Estrogen receptor- α mediates gene expression changes and growth response in ovarian cancer cells exposed to estrogen

Amanda J M O'Donnell, Kenneth G Macleod, David J Burns,
John F Smyth and Simon P Langdon

Cancer Research UK Centre, University of Edinburgh, Crewe Road South, Edinburgh EH4 2XR, UK

(Requests for offprints should be addressed to S Langdon; Email: simon.langdon@cancer.org.uk)

Abstract

Estrogens play a significant role in the development, growth, invasion and metastasis of ovarian tumors. The transcriptional program regulated by 17 β -estradiol (E_2) in human ovarian cancer cell lines was analyzed using cDNA microarrays containing 1200 cancer-related genes. Twenty-eight transcripts had at least a threefold change in expression in E_2 -treated PEO1 ovarian carcinoma cells compared with controls. These differences were confirmed by real-time quantitative PCR and shown to be dependent upon the expression of functional estrogen receptor- α (ER α). Consistent with this, these gene expression changes were blocked by the anti-estrogen tamoxifen. The use of ER α - and ER β -specific ligands allowed molecular dissection of the E_2 response and showed that ER α activation was responsible for the observed changes in gene expression, whereas ER β played no significant role. Inhibition of *de novo* protein synthesis by cycloheximide was used to distinguish between primary and secondary target genes regulated by E_2 . Actinomycin D was used to show that changes in gene expression levels induced by E_2 were a result of changes in transcription and not due to changes in mRNA stability. The results presented here demonstrate that estrogen-driven growth of epithelial ovarian carcinoma is mediated by activation of ER α -mediated, and not ER β -mediated, transcription.

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Introduction

Epithelial ovarian carcinoma is the leading cause of death from gynecological malignancies in the Western world (Greenlee *et al.* 2000). This is mainly because most ovarian tumors are not diagnosed until in an advanced metastatic stage. Although various therapeutic approaches are followed in clinical practice, most of them are not life saving, and the majority of patients have only a 10–20% overall 5-year survival rate (Boente *et al.* 1993). Insight into the molecular mechanisms involved in the etiology and progression of ovarian carcinoma is important when searching for new therapies to improve the outcome of patients with this disease.

Estrogens are major regulators of growth and differentiation in normal ovaries and in the development and progression of ovarian carcinoma, but the mechanisms of action remain unclear. The biological

effects of estrogens are mediated by two forms of estrogen receptor (ER) that are encoded by separate genes, ER α and ER β (Greene *et al.* 1985, Green *et al.* 1986, Ponglikitmongkol *et al.* 1988, Mosselman *et al.* 1996, Ogawa *et al.* 1998). The ER, a member of the nuclear receptor super-family, is an estrogen ligand-activated transcription factor that binds to an estrogen-responsive element (ERE) in the promoter region of target genes, regulating their transcription (Evans 1988, Green & Chambon 1988). There has been much speculation about the relative roles of ER α and ER β in this disease, as ER β levels are high in the normal ovary and predominate over ER α , while the converse is true in ovarian cancers (Brandenberger *et al.* 1997, Enmark *et al.* 1997, Kuiper *et al.* 1997, Pujol *et al.* 1998, Rutherford *et al.* 2000, Bardin *et al.* 2004, Lindgren *et al.* 2004). A recent study has suggested that ER β loss is an important event in the development of ovarian cancer (Bardin *et al.* 2004). In this report,

we focus on the role of ER α , the isoform that is retained in ovarian cancers.

The significance of estrogen in the etiology of ovarian carcinoma has been emphasized by the fact that anti-estrogenic intervention will inhibit the growth of ovarian carcinoma *in vitro* and *in vivo* (Langdon *et al.* 1990, 1994a), and recently it has been reported that estrogen replacement therapy induces ovarian cancer (Lacey *et al.* 2002). Clinical trials with the aromatase inhibitor letrozole, which acts by depleting levels of estrogen available to the ER, have shown clinical benefit in a sub-group of ovarian cancer patients (Bowman *et al.* 2002, Papadimitriou *et al.* 2004) as have trials using tamoxifen (Hatch *et al.* 1991, Ahlgren *et al.* 1993). These studies further support the view that certain ER-positive ovarian cancers show growth dependency on estrogen. As approximately two-thirds of all ovarian tumors express ER α at the time of diagnosis (Slotman & Rao 1988), identifying the downstream targets of estrogen will reveal putative functional pathways regulated by activated ER, and provide a greater understanding of the roles estrogens play in ovarian cancer. Estrogen regulation of protein expression has been well documented in breast cancer models but to date little is known about estrogen-regulated gene expression in ovarian cancer. Genes which have been shown to be regulated, thus far, include c-myc (Chien *et al.* 1994, Hua *et al.* 1995), progesterone receptor (Nash *et al.* 1989, Langdon *et al.* 1994b), cathepsin D (CTSD) (Galtier-Dereure *et al.* 1992, Rowlands *et al.* 1993), fibulin-1 (Clinton *et al.* 1996, Moll *et al.* 2002) and insulin-like growth factor binding proteins (Krywicky *et al.* 1993).

Here we have used the BD Atlas Human Cancer Expression Array, containing 1200 gene probes, to monitor changes in gene expression in response to the naturally occurring estrogen 17-beta estradiol (E₂), in an ER-positive, estrogen-responsive ovarian cancer cell line. The results reveal that E₂ directly regulates the transcription of a plethora of genes involved in many aspects of cellular function such as DNA repair, extracellular matrix, apoptosis and signal transduction. Using a range of different cell lines and ER α - and ER β -specific ligands, we have been able to show that changes in gene expression are related to ER α status and hormone responsiveness.

Materials and methods

Tissue culture

The ovarian cancer cell lines, PEO1, PEO1^{CDDP}, PEO4, PEO6, PEO14 and PEO16 were established

within this unit (Langdon *et al.* 1988); SKOV-3, CaOV3 and MCF-7 cells were obtained from the American Type Culture Collection (Manassas, VA, USA); OVCAR-3, OVCAR-4 and OVCAR-5 were obtained from Dr T C Hamilton (Fox Chase Institute, Philadelphia, PA, USA); 41M, 59M, OAW42, A2780, ZR-75-1 and MDA-MB-231 cells were obtained from the European Collection of Cell Cultures (ECACC; Porton Down, Wilts, UK). All cell lines were routinely cultured at 37°C, 90% humidity and 5% CO₂ in RPMI 1640 (Life Technologies, Paisley, Strathclyde, UK) containing 10% heat-inactivated FCS, 100 µg/ml streptomycin and 100 IU/ml penicillin. Where specified, cells were treated with 25 µg/ml cycloheximide (Sigma, UK) to block protein synthesis, 5 µg/ml actinomycin D (Sigma, UK) to block transcription or 0.3 µM tricostatin A (Calbiochem, La Jolla, CA, USA) to inhibit histone deacetylase activity. An ER α agonist (propyl pyrazole triol (PPT)) and an ER β -specific agonist (diarylpropionitrile (DPN)) were obtained from Tocris, Bristol, Avonmouth, UK and were used at the concentrations shown.

Western blotting

Cell lines were grown to 70% confluence in 92-cm tissue culture dishes in the presence of RPMI 1640 containing 10% FCS. The medium was removed and replaced with RPMI 1640 without phenol red but containing 5% double charcoal-stripped FCS (DCS-FCS). After a further 48–72 h, estradiol (1 nM) was added to cells. To harvest, cells were washed twice in ice-cold PBS, and lysed in 50 mM Tris pH 7.5, 150 mM NaCl, 1% NP-40, 0.5 mM EDTA, 2 mM Na₃VO₄, 10 mM NaOMe, 150 mM NaF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 0.5 mM dithiothreitol, and spun for 20 min at 16 000 *g* at 4°C. The protein content of the resulting supernatant was determined by the Bradford assay (Bio-Rad Laboratories, UK). Total protein (5–30 µg) was separated on 10% SDS-acrylamide gel and transferred onto a nitrocellulose membrane. Membranes were blocked in Tris-buffered saline (pH 7.4) containing 0.1% Tween (TBST) and 10% milk. Blots were incubated in antibodies for 1 h at room temperature. The following antibodies were used: anti-ER α (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-ER β (1:500; Upstate Biotechnology, Lake Placid, NY, USA), and anti-fibronectin, (1:200; Santa Cruz Biotechnology). Immunoreactive bands were detected by enhanced chemiluminescence (Santa Cruz Biotechnology).

Growth assays

Exponentially growing cells were harvested by trypsinization and plated in 24-well plates at a density of 5×10^4 cells/well in RPMI 1640 containing 10% FCS. After 24 h to allow for attachment, the medium was removed and replaced with RPMI 1640 without phenol red and 5% DCS-FCS; E_2 and tamoxifen were added to cells, and this was designated day 0. Cells were harvested from wells on day 5 and counted on a cell counter (Coulter Electronics Ltd, Luton, UK). Changes in growth were calculated relative to controls incubated in DCS-FCS-containing media.

Oligonucleotide array

Differential gene expression was performed using the Atlas Cancer 1.2 K cDNA Expression Array (BD, Palo Alto, CA, USA), according to the manufacturer's protocol.

cDNA synthesis

Total cellular RNA was extracted from cells in log phase growth using TRI reagent (Sigma, UK). RNA from each 75-cm² flask was treated with 20 units DNase 1 (Roche, East Sussex, UK), in 50 μ l, to remove genomic DNA contamination. Forty nanograms RNA were used per RT-PCR using a one-step RT-PCR kit (Qiagen) with product-specific primers. To detect low abundance products, RNA was first reverse transcribed with a first-strand cDNA synthesis kit (Roche) using the random primers provided. One microgram RNA yielded 10 μ l cDNA, of which 1 μ l was used for each subsequent PCR with each primer pair.

Real-time quantitative PCR

Primers were chosen with the assistance of the computer application Primer 3.0 <http://focker.wi.mit.edu/primer3/>. We performed BLASTN searches against dbEST and non redundant sequence databases of GenBank to confirm the total gene specificity of the nucleotide sequences chosen as primers. Single product amplification was checked for each primer pair by analysis of product melt curves (ROTORGENE software Cambridge, UK).

All the PCRs were performed using a Corbett Research Rotorgene 2000 instrument. The thermal cycling conditions comprised an initial Taq heat-activation step at 95°C for 10 min and 45 cycles of 95°C for 15 s, 55°C for 30 s and 72°C for 45 s. Annealing temperature was dropped to 50°C for amplification of ChIP DNA. Specific PCR amplification products were detected by the fluorescent

double-stranded DNA binding dye SYBR Green. Primer sequences used are available upon request. Experiments were performed at least three times with at least triplicates for each data point.

ERE searching

Human and mouse promoter sequences of E_2 -regulated genes were obtained from EntrezGene (NCBI; www.ncbi.nlm.nih.gov), Promoser (Boston University) and UCSC genome browser; <http://genome.ucsc.edu>. A region 4 kb upstream and 1 kb downstream of the transcriptional start site (TSS) was selected and scanned for potential ERE using MatInspector (Genomatix; www.genomatix.de). The level of conservation of a 19 bp sequence, containing the predicted ERE, was measured by using ClustalW (EBI; www.ebi.ac.uk) to align human and mouse orthologous promoters.

Luciferase reporter assay

ER transcriptional activity in PEO1 cells was measured using the Dual Luciferase Reporter Assay (Promega, UK) according to the manufacturer's protocol. The cells were co-transfected with luciferase reporter construct driven by either the consensus ERE from *Xenopus* vitellogenin A2 gene (GGTCA nnn TGACC) (*Vit*-ERE-tk-luc) (Klein-Hitpass *et al.* 1986) or the region –1908 bp to +136 bp, relative to the transcriptional start site, from the rat fibronectin promoter (PGL2F1900) (Lee *et al.* 2000), and pRL-tk (renilla luciferase) to correct for transfection efficiency. Cells, starved of estrogen for 48 h in phenol red-free medium with 5% charcoal-stripped serum, were transfected using lipofectin reagent (Invitrogen, UK) according to the manufacturer's recommendations. Cells were treated with 1 nM 17 β -estradiol 24 h after transfection and luciferase activity was measured in lysed cells 24 h later using a microplate luminometer (Berthold, Bad Wildbad, Germany).

Chromatin immunoprecipitation

Cells were grown to 95% confluence on 10-cm dishes in phenol red-free RPMI 1640 supplemented with 5% double charcoal-stripped FCS for at least 48 h. Following the addition of 17 β -estradiol for various times, cells were washed twice with PBS and cross-linked with 1% formaldehyde at room temperature for 10 min followed by 5 min incubation with 0.125 M glycine. Cells were then rinsed with ice-cold PBS and collected into PBS. Cells were washed sequentially with 1 ml ice-cold PBS, buffer I (0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA, 10 mM HEPES,

pH 6.5) and buffer II (200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM HEPES, pH 6.5), then resuspended in 0.3 ml lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1), 1× protease inhibitor cocktail (Roche) and sonicated three times for 10 s to give fragments in the range 0.8 to 2.5 kb. For each immuno precipitate, 0.1 ml lysate was diluted in dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.1) followed by immunoclearing with 2 µg sonicated salmon sperm DNA and 20 µl protein A-sepharose for 1 h at 4°C. Immunoprecipitation was performed overnight at 4°C with specific antibodies to either ERα (Santa Cruz HC20) or acetylated histone H4 (Upstate #06-866). After immunoprecipitation, 20 µl protein A-Sepharose and 2 µg salmon sperm DNA were added and the incubation was continued for another hour. Precipitates were washed sequentially for 15 min each in TSE I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), TSE II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), and buffer III (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1). Precipitates were then washed three times with TE buffer (50 mM Tris-HCl, pH 8.1; 1 mM EDTA) and extracted twice with 1% SDS, 0.1 M NaHCO₃. Eluates were pooled and heated at 65°C for at least 6 h to reverse the formaldehyde cross-linking. DNA fragments were purified with a QIAquick Spin Kit (Qiagen, UK). For real-time PCR, 5 µl from 50 µl elution were used. IP was normalized to input DNA.

Results

Expression of functional ER in human ovarian cancer cell lines

To enable us to relate E₂-directed transcription to ER status and hormone responsiveness in ovarian cancer, we first assessed the expression of ERα and ERβ in 16 ovarian cancer cell lines. Western blotting with antibodies directed against either ERα or ERβ revealed that ERα protein is expressed in PEO1, PEO1^{CDDP}, PEO4, PEO6 and SKOV3 cells at levels similar to that observed in the ERα-positive breast cell lines ZR-75-1 and MCF-7 (Fig. 1). ERβ was expressed to varying degrees in all cell lines tested.

Estrogen-responsive cell lines with functional ER are growth responsive to estradiol

Figure 2 shows E₂-induced changes in cell number relative to a control sample not exposed to E₂. Both

Figure 1 Expression of estrogen receptors in ovarian cancer cell lines. Western blot of ERα and ERβ protein expression in ovarian and breast cancer cell lines. Also shown are actin expression levels in each cell line to ensure equivalent total protein loading in each lane.

ERα-positive PEO1 and PEO4 cell lines were growth stimulated by E₂ implying the presence of functional ER. SKOV-3 cells, despite expressing ERα, did not show an increase in growth, relative to control, after treatment with E₂. PEO14 cells, negative for ERα, also displayed a lack of growth-responsiveness to E₂. A further 9 ERα-negative, ERβ-positive ovarian cell lines (PEO16, 41M, 59M, OVCAR-3, OVCAR-4, OVCAR-5, A2780, CAOV3 and OAW42) were tested for growth sensitivity to E₂ but did not respond despite expressing ERβ. These data suggest that expression of ERα is necessary for E₂-induced growth and ERβ alone does not play a significant role in the growth response.

To further test which ER isoform mediates the growth response, PEO1, PEO4, PEO14 and SKOV3 cells were treated with either E₂, which activates both ER forms, or an agonist specific for either ERα (PPT) (Stauffer *et al.* 2000) or ERβ (DPN) (Meyers *et al.* 2001) (Fig. 2B). While PPT stimulated proliferation in a similar manner to E₂, DPN failed to induce a change in proliferation until concentrations reached 1 µM. At this high concentration, DPN has been shown to cross-activate ERα (Meyers *et al.* 2001). These results again indicate that growth response to estrogen in these cell lines is mediated by ERα and not ERβ.

17β-Estradiol-mediated changes in gene expression

To identify genes regulated by E₂ in the PEO1 cell line, RNAs from untreated and E₂-treated cells were compared. RNAs from untreated cells and cells exposed to 10⁻¹⁰ M E₂ for 24 h were isolated, labeled and hybridized to BD Atlas Cancer 1.2 K cDNA microarrays and

Figure 2 Effects of estrogens and anti-estrogens on ovarian cancer cell growth. (A, B) Results are shown as percentage change in cell number, compared with control cells incubated in estrogen-stripped media (see Materials and methods), after 5 days incubation with the indicated hormone. Tamoxifen was added at 1 μ M. E₂ and the specific ER α agonist PPT and ER β agonist DPN were added at the concentrations indicated.

Figure 3 Estradiol-mediated changes in gene expression identified by cDNA microarray. (A) Gene expression profiles of PEO1 cells with and without E₂-stimulation. ³³P-Labeled cDNA from E₂-stimulated and non-stimulated PEO1 cells was hybridized to microarrays and levels of expression were detected by phosphorimage analysis. (B) AtlasImage software was used to generate a color schematic depicting changes in expression as determined by both the ratio of intensity values (upper half of boxes) and the difference in intensity values (lower half of boxes): up-regulated genes in red, down-regulated genes in blue and unchanged genes in green. (C) Bivariate comparison of gene expression fingerprints using x,y-scatterplot. Genes with equal expression values in both samples line up on the central identity line, whereas outliers correspond to up- or down-regulated genes. The two outer parallel lines represent threefold changes in expression level. Selected genes with expression changed by more than threefold were selected for verification by real-time quantitative PCR.

analyzed by phosphorimaging (Fig. 3A). BD Atlas-Image software was used to generate a color schematic normalized comparison of the two arrays to demonstrate the extent of gene expression changes in PEO1 cells upon E₂ stimulation (Fig. 3B). Figure 3C shows semi-quantitative similarities and differences between gene expression profiles of the two RNA populations. Each point in the scatterplot represents the expression values of a gene in two separate treatments, one value on the X-axis (untreated control) and the other one on the Y-axis (E₂-treated). The expression levels of the majority of genes were unaffected (lined up on the

identity line); however, 5 genes were up-regulated and 23 genes were down-regulated by more than threefold in E₂-treated cells compared with untreated control cells (Table 1). Interestingly, genes up-regulated by E₂, such as *FOSL1*, *CTSD* and *TFAP4*, are known to play a role in promoting cell proliferation. Of particular note, however, was the number of down-regulated genes, especially those involved in maintenance of the cytoskeleton, suggesting a role for E₂ signaling in invasion and metastasis.

We next selected 16 genes showing changes greater than threefold over controls and verified these changes

Table 1 cDNA expression array changes of modulated genes in PEO1 cells after stimulation with E₂ and Putative Estrogen Response Elements in these estradiol-regulated genes

Gene	Genbank	Fold-change	Putative ERE ^a	Position from TSS ^b (bp)	Conserved ^c
TNFSF7 (CD27 ligand; CD70 antigen)	L08096	3.6× up	tgaGGTCAtacTGgattag	–1488 to –1506	–
TRAP1 (Tumor necrosis factor type I receptor)	U12595	3.6× up (3.0×) ^d	gttGGTCAGgcTGgtCttg	–2597 to –2615	–
FOSL1 (FRA1)	X16707	5.0× up (6.4×)	tggGGTCAGacaGctCtgg	–1926 to –1944	+
TFAP4 (AP4 DNA binding protein)	S73885	4.0× up (4.6×)	gttGGTCAGgcTGgtCtcg	–1511 to –1529	+
CTSD (Cathepsin)	M11233	4.3× up (4.1×)	cggGGTCAGcccGgCCcag	–125 to –143	–
CCNB1 (Cyclin B1)	M25753	4.0× down	ggcGGTCAGaaTGgtttct	–1908 to –1926	–
UBL1 (Sentrin; SUMO1; GAP modifying protein)	U83117	3.7× down	gccGGTCAGagcctgCttc	–912 to –930	+
CASP4 (Caspase 4)	U28014	8.0× down (5.0)	gtaGGTCAtcaTGagtttg	–318 to –336	N
NOTCH4	U95299	3.1× down	taaGGTCAGaagtcCCtat	–3617 to –3635	–
IGFBP3	M31159	15.3× down (11.4×)	ccaGGTCAttactcCttca	–64 to –82	–
CDH6 (Cadherin 6)	D31784	5.0× down (1.6×)	ataGGTCAGtgTGcCagaa	–259 to –277	+
CYR61	AF031385	5.0× down	cccGGTCAGactGcatcac	–507 to –535	–
KRT7 (keratin 7)	X03212	3.0× down (2.5×)	acaGGTCAGcccTGcaCcca	–3850 to –3868	–
FN1 (Fibronectin)	X02761	13.2× down (9.5×)	gtgGGTCAGaaaGAttcct	–1687 to –1705	+
VIM (Vimentin)	X56134	3.0× down	ttcGGTCAGctTaACcttt	–1571 to –1589	+
KRT 13 (keratin 13)	X52426	3.7× down (11.0×)	caaGGTCAGcgTagCCctc	–379 to –397	+
TGFB1 (BIGH3)	M77349	6.6× down (5.0×)	aaagGGTCAGcaaaAaCatt	–3417 to –3399	–
DES (Desmin)	U59167	3.0× down	atgGGTCAGaaTttCcttt	–3446 to –3464	–
AKAP12 (Gravin)	M96322	7.0× down	none		
TRAM1	X63679	7.0× down (1.9×)	attGGTCAGtgTttaCtgt	–3939 to –3957	–
MMP 11 (Matrix metalloproteinase 11)	X57766	4.7× down	aggGGTCAGgagGtCCtct	–951 to –969	–
KRT4 (keratin 4)	X67683	4.7× down (18.5×)	tgcGGTCAGaaggGgCtctg	–2449 to –2467	N
PLAU (Urokinase-type plasminogen activator)	M15476	7.0× down (5.0×)	tctGGTCAGctTtAttac	–1847 to –1829	+
MMP 17 (Matrix metalloproteinase 17)	X89576	3.3× down	caaGGTCAGtctgaAgCagg	–1123 to –1141	–
EIF2B1	X95648	3.7× down	ggtGGTCAGgggagCgtct	–245 to –263	–
LCN2 (Lipocalin 2)	X99133	3.5× down (14.0×)	tgaGGTCAGtgaGACCatc	–789 to –807	–
			gttGGTCAGgcTGgCcttg	–1809 to –1827	+
GRSF1 (G-rich sequence factor 1)	U07231	3.3× down	tgaGGTCAGgcTGgtttt	–2275 to –2293	–
BENE	U017077	4.9× down (3.5×)	gggGGTCAGaagattCCtta	–8 to +7	–
			gaaGGTggaaaTGgCtcag	–879 to –897	+

^aEstrogen response elements obtained from MatInspector program; ^bPosition of ERE with respect to transcriptional start site;^cSequence similarity score >0.6 for 19 base palindrome or 1.0 for half-site in ClustalW sequence alignment. 'N' = no mouse counterpart; ^dRT-PCR verified.

using real-time quantitative RT-PCR. All 16 genes, each analyzed in at least three independent experiments, consistently showed E₂-induced expression changes in agreement with the microarray analysis results in Table 1.

17β-Estradiol-mediated changes correlate with ERα expression and growth responsiveness

The expression of a randomly selected subset of genes, identified as E₂ regulated in the PEO1 cell line, was

assessed in the growth-responsive cell line PEO4 and the non-growth-responsive SKOV3 and PEO14 cell lines. Figure 4 shows that E₂ also induced a change in expression of these genes in PEO4 cells, whereas PEO14 and SKOV3 cells were non-responsive. This implies that the expression of functional ER is necessary to mediate E₂ regulation of these genes.

To establish whether the gene expression changes induced by E₂ were ER mediated, we tested the ability of the anti-estrogen 4-hydroxy-tamoxifen (OHT) to negate the observed estrogenic effect. This compound has been shown to bind specifically to the

Figure 4 Estradiol-induced changes in gene expression in ovarian cancer cell lines correlate with ER status and estrogen responsiveness. These are abrogated by tamoxifen (OHT). Graphs show fold change in gene expression, compared with control, after incubation with 1 nM E₂ for 48 h, to confirm the trend observed with microarray analysis. Quantitation of mRNA expression was carried out by real-time RT-PCR normalized to 18s rRNA levels.

ER, causing it to undergo a conformational change and thus rendering it unable to bind E₂. Cells were treated with E₂ or anti-estrogen, either alone or in combination, over a 48 h period. Figure 4 shows that OHT effectively blocked the ability of E₂ to mediate gene expression changes in the PEO1 and PEO4 cell lines.

Assaying gene expression levels in PEO1 and PEO4 cells treated with ER α and ER β agonists demonstrated that activation of ER α alone is sufficient to cause the effects observed with E₂ treatment for all of the 8 genes tested (Fig. 5). DPN was unable to induce gene expression changes even at concentrations up to 1 μ M (data not shown). This suggests that E₂-mediated

Figure 5 Effects of the ER α and ER β specific agonists, PPT and DPN respectively, on gene expression in the PEO1 and PEO4 ovarian cancer cell lines. Quantitation of mRNA expression was carried out by real-time RT-PCR normalized to 18s rRNA levels.

gene expression changes are mediated by activation of ER α and not the consequence of activation of ER β .

mRNA expression being due to E₂-regulated transcription rate changes and not due to effects on mRNA stability.

Effects of 17 β -estradiol on mRNA stability

To establish whether changes in transcript levels of E₂-modulated genes were due to a change in transcription rate or a change in mRNA stability, the rate of decay of each transcript, in actinomycin D-treated (to block further RNA synthesis) PEO1 cells pre-incubated with 1 nM E₂ for 48 h, was measured and compared with an untreated control. RT-PCR was then used to measure the levels of target mRNA at various time points after actinomycin D treatment. Figure 6A demonstrates that up-regulation of expression of *FOSL1* and *TRAP1* genes was not a result of E₂-mediated stabilization of mRNA. Interestingly, down-regulated mRNA transcripts *IGFBP3*, *BENE*, *FN1*, *PLAU* and *TGFBI* were stabilized in the presence of E₂ (estradiol-reduced expression levels of *LCN2* were too low to accurately measure decay rate). These results are consistent with the observed changes in

ER primary target genes identified in the presence of cycloheximide

It is likely that some of these genes may be regulated because of secondary effects induced by the primary targets of ER. To identify primary targets, we analyzed the effect of E₂ in the absence and presence of cycloheximide (CHX). The inhibition of protein synthesis by CHX prevents most of the secondary gene regulation that is not transactivated directly by ER. ER protein levels remained relatively stable in the presence of CHX throughout the experiment. PEO1 cells were pretreated with CHX for 10 min prior to E₂ stimulation for a further 24 h. Six out of the subset of eight gene products tested were shown to be direct targets of E₂: E₂ induced a change in mRNA expression level in the presence of CHX (Fig. 6B). Preventing *de novo* protein synthesis greatly reduced the effects of E₂

on the expression of *PLAU* and *TRAP1* suggesting that these genes are, at least partly, regulated by transcriptional targets of ER.

Estrogen response elements in regulated genes

The promoters of some genes in this study have previously been shown to have estrogen-responsive elements, which bind estrogen receptor (e.g. *CTSD*). To investigate whether the novel estrogen-regulated genes identified in this study contain potential binding sites for ER, we performed a computer-assisted analysis of the 5'-flanking sequence of each gene. Using MatInspector professional software (www.genomatrix.gsf.de), we examined whether ER consensus sites were contained within the promoter region or the first intron of the 28 estradiol-responsive genes. To improve specificity, we used threshold values of 1.0 for core similarity and 0.85 for matrix similarity. Altogether 27 of the 28 genes investigated demonstrated at least a half-palindromic sequence GGTC A within 4kb of the transcription start site (TSS) (Table 1). Based on the hypothesis that functionally important non-coding genomic sequences will be conserved during evolution, murine orthologous counterparts for each promoter were retrieved from the UCSC genome browser and sequences aligned using ClustalW. Comparative genomic analysis showed that 10 out of 25 promoters tested contained a conserved ERE sequence (sequence similarity score across 19bp sequence >0.6, or complete conservation of half-site ERE). The genes *CASP4* and *KRT4* do not have mouse orthologs.

E₂-mediated down-regulation of FN1 in ovarian cancer cells involves recruitment of ER and histone deacetylase activity to the FN1 promoter

Of particular interest was the number of down-regulated genes identified as ER targets. FN1, although previously identified as being up-regulated in response to estradiol in cardiac fibroblasts (Mercier *et al.* 2002) and during mouse mammary gland development (Woodward *et al.* 2001), was one gene that was strikingly down-regulated in ER α -positive ovarian cancer cells. Figure 7A shows the location of a

highly conserved putative estrogen response element in human, mouse and rat genomes. In accordance with the mRNA changes observed in response to estradiol, protein levels of FN1 are also greatly reduced in the presence of E₂. Interestingly, full serum, not double charcoal stripped, possesses enough hormonal activity to reduce expression of FN1 protein. Only stripped FCS allows full de-repression of FN1 expression. Induction of E₂-responsive progesterone receptor protein is also shown together with expression of beta-actin as a loading control. To assess whether E₂ causes transcriptional repression at the *FN1* promoter, we transfected ovarian PEO1 cells with a luciferase reporter construct of the *FN1* promoter region –1908 to +136 and measured luciferase activity after treatment with E₂ and compared the results with that of *vit*-ERE-luc, which is known to be induced by E₂. Results show that, as expected, E₂ induced expression of luciferase through the *vit* ERE in ovarian cancer cells. However, the activity of the *FN1* promoter was greatly reduced by the addition of E₂. These results are in agreement with the idea that E₂ causes down-regulation of FN1 through an ERE in the promoter region. Down-regulation of the *FN1* promoter is shown in Fig. 7D to be sensitive to the histone deacetylase inhibitor trichostatin A (TSA) as *FN1* promoter driven luciferase activity and endogenous FN1 mRNA and protein levels are restored when TSA is added to E₂-treated cells. This suggests that down-regulation of FN1 occurs through recruitment of histone deacetylase activity to the promoter region. To demonstrate that E₂ recruits ER α to the promoter of *FN1*, chromatin immunoprecipitation was used. Figure 7E shows that upon E₂ stimulation, ER α is recruited to the endogenous *FN1* promoter with a concurrent decrease in the level of acetylated histone H4. Together, these results present a suggestion that E₂ treatment causes recruitment of a transcriptional repression complex of ER α and histone deacetylases to the *FN1* promoter causing a reduction in FN1 mRNA and protein levels.

Discussion

The results obtained in this study indicate that ER α mediates both the growth response and gene

Figure 6 (A) Effects of estradiol on the stability of a subset of estrogen target mRNAs. Graphs show normalized gene expression levels in PEO1 cells after incubation with actinomycin D for various times. Quantitation of mRNA expression was carried out by real-time RT-PCR. (B) Effects of cycloheximide (CHX) on E₂-induced gene expression changes. Graphs show relative gene expression levels after incubation with the treatments indicated.

Figure 7 (A) Sequence alignment of the putative ERE in the *FN1* gene. Shown are the alignments of human, mouse and rat sequences. Identity to the *vit* consensus ERE sequence is shown by capitalization. Location of ERE is shown relative to the transcriptional start site. (B) Western blot analysis of FN1 and progesterone receptor protein expression changes in response to E₂. Beta-actin expression is included as a loading control. All cells were incubated for 48 h in DCS-FCS prior to treatment with 1 nM E₂, 10% FCS or 5% DCS-FCS, as indicated, for 96 h. (C) Transfection of PEO1 cells with either a *vitellogenin*-ERE luciferase reporter or an *FN1* promoter (–1908 to +136 bp) luciferase reporter. Cells were treated either with vehicle or 1 nM E₂, as indicated, for 24 h. Relative changes in reporter activity in response to E₂ are shown. (D) De-repression of FN1 by trichostatin A (TSA). Upper panel: *FN1*-luciferase reporter transfected PEO1 cells; middle panel: quantitative RT-PCR measurement of relative levels of endogenous FN1 mRNA; bottom panel: Western blot analysis of endogenous FN1 protein levels, actin expression is also shown for normalization. (E) Quantitative PCR showing results of chromatin immunoprecipitation of the FN1 promoter via antibodies to ER α , acetylated histone H4 (achH4) or normal IgG in the presence of either vehicle (–) or 10 nM E₂ (+) for 1 h.

expression changes in ovarian cancer cells exposed to E₂. Ovarian cancer cell lines expressing only ER β are growth unresponsive to E₂. Expression levels of ER β are high in the normal ovary and, in a number of studies, this isoform predominates over ER α not only in the normal ovary but also in benign ovarian tumors (Brandenberger *et al.* 1997, Enmark *et al.* 1997, Kuiper *et al.* 1997, Pujol *et al.* 1998). ER β appears protective, as adenoviral delivery of this gene into ovarian cancer cells has been shown to inhibit cell proliferation and motility and enhance apoptosis (Bardin *et al.* 2004). Progression to ovarian cancer leads to a change in the ratio of ER α to ER β , with levels of ER α being generally higher than ER β (Brandenberger *et al.* 1997, Enmark *et al.* 1997, Kuiper *et al.* 1997, Pujol *et al.* 1998, Rutherford *et al.* 2000, Bardin *et al.* 2004, Lindgren *et al.* 2004), further suggesting that either ER β confers a protective effect and/or ER α promotes growth and invasion. This is further supported by a study comparing metastatic ovarian cancer with primary ovarian cancers wherein only ER α was found in metastatic disease with ER β being no longer present (Rutherford *et al.* 2000). These data are all consistent with the idea that different ER isoforms are performing distinct roles in the ovary – ER α function being associated with malignant growth and progression, whilst ER β plays a protective role.

We are not aware of any ER α -negative ovarian cancer cell lines that respond to estrogen and these data are in line with previous reports that the presence of this receptor at moderate to high levels is essential for a growth function. A recent clinical trial, investigating the effectiveness of the aromatase inhibitor letrozole in ovarian cancer treatment, showed a clear association between response and higher levels of expression of ER α (Bowman *et al.* 2002). However, in that trial, as in the current study with SKOV-3, it is apparent that not all ER α -positive cells respond to E₂. This may be due to disabling mutations of the ER, or the absence of critical cofactors (co-activators), or the presence of repressors, which will markedly influence the transcriptional (and growth) response (Shang *et al.* 2000). Alternatively, other growth regulatory pathways, such as the erbB2 receptor signaling pathways, may be up-regulated, essentially bypassing estrogen's control (Dowsett 2001). This may explain the lack of E₂ response in the SKOV-3 cell line, as it markedly overexpresses erbB2 (Hua *et al.* 1995).

Since the functionality of ER α will be exhibited as changes in transcription of regulated genes, gene changes were sought by use of microarray technology

after E₂ exposure. A limited number of genes have already been identified as being estrogen regulated in ovarian cancer. These include genes linked to proliferation (c-myc (Chien *et al.* 1994, Hua *et al.* 1995) and insulin-like growth factor binding proteins (IGFBPs) (Krywicky *et al.* 1993)), differentiation (progesterone receptor (Nash *et al.* 1989, Langdon *et al.* 1994b)) and to invasion (CTSD (Galtier-Dereure *et al.* 1992, Rowlands *et al.* 1993) and fibulin-1 (Clinton *et al.* 1996, Moll *et al.* 2002)). Two of these genes, namely IGFBP3 and CTSD, were present on the microarray and shown to be modulated by E₂. In the present study we identified regulators of the cell cycle (CCNB1), apoptosis (TNFSF7, TRAP1, UBL1 and CASP4), transcription (FOSL1, TFAP4, EIF2B1) and signaling (NOTCH4, IGFBP3, BENE, LCN2, GRSF1). Many of these modulated genes are also linked to either the cytoskeleton and extracellular matrix (CTSD, CDH6, CYR61, KRTs 4,7 and 13, VIM, TGFBI, DES, AKAP12, TRAM1, MMPs 11 and 17, PLAUI) and hence could be involved in tumor spread and metastasis. Other than CTSD and IGFBP3, we are only aware that CCNB1 (Zoubine *et al.* 1999), CYR61 (Tsai *et al.* 2002), FN1 (Woodward *et al.* 2001), PLAUI (Levenson *et al.* 1998) and LCN2 (Seth *et al.* 2002) have been reported as being modulated by E₂. To confirm that these changes were mediated via ER, reversals by the ER competitor OHT were demonstrated. Similarly, these gene expression changes were not seen in the ER α -negative PEO14 cell line. Use of the ER α and ER β specific agonists indicated that the E₂-modulated changes in expression could be reproduced by the ER α agonist (PPT) while the ER β specific agonist (DPN) had no effect. As for the growth changes, these data support the importance of ER α over ER β in this disease. A recent report exploring tissue-specific responsiveness to estrogen concluded that ER α mediated estrogen regulation even in tissues possessing high levels of ER β expression (Jelinsky *et al.* 2003) and our results in malignant tissue are in line with this.

Of the 8 genes studied as being either direct or indirect targets of E₂, 6 demonstrated equivalent expression changes in the presence of CHX, indicating no requirement for new protein synthesis, thereby implicating a direct action of E₂ on these target genes. For TRAP1 and PLAUI, there was some inhibition of the expression changes in the presence of CHX suggesting that expression of these genes is at least partially regulated by downstream effectors involving new protein synthesis.

The presence of an estrogen receptor binding site was sought in the promoter regions of the target genes

identified, and all but one of these genes possessed at least a half-palindromic sequence GGTCa in the 4kb region prior to the transcriptional start site, with most within a 2 kb region. Previous reports have shown that such incomplete motifs might transduce ER signaling (Tora *et al.* 1988). Indeed, very few estrogen-regulated genes contain the consensus ERE palindromic sequence (GGTCAnnnTGACC) and there is abundant evidence that half-ERE sites mediate many of estrogen's actions through co-operation with other binding sites e.g. Sp1 (reviewed in Klinge 2001, O'Lone *et al.* 2004). The use of actinomycin D suggested that the observed changes in mRNA expression were due to E₂-regulated transcription rate changes and not due to effects on mRNA stability, as the up-regulated genes (*FOSL1* and *TRAP1*) were not stabilized in the presence of both E₂ and actinomycin D and the down-regulated genes showed higher rather than lower transcript levels in the presence of both E₂ and actinomycin D.

Several genes, identified as being E₂-regulated in ovarian cancer cells, have previously been shown to be E₂ targets in other tissue types. However, the effects of E₂ observed in previous studies opposed the effects observed in this study. For example, *Cyr61*, identified here as a down-regulated E₂ target, has previously been shown to be induced by E₂ in the mammary adenocarcinoma cell line MCF-7 (Sampath *et al.* 2001). *PLAU*, also down-regulated by E₂ in this study, has been shown to be up-regulated by E₂ during fracture healing (Hatano *et al.* 2004), and is also up-regulated in response to E₂ in several breast cell lines (Seth *et al.* 2002). *FNI*, which was down-regulated in our study yet up-regulated in other cell types (Woodward *et al.* 2001, Mercier *et al.* 2002), was investigated further. We have shown that in ovarian cancer cells the promoter region of the *FNI* gene is responsive to E₂, and that as well as recruiting ERα to the promoter, a TSA-sensitive histone deacetylase activity is also recruited. Reduction in acetylation of histone H4 is known to be associated with transcriptional repression, providing a plausible mechanism for E₂-mediated down-regulation of this gene.

In conclusion, these results strongly support a role for ERα but not ERβ in the growth regulation of ovarian cancer and are consistent with the observed enhanced ratio of ERα over ERβ observed in ovarian cancers. We have identified a number of novel estrogen-regulated genes and have shown that some previously identified targets of E₂ respond differently to E₂ in ovarian cancer cells. These targets will be further explored as potential mediators of estrogen's action in this particular disease.

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