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Involvement of Heregulin-β2 in the Acquisition of the Hormone-independent Phenotype of Breast Cancer Cells[1]

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ABSTRACT

The erbB-2 receptor plays an important role in the prognosis of breast cancer. Amplification or overexpression of the *erbB-2* proto-oncogene has been detected in 30% of breast cancers and is associated with poor patient prognosis. The significance of erbB-3 and erbB-4 in breast cancer is not yet known. The discovery of the growth factor heregulin (HRG) has allowed us to investigate a number of biological events that are regulated by erbB-2, -3, and -4 signal transduction. To determine the role of HRG in breast cancer tumor progression, we have developed an *in vitro/in vivo* model. We transfected HRG cDNA into the estrogen receptor (ER)-positive breast cancer cell line, MCF-7, and studied these cells as they progressed from a hormone-dependent to -independent phenotype. The biochemical and biological characteristics presented here demonstrate that overexpression of HRG induces morphological changes in MCF-7 cells as well as erbB-2, erbB-3, and erbB-4 autophosphorylation. MCF-7/heregulin-transfected cells, which express relatively high levels of HRG, developed estrogen independence and resistance to antiestrogens *in vitro* and *in vivo*. This is consistent with a more aggressive hormone-independent phenotype. In contrast with control parental/wild-type cells, estradiol-mediated down regulation of erbB-2 expression is blocked completely in this particular model system. These results indicate that HRG plays a role in the disruption of ER function. When a transient transfection with an ERE-CAT construct was introduced into these HRG-transfected MCF-7 cells, we observed that the ER was transcriptionally inactive. This suggests that ER signaling is altered in HRG-transfected cells. We observed that overexpression of HRG induces a more aggressive, hormone-independent phenotype that is most likely directly related to the constitutive activation of the erbB-2, erbB-3, and erbB-4 receptor signaling cascade. The data presented here suggest a close cross-regulation between the erbB-2/4 receptors and ER and provide new insights into the mechanism by which breast cancer cells acquire a hormone-independent phenotype.

INTRODUCTION

In the last decade, numerous studies have indicated that growth factors and their receptors play an important role in cancer biology. The EGFR[3] family is a group of tyrosine kinases that is overexpressed frequently in a variety of carcinomas (1–4). The growth factor receptors of this class I subfamily are EGFR (5), HER2/p185$^{erbB-2/neu}$ (6–11), HER3/p160^{erbB-3} (12, 13), and HER4/p180^{erbB-4} (14). Amplification and overexpression of one of these receptors, erbB-2, have been shown to correlate with a poor prognosis in some types of adenocarcinomas, most notably breast cancer (1, 15–20). The erbB-2 receptor is overexpressed in approximately 30% of human breast cancer patients and appears to be necessary for the maintenance of the malignant phenotype (15, 20, 21). Several putative ligands such as the M_r 25,000 NEL/GF from bovine kidney (22), Neu differentiation factor from rat (23), the M_r 45,000 human HRG (24), and gp30 (25–27) have been identified. HRG exists as multiple isoforms that are alternatively spliced molecules (24, 28, 29).

The most recently identified tyrosine kinase receptor, HER4/p180^{erbB-4}, which is related structurally to p185^{erbB-2}, directly interacts with and is activated by HRG (30), in contrast to previous data that HRG may function as a ligand for erbB-2 on the basis of its ability to elevate tyrosine phosphorylation of p185^{erbB-2} in human breast cancer cells. In cell lines that express both erbB-4 and erbB-2, HRG binds to erbB-4 and induces heterodimerization with erbB-2, thereby activating both signaling pathways (31). In the absence of erbB-4, HRG fails to induce phosphorylation of erbB-2 (31). In addition, recent studies indicate that HRG binds with low affinity with erbB-3, resulting in its activation (32). HRG can also induce heterodimer formation of erbB-3/erbB-2 in the absence of erbB-4 (33, 34).

Overexpression of the erbB-2 receptor itself cannot account for an increased invasive potential, because a higher proportion of comedo-type intraductal carcinomas overexpresses the protein compared to the invasive cancer (16). Instead, increased invasive potential appears to be related to ER levels. Infiltrating ductal carcinomas may either contain or lack ER protein. ER$^+$ tumors tend to be more differentiated and less malignant in behavior, whereas ER$^-$ tumors often have a less differentiated and more malignant phenotype (35, 36). We and others have demonstrated an inverse correlation between c-erbB-2 signaling and estrogen-dependent stimulation of the ER (37, 38).

To better understand the role of HRG in breast cancer tumor progression, we generated an expression vector containing the HRG-β2 cDNA sequence and developed an *in vitro* model for studying the growth process of breast cancer cells from a hormone-sensitive to -resistant phenotype. The HRG-β2 cDNA was transfected into MCF-7 cells to evaluate whether constitutive expression of HRG-β2 could induce erbB-2/3/4 autoactivation. In addition, we examined whether this autoactivation would cause the cells to bypass the normal E_2 requirement for growth. Understanding the mechanisms through which receptors and their cognate ligands interact as well as the cross-talk and cross-regulation between the receptors will allow us to define the biological significance of their respective expression.

MATERIALS AND METHODS

Generation of an HRG-β2 Expression Vector for Human Cell Lines

The HRG-β2 cDNA 1278-bp fragment (aa.1–aa.426) was ligated into the pRC/CMV expression vector (Invitrogen Corp.) at the Hin*dIII* and *Xba*I sites. The plasmid preparation was amplified in bacteria (strain DH5α). Positive clones were picked, and the inserts were sequenced by the dideoxy chain termination method using Sequenase version 2.0 (United States Biochemical Corp., Cleveland, OH).

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[3] The abbreviations used are: EGFR, epidermal growth factor-like receptor; ER, estrogen receptor; HRG, heregulin; ECL, enhanced chemiluminescence; CCS, charcoal-stripped calf serum; PgR, progesterone receptor; FACS, fluorescence-activated cell sorting; CAT, chloramphenicol acyltransferase; ERE, estrogen response element; erbB-2/3/4, erbB-2, erbB-3 and erbB-4; OH-TAM, OH-tamoxifen; E_2, estradiol; IMEM, improved minimal essential medium; MCF-7/WT, wild-type MCF-7 cells; MCF-7/V, vector-transfected MCF-7 cells.; GUMC, Georgetown University Medical Center, Washington, D. C; GUACAC, Georgetown University Animal Care and Use Committee.

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Transient and Stable Transfections of Human Cells

The transfections were carried out using the calcium phosphate precipitation method. Stable transfectants were selected by culturing the cells in the presence 0.4 mg/ml of G418 (Life Technologies, Inc., Gaithersburg, MD) for 4–6 weeks. Clonal cell lines were generated by directly selecting G418-resistant clones, isolating them, and individually plating them. In addition, we also generated clonal cell lines by making limiting dilutions from the first passage of the pooled clone population. Cells were diluted to approximately one cell/100 μl of media. One hundred-μl cells/well were plated into 96-well plates. After 2 weeks, the wells showing growths of an individual clone were harvested and propagated.

Detection of mRNA by RNase Protection Assay

Total cellular RNA was extracted by a one-step acid guanidinium isothiocyanate-phenol procedure using RNAzol B (tel-Test, Friendswood, TX), precipitated with ethanol, and quantitated by spectrophotometry. Forty μg of the total RNA were hybridized with 120,000 counts/min of ^{32}P-labeled riboprobes for 12–16 h at 50°C (39). Samples were then digested with 40 μg/ml of RNase A for 30 min at 28°C. The RNase digestion was terminated by the addition of both proteinase K (1 μg/ml) and 1% SDS. After a phenol-chloroform-isoamyl alcohol (25:24:1) extraction, samples were precipitated with 2 μg tRNA in absolute ethanol. The RNA was redissolved in a denaturing loading buffer and electrophoresis on 6% polyacrylamide gels. Protected fragments were visualized by autoradiography and quantitated by densitometry (Huntington Station, NY).

Western Blot

Cells were lysed in lysis buffer [Tris-buffered saline (pH 6.8) containing SDS and β-mercaptoethanol] at 95°C for 5 min. Proteins were separated by SDS-PAGE (4–20% Tris-glycine gradient gel) (Novex, San Diego, CA) and electroblotted onto Hybond ECL nitrocellulose membranes (Amersham, Arlington Heights, IL). After being blocked overnight with 5% BSA in Tris-buffered saline containing 0.05% Tween 20 (pH 7.5), the blots were incubated with antiphosphotyrosine antibody (Upstate Biotechnology, Inc., Lake Placid, NY) for 1 h. After extensive washing, the blots were labeled with peroxidase-tagged sheep antimouse IgG (1:1000) for 30 min and visualized by ECL detection reagent (Amersham).

Phosphorylation Assay

MDA-MB-453 cells (200,000 cells/well) were plated in a 24-well plate. After 24 h, cells were washed with IMEM and treated with conditioned media from different cells for 30 min at 37°C. Cells were lysed in lysis buffer at 95°C for 5 min. Proteins were separated by SDS-PAGE (4–20% SDS-PAGE) followed by Western blot analysis with an antiphosphotyrosine antibody (United Biomedical, Inc., Lake Success, NY).

Immunoprecipitation

Cells were cultured in 100 mm-diameter Petri dishes in their normal growth medium. The cells were grown to 80–90% confluence. They were washed twice with PBS and lysed in 1 ml of lysis buffer. Cell lysates were then precleared with 30 μl of normal rabbit serum for 1 h at 4°C. The lysates were then immunoprecipitated with either anti-erbB-2 (Ab-3, Oncogene Science, Uniondale, NY), anti-erbB-3 (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-erbB-4 (generated in our laboratory) in combination with protein A agarose (Pharmacia, Piscataway, NJ) overnight at 4°C with gentle agitation. The immunoprecipitates were subsequently washed four times in washing buffer prior to electrophoresis on a 4–16% SDS-PAGE. The proteins were then transferred to nitrocellulose and subjected to Western blot analysis using a monoclonal antiphosphotyrosine antibody (Upstate Biotechnology, Inc.). The phosphorylated erbB-2/3/4 proteins were detected by ECL method (Amersham).

Growth Assays

Anchorage-dependent Growth Assay. Cells were plated in 24-well plates in IMEM and 10% FCS. After 24 h, media was replaced with IMEM (phenol red-free) (Biofluids, Rockville, MD) supplemented with 5% CCS for 6 days to deplete the estrogen. Cells were counted at day 0, 2, 4, and 7, after the depletion of the estrogen. All assays were performed at least three times in triplicate.

Anchorage-independent Growth Assay. Cells were grown in IMEM (phenol red-free) and 5% CCS for 5 days in a T75 flask. A bottom layer of 0.1 ml IMEM containing 0.6% agar and 10% CCS was prepared in 35 mm tissue culture dishes. After the bottom layer solidified, cells (10,000/dish) were added in a 0.8-ml top layer containing either E_2 (10^{-9} M) or TAM (10^{-7} M), 0.4% Bacto Agar, and 5% CCS. All samples were prepared in triplicate. Cells were incubated for approximately 12 days at 37°C. Colonies larger than 60 μm were counted in a cell colony counter (Ommias 3600, Imaging Products International, Inc., Charlestown, VA).

Enzyme Immunoassay

The levels of ER and PgR protein were determined by an enzyme immunoassay system (Abbott Laboratories, Chicago, IL) containing monoclonal antibodies D547 and H222. Cells were grown in 5% CCS phenol red-free media for 6 days and then treated with 10^{-9} M E_2 for 24 h. The cells were harvested and resuspended in extraction buffer [10 mM Tris (pH 7.4), 4°C, containing 1 mM EDTA, 10% glycerol, 1 mM DTT, 0.1 mM leupeptin, 0.01 mM aprotinin, and 2 μM pepstatin A] and homogenized with a glass homogenizer, followed by centrifugation at 100,000 × *g* for 30 min at 4°C. The pellet was incubated for 1 h at 4°C in extraction buffer containing 500 mM NaCl and centrifuged at 100,000 × *g* for 30 min at 4°C. The supernatants from both centrifugations were pooled, and an aliquot of these specimens was analyzed for ER content according to the manufacturer's instructions.

FACS Analysis

Cells were grown in IMEM (phenol red-free) with 5% CCS for 6 days. Cells were harvested after treatment with or without E_2 (10^{-9} M) for 24 h and then stained for 1 h with anti-erbB-2 monoclonal antibody no. 94, which recognizes an epitope on the erbB-2 extracellular domain (provided kindly by Dr. Richter King, GUMC). Then a secondary FITC-antimouse antibody was used, and the erbB-2 level in each cell was measured quantitatively by flow cytometry.

CAT Assay

Cells were plated in estrogen-deprived medium (phenol red-free) with 5% dextrin-coated charcoal-treated bovine serum for 6 days. Four h prior to transfection, the medium was changed to either estrogen-deprived IMEM or estrogen-deprived IMEM plus 10 nM E_2. Seventy-two h after the transient transfection of ERE-CAT construct (provided kindly by Dr. D. El-Ashry, GUMC) and cotransfection of *β-galactosidase* reporter gene, cells were harvested with 1 ml of TE (10 mM Tris (pH 8)-1 mM EDTA]. The cells were spun down, and the pellets were resuspended in 100 μl of ice-cold 0.25 M Tris-HCl. The cells were then lysed by three cycles of freeze-thaw and microfuged for 5′, and the supernatant was transferred to a fresh tube. Thirty to 50 μg of cell extract were mixed with 40 μCi of [^{14}C]chloramphenicol and 4 mM acetyl CoA and incubated at 37°C for 1 h. The protein was extracted with 1 ml cold ethyl acetate and desiccated in a Speed-Vac for 45 min. Finally, the sample was resuspended in 10 μl of ethyl acetate. The [^{14}C]acetyl chloramphenicol form was separated from the non-acetyl chloramphenicol form by TLC and visualized by autoradiography. To correct for differences in transfection efficiencies, equivalent amounts of *β-galactosidase* reporter gene activity were used to normalize the amounts of cell extract from each transfection utilized in subsequent CAT assays.

In Vivo Studies

Ovariectomized athymic nude mice were inoculated s.c. with either MCF-7/T6 cells (5 × 10^6), MCF-7/WT, or MCF-7/V cells in the presence or absence of estrogen pellets (0.72), tamoxifen (5 mg), or both. The slow-release pellets (60-day release) were implanted s.c. into the cervical scapular space. The sizes of the tumors were measured biweekly. When tumors reached up to 2 cm in diameter, mice were sacrificed.

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RESULTS

Construction and Transfection of Heregulin-β2 (HRG-β2) cDNA in Breast Cancer Cells

We constructed the HRG-β2 cDNA (aa.1–aa.426) in a eukaryotic expression vector, pRC/CMV, that was transfected subsequently into the ER-positive breast cancer cell line MCF-7. This cell line was chosen as a recipient because MCF-7 cells do not express HRG. They do express low levels of erbB-2/3/4 receptors. MCF-7 cells are relatively nontumorigenic and noninvasive, and they respond to estrogen and antiestrogen treatments *in vitro* and *in vivo*. This cell line is therefore useful for generating a model system to study breast cancer tumor progression from a hormone-dependent to a hormone-independent phenotype. Because MCF-7 cells demonstrate clonal heterogeneity, we carried out two independent transfections using two different sources of MCF-7 cells. More than 20 clones were isolated and characterized. Most of the clones exhibited similar phenotypes and expressed very high levels of HRG-β2. In addition, multiple clones from the vector-transfected MCF-7 cells were characterized, all of which behaved as the wild-type MCF-7 cells.

Effects of Constitutive HRG-β2 Expression on the Morphology of MCF-7 Cells

There is a marked morphological change in the stable HRG-β2-expressing clones of the MCF-7/HRG-β2 cells (MCF-7/T4 and MCF-7/T6). These cells appear larger, with larger nuclei and increased cytoplasm compared to the parental cells (MCF-7/WT) and/or transfectants with vector alone (MCF-7/V; see Fig. 1).

Expression of HRG in MCF-7/T Cells

A number of the MCF-7/T clones were selected for biochemical and biological characterization as follows.

Protein Expression. HRG protein expression was analyzed by Western blot analysis using an anti-HRG polyclonal antibody generated in our laboratory. This antibody recognizes an epitope in the extracellular domain of HRG, common to all the known isoforms. Conditioned media from the MCF-7/T (T6) cell was collected and partially purified for HRG-β2 by heparin chromatography. The anti-HRG antibody reacts with the soluble HRG protein from the transfected cells, resulting in a band at the expected molecular size of about M_r 45,000 (Fig. 2*A*).

RNA Expression. The levels of HRG-β2 mRNA expressed in these cells were determined using an RNase protection assay with a β2 riboprobe (kindly provided by Dr. Francis Kern, GUMC; Ref. 39). As expected, high expression of HRG was observed in MCF-7/T cells. These cells express very high levels of HRG mRNA as compared with MDA-MB-231 cells, which were the source of the HRG cDNA. HRG mRNA expression varied in the different clones (Fig. 2*B*). Of the three representative clones shown, the MCF-7/T6 clones express the highest level of HRG-β2. Other clones (T2, T7, and T8) also exhibit similar high levels of HRG-β2 expression. MCF-7/T4 expresses an intermediate level and MCF-7/T5 expresses a relatively low level of HRG-β2. MCF-7/WT and MCF-7/V serve as negative controls.

Detection of Bioactive HRG-β2 in MCF-7/T Cell Cultured Media. The biological activity of HRG-β2 expressed in MCF-7/T cells was determined by its ability to induce p185 tyrosine phosphorylation in MDA-MB-453 cells detected by Western blot using an antiphosphotyrosine antibody. MDA-MB-453 cells express high levels of erbB-2, as well as low and intermediate levels of erbB-3 and erbB-4 receptors. After treatment of MDA-MB-453 cells with unconcentrated conditioned media derived from the MCF-7/T (A, B, C, and E or T4, T5, T6, and T7) cells, a substantial increase in p185 tyrosine phosphorylation was observed, as shown in Fig. 2*C*. Conditioned media from MDA-MB-231 cells, concentrated 100-fold, was used as a positive control. No induction of p185 tyrosine phosphorylation was observed when cultured media from MCF-7/WT (Fig. 2*C, Lane 3*) or MCF-7/V was used. In addition, we also partially purified HRG from MCF-7/T6 condition media to determine the biochemical properties of this recombinant protein. This recombinant HRG-β2 protein produced by MCF-7/T6 cells shows the same binding affinity with heparin, and the same biochemical properties, as the native HRG produced by the MDA-MB 231 cells.

Our results indicate that the MCF-7/T6 cells produce at least 100 times more biologically active HRG than the MDA-MB-231 cells, as determined by the induction of p185 tyrosine phosphorylation and by its protein and mRNA expression.

Biochemical Characterization of MCF-7/T Cells

It has been shown previously that MCF-7 wild-type cells express no detectable levels of phosphorylated erbB-2/3/4, indicating that autophosphorylation of these receptors does not occur in these cells. Therefore, we tested the steady-state level of erbB-2/3/4 autophos-

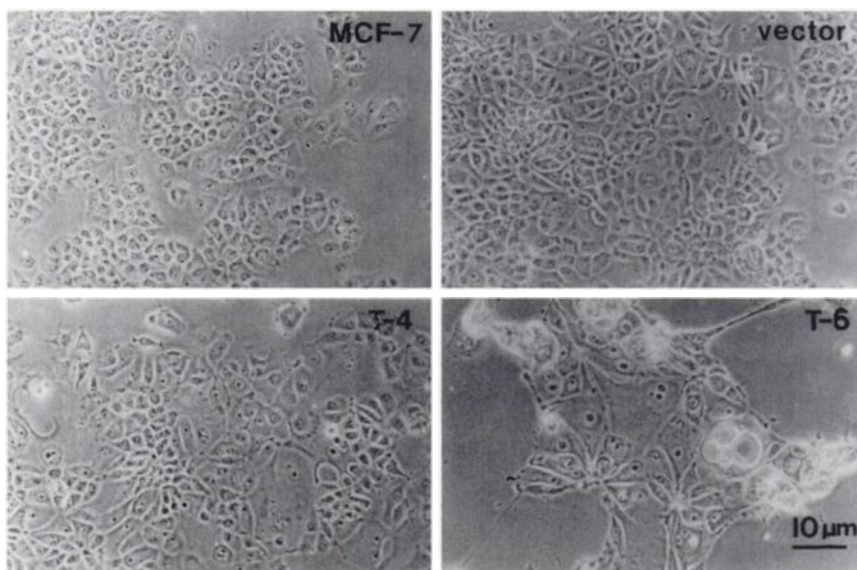

Fig. 1. Effects of constitutive HRG-β2 expression on the morphology of MCF-7 cells. Photomicrographs of MCF-7 cells (*Wt*), MCF-7 cells transfected with the pRC/CMV vector only (*V*), and MCF-7 cells transfected with HRG-β2 cDNA clones (T4 and T6).

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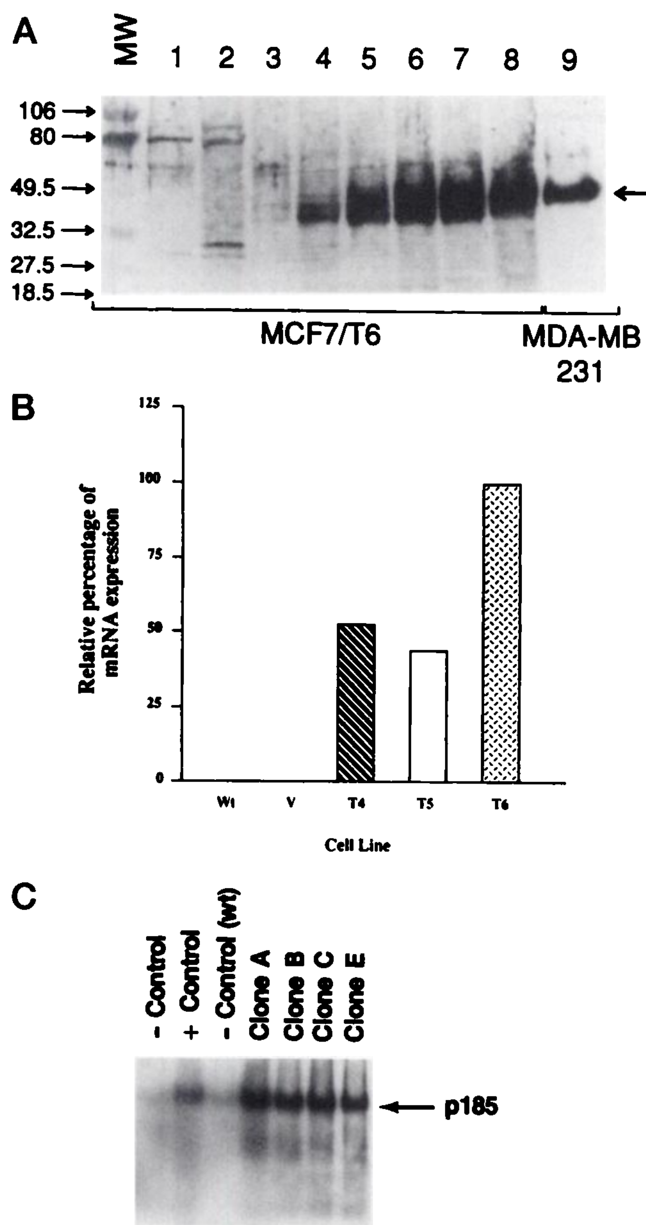

Fig. 2. Expression of HRG-β2 in MCF-7/T cells. *A*, protein expression. Expression of HRG-β2 protein produced by MCF-7/T cells exhibits similar molecular weight as the native form HRG produced by the MDA-MB-231 cells. Conditioned medium from the MCF-7/T (T6) cells was collected and purified partially by heparin chromatography to isolate the HRG-β2 isoform. The eluted protein fractions (*Lanes 1–8*) were subjected to Western blot analysis. The anti-HRG antibody (α3) reacts with the secreted HRG protein from the transfected cells, resulting in the expected molecular size of M_r 45,000 (*arrow*). *Lane 9*, secreted HRG from MDA-MB-231 cells as control. *B*, RNA expression. Expression of the HRG-β2 mRNA levels in MCF-7/T cells was determined by RNase protection assay with a HRG-β2 riboprobe (provided kindly by Dr. Francis Kern). As expected, high expression of HRG in the MCF-7/T cells was evident. Three representative clones were analyzed (T4, T5, and T6). The MCF-7/T6 clone expressed the highest level of HRG-β2 among these three clones. The MCF-7/T4 expressed an intermediate level and MCF-7/T5 expressed a relatively low level of HRG-β2. MCF-7/WT and MCF-7/V do not express detectable levels of HRG. *C*, detection of bioactive HRG-β2 in MCF-7/T cell-cultured media. MDA-MB-453 cells were plated (200,000 cells/well) in a 24-well plate. After 24 h, the serum-rich media was replaced with serum-free media overnight and then treated with conditioned media from different cells for 30 min at 37°C. Cells were then lysed in lysis buffer at 95°C for 5 min. Proteins were separated by SDS-PAGE (4–20% Tris-glycine gradient gel) followed by Western blot analysis with antiphosphotyrosine antibody. A substantial increase in tyrosine phosphorylation of p180–p185 was observed when unconcentrated conditioned media was used from MCF-7/T clones. However, no phosphorylation was observed when conditioned media from MCF-7/WT or MCF-7/V was used. The negative control was untreated MDA-MB-453 cells. The positive control was treated with 100 μl of 100× conditioned media from MDA-MB-231 cells. MCF-7/T clone appears to be able to express and secrete at least 100 times more HRG-β2 than the MDA-MB-231 cells and is able to translate the message into an active ligand form in MCF-7 cells. *Clones A, B, C,* and *D* are equivalent to T4, T5, T6, and T7.

phorylation in MCF-7/T4 as well as in the other clones including T5, T6, T7, and T8 cells, postulating that an autocrine loop could induce autophosphorylation. Immunoprecipitations were performed using specific anti-erbB-2/3/4 antibodies, followed by Western blotting analysis with an antiphosphotyrosine antibody. A significant increase of erbB-3 (Fig. 3*A*), erbB-2, and erbB-4 (Fig. 3*B*) tyrosine phosphorylation was observed in all the transfected cells (T4, T5, T6, T7, and T8). No phosphorylation was observed in the control MCF-7/V cells. Furthermore, as an additional control, when MCF-7/V cells were tested with approximately 2 ng/ml of recombinant HRG-β2, a similar increase in erbB-2/3/4 phosphorylation was observed. These results indicate that constitutive expression of HRG-β2 isoform chronically activates the erbB-2/3/4 receptor signaling pathways.

Growth Characteristics of the MCF-7/HRG-β2-Transfected Cells

Anchorage-dependent and -independent Growth of MCF-7/HRG-β2 Cells. Estrogens and progestin modulate proliferation of hormone-responsive breast cancer cells (40). Because autophospho-

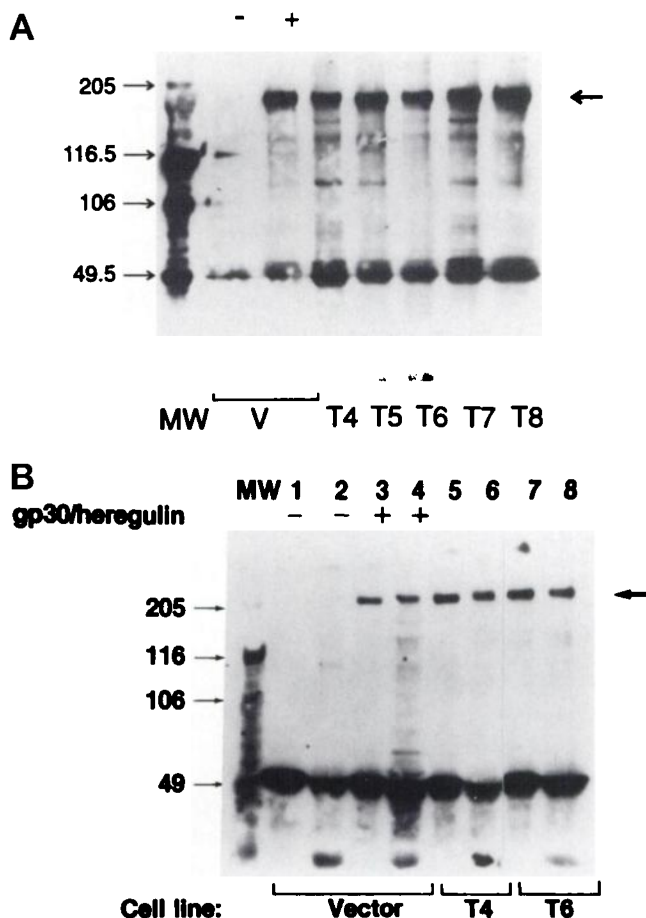

Fig. 3. Biochemical characterization of MCF-7/T cells. *A*, autophosphorylation of erbB-3 receptor in MCF-7/T cells. Cells were lysed, and immunoprecipitations were performed with specific anti-erbB-3 antibody. The precipitates were then subjected to the Western blotting with an antiphosphotyrosine antibody. MCF-7/V cells were incubated in the absence (−) or presence (+) of approximately 2 ng/ml HRG-β2 for 30 min at 37°C as control prior to immunoprecipitation. Constitutive phosphorylation of erbB-3 in MCF-7/T cells was observed. *B*, autophosphorylation of erbB-2 and erbB-4 receptors in MCF-7/T cells. Cells were lysed, and immunoprecipitations were performed with specific anti-erbB-2 (*Lanes 1, 3, 5,* and *7*) or anti-erbB-4 antibodies (*Lanes 2, 4, 6,* and *8*). The precipitates were then subjected to Western blotting with an antiphosphotyrosine antibody. MCF-7/V cells were incubated in the absence or presence of approximately 2 ng/ml HRG-β2 for 30 min at 37°C as a control prior to immunoprecipitation. Constitutive phosphorylation of erbB-2 and erbB-4 in MCF-7/T (T4 and T6) cells was observed. Similar results were obtained in other MCF-7/T cells. *Arrows* in *A* and *B*, position of the erbB receptor.

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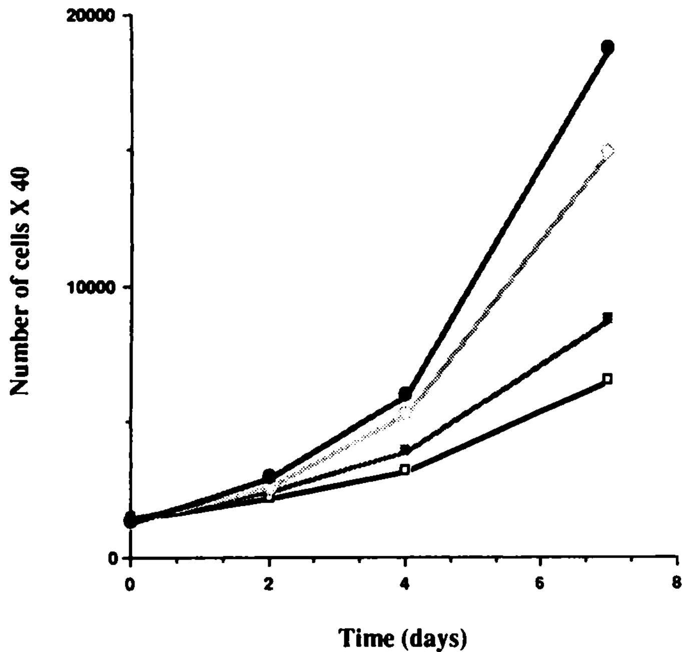

Fig. 4. Growth characteristics of MCF-7/T cells. Anchorage-dependent growth of MCF-7/T cells. Expression of HRG in MCF-7/T cells induces an estrogen-independent phenotype. Cells were plated in 24-well plates in IMEM and 10% FCS. After 24 h, medium was replaced with IMEM (phenol red-free) and supplemented with 5% CCS for 6 days. Cells were then counted at days 0, 2, 4, and 7. All samples were prepared in triplicate. SD was within 10%. The experiment was repeated three times. MCF-7/T cells proved to be estrogen independent. □, WT; ■, V; ○, T4; ●, T5.

rylation of the erbB2/3/4 receptors was observed in MCF-7/HRG-$\beta 2$ cells, we wondered how this would affect the proliferation of these cells. Anchorage-dependent growth assays were conducted. As shown in Fig. 4, MCF-7/T4 and T5 cells grow significantly faster in estrogen-depleted media than the control MCF-7/WT and MCF-7/V cells. MCF-7/T6 cells also displayed an increased growth rate (data not shown). Under estrogen-deprived conditions, the doubling time for the HRG-transfected cells is about 36–40 h, whereas the doubling time for the MCF- 7/WT and the MCF-7/V is significantly longer, about 72 h. In addition, E_2 did not induce proliferation of MCF-7/T6 cells. In contrast, growth of parental cells (MCF-7/WT and MCF-7/V) was stimulated by E_2 as expected (data not shown). These data indicate MCF-7/HRG-$\beta 2$ cells do not require estrogen for growth. To further confirm this finding, anchorage-independent assays were performed. It is well recognized that MCF-7 cells grow in an anchorage-independent fashion only in the presence of E_2. MCF-7/T6 cells exhibited an ability to grow in an anchorage-independent fashion in the absence of E_2 (Fig. 5). When these cells were treated with E_2, no further stimulation of colony formation was seen (Fig. 5). In addition, antiestrogens (OH-TAM) did not inhibit the colony formation in these MCF-7/T6 cells (Fig. 5). In the control (MCF-7/WT cells) experiment, the induction of colony formation by E_2 and inhibition of colony formation by antiestrogens were observed as expected (Fig. 5). Antiestrogenic suppression of E_2 stimulation of colony formation was also tested on the MCF-7/T6 cells. As illustrated in Fig. 5, in the presence of E_2, OH-TAM is unable to inhibit colony formation in the MCF-7/T6 cells, whereas in the parental cells OH-TAM is sufficient to inhibit the E_2-induced colony formation. Similar results were obtained using other antiestrogens, including tamoxifen and ICI compounds (data not shown). From these results, we concluded that MCF-7/T6 cells were hormone independent and resistant to antiestrogen treatments. Other clones showed similar results to those obtained with T6 cells (data not shown).

To confirm further the *in vitro* observations, we conducted *in vivo* studies using ovariectomized athymic nude mice in the absence of E_2. As shown in Table 1, the incidence of formation of large tumors was about 60% for the HRG-$\beta 2$ high-expressing cells (T2, T6, T7, and T8), and about 25% for the low and intermediate HRG-$\beta 2$-expressing cells (T4 and T5), as compared with control cells (MCF-7/WT), which do not form tumors in the absence of E_2. Supplementation of a tamoxifen pellet did not change, inhibit, or reduce tumor formation of any of the MCF-7/HRG-$\beta 2$ cells. More detailed *in vivo* studies and histology analysis will be presented in the near future. These observations demonstrate that constitutive expression of HRG-$\beta 2$ n MCF-7 cells induces tumorigenicity in MCF-7 cells.

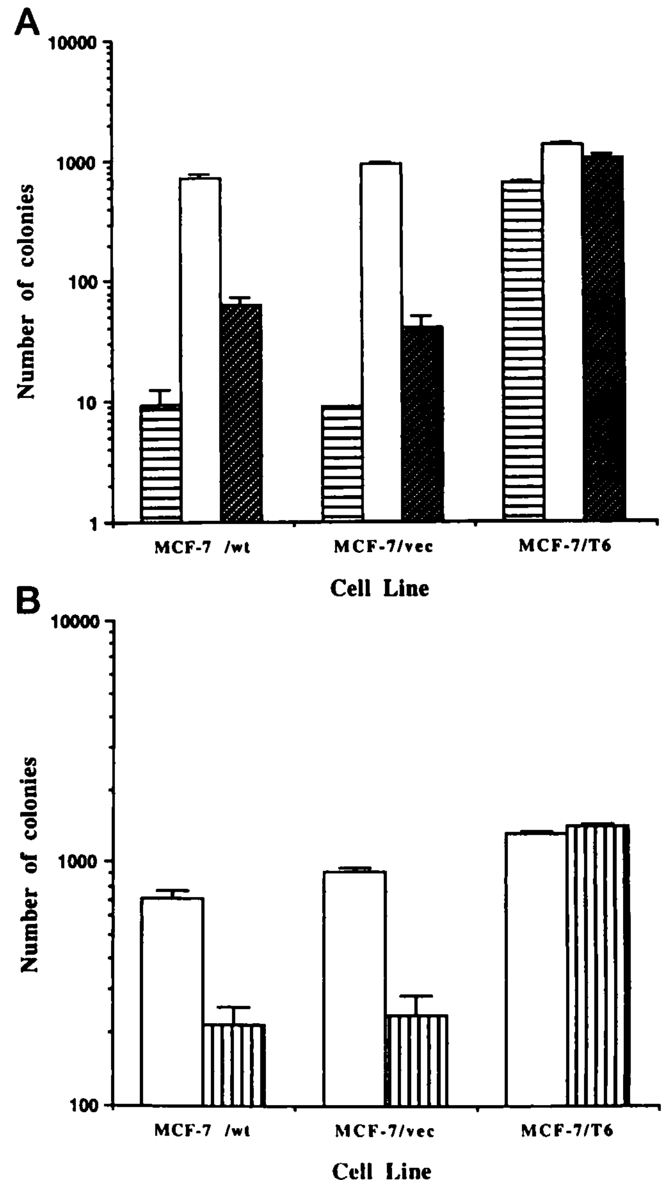

Fig. 5. Anchorage-independent growth assay. Cells (MCF-7/WT, MCF-7/V, and MCF-7/T6) were grown in IMEM (phenol red-free) and 5% CCS for 5 days. A bottom layer of 0.1 ml IMEM containing 0.6% agar and 10% CCS was prepared in 35 mm tissue culture dishes. After the bottom layer solidified, cells (10,000/dish) were added on a 0.8-ml top layer containing E_2 (10^{-9} M) (□) or OH-TAM (10^{-7} M, 0.4% Bacto Agar, and 5% CCS (■). All samples were prepared in triplicate. The cells were incubated for approximately 12 days at 37°C. Colonies larger than 60 μm were counted in a cell colony counter. ■, cells with no treatment. *A*, effect of E_2 (10^{-9} M) alone and OH-TAM (10^{-7} M) alone. *B*, effect of E_2 (10^{-9} M) alone (□) and E_2 (10^{-9} M) in the presence of OH-TAM (10^{-7} M; ▥).

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Table 1 *Tumorogenicity of MCF-7/HRG-transfected cells in athymic nude mice*

MCF-7/WT and MCF-7/T cells (5×10^6) were inoculated s.c. in ovariectomised, nude mice. Estrogen (0.7 mg/60-day release) or tamoxifen pellets (5.0 mg/60-day release) were implanted s.c.

	MCF-7/WT		MCF-7/T6	
Treatment	Incidence	Tumor size	Incidence	Tumor size
No treatment[a]	0/10	0	8/10	763 mm^3
E_2[b]	10/10	1100 mm^3	7/10	716 mm^3
Tamoxifen[c]	0/10	0	8/10	794 mm^3

[a] Measured at week 7 after inoculation (most mice were sacrificed at week 8 according to GUACAC regulations).

[b] Measured at week 6 after inoculation (most mice were sacrificed at week 7 according to GUACAC regulations).

[c] Measured at week 5 after inoculation (most mice were sacrificed at week 6 according to GUACAC regulations).

Modulation of erbB-2 and ER Expression by E_2 in MCF-7 Cells Transfected with HRG-β2

ER and PgR Expression in MCF-7/T Cells. We have shown previously that HRG modulates ER and PgR expression in MCF-7 and BT474 breast cancer cells and that ER function can be altered by HRG (38). Therefore, we examined ER/PgR expression and ER function in MCF-7/T cells. The level of ER and PgR protein was determined with an enzyme immunoassay (Abbott Laboratories). Strikingly, untreated MCF-7/T6 cells showed a substantial decrease (60%) in ER protein level, as compared with the control (MCF-7/WT and MCF-7/V) untreated cells. After serum starvation and E_2 depletion, cells were treated with 10^{-9} M E_2 for 24 h. No modulation of the ER expression level was observed when MCF-7/T6 cells were treated with 10^{-9} M E_2, as compared with a marked decrease in ER level in the estrogen-treated control wild-type MCF-7. Because *PgR* is an estrogen-regulated gene, regulation of PgR by E_2 serves as a marker for ER function. When MCF-7 cells are treated with E_2, an up regulation of PgR is seen, which implies normal ER function. In contrast, E_2 failed to regulate the levels of PgR expression in MCF-7/T6 cells as shown in Fig. 6. These results suggest a loss of ER function in the MCF-7/HRG-β2-expressing cells. We postulate that constitutive expression of HRG-β2 not only down regulates ER but also blocks E_2-mediated activation of ER, which in turn abolishes the induction of PgR by E_2. This blockage of ER function is probably one of the mechanisms by which MCF-7/T6 cells acquired an E_2-independent and anti-estrogen-resistant phenotype.

Cross-Regulation between ER and erbB-2 in MCF-7/T Cells. E_2 is a modulator of erbB-2 expression (37). As demonstrated above, ER expression and function were altered in MCF-7/HRG transfectants. Therefore, it is important to study the effect of estrogen on *erbB-2* expression, because it is an estrogen-regulated gene in MCF-7 cells. The effect of estrogen on erbB-2 expression in MCF-7/T cells was determined by FACS (FACStar). As predicted, E_2 did not affect the expression of erbB-2 in the T4 and T6 cells. In contrast, the control cells showed about a 35% reduction in erbB-2 expression after treatment with E_2 (Fig. 7). erbB-2 expression was also down regulated by E_2 in MCF-7/T5, the clone expressing the lowest level of HRG-β2, however, to a lesser extent. The inability of E_2 to down regulate erbB-2 correlates with higher levels of HRG-β2 expression. These results indicate further that the ER function in T4 and T6 cells is altered and blocked.

Mechanism Responsible for Loss of ER Function. One possible mechanism for loss of ER function is that ER is transcriptionally inactive. To determine whether ER transcriptional activity was altered in MCF-7/T cells, we transiently transfected the cells with a reporter plasmid containing an ERE upstream of a *CAT* reporter gene (provided kindly by Dr. D. El-Ashry). We then assessed the effect of E_2 on ER transcriptional activity in E_2-treated and untreated cells. As shown in Fig. 8, CAT activity in the presence of E_2 was decreased significantly in the HRG-β2-transfected (T4) cells that expressed intermediate levels of HRG-β2, whereas the CAT activity in MCF-7/T6 cells (high HRG-β2-expressing cells) was undetectable. In contrast, the control (MCF-7/WT and MCF-7/V) cells as well as MCF-7/T5 (lowest HRG-β2-expressing cells) exhibited high levels of CAT activity after E_2 treatment, indicating that the ER was still active in these cells. These results demonstrate that the transcription of ER is inactivated partially in the cells expressing intermediate levels of HRG-β2 (T4). These cells are independent of E_2 for growth, but they still respond to E_2. ER transcription is inactivated completely in cells that express high levels of HRG-β2 (T6). These results suggest that HRG-β2 promotes loss of ER function by altering ER transcription. Furthermore, it appears that, in these cells, the degree of ER transcriptional inactivation is dependent on the level of HRG-β2 expression. Our data indicate that low expression of HRG-β2 is not sufficient to completely inactivate the ER pathway, in contrast to the cells that express high levels of HRG-β2 in which the ER pathway was completely blocked.

DISCUSSION

In the last decade, numerous studies have indicated that growth factors and their receptors play an important role in cancer development and progression. The members of the EGF receptor family (EGFR, erbB-2/neu, erbB-3, and erbB-4) are often found to be overexpressed in various human cancers (1, 12, 14, 15, 41). HRG directly activates erbB-3 and erbB-4 receptors and indirectly activates the erbB-2 receptor (33, 34). HRG has been postulated to induce heterodimerization of EGFR family members, elucidating a common signaling pathway (34, 42).

More than 60% of breast cancer tumors express ER. Hormonal therapy is one of the most common therapies for breast cancer patients. The expression of ER is used to predict which patients will benefit from hormone therapy. The loss of ER expression is associated strongly with poor prognosis in breast cancer patients (43). Clinically, $ER^+/erbB\text{-}2^+$ tumors are less responsive to endocrine therapy than $ER^+/erbB\text{-}2^-$ tumors.

An inverse correlation between the erbB-2 and ER signaling pathways has been demonstrated in breast cancer cells (44, 45). E_2 appears to induce down regulation of erbB-2 in MCF-7 cells (46). Therefore,

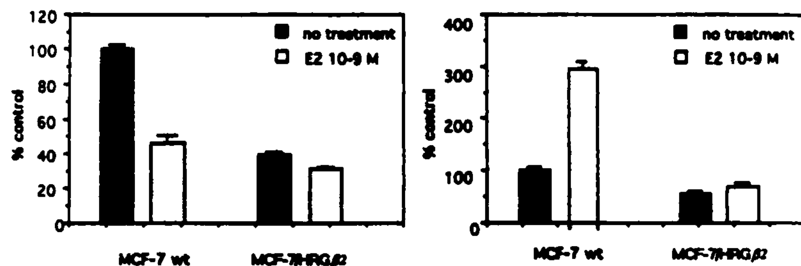

Fig. 6. Modulation of erbB-2 and ER expression by E_2 in MCF-7/T cells. ER (*left*) and PgR (*right*) expression in MCF-7/T cells. Cells were deprived of E_2 for 6 days. ER and PgR levels were measured by an enzyme immunoassay performed according to the manufacturer's protocol (Abbott Laboratories). Down regulation of ER and up regulation of PgR were abolished in the MCF-7/T cells.

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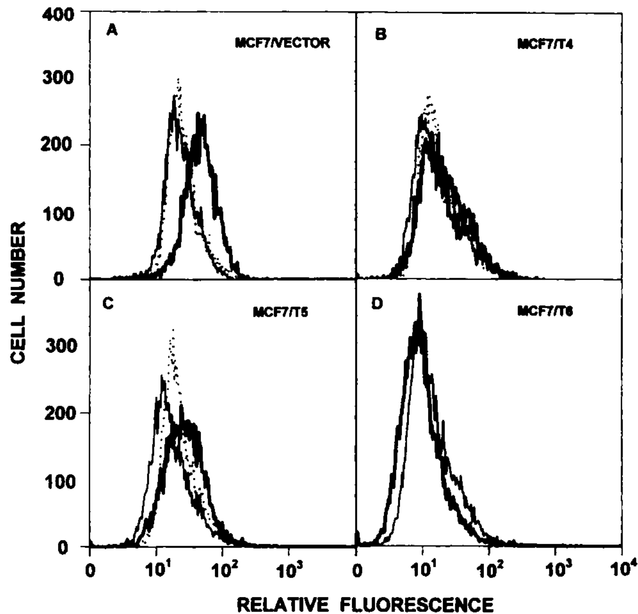

Fig. 7. Cross-regulation between ER and erbB-2 in MCF-7/T cells. Cells were deprived of E_2 for 6 days. Cells were harvested after treatment with or without E_2 (10^{-9} M) for 24 h and stained with anti-erbB-2 monoclonal antibody no. 94 (a gift from Dr. Richter King, GUMC). The erbB-2 levels were measured quantitatively by flow cytometry. *Solid bold-faced lines*, $p185^{erbB-2}$ expression under the E_2-deprived condition. *Solid light-faced lines*, $p185^{erbB-2}$ expression from the cells treated with E_2 (10^{-9} M). *Dotted lines*, $p185^{erbB-2}$ expression from the cells growing in IMEM and 10% FCS. Down regulation of $p185^{erbB-2}$ expression was blocked in MCF-7/T cells.

regulation of erbB-2 by E_2 is another avenue that can be used to determine ER function.

To study the involvement of HRG in breast cancer tumor progression, we first attempted to develop *in vitro* and *in vivo* models. We transfected HRG-β2 cDNA into MCF-7 cells, a hormone-dependent breast cancer cell line, with the assumption that this model could be relevant to the progression of tumors from a hormone-sensitive to a hormone-insensitive phenotype.

It is probable that several cellular and molecular alterations are required to change the hormone-dependent phenotype of breast cancer cells. One possible pathway is the change in cell morphology and extracellular matrix components. In the HRG-β2-transfected cells, we observed various degrees of morphological changes (Fig. 1). The morphological changes appear to correlate with the relative levels of HRG-β2 expression. Highest HRG-β2-expressing cells (T6) displayed the most extreme changes. These cells appear larger, with larger nuclei and increased cytoplasm compared to the parental cells (MCF-7/WT) and/or transfectants with vector alone (MCF-7/V). Modulation of the extracellular matrix and/or cytoskeleton components of these MCF-7/T cells is currently under investigation in our laboratory.

We have shown previously that HRG modulates breast cancer cell growth as well as ER expression and function (38). In the present study, we determined the various levels of HRG expression that are required to achieve different biological end points. More than 20 clones were characterized and graded according to their HRG-β2 expression. The level of HRG-β2 expression in T4 and T5 was lower than in T6, T2, T7, and T8. These clones expressed about 100 times more HRG than MDA-MB-231 cells, which are the original source of HRG-β2. HRG-β2 produced by MCF-7/T cells is capable of inducing tyrosine phosphorylation of a p185 protein expressed in MDA-MB-453 cells (Fig. 2*C*).

To address the potential biological role of HRG in breast cancer, anchorage-dependent and -independent growth assays were performed. Our studies show that MCF-7/HRG-β2 cells lose the "normal" estrogen requirements of ER^+ breast cancer cells and progress to a more aggressive estrogen-independent phenotype. All of the clones expressing high levels of HRG-β2 (T2, T6, T7, and T8) were capable of developing colonies in an anchorage-independent assay without E_2 supplementation. However, although the anchorage-independent growth of the clones expressing low and intermediate levels of HRG-β2 (T5 and T4, respectively) was independent of E_2 supplementation, these cells were still partially responsive to E_2 (data not shown). The E_2-independent phenotype does not appear to be achieved by a short-term exogenous treatment with HRG. We can therefore postulate that constitutive expression of HRG-β2 is necessary to maintain activation of the erbB receptor pathway to bypass the ER pathway. Given the different levels of E_2 responsiveness of the MCF-7/HRG-β2 clones, it was intriguing to determine the response of MCF-7/HRG-β2 clones to several antiestrogens. It is well established that treatment of ER^+ cells with antiestrogens can inhibit the induction of anchorage-independent growth by E_2 (47). The most commonly used antiestrogens include tamoxifen, 4-OH-tamoxifen, and ICI compounds. Interestingly, we obtained a distinct response to E_2 and antiestrogens by the different clones. The clones that expressed the highest levels of HRG-β2 (T6, T2, T7, and T8) were resistant to antiestrogens (Fig. 5) as well as independent and insensitive to E_2. The clones expressing low and intermediate levels of HRG-β2 (T5 and T4) were hormone independent yet hormone sensitive and partially insensitive to antiestrogens. In contrast, as expected, the control cells, MCF-7/V and MCF-7/WT, were dependent on E_2 for growth and sensitive to antiestrogens.

To further understand the impact of our findings and the magnitude of the effect of HRG in breast cancer tumor progression, we conducted *in vivo* studies. Ovariectomized athymic nude mice were inoculated with either MCF-7/T6 cells (5×10^6), MCF-7/WT, or MCF- 7/V cells in the presence or absence of estrogen, tamoxifen, or both. Our preliminary *in vivo* data demonstrated that the MCF-7/T6 cells, in contrast to the MCF-7/WT or the MCF-7/V, are capable of forming large tumors in the absence of estrogen. Furthermore, tamoxifen does not inhibit or block tumor formation of these MCF-7/T6 cells. Our results suggest that HRG-β2 potentiates tumorigenicity in an E_2-independent fashion. These data prompted us to postulate that this growth factor plays a critical role in the "complete transformation" of breast cancer cells. Clinically, a subset of breast cancer

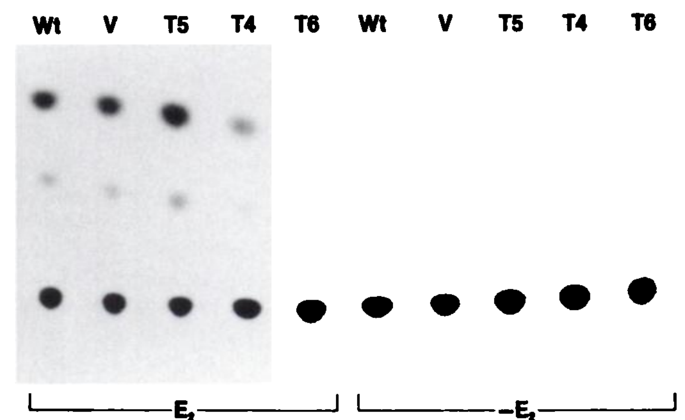

Fig. 8. Mechanism responsible for loss of ER function. An ERE-CAT construct was transfected transiently into MCF-7/WT and MCF-7/V as well as MCF-7/T cells and cotransfected with the *β-galactosidase* gene as a transfection efficiency control. These transfections were performed in estrogen-deprived medium with (*E_2*) or without (*$-E_2$*) 10 nM E_2. The CAT assays were carried out as described in "Materials and Methods." The relative CAT activities (*Lanes 1-5*) are 100, 98, 113, 62, and 12. The experiments were done three times; SD was within 10%. The transcriptional activity of ER in MCF-7/T cells was partially (T4) or almost completely abolished (T6).

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tumors is presumed to be initially ER-positive but to become ER-negative through malignant progression. These ER^- tumors no longer respond to hormonal therapy. The *in vitro* model system that we have generated appears to mimic the course of malignant progression of breast tumors in humans. The mechanism by which this event occurs is currently under investigation.

We demonstrated that it is possible to define a model that bypasses the "normal" estrogen requirements of breast cancer cells. We suggest that through the activation of an alternative pathway, it is possible to deactivate the "normal" ER pathway. It has been shown that activation of the erbB signaling pathway plays a role in breast cancer. We hypothesize that constitutive activation of the erbB-2/3/4 pathway leads to a deactivation of the ER pathway. Therefore, we predict that those cells will behave in a more aggressive, hormone-independent manner.

To confirm our hypothesis, studies were designed to determine the expression and functions of both the erbB-2/3/4 receptor and the ER pathways (Figs. 3, 6, 7, and 8). Our data indicate that MCF-7/HRG-β2 cells exhibit high levels of tyrosine phosphorylated erbB-2/3/4 receptors. This activation may be mediated through an autocrine loop. These experiments lead us to conclude that constitutive expression of HRG indeed activates the erbB-2/3/4 pathway.

Recently, it has been reported that HRG activates the erbB-3 and erbB-4 receptors in a direct fashion, whereas activation of the erbB-2 receptor is indirect (32, 33). Activation of erbB-2 is achieved presumably by transphosphorylation and/or through the induction of erbB-2/3/4 receptor heterodimerization (34). The consequent constitutive phosphorylation of tyrosine residues is probably one important element in human breast carcinoma progression.

E_2 causes a 50% decrease of the level of both erbB-2 mRNA and protein expression in MCF-7, MDA-MB-361, and BT474 human ER^+ breast cancer cells (38, 48). E_2 activates ER as well as down regulates the cellular erbB-2 content (37). In contrast, tamoxifen up regulates erbB-2 oncoprotein expression in ER-positive but not in ER-negative breast cancer cells (43, 44, 46). Expression of the *erbB-2* oncogene is under negative transcriptional control of the E_2-activated ER (45). Interestingly, we have shown that exogenous treatments with HRG have an antiestrogenic effect on erbB-2/ER^+ breast cancer cells (BT474 and MCF-7 cells). Under these conditions, E_2 is no longer able to modulate the expression of erbB-2 (38). Consequently, we tested the ability of E_2 to modulate the expression of erbB-2 in the T4 and T6 cells. Our results indicate that E_2 does not have any effect on ER or erbB-2 protein expression in the T4 and T6 cells. In addition, expression of HRG abolished the E_2-mediated up regulation of the PgR (Fig. 6). In contrast, E_2 down regulates the expression of erbB-2 (Fig. 7) and up regulates PgR (Fig. 6) in MCF-7/V and wild-type cells. From these data, we concluded that ER in the MCF-7/T6 cells is no longer active.

It is reasonable to postulate that the MCF-7/T6 cells lost their estrogenic responsiveness by expressing an "inactive ER." We performed transient transfections using an ERE-CAT construct to determine whether the ER is transcriptionally active in MCF-7/T cells. Our results indicate that ER is transcriptionally inactive in cells expressing high levels of HRG-β2 (MCF-7/T6). In contrast, partial ER activity was determined in cells expressing intermediate levels of HRG-β2 (MCF-7/T4), and no change in activity as compared with the parental MCF-7 cells was determined in cells expressing low levels of HRG-β2 (MCF-7/T5). Although T4 and T5 cells are not dependent on E_2 for growth, E_2 can still activate the ER. In contrast, the MCF-7 cells expressing high levels of HRG-β2 displayed a completely E_2-independent and tamoxifen-resistant phenotype, and no activation of ER was seen. Therefore, we postulate that this may be one of the mechanisms by which cancer cells and tumors become E_2-independent and tamoxifen-resistant. However, the exact mechanism for the loss of ER function in the MCF-7/T6 cells deserves further investigation.

HRG has different isoforms that result from alternative splicing. Biological differences among individual HRG isoforms and their tissue distribution are not known. It is plausible that different isoforms may have different affinities for homo- and heterodimers of EGF receptor family members and result in activation of different signaling pathways. Here, we have demonstrated that signaling through HRG-β2 results in suppression of the ER signaling pathway in contrast to activation of the pathway by HRG-β1, as observed by Pietras *et al.* (49). However, it is still possible that these differences could be due to different levels of HRG expression in the MCF-7 cell lines we have used, as complete inactivation of ER transcription was only observed in clones expressing high levels of HRG-β2. Our results demonstrate consistently that a high level of HRG-β2 expression correlates with their E_2-independent phenotype.

Our present results indicate that HRG-β2 plays an important role in breast cancer progression through activation of the erbB receptor family signaling pathways. The loss of estrogen responsiveness is critical and prominent in a large number of human breast cancers. Controlling the cell homeostasis may be an essential step in the malignant progression of a subset of breast carcinomas. The MCF-7/HRG-β2 cells are an important model for investigating various mechanisms of breast cancer cell progression from a hormone-sensitive to a hormone-resistant phenotype, and an excellent system for anticancer drug design.

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