

Changes in expression of steroid receptors, their downstream target genes and their associated co-regulators during the sequential acquisition of tamoxifen resistance *in vitro*

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Abstract. Tamoxifen resistance (TAMr) in breast cancer is a serious clinical dilemma, with no satisfactory explanation. We hypothesised that changes in the expression of steroid hormone receptors (ER α , ER β), their downstream target genes (PR, pS2) and their associated co-regulators (AIB-1, SRC-1, SRA, NCoR-1, SMRT and REA) could be related to the acquisition of TAMr. To test this hypothesis, we developed *in vitro* TAMr cell line models by continuous exposure of MCF-7 cells to 4-hydroxytamoxifen (4-HT) over 12 (MCF-7^{MMU1}) and 21 (MCF-7^{MMU2}) months, respectively and examined the expression of the above by Western blotting and immunohistochemistry. In addition, we further examined the changes in global gene expression in TAMr cells in comparison with TAM-sensitive cells by microarray analysis. We report here that acquisition of TAMr is associated with changes in the expression of PR, pS2 and several co-activators, but not ERs. In addition, genes associated with cell cycle, cell adhesion and extracellular matrix, were up-regulated while those associated with apoptosis or growth factors/hormones were down-regulated. Based on our results, it appears that increased co-activator expression, in concert with alterations in genes associated with controlling cell proliferation and survival contribute to TAMr in breast cancer.

Introduction

Tamoxifen (TAM) has been the primary line of therapy for ER α -positive breast cancer patients for nearly three decades and continues to be the choice of therapy for pre-menopausal

patients (1,2). Clinical studies have shown that about 70% of patients initially respond to this therapy but eventually acquire resistance (3) and the development of TAM resistance (TAMr) is a major clinical problem for long-term management of breast cancer (4).

The mechanism of action of TAM has been extensively investigated. It is now established that anti-tumour activity of TAM is mediated by binding to estrogen receptors (ER), ER α and ER β . Although the requirement of ER α in the response to TAM is undebated, the role of ER β remains controversial (5). The current thinking views it as a good prognostic marker (6).

In recent years it has become apparent that in addition to receptor binding, ER-signalling involves a complex array of co-regulatory proteins that function to enhance (co-activators) or repress (co-repressors) gene transcription (7-9). Alterations in levels of co-regulators have been implicated in breast tumourigenesis and TAMr. Overexpression of SRC-1, AIB-1, and SRA have been reported in primary breast tumours (10-12). Elevated levels of SRC-1 and AIB-1 have been associated with decreased response to endocrine therapy and poorer clinical outcome, which may ultimately result in TAMr through enhancing its agonist behaviour (13-15). TAMr tumours often have reduced levels of NCoR-1 (16-18). A role for co-repressors in TAM action has been supported by evidence that in the presence of TAM a stronger association with ER α occurs (16,19).

The aim of this work was to develop and characterise an *in vitro* model of TAMr and identify molecular changes associated with this phenomenon. Using immunohistochemistry and quantitative real-time PCR we investigated the expression of ER α , ER β , PR and pS2 in association with a small subset of 3 co-activators and 3 co-repressors in TAM sensitive MCF-7 cells and in our in-house model of acquired TAMr. Finally we used microarray analysis to explore changes in global gene expression associated with the acquisition of resistance.

Materials and methods

Development of an in vitro model of TAMr. MCF-7 cells were cultured in phenol-red free RPMI-1640 containing L-glutamine (both Invitrogen, Paisley, UK) supplemented with 5% charcoal-stripped steroid-depleted FCS (Harlan SeraLab,

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Table I. Real-time PCR primer sequences.

Gene	Accession no.	Forward primer	Reverse primer
<i>ERα</i>	M12674	dAGAGGGCATGGTGGAGATCTT	dCAAACCTCTCTCCCTGCAGATT
<i>ERβ</i>	AB006593	dTGGTCCATCGCCAGTTATCA	dAGGTGTGTTCTAGCGATCYYGCTT
<i>PR</i>	M15716	dGAACAGCGGATGAAAGAATCATC	dTTGAAGCTTGACAAACTCCTGTC
<i>pS2</i>	X00474	dATCGACGTCCCTCCAGAAGAG	dCTCTGGGACTAATCACCGTGTCT
<i>SRC-1</i>	U40396	dAACGTCAAGCGGGAAGTGTACA	dTGCCCTCATAAGCATGGCTCTT
<i>AIB-1</i>	AF012108	dTGGGAAGACCAGGAGGAGATT	dCTATTAGACCGAAGAGGCAATGTG
<i>SRA</i>	AF092038	dCCGGTGGGACGCAGC	dACTCCTACCATCCACTGACTGACC
<i>NCαR-1</i>	NM006311	dTCAGCCAGAGGTTGTAAAGGC	dTCGATAGTGATGTAATGGTCTTCATA
<i>SMRT</i>	XM006737	dTTCACACATCGTTGCCGC	dAAAATATACCCTGTAAACCAACACATC
<i>REA</i>	AF150962	dAGGGTAAGAAATGAGCCTAGTCACC	dGGTAGGGCTGTGCTGGACC
<i>RPLP0</i>	M17885	dGCTCAACATCTCCCCCTTCTC	dGATATCAAGCACTTCAGGGTTGTAGA

Loughborough), 100 U/ml penicillin and 100 U/ml streptomycin and 100 nM 4-hydroxytamoxifen (4-HT; Sigma, Poole), an active metabolite of TAM (20-22). We used 4-HT for 3 reasons: a) its binding affinity for ER α is higher than that of TAM (20,22), b) MCF-7 cells have limited capacity to metabolise TAM into its active metabolites and c) so we could directly relate our data to the results of other groups using similar models (22,23). Two separate lines were developed. MCF-7^{MMU1} was grown for 12 months in 4-HT with periodic cell characterisation. MCF-7^{MMU2} was cultured for 21 months in 4-HT after which this was removed and cells cultured for further 12 months. Parental MCF-7 cells (sensitive to 4-HT) were cultured in the same growth media as the MCF-7^{MMU1} or MCF-7^{MMU2} cells, but with ethanol vehicle only. In some cases, MCF-7^{MMU2} early (E) and late (L) were also studied which relate to MCF-7^{MMU2} cells grown in the absence of 4-HT for 13 (E) or 56 (L) passages. Bimonthly checks for *mycoplasma spp.* were negative.

Cell cycle analysis. Cells were seeded in 6-well plates (4x10⁴ cells/well) and allowed to reach log phase. Thereafter, cells were treated with either 1 nM E2 or 100 nM 4-HT for 72 h prior to overnight fixation with 70% ice-cold ethanol at -20°C. Fixed cells were then incubated with 50 µg/ml propidium iodide and 5 U/ml RNase (Sigma-Aldrich). Following overnight incubation at 4°C samples were analysed on a Coulter Epics XL flow cytometer (Beckman Coulter, High Wycombe) using the FL-3 photomultiplier to measure DNA content. Cell cycle distribution was quantified using multicycle software (Phoenix Flow Systems, San Diego, CA).

Immunohistochemistry. Cells were grown to log phase on glass slides (VWR International) and fixed in methanol:acetic (1:1). Blocking using 100 µl of normal rabbit serum (1:20) (DakoCytomation) was performed for 30 min at room temperature. Anti-ER α and PR antibodies (both DakoCytomation) were applied at 1:100 dilution and incubated at room temperature for 60 min. Anti-ER β antibody (1:200; AbCam) was incubated overnight at 4°C. Negative controls included substitution of primary antibody with rabbit serum. Three

washes [TBS (pH 7.6) for 5 min] preceded 30-min incubation in biotinylated secondary IgG (rabbit anti-mouse; 1:200; DakoCytomation). Slides were again washed before detection using streptavidin peroxidase (DakoCytomation), according to the manufacturer's recommendations. The final colour reaction was achieved using 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma) for 10 min and lightly counterstained with Mayer's haematoxylin. Slides were then dehydrated and taken through graded alcohols to xylene and finally mounted. Slides were viewed under light microscopy (Nikon Eclipse E1000) and analysed using Lucia software (version 4.51).

Western blotting. Western blotting was conducted as previously described using the same primary antibodies under identical conditions (24,25). Full-length ER β purified human recombinant protein standard (PanVera) was included in some experiments.

Real-time polymerase chain reaction. Using standard procedures (Qiagen), total RNA (10 µl) was reverse-transcribed in a final volume of 20 µl containing 1.5 µl of oligo(dT)₁₂₋₁₈ primer (0.5 µg/µl), 2 µl of 100 mM DTT, 4 µl 5X first strand buffer (25 mM Tris-HCl pH 8.3, 37.5 mM KCl, 1.5 mM MgCl₂), 1 µl dNTPs (10 mM each dNTP), 20 U of RNase inhibitor (Roche Diagnostics, Mannheim, Germany) and 200 U of Superscript II reverse transcriptase (all Invitrogen). Real-time quantitative PCR was performed using the ABI PRISM 7700 Sequence Detection System and the SYBR Green PCR Core Reagents kit according to the manufacturer's instructions (Perkin-Elmer Applied Biosystems). Primers were designed using the Primer Express program, which selected theoretically optimised primers for this system. Primer sequences are shown in Table I. An initial incubation of 50°C for 2 min was followed by denaturing at 95°C for 10 min and then 40 cycles at 95°C for 15 sec and 60°C for 1 min. PCR products were detected by bound SYBR Green double-stranded DNA fluorescence (26). Target gene expression was expressed relative to the housekeeping gene *RPLP0*. All experiments were performed in triplicate.

Table II. Effects of E2 and 4-HT on cell cycle distribution of MCF-7, MCF-7^{MMU1} and MCF-7^{MMU2}.

Cell line	Treatment	% G1	% S	% G2	
MCF-7	Con	64.05±1.38	22.53±1.80	13.43±0.82	
	E2	47.6±0.50	40.70±0.52 ^a	12.10±0.50	
	4-HT	72.2±0.28	13.40±0.42 ^a	14.45±0.78	
MCF-7 ^{MMU1}	M4	Con	61.25±1.20	17.10±0.28	21.70±0.85
		E2	58.8±0.14	20.55±0.49	20.60±0.42
		4-HT	60.65±0.49	18.95±0.42	20.50±0.78
	M7	Con	52.05±0.78	23.10±0.28	24.85±1.06
		E2	54.20±3.54	25.55±1.48	20.25±2.05
		4-HT	55.50±0.14	25.15±0.35	19.35±0.49
	M9	Con	53.90±2.69	20.25±0.78	25.85±1.91
		E2	50.80±0.42	18.90±0.99	30.30±0.57
		4-HT	51.25±1.77	20.30±0.99	29.75±1.06
M12	Con	48.60±0.85	22.10±1.70	29.15±0.92	
	E2	48.10±0.42	20.95±0.07	30.95±0.49	
	4-HT	50.10±0.71	21.20±0.85	28.65±1.49	
MCF-7 ^{MMU2}	Con	53.75±1.48	26.90±0.71	19.30±0.71	
	E2	49.00±0.57	28.65±0.92	22.40±0.42	
	4-HT	56.50±0.71	26.50±0.28	17.00±0.99	

Results are shown as percentage of cells present in each phase of the cells cycle and are the mean ± SD of 3 independent experiments. For MCF-7^{MMU1}, response to hormone was determined after 4, 7, 9 and 12 months (M) in the continuous presence of 4-HT. Significant differences in proliferation in response to E2 or 4-HT were only seen in the parental MCF-7 cells, confirming the onset of resistance. ^ap<0.05 versus control.

Microarray analysis. Total RNA was extracted from MCF-7^{MMU1} cells after 12 months in the continuous presence of 100 nM 4-HT and from parental MCF-7 controls using the Qiagen RNeasy Midi total RNA isolation kits (Qiagen, Valencia, CA) followed by further purification using two sequential Qiagen RNeasy Mini total RNA isolation columns according to manufacturer's protocol. RNA integrity was verified by amplification of the constitutively expressed gene, glyceraldehyde-3 phosphate dehydrogenase (*GAPDH*), following cDNA synthesis. Purified RNA samples were processed for oligonucleotide microarray analysis according to standard Affymetrix protocols. Arrays were washed with a non-stringent buffer (20X SSPE, 10% Tween-20) and a stringent buffer (12X MES, 5 M NaCl, 10% Tween-20) and scanned (Agilent GeneArray Scanner). Fluorescence intensities were quantified, correlated for background noise and normalized to a standard expression level, and then exported to GeneSpring (Silicon Genetics). Affymetrix Microarray Suite software calculated a percentage positive of present genes. Genes showing ≥3-fold up- or down-regulation relative to vehicle control were considered significant. Array experiments were repeated twice and for each run cells from three different flasks were pooled to prepare RNA. Array experiments were carried out at The Genomics Core Facility, The George Washington University Medical School,

Washington, DC. Genes were assigned functional categories by mining several databases including PubMed (<http://www.ncbi.nlm.nih.gov>) and associated resources therein or GeneCards (<http://www.bioinformatics.weizmann.ac.il/cards>).

Statistical analyses of data. Student's unpaired t-tests were used to determine statistical significance for flow cytometry data. Mann-Whitney U tests were used to determine statistical significance for real-time RT-PCR data. A p<0.05 was considered significant.

Results

Cells lost sensitivity to E2 and 4-HT after maintaining for 4 months in the presence of 4-HT. Two models of TAMr were developed and designated MCF-7^{MMU1} and MCF-7^{MMU2}. DNA content analysis was performed to determine the precise effects of E2 and 4-HT treatment on cell cycle distribution using flow cytometry (Table II). Compared to parental cells which were significantly growth inhibited by 4-HT, MCF-7^{MMU1} became refractory to its inhibitory properties by month 4. MCF-7^{MMU1} cells were also less responsive to the stimulatory effects of E2 as resistance developed. Following long-term culture in 4-HT, MCF-7^{MMU2} was developed. These cells retained their resistant phenotype following removal of 4-HT and growth

was similarly unaffected by adding either E2 or 4-HT to the culture medium (data not shown).

ERα and *ERβ* protein expression was unaltered but *PR* was markedly reduced during acquisition of TAMr. To determine whether TAMr was associated with altered ER expression, Western blot and immunocytochemical analyses for *ERα* and *ERβ* was performed. *ERα* was the predominant ER in wtMCF-7 cells. In MCF-7^{MMU1} cells, expression was slightly reduced while in MCF-7^{MMU2} cells, levels were comparable to wtMCF-7. *ERβ* expression was validated using 2 different antibodies and was by far the lowest protein expressed in the cell line model. Using the 14C8 antibody, *ERβ* was undetectable, even when 30 μg of protein lysate was loaded however full-length *ERβ* recombinant protein, used as a positive control could be detected. Weak *ERβ* expression was seen in both wtMCF-7 and the TAMr sublines with the 8D5 antibody, which also recognised recombinant full-length *ERβ*. Using the 8D5 antibody, *ERβ* was more strongly expressed in wtMCF-7 compared to the 4-HT-treated cells. *PR-A* was the predominant *PR* isoform in wtMCF-7 and 4-HT treated cells. *PR* expression was greatly reduced following the onset of TAMr, as shown by reduced band intensity from months 6-12, and remained low in TAMr cells compared to wtMCF-7. Immunocytochemical analysis of the same cells mirrored Western blot data (Fig. 1f). Predominantly nuclear *ERα* immunoreactivity was observed whereas *ERβ* immunoreactivity was weaker and more diffuse with patchy granular cytoplasmic staining as well as nuclear immunoreactivity (Fig. 1f). The estrogen-regulated *PR* was abundantly expressed in wtMCF-7 cells but was markedly reduced in the TAMr series (Fig. 1f).

Co-activator mRNA levels were increased but the co-repressors were unchanged during acquisition of TAMr. ERα expression was reduced in the early months of MCF-7^{MMU1} development but levels started to rise around month 9 and exceeded basal levels by month 12, while in MCF-7^{MMU1} levels remained similar to wtMCF-7 (Fig. 2a). As observed at the protein level, *ERα* expression exceeded *ERβ* and while levels of *ERβ* were low overall, these increased in the early stages of MCF-7^{MMU1} development (Fig. 2b). *PR* expression declined rapidly then reached plateau with low levels also seen in MCF-7^{MMU2} (Fig. 2c), while *pS2* levels were high in MCF-7^{MMU1} but were markedly reduced in MCF-7^{MMU2} (Fig. 2d). *AIB-1* was the most highly expressed co-activator in all cells and in MCF-7^{MMU2} (E) remained above twice the level observed in wtMCF-7 while in MCF-7^{MMU2} (L) cells, expression was similar to wtMCF-7 (Fig. 3a). Expression of *SRC-1* mirrored that of *AIB-1* (Fig. 3b). *SRA* showed a linear increase in expression in the MCF-7^{MMU1} series until month 6, after which the levels of expression decreased and appeared to plateau. No difference in expression was observed in MCF-7^{MMU2} cells, where levels were comparable to wtMCF-7 (Fig. 3c). Similar expression profiles were seen with *NCoR-1* and *SMRT* (Fig. 3d and e, respectively) throughout the MCF-7^{MMU1} series however *SMRT* expression was reduced in MCF-7^{MMU2} compared to wtMCF-7 and MCF-7^{MMU1} cells. *REA* was the most highly expressed co-repressor and in general remained constant as cells developed TAMr (Fig. 3f).

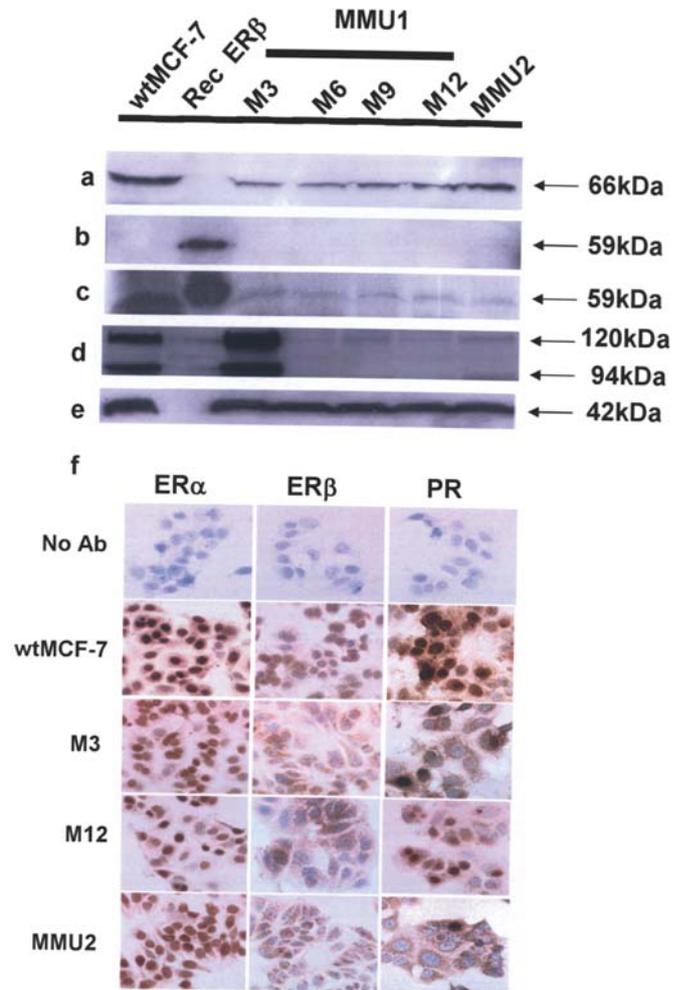


Figure 1. Western blot analysis of wtMCF-7, MCF-7^{MMU1} (months 3, 6, 9 and 12) and MCF-7^{MMU2} showed *ERα* (a) was retained as TAMr developed. *ERβ* was only detected in both wtMCF-7 and TAMr sublines using the 8D5 antibody (c), but not with an alternative *ERβ* antibody, 14C8 (b). *PR-A* and *-B* were expressed in wtMCF-7 and at a reduced level in the TAMr sublines from 3 months (M3) onwards (d). Probing with β -actin showed equivalent loading (e). Immunocytochemical data are presented in (f) and complement Western blot data. Strong uniform nuclear *ERα* immunoreactivity was observed and unaltered as resistance to TAM developed after 3 (M3) of 12 (M12) months exposure to TAM or 21 months (MMU2). *ERβ* staining was less intense and showed a granular cytoplasmic pattern of staining, consistent in all samples. *PR* staining was strongly nuclear in wtMCF-7 and demonstrated a non-uniform pattern of expression, however, expression was reduced as TAMr developed. Original magnification x200.

Global gene expression by microarray analysis of tamoxifen sensitive and resistant MCF-7 cells revealed up-regulation of proliferation promoting genes and down-regulation of apoptosis inducers. Genes differentially regulated >3-fold between MCF-7^{MMU1} and wtMCF-7 cells were considered significant. Using this criterion, 131 genes were up-regulated and 156 genes were down-regulated in MCF-7^{MMU1} cells relative to wtMCF-7 cells. Good concordance was demonstrated between repeat arrays. Differentially regulated genes were then categorised according to function by subdividing into 12 functional categories plus unknowns. The proportion of genes in each category were broadly similar, although genes associated with cell cycle, cell adhesion or extracellular matrix

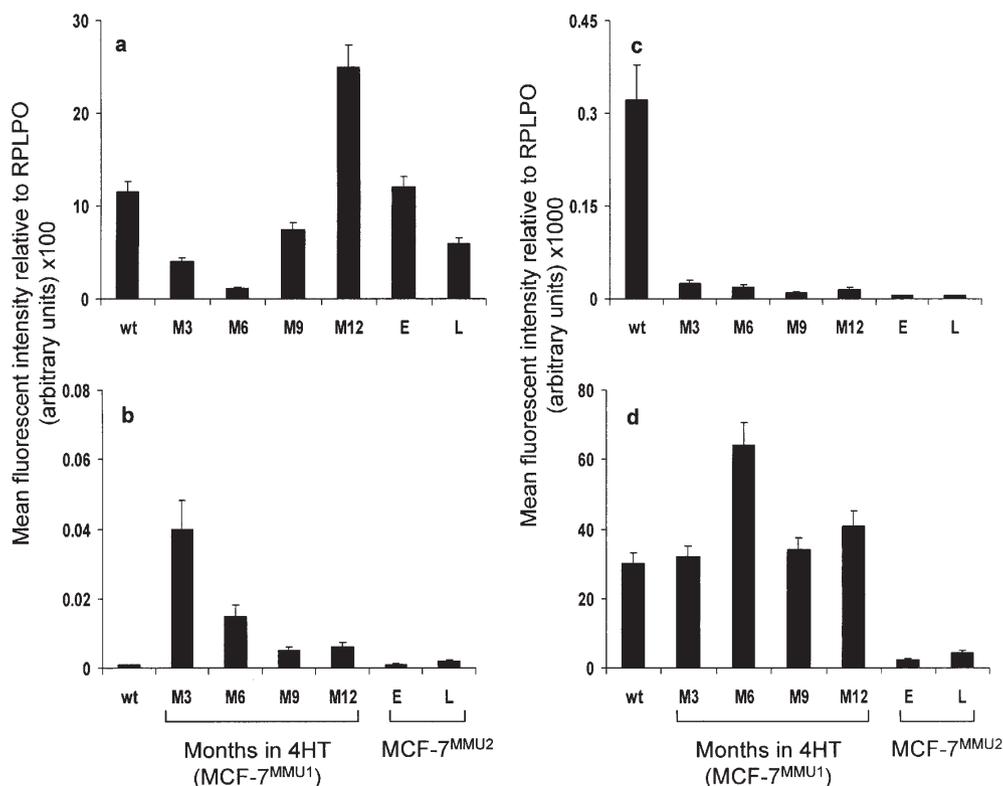


Figure 2. Real-time RT-PCR analysis of hormone receptor expression during development of 4-HT resistance. (a), ER α ; (b), ER β ; (c), PR; (d), pS2. Expression levels were calculated relative to the housekeeping gene, *RPLP0*. Data represent mean \pm SEM. * $p < 0.05$.

were generally up-regulated while those associated with apoptosis or encoding growth factors or hormones were down-regulated in MCF-7^{MMU1} cells compared with wtMCF-7 cells (Fig. 4). A selection of estrogen-regulated and cancer-associated genes together with those whose expression was mostly highly altered following TAMr are shown in Table III. Ten estrogen-regulated genes which were up-regulated included: *GREB1*, *AREG*, *CXCL12*, *CA12*, *WISP2*, *EP3*, *LIV-1* and *IGFBP5*. The cancer-associated genes which were most highly up-regulated included: *S100P*, *SOX9*, *TIMP3*, *CEACAM6*, *SGP28*, *Caveolin-1* and *AIB-1*. The most significantly up-regulated gene was *HMGCS2*, which showed a 79-fold increase in MCF-7^{MMU1} cells and the most down-regulated gene was *NPYR1* (68-fold decrease in MCF-7^{MMU1} cells).

Discussion

We have developed and characterised 2 different TAMr cell lines derived from MCF-7. These are different from other published models of TAMr in that we mirrored changes in gene and protein expression as TAMr evolved, rather than just studying the end point. Both MCF-7^{MMU1} and MCF-7^{MMU2} cells were less sensitive to the stimulatory effects of E2 and refractory to the inhibitory effects 4-HT. They retained levels and localisation of ER α and ER β that were comparable to wtMCF-7 content by Western blotting and immunohistochemistry. A reduction in PR was consistent with the development of TAMr. These general observations are consistent with other published TAMr models (27-32).

Table III. Some examples of genes up- and down-regulated in MCF-7^{MMU1} cells relative to wtMCF-7 cells following micro-array analysis.

Category	Gene	Fold change	Direction
Most altered	<i>HMGCS2</i>	79	Up
	<i>NPYR1</i>	68	Down
Cancer-related	<i>S100P</i>	46	Up
	<i>SOX9</i>	30	Up
	<i>TIMP3</i>	22	Up
	<i>CEACAM6</i>	20	Up
	<i>SGP28</i>	14	Up
	<i>Caveolin 1</i>	12	Up
	<i>AIB1</i>	3	Up
E2-responsive	<i>GREB1</i>	15	Down
	<i>WISP1</i>	10	Down
	<i>AREG</i>	9	Down
	<i>CA12</i>	9	Down
	<i>CXCL12/SDF-1</i>	8	Down
	<i>PR</i>	6	Down
	<i>EGR3</i>	4	Down
	<i>EP3</i>	4	Down
	<i>IGFBP5</i>	4	Down
	<i>LIV-1</i>	3	Down

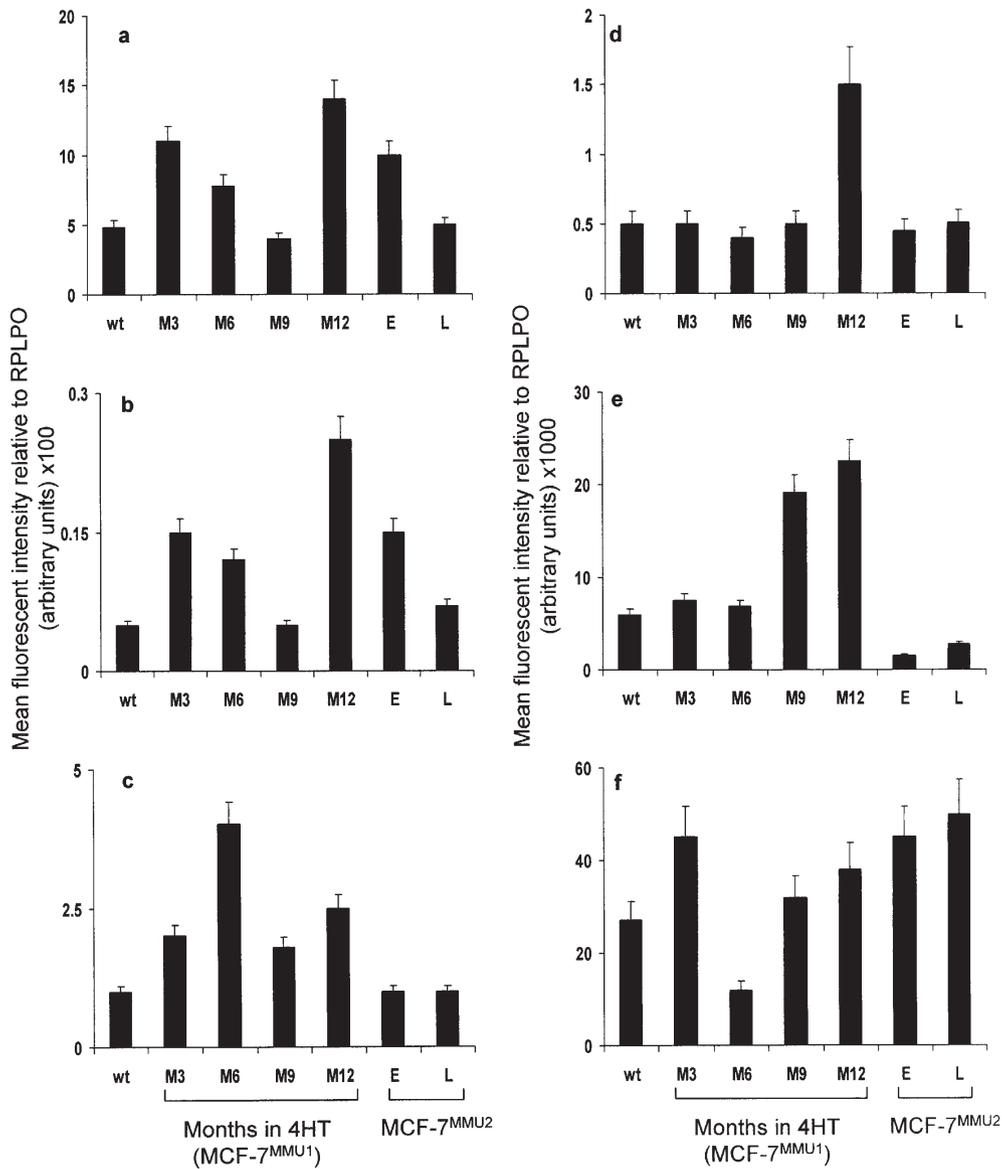


Figure 3. Real-time RT-PCR analysis of the ER co-regulators AIB-1 (a), SRC-1 (b), SRA (c), NCoR-1 (d), SMRT (e) and REA (f) during development of 4-HT resistance. Expression levels were calculated relative to the housekeeping gene, *RPLPO*. Data represent mean \pm SEM. * $p < 0.05$.

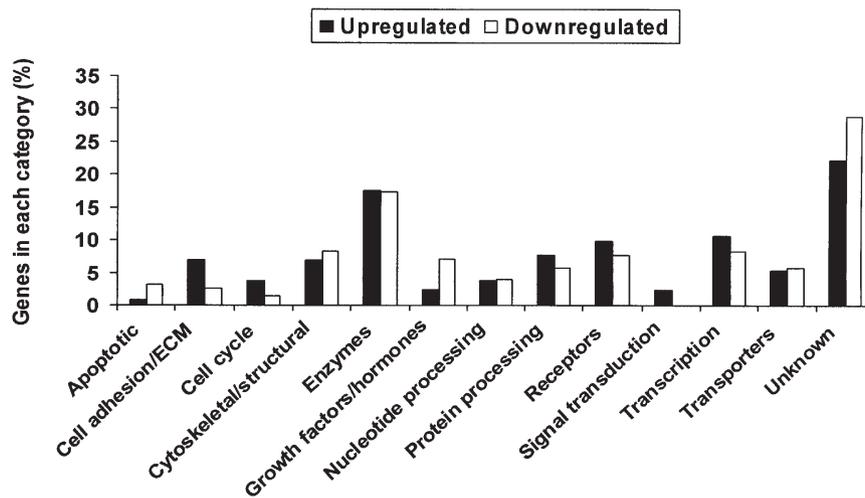


Figure 4. Functional categories of genes up- and down-regulated between MCF-7^{MMU1} and wtMCF-7 cells by microarray. Differentially regulated genes were assigned to 1 of 12 functional categories or to an unknown category by mining several databases (Materials and methods). Data are expressed as a percentage of the total number of up- or down-regulated genes.

ER α expression exceeded that of *ER β* in accord with other studies (32-36). This has also proven to be the case in a xenograft model of anti-estrogen resistance and in clinical breast cancer (37,38). Although *ER β* expression was low, we did see elevated expression at months 3 and 6, which coincided with the initial adaptation of resistance. As *ER β* is now generally regarded as good prognostic marker (6), this could suggest that this is an adaptive response to the onset of the TAMr phenotype. Interestingly, *ER β* staining was not exclusively nuclear and supports the finding of cytoplasmic *ER β* by other groups (24,39,40). This has been suggested to be due to cross-reactivity with cytoplasmic proteins (41). However, in our hands cytoplasmic staining was negated by pre-absorption of antibody with recombinant peptide (24), suggesting it is real, although its function remains unresolved but deserves further investigation.

Basal expression of *pS2* was only reduced in MCF-7^{MMU2}. Although a reduction in *pS2* expression has been reported in some studies, this did not reflect loss of ER activity since response to E2-stimulation was also observed (29,36,42). Both unaltered (28,29,36) and reduced (31,42) levels of *pS2* have been described in models of TAMr. These observations suggest that different selective mechanisms may operate during the differential acquisition of TAMr, particularly as we only observed loss of *pS2* expression in MCF-7^{MMU2} and not in MCF-7^{MMU1} cells, which were developed independently.

AIB-1 was the most highly expressed co-activator in our TAMr model and was also similarly identified by microarray analysis. Overexpression of *AIB-1* has been reported in breast cancer irrespective of *ER α* expression (10,12,43). *AIB-1* expression correlated with poorer prognosis in breast cancer and reduced response to hormonal therapy (15). Elevated levels of *AIB-1* have been reported in other breast cancer cell studies (32,33,44,45). Elevated levels in our *in vitro* TAMr model strengthen the involvement of *AIB-1* in breast carcinogenesis and its further elevation in TAMr suggests it may be contributory to this phenomenon.

SRC-1 was elevated in both MCF-7^{MMU1} and MCF-7^{MMU2} relative to wtMCF-7. Shang and Brown (46) concluded that high levels of *SRC-1* were sufficient to support the agonist activity of TAM and showed increased expression of ER-regulated genes by TAM stimulation in *SRC-1*-transfected MCF-7 cells, but not *AIB-1*-transfected cells. This is in agreement with Smith *et al* (13) who demonstrated that overexpression of *SRC-1* was associated with the enhanced agonist activity of TAM, and upheld by Xu *et al* (14), who used *SRC-1* knockout mice to demonstrate the potential involvement of *SRC-1* in TAM resistance. Fleming *et al* (47) associated *SRC-1* expression with poor response to endocrine therapy in a series of breast tumours. Therefore, increased levels of *SRC-1* in our model would support a less favourable antagonist effect of TAM on growth of MCF-7 cells. An interesting observation was the reduction of *AIB-1* and *SRC-1* levels in the MCF-7^{MMU2} cells which had been devoid of 4-HT for approximately 12 months. The results may suggest that over time the expression of co-factors involved in driving TAMr may revert back to wild-type levels. Therefore it would be interesting to re-evaluate these levels in relation to the length of time following TAM removal and hormone response.

SRA is unable to directly bind ER, and is more likely to act as an adaptor molecule, bridging the association with other co-activators (48). However it is expressed in breast cancer where it is believed to modulate ER activity (49). In our study, expression was not significantly altered in MCF-7^{MMU1} and MCF-7^{MMU2}, suggesting it has little role in development of TAMr.

REA was the most significantly expressed co-repressor and in general its expression was higher in both the MCF-7^{MMU1} and MCF-7^{MMU2} series than wtMCF-7. Higher expression of *REA* may reflect competition between *SRC-1* and *REA* for *ER α* and *ER β* (19,50). Since a consistent increase in *SRC-1* was seen in the MCF-7^{MMU1} and MCF-7^{MMU2} cells it would seem appropriate that *REA* was also elevated in response. This uniform pattern suggests that elevated levels of *REA* may be a common feature of the TAMr phenotype. However, since *REA* requires an intact F region, within the AF-2 domain (51) it is unlikely that altered expression would induce TAMr if AF-2 activity is compromised. Recent data from animal models have shown that *REA* is required for maintenance of ER activity and normal mammary gland development (52).

NCoR-1 and SMRT are not specific for ER activity, but can repress ER transcription in the presence of anti-estrogens (53,54). Decreased NCoR-1 protein expression in a mouse model of TAMr, reduced expression in association with shorter relapse-free survival in breast cancer patients and higher levels of NCoR-1 in patients without recurrence have been shown (16-18). In our model we found higher levels of *NCoR-1* in the MCF-7^{MMU1} series compared to MCF-7^{MMU2}. This could reflect the association of NCoR-1 with ER in the presence of 4-HT in the growth medium of MCF-7^{MMU1} cells as mRNA was extracted from these cells whilst in the continued presence of 4-HT, and studies have confirmed that NCoR-1 is recruited to the ER in its presence (53,54). In a study using dominant-negative NCoR mutants, Morrison *et al* (55) demonstrated TAM continued to exert antagonist properties despite loss of co-repressor expression, confirming that NCoR-1 is not solely responsible for the antagonist behaviour of TAM. Therefore, it would seem likely that altered NCoR-1 expression is not independently responsible for driving TAMr, but in association with other factors known to be involved in ER-mediated antagonism (54), may contribute to this transition. Our data would support the notion that alteration of NCoR-1 alone does not mediate TAMr since MCF-7^{MMU1} and MCF-7^{MMU2} cells showed marked differences in *NCoR-1* expression. Fewer studies have reported on the significance of SMRT in TAMr. Chan *et al* (56) found no association between SMRT and altered response to TAM in a cohort of TAMr tumours. Fleming *et al* (47) confirmed the preferential recruitment of this repressor to the ER in the presence of 4-HT. More recently basal expression of SMRT was significantly increased in toremifene-resistant cells (32). Silencing of both NCoR-1 and SMRT expression by siRNA resulted in TAM-stimulated proliferation in MCF-7 cells (57), supporting a role for these co-repressors in the TAMr phenotype.

Our data additionally illustrate the capacity of microarray technology to identify novel genes involved in TAMr as this revealed 131 up-regulated and 156 down-regulated genes associated with the TAMr phenotype. Other microarray

studies have identified numerous E2-responsive genes in MCF-7 cells (58,59). As anticipated, many of these genes were down-regulated in MCF-7^{MMU1} cells. These included, *GREB1*, *AREG*, *CXCL12*, *CA12*, *WISP2*, *EP3*, *LIV-1* and *IGFBP5*. The most significantly up-regulated gene was *HMGCS2* (79-fold increase in MCF-7^{MMU1} cells). *HMGCS2* catalyses the condensation of acetyl-CoA with acetoacetyl-CoA to form HMG-CoA and CoA in mitochondria and is a key control site of ketogenesis in various hormonal and metabolic situations. It has not previously been implicated in breast cancer. The most down-regulated gene was *NPYR1* which is expressed in breast tumours (60) and in hormone-dependent but not hormone-independent breast cancer cell lines (61). Small peptide antagonists such as those against *NPYR1* have been proposed as novel breast cancer therapies, however down-regulation of *NPYR1* in TAMr would indicate these antagonists are unsuitable in patients who relapse on TAM. In accord with other microarray studies, this study generated an enormous amount of data. As a result it is difficult to predict which might be the most important gene(s) associated with the resistant phenotype, particularly as the most significantly up-regulated gene in this study, *HMGCS2*, has no previous connection with breast cancer. Studies in this area are on-going.

In summary, loss of ER subtype expression does not contribute to TAMr in this *in vitro* model. However, increases in co-activator levels, in concert with alterations in genes associated with controlling cell proliferation and survival, as indicated by microarray analysis may underlie TAMr as this revealed a number of genes not previously associated with TAMr or indeed breast cancer, which may provide the opportunity for novel prognostic and therapeutic targets. Our data also suggest that the expression of steroid receptors, their downstream target genes and their associated co-regulators is not uniform during the acquisition of resistance. Overall, our data highlight that TAMr is a very complex and heterogeneous process that is not controlled by a trivial number of genes.

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