Estrogen regulation in human breast cancer cells of new downstream gene targets involved in estrogen metabolism, cell proliferation and cell transformation

J A Vendrell1,2, F Magnino1, E Danis1, M J Duchesne3, S Pinloche4, M Pons3, D Birnbaum5, C Nguyen6, C Theillet1 and P A Cohen1,2

1EMI 0229 INSERM, Centre de Recherche CRLC Val d’Aurelle, Montpellier, France
2CPBS-CNRS, Centre de Biotechnologie et de Pharmacologie pour la Santé, Faculté de Pharmacie, BP14491, 34093 Montpellier Cedex 5, France
3INSERM U439, 70 Rue de Navacelles, Montpellier, France
4LIRMM, 161 rue Ada, Montpellier, France
5INSERM U119 and LBT, Institut Paoli Calmette, Marseille, France
6Laboratoire TAGC, CIML, Université d’Aix-Marseille II, Marseille, France

Abstract

We explored, by cDNA mini-arrays, gene expression measurements of MVLN, a human breast carcinoma cell line derived from MCF-7, after 4 days of exposure to 17β-estradiol (E2) treatment, in order to extend our understanding of the mechanism of the pharmacological action of estrogens. We focused on 22 genes involved in estrogen metabolism, cell proliferation regulation and cell transformation. The specificity of the E2 response was reinforced by comparison with 4-hydroxytamoxifen (OH-Tam), ICI 182,780 and E2+OH-Tam expression profiles. Real-time quantitative PCR (RTQ-PCR) confirmed the variation of expression of known (TFF1, AREG, IRS1, IGFBP4, PCNA, ERBB2, CTSD, MYC) as well as novel (DLEU2, CCNA2, UGT1A1, ABCC3, ABCC5, TACC1, EFNA1, NOV, CSTA, MMP15, ZNF217) genes. The temporal response of these gene expression regulations was then investigated after 6 and 18 h of E2 treatment and this allowed the identification of different time-course patterns. Cycloheximide treatment studies indicated first that estrogen affected the transcript levels of ABCC3 and ABCC5 through dissimilar pathways, and secondly that protein synthesis was needed for modulation of the expression of the CCNA2 and TACC1 genes by estrogens. Western blot analysis performed on TFF1, IRS1, IGFBP4, amphiregulin, PCNA, cyclin A2, TACC1 and ABCC5 proteins confirmed the mini-array and RTQ-PCR data, even for genes harboring low variations of mRNA expression. Our findings should enhance the understanding of changes induced by E2 on the transcriptional program of human E2-responsive cells and permit the identification of new potential diagnostic/prognostic tools for the monitoring of estrogen-related disease conditions such as breast cancer.

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Introduction

In the reproductive system, estrogens are important regulators of growth and differentiation in the normal mammary gland and participate in the development and progression of breast carcinoma (Pike et al. 1993). The pharmacological effects of the 17β-estradiol (E2) molecule have been mainly attributed to its capacity to modify, directly or indirectly, the expression of key genes. For example, the mitogenic effects of estrogens on breast epithelial cells are, at least in part, the consequence of an increased expression of genes involved in the regulation of the cell cycle (Prall et al. 1997). Direct transcriptional activity of E2 involves the binding to either estrogen receptor
(ER)-α or ERβ (Kuiper et al. 1996, Krege et al. 1998, Ogawa et al. 1998). ERα and ERβ both recognize as ligand-bound dimers the classical estrogen response element (ERE) (Klein-Hitpass et al. 1988). ER-mediated transcription involves not only ER-DNA binding but also the complex recruitment of transcriptional regulators that influence the interaction between ER and the basal transcription machinery (McKenna et al. 1999). Besides classical interaction with the ERE, ligand-activated ERs also regulate gene expression by interacting directly with the AP-1 protein complex (Webb et al. 1999), the Sp1 protein (Safe 2001) or the nerve factor-κB (NF-κB) protein (Harnish et al. 2000). Finally, besides these so-called genomic actions of estrogens, a non-genomic action is also known (Kousteni et al. 2001).

ERα is routinely used as a prognostic and predictive marker in the management of breast cancer patients. Compared with ER-negative (ER−) breast tumors, a high percentage of ER-positive (ER+) tumors respond favorably to endocrine treatment, are associated with better prognosis and have a well-differentiated phenotype (Henry et al. 1988, Pichon et al. 1996). The marked physiological and phenotypic differences between the ER+ and ER− breast tumors may be the consequence of different gene expression profiles. A full understanding of the pharmacological effect of estrogens on downstream gene targets is therefore very important, and large-scale studies of gene expression systems such as cDNA micro-arrays and oligonucleotide chips (Cohen et al. 2000) have been used to identify E_2 downstream target genes (Klein-Hitpass et al. 1988, Soulez & Parker 2001, Inoue et al. 2002, Hodges et al. 2003).

The aim of the present study was to explore, on home-made cDNA mini-arrays, gene expression measurements of MVLN, an ERα-positive and hormone-responsive human breast carcinoma cell line derived from one of the standard models of ER+ breast cancer, the MCF-7 cell line (Demirpence et al. 1993). We explored the gene expression measurements of MVLN cells in response to three ligands of ER: E_2, the selective estrogen receptor modulator (SERM) 4-hydroxytamoxifen (OH-Tam) and the pure antagonist ICI 182,780. We searched for genes differentially expressed after E_2 treatment to extend our understanding of the mechanisms of the pharmacological action of estrogens, and to select gene candidates as potential diagnostic/prognostic tools of breast cancer. The computed data allowed us to identify new genes whose expression was specifically modulated under E_2 treatment and which might encode proteins playing a critical role in hormone-responsive tissues and cancer.

**Materials and methods**

**Cell culture**

The MVLN cell line was grown as previously described (Demirpence et al. 1993). Prior to treatment, MVLN cells were purged for 5 days in Dulbecco’s modified Eagle’s medium without phenol red supplemented with 3% steroid-depleted, dextran-coated charcoal-treated fetal calf serum. Cells were then treated for 4 days (with one medium change) under the following pharmacological conditions: steroid-depleted medium (vehicle), 1 nM E_2, 200 nM OH-Tam, 100 nM ICI 182,780, or both 1 nM E_2 and 200 nM OH-Tam (Pons et al. 1990, Demirpence et al. 1993, Badia et al. 2000). Total RNA was extracted by cesium chloride ultracentrifugation, and RNA integrity was checked by denaturing agarose gel electrophoresis.

**Culture in the presence of a protein synthesis inhibitor**

Cells were pretreated for 1 h with cycloheximide (CHX; Sigma-Aldrich, St Quentin Fallavier, France) at 30 µg/ml, then 1 nM E_2 was added (Cavailles et al. 1989). Cells were then harvested after 6 and 18 h of treatment before extracting total RNA.

**Spotted cDNA arrays**

Variations in gene expression levels were analyzed by large-scale measurement with home-made nylon cDNA mini-arrays (7.5 × 11.5 cm; 1019 human genes; 12 genes/cm²) produced in our facility (TAGC laboratory, CIML, University of Aix-Marseille II, France) as described by Bertucci et al. (1999). Spotted targets were single amplified PCR products amplified from control clones and IMAGE cDNA clones (IMAGE consortium, Hinxton, Cambridgeshire, UK). The human cDNA clones were selected on the basis of practical
<table>
<thead>
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<th>Gene</th>
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<th>Forward primers</th>
<th>Reverse primers</th>
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<td>28S</td>
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<td>5'-CGATCCATCATCCGCAATG-3′</td>
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<td>ABCC3</td>
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<td>5'-GCTCCAAGATCCTTTTAGCCAA-3′</td>
<td>5'-GGCCAAGATGAGGGCAGAGAGTA-3′</td>
</tr>
<tr>
<td>ABCC5</td>
<td>NM_005688</td>
<td>5'-TGGCAGGGCTCAGGATTTC-3′</td>
<td>5'-CTACGCCAGTGTGAGACACTGAT-3′</td>
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<td>AREG</td>
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<td>5'-CGGGAGCCGACTATGACTACTC-3′</td>
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<td>CCNA2</td>
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<td>5'-AGGAGGAACGGTGACATGCT-3′</td>
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<tr>
<td>CSTA</td>
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<td>5'-GTAGTAATTTGTTCCAGCAACAACTTG-3′</td>
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<td>5'-AAATGCTGCCTGCCTGTCTG-3′</td>
<td>5'-GCTCGGCTGCCAAGCTT-3′</td>
</tr>
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<td>DLEU2</td>
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<td>MMP15</td>
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<td>MYC</td>
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<td>TACC1</td>
<td>NM_006283</td>
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<td>5'-GGGTATCTATTGGCAAACACACACT-3′</td>
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<td>UGT1A1</td>
<td>NM_000463</td>
<td>5'-TGCTTGGTCACCCGATGAC-3′</td>
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<td>ZNF217</td>
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<td>5'-TGTTCACAAGCTGAGCCATATGTAC-3′</td>
<td>5'-GAGTCAATCTTGCAAATGTGTTAA-3′</td>
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criteria (Bertucci et al. 1999), and the genes were chosen because of proven or putative implication in cancer. The list is available at: http://tagc.univ-mrs.fr/pub/Cancer/

Hybridization of complex probe and array data analysis

Hybridization to each array with a $^{33}$P-labeled probe synthesized by reverse transcribing 5 µg total RNA was performed as previously described (Bertucci et al. 1999). Hybridization signals were scanned with a FUJI BAS 5000 beta imager (Raytest, Asnieres, France), then quantified with the HDG Analyzer software (Genomic Solution, Ann Arbor, MI, USA) by integrating all spot pixel intensities and removing a spot background value determined in the neighboring area. Intensity values were adjusted by a normalization step based on the DNA quantification of each spot and the sum of intensities detected in each experiment. Expression values were transformed as the ratio of the treated cell line gene value to that obtained with the corresponding untreated cell line (ratio called fold change or FC). ANOVA of log-transformed intensity values was used to assess the significance of the difference between treatments for each gene.

Real-time quantitative PCR (RTQ-PCR) analysis

RTQ-PCR was performed using a LightCycler (Roche, Meylan, France) in combination with the LightCycler Faststart DNA Master Sybr Green I (Roche). A specific set of primers was chosen for each gene of interest (Table 1). cDNA reverse transcription was performed on 1 µg DNase-treated total RNA with M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and 0·5 µg hexamer (Amersham Biosciences, Orsay, France) according to the manufacturer’s instructions. For each set of primers, a standard curve was made with serial dilutions from a control cDNA sample in order to evaluate the efficiency of the primers and to relatively quantify the expression level of each sample. All measurements were normalized to
the expression of the ribosomal 28S gene, considered as a stable housekeeping gene.

**Western blot**

Cells were lysed in the presence of 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM dithiothreitol, 2 mM sodium fluoride and protease inhibitor in 20 mM Tris, 150 mM NaCl (pH 7.5). For each sample, 50 µg total protein were separated on SDS-PAGE gels before transferring to polyvinylidene difluoride membrane (Sigma-Aldrich). Membranes were then incubated with the appropriate primary antibody, and detection was performed as recommended by the manufacturers with horseradish peroxidase-conjugated secondary antibody using the ECL plus Western blotting detection system (Amersham Biosciences). ABCC5 antibody was from Monosan (Uden, The Netherlands), cyclin A2 antibody from Sigma Chemical Co (St Louis, MO, USA), insulin receptor 1 substrate (IRS1) antibody from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), transforming, acidic, coiled-coil-containing protein-1 (TACC1) antibody from Upstate Biotechnology Inc. (Lake Placid, NY, USA), proliferating cell nuclear antigen (PCNA) antibody from Lab Vision Corporation (Fremont, CA, USA) and insulin-like growth factor-binding protein-4 (IGFBP4) antibody from USBiological (Swampscott, MA, USA). TFF1 (pS2) antibody was kindly provided by Dr M C Rio (Strasbourg, France).

**Results**

**Identification by cDNA arrays of known and novel genes in MVLN cells following E2 treatment**

We assessed variations in gene expression induced after 4 days of exposure to E2 of the MVLN cell line using home-made cDNA mini-arrays, as described in Materials and methods. Analysis of the hybridization signatures demonstrated that the E2 treatment of the MVLN cells induced marked changes in expression of a set of genes (Fig. 1). As it is important to reproduce cDNA array experiments to verify the reliability of the data, we performed our study on three independent cell culture replicates. Since a slight variation in gene expression might have important phenotypic consequences, we considered as regulated those genes whose expression variation (called fold change or FC) was greater than 1.7. The choice of the FC cut-off value was based on reproducibility studies of our gene expression data performed in repeated measurements, independent preparations of complex probes and independent pharmacological treatments of MVLN cell cultures (data not shown). We also considered that, by exploring independent cell culture replicates, we would be working under stringent conditions for data analysis (biological variability), thus even allowing consideration as significant gene expression variations with a low FC. Analysis of the data gave rise to 208 genes whose expression modulation was reproduced in at least two cell replicates. We then focused on 22 genes whose expression variation under E2 treatment was observed in all three cell replicates (12 up-regulations and 10 down-regulations) (Fig. 2 and Table 2). AREG, TFF1, MYC, FOS, PGR, PCNA, CTSD, IGFBP4 and ERBB2 have already been reported as E2-responsive genes in ER+ human breast cancer cells (Jakowlew et al. 1984, Cavailles et al. 1988, van der Burg et al. 1989, Russell & Hung 1992, Martinez-Lacaci et al. 1995, Jorgensen et al. 1998, Qin et al. 1999) and thus validated our study. We confirmed recent data by identifying the up-regulation of IRS1, WNT2 and CCNA2 under E2 exposure (Katoh 2001, Mauro et al. 2001, Hodges et al. 2003). Interestingly, we identified new variations of expression under E2 treatment of the following genes: CSTA, MMP15, DLEU2, NOV, ZNF217, TACC1, EFNA1, UGT1A1, ABCC3 and ABCC5. To assess the statistical significance of each of these FC values, an ANOVA was performed on log-transformed data for each gene. Except for MYC (P=0.062), PCNA (P=0.065) and MMP15 (P=0.133), the P value associated with ANOVA was below 0.05 for all genes, demonstrating a good correlation between the two analyses on this set of data. Although statistical tests like ANOVA and t-test are known to yield results different than those based on FC (Arfin et al. 2000), the good consistency we observed between both approaches is explainable by the fact that we did not select genes based only on the magnitude of their FC but also on their reproducibility in three independent experiments.
Figure 2 Gene expression variations measured by cDNA arrays in independent E2 (E2), OH-Tam, ICI 182,780 (ICI) and E2+OH-Tam experiments (exp). Each row represents a gene and each column represents a different pharmacological treatment. The results are expressed in terms of FC values with a color scale indicated at the right. The data are presented for genes whose variation of expression was reproduced in three independent E2 pharmacological treatment experiments. Expression variations observed in two independent OH-Tam experiments, two independent ICI 182,780 experiments and one E2+OH-Tam experiment are also represented. Genes highlighted in red correspond to up-regulated genes in cells under the specified pharmacological treatment compared with untreated cells (FC > +1.7). Genes highlighted in green were down-regulated (FC < −1.7) and genes highlighted in gray and in black were considered invariant.
Table 2  
E₂ OH-Tam- and ICI 182,780-regulated genes detected by cDNA array and their variation of expression

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession no.</th>
<th>Description</th>
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<tbody>
<tr>
<td>AREG</td>
<td>NM_001657</td>
<td>Amphiregulin</td>
</tr>
<tr>
<td>TFF1</td>
<td>NM_003225</td>
<td>Trefoil factor 1, pS2</td>
</tr>
<tr>
<td>FOS</td>
<td>NM_005252</td>
<td>v-fos murine osteosarcoma viral oncogene homolog</td>
</tr>
<tr>
<td>DLEU2</td>
<td>NM_006021</td>
<td>Deleted in lymphocytic leukemia 2 gene</td>
</tr>
<tr>
<td>IGFBP4</td>
<td>NM_001552</td>
<td>Insulin-like growth factor binding protein-4</td>
</tr>
<tr>
<td>WNT2</td>
<td>NM_003391</td>
<td>Wingless-type MMTV integration site family, member 2</td>
</tr>
<tr>
<td>PGR</td>
<td>NM_000926</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>IRS1</td>
<td>NM_005544</td>
<td>Insulin receptor substrate 1</td>
</tr>
<tr>
<td>MYC</td>
<td>NM_002467</td>
<td>v-myc myelocytomatosis viral oncogene homolog</td>
</tr>
<tr>
<td>PCNA</td>
<td>NM_002592</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>CTSD</td>
<td>NM_001909</td>
<td>Cathepsin D</td>
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<tr>
<td>CCNA2</td>
<td>NM_001237</td>
<td>Cyclin A2</td>
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<tr>
<td>NOV</td>
<td>NM_002514</td>
<td>Nephroblastoma overexpressed gene</td>
</tr>
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<td>Multidrug resistance-associated protein 3</td>
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<td>ZNF217</td>
<td>NM_006526</td>
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<td>NM_001657</td>
<td>Cystatin A, cysteine proteinase inhibitor</td>
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<td>NM_003225</td>
<td>V-erbB2 avian erythroblastic leukemia viral oncogene homolog 2</td>
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<td>MMP15</td>
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<td>Matrix metalloproteinase 15</td>
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Gene expression variations are expressed as the mean of the FC obtained in three independent transcriptome experiments.

*P* value calculated by ANOVA. $-$: corresponds to *F* values lower than 1, and consequently to high *P* values.

$^{a}$Gene expression variation was measured in three, one and two independent transcriptome experiments respectively for E₂, OH-Tam and ICI 182,780 treatments. ND, Not determined.
Investigation by cDNA arrays of the expression of the E<sub>2</sub>-responsive genes in the presence of estrogen antagonists

Using our cDNA arrays, we analyzed the effects of anti-estrogens, that is, the SERM OH-Tam and the pure antagonist ICI 182,780, on the expression of the 22 estrogen-responsive genes (Fig. 2 and Table 2), to assess the specificity of the E<sub>2</sub> response. None of the variations detected under the pharmacological action of E<sub>2</sub> were observed under ICI 182,780 treatment. On the contrary, ICI 182,780 seemed to exhibit a ‘reversed’ pharmacology, that is, ICI 182,780 treatment induced reproducible down-regulation of expression of the PGR and CCNA2 genes (which were up-regulated under E<sub>2</sub> treatment) and the reproducible up-regulation of two E<sub>2</sub> down-regulated gene expressions (ABCC3 and ZNF217). The OH-Tam signature was different from that of E<sub>2</sub> as the majority of the genes did not display any variation of expression, and from that of ICI 182,780 as no ‘reversed’ pharmacology was observed. Interestingly, we detected a partial agonistic action of OH-Tam, highlighted by an identical regulation of expression of a few genes under E<sub>2</sub> or OH-Tam treatment (e.g. the UGT1A1 gene which presented a reproducible decrease in expression after exposure to E<sub>2</sub> or to OH-Tam). Finally, the specificity of the E<sub>2</sub> response was reinforced by the fact that when cells were grown in the presence of both E<sub>2</sub> and OH-Tam, this treatment was able to abolish the E<sub>2</sub>-induced modulations of expression of all the genes (Fig. 2), except for UGT1A1, CTSD and DLEU2 on which OH-Tam seemed to exert an agonistic action.

Validation of array data by RTQ-PCR

We further investigated the reliability of the data obtained by cDNA arrays by RTQ-PCR on 19 genes. A differentially expressed gene detected by cDNA array analysis (FC > 1.7) was considered to be validated by RTQ-PCR if the relative expression ratio determined by this technique was equal to or greater than twofold. The resulting data are presented in Fig. 3, and demonstrate a good correlation between the two techniques (even for genes with a low FC like MMP15, PCNA, ERBB2, CTSD, CCNA2 and EEFN1). We validated 100% of the E<sub>2</sub>-induced modulations of expression, even for genes displaying an FC very close to the chosen cut-off (e.g. MMP15 (FC<sub>array</sub> = -1.78) and CCNA2 (FC<sub>array</sub> = +1.87)). We also validated the down-regulation of UGT1A1 by OH-Tam, the up-regulations of ZNF217 and ABCC3 by ICI 182,780 and the down-regulation of CCNA2 by ICI 182,780. Interestingly, the IRS1 gene, whose expression profile following OH-Tam treatment did not display an FC value above 1.7 (FC<sub>array</sub> = +1.39), but which had a significant P value (P = 0.037), was found to be up-regulated by RTQ-PCR (FC = +2.48). Taken together, we validated 87.7% of the relative gene expression modulations detected by the cDNA array experiments (50/57 measurements), demonstrating the high quality of our data. These results suggest first that, in agreement with other groups (Rajeevan et al. 2001, Galon et al. 2002), there was an excellent consistency between the cDNA results and the RTQ-PCR measurements, and secondly that transcriptome data analysis of independent cell replicates according to the FC value (as low as 1.7) gave reliable data, substantiated by ANOVA.

Time-course study of gene modulation

To explore the temporal response of gene expression regulation, measurements were performed at two different times. Briefly, MVLN cells grown in steroid-depleted medium for 5 days were then supplemented with 10<sup>-9</sup> M E<sub>2</sub> for 6, 18 and 96 h before harvesting. Gene expression variations quantified by RTQ-PCR are illustrated in Fig. 4 and revealed different groups. Nine genes (AREG, IGFBP4, TFF1, UGT1A1, EEFN1, ERBB2, ZNF217, TACC1 and ABCC5) responded after 6 h of E<sub>2</sub> treatment (Fig. 4A–C); the response was amplified over the time period studied for AREG, IGFBP4, TFF1 and UGT1A1 (Fig. 4A); the range of the response was roughly identical at the different time points tested for the EEFN1, ERBB2, ZNF217 and TACC1 genes (Fig. 4B); on the contrary, the response of only one gene, ABCC5, was maximal at 6 h of E<sub>2</sub> treatment (Fig. 4C). Expression variation of six genes (DLEU2, CCNA2, IRS1, ABCC3, CSTA and NOV) could only be detected after 18 h of E<sub>2</sub> treatment (Fig. 4D). For the DLEU2 and CCNA2 genes, the temporal response was practically identical at 96 h, whereas for the IRS1, ABCC3, CSTA and NOV genes, the magnitude of variation of expression increased between 18 and 96 h.
Finally, variation of expression of the PCNA and MMP15 genes was detectable only after 96 h of E2 treatment (Fig. 4E).

**Effect of CHX on gene modulation**

To investigate whether protein synthesis was needed for the modulation of gene expression, we analyzed the mRNA levels in MVLN cells following E2 treatment in the presence or the absence of the protein synthesis inhibitor CHX. A similar set of MVLN cells grown in the presence of CHX alone was used as control. Based on the data obtained in the presence of CHX co-treatment, the variation of expression of AREG, IGFBP4, TFF1, IRS1, DLEU2, UGT1A1, ERBB2, NOV and ABCC5 following E2 treatment did not require de novo protein synthesis (Table 3). On the contrary, the variation of expression observed for CCNA2, TACC1 and ABCC3 was totally abolished in the presence of CHX, suggesting that downstream pathways are involved in this regulation. It is interesting to note that the mechanisms of E2 regulation of the ABCC3 and ABCC5 genes under E treatment were apparently different, although these genes belong to the same gene family. Concerning EFNA1 and ZNF217 (data not shown), it was difficult to draw a conclusion because CHX treatment alone increased the level of these transcripts (such an effect of CHX has already been attributed to its capacity to inhibit labile nucleases and thereby to increase the half-life of short-lived transcripts (Almendral et al. 1988)).

**Effect of E2 treatment on protein level modulation**

We performed Western blot analyses of MVLN cell lysates following 24 and 96 h of E2 treatment to investigate the relevance of the variations of expression we selected by mini-arrays. The results obtained with the TFF1 (pS2), amphiregulin, IRS1, IGFBP4, PCNA, cyclin A2, TACC1 and ABCC5 proteins are illustrated in Fig. 5 and showed that: immunoreactive TFF1 (pS2), amphiregulin, IRS1, IGFBP4 and cyclin A2 protein levels were found to be increased in MVLN cells treated with E2 for 24 or 96 h; immunodetection of the increased PCNA protein level was visible only after 96 h of E2 treatment; the ABCC5 protein level was decreased following 96 h of exposure to estrogens (the immunoreactive signal following 24 h of E2 treatment was too faint to be able to draw a conclusion). These data are totally in accordance with the mRNA level variations of the corresponding genes (Fig. 4). We also observed a decrease in the TACC1 protein level after 96 h of E2 treatment, again validating our mRNA data (Fig. 4). Although we were not able to detect TACC1 protein level variation after 24 h of E2 treatment (whereas TACC1 mRNA level variation was detectable; Fig. 4), kinetic studies revealed that the decrease in TACC1 protein level following E2 treatment could, however, be detected after 48 and 72 h of estrogen exposure (data not shown). Taken together, our work demonstrated an excellent correlation between expression variations detected at the mRNA level and at the protein level, validating the relevance of the expression modulations we identified. Moreover, it is important to draw attention to the results obtained with the ABCC5 and cyclin A2 proteins because they demonstrate that low FC selected by transcriptome data analysis (FCarray CCNA2 = +1.87; FCarray ABCC5 = −2.28) can be validated by both RTQ-PCR and Western blotting. This observation reinforces the fact that an FC cut-off value as low as 1.7 can be chosen in transcriptome experiments involving independent cell replicates to generate reliable data.

**Discussion**

The aim of this work was to identify novel genes whose expression was specifically regulated under E2 treatment. Such genes might encode proteins playing a critical role in hormone-responsive tissues and cancer and could be new candidates as potential diagnostic/prognostic tools for hormone-dependent breast cancers. Our belief was that one of the criteria to select such candidates should be to consider genes whose estrogen regulation is persistent and, thus, could reflect the physiological exposure to endogenous estrogens. For this reason, we set up the estradiol pharmacologic treatment for 4 days.

Among the 22 selected genes were new downstream E2 gene effectors, for which the specificity of the E2 response was reinforced by the fact that it was abolished for most of them by...
Figure 3 Comparison of the gene expression variations (fold change) measured by RTQ-PCR (solid columns) and cDNA arrays (open columns) after 4 days of E2, OH-Tam or ICI 182,780 treatment of MVLN cells. The RTQ-PCR values indicated are the mean of at least three independent experiments. (A) Genes whose E2-induced regulation have already been documented. (B) Novel E2-induced gene expression regulations.
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(A) 
- **AREG**
  - Fold change: 4.20, 22.88, 142.00 at 6 h, 18 h, 96 h respectively.

- **IGFBP4**
  - Fold change: 5.64, 9.31, 10.71 at 6 h, 18 h, 96 h respectively.

(B) 
- **TFF1**
  - Fold change: 2.39, 7.80, 25.70 at 6 h, 18 h, 96 h respectively.

- **UGT1A1**
  - Fold change: -2.09, -3.21, -6.38 at 6 h, 18 h, 96 h respectively.

(C) 
- **EFNA1**
  - Fold change: -2.86, -3.57, -3.09 at 6 h, 18 h, 96 h respectively.

- **ERBB2**
  - Fold change: -3.03, -3.03, -3.23 at 6 h, 18 h, 96 h respectively.

- **ZNF217**
  - Fold change: -2.27, -2.43, -3.51 at 6 h, 18 h, 96 h respectively.

- **TACC1**
  - Fold change: -2.70, -2.51, -3.61 at 6 h, 18 h, 96 h respectively.

- **ABCC5**
  - Fold change: -6.84, -2.67, -2.10 at 6 h, 18 h, 96 h respectively.
Figure 4 Time-course study of genes under E₂ treatment. Gene expression variations were measured by RTQ-PCR after 6, 18 and 96 h of E₂ treatment of MVLN cells. The results are expressed in terms of mean FC values obtained in at least three independent experiments. (A–C) Genes whose expression variation was detectable after 6 h of E₂ treatment and with: (A) an expression which increased during the treatment; (B) an expression practically identical at the different time points studied; (C) a maximal expression at 6 h. (D) Genes whose expression variation was detected only after 18 h of treatment. (E) Genes whose expression variation was detected only after 96 h of treatment.
OH-Tam. This is in agreement with the antagonistic action of OH-Tam and suggests that these genes are regulated by E2 through ER-dependent mechanisms or pathways. Understanding the effects of estrogen antagonists is also of interest because they have been developed for anti-hormone therapy and their impact on E2 target genes is not fully understood. Treatment of the MVLN cells by ICI 182,780 globally showed a ‘reversed’ pharmacology (the most striking examples were the genes CCNA2 and \( \gamma \)NF217). This can be explained by the fact that residual estrogen activity might persist in the medium where the MVLN cells were grown. Under such conditions, the ICI 182,780 pharmacological signature might be the consequence of the inhibition of this residual estrogen activity, amplified by the decrease of the ER proteins induced by this molecule (Pink & Jordan 1996). The OH-Tam hybridization signature was different from that observed with E2 and ICI 182,780, as most of the genes did not display any expression difference or any ‘reversed’ response. This can probably be explained by the complexity of the pharmacology of the SERM OH-Tam, as cell-type and promoter-specific differences in co-regulator recruitment play a critical role in determining the cellular response to OH-Tam (Shang & Brown 2002). Thus, the subtle pharmacology of the OH-Tam molecule, added to the fact that this molecule triggers the accumulation of the ER protein in MCF-7 cells (Pink & Jordan 1996), might explain the difference in hybridization signatures observed between this molecule and ICI 182,780. Interestingly, we also highlighted a partial agonistic action of OH-Tam as revealed by the UGTA1I gene expression data. These results suggest that in MVLN cells, UGTA1I possesses an unidentified estrogen-responsive regulation mechanism on which OH-Tam acts as a partial agonist.

With the purpose of deciphering the mechanism of estrogen action on gene regulation, we performed kinetic and CHX studies on the IRS1, DLEU2, NOV, ABCC3, CSTA, AREG, TFF1, IGFBP4, UGTA1I, ERBB2, \( \gamma \)NF217, EFNA1, PCNA, MMP15, CCNA2, TACC1 and ABCC5 genes. Kinetic studies revealed that the pattern of the temporal responses varied between the genes considered. CHX studies demonstrated that inhibition of protein synthesis has no effect on the E2-induced regulation of expression of AREG, IGFBP4, ERBB2, TFF1, IRS1, DLEU2, UGTA1I, NOV and ABCC5, suggesting a direct action of E2. However, ABCC3, CCNA2 and TACC1 regulation of expression was totally abolished in the presence of CHX, suggesting a mechanism of regulation by E2 involving downstream pathways.

Besides classical interaction with the ERE, ligand-activated ERs also regulate gene expression by interacting directly with the AP-1 protein complex (Webb et al. 1999), the Sp1 protein (Safe 2001) and the NF-κB protein (Harnish et al. 2000). We used Genomatix GEMS Launcher software (München, Germany) to search for ER, Sp1, AP-1 and NF-κB binding sites in the 2 kb promoter region of ABCC3, ABCC5, CCNA2, DLEU2, EFNA1, NOV, UGTA1I, TACC1 and \( \gamma \)NF217. Genomatix software analysis led to the identification of EREs in ABCC3, ABCC5, CCNA2, DLEU2, TACC1 and ABCC5 promoters; Sp1 binding sites were present in the promoter region of ABCC3, ABCC5, CCNA2, DLEU2, EFNA1, NOV, TACC1 and UGTA1I genes; AP-1 binding sites were only identified for the ABCC3 and \( \gamma \)NF217 genes; finally, NF-κB binding sites were present in the promoter region of ABCC3, DLEU2, TACC1 and UGTA1I. Taken together, these data suggest putative mechanisms of regulation of the expression of these genes by ER-ligand complexes. However, future work will be necessary to decipher the exact mechanism.

### Table 3: Effect of CHX on E2-regulated genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>( E_2 )</th>
<th>( E_2+\text{CHX} )</th>
<th>CHX</th>
</tr>
</thead>
<tbody>
<tr>
<td>AREG</td>
<td>+23.88</td>
<td>+29.42</td>
<td>+7.88</td>
</tr>
<tr>
<td>IGFBP4</td>
<td>+9.31</td>
<td>+43.03</td>
<td>+1.12</td>
</tr>
<tr>
<td>TFF1</td>
<td>+7.80</td>
<td>+4.65</td>
<td>+1.09</td>
</tr>
<tr>
<td>IRS1</td>
<td>+4.75</td>
<td>+4.46</td>
<td>+2.50</td>
</tr>
<tr>
<td>DLEU2</td>
<td>+2.21</td>
<td>+2.57</td>
<td>+1.37</td>
</tr>
<tr>
<td>UGTA1I</td>
<td>+3.61</td>
<td>+3.33</td>
<td>+3.09</td>
</tr>
<tr>
<td>ERBB2</td>
<td>+3.33</td>
<td>+3.33</td>
<td>+1.21</td>
</tr>
<tr>
<td>NOV</td>
<td>-2.94</td>
<td>-2.38</td>
<td>+1.20</td>
</tr>
<tr>
<td>ABCC5</td>
<td>-2.67</td>
<td>-3.57</td>
<td>-1.70</td>
</tr>
<tr>
<td>CCNA2</td>
<td>+3.81</td>
<td>+1.24</td>
<td>-1.80</td>
</tr>
<tr>
<td>TACC1</td>
<td>-2.51</td>
<td>-1.47</td>
<td>+1.18</td>
</tr>
<tr>
<td>ABCC3</td>
<td>-2.74</td>
<td>+1.05</td>
<td>+1.04</td>
</tr>
</tbody>
</table>

aGene expression variations were measured by RTQ-PCR after 18 h of \( E_2 \) treatment of MVLN cells in the presence or absence of CHX. The results are expressed in terms of mean FC values, obtained in at least three independent experiments.

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of E₂ regulation, for example, by assessing estrogen responsiveness of promoter/reporter fusion constructs.

In this study, we newly identified E₂-induced down-regulation of genes potentially involved in the metabolism of estrogens (UGT1A, ABCC3 and ABCC5). Glucuronidation is a well-established metabolic route of both estradiol and tamoxifen, and the UGT1A1 enzyme is a major UDP-glucosyltransferase involved in estradiol glucuronidation. As the candidate proteins for glucuronide transporters have recently been described as the transmembrane transporters ABCCs (Konig et al. 1999), it is tempting to speculate that E₂ down-regulates UGT1A1, ABCC3 and ABCC5 genes to maintain a high intracellular estrogen level and thus to amplify its own pharmacological action. Interestingly, we also observed a slight down-regulation of UGT1A1 under OH-Tam treatment, suggesting that this phenomenon could also occur in the OH-Tam metabolism.

While the potent mitogenic effect of estrogen has been known for a long time, the mechanism of estrogen-mediated proliferation is not fully understood. Of much interest is the understanding of the relevance of estrogen target genes in estrogen-dependent growth. In this study, we selected several genes modulating cell proliferation such as IRS1.
**Identification of new human estrogen-regulated genes**

IGFBP4, CCNA2 and NOV, IRS1 and IGFBP4 are involved in the cross-talk between estrogen and insulin growth factor-dependent pathway-mediated proliferation (Corcoran et al. 1996). CCNA2 encodes for a protein reported to function at the G2/M transition. The up-regulation of this gene (observed after a 4-day E2 treatment) corroborates and completes a very recent study (Hodges et al. 2003), which showed an up-regulation of CCNA2 expression in MCF-7 cells after 24 h of E2 treatment. We also described for the first time the E2-mediated down-regulation of NOV which encodes for a negative regulator of cell proliferation. The down-regulation of the NOV gene may be involved in malignant processes, as it has been observed in fibroblasts transformed by p60 v-SRC (Scholz et al. 1996) and is associated with the progression of adrenocortical tumors (Martinerie et al. 2001). Thus, the NOV down-regulation after E2 treatment suggests that this gene could be involved in estrogen-dependent pathogenic processes.

Of much interest is also the identification of new E2 target genes potentially involved in the development by estrogens of breast carcinogenesis. WNT2 expression, which has been found associated with abnormal proliferation in human breast tissue (Huguet et al. 1994), was demonstrated to be increased following E2 exposure, confirming recent data (Katoh 2001). We also described, and this for the first time, the E2-induced down-regulation of TACC1, EFNA1 and ZNF217 genes. The TACC family members are thought to contribute to the development of cancer (Raff 2002), and TACC1 mRNA down-regulations have recently been observed in breast tumors (Conte et al. 2002). Thus, the E2-induced down-regulation we identified for the TACC1 gene could be involved in the development of estrogen-dependent breast carcinogenesis. EFNA1 protein binds to a tyrosine kinase receptor (EphA2) which has a complex function. Interestingly, EphA2 stimulation by soluble ligands reverses the malignant behavior of EphA2-transformed cells (Zelinski et al. 2001), and EphA2 expression is also down-regulated by estrogen (Zelinski et al. 2002). Thus, negative regulations of both EFNA1 and EphA2 could be a mechanism by which estrogen may promote mammary epithelial cell growth. The candidate oncogene ZNF217 has been predicted to encode alternatively spliced Krüppel-like transcription factors, which promote immortalization of human mammary epithelial cells, suggesting that aberrant expression of this gene may be selected for during breast cancer progression by overcoming senescence (Nonet et al. 2001). In this study, we identified for the first time the down-regulation of ZNF217 by estrogen in MVLN cells, and we also observed this negative regulation in the MCF-7 parental cell line (data not shown). Taken together, these data suggest ERα-dependent pathway involvement in EFNA1, TACC1 and ZNF217 expression regulation, and a potential involvement of these genes in estrogen-mediated breast tumor development.

In conclusion, this study first identified and validated new estrogen downstream targets (ABCC3, ABCC5, CCNA2, DLEU2, EFNA1, MMP15, NOV, UGT1A1, TACC1 and ZNF217), and secondly pointed out for most of these genes new estrogen-mediated down-regulations. The reliability of the data presented in this work was reinforced by validation at the mRNA level or at the protein level of the gene expression variations selected by transcriptome data analysis (even for genes displaying a low FC). Given the biological function (known or putative) of the selected genes, this work provides potential candidates first for understanding the pharmacological effects of estrogens and their consequences in estrogen-dependent diseases, and secondly for identifying new molecular markers with potential clinical applications. The MVLN cells are a very interesting model because tamoxifen-resistant, but still estrogen-dependent, cellular clones have been isolated (Badia et al. 2000). We are now studying gene expression profiling on these resistant cellular clones to delineate the molecular mechanisms associated with the development of tamoxifen resistance. These data should enhance the understanding of changes induced by ER ligands on the transcriptional program of human E2-responsive cells and permit the identification of new potential diagnostic/prognostic tools for the monitoring of estrogen-related disease conditions such as breast cancer.

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Note added by the author

While this manuscript was under revision, Frasor and colleagues published new transcriptome data from MCF-7 cells and also identified down-regulation of the ZNF217 gene upon exposure to estrogen (Frasor et al. 2003).

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