

Role for HER2/neu and HER3 in fulvestrant-resistant breast cancer

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Abstract. Tamoxifen resistance is common for estrogen receptor α (ER α) positive breast cancer. Second-line therapies include aromatase inhibitors or fulvestrant. We have shown previously that fulvestrant reversed 17 β -estradiol-induced tumor regression of tamoxifen-stimulated MCF-7 xenografts (MCF-7TAMLT) treated for >5 years with tamoxifen in athymic mice and paradoxically stimulated growth. We investigated mechanisms responsible for growth by fulvestrant in the presence of physiologic estradiol and therapeutic strategies *in vivo*. The results demonstrated that only estradiol increased expression of the estrogen-responsive genes, *c-myc*, *igf-1*, *cathepsin D*, and *pS2* mRNAs, in MCF-7E2 and MCF-7TAMLT tumors. Tamoxifen or fulvestrant decreased the estradiol-induced increase of these mRNAs in both tumor models. However, tyrosine-phosphorylated HER2/neu, HER3, phospho-extracellular-regulated kinase-1/2 (ERK-1/2), and phospho-glycogen synthetase kinase 3 α (GSK3 α) and β proteins were increased in MCF-7TAMLT tumors treated with fulvestrant compared to estradiol, control, or tamoxifen. Phospho-HER2/neu interacted with HER3 protein in MCF-7TAMLT tumors. In order to determine whether the functional interaction of HER2/neu with HER3 is critical for growth of fulvestrant-stimulated MCF-7TAMLT tumors, pertuzumab (an antibody that blocks HER2/neu-HER3 interaction) was used in an *in vivo* xenograft growth assay. Only growth of fulvestrant-treated MCF-7TAMLT xenografts was decreased significantly by 37.2% in response to pertuzumab ($P=0.004$). Pertuzumab specifically decreased the interaction of HER2/neu protein with HER3 in fulvestrant-stimulated MCF-7TAMLT tumors. These results suggested growth of MCF-7TAMLT tumors by tamoxifen or fulvestrant is potentially independent of ER α transcriptional

activity as evidenced by lack of induction of four estrogen-responsive genes. The results suggested that growth of MCF-7TAMLT tumors treated with fulvestrant in the presence of physiologic estradiol is in part mediated through enhanced signaling from the HER2/neu-HER3 pathway as pertuzumab partially inhibited growth and the interaction of HER2/neu with HER3 *in vivo*.

Introduction

Five years of tamoxifen, a selective estrogen receptor modulator (SERM), remains a valuable and cost-effective treatment for pre- and post-menopausal women with estrogen receptor α (ER α)-positive breast cancer (1). Tamoxifen is also approved for prevention of ER α -positive breast cancer in women at high risk (2). However, only 40-50% of those patients with ER α -positive breast cancer will initially respond to tamoxifen therapy (3). Furthermore, many of those responders will develop acquired resistance to tamoxifen during therapy. Second-line treatments after failure on tamoxifen include aromatase inhibitors, anastrozole (4), letrozole (5,6), and exemestane (7) or the pure antiestrogen fulvestrant (ICI 182,780, Faslodex[®]) (8,9).

Fulvestrant inhibits growth of ER α -positive breast cancer by a different mechanism compared to aromatase inhibitors. Aromatase inhibitors specially target the cytochrome p450 aromatase enzyme that converts the androgen, androstenedione to estradiol (10), the growth-promoting female hormone. In contrast, fulvestrant binds to the ER α and inhibits estradiol-mediated-receptor dimerization, activating functions 1 and 2 (AF1 and AF2) (11), and promotes proteasome-mediated degradation of the receptor complex (12). As a result, the target of estradiol, ER α is completely neutralized. Thus, the therapeutic targeting of the pure antiestrogen, fulvestrant, to completely destroy the ER α signal transduction mechanism would appear to provide an optimal treatment option for antihormonal resistant breast cancer. However, we have demonstrated previously that fulvestrant in the presence of physiologic concentrations of estradiol, as one would anticipate in post-menopausal women, paradoxically stimulates growth of a phase II tamoxifen-stimulated MCF-7 breast cancer model *in vivo* (Fig. 1) (13). Furthermore, we have shown that

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fulvestrant blocks estradiol-induced decrease of ER α mRNA and protein, suggesting that the ER α protein is not completely degraded by fulvestrant once SERM resistance develops. Based on the previous findings (13) which could have important implications for the application of fulvestrant in patients, we sought to investigate the mechanism(s) responsible for the growth promoting actions of fulvestrant in the presence of physiologic estradiol in human tamoxifen-stimulated MCF-7 tumors. Multiple antihormonal therapies are currently used exhaustively to treat metastatic breast cancer after initially completing 5 years of adjuvant tamoxifen and to date the third line use of fulvestrant produces stable disease with the majority of patients experiencing disease progressions (14,15).

It was previously reported that increased expression of both HER2/neu and amplified in breast cancer 1 (AIB1) genes in human breast tumors and MCF-7 cells genetically engineered to overexpress HER2/neu, resulted in *de novo*/intrinsic resistance to tamoxifen and patients did not respond to tamoxifen as a first-line therapy (16,17). It was also shown that the mechanism responsible for *de novo* tamoxifen resistance in genetically engineered MCF-7 breast cancer cells stably expressing the human HER2/neu gene was increased estrogenic activity of tamoxifen at ER α -regulated genes as a result of high endogenous expression of AIB1 (17). The investigators demonstrated that AIB1, a coactivator belonging to the p160 family of coactivators, bound ER α -specific promoters in response to tamoxifen treatment. In addition, gefitinib (ZD-1868, Iressa[®]), a small molecule that inhibits ATP binding to the tyrosine kinase domain of the epidermal growth factor receptor (EGFR), inhibited HER2/neu activity and tamoxifen-induced AIB1 binding to ER α -specific promoters, and reversed resistance to tamoxifen *in vivo*. However, it is not yet clear whether high expression of AIB1 and HER2/neu is important for the natural development of acquired resistance to tamoxifen. Based on these previous studies, we investigated the roles of both coactivators and corepressors and the function of ER α in the parental, tamoxifen naïve MCF-7E2 and tamoxifen-stimulated MCF-7TAMLt tumors. We also investigated the expression and function of EGFR, HER2/neu, HER3, and HER4 in both tumor models. Finally, we sought to determine the mechanism of action responsible for growth of MCF-7TAMLt tumors in response to tamoxifen and/or fulvestrant in the presence of physiologic estradiol by performing an *in vivo* growth assay with gefitinib (EGFR inhibitor), trastuzumab (HER2/neu inhibitor), or pertuzumab (HER2/neu and HER3 heterodimerization inhibitor). The results from the current study suggest that phase II tamoxifen-stimulated growth of MCF-7TAMLt tumors *in vivo* is independent of ER α transcriptional activity but could be dependent on alternate mechanisms involving the interaction of AIB1 with ER α . Furthermore, the data indicate that growth of MCF-7TAMLt tumors by the combination of estradiol plus fulvestrant is at least in part due to the HER2/neu and HER3 heterodimerization and the subsequent expression of a survival signaling pathway.

Materials and methods

Drug treatments. The following standard drug dosages were used throughout this study, unless otherwise stated. Estradiol

was given subcutaneously in 0.30-cm silastic capsules (Baxter Health Care, Mundelein, IL) as previously described (18) to achieve postmenopausal serum levels of estradiol (83.8 pg/ml in serum) (19). Tamoxifen (Sigma-Aldrich Chemicals, St. Louis, MO) at 1.5 mg/day in a total volume of 0.10 ml (10 mg tamoxifen/ml solution of 90% CMC [1% carboxymethylcellulose (Sigma-Aldrich Chemicals) in double-distilled water] and 10% PEG 400 (Sigma-Aldrich Chemicals)/Tween-80 (Sigma-Aldrich Chemicals) (99.5% polyethylene glycol 400, 0.50% polysorbate 80) was given orally by gavage. Fulvestrant (AstraZeneca, Cheshire, UK) was dissolved in 100% ethanol and then diluted in peanut oil; 5 mg/0.10 ml was injected subcutaneously twice a week. Pertuzumab (2C4), a generous gift from Genentech (San Francisco, CA) was prepared in sterile-phosphate-buffered saline (pH 7.4) to final concentration of 25 mg/ml. Three times weekly (100 mg/kg) was injected i.p. in a 0.1 ml total volume. Gefitinib, a generous gift from Dr Ron Grigg from the University of Leeds (Leeds, UK) was dissolved in 0.50% Tween-80 and a dose of 100 mg/kg total mouse body weight was given orally by gavage 5 days a week. Trastuzumab, commercially available (Genentech) in an injectable solution of 25 mg/ml in sterile-phosphate-buffered saline (pH 7.4), was injected i.p. twice a week at a dose of 35 mg/kg total mouse body weight.

Growth of MCF-7TAMLt tumors. Long-term tamoxifen-stimulated tumors (MCF-7TAMLt) were developed by retransplanting growing estradiol-dependent MCF-7 tumors into new athymic mice and treating them with tamoxifen (20,21). Select tamoxifen-stimulated MCF-7 tumors were then serially passaged into new athymic mice and treated with tamoxifen for >5 years, as described (21). The mammary fat pads of 50 ovariectomized BALB/c nu/nu athymic mice, 4-6 weeks old, were bilaterally inoculated with 1-mm MCF-7TAMLt tumor bits. Mice were fed 1.5 mg tamoxifen orally by gavage (5 days/week) until the mean tumor cross sectional reached 0.28 cm² [95% confidence intervals (CI)=0.23 to 0.33]. Tamoxifen treatment was withdrawn and groups of 10 mice were assigned randomly to the following treatment groups: no treatment control, tamoxifen, estradiol, estradiol plus fulvestrant, or fulvestrant alone. Tumors were measured weekly with Vernier calipers for ≤ 10 weeks. The tumors were then excised from the mice and snap-frozen in liquid nitrogen for later use.

In another experiment, 60 mice implanted bilaterally with 1-mm MCF-7TAMLt tumor bits were fed 1.5 mg tamoxifen orally by gavage (5 days/week) until the mean tumor cross sectional reached 0.30 cm² (95% CI=0.25 to 0.35). Tamoxifen treatment was withdrawn and groups of 10 mice were assigned randomly to the following treatment groups: no treatment, tamoxifen, tamoxifen plus gefitinib, estradiol, estradiol plus fulvestrant, or estradiol plus fulvestrant plus gefitinib, were measured weekly with Vernier calipers for up to 8 weeks.

In a third experiment, 60 mice were implanted bilaterally with 1-mm MCF-7TAMLt tumor bits and fed 1.5 mg tamoxifen orally by gavage (5 days/week) until the mean tumor cross sectional reached 0.34 cm² (95% CI=0.28 to 0.40). Tamoxifen treatment was withdrawn and groups of 10 mice were assigned randomly to the following treatment groups: no treatment, tamoxifen, tamoxifen plus trastuzumab,

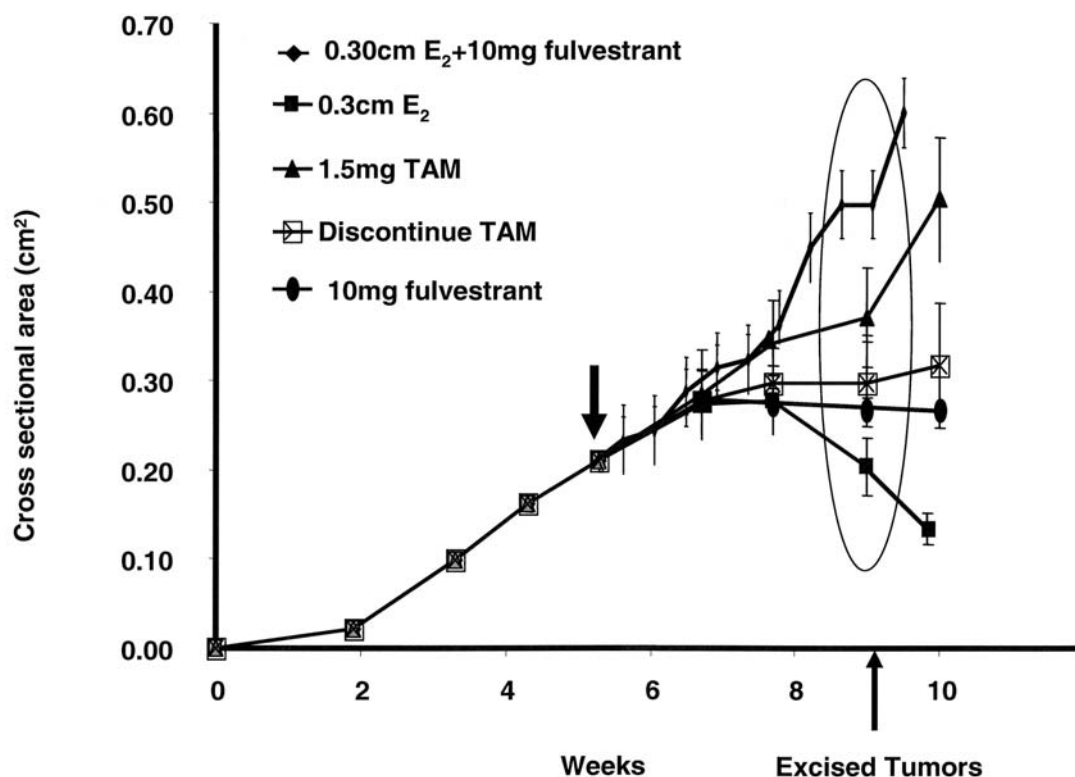


Figure 1. Representative graph for growth of MCF-7TAMLT tumors *in vivo*. MCF 7TAMLT tumor portions (1 mm²) were bitransplanted into the mammary fat pads of 50 athymic mice as described previously in Materials and methods (34). Tumors were initially treated with 1.5 mg tamoxifen (TAM) and grown to a mean cross sectional area of 0.25 cm² and then TAM was withdrawn and 10 mice per group randomly treated as follows: vehicle control, 1.5 mg tamoxifen (TAM), 0.30 cm estradiol capsules (E₂), E₂ plus fulvestrant (5 mg injected subcutaneously twice per week), or fulvestrant alone. Tumor areas were measured weekly with the use of vernier calipers up to ten weeks. Cross sectional areas of tumors were calculated using the formula: (length x width x π)/4 (i.e. $lw\pi/4$). The graph shown is a representative plot of time-dependent growth of MCF-7TAMLT tumors with TAM treatment up to 5 weeks. The bar graph represents the final mean cross sectional areas plus/minus 95% confidence intervals of MCF-7TAMLT tumors at week 10 in response to control, TAM, E₂, E₂ + fulvestrant, or fulvestrant alone.

estradiol, estradiol plus fulvestrant, or estradiol plus fulvestrant plus trastuzumab. Tumors were measured weekly with Vernier calipers for up to 8 weeks.

In a fourth experimental study, 60 mice were implanted bilaterally with 1-mm MCF-7TAMLT tumor bits and were fed 1.5 mg tamoxifen orally by gavage (5 days/week) until the mean tumor cross sectional reached 0.36 cm² (95% CI=0.35 to 0.37). Tamoxifen treatment was withdrawn and groups of 10 mice were assigned randomly to the following treatment groups: no treatment, tamoxifen, tamoxifen plus pertuzumab, estradiol alone, estradiol plus fulvestrant, or estradiol plus fulvestrant plus pertuzumab. Tumors were measured weekly with Vernier calipers for up to 9 weeks. The cross-sectional area was calculated by multiplying the length by the width by π and dividing the product by 4. The Animal Care and Use Committee of Northwestern University approved all of the procedures involving animals.

Western blot analyses. Tumors were homogenized by grinding in liquid nitrogen and resuspending in lysis buffer (1% Triton X100, 1 mM EDTA, 150 mM NaCl, 50 mM Tris base pH 7.4, 25 mg/ml phenylmethanesulfonyl fluoride, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 10 μ g/ml pepstatin, 10 μ g/ml TLCK, 10 μ g/ml TPCK, 100 mM NaF, 10 mM ortho-vanadate) (Sigma). The extract was subsequently sonicated at

level one 3 times at 1-sec intervals with a Microson sonicator (Misonix, Farmington, NY) and then centrifuged for 5 min at 5,000 x g at 4°C. The supernatant was collected and the protein concentration was measured using the Bradford assay (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein (25-50 μ g) were loaded onto a 7% polyacrylamide/bisacrylamide gel for sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred to a nitrocellulose blot for Western blot analysis. The following proteins were detected by Western blot: tyrosine1248-phosphorylated HER2/neu (1:2000 Rabbit anti-human, Upstate Biotechnology); HER2/neu (1:200 mouse anti-human, Ab-11, NeoMarkers, Fremont, CA); HER3 (1 μ g/ml mouse monoclonal anti-human HER3, clone 2F12, NeoMarkers); HER4 (1 μ g/ml rabbit anti-human HER4, Ab-2, NeoMarkers); phospho-threonine202/tyrosine204 MAPK (1:2000 mouse monoclonal anti-human phospho-p44/p42 MAPK, Cell Signaling Technology, Beverly, MA); p44/p42 MAPK (1:1000 rabbit anti-human p44/42 MAPK, Cell Signaling Technology); phospho-serine21 glycogen synthase kinase 3 α (GSK3 α) (1:500 rabbit anti-human phospho-GSK3 α (Ser21), Upstate Biotechnology); phospho-GSK3 β (Ser9) (1 μ g/ml mouse monoclonal anti-human phospho-GSK3 β (Ser9), Upstate Biotechnology); GSK3 (2 μ g/ml mouse monoclonal anti-human GSK3, clone 4G-1E, Upstate Biotechnology); and phosphotyrosine (1:1000

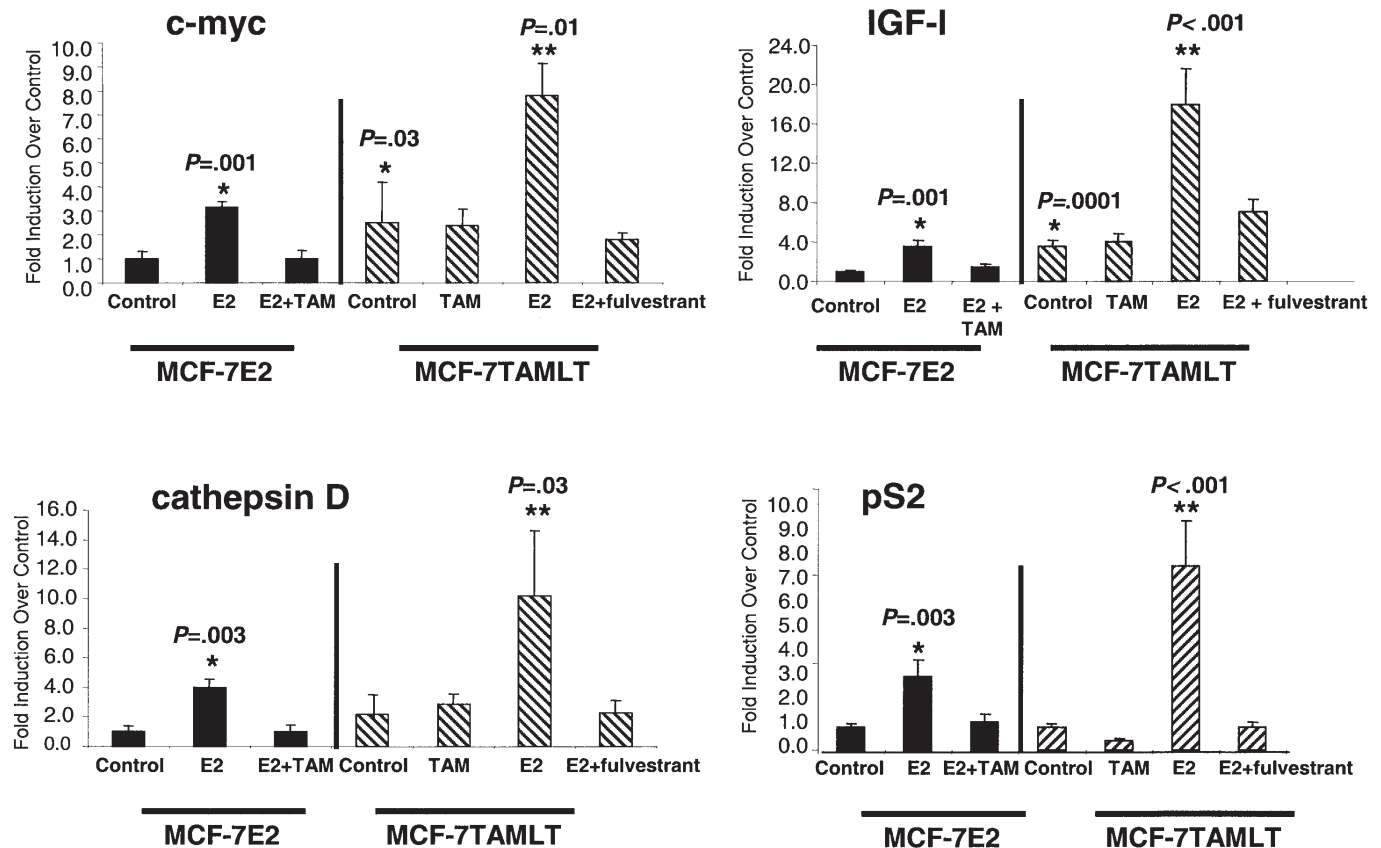


Figure 2. Expression of *c-myc*, *igf-1*, *cathepsin D*, and *pS2* mRNAs in both the parental MCF-7E2 and MCF-7TAMLT tumors. Human *c-myc*, IGF-1, *cathepsin D*, and *pS2* mRNA copy numbers were detected by real-time reverse transcription-polymerase chain reaction as described in Materials and methods. 18s RNA was used as a loading control in all samples. mRNA cycle threshold (Ct) values (which are proportional to mRNA copy number) were normalized to the Ct value for 18s RNA by subtracting the Ct value for 18s RNA from the Ct value for *c-myc* mRNA. Data shown are the mean fold induction of mRNA copy numbers of each treatment over mRNAs copy numbers for the MCF-7E2 control group and 95% confidence intervals normalized to loading control in three replicates per tumor. *Compared with MCF-7E2 control group (Student's t-test). **Compared to MCF-7TAMLT control group (Student's t-test). All statistical tests were two-sided.

mouse monoclonal anti-phosphotyrosine-P20, BD Biosciences, San Jose, CA). The appropriate secondary antibody conjugated to horseradish peroxidase was used to detect the primary antibody (goat anti-rabbit, donkey anti-goat, or goat anti-mouse IgG-HRP, Santa Cruz Biotechnology). The blot was developed using an ECL kit (Amersham Corp., Arlington Heights, IL). The membrane was exposed to Kodak X-OMAT film for 10-30 sec. Densitometry was performed using the Scion program to quantify the intensity of bands from 3 independent Western blots.

Immunoprecipitation. Tumor lysates were diluted to a concentration of 1 $\mu\text{g}/\mu\text{l}$ for a total volume of 500 μl . Antibody-specified for immunoprecipitation (4 μg) was added to tumor lysates. Samples were gently rocked at 4°C. A Protein G Plus/Protein A-Agarose suspension (40 μl) (EMD Biosciences, Calbiochem, La Jolla, CA) was added to the protein-antibody complex. The entire mixture was gently rocked for 2 h at 4°C. The protein-antibody-agarose beads complex was collected by pulsing (5 sec in a microcentrifuge at 14,000 \times g). The supernatant was decanted and pellet was washed 3 times with ice-cold lysis buffer, followed each time with pulsing and decanting of supernatant. The washed pellet was resuspended in 40 μl 2X Laemmli sample buffer and boiled for 5 min. Western blot analysis was performed thereafter as previously

described in the Western blot section. The mouse monoclonal anti-human HER2/neu antibody (Ab-11, NeoMarkers) was used for immunoprecipitation.

Real-time reverse transcription polymerase chain reaction. Total RNA was extracted from the tumors using the RNeasy mini kit (Qiagen, Stanford Valencia, CA) according to the manufacturer's instructions. The total RNA was reverse-transcribed (RT) using TaqMan reverse transcription reagents (PE Applied Biosystems, Hayward, CA) with the use of random hexamers as the primers according to the manufacturer's instructions. Primers and probes for pS2, IGF-I, *cathepsin D* were designed using Primer Express™ 1.5 software set at default parameters to select the most optimized primer and probe sets for this system. The sequences for the forward and reverse primers for human pS2 are 5'GAGGCC CAGACAGAGACGTG3' and 5'CCCTGCAGAAGTGTCT AAAATTCA3', respectively. The sequence for the pS2 probe is 5'CTGCTGTTTCGACGACACCGTTTCG 3'. The sequences for the forward and reverse primers for human IGF-I are 5'TGCTTCCGGAGCTGTGATC3' and 5'AGCTGACTTGG CAGGCTTGA3', respectively. The sequence for the human IGF-I probe is 5'AGGAGGCTGGAGATGTATTGCGCA CC3'. The sequences for the forward and reverse primers for human *cathepsin D* are 5'GTACATGATCCCCCTGTGA

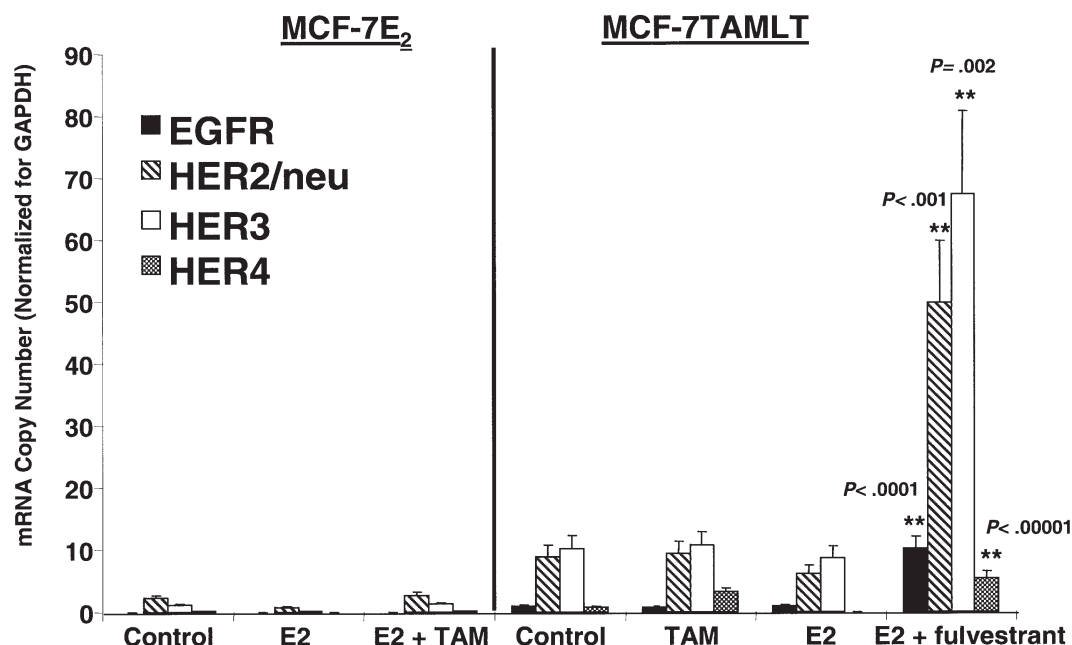


Figure 3. Expression of human EGFR, HER2/neu, HER3, and HER4 mRNAs in parental MCF-7E2 and MCF-7TAMLT tumors. Total RNA (2 μ g) from both parental MCF-7E2 and MCF-7TAMLT tumors treated as labeled was reverse transcribed to cDNA, and real-time polymerase chain reaction was performed to detect human EGFR, HER2/neu, HER3, and HER4 mRNAs as described in Materials and methods. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA was measured for an internal positive control. Copy number was normalized as described previously in Fig. 2. *Compared to MCF-7E2 control group (Student's t-test). **Compared to MCF-7TAMLT control group (Student's t-test). All statistical tests were two-sided.

GAAGGT3' and 5'GGGACAGCTTG TAGCCTTTGC3', respectively. The sequence for the human cathepsin D probe is 5'FAM-ACCCTGCCCCGCGATCACACTGA3'. The c-myc primer and probe mixture was purchased from Perkin-Elmer Applied Biosystems (PE-ABI, Stanford Valencia, CA) and used according to the manufacturer's instructions. For all real-time polymerase chain reaction, (6-carboxyfluorescein) FAM was the reporter and QSY7 was the quencher (MegaBases, Inc., Evanston, IL). The quantity of human 18s ribosomal RNA (rRNA) was also measured in each total cDNA sample for normalization. The probe and primers for 18s rRNA were purchased from PE-ABI. The PCR portion of the reaction was performed with the TaqMan PCR core reagent kit (PE-ABI). The 50- μ l PCR mixture contained 100 ng of total cDNA, 100 nM probe, and 200 nM primers. Real-time PCR was performed with the ABI-Prism 7700 sequence detection system (PE-ABI). PCR conditions were 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min.

Statistical analysis. Statistically significant differences in cross sectional areas of tumors at the end of each experiment were analyzed using a one-factor analysis of variance (ANOVA) comparing tumor cross sectional areas within treatments at the end of each study. Tumor bar graphs represent means \pm 95% confidence intervals calculated using Microsoft Excel. A two-sided Student's t-test was used to analyze differences in mRNA levels as detected by real-time PCR of the treatments to the control group. The error bars for the measurement of mRNA copy number represent 95% confidence intervals calculated using Microsoft Excel.

Results

Function of ER α in MCF-E2 and MCF-7TAMLT tumors. It was previously shown that high AIB1 and stable overexpression of HER2/neu in MCF-7 cells facilitated the interaction of AIB1 protein to promoters of estrogen responsive genes with tamoxifen treatment (17) and that this could contribute to *de novo* tamoxifen resistance. We measured expression of four known endogenous, human-specific, estrogen-responsive genes, *c-myc* (22), *igf-1* (23), *cathepsin D* (24), and *pS2* (25) in both parental MCF-7E2 and MCF-7TAMLT tumors to determine whether the direct genomic function of ER α protein contributes to tamoxifen or estradiol plus fulvestrant-stimulated growth of MCF-7TAMLT tumors. Estradiol increased expression of *c-myc*, *igf-1*, *cathepsin D*, and *pS2* mRNAs by 3.17-fold (95%CI=2.92 to 3.46, P=0.001), 3.50-fold (95%CI=3.20 to 3.80, P=0.001), 4.00-fold (95%CI=3.43 to 4.57, P=0.003), and 3.86-fold (95%CI=3.72 to 4.00, P<0.0001), respectively, in parental MCF-7E2 tumors compared to controls (Fig. 2). In each case, tamoxifen significantly decreased the estradiol-induced increase of *c-myc*, *igf-1*, *cathepsin D*, and *pS2* mRNAs to 1.00, 1.13, 1.14, and 1.24, respectively (Fig. 2). The basal expressions of *c-myc* and *igf-1* mRNAs were increased by 2.69-fold (95%CI=1.16 to 4.22, P=0.03) and by 3.53-fold (95%CI=3.25 to 4.00, P=0.0001), respectively in MCF-7TAMLT compared to MCF-7E2 tumors (Fig. 2). More importantly, estradiol increased expression of *c-myc*, *igf-1*, *cathepsin D*, and *pS2* mRNAs above control by 3.13-fold (P=0.01), 5.85-fold (P<0.001), 5.25- (P=0.03), and 8.00- (P<0.001) fold, respectively despite the fact that estradiol inhibited growth of MCF-7TAMLT tumors. In all cases, there was no statistically

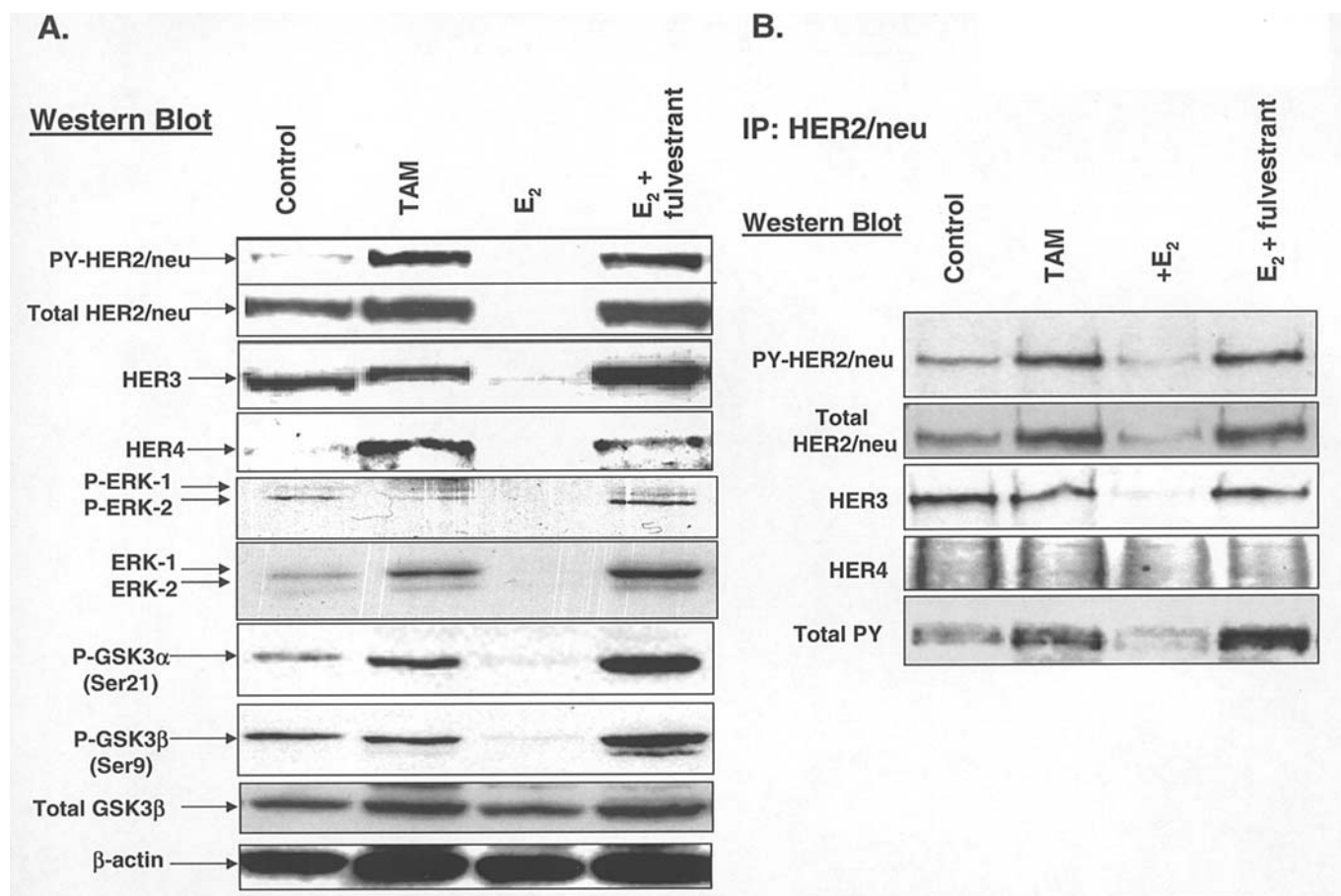


Figure 4. Expression and activity of HER receptor tyrosine kinases. (A) Western blot analyses to detect phosphorylated-tyrosine (1248)-HER2/neu, total HER2/neu, HER3, HER4, phosphorylated-ERK-1/2, total ERK-1/2, phosphorylated GSK3 α and β , and total GSK3, proteins in MCF-7TAMLT tumors, treated as labeled. Each lane was loaded with 50 μ g total protein extract the proteins described previously in Materials and methods. β -actin protein was detected by Western blot analysis for loading purposes. The experiment was repeated at least 3 times using 3 individual tumors. A representative blot is shown. (B) Total HER2/neu protein in 500 μ g total protein from MCF-7TAMLT tumor extracts was immunoprecipitated as described previously in Materials and methods. Western blot analyses were performed using the HER2/neu immunoprecipitates to detect phosphorylated tyrosine (1248)-HER2/neu, total HER2/neu, HER3, HER4, and total phosphorylated tyrosine residues of 185 kDa proteins that were precipitated with HER2/neu. The experiment was repeated at least 3 times using 3 individual tumors. A representative blot is shown.

significant increase of mRNAs in response to tamoxifen treatment when compared to controls. In addition, fulvestrant treatment decreased estradiol-induced increase of all four mRNAs (Fig. 2). The results suggested that growth of MCF-7TAMLT tumors and the interaction of AIB1 with ER α protein in response to either tamoxifen or estradiol plus fulvestrant treatment are independent of ER α transcriptional activity. These data are in contrast to the results from Shou *et al* (17) who suggested that tamoxifen was estrogenic at ER α -regulated genes in tamoxifen-resistant breast cancer due to cross-talk between HER2/neu-engineered signaling and ER α -transcriptional activity specifically due to high AIB1 expression. Clearly, the differences could result from differences in the engineered *in vitro* model that is not growth inhibited by estradiol compared to a model-developed *in vivo* where there is a natural development of drug resistance over a long period of time.

Expression and activity of HER family of receptor tyrosine kinases. A recent study has shown that overexpression of the HER2/neu oncogene in ER α -positive, AIB1 high-expressing

breast tumors might contribute to intrinsic tamoxifen resistance (26). In our *in vivo* model of MCF-7TAMLT tumors, we showed that EGFR mRNA was increased by 2.55-fold ($P < 0.00001$), HER2/neu mRNA was increased by 3.48-fold ($P = 0.001$), HER3 mRNA was increased by 5.15-fold ($P < 0.001$), and HER4 mRNA was increased by 2.25-fold ($P < 0.0001$) in MCF-7TAMLT tumors compared to the MCF7E2 control group (Fig. 3). Of note, E2 plus fulvestrant treatment of MCF-7TAMLT tumors further increased expression of EGFR by 5.24-fold ($P < 0.0001$), HER2/neu by 14.29-fold ($P < 0.001$), HER3 by 13.20-fold ($P = 0.002$), and HER4 by 2.89-fold ($P < 0.00001$) compared to the MCF-7TAMLT control group (Fig. 3). These data indicated that overexpression of the HER family of RTKs might be important for the development of tamoxifen resistant breast cancer. Moreover, high HER2/neu and HER3 mRNA expression could be responsible for growth stimulation by estradiol plus fulvestrant treatment. Thus, we investigated the roles of EGFR, HER2/neu, HER3, and HER4 receptor tyrosine kinases (RTKs) in MCF-7TAMLT tumors. In order to determine the function of HER, RTK proteins in MCF-7TAMLT tumors, expression

and interaction of HER, and downstream target proteins were investigated by Western blot analyses and immunoprecipitation. Tamoxifen or estradiol plus fulvestrant treatment of MCF-7TAMLT tumors increased tyrosine-phosphorylated HER2/neu by 6-fold over the control group while total HER2/neu protein levels were unchanged in the control, tamoxifen, or estradiol plus fulvestrant-treated groups (Fig. 4A). Total HER3, phospho-ERK-1/2, phospho-GSK3 α and β [targets of protein kinase B (Akt)] proteins were increased by 3-fold, 2-fold, and 3-fold, respectively, in estradiol plus fulvestrant-treated MCF-7TAMLT tumors compared to either the control or tamoxifen-treated tumors (Fig. 4A). Fig. 4A panel 4 showed that HER4 protein was increased by 5-fold in response to tamoxifen and by 3-fold in response to estradiol plus fulvestrant compared to the control group. Regardless of the treatment, EGFR protein was not detected by Western blot analysis (data not shown). In all cases, the growth inhibitor, estradiol, decreased HER2/neu, HER3, HER4, ERK-1/2, and phospho-GSK3 α and β proteins to almost undetectable levels in MCF-7TAMLT tumors (Fig. 4A).

HER2/neu is the preferred partner for EGFR, HER3, and HER4 (27). In order to determine whether HER2/neu interacts with EGFR, HER3 and/or HER4, co-immunoprecipitation was performed. Immunoprecipitated HER2/neu had increased phosphorylated tyrosine-1248 with tamoxifen or estradiol plus fulvestrant treatment compared to the control or estradiol group (Fig. 4B). Immunoprecipitated HER2/neu interacted with HER3 protein in the control, tamoxifen, or estradiol plus fulvestrant group (Fig. 4B). Neither EGFR (data not shown) nor HER4 protein (Fig. 4B) was detected in HER2/neu immunoprecipitates regardless of the treatment. Total phosphorylated-tyrosine proteins of 185 kDa in HER2/neu immunoprecipitates were increased by 3-fold with tamoxifen treatment and by 6-fold with estradiol plus fulvestrant treatment compared to the control group (Fig. 4B). Taken together, these results suggest that increased phosphorylation of ERK-1/2 and targets of Akt proteins (GSK3 α and β) in MCF-7TAMLT tumors-treated with estradiol plus fulvestrant could be due to increased activity from HER2/neu and HER3 heterodimers.

Growth of MCF-7TAMLT tumors in response to HER inhibitors. Recent studies have suggested that overexpression of EGFR and/or HER2/neu increased the estrogenic activities of tamoxifen at ER α -regulated genes (28-32), thus contributing to *de novo* tamoxifen-resistant breast cancer. In addition, it has been demonstrated previously that gefitinib, a specific EGFR inhibitor, blocked EGFR and HER2/neu activities and reversed resistance to tamoxifen (33). In addition to EGFR and HER2/neu, we measured expression of HER3 and HER4 since HER2/neu is the preferred partner for EGFR, HER3, or HER4 and the previous study suggested that increased activity from HER2/neu and HER3 heterodimers might contribute to the increased activity of ERK-1/2 and Akt proteins and thus explain the mechanism of growth stimulation by estradiol plus fulvestrant in MCF-7TAMLT breast tumors. In order to address these questions directly, gefitinib (an EGFR inhibitor), trastuzumab (humanized, monoclonal antibody that binds the extracellular domain of HER2/neu and promotes receptor-mediated internalization and degradation) and pertuzumab

(2C4) (monoclonal antibody that blocks HER2/neu and HER3 dimerization) were used in an *in vivo* growth assay. MCF-7TAMLT tumors grew more with tamoxifen (mean cross sectional at week 8 = 0.800 cm², 95%CI=0.635 to 0.965 cm², P=0.005) (Fig. 5A) or estradiol plus fulvestrant (mean cross sectional at week 8=0.958 cm², 95%CI=0.835 to 1.081 cm², P<0.0001) (Fig. 5A) as compared to the control group (mean cross sectional area at week 8 = 0.450 cm², 95%CI=0.291 to 0.609 cm²). Estradiol decreased growth of MCF-7TAMLT tumors (mean cross sectional area at week 8 = 0.203 cm², 95%CI=0.113 to 0.293 cm², P=0.02) compared to the control group (Fig. 5A). Moreover, fulvestrant blocked estradiol-induced growth inhibition and stimulated growth of MCF-7TAMLT (compare mean cross sectional area at week 8 of estradiol alone = 0.203 cm², 95%CI=0.113 to 0.293, to mean cross sectional area at week 8 of estradiol plus fulvestrant = 0.958 cm², 95%CI=0.835 to 1.081 cm², P<0.00001). Trastuzumab did not statistically significantly effect growth of MCF-7TAMLT tumors in response to tamoxifen or estradiol plus fulvestrant at a dose of 35 mg/kg given twice per week (data not shown). Similarly, MCF-7TAMLT tumors grew in response to tamoxifen or estradiol plus fulvestrant regardless of simultaneous treatment with 100 mg/kg gefitinib given 5 days per week (data not shown). Pertuzumab (also called 2C4) did not inhibit growth of tamoxifen-treated MCF-7TAMLT tumors (compare mean cross sectional area of tamoxifen alone at week 9 = 1.66 cm², 95%CI=1.28 to 2.04 cm², to mean cross sectional area of tamoxifen plus pertuzumab at week 9 = 1.96 cm², 95%CI=1.70 to 2.22 cm²) (Fig. 5A). In contrast, pertuzumab inhibited estradiol plus fulvestrant-stimulated growth of MCF-7TAMLT tumors by 37.2% (compare mean cross sectional of estradiol plus fulvestrant-treated group = 1.99 cm², 95% CI=1.74 to 2.24 cm² to the mean cross sectional area of estradiol plus fulvestrant plus pertuzumab = 1.25 cm², 95%CI=1.05 to 1.45 cm², P=0.004) (Fig. 5B).

To determine whether pertuzumab inhibited the tyrosine phosphorylation of HER2/neu and the subsequent interaction with HER3, a Western blot and immunoprecipitation were performed on the tumors at the end of the growth assay (week 9). Fig. 6A showed that pertuzumab (2C4) increased phospho-HER2/neu but decreased total HER2/neu protein in tamoxifen and to a greater extent estradiol plus fulvestrant-treated MCF-7TAMLT tumors. Pertuzumab blocked the HER2/neu and HER3 interaction in estradiol plus fulvestrant-treated MCF-7TAMLT tumors but not in tamoxifen-treated tumors (Fig. 6B). These results suggest that tamoxifen-stimulated growth of MCF-7TAMLT tumors *in vivo* might be independent of EGFR, HER2/neu, or HER3 pathways. Growth of MCF-7TAMLT tumors in response to estradiol plus fulvestrant might be at least in part due to HER2/neu and HER3 heterodimerization and signaling pathway.

Discussion

The results from the current study using the MCF-7TAMLT tumor model developed *in vivo* demonstrated that fulvestrant in the presence of physiologic estradiol stimulated growth of tamoxifen-resistant breast cancer in part due to an increased expression, interaction, and signaling from the HER2/neu

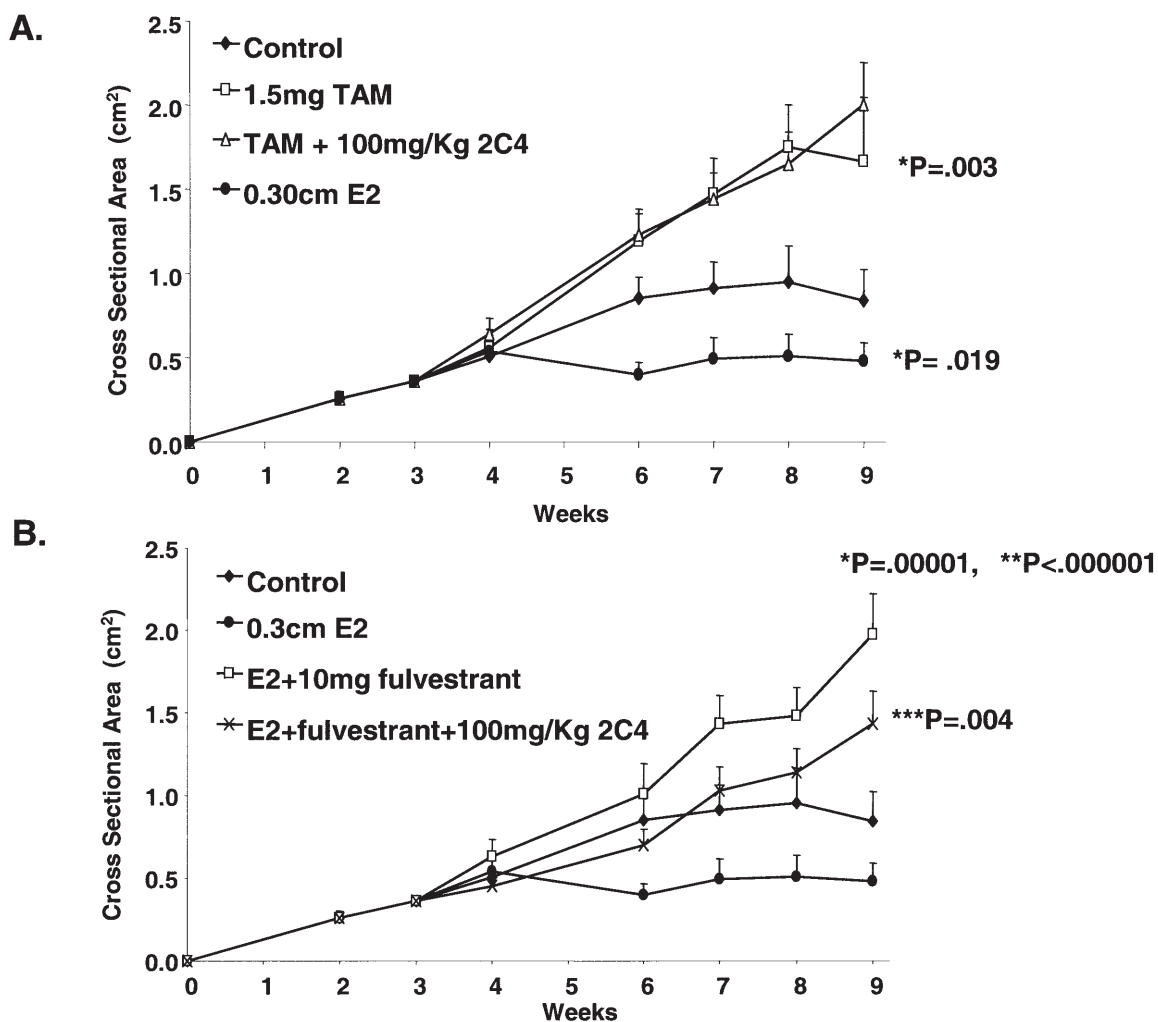


Figure 5. Growth of MCF-7TAMLT tumors in athymic mice in response pertuzumab. Estradiol (E2) was given as a 0.30-cm silastic capsule, tamoxifen (TAM) was given orally by gavage at 1.5 mg/day for 5 days, fulvestrant was given as a subcutaneous injection as a 5 mg/0.1 ml dose twice a week, and pertuzumab (2C4) was given as an i.p. injection at a dose of 100 mg/kg of mouse body weight twice a week. For both panels, tumor areas were measured weekly with vernier calipers. Cross-sectional areas were calculated by multiplying the length (l) by the width (w) and by π and dividing the product by 4 (i.e. $lw\pi/4$). Sixty athymic mice were bi-transplanted with MCF-7TAMLT tumors and grown with tamoxifen to a mean cross-sectional area of 0.36-cm². Tamoxifen was then withdrawn and groups of 10 mice were randomly assigned to the following treatments: control, tamoxifen alone, tamoxifen plus pertuzumab (2C4), estradiol alone, estradiol plus fulvestrant, or estradiol plus fulvestrant plus pertuzumab. *Compared to the control group at week 9. **Compared to estradiol alone group at week 9. ***Compared to the estradiol plus fulvestrant group at week 9. Error bars, 95% confidence intervals. All statistical tests were two-sided.

and HER3 pathway. In addition, we showed that tamoxifen-stimulated growth of tamoxifen-resistant breast tumors is independent of ER α transcriptional activity at four known estrogen-responsive genes, *c-myc*, *igf-1*, *cathepsin D*, and *pS2* and independent of EGFR, HER2/neu, or HER3 activity suggesting an alternate mechanism of action.

Resistance to adjuvant tamoxifen therapy is common for women with ER α -positive breast cancer. However, there are at least two forms of tamoxifen resistance, *de novo*/intrinsic (patients that do not respond to tamoxifen initially) or acquired resistance (patients initially respond to tamoxifen, but resistance occurs during therapy). Current studies have demonstrated that one mechanism responsible for *de novo*/intrinsic resistance to tamoxifen is high expression of both AIB1 and HER2/neu in ER α -positive breast cancer (16,17). Osborne *et al* (26) showed that post-menopausal women with ER α -positive breast cancer were less responsive

initially to tamoxifen treatment when their tumors expressed high levels of both AIB1 and HER2/neu. Using genetically-engineered laboratory models, the investigators also suggested that tamoxifen treatment induced binding of AIB1 to promoters of estrogen-responsive genes and this was promoted by the EGFR/HER2/ neu signaling pathway since gefitinib, the EGFR inhibitor, reversed this binding and tamoxifen-resistance of MCF-7 xenografts *in vivo* (17). This mechanism could be responsible for *de novo*/intrinsic resistance. However, it is not clear as yet whether high expression of both AIB1 and HER2/neu promotes acquired resistance during tamoxifen therapy. In the current study, we sought to investigate the roles of genomic ER α , EGFR, HER2/neu, HER3, and HER4 in two *in vivo* models of drug resistance: acquired resistance which results in tamoxifen-stimulated growth of MCF-7TAMLT xenografts after 5 years of treatment (20,21); and resistance to fulvestrant when given

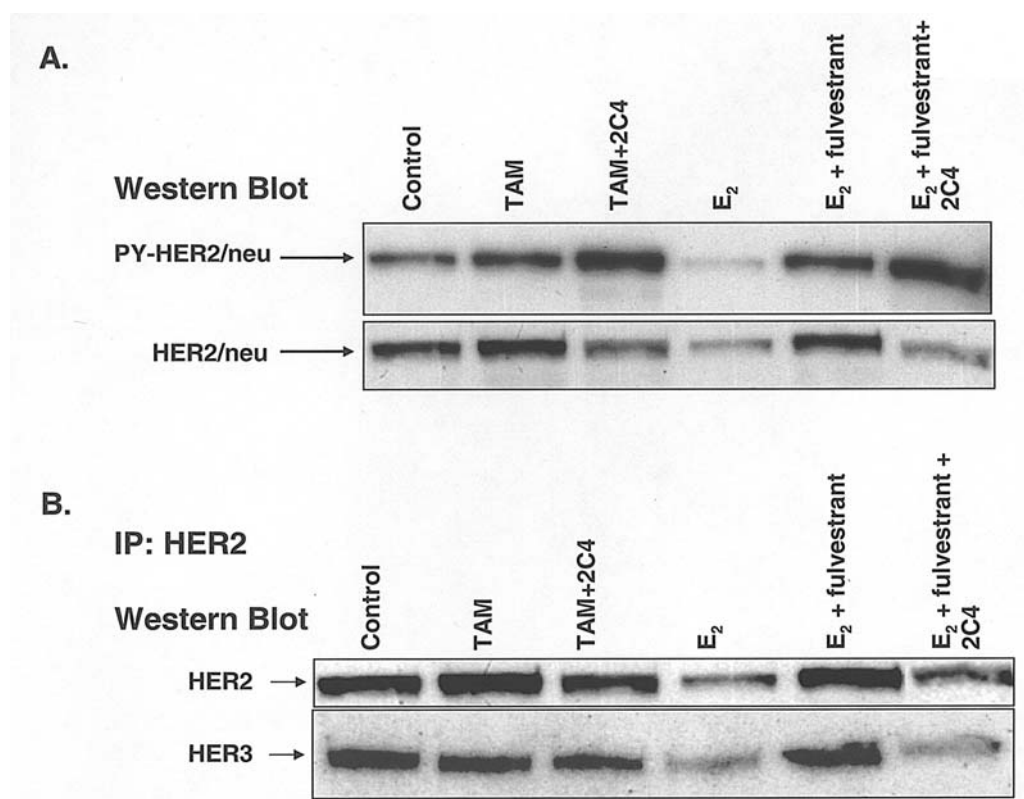


Figure 6. Effects of pertuzumab on the interaction of HER2 and HER3 in MCF-7TAMLT tumors. (A) Tumors were excised at week 9 (see Fig. 5) and protein extracted as described in Materials and methods. Western blot analysis was performed on tumor extracts to detect tyrosine1248-phosphorylated HER2/neu and Total HER2 protein. (B) Total HER2/neu protein was immunoprecipitated from tumor extracts followed by Western blot analyses to detect HER2/neu and HER3 proteins within the HER2/neu immunocomplex. The blots are representative experiments from at least 3 independent investigations.

in combination with physiologic levels of estradiol after failure on tamoxifen (34).

We have demonstrated previously that tamoxifen-stimulated MCF-7TAMLT tumors naturally expressed higher levels of HER2/neu mRNA after 5 years of treatment compared to the parental, tamoxifen naïve MCF-7E2 tumor (34). In addition, we found that treatment of tamoxifen-stimulated MCF-7TAMLT tumors with the combination of estradiol plus fulvestrant increased HER2/neu mRNA an additional 6-fold above the MCF-7TAMLT controls (34). The results from the current study showed that basal levels of EGFR, HER3, and HER4 mRNAs were also increased in MCF-7TAMLT tumors (Fig. 3). Of particular interest, both HER2/neu and HER3 mRNAs were overexpressed by ~14 and 13-fold above control in estradiol plus fulvestrant-treated MCF-7TAMLT tumors (Fig. 3). The current study demonstrated that tamoxifen or estradiol plus fulvestrant treatment did not induce expression of four known estrogen responsive genes, *c-myc*, *igf-1*, *cathepsin D*, or *pS2* above controls in our model of acquired resistance to tamoxifen (Fig. 2). More importantly, our data showed that estradiol continued to induce expression of *c-myc*, *igf-1*, *cathepsin D*, and *pS2* genes (Fig. 2) while inhibiting growth and that the antiestrogen, fulvestrant, reversed the estradiol-induced expression of these genes (Fig. 2) while promoting growth. Collectively, these results suggest that growth of MCF-7TAMLT tumors in response to tamoxifen or estradiol plus fulvestrant might be independent of ER α genomic/transcriptional activity.

Several studies (28-32) have previously suggested that overexpression of EGFR and/or HER2/neu promotes resistance to antiestrogens such as tamoxifen or fulvestrant. We demonstrated that our tumor model of acquired resistance to tamoxifen (MCF-7TAMLT) naturally expressed high basal levels of all four members of the EGFR family of RTKs including EGFR, HER2/neu, HER3, and HER4 (35). Thus, we tested the hypothesis that overexpression of EGFR RTKs promoted growth of tamoxifen-stimulated or estradiol plus fulvestrant-stimulated MCF-7TAMLT tumors by treating them with the EGFR inhibitor, gefitinib, the HER2/neu inhibitor, trastuzumab, or the blocker of HER2/neu - HER3 hetero-dimerization, pertuzumab in an *in vivo* growth assay. The results showed that growth of tamoxifen-stimulated MCF-7TAMLT tumors was not inhibited by trastuzumab (data not shown), gefitinib (data not shown), or pertuzumab (Fig. 5) *in vivo*. These findings suggest that the mechanism of growth by tamoxifen in MCF-7TAMLT tumors could also be independent of EGFR, HER2/neu, or HER3. However, it is possible that the combination therapy with gefitinib, trastuzumab, and pertuzumab could inhibit tamoxifen-stimulated growth of MCF-7TAMLT tumors. However, the conduct of the study *in vivo* is difficult and expensive with the multiple drug administrations and the accumulative toxicities and potential discomfort to the animals.

MCF-7TAMLT tumors were treated with fulvestrant in combination with physiologic estradiol in order to mimic the clinical scenario of second- or third-line treatment with

fulvestrant after failure on long-term adjuvant therapies. A single center trial demonstrated fulvestrant treatment of 42 post-menopausal women with metastatic breast cancer heavily pretreated with prior endocrine therapies produced 19% stable disease (SD) with two women being SD for 2 years (14). A phase II trial of the North Central Cancer Treatment Group of 77 post-menopausal women with metastatic breast cancer showed 10% overall response rates with fulvestrant treatment after tamoxifen and aromatase inhibitors (36). This trial demonstrated that women who had an aromatase inhibitor alone and then fulvestrant had an overall response rate of 24% versus only 9% for women who had tamoxifen, an aromatase inhibitor, and then fulvestrant. In a third trial, all 67 patients in the Perey Group (37) who had been pretreated with both tamoxifen and an aromatase inhibitor, had an objective response rate of 1.5%, with stable disease ≥ 24 weeks of 27%, for an overall clinical benefit rate of 28.4%. These clinical trials demonstrate that fulvestrant does have activity after failure on long-term endocrine therapy. However, the overall clinical benefits are small and the majority of breast tumors progress. In particular, one trial demonstrated that treating with tamoxifen first worsened the clinical benefit of fulvestrant later (36). This result agrees with our findings that tamoxifen-resistant breast cancer could be resistant to fulvestrant in combination with physiologic estradiol. The question that remains is why does fulvestrant not work in the presence of physiologic estradiol? Our results showed that both HER2/neu and HER3 mRNAs were further increased above control with estradiol plus fulvestrant treatment of MCF-7TAMLT tumors (Fig. 3). In the addition, the activities of HER2/neu and HER3 proteins as measured by phosphorylation of HER2/neu, and downstream kinases, ERK-1/2 and GSK-3 α and (targets of Akt) were hyper-activated in MCF-7TAMLT tumors-treated with estradiol plus fulvestrant as compared to control, tamoxifen, or estradiol (Fig. 4A). Furthermore, a complex of HER2/neu and HER3 proteins of 185 kDa was found to have higher levels of total phosphorylated tyrosine residues in MCF-7TAMLT tumors treated with estradiol plus fulvestrant compared to tamoxifen (Fig. 4B) indicating that the activity of heterodimers HER2/neu and HER3 could be responsible for estradiol plus fulvestrant stimulated growth of MCF-7TAMLT tumors. The results showed that only pertuzumab, an inhibitor of HER2/neu-HER3 heterodimerization, inhibited the growth of MCF-7TAMLT tumors treated with estradiol plus fulvestrant by 37.2% (Fig. 5B) and specifically blocked the interaction of HER2/neu with HER3 (Fig. 6B). These findings indicate that the mechanisms responsible for growth by tamoxifen or estradiol plus fulvestrant are distinct and that resistance to fulvestrant in combination with physiologic estradiol after failure on tamoxifen could be due to increased expression, interaction, and activity of the HER2/neu and HER3 signaling pathway.

Although the current study suggests a role for HER2/neu and HER3 signaling in estradiol plus fulvestrant-stimulated breast cancer after failure on tamoxifen, we cannot exclude the possibility of other mechanisms. For example, we demonstrated that growth of MCF-7TAMLT tumors with tamoxifen or estradiol plus fulvestrant directly correlated with interaction of ER α protein with the coactivator, AIB1 protein (data not shown). A recent study has shown AIB1

overexpressing transgenic mice have mammary hypertrophy, hyperplasia, and malignant mammary tumors and also express high levels of IGF-1 mRNA and serum IGF-1 protein (38). Another study (39) concluded that AIB1 was required for IGF-1-induced proliferation, signaling, cell survival, and gene expression in human breast cancer cells, independent of its role in estrogen signaling. Our study showed that basal levels of AIB1 mRNA and protein (data not shown), and IGF-1 mRNA (Fig. 2) were increased in MCF-7TAMLT tumors compared to the parental, tamoxifen naïve MCF-7E2 tumors. In addition, we showed that the tamoxifen or estradiol plus fulvestrant-induced interaction of ER α protein with AIB1 protein was independent of ER α transcriptional activity (Fig. 2). Thus, the current findings suggest that growth of MCF-7TAMLT tumors in response to tamoxifen or estradiol plus fulvestrant could also be due to an enhanced IGF-1 signaling pathway. This hypothesis is consistent with findings from an *in vivo* model of tamoxifen-stimulated endometrial cancer (40). In that study, trastuzumab effectively inhibited growth of tamoxifen-stimulated ECC-1 tumors by 100% while only inhibiting growth of parental ECC-1E2 tumors by 50%. The results from that study showed that both IGF-1 and IGF-1R mRNAs and the activity of the IGF-1R as measured by the extent of IRS-1 phosphorylated tyrosine residues were decreased significantly compared the parental ECC-1E2 tumors.

It was shown by Song *et al* (41) that Shc, an intracellular adaptor protein that is recruited to RTKs upon activation by ligand and phosphorylation of tyrosine residues, and the IGF-1R together serve to translocate the ER α to the cell membrane to facilitate the rapid actions of estradiol independent of ER α transcription. In our study, we showed that tamoxifen or estradiol plus fulvestrant stimulated growth of MCF-7TAMLT tumors, increased the phosphorylation of HER2/neu, phosphorylation of GSK3 α (a target of Akt), and the activity of HER2/neu with HER3 (Fig. 4) without inducing transcription of four known estrogenic responsive genes (Fig. 2), indicating a possible direct role for ER α at the cell membrane. Thus, growth stimulation by tamoxifen or estradiol plus fulvestrant could activate HER2/neu signaling by recruiting the ER α protein, which is expressed at high levels with tamoxifen or estradiol plus fulvestrant treatment, directly to the cell membrane to function as a growth stimulator. Studies are currently underway to address this hypothesis.

In summary, we suggest that acquired resistance to tamoxifen during adjuvant therapy might be independent of ER α genomic/transcriptional activity at four known estrogen-responsive genes, *c-myc*, *igf-1*, *cathepsin D*, and *pS2*. In addition, we demonstrated that fulvestrant treatment in combination with physiologic estradiol as a second-line therapy after failure on tamoxifen paradoxically promoted growth at least in part through increased expression and activity from the HER2/neu and HER3 signaling pathway as the monoclonal antibody pertuzumab which inhibits HER2/neu and HER3 heterodimerization significantly reduced growth *in vivo* by 37.2%. Finally, our findings indicate that pertuzumab or other similar inhibitors should be studied further as a possible therapeutic strategy for resistance to fulvestrant after established acquired tamoxifen resistance has occurred. Overall, our laboratory findings suggest some immediate

applications to aid patients in the clinic. If the antitumor actions of fulvestrant are impaired by physiologic estradiol in phase II antihormone resistance, then fulvestrant should be evaluated during therapy with an aromatase inhibitor. Alternatively, protocols should be developed to evaluate the apoptotic value of short-term (8 weeks) low-dose estrogen treatment for tumor debulking followed by maintenance on fulvestrant and an aromatase inhibitor to extend the period of disease control (42,43).

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