Distinct gene expression patterns in a tamoxifen-sensitive human mammary carcinoma xenograft and its tamoxifen-resistant subline MaCa 3366/TAM

Michael Becker,1 Anette Sommer,2 Jörn R. Krätzschmar,2 Henrik Seidel,2 Hans-Dieter Pohlzen,2 and Iduna Fichtner1

1Max-Delbrueck-Center for Molecular Medicine, Experimental Pharmacology, and 2Research Laboratories, Schering AG, Berlin, Germany

Abstract
The reasons why human mammary tumors become resistant to tamoxifen therapy are mainly unknown. Changes in gene expression occur as cells acquire resistance to antiestrogens. We therefore undertook a comparative gene expression analysis of tamoxifen-sensitive and tamoxifen-resistant human breast cancer in vivo models using Affymetrix oligonucleotide arrays to analyze differential gene expression. Total RNAs from the tamoxifen-sensitive patient-derived mammary carcinoma xenograft MaCa 3366 and the tamoxifen-resistant model MaCa 3366/TAM were hybridized to Affymetrix HuGeneFL and to Hu95Av2 arrays. Pairwise comparisons and clustering algorithms were applied to identify differentially expressed genes and patterns of gene expression. As revealed by cluster analysis, the tamoxifen-sensitive and the tamoxifen-resistant breast carcinomas differed regarding their gene expression pattern. More than 100 transcripts are changed in abundance in MaCa 3366/TAM as compared with MaCa 3366. Among the genes that are differentially expressed in the tamoxifen-resistant tumors, there are several IFN-inducible and estrogen-responsive genes, and genes known to be involved in breast carcinogenesis. The genes neuronatin (NNAT) and bone marrow stem cell antigen 2 (BST2) were sharply up-regulated in MaCa 3366/TAM. The differential expression of four genes (NNAT, BST2, IGFBP5, and BCAS1) was confirmed by Taqman PCR. Our results provide the starting point for deriving markers for tamoxifen resistance by differential gene expression profiling in a human breast cancer model of acquired tamoxifen resistance. Finally, genes whose expression profiles are distinctly changed between the two xenograft lines will be further evaluated as potential targets for diagnostic or therapeutic approaches of tamoxifen-resistant breast cancer. [Mol Cancer Ther 2005;4(1):151–68]

Introduction
Breast cancer is the most common type of cancer in women of the Western world. Due to advances in early detection and treatment, breast cancer survival rates have increased markedly over the past decades. After surgery, estrogen receptor α (ERα)–positive breast cancer is usually treated with endocrine therapy. Tamoxifen, a nonsteroidal antiestrogen, also termed selective estrogen receptor modulator, is the first-line therapy for premenopausal and, until recently, also for postmenopausal hormone receptor–positive women (1). For postmenopausal women, three different aromatase inhibitors are now available that might replace tamoxifen as first-line therapy in the future. Tamoxifen is effective both as adjuvant therapy and for advanced disease of hormone-responsive breast cancer and can prevent breast cancer in high-risk patients (2). In many cases, however, therapies fail and women die from recurrent, endocrine-resistant breast cancer. Prognosis of hormone-dependent breast cancer as well as treatment strategies are mainly determined by the presence of the ERα and the progesterone receptor (PR). Two thirds of the patients who present with breast cancer are ERα positive (3). Treatment of estrogen-dependent breast cancer with an antiestrogen like tamoxifen inhibits tumor growth. ERα- and PR-positive breast cancer have a better response rate than ERα- and PR-negative breast cancers (3). However, a large number of originally tamoxifen-sensitive tumors develop resistance after several months of treatment while still expressing the ERα (4).

Antagonism of tamoxifen has been attributed to the antiestrogenic activity of tamoxifen in which the active metabolite of tamoxifen, 4-hydroxytamoxifen (4-OHT), competes with E2 for binding to the ERα. Activation of the transcriptional activation domain AF-2 of the ERα, but not AF-1, is prevented by 4-OHT because coactivators can no longer bind to the antagonist-occupied ERα (5). Clinical manifestation of tamoxifen resistance is now often interpreted as a manifestation of increased tamoxifen agonism and as a switch from tamoxifen-dependent growth inhibition to growth stimulation (6, 7). The phenomenon of tamoxifen resistance is poorly understood and genetic mechanisms have been proposed, but mutations in the ERα are rare events in both patients with tamoxifen-resistant...
breast cancer and in various cell culture models of tamoxifen resistance (8, 9). In the MaCa 3366/TAM xenograft model, mutations in the ERα ligand binding domain were not identified (10, 11). Tamoxifen resistance most likely is a multicausal phenomenon. A disturbance of growth and survival pathways namely of growth factors, their receptors, extracellular proteins, proteases like kallikrein 10, immediate-early genes, transcription factors, cell cycle regulators, signal transduction molecules like BCAR1/p130Cas, phosphorylation of the ERα by protein kinase A, and alterations in the uptake, retention, and metabolism of tamoxifen might all contribute to tamoxifen resistance (12–18). Clues to the mechanisms of tamoxifen resistance could therefore be gained from an understanding of the numerous effects that tamoxifen produces at the gene expression level.

We decided to approach the question of tamoxifen resistance in a xenograft system. Xenografts of human tumors resemble the clinical situation much more closely than cell lines do (11). The xenograft tumor line MaCa 3366/TAM is one of the very few in vitro preclinical models in which antiestrogen resistance was induced in a clinically adapted manner. By direct transplantation of a ductal invasive carcinoma with moderate differentiation from a postmenopausal woman onto nude mice, the xenograft tumor line MaCa 3366 was established (19). To study tamoxifen resistance in an in vivo model, the tamoxifen-resistant xenograft tumor line MaCa 3366/TAM was developed by treatment of the tamoxifen-sensitive parental human xenograft tumor MaCa 3366 with the antiestrogen tamoxifen during successive passaging over 2 years (10, 11). MaCa 3366 and MaCa 3366/TAM are both ERα and PR positive. In both xenograft tumor lines, the PR is inducible by E2 indicating that the ERα-dependent transcriptional regulation is still intact.

In recent years, several gene expression profiling studies were performed to identify genes that are differentially expressed in human breast cancer which then allow to classify tumors and to predict outcome (20–27). In expressed in human breast cancer which then allow to classify tumors and to predict outcome (20–27). In in vitro breast cancer cell culture models, the influence of estrogen or antiestrogen treatment was also analyzed (28–37).

It was therefore our aim to compare the gene expression profile of the parental tamoxifen-sensitive mammary carcinoma xenograft model MaCa 3366 and its tamoxifen-resistant derivative MaCa 3366/TAM on a genome-wide scale to analyze the molecular events that lead to the tamoxifen-resistant phenotype in an in vivo system. By comparing the gene expression pattern of MaCa 3366, untreated or after short-term tamoxifen treatment, and MaCa 3366/TAM, either untreated, after short-term or permanent tamoxifen treatment, we expected to identify genes which respond to short-term tamoxifen treatment and those which distinguish the tamoxifen-sensitive from the tamoxifen-resistant phenotype. Different types of gene expression profiles can be envisioned which might either indicate an involvement of a particular gene in the biological process and the mechanism of tamoxifen resistance or which can potentially be exploited as marker genes of tamoxifen resistance in a diagnostic or prognostic approach. By using clustering algorithms as well as pairwise comparisons of sample groups, we focussed on those genes, which distinguish the tamoxifen-sensitive from the tamoxifen-resistant phenotype.

**Materials and Methods**

**Animal Experiment**

**Animals.** For the animal experiments, 50 female nude mice (Bom: NMRI-nu/nu) per xenograft experiment, ages 4 to 6 weeks and weighing 20 to 24 g, were used. Breeding and keeping conditions have been described (38). All animal experiments were done according to the United Kingdom Coordinating Committee on Cancer Research Guidelines for the Welfare of Animals in Experimental Neoplasia and with the permission of the responsible local authorities (G V247/98).

**Tumor Transplantation.** The s.c. transplantation of the tumor fragments (size, 4 × 4 × 4 mm³) was done under Radenarkon anesthesia (40 mg/kg i.p. Etomidat, Asta Medica, Frankfurt, Germany). The diameter of the tumors was measured once weekly using a caliper-like mechanical instrument and the tumor volume (V) was calculated according to the empirical equation \( V = (\text{length} \times \text{width}^2) / 2 \). The median volumes of each group were normalized to the initial tumor volume resulting in the relative tumor volume. In all the experiments, tumor-bearing mice received estradiol supplementation [estradiol valerate (E2D), 0.5 mg/kg once/wk i.m.]. This supplementation leads to physiologic levels of serum E2 (25-984 pg/mL) that are comparable to the human situation (25–600 pg/mL depending on the follicular phase).

**Substances.** The following substances were used: E2D (Jenapharm, Jena, Germany) and tamoxifen (Sigma, Chemie GmbH, Taukirchen, Germany).

**Treatment Modalities.** Two independent experiments were done: All MaCa 3366 and MaCa 3366/TAM transplanted animals received E2D (0.5 mg/kg) injections once a week. The last passage of MaCa 3366/TAM before the start of an experiment was cultivated without tamoxifen treatment. MaCa 3366 and MaCa 3366/TAM were either left untreated, or treated with 50 mg/kg tamoxifen 24 hours before sacrifice (short-term tamoxifen treatment). An additional group of MaCa 3366/TAM xenograft animals was treated once a week with 50 mg/kg tamoxifen during the course of the experiment starting on day 1 (permanent tamoxifen treatment; Fig. 1). As permanent tamoxifen treatment of MaCa 3366 led to complete remission of tumors, this group could not be included in the analysis. From histologic and fluorescence-activated cell sorting analyses of MaCa 3366 and MaCa 3366/TAM, the amount of murine tissue in a human breast cancer xenograft tumor was estimated to be as low as 5% to 10%.

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3. Fichtner and M. Becker, unpublished observation.
Extraction of RNA

Tumor tissue samples (size, 2 × 2 × 2 mm³) were taken from the sacrificed animals. Samples were snap frozen and stored in liquid nitrogen until use. Total RNA of tumor samples was prepared using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s recommendations. A DNase I (Qiagen) digestion step was included to eliminate DNA contamination.

Affymetrix DNA Chip Hybridization

Isolated total RNA was checked for integrity and concentration using the RNA 6000 Assay and RNA LabChips on the Agilent Bioanalyzer 2100 (Agilent Technologies, Inc., Palo Alto, CA). The cRNA was prepared as described above (39). The HuGeneFL and Hu95Av2 arrays (Affymetrix, Santa Clara, CA) were hybridized, washed, and stained with streptavidin-phyceroerythrin (Molecular Probes, Eugene, OR), biotinylated anti-streptavidin (Vector Laboratories, Burlingame, CA) and streptavidin-phyceroerythrin according to the standard antibody amplification protocol for eukaryotic targets. The arrays were scanned with a confocal scanner from Affymetrix at 570 nm.

Data Analysis

The Affymetrix arrays were normalized by employing the Maximum Order Preserving Set method. This method identifies a subset of genes which does not change its expression between two experiments. The intensity scatter plot for all genes from this restricted set is approximated by a nonlinear regression function, which is then used for normalization as described above (39). The normalized expression data sets were loaded into the CoBi database (Genedata, Basel, Switzerland) and analyzed with the Genedata Expressionist software version 5.0 (40, 41). The expression data were filtered with the coefficient of variation and on the variant data set three different fold change–based two-group tests (intergroup gap, valid value proportions, and ratio of means) were done. A ratio of means is the ratio of the mean expression values for two sample groups under investigation. A group mean is calculated as the exponentialized arithmetic mean of all logarithmized expression values within a group. This is identical to the geometric mean of all expression values. For the valid value proportions analysis, all possible pairwise combinations of a sample from the first sample group and another sample from the second sample group are created. If the first group contained m samples and the second group contained n samples, then there were m × n such sample pairs. Subsequently, for each sample pair, the ratio of their corresponding expression values is calculated and compared with a threshold which is an analysis variable. The percentage of sample pairs which pass this threshold is determined. Finally, this type of analysis returns all probe sets for which the proportion of sample pairs with above-threshold expression ratios exceeds a given percentage which is another analysis variable. The intergroup gap method identifies probe sets which have consistently higher expression values in one of the two sample groups. If expression values in the first sample group are smaller than in the second sample group, then the intergroup gap is the ratio between the smallest value of the second group and the largest value of the first group. The intergroup gap method then returns all probe sets for which this ratio is above a given threshold. Probe sets with overlapping ranges of expression values are never returned by this type of analysis. Statistical tests could not be applied because only two or three samples were present in each sample group.

Probe Sets

Profiling human genes expressed in a human breast cancer–derived xenograft model transplanted onto nude mice using the human-specific Affymetrix arrays HuGeneFL and Hu95Av2, will mostly uncover genes...
specifically expressed in human cancer cells in xenograft tumors that might comprise a mixture of human- and mouse-derived cells. Using the human-specific Affymetrix arrays in contrast to cDNA arrays has the advantage that 20 independent probes as perfect match oligonucleotides interrogate a particular gene. Unless a murine gene is 100% homologous to a human gene, this provides very high specificity compared with a continuous stretch of DNA on a cDNA array. The probe sets are usually situated either at the extreme 3′ end of the cDNA or in the 3′ untranslated region of the human genes. In the latter case, there is a very low probability that a human-specific probe set detects a murine gene because the homology between human and murine sequence is usually very low in the 3′ untranslated region. In addition, the presence of a set of 20 mismatch oligonucleotides provides a further degree of specificity as only those probe sets are incorporated in the determination of a condensed signal intensity for which the signal intensity on the perfect match is larger than on the mismatch oligonucleotide. The probe sets on the two array generations HuGeneFL and Hu95Av2 were changed by Affymetrix such that different probe sets are present on both arrays. If genes are interrogated on both types of arrays by independent probe sets or if more than one probe set interrogates a particular gene on one array then the differential expression of human genes is further corroborated.

**Unsupervised Analysis.** For the unsupervised analysis, genes with invariant expression were filtered out by the coefficient of variation analysis. HuGeneFL data were filtered with a coefficient of variation (CV) ≥ 0.03175 resulting in a selection of 503 genes. For the analysis of Hu95Av2 data, a CV ≥ 0.04050 selected 538 genes. The CV was chosen so that a manageable number of probe sets was obtained. k-Means clustering was applied and genes were sorted into 10 different clusters.

**Supervised Analysis.** To select for variant genes a CV ≥ 0.001091 (CV ≥ 0.001097 for HuGeneFL array) was applied to the data derived from the Hu95Av2 array over all hybridization samples and 4,887 (2,305 for HuGeneFL array) probe sets were selected from a total of 12386 probe sets (6,574 for HuGeneFL array). Next, three different fold change–based two-group tests [intergroup gap >1.5-fold (1.3-fold), valid value proportions 40% (40%), ratio of means 1.8-fold (1.6-fold)] were used to identify differentially expressed probe sets between MaCa 3366/TAM (untreated, short-term, and permanent tamoxifen treatment) and MaCa 3366 (untreated, short-term tamoxifen treatment). [Values in brackets for HuGeneFL array.] Those 154 [50] probe sets overlapping (intersection) and those 362 [140] probe sets identified with any of the three independently conducted, fold change–based tests were further subjected to a k-means clustering resulting in two groups consisting of 57 [37] and 144 [125] probe sets up-regulated and 97 [13] and 218 [67] down-regulated, respectively, in all MaCa 3366/TAM compared with all MaCa 3366 tumors. To compare differentially expressed genes on the two array formats, the 144 and 125 up-regulated and 218 and 67 down-regulated probe sets from the Hu95Av2 and HuGeneFL array, respectively, were subjected to manual inspection combined with a BLAST analysis of the genes corresponding to the probe set identifiers. Differential expression of several genes was detected with more than one probe set. Nonredundant genes that are regulated and present on both array types and genes present on only one array, but strongly regulated, are displayed.

**Quantitative Real-time PCR**

Six genes (BST2, NNAT, IGFBP5, ESRRG, PTK6, and BCA1) were selected and analyzed by quantitative real-time reverse transcription-PCR based on TaqMan-chemistry (Table 1). Hydroxymethylbilane synthase (HMBS) was used as housekeeping gene (Table 1). Predesigned reagents containing a specific primer pair/probe mix, premixed TaqMan Reverse Transcription Reagents and TaqMan Universal MasterMix were obtained from Applied Biosystems GmbH (Weiterstadt, Germany). For BCA1 and HMBS, new primer pair/probe combinations were designed by the Assay-by-Design-service at Applied Biosystem using the template sequences (Table 1). Pooled RNA from n = 5 animals from the five treatment groups obtained from the two independent xenograft experiments was reverse transcribed with the Reverse Transcription Reagents (Applied Biosystems) and contained 200 ng RNA in a final volume of 60 μL. The RT reaction was carried out at 25°C for 10 minutes, 48°C for 30 minutes, and 95°C for 5 minutes. The PCR was done in

### Table 1. TaqMan assays

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene description</th>
<th>Accession ID</th>
<th>Assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>BST2</td>
<td>Bone marrow stromal cell antigen 2</td>
<td>NM_004335</td>
<td>Hs00171632_m1</td>
</tr>
<tr>
<td>NNAT</td>
<td>Neuronatin</td>
<td>NM_005386</td>
<td>Hs00193590_m1</td>
</tr>
<tr>
<td>IGFBP5</td>
<td>Insulin-like growth factor binding protein 5</td>
<td>NM_000590</td>
<td>Hs00181213_m1</td>
</tr>
<tr>
<td>ESRRG</td>
<td>Estrogen-related receptor γ</td>
<td>NM_001438</td>
<td>Hs00155006_m1</td>
</tr>
<tr>
<td>PTK6</td>
<td>Protein tyrosine kinase 6</td>
<td>NM_005975</td>
<td>Hs00178742_m1</td>
</tr>
<tr>
<td>BCA1</td>
<td>Breast carcinoma amplified sequence 1</td>
<td>NM_003657</td>
<td>Assay-by-Design</td>
</tr>
<tr>
<td>HMBS</td>
<td>Hydroxymethylbilane synthase</td>
<td>NM_001900</td>
<td>Assay-by-Design</td>
</tr>
</tbody>
</table>

*NOTE: The gene symbol is the most commonly used abbreviation, usually from OMIM. The accession ID is the National Center for Biotechnology Information RefSeq or reference sequence for this gene. The assay ID is the unique name of the predeveloped or TaqMan assay from Applied Biosystems.*
96-well microtiter plates in an ABI Prism 7700 Sequence Detection System (Applied Biosystems). The reaction mixture consisted of 10 μL Taqman Universal MasterMix, 1 μL primer/probe-mix, and 2 μL of the RT reaction mix in a final volume of 20 μL. Following an initial step at 95°C for 10 minutes for denaturation of the DNA and activation of the Taq enzyme, 45 cycles consisting of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute were done. Each sample was tested in triplicate.

The relative expression of each gene was determined on the basis of the threshold cycle (CT value). With cDNA from testis, placenta, and prostate (Clontech, Palo Alto, CA) standard curves were generated. For each CT value, a corresponding ng amount of cDNA was calculated. With the housekeeping gene HMBS the quantity of cDNA was normalized. Then, the normalized target gene expression was divided by the normalized gene expression value of the calibrator (i.e. untreated MaCa 3366 xenograft) to obtain the fold change of the gene in the treatment groups relative to the untreated MaCa 3366 xenograft. The SD of the normalized target gene expression relative to HMBS is calculated from the initial SDs of the target gene and HMBS.

Results

Originating from the tamoxifen-sensitive human mammary carcinoma xenograft MaCa 3366, the tamoxifen-resistant model MaCa 3366/TAM was successfully established by the treatment of tumor-bearing nude mice with tamoxifen for 2 years during routine passaging. Tumor fragments were transplanted s.c. to female nude mice, which were supplemented with E2D to support tumor growth. For the analysis of differential gene expression, the five treatment groups were MaCa 3366 and MaCa 3366/TAM untreated and with short-term tamoxifen treatment, and MaCa 3366/TAM under permanent tamoxifen treatment (Fig. 1). In order to distinguish the effects of short-term tamoxifen treatment on gene expression from the effects of permanent tamoxifen treatment on resistance, the MaCa 3366 and the MaCa 3366/TAM xenograft mice were treated once with tamoxifen 24 hours before sacrifice (short-term tamoxifen treatment). The tumor growth curve of the five treatment groups from the second xenograft experiment are displayed (Fig. 2). Although MaCa 3366/TAM tumors grow more slowly than MaCa 3366 tumors growth of MaCa 3366/TAM tumors is independent of tamoxifen treatment. MaCa 3366 tumors treated with tamoxifen showed complete remission (data not shown).

Tumor samples from both xenograft lines were used for differential gene expression analysis. Total RNA was isolated from the tumor tissues, RNA from five animals per group was pooled, and the cRNA from the first and second xenograft experiments was hybridized to Affymetrix HuGeneFL and Hu95Av2 arrays, respectively (Fig. 1). By comparing the gene expression pattern of MaCa 3366 untreated or after short-term tamoxifen treatment and MaCa 3366/TAM either untreated, after short-term or permanent tamoxifen treatment, we expect to identify genes that respond to short-term tamoxifen treatment and genes that distinguish the tamoxifen-sensitive from the tamoxifen-resistant phenotype.

We adopted two different approaches in an attempt to define gene expression patterns segregating with a tamoxifen-resistant compared with a tamoxifen-sensitive xenograft phenotype (Fig. 1). For unsupervised analysis, k-means clustering with positive correlation as distance measure was applied to the variant gene set of 503 genes on the HuGeneFL array and 538 genes on the Hu95Av2 array and genes were sorted into 10 different clusters. Profiles for the 10 clusters on the HuGeneFL and on the Hu95Av2 array are depicted in Fig. 3A and B, respectively. When the two independent xenograft experiments were hybridized to two different array types, the corresponding clusters on the two array types were identified first and afterwards genes differentially expressed in these clusters were analyzed. Cluster 5 on HugeneFL corresponds to cluster 9 on Hu95Av2 and contains genes, which are solely sharply up-regulated in MaCa 3366/TAM treated permanently with tamoxifen. Similarly, cluster 8 on HuGeneFL corresponds to cluster 3 on Hu95Av2 and contains genes that are up-regulated in all three MaCa 3366/TAM xenografts independent of tamoxifen or length of tamoxifen treatment. Moreover, the profile of cluster 4 in the HuGeneFL data set corresponds to the profile of cluster 5 in the Hu95Av2 data set and contains genes that are significantly up-regulated after short-term tamoxifen treatment (permanent TAM) compared to untreated MaCa 3366/TAM tumors (Fig. 1). For supervised analysis, we used the corresponding gene expression data for each cluster on the two array types to estimate the gene expression pattern segregating with a tamoxifen-resistant xenograft phenotype (Fig. 1). In order to define the expression pattern of tamoxifen-resistant xenografts with the highest confidence, we used the complete set of genes and the gene expression data for each cluster on the two array types to estimate the expression pattern segregating with a tamoxifen-resistant xenograft phenotype. The expression pattern of all genes in each cluster was estimated using the complete set of genes and the gene expression data for each cluster on the two array types. For each cluster, the expression pattern was estimated using the complete set of genes and the gene expression data for each cluster on the two array types. For each cluster, the expression pattern was estimated using the complete set of genes and the gene expression data for each cluster on the two array types.
up-regulated with increasing exposure of the MaCa3366 xenografts to tamoxifen. However, as probe sets and expression values vary between the data sets on the HuGeneFL and Hu95Av2 array, a complete concordance of clusters cannot be expected. Applying \(k\)-means clustering on the Hu95Av2 array variant data set consisting of 538 genes, the following prominent gene clusters were identified: (1) genes that are remarkably up-regulated in all MaCa 3366/TAM xenografts (cluster 3: 88 genes), (2) genes that are noticeably down-regulated in all MaCa 3366/TAM xenografts (cluster 1: 104 genes), (3) genes that are up-regulated by short-term tamoxifen treatment in MaCa 3366 and MaCa 3366/TAM (cluster 6: 114 genes, cluster 7: 29 genes, cluster 10: 44 genes), (4) genes that are down-regulated by short-term tamoxifen treatment in MaCa 3366 and MaCa 3366/TAM (cluster 2: 16 genes), and (5) genes that are increasingly up-regulated in the MaCa 3366 and MaCa 3366/TAM xenograft tumors with extended exposure to tamoxifen (cluster 5: 51 genes). Surprisingly, we also found genes that are only up-regulated after permanent tamoxifen treatment in MaCa 3366/TAM (cluster 9: 33 genes).

To identify differentially expressed probe sets between MaCa 3366/TAM (untreated, short-term, and permanent tamoxifen treatment) and MaCa 3366 (untreated and short-term tamoxifen treated), a supervised analysis was done and three different fold change-based tests were applied (see Material and Methods) resulting in four groups with 57 (37) probe sets up-regulated and 97 (13) down-regulated on stringent criteria and 144 (125) probe sets up-regulated and 218 (67) down-regulated on less stringent criteria, respectively, in all tamoxifen-resistant compared with all tamoxifen-sensitive samples on the Hu95Av2 array (values for HuGeneFL in brackets). The redundancy (i.e. more than one probe set mapping to one particular gene) was removed by a BLAST analysis of the corresponding gene

![Figure 3](image-url)
identifiers as supplied by Affymetrix, and the Online Mendelian Inheritance in Man (OMIM) description was attributed to each probe set. To compare differentially expressed genes on the two different array formats, the 144 up-regulated probe sets from the Hu95Av2 and 125 up-regulated probe sets from the HuGeneFL array and the 218 down-regulated probe sets from the Hu95Av2 and 67 down-regulated probe sets from the HuGeneFL array, respectively, were subjected to a BLAST analysis and annotation by Gene Ontology (GO) and OMIM. Nonredundant genes (n = 109) were regulated >2-fold in MaCa 3366/TAM as compared with MaCa 3366. The expression values of differentially expressed nonredundant genes present on both array types or present on only one array, but regulated with a fold change >2, were ranked and the genes showing the strongest differential expression in the tamoxifen-resistant compared with the tamoxifen-sensitive human breast cancer xenograft model are displayed (Table 2A and B). If more than one probe set was given for a particular gene, then the probe sets with the highest fold change determined the rank on the list. Differences in the expression values can be explained on the one hand by different tumor growth characteristics in the xenograft mice from the two independent animal experiments which should, however, be leveled out by pooling five animals per treatment group and, on the other hand, by the fact that from array generation HuGeneFL to Hu95Av2 the number of probe sets on the array was increased from 6,574 to 12,386 explaining why some genes are only present on the Hu95Av2 array.

Functional Categories of Differentially Expressed Genes

For the 109 nonredundant genes differentially expressed and listed in Table 2A and B, either a known function or an inferred function was identified from OMIM and GO nomenclature (42, 43). GO stores controlled vocabulary organized under the three independent ontologies “biological process,” “molecular function,” and “cellular component.” The differentially expressed genes were categorized according to these ontologies (Fig. 4). In addition, we introduced three categories reflecting the biological system (i.e., “known association with cancer,” “IFN-inducible genes” (Table 2A), and “estrogen-responsive genes” (Table 2A and B). As all three GOs can be attributes of a particular gene, they can be assigned independently to it. In the GO biological process, molecular function, and cellular component, the distribution of genes to the categories was very similar for genes that are up- or down-regulated in MaCa 3366/TAM (Fig 4A and B) with three exceptions: the immune/defense response group, the IFN-inducible genes, and the lipid metabolism group. Numerous IFN-inducible genes were found that were coordinately up-regulated in MaCa 3366/TAM (Table 2A; Fig. 4A). Similarly, numerous estrogen-responsive genes were down-regulated in MaCa 3366/TAM (Table 2B; Fig. 4B). Examples for up- and down-regulated genes are displayed in Fig. 5. The graphs show the high degree of reproducibility of the two independent xenograft experiments and the reproducibility of results even if different array generations (i.e., HuGeneFL and Hu95Av2 with different probe sets are used).

IFN-Inducible Genes. Very prominently, among the genes that are up-regulated in the tamoxifen-resistant compared with the tamoxifen-sensitive breast cancer xenograft model MaCa 3366, a set of genes was discovered that was annotated as IFN-inducible (Table 2A and B; Figs. 4A and 5A, and data not shown) such as IFTM2 (34-fold), GIP2 (12-fold), GIP3 (14-fold), IFI44 (3.3-fold), IFTM1 (2.5-fold), IFIT1 (3.5-fold), MX1 (5.2-fold), NMI (2.9-fold), ISGF3G (2.4-fold), and PKR (1.9-fold) that were all increased. The gene IFI27 was found to be up-regulated 11-fold in MaCa 3366/TAM treated permanently with tamoxifen compared with the MaCa 3366 model, but it was not up-regulated in MaCa 3366/TAM untreated or treated with tamoxifen only short-term. Numerous genes were identified which are down-regulated by short-term tamoxifen treatment of MaCa 3366 and are also down-regulated in MaCa 3366/TAM (Table 2B; Figs. 4B and 5B, and data not shown). Members of this cluster of genes with decreased expression are cyclophilin D1 (2.1- to 2.3-fold depending on the probe set), GATA3 (1.9-fold), PDZK1 (4.7-fold), IGBP5 (8.4- to 10.3-fold depending on the probe set), gap junction protein 43 [GJA1, gap junction protein α1, connexin 43 (3.8-fold)], and the homeobox transcription factors MSX1 and MSX2 (2.4- and 3.2-fold, respectively). Among the genes decreased in tamoxifen-resistant xenografts, either untreated or short-term tamoxifen treated, are also the estrogen-related receptor γ (ESRRG) and insulin-like growth factor 1 receptor (IGF1R; Fig. 5B).

Several genes that are associated with breast cancer are differentially expressed, [e.g., breast carcinoma amplified sequence 1 (BCAS1)] was up-regulated 4.1-fold in MaCa 3366/TAM under permanent tamoxifen treatment compared with untreated MaCa 3366. The UDP-glycosyltransferase 2 family, member B15 (UGT2B15) is increased 3.2- to 7.9-fold depending on the probe set in MaCa 3366/TAM under permanent tamoxifen treatment compared with MaCa 3366. In addition, many genes that had not previously been implicated in tamoxifen resistance were also identified: among the genes sharply up-regulated on both array types in the tamoxifen-resistant xenograft MaCa 3366/TAM, either untreated or short-term tamoxifen treated, are bone marrow stem cell antigen-2 (BST2) and neuronatin (NNAT).

Validation of Array Data with Quantitative Reverse Transcription-PCR. To confirm the differential gene expression from the array experiments employing an independent method, we did real-time quantitative reverse transcription-PCR in xenograft RNA samples (untreated and short-term tamoxifen treated MaCa 3366, untreated, short-term, and permanent tamoxifen-treated MaCa 3366/TAM) pooled from five animals each and from two independent animal experiments. We selected four genes with strong differential expression (NNAT, BST2, BCAS1,
and IGFBP5) and two genes with a weaker differential expression (ESRRG and PTK6). The normalized gene expression levels relative to the expression in untreated MaCa 3366 as calibrator are shown in Fig. 6. TaqMan analysis clearly confirmed the increase of NNAT, BST2, and BCAS1 and the decrease of IGFBP5 in MaCa 3366/TAM (Fig. 6). NNAT and BST2 were sharply up-regulated in MaCa 3366/TAM under permanent tamoxifen treatment compared with untreated MaCa3366 as measured by TaqMan analysis in RNA derived from the first and second xenograft experiment. The down-regulation of ESRRG in MaCa 3366/TAM compared with MaCa 3366 on the Hu95Av2 array (2.2-fold) was confirmed with the Taqman assay (2-fold). However, ESRRG was not decreased in RNA from the first xenograft experiment as tested by Taqman PCR (data not shown). The increase (2.4 on Hu95Av2 array) of the breast cancer-related tyrosine kinase PTK6/Brk in MaCa 3366/TAM compared with MaCa 3366 was not confirmed by Taqman PCR (data not shown).

**Discussion**

In the past, numerous estrogen-responsive genes were identified. Many of them are down-regulated after

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**Figure 4.** Gene Ontology categories. The 54 genes that are up-regulated (A) and the 55 genes that are down-regulated (B) in MaCa 3366/TAM were categorized according to involvement in biological processes, molecular function, and according to cellular component. The GO biological process is divided into immune/defense response, development/morphogenesis, cell growth, cell signaling, metabolism, and apoptosis. The GO molecular function is divided into signal transduction, transcription, protein synthesis/modification/transport, and lipid metabolism. In addition, we introduced three categories reflecting the biological system (i.e., IFN-inducible genes, known association with cancer, and estrogen-responsive genes). The GO category cellular component is divided into the four components: membrane, nucleus, intracellular, and extracellular. One gene can be a member of more than one GO category.
Figure 5. Affymetrix expression profile of selected genes. The values are derived from probe sets on the Hu95Av2 array (black and dark gray) and/or HuGeneFL array (light gray; see Table 2A and B for reference). The gene symbol here is the OMIM abbreviation, if available. **A**, examples of genes up-regulated in MaCa 3366/TAM compared with MaCa 3366 are neuronatin (NNAT), bone marrow stromal cell antigen 2 (BST2), UDP-glycosyltransferase 2 family, member B15 (UGT2B15); breast carcinoma amplified sequence 1 (BCAS1); myxovirus resistance 1 (MX1); IFN-induced gene with tetratricopeptide repeats 1 (IFIT1); IFN-induced transmembrane protein 2 (IFITM2); and IFN-α inducible protein 27 (IFIT2). **B**, examples of genes down-regulated in MaCa 3366/TAM compared with MaCa 3366 are insulin-like growth factor binding protein 5 (IGFBP5), PDZ domain containing 1 (PDZK1), estrogen-related receptor γ (ESRRG), and insulin-like growth factor 1 receptor (IGF1R).
### Table 2. Summary of the differentially regulated genes in the human breast cancer xenograft model MaCa 3366/TAM compared with MaCa 3366

#### A. Genes that are reproducibly up-regulated in untreated or permanently tamoxifen-treated MaCa 3366/TAM compared with MaCa 3366

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Description</th>
<th>Fold increase in untreated MaCa 3366/TAM versus untreated MaCa 3366</th>
<th>Fold increase in MaCa 3366/TAM plus permanent tamoxifen treatment versus untreated MaCa 3366</th>
<th>Affymetrix probe ID</th>
<th>GO*</th>
<th>Reference</th>
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<tbody>
<tr>
<td>BST2</td>
<td>Bone marrow stromal cell antigen 2</td>
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<td>NNAT</td>
<td>Neuronatin</td>
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<td>34</td>
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<td>U25034_s_at</td>
<td>39051_at</td>
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<td>IFITM2</td>
<td>IFN-induced transmembrane protein 2 (1-8 D)</td>
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<td>12.6</td>
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<tr>
<td>GIP2</td>
<td>IFN-stimulated protein, 15 kDa</td>
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<td>12</td>
<td></td>
<td>1107_s_at</td>
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<td>GIP3</td>
<td>IFN, α-inducible protein (clone IFI-6-16)</td>
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<td>14</td>
<td></td>
<td>1358_s_at</td>
<td>i (45)</td>
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<td>GAGE8</td>
<td>G antigen 1</td>
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<td></td>
<td>542_at</td>
<td>543_g_at</td>
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<td>CRABP1</td>
<td>Cellular retinoic acid binding protein 1</td>
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<td>ACTA1</td>
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<td>PPP2K2A</td>
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<td>β-2-microglobulin</td>
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<td>6.2</td>
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<td>428_s_at</td>
<td>201_s_at</td>
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<td>BCAS1</td>
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<td>S82297_at</td>
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<td>i, c (42, 46)</td>
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<td>PROCR</td>
<td>Protein C receptor, endothelial (EPCR)</td>
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<td>NMT1</td>
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*NOTE: The expression values of differentially expressed non-redundant genes were ranked for the genes up- and down-regulated, respectively, in the tamoxifen-resistant compared to the tamoxifen-sensitive human breast cancer xenograft model. The gene symbol here is the most commonly used abbreviation, usually from OMIM, if available. Affymetrix probe ID is the unique name of the probe set on the HuGeneFL or Hu95Av2 array. Estrogen-responsive and IFN-inducible genes as well as genes associated with cancer are indicated.

*i, IFN-inducible; e, estrogen-responsive; c, known association with cancer.

(Continued on following page)
Table 2. Summary of the differentially regulated genes in the human breast cancer xenograft model MaCa 3366/TAM compared with MaCa 3366 (Cont’d)

A. Genes that are reproducibly up-regulated in untreated or permanently tamoxifen-treated MaCa 3366/TAM compared with MaCa 3366

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Description</th>
<th>Fold increase in untreated MaCa 3366/TAM versus untreated MaCa 3366</th>
<th>Fold increase in MaCa 3366/TAM plus permanent tamoxifen treatment versus untreated MaCa 3366</th>
<th>Affymetrix probe ID</th>
<th>GO*</th>
<th>Reference</th>
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<td>ISGF3G</td>
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<td>(47)</td>
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<td>IFITM1</td>
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<td>(48)</td>
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<td>LGALS3BP</td>
<td>Lectin, galactoside-binding, soluble, 3 binding protein</td>
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<td>(42)</td>
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<td>GTF2E2</td>
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<td>CTSB</td>
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(Continued on following page)
Table 2. Summary of the differentially regulated genes in the human breast cancer xenograft model MaCa 3366/TAM compared with MaCa 3366 (Cont’d)

B. Genes that are reproducibly down-regulated in untreated or permanently tamoxifen-treated MaCa 3366/TAM compared with untreated MaCa 3366 xenograft tumors

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Description</th>
<th>Fold decrease in untreated MaCa 3366/TAM versus untreated MaCa 3366</th>
<th>Fold decrease in MaCa 3366/TAM plus permanent tamoxifen treatment versus untreated MaCa 3366</th>
<th>Affymetrix probe ID</th>
<th>GO*</th>
<th>Reference</th>
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<td>Elongation of very long chain fatty acids-like 2</td>
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<td>(59)</td>
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<td>DFKZ5644H1916</td>
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<td>41229_at</td>
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<td>Homologue of mouse quaking QKI (KH domain RNA binding protein)</td>
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<td>OSBP1A</td>
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<td>PDC1V</td>
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</tr>
<tr>
<td>QKI</td>
<td>Homologue of mouse quaking QKI (KH domain RNA binding protein)</td>
<td>2.4</td>
<td>2.7</td>
<td>39760_at</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL1R1</td>
<td>Interleukin 1 receptor, type 1</td>
<td>2.4</td>
<td>2.8</td>
<td>1368_at</td>
<td></td>
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<tr>
<td>CCND1</td>
<td>Cyclin D1</td>
<td>2.3</td>
<td>2.3</td>
<td>2017_s_at</td>
<td>e</td>
<td>(42, 65)</td>
</tr>
<tr>
<td>ESRG</td>
<td>Estrogen-related receptor γ</td>
<td>2.2</td>
<td>2.2</td>
<td>38418_at</td>
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<td></td>
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<tr>
<td>TM4SF1</td>
<td>Transmembrane 4 superfamily member 1</td>
<td>2.2</td>
<td>1.7</td>
<td>31843_at</td>
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(Continued on following page)
### Table 2. Summary of the differentially regulated genes in the human breast cancer xenograft model MaCa 3366/TAM compared with MaCa 3366 (Cont’d)

#### B. Genes that are reproducibly down-regulated in untreated or permanently tamoxifen-treated MaCa 3366/TAM compared with untreated MaCa 3366 xenograft tumors

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Description</th>
<th>Fold decrease in untreated MaCa 3366/TAM versus untreated MaCa 3366</th>
<th>Fold decrease in MaCa 3366/TAM plus permanent tamoxifen treatment versus untreated MaCa 3366</th>
<th>Affymetrix probe ID</th>
<th>GO*</th>
<th>Reference</th>
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<tr>
<td>SEMA3C</td>
<td>Semaphorin 3C</td>
<td>2.2</td>
<td>2.0</td>
<td>377_g_at</td>
<td>c</td>
<td>(42)</td>
</tr>
<tr>
<td>ZNF43</td>
<td>Zinc finger protein 43 (HTF6)</td>
<td>2.1</td>
<td>2.0</td>
<td>33992_f_at</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RBPI</td>
<td>Retinol binding protein 1, cellular</td>
<td>2.1</td>
<td>2.1</td>
<td>38634_at</td>
<td>c</td>
<td>(42)</td>
</tr>
<tr>
<td>GATA3</td>
<td>GATA binding protein 3</td>
<td>2.4</td>
<td>1.9</td>
<td>X07438_s_at</td>
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<tr>
<td>IRF3</td>
<td>IFN regulatory factor 3</td>
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<td>2.0</td>
<td>371_at</td>
<td></td>
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<tr>
<td>AIM1</td>
<td>Absent in melanoma 1</td>
<td>2.0</td>
<td>2.3</td>
<td>32112_s_at</td>
<td>c</td>
<td>(42)</td>
</tr>
<tr>
<td>MSX1</td>
<td>Msh homeo box homologue 1</td>
<td>1.7</td>
<td>1.8</td>
<td>U83115_at</td>
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<tr>
<td>ME1</td>
<td>Malic enzyme 1, NADP(+)-dependent, cytosolic</td>
<td>1.9</td>
<td>2.4</td>
<td>40199_at</td>
<td>e</td>
<td>(60)</td>
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<td>LBR</td>
<td>Lamin B receptor</td>
<td>1.9</td>
<td>1.6</td>
<td>837_s_at</td>
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<tr>
<td>BCAS2</td>
<td>Breast carcinoma amplified sequence 2</td>
<td>1.9</td>
<td>1.7</td>
<td>288_s_at</td>
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<tr>
<td>PKIA</td>
<td>Protein kinase (cAMP-dependent, catalytic) inhibitor α</td>
<td>1.9</td>
<td>2.2</td>
<td>34286_at</td>
<td>c</td>
<td>(42)</td>
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<tr>
<td>#23612</td>
<td>Human clone 23612 mRNA sequence</td>
<td>1.5</td>
<td>1.4</td>
<td>S76965_at</td>
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<td>EMP1</td>
<td>Epithelial membrane protein 1</td>
<td>2.6</td>
<td>1.6</td>
<td>U90902_at</td>
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<tr>
<td>LENG4</td>
<td>Leukocyte receptor cluster (LRC) member 4</td>
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<td>1.8</td>
<td>37762_at</td>
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<tr>
<td>AGPS</td>
<td>Alkylglycerone phosphate synthase</td>
<td>1.4</td>
<td>1.3</td>
<td>38370_at</td>
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<td>HDAC2</td>
<td>Histone deacetylase 2</td>
<td>1.6</td>
<td>1.8</td>
<td>36202_at</td>
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<td>IGFR1</td>
<td>Insulin-like growth factor 1 receptor α chain</td>
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<td>2.1</td>
<td>34170_at</td>
<td>c</td>
<td>(42)</td>
</tr>
<tr>
<td>COX7A2</td>
<td>Cytochrome c oxidase subunit VIIa polypeptide 2 (liver)</td>
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<td>1.8</td>
<td>41760_at</td>
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<tr>
<td>TM4SF6</td>
<td>Transmembrane 4 superfamily member 6</td>
<td>1.7</td>
<td>1.4</td>
<td>39362_r_at</td>
<td></td>
<td></td>
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<tr>
<td>ORCML</td>
<td>Origin recognition complex, subunit 3-like</td>
<td>2.8</td>
<td>1.8</td>
<td>36895_at</td>
<td></td>
<td></td>
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<tr>
<td>CD24</td>
<td>CD24 antigen</td>
<td>1.6</td>
<td>1.1</td>
<td>266_s_at</td>
<td>c</td>
<td>(42)</td>
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<tr>
<td>ADAM17</td>
<td>A disintegrin and metalloproteinase domain 17</td>
<td>1.6</td>
<td>1.6</td>
<td>L33930_s_at</td>
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<tr>
<td>PTPRK</td>
<td>Protein tyrosine phosphatase, receptor type, K</td>
<td>1.7</td>
<td>1.8</td>
<td>41601_at</td>
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<td>ACAA2</td>
<td>Acetyl-coenzyme A acyltransferase 2 (mitochondrial 3-oxoacyl-coenzyme A thiolase)</td>
<td>1.6</td>
<td>1.8</td>
<td>41530_at</td>
<td></td>
<td></td>
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<tr>
<td>AREG</td>
<td>Amphiregulin</td>
<td>1.3</td>
<td>1.3</td>
<td>D16294_at</td>
<td></td>
<td></td>
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<tr>
<td>TRIM16</td>
<td>Tripartite motif-containing 16</td>
<td>1.3</td>
<td>1.2</td>
<td>34898_at</td>
<td>e</td>
<td>(42, 67)</td>
</tr>
<tr>
<td>LIPF</td>
<td>Lipase, gastric</td>
<td>2.5</td>
<td>2.1</td>
<td>38881_i_at</td>
<td>e</td>
<td>(68)</td>
</tr>
<tr>
<td>NR2F2</td>
<td>Nuclear receptor subfamily 2, group F, member 2</td>
<td>1.5</td>
<td>1.5</td>
<td>M64497_at</td>
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short-term tamoxifen treatment and after permanent tamoxifen treatment in the tamoxifen-resistant breast cancer xenograft MaCa 3366/TAM. Members of this group of genes which previously were shown to be estrogen-responsive and which are down-regulated in MaCa 3366/TAM are cyclin D1 (65), GATA3 (66), PDZK1 (59), gap junction protein 1 GJA1 (64), and the homeobox transcription factors MSX1 and MSX2 (60). MSX2 was shown to be induced by E2 in the human breast cancer cell line MCF-7 (61). The growth factor amphiregulin, which modulates growth by binding to the epidermal growth factor receptor, was sharply down-regulated in MaCa 3366/TAM. It is well known that estrogen induces the expression of amphiregulin in human breast carcinoma cells and that 4-OHT interferes with the induction of amphiregulin by estrogen in cell lines (67). Down-regulation of IGFBP5 (62, 63) was confirmed by real-time PCR (Fig. 6). The IGF1R was down-regulated in MaCa 3366/TAM. In a comparative study of different antiestrogen-resistant cell lines, reduced levels of IGF1R were detected and it was proposed that this protein might be a general marker for antiestrogen resistance (14).

NNAT was found to be sharply up-regulated in MaCa 3366/TAM compared with MaCa 3366 (Table 2A; Fig. 5). By real-time PCR, the strong differential expression was confirmed (Fig. 6). NNAT is highly expressed in the brain of the murine fetus and neonate and at much lower levels in the brain of the adult (69). The function of NNAT is unknown, but its functioning as a regulator of ion channels during brain development has been proposed (70). Thus far, no association of NNAT expression with breast cancer and tamoxifen-resistant breast cancer in particular has been shown.

Breast carcinoma amplified sequence 1 (BCAS1) was up-regulated (4.1-fold on the Hu95Av2 array) in MaCa 3366/TAM treated permanently with tamoxifen compared with MaCa 3366 (Table 2A). By real-time PCR, the up-regulation was confirmed (Fig. 6). BCAS1 also known as novel amplified in breast cancer-1 (NABC1) lies in 20q13.3, a region often amplified in breast carcinoma, and is associated with aggressive tumor behavior. BCAS1 was found to be overexpressed in breast carcinomas and certain breast cancer cell lines on the RNA and protein level (71, 72). Surprisingly, BCAS1 mRNA is expressed at high levels only in normal brain and prostate (72). Currently, the function of BCAS1 is unknown; however, overexpression of human BCAS1 in NIH-3T3 cells did not affect the growth rate or anchorage-independent growth suggesting that BCAS1 is not a typical proto-oncogene (72).
Metabolism of steroid hormones are subject to glucuronidation which is a major pathway for the elimination of steroids in liver and several other steroid target tissues. Glucuronidation is catalyzed by UDP-glycosyltransferases, which transfer glucuronic acid to a wide variety of endogenous and exogenous compounds. The UDP-glycosyltransferase 2 family, member B15 (UGT2B15) is up-regulated 3.2- to 7.9-fold depending on the probe set in MaCa 3366/TAM under permanent tamoxifen treatment compared with MaCa 3366 (Table 2A; Fig. 5). It was shown that recombinant UGT2B15 selectively glucuronidates cis-4-OHT, the active metabolite of tamoxifen (73). It is of interest that cis-4-OHT existed at a higher concentration than trans-4-OHT in a MCF-7 breast carcinoma xenograft model of tamoxifen-administered mice who had become resistant to tamoxifen in the course of tamoxifen treatment (74).

In addition, it was shown that acquired resistance to tamoxifen is associated with markedly reduced cellular levels of tamoxifen together with an accumulation of the less antiestrogenic cis-4-OHT in human breast cancer patients who had become resistant to tamoxifen (75). Possibly, tamoxifen administration induces the expression of UGT2B15, which metabolizes and inactivates cis-4-OHT. Interestingly, the phenol sulfotransferase SULT1A that was seen for MX1 and BST2 in MaCa 3366/TAM as compared with the parental xenograft (45), is still completely unknown (79). -4-OHT existed at a higher concentration than trans-4-OHT in a MCF-7 breast carcinoma xenograft model of tamoxifen-administered mice who had become resistant to tamoxifen in the course of tamoxifen treatment (74).

Many IFN-inducible genes were up-regulated in MaCa 3366/TAM as compared with the MaCa 3366 xenograft (Table 2A). Via activation of the JAK signal transducer and activator of transcription (STAT) pathway, IFNs but also other cytokines like IL-6 elicit multiple biological responses, which are mediated by the proteins encoded by IFN-inducible genes. Phosphorylated STAT proteins bind DNA sequences in the promoters of IFN-inducible genes. Phosphorylated STAT1 and STAT3 are post-transcriptionally activated (e.g., by growth factor receptor or receptor tyrosine kinase mediated phosphorylation). Additionally, it is well known that STAT3 and to some extent also STAT1 can be activated in breast carcinomas (85) and that constitutively active mutants of STAT3 are oncogenic (86–89). Activated STAT3 promotes the growth and survival of tumor cells thereby contributing to malignancy (86, 89). STAT1 and STAT3 can be activated by various receptor and non-receptor tyrosine kinases (e.g., the epidermal growth factor receptor and c-Src; refs. 86, 88). Moreover, it was shown in vitro that E2-mediated inhibition of IL-6–induced STAT3 activation was reversed by tamoxifen indicating that under permanent tamoxifen treatment STAT3 could indeed be hyperactivated (90, 91). One may assume that heterodimers of the transcription factors STAT1/STAT2 and STAT11/STAT3, which bind to IFN-stimulated response elements (76) are involved in the up-regulation of MX1 and BST2. Thus, the up-regulation seen for MX1 and BST2 in MaCa 3366/TAM could be explained by an activated STAT pathway in these cells. Activation of STAT3 in MaCa 3366/TAM might be responsible for the up-regulation of the numerous IFN-inducible genes that contain an IFN-stimulated response element in their promoter.
In conclusion, this study provides a detailed analysis of the genes that are up- and down-regulated in an *in vivo* model of tamoxifen resistance. More than 100 transcripts are changed in abundance in MaCa 3366/TAM as compared with MaCa 3366. Our future studies will aim at identifying which of the genes in the tamoxifen-resistant xenograft model contribute most to the phenotype. Among the genes that are differentially expressed in the tamoxifen-resistant tumors, there are several IFN-inducible genes, estradiol-regulated genes, and genes known to be involved in breast carcinogenesis. The genes *NNAT* and *BST2* were strongly increased in MaCa 3366/TAM. The differential expression of four genes (*NNAT*, *BST2*, *IGFBP5*, and *BCAS1*) was confirmed by TaqMan PCR. To examine a larger number of differentially expressed genes, ideally in tissue samples obtained from tamoxifen-treated patients and patients who became resistant in the course of tamoxifen therapy, we are currently designing TaqMan Low Density Arrays with which we will determine whether overexpression of these genes is a hallmark of tamoxifen-resistant breast cancer. Very recently, based on gene expression profiling, a two-gene expression ratio involving the expression ratio of *HOXB13* versus *ILI7BR* was shown to predict clinical outcome in breast cancer patients treated with tamoxifen (92) strongly supporting this concept. An extension of this approach should help to pinpoint which genes might be predictive of tamoxifen-resistant growth.

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References


Distinct gene expression patterns in a tamoxifen-sensitive human mammary carcinoma xenograft and its tamoxifen-resistant subline MaCa 3366/TAM

Michael Becker, Anette Sommer, Jörn R. Krätzschmar, et al.


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