A Central Role for HER3 in HER2-Amplified Breast Cancer: Implications for Targeted Therapy

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Abstract

Epidermal growth factor receptor (EGFR) and HER3 each form heterodimers with HER2 and have independently been implicated as key coreceptors that drive HER2-amplified breast cancer. Some studies suggest a dominant role for EGFR, a notion of renewed interest given the development of dual HER2/EGFR small-molecule inhibitors. Other studies point to HER3 as the primary coreceptor. To clarify the relative contributions of EGFR and HER3 to HER2 signaling, we studied receptor knockdown via small interfering RNA technology across a panel of six HER2-overexpressing cell lines. Interestingly, HER3 was as critical as HER2 for maintaining cell proliferation in most cell lines, whereas EGFR was dispensable. Induction of HER3 knockdown in the HER2-overexpressing BT474M1 cell line was found to inhibit growth in three-dimensional culture and induce rapid tumor regression of in vivo xenografts. Furthermore, preferential phosphorylation of HER3, but not EGFR, was observed in HER2-amplified breast cancer tissues. Given these data suggesting HER3 as an important therapeutic target, we examined the activity of pertuzumab, a HER2 antibody that inhibits HER3 signaling by blocking ligand-induced HER2/HER3 heterodimerization. Pertuzumab inhibited ligand-dependent morphogenesis in three-dimensional culture and induced rapid tumor regression of in vitro xenografts. Importantly, these activities of pertuzumab were distinct from those of trastuzumab, a monoclonal antibody currently used for treatment of HER2-amplified breast cancer patients. Our approach used gene silencing techniques, which allowed for detailed investigation of the role of EGFR and HER3 in HER2-amplified breast cancer cell lines. Our data suggest that inhibition of HER3 may be more clinically relevant than inhibition of EGFR in HER2-amplified breast cancer and also suggest that adding pertuzumab to trastuzumab may augment therapeutic benefit by blocking HER2/HER3 signaling. [Cancer Res 2008;68(14):5878–87]

Introduction

HER2 is the target of the therapeutic agents trastuzumab and lapatinib, which are used to treat HER2-positive breast cancer (1–4). In normal tissue, HER2/ErbB2 is known to function as a common coreceptor for the other HER/ErbB receptors (5). When epidermal growth factor receptor (EGFR) or HER3 binds their respective cognate ligands, HER2 is frequently observed to be recruited to these ligand-receptor complexes. Tumors harboring HER2 gene amplification produce an excess of HER2 at the cell membrane resulting in constitutive signaling (6). Although HER2 homodimerization is frequently cited as the source of the oncogenic signal, HER2-containing heterodimers are also implicated. Because EGFR is expressed in HER2-positive breast cancer, synergistic interaction between EGFR and HER2 could be important for oncogenesis (7–10). In support of this hypothesis, potential cooperativity of EGFR and HER2 in mammary tumorigenesis was reported in genetically engineered mouse models (11, 12). HER3 has also been implicated as a partner with HER2 in mediating neoplastic transformation. The HER2/HER3 heterodimer was found to mediate the most mitogenic signal in vitro and was sufficient to induce cellular transformation of a mouse fibroblast cell line that expressed nontransforming levels of HER2 (13, 14).

Because HER3 lacks intrinsic kinase activity, the generation of specific HER3-directed small-molecule inhibitors is challenging. Nevertheless, alternative approaches, such as therapeutic monoclonal antibodies that specifically inhibit the ability of HER2 to associate with other HER family members, have been described (15). The standard of care in first-line metastatic and adjuvant HER2-positive breast cancer is a regimen containing trastuzumab (Herceptin) that binds to subdomain IV of the extracellular region of HER2 (16). By contrast, another HER2 antagonistic antibody, pertuzumab (2C4), binds to the subdomain II dimerization arm of HER2 and inhibits formation of ligand-induced HER2 heterodimers (15, 17). Pertuzumab could have a distinct therapeutic role if dimerization of HER2 with EGFR or HER3 is a significant driver of HER2-positive breast cancer. Similarly, a significant contribution from EGFR could have implications for dual EGFR/HER2 small-molecule inhibitors.

In the present study, we sought to further investigate the nature of the oncogenic unit in HER2-amplified breast cancer cell lines. Our approach used gene silencing techniques, which allowed for detailed investigation of the role of EGFR and HER3 in a panel of HER2-amplified breast cancer cell lines using the same experimental conditions. Our results show that knockdown of EGFR expression has no effect on basal level mitogenesis. Interestingly, HER3 knockdown decreases proliferation to the same extent as HER2 knockdown in most cell lines. Moreover, phospho-HER3 is readily detected in human breast cancers that overexpress HER2. These results confirm and extend the notion that HER2/HER3 is the major oncogenic unit in breast cancers with HER2 gene amplification (18). Taken together with emerging data from the clinic, these results suggest that targeting EGFR in HER2-positive breast cancer will not likely lead to substantial clinical benefit, and approaches that target HER2/HER3 signaling may be warranted.
Materials and Methods

Cell lines and small interfering RNA transfection. BT474M1 is a subclone of BT474 that was obtained from California Pacific Medical Center. All other cell lines were obtained from the German Collection of Microorganisms and Cell Cultures or American Type Culture Collection. The cell lines were maintained at 37°C and 5% CO2 in high-glucose DMEMHam’s-F-12 (BT474, BT474M1, and SKBR3) or RPMI 1640 (EFM192A, HCC1419, HCC1954, and ZR-75-30), and all were supplemented with 10% fetal bovine serum (FBS) and 2 mmol/L L-glutamine. Tetracycline (Tet)-free FBS (10%; HyClone) was used for maintaining BT474M1 cells that were infected with the inducible short hairpin RNA (shRNA) constructs. Reverse transfection was performed by seeding 107 cells per well into 96-well microtiter plates containing a preincubulated mixture of pooled small interfering RNA (siRNA) oligos at 100 nmol/L final concentration and Lipofectamine 2000 (Invitrogen) diluted in Opti-MEM (Invitrogen) as per the manufacturer’s recommendations. Four predesigned oligonucleotides (sequence obtained from Dharmacon) at equal concentrations were added to generate a pool that targeted each gene of interest.

Cell proliferation determined by [3H]thymidine incorporation into DNA. After an overnight incubation following transfection, the cells were treated with 25 µg/mL HER2 antibody (trastuzumab or pertuzumab; Genentech). After an additional 24-h incubation, the cells were pulsed with 1 µCi/well [3H]thymidine for 18 h and harvested onto UniFilter GF/C plates using a Filtermate 196 harvester (Perkin-Elmer). MicroScint 20 liquid scintillation mixture was added to the dried filter plates, which were sealed and counted in a TopCount (Perkin-Elmer).

Preparation of tumor samples, Western blot analysis, and antibodies. Frozen breast cancer tissues were obtained from ILShio. HER2 immunohistochemistry was performed on frozen sections using reagents from the HercepTest kit. All frozen tumor samples were homogenized in lysis buffer containing 50 mmol/L Tris-HCl (pH 7.6), 150 mmol/L NaCl, 1% Triton X-100, Protease Inhibitor Cocktail tablet (Roche), and Phosphatase Inhibitor Cocktail I and II (Sigma). Analysis of protein expression of cell lines was performed by lysing cells in radioimmunoprecipitation assay buffer (Sigma) supplemented with protease inhibitors (Roche) and phosphatase inhibitor cocktail (Calbiochem). Tris-acetate gel (3–8%; Invitrogen) was used to resolve the proteins. The gel was electrophoresed onto nitrocellulose followed by immunodetection using standard procedures. Antibodies used for Western blotting were as follows: anti-EGFR (MLB), anti-phospho-EGFR (Cell Signaling), anti-HER2 (Ab-17; NeoMarkers), anti-phospho-HER2 (Cell Signaling), anti-HER3 (Santa Cruz Biotechnology), anti-phospho-HER3 (Cell Signaling), anti-α-tubulin (Sigma), and anti-β-actin (Santa Cruz Biotechnology).

Inducible shRNA cell lines. Hairpin oligonucleotides used in this study are as follows: HER3 shRNA-1, 5′-GATCCCCAAGAGGATGTCAACGGT-3′ (antisense); and 5′-TATTCAAGAGATAACCGTTGACATCCTCTTTTTTTGAAA-3′ (sense). The complementary double-stranded shRNA oligonucleotides were inserted into our Tet-inducible viral gene transfer vector using RagII and HindIII restriction enzyme sites as described (19). Our vector system is composed of shuttle plasmid and an enhanced green fluorescent protein (eGFP) expressing viral vector backbone that contains a codon-optimized Tet repressor-internal ribosomal entry site-eGFP cassette to enable Tet-regulated shRNA expression. The luciferase shRNA construct was described previously (19). Fluorescence-activated cell sorting was used to select eGFP-positive cells that were collected and pooled 3 d after infection. Expanded pools were treated with 25 ng/mL doxycycline (BD Clontech) for 3 d, and endogenous HER3 knockdown was assessed by Western blot.

Three-dimensional cell culture. For three-dimensional cultures, BT474M1 cells were trypsinized from monolayer cultures and plated on top of commercially available laminin-rich matrix produced from Engelbreth-Holm-Swarm tumors (Matrigel, BD Biosciences) according to the protocol described by Deb Nath and colleagues (2003). Cell lines were maintained in modified Dulbecco’s medium and 10% Tet-free FBS conditioned with 5% Matrigel. Cells were treated with 20 µg/mL trastuzumab, 25 µg/mL pertuzumab, 1 nmol/L HERGbeta1-7,77-244, and 200 ng/mL doxycycline on day 0 as indicated. Culture medium was refreshed every 3 d and acinar growth and morphogenesis were analyzed on day 10. Cell number was assayed using CellTiter-Glo (Promega) according to the manufacturer’s instructions. Acinar morphogenesis was quantified by counting budding sites for 100 acini per treatment.

In vivo tumor models. For xenograft studies, 7- to 8-wk-old female beige nude XID mice were purchased from Harlan Sprague Dawley and maintained according to the Institute of Laboratory Animal Resources Guide for the Care and Use of Laboratory Animals. Mice were inoculated in the mammary fat pad with 5 × 106 BT474M1 lacZ shRNA or HER3 shRNA cells in 50% phenol red-free Matrigel (Becton Dickinson Bioscience). One day before cell inoculation, mice were implanted s.c. with a 0.36-mg, 60-d sustained release 17β-estradiol pellet (Innovative Research of America) to maintain serum estrogen level. When tumors reached a mean volume of 250 to 350 mm3, mice with similarly sized tumors were grouped into treatment cohorts. For shRNA-mediated gene knockdown studies, mice received either 5% sucrose only or 5% sucrose plus 1 mg/mL doxycycline (Clontech Laboratories, Inc.) in the drinking water for control and knockdown cohorts, respectively. I.p. injection of trastuzumab or/and pertuzumab in dilution buffer was performed as indicated. Tumors were measured with calipers twice a week and tumor volumes were calculated as follows: (longer measurement × shorter measurement2) × 0.5. Between 5 and 10 mice were used for each treatment group and results are presented as mean tumor volume. To establish the MDA-MB-175 tumor model, 20 million cells were inoculated into the gonadal fat pad of beige nude XID mice. The tumors generated were subsequently transplanted into the mammary fat pad of a new cohort of mice. After 12 sequential in vivo passages, the tumors were allowed to grow to a mean tumor volume of 100 to 200 mm3 before HER2 antibody treatment as described.

Analysis of xenografts. An independent group of mice was inoculated with BT474M1 shRNA pools as described above for 2 d before analysis. Total RNA was prepared from 30 mg of tissue and the Qiagen RNAeasy Mini kit. cDNA was prepared using the cDNA High-Capacity Kit (Applied Biosystems). Gene expression was quantified using real-time quantitative PCR (Taqlman, ABI PRISM 7900, Applied Biosystems). The HER3 and the endogenous control glyceraldehyde-3-phosphate dehydrogenase primer/probe sets were obtained from Applied Biosystems (4). Taqman analysis was performed in quadruplicate in a standard 384-well plate format according to the protocol for Taqman Universal PCR Master Mix (2X) (Applied Biosystems). Phospho-Akt1 was determined using a solid-phase sandwich ELISA (PathScan Phospho-Akt 1 ELISA kit, Cell Signaling). The levels of phospho-Akt1 of HER2-amplified cell lines and xenograft tumors were corrected with the total amount of Akt1 (PathScan Total Akt1 ELISA kit, Cell Signaling) and presented as the means of three independent experiments or as the average of five tumors. For immunohistochemistry, tumors were formalin fixed and paraffin embedded and a routine H&E slide was first evaluated. Immunohistochemical staining was done on 4-µm-thick paraffin sections using anti-Ki-67 (clone MIB-1, mouse anti-human with the DAKO ARC kit), anti-phospho-S6 235/236 (clone 91B2, rabbit anti-human; Cell Signaling), and anti-cleaved caspase-3 (rabbit anti-human and anti-mouse) antibodies with a standard avidin-biotin horseradish peroxidase detection system according to the instructions of the manufacturer. Tissues were counterstained with hematoxylin, dehydrated, and mounted. In all cases, antigen retrieval was done with the DAKO Target Retrieval kit as per instructions of the manufacturer.

Results

A critical role for HER3 in HER2-overexpressing breast cancer cell lines. To clarify the relative contributions of EGFR and HER3 toward HER2-driven proliferation, we examined the effect of RNA interference (RNAi)-mediated gene knockdown of HER receptors in a panel of breast cancer cell lines that express high levels of HER2 using a [3H]thymidine incorporation assay. Transfection of a pool of four siRNA duplexes targeting HER2 resulted in a 3- to 5-fold reduction of cell proliferation in five of six
HER2-overexpressing cell lines when compared with cells transfected with a nontargeting (negative control) siRNA oligonucleotide (Fig. 1A; Supplementary Fig. S1). This result is expected and consistent with the known role of HER2 as a driver of proliferation in this breast cancer subtype. Interestingly, transfection of a HER3 siRNA pool inhibited proliferation to the same magnitude as did HER2 knockdown in four of five cell lines. In the one cell line that did not respond to HER2 knockdown, HER3 knockdown inhibited

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**Figure 1.** HER3, but not EGFR, plays a critical role in proliferation of HER2-overexpressing cell lines. A, the proliferation of a panel of HER2-amplified breast cancer cell lines was measured by [3H]thymidine uptake assay. BT474, HCC1419, SKBR3, ZR-75-30, EFM192A, and HCC1954 were transfected with either a nontargeting control (NTC) siRNA oligonucleotide (white columns) or a pool of four siRNA oligonucleotides against EGFR (hatched columns), HER2 (gray columns), or HER3 (black columns). The extent of proliferation under each condition was plotted as a percentage of the normalized nontargeting control value for each cell line. Columns, mean; bars, SE. B, to verify that HER3 siRNA does not directly affect HER2, the HER2 expression and phosphorylation status was compared in nontargeting control and HER3 siRNA-transfected cells. The effect of RNAi-mediated gene knockdown of HER2 (C) or EGFR (D) on HER3 phosphorylation was examined using the phospho-HER3 (P-HER3) antibody (Tyr1289). Equal protein loading was confirmed by either α-tubulin or β-actin Western blot.
proliferation. In contrast, EGFR knockdown in the same cell lines did not affect proliferation. Overall, these results show that HER3 is just as important as HER2 in several HER2-overexpressing cell lines. The data further indicate that EGFR has little, if any, contribution to the growth of these HER2-overexpressing cell lines in vitro.

We next examined the expression of HER family receptors by Western blot in relation to siRNA knockdown. In both untransfected cells and cells transfected with the nontargeting control siRNA, HER3 is expressed and is phosphorylated at Tyr1289 in all cell lines examined, whereas EGFR was only detected in HCC1954 and SKBR3 (Supplementary Fig. S2; data not shown). Knockdown of HER3 was clearly evident by Western blot in the presence of HER3 siRNA, whereas neither HER2 protein levels nor HER2 phosphorylation (Tyr1248) was affected (Fig. 1B), indicating that HER3 siRNA oligonucleotides do not affect HER2 through some off-target effect. HER3 knockdown reduced phospho-Akt by 50% in all cell lines (Supplementary Fig. S3), thus supporting the previously reported role of HER3 as a potent activator of the phosphatidylinositol 3-kinase pathway (20). In contrast, knockdown of HER2 but not EGFR caused a significant decrease of HER3 phosphorylation (Fig. 1C and D), further supporting the central role of HER3 in most HER2-overexpressing breast cancer cell lines.

A role for HER3 is evident in three-dimensional culture. To investigate whether HER3 has a significant role beyond proliferation in monolayer cell culture, we sought to use the three-dimensional culture system. Mammary epithelial cells grown in three-dimensional culture can organize into structures resembling their in vivo architecture. A Tet-inducible shRNA system (19) was used to generate an inducible HER3 knockdown cell line (BT474M1-shHER3) and a negative control cell line (BT474M1-shlacZ). Addition of doxycycline at 25 ng/mL mediates knockdown of HER3 protein and inhibits growth of BT474M1-shHER3 cells but does not affect growth of BT474M1-shlacZ control cells in two-dimensional culture (Supplementary Fig. S4). When BT474M1 cells were plated onto laminin-rich, reconstituted basement membrane, they proliferated rapidly and formed solid acini similar to BT474 (data not shown). As shown in Fig. 2A, addition of doxycycline during plating significantly decreased the size of BT474M1-shHER3 acini. This effect was confirmed by quantitation with CellTiter-Glo, which revealed a 3-fold reduction in doxycycline-treated BT474M1-shHER3 cultures (Fig. 2B). Thus, the critical role of HER3 for growth is also observed in a three-dimensional cellular context.

In vivo knockdown of HER3 causes tumor regression. We next sought to determine whether specific inhibition of HER3 signaling would attenuate growth of BT474M1 cells in vivo. Mice inoculated with BT474M1-shHER3 or shlacZ cells were administered 5% sucrose water with or without 1 mg/mL doxycycline. As shown in Fig. 3A, the administration of doxycycline induced significant tumor regression of BT474M1-shHER3 but not of sucrose-treated BT474M1-shHER3 xenografts or the control line BT474M1-shlacZ (Supplementary Fig. S5). HER3 gene knockdown in xenograft tumors was verified by quantitative reverse transcription-PCR (qRT-PCR) using human-specific HER3 primers 48 h after doxycycline treatment (Fig. 3B). In agreement with our in vitro data, phospho-Akt levels were also decreased in doxycycline-treated shHER3 xenograft tumors (Fig. 3C). By contrast, the level of HER2 expression was unchanged (data not shown). Furthermore, immunohistochemical analysis revealed a decrease in phosphorylation of S6 in doxycycline-treated tumors compared with untreated controls (Fig. 3D). Moreover, HER3 knockdown was associated with a reduction in the proliferative marker Ki-67 but
not the apoptotic marker cleaved caspase-3, suggesting that tumor regression was primarily due to inhibition of proliferation. These data show that HER3 is critically important for the maintenance of HER2-overexpressing tumor cells in vivo.

**Pertuzumab and trastuzumab have distinct activity in vitro and in vivo.** Interfering with the HER3 component of signaling could have therapeutic efficacy in HER2-positive breast cancer. Pertuzumab hinders the interaction of HER2 and HER3 by binding to the dimerization arm of HER2 (subdomain II; ref. 17) and has been shown to attenuate the growth of HER2-amplified xenograft tumors (15). In comparison, trastuzumab binds to subdomain IV of HER2 and abrogates signaling without affecting ligand-driven HER2-HER3 dimerization. Trastuzumab and pertuzumab were examined on BT474M1 cells in three-dimensional culture and observed to differ in their ability to inhibit growth of acini, with trastuzumab exhibiting more robust growth inhibition (Supplementary Fig. S6). We next investigated the effect of pertuzumab and trastuzumab on morphogenesis induced by the HER3 ligand heregulin. As shown in Fig. 4A, untreated BT474M1 acini underwent budding/branching morphogenesis in the presence of the HER3 ligand heregulin. Pertuzumab robustly inhibited this branching phenotype, whereas trastuzumab had no effect. In agreement, induction of HER3 knockdown in the presence of heregulin also inhibited branching (Fig. 4B and C). Thus, pertuzumab is particularly efficient at inhibiting heregulin-mediated branching, whereas trastuzumab is more efficient at growth inhibition in the absence of heregulin.

We next examined whether pertuzumab would effectively inhibit tumorigenesis in vivo using the MDA-MB-175 breast cancer cell line. This model is driven by an autocrine loop via high expression of heregulin and is sensitive to pertuzumab in vitro (21). As shown in Fig. 5A, pertuzumab but not trastuzumab treatment caused rapid tumor regression, showing the differential activities of these antibodies in vivo. Moreover, simultaneous treatment with trastuzumab and pertuzumab seemed to trend toward greater tumor regression than with pertuzumab treatment alone (Fig. 5A, red

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**Figure 3.** HER3 knockdown mediates tumor regression in vivo. The volume of BT474M1-shHER3 (A) tumors was measured for 26 d after initial treatment with vehicle (black line) or with doxycycline to activate the hairpin (red line). In a separate experiment, five BT474M1-shHER3 xenograft tumors that were treated with either sucrose or doxycycline for 48 h were collected. B, the mRNA level of HER3 was assessed by qRT-PCR and found to be decreased in doxycycline-treated BT474M1-shHER3 cells. Vh, sucrose. C, the level of phospho-Akt (pAkt) normalized to total Akt (tAkt) was determined by ELISA. Points, mean; bars, SE. D, immunohistochemical analysis for phospho-S6, Ki-67, and cleaved caspase-3 was also performed on the same set of BT474M1-shHER3 xenograft tumors.
lines), although the data are not statistically significant. Next, we examined whether combination treatment of trastuzumab and pertuzumab may also have distinctive effects in the BT474M1 xenograft model (Fig. 5B). Although initial tumor regression was comparable with trastuzumab, pertuzumab, or the combination of both antibodies, subsequent tumor regrowth was most evident with pertuzumab (at 5 mg/kg). Moreover, the trend toward tumor regrowth was not observed with trastuzumab plus pertuzumab in combination (albeit not statistically significant). Consistent with our observations, a combinatorial effect of trastuzumab and pertuzumab has been noted in other HER2-overexpressing xenograft models (22).

HER3 is preferentially phosphorylated in HER2-overexpressing breast cancer. To determine whether the role of HER3 in cell
line and xenograft models is likely to be relevant for HER2-amplified breast cancer patients, we next examined the phosphorylation status of HER3 in a panel of human breast cancers. HER2 status was determined in frozen tumors by immunohistochemistry and was also verified by Western blot. A representative subset of the tumor samples is shown in Fig. 6A. Interestingly, the ratio of phospho-HER3 (Tyr1289) to total HER3 as determined by densitometry was significantly higher in HER2-overexpressing breast cancers compared with HER2-negative breast cancers, whereas the extent of EGFR phosphorylation (Tyr1068) was equivalent (Fig. 6B).

In agreement with the data obtained from our experimental models, HER2 overexpression in human breast cancers is associated with activation of HER3, not activation of EGFR. These observations highlight the importance of considering therapeutic strategies that disrupt the critical HER2/HER3 heterodimer in HER2-positive breast cancer.

**Discussion**

Activation of the ErbB/HER network is tightly regulated throughout development and in the adult (6, 23). Receptor components of the pathway, such as EGFR or HER2, are frequently activated in various solid tumors. A special case of inappropriate ErbB/HER activation occurs in human breast cancers containing HER2 gene amplification. In these tumors, HER2 receptors are constitutively activated. Since this discovery, the nature of this "constitutively activated" receptor has been controversial. Early reports argued that EGFR coexpression was required for fibroblast transformation by erbB2/HER2 (24). However, these results were challenged when a variant of 3T3 cells lacking EGFR expression, termed NR6, was shown to be transformed by erbB2/HER2 alone (25). This and many other studies gave rise to a model that HER2-amplified cancers were driven by signals emanating from HER2 oligomers.

A renewed interest in the role of EGFR in HER2-amplified tumors arose with the advent of small-molecule tyrosine kinase inhibitors that were designed to specifically inhibit EGFR (26, 27). Using these compounds, several investigators observed potent antitumor activity of these agents when used on HER2-overexpressing cell lines (28, 29). These results prompted the initiation of clinical trials that tested these agents in patients whose tumors overexpressed HER2. To date, the results of these studies have been largely disappointing (30). In contrast, a dual HER2-EGFR kinase inhibitor, lapatinib, is clinically active in HER2-positive breast cancer yet displays little activity against breast cancer cell lines that overexpress EGFR (4, 31). To complicate matters further, more recent investigations have described an essential role for kinase-dead HER3 in selected HER2-amplified cell lines (18, 32). In the first study, a dominant-negative form of HER3 was used to suppress signaling and proliferation. In agreement, Holbro and colleagues (18) used a novel and elegant approach of introducing an artificial transcription factor to delineate a role for HER3.

We embarked on the present study to help further clarify whether HER2 itself or HER2 heterodimers were responsible for oncogenic signaling in HER2-amplified breast cancers. Our approach was to use RNA silencing of HER/ErbB receptors with a panel of HER2-overexpressing cell lines. Here, we show that signaling emanating from HER2/HER3 is required for the proliferation of HER2-amplified breast cancer cells in vitro and in vivo. In contrast, EGFR does not seem to play a significant role. We also provide evidence that the phenotypic effect of pertuzumab in vitro and in vivo is both distinct from the activity of trastuzumab and overlaps with the effect of HER3 knockdown. Because neither HER2 expression levels nor phosphorylation status was changed in HER3 knockdown cell lines or xenografts, signaling emanating from HER2 oligomers is insufficient for proliferation in vitro and tumor maintenance in vivo. Rather, the data suggest that the HER2/HER3 heterodimer may be the key oncogenic unit.

The current study confirms and extends the role of HER3 in HER2-positive breast cancer in four ways. First, we used a panel of
HER2-overexpressing cell lines to show that the effect of HER3 knockdown is a general phenomenon and not a peculiarity of one or two cell lines. Second, we have shown for the first time that the role of HER3 is also clearly evident in three-dimensional culture and an in vivo xenograft model. The finding of tumor regression on HER3 knockdown in vivo suggests that HER3 could be a relevant drug target in HER2-amplified breast cancer. Third, our data suggest that EGFR is dispensable in HER2-amplified breast cancers. Fourth, in agreement with our in vitro findings, the phosphorylation status of HER3 was significantly elevated in HER2-positive versus HER2-negative breast cancer, whereas phospho-EGFR was not. Although HER3 phosphorylation has been previously described in cell lines, we now provide evidence that HER3 signaling may be active in human breast cancer. These data highlight the potential relevance of HER3 as a therapeutic target.

Figure 6. HER3 is activated in HER2-amplified breast tumor samples. Protein lysates were extracted from 26 breast tumor samples for immunoblotting and phosphorylation statuses of HER family receptors were analyzed. A, representative Western blots of samples. TGFα, transforming growth factor α. B, the extent of phosphorylation of HER3 (top) and EGFR (bottom) normalized to total receptor level for the entire sample set was quantified by densitometry.
a frequently observed gain-of-function mutation in the kinase domain of PIK3CA (33), which could potentially affect response to knockdown. Interestingly, HER3 knockdown caused a decrease in levels of phospho-Akt in EFM192A despite the lack of proliferative phenotype, suggesting that compensatory pathways might bypass Akt to drive cell proliferation in some cases.

The HER2 antibody pertuzumab is one approach to directly block the HER2/HER3 interaction by binding to the domain II dimerization arm of HER2. We found that pertuzumab ablated heregulin-mediated morphogenesis of BT474M1 acini in three-dimensional culture. It is not entirely clear how heregrulin-induced branching might be related to oncogenesis. Nevertheless, it has been speculated in the literature that heregrulin-induced morphogenesis causes cell invasion and metastasis in vivo (34). Moreover, this process may depend on modulation of both actin- and microtubule-based cytoskeleton structures (35). In addition to the effect on morphogenesis, pertuzumab treatment inhibited tumor growth in vivo in the heregrulin-expressing MDA-MB-175 xenograft model. These activities of pertuzumab are distinctly different from those of trastuzumab and are consistent with previous findings (15).

Although the effect of HER3 knockdown in the presence of heregrulin parallels the inhibition of branching morphogenesis by pertuzumab, the inhibition of growth with HER3 knockdown in the absence of heregrulin was more similar to the effect of trastuzumab. This observation suggests that there could be therapeutic value in the combination of pertuzumab and trastuzumab in HER2-amplified breast cancer. Indeed, encouraging clinical activity of combination antibody therapy was recently reported from a phase II metastatic breast cancer study where pertuzumab was added to trastuzumab in patients whose tumors had progressed on trastuzumab therapy (36). The potential of combined trastuzumab and pertuzumab was explored in both the BT474M1 and MDA-MB-175 models. Treatment of BT474M1 xenografts with both trastuzumab and pertuzumab trended toward more sustained tumor growth inhibition over time compared with either treatment alone. MDA-MB-175 tumors are dependent on a paracrine heregrulin loop that activates HER2/HER3 signaling, thus are highly sensitive to pertuzumab treatment. These tumors are not responsive to trastuzumab treatment alone because they do not express a high level of HER2 (HER2 1+ by immunohistochemistry). Addition of trastuzumab to pertuzumab in MDA-MB-175 tumors resulted in a nonsignificant trend toward improved tumor response. Although any combinatorial effect may seem to be minimal in these models, we speculate that this observation may be due to the fact that single-agent pertuzumab (in both MDA-MB-175 and BT474M1) and single-agent trastuzumab (in BT474M1) were so effective on their own. More in-depth analysis of the potential additive effect is probably best carried out in other models that may be less sensitive to single agents. For example, there is an early indication from another laboratory that there may be more significant combinatorial activity in other models (22, 35). It should also be noted that the mechanism of these therapeutic antibodies is complex and includes effects on signaling as well as engagement of Fcγ receptors (FcγR) on immune cells that can mediate tumor cell killing [antibody-dependent cellular cytotoxicity (ADCC)]. For example, it has recently been reported that specific polymorphisms in FcγRIIIa and FcγRIIIb that mediate greater efficiency of FcγR engagement are associated with improved outcome on trastuzumab (37). In the future, it will be interesting to understand whether any potential additive effects of the two antibodies are indeed mediated by enhanced ADCC or enhanced signaling inhibition or both.

The central role of HER3 signaling in HER2-amplified breast cancer suggests that HER3 is a promising therapeutic target. Furthermore, the lack of evidence for a role of EGFR in HER2 signaling suggests that targeting EGFR in HER2-amplified breast cancer is not likely to provide clinical benefit and perhaps would be best avoided to minimize toxicities, such as skin rash, which are attributed to EGFR inhibition. Blocking the HER2/HER3 receptor complex with pertuzumab has an effect on tumor cells in three-dimensional culture and in vivo xenografts, which is distinct from the effect of trastuzumab. These data suggest that combination treatment with trastuzumab and pertuzumab may be particularly efficacious in HER2-amplified breast cancer. A clinical trial to examine trastuzumab plus pertuzumab (with chemotherapy) in first-line HER2-amplified metastatic breast cancer patients is currently under way.

Disclosure of Potential Conflicts of Interest

All authors are employees of, and have ownership interest in, Genentech, Inc.

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