Journal of Steroid Biochemistry & Molecular Biology 114 (2009) 21–32

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

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

# Use of global gene expression patterns in mechanistic studies of oestrogen action in MCF7 human breast cancer cells☆

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ARTICLE INFO

*Article history:*
Received 25 November 2008
Accepted 31 December 2008

*Keywords:*
Global gene expression
Microarray
Oestrogen
Long-term oestrogen deprivation
Antioestrogen
Fulvestrant
Antioestrogen resistance
Parabens
Environmental oestrogens
Breast cancer

ABSTRACT

Over the years, the MCF7 human breast cancer cell line has provided a model system for the study of cellular and molecular mechanisms in oestrogen regulation of cell proliferation and in progression to oestrogen and antioestrogen independent growth. Global gene expression profiling has shown that oestrogen action in MCF7 cells involves the coordinated regulation of hundreds of genes across a wide range of functional groupings and that more genes are downregulated than upregulated. Adaptation to long-term oestrogen deprivation, which results in loss of oestrogen-responsive growth, involves alterations to gene patterns not only at early time points (0–4 weeks) but continuing through to later times (20–55 weeks), and even involves alterations to patterns of oestrogen-regulated gene expression. Only 48% of the genes which were regulated ≥2-fold by oestradiol in oestrogen-responsive cells retained this responsiveness after long-term oestrogen deprivation but other genes developed *de novo* oestrogen regulation. Long-term exposure to fulvestrant, which resulted in loss of growth inhibition by the antioestrogen, resulted in some very large fold changes in gene expression up to 10,000-fold. Comparison of gene profiles produced by environmental chemicals with oestrogenic properties showed that each ligand gave its own unique expression profile which suggests that environmental oestrogens entering the human breast may give rise to a more complex web of interference in cell function than simply mimicking oestrogen action at inappropriate times.



## 1. Introduction

Oestrogen plays an important role in the development [1,2] and the progression [3] of breast cancer, and the ablation of oestrogen action either through the inhibition of oestrogen synthesis (aromatase inhibitors) or the antagonism of oestrogen action (antioestrogens) remains the basis of the successful use of endocrine therapy as a treatment for breast cancer [4]. Although initially effective, tumour progression seems to lead inevitably to growth independent of oestrogen or antioestrogen and failure of endocrine therapy is a major problem in the clinical management of breast cancer [3,4]. Over the years, the MCF7 human breast cancer cell line [5,6] has provided a model system to identify cellular and molecular mechanisms in oestrogen regulation of cell growth and in events leading to oestrogen and antioestrogen independent growth [3,4,7,8]. Although extensive molecular studies have identified functional involvement of many single gene products [3,4,7,8], of cross-talk with growth factor signaling pathways [7–15] and with apoptotic pathways [16,17], the application of expression microarray technology [18] now offers the opportunity to extend these molecular studies to identify wider changes in global gene expression brought about by oestrogen and antioestrogen in these cells [19–33].

Long-term growth of MCF7 cells under conditions of oestrogen deprivation provides a model system to study development of oestrogen independent growth since it results in upregulation of growth such that the cells eventually grow at the same rate in the absence of oestrogen as they originally grew only in the presence of oestrogen [15,34–41]. Targeting of any one signaling pathway by blockade of the oestrogen receptor (ER) [34,41], the insulin-like growth factor receptor type 1 (IGF1R) [38] or mitogen-activated protein kinase (MAPK) [40] pathways is no longer sufficient to completely inhibit the growth of these cells and global gene expression profiling can provide a wider view of the extent of the signaling pathways altered. Cell culture studies have demonstrated that MCF7 cells seem to be able to circumvent any form of imposed growth inhibition and accordingly, continuous culture in the presence of antioestrogen results also in progression to growth resistant to the presence of tamoxifen [41–45] or fulvestrant [41,46–48]. Since in this model system, fulvestrant results in loss of oestrogen receptor whilst tamoxifen does not [41], molecular mechanisms are likely to differ in the development of growth resistant to

☆ Lecture presented at the '18th International Symposium of the Journal of Steroid Biochemistry and Molecular Biology', 18–21 September 2008, Seefeld, Tyrol, Austria.

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doi:10.1016/j.jsbmb.2008.12.017

distinct antioestrogens and global gene expression profiling offers the opportunity of identifying prognostic markers.

Many environmental compounds which can mimic oestrogen action are now known to be able to enter the human breast [49] but the extent of their contribution to the development and progression of breast cancer remains to be established. Such environmental oestrogens may enter the human body through diet as phytoestrogens in edible plant material or xenoestrogens in animal fat or through topical application of consumer products including cosmetics [49]. Although a bewildering array of compounds with varied chemical structures have been shown to bind to oestrogen receptors and to be able to regulate the expression of oestrogen-regulated reporter genes and a few endogenous oestrogen-regulated genes such as pS2 [49–51], the extent of similarity in their global gene expression profiles could have implications for their overall effects in human breast cancer cells.

## 2. Outline of methods

### 2.1. Culture of stock oestrogen-maintained MCF7 cells

MCF7 McGrath human breast cancer cells were kindly provided by C.K. Osborne at passage number 390 in 1987 [6]. This cell line is dependent on oestrogen for growth [52]. Stock MCF7 cells were grown as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) foetal calf serum (FCS), 10 μg/ml insulin and $10^{-8}$ M 17β-oestradiol (*oestrogen-maintained medium*) as described previously [41].

### 2.2. Long-term adaptation of MCF7 cells to oestrogen deprivation

A new vial of cells was thawed from liquid nitrogen at the start of each experiment, which ensured that control cells were of similar starting passage number and that cells of the starting passage number would be available for comparison at any time. Freshly thawed cells were grown for 2 weeks as stock oestrogen-maintained cultures (see above) and then trypsinised from the plate, washed and replated into an *oestrogen-deprived medium* comprised of phenol red-free [53] RPMI 1640 medium containing 5% dextran-charcoal stripped FCS (DC-FCS) [54] as described previously [41].

### 2.3. Long-term adaptation of MCF7 cells to growth with fulvestrant

Stock oestrogen-maintained MCF7 cells (see above) were replated into DMEM supplemented with 5% (v/v) FCS, 10 μg/ml insulin, $10^{-10}$ M 17β-oestradiol and $10^{-7}$ M fulvestrant (Faslodex, ICI 182,780) (gift from A. Wakeling, AstraZeneca). Parallel cultures of MCF7 cells were maintained for one year in the oestrogen-maintained medium (see above) and in the oestrogen-maintained medium supplemented with fulvestrant [41].

Following one year of growth under the oestrogen-deprived conditions described above, cells were replated into phenol red-free RPMI 1640 medium containing 5% DC-FCS and $10^{-7}$ M fulvestrant. Parallel cultures of oestrogen-deprived (for one year) MCF7 cells were maintained for a further one year in the oestrogen-deprived medium (see above) and in the oestrogen-deprived medium supplemented with fulvestrant [41].

### 2.4. Cell proliferation assays

Cell proliferation assays were carried out and cell counts performed by counting released nuclei on a ZB1 Coulter Counter as described previously [41,55]. Doubling time of the cells was calculated as described previously [56].

### 2.5. Expression microarray analysis

RNA was prepared using the RNeasy system (Qiagen) from independent replicate cultures of cells as described previously [33]. Microarray analysis was carried out using the Amersham Codelink 20K human expression microarrays (19,947 human gene targets) using separate arrays for each RNA sample. cDNA synthesis, preparation of biotinylated cRNA, fragmentation of cRNA, hybridization to arrays, washing of the arrays after hybridization and staining of the arrays with Cy5-streptavidin were all carried out according

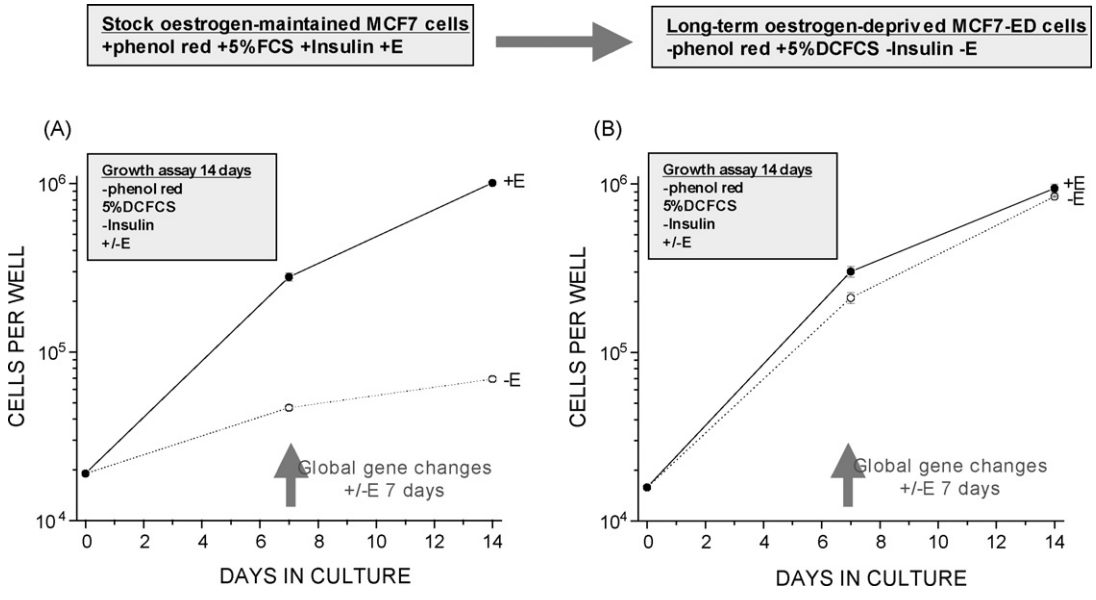



**Fig. 1.** The cell culture model of long-term oestrogen deprivation for studying loss of oestrogen responsive growth in MCF7 human breast cancer cells. Stock oestrogen-maintained MCF7 cells show a growth response to $10^{-8}$ M 17β-oestradiol when grown short-term (14 days) in oestrogen-deprived medium (A). Long-term growth in an oestrogen-deprived medium results in the loss of any short-term growth response to 17β-oestradiol (B). Changes in global gene expression were compared between the oestrogen-maintained (A) and oestrogen-deprived (B) cells after growth of the cells in oestrogen-deprived medium with or without added 17β-oestradiol at $10^{-8}$ M for 7 days.

**Table 1**
The percentage similarity between each of the functional groupings of genes used in the analysis of global gene expression profiles in Figs. 2 and 9.

| FUNCTIONAL GROUPING | No genes in each grouping | Metabolism | Developmental | Transcription | Signal transduction | Receptor activity | Transport | Cell signalling | Cell differentiation | Protein kinase activity | Receptor binding | Cell cycle | Cell proliferation | Cell adhesion | Cell recognition | Cell death | Stress response |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *4742* | *4742* | *3167* | *2094* | *2053* | *1954* | *1706* | *1060* | *1010* | *991* | *790* | *577* | *569* | *540* | *451* | *412* | *183* |
| Metabolism | *4742* | | 70 | 66 | 17 | 15 | 41 | 26 | 37 | 20 | 23 | 28 | 24 | 9 | 9 | 26 | 39 |
| Developmental | *3167* | | | 100 | 30 | 31 | 8 | 55 | 100 | 24 | 44 | 64 | 72 | 30 | 14 | 34 | 25 |
| Transcription | *2094* | | | | 18 | 17 | 5 | 29 | 35 | 16 | 27 | 62 | 59 | 12 | 6 | 23 | 19 |
| Signal transduction | *2053* | | | | | 52 | 11 | 47 | 21 | 36 | 55 | 21 | 87 | 22 | 12 | 34 | 23 |
| Receptor activity | *1954* | | | | | | 11 | 55 | 20 | 20 | 97 | 10 | 35 | 30 | 23 | 30 | 8 |
| Transport | *1706* | | | | | | | 8 | 5 | 3 | 13 | 3 | 4 | 3 | 5 | 5 | 6 |
| Cell signalling | *1060* | | | | | | | | 16 | 9 | 41 | 11 | 31 | 15 | 9 | 16 | 6 |
| Cell differentiation | *1010* | | | | | | | | | 5 | 14 | 8 | 18 | 15 | 5 | 12 | 7 |
| Protein kinase activity | *991* | | | | | | | | | | 17 | 20 | 16 | 4 | 3 | 13 | 22 |
| Receptor binding | *790* | | | | | | | | | | | 7 | 22 | 16 | 9 | 14 | 7 |
| Cell cycle | *577* | | | | | | | | | | | | 30 | 2 | 2 | 12 | 4 |
| Cell proliferation | *569* | | | | | | | | | | | | | 7 | 4 | 14 | 5 |
| Cell adhesion | *540* | | | | | | | | | | | | | | 14 | 3 | 2 |
| Cell recognition | *451* | | | | | | | | | | | | | | | 3 | 3 |
| Cell death | *412* | | | | | | | | | | | | | | | | 10 |
| Stress response | *183* | | | | | | | | | | | | | | | | |

The functional grouping is shown in the top row and left-hand column, with the total number of genes in each grouping given in italics in the second row down and the second column from the left.

to the detailed protocols supplied by the manufacturer (Amersham Biosciences, UK). The arrays were scanned with a GenePix Array Scanner 4000B. Analysis of array data using GeneSpring was as described previously and included normalization of each array per chip and per gene [33]. Mean relative values of gene expression were calculated for each treatment over independent replicate experiments and a mean fold change with standard error (SE) was calculated as compared to the control value indicated.

## 3. Oestrogen regulation of gene expression in MCF7 cells

Hundreds of publications report that growth of MCF7 human breast cancer cells is responsive to oestrogen. If the cells are grown in a phenol red-free medium [53] and dextran charcoal-stripped serum (DCFCS) [54] (oestrogen-deprived medium), then cell growth is low in the absence of added oestrogen and is stimulated by 17β-oestradiol [52] (Fig. 1A). In our laboratory, these cells are maintained as stock cultures in a phenol red-containing medium with unstripped foetal calf serum (FCS), 10 μM insulin and $10^{-8}$ M 17βoestradiol (oestrogen-maintained medium), and so one approach to studying oestrogen regulation of gene expression is to place the cells into oestrogen-deprived medium for up to 1 week and then to analyse global gene expression patterns after the early hours of oestrogen readdition, as has already been reported by others [25]. We have taken a more long-term approach of investigating the gene changes after transfer to the oestrogen-deprived medium for 7 days with or without added oestradiol when there is a clear growth response to oestradiol (Fig. 1A). Using the Amersham Codelink 20K arrays and three biological replicates of cells grown with or without oestradiol for 7 days, we have found that a total of 7.6% of the genes on the array were regulated by ≥2-fold after 7 days with or without oestradiol. In taking a global view, more genes were downregulated by oestradiol than upregulated, with 62% of the regulated genes being downregulated. Overall 4.7% of the genes on the array were downregulated and 2.9% were upregulated (Fig. 2 track 1). If the genes were then subdivided into different functional groupings, a similar small percentage of genes were regulated by

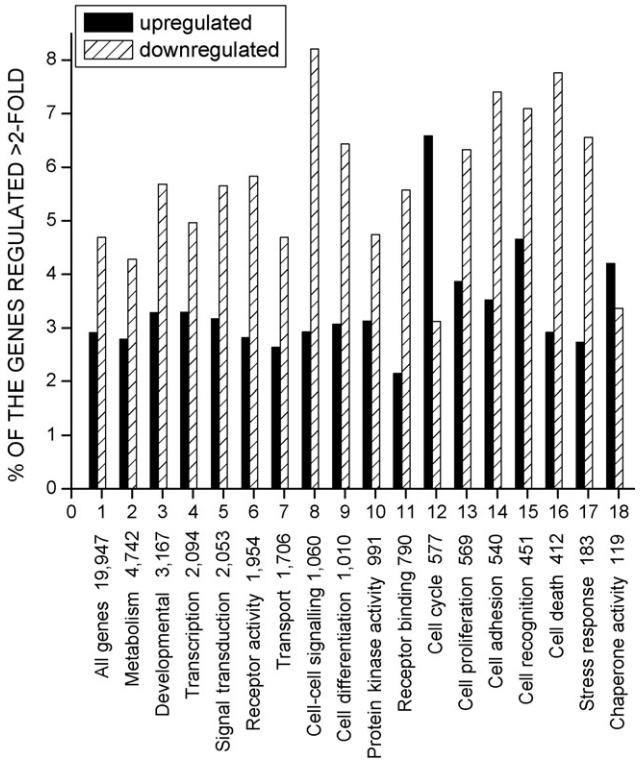



**Fig. 2.** Percentage of genes upregulated or downregulated by ≥2-fold in oestrogen-maintained MCF7 human breast cancer cells grown in oestrogen-deprived medium with or without $10^{-8}$ M 17β-oestradiol for 7 days. Regulation is given as a percentage of the total genes (19,947 genes) on the expression microarray (track 1) and of the genes when subdivided into a range of functional groupings within GeneSpring (tracks 2–18). The number of genes in each functional grouping is shown on the *x*-axis and the proportional overlap of gene identity between each of the groups has been collated in Table 1.

**Table 2**
List of genes which were upregulated (+ values) or downregulated (− values) ≥10-fold by oestradiol and with a level of significance of $P < 0.05$ in MCF7 human breast cancer cells as maintained in our laboratory and after growth in oestrogen-deprived medium with or without oestradiol at $10^{-8}$ M for 7 days.

| Fold change | Gene | Gene function | Genbank number |
|---|---|---|---|
| Genes upregulated | | | |
| 35 | PDZ domain containing 1 | Cell signaling (?) | NM_002614 |
| 32 | GREB1 protein | Unknown | NM_014668 |
| 30 | Neuropeptide Y receptor Y1 | GPCR | NM_000909 |
| 18 | Interleukin 20 | Cytokine | NM_018724 |
| 15 | Transmembrane protease, serine 3 | Protease | NM_032404 |
| 15 | ProteinLOC143381 | Unknown | AK056108 |
| 10 | Trefoil factor 1 (pS2) | Growth factor | NM_003225 |
| Genes downregulated | | | |
| −32 | Chemokine ligand 26 | Chemotaxis | NM_006072 |
| −29 | Ubiquitin D | Proteolysis | NM_006398 |
| −28 | Vestigial-like 1 (Drosophila) | Transcription | NM_016267 |
| −22 | Lymphotoxin beta (TNF member 3) | Signal Transduction | NM_009588 |
| −19 | RAS, dexamethasone-induced 1 | Signal transduction | NM_016084 |
| −19 | Uroplakin 3A | Transmembrane | NM_006953 |
| −15 | Integrin alpha M (CD11b) | Cell adhesion | NM_000632 |
| −14 | Solute carrier family 19, member 3 | Transport | NM_025243 |
| −13 | Short-chain dehydrogenase/reductase 1 | Metabolism | NM_004753 |
| −13 | Microtubule-associated protein 1B | Tubulin binding | AK055112 |
| −12 | Aldehyde dehydrogenase 1 family, A3 | Metabolism | NM_000693 |
| −12 | Normal mucosa of oesophagus specific 1 | Unknown | NM_032413 |
| −12 | Protein MGC14161 | Unknown | NM_032892 |
| −12 | B lymphocyte gene 1 | Transcription | NM_007005 |
| −11 | Kraken-like | Unknown | NM_014509 |
| −11 | Protein FLJ10901 | Unknown | NM_018265 |
| −11 | Sister of mammalian grainyhead | Transcription | AL137763 |
| −10 | Chromosome 20 ORF114 | Unknown | NM_033197 |

≥2-fold in each grouping and a similar increased proportion were downregulated rather than upregulated in most of the groupings (Fig. 2 tracks 2–18). Since higher proportions of genes were not altered in any one grouping, this suggests a very widespread action of oestradiol on expression of genes with a varied range of different cellular functions. Table 1 shows the extent of similarity between each of the groupings of genes used and demonstrates that the similarity observed across all the functional groupings was not the result of reiteration of similar gene contents. For example, 17% of the genes in the signal transduction grouping were also represented in the metabolism grouping (348 out of 2053 genes), only 5% of the genes in the cell differentiation grouping were reiterated in the transport grouping (54 out of 1010 genes) and only 2% of the genes in the cell adhesion grouping were also found in the cell cycle grouping (11 out of 540 genes). Table 2 gives a list of genes which were regulated ≥10-fold by oestradiol and with a level of significance of $P < 0.05$ in MCF7 cells as grown in our laboratory. The greatest fold induction was 35-fold for upregulation and 32-fold for downregulation and assessing the known functions of these genes it would seem that even in this category of high fold induction the genes are found involved in a varied range of cellular functions. Although technically more easily detected, it remains in question as to whether high level of expression or high fold induction influences cell growth to any greater extent than lower levels. However, the fact that regulated genes fall across a wide range of functional groupings demonstrates the very varied actions of oestradiol in regulating cell growth. The significance of the small but similar proportion of genes influenced in each functional group remains to be understood but could reflect complementarity in function between genes within the groupings.

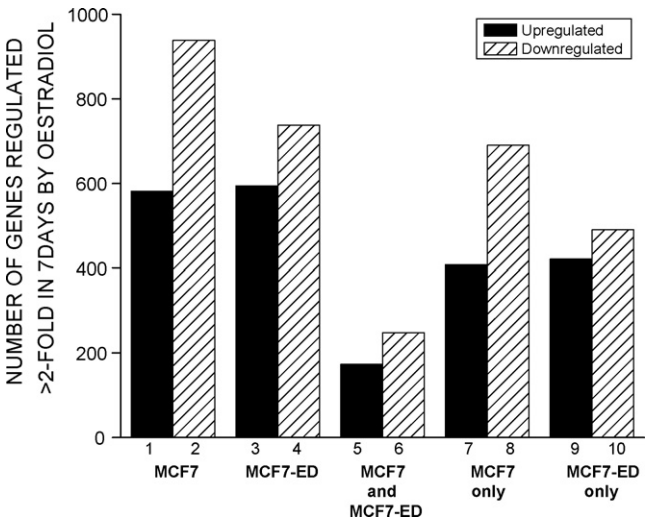



**Fig. 3.** Number of the genes on the expression microarray (out of a total of 19,947 genes) which were upregulated or downregulated by ≥2-fold by $10^{-8}$ M 17β-oestradiol in oestrogen maintained (MCF7) or oestrogen-deprived (MCF7-ED) MCF7 human breast cancer cells (tracks 1–4). Cells were grown for one year as stock cultures in oestrogen-maintained medium (MCF7) or oestrogen-deprived medium (MCF7-ED) and then for a further 7 days in the oestrogen-deprived medium with or without added 17β-oestradiol at $10^{-8}$ M. Using a cut-off of ≥2-fold, only some genes were regulated by oestradiol in both MCF7 and MCF7-ED cells (tracks 5–6). Some genes lost oestrogen regulation after long-term oestrogen deprivation (tracks 7–8) whilst others developed *de novo* oestrogen regulation (tracks 9–10).

## 4. Alteration to global gene expression patterns following long-term oestrogen deprivation

It has been known now for many years that MCF7 cells, which are responsive to oestrogen for growth (Fig. 1A), can adapt and learn to grow in the long-term absence of oestrogen such that their growth rate in the absence of oestradiol rises to equal that of the previous growth rate only in the presence of oestradiol (Fig. 1B) [15,34–41]. This growth adaptation has been observed in other oestrogen-responsive human breast cancer cell lines [55,57,58] and events have been shown to be reproducible [55]. It does involve clonal selection but with a high frequency suggesting epigenetic changes across the cell population and not outgrowth of a single mutated clone [55]. Oestrogen receptor alpha (ERα) levels are not lost but rather upregulated and remain downregulatable by readdition of oestradiol [41] in line with the known downregulation of ER by oestradiol [59]. Furthermore, this loss of oestrogen responsiveness relates only to growth and is not mirrored by loss of oestrogen responsive gene expression [15,34–41]. Levels of ERβ seem to be unaffected [41]. If these cells are transferred to a serum-free medium, then oestrogen responsive growth is restored and occurs with an altered dose–response relationship such that the cells will grow at lower concentrations of oestradiol than the stock oestrogen-maintained cells. This phenomenon has been termed "hypersensitivity to oestrogen" and occurs not only in cell culture [60] but is also a property of the cells when placed back *in vivo* in xenograft tumours [61]. However, although it is associated with increased transcription [36], hypersensitivity is found in cell culture only in serum-free medium and only in terms of oestrogen-regulated cell growth and not oestrogen-regulated gene expression [36,62]. Through the years, a variety of single gene targets and signaling pathways have been implicated in the growth adaptation to long-term oestrogen deprivation [9–17,38–40]. In order to obtain a wider view of gene expression changes, we have used the Amersham Codelink 20K expression microarrays to ask questions of the extent of global gene expression changes as the cells undergo this adaptive process.

### 4.1. Alterations to oestrogen-regulated gene expression

Long-term oestrogen deprivation resulted in alterations to oestrogen-regulated gene expression. Overall, a similar 7% of the genes on the array were altered ≥2-fold by oestradiol over 7 days in the oestrogen-maintained MCF7 and in the oestrogen-deprived MCF7-ED cells, and in each case, more genes were downregulated by oestradiol than upregulated (Fig. 3 tracks 1–4). However, the proportion of the genes downregulated by ≥2-fold was reduced from 62% in the oestrogen-maintained cells (Fig. 3, tracks 1–2) to 55% in the oestrogen-deprived cells (Fig. 3 tracks 3–4). Furthermore, it was not the same genes which showed oestrogen regulation in both cases and only 28% of the genes regulated ≥2-fold by oestradiol in the oestrogen-maintained cells were also regulated by ≥2-fold in the oestrogen-deprived cells (Fig. 3 tracks 5–6): some genes lost oestrogen regulation (Fig. 3 tracks 7–8) whilst others developed *de novo* oestrogen regulation (Fig. 3 tracks 9–10) after long-term oestrogen deprivation. Fig. 4 illustrates these differences in patterns of alterations to oestrogen-regulated gene expression for a few highly regulated genes. Although fold-regulation was reduced, GREB1 protein remained upregulated and uroplakin3A remained downregulated by oestradiol after long-term oestrogen deprivation. However, the upregulation of interleukin 20 and the downregulation of lymphotoxin beta (TNF3) in the oestrogen-maintained cells were no longer observed in the long-term oestrogen-deprived cells. By contrast, vimentin and folate hydrolase were not found to be oestrogen regulated in the stock oestrogen-maintained cells but developed oestrogen responsiveness after long-term oestrogen deprivation (Fig. 4). Using a 1900 cDNA microarray, long-term oestrogen deprivation has previously been reported to result in altered short-term transcriptional responses to oestradiol up to 24 h [32], and our larger 20K array together with the longer time frame of 7 days of oestradiol exposure shows even more extensive changes to oestrogen-regulated

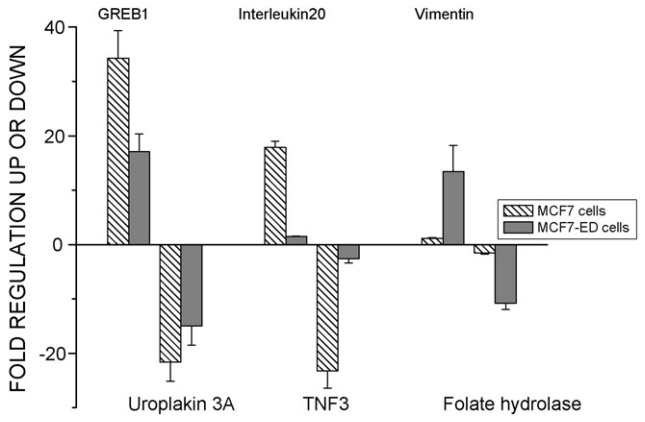



**Fig. 4.** Global gene expression profiles reveal differences in oestrogen-regulation of individual genes after long-term oestrogen deprivation in MCF7 human breast cancer cells as grown under conditions described in Fig. 3. GREB1 protein (NM_014668) was upregulated and uroplakin 3A (NM_006953) downregulated by oestradiol in both oestrogen-maintained and oestrogen-deprived cells. However, interleukin 20 (NM_018724) was upregulated and lymphotoxin beta (TNF superfamily member 3) (NM_009588) downregulated by oestradiol only in the oestrogen-maintained cells and after long-term oestrogen deprivation the regulation was lost. By contrast vimentin (NM_003380) and folate hydrolase (NM_004476) were genes which were not regulated in the stock oestrogen-maintained cells but developed *de novo* regulation by oestradiol after long-term oestrogen deprivation.

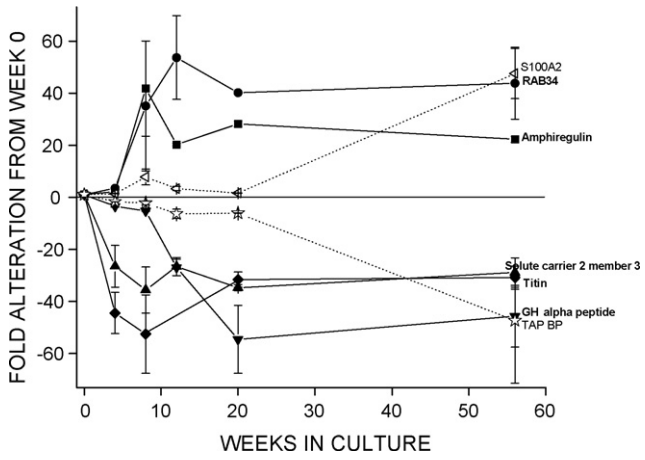



**Fig. 6.** Time course of the alterations to gene expression in MCF7 human breast cancer cells following long-term oestrogen deprivation. Microarray expression profiles were carried out by growing cells for 7 days in oestrogen deprived medium following 0, 4, 8, 12, 20 or 55 weeks in the oestrogen-deprived medium. Values were normalized per chip and per gene and fold alteration is shown as mean fold change from time 0 weeks over biological replicates. Expression of the genes Ras oncogene family member RAB34 (NM_031934) and amphiregulin (NM_001657) were upregulated at an earlier time than S100 calcium binding protein A2 (NM_005978). Expression of the genes for titin (X69490) and solute carrier 2 member 3 (NM_003039) were downregulated prior to glycoprotein hormone alpha peptide (NM_000735) and that was followed later by TAP binding protein-related gene (NM_018009).

gene expression. Lists of genes altered by ≥2-fold by oestradiol in both oestrogen-maintained MCF7 and oestrogen-deprived MCF7-ED cells have been recorded in a Ph.D. thesis as have those genes altered by ≥2-fold by oestradiol in only MCF7 cells (and not in MCF7-ED cells), and in only MCF7-ED cells (and not in MCF7 cells) [63].

### 4.2. Alterations to gene expression with increasing time of oestrogen-deprivation

In order to investigate the time course of alterations to gene expression as the cells undergo growth adaptation in the long-term absence of oestrogen, expression arrays were carried out for RNA samples taken after 0, 4, 8, 12, 20 and 55 weeks of growth in the oestrogen-deprived medium (Fig. 5A). Comparing the number of genes altered by ≥2-fold between each of the two consecutive time points (Fig. 5B), it was found that there were more gene changes in the early time periods than in the later times but there were still large numbers of alterations to gene expression even between 20 and 55 weeks. Proportionally more genes were downregulated between weeks 0 and 4 but this was followed by phases where proportionally more genes were upregulated between 4–8 and 8–12 weeks. There remained genes altered at later time frames but numbers were less and the proportion downregulated or upregulated was similar (Fig. 5). Fig. 6 illustrates the varied patterns of alterations to gene expression over the one year time course for a few highly regulated genes. It was evident that some genes, such as the Ras oncogene family member RAB34 and amphiregulin were upregulated at relatively early times whilst the S100 calcium binding protein A2 was upregulated at a later time point (Fig. 6). Titin and solute carrier 2 member 3 were downregulated at earlier time points than the TAP binding protein-related gene (Fig. 6).

Gene profile reproducibility was tested when comparisons were made not just between biological replicates of cells from the same time course of long-term oestrogen deprivation but between

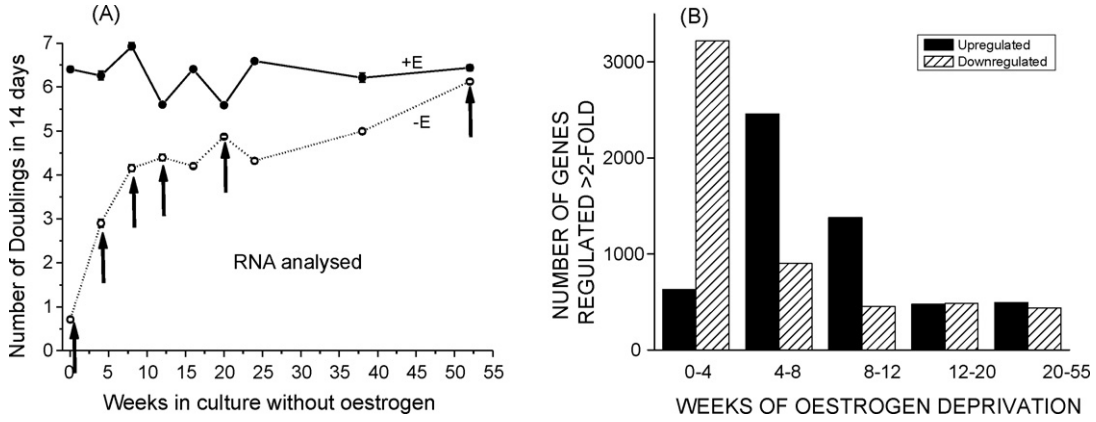



**Fig. 5.** Changes in global gene expression patterns over time during long-term oestrogen deprivation of MCF7 human breast cancer cells. (A) Time course of the changes in growth response to oestradiol during long-term oestrogen deprivation. Cells were grown for increasing periods of time in oestrogen-deprived medium and the number of doublings over 14 days determined in the same oestrogen-deprived medium with (+E) or without (−E) $10^{-8}$ M 17β-oestradiol. Error bars represent the standard error of all 9 values of triplicate estimates of cell numbers after 0 and 14 days. Microarray expression profiles were carried out by growing cells for 7 days in oestrogen deprived medium following 0, 4, 8, 12, 20 or 55 weeks in the oestrogen-deprived medium. (B) Number of the genes on the expression microarray (out of a total of 19,947 genes) which were upregulated or downregulated ≥2-fold between each of the two consecutive time points.

**Table 3**
List of genes which were upregulated (+ values) or downregulated (− values) ≥2-fold between 1 week and one year of oestrogen deprivation.

| Genbank | Description | Function | MCF7-ED | Time course 55 wk |
|---|---|---|---|---|
| NM_005032 | Plastin 3 (T isoform) | Actin cytoskeleton | 3.0 | 8.3 |
| NM_016629 | Tumour necrosis factor receptor superfamily, member 21 | Apoptosis | −2.9 | −2.2 |
| NM_001188 | BCL2-antagonist/killer 1 (BAK1) | Apoptosis | −3.6 | −3.9 |
| NM_004848 | Chromosome 1 open reading frame 38 | Cell adhesion | −4.2 | −2.0 |
| NM_078467 | Cyclin-dependent kinase inhibitor 1A (p21, Cip1) | Cell cycle | −2.9 | −4.9 |
| NM_003225 | Trefoil factor 1 (pS2) | Cell proliferation | 2.5 | 16.2 |
| NM_007286 | Synaptopodin | Cytoskeleton | −4.2 | −11.6 |
| NM_004753 | Short-chain dehydrogenase/reductase 1 | Fatty acid metabolism | −2.1 | −33.1 |
| NM_004411 | Dynein, cytoplasmic, intermediate polypeptide 1 | Microtubule based process | −2.2 | −3.5 |
| NM_020992 | PDZ and LIM domain 1 (elfin) | Oxidative stress | −2.5 | −2.1 |
| BC008915 | Serine (or cysteine) proteinase inhibitor, clade A member 5 | Protease inhibitor | −6.8 | −7.2 |
| NM_030952 | Ortholog of rat SNF1/AMP-activated protein kinase | Protein phosphorylation | −3.7 | −2.5 |
| NM_021643 | Tribbles homolog 2 | Protein phosphorylation | −4.4 | −9.3 |
| NM_005607 | PTK2 protein tyrosine kinase 2 | Protein phosphorylation | −10.7 | −7.2 |
| NM_001710 | B-factor, properdin | Proteolysis | −21.8 | −31.2 |
| NM_001124 | Adrenomedullin | Signal transduction | 7.4 | 12.0 |
| NM_000125 | Oestrogen receptor 1 (ERα) | Signal transduction | 4.6 | 5.3 |
| NM_015149 | RalGDS-like gene | Signal transduction | −2.1 | −10.5 |
| NM_014353 | RAB26, member RAS oncogene family | Signal transduction | −2.4 | −4.7 |
| NM_005567 | Lectin, galactoside-binding, soluble, 3 binding protein | Signal transduction | −2.9 | −31.7 |
| NM_003706 | Phospholipase A2, group IVC (cytosolic, Ca-independent) | Signal transduction | −4.2 | −13.8 |
| AB033022 | K1AA1196 protein | Transcriptional regulation | −2.2 | −2.8 |
| NM_032211 | Lysyl oxidase-like 4 | Transport | −2.0 | −5.5 |
| NM_006598 | Solute carrier family 12 (K/Cl transporters), member 7 | Transport | −3.0 | −5.9 |
| NM_015516 | Hypothetical protein, estradiol-induced | Unknown | 4.3 | 3.5 |
| NM_015400 | DKFZP586N0721 protein | Unknown | −2.0 | −5.0 |
| NM_018265 | Hypothetical protein FLJ10901 | Unknown | −2.1 | −16.3 |
| AL049949 | Hypothetical protein FLJ90798 | Unknown | −2.1 | −2.8 |
| NM_018009 | TAP binding protein related | Unknown | −2.1 | −40.6 |
| AK022375 | cDNA FLJ12313 fis, clone MAMMA1002041 | Unknown | −2.5 | −4.2 |
| NM_024738 | Hypothetical protein FLJ21415 | Unknown | −3.0 | −2.1 |
| BE788111 | Homo sapiens cDNA clone IMAGE:3882633 | Unknown | −3.3 | −12.4 |
| AL713742 | DKFZP434C171 protein | Unknown | −3.5 | −4.2 |
| NM_004209 | Synaptogyrin 3 | Unknown | −4.0 | −6.5 |
| NM_032413 | Normal mucosa of esophagus specific I | Unknown | −5.6 | −26.2 |
| ALI63248 | EST | Unknown | −2.0 | −3.5 |
| AW955692 | Ubiquiitin specific protease 2 | Unknown | −2.8 | −5.1 |

Genes are listed which were altered in the same direction and with a level of significance of $P < 0.05$ in two independent time courses (MCF7-ED (Fig. 1) and time course 0–55 weeks (Fig. 5)) of one year carried out from the same starting passage number of MCF7 human breast cancer cells [63].

independent time courses of one year carried out from the same starting passage number of MCF7 cells. Table 3 lists the genes which were altered by ≥2-fold in the same direction and with a level of significance of $P < 0.05$ [63]. This list was selected to contain the identity only of those genes which were upregulated in both one year time courses or downregulated in both one year time courses, but even then, the fold changes varied considerably. This revealed the large degree to which there was variability in gene changes with independent time courses of long-term oestrogen deprivation. This would suggest that there is no single gene target, no single functional group and no single signaling pathway which is consistently altered in these experimental conditions to explain this adaptation to growth without oestrogen. Upregulation of the gene for adrenomedullin was one of the higher most consistent alterations (Table 3), and its overexpression in cancer cells is already thought to stimulate cell proliferation, to inhibit apoptosis, to enable tumour cells to evade immune surveillance and to act as an angiogenic factor promoting neovascularisation due to its upregulation under hypoxic conditions [64–67] which suggests that it might play a functional role in cellular adaptation to long-term oestrogen deprivation. The gene for ERα was also consistently upregulated after long-term oestrogen deprivation, in line with previous publications using this model system [36,41] and the known downregulation of ERα by oestrogen [59]. Since pS2 is a well established oestrogen-regulated gene [68], its expression would have been expected to be consistently low after long-term oestrogen deprivation in both time courses but it was not, which might explain why sometimes oestrogen deprivation is reported to result in low pS2 [34,41] and sometimes it is not [69].

Interestingly, the list of genes in Table 3 also showed altered expression of a gene for a member of the tribbles family of signaling regulator proteins which appear to act as "bottle necks" with scaffold-like regulatory function in the linking of independent signal processing systems and which have been implicated in insulin resistance [70]. This raised the potential question that its consistent alteration might result from removal of insulin from the culture medium rather than oestrogen deprivation. The oestrogen deprivation model of loss of oestrogen responsive growth does involve more manipulations than simply withdrawing oestrogen from the MCF7 cells [41]. It involves transferring the cells to a medium lacking phenol red [53] containing charcoal stripped serum [54] and withdrawing insulin as well as oestradiol [6,41]. It is therefore likely that the gene changes observed in this model are unduly complicated by all these manipulations which mask the specific alterations associated with oestrogen deprivation.

When expression microarray profiles of oestrogen-maintained MCF7 cells were compared between cells grown for 1 week in oestrogen-maintained medium and cells grown for 1 week in oestrogen-deprived medium with or without added oestradiol, there were more gene alterations associated with moving the cells into the oestrogen-depleted medium than associated with the presence or absence of oestradiol in the oestrogen-deprived medium. Transfer for 7 days to oestrogen-deprived medium but keeping oestradiol present resulted in about one-third of the genes (6671) on the array altering expression by ≥2-fold, of which 77% were downregulated, but the difference between growing the cells in the oestrogen-deprived medium for 7 days with or without added oestradiol resulted in only 1516 genes altered by ≥2-fold,

of which only 62% were downregulated. Furthermore, this transfer into charcoal-stripped serum without insulin also influenced the oestrogen regulation of gene expression. Fig. 7 illustrates the patterns of alterations for a few highly regulated genes. The genes GREB1 protein, neuropeptide Y receptor Y1 and transmembrane protease serine 3 retained oestrogen regulation, albeit with reduced upregulation, in the oestrogen-deprived medium. However, the genes for insulin-like growth factor binding protein 5 (IGFBP5), solute carrier family 1 (glutamate transporter) member 2 and sulphatase 1 lost oestrogen responsiveness in the oestrogen-deprived medium whilst the genes for PDZ domain containing 1, interleukin 20 and pS2 were regulated to a greater extent by oestradiol in the oestrogen-deprived medium. Since transfer to the medium with charcoal-stripped serum and lacking added insulin resulted in more changes to global gene expression than simply addition or removal of oestradiol, the underlying cell biological model now needs refining in order to identify the extent to which oestradiol removal is involved in the observed processes of growth adaptation (Fig. 1) and in order to separate the events associated with the switch in serum/insulin environment from those events more specifically associated with adaptation to oestrogen deprivation.

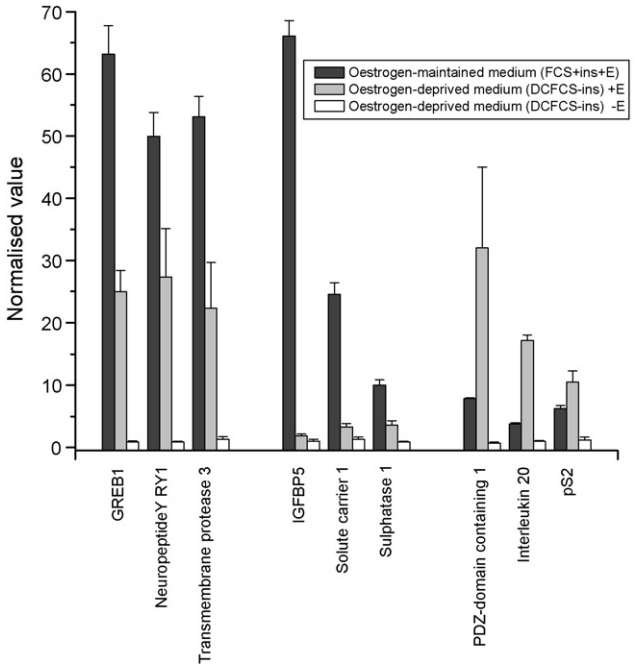
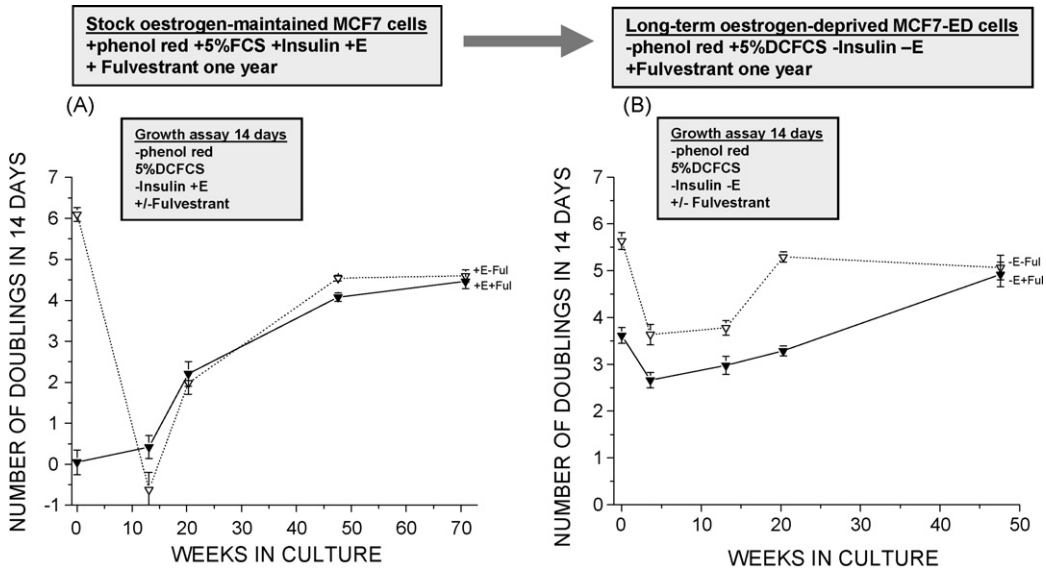



**Fig. 7.** Effect on oestrogen regulation of gene expression following the transfer of oestrogen-maintained MCF7 cells into oestrogen-deprived medium. Global gene expression patterns were investigated in oestrogen-maintained MCF7 cells replated at the same density and grown for 7 days in oestrogen-maintained medium or in oestrogen-deprived medium with (+E) or without (−E) added 17β-oestradiol at $10^{-8}$ M. Values were normalized per chip and per gene. Expression levels of the genes for GREB1 protein (NM_014668), neuropeptide Y receptor Y1 (NM_000909) and transmembrane protease serine 3 (NM_032404) were all reduced following growth in oestrogen-deprived medium but the expression remained upregulated by oestradiol. Expression levels of the genes for insulin growth factor binding protein 5 (IGFBP5) (L27560), solute carrier family 1 (glutamate transporter) member 2 (AK057674) and sulphatase 1 (AB029000) were also reduced following growth in oestrogen-deprived medium but oestradiol-induced expression was substantially reduced. Expression of the genes for PDZ domain containing 1 (NM_002614), interleukin 20 (NM_018724) and trefoil factor 1 (pS2) (NM_003225) were all increased with oestradiol in the oestrogen-deprived medium to a greater level of expression than in the oestrogen-maintained medium.

## 5. Alteration to global gene expression patterns following long-term exposure to fulvestrant

Many of the published models for the study of the development of antioestrogen (tamoxifen, fulvestrant) resistant growth in MCF7 cells also suffer with the same complication of a parallel change to culture conditions in conjunction with addition of the antioestrogen [41]. Often the model involves transfer to charcoal-stripped serum, ceasing the addition of insulin or oestradiol and then adding antioestrogen in addition. In anticipation of the compounding effects of these multiple culture manipulations on molecular alterations in the cells, we have developed cell culture models of antioestrogen resistant growth where the effects of addition of tamoxifen or fulvestrant could be separated from the effects of alterations to other culture parameters [41]. This was achieved in two independent ways. In the first model [41], MCF7 cells were kept long-term in stock oestrogen-maintained medium (FCS, insulin and oestradiol) with or without antioestrogen. This model has the benefit of mirroring the situation *in vivo* where oestradiol and insulin would normally remain present throughout antioestrogen administration. In the second model [41], MCF7 cells were initially grown in the oestrogen-deprived medium (DCFCS lacking insulin or oestradiol) for one year which allowed time for growth adaptation and upregulated basal growth rate. These long-term adapted cells were then maintained for a further one year with or without the addition of a single antioestrogen. This allowed for the effects of growth adaptation to culture conditions to be separated from the effects of the subsequent addition of antioestrogen [41].

Use of this model system to study the molecular basis of growth resistance to fulvestrant has shown that in both models the cells adapt to the imposed growth inhibition and learn to grow in the presence of fulvestrant over the period of one year [41]. The cells in the oestrogen-maintained medium gave an initial strong growth inhibition by fulvestrant which was lost in the long-term (Fig. 8A) and in accordance with the action of fulvestrant on degradation of the ER [71–73], ER levels fell to undetectable levels in the long-term [41]. The cells grown in the oestrogen-deprived medium exhibited a reduced growth inhibitory response to fulvestrant and this was also lost in the long-term (Fig. 8B), but in contrast retained a low level of ERα even in the long-term [41]. Use of the Amersham Codelink 20K expression microarrays revealed that a similar 22% of the genes on the array were altered by ≥2-fold by long-term exposure to fulvestrant in both models, but in contrast to the greater proportion of genes being downregulated by oestradiol, more genes were upregulated by one year of exposure to fulvestrant in either of the model systems (Fig. 8). Increase in the threshold of fold induction showed that some genes were regulated to very high fold levels with a few regulated by ≥10,000-fold in both model systems (Fig. 8, Table 4). However, despite the similarity in the overall number of genes altered in the two model systems, it was not all the same genes altered in each case. In fact, only 48% of the 4343 genes regulated ≥2-fold by fulvestrant in the oestrogen-maintained cells were also regulated by ≥2-fold by fulvestrant in the oestrogen-deprived cells (Fig. 8). Lists of the genes altered by long-term exposure to fulvestrant by ≥2-fold and with a level of significance $P < 0.05$ in both models have been recorded in a Ph.D. thesis, as have those genes altered by fulvestrant only in the oestrogen-maintained model and only in the oestrogen-deprived model [63]. Subdivision of the genes into the same functional groupings as for oestradiol (Table 1, Fig. 2) showed again a similar proportion of genes up- and downregulated across each functional grouping for both models (Fig. 9). This suggests that, as for oestradiol, long-term exposure to fulvestrant also influences a wide range of different cellular functions and that only a similar proportion of genes in each functional group is affected rather than all of one functional grouping as might have been anticipated/hoped. Table 4 lists the genes which were regulated ≥100-fold in the same direction in both models. Whilst the magnitude of the fold regulation may or may not be relevant to function, it might be hoped that some of these genes regulated in




| Fold regulation | Oestrogen maintained MCF7 | | | Oestrogen-deprived MCF7 | | |
|---|---|---|---|---|---|---|
| | Total | Upregulated | Downregulated | Total | Upregulated | Downregulated |
| 2-fold | 4343 | 2746 | 1597 | 4264 | 2784 | 1480 |
| 10-fold | 1281 | 896 | 385 | 835 | 505 | 330 |
| 100-fold | 479 | 317 | 162 | 133 | 82 | 51 |
| 1,000-fold | 160 | 91 | 69 | 33 | 15 | 18 |
| 10,000-fold | 15 | 3 | 12 | 8 | 0 | 8 |

**Fig. 8.** The cell culture model for studying loss of response to fulvestrant following long-term exposure to the antioestrogen in either oestrogen-maintained or oestrogen-deprived MCF7 human breast cancer cells. Cells were grown for one year under either oestrogen-maintained or oestrogen-deprived conditions and then for a further year in the presence or absence of $10^{-7}$ M fulvestrant [41]. The inhibitory response to fulvestrant in the oestrogen-maintained (A) and oestrogen-deprived (B) cells was lost with time over the course of one year of continuous exposure to the antioestrogen. Changes in global gene expression were investigated following growth of oestrogen-maintained cells for one year with or without fulvestrant and were compared with those following growth of oestrogen-deprived cells for one year with or without fulvestrant. The total number of genes (out of a total of 19,947 genes) which were altered in their expression both up or down are shown using cut-off values of 2-fold to 10,000-fold, together with subdivision into the numbers of genes either upregulated or downregulated.

**Table 4**
List of genes which were upregulated (+ values) or downregulated (− values) ≥100-fold in the same direction and with a level of significance of $P < 0.05$ following long-term (one year) exposure to fulvestrant in either the oestrogen-maintained or the oestrogen-deprived culture model [41].

| Fold change, MCF7 cells | Fold change, MCF7-ED cells | Gene | Genbank number |
|---|---|---|---|
| Upregulated >100-fold by fulvestrant in both models | | | |
| 2,013 | 197 | G protein gamma 4 | NM_004485 |
| 1,338 | 145 | Ubiquitin-activating enzyme E1-like | NM_003335 |
| 646 | 222 | Defensin beta 1 | NM_005218 |
| 642 | 151 | Heparanase | NM_006665 |
| 542 | 212 | Vestigial like 1 (Drosophila) | NM_016267 |
| 517 | 167 | B lymphocyte gene 1 | NM_007005 |
| 182 | 223 | Tensin | NM_022648 |
| 172 | 1,288 | Antileukoproteinase | NM_003064 |
| 168 | 431 | Heparan sulfate proteoglycan 2 (perlecan) | NM_005529 |
| 141 | 245 | Adaptor-related protein complex 1, sigma 3 subunit | BC021898 |
| 140 | 302 | UDP glycosyltransferase 1 family, polypeptide A10 | NM_007120 |
| 140 | 139 | Peroxisome proliferative activated receptor, gamma | NM_005037 |
| 131 | 190 | Bridging integrator 1 | NM_004305 |
| 128 | 531 | Myxoid liposarcoma associated protein 4 | NM_018192 |
| Downregulated >100-fold by fulvestrant in both models | | | |
| −16,264 | −400 | Transmembrane protease, serine 3 | NM_032404 |
| −15,716 | −10,331 | Sarcosine dehydrogenase | AF095735 |
| −5,581 | −409 | Rac/Cdc42 guanine nucleotide exchange factor 6 | D25304 |
| −4,904 | −5,235 | Complement factor H related 3 | NM_021023 |
| −2,807 | −2,269 | Clade F member 1 | NM_002615 |
| −1,422 | −33,092 | Guanylate cyclase 1, soluble, alpha 3 | NM_000856 |
| −1,061 | −205 | Solute carrier family 21(prostaglandin transporter)member 2 | NM_005630 |
| −1,014 | −190 | NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4 | U94586 |
| −651 | −448 | Relaxin 2 (H2) | NM_005059 |
| −427 | −4,456 | Clade I (neuroserpin) | NM_005025 |

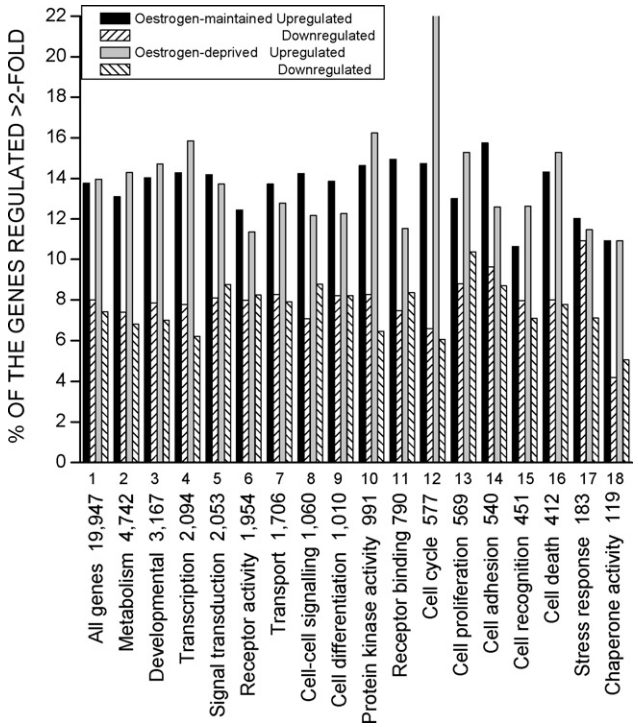



**Fig. 9.** Percentage of genes upregulated or downregulated by ≥2-fold in oestrogen-maintained MCF7 and in oestrogen-deprived MCF7-ED human breast cancer cells after each was grown for one year with or without $10^{-7}$ M fulvestrant. Regulation is given as a percentage of the total genes (19,947 genes) on the expression microarray (track 1) and of the genes when subdivided into a range of functional groupings within GeneSpring (tracks 2–18). The number of genes in each functional grouping is shown on the *x*-axis and the proportional overlap of gene identity between each of the groups has been collated in Table 1.

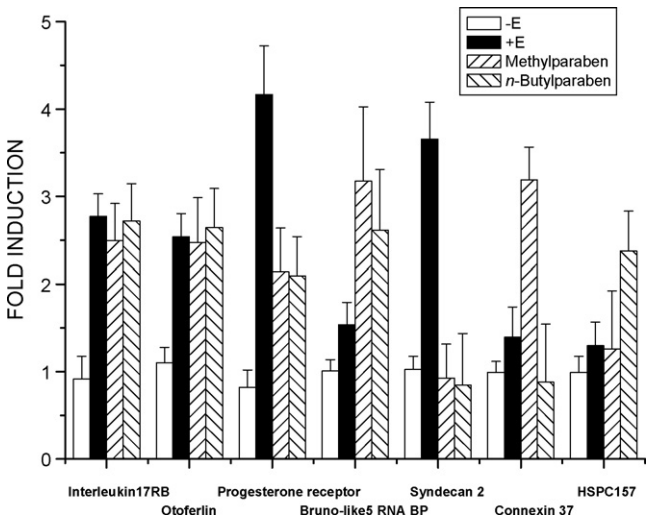



**Fig. 10.** Global gene expression profiles reveal differences in expression of individual genes upregulated by 17β-oestradiol, methylparaben and *n*-butylparaben under the growth conditions previously published [33]. Values from three biological replicate arrays were normalized per chip and per gene and expression levels are shown as mean fold change with the standard error (SE) of the triplicate values. Interleukin 17receptorB (IL17RB) (NM_018725) and otoferlin (OTOF) (NM_004802) were upregulated to a similar extent by 17β-oestradiol, methylparaben and *n*-butylparaben. Progesterone receptor (PR) (NM_000926) was upregulated to a greater extent by 17β-oestradiol than by the parabens. Bruno-like5/CELF5 RNA binding protein (CELF5) (NM_021938) was upregulated to a greater extent by parabens than by 17β-oestradiol. Syndecan 2 (SDC2) (J04621) was upregulated only by 17β-oestradiol. Connexin 37 (CX37) (NM_002060) was upregulated only by methylparaben. HSPC157 protein (NM_014179) was upregulated more by *n*-butylparaben than by 17β-oestradiol or methylparaben.

the same direction in both models might prove to be molecular markers of long-term exposure of MCF7 cells to fulvestrant.

## 6. Alteration to global gene expression following exposure to other oestrogenic compounds

Although most studies have concentrated on the actions of the physiological oestrogen, 17β-oestradiol, and the therapeutically important antioestrogens, tamoxifen and fulvestrant, the human breast is exposed variably in the modern world to a range of other compounds which possess also oestrogenic properties. This includes not only other physiological and pharmaceutical oestrogens but also phytoestrogens consumed in edible plant material [74] and xenoestrogens either consumed in the diet as pollutant chemicals in animal fat or applied topically to human skin in the form of personal bodycare products [49,51,75]. The alkyl esters of *p*-hydroxybenzoic acid (parabens) are one group of environmental oestrogens to which the human population is exposed through their widespread use as preservatives in cosmetic, food and pharmaceutical consumer products [76,77]. Methylparaben, ethylparaben, *n*-propylparaben, *n*-butylparaben and isobutylparaben are the most widely used esters and these have now all been shown to possess oestrogenic activity in assay systems *in vitro* and *in vivo*, with oestrogenic activity increasing with linear length of the alkyl chain and with branching in the alkyl chain [51].

Although parabens have been shown to increase the expression of oestrogen-regulated reporter genes and of endogenous oestrogen-regulated genes (such as pS2) in a manner similar to that of 17β-oestradiol [51], an important question remains as to their wider similarity of action on global gene expression patterns. Using the Amersham Codelink 20K expression microarrays, we have shown that as for oestradiol, more genes were downregulated than upregulated by both methylparaben and *n*-butylparaben but the identity of the genes upregulated or downregulated was not the same [33]. Of the genes upregulated by 17β-oestradiol, only 39% were also upregulated by methylparaben and 27% by *n*-butylparaben [33]. Of the genes downregulated by 17β-oestradiol, 62% were also downregulated by methylparaben and 46% by *n*-butylparaben [33]. Some individual genes could be identified with a similar pattern of response to 17β-oestradiol and both parabens such as interleukin 17RB and otoferlin (Fig. 10) [33]. However, other genes could be identified which were regulated differently by 17β-oestradiol and the two parabens such as progesterone receptor which was regulated to a greater extent by oestradiol than the parabens, and Bruno-like5 (CELF5) RNA binding protein which was regulated to a lesser extent by oestradiol than the parabens (Fig. 10) [33]. Yet other genes could be identified which were regulated only by 17β-oestradiol (syndecan 2), only methylparaben (connexin 37) or only *n*-butylparaben (HSPC157 protein) (Fig. 10) [33]. Clearly, therefore, although parabens can mimic the action of oestradiol on the expression of some genes, the extent of their mimicry is not perfect across global patterns of gene expression, and gene expression profiles even differed between the two individual parabens. This is in agreement with global gene expression studies published for other environmental oestrogens [78–81] and demonstrates that oestrogenic compounds, even of such similar structure as these two parabens [51], can each give rise to a unique profile of gene expression and such differences could be expected to result in different consequences to cell regulation and even in different toxicological responses.

## 7. Conclusions

- Studies using global gene expression profiling have shown that oestrogen action in MCF7 human breast cancer cells involves the

coordinated regulation of hundreds of genes. In contrast to previous studies focused on a few single upregulated genes, global gene profiles show that more genes are downregulated than upregulated by oestradiol. However, since a similar percentage of genes are regulated either up or down across a wide range of different functional groupings, this demonstrates that there is no single gene target and no single signaling pathway responsible for oestrogen action but rather that oestradiol influences a wide range of varied cell functions.

- Long-term oestrogen deprivation, which results in loss of oestrogen-responsive growth, involves complex alterations to gene patterns over the time course of a year. The number of genes altered in expression by ≥2-fold is greatest in the early weeks and decreases with time but alterations to gene expression were still observed at the last time point studied of 20–55 weeks. Events in the first 4 weeks seemed to involve proportionally more genes being downregulated than upregulated but this was followed by a period between 4 and 12 weeks where proportionally more genes were upregulated. Later time points showed lower numbers of alterations to expression of genes and more similar proportions of up and down regulation. Long-term oestrogen deprivation also resulted in alterations to the patterns of oestrogen-regulated genes. Only 48% of the genes which were oestrogen-regulated in the oestrogen-responsive cells retained this responsiveness after the long-term oestrogen deprivation, and in addition, other genes developed *de novo* oestrogen regulation after oestrogen deprivation. The significance of the general decrease in the proportion of genes downregulated by oestradiol following progression to oestrogen independent growth remains to be established.
- Long-term exposure to fulvestrant, which also results in growth resistance to the antioestrogen, involves even larger changes to gene expression both in terms of the number of genes altered ≥2-fold and in terms of the magnitude of the response.
- Environmental chemicals with oestrogenic properties do not give global gene expression profiles identical to either oestradiol or to other xenoestrogens even of similar chemical structure. Since many environmental oestrogenic chemicals are now known to enter the human breast, this suggests a more complex web of interference in cell function than simply mimicking oestrogen action at inappropriate times.

## Acknowledgments

We thank the Breast Cancer Campaign and the Felix Trust for financial support.

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