HORM CANC (2012) 3:26–36
DOI 10.1007/s12672-011-0083-5

Estrogen Receptor-beta Mediates the Protective Effects of Aromatase Induction in the MMTV-Her-2/neu x Aromatase Double Transgenic Mice

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Published online: 18 October 2011

Abstract Breast cancers amplified for the tyrosine kinase receptor Her-2/neu constitute ~30% of advanced breast cancer cases, and are characterized by hormone independence and aggressive growth, implicating this pathway in breast oncogenesis. The induction of Her-2/neu leads to tumor development in 60% of transgenic mice. We have previously examined the effects of estrogen in the MMTV-Her-2/neu background by generating the MMTV-Her-2/neu x aromatase double transgenic mouse strain. MMTV-Her-2/neu x aromatase mice developed fewer mammary tumors than the Her-2/neu parental strain. Our present data show the induction of several estrogen-related genes, including the tumor suppressors BRCA1 and p53, and a decrease in several angiogenic factors. The phosphorylated forms of MAPK p42/44 and AKT were lower in the MMTV-Her-2/neu x aromatase double transgenic mice compared to the MMTV-Her-2/neu parental strain; conversely, phospho-p38 levels were higher in the double transgenic strain. The ERβ-selective antagonist THC reversed these changes. The regulation of these factors by ERβ was confirmed in clones of MCF7 breast cancer cells overexpressing Her-2/neu in combination with ERβ, suggesting that ERβ may play a direct role in regulating MAPK and AKT pathways. In summary, the data suggest that ERβ may play a major role in decreasing tumorigenesis and that it may affect breast cancer cell proliferation and survival by altering MAPK and AKT activation as well as modulation of tumor suppressor and angiogenesis factors. Treatment with selective ERβ agonist may provide therapeutic advantages for the treatment and prevention of breast cancer.

Keywords Estrogen receptor beta · Breast cancer · Aromatase · Her2/neu

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Introduction

Breast cancer is largely a hormone-dependent malignancy in which estrogen plays a critical role in breast cancer development. The actions of estrogen through its receptor (ER) have been shown to be instrumental in normal mammary gland programming as well as involved in the initiation of mammary neoplasia. Mice lacking the ERα subtype (ERKO) exhibited stunted mammary development with only rudimentary mammary ducts [1]. On the other hand, the ERβ knockout mice exhibited normal mammary development [2]. The data suggest that the ERα subtype is

responsible for the estrogenic actions resulting in mammary ductal growth and proliferation. The effectiveness of tamoxifen, a selective ER modulator (SERM) with antagonist activity in breast tissue, in the treatment of breast cancer attests to the importance of ER in breast tumorigenesis [3, 4].

Local estrogen production due to in situ induction of aromatase, which catalyzes the conversion of androgens to estrogens, could play an important role in mammary oncogenesis. Aromatase expression was found to be increased adjacent to tumors and in tumor-bearing breast quadrants [5, 6]. These observations led to the development and clinical use of aromatase inhibitors (AI) [7–10]. These drugs not only target ER-mediated carcinogenesis, but also inhibit estrogen biosynthesis and may reduce the genotoxic effects of estrogen [11, 12]. In our previous studies, induction of aromatase in transgenic mice (MMTV-aromatase) resulted in induction of cell cycle-promoting factors and increased mammary epithelial cell proliferation, leading to ductal hyperplasia and dysplasia [13, 14]. Treatment with AI resulted in the abrogation of these precancerous lesions [15, 16].

While the majority of breast tumors express ER and about 70% respond to the antiestrogen tamoxifen, prolonged treatment leads to drug resistance in spite of continued ER expression. Hormone independence of breast cancer could occur with disease progression and is generally associated with induction of growth factor signaling such as the Her-2/neu pathway. Her-2/neu, a tyrosine kinase receptor belonging to the epidermal growth factor receptor (EGFR) family, is amplified in about 30% of advanced breast cancers [17]. Anti-estrogen drugs, such as tamoxifen, which are used in the treatment of ER-positive breast cancers, are ineffective in the treatment of Her-2/neu-positive compared to Her-2/neu-negative malignancies [8]. Similarly, aromatase inhibitors (AIs), which block estrogen production, are effective in the treatment of ER-positive breast cancers, but exhibit reduced efficacy in Her-2/neu-positive tumors. Nonetheless, AIs are more effective in Her-2/neu-positive tumors that are positive for ER and/or progesterone receptor (PR) compared to Her-2/neu negative for these steroid receptors [8, 18].

The roles of estrogen and growth factor pathways in breast cancer have been examined extensively using animal transgenic models. The MMTV-Her-2/neu transgenic mouse model exhibits tumor formation in 60% of mice [19]. The MMTV-aromatase transgenic mice, which produce elevated levels of estrogen in mammary glands that is associated with increased ER expression, develop mammary hyperplasia as well as dysplastic lesions characteristic of pre-malignant transformation, but do not progress into tumors unless primed by carcinogens such as DMBA [13, 20]. To examine the interaction between Her-2/neu and aromatase induction in breast cancer, we developed double transgenic mice overexpressing both these factors (MMTV-Her-2/neu x aromatase) [21]. Our recent evidence, using Her-2/neu x aromatase double transgenic mice, demonstrated that induction of aromatase in the Her-2/neu background resulted in drastically and significantly reducing tumor development [21]. The data suggest that estrogen induction due to aromatase over-expression is protective against Her-2/neu-driven mammary tumors. In addition, we have observed that ERβ is induced in double transgenic mice and that treatment with the ERα agonist and ERβ antagonist THC induces ductal proliferation in these mice [21]. The data suggested that ERβ induction in the double transgenic mice may play a protective role in mammary tumor development, which is consistent with other published evidence showing a protective role for ERβ in breast cancer cell lines [22, 23]. The protective role of estrogen and progesterone was also seen in the MMTV-Her-2/neu transgenic mice and a p53-null mammary transplant model [24]. In addition, evidence from in vitro studies demonstrated that transfection of ERβ but not ERα resulted in the nuclear translocation of the tumor suppressor p53, leading to increased p53 transcriptional activity, which suggests that ERβ induction may directly activate p53 [25]. These studies combined with ours support the protective role of estrogen exposure in mammary oncogenesis.

In this report, we have further examined the protective role of aromatase in the Her-2/neu x aromatase double transgenic mice and the protective role of ERβ in MCF7 breast cancer cell clones that overexpress Her-2/neu, aromatase and ERβ. Our results suggest that ERβ may negatively affect breast cancer cell proliferation through the modulation of MAPK and angiogenic pathways as well as induction of estrogen-responsive tumor suppressor genes. The data also suggest that selective modulators of ERβ may be used in the treatment of Her-2/neu positive breast cancers.

Materials and Methods

Reagents Tetrahydrochrysene (THC) and 2,3-bis(4-hydroxyphenyl)—propionitrile (DPN) were purchased from Sigma-Aldrich (St Louis, MO) and Tocris (Ellisville, MO), respectively. The antibiotic Blastidin was purchased from Invitrogen (Carlsbad, CA).

Transgenic Mice and Treatment The generation and maintenance of transgenic mice were described previously [21]. Briefly, the aromatase×HER-2/neu mice were obtained by mating the parental strains. Female mice positive for both HER-2/neu and aromatase transgene along with individual parental types of the same genetic background (FVB/N) were used for the various analyses. Experimental and controls groups consisted of at least 90 animals per group. Tumorigenesis was observed for 16 months (almost twice

the mean tumor incidence period ~28 weeks). Tumor-free survival was assessed by the Kaplan-Meier plot and statistical analysis by the generalized Wilcoxon test.

Double transgenic mice (n=6) were treated with the selective ERβ antagonist THC at 500 μg/day/mouse. THC was administered daily by subcutaneous injections for 3 weeks beginning at the age of 5 weeks. The control group (n=6) received vehicle only for 3 weeks. At the end of the experimental period, mice were killed, and mammary tissue was used for biochemical analyses as described below. Mice were housed in a centralized animal facility accredited by the AAALAC and USDA, and maintained according to the recommendations established in the NIH Guide for the Care and Use of Laboratory Animals.

Generation of MCF-7 Clones Overexpressing Her-2/neu and Combination of Aromatase and/or ERβ All cells were cultured in RPMI media containing 10% FBS. MCF7Her-2/neu was obtained from Dr. Kaladhar Reddy, Wayne State University, WI. Nucleofection-based transfection (Amaxa, Gaithersburg, MD) was performed with expression vectors containing aromatase or ERβ cDNA in conjunction with the antibiotic Blastidin for selection of transfected cells. Clones were picked and cultured in appropriate media containing Blastidin. Her-2/neu, aromatase, and ERβ expression was determined by real-time PCR. Three distinct clones were finally obtained: MCF7Her-2-ERβ, MCF7Her-2-aromatase, and MCF7Her-2-aromatase-ERβ.

Real-time RT-PCR RNA was isolated from tissue and cells using Qiagen lipid tissue and RNeasy, respectively, according to manufacturer's protocols (Qiagen, Valencia, CA). An in-column DNase treatment was carried as per Qiagen's protocol to eliminate trace DNA contamination. Pathway-specific RT-PCR arrays (RT^2 Profiler™) were obtained from SuperArray (Frederick, MD). The reverse transcription (RT) protocol was carried out by single shot (Clontech, Mountain View, CA). Real-time RT-PCR was carried using smartmix PCR beads (Cepheid, Sunnyvale, CA) with 0.25 X SybrGreen in the Cepheid SmartCycler.

Protein Analysis Protein extracts from mammary glands were prepared by homogenizing the tissue in lysis buffer. Equal amounts (generally 60–75 μg) of protein from each sample were separated on a denaturing polyacrylamide gel and transferred to a nylon membrane. The protein-bound membranes were then incubated for at least 4 h at room temperature with Tris-buffered saline (TBS) containing 0.05% Triton X-100 (TBST) and 5% nonfat dry milk to block non-specific antibody binding. The membranes were then incubated with respective primary antibodies in TBST milk overnight at 4°C, and specific binding was visualized by using species-specific IgG followed by enhanced chemiluminescent detection (ECL kit; Amersham, Piscataway, NJ) and exposure to ECL X-ray film. Specific antibodies were obtained from different commercial sources: β-actin (Santa Cruz Biotech, Santa Cruz, CA); p53 and pRB (Santa Cruz Biotech, Santa Cruz, CA); cyclin D1, p21, and p27 (Labvision-Neomarkers, Fremont, CA); ERα and ERβ (Upstate Cell Signaling Solutions, Charlottesville, VA); pRb (BD Biosciences, San Jose, CA); phospho-AKT, phospho-p42/44, and phospho-p38 (Cell Signaling Technologies, Danvers, MA); and vincuillin (Sigma-Aldrich, St Louis, MO). *Chromatin Immunoprecipitation (ChIP) Assay.* ChIP assays were performed using MCF-7, MCF7Her-2-ARO, and MCF7Her-2-ARO-ERβ (2×10^6 cells). Chromatin was cross linked using 1.0% formaldehyde for 10 min at 37°C, and the chromatin was prepared as described previously [26]. Fragmented chromatin was immunoprecipitated using ERβ antibodies, and ERβ recruitment was verified by PCR using target gene-specific primers for the p53 promoter: forward 5′- GGATCCAGCTG AGAG CAAAC 3′ and reverse 5′- GTGTCACCGTCGTGGAAAG 3′. For in vivo ChIP assays, mammary gland tissues were used from HER-2/neu and HER-2/neu x aromatase transgenic mice. Mammary gland was harvested immediately after euthanizing the mice. Tissues were minced finely and crosslinked using paraformaldehyde as described earlier [27]. Homogenate was filtered through a 100-μm nylon membrane strainer, required cells were pooled, and chromatin was prepared as described previously [26]. Fragmented chromatin was immunoprecipitated using ERβ antibody, and ERβ recruitment was verified by PCR using target gene-specific primers. p53 primers used in the ERβ ChIP are: mp53-330F 5′- GGCGGTCCACTTACGATAAA 3′ and mp53-10R 5′-AACTTTAGCCAGGGTGAGCA 3′

Immunocytochemistry (IHC) IHC was performed as described previously [28]. MCF-7 and its derived cells were transfected with vectors that express ERβ in fusion with GFP. Transfected cells plated on coverslips were fixed with a modified Zamboni's fixative and incubated with primary antibodies (rabbit polyclonal anti-p53; AbD Serotec, Oxford, UK) followed by Alexa568-conjugated anti-rabbit antibodies (Invitrogen; Carlsbad, CA). Confocal scanning analysis was performed on the mounted coverslips using an Olympus FV300 laser scanning confocal microscope in accordance with established methods, utilizing sequential laser excitation to minimize fluorescent emission bleed-through.

Statistical Analysis

Data from at least three separate experiments were polled and analyzed using Student's t-test. When individual experi-

ments were analyzed, the results were indistinguishable from those obtained from the pooled data. Differences were considered to be statistically significant at $P<0.05$.

Results

Aromatase Overexpression Increases Tumor-Free Survival in the Her-2/neu Transgenic Background

Our previous data revealed that the MMTV-aromatase transgenic mice develop preneoplastic lesions but not mammary tumors [13]. Sub-carcinogenic DMBA treatment resulted in tumor development in these mice [20]. To examine the effects of in situ estrogen production due to aromatase overexpression on growth factor-derived mammary tumorigenesis, we crossed aromatase transgenic mice with MMTV-Her-2/neu transgenic mice [21]. These transgenic mice exhibited significantly lower tumor development (<5%) than the Her-2/neu parental strain (60%). The data suggested that aromatase overexpression had protective effects on Her-2/neu-induced mammary tumors [21]. In this study, we examined tumor-free survival in the double transgenic mice compared to the parental strains and the underlying biochemical events that may lead to reduced tumor formation. Figure 1 shows that the tumor-free survival rate is increased in the MMTV-Her-2/neu x aromatase mice as compared to the MMTV-Her-2/neu strain.

Aromatase Overexpression Results in an Anti-Tumorigenic Gene Expression Profile

The observed differences between the Her-2/neu x aromatase double transgenic mice and the MMTV-Her-2/neu mice were not due to alterations in transgene expression, as we have shown that there were no significant change in the transgenic expression of aromatase or Her-2/neu expression at the RNA or protein levels [21]. To examine the biochemical changes between the double transgenic and the Her-2/neu mice, we performed pathway-specific (estrogen-responsive and breast cancer-related genes) real-time RT-PCR array analysis on RNA from mammary glands of these mice. The data for the RT-PCR arrays are summarized in supplementary Table 1. Validation of some of these genes was carried out using standard real-time RT-PCR. Figure 2a shows induction in expression of several anti-tumorigenic factors, notably BRCA1 (38 fold), in the double transgenic strain compared to the Her-2/neu parental strain.

Because angiogenesis is associated with tumor development, we examined a panel of angiogenic factors in the transgenic mice. The data showed a decrease in angiopoietin 1, TIE1, TIE2, and flt 1 in the double transgenic mice compared to the Her-2/neu parental strain, which suggests that decreased expression of angiogenic factors due to aromatase induction may lead to a decrease in angiogenesis and consequently decreased mammary oncogenesis in the double transgenic mice (Fig. 2b). We have also examined a panel of apoptosis-related genes (Fig. 2c). Interestingly, the pro-apoptotic factor Bax and to a lesser degree Bak were decreased in the MMTV-Her-2/neu x aromatase mice compared to the MMTV-Her-2/neu parental strain. On the

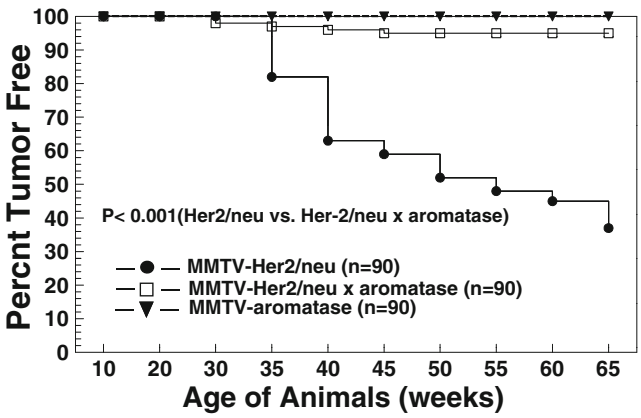

Fig. 1 Increased survival of MMTV-Her-2/neu x aromatase double transgenic mice compared to MMTV-Her2/neu mice: Tumor-free survival is represented as Kaplan-Meier curve. No tumors are seen in MMTV-aromatase mice in the absence of carcinogens such as DMBA exposure [20]

Table 1 Summary of PCR array gene expression

Gene	Her2Aro/Her2
Bc12	3.7
BRCAI	3.8
Ccndl	0.19
Ccne2	0.08
CIdn7	0.07
FasI	2.1
EGFR	0.5
Itga6	0.2
Itgb4	0.35
Jun	0.09
Kit	0.33
KIklb4	6.5
Mud	0.27
Nfyb	0.28
Ngfb	0.47
Ngfr	3.03
Nmel	0.47
Pappa	0.27
PgR	0.57
Tiel	0.38
VEGFa	0.13

Change in gene expression for Her2/Aro double transgenic mouse relative to parental Her2 strain

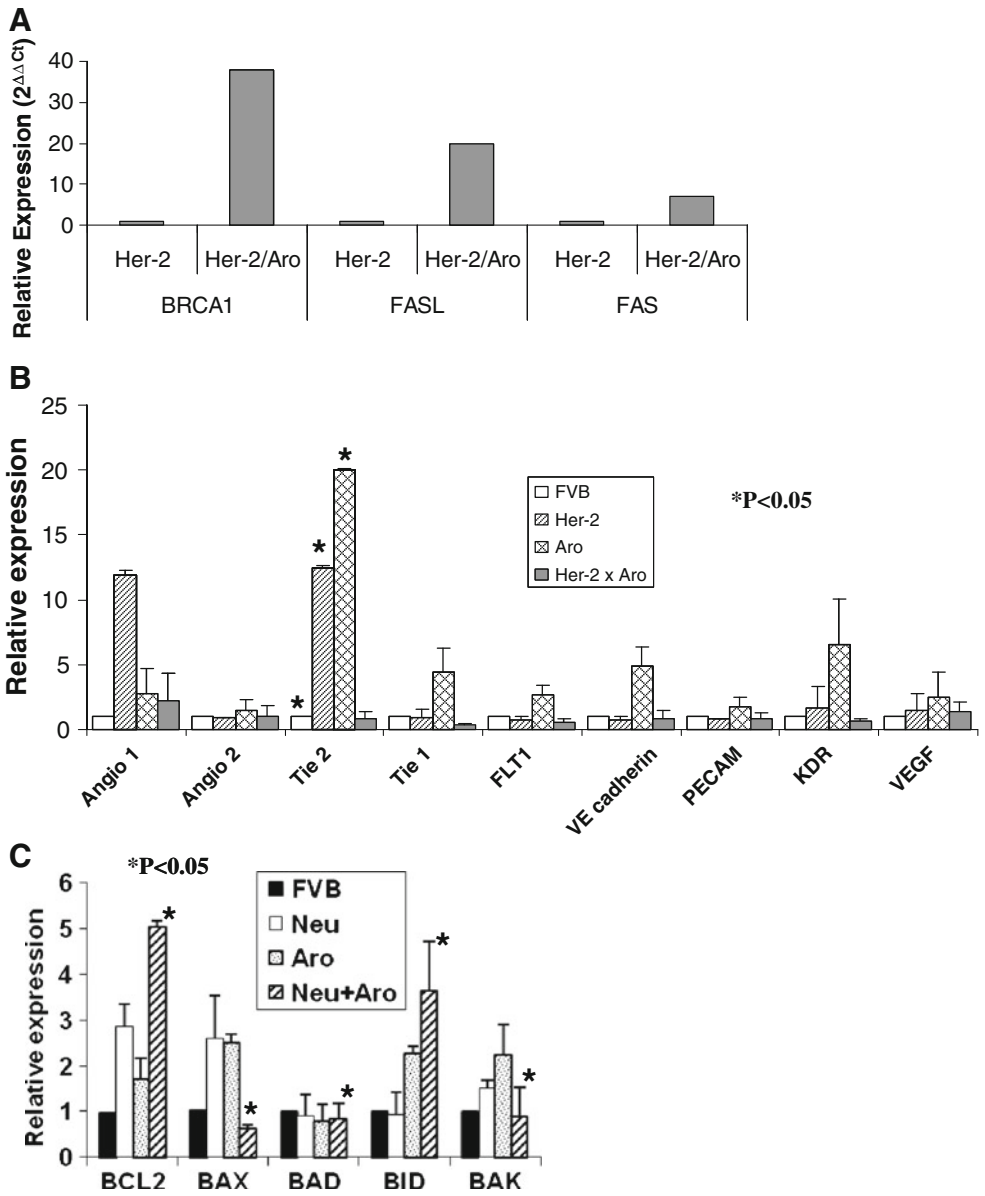

Fig. 2 The expression of angiogenic factors is decreased in MMTV-Her-2/neu x aromatase double transgenic mice. RNA isolated from the MMTV-Her-2/neu x aromatase double transgenic mice, parental strains, and FVB wild-type mice was subjected to real-time RT-PCR to examine the expression of BRCA1, FAS, and its ligand (**a**), a panel of angiogenic factors (**b**), and a panel of apoptosis-related genes (**c**). Note the strong induction of BRCA1 mRNA and decrease in Angio 1, TIE1, and TIE2 in the MMTV-Her-2/neu x aromatase mice compared to the parental strain MMTV-Her-2/neu (*$P<0.01$)

other hand, a slight increase in the pro-survival factor Bcl2 was observed in the double transgenic mice (Fig. 2c). These observations from the gene expression experiment suggested that these apoptosis-related factors may not be involved in the reduction of tumor formation observed due to aromatase induction.

ERβ affects MAPK Activation in MMTV-Her-2 x Aromatase Double Transgenic Mice

The decreased mammary tumorigenesis in the MMTV-Her-2/neu x aromatase double transgenic mice suggests that estrogenic signaling is protective against HER-2/neu-driven breast cancer. In support of this, ERβ levels were drastically increased, whereas ERα was decreased in the double transgenic mice, effectively decreasing the ERα/ERβ ratio (Fig. 3a and [21]). Given potential nongenomic activities of ER and cross-interaction with growth factor signaling pathways, we examined the total tyrosine phosphorylation as well as phosphorylation levels of the MAPK factors p38 and p42/44 as well as AKT. As shown in Fig. 3, both the MMTV-Her-2/neu x aromatase and the MMTV-Her2/neu mice showed a significant increase in phospho-tyrosine proteins. Interestingly, examination of specific Her2/neu downstream kinases showed an increase in the phosphorylation of p38 and a decrease in the phosphorylation of p42/44 and AKT in the double transgenic mice compared to the Her-2/neu parental strain (Fig. 3). To examine whether ERβ is involved in the regulation of these factors, we treated the MMTV-Her-2/neu x aromatase double transgenic mice with the ERβ-selective antagonist THC. This treatment

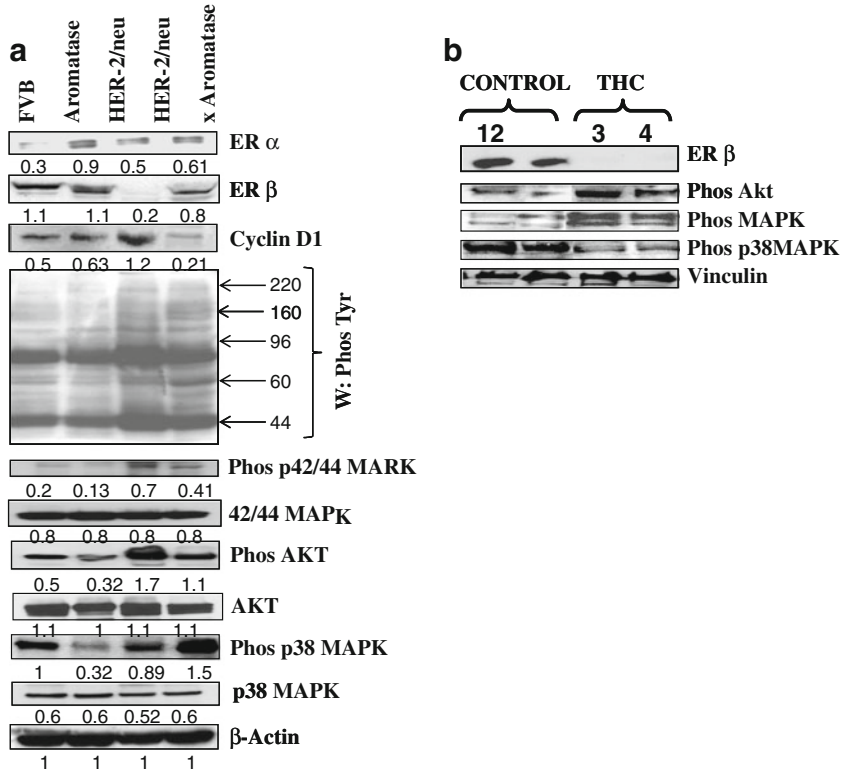

Fig. 3 ERβ antagonist THC reverses the decrease in the levels of phosphorylated MAPK p42/44 and AKT and the increase in phosphorylated p38 levels in MMTV-Her-2/neu x aromatase double transgenic mice. **a** Total protein samples were isolated from the mammary glands of MMTV-Her-2/neu x aromatase and parental strains. The protein levels of cyclin D1, ERα, and ERβ were examined by Western blotting (*upper panels*). Total tyrosine phosphorylation was examined using anti-phospho-tyrosine antibody (*middle panel*). Posphorylation levels of MAPK p42/44 and p38 as well as AKT were examined by Western blotting (*lower panels*) using antibodies specific against the phosphorylated forms as described in Materials and Methods. **b** MMTV-Her-2/neu x aromatase mice were treated with THC (500 μg/day/mouse) for 3 weeks. Mammary gland total protein was isolated and subjected to Western blot analysis using antibodies specific to phosphorylated AKT, p42/44, and p38. Two representative samples for each control and THC-treated mice are shown

diminished levels of ERβ to almost undetectable levels, suggesting that THC not only antagonizes ERβ activity, but also results in its degradation, possibly due to altered protein confirmation of THC-bound ERβ. THC treatment led to increased levels of phospho-AKT and phospho-p42/44 as well as decreased phospho-p38 levels (Fig. 3b).

ERβ Expression Reduces Cellular Proliferation and Leads to Regulation of Cell Cycle Progression as well as Changes in the Expression of cell Cycle Factors and Tumor Suppressors

To examine the roles of aromatase and ERβ on the Her-2/neu signaling pathway in vitro, we generated MCF7 cells that overexpress Her-2/neu as well as aromatase and/or ERβ (designated MCF7Her-2-ERβ, MCF7Her-2-aro, and MCF7Her-2-aro-ERβ). To examine the role of ERβ in cellular proliferation in the MCF7-Her-2-aro-ERβ, we treated these cells with the ERβ-selective agonist DPN at various concentrations. The data in Fig. 4 show a 50% decrease in proliferation in cells treated with 100 nM of DPN and a 70% reduction in proliferation in cells treated with 500 nM DPN (Fig. 4a). We have also examined the effects of DPN in the presence or absence of the ERβ antagonist THC on cell cycle transition. The data showed an over three-fold decrease in S phase cells with DPN treatment, which was reversed when THC was added along with DPN (Fig. 4b).

Because our data suggested that ERβ may affect the cell cycle and cellular proliferation, we next examined whether the overexpression of ERβ affects the expression of estrogen-responsive cell cycle factors, including tumor suppressors, by Western blotting. Figure 5a shows that a decrease in the ERα/ERβ ratio corresponds to suppression of cyclin D1 expression and RB phosphorylation as well as an increase in the levels of the CDK inhibitors p21 and p27, which block cyclin D1 and E-dependent progression from G1 to S phase. Interestingly, the tumor suppressor p53 was notably increased in the MCF7 clones overexpressing ERβ (Fig. 5a). Nuclear localization of p53 was also increased because of transfection of ERβ-GFP into MCF7Her-2-aro cells (Fig. 5b). Co-localization of ERβ with p53 was also observed (Fig. 5b). Our in vivo data also showed that the

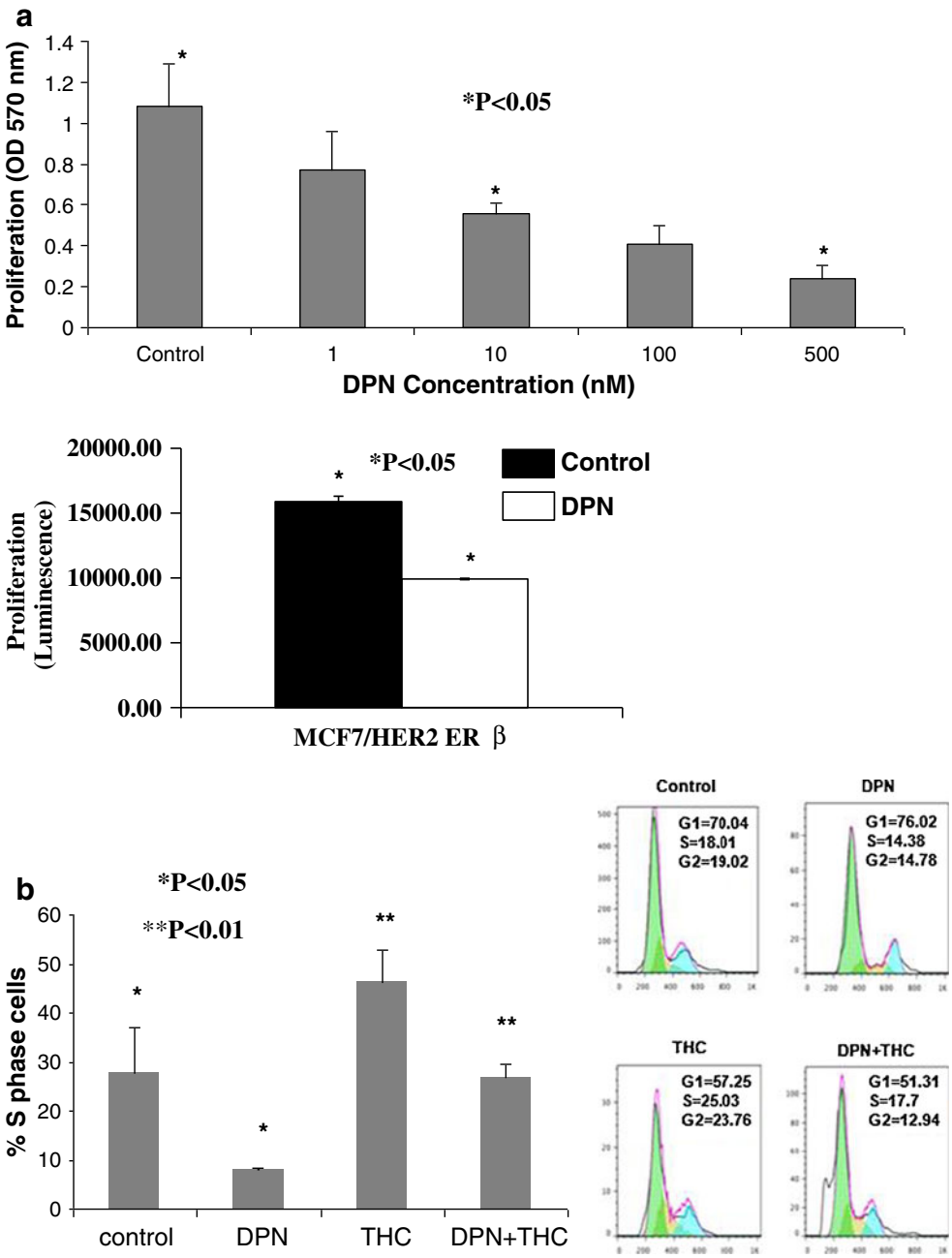

Fig. 4 Induction of ERβ activity by selective agonist DPN leads to decreased cellular proliferation and cell cycle progression. **a** DPN was used at the indicated concentrations in the treatment of MCF7Her-2-aro-ERβ cells. MTT proliferation assay was used as described in Materials and Methods. The lower figure in a represents proliferation of MCF7Her-2-ERβ cells treated with 1 μM DPN (dose derived from above proliferation assay, CellTiter-Glo method, Promega). **b** Cell cycle effects of DPN were examined in MCF7Her-2-aro-ERβ cells using PI staining and flow cytometry as indicated in Materials and Methods. (*$P<0.001$, **$P<0.01$)

mammary glands of MMTV-Her-2/neu x aromatase double transgenic mice, which contain elevated ERβ levels, exhibited a drastic increase in p53 compared to the MMTV-Her-2/neu parental strain and that treatment with the ERβ antagonist THC resulted in decreasing p53 levels (Fig. 5c). Furthermore, ChIP analysis has shown that the binding of ERβ to the p53 promoter is induced in the MCF7Her-2-aromatase-ERβ clone as well as in the mammary glands of MMTV-Her-2/neu x aromatase mice compared to the MMTV-Her-2/neu parental strain, suggesting that aromatase induction leads to elevated levels of ERβ, which in turn directly bind to the p53 promoter and enhance the transcriptional expression of p53 (Fig. 5d).

Induction of ERβ Leads to Increased Levels of Phosphorylated p38 and in MCF7Her-2 cells Overexpressing ERβ

To determine whether the regulation of MAPK observed in the MMTV-Her-2/neu x aromatase double transgenic mice also occurred in our vitro models, we examined the levels of phosphorylated p38 in the different MCF7Her-2 clones [22]. Our data in Fig. 6a shows that phosphorylated p38 levels were consistently increased in the MCF7Her-2 cell lines overexpressing ERβ. The levels of phosphorylated p42/44, on the other hand, were not different between the strains (data not shown). We also analyzed the effects of the ERβ antagonist

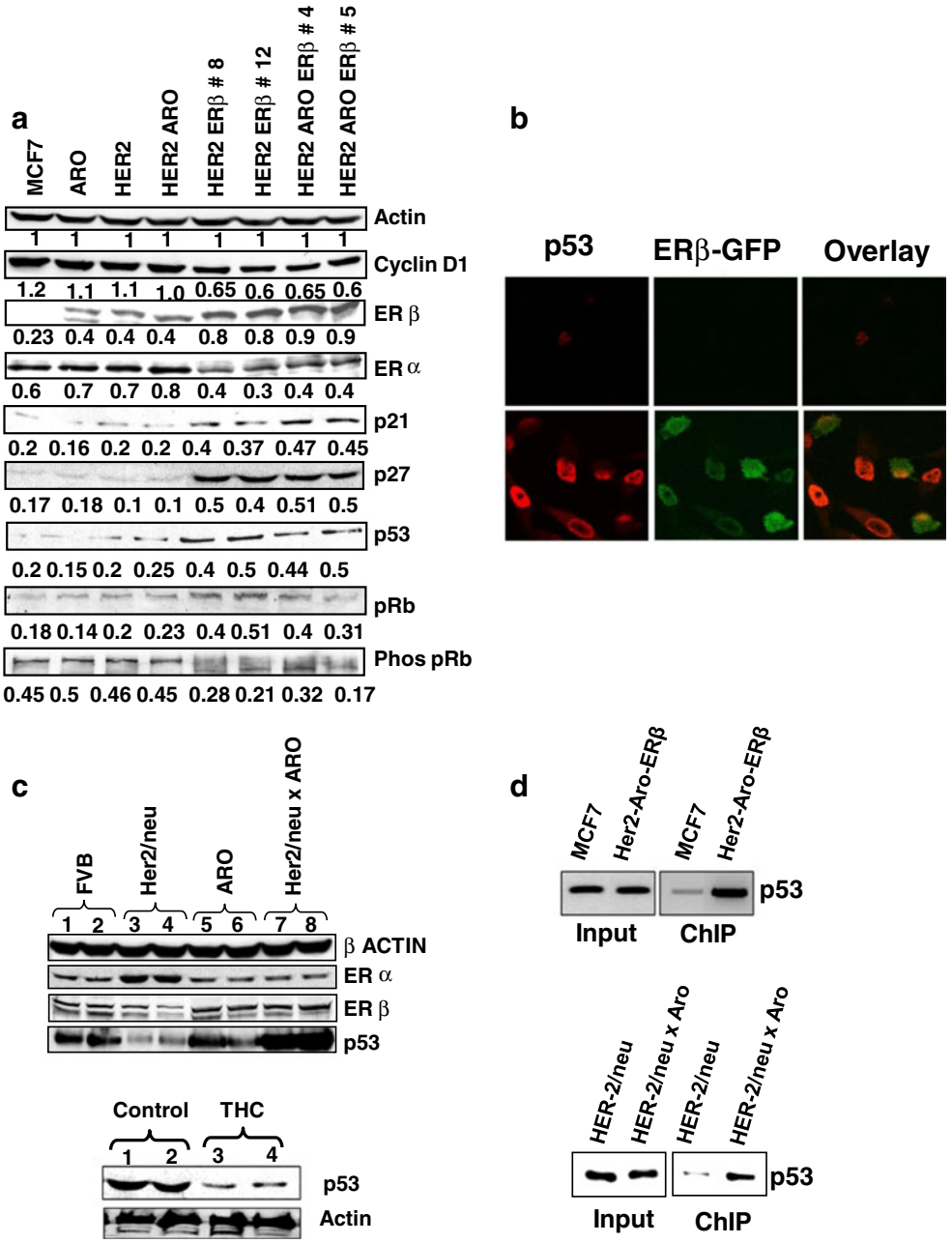

Fig. 5 ERβ induction alters cell cycle factor expression profile in vitro and induces the expression of p53 in vitro and in vivo. Western blot analysis was carried out on clones of MCF7Her-2 overexpressing aromatase and/or ERβ along with parental MCF7 clones using antibodies against cell cycle factors and tumor suppressors (**a**). Nuclear co-localization of ERβ and p53 was visualized using a laser scanning confocal microscope (**b**). *Upper panels*: MCF-7Her-2-aro; *lower panels*: MCF7Her-2-aro transfected with ERβ-GFP (**b**). The levels of ERα, ERβ, and p53 were examined in two representative samples from the MMTV-Her-2/neu x aromatase mice and parental strains by Western blotting (**c**). Note the induction of p53 in the double transgenic mice (**c**). The effect of modulating ERβ activity in MMTV-Her-2/neu x aromatase mice by THC on p53 expression was also examined using Western blot analysis (c, *bottom*). ChIP analysis was carried out to determine whether ERβ may induce the transcriptional activation by binding to the proximal promoter region of p53 (**d**). Increased ERβ binding to the p53 promoter was observed in the MCF7Her-2-aro-ERβ clone (*top*, d) and in mammary glands of MMTV-Her-2 x aromatase double transgenic animals compared to the parental strain MMTV-Her-2/neu (*bottom*, d)

THC on the expression of the phosphorylated forms of MAPK p38, p42/44, and AKT in MC7 Her-2-aromatase-ERβ. THC treatment resulted in a 60% reduction in the levels of phosphorylated p38 and an approximately four-fold induction in phosphorylated p42/44 and AKT (Fig. 6b), a pattern similar to the THC treatment of the MMTV-Her-2/neu x aromatase double transgenic mice (Fig. 3b).

Discussion

Our current and previous data suggest that estrogen induction via in situ aromatase overexpression protects against mammary oncogenesis driven by HER-2/neu [19] and transforming growth factor alpha (TGFα) [29]. In this study we have examined the mechanism underlying the protection conferred by estrogen induction on mammary tumor development in the MMTV-HER-2/neu background using MMTV-aromatase x HER-2/neu double transgenic mice.

Similarly published results by Yao et al. have shown that treatment of tamoxifen-resistant MCF-7 breast cancer cells with physiological levels of estrogen resulted in a transient apoptosis-induced tumor regression in a xenograft model [30]. The data underscore the complex role of estrogen in the development of breast cancer and suggest that although the mitogenic properties of estrogen may play an important

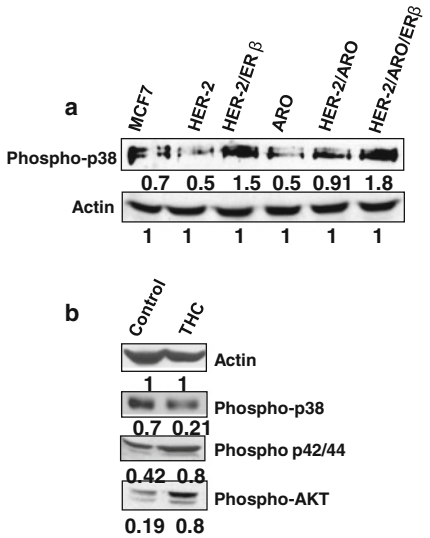

Fig. 6 ERβ regulates the levels of phosphorylated p38 MAPK, p42/44 MAPK and AKT. The levels of phosphorylated p38 MAPK were examined in the MCF7Her-2 clones (**a**). Note the induction of phosphorylated p38 MAPK in the clones overexpressing ERβ (**a**). The effect of THC on the phosphorylation levels of p38 MAPK, p42/44 MAPK, and AKT was determined by Western blotting (**b**)

role in breast oncogenesis, this steroid hormone may antagonize the tumorigenic actions of growth factors.

The decrease in the expression of angiogenic factors, such as TIE2 in MMTV-HER-2/neu x aromatase mice, may be a factor in reduced oncogenesis [31]. Recent studies have demonstrated that ERβ inhibited the proliferation of T47D breast cancer cells in vitro, and interfered with their growth and angiogenesis in a xenograft model, suggesting a negative influence by ERβ on angiogenesis [22].

The drastic induction of ERβ in the double transgenic mice suggests that this ER subtype may mediate the anti-tumor protective effects of estrogen through gene regulation with potential cross-talk with the Her-2/neu growth factor pathway. Our previous studies have shown that selective inhibition of ERβ activity by THC diminished its levels, and resulted in the induction of mammary gland ductal growth and elevated levels of cyclin D1, a cell cycle marker associated with breast tumorigenesis, suggesting that the estrogenic protective effects may be mediated by ERβ in these mice [21]. In this study, we have shown that the Her-2/neu x aromatase double transgenic mice exhibited decreased phosphorylation of RB, AKT, and P42/p44, changes that are associated with decreased cellular proliferation. Treatment with THC resulted in the reversal of the effects on these factors with increased phosphorylation of p42/p44 and AKT, implicating ERβ in the regulation of these mitogenic signaling pathways (Fig. 6b). Furthermore, the phosphorylation of p38, a MAPK that has been implicated in apoptosis in breast cancer cells overexpressing EGFR or Her-2/neu [32], was elevated in untreated double transgenic strain (compared to the Her-2/neu parental strain), but decreased drastically with THC treatment (Fig. 6b). The post-translational regulation of these factors by ERβ in our in vivo and in vitro model systems suggests regulatory cross-talk pathways between estrogenic signaling and MAPK, leading to decreased MAPK activity associated with oncogenesis. ERα, and in our study ERβ, cytosolic interactions with MAPK have been documented by several studies [14, 32, 33]. A recent study has shown that the Her-2/neu pathway can negatively regulate ERβ, but not ERα, activity by PI3/AKT-dependent phosphorylation, leading to diminished affinity to the transcriptional coactivator CBP [34]. Combined, the data suggest that a negative feedback loop may exist between ERβ activity and PI3/AKT signaling.

Tumor suppressors such as BRCA1 and p53 as well as apoptotic factors like Fas/Fasl were induced in the MMTV-HER-2/neu x aromatase mice, suggesting that these estrogen-responsive factors may mediate the protective effects observed in the double transgenic mice. Loss of tumor suppressors BRCA1 and p53 has been implicated in breast cancer, suggesting that these factors play important roles in protecting against mammary tumorigenesis [35–38]. In this study, we present data showing that ERβ may directly influence the expression of p53 and BRCA1, both of which are estrogen-responsive factors. Increased p53 levels were evident in the double transgenic strain as well as in MCF7Her-2/neu clones overexpressing ERβ, in which p53 nuclear localization was increased compared to the MCF7Her-2/neu parental strain. The binding of ERβ on the p53 proximal promoter was demonstrated by ChIP assays in vitro and in vivo, suggesting that ERβ may directly induce the transcription of this tumor suppressor. A drastic increase in the levels of ERβ bound to the p53 promoter was observed in the mammary glands of the MMTV-Her-2 x aromatase double transgenic mice compared to the MMTV-Her-2 parental strain. Our data also showed that ERβ may not only influence p53 expression, but also its translocation into the nucleus. P53 was evidently localized in the nuclei of MCF7Her-2-aromatase-ERβ clone compared to MCF7Her-2-aromatase, which showed only residual p53. Consistent with our observations, a previous study has shown that transient transfection of ERβ into MCF-7 cells resulted in p53 nuclear translocation and consequently increased transcriptional activity [25]. The induction of p53 and BRCA1 may lead to increased genomic stability in the MMTV-Her-2/neu x aromatase double transgenic mice, which could be a contributing factor to decreased tumorigenesis. Interestingly, estrogen as well as the combination of estrogen and progesterone treatment has been shown to have protective effects against Her-2/neu-driven breast cancer in transgenic mice, which is consistent with our observations [24]. A similar protective effect was observed in transplanted models of p53 knockout

mammary glands [24]. Our data not only support the role of p53 in estrogen-mediated protection, but also further shows that the presence of continuous estrogen stimulation during early stages of tumor initiation induces p53. The induction of p53 appears to be mediated by ERβ, as shown by our in vivo and in vitro ChIP data.

The protective role of estrogen was demonstrated in studies that provide evidence that prepubertal dose of phytoestrogens, which have affinity to ERβ like genstein, may confer protective effects for the mammary gland by reducing the number of terminal end buds, which are targets for malignant transformation, as well as reducing cellular proliferation, increasing apoptosis, and inducing BRCA1 expression [39, 40]. Estrogenic exposure of the mammary tissue in the MMTV-Her-2/neu x aromatase mice occurs early because of aromatase overexpression, which may lead to epigenetic changes and mammary gland remodeling conferring protection against Her-2/neu driven tumorigenesis. This raises the possibility that early epigenetic modifications due to increased estrogen signaling in mice harboring aromatase transgene may cause long-term alterations in the expression of ERα and ERβ. The exact mechanism of these regulatory events will need to be determined in future studies. Future studies should also address how potential epigenetic changes influenced by ERβ may lead to mammary remodeling and confer protection against tumor development.

In summary, the data presented in this report shed new light on the protective effects of estrogen exposure on Her-2/neu-induced mammary tumorigenesis and the role of ERβ in mediating these effects. ERβ seems to exert direct effects on the activation of the MAPK and AKT pathways as well as the induction of tumor suppressor genes. These changes are also associated with a decrease in several angiogenic factors. The data suggest that modulating ERβ activity may be used as a chemoprotective strategy against Her-2/neu and EGFR-driven breast cancer. Our ongoing studies with our Her-2/neu/ x aromatase x ERβ knockout models should shed definite light on the critical role of ERβ in mediating the protective actions of estrogen.

Acknowledgments We would like to acknowledge the University of Texas Health Science Center/San Antonio Cancer Institute Flow Cytometry core facility. This was work was supported by NIH/NCI grants R01 CA75018 and P30 5474 to RRT.

Conflict of Interest The authors have no conflicts of interest to declare.

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