fig. 6; van der Burg *et al.* 1989, Weisz *et al.* 1990) and suggests that the negative regulation of transcription factors that control growth and differentia-

tion may be a key event leading to the E2-dependent suppression of proliferation in MDA-MB-231 cells that re-express ER α .

Overall, these data reveal the diverse gene networks and metabolic and cell regulatory pathways through which E2 exerts its effects on MDA-MB-231 breast cancer cells that re-express ER α , and provide novel mechanistic insights into the anti-proliferative effect of E2 in these cells.

Discussion

We have used gene expression profiling to obtain a holistic view of the transcriptional responses associated with the effects of estrogen in ER-negative MDA-MB-231 breast cancer cells that re-express ER α . The genes we have identified are likely to be regulated directly by E2. Evidence for this comes from: (1) the dependence of their regulation on E2; (2) the requirement for ER α and (3) the presence of consensus EREs within 3000 bp upstream of their transcriptional start sites. Moreover, the molecular functions of many of the E2-responsive genes that we have identified, including chromosome replication, cell cycle regulation, cell survival and growth factor signalling, provide novel insights into the mechanisms underlying the E2-induced suppression of

Table 5 Opposing transcriptional responses to E2 in ER-positive MCF-7 cells versus MDA-MB-231 cells that re-express ER α

Gene name	Response to E2 in MDA-MB-231 cells that re-express ER α		Response to E2 in ER-positive MCF-7 cells
cell division cycle 20 homolog	CDC20	down-regulated ¹	up-regulated (Lobenhofer <i>et al.</i> 2002 Frasor <i>et al.</i> 2003)
cell division cycle 2 (CDK1)	CDC2	down-regulated ¹	up-regulated (Frasor <i>et al.</i> 2003)
CDC28 protein kinase regulatory subunit 1B	CKS1B	down-regulated ¹	up-regulation of CDC28 protein kinase 2 (Lobenhofer <i>et al.</i> 2002)
budding inhibited by benzimidazoles 1 homolog	BUB1	down-regulated ¹	up-regulated (Frasor <i>et al.</i> 2003)
minichromosome maintenance deficient 2	MCM2	down-regulated ¹	up-regulated (Frasor <i>et al.</i> 2003)
minichromosome maintenance deficient 3	MCM3	down-regulated ¹	up-regulated (Lobenhofer <i>et al.</i> 2002; Frasor <i>et al.</i> 2003)
minichromosome maintenance deficient 7	MCM7	down-regulated ¹	up-regulated (Lobenhofer <i>et al.</i> 2002)
flap structure-specific endonuclease 1	FEN1	down-regulated ¹	up-regulated (Lobenhofer <i>et al.</i> 2002)
replication factor C 1	RFC1	down-regulated ¹	up-regulation of RFC3 (Lobenhofer <i>et al.</i> 2002) and RFC4 (Frasor <i>et al.</i> 2003)
chromosome segregation 1-like	CSE1L	down-regulated ¹	up-regulated (Lobenhofer <i>et al.</i> 2002)
serine/threonine kinase 6	STK6	down-regulated ¹	up-regulated (Frasor <i>et al.</i> 2003)
baculoviral IAP repeat-containing 5 (survivin)	BIRC5	down-regulated ¹	up-regulated (Frasor <i>et al.</i> 2003)
E-cadherin	CDH1	up-regulated ¹	down-regulated (Osterreich <i>et al.</i> 2003)

¹Quantitative gene expression data are shown in Table 4.

proliferation in ER-negative breast cancer cells that re-express ER α (Garcia *et al.* 1992, Levenson & Jordan 1994). Importantly, our data reveal that several key regulators of cell proliferation and survival are regulated in opposite directions when compared with their behaviour in ER-positive MCF-7 breast cancer cells. Therefore, these data go some way towards explaining the paradoxical effects of estrogens in ER-negative breast cancer cells in which ER α has been re-expressed.

An important question arising from our studies is how E2-bound ER α targets the same genes with opposing transcriptional outcomes in ER-negative and ER-positive breast cancer cells. Transfection of functional ER α into MDA-MB-231 cells does not alter gene expression significantly in the absence of exogenous E2 (Fig. 4; Lazennec & Katzenellenbogen 1999), indicating that re-expression of ER α *per se* does not alter the transcriptional status of these genes. Since ER-mediated transcriptional regulation involves a plethora of coregulator proteins (Moggs & Orphanides 2001, Hall *et al.* 2001, McKenna & O'Malley 2002, Tremblay & Giguere 2002), it is possible that cell type-specific differences in transcriptional responses to estrogens are due to differences in the expression levels, accessibility, or localisation of critical cofactors. Precedent exists for this mechanism: higher levels of steroid receptor coactivator 1 (SRC-1) expression in Ishikawa endometrial cells, compared with MCF-7 breast cancer cells, result in opposing cellular responses to the selective estrogen receptor modulator tamoxifen (Shang & Brown 2002). Furthermore, the altered localisation of Retinoid $\times$ receptor alpha (RXR α) in MDA-MB-231

cells versus MCF-7 cells has been associated with the differential responsiveness of these cell lines to retinoids (Tanaka *et al.* 2004). RXR α is localized throughout the nucleoplasm in the retinoid-responsive MCF-7 breast cancer cell line, whereas it is found in the splicing factor compartment of the retinoid-resistant MDA-MB-231 breast cancer cell line. Interestingly, previous studies have shown that hydroxytamoxifen can reverse the suppression of proliferation by E2 in MDA-MB-231 cells that re-express ER α (Garcia *et al.* 1992, Lazennec & Katzenellenbogen 1999). Since hydroxytamoxifen normally suppresses proliferation in ER-containing breast cancer cells, these observations are consistent with MDA-MB-231 cells lacking the full complement of cofactors that are required for appropriate regulation of proliferation by E2 and anti-estrogens.

Another factor that may contribute to the contrasting ER-mediated transcriptional effects seen in MDA-MB-231 and MCF-7 cells is the DNA methylation status and chromatin structure of the gene regulatory regions. Indeed, DNA methylation status determines the expression levels of ER α in breast cancer cells: silencing of the ER α gene in MDA-MB-231 cells occurs through epigenetic alterations that include the hypermethylation of CpG island DNA sequences in the gene promoter region (Ottaviano *et al.* 1994). Consistent with the existence of an epigenetic silencing mechanism in MDA-MB-231 cells, the ER α gene can be reactivated by the DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine (Ferguson *et al.* 1995), and the histone deacetylase inhibitor, trichostatin A (Yang *et al.* 2000), and a combination of these inhibitors results in the

Figure 6 Quantitative real-time PCR analysis of opposing transcriptional responses in MCF-7 versus MDA-MB-231 breast cancer cells that re-express ER α . MDA-MB-231 cells were transfected with either Ad-LacZ or Ad-ER α before treatment with either vehicle control (0.01% ethanol) or 10^{-8} M E2 for 48 h. MCF-7 cells were treated with either vehicle control (0.1% ethanol) or 10^{-9} M E2 for 4, 8, 24 and 48 h. E2-dependent changes in gene expression are shown relative to time-matched vehicle controls. $\Delta\Delta$ Ct was calculated by normalising to the control gene RPLP0/36B4 (Accession number: NM_001002; Laborda 1991) and comparative Ct values are shown as log₂ fold changes.

synergistic reactivation of ER α (Yang *et al.* 2001). It is, therefore, likely that differences in the direction of E2-induced gene regulation between MDA-MB-231 and MCF-7 cells may be due to differences in the epigenetic status of target genes.

The poor prognosis of ER-negative breast cancers, together with their unresponsiveness to anti-estrogen therapy, creates an urgent need for novel targeted therapies that do not rely on inhibition of ER α (Rochefort *et al.* 2003). Alternative approaches have met with some success. For example, over-expression of the human epidermal growth factor-2 (HER2) oncogene in human breast cancers has been associated with a more aggressive progression of disease, and a monoclonal antibody (trastuzumab) directed against the extracellular domain of HER2 is therapeutically active in a proportion of HER2-positive breast tumours (Menard

et al. 2003). We have identified the transcriptional networks through which ER α is able to inhibit the proliferation of an ER-negative cell line. Targeting of these, or similar, pathways may lead to the development of novel approaches for the control of ER-negative breast tumours.

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