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The PPAR γ agonist efatutazone delays invasive progression and induces differentiation of ductal carcinoma in situ

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Abstract

Purpose—Ductal carcinoma in situ (DCIS) is a pre-invasive lesion of the breast considered a precursor of invasive ductal carcinoma. This study aimed to determine whether activated PPAR γ acts as a tumor suppressor in human DCIS progression.

Methods—We utilized the high-affinity PPAR γ agonist, efatutazone, to activate endogenous PPAR γ in a well-defined model for the progression of basal (triple negative) DCIS, MCFDCIS cells, cultured under 2D and 3D conditions. We studied the effects of activated PPAR γ on DCIS progression in MCFDCIS xenograft and C3(1)/Tag transgenic mice treated with 30 mg/kg of efatutazone.

Results—In vitro, efatutazone did not alter the MCFDCIS cell proliferation but induced phenotypic and gene expression changes, indicating that activated PPAR γ is able to differentiate MCFDCIS cells into more luminal and lactational-like cells. In addition, MCFDCIS tumorsphere formation in 3D was reduced by PPAR γ activation. In vivo, efatutazone-treated MCFDCIS tumors exhibited fat deposition along with upregulation of PPAR γ responsive genes in both epithelial and stromal compartments, suggesting features of milk-producing mammary epithelial cell differentiation. The efatutazone-treated lesions were less invasive with fewer CD44+/p63+ basal progenitor cells. PPAR γ activation downregulated Akt phosphorylation in these tumors, although the ERK pathway remained unchanged. Similar trends in gene expression changes consistent with lactational and luminal cell differentiation were observed in the C3(1)/Tag mouse model after efatutazone treatment.

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Compliance with ethical standards

Ethical standards The experiments in this study comply with the current laws of the country in which they were performed. The data sets during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interest The authors do not have a financial relationship with Daiichi Sankyo pharmaceutical company and declare that they have no conflict of interest.

Conclusions—Our data suggest that activation of the PPAR γ pathway differentiates DCIS lesions and may be a useful approach to delay DCIS progression.

Keywords

Efatutazone; PPAR γ ; DCIS; Breast cancer

Introduction

In 2016, over 60,000 ductal carcinoma in situ (DCIS) breast cancer cases were diagnosed in the U.S. [1]. DCIS is a precursor of Invasive Ductal Carcinoma (IDC) evolving through sequential stages, dependent upon genetic and/or epigenetic changes [2–7]. A significant portion of DCIS lesions will not progress to IDC [8–12], and to date, no diagnostic test is able to definitively predict DCIS outcome of patients. Thus, the extent of treatment of patients with DCIS is the subject of debate and the development of possible therapeutic strategies to prevent the progression of DCIS to more advanced disease is of clinical interest [13].

Peroxisome proliferator-activated receptor gamma (PPAR γ) is a member of the nuclear receptor family of ligand-activated transcription factors that bind to a defined response element in the promoter of target genes [14]. The endogenous ligands for PPAR γ are prostaglandins, fatty acids, and therapeutic modulators that include the thiazolidine (TZD) drugs such as rosiglitazone [14]. Activation of PPAR γ has profound effects on cellular differentiation, proliferation, apoptosis, metabolism, angiogenesis, and the inflammatory response in normal and cancer tissues [14]. PPAR γ influences multiple cellular signaling pathways [15–17]. PPAR γ functions as a tumor suppressor in rodent mammary tumors [18–20]. Consistent with this, the first-generation TZDs have some reported antiproliferative effects in mammary cancer cells [21]. However, in clinical trials, no significant benefit from these first generation TZDs was observed [22, 23]. More recently, newer, more potent, selective high-affinity, and anti-proliferative third-generation TZD agonists have been developed such as efatutazone (CS7017/RS5444) [16]. Efaturazone has demonstrated efficacy in the treatment of advanced malignancies [24]. The previous works in rodent models of breast cancer progression for the different subtypes of breast cancer have indicated that efaturazone plays a role in the proliferation, differentiation, inflammatory modulation, and metabolism of breast cancer cells [19, 25]. In the current study, we examined the effect of efaturazone on the progression of human DCIS to IDC cells on proliferation and differentiation in vitro, on tumorsphere formation in 3D, and tumor progression in vivo. For this study, we used the human MCFDCIS line which is derived from the normal mammary epithelial line MCF10A [26]. MCFDCIS cells have the rare ability to form DCIS lesions in vivo with gene expression patterns and pathology similar to human DCIS samples and undergo transition to IDC after transplantation in immune compromised mice [26–28]. Here, we report that short-term treatment with efaturazone delays progression of human DCIS, which causes features of luminal and mammary epithelial cell lactational differentiation, loss of basal progenitor cell characteristics, and reduction in Akt signaling. We propose that PPAR γ activation could be a useful approach to prevent progression of a subset of the early stage breast cancers.

Materials and methods

Cell lines and cell culture

MCF10A and MCFDCIS cell lines were obtained from Dr. Susette Mueller (Georgetown University) and were cultured as described [29, 30].

In vitro drug sensitivity assays

Effects on cell phenotype were analyzed in cells treated for 72 h with different concentrations of efatutazone (Daiichi Sankyo, Tokyo, Japan). Cell morphology was examined with an Olympus IX-71 inverted epifluorescence microscope. Cell proliferation was quantified using the crystal violet staining method [31]. Lipid droplet production was assessed microscopically and after staining with Oil Red O (Sigma-Aldrich Corp., MO, US). Differential expression of genes involved in lipid production and mammary cell differentiation were investigated using quantitative real-time PCR (below).

Matrigel tumorsphere formation assay

Matrigel tumorsphere formation assay was conducted as described [29, 30]. Tumorspheres were photographed at day 8 using an Olympus IX-71 Inverted epifluorescence microscope and the percentage of tumorsphere formation (diameter $\geq 100 \mu\text{m}$) was determined using Fiji software.

Animal models

Athymic nude mice were purchased from Envigo (Envigo, MD, US) and C3(1)/SV40 T-antigen transgenic mice (C3(1)/Tag) were donated by Dr. Jeffrey E. Green (National Cancer Institute, MD) [32]. Animals were housed in a pathogen-free environment with controlled temperature and humidity. All animal experiments were conducted in accordance with protocols approved by the Georgetown University IACUC.

In vivo drug treatment experiments

MCFDCIS xenografts in mice were performed as described [28]. Once MCFDCIS tumors became palpable, i.e., 2–3 weeks after tumor cell injection, efatutazone (Daiichi Sankyo, Tokyo, Japan) (30 mg/kg, or vehicle (dimethyl sulfoxide) control diluted in 0.5% methylcellulose and 0.2% Tween 80 was administered daily by oral gavage for 3 weeks. Similarly, 12-week-old female C3(1)/Tag mice were treated daily by oral gavage with efatutazone or vehicle control for 4 weeks. Mice were regularly monitored for signs of toxicity and the formation of palpable tumors. Animal weight and tumor area were measured twice per week. MCFDCIS xenograft and C3(1)/Tag mice were euthanized after 3 and 4 weeks of drug treatment, respectively. Tumor samples were fixed in 10% formalin and embedded in paraffin for histopathology or frozen for gene and protein expression analyses.

RNA isolation and quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) analyses of total RNA obtained from cells were performed as previously described [33]. Primers were designed using the IDT real-time PCR tool (Integrated DNA Technologies, Inc., IA) (Table 1). Gene expression levels were

quantified using the comparative cycle threshold method ($-C_T$), with relative mRNA levels determined as 2^{-C_T} normalized to human GAPDH, $\beta 2$ -microglobulin, or mouse actin as housekeeping genes.

Histology and immunohistochemistry analyses

Paraffin-embedded tissues were sectioned and stained by the Lombardi Comprehensive Cancer Center Histopathology & Tissue Shared Resource using standard protocols [34]. Primary antibodies (CD44, p63, Cytokeratin 8 (CK8), and PCNA) used for IHC are detailed in Table 2. Images were captured using an Olympus BX40 microscope. The percentage of tumor cells with nuclear staining for p63 and PCNA were scored within DCIS lesions, while CD44- and CK8-positive cells were quantified based on a complete membranous staining in five representative fields using Fiji software.

Protein extraction and western blot analysis

Protein lysates were isolated from frozen tumor tissues, and western blot analyses were carried out as previously described [35]. Primary antibodies (phospho-Akt (Ser473), Akt, phospho-ERK1/2 (Thr202/Tyr204), ERK1/2, and actin) used for western blot analysis are listed in Table 2. Relative band intensities were quantified by densitometry using Fiji software.

DNA microarray analysis

Total RNA was prepared from tumors as described above. RNA with an Integrity Number greater than 7.0 was used for microarray analysis which was carried out by the UCLA Neurosciences Genomics Core using Illumina HumanHT-12 v4 Expression BeadChip representing 47,000 well-annotated genes according to the manufacturer's instructions (Illumina, Inc., San Diego, CA). GenePattern open-source software (<http://software.broadinstitute.org/cancer/software/genepattern#>) was utilized to quantile-normalize the microarray expression data. Differential gene expression profiles were assessed by comparing changes between paired samples and ranking genes by their log2 fold changes.

Statistics

Statistical analyses were conducted using GraphPad Prism 5 software (Graph-Pad Software, Inc., San Diego, CA, USA). Comparisons among groups were performed using the unpaired Student's t test or the χ^2 test, and were considered statistically significant if p values < 0.05 .

Results

Effects of PPAR γ agonist efatutazone on human DCIS cells in vitro

We compared the effect of efatutazone on normal human epithelial cells (MCF10A) vs human MCFDCIS cells in vitro and did not observe gross changes in the cuboidal, epithelial morphology of either cell line at concentrations between 0.001 and 1 μ M, (Fig. 1a). However, we observed that $> 4\%$ of the MCFDCIS cells had visible lipid droplet accumulation (see arrows Fig. 1a, b). Identification of lipid droplets was confirmed with Oil Red O staining (Fig. S1a, b). Significantly less lipid accumulation was observed at any

concentration in the MCF10A line (Fig. 1a, b). Despite the effect on lipid droplet accumulation, the proliferation rate of the MCFDCIS or MCF10A cells after efatutazone treatment as measured by crystal violet staining (Fig. S1c) or real-time qPCR of PCNA mRNA levels (Fig. S1d) was variable and did not reach significance vs control for MCFDCIS cells at most of the concentrations used. Lipid accumulation is characteristic of activation of the PPAR γ pathway [36] and occurs in normal mammary epithelial cells as they differentiate into milk-producing cells [37, 38]. Consistent with the predominant effect of efatutazone being on the differentiation rather than proliferation of MCF10A or MCFDCIS cells, we found a robust induction of the PPAR γ -responsive gene fatty acid-binding protein 4 (FABP4) even at the lowest concentration of efatutazone (0.001 μ M) (Fig. 1c). Other PPAR γ responsive genes pyruvate dehydrogenase kinase 4 (PDK4) and perilipin 2 (PLIN2) [36] trended towards increased expression after efatutazone treatment in both cell lines, but the changes were not significant due mainly to the variability of the baseline expression of these genes (Fig. S1e, f). We next compared the pattern of mRNA expression of mammary epithelial cytokeratin differentiation markers after efatutazone treatment. Although we saw no significant changes in MCF10A cells, in MCFDCIS cells, we observed loss of expression of the basal cytokeratin markers CK6a, CK6b, and CK17 while gaining expression of the luminal marker CK8 [39] (Fig. 2). Interestingly, in MCFDCIS cells, efatutazone also increased the expression of other luminal markers such as progesterone receptor (PgR) and mucin 1 (MUC1)(Fig. 2). Despite the induction of PgR, we detected only low levels of estrogen receptor (ESR1) mRNA levels in the MCFDCIS cells that were unaltered by efatutazone treatment (Fig. 2). Overall, these data suggest that in vitro basal characteristics of MCFDCIS cells can be partially reverted to a more luminal and lactational-like phenotype by efatutazone treatment.

We and others have previously determined that reduction in the levels of MCFDCIS breast cancer initiating cells (BCIC) parallels changes in differentiation and inhibits the ability of the MCFDCIS cells to progress to invasive disease [28]. To determine the effect of efatutazone on the BCIC population and tumorigenic potential of MCFDCIS cells, we next examined tumorsphere formation. Tumorspheres are developed as an outgrowth of BCICs in culture and can be distinguished from aggregate formation (see arrows in Fig. 3a). At the lowest concentration of efatutazone (0.01 μ M), there was no significant reduction in number of tumorspheres (defined as > 100 μ m diameter) (Fig. 3a, b). However, at higher concentrations (0.1 μ M and above), the number of tumorspheres formed dropped significantly by greater than 30% ($p < 0.05$, Student's t test) (Fig. 3b), suggesting that efatutazone induces a loss of BCIC from the overall MCFDCIS population.

Effects of efatutazone on progression of DCIS in vivo

To examine effect of efatutazone on MCFDCIS tumor development and progression to invasive disease in vivo, MCFDCIS cells were injected subcutaneously into nude mice treated with either 30 mg/kg of efatutazone or vehicle daily after MCFDCIS tumors became palpable, i.e., 2–3 weeks after the MCFDCIS cells were implanted. This dose is considered an appropriate dose for this type of studies based on the previous efficacy and toxicity studies [16, 19, 40], and we observed no decrease in body weight of the mice during the study (Fig. S2a). Over 40 days, tumors in the efatutazone or vehicle treated groups grew

rapidly (Fig. S2b). The overall mass and volume of the MCFDCIS tumors was reduced only slightly by efatutazone (Fig. S2b, d), although the glands were thicker after efatutazone treatment possibly due to lipid deposition (Fig. S2c). However, histopathology of the tumors harvested after 3 weeks of efatutazone treatment showed less invasion and necrosis in the efatutazone-treated animals indicating delayed tumor progression (Fig. 4a, b). In addition, the efatutazone-treated animals had lesions with lipid-filled droplets in them (arrowed Fig. 4a, b, and d). The efatutazone-treated MCFDCIS tumors had significant reduced CD44 and p63 staining and a tendency for an increase in CK8 staining but no change in PCNA staining after efatutazone treatment (Fig. 4c). These changes coupled with observed increases in FABP4 and PLIN2 mRNA expression in both epithelial and stromal compartments (Fig. 5a, b) are consistent with efatutazone inducing differentiation into milk-producing and more luminal-like mammary epithelial cells with possible loss of BCIC within the MCFDCIS lesions in vivo.

To study genes that might be involved in efatutazone effects on MCFDCIS lesions, we analyzed changes in human global gene expression patterns in tumors from treated vs untreated animals (Fig. S2e). A number of genes were upregulated by efatutazone treatment including the PPAR γ -responsive gene FABP4 as well as genes that play a predominant role in inflammation such as S100A7, S100A8 [41], and LCN2 [42]. Conversely, genes downregulated by efatutazone are involved in cell proliferation and motility (e.g., IGFBP6 and MYLC2PL) [43].

PPAR γ has been shown to regulate a number of cellular signaling pathways related to proliferation and differentiation such as ERK and Akt [25, 44–46]. To determine which signaling pathways were influenced in our study, we analyzed tumor cell lysates from eight different tumors (Fig. 6). Efutazone was able to prevent the activation of the Akt pathway as evidenced by > 50% reduction in phospho-Akt (Ser 473) (Fig. 6a). In contrast, the phospho-ERK (Thr202/Tyr204) levels were unaltered by the administration of drug (Fig. 6b).

Finally, we determined if the effects of efatutazone on DCIS progression could be observed in a different model of DCIS progression. The C3(1)/Tag-driven model of mouse mammary tumorigenesis represents a basal (triple negative) model of mammary cancer progression in mice which progress through hyperplastic alveolar nodules at about 8–12 weeks of age that represent DCIS-like in situ lesions which will progress to invasive cancer 100% of the time [32]. Female C3(1)/Tag mice at 12 weeks of age were treated for 4 weeks with efatutazone or vehicle control with no change in weight (Fig. S3a). The mammary glands (MG2 and MG4) (Fig. S3b) were examined for histopathology and gene expression changes (Fig. S3c, d). Similar to the MCFDCIS model, we found that there was a trend towards delayed DCIS progression of hyperplastic-like lesions (Fig. S3c), although this only reached significance at $p < 0.06$ because of the variable rate of conversion to DCIS in the vehicle-treated controls. Nevertheless, the mammary glands showed changes in gene expression consistent with differentiation into milk-producing and luminal epithelial cells similar to the MCFDCIS tumors (Fig. S3d).

Discussion

Here, we show that activation of PPAR γ with efatutazone can cause delay in progression of human DCIS to invasive breast cancer in vivo. Efaturazone treatment causes the basal MCFDCIS cells in vitro and in vivo to become more luminal and exhibit signs of normal mammary epithelial cell lactational differentiation including fat deposition. The previous studies have shown that PPAR γ agonist inhibition of mammary cancer proliferation can be a contributing factor to anti-tumorigenic effects [21, 25]. However, efaturazone had no significant effect on MCFDCIS proliferation in vitro and in vivo. This is also consistent with the overall size of the tumors being unaltered by efaturazone treatment. Thus, it is likely that the effects of PPAR γ activation on differentiation of the MCFDCIS cells are the major contributory factor to the slowing of the progression rate of DCIS in the tumors in vivo.

Efaturazone has been shown previously to cause luminal differentiation associated with increased ESR1 in basal mammary epithelial cells in mice with a loss of full length BRCA1 expression plus loss of one germline copy of p53 [25]. Increased ESR1 expression has also been observed with PPAR γ agonists in other rodent studies [19]. We did not observe significant changes in expression of the ESR1 gene after efaturazone treatment, suggesting that changes in ESR1 with efaturazone may be only in rodent mammary epithelium. Our data on the effect of efaturazone in reducing tumorsphere formation and changes in gene expression in MCFDCIS lesions argue that activation of PPAR γ causes loss or differentiation of a tumor initiating cell population. Thus, overall differentiation changes, which may cause the loss of more aggressive progenitor populations, likely contribute to the effect of PPAR γ activation on DCIS progression and are possibly mediated by loss of Akt activation. Decreased Akt phosphorylation has been previously reported in a mouse model of BRCA1 mutation after efaturazone treatment [25]. Conversely, the absence of ERK activation and proliferative changes after efaturazone treatment in MCFDCIS cells may be related, since ERK activation by PPAR γ has been associated with proliferation of other cancers [47].

Analysis of overall human gene expression changes in the MCFDCIS tumors in efaturazone treated mice showed changes in gene expression related to lipid production as well as downregulation of genes in the IGF (insulin like growth factor) pathway such as IGFBP6 that have been associated with PPAR γ activation and effects [48]. Interestingly, changes of expression of genes involved in the inflammatory response pathways such as S100A7, S100A8, and LCN2 were also induced by efaturazone. Thus, effects on the immune environment may also play a role in mammary epithelial cells differentiation in the presence of efaturazone. Our study taken together with the previous studies on PPAR γ effects in rodent mammary cancer [25] indicates that a broad range of basal type breast cancer is responsive to efaturazone treatment at early stages.

We observed minimal toxicity for efaturazone in this short duration study which is consistent with the previous safety profile reports [24]. Although efaturazone failed to eradicate DCIS lesions during the time period of our observations, it would be interesting to assess the effect of higher doses, more prolonged treatment, and the effects of efaturazone in combination

with other preventive agents such as tamoxifen on reducing overall incidence of invasive breast cancer in human DCIS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Efaturazone treatment enhances features of mammary epithelial cell lactational differentiation. **a** Representative phase-contrast images of MCF10A (upper panel) and MCFDCIS cells (lower panel) treated 72 h with vehicle or increased concentrations of efaturazone. Scale bar = 100 μ m. Cells containing visible lipid droplets are indicated by arrows. **b** Quantification of the percentage of MCF10A and MCFDCIS cells with visible lipid droplets in the presence of increased concentrations of efaturazone using Fiji software. **c** qRT-PCR analysis of human FABP4 mRNA levels in MCF10A (upper panel) and MCFDCIS cells (lower panel) treated 72 h with vehicle or increased concentration of

efatutazone. Mean $\pm$ SEM (Student's t test; $n = 3$; $*p < 0.05$ vs vehicle; $**p < 0.01$ vs vehicle; $***p < 0.001$ vs vehicle)

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Fig. 2.

Efatutazone affects the expression of differentiation markers in MCFDCIS cells. Fold changes of human CK6a & b, CK17, CK8, MUC1, PgR, and ESR1 mRNAs in MCFDCIS cells treated 72 h with 0.01 μ M efatutazone analyzed by qRT-PCR and compared to cells treated with vehicle. Mean $\pm$ SEM (Student's *t* test; *n* = 3; **p* < 0.05 vs vehicle; ***p* < 0.01 vs vehicle)

Fig. 3.

Efaturazone treatment leads to a reduction of MCFDCIS tumorsphere formation. **a** Representative phase-contrast images of MCFDCIS cultivated on Matrigel for 8 days in the presence of vehicle (*a*), 0.01 μM (*b*), 0.1 μM (*c*), and 1 μM efaturazone (*d*). Tumorspheres with diameter 100 μm are indicated by arrows. Scale bar = 200 μm. **b** Quantification of the percentage of MCFDCIS tumorspheres with diameter 100 μm treated with increased concentrations of efaturazone using Fiji software. Mean ± SEM (Student's *t* test; *n* = 5; **p* < 0.05 vs vehicle; ***p* < 0.01 vs vehicle)

Fig. 4.

Efatutazone-treated DCIS lesions are less invasive with fewer CD44+/p63+ basal progenitor cells. **a** Representative hematoxylin and eosin-stained images of tissue sections from MCFDCIS xenograft tumors treated 3 weeks with vehicle or 30 mg/kg efatutazone. (*a, b*) DCIS lesions, INV invasive lesions. DCIS lesions containing lipid droplets are indicated by arrows. Scale bar = 200 μ m. (*c, d*) Lipid droplets are indicated by arrows. Scale bar = 100 μ m. **b** Percentage of DCIS, invasive, and necrotic lesions determined by microscopic area measurements using Fiji software. (χ^2 test; $n = 6$; $**p < 0.01$ vs vehicle). **c** Representative IHC images of CD44, p63, CK8, and PCNA staining in xenografted tumor

tissues obtained from MCFDCIS cells and treated with 30 mg/kg efatutazone (lower panel) or not (upper panel). The percentage of positive cells is indicated (upper right corner). Mean $\pm$ SEM (Student's *t* test; *n* = 4; **p* < 0.05 vs vehicle; ***p* < 0.01 vs vehicle). Scale bar = 200 μ m

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Fig. 5. Efaturazone regulates the expression of genes associated with lipid accumulation in MCFDCIS xenograft tumors. qRT-PCR analysis of **a** human and **b** mouse FABP4, PLIN2, and PDK4 mRNA levels in MCFDCIS tumor tissues treated 3 weeks with vehicle or 30 mg/kg efaturazone. Mean $\pm$ SEM (Student's *t* test; *n* = 10; **p* < 0.05 vs vehicle; ****p* < 0.001 vs vehicle)

Fig. 6.

Efatutazone treatment downregulates Akt phosphorylation in MCFDCIS xenograft tumors. **a** Representative western blots showing phospho-Akt (Ser 473), Akt, and **b** phospho-ERK1/2 (Thr202/Tyr204), ERK1/2, and actin expression in protein lysates obtained from MCFDCIS xenograft tumors treated 3 weeks with vehicle or 30 mg/kg efatutazone (left panels). The ratio of phospho-Akt to total Akt and phospho-ERK1/2 to total ERK1/2 was quantified using Fiji (right panels). Mean $\pm$ SEM (Student's *t* test; *n* = 8; **p* < 0.05 vs vehicle)

Table 1

Primers used for qRT-PCR analyses

Gene	Forward primer	Reverse primer
<i>hPCNA</i>	5'- GTCTCTTTGGTGCAGCTCA -3'	5'- ATCTTCGGCCCTTAGTGTAAATG -3'
<i>hFABP4</i>	5'- CATGTGCAGAAATGGGATGG -3'	5'- AACTTCAGTCCAGGTCAACG -3'
<i>hPDK4</i>	5'- TGAGACTCGCCAACATTCTG -3'	5'- TCTGGTCATCTGGGCTTTTC -3'
<i>hPLIN2</i>	5'- GCTCCATTCTACTGTTACCTG -3'	5'- CTCCTTTTCCACTCTACCCATG -3'
<i>hMUC1</i>	5'- TCCTCACAGTGCTTACAGTTG -3'	5'- AGAAAGAGACCCCACTAGACA -3'
<i>hCK8</i>	5'- AAGGCACAGTACGAGGATATTG -3'	5'- AGATCTCAGTCTTTGTGCGC -3'
<i>hESR1</i>	5'- ATCTCGGTTCCGCATGATGAATCTGC -3'	5'- TGCTGGACAGAAATGTGTACTACTCCAGA -3'
<i>hPgR</i>	5'- ATTACCAGTGTTCCCGTCTTC -3'	5'- GTGCTCCAATAGTTCCTGTAC -3'
<i>hCK6a</i>	5'- TCCCCTCAACCTGCAAATC -3'	5'- CCACTTTGTTTCCAGAACCTTG -3'
<i>hCK6b</i>	5'- AGCTGAGAAACATGCAGGAC -3'	5'- GCTTGCAGTTCAACCTTGTTT -3'
<i>hCK17</i>	5'- AACAAAGATCCTCACAGCCAC -3'	5'- CCATTGATGTCGGCCTCC -3'
<i>hGAPDH</i>	5'- CCCACATGGCCTCCAAGGAGTA -3'	5'- GTCTACATGGCAACTGTGAGGAGG -3'
<i>hβ2m</i>	5'- CTTAGCTGTGCTCGCGTACTCT -3'	5'- CCATTCTCTGCTGGATGACGTGAGT -3'
<i>mFABP4</i>	5'- GACAGGAAGGTGAAGAGCATC -3'	5'- GTCACGCCTTTCATAACACATTC -3'
<i>mPDK4</i>	5'- AGTGACTCAAAGACGGGAAAC -3'	5'- GTGTGAGGTTAATTCTGGCG -3'
<i>mPLIN2</i>	5'- GGATAAGCTCTATGTCTCGTGG -3'	5'- GTCTGGCATGTAGTCTGGAG -3'
<i>mCK8</i>	5'- TGTGAGCCCTTGAAGC -3'	5'- GGTCTCCAGCATCTTGTCTG -3'
<i>mCK18</i>	5'- ACACCAACATCACAAGGCTG -3'	5'- TTCCACAGTCAATCCAGAGC -3'
<i>mESR1</i>	5'- AACCGCCCATGATCTATTCTG -3'	5'- AGATTCAAGTCCCCAAAGCC -3'
<i>mPgR</i>	5'- TGAGCCTGATGGTGTGTTGG -3'	5'- ACAGCGAGTAGAATGACAGC -3'
<i>mACTIN</i>	5'- ATTTCTGAATGGCCAGGTC -3'	5'- GCTGCCTCAACACCTCAA -3'

MUC1 mucin 1, *CK* cytokeratin, *ESR1* estrogen receptor 1, *PgR* progesterone receptor, *β2m* β2-microglobulin

Table 2

Antibodies used for immunohistochemistry and western blot analyses

Antibody	Source	References	Clone	Species	Dilution
<i>CD44</i>	ThermoFisher Scientific Inc.	MA1-39422	SPM521	Mouse	1:75
<i>P63</i>	Santa Cruz Biotechnology	sc-8431	4A4	Mouse	1:400
<i>Cytokeratin 8</i>	Abcam	ab59400		Rabbit	1:100
<i>PCNA</i>	Santa Cruz Biotechnology	sc-7907	FL-261	Rabbit	1:300
<i>Phospho-Akt (Ser473)</i>	Cell Signaling	4051	587F11	Mouse	1:1000
<i>Total Akt</i>	Cell Signaling	9272		Rabbit	1:1000
<i>Phospho-ERK1/2 (Thr202/Tyr204)</i>	Cell Signaling	9101		Rabbit	1:1000
<i>Total ERK1/2</i>	Cell Signaling	9102		Rabbit	1:1000
<i>Actin</i>	Millipore	MAB1501	C4	Mouse	1:3000