High N-Acetyltransferase 1 Expression is Associated with Estrogen Receptor Expression in Breast Tumors, but is not Under Direct Regulation by Estradiol, 5 α -androstane-3 β , 17 β -Diol, or Dihydrotestosterone in Breast Cancer Cells

Xiaoyan Zhang,¹ Samantha M. Carlisle, Mark A. Doll, Robert C. G. Martin, J. Christopher States, Carolyn M. Klinge, and David W. Hein

Departments of Pharmacology and Toxicology (X.Z., S.M.C., M.A.D., J.C.S., D.W.H.), Surgery (R.C.G.M.), Biochemistry and Molecular Genetics (C.M.K.), and James Graham Brown Cancer Center, University of Louisville School of Medicine, Louisville, Kentucky

Received December 7, 2017; accepted January 12, 2018

ABSTRACT

N-acetyltransferase 1 (NAT1) is an enzyme that metabolizes carcinogens, which suggests a potential role in breast carcinogenesis. High *NAT1* expression in breast tumors is associated with estrogen receptor α (ER α +) and the luminal subtype. We report that *NAT1* mRNA transcript, protein, and enzyme activity were higher in human breast tumors with high expression of ER α /*ESR1* compared with normal breast tissue. There was a strong correlation between *NAT1b* promoter and NAT1 protein expression/enzyme activity. High *NAT1* expression in tumors was not the result of adipocytes, as evidenced by low perilipin (*PLIN*) expression. *ESR1*, *NAT1*, and *XPB1* expression were associated in tumor biopsies. Direct regulation of *NAT1* transcription by estradiol (E₂) was investigated in ER α (+) MCF-7 and T47D breast cancer cells. E₂ did not increase *NAT1* transcript expression but

increased progesterone receptor expression in a dose-dependent manner. Likewise, *NAT1* transcript levels were not increased by dihydrotestosterone (DHT) or 5 α -androstane-3 β , (3 β -adiol) 17 β -diol. Dithiothreitol increased levels of the activated, spliced *XPB1* in ER α (+) MCF-7 and T47D breast cancer cells but did not affect *NAT1* or *ESR1* expression. We conclude that *NAT1* expression is not directly regulated by E₂, DHT, 3 β -adiol, or dithiothreitol despite high *NAT1* and *ESR1* expression in luminal A breast cancer cells, suggesting that *ESR1*, *XPB1*, and *NAT1* expression may share a common transcriptional network arising from the luminal epithelium associated with better survival in breast cancer. Clusters of high-expression genes, including *NAT1*, in breast tumors might serve as potential targets for novel therapeutic drug development.

Introduction

N-acetyltransferase 1 (NAT1) genetic polymorphism is associated with breast cancer risk and mortality (Zheng et al., 1999; Millikan, 2000; Andres et al., 2015). Recent meta-analyses suggest the associations of NAT1 genetic polymorphisms with pancreatic (Zhang et al., 2015) and urinary bladder (Dhaini et al., 2017) cancers. Congenic rats with higher arylamine N-acetyltransferase 2 activity (homologous to human NAT1) exhibit greater carcinogen-induced mammary tumor susceptibility (Stepp et al., 2017). Overexpression of NAT1 enhances breast cancer cell proliferation and was associated with a gene signature of epithelial-to-mesenchymal transition in breast tumors (Adam et al., 2003; Savci-Heijink et al., 2016). Small molecule inhibition of NAT1 decreases proliferation and invasiveness (Tiang et al., 2010),

and both genetic and small-molecule inhibition of NAT1 reduce anchorage-independent growth (Stepp et al., 2018) in human breast cancer cells. Thus, the role of NAT1 in breast carcinogenesis or progression remains unclear and requires further investigation.

Human NAT1 is located in chromosome 8p 21.3. *NAT1* has two alternative promoters (*NATa* and *NATb*) and the NAT1 open reading frame is contained within the 3' exon. The *NATb* promoter is located 11.8 kb upstream of the *NAT1* coding exon (Husain et al., 2004, 2007), whereas an alternative *NATa* promoter is located 51.5 kb upstream of the *NAT1* coding exon (Barker et al., 2006). *NATb* is the major promoter for *NAT1* and is responsible for ubiquitous *NAT1* expression (Husain et al., 2004). However, *NATb* lacks a TATA box, and thus correlates with low-to-moderate *NAT1* expression (Husain et al., 2004). SP1 detected in the minimal *NATb* region is expressed ubiquitously and is probably responsible for wide *NATb* activity in every tissue throughout the human body (Sadrieh et al., 1996; Windmill et al., 2000). *NATa* is most active in specific tissues such as kidney, liver, lung, and trachea (Barker et al., 2006). In human breast tissues, *NAT1*

Supported by National Institutes of Health United States Public Health Service Grants [R01-CA-034627, R01-DK-053220, and T32-ES-011564].

¹Current affiliation: Sanofi US, Bridgewater, New Jersey.
<https://doi.org/10.1124/jpet.117.247031>

ABBREVIATIONS: 3 β -adiol, 5 α -androstane-3 β , 17 β -diol; AR, androgen receptor; DHT, dihydrotestosterone; DTT, dithiothreitol; E₂, estradiol; ER α , estrogen receptor α ; FBS, fetal bovine serum; NAT1, N-acetyltransferase 1; 4-OHT, 4-hydroxytamoxifen; PGR, progesterone receptor; PLIN, perilipin; RISC, RNA-induced silencing complex; RSEM, RNA-Seq by expectation maximization; XPB1, X-box binding protein 1.

TABLE 1

Primers and probes for quantitative real-time RT-PCR

Product (RT-PCR)	Forward Primer	Reverse Primer	Probe
Total <i>NAT1</i>	5'AATTCAAGCCAGGAAGAAGCA 3'	5'TCCAAGTCCAATTTGTTCTCTAGACT 3'	5'FAMCAATCTGTCTTCTGGATTAA 3'MGBNFQ
<i>NATb</i>	5'TGGTTGCCGGGTGAAATAAC 3'	5'TCCAAGTCCAATTTGTTCTCTAGACT 3'	5'FAMCAATCTGTCTTCTGGATTAA 3'MGBNFQ
<i>NATa</i>	5'GGGGATAATCGGACAATACAAC 3'	5'TCCAAGTCCAATTTGTTCTCTAGACT 3'	5'FAMCAATCTGTCTTCTGGATTAA 3'MGBNFQ

expression is detectable at the mRNA, protein, and enzyme activity levels (<https://www.gtexportal.org/home/gene/NAT1>), but whether expression is driven by *NATb* or *NATa* remains unknown.

NAT1 was reported to be overexpressed in breast tumors, and high *NAT1* expression was mainly found in luminal tumors characterized by high estrogen receptor (*ERα/ESR1*) expression (Perou et al., 2000; Sorlie et al., 2001; Adam et al., 2003). Along with other gene signatures, including *ESR1* and progesterone receptor (*PGR*), high *NAT1* expression in luminal-type breast carcinomas is associated with better prognosis and longer relapse-free survival (Perou et al., 2000; Sorlie et al., 2001). Immunohistochemical staining showed *NAT1* levels correlated with *ERα* positivity and that expression of *NAT1* in *ERα* (–) breast cancer was low (Tozlu et al., 2006). Further, *NAT1* expression was higher in breast cancer cell lines compared with other cancer cell lines (Ross et al., 2000). *NAT1* expression was also significantly associated with increased overall survival and with disease-free survival in breast cancer patients who had received tamoxifen as an adjuvant therapy (Endo et al., 2013). Functionally, overexpression of *NAT1* increased the proliferative capacity of the HB4a normal breast epithelium-derived cells in vitro, possible by upregulating the methionine salvage pathway (Adam et al., 2003; Witham et al., 2017). Interestingly, the correlation between *NAT1* and *ERα* observed in primary tumors is lost in metastatic tumors from the same patients (Butcher and Minchin, 2012). Despite these observations, the mechanism for the correlation between *NAT1* and *ERα* expression in luminal breast cancer is not yet clear.

Analysis of published microarray studies revealed a positive correlation between overexpression of *NAT1* and *ER* positivity (Wakefield et al., 2008). One study showed a 2-fold increase in *NAT1* expression in estradiol (*E₂*)-treated MCF-7 cells (Finlin et al., 2001). In androgen receptor (AR)-positive 22Rv1 and LNCaP prostate cancer cells, *NAT1* expression was induced by the synthetic androgen R1881, although the increased *NAT1* transcription was not direct but was mediated instead by a secondary effect of upregulating the expression of heat shock factor (HSF1) that bound to *NAT1* promoter to increase transcription (Butcher and Minchin, 2010). Interestingly, *NAT1* was associated with AR expression as well as Trefol factor 3, apolipoprotein D, and activated leukocyte cell adhesion molecule in a subset of *ERα* (–)/*PGR* (–) human breast tumors, but no study has examined androgen regulation of *NAT1* expression in breast cancer (Doane et al., 2006).

The association between *ERα* and *NAT1* expression described above was reviewed (Butcher and Minchin, 2012). We investigated *NAT1* expression in matched tumor and normal human breast tissue specimens and in *ERα* (+) MCF-7 and T47D breast cancer cell lines to assess the regulation of *NAT1* expression by *E₂* and selective ER modulators.

Material and Methods

Patients and Human Samples. De-identified breast tumor and normal tissues of newly diagnosed breast cancer patients were obtained from Louisville Repository Tissue Network and University of Louisville Department of Surgery. The samples were collected and stored in liquid nitrogen immediately after surgery. The *ER* (*ERα*) status was clinically provided from immunohistochemical staining analyses. All procedures were performed under the approval of University of Louisville Institutional Review Board.

RNA Preparation and Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction. Total RNA was extracted from frozen breast tissue specimens using RNeasy kit (Qiagen, Valencia, CA) following the manufacturer's instructions. The purity of extracted RNA was determined by measuring the absorbance at 280/260 nm. Complementary DNA was synthesized from 1 μg RNA using the Superscript III Reverse Transcriptase kit (Invitrogen/Thermo Fisher Scientific, Carlsbad, CA). Total *NAT1*, *NATb*, and *NATa* mRNA transcripts were measured by quantitative real-time reverse transcription–polymerase chain reaction (RT-PCR) using a probe and a forward primer designed in the 79-bp exon region, and the reverse primer in the protein coding exon as shown in Table 1 (Husain et al., 2004; Barker et al., 2006). *ERα* (*ESR1*, Hs00174860_m1), perilipin (*PLIN*, Hs00160173_m1), progesterone receptor (*PGR*, Hs00172183_m1), and X-box binding protein 1 (*XBPI*, Hs00964359_m1) were measured using ABI predesigned primers and probes (Applied Biosystems, Foster City, CA). 18S rRNA (Applied Biosystems) was used as a reference to normalize the RT-PCR results.

Measurement of *NAT1* N-Acetyltransferase Activity in Tumor and Normal Breast Tissue Samples. Breast tumor and normal tissues were homogenized in 20 mM sodium phosphate, pH 7.4, containing EDTA (1 mM), dithiothreitol (DTT: (1 mM) and protease inhibitors (1 μg/ml), phenylmethylsulfonyl fluoride (100 μM), and pepstatin (0.75 μM) using a Tissue Tearor (Biospec Products, Bartlesville, OK), and centrifuged at 100,000g for 1 hour at 4°C. Total cellular protein was measured using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). *NAT1* enzyme activity in human mammary tissues was detected using *p*-aminobenzoic acid as substrate. Reactions containing suitable diluted cell cytosol protein, *p*-aminobenzoic acid (100 μM), and 1 mM acetyl-coenzyme A were incubated at 37°C for 30 minutes. Reactions were stopped by adding acetic acid. Reactants and products were separated by high-performance liquid chromatography (Beckman-Coulter, Fullerton, CA) and quantified by their absorbance at 280 nm (Hein et al., 2006).

Culture of the Human Breast Cancer Cell Lines. MCF-7 and T47D cells were obtained from ATCC (Manassas, VA). MCF-7 cells were cultured in modified Iscove's modified Eagle's medium (IMEM) (Invitrogen) supplemented with 10% fetal bovine serum (HyClone, Logan, UT). T47D cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS; HyClone). Penicillin (100 IU/ml) and streptomycin (100 μg/ml) were added to complete medium just prior to use. Cell culture media was routinely changed every 3–4 days. For experiments, cells were grown in phenol red-free IMEM (MCF-7) or RPMI 1640 (T47D) supplemented with 5% FBS charcoal/dextran-stripped to remove hormones.

Hormone Treatment of the Human Breast Cancer Cells. T47D and MCF-7 cells were grown in phenol red-free medium

Fig. 1. *ESR1* mRNA expression in ER α (+) and ER α (-) human breast tumors. Quantitative real-time RT-PCR was used to measure *ESR1* mRNA expression that is shown for individual breast tumors and was normalized relative to expression in MCF-7 cells, i.e., set to 1, far right. Breast tumors listed on the abscissa are grouped into ER α (+) and ER α (-) on the basis of immunohistochemical staining information. Inserted graph: Each bar represents the mean $\pm$ S.E.M. of *ESR1* mRNA in ER α (+) ($n = 16$) and ER α (-) ($n = 5$) breast tumor samples.

containing 5% charcoal/dextran-stripped FBS for 48 or 72 hours before each experiment. Different concentrations of E₂ (Sigma-Aldrich, St. Louis, MO) ranging from 10^{-10} to 10^{-7} M were tested. In additional experiments, cells were treated with 10 nM E₂, 100 nM/1 μ M 4-hydroxytamoxifen (4-OHT; Sigma-Aldrich), or 100 nM ICI 182,780 (fulvestrant; Tocris, Ellisville, MO) alone or in combination for 6 or 24 hours. In other experiments, cells were treated with dihydrotestosterone (DHT), 5 α -androstane-3 β , 17 β -diol (3 β -adiol), both from Sigma-Aldrich. The selective AR modulator/antiandrogen bicalutamide (Casodex) was generously provided by AstraZeneca (Macclesfield, UK) (Teng et al., 2013). RNA was extracted as described above and quantitative real-time RT-PCR was performed to determine *NAT1* and *PGR* mRNA expression.

Dithiothreitol Treatment of the Human Breast Cancer Cells. MCF-7 cells were treated with 5 mM dithiothreitol (DTT; Sigma-Aldrich) for 4 hours when the cells had grown to 70–80% confluence in six-well plates. Cells were collected and RNA was extracted using RNeasy kit (Qiagen). cDNA was synthesized using Superscript III Reverse Transcriptase kit (Invitrogen). PCR was performed under the following conditions: 94°C for 3 minutes, followed by 30 cycles of 94°C for 30 seconds, 62°C for 1 minute, 72°C for 1.5 minutes, and then 72°C for 5 minutes. The *XBPI* forward primer sequence was 5'-GAAGCCAAGGGGAATGAAGTGAGG-3' and the reverse primer sequence was 5'-CATGGGGAGGAGATGTTCTGGAGGGG-3'.

Analysis of *ESR1*, *FOXA1*, *GATA3*, *NAT1*, and *XBPI* RNA Expression between ER α (+) and ER α (-) Breast Tumor Samples. Publicly available data from The Cancer Genome Atlas (Cancer Genome Atlas Research Network et al., 2013) for the breast invasive carcinoma cohort were downloaded and analyzed ($N = 1043$) using FirebrowseR (Deng et al., 2017), an R client to the Broad Institute's RESTful Firehose Pipeline. Differences in RNA expression of *ESR1*, *FOXA1*, *GATA3*, *NAT1*, and *XBPI* between primary solid breast tumor samples stratified by ER α status (+ or -) were evaluated statistically by Mann-Whitney for each gene. RNA expression values are reported in RSEM from the Cancer Genome Atlas Research Network (RNA-Seq by Expectation-Maximization) (Li and Dewey, 2011).

Results

***ESR1* Expression in ER α (+) and ER α (-) Breast Tumor Samples.** *ESR1* mRNA was measured in 21 breast tumors and mRNA measurement in ER α (+) MCF-7 cells was used as a reference (Fig. 1). As expected, *ESR1* expression differed significantly ($P < 0.0025$) between ER α (+) versus

ER α (-) breast tumors with higher expression in ER α (+) tumors. For subsequent figures, the labeling of ER α status was consistently determined the same way.

***NAT1* mRNA Expression Correlates with *NAT1* Enzymatic Activity in the Breast Samples.** *NAT1* expression (both mRNA and catalytic activity for *p*-aminobenzoic acid N-acetylation) was measured in the breast tumor and normal breast tissues. Both *NAT1* mRNA (Fig. 2A) and N-acetyltransferase activity (Fig. 2B) were significantly higher in ER α (+) breast tumors compared with ER α (-) tumors, normal breast tissues, and MCF-7 cells. However, as observed in the tumor sample no. 3, there were exceptions to high *NAT1* mRNA and N-acetyltransferase activity. We also observed that breast tumor tissue from patient no. 9 had high *NAT1* mRNA (Fig. 3A) but not high *NAT1* N-acetyltransferase activity, a result that might be a consequence of *NAT1* degradation, or other mechanisms resulting in reduced enzyme activity. The Pearson correlation coefficient between *NAT1* mRNA and enzyme activity in 19 breast tumors was 0.709 ($p < 0.0001$).

Perilipin Expression in ER α (+) and ER α (-) Breast Tumor Samples. Adipose cells account for 90% of the total mass of the normal breast tissue (Nishidate et al., 2004). Perilipin (*PLIN*) is expressed exclusively in adipocytes in mammary gland (Saito-Hisaminato et al., 2002). To evaluate the relative purity of the breast tumor/tissue samples in our study, *PLIN* expression was measured in the same set of matched tumor/normal samples. Breast tumor samples had very low *PLIN* mRNA expression; in contrast, matched normal samples had much higher *PLIN* mRNA expression confirming that *NAT1* expression was contributed primarily by breast tumor cells in our tumor samples (Fig. 3).

***NATa* and *NATb* Promoter Expression in the Breast Samples.** To better evaluate if the high *NAT1* expression in these breast tumors was at the transcriptional level, the promoter utilization was examined. *NAT1* is transcribed from a major promoter, *NATb*, and an alternative promoter, *NATa*, resulting in mRNAs with distinct 5'-untranslated regions (UTR) (Millner et al., 2012). Quantitative real time RT-PCR was used to measure *NATa*, *NATb*, and *NAT1* mRNA expression in breast tumors. *NATa* was not detected or was used at very low levels in these breast tumors (data not

Fig. 2. *NAT1* mRNA levels and N-acetyltransferase activity in breast tumor/normal breast samples. (A) Quantitative real-time RT-PCR was used to measure *NAT1* mRNA expression in the same breast tumor samples as in Fig. 1. (B) *NAT1* N-acetyltransferase activity was measured in the tumor samples using *p*-aminobenzoic acid as a substrate. No enzyme activity data for sample 1. The y-axis was divided into two segments to accommodate the wide range of values. Each bar represents the mean $\pm$ S.E.M. of *NAT1* mRNA expression in ER α (+) ($n = 16$) and ER α (-) ($n = 5$) and *NAT1* N-acetyltransferase activity in ER α (+) ($n = 15$) and ER α (-) ($n = 5$) breast tumor samples.

shown). As shown in Fig. 4, *NAT1b* mRNA expression was high and correlated with *NAT1* mRNA expression (Fig. 2A) in breast tumors ($R^2 = 0.60$, $P < 0.0001$). Thus, we conclude that the high level of *NAT1* transcripts is primarily from the major *NAT1b* promoter. ER α (+) breast tumors had a significantly higher *NAT1b* mRNA expression than the ER α (-) breast tumors ($P = 0.0275$).

***NAT1* Expression Is Not E₂-Regulated in ER α (+) Breast Cancer Cells.** Because *NAT1* mRNA expression (Fig. 2A) correlated with ER α expression (Fig. 1), we determined if E₂ stimulated *NAT1* expression in ER α (+) breast cancer cell lines. As a positive control, we observed that E₂ increased *PGR* mRNA expression in a concentration-dependent manner in MCF-7 cells; however, *NAT1* expression was not increased by E₂ (Fig. 5A). The active metabolite of tamoxifen, 4-OHT, inhibited E₂-induced *PGR* expression but did not affect *NAT1* expression (Fig. 5, B and C). These data indicate that E₂ does not directly stimulate *NAT1* transcription in these breast cancer cells.

Because *NAT1* was associated with AR expression in a subset of ER α (-)/PGR(-) human breast tumors (Doane et al., 2006), we determined if dihydrotestosterone (DHT) increased *NAT1* expression in AR(+) MCF-7 cells (Fig. 5C). For these

experiments, cells were treated for 24 hours. DHT did not increase *NAT1* but did increase *PGR* expression, although the increase was small compared with E₂ and may be attributable to metabolism of DHT to E₂ in MCF-7 cells (Maggiolini et al., 2001). In postmenopausal women, DHT can be metabolized to 5 α -androstane-3 β , 17 β -diol (3 β -adiol) which binds and activates ER α and ER β (Maggiolini et al., 1999; Weihua et al., 2002; Sikora et al., 2009; Sharma et al., 2012). 3 β -Adiol did not stimulate *NAT1* expression, but significantly increased *PGR* expression in an ER-dependent manner, since the stimulation was inhibited by concomitant treatment of the cells with 4-OHT but not the AR antagonist bicalutamide. Together, these data indicate that *NAT1* expression is not regulated by either estrogens or DHT in MCF-7 cells.

Similarly, in ER α (+) T47D breast cancer cell line, *PGR* expression was significantly ($p = 0.0002$) increased by E₂ in a concentration-dependent manner (Fig. 6A) and this activation was inhibited by selective ER modulator 4-OHT and the pure ER antagonist ICI 182,780 (fulvestrant) (Fig. 6B). We also tested the effect of E₂ and 4-OHT in ER α (-)/PGR(-) MDA-MB-231 triple-negative breast cancer cells and observed no effect of either treatment on *NAT1* or *PGR* expression in these cells (data not shown). Taken together, our results indicate

Fig. 3. (A) Perilipin (*PLIN*) mRNA expression in the breast tumor/normal tissue samples. *PLIN* mRNA was measured by quantitative real-time RT-PCR in breast tumor and normal breast tissues and normalized to expression in MCF-7 cells. The tumor/breast sample numbers correspond to those in Figs. 1 and 2. The y-axis was divided into three segments owing to the low level of *PLIN* expression in tumor in comparison with normal breast tissue. (B) Each bar is the mean $\pm$ S.E.M. of *PLIN* mRNA expression in breast normal ($n = 21$) and tumor tissues ($n = 21$).

that E_2 does not stimulate *NAT1* mRNA expression in either $ER\alpha$ (+) breast cancer cell line or in MDA-MB-231 triple-negative breast cancer cells.

***XBP1* Expression in Breast Tumors and *NAT1* Expression Regulation by DTT.** The alternatively spliced transcription factor *XBP1* was more highly expressed in $ER\alpha$ (+) than $ER\alpha$ (-) breast tumors and was reported to be associated with *NAT1* expression (Finlin et al., 2001). We examined *XBP1* expression and evaluated the correlation between *XBP1* and *NAT1* expression in the breast tumor

samples. The primer and probe used for quantitative real time RT-PCR measurements was designed across exon 1 and 2 and not in the exon 4 and 5 region, in which 26 bp was removed to become the spliced and activated *XBP1* (Lemin et al., 2007). Accordingly, both unspliced and spliced *XBP1* mRNA expression were measured in this quantitative RT-PCR assay. In agreement with the previous report (Finlin et al., 2001), we confirmed in a separate set of breast tumors that *XBP1* mRNA was significantly ($p = 0.0026$) higher in $ER\alpha$ (+) than $ER\alpha$ (-) breast tumors (Fig. 7).

Fig. 4. *NATb* utilization in breast samples. Quantitative real-time RT-PCR was used to measure *NATb* expression in 21 breast tumors and expression in MCF-7 cells was used as a reference control. The tumor/breast sample numbers correspond to those in Figs. 1–3. The y-axis was broken into two segments. Inset graph: Each bar represents the mean $\pm$ S.E.M. of *NATb* mRNA in $ER\alpha$ (+) ($n = 16$) and $ER\alpha$ (-) ($n = 5$) breast tumor samples.

Fig. 5. *NAT1* and *PGR* expression in MCF-7 cells. MCF-7 cells were incubated in “serum starvation” medium (5% charcoal-stripped FBS) for 48 hours prior to treatment with vehicle control (DMSO) or the indicated concentrations of E₂ for 6 hours (A); E₂ alone or in combination with 4-OHT for 6 hours (B); or with E₂, DHT, 3β-adiol, 4-OHT, or bicalutamide, alone or together, as indicated, for 24 hours (C). *NAT1* and *PGR* mRNA expression were measured by quantitative real-time RT-PCR using 18S as a reference gene. Values of *NAT1* and *PGR* expression were normalized to vehicle (DMSO)-treated cells and are the mean ± S.E.M. for three separate experiments. **P* < 0.05 vs. DMSO control. #*P* < 0.05 vs. the same treatment without antagonist.

DTT activates the uncoupled protein response (UPR) and stimulates *XBP1* splicing (Lemin et al., 2007). We used agarose gel electrophoresis to separate the *XBP1* spliced and unspliced products. DTT increased the amount of activated spliced *XBP1* in ERα (+) MCF-7 and T47D cells (Fig. 8). Despite the correlation of *ESR1*, *XBP1*, and *NAT1* expression in ERα (+) breast tumors (Doane et al., 2006), no significant (*P* > 0.05) effect of DTT on *NAT1* or *ESR1* (data not shown) mRNA expression was observed (Fig. 8).

***ESR1*, *FOXA1*, *GATA3*, *NAT1*, and *XBP1* RNA Expression in ERα (+) and ERα(−) Breast Tumor Samples.** *ESR1*, *FOXA1*, *GATA3*, *NAT1*, and *XBP1* mRNA

expression was significantly higher (*P* < 0.0001 for all) in ERα (+) primary solid breast tumor samples (*n* = 806) compared with ERα (−) primary solid breast tumor samples (*n* = 237) (Fig. 9).

Discussion

We observed that *NAT1* expression is higher in ERα (+) breast tumor tissues than in the associated normal breast tissue and that *NAT1* is not highly expressed in ERα (−) breast tumors, consistent with previous studies (Dolled-Filhart et al., 2006; Ring et al., 2006; Endo et al., 2013).

Fig. 6. *NAT1* and *PGR* expression in T47D cells. T47D cells were incubated in “serum starvation” medium (5% charcoal-stripped FBS) for 48 hours prior to treatment with vehicle control (DMSO) or the indicated concentrations of E₂ for 6 hours. *NAT1* expression increased significantly (*P* = 0.0002) with respect to E₂ concentration (A); E₂ alone or in combination with 4-OHT or ICI 182,780 (ICI) for 6 hours (B). *NAT1* and *PGR* mRNA expression were measured by quantitative real-time RT-PCR using 18S as a reference gene. Values of *NAT1* and *PGR* expression were normalized to vehicle (DMSO)-treated cells and are the mean ± S.E.M. for three separate experiments. **P* < 0.05 vs. DMSO control. #*P* < 0.05 vs. the same treatment without antagonist.

Fig. 7. *XBP1* mRNA expression in breast tumor samples. *XBP1* mRNA expression was measured using quantitative real-time RT-PCR in 20 breast tumors with the indicated ER α expression status. The tumor/breast sample numbers correspond to those in Figs. 1–4. *XBP1* mRNA expression in MCF-7 was used as a reference control. Inserted graph: Each bar represents the mean $\pm$ S.E.M. of *XBP1* mRNA in ER α (+) ($n = 16$) and ER α (–) ($n = 5$) breast tumor samples.

We also observed that *NAT1* mRNA correlates positively with *NAT1* enzyme activity in ER α (+) breast tumor tissues. Although patients nos. 14, 15, and 16 were defined as ER α (+), on the basis of immunohistochemistry, the *ESR1* mRNA levels in these tumors was less than the ER α (+) MCF-7 breast cancer cell line, suggesting the possibility of RNA degradation. Additional explanations for this lack of correlation between *ESR1* mRNA and protein expression in these three tumor samples could be owing to protein stability in the presence of ligand(s) that stabilize ER α protein, ER α phosphorylation, or false positive information on ER status (Lupien et al., 2007). Interestingly, we note that *NAT1* mRNA level and enzyme activity were also lower in these patients' breast tumors compared with other tumors classified as ER α (+), instead resembling the ER α (–) breast tumors examined. These data support the suggestion that *NAT1* is expressed in association with *ESR1* in breast tumors.

Alternative promoter usage is one of the mechanisms by which gene expression is differentially regulated in tissues (Chen et al., 2005). *NATb* is active in all tissues tested, whereas *NATa* is active in certain tissues, including kidney, lung, trachea, and liver, that have the highest exposure potential to environmental chemicals (Barker et al., 2006). The *NATa* promoter is activated in the ER α (+) ZR-75-1 breast cancer cell line, which has relatively high *NAT1* activity and mRNA levels (Yan et al., 2007). In our study, we clearly demonstrated that the *NATb*, not the *NATa*, promoter was active and responsible for the high *NAT1* expression in ER α (+) breast tumors. Thus, we conclude that alternative promoter usage was not responsible for *NAT1* overexpression in breast tumors.

Despite the strong correlation between *ESR1* and *NAT1* expression in breast tumors, our studies indicated that there was no direct effect of E₂ or 4-OHT on *NAT1* expression in

either MCF-7 or T47D luminal breast cancer cells in vitro. Previous studies reported that *NAT1* expression was increased ~2-fold by 10 nM E₂ and significantly reduced by 6 μ M tamoxifen in MCF-7 cells (Finlin et al., 2001). In this study, *NAT1* gene expression was measured after 48 hours of tamoxifen treatment; thus, *NAT1* expression might have been downregulated by some cross-signaling pathway in addition to, or instead of, a direct effect on ER α transactivation of *NAT1* expression, because transcriptional regulation usually occurs within 24 hours (Heintz et al., 1983). Although the synthetic androgen R1881 increased *NAT1* expression by an unknown secondary mechanism in AR (+) 22Rv1 and LNCaP prostate cancer cells (Butcher et al., 2007; Butcher and Minchin, 2010), we observed no effect of DHT on *NAT1* expression in MCF-7 cells. The difference in androgen regulation of *NAT1* expression between the breast and prostate cancer cell lines may be attributed to tissue-specific differences in additional transcription factors and coregulators needed to induce *NAT1* expression or to differences in chromatin architecture and epigenetic signaling, including micro-RNA and long noncoding RNA expression.

Likewise to *NAT1*, *GATA3* is another gene highly expressed in breast tumors that is positively correlated with ER α expression (Licata et al., 2010). Like *NAT1*, *GATA3* is not directly regulated by E₂ or ER α , indicating that a set of genes expressed at high levels along with *ESR1*, including *GATA3* and *NAT1*, might be involved in a complex regulatory pathway during breast cancer development (Hoch et al., 1999). Overexpression of the luminal/*ESR1*(+) cluster genes *GATA3* and *NAT1* is characteristic and specific for breast cancer, because there is no similar pattern of high luminal/*ESR1*(+) gene expression in other cancer types. Theodorou et al. (2013) show that ER α -chromatin binding in MCF-7 cells is mediated by

Fig. 8. DTT stimulates *XBP1* splicing in ER α (+) breast cancer cell lines T47D and MCF-7. The cells were treated with 5 mM DTT for 4 hours. Products from the RT-PCR reaction were separated by 1% agarose gel electrophoresis and the bands visualized by ethidium bromide staining. The unspliced *XBP1* PCR product is 125 bp and the spliced *XBP1* product is 99 bp.

Fig. 9. RNA expression of *ESR1*, *FOXA1*, *GATA3*, *NAT1*, and *XBP1* was increased in estrogen receptor positive primary solid breast tumor samples. Publicly available data from The Cancer Genome Atlas for the breast invasive carcinoma cohort was downloaded and analyzed ($N = 1043$). Samples were stratified by estrogen receptor status and differences in RNA expression were evaluated by Mann-Whitney for each gene. In the boxplots the solid black line represents the median, the upper hinge represents the 75th quartile, the lower hinge represents the 25th quartile, the upper whisker represents the largest observation less than or equal to upper hinge + $1.5 \times$ interquartile range (IQR), the lower whisker represents the smallest observation greater than or equal to the lower hinge - $1.5 \times$ IQR, and outliers are plotted as open circles. White boxplots show ER positive samples ($n = 237$) and gray boxplots show ER negative samples ($n = 806$). N = negative, P = positive, *** $P < 0.001$.

GATA3 and *FOXA1* binding to the *cis*-regulatory elements that drive transcription of the *ESR1* target genes prior to $ER\alpha$ binding; thus acting as pioneer factors. However, in the absence of *GATA3*, chromatin accessibility at potential $ER\alpha$ -binding elements may be altered, resulting in a rewired $ER\alpha$ -binding profile and expression program. Their data suggests *GATA3* is one of the central components of the $ER\alpha$ complex that determines the binding potential and transcriptional targets in breast cancer cells. Thus, the correlation observed between *ESR1* and *GATA3* is not because the *ESR1* product ($ER\alpha$) interacts with *GATA3* to induce its expression, but rather because *GATA3* interacts with $ER\alpha$ DNA binding sites to shape enhancer accessibility that leads to downstream effects on other genes not influenced by $ER\alpha$.

XBP1 is a transcription factor associated with endoplasmic reticulum stress and the unfolded protein response when cells are exposed to hypoxia, low glucose, or low pH that is upregulated in endocrine-resistant breast cancer (Koong et al., 2006). Tumor cells are often hypoxic and it is reasonable to hypothesize that *XBP1* activation is involved in the regulation of genes overexpressed in tumors compared with normal tissues (Koong et al., 2006). *XBP1* binds to and activates $ER\alpha$ in a ligand-independent manner to initiate downstream gene expression, such as the antiapoptotic gene *BCL2* and several other genes associated with the control of

breast cancer cell cycle and apoptosis (Gomez et al., 2007). *XBP1* as an estrogen-responsive gene was clearly induced ~6- to 8-fold by E_2 and downregulated by tamoxifen in MCF-7 cells (Finlin et al., 2001; Wang et al., 2004). Here we validated previous reports that *XBP1* was more highly expressed in $ER\alpha$ (+) breast tumors (Bertucci et al., 2005; Tozlu et al., 2006; Lien et al., 2007; Guedj et al., 2012). Also, *XBP1* can bind and activate $ER\alpha$ to initiate transcription of gene expression in a ligand-independent manner in breast cancer cells (Ding et al., 2003). Here we demonstrated that DTT induced the unfolded protein response in MCF-7 cells, as evidenced by increased amounts of spliced (active) *XBP1*, but *XBP1* activation did not increase *NAT1* expression in MCF-7 cells, despite *XBP1* binding sites in the upstream *NAT1* promoter (Butcher et al., 2007). We conclude that *XBP1* does not regulate *NAT1* expression in MCF-7 cells.

Evidence has shown that breast tumors with high expression of genes, including *NAT1*, probably arise from the luminal epithelium in the mammary gland duct (Dressman et al., 2001; Usary et al., 2004; Farmer et al., 2005; Badve et al., 2007). *ESR1*, *NAT1*, *GATA3*, and *FOXA1* are primarily expressed in luminal epithelial cells in the lumen of the duct and not in the myoepithelial cells that compose the outer layer (Tong and Hotamisligil, 2007). Interestingly, miR-1290 was identified to be low in $ER\alpha$ (+) breast tumors and demonstrated

Fig. 10. Model of *NAT1* transcriptional regulation and correlation with *ESR1* ($ER\alpha$), *XBP1*, *FOXA1*, and *GATA3* expression in $ER\alpha$ + breast tumors and in MCF-7 cells. Known regulators of *NAT1* transcription acting through the *NATb* major promoter are SP1 and glucocorticoid receptor (GR). The AR-mediated increase in heat shock factor 1 (HSF1) was reported to be responsible for androgen-induced *NAT1* transcription in 22Rv1 and LNCaP prostate cancer cells (Butcher et al., 2007). Although miR-1290 was demonstrated to target *NAT1* and decrease *NAT1* transcript and protein levels, whether E_2 - $ER\alpha$ inhibits miR-1290 expression in breast cancer cells or tumors is not yet known.

to decrease the expression of *FOXA1* and *NAT1*, suggesting a mechanism of *NAT1* upregulation in $ER\alpha$ (+) breast cancer (Endo et al., 2013). Subsequent studies showed miR-1290 targets sites on the *NAT1* 3'-UTR (Endo et al., 2014). Targeting these genes might interrupt the tumor proliferation pathway and offers guidance for target screening and development of novel breast cancer drugs (Endo et al., 2011; Sim et al., 2014). However, there are no reports of E_2 regulation of miR-1290 expression (Klinge, 2012; Muluhngwi and Klinge, 2015, 2017). Although miR-1290 is not included in the BreastMark miRNA survival analysis database (<http://glados.ucd.ie/BreastMark/index.html>), analysis of all breast tumors in the Kaplan-Meier Plotter showed high miR-1290 was associated with lower overall survival (http://kmplot.com/analysis/index.php?p=service&cancer=breast_mirna). Since miR-1290 reduced *NAT1* expression (Endo et al., 2013), this observation is consistent with a positive association of *ESR1* and *NAT1* in breast tumors.

In summary, *NAT1* mRNA transcript levels, protein, and enzyme activity were all higher in human breast tumors with high expression of $ER\alpha$ /*ESR1* compared with normal breast tissue. High *NAT1* expression in tumor cells was not attributable to adipocytes as evidenced by low *PLIN* mRNA expression in breast tumor samples. E_2 , DHT, and 3 β -adiol did not increase *NAT1* transcript levels in $ER\alpha$ (+) breast cancer cells. DTT increased levels of the activated spliced *XBP1* level in these cells, but did not increase *NAT1* expression. SP1 binds the *NATb* promoter and is responsible for basal *NAT1* transcription (Sadrieh et al., 1996; Windmill et al., 2000). Interestingly, E_2 increases SP1 transcription in MCF-7 breast cancer cells [Nuclear Receptor Signaling Atlas (NURSA) Transcriptome, www.nursa.org] and in U-2 OS osteosarcoma cells (Hu et al., 2017). Thus, we speculate that E_2 - $ER\alpha$ upregulation of SP1 expression may indirectly increase *NAT1* transcription, although further studies are needed to explore this suggestion. We have assembled the data presented and reviewed here in a model (Fig. 10). Overall, the lack of direct regulation of *NAT1* expression by E_2 or DTT despite high *ESR1* and *XBP1* expression suggest that *ESR1*, *XBP1*, and *NAT1* expression share a common transcriptional network in breast cancer that remains to be fully elucidated. Clusters of high-expression genes, including *NAT1*, in breast tumors might serve as potential targets for development of novel drugs and therapeutic interventions for breast cancer patients.

Acknowledgments

The authors thank Katherine Bourcy, David Barker, and Amar Singh for advice and technical support.

Authorship Contributions

Participated in research design: Zhang, States, Klinge.
Conducted experiments: Zhang, Carlisle, Doll.
Contributed new reagents or analytic tools: Zhang, Carlisle, Martin, Klinge.
Performed data analysis: Zhang, Carlisle, Klinge.
Wrote or contributed to the writing of the manuscript: Zhang, Carlisle, Klinge, States, Hein.

References

- Adam PJ, Berry J, Loader JA, Tyson KL, Craggs G, Smith P, De Belin J, Steers G, Pezzella F, Sachsenmeier KF, et al. (2003) Arylamine N-acetyltransferase-1 is highly expressed in breast cancers and conveys enhanced growth and resistance to etoposide in vitro. *Mol Cancer Res* 1:826–835.
- Andres SA, Bickett KE, Alatoum MA, Kalbfleisch TS, Brock GN, and Wittliff JL (2015) Interaction between smoking history and gene expression levels impacts survival of breast cancer patients. *Breast Cancer Res Treat* 152:545–556.
- Badve S, Turbin D, Thorat MA, Morimiya A, Nielsen TO, Perou CM, Dunn S, Huntsman DG, and Nakshatri H (2007) FOXA1 expression in breast cancer—correlation with luminal subtype A and survival. *Clin Cancer Res* 13:4415–4421.
- Barker DF, Husain A, Neale JR, Martini BD, Zhang X, Doll MA, States JC, and Hein DW (2006) Functional properties of an alternative, tissue-specific promoter for human arylamine N-acetyltransferase 1. *Pharmacogenet Genomics* 16:515–525.
- Bertucci F, Finetti P, Rougemont J, Charafe-Jauffret E, Cervera N, Tarpin C, Nguyen C, Xerri L, Houlgatte R, Jacquemier J, et al. (2005) Gene expression profiling identifies molecular subtypes of inflammatory breast cancer. *Cancer Res* 65:2170–2178.
- Butcher NJ and Minchin RF (2010) Arylamine N-acetyltransferase 1 gene regulation by androgens requires a conserved heat shock element for heat shock factor-1. *Carcinogenesis* 31:820–826.
- Butcher NJ and Minchin RF (2012) Arylamine N-acetyltransferase 1: a novel drug target in cancer development. *Pharmacol Rev* 64:147–165.
- Butcher NJ, Tetlow NL, Cheung C, Broadhurst GM, and Minchin RF (2007) Induction of human arylamine N-acetyltransferase type I by androgens in human prostate cancer cells. *Cancer Res* 67:85–92.
- Cancer Genome Atlas Research Network; Weinstein JN, Collisson EA, Mills GB, Shaw KR, Ozenberger BA, Ellrott K, Shmulevich I, Sander C, and Stuart JM (2013) The cancer genome atlas pan-cancer analysis project. *Nat Genet* 45:1113–1120.
- Chen M, Beaven S, and Tontonoz P (2005) Identification and characterization of two alternatively spliced transcript variants of human liver X receptor alpha. *J Lipid Res* 46:2570–2579.
- Deng M, Brägelmann J, Kryukov I, Saraiva-Agostinho N, and Perner S (2017) FirebrowserR: an R client to the Broad Institute's Firehose pipeline. *Database (Oxford)* 2017:baw160.
- Dhaini HR, El Hafi B, and Khamis AM (2017) *NAT1* genotypic and phenotypic contribution to urinary bladder cancer risk: a systematic review and meta-analysis. *Drug Metab Rev Dec* 19: 1–12 DOI: 10.1080/03602532.2017.1415928 (published ahead of print).
- Ding L, Yan J, Zhu J, Zhong H, Lu Q, Wang Z, Huang C, and Ye Q (2003) Ligand-independent activation of estrogen receptor alpha by XBP-1. *Nucleic Acids Res* 31: 5266–5274.
- Doane AS, Danso M, Lal P, Donaton M, Zhang L, Hudis C, and Gerald WL (2006) An estrogen receptor-negative breast cancer subset characterized by a hormonally regulated transcriptional program and response to androgen. *Oncogene* 25: 3994–4008.

- Dolled-Filhart M, Rydén L, Cregger M, Jirstrom K, Harigopal M, Camp RL, and Rimm DL (2006) Classification of breast cancer using genetic algorithms and tissue microarrays. *Clin Cancer Res* **12**:6459–6468.
- Dressman MA, Walz TM, Lavedan C, Barnes L, Buchholtz S, Kwon I, Ellis MJ, and Polymeropoulos MH (2001) Genes that co-cluster with estrogen receptor alpha in microarray analysis of breast biopsies. *Pharmacogenomics J* **1**:135–141.
- Endo Y, Toyama T, Takahashi S, Sugiura H, Yoshimoto N, Iwasa M, Kobayashi S, Fujii Y, and Yamashita H (2011) High estrogen receptor expression and low Ki67 expression are associated with improved time to progression during first-line endocrine therapy with aromatase inhibitors in breast cancer. *Int J Clin Oncol* **16**:512–518.
- Endo Y, Toyama T, Takahashi S, Yoshimoto N, Iwasa M, Asano T, Fujii Y, and Yamashita H (2013) miR-1290 and its potential targets are associated with characteristics of estrogen receptor α -positive breast cancer. *Endocr Relat Cancer* **20**:91–102.
- Endo Y, Yamashita H, Takahashi S, Sato S, Yoshimoto N, Asano T, Hato Y, Dong Y, Fujii Y, and Toyama T (2014) Immunohistochemical determination of the miR-1290 target arylamine N-acetyltransferase 1 (NAT1) as a prognostic biomarker in breast cancer. *BMC Cancer* **14**:990.
- Farmer P, Bonnefoi H, Becette V, Tubiana-Hulin M, Fumoleau P, Parsimont D, Macgrogan G, Bergh J, Cameron D, Goldstein D, et al. (2005) Identification of molecular apocrine tumours by microarray analysis. *Oncogene* **24**:4660–4671.
- Finlin BS, Gau CL, Murphy GA, Shao H, Kimel T, Seitz RS, Chiu YF, Botstein D, Brown PO, Der CJ, et al. (2001) RERG is a novel ras-related, estrogen-regulated and growth-inhibitory gene in breast cancer. *J Biol Chem* **276**:42259–42267.
- Gomez BP, Riggins RB, Shajahan AN, Klimach U, Wang A, Crawford AC, Zhu Y, Zwart A, Wang M, and Clarke R (2007) Human X-box binding protein-1 confers both estrogen independence and antiestrogen resistance in breast cancer cell lines. *FASEB J* **21**:4013–4027.
- Guedj M, Marisa L, de Reynies A, Orsetti B, Schiappa R, Bibeau F, MacGrogan G, Lerebours F, Finetti P, Longy M, et al. (2012) A refined molecular taxonomy of breast cancer. *Oncogene* **31**:1196–1206.
- Hein DW, Doll MA, Nerland DE, and Fretland AJ (2006) Tissue distribution of N-acetyltransferase 1 and 2 catalyzing the N-acetylation of 4-aminobiphenyl and O-acetylation of N-hydroxy-4-aminobiphenyl in the congenic rapid and slow acetylator Syrian hamster. *Mol Carcinog* **45**:230–238.
- Heintz N, Sive HL, and Roeder RG (1983) Regulation of human histone gene expression: kinetics of accumulation and changes in the rate of synthesis and in the half-lives of individual histone mRNAs during the HeLa cell cycle. *Mol Cell Biol* **3**:539–550.
- Hoch RV, Thompson DA, Baker RJ, and Weigel RJ (1999) GATA-3 is expressed in association with estrogen receptor in breast cancer. *Int J Cancer* **84**:122–128.
- Hu Q, Li S, Chen C, Zhu M, Chen Y, and Zhao Z (2017) 17 β -Estradiol treatment drives Sp1 to upregulate MALAT-1 expression and epigenetically affects physiological processes in U2OS cells. *Mol Med Rep* **15**:1335–1342.
- Husain A, Barker DF, States JC, Doll MA, and Hein DW (2004) Identification of the major promoter and non-coding exons of the human arylamine N-acetyltransferase 1 gene (NAT1). *Pharmacogenetics* **14**:397–406.
- Husain A, Zhang X, Doll MA, States JC, Barker DF, and Hein DW (2007) Functional analysis of the human N-acetyltransferase 1 major promoter: quantitation of tissue expression and identification of critical sequence elements. *Drug Metab Dispos* **35**:1649–1656.
- Klinge CM (2012) miRNAs and estrogen action. *Trends Endocrinol Metab* **23**:223–233.
- Koong AC, Chauhan V, and Romero-Ramirez L (2006) Targeting XBP-1 as a novel anti-cancer strategy. *Cancer Biol Ther* **5**:756–759.
- Lemin AJ, Saleki K, van Lith M, and Benham AM (2007) Activation of the unfolded protein response and alternative splicing of ATF6 α in HLA-B27 positive lymphocytes. *FEBS Lett* **581**:1819–1824.
- Li B and Dewey CN (2011) RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* **12**:323.
- Licata LA, Hostetter CL, Crismale J, Sheth A, and Keen JC (2010) The RNA-binding protein HuR regulates GATA3 mRNA stability in human breast cancer cell lines. *Breast Cancer Res Treat* **122**:55–63.
- Lien HC, Hsiao YH, Lin YS, Yao YT, Juan HF, Kuo WH, Hung M-C, Chang KJ, and Hsieh FJ (2007) Molecular signatures of metaplastic carcinoma of the breast by large-scale transcriptional profiling: identification of genes potentially related to epithelial-mesenchymal transition. *Oncogene* **26**:7859–7871.
- Lupien M, Jeyakumar M, Hébert E, Hilmi K, Cotoir-White D, Loch C, Auger A, Dayan G, Pinard G-A, Wurtz J-M, et al. (2007) Raloxifene and ICI182,780 increase estrogen receptor- α association with a nuclear compartment via overlapping sets of hydrophobic amino acids in activation function 2 helix 12. *Mol Endocrinol* **21**:797–816.
- Maggiolini M, Carpino A, Bonfiglioli D, Pezzi V, Rago V, Marsico S, Picard D, and Andò S (2001) The direct proliferative stimulus of dehydroepiandrosterone on MCF7 breast cancer cells is potentiated by overexpression of aromatase. *Mol Cell Endocrinol* **184**:163–171.
- Maggiolini M, Donzé O, Jeannin E, Andò S, and Picard D (1999) Adrenal androgens stimulate the proliferation of breast cancer cells as direct activators of estrogen receptor α . *Cancer Res* **59**:4864–4869.
- Millikan RC (2000) NAT1*10 and NAT1*11 polymorphisms and breast cancer risk. *Cancer Epidemiol Biomarkers Prev* **9**:217–219.
- Miller LM, Doll MA, Cai J, States JC, and Hein DW (2012) NATb/NAT1*4 promotes greater arylamine N-acetyltransferase 1 mediated DNA adducts and mutations than NATa/NAT1*4 following exposure to 4-aminobiphenyl. *Mol Carcinog* **51**:636–646.
- Mulhngwi P and Klinge CM (2015) Roles for miRNAs in endocrine resistance in breast cancer. *Endocr Relat Cancer* **22**:R279–R300.
- Mulhngwi P and Klinge CM (2017) Identification of miRNAs as biomarkers for acquired endocrine resistance in breast cancer. *Mol Cell Endocrinol* **456**:76–86.
- Nishidate T, Katagiri T, Lin ML, Mano Y, Miki Y, Kasumi F, Yoshimoto M, Tsunoda T, Hirata K, and Nakamura Y (2004) Genome-wide gene-expression profiles of breast-cancer cells purified with laser microbeam microdissection: identification of genes associated with progression and metastasis. *Int J Oncol* **25**:797–819.
- Perou CM, Sørliet T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, et al. (2000) Molecular portraits of human breast tumours. *Nature* **406**:747–752.
- Ring BZ, Seitz RS, Beck R, Shastene WJ, Tarr SM, Cheang MC, Yoder BJ, Budd GT, Nielsen TO, Hicks DG, et al. (2006) Novel prognostic immunohistochemical biomarker panel for estrogen receptor-positive breast cancer. *J Clin Oncol* **24**:3039–3047.
- Ross DT, Scherf U, Eisen MB, Perou CM, Rees C, Spellman P, Iyer V, Jeffrey SS, Van de Rijn M, Waltham M, et al. (2000) Systematic variation in gene expression patterns in human cancer cell lines. *Nat Genet* **24**:227–235.
- Sadrieh N, Davis CD, and Snyderwine EG (1996) N-acetyltransferase expression and metabolic activation of the food-derived heterocyclic amines in the human mammary gland. *Cancer Res* **56**:2683–2687.
- Saito-Hisaminato A, Katagiri T, Kakiuchi S, Nakamura T, Tsunoda T, and Nakamura Y (2002) Genome-wide profiling of gene expression in 29 normal human tissues with a cDNA microarray. *DNA Res* **9**:35–45.
- Savci-Heijink CD, Halfwerk H, Koster J, and van de Vijver MJ (2016) A novel gene expression signature for bone metastasis in breast carcinomas. *Breast Cancer Res Treat* **156**:249–259.
- Sharma D, Handa RJ, and Uht RM (2012) The ER β ligand 5 α -androstane, 3 β ,17 β -diol (3 β -diol) regulates hypothalamic oxytocin (Oxt) gene expression. *Endocrinology* **153**:2353–2361.
- Sikora MJ, Cordero KE, Larios JM, Johnson MD, Lippman ME, and Rae JM (2009) The androgen metabolite 5 α -androstane-3 β ,17 β -diol (3 β -diol) induces breast cancer growth via estrogen receptor: implications for aromatase inhibitor resistance. *Breast Cancer Res Treat* **115**:289–296.
- Sim E, Abuhammad A, and Ryan A (2014) Arylamine N-acetyltransferases: from drug metabolism and pharmacogenetics to drug discovery. *Br J Pharmacol* **171**:2705–2725.
- Sørliet T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, et al. (2001) Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci USA* **98**:10869–10874.
- Stapp MW, Doll MA, Carlisle SM, States JC, and Hein DW (2018) Genetic and small molecule inhibition of arylamine N-acetyltransferase 1 reduces anchorage-independent growth in human breast cancer cell line MDA-MB-231. *Mol Carcinog* DOI: 10.1002/mc.22779 (published ahead of print).
- Stapp MW, Doll MA, Samuelson DJ, Sanders MA, States JC, and Hein DW (2017) Congenic rats with higher arylamine N-acetyltransferase 2 activity exhibit greater carcinogen-induced mammary tumor susceptibility independent of carcinogen metabolism. *BMC Cancer* **17**:233.
- Teng Y, Manavalan TT, Hu C, Medjakovic S, Jungbauer A, and Klinge CM (2013) Endocrine disruptors fludionoxil and fenhexamid stimulate miR-21 expression in breast cancer cells. *Toxicol Sci* **131**:71–83.
- Theodorou V, Stark R, Menon S, and Carroll JS (2013) GATA3 acts upstream of FOXA1 in mediating ESR1 binding by shaping enhancer accessibility. *Genome Res* **23**:12–22.
- Tiang JM, Butcher NJ, and Minchin RF (2010) Small molecule inhibition of arylamine N-acetyltransferase type I inhibits proliferation and invasiveness of MDA-MB-231 breast cancer cells. *Biochem Biophys Res Commun* **393**:95–100.
- Tong Q and Hotamisligil GS (2007) Developmental biology: cell fate in the mammary gland. *Nature* **445**:724–726.
- Tozlu S, Girault I, Vacher S, Vendrell J, Andrieu C, Spyros F, Cohen P, Lidereau R, and Bieche I (2006) Identification of novel genes that co-cluster with estrogen receptor α in breast tumor biopsy specimens, using a large-scale real-time reverse transcription-PCR approach. *Endocr Relat Cancer* **13**:1109–1120.
- Usary J, Llac V, Karaca G, Presswala S, Karaca M, He X, Langerød A, Kåresen R, Oh DS, Dressler LG, et al. (2004) Mutation of GATA3 in human breast tumors. *Oncogene* **23**:7669–7678.
- Wakefield L, Robinson J, Long H, Ibbitt JC, Cooke S, Hurst HC, and Sim E (2008) Arylamine N-acetyltransferase 1 expression in breast cancer cell lines: a potential marker in estrogen receptor-positive tumors. *Genes Chromosomes Cancer* **47**:118–126.
- Wang DY, Fulthorpe R, Liss SN, and Edwards EA (2004) Identification of estrogen-responsive genes by complementary deoxyribonucleic acid microarray and characterization of a novel early estrogen-induced gene: EEIG1. *Mol Endocrinol* **18**:402–411.
- Weihua Z, Lathe R, Warner M, and Gustafsson J-Å (2002) An endocrine pathway in the prostate, ER β , AR, 5 α -androstane-3 β ,17 β -diol, and CYP17B1, regulates prostate growth. *Proc Natl Acad Sci USA* **99**:13589–13594.
- Windmill KF, Gaedigk A, Hall PM, Samarantunga H, Grant DM, and McManus ME (2000) Localization of N-acetyltransferases NAT1 and NAT2 in human tissues. *Toxicol Sci* **54**:19–29.
- Witham KL, Minchin RF, and Butcher NJ (2017) Role for human arylamine N-acetyltransferase 1 in the methionine salvage pathway. *Biochem Pharmacol* **125**:93–100.
- Yan LZ, Hsiung HM, Heiman ML, Galski RA, Emmerson PJ, Hertel J, Flora D, Edwards P, Smiley D, Zhang L, et al. (2007) Structure-activity relationships of beta-MSH derived melanocortin-4 receptor peptide agonists. *Curr Top Med Chem* **7**:1052–1067.
- Zhang K, Gao L, Wu Y, Chen J, Lin C, Liang S, Su J, Ye J, and He X (2015) NAT1 polymorphisms and cancer risk: a systematic review and meta-analysis. *Int J Clin Exp Med* **8**:9177–9191.
- Zheng W, Deitz AC, Campbell DR, Wen WQ, Cerhan JR, Sellers TA, Folsom AR, and Hein DW (1999) N-acetyltransferase 1 genetic polymorphism, cigarette smoking, well-done meat intake, and breast cancer risk. *Cancer Epidemiol Biomarkers Prev* **8**:233–239.

Address correspondence to: Dr. David W. Hein, Kosair Charities CTR-Room 303, University of Louisville Health Sciences Center, 505 South Hancock Street, Louisville, KY 40202. E-mail: d.hein@louisville.edu