Impact of Estrogen Deprivation on Gene Expression Profiles of Normal Postmenopausal Breast Tissue In vivo

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Abstract

Aromatase inhibitors play a key role in the clinical management of hormone receptor–positive breast cancer and have potential utility as chemopreventive agents. Further understanding of the molecular effects of estrogen and its deprivation in normal breast tissue may allow the development of biomarkers of risk of breast cancer and help to predict the value of chemoprevention with aromatase inhibitors. Core biopsies of normal breast tissue were taken before and after letrozole treatment from postmenopausal women in the LITMaS pilot prevention study. RNA was extracted from these samples and used for cDNA microarray analysis. Gene expression changes induced by letrozole treatment were much less extensive than observed in estrogen receptor–positive malignant tissue; however, overall, they correlated to a highly significant degree ($r = 0.511; P < 10^{-20}$). As well as some classically estrogen-associated genes, many genes associated with extracellular matrix remodeling were affected by estrogen deprivation in the normal breast in vivo. These data indicate for the first time that gene expression of normal breast tissue remains dependent on endogenous estrogens after the menopause. The modest degree of gene change suggests that intermediate markers of chemoprevention may be difficult to identify. (Cancer Epidemiol Biomarkers Prev 2008;17(4):855–63)

Introduction

Aromatase inhibitors (AI) act to prevent the conversion of androgens to estrogens by the aromatase enzyme and have been shown to have superior efficacy to the selective estrogen receptor (ER) modulator, tamoxifen, for the treatment of hormone receptor–positive breast cancer in the adjuvant setting (1–4). AIs are now considered to be the standard of care for the hormonal treatment of early breast cancer in postmenopausal women in both the neoadjuvant and adjuvant context as well as being established therapy for metastatic disease.

Adjuvant tamoxifen treatment of patients with primary breast cancer has been found to cause an ~50% reduction in the incidence of contralateral breast cancer in hormone receptor–positive patients (5). Tamoxifen was also found to reduce breast cancer incidence in chemoprevention trials. In an overview of these, there was a 46% relative reduction (95% confidence interval, 36–58; $P < 0.0001$) in the incidence of ER-positive breast cancer in those taking tamoxifen compared with placebo (6). However, tamoxifen has not been widely used as a chemopreventive agent mainly due to toxicity issues, including increased incidence of endometrial cancer and thromboembolism (7). Data from trials of AIs versus tamoxifen as adjuvant treatment for early breast cancer showed that the incidence of contralateral breast cancer was significantly reduced by AIs compared with tamoxifen (1–3). The improved efficacy and modified toxicity profile of AIs over tamoxifen for the treatment of established disease and their shown effect on incidence of contralateral breast cancer has led to the initiation of large-scale breast cancer prevention trials, for example, IBIS-II, of AIs in a chemopreventive role.

The effect of AIs on the normal breast of postmenopausal women is largely uncharacterized. It is known that exogenous estrogens lead to significant changes in breast density and breast cancer risk, particularly when combined with progestins (8–11); however, this may not extend to the very low levels of endogenous estrogens in postmenopausal women. If the tissue remains sensitive to endogenous estrogens, the changes exerted by an AI may be indicative both of the estrogen-associated risk of breast cancer development for the individual and the potential for this to be reduced by an AI.

In our previously published pilot prevention study (LITMaS: Letrozole Intermediate Tissue Marker Study; ref. 12), 32 postmenopausal women without active breast disease had core biopsies taken from the normal breast before and after 3 months of letrozole treatment. Expression of the proliferation marker Ki67, which is markedly suppressed by AIs in ER-positive breast carcinomas, was assessed to determine whether such suppression occurred in normal breast tissues. Pretreatment Ki67 levels were low and, although levels were reduced posttreatment, the change was not statistically significant.

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In this study, tissue from the original snap-frozen LITMaS samples was used to study the effects of estrogen deprivation on the gene expression profile of normal postmenopausal breast tissue. RNA extracted from these samples was used for cDNA microarray analysis. Data obtained were interrogated to identify significant gene expression changes in samples taken pre- and post-letrozole treatment. Results obtained were compared with gene expression profiling data from a parallel study within our institution of paired biopsies of ER-positive breast tumors taken pre- and post-AI treatment (13). Although DNA microarray analysis, enabling the RNA expression of many thousands of genes to be assessed simultaneously, has been used extensively in the field of breast cancer research, notably for the identification of distinct molecular subtypes of tumors (14) and in attempts to derive prognostic (15, 16) and predictive signatures (17), to our knowledge this is the first study using this technology for assessing the responsiveness of postmenopausal normal breast tissue.

Materials and Methods

Patients. Postmenopausal women were recruited from benign breast disease clinics, ductal carcinoma in situ or lobular carcinoma in situ follow-up clinics at the Royal Marsden Hospital, or the regional breast screening unit. They were approached via a stepwise recruitment system with mail shots, detailed follow-up literature on request, and telephone contact. They were ineligible if (a) there had been previous invasive malignancy to either breast; (b) radiotherapy, as part of ductal carcinoma in situ management, had been completed <4 wk previously; (c) taking any prior or concomitant hormonal therapy (e.g., tamoxifen); (d) taking hormone replacement therapy <3 mo before entry into the study; (e) >80-y-old and Eastern Cooperative Oncology Group performance status <2; (f) having any other medical complaint or taking medication that may have interfered with the use of letrozole; or (g) unable to comply with the study protocol. Approval for the study was obtained from the Royal Marsden Research Ethics Committee, and written consent was obtained from all subjects after they had reviewed the patient information sheet. Participants received 2.5 mg of letrozole (Femara, Novartis AG) daily for 12 wk with tissue samples taken at the beginning and end of treatment.

Tissue Collection. Core-cut biopsies were taken from the study breast (i.e., the contralateral side to that affected by benign disease, ductal carcinoma in situ or lobular carcinoma in situ) in regions assessed by ultrasound imaging as likely to provide tissue containing normal glandular epithelium. A 14-gauge BIP High Speed Core Cut needle was used to take up to seven cores under local anesthetic with one immediately snap frozen in liquid nitrogen and stored at −70°C. The rest were fixed in 10% neutral buffered formalin and then embedded in a single paraffin wax block. Sections of 3-μm thickness were taken from these blocks and reviewed by a consultant histopathologist to exclude malignant disease.

RNA Extraction. Due to the small volumes and lipid-rich nature of the frozen cores of normal breast tissue, standard commercial kits for RNA extraction were not suitable. Immediately before RNA extraction ~5 mg of the tissue were pulverized in 600 μL lysis buffer (RNeasy Lipid Tissue Mini Kit, Qiagen Instruments AG) in an Eppendorf on dry ice using an Eppendorf pestle (Sigma-Aldrich). The sample was further homogenized by being centrifuged through a column-based shredder (Qiagen) before subsequent RNA extraction using a combination of RNeasy Lipid Tissue Mini and RNeasy Micro kits (Qiagen) according to the manufacturer’s instructions. The quality and quantity of the total RNA were measured using the Bioanalyzer 2100 Pico Kit (Agilent Technologies).

RNA Amplification and Microarray Hybridizations. Total RNA was subjected to two rounds of amplification based on linear T7 amplification methods (18) using Amino Allyl MessageAmp II aRNA Amplification Kit (Ambion) as per the manufacturer’s protocol. Reference RNA was generated from a pool of RNAs extracted from eight independent normal breast tissue samples and similarly subjected to two rounds of amplification. Amplified sample and reference aRNAs, 2.5 μg, were individually labeled with either Cy3 or Cy5 dyes (Amersham, GE Healthcare) and hybridized to in-house Breakthrough cDNA microarrays (13). A single dye swap experiment was done for each study sample. The human microarrays were spotted with 19,958 IMAGE cDNA clones acquired from the Cancer Research UK Microarray Facility (Institute of Cancer Research) and the Medical Research Council Geneservice.

Microarray Preprocessing. Spots in the hybridization image with extremely low intensities or hybridization artifacts were flagged in Genepix Pro 5.1 (Molecular Devices) and removed from further analysis. For the remaining spots, raw intensity values were logged (base 2) then converted to ratios relative to expression in the reference channel (M values) and average log intensity (A values) was calculated for each spot. Loess regression was used to correct for spatial- and intensity-dependent bias across the slide. Replicate hybridizations were averaged and the data were then filtered to remove genes with intensity values below the median in 20 or more of the 28 hybridizations. To remove genes that showed little signal variation across hybridizations, genes with an interquartile range of <0.25 across hybridizations were discarded. The data were then “collapsed” by IMAGE ID, and the mean of multiple probes for the same gene was calculated, leaving data from 5,750 genes for use in further analysis.

Data Analysis. We identified genes that were differentially expressed between pretreatment and posttreatment samples using paired significance analysis of microarrays (SAM) (19). Gene set enrichment analysis (20) was done within the SAM software using the Segal cancer module gene sets (21). Heatmaps were generated using Gene Cluster version 2.1 (22) and visualized using Java Treeview 1.0.12 software.

Results

Derivation of Data Set. Of the 27 patients who completed the LITMaS study with a demonstrable significant drop in circulating estradiol levels
A. Genes up-regulated by letrozole treatment

- **COL3A1**: Collagen, type III, α1
- **FOS**: Fos
- **RH11**: RNase/angiojenin inhibitor 1
- **ODF2L**: Outer dense fiber of sperm tails 2-like
- **COL1A1**: Collagen, type I, α1
- **CILP**: Cartilage intermediate layer protein
- **CTGF**: Connective tissue growth factor
- **GADD45A**: Growth arrest and DNA-damage-inducible, α
- **COL5A2**: Collagen, type V, α2
- **IFT122**: Intraflagellar transport 122 homologue

Gene ID Description UniGene number q (%) Fold change

<table>
<thead>
<tr>
<th>Gene ID</th>
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<th>q (%)</th>
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<td>Hs.445827</td>
<td>0.00</td>
<td>1.43</td>
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</tbody>
</table>

B. Genes down-regulated by letrozole treatment

- **OLFM2B**: Olfactomedin-like 2B
- **APOLD1**: Apolipoprotein L domain containing 1
- **COQ4**: Coenzyme Q4 homologue
- **COL�A2**: Collagen, type I, α2
- **DNAJB1**: DnaJ Hsp40 homologue, B1
- **PDGFR**: Platelet-derived growth factor receptor-like
- **OLFML2B**: Olfactomedin-like 2B
- **COL6A3**: Collagen, type VI
- **COL5A2**: Collagen, type V
- **COL3A1**: Collagen, type III
- **GADD45A**: Growth arrest and DNA-damage-inducible, α
- **COL1A1**: Collagen, type I, α1
- **COL5A2**: Collagen, type V, α2
- **IFT122**: Intraflagellar transport 122 homologue
- **COL6A3**: Collagen, type VI
- **COL5A2**: Collagen, type V
- **COL3A1**: Collagen, type III

**Table 1. Paired SAM analysis of genes changing in response to letrozole treatment**

<table>
<thead>
<tr>
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<th>q (%)</th>
<th>Fold change</th>
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**Post-letrozole** (12), sufficient tissue was available from 22 paired samples for RNA extraction. RNA of acceptable quality and quantity was obtained for 17 paired samples. One sample subsequently failed to amplify successfully, with an additional three samples failing during hybridization, resulting in a total of 13 pairs of samples available for analysis.

**Paired SAM Analysis of Genes Changing in Response to Letrozole.** Statistical analysis was done to identify genes showing significant differences in expression between pretreatment and posttreatment samples. After preprocessing (normalization and filtering) of raw gene expression data, 5,906 genes were used in further statistical analysis. Table 1 shows the top up- and down-regulated genes in a paired SAM statistical analysis between pretreatment and posttreatment samples. The results are ranked according to q value and fold change. A complete list is shown in Supplementary Table S1. The most consistently down-regulated genes had much higher q values (~40%) compared with up-regulated genes (q values ~0-5%), indicative of a greater variability in their responses. Genes identified as being up-regulated upon AI treatment included COL3A1, COL1A1, COL5A2, and COL6A3 and those identified as being down-regulated included STC2, ESR1, AGR2, and FOXA1.

**Supervised Hierarchical Clustering.** The top 23 up-regulated genes and the top 22 down-regulated genes (derived from the SAM list with the lowest q values and greatest fold changes) were used for supervised hierarchical clustering. Consistent with the use of genes previously selected to discriminate treated from nontreated samples, one sample cluster consists of predominantly pretreatment samples, with the other cluster predominantly posttreatment samples, with the other cluster consisting of a predominantly postletrozole sample.
predominantly posttreatment samples. However, separation of pretreatment and posttreatment samples is not complete. Visual representation of the data as a heatmap (Fig. 1) shows two key sets of functionally related genes that distinguish pretreatment and posttreatment samples. The first of these gene clusters relates to extracellular matrix function (including COL5A2, COL6A3, COL1A2, and COL3A1) with an additional cluster relating to ER expression and classically estrogen-dependent genes (including AGR2, ESR1, AZGP1, FOXA1, STC2, KRT17, and KRT19).

**Pathway Analysis of Differentially Expressed Genes.** In addition to studying transcriptional changes at an individual gene level, consideration of gene expression changes across coregulated biological pathways and gene sets may enable the identification of pathways, which as a whole are significantly differentially expressed pretreatment and posttreatment despite only modest, but potentially biologically meaningful, individual gene changes. Gene set enrichment analysis was done to identify any gene sets differentially expressed between the pretreatment and posttreatment samples. A number of gene sets were found to be significantly enriched for differential expression between pretreatment and posttreatment samples.
of gene sets were identified as being either significantly up- or down-regulated upon AI treatment using a false discovery rate cutoff of 0.3. Table 2 lists all named gene sets from within the gene set enrichment analysis results, with the complete module list available in Supplementary Table S2. The gene sets shown to be up-regulated by AI treatment are all gene sets or pathways in which collagens and other extracellular matrix molecules, for example, CTGF and LUM, are dominant members. Despite no down-regulated genes showing significance individually, when gene sets and pathways are considered collectively, statistically significant down-regulated gene sets are revealed. Among the gene sets identified, several share the common theme of cancer expression clusters, notably breast cancer, with the highly relevant steroid hormone metabolism pathway, including multiple hydroxysteroid dehydrogenase genes, also being highlighted.

<table>
<thead>
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<th>Table 2. Gene set enrichment analysis</th>
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<tr>
<td>Gene set name</td>
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<td>A. Gene sets up-regulated upon AI treatment</td>
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<td>Ovary genes</td>
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<td>Placenta genes</td>
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<td>ECM and collagens</td>
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<td>Cell line expressed genes</td>
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<td>Adhesion molecules</td>
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<td>Bone remodeling</td>
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<td>B. Gene sets down-regulated upon AI treatment</td>
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<td>Blood cells and cancer expression clusters</td>
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<td>Vesicular transport/synapse genes</td>
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<td>ROS metabolism</td>
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Abbreviations: ECM, extracellular matrix; FDR, false discovery rate; ROS, reactive oxygen species.

Figure 2. Changes in gene expression upon AI treatment for individual patient samples for COL3A1 (A), COL5A2 (B), ESR1 (C), and STC2 (D).
Changes in Gene Expression upon AI Treatment for Individual Patients. Changes in gene expression upon AI treatment for individual patient samples are illustrated in Fig. 2 for two significantly up-regulated genes from the extracellular matrix cluster (COL3A1 and COL5A2) and two down-regulated genes from the estrogen-related cluster (ESR1 and STC2). For the illustrated significantly up-regulated genes, it can be seen that increases in gene expression upon AI treatment are observed consistently across the majority of patient samples, but that the magnitude of the changes varies between individual patients. For the down-regulated genes, decreases in gene expression are again consistently seen for most patients. However, it is notable that for one patient, gene expression levels of ESR1, and also other top down-regulated genes, have increased in contrast to the trend exhibited generally. It can also be noted that individual samples with high pretreatment levels of these estrogen-related genes show the most marked down-regulation of gene expression.

Correlation of AI-Responsive Genes with Baseline ESR1 Expression. The correlation between degree of response to AI treatment and pretreatment ESR1 expression level for individual patient samples was examined for a number of the genes identified by SAM analysis. Of the up-regulated genes (Fig. 3A), both COL3A1 and COL5A2 exhibited a significant positive correlation between degree of response to AI treatment and pretreatment ESR1 level; however, this was not the case for all up-regulated genes, for example, COL1A1. For the down-regulated genes studied, a statistically significant negative correlation was seen between pretreatment ESR1 expression in each sample and degree of response to estrogen deprivation (Fig. 3B).

Comparison of Gene Expression Changes in Normal Postmenopausal Breast Tissue with Breast Tumor Tissue following AI Therapy. The data obtained in this study on gene expression changes in normal breast tissue following AI treatment were compared with analogous data obtained by us from malignant tissues using the same microarray platform (13). Visual inspection of the up- and down-regulated genes identified by SAM in each study revealed considerable overlap between the genes exhibiting responses to estrogen deprivation across both normal and tumor tissue. Among the up-regulated genes, a substantial number of genes were common to both studies, including COL3A1, RNH1, ODF2L, CILP, CTGF and COL5A2, with down-regulated genes including AZGP1, STC2, and AGR2 also being identified in both settings. The commonality is particularly striking among the up-regulated genes with 9 of the top 10 genes identified as being up-regulated upon AI treatment in
normal breast tissue also shown to be up-regulated in malignant tissue (with a local false discovery rate of <1%). To further explore the global similarity between the gene changes in normal postmenopausal tissue and breast cancers, the median differences in gene expression between the pretreatment and posttreatment samples were ranked for each study. A highly significant correlation between the treatment-induced gene changes in both studies was observed (Spearman’s ρ correlation coefficient was determined).

Changes in the extracellular matrix components were also observed following neoadjuvant treatment of breast carcinomas (23), also not microdissected, and were noted in this study to be consistent with earlier work suggesting that decreases in tumor cellularity and increases in fibrosis may result from long-term letrozole treatment (24). Extracellular membrane remodeling is a function commonly ascribed to stromal cells (25), thus emphasizing the unique insights an in vivo study such as this provides. It has been reported that in some cases, genetic alterations in stromal cells may precede the malignant conversion of tumor cells (26).

Both SAM analysis (Table 2B) and the heatmap (Fig. 1) reveal some, but not all, genes classically associated with estrogen in breast tumors to be down-regulated upon AI treatment (STC2, ESR1, AGR2, and FOXA1). Although the false discovery rate for such genes is high, as noted above the presence of so many related genes and the rationale for the down-regulation of these genes suggest that these reflect a real biological change in response to estrogen deprivation. Gene set enrichment analysis revealed multiple breast cancer expression clusters, one of which includes the classically estrogen-regulated gene AZGP1, as significantly changing upon estrogen deprivation, emphasizing that the changes detected reflect genuine biological pathways. It is of particular interest that the steroid metabolism pathway, in which several isoforms of hydroxysteroid dehydrogenase are present, was identified as significantly differentially expressed upon AI treatment as 17β-hydroxysteroid dehydrogenase is responsible for the interconversion of androstenedione/testosterone and estrone/estradiol. STC2 and AGR2 have been shown to be regulated by estrogen...
The transcription factor FOXA1 associated with a poor response to endocrine therapy in vitro (30). The transcription factor FOXA1 has been previously identified as being coexpressed with ESR1 in breast cancer (31). ESR1 is clearly of critical relevance in hormone-sensitive breast cancer and the observation that ESR1 mRNA levels decrease upon AI treatment is consistent with a previous study conducted using immunohistochemistry that showed reduction of ER protein levels upon AI treatment (32) although we have not seen this in our own neoadjuvant studies (33).

Estrogen is a key driver of proliferation, and AI treatment of tumors induces a marked antiproliferative response (33). Consistent with the lack of a statistically significant reduction in Ki67 upon AI treatment noted in the original LITMaS study (12), pathway analysis did not reveal any significant effects of estrogen deprivation on proliferation-associated genes in this study.

Response of breast cancers to AIs varies markedly: although some patients respond well to therapy, up to 50% of treated patients derive little or no benefit from treatment (7). It is possible that similar variation may be seen in the biological response of the normal breast to AIs. The data presented here support that concept but the number of sample pairs was low and does not allow the determination of any putative determinants of the variability other than an indication that quantitative expression of ER may be important. It could be seen that for many of the genes considered, the patients with the highest pretreatment levels of ESR1 showed the greatest magnitude of change in gene expression upon estrogen deprivation (Fig. 3). It is unsurprising that the responses of classically estrogen dependent genes are highly correlated with pretreatment ESR1 levels for individual patients but it was notable that this was not the case for all genes, indicating that ER expression is not the only determinant of response to estrogen deprivation in normal breast tissue.

The commonality of the gene changes between normal and malignant breast tissue following AI treatment (13, 23) not only provides additional confidence that the genes identified here are indeed significantly altered by estrogen deprivation but also suggests that estrogen regulation of gene expression is largely maintained during carcinogenesis. Overlap between the two tissue types is not complete, with some of the top genes identified as changing in response to AI treatment in tumors, for example, TFF1, PDZK1, CCND1, and other classically estrogen-regulated genes (27), not being revealed to be changing significantly in response to AI treatment in normal breast tissue. However, to what extent this reflects the limited statistical power of this study rather than genuine differences in the responses of individual genes is difficult to ascertain.

The gene expression changes in response to estrogen deprivation in normal breast tissue indicate that postmenopausal normal breast tissue retains sensitivity to the low levels of endogenous estrogen. This suggests the case that treatment with AIs may be a viable chemoprevention strategy by an effect on normal tissue as well as subclinical malignant disease. The magnitude of the expression changes upon treatment seems to be considerably lower in the normal breast tissue compared with the tumor. ERα is expressed within only a small proportion of epithelial cells in normal breast (34). In the absence of malignancy, ER-positive cells are non-proliferative (35) and hormone-mediated epithelial cell proliferation is believed to depend on paracrine signaling with stromal cells (25). Upon malignant conversion, ERα expression increases and ER-positive cells acquire proliferative ability (36). These key differences in both the number and properties of ER-positive cells between normal and malignant tissues may explain the differences observed in magnitude of response between the two tissue types. Different signaling mechanisms may also exist in the normal breast to allow downstream responses to subtler gene expression changes. It is also possible that if microdissection of the core biopsies of normal tissue had been undertaken to enrich for epithelial cells, the relative expression of genes may have been increased. We did pilot studies of microdissection but rejected this because of the major losses incurred by the process in these tissues. One consequence of the modest degree of gene expression change, however, may be that intermediate markers of chemoprevention may be difficult to identify. However, it may be significant that the women studied here were not selected according to risk of breast cancer; high-risk women might show greater estrogen-dependent transcriptional activity and consequently greater changes with estrogen deprivation. This possibility merits study.

Conclusions
To our knowledge, this is the first study to investigate at a transcriptional level the importance of endogenous estrogen in normal postmenopausal breast tissue. The data suggest that extracellular matrix remodeling and some classically estrogen-associated genes are among those affected by estrogen deprivation in the normal breast in vivo. Comparison of the responses observed in normal breast tissue with those identified in a comparable study of breast carcinomas indicates that the transcriptional responses of the two tissue types show qualitative similarities with a high overall correlation in gene expression changes and common genes identified across the two data sets. However, the extent of the changes seen in normal breast tissue upon estrogen deprivation is markedly lower than seen in malignant tissue. The study shows the utility and feasibility of performing molecular profiling on normal breast tissue samples pretreatment and posttreatment and suggests that extending this work into a larger set of samples, including those from high-risk women, would be worthwhile.

Acknowledgments
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