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Estrogenic Promotion of ErbB2 Tyrosine Kinase Activity in Mammary Tumor Cells Requires Activation of ErbB3 Signaling

Bolin Liu,[1] Dalia Ordonez-Ercan,[2] Zeying Fan,[1] Xiaoping Huang,[1] Susan M. Edgerton,[1] XiaoHe Yang,[3] and Ann D. Thor[1]

[1]Department of Pathology, University of Colorado Denver School of Medicine, Aurora, Colorado; [2]Medical Oncology, Dana-Farber Cancer Institute, Boston, Massachusetts; and [3]Department of Pathology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma

Abstract

Increasing evidence suggests molecular interactions between erbB2 and other receptor tyrosine kinases, and estrogenic compounds and their cognate receptors. We have recently reported that downregulation of erbB3 abrogates erbB2-mediated tamoxifen resistance in breast cancer cells. On the basis of these data, we hypothesized that erbB3 may play a major role connecting these two sentinel pathways. Interactions were studied using mammary/breast cancer cell lines from wild-type rat c-*neu* gene transgenic mice and humans. Estradiol promoted cell proliferation and activated erbB2/*neu* tyrosine kinase, Akt, and mitogen-activated protein kinase signaling exclusively in mammary and breast epithelial cell lines with coexpression of both erbB2 and erbB3. Estradiol action was independent of the transgene promoter (MMTV-LTR) activity, both *in vitro* and *in vivo*, as well as c-*neu* transgene or endogenous erbB2 gene expression. Estrogen induction of cell growth promotion, erbB2/*neu* activation, and downstream signaling was abrogated by blockade of estrogen receptor (ER) with the pure ER antagonist ICI 182,780 or knockdown of erbB3 expression *via* specific siRNA. These data suggest that activation of both ER and erbB2/erbB3 signaling is requisite for estrogen-induced mitogenesis and erbB2/*neu* tyrosine kinase activation. (Mol Cancer Res 2009;7(11):1882–92)

Introduction

Genetic, hormonal, and environmental factors that promote breast (mammary) cancer have been identified. Both endogenous and exogenous steroid hormones are believed to play a crucial role in mammary/breast cancer development (1-3). The effects of estrogen (17-β-estradiol, E2) are particularly profound on the developing mammary gland. E2-induced growth and tumorigenesis are typically mediated through nuclear ligand–activated transcription factors, the estrogen receptors (ER) α and β, nonclassic genomic mechanisms and nongenomic pathways (reviewed in detail elsewhere refs. 4, 5). There is compelling evidence of bidirectional cross-talk between E2/ER and the erbB receptor tyrosine kinase (RTK) signal transduction pathways. E2-induced transcriptional upregulation of various growth factors [such as the epidermal growth factor (EGF), heregulin (HRG)] and the RTK EGF receptor (EGFR) have been well studied (6-8). E2 induction may therefore promote interactions between these ligands and receptors, activating downstream signaling pathways such as MEK/mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase/Akt.

The type I RTKs (the erbB or EGFR family) are important receptors that are critical to important biological functions of the mammary gland including: development, differentiation, proliferation, and survival (9). Dysregulation of RTKs or their ligands, which may occur through hormonal upregulation, transcriptional alterations, gene duplication or deletion, or mutational events, have been implicated in diverse types of human cancers including breast carcinoma (9, 10). ErbB2 (also called HER2/*neu*) is the most often studied RTK in breast cancers. Approximately one third of invasive ductal carcinomas of the breast have *erbB2* gene amplification and/or overexpression of the encoded protein erbB2 (11-13). *ErbB2* alterations are a marker of poor prognosis for breast cancer patients, and if these patients are treated with an anti-erbB2 agent such as trastuzumab, their survival can be significantly improved (14).

To more thoroughly investigate the role of specific RTKs or their aberrant forms in mammary tumorigenesis, numerous transgenic mouse strains have been generated (15-17). A transgenic mouse (FVB-TgN) bearing the wild-type (wt)-rat c-*neu*, under control of the mouse mammary tumor virus promoter (MMTV-LTR), was generated nearly 2 decades ago. These mice have been widely studied, develop mammary tumors after a relatively long latency, with a median of 36 weeks (16).

We have previously shown that short-term E2 treatment of young, nontumor-bearing transgenic mice modifies gland development and promotes mammary tumorigenesis in a dose-, age-, and duration-dependent manner (18). E2-treated mice developed mammary tumors of higher histologic tumor grade, with an increased proliferative rate, more tumors per animal, and a shortener latency. Cell lines derived from E2 treated, compared with control untreated mice, showed higher proliferative rates and clonogenicity *in vitro* as well (18, 19). When

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these transgenic mice were treated with short-term tamoxifen, a selective estrogen response modifier, c-*neu*-associated mammary tumorigenesis was abrogated in most mice. For those who developed tumors, the latency interval was significantly prolonged (20). These data suggest that estrogen-associated conditioning of the mammary gland, or interactions between c-*neu* and estrogen, play a major role in erbB2-associated tumorigenesis.

To study these relationships, we derived over 100 novel immortalized cell lines from mammary tumors that arose spontaneously in these transgenic mice. These have been passaged and studied *in vitro*, and compared with the tumors from which they were derived. The majority expressed significant amount of the transgene-encoded rat c-neu/erbB2, as well as mouse ERβ and erbB3 (20, 21). We have also shown that the wt rat c-neu/erbB2 and mouse erbB3 form a stable complex, with physical and functional interactions that may be promoted by HRG (21).

The studies described herein focus on interactions between estrogen, ER, and the RTKs erbB2 and erbB3. These have been evaluated in neoplastic mammary tumors and derived cell lines from the rat c-*neu* transgenic model as well as two human breast cancer cell lines. The data indicate similar interactions in these human and mouse models. We also evaluated whether E2 could activate the MMTV promoter, and thus enhance oncogenicity in MMTV-driven model systems. It is widely known that mammary tumors in MMTV promoter associated models such as ours are enhanced by pregnancy (15). MMTV is responsive to prolactin, progesterone, androgens, and glucocorticoids through its hormonal regulatory element (22-24). The MMTV promoter has not been shown to contain an estrogen response element (ERE), nor has promoter activity been shown to be upregulated by E2 (25).

These studies have significance, because of the broad use of this model and others driven by MMTV in studies of mammary tumorigenesis. We believe that the wt-rat c-*neu* transgenic mouse fulfills the criteria recommended by Daniel Medina (26) for a transgenic model with high relevance to human breast cancer. Derived lines and the mouse model provide unique vehicles to study mechanisms of mammary tumorigenesis and explore interactions between hormonal and RTK signaling pathways.

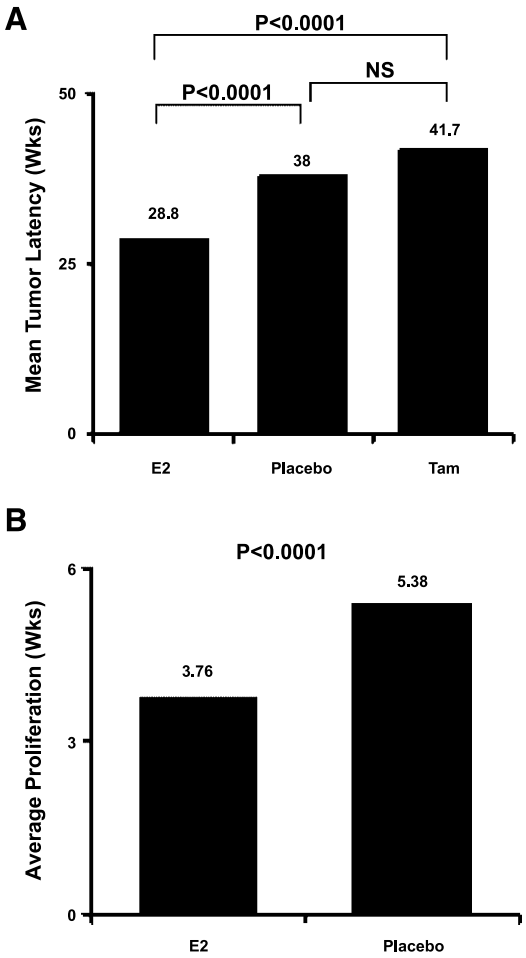

FIGURE 1. E2 promotes mammary tumor growth *in vivo*. The wt-rat c-*neu* transgenic mice were implanted with an E2 (n = 117), placebo (n = 82), or tamoxifen pellet (n = 64) at 8 wk of age. Mice were checked twice weekly for tumor formation. **A.** Tumor latency was calculated from the date of the first palpable tumor. Mean data by group for tumor latency is shown above column. Compared with placebo or tamoxifen, E2 treatment significantly shortened mammary tumor latency. **B.** Mice were visually checked for tumors twice weekly until 60 wk. Once palpable, tumors were measured by caliper twice weekly. The interval of growth from the first date palpable to the time the tumor reached 1.2 cm in greatest dimension was recorded as the tumor growth rate. In each subgroup, the average of time it took to reach 1.2 cm was calculated and is shown in **B**. Differences in tumor growth for the E2 (n = 23) and placebo (n = 20) mice were significant, $P < 0.0001$.

Results

E2 Promotes Mammary Tumor Cell Growth Both In vivo *and* In vitro

We previously reported that short-term E2 treatment (during the early reproductive period, with a peak window of risk determined by timing studies to be 8 to 16 weeks of age) alters mammary gland development and strongly promotes mammary tumor development in the wt rat *c-neu* transgenic model (18). Transgenic mice implanted with an E2 pellet develop mammary tumors with significantly shorter latency compared with either control-untreated or tamoxifen-treated mice (the number of mice used for this study is greater than previous reports; Fig. 1A). In addition, tumors arising in the mammary glands of E2-treated mice grow faster than those which arise in control or tamoxifen-treated mice (Fig. 1B).

We have previously shown that the derived mammary cell lines express mouse ERβ but not ERα (20, 21). *In vitro* cell proliferation assays using tumor-derived cell lines shows that some, but not all, show enhanced cell growth with estrogen or corticosterone (Fig. 2A). E2 also promoted anchorage-dependent colony formation (Fig. 2B). Cell lines that were most responsive to E2, such as 78617, 85815, and 85819, exhibited the highest expression of mouse *erbB3* and the rat *erbB2/neu* transgene (21). For example, 78717 and 85819, which coexpressed erbB2 and erbB3, were responsive to E2 for both cell growth and anchorage-dependent colony formation (Fig. 2A and B). E2 did not induce cell growth in the mouse mammary tumor cell line 78423, which expressed neither the mouse *erbB3* nor the rat *erbB2/neu* encoded proteins

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(21). E2 thus seems to promote both proliferation and anchorage-dependent clonogenicity in lines with coexpression of erbB2 and erbB3.

E2 Promotes Mammary Tumorigenesis Independent of MMTV Activity

We hypothesized that corticosterone-induced mitogenesis was due, at least in part, through activation of the MMTV-associated hormonal regulatory element (22, 23). Although MMTV is reportedly not induced by E2 (25), we performed three experiments to validate these findings in our laboratory.

First, we used MCF-7 cells transfected with a MMTV-driven luciferase (MMTV-Luc)–containing vector, so that we could study the direct effect of E2 on MMTV activation. E2 had no effect on luciferase activity (and thus MMTV activation), whereas corticosterone (a positive control) dramatically activated the MMTV promoter (Fig. 3A). To show that the failure of MMTV to be activated by E2 was not due to a lack of biological function of our assay system, MCF-7 cells transfected with an ERE-Luc–containing vector was used as an additional control. E2 significantly increased, tamoxifen decreased, and corticosterone had no effect on luciferase activity in these ERE-Luc vector–transfected cells (Fig. 3A).

We then did *in vitro* cell culture experiments using a representative mammary tumor–derived cell line (78717) to determine if the transgene would be upregulated in response to these same factors. As expected, corticosterone upregulated the levels of both mRNA and protein of the transgene rat *c-neu/erbB2*. In contrast, E2 had no effect on transgene mRNA or its encoded protein expression (Fig. 3B).

To further exclude indirect interactions between E2 and the MMTV promoter *in vivo*, MMTV-*neu* transgenic mice were implanted with a single estrogen or placebo pellet at 8 weeks of age. Mammary glands were harvested at 15 or 25 weeks of age. Total RNA was generated from the whole mammary glands. We then used semiquantitative reverse transcription-PCR (RT-PCR) methods to evaluate transgene expression. E2 treatment had no significant effect on rat c-*neu/erbB2* transgene expression in the mammary glands, whereas the E2-inducible mouse *EGFR* gene (which bears an ERE in its promoter; refs. 27,

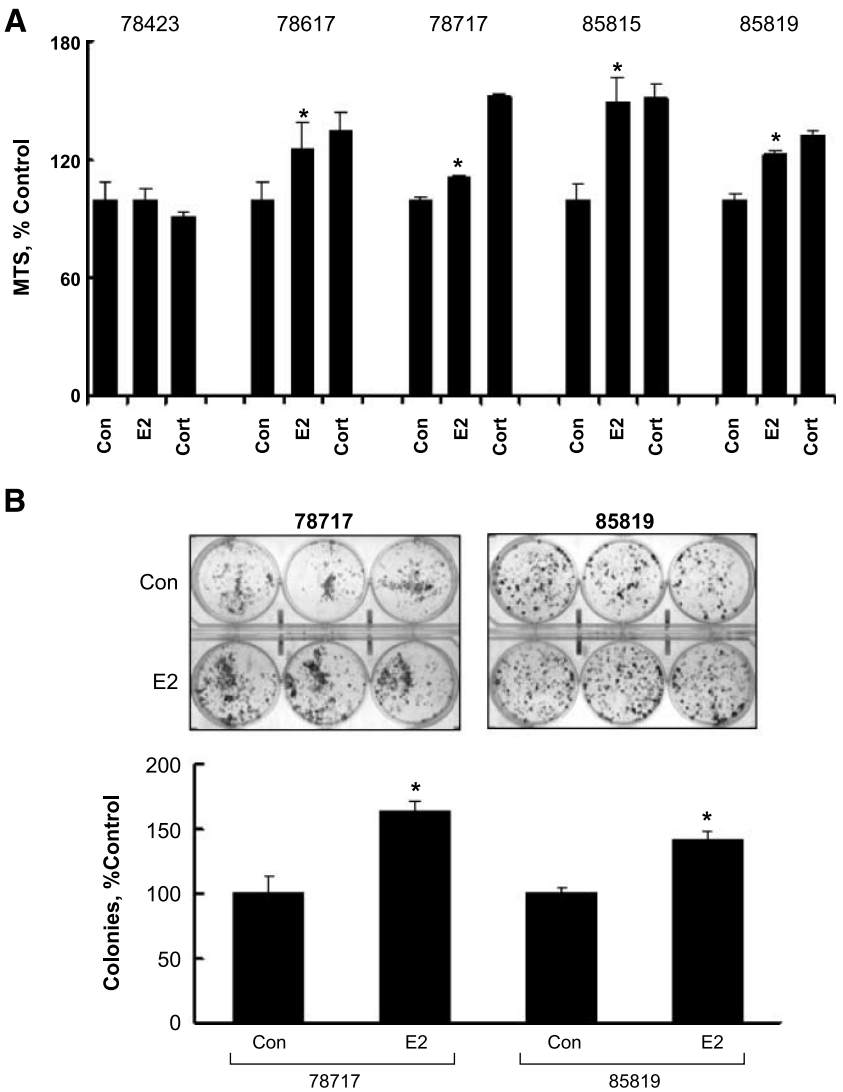

FIGURE 2. E2 promotes proliferation and colony formation in mammary tumor–derived cells with coexpression of both erbB2 and erbB3. **A.** The indicated mammary tumor–derived cell lines (2×10^3) in 0.1 mL culture media with 10% FBS were plated onto 96-well plates. After 24-h incubation, cells were grown in 0.1 mL phenol red–free media with 2.5% C-FBS as control (*Con*) or media containing 10^{-8} mol/L E2 or corticosterone (*Cort*). Cells were incubated for another 72 h, and the percentages of surviving cells from each group relative to controls, defined as 100% survival, were determined by reduction of MTS. Data shown are representative of three independent experiments. Columns, mean; bars, SD. *, $P <$ 0.02 versus controls. **B.** Mammary tumor cells (78717 and 85819) were plated in triplicates onto six-well plates. After 24-h incubation, the cells were grown in phenol red–free media with 2.5% C-FBS as control or in media containing 10^{-8} mol/L E2 for 2 wk. The pictures of six-well plates with colonies were taken by a digital camera, and the bar graph underneath was obtained by the percentages of colony numbers from each cell line relative to controls, defined as 100%, measured by EAGLE EYE II. Data shown are representative of three independent experiments. Columns, mean; bars, SD. *, $P < 0.02$ versus controls.

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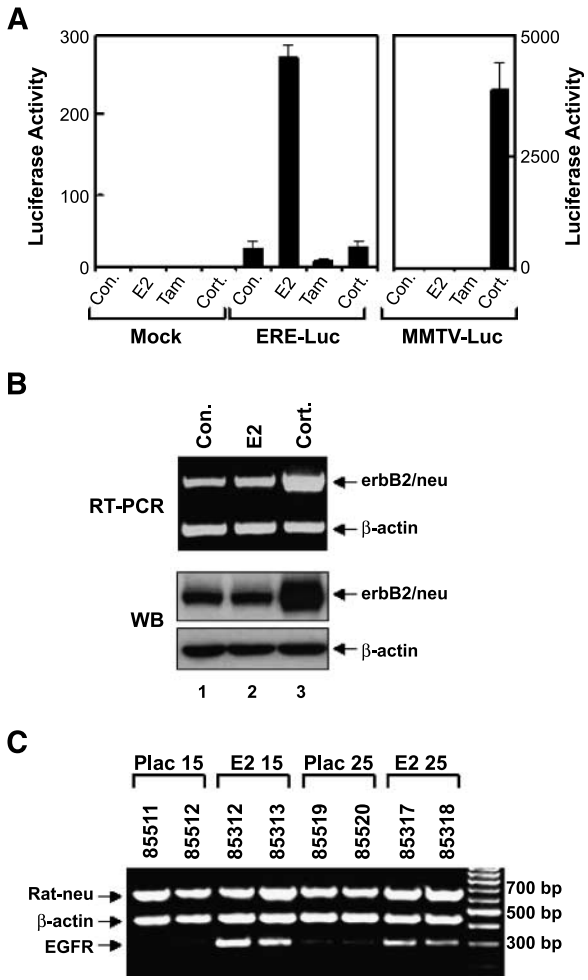

FIGURE 3. E2 promotes mammary tumorigenesis independent of MMTV activity *in vitro* and *in vivo*. **A.** Effect of E2 on luciferase activity driven by MMTV promoter. Human breast cancer MCF-7 cells were transiently transfected with mock or luciferase-containing expression vectors ERE-Luc or MMTV-Luc for 24 h. Culture media were replaced by phenol red–free media supplemented with 2.5% C-FBS either alone (*Con*) or containing 10^{-8} mol/L E2, 2 μmol/L tamoxifen (*Tam*), or 10^{-8} mol/L corticosterone for another 18 h. Cells were collected, lysed, and subjected to luciferase activity measurement with Luciferase Assay Systems (Promega Corp.). **B.** *In vitro* studies of novel tumor-derived cell line showed no evidence that E2 upregulated expression of the transgene. Mammary tumor–derived 78717 cells were treated with 10^{-8} mol/L of E2 or corticosterone for 24 h. After collection, half of the cells were subjected to RNA extraction and RT-PCR analysis with the specific primers for rat *neu/erbB2* or mouse *β-actin* (*top*); the remaining cells were used for Western blot (*WB*) analysis with the specific antibodies directed against erbB2/neu or β-actin (*bottom*). Compared with control cells (*Con, lane 1*), corticosterone (*Cort, lane 3*), but not E2 (*lane 2*), upregulated both mRNA and protein expression levels of the transgene rat *c-neu/erbB2*. **C.** *In vivo* studies of the transgene expression in benign mammary glands with or without E2 pretreatment. The transgenic mice bearing wt-rat c-*neu/erbB2* under control of MMTV promoter were implanted with a placebo (*Plac*) or E2 pellet at 8 wk of age. The benign mammary glands were collected at 15 or 25 wk of age. Total RNA were extracted from the mammary glands and followed by RT-PCR analysis with specific primers for rat c-*neu/erbB2*, mouse *β-actin*, or mouse *EGFR*. The PCR products were separated on a 1.2% agarose gel containing ethidium bromide and visualized under UV light.

28) showed enhanced expression in mammary glands harvested at 15 or 25 weeks of age from mice treated with an E2 pellet, but not from mice treated with a placebo pellet (Fig. 3C). *EGFR* induction was highest in the 15-week glands and was less significant in the 25-week glands, which is consistent with lower E2 levels 17 weeks postimplantation (the pellets have been shown to release hormones for ~8 weeks postimplantation; ref. 18).

Having ruled out upregulation of the transgene by E2 *in vivo*, we next sought to survey what other genes or mechanisms might be responsible for the progrowth effects of E2 in this model system. We used harvested mammary glands to derive RNA as described above, then performed two mouse expression microarrays for genes associated with steroid signaling or oncogenesis. Our data showed numerous progrowth/procancer genes that were upregulated at least 2-fold by E2 (Table 1). In particular, E2 induced numerous growth factors and growth factor receptors, oncogenes, adhesion molecules, factors associated with angiogenesis, factors that modulate metal homeostasis, etc. (Table 1). E2 had no effect on the expression of endogenous mouse *erbB2*, whereas mouse *EGFR* was upregulated 2.8-fold by 15 weeks and 2.3-fold by 25 weeks. These expression microarray data not only confirm our RT-PCR data (Fig. 3C), these are also consistent with E2-associated signaling data reported by others (27, 28).

In aggregate, both our *in vitro* and *in vivo* data support the reported literature and indicate that E2 does not activate, directly or indirectly, the MMTV promoter. Rather, E2 likely promotes mammary tumorigenesis via multiple mechanisms, independent of MMTV activity in this transgenic mouse model and tumor-derived lines.

*E2 Promotes Mammary Tumor Cell Growth through Activation of erbB2/*neu *RTK*

It has been reported that E2 induces the synthesis and secretion of peptide growth factors, such as EGF, transforming growth factor (TGF)-α, and HRG, in mammary glands (6-8). Our expression microarray data also show that E2 upregulates the expression of numerous growth-promoting factors in the mammary glands of these transgenic mice (Table 1). Many of these growth factors are stromal or epithelial derived, and known to bind to epithelial cell membrane receptors (including other RTK family members) to regulate cell growth via autocrine and/or paracrine mechanisms (29, 30).

Mammary tumors and tumor-derived cell lines from the wt rat *c-neu/erbB2* transgenic mice exhibited frequent coexpression of both the transgene rat *c-neu/erbB2* and the mouse *erbB3* gene. We have shown that these two species receptors form functional, stable complexes and that the erbB2/erbB3 heterodimer can be upregulated by HRG (21). In this study, we show that E2 has a marked progrowth effect only on cells with coexpression of both erbB2 and erbB3 (Fig. 2A). We hypothesize that E2 may transcriptionally upregulate HRG, and that this ligand plays an active role in the dimerization of these RTK members, which subsequently activates erbB2 tyrosine kinase activity and the downstream signaling transduction pathways. To test this hypothesis, we took several experimental approaches. First, we studied whether E2 could activate erbB2 tyrosine kinase activity. Two mammary tumor cell lines with coexpression of erbB2 and erbB3 were treated with either E2 or tamoxifen. Western blot analyses were done to detect the tyrosine phosphorylation levels of both erbB2 and erbB3. E2 increased and tamoxifen reduced the levels of P-erbB2 and

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P-erbB3 in both cell lines, although the total protein levels of erbB2 and erbB3 remained unchanged (Fig. 4A). We then showed that the E2-induced phosphorylation of erbB2 and erbB3 was associated with the activation of Akt and MAPK in a dose-dependent manner only in 78617 and 85815 cells with coexpression of erbB2 and erbB3 (Fig. 4B). In contrast, E2 had no such effect on the activity of either Akt or MAPK in 78423 cells lacking both erbB2 and erbB3 expression (Fig. 4B). These data were further confirmed by studies on two human breast cancer cell lines. E2 promoted and tamoxifen inhibited cell proliferation in the ERα-positive MDA-MB-361 cells, but not the ER-negative (both ERα and ERβ) SKBR3 cells (Fig. 4C), which correlated with their effects on the erbB receptor tyrosine phosphorylation and the downstream signaling, i.e., E2 increased and tamoxifen reduced the levels of P-erbB2, P-erbB3, P-Akt, and P-MAPK only in MDA-MB-361 cells (Fig. 4D). Thus, our data suggest that E2 may promote ERα- and/or ERβ-positive breast/mammary cancer cell growth through activation of erbB2/*neu* RTK.

To investigate whether erbB2 tyrosine kinase activation is a requisite component of the E2-associated cell growth, a specific erbB2 tyrosine kinase inhibitor AG825 was used. When this inhibitor was added with E2 in a cell proliferation assay, the cell growth promotion was significantly diminished (Fig. 5A). When we added a negative control compound AG9 (with a similar chemical structure to AG825 but without inhibitory effect on erbB2 tyrosine kinase activity) with E2 in the same cell proliferation assay system, E2-induced cell growth was again observed (Fig. 5A, the negative control). To access the biological function of ERβ in E2-mediated stimulatory effects on these mammary tumor cells, the pure ER antagonist ICI 182,780 was used in our studies. ICI 182,780 not only dramatically inhibited E2-promoted cell growth, it also abrogated E2-induced activation of erbB2/erbB3 receptors and the downstream signaling Akt and MAPK (Fig. 5B). These data indicate that ERβ blockade inhibits the E2 effects.

E2 Promotion of erbB2 Activation Requires erbB3 Expression

To explore whether E2-induced cell growth promotion requires erbB3 (ligand-induced erbB3/erbB2 heterodimerization), we used siRNA technology to specifically knockdown erbB3 *in vitro*. The erbB3 siRNA, but not control siRNA, dramatically reduced erbB3 expression levels, whereas erbB2 levels

Table 1. Differentially Upregulated Genes (>2.0-fold, $P < 0.05$) from Benign Mammary Glands of the MMTV-*neu* Transgenic Mice Exposed to E2 from Age of 8 wk, Determined by Breast Cancer & Estrogen Receptor Signaling cDNA MicroArray

Gene name	Protein	E2/control (wk)*	
		15	25
Adhesion			
E-cadherin	Cadherin 1	3.0	NS
Angiogenesis			
uPA(Plau)	Urokinase (plasminogen activator)	NS	2.3
Apoptosis			
Bcl-2	B-cell leukemia/lymphoma 2	2.0	NS
Bcl-w	Bcl2-like 2	2.2	NS
Fas	Fas antigen	2.0	NS
Cell cycle			
Cyclin E2	Cyclin E2	2.0	NS
$P21^{waf1/cip1}$	Cyclin-dependent kinase inhibitor	2.0	NS
$P27^{kip1}$	Cyclin-dependent kinase inhibitor	2.2	NS
$P16^{ink4a}$	CDK4 and CDK6 inhibitor	2.6	NS
Cytoskeleton organization			
Krt1-19	Keratin complex 1, acidic, 19	NS	2.1
Krt2-19	Keratin complex 2, basic, 19	2.3	NS
Extracellular matrix remodeling			
Klk5	Kallikrein 5	2.1	NS
Growth factors†			
αFGF	Fibroblast growth factor 1	2.2	NS
NGFα	Nerve growth factor α	2.7	NS
TGF-α	Transforming growth factor α	2.1	NS
Transmembrane proteins			
EGFR	Epidermal growth factor receptor	2.8	2.3
IL-6R	Interleukin 6 receptor, α	2.2	NS
MUC1	Mucin 1, transmembrane	2.0	2.4
Tie 1	Tyrosine kinase receptor 1	NS	2.0
Metal homeostasis			
MT3	Metallothionein 3	2.2	NS
Cox17 (copper metallochaperone)	Cytochrome c oxidase, subunit XVII	2.2	NS
Other			
c-Jun	Jun oncogene	2.0	NS
Tob2	Transducer of erbB2, 2	3.3	NS
Vim	Vimentin	2.0	2.3

Abbreviation: NS, not significant.
*The ratio of normalize group average signal of E2 vs control (placebo) at two time points.
†*Heregulin* (*HRG*) not in the array.

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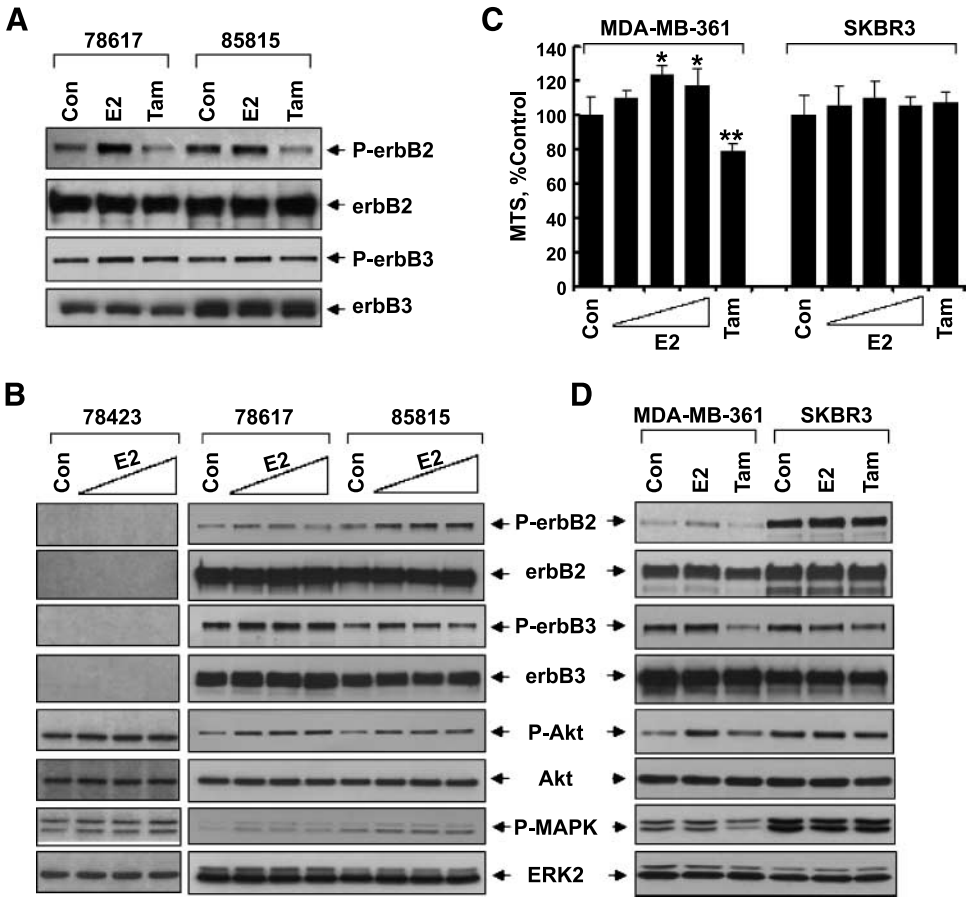

FIGURE 4. E2 induces tyrosine phosphorylation of erbB2 and erbB3, and activates the downstream signaling molecules Akt and MAPK in mammary tumor cells with coexpression of both erbB2 and erbB3. **A.** Modulation of erbB2 and erbB3 phosphorylation levels by E2 and tamoxifen in mouse mammary tumor cells. The mammary tumor cells (78617 and 85815) were cultured with phenol red–free media supplemented with 2.5% C-FBS for 48 h before being treated with vehicle (*Con*), E2 (10^{-8} mol/L), or tamoxifen (*Tam*; 2.5 μmol/L) for 8 h. Cells were collected, lysed, and subjected to Western blot analyses with specific antibodies directed against P-erbB2, erbB2, P-erbB3, or erbB3. **B.** Activation of Akt and MAPK by E2 in the mammary tumor cell lines with coexpression of both erbB2 and erbB3. The indicated mammary tumor cell lines were cultured with phenol red–free media supplemented with 2.5% C-FBS for 48 h before being treated with either vehicle or a series concentrations of E2 (1×10^{-9} mol/L, 1×10^{-8} mol/L, 1×10^{-7} mol/L) for 8 h. Cells were collected, lysed, and subjected to Western blot analyses with specific antibodies directed against P-erbB2, erbB2, P-erbB3, erbB3, P-Akt, Akt, P-MAPK, or ERK2. **C.** Growth regulation of human breast cancer cells by E2 and tamoxifen. The indicated human breast cancer cell lines (5×10^3) in 0.1 mL culture media with 10% FBS were plated onto 96-well plates. After 24-h incubation, cells were grown in 0.1 mL phenol red–free media with 2.5% C-FBS as control, or same media containing a series concentrations of E2 (1×10^{-9} mol/L, 1×10^{-8} mol/L, 1×10^{-7} mol/L; E2), or tamoxifen (2.5 μmol/L). Cells were incubated for another 72 h, and the percentages of surviving cells from each group relative to controls, defined as 100% survival, were determined by reduction of MTS. Data shown are representative of three independent experiments. Columns, mean; bars, SD. *, $P < 0.001$; **, $P < 0.002$ versus controls. **D.** Modulation of erbB2/erbB3 phosphorylation and the downstream signaling Akt and MAPK by E2 and tamoxifen in human breast cancer cells. MDA-MB-361 and SKBR3 cells were cultured with phenol red–free media supplemented with 2.5% C-FBS for 48 h before being treated with either vehicle, or E2 (10^{-8} mol/L), or tamoxifen (2.5 μmol/L) for 8 h. Cells were collected, lysed, and subjected to Western blot analyses with specific antibodies directed against P-erbB2, erbB2, P-erbB3, erbB3, P-Akt, Akt, P-MAPK, or ERK2.

remained unchanged in all three mammary tumor cell lines (Fig. 6A). These data indicate that the siRNAs used were both specific and efficient in their downregulation of erbB3 expression as we reported (31). E2 significantly stimulated cell proliferation in the control siRNA-transfected mammary tumor cells, whereas it lost its ability to promote cell growth in erbB3 siRNA–transfected mammary cells (Fig. 6B). These data indicate that the expression of erbB3 is required for E2-induced cell growth promotion in these mammary tumor cells. In aggregate, our results suggest that E2 may upregulate HRG, which promotes ligand-dependent erbB2/erbB3 heterodimerization, and activates erbB2 tyrosine kinase activity and downstream signaling transduction pathways to promote mammary tumor cell growth in this model system (Fig. 6C). We believe that cross-talk, which activates erbB2 tyrosine kinase, Akt, and MAPK, is independent of the MMTV promoter, and requires activation of erbB3 signaling.

Discussion

Several mechanisms have been proposed by which estrogen may exert a procarcinogenic effect including the following: (*a*) increased cellular proliferation, enhancing the susceptibility for a genotoxic event to produce mutations, resulting in accumulated genetic errors (32, 33); (*b*) tumor initiation, possibly via intermediate metabolites of estrogen (34); (*c*) tumor promotion via the induction of terminal end bud development (35); or (*d*) regulatory control of other genes, particularly the genes

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involved in cell cycle progression, such as c-*myc* and cyclin D1, which are rapidly upregulated in response to estrogen (5).

We have previously shown that E2 promotes and the antiestrogen tamoxifen reduces mammary tumorigenesis in MMTV-*neu* transgenic mice (18). In this report, we explored mechanisms involved in his biological phenomenon. We confirmed that E2 does not activate the MMTV-LTR and thereby upregulate transgene expression, with evidence derived from three experimental systems: (*a*) luciferase activity assays that show E2 does not activate the MMTV promoter *in vitro*; (*b*) cell lines with MMTV linked *neu/erbB2* expression show no upregulation of *neu/erbB2* mRNA or protein upon E2 treatment, although E2 promoted cell proliferation and colony formation (Fig. 2); and (*c*) benign mammary glands harvested from E2-treated MMTV-*neu* transgenic mice had no significant increase in transgene expression, whereas they showed increases in *EGFR*, which has an ERE in its promoter.

Recent studies suggest that E2 induction of erbB2 signaling (activation) results in downstream Akt and MAPK activation in MCF-7 human breast cancer cells (6, 36). We observed a

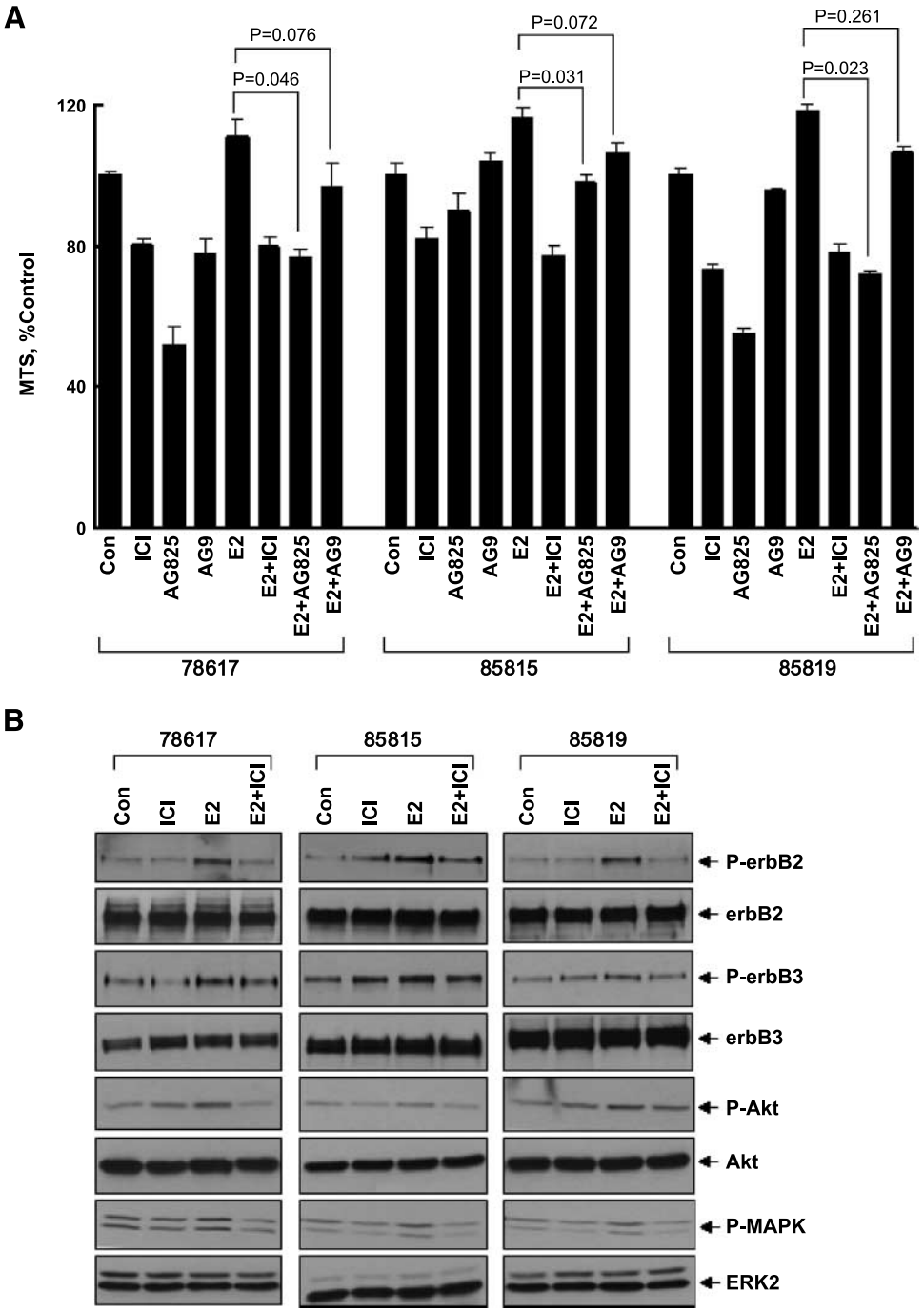

FIGURE 5. Both ER antagonist and erbB2 tyrosine kinase inhibitor abrogate E2-induced mammary tumor cell growth. **A.** Mammary tumor–derived cell lines were plated onto 96-well plates. After 24-h incubation, cells were grown in either phenol red–free media with 2.5% C-FBS as control (*Con*), or same media containing 1 μmol/L ICI 182,780 (*ICI*), 10 μmol/L AG825, 10 μmol/L AG9, 1×10^{-8} mol/L E2, or combinations of E2 and ICI, E2 and AG825, or E2 and AG9. Cells were incubated for another 72 h, and the percentages of surviving cells from each group relative to controls, defined as 100% survival, were determined by reduction of MTS. Data shown are representative of three independent experiments. Columns, mean; bars, SD. **B.** The indicated mammary tumor cell lines were cultured with phenol red–free media supplemented with 2.5% C-FBS for 48 h before being treated with vehicle, ICI 182,780 (1 μmol/L), E2 (10^{-8} mol/L), or combinations of ICI and E2 for 8 h. Cells were collected, lysed, and subjected to Western blot analyses with specific antibodies directed against P-erbB2, erbB2, P-erbB3, erbB3, P-Akt, Akt, P-MAPK, or ERK2.

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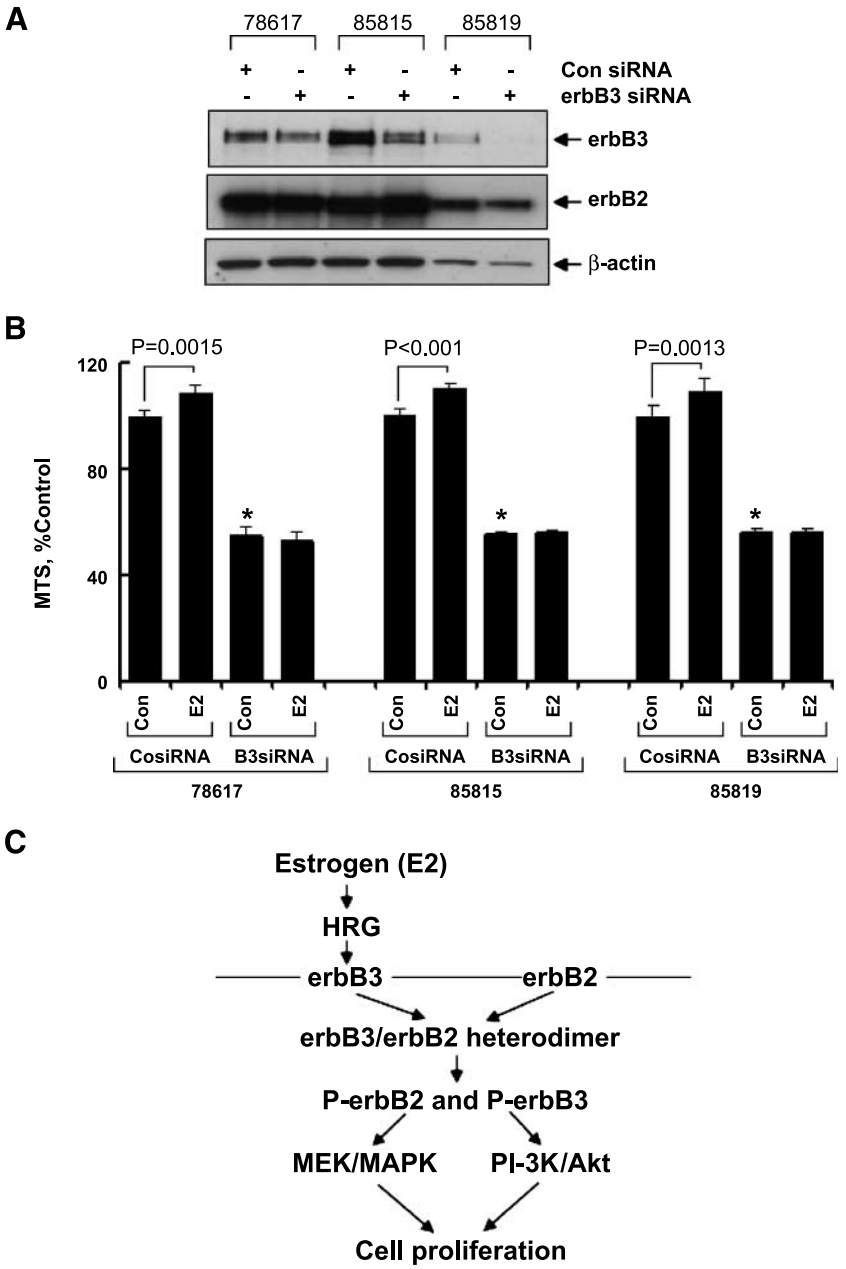

FIGURE 6. Expression of erbB3 is required for E2-induced growth promotion in mammary tumor cells with coexpression of both erbB2 and erbB3. **A.** Mouse mammary tumor cell lines (78617, 85815, and 85819) were transiently transfected with either control siRNA or mouse erbB3–specific siRNA. After 48 h, cells were collected, lysed, and subjected to Western blot analyses with specific antibodies directed against erbB3, erbB2, or β-actin. **B.** 78617, 85815, and 85819 cells were transfected with either control siRNA (*CosiRNA*) or erbB3 siRNA (*B3siRNA*) for 24 h. The cells were then plated onto 96-well plates. After 24-h incubation, cells were grown in either phenol red–free media with 2.5% C-FBS as control (*Con*) or same media containing 10^{-8} mol/L E2. Cells were incubated for another 60 h, and the percentages of surviving cells from each group relative to controls, defined as 100% survival, were determined by reduction of MTS. Data shown are representative of three independent experiments. Columns, mean; bars, SD. *, $P < 0.00001$ versus controls with control siRNA transfection and without E2 treatment. **C.** Schematic diagram showing the pathways for E2-induced growth promotion in mammary tumor cells with coexpression of both erbB2 and erbB3.

similar phenomenon in our mouse model system, with E2 induction of transgene activation (but not protein upregulation). Having excluded E2 as a direct or indirect modulator of MMTV-associated gene transcription, we wanted to study the mechanisms/factors that played a major role in the cross-talk between these two signaling pathways. We previously reported that majority of mammary tumors and tumor-derived cell lines derived from this transgenic model showed coexpression of the transgene rat *c-neu/erbB2* and endogenous mouse *erbB3*, and that these two erbB receptors form stable physical and functional interactions in this model (21). The erbB2/erbB3 heterodimer is believed to be the most biologically active and protumorigenic (37, 38). We have also reported that downregulation of erbB3 abrogates erbB2/*neu*-mediated tamoxifen resistance (31). Others have reported a high incidence of erbB3 expression in *erbB2*-altered breast/mammary cancers as well (39, 40). In aggregate, these findings suggest that erbB3 plays a major role in mammary/breast tumors with erbB2 activation and it may be an important mediator of cross-talk between estrogenic and RTK signaling pathways.

Our data obtained from both mouse mammary tumor–derived cell lines and human breast cancer cells indicate that E2 induces cell growth and activation of erbB2, Akt, and MAPK. Furthermore, these effects occur only in tumor cells with coexpression of erbB2 and erbB3 in cell lines that express ERs (Fig. 4). ErbB3 knockdown experiments by specific siRNA confirm that erbB3 expression is an important component of E2-induced mammary tumor cell growth promotion (Fig. 6A

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and B). A logical model is that E2 induces HRG, which then binds to erbB3 receptors on the cell surface. The ligand-bound erbB3 then forms a functional, stable heterodimer with erbB2, a prerequisite for erbB2 tyrosine kinase activation (31). Activated erbB2 tyrosine kinase induces downstream signaling, including Akt and MAPK (Fig. 6C). Alternative models are also feasible. For example, E2 is known to induce EGF and TGF-α, which might bind to EGFR, promote EGFR:erbB2 heterodimers, and activate downstream signaling. In our studies, EGFR levels were variable, but often minimal to undetectable in the majority of mammary tumors and derived lines (21). A lack of significance for TGF-α/EGFR signaling in E2-induced cell growth promotion is also supported by reported data from MCF-7 cells (6).

Another interesting component of our data is the apparent importance of ERβ in E2 activation of the transgene. ER knockout models have shown that ERα is the predominant mediator of E2-induced mitogenesis (41); however, ERβ also exerts progrowth effects on mammary gland development and tumor formation (42). In humans, cross-talk between the erbB RTKs and ER signaling has been implicated in both tumorigenesis and resistance to endocrine therapy (43, 44). Although most studies of this phenomenon have focused on RTK interactions with ERα, this is largely because most of the reagents used to detect ER preferentially recognize ERα. Much less is known about ERβ, particularly in the erbB2-overexpressing breast cancers that are typically ERα negative but may express ERβ. In the transgenic model system and derived ERβ-positive tumor lines, only those with erbB2 and erbB3 upregulation showed significant mitogenesis in response to E2 (Fig. 2). The role of ER in this process is clearly important, because it was abrogated by the addition of tamoxifen (20) or a pure ER antagonist ICI 182,780 (Fig. 5). Further studies to better delineate the role of ERβ in *erbB2*-altered human breast cancers may be of value, as it seems to have biological relevance at least in model systems.

In summary, we have shown that E2 promotes mammary tumor growth in the wt rat c-*neu* transgenic mice and induces proliferation in derived tumor cell lines that coexpress erbB2 and erbB3. At the molecular level, E2 induces upregulation of many procarcinogenic genes, activates erbB2 tyrosine kinase activity through ligand-dependent erbB3/erbB2 heterodimerization, and results in activation of the phosphoinositide 3-kinase/Akt and MEK/MAPK signaling pathways. Furthermore, both inhibition of erbB2 tyrosine kinase activity by a specific inhibitor and knockdown of erbB3 by specific siRNA abrogated E2-induced mammary tumor cell growth. These data strongly suggest that erbB3/erbB2 dimerization may be essential for E2-induced cross-talk, which can promote the growth of breast/mammary tumor cells. Finally, we have confirmed that E2 does not activate, directly or indirectly, the MMTV promoter and it does not affect transgene c-*neu* expression *in vitro* or *in vivo*.

Materials and Methods

Reagents

For *in vivo* mice implantation, placebo, estrogen, and tamoxifen pellets were obtained from Innovative Research of America. For *in vitro* cell culture experiments, E2, 4-Hydroxytamoxifen (for convenience, described as tamoxifen herein), and Corticosterone were obtained from Sigma Co. AG825 and AG9 were purchased from Calbiochem Co. ICI 182,780 was from Tocris Bioscience. Both mouse erbB3 siRNA and control siRNA were purchased from Santa Cruz Biotechnology, Inc.

Antibodies used for Western blot analysis were from following sources: erbB2 (c-*neu* Ab-3, Oncogene Research Products), phospho-erbB2 (P-erbB2, clone PN2A, NeoMarkers, Inc.), erbB3 (Ab-7, NeoMarkers, Inc.), phospho-erbB3 (P-erbB3, clone 21D3, Cell Signaling Technology, Inc.), phospho-Akt (Ser473) and Akt (Cell Signaling Technology, Inc.), total ERK2 (polyclonal antibody C-14, Santa Cruz Biotechnology, Inc.), phospho-MAPK (P-MAPK, E10 monoclonal antibody, Cell Signaling Technology, Inc.), and β-actin (clone AC-74, monoclonal antibody, Sigma Co.). All other reagents were purchased from Sigma. unless otherwise specified.

Cells and Cell Culture

Murine mammary tumor cell lines 78423, 78617, 78717, 85815, and 85819 were established from mammary tumors derived from wt rat c-*neu* transgenic mice and described previously (20, 21). Human breast cancer cell lines MCF-7, MDA-MB-361, and SKBR3 were obtained from the American Type Culture Collection and maintained in DMEM and Ham's F-12 medium (DMEM/F12 1:1, v/v; Invitrogen Corp.) supplemented with 10% fetal bovine serum (FBS; Invitrogen Corp.). All the cell lines were cultured in a 37°C humidified atmosphere containing 95% air and 5% CO_2 and were split twice a week as described (20).

Mice and Treatment Groups

All animal care was done in accordance with institutional guidelines in our Association for the Assessment and Accreditation of Laboratory Animal Care-approved mouse facilities. All protocols and experiments were reviewed and approved by our Institutional Animal Care and Use Committee. Virgin female FVB-TgN (MMTV-*neu*) transgenic mice (wt rat c-*neu*/*erbB2*) were obtained from Jackson Labs at 4 to 5 wk of age. Mice were placed on a casein (milk protein based) diet, Purina 5K96 (Ralston Purina Co.), to exclude potential interactions between dietary phytoestrogens and other hormonal agents in these studies.

For our studies of mammary tumorigenesis, the MMTV-*neu* transgenic mice were implanted with a single, 60-d constant release pellet in the lateral neck at 8 wk of age. A placebo pellet was implanted in 82 mice. A single 0.5-mg estrogen pellet was implanted in 117 virgin mice. A single 5.0-mg tamoxifen pellet was implanted in 64 mice. Mice were checked twice weekly for tumor formation. Tumor latency was calculated from the date of the first palpable tumor. These data were used for Fig. 1A. Tumors were measured twice weekly using calipers to evaluate tumor growth rates as shown in Fig. 1B. Once tumors reached 1.2 cm in greatest dimension, animals were euthanized to prevent undue stress to the animals. Histologic examination of all palpable or visible tumors was preformed to confirm tumorigenesis and evaluate tumor histology. Histologic patterns and heterogeneity of these and other mammary tumors have been reported elsewhere (45).

For gene expression studies involving benign mammary tissues, an additional group of 34 MMTV-*neu* transgenic mice were used. A group of eight mice were euthanized at 8 wk of

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age to provide untreated control tissues. The remaining 26 mice were divided into two groups and implanted with either a 0.5-mg estrogen pellet (12 mice) or a similar placebo pellet (14 mice) at 8 wk of age, similar to the above experiment. A group of six mice from each group were euthanized at 15 wk of age, and the remaining mice (six estrogen, eight placebo) were euthanized at 25 wk of age. The entire gland from all mammary glands (except the L4 gland from the 15- and 25-wk mice) were placed in RNAlater (Ambion, Inc.) at the time of euthanasia and stored at −80°C.

RNA Extraction, Gene Expression by Microarray, cDNA Synthesis, and RT-PCR Analysis

Total RNA was extracted using a modified chloroform/phenol procedure (TRIZOL, Invitrogen Corp.). Briefly, vials containing the RNAlater and mammary glands were thawed; RNAlater was removed and replaced with 2 mL Trizol. Vials were homogenized followed by centrifugation to remove insoluble matter and fat. The RNA was extracted from the supernatant by the use of chloroform followed by precipitation with isopropanol and ethanol. RNA was cleaned using the Qiagen RNeasy kit (Qiagen, Inc.) following manufacturer directions. Microarrays were done using the GEArray Q Series Mouse Breast Cancer and Estrogen Receptor Signaling Gene Array (MM-020-12, Superarray Bioscience Corp.) according to manufacturer instructions. Two-point 5 μg of RNA were used for each array. The arrays were incubated using an Amersham Pharmacia Hybridization shaker/oven (G.E. Healthcare). All membranes were scanned and the intensity of all signals of densitometry analysis was done using ScanAlyze from Stanford University.[4]

The first strand of cDNA was generated using reverse transcriptase (Roche Diagnostics Corp.) following the manufacturer's protocol. It was subsequently amplified by PCR using the Expand High Fidelity PCR System (Roche) and the following primer sets: rat *neu* forward primer, 5′-GGA AGT ACC CGG ATG AGG AGG GCA TAT G-3′ and the reverse primer, 5′-CCG GGC AGC CAG GTC CCT GTG TAC AAG CCG-3′; mouse *EGFR* forward primer, 5′-GGA GGA AAA GAA AGT CTG CC-3′ and the reverse primer, 5′-CCC ATA GTT GGA TAG GAT GG-3′; *β-actin* forward primer, 5′-GCA CCA CAC CTT CTA CAA TGA GC-3′, and the reverse primer, 5′-GAC GTA GCA CAG CTT CTC CTT AAT G-3′.

Western Blot Analysis

Protein expression levels were determined by Western blot analysis as previously described (20, 21, 31). Briefly, cells were lysed in a buffer containing 50 mmol/L Tris (pH 7.4), 50 mmol/L NaCl, 0.5% NP40, 50 mmol/L NaF, 1 mmol/L Na_3VO_4, 1 mmol/L phenylmethylsulfonyl fluoride, 25 μg/mL leupeptin, and 25 μg/mL aprotinin. The lysates were centrifuged and the supernatants were collected for protein concentration determination by the Coomassie Plus protein assay reagent (Pierce Chemical Co.). Equal amounts of cell lysates were resolved by SDS-PAGE, and Western blot analysis with specific antibodies as described in the figure legends.

Transient Transfections and Luciferase Assays

MCF-7 cells were plated in six-well plates to be 60% to 70% confluent on the day of transfections with ERE- or MMTV-dependent luciferase plasmid (1 μg/well, kind gifts from Dr. Donald P. McDonnell, Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC). Transient transfections were done with the FuGENE-6 transfection kit (Roche Diagnostics Corp.) as previously described (31). Twenty-four hours posttransfection, cell culture media was replaced with phenol red–free media supplemented with 2.5% charcoal-stripped FBS (C-FBS; Cocalico Biologicals, Inc.). Cells were then either untreated or treated with E2, tamoxifen, or corticosterone for another 18 h. Cells were collected, lysed, and subjected to luciferase activity measurement with Luciferase Assay Systems (Promega Corp.).

Gene Knockdown by RNA Interference

To diminish erbB3 expression, mammary tumor–derived cell lines were transiently transfected with Lipofectamine 2000 reagent (Invitrogen Corp.) with mouse-specific erbB3 siRNA or control siRNA as previously described (31).

Clonogenic Assay

Clonogenic assays were done as described previously (31). In brief, cells were seeded into six-well plates in triplicates at a density of 500 cells per well in 2 mL of media containing 10% FBS. After 24-h incubation, media was replaced with phenol red–free media containing C-FBS with or without E2 and continued cultured for 14 d in a 37°C humidified atmosphere containing 95% air and 5% CO_2. The cell colonies were stained for 15 min with a solution containing 0.5% crystal violet and 25% methanol, followed by three rinses with tap water to remove excess dye. The colony numbers were counted by gel documentation system EAGLE EYE II (Stratagene).

Cell Proliferation Assay

A CellTiter96 AQ non radioactive cell proliferation kit (Promega Corp.) was used to determine the number of viable cells as described previously (20, 21, 31). In brief, cells were plated onto 96-well plates. After 24 h, cells were grown in either 100 μL fresh media as control, or 100 μL fresh media containing different treatment reagents as described in the figure legends. Cells were incubated at 37°C in a 5% CO_2 atmosphere for 72 h and then cell proliferation assay was done to measure the conversion of MTS by dehydrogenase enzymes in living cells into a water-soluble formazan. The absorbance of formazan at 490 nmol/L was recorded by a microplate reader. The percentages of cells surviving from each group relative to controls, defined as 100% survival, were determined by reduction of MTS.

Statistical Analyses

The statistical analyses of *in vivo* and *in vitro* experimental data were done using a two-sided Student's *t* test. Fold differences for gene microarrays were calculated by comparing the averages of all animals tested in each group.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

[4] http://graphics.stanford.edu/software/scanalyze/

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References

1. Bergkvist L, Adami HO, Persson I, Hoover R, Schairer C. The risk of breast cancer after estrogen and estrogen-progestin replacement [see comment]. N Engl J Med 1989;321:293–7.

2. Bernstein L, Yuan JM, Ross RK, et al. Serum hormone levels in premenopausal Chinese women in Shanghai and white women in Los Angeles: results from two breast cancer case-control studies. Cancer Causes Control 1990;1:51–8.

3. Grabrick DM, Hartmann LC, Cerhan JR, et al. Risk of breast cancer with oral contraceptive use in women with a family history of breast cancer [see comment]. JAMA 2000;284:1791–8.

4. Schiff R, Massarweh S, Shou J, Osborne CK. Breast cancer endocrine resistance: how growth factor signaling and estrogen receptor coregulators modulate response. Clin Cancer Res 2003;9:447–54S.

5. Butt AJ, McNeil CM, Musgrove EA, Sutherland RL. Downstream targets of growth factor and oestrogen signalling and endocrine resistance: the potential roles of c-Myc, cyclin D1 and cyclin E. Endocr Relat Cancer 2005;Suppl 1: S47–59.

6. Keshamouni VG, Mattingly RR, Reddy KB. Mechanism of 17-β-estradiol-induced Erk1/2 activation in breast cancer cells. A role for HER2 AND PKC-δ. J Biol Chem 2002;277:22558–65.

7. DiAugustine RP, Petrusz P, Bell GI, et al. Influence of estrogens on mouse uterine epidermal growth factor precursor protein and messenger ribonucleic acid. Endocrinology 1988;122:2355–63.

8. Nelson KG, Takahashi T, Lee DC, et al. Transforming growth factor-α is a potential mediator of estrogen action in the mouse uterus. Endocrinology 1992; 131:1657–64.

9. Olayioye MA, Neve RM, Lane HA, Hynes NE. The ErbB signaling network: receptor heterodimerization in development and cancer. EMBO J 2000; 19:3159–67.

10. deFazio A, Chiew YE, Sini RL, Janes PW, Sutherland RL. Expression of c-erbB receptors, heregulin and oestrogen receptor in human breast cell lines. Int J Cancer 2000;87:487–98.

11. Muss HB, Thor AD, Berry DA. c-erbB-2 expression and response to adjuvant therapy in women with node-positive early breast cancer. N Engl J Med 1994;330:1260–6.

12. Thor AD, Berry DA, Budman DR, et al. erbB-2, p53, and efficacy of adjuvant therapy in lymph node-positive breast cancer [comment]. J Natl Cancer Inst 1998;90:1346–60.

13. Liu E, Thor A, He M, Barcos M, Ljung BM, Benz C. The HER2 (c-erbB-2) oncogene is frequently amplified in *in situ* carcinomas of the breast. Oncogene 1992;7:1027–32.

14. Wolff AC, Hammond ME, Schwartz JN, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. J Clin Oncol 2007;25:118–45.

15. Bouchard L, Lamarre L, Tremblay PJ, Jolicoeur P. Stochastic appearance of mammary tumors in transgenic mice carrying the MMTV/c-neu oncogene. Cell 1989;57:931–6.

16. Guy CT, Webster MA, Schaller M, Parsons TJ, Cardiff RD, Muller WJ. Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. Proc Natl Acad Sci U S A 1992;89:10578–82.

17. Muller WJ, Sinn E, Pattengale PK, Wallace R, Leder P. Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. Cell 1988;54:105–15.

18. Yang X, Edgerton SM, Kosanke SD, et al. Hormonal and dietary modulation of mammary carcinogenesis in mouse mammary tumor virus-c-erbB-2 transgenic mice. Cancer Res 2003;63:2425–33.

19. Jeruss JS, Liu NX, Chung Y, et al. Characterization and chromosomal instability of novel derived cell lines from a wt-erbB-2 transgenic mouse model. Carcinogenesis 2003;24:659–64.

20. Liu B, Edgerton S, Yang X, et al. Low-dose dietary phytoestrogen abrogates tamoxifen-associated mammary tumor prevention. Cancer Res 2005;65: 879–86.

21. Kim A, Liu B, Ordonez-Ercan D, et al. Functional interaction between mouse erbB3 and wild-type rat c-neu in transgenic mouse mammary tumor cells. Breast Cancer Res 2005;7:R708–18.

22. Chalepakis G, Arnemann J, Slater E, Bruller HJ, Gross B, Beato M. Differential gene activation by glucocorticoids and progestins through the hormone regulatory element of mouse mammary tumor virus. Cell 1988;53: 371–82.

23. Darbre P, Dickson C, Peters G, Page M, Curtis S, King RJ. Androgen regulation of cell proliferation and expression of viral sequences in mouse mammary tumour cells. Nature 1983;303:431–3.

24. Haraguchi S, Good RA, Engelman RW, Day NK. Human prolactin regulates transfected MMTV LTR-directed gene expression in a human breast-carcinoma cell line through synergistic interaction with steroid hormones. Int J Cancer 1992; 52:928–33.

25. Otten AD, Sanders MM, McKnight GS. The MMTV LTR promoter is induced by progesterone and dihydrotestosterone but not by estrogen. Mol Endocrinol 1988;2:143–7.

26. Medina D. Biological and molecular characteristics of the premalignant mouse mammary gland. Biochim Biophys Acta 2002;1603:1–9.

27. Mukku VR, Stancel GM. Regulation of epidermal growth factor receptor by estrogen. J Biol Chem 1985;260:9820–4.

28. Lingham RB, Stancel GM, Loose-Mitchell DS. Estrogen regulation of epidermal growth factor receptor messenger ribonucleic acid. Mol Endocrinol 1988; 2:230–5.

29. Dickson RB, Lippman ME. Estrogenic regulation of growth and polypeptide growth factor secretion in human breast carcinoma. Endocr Rev 1987;8:29–43.

30. Reddy KB, Mangold GL, Tandon AK, et al. Inhibition of breast cancer cell growth *in vitro* by a tyrosine kinase inhibitor. Cancer Res 1992;52:3636–41.

31. Liu B, Ordonez-Ercan D, Fan Z, Edgerton SM, Yang X, Thor AD. Downregulation of erbB3 abrogates erbB2-mediated tamoxifen resistance in breast cancer cells. Int J Cancer 2007;120:1874–82.

32. Ethier SP. Growth factor synthesis and human breast cancer progression. J Natl Cancer Inst 1995;87:964–73.

33. Nandi S, Guzman RC, Yang J. Hormones and mammary carcinogenesis in mice, rats, and humans: a unifying hypothesis. Proc Natl Acad Sci U S A 1995; 92:3650–7.

34. Cavalieri EL, Stack DE, Devanesan PD, et al. Molecular origin of cancer: catechol estrogen-3,4-quinones as endogenous tumor initiators. Proc Natl Acad Sci U S A 1997;94:10937–42.

35. Fishman J, Osborne MP, Telang NT. The role of estrogen in mammary carcinogenesis. Ann N Y Acad Sci 1995;768:91–100.

36. Stoica GE, Franke TF, Wellstein A, et al. Estradiol rapidly activates Akt via the ErbB2 signaling pathway. Mol Endocrinol 2003;17:818–30.

37. Alimandi M, Romano A, Curia MC, et al. Cooperative signaling of ErbB3 and ErbB2 in neoplastic transformation and human mammary carcinomas. Oncogene 1995;10:1813–21.

38. Wallasch C, Weiss FU, Niederfellner G, Jallal B, Issing W, Ullrich A. Heregulin-dependent regulation of HER2/neu oncogenic signaling by heterodimerization with HER3. EMBO J 1995;14:4267–75.

39. Bieche I, Parfait B, Laurendeau I, Girault I, Vidaud M, Lidereau R. Quantification of estrogen receptor α and β expression in sporadic breast cancer. Oncogene 2001;20:8109–15.

40. Siegel PM, Ryan ED, Cardiff RD, Muller WJ. Elevated expression of activated forms of Neu/ErbB-2 and ErbB-3 are involved in the induction of mammary tumors in transgenic mice: implications for human breast cancer. EMBO J 1999;18:2149–64.

41. Hewitt SC, Harrell JC, Korach KS. Lessons in estrogen biology from knockout and transgenic animals. Annu Rev Physiol 2005;67:285–308.

42. Hou YF, Yuan ST, Li HC, et al. ERβ exerts multiple stimulative effects on human breast carcinoma cells. Oncogene 2004;23:5799–806.

43. Osborne CK, Shou J, Massarweh S, Schiff R. Crosstalk between estrogen receptor and growth factor receptor pathways as a cause for endocrine therapy resistance in breast cancer. Clin Cancer Res 2005;11:865–70s.

44. Normanno N, Di Maio M, De Maio E, et al. Mechanisms of endocrine resistance and novel therapeutic strategies in breast cancer. Endocr Relat Cancer 2005;12:721–47.

45. Kosanke S, Edgerton SM, Moore DI, et al. Mammary tumor heterogeneity in wt-ErbB-2 transgenic mice. Comp Med 2004;54:268–75.

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Estrogenic Promotion of ErbB2 Tyrosine Kinase Activity in Mammary Tumor Cells Requires Activation of ErbB3 Signaling

Bolin Liu, Dalia Ordonez-Ercan, Zeying Fan, et al.

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