Trastuzumab Therapy for Tamoxifen-Stimulated Endometrial Cancer

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Abstract
A novel in vivo model of tamoxifen-stimulated endometrial cancer was developed and the role of HER-2/neu investigated by using trastuzumab. Tamoxifen-stimulated tumors (ECC-1TAM) were growth stimulated by 17β-estradiol (E2), tamoxifen, or raloxifene. Trastuzumab inhibited growth of E2-stimulated ECC-1E2 tumors by 50% and tamoxifen-stimulated ECC-1TAM tumors by 100%. ECC-1 tumors expressed functional estrogen receptor α (ERα) as measured by induction of pS2 and c-myc mRNAs. E2 induced pS2 and c-myc mRNAs up to 40-fold in ECC-1E2 and ECC-1TAM. Tamoxifen induced pS2 and c-myc mRNAs up to 5-fold in ECC-1E2 tumors and up to 10-fold in ECC-TAM tumors. Trastuzumab blocked E2-induced pS2 mRNA (P < 0.01) in ECC-1E2 by 50% and tamoxifen-induced c-myc mRNA (P < 0.1) in ECC-1TAM tumors by 70%. Trastuzumab decreased phosphorylated and total HER-2/neu protein in ECC-1E2 and ECC-1TAM tumors. However, only phospho-ERK-1/2 and not phospho-Akt protein was decreased by trastuzumab in tamoxifen-treated ECC-1TAM tumors. The insulin-like growth factor (IGF-I) signaling pathway also activates extracellular signal-related kinase (ERK)-1/2 and could block the efficacy of trastuzumab in ECC-1E2 tumors. The results showed that IGF-I, IGF-IR mRNAs, and phospho-insulin receptor substrate-1 (IRS-1) protein were decreased in ECC-1TAM compared with ECC-1E2 tumors. The results show that trastuzumab is an effective therapy for both E2-stimulated and tamoxifen-stimulated endometrial cancer. The data suggest estrogenic activities of E2 and tamoxifen at ERα-regulated pS2 and c-myc genes are in part mediated by HER-2/neu. However, trastuzumab is a better growth inhibitor of ECC-1TAM tumors where there is diminished IGF-I signaling allowing for complete blockade of the downstream phospho-ERK-1/2 signal. (Cancer Res 2005; 65(18): 8304-13)

Introduction
Tamoxifen has been the standard of care for estrogen receptor α (ERα)-positive breast cancer in premenopausal and postmenopausal women for over 15 years (1). Five years of adjuvant tamoxifen therapy reduces the incidence of contralateral breast cancer (2), improves overall survival (2), and also reduces the risk of breast cancer in women at high risk (3). However, tamoxifen is not a pure antiestrogen but a selective estrogen ERα modulator (4, 5). It has antiestrogenic affects on the breast epithelium but estrogenic affects on bone density (6, 7), circulating cholesterol and cardiovascular events (8), and the uterus (9). As a result of tamoxifen's selective estrogen receptor modulator activity around a woman's body, it increases the incidence of endometrial cancer by 0.1% in postmenopausal women (9). The exact mechanism by which tamoxifen induces endometrial cancer during breast cancer therapy is as yet unclear.

Numerous studies during the last 10 years have focused on formation of DNA adducts by metabolites of tamoxifen in the endometrium of rats and humans. Recently, Liehr showed that 17-estradiol (E2) is a dual mutagen and carcinogen in the immature rat lacking expression of ER (10). Tamoxifen might also be a mutagen/carcinogen as it has estrogenic activity in the uterus. However, the data to date shows that tamoxifen is a potent rat liver carcinogen causing the formation of tamoxifen-DNA adducts and liver cancer (11). However, little conclusive data exists showing a direct affect of tamoxifen or its metabolites on DNA adduct formation in the rat uterus to cause endometrial cancer. Most studies lack convincing evidence for tamoxifen-DNA adduct formation in rat and human endometrium (11–18), suggesting that the estrogenic action of tamoxifen at ERα-regulated genes is likely to be the more important factor in development of endometrial cancer in women undergoing tamoxifen therapy.

Shang and Brown (19) showed, using in vitro cell culture models, that tamoxifen is estrogenic in endometrial cancer cells by stimulating the recruitment of coactivators, in particular SRC-1, a member of the p160 family of coactivators, to a certain subset of genes regulated by the ERα at the transcriptional level. The investigators concluded that the SERM activity of tamoxifen in the endometrium could be due to differences in the recruitment of coregulators to certain ER-regulated promoters. Furthermore, Shah and Rowen (20) recently showed that the nonreceptor tyrosine kinase, src, enhances the agonist activity of tamoxifen in endometrial cancer cells by phosphorylation of Ser167 and stabilization of ER promoter interaction. However, neither of these studies has been confirmed using preclinical in vivo models of tamoxifen-stimulated endometrial tumors.

In addition, resistance to adjuvant tamoxifen therapy for ERα-positive breast cancer is a very common occurrence. Resistance to tamoxifen is defined as the lack of tumoristasis and/or regression or might be evidenced by growth stimulation. Subsets of breast tumors that do not initially respond (i.e., are not growth arrested or do not regress) to tamoxifen are intrinsically resistant to tamoxifen therapy. In contrast, acquired resistance to tamoxifen during ≥5 years of adjuvant tamoxifen therapy (21) could result from growth stimulation in response to continuous treatment with tamoxifen. Osborne et al. (22) showed that postmenopausal women with ERα-positive breast cancer do not initially respond to tamoxifen therapy when their tumors overexpress both HER-2/neu, a member of the epidermal growth factor receptor family of receptor tyrosine kinases, and the gene amplified in breast cancer-1 (AIB1), a member of the p160 family of coactivators.
More recently, Shou et al. (23) concluded that tamoxifen's estrogenic activity is partly due to crosstalk between HER-2/neu and ERα in MCF-7 cancer cells that express high levels of AIB1 and stably overexpress HER-2/neu. These studies showed that overexpression of HER-2/neu and AIB1 in ERα-positive breast cancer cells can cause intrinsic resistance to tamoxifen. However, only ~10% of all ERα-positive breast cancer cells overexpress HER-2/neu. Therefore, it is unclear whether HER-2/neu plays a role in the development of acquired resistance to tamoxifen during the 5 years of adjuvant therapy.

Studies suggest that overexpression of HER-2/neu leads to tamoxifen-resistant breast cancer (22, 24). A recent report showed that src tyrosine kinase potentiates the agonist activity of tamoxifen in endometrial cancer cells (20). In addition, overexpression of HER-2/neu correlates with high-grade endometrial cancer (25–27). However, exact mechanisms leading to tamoxifen-stimulated breast and endometrial cancers are not well understood. Currently, inhibitors to epidermal growth factor receptor (i.e., gefitinib), HER-2/neu (i.e., trastuzumab), Ras/mitogen-activated protein kinase (i.e., farnesyltransferase inhibitor, tipifarnib), and protein kinase B (Akt; i.e., an mammalian target of rapamycin inhibitor, CCI 779) are being investigated either alone or in combination with tamoxifen to prevent and/or treat resistance to tamoxifen therapy. The question that remains to be answered is if HER-2/neu is important for tamoxifen-resistant breast cancer, then does HER-2/neu play a role in the development of endometrial cancer during long-term tamoxifen therapy? We have developed a novel in vivo model of tamoxifen-stimulated endometrial cancer (ECC-ITAM) by treating tamoxifen-naïve, E2-stimulated ECC-1E2 tumors with E2 plus tamoxifen for 24 weeks. We investigated the role of HER-2/neu in the growth of both ECC-1E2 and ECC-ITAM tumors using the humanized monoclonal antibody to HER-2/neu, trastuzumab.

Materials and Methods

Cell culture. The human endometrial cancer cell line, ECC-1, was a generous gift from Dr. Myles Brown at the Dana-Farber Cancer Institute, Harvard Medical School. ECC-1 cells were maintained at 37°C in a 95% humidified/5% CO₂ atmosphere in phenol red–containing DMEM supplemented with 10% fetal bovine serum, 6 ng/mL insulin solution, 1% t-glutamine, 1% nonessential amino acids, 1% of a combination of penicillin streptomycin, and ant myecotic (Life Technologies, Long Island, NY).

Growth of ECC-1E2 tumors in vivo. The ECC-1E2 tumors used in these experiments were derived by bilateral inoculation of 0.10 mL of 1 × 10⁶ suspended ECC-1 cells in sterile PBS into the mammary fat pads of 4- to 6-week-old ovariectomized BALB/c nu/nu athymic mice (Harlan Sprague-Dawley, Madison, WI). ECC-1 cells-inoculated into athymic mice were supplemented with 0.30-cm E2 (Sigma, St. Louis, MO) silastic capsules (Baxter Health Care, Mundelein, IL) to achieve E2-stimulated tumor growth (ECC-1E2; refs. 28–33). Tumors were maintained by serial passage of solid tumors into athymic mice as described previously (29). Thirty mice were inoculated with parental ECC-1E2 tumors and groups (10 mice) were treated in the following manner: vehicle, 0.30-cm E2 capsule, 1.5 mg/d tamoxifen, 10 mg fulvestrant (dissolved in 100% ethanol and diluted in sterile-filtered peanut oil, 5 mg/0.10 mL injected s.c., twice weekly), and 0.30-cm E2 + 30 mg/kg trastuzumab (15 mg/kg injected ip, twice weekly).

Growth of ECC-ITAM tumors in vivo. Long-term tamoxifen-stimulated tumors (ECC-ITAM) were developed by retransplanting the surviving E2 + 1.5 mg tamoxifen-treated ECC-1E2 tumors into 40 new athymic mice, and treating groups (10 mice) with vehicle, 1.5 mg/d tamoxifen, 0.30-cm E2 capsules, or 1.5 mg/dRaloxifene (obtained from the pharmacy; refs. 29, 30, 34) for 12 weeks. In additional experiments, 50 animals were bitransplanted with ECC-ITAM tumors and grown to a mean cross-sectional area of 0.31 cm² and randomly separated into groups of 10 mice that were then subsequently treated as follows: vehicle, 1.5 mg tamoxifen, 0.30-cm E2 capsules, 10 mg fulvestrant (5 mg given sc., twice weekly), and 1.5 mg tamoxifen + 30 mg/kg trastuzumab. In a final in vivo experiment, 30 mice were bilaterally transplanted with 1-mm tamoxifen-treated ECC-ITAM tumors from Fig. 1D and 10 mice per group were treated as follows: control, 1.5 mg tamoxifen, and 1.5 mg tamoxifen plus 5 mg fulvestrant (twice weekly).

Real-time reverse transcription-PCR for human pS2, c-myc, HER-2/neu, insulin-like growth factor-1, and insulin-like growth factor-IR mRNAs in tumors. Total RNA is extracted from the tumors using the RNeasy Mini Kit (Qiagen, Stanford Valencia, CA) according to the manufacturer's instructions. The total RNA is reverse transcribed 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 human pS2, HER-2/neu, insulin growth factor-1 (IGF-I), and IGF-IR are designed using Primer ExpressTM1.5 software set at default variables 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′-AGGCCGACAGACAGTGTG-3′ and 5′-CCCTGCA-GAAGTGTCTAAAATCTCA-3′, respectively. The sequence for the pS2 probe is 5′-CTCTGTTTGCAGACACCTGTTCG-3′ where the FAM is the reporter and QSY7 is the quencher (MegaBases). The sequences for the forward and reverse primers for human HER-2/neu are 5′-ACTGCAGAGCTGCGGATT-3′ and 5′-ACGCCGACGCA-TAGTTGT-3′, respectively. The sequence for the human HER-2/neu probe is 5′-TGCAAGGACACCATCCCTTGA-3′ where the FAM is the reporter and QSY7 is the quencher (MegaBases). The probe and primers for human IGF-IR were purchased from Perkin-Elmer Applied Biosystems (PE-ABI, Stanford Valencia, CA) and used according to the manufacturing instructions. The sequences for the forward and reverse primers for human HER-2/neu are 5′-ACTGCAGAGCTGCGGATT-3′ and 5′-ACGCCGACGCA-TAGTTGT-3′, respectively. The sequence for the human IGF-I probe is 5′-AGAGGCGTAGGTATGTAGCGCACC-3′ where the FAM is the reporter and QSY7 is the quencher (MegaBases). The probe and primers for human IGF-IR were purchased from Perkin-Elmer Applied Biosystems (PE-ABI, Stanford Valencia, CA). The quantity of human 18s RNA was also measured in each total cDNA sample for normalization purposes. The probe and primers for 18s RNA were purchased from Perkin-Elmer Applied Biosystems (PE-ABI, Stanford Valencia, CA). The PCR conditions were 50°C for 2 minutes, 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Western blot analyses. Tumors were homogenized by grinding in liquid nitrogen and resuspending in lysis buffer [1% Triton X-100, 1 mmol/L EDDTA, 150 mmol/L NaCl, 50 mmol/L Tris base (pH 7.4), 25 mg/mL phenylmethylsulfonyl fluoride, 10 (g/mL leupeptin, 10 mg/mL aprotinin, 10 mg/mL pepstatin, 10 mg/mL TLCK, 10 mg/mL N-tosyl-L-phenylalanine chloromethyl ketone, 100 mmol/L NaF, 10 mmol/L ortho-vanadate; Sigma]. The extract was subsequently sonicated and then centrifuged for 5 minutes at 5,000 × g at 4°C. The supernatant was collected and protein concentration was measured using the Bradford assay (Bio-Rad Laboratories, Inc., Santa Cruz, CA). Equal amounts of protein (25–50 μg) were loaded onto a 7% polyacrylamide/bisacrylamide gel for SDS-PAGE followed by Western blotting. The following proteins were detected by Western blot: Tyr539, phosphorylated HER-2/neu [1:2000 Rabbit anti-human, Upstate Biotechnology, New York, NY], HER-2/neu (1:200 mouse anti-human, Ab-11, NeoMarkers, Fremont, CA), Ser473-phosphorylated Akt (1:1000 rabbit anti-human, Cell Signaling Technology, Beverly, MA), Akt (1:1000 rabbit anti-human, Cell Signaling Technology), phosphorylated-ERK1/2 (1:1000 mouse anti-human, Cell Signaling Technology), extracellular signal-related kinase (ERK)-1/2 (1:1000 mouse anti-human, Cell Signaling Technology), β-actin...
Figure 1. Development of tamoxifen (TAM)–stimulated endometrial cancer in vivo and the effect of trastuzumab on growth. A, thirty athymic mice were bitransplanted with 1-mm ECC-1E2 tumors. Mice were randomly treated as follows: 10 mice received no treatment as Controls, 20 mice received 0.30-cm E2 capsules that were implanted intrascapularly under the skin for silastic release of E2. The capsules were replaced every 8 weeks to maintain a blood concentration of 83 to 100 pg/mL. Of the 20 mice receiving E2, 10 were also fed 1.5 mg/d tamoxifen orally by gavage 5 days a week. The length and width of tumors were measured weekly up to 24 weeks using Vernier calipers. B, at week 24, the E2 plus tamoxifen tumors were excised and retransplanted into 40 new athymic mice and 10 mice per group were treated as follows: Control, 1.5 mg/d tamoxifen, 0.30-cm E2 capsules, or 1.5 mg/d raloxifene (RAL, fed orally by gavage, 5 days a week). The tumors were grown up to 12 weeks and measured weekly as described above. C, fifty athymic mice were bitransplanted with 1-mm ECC-1E2 tumors and treated with 0.30-cm E2 capsules until they reached a mean cross-sectional area of 0.28 cm² at week 4. Then, 10 mice per group were randomly treated as follows: Control, E2, tamoxifen, 5 mg/0.1 mL fulvestrant injected twice per week s.c., or E2 plus 15 mg/kg trastuzumab injected i.p. twice per week. The tumor sizes were measured weekly as described in (A) up to 9 weeks. D, fifty athymic mice were bilaterally transplanted with 1-mm tamoxifen-treated ECC-1TAM tumors from (B) and continued to be treated with tamoxifen until the mean cross-sectional area of the tumors reached 0.31 cm². Then, 10 mice per group were randomly treated as follows: Control, tamoxifen, E2, fulvestrant, or tamoxifen plus trastuzumab. The tumor sizes were measured as described in (A) up to 11 weeks. The cross-sectional area of each tumor was calculated using the following formula: cross-sectional area = (length × width × π)/4. Points, mean cross-sectional areas of tumors; bars, ±SE. *, statistical significance of mean cross-sectional areas of tumors at the end of each growth study compared with Control. **, statistical significance of mean cross-sectional areas of tumors at the end of each growth study compared with the E2-treated group in (C) or to the tamoxifen-treated group in (D).

Results

Growth of ECC-1E2 and ECC-ITAM tumors in vivo. To elucidate the mechanism of action by which tamoxifen stimulates growth of endometrial cancer cells under physiologic conditions to mimic the clinical situation, we developed a novel tamoxifen-stimulated endometrial cancer model in vivo by treating the parental ECC-1E2 tumors originated from cell lines (ECC-1) derived from the human EnCa101 tumor with postmenopausal levels of E2 plus tamoxifen for 24 weeks. The results showed that the parental ECC-1E2 tumors grew faster in response to E2 (P < 0.0001) compared with control (Fig. 1A). At 24 weeks, ECC-1E2 tumors treated with E2 plus tamoxifen were larger (P < 0.0001) than control (Fig. 1A). Upon serial bitransplantation of ECC-1E2 tumors treated with E2 plus tamoxifen into new generations of athymic mice, these tumors were growth stimulated more by E2 alone (P < 0.01), tamoxifen alone (P = 0.48), or raloxifene alone (P = 0.006) compared with control (Fig. 1B, C, D).
Comparison of tamoxifen (Tam) and tamoxifen + fulvestrant treated groups (Fig. 1D). The tumor volume was increased with Tam treatment in parental ECC-1E2 tumor by 1.5 mg tamoxifen (P = 0.35) compared with control (Fig. 1B). These data show the development of a novel in vivo model of tamoxifen-stimulated endometrial tumor that continues to grow in response to E2 and is cross-resistant with raloxifene.

Growth of ECC-1E2 and ECC-1TAM tumors in response to trastuzumab. We investigated the consequences of using a HER-2/neu inhibitor, trastuzumab, on growth of ECC-1E2 and ECC-1TAM tumors in vivo. The results showed that ECC-1E2 tumors that have reached an initial mean cross sectional area of 0.28 cm² at week 4 did not grow in response to vehicle control, tamoxifen, or fulvestrant compared with the E2-treated group (P = 0.02; Fig. 1C). Interestingly, trastuzumab partially blocked E2-induced growth of ECC-1E2 tumors by almost 50% (P = 0.06) compared with tumors treated with E2 alone (Fig. 1C).

ECC-1TAM tumors that were initially a mean cross sectional area of 0.31 cm² at week 6 grew with E2 (P < 0.01) or tamoxifen (P = 0.1) compared with control (Fig. 1D). Interestingly, fulvestrant modestly stimulated growth of ECC-1TAM tumors compared with control. More importantly, tamoxifen-stimulated ECC-1TAM tumors were completely growth inhibited by trastuzumab (P = 0.002) compared with tamoxifen alone (Fig. 1D). These results suggest for the first time that tamoxifen stimulates growth of endometrial cancer at least in part through HER-2/neu.

To determine whether tamoxifen stimulated growth of ECC-1TAM tumors through Erk, a separate experiment was done using the combination of tamoxifen plus fulvestrant. The results showed that fulvestrant inhibited growth of tamoxifen-treated ECC-1TAM tumors (Fig. 2) indicating that the growth stimulatory effect of tamoxifen is mediated by Erk.

Expression of estrogen-responsive genes, pS2 and c-myc, in ECC-1E2 and ECC-1TAM tumors. The results from the growth studies showed that fulvestrant blocked tamoxifen-stimulated ECC-1TAM tumors (Fig. 2) thus suggesting that tamoxifen is estrogenic through Erk. In addition, Shang and Brown previously showed that tamoxifen is estrogenic at Erk-mediated promoters for c-myc and IGF-I genes (19). Therefore, we investigated Erk transcriptional activity at pS2 (classic promoter-driven gene) and c-myc (tethered-promoter driven gene) in both ECC-1E2 and ECC-TAM tumors. The results in Fig. 3A showed that E2 increased pS2 mRNA expression in ECC-1E2 (25.06-fold over control, P < 0.0001) and ECC-1TAM (34.59-fold over control, P < 0.01) tumors. Trastuzumab inhibited E2-induced pS2 mRNA expression (P < 0.1) by almost 50% in parental ECC-1E2 tumors (Fig. 3A). Tamoxifen induced pS2 mRNA expression by 3.94-fold over control (P = 0.045) in ECC-1E2 tumors and 7-fold in ECC-1TAM tumors over control (P = 0.045; Fig. 3A) and trastuzumab had no effect on tamoxifen-mediated increase of pS2 mRNA expression in ECC-1TAM tumors (Fig. 3A).

mRNA for the c-myc gene was increased with E2 treatment in both ECC-1E2 and ECC-1TAM tumors by 41.72-fold (P = 0.0001) and 29.98-fold (P < 0.01), respectively, compared with controls (Fig. 3B). Tamoxifen induced expression of c-myc mRNA in ECC-1E2 by only 3.32-fold (P = 0.25) while increasing c-myc mRNA by 10.06-fold (P < 0.001) in ECC-1TAM tumors compared with control. Interestingly, trastuzumab inhibited the tamoxifen-induced increase of c-myc mRNA in ECC-1TAM tumors by 70% (P < 0.1; Fig. 3B). These results confirm that tamoxifen is estrogenic at Erk-regulated genes pS2 and c-myc in ECC-1 endometrial tumors. More importantly, the data suggest that estrogenic affects that correlate directly to growth stimulation by either E2 in ECC-1E2 tumors or tamoxifen in ECC-1TAM tumors at specific genes such as pS2 or c-myc, respectively, are at least in part regulated by HER-2/neu.

Effectiveness of trastuzumab in inhibiting HER-2/neu, mitogen-activated protein kinase, and Akt activities. HER-2/neu was not overexpressed at the mRNA level in ECC-1TAM tumors compared with ECC-1E2 tumors (data not shown). Thus, an increase in HER-2/neu expression could not explain the effectiveness of trastuzumab in blocking growth of ECC-1TAM tumors compared with ECC-1E2 tumors. To determine the effect of trastuzumab on HER-2/neu activity, we measured tyrosine-phosphorylated HER-2/neu, total HER-2/neu, and downstream effectors, phosphorylated ERK-1/2, total ERK-1/2, phosphorylated Akt, and total Akt protein levels by Western blot analyses. The results showed that total HER-2/neu protein levels were unchanged in ECC-1TAM tumors compared with ECC-1E2 tumors (Fig. 3C).

However, basal levels of phospho-HER-2/neu protein were increased in ECC-1TAM by 2.7-fold versus ECC-1E2 tumors (Fig. 3C) as determined by densitometry of three independent Western blots. However, trastuzumab decreased...
tyrosine-phosphorylated HER-2/neu and total HER-2/neu protein to almost undetectable levels in both ECC-1E2 and ECC-ITAM (Fig. 3C). Furthermore, trastuzumab had little effect on phospho-ERK-1/2, total ERK-1/2, phospho-Akt, or total Akt protein levels in ECC-1E2 tumors (Fig. 3C). In contrast, phospho-Akt protein was increased in ECC-ITAM tumors treated with tamoxifen compared with control but this increase was not affected by trastuzumab. Interestingly and in contrast to ECC-1E2 tumors, E2 or fulvestrant increased phospho-ERK-1/2 levels in ECC-ITAM tumors compared with control (Fig. 3C). More importantly,

![Diagram showing expression of estrogen-responsive genes, HER-2/neu, Akt, and ERK-1/2 proteins in ECC-1E2 and ECC-1TAM tumors.](image)

**Figure 3.** Expression of estrogen-responsive genes, HER-2/neu, Akt, and ERK-1/2 proteins in ECC-1E2 and ECC-1TAM tumors. A, ECC-1E2 tumors treated with Control, E2, tamoxifen (TAM), fulvestrant (F), or E2 + trastuzumab were excised at week 9 (see Fig. 1C). ECC-1TAM tumors treated with Control, tamoxifen, E2, fulvestrant, or tamoxifen + trastuzumab were excised at week 11 (see Fig. 1D). Total RNA was extracted from both sets of tumors as described in Materials and Methods. Total RNA was reverse transcribed to total cDNA and real-time PCR was done using the Applied Biosystems ABI 7700 Taqman PCR instrument to detect expression of the human pS2 mRNA as described in Materials and Methods. 18S RNA was used as a loading control in all samples. pS2 mRNA cycle threshold (Ct) values were normalized to C1 values for 18S RNA by subtracting the C_{18S} from the C_{pS2}. Columns, mean pS2 mRNA copy numbers relative to Control in three independent tumors with three replicates per tumor. *, statistical significance of pS2 mRNA copy number compared with Control. **, statistical significance of pS2 mRNA copy number of E2 + trastuzumab compared with E2 alone in ECC-1E2 tumors. B, the same sets of cDNA and protocol used to detect the human c-myc mRNA was used to detect the human c-myc mRNA in ECC-1E2 and ECC-1TAM tumors. *, statistical significance of c-myc mRNA copy number compared with Control. **, statistical significance of c-myc mRNA copy number of tamoxifen + trastuzumab compared with tamoxifen alone in ECC-1TAM tumors. C, total protein was extracted from ECC-1E2 (at week 9, Fig. 1C) and ECC-1TAM (at week 11, Fig. 1D) tumors as previously described in Materials and Methods. Extracts were vigorously vortexed followed by 5-second ultrasonic pulses to disrupt cellular membranes. The total mixture was centrifuged for 5 minutes at 5,000 × g at 4 °C. Fifty micrograms of supernatant from each sample were boiled in 2× Laemmli buffer and loaded onto a 7% polyacrylamide/bisacrylamide gel followed by SDS-PAGE. Western blot analyses to detect tyrosine (Y1248) phosphorylated HER-2/neu, total HER-2/neu, phosphorylated Ser117A- Akt, total Akt, phosphorylated ERK-1 and ERK-2, total ERK-1 and ERK-2, and β-actin proteins were done as described in Materials and Methods. The experiment was repeated at least thrice using three independent tumors. *, 2.7 ± 0.3-fold increase of basal levels of PY-HER2 protein as determined by densitometry in ECC-1TAM tumors compared with ECC-1E2 tumors.
trastuzumab decreased phospho-ERK-1/2 protein levels in ECC-1TAM compared with tamoxifen alone (Fig. 3C). These results suggest that trastuzumab is effective at down-regulating HER-2/neu protein and tyrosine phosphorylation of HER-2/neu in ECC-1E2 and ECC-1TAM tumors in the absence of HER-2/neu overexpression. However, the difference in trastuzumab's efficacy on growth of ECC-1TAM versus ECC-1E2 tumors might be due to differences in the regulation of activity of HER-2/neu and ERK-1/2, as the phosphorylation of HER-2/neu was elevated in ECC-1TAM tumors and ERK-1/2 was inhibited by trastuzumab only in ECC-1TAM tumors.

Measurement of insulin-like growth factor-I and insulin-like growth factor receptor mRNA and insulin receptor substrate-1 protein expression. Both HER-2/neu and insulin-like growth factor-I receptor (IGF-IR) activate ERK-1/2 and Akt. Thus, trastuzumab would be less effective in blocking the activity of ERK-1/2 or Akt from HER-2/neu if the IGF-IR signaling pathway was also active. A recent study has shown that the IGF-IR signaling pathway blocks the effectiveness of trastuzumab to inhibit growth of breast cancer cells and possibly lead to resistance (35). In addition, the IGF-IR pathway is increased in response to E2 and leads to activation of ERK-1/2 and Akt signaling in breast cancer cells (36). Therefore, we measured mRNA expression of both human IGF-I and IGF-IR in ECC-1E2 and ECC-1TAM tumors. In addition, to measure the cellular activity of the IGF-I/IGF-IR signaling pathway in the tumors, we measured tyrosine-phosphorylated IRS-1, and total IRS-1, a downstream adaptor protein that is recruited to the activated IGF-IR upon IGF-I ligand binding (37). The results showed that E2 increased IGF-I mRNA in ECC-1E2 tumors (4.7-fold, \( P < 0.01 \)).
Table 1. Summary of growth and signaling for ECC-1 tumors

<table>
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<tr>
<th>Experiment treatment</th>
<th>Growth</th>
<th>pS2* mRNA</th>
<th>c-myc* mRNA</th>
<th>Phospho-HER-2/neu</th>
<th>Phospho-Akt</th>
<th>Phospho-ERK-1/2</th>
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<td>+++</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>++++</td>
<td>7.00</td>
<td>10.06</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Tamoxifen + trastuzumab</td>
<td>–</td>
<td>4.89</td>
<td>3.25</td>
<td>+</td>
<td>++</td>
<td>–</td>
<td>++</td>
</tr>
<tr>
<td>Fulvestrant</td>
<td>++</td>
<td>4.58</td>
<td>1.08</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>++</td>
</tr>
</tbody>
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*Fold induction of mRNA compared with control.

and ECC-ITAM tumors (3.0-fold, \( P = 0.45 \)) compared with control (Fig. 4A). More importantly, IGF-1 mRNA was decreased in ECC-ITAM control (60% less, \( P = 0.02 \)), E2-treated (70% less, \( P = 0.14 \)), tamoxifen-treated (67% less, \( P = 0.11 \)), or fulvestrant-treated tumors (70% less, \( P = 0.04 \)) compared with ECC-1E2 tumors (Fig. 4A). IGF-IR mRNA was increased by E2 in ECC-1E2 tumors (5.51-fold, \( P = 0.03 \)) and ECC-ITAM tumors (3.5-fold, \( P = 0.04 \)) compared with controls (Fig. 4B). Similarly to the IGF-1 mRNA results, IGF-IR mRNA was decreased in ECC-ITAM control tumors (56% less, \( P = 0.02 \)), E2-treated (150% less, \( P = 0.21 \)), tamoxifen-treated (50% less, \( P = 0.03 \)), or fulvestrant-treated (70% less, \( P < 0.01 \); Fig. 4B). The activity of the IGF-1/IGF-IR signaling pathway was assessed by measuring tyrosine phosphorylation of IRS-1. E2, tamoxifen, or fulvestrant increased phosphorylated IRS-1 protein in ECC-1E2 tumors (Fig. 4C). In addition, E2 induced phosphorylated IRS-1 protein in ECC-ITAM tumors whereas tamoxifen had no effect on phospho-IRS-1 status compared with control (Fig. 4C). However, the overall extent of phosphorylated IRS-1 was less in ECC-ITAM tumors compared with ECC-1E2 tumors as determined by densitometry of three independent Western blots (Fig. 4D). These results taken together suggest that the effectiveness of trastuzumab to inhibit growth of ECC-ITAM tumors by blocking the activities of HER-2/neu, downstream ERK-1/2, and ERα-mediated c-myc mRNA expression could be due to the diminished IGF-1/IGF-IR signaling pathway (results summarized on Table 1 and conclusions summarized in Fig. 5).

**Discussion**

The results show, for the first time, that trastuzumab can completely block the growth of tamoxifen-stimulated endometrial cancer by inhibiting HER-2/neu signaling through ERK-1/2 and by blocking tamoxifen-induced transcription of c-myc (summarized in Table 1 and Fig. 5). We also showed that trastuzumab is more efficacious in tamoxifen-stimulated endometrial cancer than the parental E2-stimulated endometrial cancer possibly due to increased activity of HER-2/neu and decreased expression and signaling of the IGF-1/IGF-IR signaling pathway. These results strongly indicate that enhanced crosstalk between HER-2/neu through ERK-1/2 and ERα contributes to the natural development of tamoxifen-stimulated endometrial cancer.

Five years of adjuvant tamoxifen therapy has been the standard of care for ERα-positive breast cancer for over 15 years (1). The major serious side effect of tamoxifen treatment is the increased risk of endometrial cancer by 0.1% (9). To date, little is known about the mechanism of tamoxifen-stimulated endometrial cancer cell growth. However, Shang and Brown (19) showed that tamoxifen was more estrogenic in endometrial cancer cells compared with MCF-7 breast cancer cells in vitro by inducing transcription of ERα-regulated genes, c-myc and IGF-1. Our study examined the function of the ERα in a novel model of tamoxifen-stimulated endometrial cancer developed in vivo under physiologic conditions by measuring expression of two estrogen-responsive genes, pS2 and c-myc. The results clearly showed that tamoxifen treatment increased expression of pS2 and c-myc up to 4-fold in parental ECC-1E2 tumors and up to 10-fold in ECC-ITAM tumors (Fig. 3A and B). These data showed that tamoxifen is a weak agonist initially in tamoxifen-naive ECC-1E2 tumors and then a stronger agonist in tamoxifen-stimulated ECC-ITAM tumors. The results suggested that the increased tamoxifen-induced transcriptional activity might play an important role in the development of tamoxifen-stimulated endometrial cancer. More importantly, our data showed that trastuzumab, the HER-2/neu inhibitor, blocked tamoxifen-induced expression of c-myc by 70% in ECC-ITAM tumors, indicating that the increased agonist activity of tamoxifen on c-myc mRNA expression in these tumors might be due to the activity of the HER-2/neu signaling pathway.

In addition to the estrogenic activity of tamoxifen at ERα-regulated genes, tamoxifen could potentially exert nongenomic activity by binding to membrane-associated ERα-activating ERK-1/2 and/or Akt directly. It has been shown previously that E2 activates ERK-1/2 or Akt within minutes of treatment suggesting a nongenomic activity for ERα (38). Moreover, Song et al. showed that ERα is recruited to the membrane by interacting with Shc and IGF-IR where it can bind E2 or tamoxifen (39). Thus, the increase in phospho-ERK-1/2 by E2 or fulvestrant or phospho-Akt by tamoxifen or fulvestrant in ECC-ITAM tumors (Fig. 3C) could be a result of interaction with membrane ERα. Interestingly,
trastuzumab inhibited growth of tamoxifen-stimulated ECC-1TAM tumors (Fig. 1D), induction of c-myc mRNA (Fig. 3B), and phospho-ERK-1/2 (Fig. 3C) but did not block tamoxifen-induced phospho-Akt (Fig. 3C). Therefore, the function of the membrane ERα in activating Akt in terms of growth is unclear for tamoxifen-stimulated ECC-1TAM tumors. However, E2, tamoxifen, or fulvestrant stimulated growth and induced phospho-ERK-1/2 in ECC-1TAM tumors, suggesting that a direct interaction of ligand with membrane ERα is possible. In addition, fulvestrant had little effect on pS2 and c-myc mRNA expression (Fig. 3A and B), increased phospho-HER-2/neu, phospho-Akt, and -ERK-1/2 (Fig. 3C), and modestly increased growth of ECC-1TAM tumors (Fig. 1D). These results suggest that fulvestrant might act through membrane ERα. This result is not surprising as previously we reported that fulvestrant in combination with E2 stimulated growth and HER-2/neu signaling of tamoxifen-stimulated ECC-1TAM tumors independent of ERα transcriptional activity (40).

Surprisingly, the results from the present study also showed that trastuzumab therapy is effective in blocking tamoxifen-stimulated endometrial tumor growth in the absence of HER-2/neu overexpression. However, the basal levels of phospho-HER-2/neu were increased in ECC-1TAM tumors by 2.7-fold (Fig. 3C) suggesting that hyperactivity of HER-2/neu could be important for the complete growth inhibitory effect of trastuzumab. Clinical studies have shown that trastuzumab therapy is only beneficial in women with HER-2/neu overexpressing or amplified breast tumors (41, 42). However, the tests for determining HER-2/neu status may need further validation as trastuzumab therapy is effective in only 40% of patients with high HER-2/neu expression (42). In addition, it might be more reasonable to measure the active HER-2/neu protein and the extent of phosphorylation of downstream effector molecules such as Akt and ERK-1/2 (43–45). Keeping this in mind, there are other signaling pathways in addition to HER-2/neu that converge on Akt and ERK-1/2. The hyperactivity of the IGF-I/IGF-IR signaling pathway has previously been shown to result in resistance to trastuzumab therapy in breast cancer cells (35). This pathway also converges on the Akt (46) and ERK-1/2 (47) proteins leading to activation of these intracellular molecules. Our study found that the parental E2-stimulated ECC-1E2 tumors expressed constitutively active Akt and ERK-1/2 proteins and whereas trastuzumab decreased HER-2/neu protein, it had little effect on the phosphorylation status of ERK-1/2 or Akt proteins. However, trastuzumab partially inhibited E2-induced growth (Fig. 1C) and expression of pS2 mRNA in E2-stimulated ECC-1E2 tumors (Fig. 3A) suggesting that growth and ERα activity are in part regulated by HER-2/neu independent of ERK-1/2 and Akt activities. More importantly, the data clearly showed that IGF-1 and IGF-IR mRNAs were decreased in ECC-1TAM tumors compared with ECC-1E2 tumors (Fig. 4A).
and B) and that this translated to decreased signaling of HER-2/neu, ERK-1/2, and IGF-IR. Therefore, it is reasonable to conclude that the efficacy of trastuzumab to completely inhibit growth of ECC-ITAM tumors could be due to an overall increase of HER-2/neu signaling and the decrease of signaling from the IGF-I/IGF-IR pathway thereby allowing trastuzumab to block the downstream activity of ERK-1/2-originated from HER-2/neu.

ERK-1 and ERK-2 are serine/threonine kinases that are activated by phosphorylation cascades originated from receptor tyrosine kinases such as HER-2/neu and/or IGF-IR. Once activated, they translocate into the nucleus, where they phosphorylate transcription factors, including EBox (45) and Sp1 (48–50). Activation of EBox and Sp1 leads to transcription of c-myc (51), which is associated with increased cellular proliferation and tumor growth (52). Transcription of the c-myc gene is regulated by EBoxes through tethering to the Sp1 transcription factor (53) which binds directly to Sp1 sites within the c-myc promoter (51). Recent studies have shown that phosphorylation of Sp1 by ERK-1/2 and/or jun kinase leads to transcription of target genes (50). Thus, it is reasonable to conclude that trastuzumab’s inhibitory effect on tamoxifen-induced increase of c-myc mRNA could be due to a decrease of Sp1 activity through inactivation of ERK-1/2. Further studies are currently under way to elucidate the exact mechanism by which trastuzumab inhibits c-myc transcription.

In conclusion, the results from the current study suggest that trastuzumab is effective at blocking growth of tamoxifen-stimulated endometrial cancer when the basal activity of HER-2/neu is enhanced. Furthermore, complete blockade of growth of ECC-ITAM tumors seems achieved by inhibiting activities of HER-2/neu, ERK-1/2, and tamoxifen-induced expression of c-myc mRNA in an environment where the IGF-1/IGF-IR signaling pathway is diminished. These data suggest that the decreased expression and activity of the IGF-1/IGF-IR signaling pathway in ECC-ITAM tumors improves the overall efficacy of trastuzumab to inhibit growth of tumors expressing low levels of the IGF-1 ligand and receptor. To address this hypothesis, studies are currently under way to investigate whether overexpression of the IGF-IR is necessary and/or sufficient to block the efficacy of trastuzumab to inhibit growth of both breast and endometrial cancer cells. Based on the current results, we suggest that measuring the complex network of signals such as phospho-HER-2/neu, phospho-ERK-1/2, and phospho-IRS-1 status within tumors might improve response rates of patients having tamoxifen-stimulated breast and endometrial cancer to trastuzumab treatment and possibly lead to prevention of tamoxifen-resistant breast cancer and tamoxifen-stimulated endometrial cancer. There is clinical significance for trastuzumab therapy for tamoxifen-stimulated endometrial cancer as a single case report shows (54).

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References


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