Ishikawa cells exhibit differential gene expression profiles in response to oestradiol or 4-hydroxytamoxifen

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Abstract

In this study, the oestrogen agonist/antagonist action of 4-hydroxytamoxifen (OHT; 1×10^{-6} M) and 17β -oestradiol (E_2 ; 1×10^{-8} M) were assessed on the oestrogen receptor (ER)-positive epithelial cell line (Ishikawa) with respect to cell proliferation, and to gene and protein expression. qRT-PCR and western blotting confirmed that Ishikawa cells expressed both ER isoforms and that there was no change in transcript levels in response to either ligand. Gene expression profiles, using oligonucleotide arrays representing $\sim 19\,000$ human genes, showed that the expression of 716 and 534 genes were changed differentially by treatment with either OHT or E_2 respectively, at the 24-h time point, with modulation of 46 genes common to both ligands, whereas 335 (OHT) and 240 (E_2) genes showed expression changes unique to ligand, with 13 common alterations at 48 h. Both OHT and E_2 had demonstrable oestrogen agonist actions on Ishikawa cells, exemplified by increased proliferation and expression of known oestrogen-responsive genes, such as creatine kinase B and by the induction of alkaline phosphatase activity. Additionally, the data indicate that the two oestrogen agonists generated not only common gene expression changes but also unique ligand-specific profiles, raising the intriguing possibility that tamoxifen has E_2 -independent effects on the uterine epithelium.

Endocrine-Related Cancer (2007) 14 337–350

Introduction

Tamoxifen has been the leading adjuvant breast cancer treatment for more than two decades. It is licensed as a chemopreventive agent in the US following findings of a 49% reduction in invasive breast cancer in treated women (Fisher *et al.* 1998) and has proven to be an extremely effective treatment for oestrogen receptor (ER)-positive breast cancer; however, it is not without adverse side effects.

Epidemiological evidence has shown that tamoxifen treatment confers a two- to sevenfold increased risk of developing endometrial pathologies (Cohen *et al.* 1993, McGonigle *et al.* 2006), including endometrial cancer (Fisher *et al.* 1998, Cohen 2004). Type 1 endometrial tumours are associated with a history of unopposed oestrogen exposure (Amant *et al.* 2005) and the aberrant proliferation of the endometrium in post-menopausal women receiving tamoxifen treatment could be due to the oestrogen-like agonistic action of tamoxifen in this tissue (White 2001). In this context, the role of tamoxifen in

terms of carcinogenesis is considered to be that of a tumour promoter, whereby the accumulation of genetic insults increases the likelihood of damage occurring in critical genes involved in or leading to a proliferative response in the endometrial epithelial cell.

In recent years, some of the molecular genetic alterations associated with both Type I and Type II endometrial carcinomas have been elucidated and include mutations in PTEN, K-RAS, TP53, CTNNB1 (β -catenin), and the presence of microsatellite instability (MI), a marker of inactivation of DNA mismatch repair (Prasad *et al.* 2005). As atypical hyperplasia is the precursor lesion in endometrioid carcinoma Type 1 (Levine *et al.* 1998), elucidation of the non-genotoxic effect of tamoxifen, particularly with respect to uterine endometrial cell proliferation, would be highly informative.

Although the tissue-specific activity of tamoxifen has been well documented (Lonard & Smith 2002, Shang & Brown 2002), the molecular mechanisms

remain unclear. Tamoxifen binds to and activates, both ER isoforms (α and β) in a tissue-specific manner (Watanabe *et al.* 1997) and its ability to exert a tissue-specific agonist/antagonist effect led to its classification as the prototypic selective ER modulator (SERM; Cole *et al.* 1971, Ward 1973). Transcriptional activation of oestrogen-regulated genes is thought to occur through the association of the ligand-bound ER directly with oestrogen response elements (EREs) in the promoter region of target genes (McDonnell & Norris 2002), or indirectly through interaction with other transcription factor complexes such as AP1 or SP1 (Kushner *et al.* 2000, Shang & Brown 2002). However, the efficacy of the activated ER is influenced further by post-translational modifications, such as site-specific phosphorylation or ubiquitination (Shang 2006). Several *in vitro* studies have shown that oestradiol (E₂) and related SERMs differentially regulate target gene expression via ER α or ER β (Kian *et al.* 2004, Stossi *et al.* 2004, Monroe *et al.* 2005). Recently, it has been demonstrated, in various human cell types, that interaction between growth factor receptors and a membrane-associated ER α isoform (Kato 2001) leads to rapid non-genomic signalling through cytoplasmic phosphorylation cascades (Pedram *et al.* 2002, Song & Santen 2006). Genomic activation of transcription by ligand-bound ER is influenced further by the recruitment of co-regulator proteins in a promoter-specific manner (Klinge *et al.* 2004). The expression of proteins such as nuclear receptor interacting protein (NRIP) and nuclear receptor co-repressor 2/silencing mediator for retinoid and thyroid hormone receptors (SMRT) have been shown to be cell-type specific (Shang & Brown 2002) and may add to the selective regulation of ER transcriptional activity by tamoxifen (Smith *et al.* 1997, Keeton & Brown 2005). Each of these cell-specific mechanisms could play a critical role in the transcriptional behaviour of a target cell in response to SERMs, by potentially modulating the agonist/antagonist effect of each compound.

To assist the search for a SERM that acts as an oestrogen antagonist in the breast, but shows minimal agonistic action in the uterus, it is essential to gain an understanding of the mechanistic actions of tamoxifen in these target tissues, particularly in the human uterine epithelial cell. Current opinion suggests that the tissue-specific action of tamoxifen is due to the regulation of ER-mediated gene transcription (reviewed Shang 2006). This hypothesis is supported by several gene expression studies performed using normal (Mutter *et al.* 2001, Pole *et al.* 2005) or cancerous primary endometrial cell cultures (Pole *et al.* 2004, Wu *et al.*

2005), and human breast or uterine cell lines (MCF-7; (Frasor *et al.* 2004) ECC-1; (Dardes *et al.* 2002)), which show evidence of a differential transcriptional response to different oestrogens. Nevertheless, the effect of tamoxifen treatment on gene expression patterns in ER-positive human uterine endometrial epithelial cells has not been fully elucidated.

Data from previous studies using primary uterine cultures, derived from clinical samples, have been difficult to interpret due to patient variability (Pole *et al.* 2004). In addition, the proliferative response of endometrial cells in culture to SERMs has been shown to be variable, largely due to conditional differences between dose, time of exposure or media components (Shah *et al.* 2004). In this study, we used an established human uterine-derived epithelial cell line (Ishikawa; (Anzai *et al.* 1989)), which is oestrogen-responsive (Holinka *et al.* 1986, Robertson *et al.* 2002) and shares many phenotypic features in common with normal human endometrial epithelial cells (Lessey *et al.* 1996) to investigate the variation and extent of gene expression changes following tamoxifen treatment. Global gene expression profiles were determined and compared following treatment with 4-hydroxytamoxifen (OHT, 10⁻⁶ M) or 17 β -oestradiol (E₂, 10⁻⁸ M). The altered expression of key oestrogen-responsive genes and proteins were investigated and the ability of OHT or E₂ to influence Ishikawa cell proliferation was assessed.

Materials and methods

Cell culture

Ishikawa cells (ECACC, Wiltshire, UK) were routinely maintained in phenol-red-free RPMI 1640 (Invitrogen) medium containing 10% foetal calf serum (FCS, Hyclone, Northumberland, UK) and 2 mM glutamine (Gmax, Sigma) and incubated at 37 °C with 5% CO₂ in vented flasks (BDBiosciences, Oxford, UK). For all dosing experiments, the medium was replaced with RPMI 1640 containing 10% charcoal-stripped FCS (CSS, Hyclone) and 2 mM glutamine (Gmax) for 72 h prior to treatment with 10⁻⁸ M 17 β -oestradiol (E₂; Sigma), reported to significantly increase proliferation in MCF7 cells (Vanparys *et al.* 2006), 10⁻⁶ M (OHT; Sigma), which was shown to display both agonist and antagonist behaviour (Bramlett & Burris 2003) and induce proliferation (Horner-Glister *et al.* 2005) in ER+ Ishikawa cells, or ethanol (0.1%) as the vehicle control. All experiments were performed in triplicate on Ishikawa cells between passage number 3 and 15, and repeated three times.

Cell proliferation assay

Ishikawa cells were seeded at 10^4 cells per well in six-well plates in RPMI 1640 medium supplemented with 10% CSS and 2 mM glutamine for 72 h prior to treatment with 17β -E₂ (10^{-8} M) or OHT (10^{-6} M), or ethanol (0.1%) as the vehicle control in the same media. Media containing dose or ethanol was replenished daily and viable cell counts taken using the trypan blue exclusion method at 24, 48 and 72 h using a Neubauer-improved haemocytometer.

Confirmation of ER isoform expression

Ishikawa cells were seeded to 75 cm² vented flasks at 10^6 cells per flask in either maintenance or dosing medium (described above) for 72 h. After this time, cells were harvested following incubation with trypsin/EDTA (Invitrogen) for ~5 min and counted using a haemocytometer. Viability was determined using trypan blue exclusion. The cells were collected by centrifugation at 1500 *g* for 5 min at 4 °C and the cell pellets were used for either RNA extraction or western blot analyses.

Western blot analysis

Whole cell lysates were prepared using protein extraction buffer according to the method of Song *et al.* (2002). Protein concentration was determined using the Bicinchoninic acid kit for protein determination (Sigma). A standard curve was created by dilutions of BSA (200–1000 µg/ml), which were used as a reference. SDS–PAGE and blotting performed as previously described (Horner-Glister *et al.* 2005). Human recombinant proteins for either ER α or ER β (10 ng; Calbiochem, Nottingham, UK) were used as positive controls and run alongside a standard molecular weight ladder (BioRad). For ER α determination, blots were blocked with 5% non-fat milk powder-PBS and probed using H-184 rabbit polyclonal antibody (sc-7207 diluted 1:1000 in 3% non-fat milk powder-PBS, incubated overnight at 4 °C) followed by HRP-conjugated anti-rabbit secondary antibody (sc-2030, 1:2500, 1 h at room temperature; Santa Cruz Biotechnologies Inc., Heidelberg, Germany). ER β blots were blocked with 5% BSA-TBS and probed using GR-39 mouse monoclonal antibody (Oncogene Research Products, Nottingham, UK; diluted 1:500 in 3% BSA-TBS, incubated overnight at 4 °C) followed by anti-mouse secondary antibody (sc-2031, 1:2500, 1 h at room temperature; Santa Cruz Biotechnologies Inc.; 1:2500, 1 h at room temperature). West Pico Super Signal chemiluminescence detection reagent (Pierce,

Perbio Science UK, Northumberland) was applied for 1 min and images captured using the GeneGnome chemiluminescence detection system (Syngene, Cambridge, UK). Blots were washed well with PBS and incubated overnight at 4 °C in PBS before being re-probed for actin (I-19, sc-1616, Santa Cruz Biotechnology Inc.; diluted 1:1000 in 3% non-fat milk powder-PBS, incubated for 1 h at room temperature) followed by 1 h at room temperature with donkey anti-goat secondary antibody (sc-2033, 1:5000, 1 h at room temperature) and densitometry was performed using the GeneTools software (Syngene).

Alkaline phosphatase (AP) assay

Cells grown for 48 h in RPMI 1640 medium containing 10% CSS and 2 mM glutamine described above were trypsinised and seeded into 12-well plates at 7.5×10^3 cells per well for a further 24 h. The medium was then replaced with medium containing either: vehicle alone, E₂ (10^{-8} M), OHT (10^{-6} M), Faslodex (10^{-6} M), E₂ + OHT, or E₂ + Faslodex and the plates incubated at 37 °C and the medium replenished every 48 h. After 1, 3 and 5 days, the cells were washed with PBS and then lysed by freezing at –80 °C for 30 min. The alkaline phosphatase activity was determined using a 1-Step PNPP Kit (Pierce) following the manufacturers instructions. Briefly, after thawing the frozen cells, 1-Step PNPP solution (500 µl) was added to each well and mixed with the cell extracts. The plates were then incubated at RT for 1 h on an orbital shaker. Stop solution (2 N NaOH, 100 µl) was added to each well, mixed and the lysates transferred to microfuge tubes. These were centrifuged at 12 000 *g* for 2 min at RT and the supernatant (200 µl) transferred to a 96-well plate for colorimetric assessment. The absorbance was measured at 405 nm using a FluorOptima plate reader (BMG LabTech Ltd, Aylesbury, UK).

Time course for gene expression using qRT-PCR analysis

Ishikawa cells were seeded to six-well plates at 5×10^4 cells per well in dosing medium for 72 h, prior to dosing. Cells were dosed with vehicle only, OHT or E₂ at time 0 and harvested at 6, 12, 18 and 24 h, and used for RNA extraction and RT-PCR. In addition, cultures were seeded at 1×10^4 cells per well, dosed in the same manner as above with dose replenished every 48 h and harvested at day 1, 3 and 5 after dose for analysis of alkaline phosphatase, placental-like 2 (ALPPL2) expression in accordance with the alkaline phosphatase assay.

RNA extraction

Total RNA was extracted using the RNeasy Kit (Qiagen) as described by the manufacturer, including the on-column RNase-free DNase I treatment. RNA was eluted in 50 µl nuclease-free water, and diluted in the ratio of 1:100 before the concentration and purity of the RNA was determined spectrophotometrically by the A_{260/280} ratio and by using the Agilent Bio-Analyser (Agilent Technologies UK Ltd, West Lothian, UK). Only RNA with an A_{260/280} ratio of 1.9–2.1 and with no evidence of peak degradation (18S/28S) was used in this study.

Synthesis of first strand cDNA

Total RNA was reverse transcribed according to the manufacturer's instructions and all reagents were obtained from Roche Diagnostics: one microgram was primed using random hexamer primers (0.08 A₂₆₀ units) at 65 °C for 10 min then placed on ice. To a final volume of 20 µl, the following was added per reaction: reaction buffer (1×), dNTP (10 mM), Transcriptor Reverse Transcriptase (10 Units) and Protector RNase inhibitor (20 Units), and incubated at 25 °C for 10 min followed by 55 °C for 30 min.

qRT-PCR

Real-time PCR was performed using the LightCycler 2.0 system and software (Roche Molecular Biochemicals). Preliminary results showed the GAPDH housekeeping gene to be up-regulated in response to E₂ at 24 h (data not shown). Therefore, we used the Housekeeping Gene Selection Kit (Roche) to select an alternative and found that 5-aminolevulinic acid synthase (ALAS) expression was not affected by any of the treatments, and was used throughout the study. One microlitre of cDNA was amplified using sequence-specific, intron-spanning primers designed using the Roche Universal ProbeLibrary Assay Design Centre software unless otherwise stated. PCR was performed in a capillary format using DNA Master FastStart SYBR green I (Roche) according to the manufacturer's instructions, to a final volume of 20 µl containing cDNA (1 µl), specific primers (10 pmol each forward and reverse primer) and FastStart DNA Master SYBR green I mix (1×) containing FastStart DNA Taq Polymerase for Hotstart PCR. Samples were taken through a pre-incubation step, amplification cycles (including denaturation, annealing and extension segments >45 cycles) and melting curve analysis. Fluorescence was acquired as a single reading at the end of the extension segment of each cycle, and continuously during the melting curve analysis. The

size of amplicons generated was confirmed by agarose gel electrophoresis (2% agarose in 1×TAE buffer containing ethidium bromide) alongside a 100 bp DNA ladder (Promega). Each PCR run included 3× calibrator cDNA control samples prepared from untreated Ishikawa RNA and a no-template, negative control. Serial dilutions of the calibrator cDNA were used to create co-efficiency files for each primer set versus ALAS (housekeeping gene) and to normalise across PCR runs. Gene expression was quantified using the RelQuant software (Roche). Primer sequences were: ESR1 (F: GGCTACATCATCTCGGTTCC, R: TCAGGGTGCTGGACAGAAA), ALPL2 (F: TGTTACCGAGAGCGAGAGC, R: GTGGGTCTCTCCGTCCAG), CKB (F: CTTCAGAAGCGAGGCACAG, R: ACTCCGTCCACCACCATCT), CASPASE 3 (F: TGTGAGGCGGTTGTAGAAGA, R: GGGCTCGCTAACTCCTCAC), CABLES1 (F: TCGCGACAGTACCCAAGTC, R: TCAAACCTACTGCA-CCAGTTG), NEDD8 (F: TCTACAGTGGCAAGCAATGA, R: GCCTAAGACCACCTCCTCCT), VINEXINB (F: TCAGATACACTGGACCCCGTA, R: CATGACATCCACCCTGTCC), GREB1* (F: CCAAGAATAACCTGTTGGCCCTGC, R: GACATGCCTGCGCTCTCATACTTA) *(Rae et al. 2005). All oligos were synthesised by Sigma-Genosys.

Microarray analysis

Ishikawa cells were seeded in triplicate 175 cm² flasks at either 2.5 × 10⁶ (24 h) or 2 × 10⁶ (48 h) cells per flask in dosing medium for 72 h. Cells were dosed with 10^{−8} M E₂, 10^{−6} M OHT or ethanol, and then harvested at 24 and 48 h by trypsinisation. The cell number was determined using a haemocytometer, cells pelleted by centrifugation at 1500 g for 5 min at 4 °C and RNA extracted.

Microarray analyses using Cy3, Cy5 forward and reverse dye bias labelling were performed on spotted oligo arrays obtained from the Medical Research Council Human Genome Mapping Project Resource Centre (MRC HGMP-RC, Cambridge, UK). These arrays represent ~19 000 human genes, each gene being present in duplicate and the full set printed on two separate slides. Triplicate experiments were performed at 24 and 48 h for both E₂ and OHT. In total, 12 slides were used per treatment, per time point. Labelled cDNA was prepared from total RNA extracted from treated (E₂ or OHT) or control Ishikawa cells by the annealing of 8 µg/µl oligo dT₂₅ at 70 °C for 8 min, with a graduated reduction of temperature to 42 °C over 30 min. Subsequently, to the reaction mixture was added: RNasin (20 Units, Promega), dithiothreitol (0.1 M), reaction buffer (5×), Superscript II reverse transcriptase

(2 Units, Roche), dNTP mix containing a final concentration of 0.5 mM each, except 0.2 mM dTTP (Pharmacia), 0.1 mM Cy3 (control) or Cy5 (test) fluor-dUTP (Amersham) and DEPC-treated Ultra Pure H₂O to a final volume of 41 µl followed by incubation at 42 °C for 1 h. Dye Bias (reverse) labelling was performed on the same batch of RNA by swapping sample labelling where Cy3 becomes test in a separate reaction. RNA was hydrolysed by the addition of EDTA (0.5 M), SDS (10% w/v) and NaOH (3 M) and 10 min incubation at 70 °C. After cooling, the mixture was neutralised by the addition of HCl (2 M), Tris–HCl (1 M, pH 7.5) and tRNA (4 µg/ml, Invitrogen) to a final volume of 60 µl. Labelled probes were purified using re-hydrated Centri-Sep columns (Cambio Ltd, Cambridge, UK). Next, PolyA (1 µg) was added to one of the probes and human Cot 1 DNA (10 µg, Invitrogen) to the other to avoid non-specific binding of Alu fragments to the target sequence. Samples were dried down using a SpeedVac before being prepared for hybridisation. The following mix was added to one dried probe per control/treated pair: 21 µl hybridisation buffer prepared by adding formamide (1 ml, Sigma), 50× Denhardt's solution (100 µl, Sigma), ultra pure H₂O (200 µl), 10% SDS (100 µl, freshly made) and filtered through a 0.45 µm disc (Millipore UK Ltd, Watford, UK) using a syringe and a 20× saline phosphate EDTA solution (9 µl) containing NaCl (3 M), NaH₂PO₄ (1 mM) EDTA and adjusted to pH 7.4 and mixed by gentle vortexing. This was then added to the other dried probe, mixed and heat-denatured at 100 °C for 2 min and allowed to cool to 42 °C. The combined test+control samples were then hybridised to paired spotted oligonucleotide microarray slides by incubation inside humidified microarray chambers, which were sealed and immersed in a water bath at 42 °C overnight. A series of stringent washes were performed as follows: pre-heated 1×SSC (0.15 M NaCl and 0.015 M sodium citrate) solution containing 0.03% SDS (2×10 min) at 50 °C, followed by 0.2×SSC, then 0.05×SSC (both 2×5 min) at RT. Slides were quickly transferred to a centrifuge and centrifuged at 200 g for 10 min to dry and avoid background staining.

Scanning and analysis of cDNA microarrays

Information on the location and identity of the genes represented on the slides was contained in .gal files obtained from HGMP. These were loaded onto the scanner prior to use. The pixel intensity for hybridization was determined using an Axon 4000A scanner and GenePix software (Axon Instruments, Union City, CA, USA) version 3.0.6.

Statistical analysis

Microarray data

The data were normalised, condensed and analysed statistically to a final measure of differential gene expression with a false positive detection rate of 5% as previously described (Zhang & Gant 2004) based on the program accessible at <http://www.le.ac.uk/mrctox/microarray-lab>. Changes in gene expression were expressed as the normalised log₂ ratio of median fluorescence. Genes having a value $P < 0.05$ in a two-tailed *t* test were regarded as being significantly changed in expression, when compared with the control. Other statistical analysis was performed using Minitab release 14.13 (Minitab Inc., PA, USA). Differences between groups were tested using ANOVA with Fisher's test for significance at the 5% level.

Analysis of gene function

The Gene-Ontology database (GO: <http://www.geneontology.org>) was used to annotate the gene expression profiles to allow further analysis. Gostat (<http://gostat.wehi.edu.au/>) computes GO statistics of a list of genes selected from a microarray and displays statistically over-represented GO terms within a group of genes. Probability of significance of pathway was determined by DAVID 2.1 beta, functional annotation program (<http://david.abcc.ncifcrf.gov/>).

Results

ERα and β protein expression in Ishikawa cells

Western blot analysis of whole cell lysates with human ERα and ERβ recombinant proteins as positive controls, detected independently with isoform-specific antibodies, showed the presence of both ERα and ERβ at their expected molecular masses of 66 and 53 kDa respectively (Fig. 1A and B). Both proteins were expressed when cells were maintained in culture media containing either normal FCS (lane 3) or CSS (lane 4). Additional lower molecular mass ERβ isoforms (1B) were also detected as has been shown previously for Ishikawa cells (Taylor *et al.* 2002).

Effect of OHT and E₂ on Ishikawa cell proliferation

Ishikawa cells treated with ethanol only (controls) proliferated at a steady rate and at 3.8×10^5 cells/well at the 72-h time point, had not yet reached plateau phase. By contrast, Ishikawa cells treated with either OHT or E₂ initially mirrored the growth pattern of controls (Fig. 2); however, the rate of proliferation was significantly increased by the addition of either OHT or E₂ during

Figure 1 The presence of ER α (A) and ER β (B) was confirmed by Western blot analysis on Ishikawa, whole cell lysates resolved on 10% SDS–PAGE. The molecular mass ladder in Lane 1 indicates the position of the 55 kDa band. Recombinant proteins for either ER α or ER β protein were included as positive controls (Lane 2) and indicate products of the expected size for each protein. Ishikawa cells were grown in media containing 10% FCS (Lane 3) or 10% charcoal-stripped-FCS (Lane 4) for 72 h (media replenished at 48 h), prior to harvesting and western blot analysis.

the second and third days of culture ($P < 0.001$). By 72 h of stimulation, the cell numbers for cultures treated with OHT or E₂ were almost double that of the controls at 6.53×10^5 and 6.93×10^5 cells/well respectively.

Expression of oestrogen-responsive genes

In order to choose a relevant time point for global gene expression analysis, quantitative real-time RT-PCR was used to monitor the expression of ER α and selected oestrogen-responsive genes at 6, 12, 18 and 24 h. The levels of ER α increased in cultured Ishikawa cells by approximately threefold during the 24 h period, but neither E₂ nor OHT affected the basal

Figure 2 Effects of OHT and E₂ on cell proliferation. Cells were seeded in six-well plates and serum starved in RPMI 1640 containing charcoal-stripped FCS for 72 h prior to dosing. Control cells were treated with vehicle (ethanol) only. Viable cell counts were performed daily on triplicate wells using trypan blue exclusion. $\blacktriangle$, controls; $\square$, OHT; $\bullet$, E₂. Results represent the mean $\pm$ S.D. of three experiments. († Significantly different from time 0; * significantly different from controls at the corresponding time point by ANOVA $P < 0.001$).

ER α transcript levels (Fig. 3A). By contrast, both E₂ and OHT significantly increased the presence of CKB transcripts at 24 h (by 2.7- and 2.1-fold respectively when compared with the control; $P < 0.001$; Fig. 3B), whilst E₂ increased GREB1 transcript levels at 12, 18 and 24 h (by 1.8-, 1.9- and 2.5-fold respectively; $P < 0.001$; Fig. 3C). The effect of OHT on GREB1 transcript levels was not significant.

AP activity and expression of ALPPL2

AP activity was used as a protein marker assay for oestrogen agonist and antagonist activity. Figure 4A shows an induction of AP activity in response to both OHT (2.5-fold) and E₂ (13.4-fold) at 5 days after treatment. Faslodex (ICI 181780; 10^{-6} M), a pure anti-oestrogen, was included as a negative control and no induction of AP activity was observed (Fig. 4A and C). Cultures were dosed with E₂ + OHT or E₂ + Faslodex for 5 days to assess the oestrogen antagonistic potential of OHT in this cell line (Fig. 4C). E₂ treatment resulted in a dramatic increase in AP activity (1.9-fold on day 3 to 13.4-fold on day 5), which was significantly ($P < 0.001$) higher than control, whilst OHT exhibited a partial oestrogen agonist effect at 5 days (1.1- to 2.5-fold). Both OHT and Faslodex were able to significantly ($P < 0.001$) antagonise the oestrogenic action of E₂ in these cells, resulting in the abrogation of increased AP activity induced by E₂. Most notably, the AP activity measured when co-dosed with E₂ + OHT was significantly different from controls, demonstrating the partial oestrogen agonist action of OHT in this cell type, which remains in the presence of E₂.

We were interested to see if this increased activity would coincide with the altered expression of any specific alkaline phosphatase gene which might be present on the array. The only one to be significantly up-regulated on the array was ALPPL2 in response to E₂ at 48 h (by 3.7-fold). Using real-time PCR (Fig. 4B), we observed a significant ($P < 0.001$) up-regulation of ALPPL2 in response to E₂ at 1, 3 and 5 days following treatment. In addition, OHT also induced a significant increase in expression at day 5.

Microarray analysis

Figure 5 summarises the total number of significant changes ($P < 0.05$) in gene expression in Ishikawa cells treated for 24 or 48 h with either E₂ or OHT. Results indicate that the cellular response to E₂ and OHT is quite different. At 24 h, E₂ significantly ($P < 0.05$) changed the expression of 534 genes, while OHT changed 716 in a manner unique to each ligand, whilst the altered expression of only 46 genes was common to both.

Figure 3 LightCycler RT-PCR on cDNA from Ishikawa cells dosed with E₂ or OHT for 6, 12, 18 and 24 h for (A) ER α , and the oestrogen-responsive genes (B) CKB and (C) GREB1. $\blacktriangle$, controls; $\square$, OHT; $\blacksquare$, E₂ (* P < 0.001 ANOVA when compared with control). Expression was normalised to ALAS and relative quantification was performed using the LightCycler RelQuant software.

Similarly, at 48 h, E₂ induced 240 and OHT 335 unique gene changes, whilst only 13 were significantly changed by both treatments. Overall, the number of genes up- or down-regulated by OHT at 24 h was approximately equal ($\uparrow$ 49%, $\downarrow$ 51%), whilst at 48 h with OHT and with E₂ at both time points, there were more up-regulated genes than down ($\sim$ $\uparrow$ 60%, $\downarrow$ 40%). Data have been deposited in accordance with Microarray Gene Expression Data Society's MIAME recommendations in the GEO database (<http://www.ncbi.nlm.nih.gov/geo>). The accession numbers are as follows: GSE3762, Platforms GPL2914 & GPL3218 and Samples GSM86306-86353. Individual gene changes are also available as supplemental data for Fig. 5.

qRT-PCR validation of microarray data

In order to validate the microarray data, the expression of selected genes altered by E₂ or OHT in the array data were confirmed using qRT-PCR, and normalised to ALAS expression. The fold change in gene expression (compared to control) was taken from the PCR and array experiments and tabulated (Table 1). Comparison of the two experimental data sets showed excellent correlation with a regression coefficient of 0.91. In each case, the direction of altered expression was consistent (i.e. genes up-regulated in the array data were shown to be over-expressed when compared with control by qRT-PCR).

Microarray data analysis: oestrogen-responsive genes

Array data were compared with known oestrogen-responsive genes on the Dragon Oestrogen Responsive Gene Database v 2.0 (ERGDB; Tang *et al.* 2004). Out of the 1069 oestrogen-responsive genes present in the above database, 900 were also represented on the array used in this study.

We investigated the significance of an identifiable ERE motif in the promoter region of this group of genes, as an indication of potential for classical ER-mediated transcriptional activation by either OHT or E₂ (individual gene changes provided as supplemental data). The data indicate that at 24 h, the expression of two genes with a known ERE was changed after E₂ or OHT treatment. These were, gene-regulated by oestrogen in breast cancer; GREB1 (up-regulated E₂; 2.35-fold, OHT; 1.29-fold) and proteasome subunit β -type 6; PSMB6 (proteasome subunit beta, type 6) (down-regulated E₂; 0.68-fold, OHT 0.48-fold). After 48 h, only glyceraldehyde-6-phosphate dehydrogenase was up-regulated following both E₂ and OHT treatment (1.26 and 2.46 respectively). Out of the oestrogen-responsive genes induced by E₂ at 24 h, 68% contained an ERE and this increased to 81% at 48 h. Only three genes without an ERE were altered at 48 h; one of these (PLK1; polo-like kinase 1) was significantly up-regulated at both time points with E₂ (24 h, 1.32-fold; 48 h, 1.41-fold),

Figure 4 Alkaline phosphatase activity and expression of ALPPL2. Ishikawa cells were serum-starved for 72 h before dosing with either OHT (10^{-6} M), E₂ (10^{-8} M) or ethanol (control) on days 0, 2 and 4. (A) Alkaline phosphatase activity was assessed at days 1, 3 and 5. □, OHT; ■, E₂; ♦, Faslodex. (B) Expression of ALPPL2 was determined at days 1, 3 and 5. ▲, controls; □, OHT; ■, E₂. (* $P < 0.001$ ANOVA when compared with controls). (C) Ishikawa cells were serum-starved for 72 h before dosing with either OHT (10^{-6} M), E₂ (10^{-8} M), 10^{-6} M Faslodex or the combinations OHT + E₂, and Faslodex + E₂ on days 0, 2 and 4. Control cultures were treated with ethanol only. Alkaline phosphatase activity was assessed at day 5. (*Significantly different from control at the corresponding time point; †significantly different from E₂ alone: ANOVA $P < 0.001$).

whilst the transcription factor subunit RUNX1 was down-regulated at 48 h with both treatments (E₂; 0.91-fold, OHT; 0.76-fold). The number of OHT-induced oestrogen-responsive genes with an ERE was 64% at 24 h, which was only marginally higher at 48 h (70%). It is of interest to note that the ERGDB is not an exhaustive list of oestrogen-responsive genes, for example TRIM16 (also known as oestrogen-responsive B-box protein EBBP (oestrogen responsive B box protein)), which was up-regulated with both E₂ and OHT in our array data at 24 h (1.43 and 1.32 respectively), and has been reported to be oestrogen-responsive, and tamoxifen-regulated in human mammary epithelial cells (Liu *et al.* 1998) is not included in this database.

Microarray data analysis: functional annotation

The allocation of significantly changed genes according to the Gene Ontology project (<http://www.geneontology.org/>), allowed the data to be ranked according to the importance of functional information associated with the individual genes. This approach, is complemented by GoStat analysis, a method which was developed to find statistically over-represented

GO terms within a group of genes. Many of the more general GO terms including cellular biosynthesis and lipid metabolism featured in both analyses and those terms associated with cell cycle, mitosis and cell proliferation were most prominent. Terms unique to OHT were not so informative with ribonucleotide metabolism and biosynthesis predominating, although protein folding and localisation and cell motility also featured. Since both OHT and E₂ increased cell proliferation relative to controls, further analysis of the archetypal GO terms 'cell cycle' and 'cell death' are presented (Table 2). Dosing of Ishikawa cells with OHT for 24 h resulted in more down-regulated genes (71%), including cyclins B1, E₂, G2 and cell division cycle CDC7 and CDC16, than up-regulated genes. By contrast, following E₂ treatment, a higher number was up-regulated than down (55%) including cyclin D2, BRCA2 and MAPK13, whilst it shared the down-regulation of cyclins B1 and G2 with OHT treatment. In agreement with the cell proliferation assay, none of the cell death associated genes were altered with OHT at 24 h (individual gene changes provided as supplemental data).

Figure 5 Venn diagram showing the number of significant gene changes ($P < 0.05$) in response to 24 or 48 h treatment with either E_2 (10^{-8} M) or OHT (10^{-6} M) in Ishikawa cells. The numbers given within each of the circles represents the total number of significantly changed genes, which are unique to that time and treatment, and the manner in which they are regulated (up or down when compared with the control). Overlaps indicate the number of commonly changed genes between time/ligand. No genes were significantly changed at both time points, and by both ligands.

Discussion

The aim of this study was to define the transcriptional response of a model system that represents the human uterine epithelial cell to treatment with E_2 or the

SERM, tamoxifen, in order to evaluate the differential effects of oestrogenic compounds on this cell type. In breast cancer patients, tamoxifen is thought to have an oestrogen agonist action on the uterus (White 2001)

Table 1 Validation of microarray data by quantitative real-time PCR

Gene	Accession no.	Microarray fold change $\pm$ s.d.	RT-PCR fold change $\pm$ s.d.
A. OHT			
GREB1	NM_014668	1.29 $\pm$ 0.16	1.25 $\pm$ 0.20 NS
CKB	NM_001823	1.72 $\pm$ 0.90 NS	2.14 $\pm$ 0.20
CCNG2	NM_004354	0.73 $\pm$ 0.07	0.75 $\pm$ 0.01
NEDD8	NM_006156	0.57 $\pm$ 0.09	0.68 $\pm$ 0.28 NS
VINEXIN β	NM_005775	0.39 $\pm$ 0.08	0.68 $\pm$ 0.20 NS
B. E_2			
CABLES1	AK025627	2.39 $\pm$ 0.24	2.09 $\pm$ 0.13
GREB1	NM_014668	2.35 $\pm$ 0.27	2.52 $\pm$ 0.34
CKB	NM_001823	1.84 $\pm$ 0.39	2.70 $\pm$ 0.11
CCNG2	NM_004354	0.67 $\pm$ 0.06	0.62 $\pm$ 0.01
CASP3	NM_004346	0.67 $\pm$ 0.02	0.80 $\pm$ 0.14 NS

s.d., standard deviation; NS, not significant. A selection of genes, whose expression was significantly changed by microarray analysis was confirmed using SYBR green I real-time PCR on the LightCycler system all changes were significant relative to controls $P < 0.05$ except those labelled NS. Data are presented as fold-change relative to control where values < 1 are down-regulated, and those > 1 are up-regulated.

Table 2 Gene ontology analysis of effects of treatment with either 4-hydroxytamoxifen (OHT) or oestradiol (E₂) on changes in gene expression associated with terms cell cycle or cell death

Time	GO term	P value*	OHT		E ₂	
			Up	Down	Up	Down
24 h	Cell cycle	1.37×10^{-4}	14	34	22	18
	Cell death	—	0	0	12	10
48 h	Cell cycle	5.25×10^{-4}	9	7	13	5
	Cell death	2.05×10^{-2}	2	6	11	3

*Probability of significance of pathway determined by DAVID 2.1 beta, functional annotation program (<http://david.abcc.ncifcrf.gov>). Individual gene identity available as supplementary data.

and the Phase 1 metabolite, OHT shows in the order of 100-fold greater oestrogen agonist activity in the uterus than tamoxifen (Furr & Jordan 1984). Here, we present evidence of a differential response in terms of transcript levels following treatment of Ishikawa cells with either OHT or E₂ at 24 and 48 h.

The Ishikawa cells maintained their ER α and ER β expression in culture and unlike MCF-7 cells, continued to grow well in medium supplemented with CSS, in the absence of E₂. The oestrogen-responsive nature of these cells was demonstrated by a significant increase in the rate of proliferation in response to both E₂ and OHT, in agreement with other studies (Croxtall *et al.* 1990, Shah *et al.* 2004, 2005, Vivacqua *et al.* 2006), a marked increase in alkaline phosphatase activity and increased expression of known oestrogen-responsive genes, such as *CKB* and *GREB1*. There is good agreement that alkaline phosphatase activity in Ishikawa cells is positively regulated by oestrogens in an ER-dependent manner and is considered as a very sensitive means to assess ER agonist and antagonist activity (Holinka *et al.* 1986, Littlefield *et al.* 1990, Kasiotis *et al.* 2006). This was confirmed by our studies where both OHT and E₂ independently increased AP activity, demonstrating the partial oestrogen agonist activity of OHT. It is of interest that this level of activity remains in the presence of E₂, where OHT is also acting as an efficient antagonist. By contrast, co-dosing with the pure anti-oestrogen Faslodex, which binds to and degrades the ER (Wakeling 2000) rather than compete for the occupancy of the receptor, abolished the E₂-induced AP activity to below that of controls, suggesting that the effect of OHT on AP activity maybe through a subtly different mechanism than with E₂. There are four alkaline phosphatase isozymes: placental, placental-like, intestinal and tissue non-specific (liver/bone/kidney). We show here that the specific gene likely to be involved in the induction of alkaline phosphatase activity in Ishikawa cells is ALPPL2. The expression of ALPPL2 was up-regulated on the microarray in response to both OHT and E₂, and this was confirmed by qRT-PCR

analysis over 5 days resulting in a significant increase in expression, albeit to different extents, mirroring the effect observed in the AP assay.

Diverse gene expression profiles are dictated or influenced by ligand binding

The magnitude of gene expression changes, which were largely unique to ligand at either time point investigated and the minimal overlap between profiles, implies that OHT does not directly mimic E₂, but rather orchestrates a cellular response of it own, despite acting via the same receptors. In addition, this study highlights the extent of influence that oestrogenic compounds have on the gene expression profile of uterine epithelial cells and gives weight to the hypothesis that the differential effects seen in the uterine endometrium might be mediated by specific OHT-regulated gene transcription pathways (Shang 2006).

Gene ontology: analysis of gene expression.

Analysis of gene expression changes by programs such as DAVID <http://niaid.abcc.ncifcrf.gov>/or GoMiner <http://discover.nci.nih.gov/gominer/> showed a number of significant GO categories and enrichment common to both OHT and E₂. Out of these, the terms protein biosynthesis, cellular protein metabolism and cell cycle are consistent with the induction of cell proliferation. In response to E₂, CASPASE 3 (CASP3), which is intimately involved in cell death decisions was one gene shown to be down-regulated at 24 h. In some tissues, including neuronal cells and cardiac myocytes, E₂ protects against cell death, in part, by down-regulating CASPASE 3 expression (Pelzer *et al.* 2000, Rau *et al.* 2003). Such protection may play a role in the actions of E₂ on Ishikawa cells but there is no evidence for this following OHT treatment at this time point, suggesting that E₂ and OHT augment uterine epithelial cell proliferation and survival through subtly different mechanisms.

Differential expression of key genes in a uterine cell line

The transcriptional behaviour of *GREB1* (gene-regulated by oestrogen in breast cancer) was particularly prominent in this dataset as up-regulated by both OHT and E_2 at 24 h on the array. *GREB1* was identified as an early response gene-regulated by $ER\alpha$ in the three ER-positive breast cancer cell lines, MCF-7, T47D and BT-474, where it was one of only three genes to be significantly induced in all three cell lines (others included pS2 and SDF-1), and by contrast also repressed by OHT or Faslodex (Rae *et al.* 2005). In addition, the ERE present in the *GREB1* promoter was shown to be directly targeted by the $ER\alpha$ following E_2 stimulation (Lin *et al.* 2004). Although the cellular function of *GREB1* has yet to be fully defined, there is increasing evidence, in particular its pattern of expression and regulation by E_2 , to imply that it might be critically involved in the regulation of hormonal cancers (Ghosh *et al.* 2000, Rae *et al.* 2005). The negative cell cycle regulator cyclin G2 (CCNG2), which was recently identified as a primary ER target gene in MCF-7 breast cancer cells where it was robustly down-regulated by oestrogen but not OHT (Stossi *et al.* 2006), was down-regulated by both OHT and E_2 in Ishikawa cells demonstrating a further clear difference in response between breast and endometrial cells.

We have previously shown that OHT is capable of stabilising $ER\alpha$ in Ishikawa cells, and inducing ligand-mediated degradation of $ER\beta$ protein, but not $ER\alpha$ (Horner-Glister *et al.* 2005). This effect was shown to be cell-type specific, since in breast cancer cells, $ER\alpha$ protein is targeted for rapid degradation via the ubiquitin–proteasome pathway in response to E_2 (Dowsett & Ashworth 2003). The ubiquitin-like protein neural precursor cell-expressed developmentally down-regulated (NEDD8) is essential for protein processing and cell-cycle progression, and has been linked to ubiquitination of $ER\alpha$ (Fan *et al.* 2003) and an intact NEDD8 pathway is essential for $ER\alpha$ ubiquitination and degradation. This gene was significantly down-regulated in our study in response to OHT at 24 h, but not by E_2 , suggesting that ER degradation could be partly responsible for the differential effects of OHT and E_2 in this cell type. *VINEXIN β* (SCAM-1; SH3 containing adaptor molecule); a focal adhesion protein, which regulates the anchorage dependence of ERK2 activation (Suwa *et al.* 2002) was one of the most down-regulated genes following OHT treatment at 24 h. Phosphorylation controls RAR γ -mediated transcription by triggering the dissociation of *VINEXIN β* from the focal adhesion plaque (Bour *et al.* 2005). As a member of the nuclear

receptor superfamily, the retinoid receptor shares some homology and characteristics with the ER. Indeed, *VINEXIN α* binds *in vitro* to $ER\alpha$ and $ER\beta$ and stimulates the ligand-induced transactivation function of these receptors (Tujague *et al.* 2004). Down-regulation of *VINEXIN β* could therefore influence the downstream transcription activity of OHT-associated ER, which presumably involves the recruitment of a different repertoire of nuclear transcription factors, adaptors or co-regulators.

Differential expression of genes in breast MCF-7 cells versus Ishikawa cells

Gene expression studies in human MCF-7 cells have shown that some genes respond rapidly within 2 h whilst others are only maximally induced after 24 to 48 h (Frasor *et al.* 2003). More recently, meta-analysis of heterogeneous *in vitro* and *in vivo* datasets showed a similar response in expression profiles in breast derived T-47D and MCF-7 cells to E_2 (Lin *et al.* 2004). The highest ranked ER direct target genes were *NR1P1*, *GREB1* and *ABCA3*. As indicated above, in Ishikawa cells at 24 h, *GREB1* was up-regulated by E_2 and OHT whilst *NR1P1* was up-regulated only by E_2 . There was no significant change in *ABCA3* gene expression. While many have used MCF-7 cells to elucidate the role of the ER in response to oestrogen stimulation (Lin *et al.* 2004, Stossi *et al.* 2006), the present results suggest these may be confined only to breast cancer cells and not applicable to other oestrogen-responsive tissues, such as the uterus.

Conclusions

OHT behaves largely as an oestrogen agonist in this cell type, but also displays oestrogen antagonist activity in the presence of E_2 . The data presented strongly suggest that the effect of OHT or E_2 on the gene expression profile of human uterine epithelial cells is highly diverse and ligand-specific, and does not necessarily mimic that seen in breast cancer cells, supporting the hypothesis that OHT might be capable of influencing the transcriptional response of a specific subset of genes in the uterus.

Acknowledgements

We would like to thank Emma Horner-Glister, Anthony H Taylor and Thierry Bailhache for their helpful discussion and review of the manuscript. We also thank Tim Gant and ShuDong Zhang for advice and assistance on microarray and statistical analyses. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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