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Featured Article

Human Epidermal Growth Factor Receptor 2 Status Modulates Subcellular Localization of and Interaction with Estrogen Receptor α in Breast Cancer Cells

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ABSTRACT

Purpose: Approximately two-thirds of breast cancer patients respond to endocrine therapy, and this population of patients is estrogen receptor (ER) positive. However, a significant proportion of patients do not respond to hormone therapy. ER hormone responsiveness is widely believed to be influenced by enhanced cross-talk of ER with overexpressed human epidermal growth factor receptor 2 (HER2), and a subgroup of ER-positive tumors coexpress high HER2.

Experimental Design: Breast cancer cells with or without HER2 overexpression were analyzed for ER status, subcellular localization, and interactions with HER2 signaling components by biochemical and immunological methods. Experiments explored the regulatory interactions between the HER2 and ER pathways and the sensitivity of breast cancer cells to tamoxifen.

Results: Stable or transient or natural HER2 overexpression in ER-positive breast cancer cells promoted the nucleus-to-cytoplasm relocalization of ER, enhanced interactions of ER with HER2, inhibited ER transactivation function, and induced resistance to tamoxifen-mediated growth inhibition of breast cancer cells. In addition, HER2 up-regulation resulted in ER interaction with Sos, a component of Ras signaling, and hyperstimulation of the mitogen-activated protein kinase extracellular signal-regulated kinase 1/2 (ERK1/2). Conversely, down-regulation of HER2 by the anti-HER2 monoclonal antibody Herceptin led to suppression of ERK1/2 stimulation, restoration of ER to the nucleus, and potentiation of the growth-inhibitory action of tamoxifen.

Conclusion: The results presented here show for the first time that ER redistribution to the cytoplasm and its interaction with HER2 are important downstream effects of HER2 overexpression, that ERK1/2 is important for ER

cytoplasmic localization, and that subcellular localization of ER may play a mechanistic role in determining the responsiveness of breast cancer cells to tamoxifen.

INTRODUCTION

Breast cancer is one of the most common malignancies in the United States, affecting one in nine women during their lifetime. Localized breast cancer, before metastasis, can be cured by surgery. The high mortality rate associated with breast cancer, however, is due to a propensity for the tumor to metastasize while the primary tumor is small and undetected. The process of breast cancer metastasis requires, among other steps, changes in cell signaling pathways, enhanced cell survival, and development of a hormone-independent state. The development of human breast cancer is promoted by estrogen stimulation of mammary epithelial cell growth. The biological effects of estrogen are mediated by its binding to the structurally and functionally distinct estrogen receptors (ER α and ER β). ER α (ER) is the major estrogen receptor in the human mammary epithelium (1), and targeted disruption of estrogen signaling inhibits the mammary gland development (2, 3). The principal target of estrogen, ER is found in 60–70% of breast tumors at presentation, together with a profile of ER-regulated genes. These tumors are generally responsive to antiestrogen therapy (4). Several studies have demonstrated that approximately two-thirds of breast cancer patients respond to antihormone therapy and that tumors in these patients are ER positive; *i.e.*, ER is localized in the cell nucleus. Regardless of the presence of ER, a significant proportion of patients do not respond to antihormone therapy, and most who do initially respond will eventually develop acquired hormone-independent disease (5, 6). At present, it is also not known whether a correlation exists between the between the ER location and tamoxifen sensitivity.

The molecular mechanisms leading to breast cancer progression and advanced malignancy are not completely understood at present but are widely believed to involve intracellular growth-factor-triggered signaling cascades. Studies have established that the human epidermal growth factor receptor (HER) family of receptor tyrosine kinases and their ligands have an essential role in the regulation of proliferation of mammary epithelial cells and in the pathogenesis of breast cancer (7, 8). For example, overexpression of the HER2 receptor gene product is frequently associated with a decrease in disease-free survival, poor prognosis, increased metastasis, and an aggressive clinical course of human breast cancer (9–13). Among the various factors that could participate in the development of antihormone-resistant breast tumors, deregulation of HER2 expression/signaling has emerged as one of the most recognizable molecular dysfunctions in breast tumors. A variety of *in vitro* and *in vivo* models have shown that suppression of HER2

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enhances the antiproliferative effects of tamoxifen (14–18), thus demonstrating a causal association between HER2 overexpression and acquisition of resistance to tamoxifen. Although a subgroup of ER-positive tumors coexpress high HER2 levels, the mechanism by which HER2 influences the ER pathway in breast cancer cells remains poorly understood.

It is generally accepted that estrogen activates components of growth factor signaling pathways, *i.e.*, extracellular signal-regulated kinase 1/2 (ERK1/2) and Shc/Grb2/Sos, in ER-positive breast cancer cells (19–22). These findings are significant because ERK1/2 activity has been shown to be frequently up-regulated in human breast tumors. Furthermore, the ERK1/2 pathway has been shown to regulate the expression of genes with roles in the invasiveness of breast cancer cells (23–26). In this context, recent studies have demonstrated the presence of hyperstimulation of ERK1/2 in HER2-overexpressing, tamoxifen-resistant MCF-7 breast cancer cells and that inhibition of ERK1/2 enhanced tamoxifen-mediated growth inhibition (18). ER signaling has also been shown to regulate cell growth, presumably as a result of cross-talk between growth factors and ER. For example, HER2 promotes the development of the hormone-independent state and a more aggressive phenotype in breast cancer cells (14–18). Estrogen stimulation of growth by ERK1/2 can also occur via a nongenomic action of ER because estrogen-induced ERK1/2 activation and DNA synthesis can be effectively blocked by the mitogen-activated protein kinase kinase (MEK1) inhibitor PD 98059 (19, 22). In brief, enhanced cross-talk of ER with growth factor signaling components may be involved in the development of a hormone-independent phenotypes in breast cancer cells. This study was undertaken to further explore the regulatory interactions between the HER2 and ER pathways in breast cancer cells.

MATERIALS AND METHODS

Cell Cultures and Reagents. MCF-7, MCF-7/HER2 (14, 27, 28), and SKBR3 (28) human breast cancer cells (23) were maintained in DMEM-F12 (1:1) supplemented with 10% FCS. The following antibodies were used: (a) anti-ER α and anti-Grb2 (Upstate Biotechnology, Lake Placid, NY); (b) anti-HER2, anti-Sos, and anti-ERK1/2 antibodies (Neomarkers, Fremont, CA); (c) anti-phospho-ERK1/2 (Thr²⁰²/Tyr²⁰⁴, P-ERK1/2) and anti-phospho-ER α (Ser¹¹⁸; Transduction Laboratories, Franklin Lakes, NJ); (d) antiactin and antivinculin (Sigma Chemical Co., St. Louis, MO); and (e) antimouse and antirabbit horseradish peroxidase-conjugated secondary antibodies (Amersham, Piscataway, NJ). The following reagents were also used: (a) anti-HER2 monoclonal antibody Herceptin (Genentech, San Francisco, CA); (b) the pure antiestrogen ICI 182780 (Tocris, Ellisville, MO); (c) the steroid hormone 17 β -estradiol (E2) and charcoal-stripped serum (DCC serum; Sigma).

Reverse Transcription-PCR. Isolated RNA was reverse-transcribed and amplified with the ONE-STEP RT-PCR System (Promega). The primer sequences used were as follows: PS2 forward (5'-ATA CCA TCG ACG TCC CTC CA-3') and PS2 reverse (5'-CAC CTC AGA CAC GCT T-3'), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward (5'-CCA TCT TCC AGG AGC GAG ATC-3') and GAPDH reverse

(5'-CGT TCA GCT CAG GGA TGA CC-3'). Amplification was conducted in 20- μ l reactions, each containing 50 ng of total RNA, 0.2 mM deoxynucleotide triphosphates, 1 mM MgCl₂, 1 μ M each primer, 0.5 units of AMV, 0.5 units of *Tfl* DNA polymerase, and reaction buffer. The cycling conditions included an initial incubation at 48°C for 45 min and a second incubation at 94°C for 2 min, followed by 35 cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 68°C. The final extension was for 7 min at 68°C. The reverse transcription-PCR products were 156 bp for PS2 and 451 bp for GAPDH. Reverse transcription-PCR products were separated on 2% agarose gels.

Metabolic Labeling of Cells. Cells (3×10^5) were plated in F-12/DMEM in each well of a 6-well dish. After 24 h, cultures were washed with phosphate-free medium and incubated for up to 15 h in phosphate-free F-12/DMEM containing 0.4 mCi/ml ³²P in the presence or absence of 100 nM Herceptin. After 16 h, cells were washed three times with PBS and then lysed in 400 μ l of RIPA buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% NP-40, 0.1% SDS, 0.1% sodium deoxycholate, 1 \times protease inhibitor cocktail (Roche Biochemical), and 1 mM sodium vanadate] for 15 min on ice. The lysates were centrifuged at 12,000 rpm in an Eppendorf microfuge for 10 min. For labeling with [³⁵S]methionine, the cells were washed with methionine-free medium and incubated for up to 16 h with methionine-free F-12/DMEM containing 0.15 mCi/ml ³⁵S with or without 100 μ M Herceptin. Lysates containing equal amounts of perceptible trichloroacetic acid counts were immunoprecipitated with the anti-HER2 or anti-ER monoclonal antibodies, resolved on 10% SDS-polyacrylamide gels, and analyzed by autoradiography.

Cell Extracts, Immunoblotting, and Immunoprecipitation. To prepare cell extracts, cells were washed three times with PBS and then lysed in RIPA buffer for 15 min on ice. The lysates were centrifuged in an Eppendorf centrifuge at 4°C for 15 min. Cell lysates containing 200 μ g of protein were resolved on SDS-polyacrylamide gels (8% acrylamide), transferred to nitrocellulose membranes, probed with the appropriate antibodies, and developed by either the enhanced chemiluminescence method or the alkaline phosphatase-based color reaction method. Immunoprecipitation was performed for 2 h at 4°C with 1 μ g antibody/mg of protein (28).

Reporter Gene Assays. For reporter gene transient transfections, MCF-7 and MCF-7/HER2 cells were cultured in 6-well plates (10⁵ cells/well) in MEM, without phenol red, containing 10% DCC serum for 48 h and then transfected with 100 ng of estrogen response element-luciferase reporter plasmid per well using Fugene-6 reagent according with the manufacturer's instructions (Roche Molecular Biochemicals). After 24 h, cells were treated with or without 10⁻⁹ M estrogen for 16 h. Cells were then lysed with passive lysis buffer, and the luciferase assay was performed with a luciferase reporter assay kit (Promega, Madison, WI); luciferase activity was measured with a Lumant Luminometer. The activity of β -galactosidase reporter was used to correct the transfection efficiencies. Each transfection was performed in triplicate wells (28). Each experiment was repeated three to six times, and transfection efficiency varied between 30 and 50%.

Northern Blot Hybridization. Total cytoplasmic RNA was isolated with use of Trizol reagent, and 20 μ g of RNA was

analyzed by Northern hybridization using a 496-nucleotide fragment of the PS2 full-length cDNA. rRNA (28S and 18S) was used to assess the integrity of the RNA, and the blot was routinely reprobated with human GAPDH cDNA for RNA loading and transfer control as described previously (28).

Transient Transfection with Human HER2. The breast cancer cell lines MCF-7 and ZR-75 growing on glass coverslips in DMEM-F12 (1:1) supplemented with 10% FCS were transfected with human HER2 plasmid (kindly provided by Dr. Mien-Chie Hung; University of Texas M. D. Anderson Cancer Center, Houston, TX) using Fugene-6 reagent according with the manufacturer's instructions (Roche Molecular Biochemicals). Thirty-six h after transfection, cells were fixed with methanol at -20°C for 6 min and then prepared for confocal study as described below.

Immunofluorescence and Confocal Studies. Cellular localization of different proteins was determined by indirect immunofluorescence as described previously (29, 30). Briefly, cells grown on glass coverslips were fixed in either ice-cold methanol for 6 min or 4% paraformaldehyde in PBS at room temperature for 15 min. Cells were incubated with the respective primary antibodies for 2 h at room temperature, washed three times in PBS, and then incubated with 546-Alexa- (red) or 488-Alexa-labeled (green) secondary antibodies (Molecular Probes, Eugene, OR). We used 488-Alexa-phalloidin to localize F-actin. The DNA dye Topro-3 (Molecular Probes), used to costain the DNA, gives an emission in the far-red segment of the light spectrum and was color-coded in blue. For controls, cells were treated only with the secondary antibodies or pretreated with the peptide against which the antibodies had been raised. Confocal analysis was performed with a Zeiss laser-scanning confocal microscope with established methods (29). Each image represents Z-sections at the same cellular level and magnification. Colocalization of two proteins as a result of red and green overlapping pixels is indicated by a yellow color.

Cell Growth Assay. MCF-7/HER2 cells were plated in 24-well plates maintained in DMEM-F12 (1:1) supplemented with 10% FCS. After an overnight incubation, cells were treated with various concentrations of the antiestrogen Tamoxifen (Sigma), the anti-HER2 antibody Herceptin, or both agents. Cells were allowed to grow at 37°C in an atmosphere of 5% CO_2 . After 4 days, the medium was removed, and 1 ml of the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Thiazolyl blue; Sigma) was added. After a 1-h incubation at 37°C in a 5% CO_2 atmosphere, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was removed, and isopropanol-1 N HCl (96:4 v/v) was added. The absorbance at 570 nm was measured on a Bio-tek Instruments (Winooski, VT) automated microtiter plate reader. Identical concentrations and combinations were tested in four separate wells/assay, and the assay was performed four times (16).

RESULTS AND DISCUSSION

Evidence of Cytoplasmic Localization of ER in HER2-Overexpressing Cells. It is increasingly accepted that a causal association exists between HER2 overexpression and acquisition of resistance to tamoxifen, both in human breast cancer cell lines (14, 16, 18) and in clinical studies of patients with ER-

positive, hormone-dependent tumors (9–13). However, there are few molecular explanations for this well-recognized cellular phenomenon in HER2-overexpressing ER-positive breast cancer cells. To explore the potential effect of HER2 deregulation on the ER pathway in breast cancer cells, we used MCF-7 and a well-characterized clone of MCF-7/HER2 breast cancer cells with aggressive phenotypes (14, 18, 27, 28, 30). HER2 overexpression in MCF-7 cells (Fig. 1A) was accompanied by a significant inhibition of both baseline and E2-induced stimulation of ER transactivation function as assessed in an estrogen response element–luciferase reporter system (Fig. 1B) and by suppression of ER target gene PS2 mRNA (Fig. 1C). These results suggested that HER2 overexpression antagonizes genomic responses to ER transactivation in breast cancer cells. Consistent with these results, we also found that estrogen induces expression of endogenous ER target PS2 mRNA in a tamoxifen-sensitive manner in MCF-7 but not in MCF-7/HER2 cells (Fig. 1D). In addition, tamoxifen was able to inhibit the basal levels of PS2 in exponentially growing MCF-7 cells (Fig. 1E). Because subcellular localization of several other proteins (*i.e.*, FKHR and 14-1-1) has been shown to affect their nuclear functions and because there was no effect of HER2 on the steady-state level of ER, we next explored whether HER2 overexpression alters ER subcellular localization. Results from confocal scanning microscopy indicated that HER2 deregulation was accompanied by the presence of ER in the cytoplasmic compartment with a concurrent reduction in the level of nuclear ER (Fig. 1F), suggesting that exclusion of ER from the nucleus may well act to deprive the antiestrogenic agents of their target in the proper cellular compartment. Estrogen treatment was able to translocate ER to the nucleus in MCF-7 but not in MCF-7/HER2 cells (Fig. 1F). This potential mechanism may be related to the reduced tamoxifen response in HER2-overexpressing breast tumors. To evaluate whether the observed phenomenon is restricted to the MCF-7/HER2 clone or whether it could be demonstrated in other ER-positive breast cancer cells, we transiently transfected HER2 into ER-positive MCF-7 and ZR-75 breast cancer cells. Our results demonstrated that HER2 overexpression did indeed promote ER translocation and colocalization with HER2 in the cytoplasm (Fig. 1G). Because MCF-7/HER2 cells have the same expression of ER as MCF-7 cells (Fig. 1A), there was no apparent effect of the altered ER localization on its steady-state level. A recent study showed that peroxisome proliferator-activated receptor- γ agonists induce proteasome-dependent degradation of ER α (31). However, HER2 overexpression antagonizes the sensitivity of peroxisome proliferator-activated receptor- γ to its ligand (32), which may explain, at least in part, why cytoplasmic ER in MCF-7/HER2 cells is not subject to degradation.

Hyperstimulation of ERK1/2 in HER2-Overexpressing Cells. The hyperstimulation of ERK1/2 kinase, a downstream signaling effector of several receptor tyrosine kinases, is a common event associated with the progression of breast tumors and breast cancer cells to more invasive phenotypes (23–26). As expected, HER2-overexpressing cells have elevated levels of activated ERK1/2 as determined by use of an anti-ERK1/2 antibody that specifically recognizes ERK1/2 phosphorylated on Thr²⁰²/Tyr²⁰⁴ either in a Western blot assay (Fig. 2A) or by confocal microscopy (Fig. 2B). The ERK1/2 pathway was rapidly stimulated in MCF-7

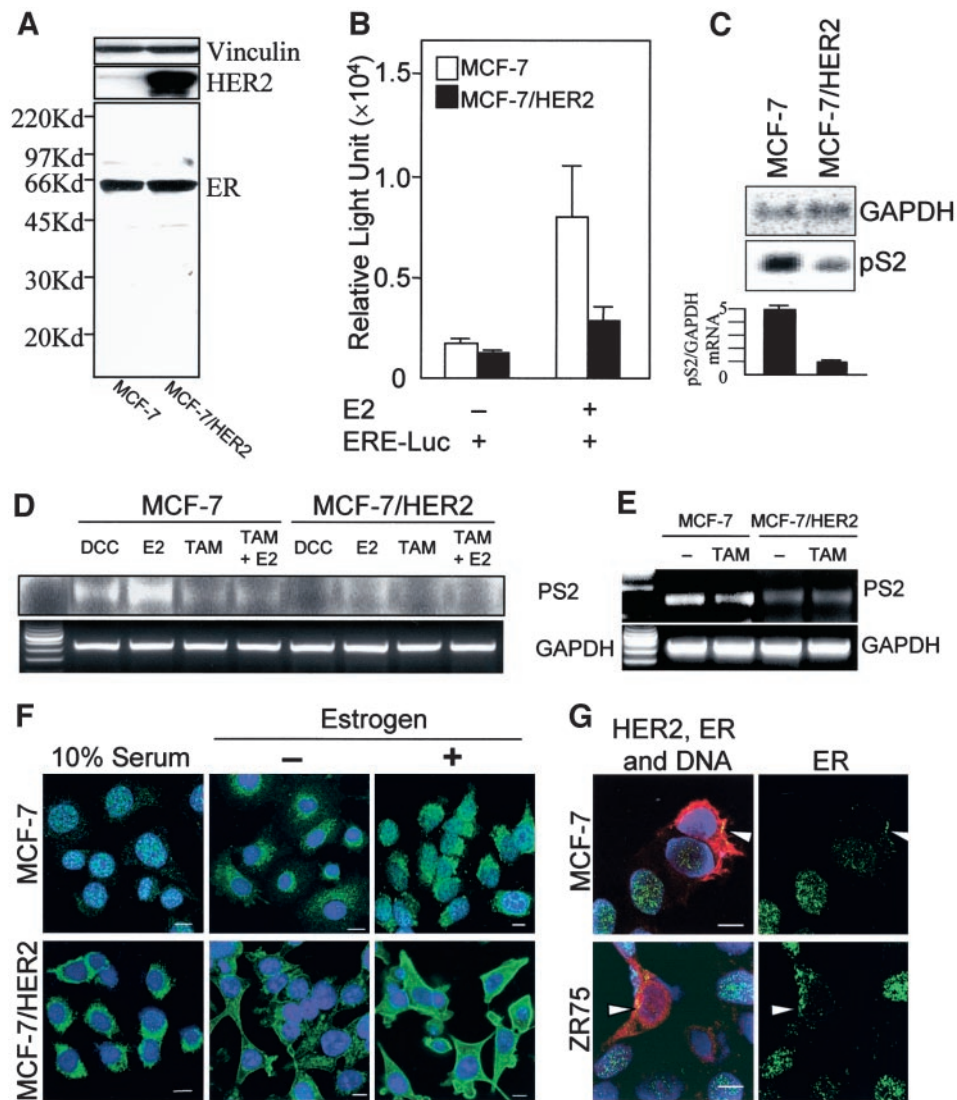


Fig. 1 Subcellular localization of human epidermal growth factor receptor 2 (*HER2*) and estrogen receptor (*ER*) in *HER2*-overexpressing breast cancer cells. **A**, the status of *HER2* and *ER* expression in MCF-7 and MCF-7/*HER2* cells was tested by Western blot. Equal loading was indicated by antivinculin antibody ($n = 3$). **B**, effect of *HER2* overexpression in MCF-7 cells on estrogen response element–luciferase (*ERE-Luc*) activity. MCF-7 and MCF-7/*HER2* cells were cultured in 6-well plates (10^5 cells/well) in MEM, without phenol red, containing 10% charcoal-stripped serum (DCC serum) for 48 h and then transfected with 100 ng estrogen response element–luciferase reporter plasmid/well, using Fugene-6 reagent. After 24 h, cells were treated with or without 10^{-9} M 17 β -estradiol (*E2*) for 16 h, and promoter activity was measured ($n = 4$). Bars, SE. **C**, Northern blot of mRNA levels of the *ER* target gene *PS2* in MCF-7 and MCF-7/*HER2* cells. The mean \pm SE (bars) of the *PS2*:glyceraldehyde-3-phosphate dehydrogenase mRNA ratio (*pS2/GAPDH*) is shown in the bottom panel with each measurement performed three times. **D**, reverse transcription-PCR of *PS2* in MCF-7 and MCF-7/*HER2* cells. Cells were cultured in MEM, without phenol red, containing 10% DCC serum for 48 h and then treated with or without 10^{-9} M 17 β -estradiol (*E2*) for 16 h after being pretreated with or without 10^{-8} M 4-hydroxytamoxifen (*TAM*) as indicated. **E**, reverse transcription-PCR of *PS2* in MCF-7 and MCF-7/*HER2* cells in regular DMEM containing 10% fetal bovine serum. Cells were treated with or without 10^{-8} M 4-hydroxytamoxifen (*TAM*) as indicated. **F**, confocal microscopy shows the presence of *ER* (green) in the cytoplasmic compartment and a concurrent reduction in the level of nuclear *ER* in MCF-7/*HER2* cells compared with MCF-7 cells (blue, DNA counterstain). Cells grown in DCC serum (–) were stimulated with 10^{-9} M estradiol (+) to examine the effects on *ER* localization. Cellular localization of different proteins was determined by indirect immunofluorescence. Bars, 10 μ m ($n = 4$). **G**, MCF-7 and ZR-75 breast cancer cells growing on glass coverslips were transfected with human *HER2*. *ER* (green) was translocated from the nucleus and colocalized with *HER2* (red) in *HER2*-transfected cells (blue, DNA counterstain). Bars, 10 μ m ($n = 3$).

cells by 10^{-9} M *E2*, and maximum activation was attained by 5 min after *E2* stimulation (Fig. 2C). A comparative analysis of *E2* stimulation for 5 min indicated that ERK1/2 phosphorylation was further induced over already elevated baseline ERK1/2 levels in

MCF-7/*HER2* cells (Fig. 2D). The specificity of *E2* stimulation of ERK1/2 was confirmed by pretreating the cells with 10^{-8} M of the pure antiestrogen ICI 182780 for 30 min (Fig. 2D). ICI 182780 destroys the *ER* and decreases ERK1/2. Of interest, the ERK1/2

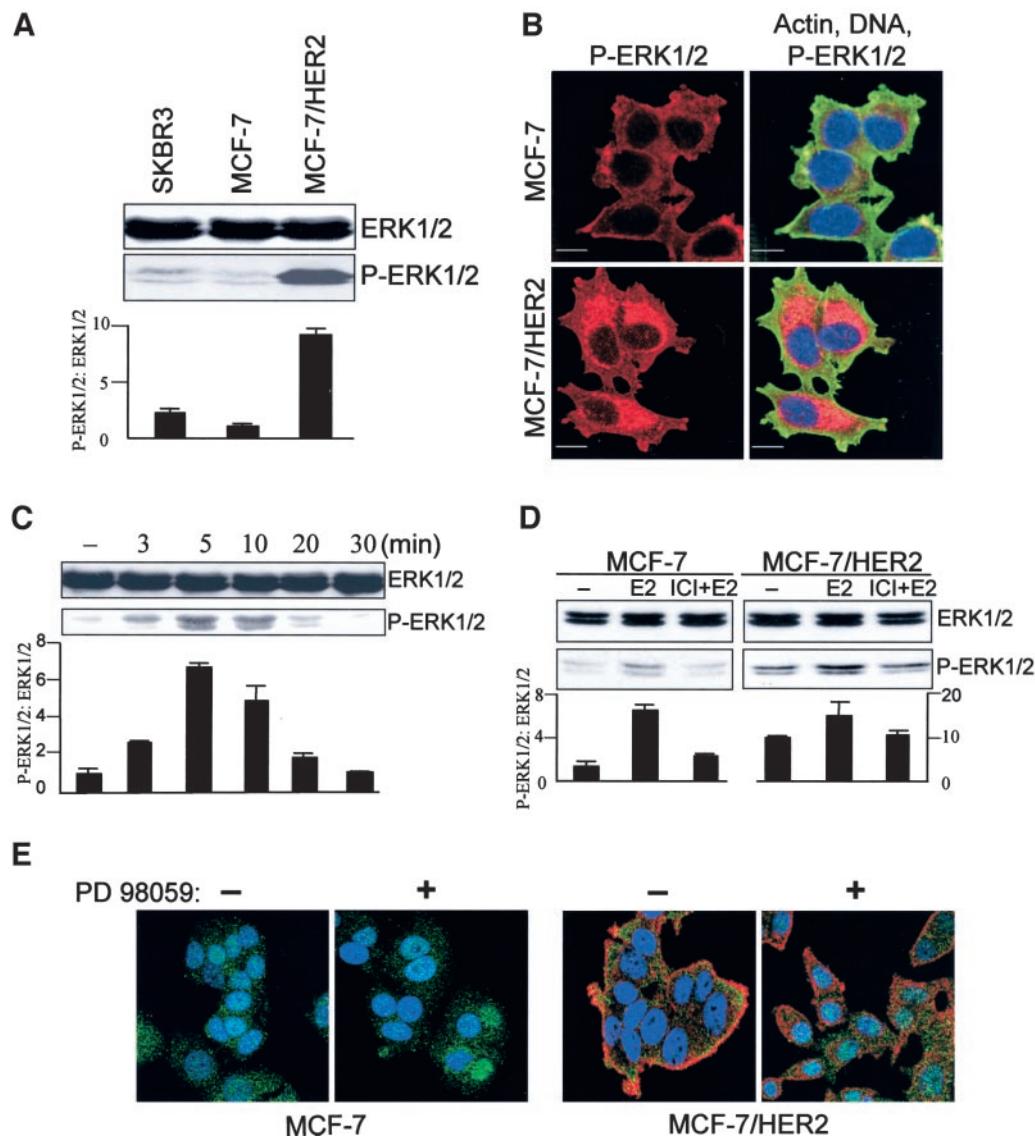


Fig. 2 Human epidermal growth factor receptor 2 (HER2) overexpression and stimulation of extracellular signal-regulated kinase 1/2 (ERK1/2) in breast cancer cells. The levels of total and activated ERK1/2 in MCF-7 and MCF-7/HER2 cells were detected by both Western blot (A) and confocal microscopy [B; red, phospho-ERK1/2 (P-ERK1/2); green, actin; blue, DNA]. Bars, 10 μ m. Cells growing in DMEM-F12 (1:1) supplemented with 10% FCS were used in the experiments shown in A and B ($n = 4$). C, activation of the ERK1/2 pathway by 10^{-9} M 17 β -estradiol (E2) in MCF-7 cells as indicated by Western blot of phospho-ERK1/2. MCF-7 cells were cultured in MEM, without phenol red, containing 10% charcoal-stripped serum (DCC serum) for 48 h and then treated with 10^{-9} M E2 for different times as indicated ($n = 2$). D, comparative Western blot analysis of E2-stimulated (10^{-9} M) ERK1/2 phosphorylation. MCF-7 and MCF-7/HER2 cells were cultured in MEM, without phenol red, containing 10% DCC serum for 48 h and then treated with 10^{-9} M E2 for 5 min with or without pretreatment with 10^{-8} M ICI 182780 (ICI) for 30 min. The bottom panel shows a bar graph of the phospho-ERK1/2:ERK1/2 ratios (P-ERK1/2:ERK1/2; $n = 4$). E, confocal microscopy [red, HER2; green, estrogen receptor; blue, DNA] was used to examine the effect of the ERK1/2 inhibitor PD 98059 (20 μ M for 3 h) on estrogen receptor localization in exponentially growing cells.

inhibitor PD 98059 was able to partially restore nuclear ER in HER2-overexpressing MCF-7 cells, implying a functional role for ERK1/2 signaling in ER cytoplasmic localization (Fig. 2E). Overall, these findings support the notion of a close relationship between ERK1/2 hyperstimulation and HER2 overexpression in breast cancer cells.

Evidence of HER2 Interaction with ER in HER2-Overexpressing Cells. Because E2 has been shown to activate components of the Ras signaling pathway in breast cancer cells

(19–22) and because ER is localized predominantly in the cytoplasmic compartment in MCF-7/HER2 cells, we next explored the possibility whether ER could interact with HER2 and participate in ERK1/2 activation. To investigate this possibility, lysates from exponentially growing cells were immunoprecipitated with anti-ER antibody and sequentially immunoblotted with HER2, Sos, Grb2, or ER antibodies (Fig. 3A). Of interest, HER2 and Sos proteins were selectively associated with ER in MCF-7/HER2, whereas there was no change in the level of

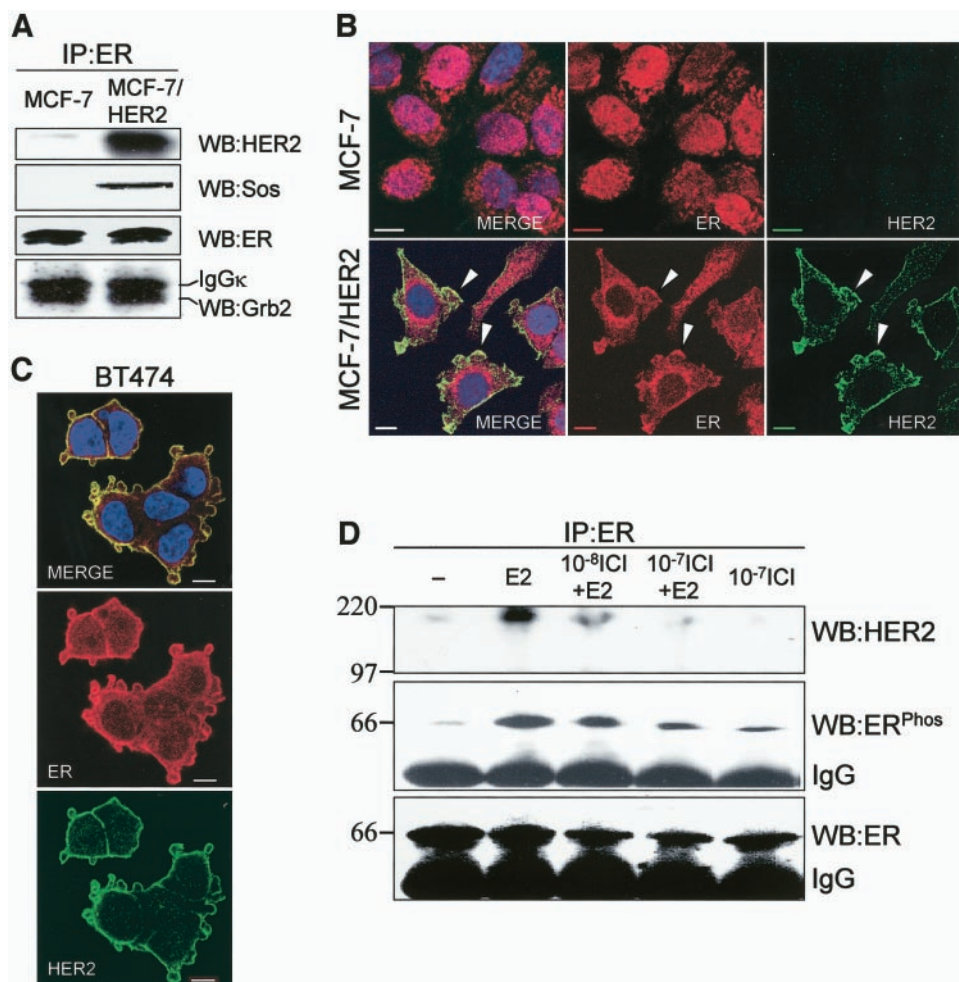


Fig. 3 Estrogen receptor (ER) interaction with human epidermal growth factor receptor 2 (HER2) and Sos in HER2-over-expressing MCF-7 cells. **A**, lysates from exponentially growing MCF-7 and MCF-7/HER2 cells were immunoprecipitated (IP) with an anti-ER antibody and sequentially immunoblotted with HER2, Sos, and Grb2 or ER antibodies. In a reverse experiment, lysates were immunoprecipitated with an anti-HER2 monoclonal antibody and blotted with ER or HER2 antibodies. **WB**, Western blot. **B**, confocal microscopic analysis of colocalization (yellow) of ER (red) and HER2 (green) in MCF-7 and MCF-7/HER2 cells (blue, DNA counterstain). **Bars**, 10 μ m. **C**, confocal microscopic studies of ER cytoplasmic localization and colocalization with HER2 in BT474 cells. **Bars**, 10 μ m. Cells growing in DMEM-F12 (1:1) supplemented with 10% FCS were used for **A**, **B**, and **C**. **D**, immunoprecipitation (IP) with anti-ER antibody and Western blot (WB) of MCF-7/HER2 cells treated with 17 β -estradiol (E2) and/or ICI 182780 (ICI). MCF-7/HER2 cells were treated with 10⁻⁹ M estradiol for 30 min with or without pretreatment with 10⁻⁷ or 10⁻⁸ M ICI 182780 for 60 min as indicated. Experiments in **A–D** were repeated three to four times, and similar results were obtained.

ER-associated Grb2 (Fig. 3A, upper panel). We further validated the observed ER-HER2 interaction in MCF-7/HER2 cells by confocal scanning microscopy (Fig. 3B). We next extended these observations to ER-positive BT-474 breast cancer cells, which naturally express high HER2 levels. Our results showed that ER was also localized predominantly in the cytoplasm and colocalized with HER2 on the cellular membrane in BT474 cells (Fig. 3C) and raised the possibility of a causal role of HER2 signaling in ER translocation.

To investigate whether E2 stimulation might affect ER-HER2 interaction in MCF-7/HER2 cells, we next examined the effect of E2 with or without pretreatment with ICI 182780 on ER-HER2 interactions and the state of ER phosphorylation on the mitogen-activated protein kinase consensus phosphorylation site at Ser¹¹⁸ (33). Our results indicated that E2 stimulation further enhanced the interaction between ER and HER2 over an easily detectable baseline interaction and in parallel stimulated ER phosphorylation on Ser¹¹⁸ (Fig. 3D). E2-enhanced ER-HER2 interaction was sensitive to the pure antiestrogen ICI 182780, which effectively blocked ER phosphorylation on Ser¹¹⁸ (Fig. 3D, lower panel). Because ICI 182780 effectively inhibited E2-mediated stimulation of mitogen-activated protein

kinase (Fig. 2D), ER activation, and ER-HER2 interactions (Fig. 3D), these findings suggested an apparent role for mitogen-activated protein kinase stimulation in the observed ER-HER2 interactions in MCF-7/HER2 cells.

HER2 Down-Regulation Promotes Nuclear Localization of ER. Together, the results illustrated in Figs. 1–3 suggested that HER2 overexpression leads to cytoplasmic localization of ER and its interaction with HER2. To further establish a role for HER2 in the observed cytoplasmic localization of ER in MCF-7/HER2 cells, we next examined the effect of HER2 down-regulation by the anti-HER2 monoclonal antibody Herceptin on the subcellular localization of ER in MCF-7/HER2 cells. As expected, treatment of cells with 100 nM Herceptin for 16 h down-regulated the amount of phosphorylated HER2 bound by ER in MCF-7/HER2 cells metabolically labeled with ³²P (Fig. 4A) and, hence, down-regulated the level of [³⁵S]methionine-labeled HER2 in MCF-7/HER2 cells (Fig. 4B). Herceptin treatment also reduced the level of activated ERK1/2 in MCF-7/HER2 cells (Fig. 4C). Importantly, Herceptin treatment restored the primary localization of nuclear ER in a significant proportion of MCF-7/HER2 cells (93% of Herceptin-treated cells compared with 12% in untreated cells; Fig. 4D). These results raised the possibility that Herceptin treatment and

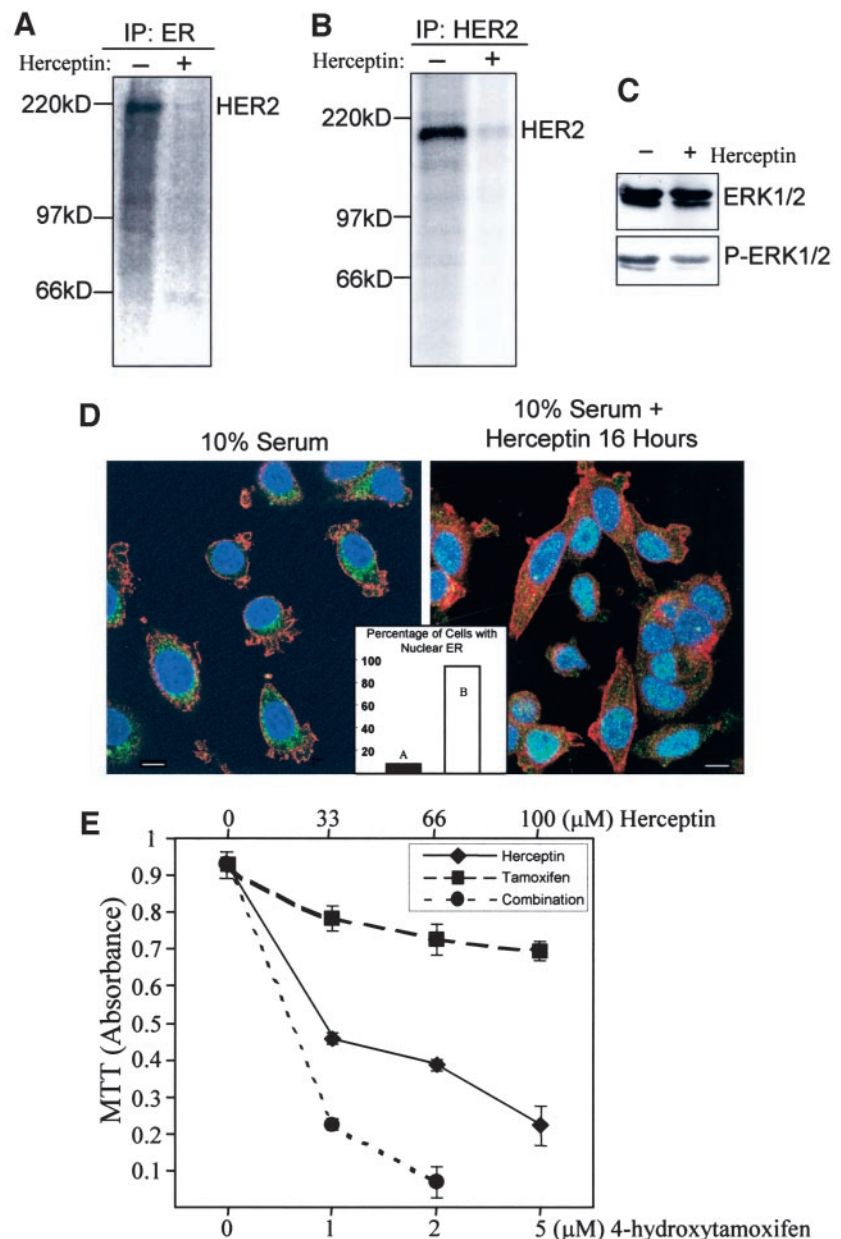


Fig. 4 Effect of Herceptin on estrogen receptor (ER) subcellular localization. Immunoprecipitation (IP) of ^{32}P -labeled MCF-7/HER2 cells with anti-ER antibody (A) or [^{35}S]methionine-labeled MCF-7/HER2 cells with anti-human epidermal growth factor receptor 2 (HER2) antibody (B) with or without Herceptin treatment. C, Western blot for activated extracellular signal-regulated kinase 1/2 (ERK1/2). P-ERK1/2, phospho-ERK1/2. In A, B, and C, + indicates treatment with 100 nM Herceptin for 16 h; - indicates no Herceptin treatment. D, confocal microscopic analysis of HER2 (red) and ER (green) localization in MCF-7/HER2 cells with and without Herceptin treatment (blue, DNA). Inset, quantitation of cells with nuclear ER ($n = 200$ cells/group). Bars, 10 μm . E, effect of tamoxifen alone (■), Herceptin alone (◆), or the combination of the two agents (●) on the proliferation of MCF-7/HER2 cells as measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay at 570 nm. Identical concentrations and combinations were tested in four separate wells per assay in three independent experiments. Representative results are shown. Bars, SE.

restoration of nuclear ER may promote the growth-inhibitory action of tamoxifen in MCF-7/HER2 cells, which were previously reported to be tamoxifen resistant (14, 16, 18). Indeed, the combination of tamoxifen with Herceptin provided a beneficial growth-inhibitory action compared with treatment with individual agents alone (Fig. 4E). Because inhibition of ERK1/2 has also been shown to augment the action of tamoxifen (18) and because Herceptin treatment was accompanied by inhibition of ERK1/2 as well as nuclear localization of ER (this study), these findings suggested that Herceptin-mediated down-regulation of ERK1/2 may be associated with increased sensitivity to tamoxifen, presumably because of nuclear localization of ER. These observations may provide a potential rationale for ongoing and planned clinical studies combining Herceptin and hormone-based therapies (34). In brief, these

findings suggest that HER2 down-regulation by Herceptin might promote the action of tamoxifen by inhibiting the ER-HER2 interaction and/or ERK1/2 activation. These findings are consistent with a mechanistic role for the subcellular localization of ER in the determination of the responsiveness of breast cancer cells to tamoxifen.

In summary, the results presented here show for the first time that ER redistribution to the cytoplasm is an important downstream effect of HER2 overexpression and that HER2 interaction with the ER could explain, at least in part, the previously noted tamoxifen resistance of HER2-overexpressing breast cancer cells (35). Because recent studies have also suggested that ER could be sequestered in the cytoplasm by the deregulation of MTA1 in MCF-7/HER2 cells (36), our current

studies raise the possibility of existence of multiple complementary and overlapping mechanisms that might be working to influence the subcellular localization of ER. The finding that HER2 deregulation in ER-positive breast cancer cells enhanced both cytoplasmic sequestration of ER and ERK1/2 activation raises the possibility of cross-talk between HER2 and ER in the cytoplasmic compartment, which could affect the action of antiestrogens in the nucleus. The observed hyperstimulation of ERK1/2 may be an important event because these results provide a new explanation for the aggressiveness of HER2-overexpressing, ER-positive breast cancer cells. Herceptin, through down-regulation of HER2-mediated growth factor receptor signaling, restores ER in the nucleus, and this, in turn, might account for the potentiation of the growth-inhibitory effect by the combined action of Herceptin and antiestrogen therapy.

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