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Heregulin negatively regulates transcription of ErbB2/3 receptors via an AKT-mediated pathway

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Abstract

Despite the importance of the ErbB2/3 heterodimer in breast cancer progression, the negative regulation of these receptors is still poorly understood. We demonstrate here for the first time that the ErbB3/4 ligand Heregulin (HRG) reduced both ErbB2 and ErbB3 mRNA and protein levels in human breast cancer cell lines. In contrast, EGFR levels were unaffected by HRG treatment. The effect was rapid with a decline in steady state mRNA levels first noted two hours after HRG treatment. HRG reduced the rate of transcription of ErbB2 and ErbB3 mRNA, but did not affect ErbB2 or ErbB3 mRNA stability. To test if ErbB2 kinase activity was required for the HRG-induced downregulation, we treated cells with the ErbB2/EGFR inhibitor lapatinib. Lapatinib diminished the HRG-induced decrease in ErbB2 and ErbB3 mRNA and protein, suggesting that the kinase activity of EGFR/ErbB2 is involved in the HRG-induced receptor down-regulation. Further, HRG-mediated decreases in ErbB2/3 mRNA transcription are reversed by inhibiting the AKT but not MAPK pathway. To examine the functional consequences of HRG-mediated decreases in ErbB receptor levels, we performed cell cycle analysis. HRG blocked cell cycle progression and lapatinib reversed this block. Our findings support a role for HRG in the negative regulation of ErbB expression and suggest that inhibition of ErbB2/3 signaling by ErbB2 directed therapies may interfere with this process.

Keywords

ErbB2; ErbB3; HRG; HER2; HER3; Breast Cancer

Introduction

The ErbB receptor tyrosine kinase family is comprised of four members: EGF receptor (ErbB1), ErbB2, ErbB3 and ErbB4 (Yarden and Sliwkowski, 2001). In spite of having significant structural homology, these receptors show ligand specificity. For example, Heregulin binds to ErbB3 and 4 and does not bind to EGFR (Breuleux, 2007). ErbB2 is an orphan receptor that lacks a known ligand. It is the preferred heterodimerization partner of the other ErbB receptors (Graus-Porta et al., 1997). ErbB2 is activated by homodimerization

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due to overexpression or heterodimerization due to ligand stimulation of another ErbB receptor. The ErbB3 receptor has extremely weak kinase activity (Kim et al., 1998; Sierke et al., 1997) and transmits growth factor signals by heterodimerization with other ErbB receptors. The ligand- induced ErbB2-ErbB3 complex phosphorylates downstream substrates, resulting in activation of multiple pathways, including MAPK and PI3K-AKT, that regulate cell proliferation and cell survival (Citri et al., 2003). The ErbB2 /ErbB3 heterodimer forms the strongest signaling dimer in this family and drives breast cancer cell growth (Alimandi et al., 1995; Zhang et al., 1996).

Therapies targeting ErbB2, such as the humanized monoclonal antibody Trastuzumab (Carter et al., 1992; Romond et al., 2005; Slamon et al., 2001) and the EGFR/ErbB2 receptor tyrosine kinase inhibitor lapatinib (Geyer et al., 2006; Rusnak et al., 2001), are currently in clinical use. Trastuzumab inhibits growth of ErbB2 positive cells in part by down-regulating surface ErbB2 receptors (Junttila et al., 2009) and inducing antibody dependent cytotoxicity (Hudis, 2007). Lapatinib inhibits EGFR and ErbB2 kinase activity, decreasing phosphorylation of substrates of these receptors and reducing downstream signaling (Xia et al., 2002; Zhou et al., 2004). ErbB-directed therapies have improved the outcome of patients with ErbB2 positive tumors. However, a significant number of patients do not initially benefit or become resistant after chronic exposure to these drugs (Esteva et al., 2010; Nahta et al., 2006). In many cases, resistance is caused by increased levels and activity of ErbB2 or ErbB3. For example, lapatinib increases ErbB2 protein levels via a post-transcriptional mechanism (Amin et al., 2010). Inhibition of ErbB2 kinase activity by lapatinib or of AKT by AKT inhibitors (Chakrabarty et al., 2012) results in stimulation of ErbB3 transcription (Garrett et al., 2011; Myatt and Lam, 2007).

As inhibition of ErbB2 kinase activity increased ErbB3 and ErbB2 levels, we questioned whether lapatinib treatment might be disrupting a normal mechanism of ErbB negative regulation. Ligand-induced negative regulation of receptor levels is a very common feature of nuclear receptor transcription factors. For instance, transcription of the nuclear receptors ER, PR and GR is negatively regulated by their cognate ligands (Geng and Vedeckis, 2011; Hatsumi and Yamamuro, 2006). In the current study, we examined the negative regulation of ErbB2 and ErbB3 by the ErbB3 ligand HRG. We found that HRG down-regulated ErbB2 and ErbB3 both at the mRNA and protein levels. HRG decreased the rate of transcription of these genes. ErbB2 kinase inhibitor treatment rescued the HRG mediated ErbB2 and ErbB3 down- regulation. The AKT, but not the MAPK pathway, was primarily involved in the HRG induced ErbB2 and ErbB3 negative regulation.

Materials and Methods

Reagents and Antibodies—Heregulin $\beta 1$ (HRG $\beta 1$) was obtained from R & D Systems Inc. (Minneapolis, MN). Lapatinib was purchased from LC Laboratories (Woburn, MA), GSK690693 from Selleckchem (Houston, TX) and U0126 from Cell Signaling Technology (Danvers, MA). Antibodies were obtained from the following sources: rabbit anti-ErbB1, rabbit anti-ErbB2 (C-18), rabbit anti-ErbB3 (C-17) and mouse anti-phospho p70S6K (Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-GAPDH (Calbiochem, Billerica, MA); rabbit monoclonal anti-phospho ErbB2 (Tyr1221/1222), rabbit monoclonal anti-phospho

ErbB3 (Tyr1289), mouse anti- ERK, rabbit anti- phospho p44/42 ERK (T202/Y204) and rabbit anti- phospho p70 S6K (Thr389) (Cell Signaling).

Cell Culture—BT474 cells were a gift from Dr. Stuart Martin, University of Maryland School of Medicine. AU565 cells were obtained from the American Type Culture Collection (Manassas, VA). BT474 and AU565 cell lines were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air in RPMI 1640 (Biofluids, Rockville, MD) and 10% FBS (Sigma, St. Louis, MO). LTLT-Ca cells were a gift from Dr. Angela Brodie, University of Maryland School of Medicine and maintained as described (Jelovac et al., 2005).

Cytosol extraction and Western Blot Analysis—Cells (200,000 per well in 6-well plates) were washed with PBS twice and collected by scraping in PBS, followed by spinning at 2000g for 5 min and were stored at -80°C until use. Cells were lysed in RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% Sodium Deoxycholate, 0.1% SDS) containing protease inhibitor cocktail (Complete™, Roche, Indianapolis, IN) and Phosphatase inhibitor Cocktails 1 and 2 (Calbiochem). Lysates were spun at 16,000g at 4°C for 20 min. Protein was estimated using a Bradford reagent (BioRad, Richmond CA). Equal amounts of protein (10µg) were run on 4-12% denaturing NuPAGE gels (Invitrogen, Carlsbad, CA) followed by Western blotting. Western blotting was performed as previously described (Ghosh et al., 2013b). Images were quantified using VisionWorksLS analysis software (UVP LLC, Upland, CA).

RNA isolation and qRT-PCR—Cells were seeded into 35 mm plates at 200,000 cells per plate and harvested in 1 ml Trizol reagent (Invitrogen) at the times indicated. Total RNA was isolated according to the manufacturer's instructions. First-strand cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen). Relative levels of target mRNAs were quantitated using PerfeCTa™ SYBR® Green SuperMix for IQ™ (Quanta Biosciences, Gaithersburg, MD) on an iCycler IQ® System (BioRad). The following primers were used: **ErbB1** (forward 5'- GGA GAA CTG CCA GAA ACT GAC C - 3' reverse 5'- GCC TGC AGC ACA CTG GTT G - 3'), **ErbB2** (forward 5' – AAA CCT GGA ACT CAC CTA - 3', reverse 5' - ATA GTT GTC CTC AAA GAG C - 3'), **ErbB3** (forward 5'-GTC TGT GTG ACC CAC TGC AAC T - 3', reverse 5' - TTG GCA TTC CGG GTG GCA G - 3'), **ErbB4** (forward 5' - GGC TGC TGA GTT TTC AAG GAT G - 3', reverse 5' - TCC CAG TCC AAA TGA CAG CAA GT - 3'). **GAPDH** (forward 5'- CCA CCC ATG GCA AAT TCC - 3', reverse 5'- TCG CTC CTG GAA GAT GGT G - 3') was measured as an internal control.

mRNA stability assays—Cells were seeded in 35 mm plates (200,000 cells/ plate). The following day, cells were serum-starved overnight, then incubated with or without HRG (50 ng/ml) for 24 hours, followed by addition of Actinomycin D (Act D) (5 µg/ml). Cells were harvested at sequential times (0, 2, 4, 6 and 8 h). Total RNA was extracted with Trizol as per the manufacturer's protocol. First-strand cDNA was synthesized using SuperScript III reverse transcriptase, followed by qPCR with ErbB2, ErbB3 and GAPDH primers as described above. Data from the Act D assays were processed using Prism 3.03 software to

calculate the time required for each mRNA to reach one-half of its initial abundance and p-value.

Click-iT nascent RNA capture assay—LTLT-Ca cells were seeded in 60 mm plates (500,000 cells per plate). The following day, cells were serum-starved in IMEM containing 0.5% charcoal–dextran stripped FBS (Sigma) for 24 h. Cells were treated with or without HRG (50ng/ml) for 0, 1, 2 and 4 hr. A pulse of Ethenyl Uridine (0.5 μ M) was added 1 h before harvesting cells in 1 ml Trizol. Total RNA was extracted as per the manufacturer's protocol (Trizol, Invitrogen) and 5 μ g RNA from each sample was biotinylated for 30 min at RT following the manufacturer's instructions (Click-iT® Nascent RNA Capture Kit, Invitrogen). RNA was precipitated by adding chilled ethanol in the presence of ultra-pure glycogen and 7.5 M ammonium acetate at -70°C overnight, followed by washing with 70% ethanol twice and reconstituted in DEPC water. Biotinylated RNA (1 μ g) was heated in Click-iT reaction binding buffer containing RNaseOUT at 68-70°C 5 min and incubated with Dynabeads (50 μ l) for 30 min at room temperature with intermittent vortexing. Beads immobilized on a DynaMagnet were washed five times with Click- iT reaction wash buffers 1 and 2. Beads were resuspended in 10.5 μ l of Click-iT reaction wash buffer 2. First-strand cDNA was synthesized by SuperScript III reverse transcriptase using RNA immobilized on the resuspended beads, followed by qRT-PCR in duplicate with ErbB2, ErbB3 and GAPDH primers as described above.

Cell Cycle analysis—Cells were serum starved for 24 h followed by treatment with HRG and/or lapatinib. At harvest, cells were trypsinized and washed twice with PBS. Cells were resuspended, fixed in cold 70% ethanol, and stained in hypotonic propidium iodide (PI) solution (0.05 mg/ml in PBS with 200 μ g/ml DNase-free RNase) for 2 hours on ice. Samples were excited at 488 nm and PI fluorescence emission was measured with a 575 band pass filter in a FACScan. Cell cycle distribution analysis on 10,000 cells per group was performed using FlowJo. .

Statistical Analysis—Data were analyzed using a two-tailed Students t-test. Differences with a $p < 0.05$ were deemed significant.

Results

HRG decreases ErbB2 and ErbB3 protein and mRNA levels

Although overexpression of the ErbB2/3 heterodimer promotes breast cancer progression, the negative regulation of these receptors is incompletely understood. We have previously shown that ligands regulate nuclear hormone receptor levels through effects on transcription, resulting in altered sensitivity of target tissues to hormones (Awasthi et al., 2007; Geng and Vedeckis, 2011; Hatsumi and Yamamuro, 2006). To establish if a similar mechanism exists to control ErbB receptor levels, we first determined the effect of HRG β 1 on ErbB2 and ErbB3 protein and mRNA levels in AU565, BT474 and LTLT-Ca cell lines. AU565 cells are ER and PR negative with high expression of ErbB2. BT474 cells express ER, PR and high levels of ErbB2. LTLT-Ca cells were derived from aromatase transfected MCF-7 cells made tamoxifen resistant by passage in mice in the presence of letrozole (Sabnis et al.,

2009). They are ER and PR positive and have higher expression of ErbB2 than the parental MCF-7 cells. We observed that HRG decreased ErbB2 protein in AU565 and LTLT-Ca cells and ErbB3 protein levels in all three cell lines 24 h after treatment (Figure 1A). ErbB1 levels were not decreased. In keeping with this finding, ErbB2 mRNA was significantly decreased in all three cell lines at the 24 hour time point. ErbB3 mRNA was decreased in BT474 and LTLT-Ca cell lines. In contrast, the level of ErbB3 mRNA remained unchanged in AU565 cells 24 hours after treatment (Figure 1B). ErbB1 mRNA levels were unaffected by HRG treatment. In keeping with previously published data (Mill et al., 2011), we were unable to detect either ErbB4 protein or mRNA in any of these cell lines (data not shown). In addition, treatment with the EGFR ligand EGF had no effect on ErbB2/3 mRNA or protein levels in any of the cell lines tested 24 hours after treatment (Fig. S1A, B). As expected, EGF decreased EGFR mRNA and protein levels (King et al., 1988; King and Sartorelli, 1986).

We next studied the kinetics of the HRG-induced down regulation. We measured ErbB2/ ErbB3 protein and mRNA levels at different time points after HRG addition. Both ErbB2 and ErbB3 protein levels decreased starting 2 h after HRG treatment in LTLT-Ca cells (Figure 2 A). Levels of ErbB2 or ErbB3 were not changed just by incubating cells an additional 6 or 24 hours in the absence of HRG (data not shown). In AU565 cells, protein levels of ErbB2 and ErbB3 were decreased starting at 6 hours after HRG treatment (Fig 2B). Protein levels of ErbB2 and ErbB3 were not changed in untreated cells at these time points (Fig. S2). We next examined the kinetics of HRG-induced changes in mRNA levels. ErbB2 and ErbB3 mRNA levels in LTLT-Ca cells were significantly ($p<0.05$) decreased starting 2 hours after HRG treatment (Figure 2C, upper panel). In AU565 cells, a decline in ErbB2 mRNA was noted one hour after HRG treatment with a significant decline ($p=0.01$) 4 hours after HRG treatment. Levels continued to decline until 24 hours. In contrast, although ErbB3 mRNA levels were significantly decreased two hours after HRG treatment ($p<0.05$) and were 40% of control values after 6 hours of treatment ($p=0.02$), mRNA levels of ErbB3 rose back to control levels at 24 hours (Figure 2C, lower panel). This observation is consistent with the finding shown in Figure 1B.

Heregulin $\beta 1$ does not decrease mRNA stability

To determine the mechanism of the HRG- induced down regulation of ErbB2 and ErbB3 steady state mRNA levels, we examined ErbB2 and ErbB3 mRNA stability in LTLT-Ca and ErbB2 stability in AU565 cells using Actinomycin D. We found that the half-life of ErbB2 mRNA was approximately 8 hours in both LTLT-Ca and AU565 cells, in keeping with previously published data (Pasleau et al., 1993). HRG did not significantly alter the stability of ErbB2 mRNA (Figure 3A, B). ErbB3 mRNA half- life in LTLT-Ca cells in the absence of HRG was 4.4 hours. HRG had no significant effect on the stability of ErbB3 mRNA (Figure 3C). Thus, changes in mRNA stability could not account for the decreased steady state levels of ErbB2 and ErbB3 mRNA after HRG treatment.

Heregulin $\beta 1$ decreases the rate of transcription of ErbB2 and ErbB3 mRNA

As we found that HRG did not affect ErbB2 or ErbB3 mRNA stability, we next examined the effect of HRG on ErbB2 and ErbB3 mRNA synthesis using Click-iT technology. This

technique measures the rate of incorporation of a Uridine analogue Ethenyl uridine (ENU) into newly transcribed RNA, which is then conjugated to biotin and purified. The amount of newly synthesized RNA was estimated using the Click-it nascent mRNA capture assay. As shown in Figure 4A, we found that HRG significantly decreased the accumulation of newly synthesized ErbB2 mRNA transcripts in LTLT-Ca cells. A decrease was observed 2 hours after treatment. Four hours after HRG treatment, ENU incorporation was decreased by 40%. Incorporation of ENU into ErbB3 mRNA was decreased 1 h after HRG addition and decreased to 80% of control at 4 hours (Figure 4B). These findings suggest that the inhibition of ErbB2 and ErbB3 mRNA synthesis by HRG was the major driver of the observed decrease in ErbB2 and ErbB3 steady state mRNA levels.

To further determine a role for the endogenous ErbB2 promoter in the changes in ErbB2 protein levels, we tested the effect of HRG on ErbB2 protein in Her2-18 cells. These MCF-7 derived cells overexpress ErbB2 due to transfection with the coding sequence for *ERBB2* under the control of a CMV promoter. HRG did not affect the expression of ErbB2 protein (Figure 4C), suggesting that HRG may affect the activity of the endogenous *ERBB2* promoter.

An ErbB1/ErbB2 kinase inhibitor rescues HRG mediated ErbB2/3 down-regulation

We next examined signaling pathways that might be involved in HRG-mediated ErbB2/3 downregulation. As HRG induces ErbB2/3 dimerization and activation of ErbB2 kinase activity, we examined the effect of reduction of ErbB2 kinase activity on ErbB2 and ErbB3 levels. LTLT-Ca cells were treated with the ErbB1/ErbB2 dual kinase inhibitor lapatinib for 24 h followed by HRG addition. Cells were harvested at time points up to 24 hours after HRG treatment. The effectiveness of both the HRG and lapatinib treatment was monitored by measuring the phosphorylation of ErbB3. As expected, HRG induced phosphorylation of ErbB3 after 30 min and this phosphorylation diminished gradually over time (Figure 5A, left panel 3). Lapatinib inhibited the HRG- induced phosphorylation of ErbB3 (Figure 5A, right panel 3). We found that HRG reduced ErbB2 protein levels at 6 and 24h after treatment. Lapatinib abrogated the HRG- induced decrease in ErbB2 protein (Figure 5A, top panel). In fact, lapatinib increased ErbB2 levels even in the absence of HRG in agreement with previous findings (Amin et al., 2010). Similarly, lapatinib by itself increased ErbB3 protein as previously reported (Garrett et al., 2011). Lapatinib treatment abrogated the HRG-induced decrease of ErbB3 protein (Figure 5A, second panel). Similar results for ErbB2 protein were observed in AU565 cells (Supporting information, Figure S3).

We next examined the ability of lapatinib to affect the HRG-induced decreases in ErbB2 and ErbB3 mRNA in LTLT-Ca cells. As expected (Figure 2), ErbB2 mRNA was decreased starting 4 hours after HRG treatment relative to untreated controls. Lapatinib significantly ($p < 0.05$) reduced the HRG-induced decrease at 24 h. Lapatinib alone did not increase levels of ErbB2 mRNA (Figure 5B, C). In contrast, lapatinib increased levels of ErbB3 mRNA in the absence of HRG in agreement with previously published work (Amin et al., 2010; Garrett et al., 2011). Lapatinib abrogated the HRG-induced decrease of ErbB3 mRNA at 6 ($p = 0.02$) and 24 hours ($p < 0.05$) (Figure 5 B,C). These findings suggest that ErbB2 kinase activity is an important component of the HRG- induced decrease in ErbB2/3 mRNA.

AKT is involved in HRG- induced ErbB2/3 downregulation

We next sought to delineate the pathway downstream of ErbB2 responsible for HRG-mediated ErbB2/3 negative regulation. ErbB2 signals through both the PI3K-AKT and MAPK pathways. We serum-starved LTLT-Ca cells in the presence or absence of the MEK inhibitor U0126 or the AKT inhibitor GSK690693 for 24 hours. Cells were then treated with HRG for up to 24 hours. The MEK inhibitor U0126 at a 10 μ M concentration reduced both basal (Aksamitiene et al., 2010; Jelovac et al., 2005) and HRG-induced ERK phosphorylation as expected (Figure 6A, panels 3 and 4). Inhibition of MEK activity reduced the HRG-induced decrease of both ErbB2 and ErbB3 protein observed 6 hours after HRG treatment. However, inhibition of MEK activity did not prevent the HRG-induced decrease in ErbB2 and ErbB3 protein levels 24 hours after HRG treatment (Figure 6A). The MEK inhibitor did not significantly affect the HRG- induced decrease of either ErbB2 or ErbB3 mRNA (Figure 6B, C). These findings suggest that a MEK regulated change in protein stability may be responsible for HRG-induced decreases in ErbB2 and ErbB3 at early time points. However, MEK inhibition did not affect HRG-induced changes in mRNA levels, which contributed in large part to the HRG-induced decreases in ErbB2 and ErbB3 protein levels observed 24 hours after treatment.

We next examined the effect of the AKT pathway on HRG's reduction of ErbB levels. The AKT inhibitor GSK690693 at 10 μ M reduced HRG -induced phosphorylation of the AKT substrate p70S6K as expected (Figure 7A, panels 3 and 4). GSK690693 reduced the ability of HRG to decrease ErbB2 or ErbB3 protein levels after 24 hours of treatment (Figure 7A, panels 1 and 2). The AKT inhibitor completely blocked the HRG-induced downregulation of ErbB2 mRNA at all times tested (Figure 7 B, C). Inhibition of AKT activity significantly reduced HRG's ability to decrease ErbB3 mRNA 4 ($p<0.05$), 6 ($p<0.01$) and 24 hours ($p<0.05$) after treatment (Figure 7B, C), although the degree of rescue varied. Thus, inhibition of AKT kinase activity rescued the HRG-mediated decreases in ErbB2 and ErbB3 mRNA.

Cell Cycle Analysis

To determine if HRG-induced downregulation of ErbB2/3 receptors ultimately affected cell cycle progression, cells were serum starved for 24 h followed by treatment with HRG (50 ng/ml) and/or lapatinib (1 μ M) for up to 72 hrs. Flow cytometry results indicated that HRG decreased the percentage of cells in the S phase of the cell cycle from 25.8 to 19.2 ($p<0.01$) in LTLT-Ca cells and from 23.1 to 12.2 percent ($p<0.05$) in AU565 cells (Figure 8A, B). HRG increased the percentage of cells in the G2/M phase from 10.4 to 20.7 ($p=0.07$) in LTLT-Ca cells and from 3.8 to 12.1 ($p=0.01$) in AU565 cells in keeping with previous data indicating that HRG decreases growth of ErbB2 overexpressing cells (Daly et al., 1999; Yoo and Hamburger, 1998) and Supporting information (Figure S4). We also examined the effects of lapatinib treatment on cell cycle progression. In AU565 cells (Figure 8B), lapatinib decreased the percentage of S phase cells from 23.1 to 1.4 ($p<0.01$) as reported (Xia et al., 2007). However, lapatinib ameliorated the HRG-induced decrease in S phase cells in AU565 cells (24.3 for lapatinib+ HRG treated cells vs 12.2% for HRG only, $p=0.01$). In LTLT-Ca cells, lapatinib alone did not significantly change the percentage of S phase cells from untreated controls (29.9 for lapatinib vs 25.8 for untreated cells). However,

lapatinib reversed the HRG-induced decrease in S phase cells (32.4 for lapatinib and HRG vs 19.2 for HRG alone ($p=0.01$)) (Figure 8).

Discussion

Although growth of ErbB2 positive tumors depends on the ErbB2/3 heterodimer (Geng and Vedeckis, 2011), 50% of ErbB-2 positive breast cancers show primary resistance or become resistant to ErbB2-2 targeted drugs (Esteva et al., 2010; Nahta et al., 2006). A complex network of compensatory mechanisms dampens the response to ErbB2 directed therapies. A greater understanding of factors that negatively regulate these receptors in a physiological setting is needed. Based on our prior work on the role of ligand repression of nuclear hormone receptors (Awasthi et al., 2007), we examined the role of the ErbB3/4 ligand HRG in regulation of ErbB2 and 3 expression.

We have observed, for the first time, that HRG $\beta 1$ caused a significant decrease in ErbB2 and ErbB3 protein and mRNA in breast cancer cell lines. Both the kinetics of this down-regulation as well as the degree of decrease at the protein and mRNA level varied amongst the cell lines. This highlights the importance of cellular context for the HRG-induced effects. The observation that HRG decreased ErbB2/3 levels was unexpected as ErbB2 and ErbB3 do not undergo classical ligand mediated receptor downregulation (Waterman and Yarden, 2001). The decrease in ErbB protein was due in large part to a previously unreported effect of HRG on RNA transcription. This is in keeping with the observation that HRG decreased steady state levels of *ERBB3* mRNA in ovarian cancer cell lines (Makhija et al., 2010). However, that study did not determine if the HRG-induced decrease in steady-state mRNA levels was due to decreased transcription or increased mRNA degradation. Our studies of mRNA stability indicated that *ERBB2* and *ERBB3* mRNA were not destabilized by HRG treatment. The half-life observed for *ERBB2* mRNA was in keeping with previously published data (Grooteclaes et al., 1994). In addition, we found a half-life of *ERBB3* mRNA of approximately 4 hours. To the best of our knowledge, this is the first report of *ERBB3* mRNA half-life in breast cancer cells.

To determine the rate of *ERBB2/3* mRNA transcription, we performed nascent mRNA capture assays using Click-iT technology in LTLT-Ca cells. This cell line has been used in a extensively in pre-clinical studies to model therapy for breast cancer patients (Chumsri et al., 2011). A decrease in the rate of transcription of both *ERBB2* and *ERBB3* was noted after HRG treatment. Effects of HRG on transcription rate were not examined in AU565 cells, but as steady-state mRNA levels were decreased and stability unchanged, we predict that there will also be changes in transcription although with different kinetics. The HRG-induced decrease appeared to involve the endogenous *ERBB2* promoter, as HRG did not affect ErbB2 protein levels in Her2 -18 cells which overexpress *ERBB2* under the control of a CMV promoter. Although HRG -induced repression of ErbB receptor transcription has not previously been demonstrated, transcription of many other receptors such as the Estrogen and Progesterone receptors are regulated by their cognate ligands (Awasthi et al., 2007; Geng and Vedeckis, 2011; Hatsumi and Yamamuro, 2006).

Further work is needed to determine the cis and trans factors responsible for the HRG-induced decreases in *ERBB2* and *ERBB3* transcription. One possible candidate to mediate the HRG-induced repression may be ErbB2 itself, as ErbB2 kinase activity was necessary for the HRG-induced down regulation of *ERBB2* and *ERBB3* mRNA. Early studies demonstrated that ErbB2 negatively regulates its own expression (Zhao and Hung, 1992). In addition, the kinase activity of ErbB2 is needed for its nuclear localization and transcriptional effects (Kim et al., 2009). Other proteins such as C-myc, the adenovirus 5 E1A gene product, estrogen receptor, PEAS3, FOXP3, GATA4 and the ErbB3 binding protein EBP1 all repress *ERBB2* transcription (Ghosh et al., 2013a; Russell and Hung, 1992; Suen and Hung, 1991; Yu et al., 1990). The phosphorylation and activity of many of these proteins is regulated by HRG and changes in their activity may be responsible for the repression of transcription that we observed.

Inhibiting ErbB2 kinase activity by lapatinib interfered with HRG-mediated ErbB2 and ErbB3 downregulation at both the protein and mRNA levels. These findings are consistent with the observation that HRG downregulation of *ERBB3* mRNA in ovarian cancer cells was blocked by the monoclonal antibody Pertuzumab that inhibits ErbB2-ErbB3 dimerization (Makhija et al., 2010). Lapatinib rescued the HRG-induced decreases in *ERBB3* mRNA and also increased *ERBB3* mRNA in the absence of HRG as reported (Amin et al., 2010; Garrett et al., 2011). In contrast, lapatinib by itself did not affect *ERBB2* mRNA levels similar to results reported by (Amin et al., 2010). However, lapatinib was able to block HRG-induced decreases in *ERBB2* mRNA suggesting that lapatinib may alter the activity of proteins that mediate HRG's repression of *ERBB* mRNA transcription. At the protein level, lapatinib alone increased ErbB2 and ErbB3 in keeping with previously published data (Amin et al., 2010; Scaltriti et al., 2009). As lapatinib alone failed to increase *ERBB2* mRNA, the increase in ErbB2 protein levels in the absence of HRG is likely due to the stabilization of ErbB2 protein as previously noted (Scaltriti et al., 2009). In addition, at this point we cannot rule out the role of EGFR in these effects as lapatinib is a dual EGFR/ErbB2 inhibitor. However, we believe EGFR would not play a major role in the HRG induced effects as EGFR expression is quite low in MCF-7 cells and its derivatives (de Cremoux et al., 2003; Gilani et al., 2012) and experiments were performed in serum-free media when EGFR is not activated. Finally, we found that EGF does not affect ErbB2 or ErbB3 levels in keeping with previous studies (Baulida et al., 1996; Kim et al., 1998).

Our data suggest that HRG reduced *ERBB2/3* mRNA levels through the AKT signaling pathway as pharmacologic inhibition of this pathway rescued HRG's ability to decrease ErbB2 and ErbB3 mRNA levels in the LTLT-Ca cell line. We were unable to inhibit AKT activity (as judged by p70S6 kinase phosphorylation) to a sufficient extent to evaluate the effect of suppression of AKT on ErbB levels in AU565 cells. Similarly, the PI3K inhibitor GDC-0941 prevented HRG-induced decreases in steady state levels of ErbB3 mRNA in ovarian cancer (Makhija et al., 2010). Our data are consistent with the model proposed by Garrett (Garrett et al., 2011), in which lapatinib increases ErbB3 mRNA levels by blocking AKT suppression of FoxO3a dependent *ERBB3* transcription. However, AKT regulation of *ERBB2* transcription was not reported in that study. As the MEK inhibitor had no effect on the ability of HRG to reduce ErbB2/3 mRNA levels, our pharmacologic studies suggest that

ligand-induced negative regulation of *ERBB2* and *ERBB3* transcription is primarily downstream of ErbB2- PI3K-AKT signaling.

However, it is of interest that the MEK inhibitor reduced the ability of HRG to decrease ErbB2 and ErbB3 protein, but not mRNA, at early time points (Shtiegman and Yarden, 2003). Although not formally proven in our study, we suggest that the HRG-induced changes in protein levels at early time points may have been due in part to protein destabilization. For example, HRG destabilizes ErbB3 protein at early time points via upregulation of the Neuregulin receptor degradation protein-1 (Nrdp-1) (Cao et al., 2007). CHIP and EBP1, which are also HRG regulated proteins, have also been demonstrated to ubiquitinate and destabilize ErbB2 (Lu et al., 2011; Xu et al., 2002). However, the effect of MAPK on the ability of these proteins to destabilize ErbB2 has not been reported.

The HRG-induced decreases in ErbB2/3 protein correlated with our cell cycle data indicating that HRG decreased the percentage of cells in the S phase of the cell cycle. Thus, the changes in ErbB levels translated into changes in cell growth. This is of interest as the mechanism of ErbB inhibition varied amongst the cell lines with changes in RNA and protein levels (LTLT-Ca), mRNA only (BT-474) or protein only (AU565) observed at later times points. Our finding that HRG results in growth inhibition is in keeping with reports from us (Yoo and Hamburger, 1998) (Supplementary information, Figure S4) and others (Daly et al., 1997) that HRG decreases growth of ErbB2 overexpressing breast cancer cell lines. Lapatinib greatly decreased the number of AU565 cells in S phase as expected (Xia et al., 2002). The lapatinib induced decrease in LTLT-Ca cells was not significant, perhaps due to the relatively lower expression of ErbB2 in this cell line. However, quite surprisingly in both cases, the percentage of S phase cells increased in cells treated with both HRG and lapatinib as compared with cells treated with HRG alone. Thus, lapatinib appeared to overcome the HRG mediated growth inhibition, in keeping with its ability to rescue HRG-induced receptor down-regulation. The ability of lapatinib to block the HRG-mediated decreases in ErbB2 and ErbB3 expression may lead to residual activation of ErbB2-ErbB3 signaling, ultimately reducing lapatinib's anti- proliferative effect. Interestingly, Xia et al (Xia et al., 2013) observed that HRG, when it was not growth inhibitory, could reverse lapatinib's anti-proliferative effect. This study, like ours, emphasizes the importance of HRG-lapatinib interactions in determining efficacy of ErbB directed drugs.

Taken together, our data suggest that ErbB2 and ErbB3 levels are regulated in a physiological manner by HRG which maintains signaling homeostasis by controlling ErbB2/3 transcription. Activated ErbB2 and AKT facilitate this downregulation. Treatment of breast cancer patients with drugs inhibiting ErbB2 downstream signaling may interfere with HRG- induced downregulation of ErbB2/3 receptors, making these drugs less effective. The current study suggests a need to develop drugs which can disrupt hyperactivation of ErbB2/3 signaling, without interfering with HRG induced decreases in ErbB2/3 levels.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Heregulin β 1 mediated down-regulation of ErbB2 and ErbB3 mRNA and protein
Cells were starved in medium containing 0.5% FBS for 24 h followed by treatment with 50ng/ml of Heregulin β 1 for an additional 24h. **(A)** Cell lysates were subjected to immunoblot analysis with ErbB2, ErbB3, ErbB1 and GAPDH antibodies. Due to the relatively lower expression of ErbB2 and EGFR in LTLT-Ca cells (de Cremoux et al., 2003), three times the amount of protein was analyzed on these blots as compared to AU565 and BT474 cells. Representative of 5 independent experiments. The numbers below the ErbB1, ErbB2, and ErbB3 western blots represent relative densities normalized to GAPDH. **(B)** RNA was extracted and the relative expression of ErbB2, ErbB3 and ErbB1 mRNA was assayed by real-time quantitative PCR as described. GAPDH was used as a normalization control. The results shown are the average of four independent experiments. Error bars represent SEM. The data are expressed relative to the value from the corresponding vehicle treated cells which is set as 100%. *=differences were significant at $P < 0.05$, ** differences were significant at $p < 0.01$

Figure 2. Time-dependent changes in ErbB2 and ErbB3 protein and mRNA after HRG treatment

Cells were starved in medium containing 0.5% FBS for 24 h. Cells were treated with Heregulin $\beta 1$ (50 ng/ml) for the indicated times. (A, B) LTLT-Ca (A) or AU565 (B) cell lysates were subjected to immunoblot analysis with ErbB2, ErbB3, and GAPDH antibodies. The numbers below the ErbB2 and ErbB3 western blots represent relative densities normalized to GAPDH. (C) RNA was extracted from LTLT-Ca and AU565 cells and the relative expression of ErbB2 and ErbB3 mRNA was assayed by real-time quantitative PCR. GAPDH was used as a normalization control. The results shown are the average of 3 independent experiments. Error bars represent SEM. The data are expressed relative to the value from the corresponding vehicle treated cells which is set as 100%. * = differences were significant at $P < 0.05$, ** differences were significant at $p < 0.01$.

Figure 3. Effect of HRG on ErbB2/ErbB3 mRNA stability

The stability of ErbB2 mRNA was analyzed in LTLT-Ca (A) and AU565 (B) cells. The stability of ErbB3 mRNA was measured in LTLT-Ca cells (C). Cells were serum-starved overnight followed by HRG (50ng/ml) treatment for another 24 h. Cells were then treated with ActD for the indicated times. Total cellular RNA was isolated and the levels of ErbB2 and ErbB3 were measured by RT-qPCR analysis. GAPDH was used as a normalization control. Data from Act D assays were processed by Prism 3.03 software to calculate the time required for each mRNA to reach one-half of its initial abundance and P-value. Values are the means $\pm$ SEM of triplicates. Results are representative of 3-7 independent experiments.

Figure 4. HRG decreases the rate of ErbB2 and ErbB3 transcription

LTLT-Ca cells were starved for 24h in 0.5% FBS followed by HRG (50 ng/ml) treatment for the indicated times. Ethenyl uridine was added to the medium 1 h before harvest of the cells. Total RNA was isolated and conjugated to Biotin. Biotinylated RNA was purified by Streptavidin conjugated magnetic beads followed by cDNA synthesis. Nascent ErbB2 (**A**) and ErbB3 (**B**) mRNA were measured by RT- qPCR. GAPDH was used as a normalization control. Data are expressed as fold change relative to corresponding control. Mean $\pm$ SEM of duplicate experiments are shown. *—differences were significant at $P < 0.05$ (**C**) Her2-18 cells (MCF-7 cells stably transfected with ErbB2) were treated with HRG (50 ng/ml) for 24 h and cell lysates were subjected to Western blot analysis for ErbB2 and GAPDH.

Figure 5. Lapatinib rescues ErbB2 and ErbB3 from the inhibitory effect of HRG

LTLT-Ca cells were starved in medium containing 0.5% FBS and lapatinib (1 μ M) for 24 h. Cells were then refed with media containing 0.5% FBS with HRG (50ng/ml) and lapatinib (1 μ M) or vehicle control for the indicated times. **(A)** Cell lysates were subjected to immunoblot analysis for ErbB2, ErbB3, p-ErbB3 and GAPDH as indicated. Data are representative of 3 independent experiments. Although the DMSO and lapatinib treated groups are presented in separate panels, samples were run on the same gel and blotted at the same time. The numbers below the ErbB2 and ErbB3 western blots represent relative densities normalized to GAPDH. **(B)** Total RNA was subjected to qRT-PCR to measure ErbB2 and ErbB3 mRNA. GAPDH was used for normalization. Data are expressed as percent relative to DMSO treated cells at time 0. Data are representative of 3 independent experiments. *=differences between HRG and HRG and lapatinib treated groups were significant at $P<0.05$, ** differences were significant at $p<0.01$. **(C)** To better visualize the data, the value of each individual treatment group (vehicle or lapatinib) at time 0 prior to HRG addition was set as 100%.

Figure 6. Effect of a MEK inhibitor on HRG-induced changes in ErbB2 and ErbB3

LTLT-Ca cells were starved in medium containing 0.5% FBS and UO126 (10 μ M) or vehicle control for 24 h. Cells were then refed with media containing 0.5% FBS with HRG (50ng/ml) and UO126 (10 μ M) for the indicated times. (A) Cell lysates were subjected to immunoblot analysis for ErbB2, ErbB3, pERK, total ERK and GAPDH as indicated. Data are representative of 3 independent experiments. Although the DMSO and UO126 treated groups are presented in separate panels, samples were run on the same gel and blotted at the same time. The numbers below the ErbB2 and ErbB3 western blots represent relative densities normalized to GAPDH. (B) Total RNA was subjected to qRT-PCR to measure ErbB2 and ErbB3 mRNA. GAPDH was used for normalization. Data are expressed as percent relative to DMSO treated cells at time 0. Data are representative of 3 independent experiments. (C) To better visualize the data, the value of each individual treatment group (vehicle or UO126) at time 0 prior to HRG addition was set as 100%.

Figure 7. Effect of an AKT inhibitor on HRG-induced changes in ErbB2 and ErbB3
LTLT-Ca cells were starved in medium containing 0.5% FBS and GSK 690693 (10 μ M) for 24 h. Cells were then refed with media containing 0.5% FBS with HRG (50ng/ml) and GSK 6900693 (10 μ M) or vehicle control for the indicated times. (A) Cell lysates were subjected to immunoblot analysis for ErbB2, ErbB3, phospho p70 S6K, total p70 S6K and GAPDH as indicated. Data are representative of 3 independent experiments. Although the DMSO and GKS690693 treated groups are presented in separate panels, samples were run on the same gel and blotted at the same time. The numbers below the ErbB2 and ErbB3 western blot represent relative densities normalized to GAPDH. (B) Total RNA was subjected to qRT-PCR to measure ErbB2 and ErbB3 mRNA. GAPDH was used for normalization. Data are expressed as percent relative to DMSO treated cells at time 0. Data are representative of 3 independent experiments. *=differences between HRG and HRG and GSK 690693 treated groups at each time point were significant at $P < 0.05$, ** differences were significant at $p < 0.01$. (C) To better visualize the data, the value of each individual treatment group (vehicle or GSK690693) at time 0 prior to HRG addition was set as 100%.

Figure 8. Effect of HRG and lapatinib on cell-cycle distribution

LTLT-Ca or AU565 cells were serum starved overnight and then treated with HRG (50 ng/ml), lapatinib (1 μ M) or the combination for 72 hrs (LTLT-Ca) or 48 hrs (AU565). Cells were harvested and fixed in 70% ethanol, then stained with propidium iodide and analyzed by flow cytometry. To better visualize the data, the error bars are directed down only for G1 and S phases and up only for the G2/M phase. Average of two experiments, 4 points.