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Anti-HER-3 MAbs Inhibit HER-3-Mediated Signaling in Breast Cancer Cell Lines Resistant to Anti-HER-2 Antibodies

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Two members of the EGF receptor family, HER2 and HER3, act as key oncogenes in breast cancer cells. A MAb against HER2, trastuzumab, interferes with HER2 signaling and istherapeutically effective in humans. Here, we explored the biologic effects of an antibody against HER3 (α-HER3^{ECD}) in the invasive breast cancer cell lines MCF-7^{ADR} and MDA-MB-468. Pretreating the breast cancer cells with α-HER3^{ECD} prior to Heregulin stimulation caused significant reduction of the migratory and proliferative properties. This reduction is due to a substantial decrease in the tyrosine phosphorylation content of HER2 and to a modification of the HER2/HER3 association, which ultimately inhibits the activity of the downstream effectors phosphatidyinositol-3-OH-kinase and c-jun-terminal kinase. Furthermore, HER3 is internalized and not activated by HRG after pretreatment with α-HER3^{ECD}. Our data reinforce the notion that HER3 could be a key target in cancer drug design and show the great potential of anti-HER3 antibodies for the therapy of breast cancer and other malignancies characterized by overexpression of HER3.

Key words: HER2; HER3; trastuzumab; breast cancer; antibody therapy

Breast cancer is the most common malignancy among women in the United States, accounting for one million new cases every year. It constitutes 18% of all cancers in women and 30% of all cancers diagnosed.[1] During the last decade, intensive efforts have been made to substantially improve the prognostic techniques and therapeutic strategies to fight breast cancer.[2] Analysis of the disease course and recurrence potential of individual patients through microarray-based investigation of predictor genes is becoming increasingly accurate; more selective chemotherapeutic protocols can be designed based on genomic data, to improve the selectivity for cancer cells and reduce toxicity.[3] In this perspective, molecular characterization of predictor genes and signaling pathways controlling cancer growth is instrumental to new drug design and the refinement of cancer chemotherapy for individual patients.

A member of the EGFR family, HER2 (known as ErbB2 or neu), has been identified as one of the key oncogenes in invasive breast cancer.[4,5] HER2 gene amplification is found in 20–30% of all diagnosed breast cancer patients and correlates with a very poor prognosis. Another member of the EGFR family, HER3 (ErbB3), is consistently overexpressed together with HER2 in invasive breast cancer, and a correlation between expression of HER2/HER3 and progression from a noninvasive to an invasive stage has been shown.[6–8] HER3 is a receptor tyrosine kinase reported to have weak kinase activity.[9] Moreover, HER3 forms with HER2 a high-affinity complex for its natural ligand, HRG.[10] Upon HRG stimulation, HER2/HER3 heterodimers deliver the most potent and long-lasting proliferative signal among the possible combinations of pairs of EGFR family members. This characteristic is due to the ability of HER2/HER3, but not other combinations, to signal within the endosome after ligand-induced internalization.[11,12] Additionally, the most invasive breast cancer cell lines, which lack estrogen receptor and consequent responsiveness to hormone therapy, overexpress HRG in an autocrine manner.[13–16] Together, these data indicate that not HER2 alone but HER2/HER3 complexes play a crucial role in breast cancer progression. However, while HER2 was the objective of intensive anticancer drug-design studies, HER3, due to its reportedly impaired tyrosine kinase activity, has not been a primary target for pharmacologic research.

Antibodies have for many decades been anticipated as the ideal molecules for the therapy of cancer and infectious diseases.[17] Many therapeutic antibodies have been designed to exert their effect when coupled to immunotoxins, drugs or radioisotopes.[18,19] Meanwhile, the effector mechanism of some naked antibodies has been elucidated and proven to be of great clinical benefit. The development of a MAb against HER2, 4D5, and its subsequent humanized counterpart trastuzumab (HC), approved by the FDA in 1998, has been a fundamental achievement in the treatment of HER2-overexpressing metastatic breast carcinoma.[20–22] Multinational studies have employed HC in protocols of combined therapy, significantly prolonging the survival of patients.[23–25] Moreover, a second-generation antibody against HER2, 2C4 (pertuzumab), has been developed, which shows antitumor activity *in vitro* and *in vivo* against several breast and prostate tumor models that do not overexpress HER2.[26,27]

Our aim was to determine whether HER3 has a physiologically relevant HER2-independent role in transmitting proliferative and migratory signals in breast cancer cell lines. Therefore, we analyzed the inhibitory potential of α-HER3^{ECD} on breast cancer cell growth and motility and dissected the effects of α-HER3^{ECD} and HC on HRG-mediated signaling. We used breast cancer cell lines lacking HER2 amplification, which results in HER2 levels that are too low to confer HC sensitivity. Our data indicate that HER3 represents a potential novel therapeutic target in breast cancer treatment.

Material and methods

Media were purchased from GIBCO (Carlsbad, CA). FBS and collagen types I and IV were from Sigma (St. Louis, MO). Hybond ECL membranes and $^{[32]}$P-γ-ATP were purchased from Amersham (Piscataway, NJ). WT was from Calbiochem (San Diego, CA), and HC was from Roche (Indianapolis, IN). Antibodies raised against the following proteins were used: HER2 (mouse

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Abbreviations: BrdU, bromodeoxyuridine; DAPI, 4′,6-diamidino-2-phenylindole; ECL, enhanced chemiluminescence; EGFR, epidermal growth factor receptor; HC, Herceptin; HRG, Heregulin; HRP, horseradish peroxidase; IP, immunoprecipitation; JNK, c-jun-terminal kinase; MAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; PI_3-K, phosphatidylinositol-3-OH-kinase; PY, phosphotyrosine; TNF, tumor necrosis factor; WB, Western blotting; WT, wortmannin.

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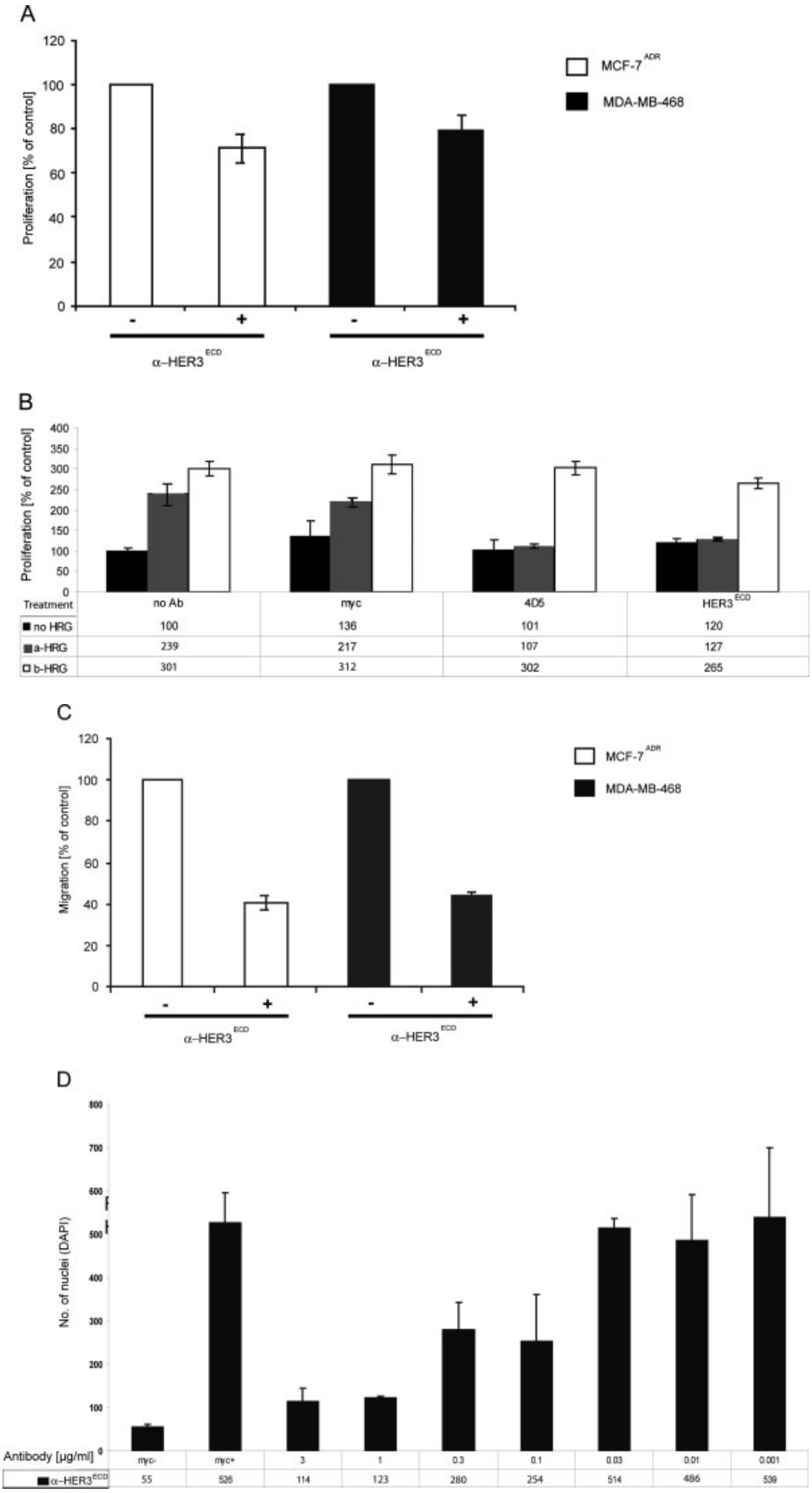
A
Proliferation [% of control]
120
100
80
60
40
20
0
MCF-7 ADR
MDA-MB-468
-
+
-
+
α–HER3 ECD
α–HER3 ECD
B
Proliferation [% of control]
400
350
300
250
200
150
100
50
0
Treatment | no Ab | myc | 4D5 | HER3 ECD
no HRG | 100 | 136 | 101 | 120
a-HRG | 239 | 217 | 107 | 127
b-HRG | 301 | 312 | 302 | 265
C
Migration [% of control]
120
100
80
60
40
20
0
MCF-7 ADR
MDA-MB-468
-
+
-
+
α–HER3 ECD
α–HER3 ECD
D
No. of nuclei (DAPI)
800
700
600
500
400
300
200
100
0
Antibody [µg/ml] | myc- | myc+ | 3 | 1 | 0.3 | 0.1 | 0.03 | 0.01 | 0.001
α–HER3 ECD | 55 | 526 | 114 | 123 | 280 | 254 | 514 | 486 | 539

FIGURE 1.

MAb 2-13D), HER3 (MAb 2F12; UBI, Lake Placid, NY), HER3 [MAb 105.5 (α-HER3^{ECD}), UBI], SHC (pAb28, MAb; Affiniti, Nottingham, UK), GRB2 (MAb 2F12, UBI), p85 (MAb UB93-3, UBI) and PY (MAb 4G10, UBI). HRP-coupled secondary antibodies were purchased from Bio-Rad (Hercules, CA). Transwell chambers (0.3 cm^2, 8 μm) were purchased from Costar (Cambridge, MA). Thin-layer chromatography plates (Silica Gel 60) precoated with oxalate were purchased from Merck (Darmstadt, Germany). Recombinant human β-HRG was purchased from R&D Systems (Minneapolis, MN). Recombinant human GST-HRG fusion protein (α-HRG), GST-GRB2 and GST-c-Jun were produced in *Escherichia coli* and purified as described[28] or using standard methods. Both α- and β-HRG were used according to the different sensitivities of the 2 cell lines, with 5 μg/ml α-HRG for MCF-7^{ADR} and 10 ng/ml β-HRG for MDA-MB-468 being optimal conditions. The cell line MDA-MB-468 and the hybridoma cell line 4D5 were obtained from the ATCC (Rockville, MD). MCF-7^{ADR} was obtained from the DKFZ (Heidelberg, Germany). All cell lines were cultured according to the suppliers' protocols.

WB

MCF-7^{ADR} and MDA-MB-468 cells were either left untreated or pretreated with HC (10 μg/ml), α-HER3^{ECD} (10 μg/ml) and WT (100 nM) for 30–60 min following stimulation with 5 μg/ml recombinant human HRG for 5 min at 37°C. After HRG stimulation, cells were lysed on ice in lysis buffer [50 mM HEPES (pH 7.5), containing 150 mM NaCl, 1 mM EDTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1 mM sodium fluoride, 1 mM PMSF, 2 mM sodium orthovanadate, 5 mM β-glycerolphosphate, 10 mg/ml aprotinin]. Crude lysates were centrifuged at 13,000*g* for 20 min at 4°C. For immunoprecipitations, the appropriate antibody and protein A-Sepharose (Pharmacia, Piscataway, NJ) were added to the cleared lysate and incubated for 3 hr at 4°C. Immunoprecipitates were washed with washing buffer [20 mM HEPES (pH 7.5), containing 150 mM NaCl, 1 mM EDTA, 1 mM sodium fluoride, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100]. Sample buffer containing SDS and 2-mercaptoethanol was added, and samples were denatured by heating at 95°C for 4 min. Proteins were fractionated by SDS-PAGE and electrophoretically transferred to nitrocellulose filters. For immunoblot analysis, nitrocellulose filters were first incubated with mouse MAb or rabbit polyclonal primary antibody for 3 hr at 4°C. Next, HRP-coupled goat antimouse or goat antirabbit secondary antibody was added, followed by an ECL substrate reaction (Amersham). The substrate reaction was detected on Kodak (Rochester, NY) X-Omat film. Filters used more than once with different antibodies were stripped according to the manufacturer's protocol, blocked and reprobed.

Where indicated in the text, percent reduction was calculated by quantitation of the bands using IMAGE software (Scion, Frederick, MD).

In vitro *kinase assay*

JNK and PI$_3$-K assays were performed as described previously.[29,30]

Cell surface biotinylation

Cell surface biotinylation was essentially performed as described.[31]

Proliferation and migration assay

Proliferation was measured for the indicated time points either with a BrdU incorporation assay (Roche) or with an alamar blue (Biosource, Camarillo, CA) proliferation assay, according to the manufacturer's protocol. Migration assay (chemotaxis) was performed as follows. Briefly, 1 to 2 × 10^5 serum-starved cells were plated on transwell chambers precoated with 4 μg collagen type 4 or 3 μg collagen type 1. Either conditioned NIH-3T3 medium or, where indicated, β-HRG was used as a chemoattractant.[32] Following 8–16 hr of incubation, nonmigrating cells were removed with cotton swabs and invading cells were fixed and stained with either crystal violet or DAPI. Crystal violet–stained cells were counted under bright-field illumination, whereas DAPI-stained cell nuclei were counted under fluorescence illumination using an Axiovert-135 inverted microscope (Zeiss, Thornwood, NY). Counts from 4 filters for each strain were pooled and compared among different strains using ANOVA.

Results

We analyzed the effects of a MAb that specifically antagonizes HRG binding to HER3.[33] Cell lines MCF-7^{ADR} and MDA-MB-468 were chosen on the basis of their lack of HER2 overexpression, their different relative ratios of HER2:HER3 and their inherent migratory properties, with MDA-MB-468 being the most invasive cell line. HER2:HER3 ratios were about 1:3 and 3:1 in MDA-MB-468 and MCF-7^{ADR}, respectively, based on WB (not shown).

Pretreatment with α-HER3^{ECD} decreases the migratory and proliferative potential of breast cancer cells

We asked whether HER3 crosslinking at the surface of living breast cancer cells could influence their functional properties. We therefore compared the proliferative and migratory capacity of MCF-7^{ADR} and MDA-MB-468 cells pretreated for 1 hr with α-HER3^{ECD} to those of mock-treated controls. As shown in Figure 1*a,* pretreatment with α-HER3^{ECD} caused, after 24 hr, a 20% reduction in the proliferation of both cell lines, as assessed by BrdU incorporation. We then repeated the experiment using metabolic activity as a read-out (see Material and methods). We included α-myc antibody as a negative control and 4D5 (the mouse homologue to trastuzumab) as a positive control and measured proliferation for 72 hr in the absence or presence of α- and β-HRG, respectively (Fig. 1*b*). In the presence of α-HRG, 4D5 and α-HER3^{ECD} reduced proliferation by 94% and 81% ($p < 0.001$), respectively, whereas α-myc showed no inhibitory effect. In the presence of β-HRG, α-HER3^{ECD} exhibited an inhibitory effect of 20% on proliferation in MCF-7 cells ($p < 0.001$), whereas 4D5 was ineffective. An even stronger inhibitory effect of α-HER3^{ECD} was observed on cell migration in response to chemoattractants (see Material and methods), which was reduced by 60% in both cell lines (Fig. 1*c*). We then explored the inhibitory potential of α-HER3^{ECD} on β-HRG-dependent migration

FIGURE 1 – The proliferative and migratory properties of breast cancer cell lines are inhibited by α-HER3^{ECD}. *(a)* Serum-starved MCF-7^{ADR} and MDA-MB-468 cells were pretreated with α-HER3^{ECD} for 1 hr and subsequently stimulated with HRG for 16 hr. BrdU incorporation was determined as described. Results are the average of triplicate samples from at least 4 independent experiments. *(b)* MCF-7 cells were seeded in FCS-containing medium on 96-well plates overnight. Cells were preincubated in quadruplicate with the indicated antibodies diluted in medium without FCS for 1 hr at 37°C and then stimulated with 30 ng/ml α- or 20 ng/ml β-HRG. After 72 hr, alamar blue was added, the plate was incubated at 37°C in the dark and absorbance was measured at 590 nm. Comparisons were made using one-way ANOVA, with $p < 0.05$ being considered statistically significant. *(c)* Serum-starved MCF-7^{ADR} and MDA-MB-468 cells were pretreated with either α-myc (negative control) or α-HER3^{ECD} for 1 hr, detached and transferred to the upper well of a Boyden chamber. Chemotaxis assay was carried out for 16 hr. Migrating cells present on the lower side of the membrane were stained and counted. Comparisons were made using Student's *t*-test, with $p < 0.05$ being considered statistically significant. *(d)* Serum-starved MCF-7 cells were preincubated by adding the indicated amount of antibody to the cell suspension for 45 min at 37°C. Cells were then placed in the top chamber of collagen 1-coated transwells. Medium alone or containing the ligand β-HRG was used in the bottom chamber as a chemoattractant. Cells were left to migrate for 8 hr at 37°C, stained with DAPI and counted. Results are the average of triplicate samples of counted DAPI-positive cells from 2 independent experiments. All data are expressed as means ± SD, represented as error bars.

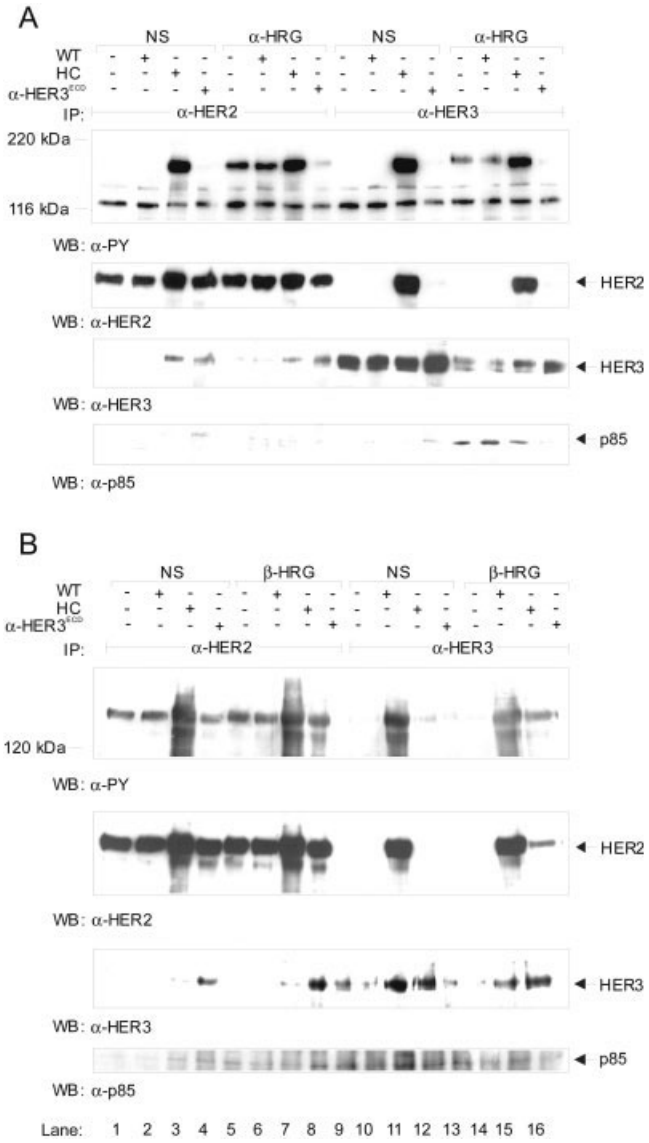

FIGURE 2 – α-HER3^{ECD} interferes with receptor tyrosine phosphorylation and dimerization of HER3 and HER2. Serum-starved breast cancer cells, *(a)* MCF-7^{ADR} and *(b)* MDA-MB-468, were pretreated with 10 μg/ml of either HC or α-HER3^{ECD} for 60 min. Cell stimulation with HRG was carried out for 5 min (see Material and methods). Cell lysates were subjected to IP using anti-HER2 (α-HER2) or anti-HER3 (α-HER3) MAbs. The level of tyrosine phosphorylation was analyzed by WB with anti-PY MAb (α-4G10, upper panels). Reblotting with α-HER2 and α-HER3 antibodies shows equal protein loading (middle and bottom panels). NS, nonstimulated cells; arrow, coprecipitating proteins.

(Fig. 1*d*). Since it has been shown that active migration may depend on protein, but not DNA, synthesis[34,35] and to exclude the possibility of measuring inhibition of proliferation, we used serum-starved MCF-7 cells, titrated with different concentrations of α-HER3^{ECD}, and measured their migratory capabilities after 8 hr (Fig. 1*d*). Compared to α-myc-treated MCF-7 cells, α-HER3^{ECD} inhibited β-HRG-mediated migration by 80%. A reduction of 50% was observed between 100 and 300 ng/ml of α-HER3^{ECD}, whereas the inhibitory effect was lost at 30 ng/ml. Taken together, the data show that α-HER3^{ECD} inhibits proliferative and migratory properties of tumor cells, irrespective of the absence or presence of HRG.

HER3 crosslinking by α-HER3^{ECD} decreases receptor tyrosine phosphorylation

To investigate the molecular basis of the inhibitory effects of α-HER3^{ECD} on cell migration and proliferation, we analyzed the effects of α-HER3^{ECD} pretreatment on the tyrosine phosphorylation state of the HER2 and HER3 receptors with and without stimulation with the specific ligand HRG. The effect of pretreatment with α-HER3^{ECD} was compared to that of pretreatment with HC. Receptor immunoprecipitation was performed and the PY content of HER2 and HER3 analyzed by WB with an anti-PY antibody. As shown in Figure 2*a,* pretreatment of MCF-7 cells with α-HER3^{ECD} modestly decreased the tyrosine phosphorylation level of HER3 and HER2 (3% and 13% of control, respectively) after HRG stimulation. In MDA-MB-468, where HER2 is more abundant than HER3, pretreatment with α-HER3^{ECD} reduced tyrosine phosphorylation of HER2 by 84%, while an increase of HER3 tyrosine phosphorylation and of HER2/HER3 association could be observed (Fig. 2*b,* lanes 8 and 16). In contrast, HC upregulated receptor tyrosine phosphorylation and promoted the association of HER3 and HER2, independent of HRG stimulation, in both cell lines (Fig. 2*a,b*). These results show that HER2/HER3 receptor signaling was significantly modified by α-HER3^{ECD}.

HER3 crosslinking interferes with activation of downstream targets

We subsequently asked whether α-HER3^{ECD} has an effect on the known substrates of HER3, namely SHC and PI$_3$-K, which are, respectively, effector proteins responsible for MAPK cascade activation and lipid signaling.[12,28] To address this question, we immunoprecipitated SHC and the regulatory subunit of PI$_3$-K, p85, under the experimental conditions described above and assessed the tyrosine phosphorylation of these effectors. As shown in Figure 3, α-HER3^{ECD} significantly decreased the tyrosine phosphorylation of SHC (lower arrow) after HRG stimulation in MCF-7^{ADR} and MDA-MB-468 (49% and 66% of control, respectively; Fig. 3*a,b,* compare lanes 10 and 12). Interestingly, the association of SHC with the tyrosine-phosphorylated receptors (upper arrow) was strongly inhibited (37% of control) in MCF-7^{ADR} cells (Fig. 3*a*) but less so in MDA-MB-468 (82% of control) (Fig. 3*b*). The amount of immunoprecipitated SHC did not significantly change under these experimental conditions (Fig. 3*a,b,* bottom panels). Immunoprecipitates of the regulatory subunit of PI$_3$-K yielded similar results. Binding of tyrosine-phosphorylated HER3 to p85 was decreased in MCF-7^{ADR} while an increase was observed in MDA-MB-468 (Fig. 3*a,b,* lanes 4 and 6). HC enhanced binding of the effector proteins SHC and PI$_3$-K in both cell lines (lane 5). These data show that α-HER3^{ECD} interferes with SHC function by inhibiting its tyrosine phosphorylation. The association of SHC and PI$_3$-K with HER3 is also substantially modified by α-HER3^{ECD} with respect to control treatment in both cell lines: in MCF-7^{ADR}, where HER3 is more abundant than HER2, the association with the effectors is decreased, while in MDA-MB-468, where HER3 is present at a lower relative amount than HER2, α-HER3^{ECD} promotes the association of SHC and PI$_3$-K under both resting and stimulated conditions.

Since SHC associates with the adaptor molecule GRB2 after HRG stimulation, we explored the effect of the reduced phosphorylation of SHC in MCF-7 cells by measuring GRB2 binding (Fig. 3*c*). We performed GST-pulldown assays in cells using GST-GRB2 fusions and the same experimental design as before. Indeed, the reduced tyrosine phosphorylation of SHC observed in MCF-7^{ADR} resulted in decreased binding of GRB2 to SHC (Fig. 3*c,* bottom panel, compare lanes 4 and 6) and complete inhibition of its association with HER2 (Fig. 3*c,* middle panel).

α-HER3^{ECD} decreases JNK and PI$_3$-K signaling

The adaptor protein SHC regulates MAPK signaling pathways downstream of growth factor receptors, activating JNK and ERK2, respectively.[36,37] To investigate the effect of α-HER3^{ECD}

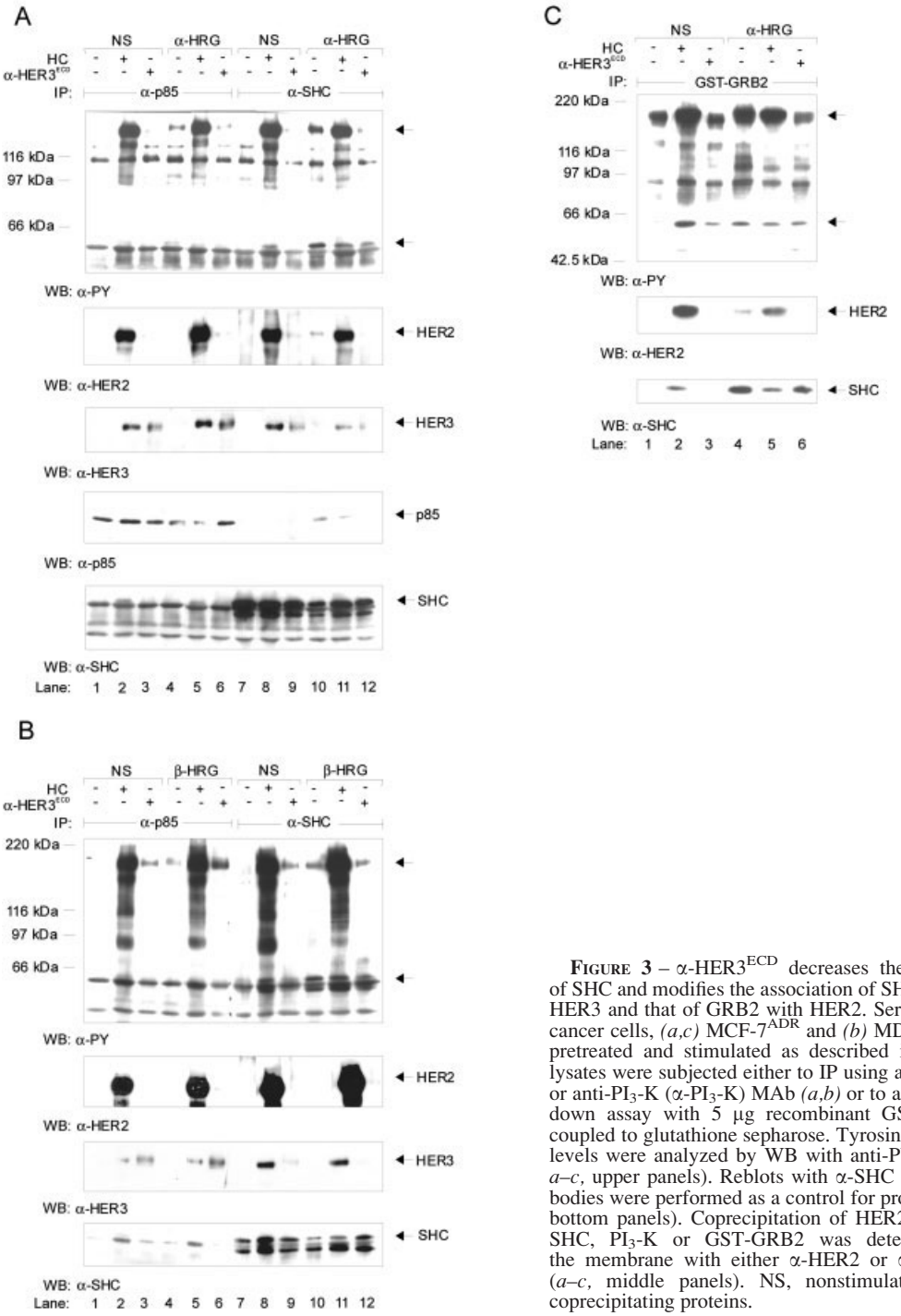

FIGURE 3 – α-HER3^{ECD} decreases the phosphorylation of SHC and modifies the association of SHC and PI_3-K with HER3 and that of GRB2 with HER2. Serum-starved breast cancer cells, *(a,c)* MCF-7^{ADR} and *(b)* MDA-MB-468, were pretreated and stimulated as described in Figure 1. Cell lysates were subjected either to IP using anti-SHC (α-SHC) or anti-PI_3-K (α-PI_3-K) MAb *(a,b)* or to a GST-GRB2 pull-down assay with 5 μg recombinant GST-GRB2 protein coupled to glutathione sepharose. Tyrosine phosphorylation levels were analyzed by WB with anti-PY MAb (α-4G10; *a–c,* upper panels). Reblots with α-SHC and α-PI_3-K antibodies were performed as a control for protein loading (*a,b,* bottom panels). Coprecipitation of HER2 and HER3 with SHC, PI_3-K or GST-GRB2 was detected by probing the membrane with either α-HER2 or α-HER3 antibody (*a–c,* middle panels). NS, nonstimulated cells; arrow, coprecipitating proteins.

on MAPK signaling, we performed kinase assays in MCF-7^{ADR} and MDA-MB-468 cells under the experimental conditions described above. Pretreatment with α-HER3^{ECD} caused a decrease of JNK activity in both cell lines (Fig. 4*a,b,* compare lanes 5 and 8). Despite the lower inhibitory effect on the upstream signals, the ability of α-HER3^{ECD} to decrease JNK activity was stronger in MDA-MB-468 than in MCF-7^{ADR}. HC, however, only reduced JNK activity in MCF-7^{ADR} (Fig. 4*a*). ERK2 activity was also significantly decreased (data not shown).

Since involvement of PI_3-K in carcinoma invasion has been demonstrated,[38] we investigated the inhibitory properties of α-HER3^{ECD} on PI_3-K activity (Fig. 4*a,b,* bottom panels). In both cell lines, α-HER3^{ECD} resulted in a strong decrease of PI_3-K activity upon HRG stimulation. In MDA-MB-468, HC exerted an even greater inhibitory effect on PI_3-K activity than α-HER3^{ECD}, which could be due to the 3-fold higher expression of HER2. WT, a widely used inhibitor of PI_3K, was also used as a control pretreatment (Fig. 4*a,b,* lanes 2 and 6).

Taken together, these results have potential therapeutic significance because they show that α-HER3^{ECD} differentially modifies early signaling events (*i.e.,* receptor phosphorylation, PI_3-K and SHC association) according to the HER2:HER3 ratio but invariably decreases the activity of downstream effectors regardless of this ratio.

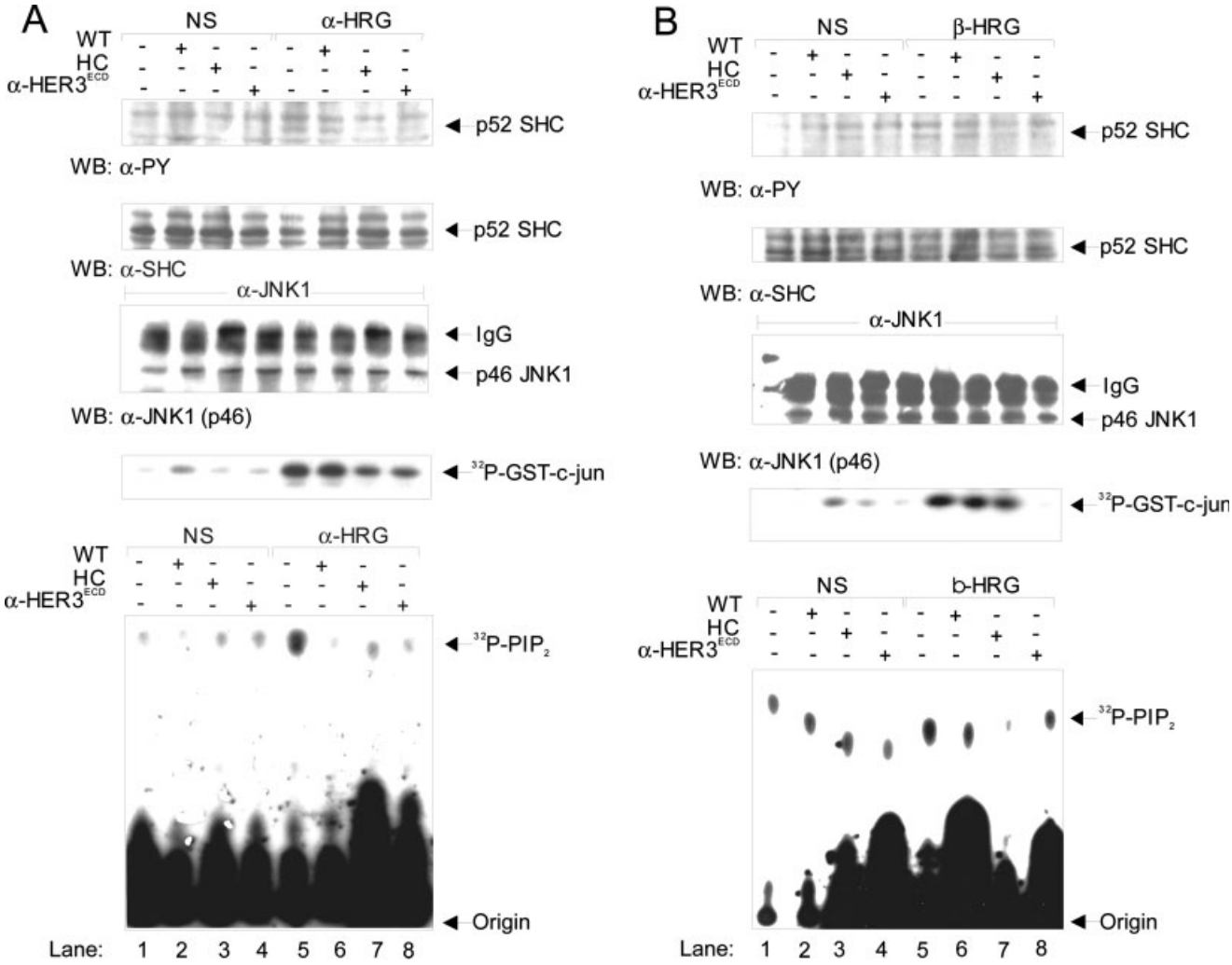

FIGURE 4 – α-HER3^{ECD} inhibits JNK1 and PI$_3$-K activity. Serum-starved breast cancer cells, *(a)* MCF-7^{ADR} and *(b)* MDA-MB-468, were pretreated with 10 μg/ml of either HC or α-HER3α for 60 min or with 100 nM/ml WT for 30 min and stimulated as described in Figure 1. Cell lysates were used for IP with anti-JNK-1 (α-JNK-1) polyclonal antibody or α-4G10 MAb, and PI$_3$-K assays were performed (see Material and methods). Whole-cell lysates were additionally probed with α-PY antibody to confirm the effect of α-HER3^{ECD} on SHC tyrosine phosphorylation and, after stripping, reprobed with α-SHC antibody to determine equal loading of proteins (*a–c,* middle and upper lower panels). α-JNK1 immunocomplexes were subjected to JNK kinase assays (*a–c,* upper panels). Phosphorylated c-jun is indicated by an arrow. An anti-JNK1 antibody (α-JNK1) was used to confirm equal protein loading. PI$_3$-K activity is strongly diminished after α-HER3^{ECD} pretreatment, due to the inhibition of PI$_3$-K binding to HER3 (*a–c,* lower panels). Phosphorylated phosphatidylinositol is indicated by an arrow.

α-*HER3^{ECD} interferes with receptor recycling*

We then tried to dissect the molecular mechanisms leading to α-HER3^{ECD}-induced inhibition of HER3 tyrosine phosphorylation. HER2 and HER3 are endocytosed and recycled after HRG stimulation.[12,39] We hypothesized that α-HER3^{ECD} could interfere with receptor recycling; thus, we determined the surface expression of HER3 after incubation with α-HER3^{ECD} for different time periods and subsequent stimulation with HRG for 5 min. To selectively analyze the number of HER3 molecules at the cell surface, the monolayer was biotinylated and the anti-HER3 immunoprecipitates were probed with streptavidin. In parallel, the PY content was assessed by WB in whole-cell lysates.

As shown in Figure 5*a* (upper panel), α-HER3^{ECD} caused a long-lasting decrease in the surface expression of HER3 (18% of control). The PY content of the receptors after HRG stimulation was, at all pretreatment time points, indistinguishable from that of control untreated cells (Fig. 5*a,* lower panel). Treatment with HC had strikingly different effects on HER2 recycling, resulting in strong stabilization of HER2 at the cell surface and a high level of tyrosine phosphorylation (Figs. 2*a*,5*b*). For comparison, we treated cells for equivalent time periods with the physiologic ligand HRG. This treatment reduced the amount of HER3 on the cell surface (though a relative increase in surface HER3 was reproducibly observed after 2 hr incubation; Fig. 5*a,* lane 9), but the receptors, as expected, were still highly phosphorylated after 2 hr of incubation with the ligand. The effects of HRG pretreatment on HER2 downregulation were modest, and a significant fraction of the receptor molecules were still on the cell surface after 3 hr of treatment (Fig. 5*b,* lanes 8–10). Mechanistically, we hypothesize that the strong general inhibitory effects of α-HER3^{ECD} on HRG signaling could be due, at least in part, to increased HER3 endocytosis, which prevents the receptor from forming active signaling complexes with HER2 at the cell surface.

Discussion

HER2/HER3 heterodimers are fundamental regulators of breast cancer cell growth.[15,16,40] Clinical studies have shown that overex-

pression of HER2 and HER3 is associated with lack of response to hormonal therapy, high malignancy and poor prognosis.[4,41] The most invasive breast cancer cell lines, however, lack estrogen receptor and endogenously express HRG, which promotes their growth through an autocrine loop.[13–16] Our results indicate that an antibody directed against the extracellular domain of HER3 efficiently decreases the tyrosine phosphorylation of HER2 after HRG stimulation in the breast cancer cell lines MCF-7^{ADR} and MDA-MB-468. The antibody treatment inhibits the activation of downstream effectors and ultimately limits the proliferation and motility properties of the cells. α-$HER3^{ECD}$ accelerates the endocytosis of HER3, resulting in clearance of HER3 from the cell surface and potentially preventing HRG-mediated HER2/HER3 heterodimerization. The inhibitory effects of α-$HER3^{ECD}$ are strikingly potent; this antibody counteracts the activity of the HER2/HER3 complex, the most active signal transducer among the EGFR family members, characterized by a slow endocytosis rate and the ability of signaling throughout the internalization process. The active mechanism of the therapeutic anti-HER2 antibody HC is, in our cell system, substantially different since HC induces hyperphosphorylation of HER2 and does not abrogate the transphosphorylation activity of HER3 by HER2 after HRG stimulation. HC has been reported to sensitize breast cancer cells to TNF-α, Trance and various chemotherapeutics and to induce cell cycle arrest due to $p27^{kip}$ expression.[42–44] Moreover, Nagata *et al*.[45] have suggested that PTEN activation contributes to trastuzumab's antitumor activity, thereby explaining the therapeutic efficacy of trastuzumab. They showed that trastuzumab treatment increased PTEN membrane localization and its phosphatase activity by reducing PTEN tyrosine phosphorylation *via* Src inhibition. Still, these effects are not sufficient to provide tumor clearance in living organisms. Clynes *et al*.[46] showed that the primary effect of HC is to stimulate the immune system by binding to the FcγR receptor. However, inhibition of the growth and invasion promoting signals within the tumor cell is predicted to increase the chances of the immune system to efficiently fight the malignancy. Given that the inhibitory effects of α-$HER3^{ECD}$ on HER2/HER3 signaling are stronger than those of HC, the former antibody could be an even more effective chemotherapeutic agent, provided that it has an equivalent ability to activate the effector cells of the immune system.

An important feature of α-$HER3^{ECD}$ is its ability to completely block JNK activity (Fig. 4). JNK is involved in the control of cell migration,[47] and several lines of evidence have also demonstrated the specific importance of JNK signaling in cancer cell motility and growth.[48] Very invasive breast cancer cell lines were shown by microarray analysis to overexpress JNK.[49] Additionally, the prolylisomerase PIN1, overexpressed in breast cancer, cooperates with Ras in enhancing JNK activity; this, in turn, controls the cyclin D1 promoter and increases transcription of cyclin D1.[50] Cyclin D1 is overexpressed in >50% of diagnosed breast carcinomas and has a causative role in the pathogenesis of breast cancer induced by *neu,* the oncogenic variant of HER2.[51] α-$HER3^{ECD}$ abrogates JNK activity more efficiently than HC by interfering with SHC binding to the C-terminal domain of HER3. Both α-$HER3^{ECD}$ and HC, however, efficiently inhibit PI_3-K activity, another important determinant of the proliferation and invasiveness of breast cancer cells. The generation of the new anti-HER2 antibody 2C4 (pertuzumab) has opened new avenues in antibody-mediated cancer therapy. 2C4 inhibits ligand-dependent HER2 signaling in both low- and high-HER2-expressing systems, thereby increasing the potential of HER2-targeted therapy.[26,27] The cell lines used in our study do not have HER2 amplification, which is representative of the majority of human breast cancers. However, although they are characterized by a different HER2:HER3 ratio, namely 1:3 for MCF-7^{ADR} and 3:1 for MDA-MB-468, α-$HER3^{ECD}$ effectively inhibited receptor tyrosine phosphorylation, proliferation and migration in both cases. This finding is of potential significance for the prospective therapeutic use of anti-HER3 antibodies in addition to anti-HER2 antibodies because beneficial effects could be expected in a wide spectrum of clinical cases presenting different relative amounts of HER2 and HER3. It would be most interesting to see if the α-$HER3^{ECD}$ *in vitro* data can be translated to an *in vivo* proof-of-concept experiment. Currently, more efficient blocking MAbs against HER3 are being generated and will be tested in an *in vivo* setting in the future.

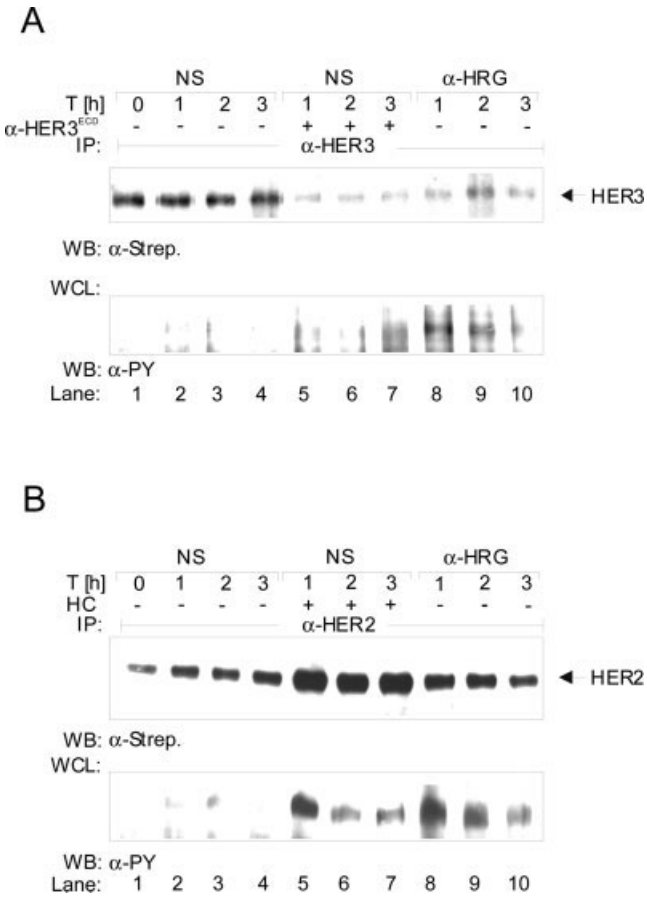

Figure 5 – Effect of α-$HER3^{ECD}$ and HC on the endocytosis of HER3 and HER2 after HRG stimulation. *(a,b)* Serum-starved MCF-7^{ADR} cells were either incubated at 4°C with 10 μg/ml HC, 10 μg/ml α-$HER3^{ECD}$ or 5 μg/ml α-HRG for 1 hr, then washed 2 times with PBS and incubated at 37°C for the indicated time points. Antibody-treated cells were subsequently stimulated with 5 μg/ml HRG for 5 min. At the end of incubation, cells were biotinylated and lysed (see Material and methods). Cell lysates were subjected to IP using anti-HER2 (α-HER2) or anti-HER3 (α-HER3) MAb (*a,b,* upper panel). Tyrosine phosphorylation level of whole-cell lysates was analyzed by WB with anti-PY MAb (α-4G10; *a,b,* lower panels). NS, nonstimulated cells.

Taken together, our data show that HER3, in association with HER2, plays a fundamental role in the control of breast cancer cell growth and invasiveness. Moreover, HER3 emerges as an important determinant of breast cancer cell behavior as well as a target for drug design. Our findings provide a strong biologic basis for the idea that counteracting aberrant HER3 signaling could be a primary objective in antibody-based breast cancer therapy.

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