Gene expression changes during the development of estrogen-independent and antiestrogen-resistant growth in breast cancer cell culture models

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We have established estrogen-independent and antiestrogen-resistant cell lines from hormone-dependent MCF-7 breast cancer cells by long-term culture in the absence of estrogen, or in the presence of antiestrogen toremifene, respectively. By using a cDNA microarray we compared gene expression profiles among estrogen-independent, antiestrogen-resistant and long-term estrogen-treated MCF-7 cells. We also determined how the expression of the differentially expressed genes has developed during the long-term culture of the cell lines. Of the screened 1176 cancer-related genes, FOSL1, TIMP1, L1CAM, GDF15, and MYBL2 were found to be differentially expressed between the cell lines. A change in FOSL1 and TIMP1 expression could be attributed to the development of antiestrogen resistance, whereas induced L1CAM expression was implicated in the development of estrogen-independent growth of the cells. Estrogen regulated genes GDF15 and L1CAM became regulated by toremifene in the later passage number of toremifene-resistant cells, which might be an indication of the developed estrogen-agonistic activity of toremifene in these cells. Our findings suggest a pattern where the hormone-responsive cancer cells, which survive E_2 deprivation and/or antiestrogen treatment, first acquire necessary changes in gene expression for transition to maximal growth in the new hormonal environment. Then, after prolonged treatment with antiestrogen, the antiestrogen-resistant cells may eventually generate an E_2-agonistic response to antiestrogen, probably acquiring additional growth advantage. *Anti-Cancer Drugs* 20:51–58 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

The ability to reduce breast tumor growth through the administration of antiestrogens has played an important role in the endocrine therapy of hormone-dependent breast cancer (reviewed in Refs [1,2]). Although the initial response rates to antiestrogen are high among ER-positive tumors, the majority of the patients that respond will eventually develop antiestrogen resistance. A number of mechanisms leading to the development antiestrogen resistance *in vivo* have been proposed including estrogen receptor (ER) mutations (reviewed in Ref. [3]), alterations in the expression levels of ERα, ERβ and progesterone receptor, and their coactivators and corepressors [4–11]. Growing evidence *in vivo* suggests that high expression and/or activation of epidermal growth factor receptor and v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma-derived oncogene homolog (avian) (ERBB2) can confer resistance by the activation of downstream signaling pathways. Among these activated pathways are the cell survival pathway mediated by PI3 kinase and AKT/PKB, and the cell proliferation pathway mediated by the mitogen-activated protein kinase ERK1/2 [11–13]. The involvement of growth factor signaling in antiestrogen resistance is also supported by in-vitro studies with breast cancer cell lines [14–17].

The in-vitro studies elucidating mechanisms of antiestrogen-resistant growth emerge from breast cancer cell culture models, in which cells that are resistant to different antiestrogens have been established from hormone-dependent parental cells by long-term culture. Most of these cell lines have been obtained by in-vitro selection of the MCF-7 breast cancer cells by tamoxifen [15,18–22], but cell lines that are resistant to other antiestrogens or pure antiestrogens have also been established [23–28]. Another feature of hormone-dependent breast cancers is the development of estrogen-independent (EI) growth of cancer cells in the absence of estrogen. Several groups have used in-vitro MCF-7 breast cancer model systems to characterize the adaptive changes occurring in the development of EI phenotype [29–33].

We have earlier established three MCF-7 cell line-derived sublines by long-term (9 months) culture in the absence

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of estrogen (E_2), or in the absence of E_2 and presence of antiestrogen toremifene (TOR). These sublines serve as in-vitro models of EI or EI and toremifene-resistant (TR) breast cancer, respectively. The third, long-term estrogen-treated cell line (LE) was established by 9 months culture of MCF-7 cells in the presence of E_2 [28]. In this study, we compared gene expression profiles among TR, EI, and LE cells. Of the 1176 cancer-related genes screened by cDNA microarray we found five differentially expressed genes. In addition to these three cell lines, the original (pMCF-7) cells were included in the subsequent verification of the microarray result and further studies by real-time RT-PCR. We have frozen earlier passage numbers of the studied cells during the long-term culture. This enabled us to determine how the expression of the differentially expressed genes has developed as the cells have adapted to grow in these different hormonal environments.

Materials and methods

Hormones and reagents

17β-estradiol (E_2) and insulin were purchased from Sigma (St Louis, MO, USA). The antiestrogen toremifene citrate was kindly provided by Orion Pharma (Turku, Finland). Stock solutions were prepared by dissolving the hormones in 96% ethanol, stored at −20°C and added to culture media to yield ethanol concentration not exceeding 0.1%. The antibiotics, penicillin and streptomycin, were obtained from GIBCO (Invitrogen Corporation, Paisley, UK).

Cell growth experiments

The establishment of LE, TR and EI MCF-7 sublines has been described earlier [28]. Briefly, estrogen and antiestrogen-sensitive, estrogen- and progesterone receptor-positive MCF-7 human breast cancer cells were used as parent cells to generate these sublines. LE cells have received vehicle (96% ethanol) + 1 nmol/l E_2, TR cells 1 μmol/l TOR and EI cells vehicle only continuously for 9 months. In this study, parent MCF-7 cells (pMCF-7) and the MCF-7-derived sublines with different passage numbers were routinely cultured in 75 cm^2 flasks at 37°C in a humidified atmosphere of 5% CO_2: 95% air in phenol red-free Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F-12; Sigma) supplemented with 5% dextran charcoal-treated fetal bovine serum (DCC-FBS; GIBCO), penicillin (100 IU/ml), streptomycin (100 μg/ml) and insulin (10 ng/ml). The media with appropriate hormones were changed every 2 or 3 days. All disposable cell culture materials were purchased from Nunc (Apogent Technologies Inc., Portsmouth, NH, USA).

In the cDNA microarray experiments, LE, TR and EI cells were plated on the flasks and grown with appropriate hormones for one passage. After reseeding, the LE and TR cells were grown without hormones, and EI cells without vehicle, for 6 days to eliminate any residual hormonal effects on the gene expression, and then subjected to TRIzol reagent (Gibco, Invitrogen, CA, USA) for RNA extraction according to the manufacturer's instructions. In the real-time RT-PCR experiments, pMCF-7, LE, TR and EI cells were grown as explained above in the cDNA microarray experiment (i.e., hormonal deprivation for 6 days) before RNA extraction. Parallel to this, pMCF-7, LE, TR and EI cells were grown for 1 week with appropriate hormones and then subjected to RNA extraction to obtain RNA samples from the cells that have been grown routinely in normal growth conditions (i.e., no hormonal deprivation).

cDNA microarray

Atlas Human Cancer 1.2 Array (Clontech, CA, USA) containing 1176 cancer-related genes was used to study the basic gene expression differences among LE, TR, and EI cells. The integrity of the RNA samples was assessed by electrophoresis on a denaturing 1% agarose gel, and the concentration and purity of the RNA was determined using a spectrophotometer (GeneQuant II, Pharmacia Biotech Ltd, UK). The microarray experiments were performed according to the manufacturer's protocol. Briefly, 50 μg of total RNA from each sample was reverse transcribed to [α-^{33}P]dATP-labelled cDNA with the Atlas Pure Total RNA Labeling System. The radiolabelled cDNAs were hybridized to the array membranes overnight, and the phosphorimaging screen was exposed with the membranes overnight. The phosphorimaging screen was scanned with a Storm Phosphorimager (Molecular Dynamics, Sunnyvale, California, USA) at a resolution of 100 μm. Imagequant files from scans were analyzed using ArrayVision software (Imaging Research, St Catharines, Ontario, Canada). A template, which contains the spot layout of the array, was overlaid on the phosphorimage, and the pixel intensity of each spot on the array was determined. Spot intensities were background subtracted and globally normalized.

Real-time RT-PCR

The RNA samples were reverse transcribed to cDNA with High Capacity Archive Kit (Applied Biosystems, California, USA) following the instructions of the manufacturer. Real-time RT-PCR was done with the SYBR Green PCR Master Mix Kit in an ABI PRISM 7000 Detection System according to the manufacturer's instructions (Applied Biosystems). The following PCR conditions were used: denaturation at 95°C for 10 min followed by 40–50 cycles at 95°C for 15 s (denaturation) and 60°C for 1 min (elongation). The data were analyzed by ABI PRISM 7000 SDS Software (Applied Biosystems). The final results, expressed as *N*-fold relative differences (ratio) in gene expression between the studied samples and the control (i.e., calibrator) sample, were calculated according to the following equation [34]: $\text{Ratio} = [(E_{\text{target}})^{\Delta CP\ \text{target}\ (\text{control}-\text{sample})}]/[(E_{\text{ref}})^{\Delta \text{CP}\ \text{ref}\ (\text{control}-\text{sample})}]$. E_{target} is the real-time PCR

efficiency of target gene transcript; E_{ref} is the real-time PCR efficiency of a reference gene transcript; ΔCP_{target} is the *CP* (crossing point) deviation of control–sample (subtraction) of the target gene transcript; ΔCP_{ref} is the *CP* deviation of control–sample of reference gene (RPLP0; ribosomal protein, large, P0) transcript. Real-time PCR efficiencies (*E*) were calculated, according to $E = 10^{(-1/slope)}$. The following primers (TAG, Copenhagen, Denmark) were used: MYBL*2* forward primer (f) 5′-GCCG AGATCGCCAAGATG-3′ and MYBL*2* reverse primer (r) 5′-TGATGGTAGAGTTCCAGTGATTCTTC-3′. FOSL1 (f) 5′-GGAGGAAGGAACTGACCGACTT-3′ and FOSL1 (r) 5′-TGCAGCCCAGATTTCTCATCT-3′. GDF15 (f) 5′-TGCCCGCCAGCTACAATC-3, and GDF15 (r) 5′-TCTTTGGCTAACAAGTCATCATAGGT-3′. L1CAM (f) 5′-CCACAGATGACATCAGCCTCAA-3′ and L1CAM (r) 5′-GGTCACACCCAGCTCTTCCTT-3′. TIMP1 (f) 5′-GATACTTCCACAGGTCCCACAAC-3′ and TIMP1 (r) 5′-GCAAGAGTCCATCCTGCAGTTT-3′. RPLP0 (f) 5′-AATCTCCAGGGGCACCATT-3′ and RPLP0 (r) 5′-CG CTGGCTCCCACTTTGGT-3′. The primers were designed using Primer Express software for ABI PRISM 7000 detection system (Applied Biosystems).

Statistical analysis

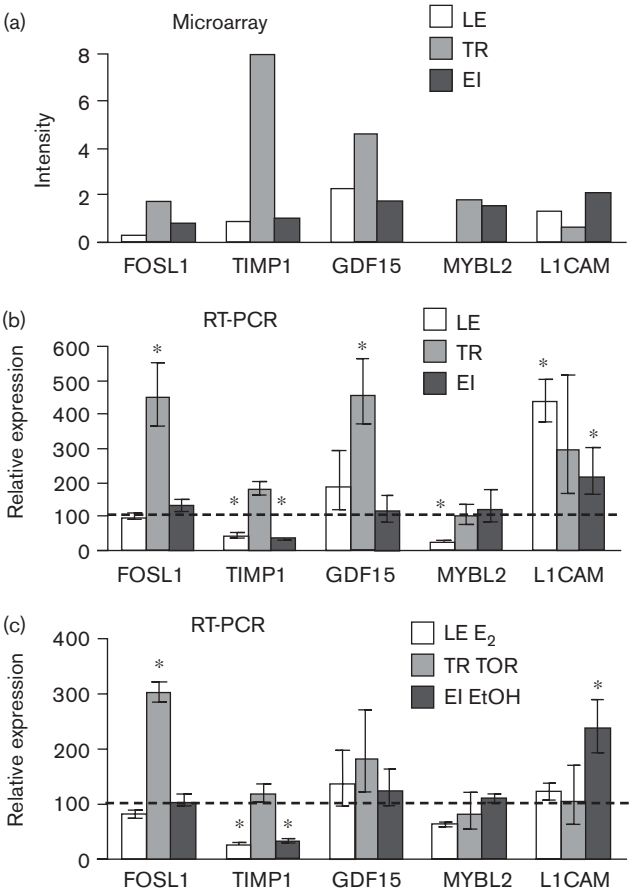
All experiments were repeated three times. The quantitative data are expressed as geometric mean ± geometric standard error. Comparisons were made by the Mann–Whitney *U* test and *P* value of less than 0.05 was considered as statistically significant difference.

Results

Differentially expressed genes in LE, TR and EI cells

The cDNA microarray method was used to reveal differences in basal gene expression between long-term E_2-treated (LE) cells, toremifene-resistant (TR) and estrogen-independent (EI) cells (Fig. 1a). To find differences in basal gene expression, LE and TR cells were deprived of hormone for 6 days (E_2 and TOR-deprived, respectively). Five genes (FOSL1, TIMP1, L1CAM, GDF15, and MYBL*2*) were found to be differentially expressed among the three sublines. FOSL1, TIMP1, and GDF15 were upregulated in TR cells whereas L1CAM was downregulated in these cells. MYBL*2* proved to be downregulated in LE cells. The cDNA microarray results were verified by real-time RT-PCR, both in the hormone-deprived cells (Fig. 1b) and in the cells that were grown with their appropriate hormones (LE cells with E_2 and TR cells with TOR) (Fig. 1c). In the real-time RT-PCR experiments the expression level of the genes in LE, TR and EI cells was calculated relative to their expression level in original pMCF-7 cells that were grown in the presence of estrogen. By comparing the gene expressions between the hormone-deprived cells (Fig. 1b) and the cells grown with the appropriate hormones (Fig. 1c), it was evident that the expression of FOSL1 and TIMP1 was not changed. However, the expression of GDF15 was downregulated by TOR in TR cells. Similarly, the expression of L1CAM was downregulated by TOR in TR cells, and it was also downregulated by E_2 in LE cells. MYBL*2* was upregulated by E_2 in LE cells.

Fig. 1



Relative expression of five differentially expressed genes in the long-term estrogen-treated (LE), toremifene-resistant (TR), and estrogen-independent (EI) cells. Basal expression differences studied by cDNA microarray (a), verification of the cDNA microarray result by real-time RT-PCR (b), and expression in cells grown with the appropriate hormones studied by real-time RT-PCR (c), are shown. The cDNA microarray results represent intensity values obtained from the microarray filter. Intensity value 0 is equivalent to the level of background intensity in the filter. In the real-time RT-PCR experiments, the expression of each gene was calculated relative to its expression in original (pMCF-7) cells. The expression of each gene in pMCF-7 cells was given the arbitrary value 100 (broken line). pMCF-7 cells were grown in the presence of estrogen. The values represent the geometric average and geometric standard error (*$P<0.05$). E_2, estradiol; EtOH, ethanol; TOR, toremifene.

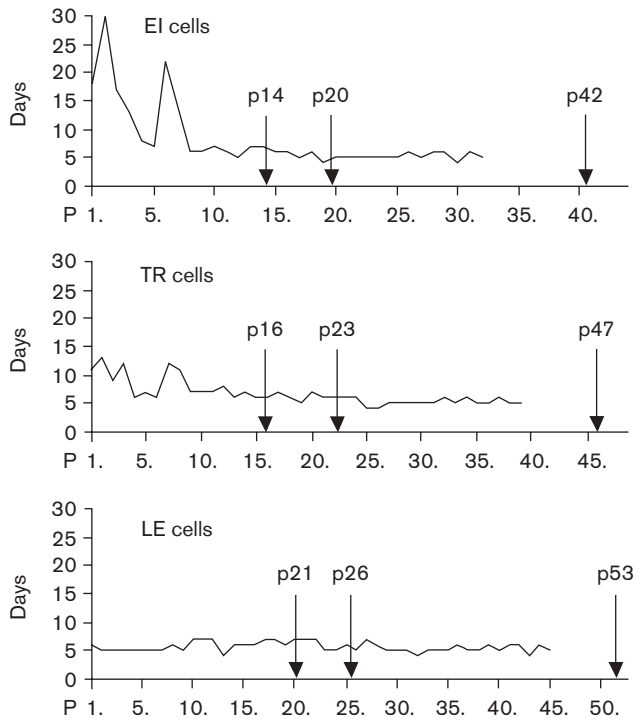
Gene expression changes during the acquisition of estrogen-independent growth and antiestrogen resistance

Several different passage numbers of the sublines were used in this study. The time to reach confluence, after constant number of cells seeded to flasks, was measured during the 9-month culture period to follow the growth

rate of the sublines. The cell passages used in this study, and their growth rates, are shown in Fig. 2. The growth rate study did not include the last passages of the cell lines. However, we observed no changes in the growth rate of these cells when compared with the cells representing earlier passages, and therefore it is apparent that all the passages used in the study represented proliferating cells that have similar growth rates.

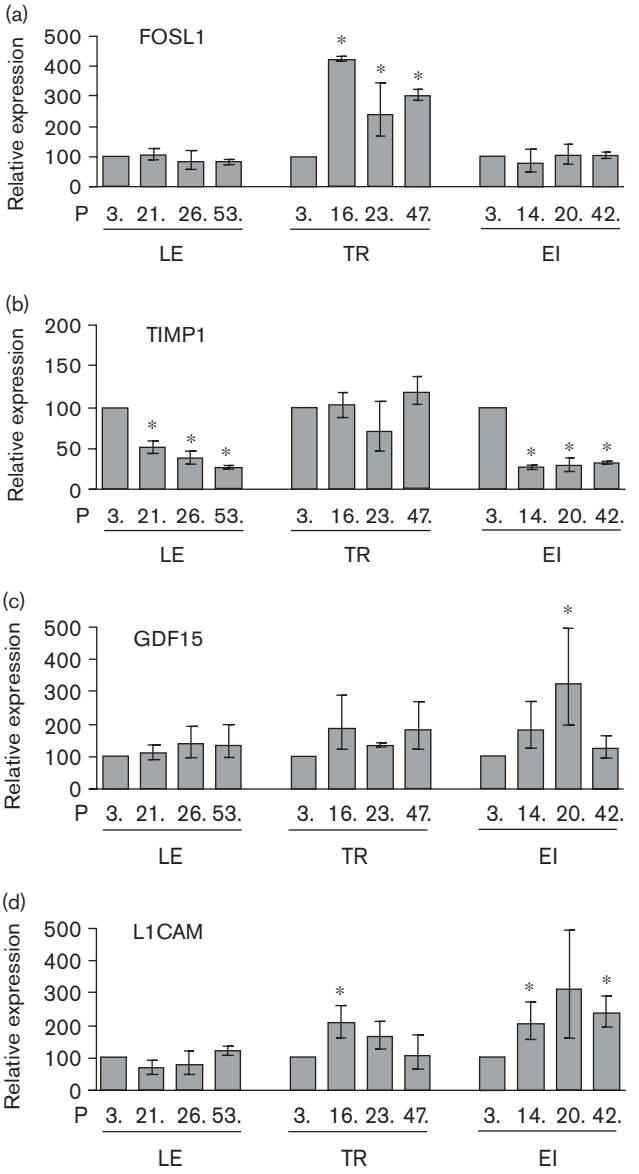
We wanted to determine how the changes in the expression pattern of the five genes have developed as the cells have adapted to grow in different hormonal environments during the long-term culture. The expression levels of the genes were studied in LE, TR, and EI cells grown with the appropriate hormones. Four time points from each subline were studied including original pMCF-7 cells, two passages from the middle of the subculture period, and passages that represented the sublines after 9 months culture. FOSL1 expression level remained unchanged in LE and EI cells, but its induction was evident already in passage number 16 (p16) in TR cells (Fig. 3a). TIMP1 mRNA levels decreased in LE and EI cells as the passage number increased, but in TR cells its mRNA level remained at the same level as in pMCF-7

Fig. 2



Growth rate of the estrogen-independent (EI), toremifene-resistant (TR) and long-term estrogen-treated (LE) cell lines during the 9-month cell culture period. The *y*-axis shows the time (days) to reach confluence after a constant number of cells were reseeded to flasks. The *x*-axis shows the growing passage (P) numbers of the cell lines. The passage numbers that were used in this study are shown for each cell line (arrows).

Fig. 3



The expressions of FOSL1 (a), TIMP1 (b), GDF15 (c), and L1CAM (d) were studied by real-time RT-PCR in long-term estrogen-treated (LE), toremifene-resistant (TR), and estrogen-independent (EI) cells representing different passage numbers. The cell lines were grown with the appropriate hormones. The growing passage (P) numbers are shown (*x*-axis), starting from the original (pMCF-7) cells representing passage number 3. The expressions of the genes in different cell lines were calculated relative to the expression of the gene in pMCF-7 cells, which were given the arbitrary value 100. The values represent the geometric average ± geometric standard error (*$P < 0.05$).

cells (Fig. 3b). The expression of GDF15 remained modestly induced in the last passage of TR cells, but not in the last passage of EI cells (nonsignificantly) (Fig. 3c). On the contrary, L1CAM expression remained upregulated in the last passage of EI cells, but in TR cells its expression fell back to the level observed in pMCF-7 cells

(Fig. 3d). No changes in MYBL*2* gene expression were seen during the long-term culture in LE, TR, or EI cells (data not shown).

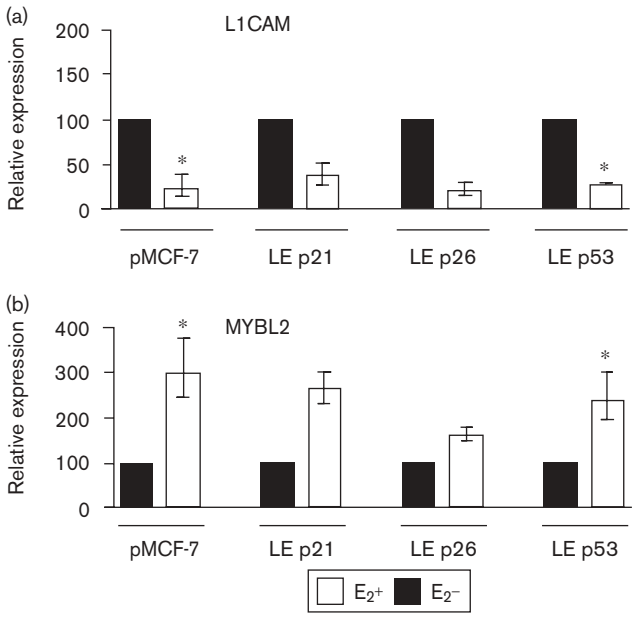
Estrogen-regulated genes

The cDNA microarray and real-time RT-PCR results indicated that L1CAM and MYBL*2* were regulated by E_2 in LE cells, whereas GDF15, FOSL1, and TIMP1 expression was not altered by the hormone. We assessed the E_2 regulation of L1CAM and MYBL*2* in the pMCF-7 cells and in three passages of LE cells (p21, p26, and p53). L1CAM and MYBL*2* proved to be E_2-regulated in the original pMCF-7 cells and in the long-term cultured LE cells representing passage numbers 21, 26, and 53 (Fig. 4a, b). It was also evident that the E_2 regulation of these genes remained relatively similar throughout the subculture period.

Toremifene-regulated genes

The cDNA microarray and real-time RT-PCR results indicated that GDF15 and L1CAM were regulated by TOR in TR cells. We wanted to study whether this TOR regulation existed already in the original pMCF-7 cells, or had evolved during the long-term culture. Therefore we assessed the TOR regulation of these genes in pMCF-7 and three passages of LE cells (p21, p26 and p53), and in three passages of TR cells (p16, p23 and p47). The expression of both GDF15 and L1CAM was regulated in TR cells by TOR in the last passage of the cells only (Fig. 5b, d GDF15 text is missing from Fig. 5b. Similarly L1CAM text is missing from Fig. 5c, d. GDF15 text is correctly in Fig. 5a). GDF15 was modestly regulated by TOR in pMCF-7 cells and all passages of LE cells (Fig. 5a), whereas the expression of L1CAM was mostly unaltered by TOR in these cells (Fig. 5c). Interestingly, TOR regulation of L1CAM in TR cells was essentially similar to its regulation by E_2 in pMCF-7 and LE cells (compare with Fig. 4a).

Fig. 4



Estrogen (E_2) regulation of L1CAM (a) and MYBL2 (b) expression in original (pMCF-7) cells and in long-term estrogen-treated (LE) cells representing different passage numbers. The expressions were studied by real-time RT-PCR in the cells that were grown routinely with E_2 (E_2+), and in the cells that were E_2-deprived for 6 days (E_2-). The expressions of the genes in (E_2+) – samples were calculated relative to the (E_2-) – samples, which were given the arbitrary value 100. The values represent the geometric average ± geometric standard error (*$P<0.05$).

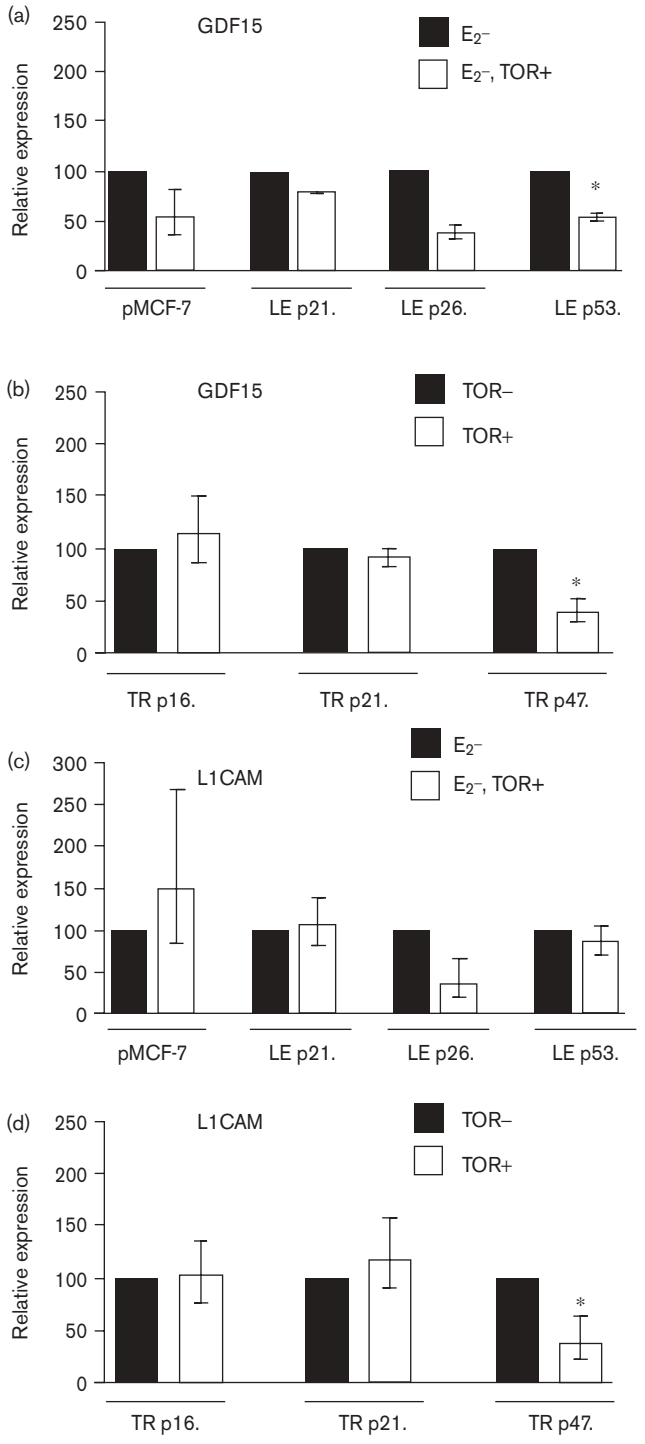
Discussion

Cell culture models to study EI growth and antiestrogen resistance exist, but they are relatively few in number, and almost all are based on the MCF-7 human breast cancer cell line [35]. Large-scale gene expression studies from these cell lines are limited, and comparing the results from these studies is complex as the cell sublines often show different characteristics that originate from variations in cell culture conditions and hormone treatments; for example, the different antiestrogens used [18,36–38]. Two different MCF-7 cell-derived models of estrogen independence and antiestrogen resistance have been compared by De Cremoux *et al.* [39]. The conclusion of the study was that the two cell model systems displayed some similar but also markedly different gene expression characteristics, implying that more gene expression studies of antiestrogen-resistant cell lines are needed for elucidating the mechanisms involved in the development of acquired resistance to antiestrogens.

We have previously established MCF-7 human breast cancer cell variants that serve as in-vitro models of E_2-independent growth (EI cells), and both E_2-independent and antiestrogen-resistant (toremifene) growth (TR cells) [28]. These cell culture models facilitated our study of the gene expression changes that may contribute to the development of E_2-independent growth, acquired resistance to antiestrogen treatment and ultimately development of possible antiestrogen-stimulated growth of breast cancer cells. Of the 1176 genes that were screened, we found five genes (FOSL1, TIMP1, L1CAM, GDF15, and MYBL*2*) that were differentially expressed between the cell lines and differentially regulated by E_2 and/or TOR.

Altered expression of FOSL1 and TIMP1 was observed in the development of E_2-independent and antiestrogen-resistant TR cells whereas L1CAM upregulation was characteristic of EI cells. The induction of FOSL1 and TIMP1 genes was evident already in the early passage number of TR cells, and E_2 or TOR treatment of TR cells

Fig. 5



Toremifene (TOR) regulation of GDF15 and L1CAM expression was studied by real-time RT-PCR in original (pMCF-7), long-term estrogen treated (LE) and toremifene-resistant (TR) cells that represented different passage numbers. E_2-deprived (E_2-) pMCF-7 and LE cells, and TOR-deprived (TOR-) TR cells were treated with 1 μmol/l TOR. GDF15 regulation by TOR is shown in pMCF-7 and LE cells (a) and in TR cells (b). Similarly, L1CAM regulation by TOR is shown in pMCF-7 and LE cells (c) and in TR cells (d). The values represent the geometric average ± geometric standard error (*$P < 0.05$).

had no effect on the expression of these genes. FOSL1 is a transcription factor implicated in multiple human cancers including breast cancer. Elevated FOSL1 expression has been detected in breast epithelioid carcinoma cells [40,41], and a higher expression of FOSL1 is seen, particularly in ER-negative breast cancer cell lines [42,43]. It could be hypothesized that a state that resembles ER negativity, and thus facilitates FOSL1 upregulation, is achieved in our ER-expressing TR cells by the presence of antiestrogen in addition to E_2 deprivation in the culture medium. Long-term E_2 deprivation alone (EI cells) apparently did not cause FOSL1 or TIMP1 induction. TIMP1 expression was downregulated in LE and EI cells as the passage number increased, but maintained in TR cells at the level of original (pMCF-7) cells. Belguise *et al.* [43] have shown a correlation with FOSL1 expression and the expression of some genes implicated in malignant progression and these genes included TIMP1. The authors suggested that FOSL1 upregulated TIMP1 expression in breast cancer cells, though modestly. This regulation could also explain higher TIMP1 expression in our TR cells.

We have previously shown that the growth of the antiestrogen-resistant TR cells is stimulated by TOR [28]. The results of this study also suggested that TOR might function as an E_2 agonist in TR cells. Supporting this hypothesis was the expression pattern of L1CAM and GDF15 in the sublines. L1CAM was found to be E_2-regulated gene in E_2-dependent LE and original pMCF-7 cells, and it became similarly regulated by TOR in the late passage of TR cells. GDF15 seemed to be regulated by TOR in pMCF-7 and LE cells; however, in TR cells the regulation of GDF15 was lost in the passages representing the mid-phase of the long-term culture of TR cells, being retained again in the last passage of the cells. Taken together, E_2 agonism of TOR has probably developed late during the establishment of antiestrogen-resistant phenotype as the E_2 agonism was not observed in the earlier passage numbers of the TR cells. GDF15, a divergent member of transforming growth factor-β superfamily, and the cell adhesion molecule, L1CAM, are both implicated in breast cancer. GDF15 is upregulated by v-akt murine thymoma viral oncogene homolog/protein kinase β (AKT/PKB) in MCF-7 cells conferring antiestrogen resistance [44,45]. L1CAM has been shown to play a role in epithelial–mesenchymal transition-like events in MCF-7 cells in which its overexpression increases motility of the cells and promotes the scattering of epithelial cells from compact colonies [46].

These results show that the gene expression changes that accompany EI growth are different from those observed in the development of antiestrogen-resistant growth. Our findings also suggest a pattern in which the hormone-responsive cancer cells that survive from the E_2 deprivation and/or antiestrogen treatment of several weeks or

months, first acquire necessary changes in gene expression for transition to maximal growth in the new hormonal environment. Then, after prolonged treatment with antiestrogen, the antiestrogen-resistant cells may eventually generate E_2-agonistic response to antiestrogen, probably acquiring additional growth advantage.

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