Tamoxifen induces a pluripotency signature in breast cancer cells and human tumors

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ARTICLE INFO

Article history:
Received 6 May 2015
Accepted 20 May 2015
Available online 5 June 2015

Keywords:
Breast cancer
Tamoxifen
Pluripotency
Endocrine resistance
Transcriptomic analysis
Cell lines
Patient data

ABSTRACT

Tamoxifen is the treatment of choice in estrogen receptor alpha breast cancer patients that are eligible for adjuvant endocrine therapy. However, ~50% of ER\textsuperscript{a}-positive tumors exhibit intrinsic or rapidly acquire resistance to endocrine treatment. Unfortunately, prediction of \textit{de novo} resistance to endocrine therapy and/or assessment of relapse likelihood remain difficult. While several mechanisms regulating the acquisition and the maintenance of endocrine resistance have been reported, there are several aspects of this phenomenon that need to be further elucidated. Altered metabolic fate of tamoxifen within patients and emergence of tamoxifen-resistant clones, driven by evolution of the disease phenotype during treatment, appear as the most compelling hypotheses so far. In addition, tamoxifen was reported to induce pluripotency in breast cancer cell lines, in vitro. In this context, we have performed a whole transcriptome analysis of an ER\textsuperscript{a}-positive (T47D) and a triple-negative breast cancer cell line (MDA-MB-231), exposed to tamoxifen for a short time frame (hours), in order to identify how early pluripotency-related effects of tamoxifen may occur. Our ultimate goal was to identify whether the transcriptional actions of tamoxifen related to induction of pluripotency are mediated through specific ER-dependent or independent mechanisms. We report that even as early as 3 hours after the exposure of breast cancer cells to tamoxifen, a subset of ER\textsuperscript{a}-dependent genes associated with developmental processes and pluripotency are induced and this is accompanied by specific phenotypic changes (expression of pluripotency-related proteins). Furthermore...
1. Introduction

The discovery of estrogen receptors (ERs) (Jensen et al., 1967a, 1967b) and the introduction of selective estrogen receptor modulators (SERMs) after the early ‘70s, with tamoxifen as the leading molecule, have revolutionized our concept of targeted and personalized therapy for the treatment of breast cancer patients (Jensen and Jordan, 2003; Jordan, 1999, 2001c, 2006). Tamoxifen has been shown to be highly effective in ERα-positive patients (Early Breast Cancer Trialists’ Collaborative, 1998, 2005), being the gold standard therapy for this group. Indeed, tamoxifen adjuvant therapy for five or more years leads to significant enhancement of disease-free or overall survival; however, about 50% of patients develop secondary resistance to endocrine therapy, experience relapse and subsequently die from the disease (Early Breast Cancer Trialists’ Collaborative, 1998, 2005), although, in ERα-positive patients, tamoxifen may provide a very long period of protection, even after the cessation of therapy (Cuzick et al., 2015). Tamoxifen resistance has been attributed to either a variability of tamoxifen metabolic fate by individual patients, concurring by the identification of low, normal and increased tamoxifen metabolizers (Dehal and Kupfer, 1997; Goetz et al., 2005), or to the evolution of the disease phenotype during treatment, with the emergence of tamoxifen-resistant clones.

Some reports, related to failure of tamoxifen treatment in breast cancer, have identified that long term exposure to the drug may induce the emergence of tamoxifen-resistant ERα-positive cell clones, on which estrogen may induce apoptosis (Ariazi et al., 2011) and the emergence of a stem cell-like population of resistant cells in cell lines (Ao et al., 2011; Iliopoulos et al., 2009) or cells deriving from primary tumors (Kok et al., 2009; Manni et al., 1985). Additionally, it was reported that tamoxifen-resistance may originate from cell populations within the cell culture with stem cell characteristics (Lin et al., 2013; Liu et al., 2013; Martinez-Outschoorn et al., 2011). However, the time-frame needed for tamoxifen to achieve this cell transformation is yet unclear. Interestingly, in addition to ER-related effects, tamoxifen has been reported to exert ER-unrelated actions (Frasor et al., 2006), such as effects on body weight, bone growth, pituitary function (Fitts et al., 2011) and induction of cell cycle arrest and apoptosis (Morad et al., 2013). Therefore, the induction of pluripotency by the drug may also be mediated through similar, ER-unrelated actions.

A large number of studies (~1700 hits on Medline) have dealt with transcriptional changes induced by tamoxifen (see Dimitrakopoulou et al., 2014; Fan et al., 2014; Frasor et al., 2006; Lebedeva et al., 2012; Oyama et al., 2011, for specific examples in breast cancer cells). However, none of the aforementioned studies has described a specific hint on early tamoxifen-induced pluripotency. In view of the above and in order to identify how early pluripotency-related effects of tamoxifen may occur, we have performed a whole transcriptome analysis of an ER-positive and a triple-negative breast cancer cell line, shortly exposed to tamoxifen (3 h), in order to decipher direct actions of the agent on the transcriptome. Our specific aim was to identify whether early/direct tamoxifen transcriptional actions related to induction of pluripotency, were mediated through specific ERα-dependent, or ER-unrelated transcription. Our data show that early exposure to tamoxifen triggers modifications of an ERα-dependent subset of genes, related to developmental processes and pluripotency. Furthermore we report an association between the level of expression of pluripotency-related genes in ERα-positive breast cancer tissues (prior to treatment) and disease relapse after tamoxifen administration, in a retrospective cohort of patients. Finally, in a small group of ERα-positive breast cancer human tumors with disease relapse after surgery and tamoxifen treatment, we identified an increase of ALDH1A1 expression and a modification of its intracellular distribution.

2. Material and methods

2.1. Cell lines

The human breast cancer cell lines T47D and MDA-MB-231 were obtained from DSMZ (Braunschweig, Germany) and were cultured in RPMI 1640, supplemented with 10% fetal bovine serum, at 37 °C, with 5% CO2. All chemicals were purchased from Sigma (St Louis, MO), unless stated otherwise.

2.2. Whole transcriptome assay and analysis

After a 4 h incubation in a medium containing 10% charcoal stripped FBS, cells were incubated in the same conditions,
with or without equimolar concentrations of E2 (estradiol) or Tamoxifen (10⁻⁶ M) for 3 h. We have used this pharmacological concentration of estradiol, in order to compare the transcriptional effects of tamoxifen on an equimolar basis, while the short time-frame used permits to distinguish the direct effect of the drugs on the transcriptome, excluding indirect interactions, mediated by tamoxifen transcribed genes/proteins. Total RNA was isolated using Nucleospin RNA II columns (Macherey-Nagel, Düren, Germany), according to the manufacturer’s instructions. RNA was labeled and hybridized according to the Affymetrix protocol (Affymetrix Gene-Chip Expression Analysis Technical Manual, Santa Clara, CA), using the HG-U133 Plus 2.0 chip, analyzing a total of 54,675 transcripts. Signals were detected by an Affymetrix microarray chip reader. Gene array data have been stored at the NIH Gene Expression Omnibus (GEO) repository (Accession No. GSE60882). RMA normalization of data was performed using GeneSpring GX V11.0 (Agilent, Foster City, CA). One sample per cell line and treatment were used in the analysis. Any transcript modified by at least a factor of 1.5 in either direction (E2/vehicle, Tamoxifen/vehicle) were retained. Next, transcripts modified by E2, whose action was reverted by tamoxifen, were further identified. Finally, we have isolated transcripts, significantly modified by tamoxifen and not influenced by E2, in each cell line.

Data were analyzed, according to the presence of estrogen receptors in each cell line (Kampa et al., 2012; Notas et al., 2012; Pelekanou et al., 2012): in details, T47D and MDA-MB-231 cells express ERα, ERα36, ERα46 and GPR30. In addition, ERα is further expressed by T47D cells. Therefore, transcripts commonly expressed by the two cell lines were attributed to the action of tamoxifen on the commonly expressed estrogen receptors (ERα, ERα36, ERα46 and GPR30, annotated as “other ER-related”). Unique genes identified in T47D cells were attributed to the interaction of the agent with ERα (ERα-related). Finally, transcripts uniquely modified in MDA-MB-231 cells were retained as expressing a non-ER-related effect of tamoxifen (see Figure 1B).

Data in each list were further analyzed (separately for up- and down-regulated transcripts) using the online resource DAVID (Huang da et al., 2009a, b; Huang da et al., 2009c), for the extraction of significantly modified GO terms and pathways. The same tool was further used for functional annotation clustering (FAC) of significantly enriched terms. Gene Ontology (GO) terms analysis and grouping was also performed with the online resource REVIGO (Supek et al., 2011). Furthermore, the signed list of significantly modified transcripts was introduced to the web resource TFaCtS (Essaghir et al., 2010) for the detection of putative transcription factors activation/signature.

2.3. Re-analysis of a publically-available dataset and cases from the Cancer Genome Atlas repository

The publically available dataset GSE9195, containing 77 breast cancer patients treated with tamoxifen (Loi et al., 2008), was re-analyzed. According to provided clinical data, this dataset can be divided in responders (no secondary tumor or metastasis, n = 67) and non-responders (relapsed patients, n = 10). The original data (Affymetrix CEL files) were retrieved from the Gene Expression Omnibus (GEO), preprocessed and normalized using the GeneChip robust multi-array analysis (gcRMA) method (Wu et al., 2004). For the gene differential expression analysis, the Significance Analysis for Microarrays (SAM) (Tusher et al., 2001) and the limma (Smyth, 2005) methods were used, with the False Discovery Rate (FDR) set to 0.1.

Additionally, 160 publically-available RNASeq cases of breast cancer patients, treated with tamoxifen were retrieved from the Cancer Genome Atlas site (http://cancergenome.nih.gov/), through the R-package TCGA-Assembler v 1.0.2 (Zhu et al., 2014). This routine retrieves, normalizes and aligns data to the human genome. The Hg19 release of the human genome was used. In addition, proteome data and clinical annotations of samples are also retrieved, using routines described in https://tcga-data.nci.nih.gov/tcgafiles/ftp_auth/distro/ftpusers/anonymous/tumor/brcacgc.ucsf.edu/illuminahiseq_rnaseqv2/rnaseq/v2/unc.edu_BRCAnlluminaHiSeq_RNASeqV2 mage-tab.1.7.0/DESCRIPTION.txt. Data are reported by their official gene symbol, as normalized counts per gene. These data, after Log2-transformation, were introduced to the GenePattern program (http://genepattern.broadinstitute.org), and subjected to a marker selection of differentially expressed genes in Tamoxifen-responders (n = 130) and non-responders (n = 13, non-available information in 17 cases). 678 genes with an FDR < 0.25 were retained, in order to significant populate our initial lists and subjected in GSEA analysis (Subramanian et al., 2005) in GenePattern, using all canonical pathways, GO terms and transcription factor databases, from the Broad Institute. At this analysis, only hits with both a p-value and an FDR-value < 0.01 were retained.

2.4. Real-Time PCR

Real-Time PCR was performed as described previously (Notas et al., 2012). Primers were selected from qPrimer Depot (http://primerdepot.nci.nih.gov) (Supplementary Table 1) and synthesized by VBC Biotech (Vienna, Austria). Changes were normalized according to 18S RNA expression.

2.5. Mammosphere formation assay

Mammosphere assay was performed as described previously (Shaw et al., 2012). Briefly, T47D cells (70–80% confluent) were detached by trypsinization, centrifuged at 580 × g for 2 min, resuspended in 2 ml of ice-cold PBS and passed several times through a 25 G syringe needle. Separated cells were subsequently measured with a haemocytometer and were seeded in 6-well plates (600 cells/cm²), previously coated with 1 ml/ well of 1.2% poly-(2-hydroxyethyl methacrylate) (pHEMA) solution in absolute ethanol, for 48 h at 40 °C. Cells were incubated in the presence of Tamoxifen (final concentration 10⁻⁶ M) or vehicle, in a humidified atmosphere at 37 °C and 5% CO₂, for 7 days, without disturbing the plates or replenishing the medium. Then, the number of mammospheres (defined as a cellular mass of at least 10 cells) was counted, with a Leica DMIRE2 inverted microscope, at 40× magnification.
Short-term incubation of ERα-positive and negative breast cancer cells with tamoxifen, results in massive transcriptional changes. T47D and MDA-MB-231 breast cancer cells were incubated for 3 h with 10⁻⁶ M tamoxifen and transcriptional changes were identified with the Affymetrix HU133plus2 microarray. In A, the number of up- and down-regulated genes in each closed area of the Venn diagram (shown in B) is depicted. In B, the strategy for the identification of ERα, other ERs and ER-independent action of tamoxifen is presented. Panels C–E present analysis (in terms of p-value, in Logarithmic scale) of significantly modified GO-terms, analyzed separately for each category presented in B, with the online resource DAVID (data are presented in Supplemental Table 3). Finally, panel F presents the 173 genes found in category “Anatomical Structure Morphogenesis” (GO:0009653), identified with the blue arrow in panel C. Pluripotency-related genes are identified in color.
For secondary mammosphere generation, primary mammospheres were collected, washed twice with 1 ml PBS and centrifuged at 115 g for 5 min. Supernatant was discarded and mammospheres were resuspended in 300 μl of 0.5% trypsin/0.2% EDTA and incubated at 37 °C for 2 min. Disaggregated cells were collected, after trypsin neutralization with 1 ml of serum-containing medium. Cells were then collected by centrifugation at 580 g for 5 min, resuspended in 200 ml of ice-cold PBS, counted with a haemocytometer and seeded in pHMA coated 6-well plates (600 cells/cm²), as above, for seven additional days.

2.6 Clinical specimens

Specimens from both the primary and relapsing tumor of five breast cancer patients, who relapsed under tamoxifen monotherapy, were retrieved from the archive of the Department of Pathology, University Hospital of Heraklion. The duration of Tamoxifen administration was ranging from 1.2 to 5 years. Additionally, tissue samples from two patients, treated with Tamoxifen monotherapy, who did not relapse, were retrieved from the same archive, and used as controls. The duration of treatment and the grade of tumors are shown in Table 1. Five (5) sequential 3 mm slides were cut from these tumors, avoiding areas of necrosis. One was stained with Hematoxylin-Eosin and four were stained for Sox2, Nanog, Myc and ALDH1A1. This part of the study was approved by the University Hospital Research and Ethical Committee.

2.7 Immunohistochemistry-immunocytochemistry

Tumor sections were deparaffinized in xylene and rehydrated through a series of graded ethanol concentrations into Tris buffered saline (TBS, pH 7.4). Heat-mediated epitope retrieval was performed by three cycles (5 min) of citrate buffer (0.01 M, pH 6.0) incubation in a microwave oven (500 W). Sections were incubated at room temperature for one hour with 3% BSA in TBS, and then overnight at 4 °C with the primary antibody (antibody list and dilutions used are presented in Supplemental Table 2). For the detection of tissue antibody binding, the UltraVision LP Detection System: HRP Polymer Quanto (Thermo Scientific, Cheshire, UK) was used, with 3′-Diaminobenzidine (DAB) as chromogen. Stained sections were lightly counterstained in Harris hematoxylin for 10 s, hydrated and mounted in Permount (Fisher Scientific, Fair Lawn, NJ). Internal controls for specificity of immunostaining included replacement of primary antibody (antibody list and dilutions used are presented in Supplemental Table 2).

Slides were evaluated for the presence (percentage of positive cells) and the intensity of staining (expressed in a scale of 1–3). The Histology score (H-score, McCarty et al., 1985) was used for the analysis of results by the formula (%1 + %2 + %3), ranging from 0 to 300.

For the detection of immunoreactive proteins in breast cancer cells, treated or not (control) with 10⁻⁶ M tamoxifen for 24 and 48 h, the same antibodies were used, at dilutions

<table>
<thead>
<tr>
<th>Table 1 – Immunohistochemical results and IHC characteristics of patient’s samples.</th>
<th>Membrane</th>
<th>Cytoplasmic</th>
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<tbody>
<tr>
<td>Case #</td>
<td>Grade</td>
<td>Histology</td>
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<tr>
<td>Op 1</td>
<td>3</td>
<td>Invasive ductal</td>
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<td>Relapse</td>
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For the detection of immunoreactive proteins in breast cancer cells, treated or not (control) with 10⁻⁶ M tamoxifen for 24 and 48 h, the same antibodies were used, at dilutions...
described in Supplemental Table 2. Cells were fixed with 2% paraformaldehyde and thereafter processed as detailed above.

2.8. Statistical analysis

Statistical analysis was performed with the use of appropriate parametric and non-parametric tests, as described in the Results section, with the Statistical Package for Social Sciences (SPSS, v 21, IBM, SPSS Inc, Chicago, IL). For multiple comparisons, the Bonferroni adjustment was always applied. The statistical significance was set at 0.05.

3. Results

3.1. Analysis of tamoxifen-modified genes reveals interaction with major cellular functions in ERα positive and triple-negative cells

Tamoxifen induces a massive early transcriptional effect in breast cancer cells (Figure 1A, Supplemental Table 3). In T47D cells, 2620 transcripts were up-regulated and 569 were down-regulated. Of these, 68 and 226 respectively were also modified by E2 at the same direction. In contrast, in MDA-MB-231 cells (lacking ERα) tamoxifen up-regulates 868 transcripts (only one is modified by E2 in the same direction). In this cell line, tamoxifen down-regulates more transcripts than in T47D cells (793 versus 569). Only 69 and 19 transcripts were commonly up- and down-regulated in the two lines.

In T47D cells, analysis of the extracted GO terms revealed that tamoxifen down-regulates processes related to cellular metabolism, translation, cell cycle progression, gene expression and immunoglobulin synthesis. In addition tamoxifen up-regulates genes involved in transcription, regulation of apoptosis, and kinases activity. Interestingly, an up-regulation was noted in genes related to the cellular stress response, nuclear transfer of proteins, transcription factor activity and actin cytoskeleton organization (Supplemental Table 3). In MDA-MB-231 cells, tamoxifen down-regulates genes related to focal adhesion, vesicles, including carriers, actin and caveolin, mitochondrial catabolizing enzymes (especially drug metabolizing CYP isoforms) and actin binding. In contrast, genes related to the hydrolysis of polyubiquitinated proteins, protein catabolism, microtubules, autophagy, mitochondrial proteins, transporters and solute carriers were found up-regulated by tamoxifen (Supplemental Table 3).

In Figure 1C–E the major GO-categories, significantly modified by Tamoxifen are presented based on their classification as ERα-related, other ER-related and non ER-related effects (see Materials and Methods, Figure 1B and Supplemental Table 3). As expected, the p-value of ERα-related categories is by far more significant, as compared to that of the other two categories. In the ERα-related category we could identify functions related to metabolism, transport, transcription and, interestingly, anatomical morphogenesis. In the other ER-related category, tamoxifen positively modifies functions linked to signal transduction, cell cycle and macromolecule modifications. The majority of these functions have been previously reported in a number of excellent transcriptomic analyses of tamoxifen action in breast cancer cells (see Dimitrakopoulou et al., 2014; Fan et al., 2014; Frasor et al., 2006; Lebedeva et al., 2012; Oyama et al., 2011, for specific examples). Finally, ERα-independent tamoxifen actions comprise biosynthetic and metabolic processes of macromolecules (proteins nucleic acids), regulation of apoptosis, and organelle organization.

3.2. Exposure of breast cancer cell lines to tamoxifen leads to early induction of genes and processes related to differentiation-dedifferentiation and cancer stem cell induction

As described above, an ERα-related GO-term significantly increased by tamoxifen (p = 5.3 × 10⁻⁸, FDR = 9.9 × 10⁻⁵) is related to “anatomical structure morphogenesis” (GO:0009653). This category comprises 179 modified genes (presented in Figure 1F), some of which are related to pluriptotency (depicted in color in Figure 1F). Additionally, in both cell lines, a constant finding of early transcriptome analysis is the induction of aldehyde dehydrogenase isoforms (ALDH1A3, 3A2, 6A1, 16A1 in T47D, and ALDH1A2 and 1L1 in MDA-MB-231 cells). This enzyme is considered a marker of sterness (Debeb et al., 2012; Lagadec et al., 2012). Additionally, in our set of data, Sox2, 4, 6 and 9 (also involved in the expression or the maintenance of stem cell phenotype, Chambers and Tomlinson, 2009), were identified in the ERα-related tamoxifen up-regulated genes, together with Oct1 (POU2F1). The latter is another member of the octamer/POU-family of transcription factors, considered an embryonic transcription factor and implicated in dedifferentiation and carcinogenesis (Bellance et al., 2012; reviewed in Kasper, 2008; Wang et al., 2013). The same is also observed for Myc, another transcription factor related to the induction or the maintenance of pluripotency (see Gifford and Meissner, 2012, for a recent review).

3.3. Verification of tamoxifen induced stem characteristics of breast cancer cell lines

We have concentrated to the ERα-positive cell line T47D, as pluripotency-related genes were found in the ERα-related category. In order to confirm whether tamoxifen could reproduce the sequence of events described above, we performed a time-course expression analysis of selected pluripotency-related genes (Myc, Sox2, Oct1, KLF4 and ALDH1A1), in T47D cells, by qRT-PCR, extended up to 24 h. Our data (Figure 2A) show that Sox2 and ALDH1A1 present an early moderate and massive increase respectively, after 3 h of tamoxifen treatment. In addition, a massive increase of Myc transcription (~12-fold) was observed at 6 h, together with a moderate (~3) and a higher (~6) increase of Oct1 and KLF4 respectively. This early increase returned to basal levels at 12 h, followed by a second wave of sustained transcription of Myc, Sox2 and Oct1 extending beyond the 24 h, while a similar, yet lesser, secondary effect was observed with KLF4. The above results show that, indeed, some pluripotency-related genes are early transcribed after tamoxifen treatment. It is interesting that the succession of pluripotency-related gene amplification follows the cascade previously described for the induction of pluripotency (Chambers and Tomlinson, 2009; Gifford and
Meissner, 2012). Our data were further verified by immunocytochemistry: T47D cells were incubated with 10^{-6} M tamoxifen, and stained for Myc, Nanog, Sox2 and ALDH1A1. Due to the fact that protein translation may require more time than transcription and the expression of the proteins should persist significantly longer in order to induce phenotypic changes, we have performed IHC analysis at 24 h and 48 h. A significant increased nuclear immunoreactivity for Myc and Sox2 24 h after tamoxifen incubation was observed (Figure 2B). Sox2 increase was further sustained after 48 h of incubation, while the other markers return to control levels. ALDH1A1 staining was also significantly enhanced by tamoxifen. In addition, a redistribution of ALDH1A1 immunoreactivity from a submembrane staining to the whole cytoplasmic compartment was evidenced (Supplemental Figure 1).

Finally, another evidence of pluripotency induction is the capacity of ERα-positive breast cancer cells to form mammospheres (Figure 2C). When T47D cells were incubated in a low attachment plate and at a low density, they spontaneously formed mammospheres, depending on the presence of
stem-like cells. Tamoxifen, under basal conditions, significantly decreased the formation of mammospheres, after 7 days of treatment (p = 0.001). However, after dispersion of primary mammospheres and incubation of derived cells under the same low attachment conditions for another 7 days, in the absence of any agent, cells that were previously exposed to tamoxifen generated significantly higher numbers of secondary mammospheres (p = 0.002), indicative of the emergence of a higher number of stem-like cells after tamoxifen treatment.

In conclusion, both transcriptome and phenotypic data suggest that tamoxifen treatment of hormone-sensitive ERα-positive breast cancer cells results in an early emergence of a number of stem-like characteristics that are possibly related to a more aggressive cellular phenotype. The arising question, in view of these data is whether such an aggressive phenotype could also be reflected in patients’ samples, not-responding to tamoxifen.

3.4. Patients who relapse under tamoxifen, have a discrete gene-expression signature, suggestive of cell pluripotency

In order to investigate whether tamoxifen treatment-escape could be reflected in the gene signature of patients’ samples, we re-analyzed gene set GSE9195 (Loi et al., 2008), performed with the same gene-array technology (Affymetrix HG-U133 Plus 2.0) and assaying gene expression in primary tumors of patients under tamoxifen monotherapy. Samples from patients with disease relapse were identified and analyzed versus the samples from patients that did not (control). A number of tests (T-test, limma, SAM) were applied and for reasons of stringency, only genes identified by all three tests were retained for further evaluation. Thirty three (33) probe sets, corresponding to 21 genes (BMF, IQGAP3, OTOR, LRRC69, ART3, TMPO-AS1, WDR72, KIF22, CXXC4, ARHGAP11B, UGT8, FAM19A3, EN1, KRT86, NELL2, DHTKD1, PRR4, MAGEC2, Figure 3A) were found to be differentially expressed in tamoxifen-relapsing cases. The products of these 21 genes are involved in different metabolic processes (ART3, UGT8, DHTKD1, Bosio et al., 1996; Friedrich et al., 2006; Xu et al., 2013), signaling (IQGAP3, CXXC4, Hino et al., 2001; Wang et al., 2007), proteins involved in intracellular molecular motors and signaling thereof (KIF22, ARHGAP11B, Miki et al., 2001; Riley et al., 2002; Smith et al., 2010), proteins acting as specific neural chemokines or neurokinines (FAM13A3, NELL2, Tom Tang et al., 2004; Watanabe et al., 1996), encoding for proteins involved in apoptosis (BMF, Puthalakath et al., 2001), or keratin family (KRT86), involved in skin annexes formation (Schweizer et al., 2006). Finally, TMPO-AS1 is a LncRNA. Gene set enrichment analysis for Gene Ontology (GO) term association, using the GOstats package in Bioconductor, revealed two GO biological processes related to “stemness”: “stem cell differentiation” (GO:0048863, p-value: 0.0065) and “stem cell maintenance” (GO:0019827, p-value: 0.0091), involving EZH2, SMARCA4 and SOX4 genes. A reverse analysis of these 21 genes for the identification of involved transcription factors with the web resource T-Facts (www.tfacts.org, Essaghir et al., 2010) revealed five transcription factors (Figure 3B), significantly involved in tamoxifen-resistance of patients. Finally, introducing these transcription factors in the web resource SNOW (http://babelomics.bioinfo.cifp.es/functional.html, Medina et al., 2010), it becomes evident that they participate in a network of protein—protein interactions, involved in major cellular events (Figure 3C).

In another approach, we retrieved public data from the Cancer Genome Atlas (http://cancergenome.nih.gov/), and identified patients who received tamoxifen treatment and relapsed or not. One hundred and sixty (160) cases were selected, together with their clinical data. Thirteen cases did not respond to therapy and relapsed, 130 responded to therapy, while no data were available for the rest of patients. Gene expression and clinical data are presented in Supplemental Table 4. There was no difference in the average follow-up of responders versus non responders (median 209 days, mean 204.3 days 95%CI 186.5 to 222.1 for responders versus median 235, mean 226.3 days 95%CI 136.0 to 316.6 for non-responders). Six hundred seventy four (674) genes were differentially expressed (Supplemental Table 4), between tamoxifen-responders and non-responders. A GSEA analysis of these differentially expressed genes yielded a number of GO-enriched pathways (Supplemental Table 4). SOX2-related pathway (with 7 significant up-regulated genes, HNRNPL, HNRNPA2B1, FUS, TRA2A, MED12, NBF646, FANCJ) is the pathway which is the most significantly enhanced in relapsing patients (Supplemental Table 4, Figure 4A). Interestingly, a re-analysis of the GSE9195 set, using a metagene based on the mean value of the six genes that were also mapped in the Affymetrix HU133plus2 array (FANCC, FUS, MED12, PTBP1, TRA2A, NBF646) revealed a significant difference (p = 0.049) among Tamoxifen responders and non-responders. In addition, GSEA revealed that EZF and POU3F2 (Figure 4B) transcription factor signatures are also significantly modified in tamoxifen non-responders. These data suggest that tamoxifen non-responders express, even before therapy, a set of molecular signatures which might be related to a more de-differentiated (stem like) phenotype.

3.5. Increased cytoplasmic localization of ALDH1A1 in breast cancer tumor specimens relapsed after tamoxifen therapy

In a final approach, we examined whether a phenotypic modification is also evidenced in patients’ samples, who relapsed after tamoxifen monotherapy. As a proof of concept, we retrieved from the archives of the Departments of Pathology a small set of five patients who underwent breast surgery for ER positive breast cancer, were treated by tamoxifen post-therapy, a set of molecular signatures which might be related to a more de-differentiated (stem like) phenotype.
proteins, directly related to cell stemness. Two more patients who responded well to tamoxifen treatment, without relapsing were also studied as a control. One relapsing and a non-relapsing case are presented in Figure 5A.

An interesting finding, reported here for the first time, is the change in the pattern of ALDH1A1 immunoreactivity in relapsing tumors (Figure 5B): in primary tumors, ALDH1A1 immunoreactivity was primarily detected at the periphery, under the plasma membrane. The intensity was significantly higher in primary tumors that later relapsed, as compared to non-relapsed tumors (H-score 138 ± 32 and 60 ± 10 respectively, p = 0.008). In relapsing tumors, this submembrane staining was significantly decreased after tamoxifen therapy (H-score 14 ± 7, p = 0.03), while cytoplasmic staining was significantly intensified (H-score 182 ± 27, as compared to 32 ± 20 in primary tumors, p = 0.001). ALDH1A1 did not stain non-cancerous tissue in all cases we investigated.

In contrast, neither Sox2, nor Nanog or Myc immunoreactivity was conclusive in our samples: in two out of the five cases, nuclear Sox2 protein was enhanced after tamoxifen treatment; in contrast, in 3 cases, Sox2 nuclear protein staining was decreased, while no correlation with Nanog or Myc was identified. Nanog, on the other hand was usually negative in the majority of samples (4/5) after tamoxifen treatment, while c-Myc immunoreactivity was light and was also present in the cytosol, in relapsed tumors. In non-relapsing tumors, a great heterogeneity of staining was also observed. In conclusion, these three markers could not discriminate between primary and relapsing tumor samples, in our small series of patients.

Figure 3 — Primary tumors of tamoxifen-resistant breast cancer patients present early elements of pluripotency. A. Heat-map of the 21 genes, being differentially expressed between tamoxifen-responders and tamoxifen-relapsing patients (0 and 1 in the tumor identification). Presented data were derived from the re-analysis of the set GSE9195, as described in Material and Methods and Results sections. B. Identified transcription factors differentially modified in tamoxifen-sensitive and relapsing tumor specimens of GSE9195 data-set, by the use of the T-Facts web resource (www.tfacts.org). See Material and Methods for further details. C. Network of protein–protein interactions of the identified transcription factors presented in B, through the web resource SNOW (http:// babelomics.bioinfo.cipf.es/functional.html). See Results for further details.
Figure 4 — Tamoxifen-resistant and sensitive breast cancer primary tumors express elements of pluripotency. One hundred sixty three tamoxifen-treated breast cancer cases were retrieved from the Cancer Genome Atlas collection (www.cancergenome.nih.gov). In A, gene set enrichment analysis of the “Sox-targets” GO category is presented, while in B, GSEA of genes related to POU3F2 transcription factor are depicted. See Material and Methods and Results sections for additional details.

Figure 5 — Immunohistochemistry of tumor specimens in tamoxifen-responders and relapsing tumors present elements of pluripotency. A. Immunohistochemical determination of Myc, Nanog, Sox2 and ALDH1A1 in a case of tamoxifen relapsing (#1 in Table 1) tumor. In the first row the initial tumor is presented, while in the middle row the relapsing tumor is shown. The lower row of the Figure presents the immunohistochemical analysis of a tamoxifen-sensitive (non-relapsed) tumor specimen (#1 in Table 1). ×400, bar = 50 μm. B. ALDH1A1 presents a specific intracellular redistribution in the cytosol of tamoxifen resistant patients’ tumors. Membrane localization of ALDH1A1 immunopositivity (evidenced mainly in left panel, before Tamoxifen treatment) is marked by black arrows. A typical case is presented (#2 in Table 1). Bar = 50 μm.
4. Discussion

Breast cancer is a primary cause of death in females, worldwide. Most breast tumors are ERα positive (~70%) and therefore therapies targeting ERα are applied, either with selective estrogen receptor modulators (SERMs), such as tamoxifen (Jordan, 2000, 2001a, b, 2006), or through deprivation of locally produced estrogen. However, about half of ERα-positive tumors exhibit intrinsic or rapidly acquire resistance to endocrine treatment. It has been difficult to predict de novo resistance to endocrine therapy and/or assess the likelihood of early relapse. Regulation of acquisition and maintenance of endocrine resistance have been reported (Palmieri et al., 2014; Patani and Martin, 2014), but several aspects of this phenomena require further elucidation. Indeed, tamoxifen was reported to have pleiotropic effects; in addition to ERα inhibition, a direct effect on other estrogen receptors, as well as ER-unrelated drug interactions was also described. Indeed, ERα is reported to have pleiotropic effects; in addition to ERα inhibition, a direct effect on other estrogen receptors, as well as ER-unrelated drug interactions was also described. Indeed, in ERα-positive cells, the majority of tamoxifen significantly modified transcripts were different from those modified by estradiol, suggesting a specific E2-antagonistic effect of the agent. However, a minority of genes (68 up-regulated and 226 down-regulated) were modified by estradiol and tamoxifen to the same direction, suggesting either a parallel action of both agents such as a partial ERα agonist effect of tamoxifen as reported previously (Jordan, 1999; Jordan and Brodie, 2007; Komm and Mirkin, 2014), or a similar effect through completely different mechanisms. Such an effect is probably not related to the action of tamoxifen on other estrogen receptors (for example ERβ, GPR30), since only one transcript is also modified by estradiol, to the same direction in MDA-MB-231 cells, bearing also these latter receptors (Notas et al., 2012).

It should be noted that the high concentration of estradiol used in our study might overestimate the inhibitory effects of tamoxifen, but still these different effects were seen with equimolar concentrations of the two substances. Therefore, we acknowledge that the effect of tamoxifen (and subsequently estradiol) might be exerted on a number of functions, not directly related to classical estrogen receptors, but possibly related to other, extranuclear or not, actions of estrogen and SERMs. These possibilities are not mutually exclusive, as ligand activation of a few ERα cofactors/co-regulators, at high concentrations may favor the interaction of these proteins with the receptor. However, the data still show that tamoxifen, through an action on estrogen receptors, modulate a large number of basic cellular processes, such as cell cycle, proliferation and apoptosis, basic signaling mechanisms, metabolic processes, transport, reported previously in numerous publications and, as we stress here, differentiation processes, involving maintenance or acquisition of pluripotency.

Early after tamoxifen introduction in breast cancer therapy, an effect on cell pluripotency was observed; although a significant reduction of colony formation of breast cancer cell lines in soft agar under tamoxifen treatment was reported (Osborne et al., 1985), a stimulatory effect of the agent was observed in some cases of primary tumor single cell clonogenicity assays (Manni et al., 1985), attributed to different mechanisms of action of E2 and tamoxifen (Nomura et al., 1990). More recently, the introduction of low adhesion substrates and the development of mammosphere formation assays permitted a better detection of stem cell populations within the mass of primary breast tumors (Kok et al., 2009). These cells however, were reported to exist in a quiescent state, not-related to cell proliferation, EMT or metastasis. Mammospheres may be expanded by estrogen treatment (Fillmore et al., 2010) and although short-term tamoxifen treatment did not modify the proliferative capacity of breast cancer cells (MCF7), data presented here, show that a seven-days incubation of T47D breast cancer cells with tamoxifen resulted in decreased primary (during tamoxifen treatment) but increased secondary (after tamoxifen was removed from the culture medium) mammosphere-generating potential. Contrariwise, although pluripotent MCF7-derived cells have been reported to retain their sphere forming capacity after 7 days tamoxifen treatment, an attenuated expansion potential after even longer exposure was shown (Ao et al., 2011). This discrepancy may be due to the different cell lines utilized (MCF7 in previous study versus T47D in the present investigation), and the different p53 status of cells (non-mutated versus mutated respectively), an element clearly involved in tamoxifen-induced pluripotency (Martinez-Outschoorn et al., 2011; Mosoyan et al., 2013).

Tamoxifen has also been used as a trigger to induce breast cancer stem cell formation. In ER-Src-transformed breast epithelial cells, it was reported that tamoxifen induces IL6 production and secretion, via NFκB signaling, relaying inflammation to the transformation of breast epithelial cells (Iliopoulos et al., 2009), through a specific miR-200b-Suz12-cadherin pathway (Iliopoulos et al., 2010). The authors propose that IL6 is the necessary and sufficient switch for the transformation of non-stem to stem cells in vitro and in the tumor mass of breast cancer xenografts (Iliopoulos et al., 2011). In addition, a number of recent reports suggest that acquired tamoxifen resistance promoted the emergence of cell clones with stem cell characteristics (Lin et al., 2013; Liu et al., 2013; Martinez-Outschoorn et al., 2011). Treatment of MCF7 cells with tamoxifen in the presence of E2, leads to an increase of a mammosphere producing cell population in vitro and induction of mouse mammary tumors in vivo (Raffo et al., 2013), through stroma–epithelial interaction, with a parallel shift of mitochondrial metabolism of epithelial cells (Martinez-Outschoorn et al., 2011). Under these settings, stem-related genes (Nanog, Sox2, Oct4) were found to be unmodified by tamoxifen (Stone et al., 2012). However, our data showed a double direct effect of tamoxifen on breast cancer cells: (1) Three to six hours after exposure to tamoxifen, pluripotency-related genes are massively expressed at the protein level (Myc, Sox2, KLF4, Oct1, ALDH1A1). A recent study (Sridharan et al., 2009) also suggested that c-Myc acts early during reprogramming, repressing the expression of differentiation genes. c-Myc might therefore function before other stem cell-specific factors (Oct4/Sox2/Klf4, reviewed in Chambers and Tomlinson, 2009; Huangfu et al., 2008). Very recent data (discussed in Dang, 2014; Sabo et al., 2014; Walz et al., 2014) indicate that the magnitude of response to Myc paralleled the occupancy of each Myc-related promoter, leading to a positive or negative regulation of transcription. ALDH1A1 is one of the genes directly regulated by Myc (see
below (Rogulski et al., 2005). (2) This early effect of tamoxifen is followed by a second, sustained wave of transcriptional increase, exceeding 24 h, verified by our immunocytochemical data, showing an enhanced expression of Myc, Nanog and Sox2 proteins in 24 and 48 h tamoxifen-treated T47D cells. Interestingly, Sox and Oct genes are positive regulators of their own transcription and the transcription of other stem related genes, acting also as rheostats of stem cell proliferation (at low concentrations) or differentiation (Rizzino, 2009), while they subsequently activate Nanog transcription (Boyer et al., 2005), a result, explaining our immunocytochemical data. Finally, it is to note that, although Nanog does not present a significant increase, its intracellular (nuclear) levels tend also to increase at 24 h. This non-significant change might be attributed to high intracellular Nanog levels in our cell preparation (see Figure 2B). Our findings show for the first time that several molecular signatures, related to the induction of pluripotency in breast cancer cells, can be induced extremely early after exposure to tamoxifen. Thus, early identification of such phenomena could be of clinical importance for patients that will present with early or late endocrine resistance. In this respect they subscribe to a number of studies which have identified gene signatures related to the outcome of breast cancer patients (Fraros et al., 2006; Karlsson et al., 2013; Loi et al., 2008; Ma et al., 2004; Sieuwerts et al., 2014; Sikora et al., 2014).

Furthermore in an attempt to identify a (molecular and/or cellular) signature, predictive of tamoxifen resistance in breast cancer patients’ samples, we have analyzed two public set of data: (1) Re-analysis of 77 cases of set GSE9195 (Loi et al., 2008) and (2) 160 cases of tamoxifen-treated patients, from the Cancer Genome Atlas repository, in which clinical and RNA-Seq data were available. Previous investigations have advanced a 47 gene signature related to tamoxifen resistance: the top-ranked selected genes were ESR1, MET, FOS, SNCG, IGFBP4, and BCL2; additionally, a reduced expression of ESR1/ERα, IGFBP4, SNCG, BCL2, and FOS in the relapsing group was observed (Vendrell et al., 2008). Contrariwise, in a recent study, a three-gene signature (BCL2, BCAR3 and NAT1) was proposed as a molecular marker of adjuvant tamoxifen beneficial therapy (Sieuwerts et al., 2014). In our study, analysis of the first data set (Loi et al., 2008) revealed 21 genes, differentially expressed in relapsing over non-relapsing patients; some of these genes and related to stemness. Interestingly, further analysis of this 21-gene set identified ESR1 (Erα), Myc and E2F among the significantly modified transcription factors. In the second set (The Cancer Gene Atlas), E2F is retained as a significantly modified transcription factor, between tamoxifen-sensitive and relapsing patients, together with Oct3 (POU3F, Oct7), a factor also directly related to pluripotency. Therefore our data closely related to previously published ones (Vendrell et al., 2008), as we also identify ESR1 and E2F (Erα and Fos-modified factor in breast cancer cells) (Petz et al., 2002) and Myc, as an early tamoxifen-modified gene in our cells, suggest that, even before the initiation of tamoxifen treatment, specific molecular signatures may identify patients with increased risk for future tamoxifen resistance and tumor relapse. This element should be further exploited and analyzed in larger series of tamoxifen resistant patients. However, an element not taken into account in all these studies (including ours) is a possible specific effect of tamoxifen metabolites in mediating some of the resistance-related phenomena, including transcription and the induction of stemness (see Barnadas et al., 2011; Brauch and Jordan, 2009; de Souza and Olopade, 2011; Huber-Wechselberger et al., 2012, for reviews). Analysis of tamoxifen metabolites in patients that developed resistance to tamoxifen and further study of their effect on the patients’ transcriptome could provide further insights in endocrine therapy resistance.

Our final goal in this study was to examine whether tumors of tamoxifen monotherapy-relapsing patients express elements of pluripotency, before the initiation of treatment, which are modified after relapse. As a proof of concept, we assayed Myc, Sox2, Nanog and ALDH1A1, as markers of the presence of pluripotent cells in the tumor mass. Interestingly, we identified a higher expression of total ALDH1A1 immunoreactivity in primary tumors of relapsing patients, which is further accentuated in the relapsing tumor. In addition, ALDH1A1 was not found as a prominent gene in the two data-sets analyzed, a fact that can be attributed to increased RNA stability or decreased degradation of this molecule, mechanisms widely present in biological systems (Opitz et al., 2010). Contrariwise, we could not identify a significant pattern of expression of Myc, Sox2 or Nanog between relapsing and non-relapsing samples, nor any significant change in primary and relapsing tumors. ALDH1 was found in different series of breast tumors (Marcato et al., 2011), and, as it metabolizes different substrates, it is considered a factor of drug resistance of tumor cells (Abdullah and Chow, 2013). It has been proposed that the ALDH1A1 phenotype is an independent predictor of early tumor relapse characteristic (incidence of early local recurrence and distant metastasis) of invasive ductal carcinoma (Zhong et al., 2013). In addition, in a recent meta-analysis (Liu et al., 2014) it was reported that higher expression of ALDH1A1 was associated with larger tumor size, higher histological grade, greater possibility of lymph node metastasis, higher level expression of epidermal growth factor receptor 2 (HER2) levels and lower expression of estrogen receptor/progesterone receptor levels. Finally, the prognosis of breast cancer patients with ALDH1A1-positive tumors was poorer compared to ALDH1A1-negative patients. Therefore, our data showing an increased ALDH1A1 expression in relapsing patients, if verified, suggest that this molecule could be an early personalized indicator of future tamoxifen resistance. However, our data in breast cancer patients should be taken with caution, and further studies, with a significant higher number of samples is needed, before any valid conclusion may be drawn.

A final observation, reported here for the first time, is the change in the pattern of ALDH1A1 distribution in cells and tumor specimens after exposure to tamoxifen: ALDH is preferentially concentrated under the cell membrane in the primary tumors of relapsing patients, while, in relapsed tumors, the enzyme immunoreactivity is homogenously distributed in the cytoplasm. ALDH is a metabolic enzyme, responsible for the reduction of aldehydes (Allahverdiyev et al., 2012). In this respect, it may metabolize a number of therapeutic agents (Abdullah and Chow, 2013) and therefore regulate drug resistance in cancer (Marcato et al., 2011). Whether this alteration of ALDH1A1 distribution reflects a
subsequent change of the metabolic profile of breast cancer cells and tumors, or it is an element of tamoxifen treatment per se, needs further clarification.

In conclusion, our data show for the first time that tamoxifen treatment of breast cancer cells initiates an early induction of pluripotency-related genes. Furthermore, ERα-positive tumors that relapsed under tamoxifen, expressed at diagnosis higher levels of a number of several pluripotency related genes that could potentially be augmented by tamoxifen, thus leading to the disease relapse. Our results demonstrate the need for further prospective studies in this field that could develop molecular tools for the early identification of patients that are potential tamoxifen non-responders. These patients could then be offered a more efficient/personalized breast cancer therapy.

Acknowledgments

This work was partially supported by the European Union-FP7 Marie Curie Actions-Career Reintegration Grants FCIG-GA-2011-303723 (to VP), the University of Crete Research Committee funds (No. 359 & 2722, to EC) and the European Union Programs Regional Potential/Translational Potential Grant 285948 (to GN and EC).

Appendix A.

Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molonc.2015.05.008.

REFERENCES


tamoxifen-resistance indicate a potential role of cells with stem-like properties. Breast Cancer Res. 15, R119.


