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Tumor and Stem Cell Biology

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Heterotrimerization of the Growth Factor Receptors erbB2, erbB3, and Insulin-like Growth Factor-I Receptor in Breast Cancer Cells Resistant to Herceptin

Xiaoping Huang[1], Lizhi Gao[1], Shuiliang Wang[1], James L. McManaman[2], Ann D. Thor[1], XiaoHe Yang[3], Francisco J. Esteva[4], and Bolin Liu[1]

Abstract

Primary and acquired resistance to the breast cancer drug trastuzumab (Herceptin) is a significant clinical problem. Here, we report enhanced activation of downstream signaling pathways emanating from the growth factor receptors erbB2, erbB3, and insulin-like growth factor-I receptor (IGF-IR) in trastuzumab-resistant breast cancer cells. Interactions between IGF-IR and erbB2 or erbB3 occur exclusively in trastuzumab-resistant cells, where enhanced erbB2-erbB3 interactions are also observed. Moreover, these three receptors form a heterotrimeric complex in resistant cells. erbB3 or IGF-IR knockdown by short hairpin RNA–mediated strategies upregulates $p27^{kip1}$, inactivates downstream receptor signaling, and resensitizes resistant cells to trastuzumab. Our findings reveal a heterotrimer complex with a key role in trastuzumab resistance. On the basis of our results, we propose that trastuzumab resistance in breast cancer might be overcome by therapeutic strategies that jointly target erbB3, erbB2, and IGF-IR. *Cancer Res; 70(3); 1204–14. ©2010 AACR.*

Introduction

Trastuzumab (Herceptin), a humanized monoclonal antibody (mAb) against erbB2 receptor, was the first erbB2-targeted therapy approved by the Food and Drug Administration (1). Trastuzumab has been successfully used in early-stage and metastatic breast cancer therapy of patients with erbB2-overexpressing tumors as monotherapy (2, 3) and in combination with other agents (4–6). Unfortunately, not all breast cancer patients whose tumors overexpress erbB2 respond to trastuzumab, and the majority of patients who achieve an initial response to trastuzumab-based regimens develop resistance within 1 year (7, 8). These observations indicate that both primary and acquired resistances to trastuzumab are common.

Several mechanisms contributing to trastuzumab resistance have been reported (9–11). These can be divided into two general categories. (*a*) Inability or reduced capacity of trastuzumab binding to erbB2—trastuzumab is unable to interfere with the erbB2 heterodimers by epidermal growth factor receptor (EGFR) or erbB3 (12–15). Elevated expression of membrane-bound glycoprotein mucin-4 directly interacts with erbB2 and sterically hinders erbB2 from binding to trastuzumab (16, 17). A truncated form of erbB2 receptor (called p95ErbB2) through either an alternative initiation of protein translation or cleavage by proteases does not bind to trastuzumab (18, 19). (*b*) Activation of downstream signaling pathways leads to trastuzumab resistance. Studies have identified the phosphoinositide 3-kinase (PI3K)/Akt signaling as the major determinant of trastuzumab resistance (20, 21). PTEN activation is critical for trastuzumab-induced tumor inhibition, and loss of PTEN function predicts trastuzumab resistance (7, 22). As an important tumor suppressor, PTEN antagonizes PI3K function and negatively regulates Akt activities (23). The fact that loss of PTEN function leads to activation of PI3K/Akt signaling further emphasizes the importance of PI3K/Akt pathway in trastuzumab resistance. In addition, reduced cyclin-dependent kinase (CDK) inhibitor $p27^{kip1}$ expression and elevated CDK2 activity have been reported in trastuzumab-resistant breast cancer cells (24). Cellular localization of $p27^{kip1}$ might be important, as trastuzumab-resistant cells showed loss of nuclear $p27^{kip1}$ (25). The receptor tyrosine kinase (RTK) Met activation or overexpression protects erbB2-overexpressing breast cancer cells against trastuzumab (26). Trastuzumab-induced growth inhibition was lost in breast cancer cells that overexpressed both insulin-like growth factor-I receptor (IGF-IR) and erbB2 (27). However, the expression of IGF-IR per se does not predict trastuzumab resistance in erbB2-overexpressing breast cancer patients (28), suggesting that activation of IGF-IR signaling may be more important than its expression for development of resistance to trastuzumab. Indeed, IGF-I signaling

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resulted in elevated expression of the $p27^{kip1}$ ubiquitin ligase SKP2, leading to decreased $p27^{kip1}$ and loss of growth arrest in the presence of trastuzumab (29). Furthermore, cross-talk occurs between IGF-IR and erbB2, and IGF-IR physically interacts with erbB2 and induces activation of erbB2 in trastuzumab-resistant, but not trastuzumab-sensitive, breast cancer cells (30).

Given the reportedly complex interactions between the RTK ligands, receptors, and signaling pathways, it is perhaps not surprising that erbB2-overexpressing breast cancers have shown variable responses to trastuzumab. Overexpression of other erbB receptors may be one mechanism of trastuzumab resistance (31–33). Thus, novel strategies simultaneously targeting multiple receptors or signaling pathways have drawn great interest for breast cancer therapy. Coexpression of erbB3 and erbB2 is frequently observed in breast cancers (34) and cell lines (35). erbB2 requires erbB3 to promote breast cancer cell proliferation (36), and erbB3 plays a critical role in breast cancer development driven by *erbB2* amplification/overexpression (37). Elevated expression of erbB3 and its association with erbB2 promote mammary tumorigenesis in c-*neu* transgenic models (38, 39). We have recently shown that erbB3 contributes to erbB2-mediated antiestrogen resistance (40). Ligand-induced dimerization between erbB3 and erbB2 is one likely mechanism of trastuzumab resistance (41). Considering the importance of both IGF-IR and erbB3 in erbB2-mediated breast cancer development, it is conceivable to predict that cotargeting IGF-IR and erbB3 signalings may ultimately enhance the therapeutic efficacy of trastuzumab in erbB2-overexpressing breast cancers; however, the relationship between erbB3 and IGF-IR in trastuzumab resistance remains unknown. Our current studies suggest that both erbB3 and IGF-IR work cooperatively with erbB2, and these three RTKs may interact with each other to form more complicated, multiple receptor complexes, which promote activation of the downstream signalings contributing to trastuzumab resistance.

Materials and Methods

Reagents and antibodies. Trastuzumab (Herceptin, Genentech, Inc.) was obtained from the University of Colorado Hospital pharmacy. MISSION Non-target short hairpin RNA (shRNA), which does not target human and mouse genes, control vector (pLKO.1-ConshRNA), pLKO.1 containing human IGF-IR shRNA (pLKO.1-IGF-IRshRNA), and pLKO.1 containing human *erbB2* shRNA (pLKO.1-ErbB2shRNA) were purchased from Sigma. pLKO.1 containing human *erbB3* shRNA (pLKO.1-ErbB3shRNA) and lentivirus packaging plasmids pCMV-VSVG and pCMV-ΔA.9 were kindly provided by Dr. Haihua Gu (Department of Pathology, University of Colorado Denver).

Antibodies were from the following sources: erbB2 (EMD Biosciences); erbB3 polyclonal antibody (Abcam); erbB3 and phosphorylated erbB2 (P-erbB2) mAbs (LabVision); IGF-IRβ, phosphorylated erbB3 (P-erbB3), phosphorylated Src (P-Src; Tyr^{416}), Src, caspase-8, caspase-9, caspase-3, phosphorylated mitogen-activated protein kinase (P-MAPK), MAPK, Akt, and phosphorylated Akt (P-Akt; Ser^{473}; Cell Signaling Technology); $p27^{kip1}$ (Santa Cruz Biotechnology); poly(ADP-ribose) polymerase (PARP; BIOMOL Research Laboratories); and β-actin (Sigma). All other reagents were purchased from Sigma unless otherwise specified.

Cells and cell culture. Human breast cancer cell lines SKBR3 and BT474 were obtained from the American Type Culture Collection and maintained in DMEM/F-12 (1:1) medium (Sigma) containing 10% fetal bovine serum (Sigma). Both cell lines and their trastuzumab-resistant sublines were cultured in a 37°C humidified atmosphere containing 95% air and 5% CO_2 and split twice a week.

Production of lentivirus containing specific shRNA. The lentiviral vector pLKO.1-ConshRNA, pLKO.1-ErbB3shRNA, pLKO.1-IGF-IRshRNA, or pLKO.1-ErbB2shRNA and lentivirus packaging plasmids pCMV-VSVG and pCMV-ΔA.9 were cotransfected into 293T cells with FuGene 6 (Roche Diagnostics). After 24 h, the culture media were replaced with fresh medium. The virus in conditioned medium was harvested in 3 consecutive days and filtered with low-protein binding filters (Millex-HV, 0.45-mm polyvinylidene difluoride; Millipore Corp.) before they were aliquoted and stored at −80°C freezer.

Gene silencing with the lentivirus encoding specific shRNA. Before infection, the ConshRNA, ErbB3shRNA, IGF-IRshRNA, or ErbB2shRNA lentivirus-containing media (5 mL) were thawed completely at room temperature. Another 5 mL of fresh medium containing polybrene (8 μg/mL) were added into the virus-containing media, which were used to replace the culture media of interested cells. After 24 h, the virus-infected cells were selected by puromycin (1 μg/mL) for 48 h and subjected to required assays.

Immunoprecipitation and Western blot analysis. Immunoprecipitation and Western blot assays were performed as described (39). Briefly, equal amounts of cell lysates were incubated with primary antibody for 2 h at 4°C followed by incubation with protein A or G–agarose (Roche Diagnostics) at 4°C overnight. The immunoprecipitates or equal amounts of cell lysates were boiled in SDS sample buffer, resolved by SDS-PAGE, transferred to nitrocellulose (Bio-Rad), and probed with primary antibody. After the blots were incubated with horseradish peroxidase–labeled secondary antibody (Jackson ImmunoResearch), the signals were detected using the enhanced chemiluminescence reagents (Amersham Life Science).

Detection of protein complex with native PAGE analysis. Breast cancer cells were cultured in the absence of trastuzumab for 24 h. After collection and lysis, equal amounts of cell lysates were prepared in sample buffer without SDS and resolved by a native PAGE in Tris-glycine running buffer without SDS. After transferring proteins from the native PAGE to nitrocellulose membranes, the membranes were probed with primary antibody as indicated in the figure legends and followed by Western blot analysis.

Cell proliferation assay. The CellTiter96 AQ Non-Radioactive Cell Proliferation kit (Promega) was used to determine cell viability as described (39, 40, 42). Briefly, cells were plated

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onto 96-well plates for 24 h. Cells were then grown in either control medium or the same medium containing trastuzumab and incubated for another 72 h. After reading at 490 nm with a microplate reader, the percentages of surviving cells from each group relative to controls, defined as 100%, were determined by reduction of MTS.

Quantification of apoptosis. An apoptosis ELISA kit (Roche Diagnostics) was used to quantitatively measure cytoplasmic histone-associated DNA fragments as previously reported (40).

Statistical analyses. Statistical analyses of all experimental data were performed using a two-sided Student's t test. Significance was set at $P < 0.05$.

Results

Coexpression of three RTKs—erbB2, erbB3, IGF-IR—and their interactions in trastuzumab-sensitive and trastuzumab-resistant breast cancer cells. To investigate the molecular mechanisms of trastuzumab resistance, we focused on the role of erbB3 and its relationship with IGF-IR in trastuzumab-resistant breast cancer cells. SKBR3 is a trastuzumab-sensitive breast cancer cell line, and its resistant subline SKBR3-pool2 (pool2) was developed through continuously exposing SKBR3 cells to trastuzumab (24). BT474 is another trastuzumab-sensitive cell line, and we have developed its resistant subline BT474-HR20 (HR20) through continuously culturing BT474 cells by gradually increasing the concentrations of trastuzumab for 4 months. Both pool2 and HR20 cells are now maintained well in the presence of 20 μg/mL trastuzumab. We first performed cell proliferation (MTS) assays and were able to confirm the resistant phenotype of pool2 and HR20 cells (Fig. 1A). We then used Western blots to generate the expression profile of RTKs and to examine the downstream signaling in these breast cancer cells (Fig. 1B). In general, the levels of erbB2, erbB3, and IGF-IR were similar in all lines, although erbB3 was slightly higher in pool2 than SKBR3 cells. The levels of P-Akt were dramatically increased in HR20 cells as compared with BT474 cells, but P-MAPK levels remained the same. In contrast, P-MAPK levels were considerably higher in pool2 as compared with SKBR3 cells, and there was little increase in P-Akt levels. Interestingly, the levels of P-Src were significantly increased in pool2 and HR20 cells as compared with their corresponding control counterparts (Fig. 1B), suggesting that Src kinase activity was enhanced in the resistant cells. Thus, both trastuzumab-sensitive and trastuzumab-resistant breast cancer cells exhibit coexpression of erbB2, erbB3, and IGF-IR. Hence, multiple and distinct patterns of signaling pathway activation were observed in pool2 and HR20 cells.

Because the interaction between IGF-IR and erbB2 is the major molecular mechanism resulting in trastuzumab resistance in pool2 cells (30), we wondered if the association of these two receptors also exists in HR20 cells, and what is the role of erbB3 in the cross-talk of IGF-IR and erbB2. To answer these questions, immunoprecipitation with an anti–IGF-IR antibody was carried out. A negative control immunoprecipitation with a rabbit IgG was also performed using the same lysates of pool2 and HR20 cells. The immunoprecipitates were separated by SDS-PAGE and followed by Western blot analyses of erbB2, erbB3, or IGF-IR. A strong interaction between IGF-IR and erbB2 was found in pool2 and HR20 cells, confirming previous studies (30). Importantly, our data revealed, for the first time, a clear association between IGF-IR and erbB3 only in pool2 and HR20 cells (Fig. 1C). Thus, interactions between IGF-IR and erbB2 and between IGF-IR and erbB3 were exclusively observed in trastuzumab-resistant cells. We next investigated the potential interactions of erbB2 and erbB3 in the trastuzumab-sensitive and trastuzumab-resistant cells. A similar coimmunoprecipitation assay was performed on the four cell lines. Weak but clear associations of erbB2 and erbB3 were observed in SKBR3 and BT474 cells; interestingly, increased interactions between these two receptors were found in pool2 and HR20 cells (Fig. 1D). These data show that any two of the three RTKs are able to exhibit strong interactions in trastuzumab-resistant cells. To further confirm the results, immunofluorescent staining analyses were performed to examine the colocalization of the receptors on single-cell level. After hybridization with specific primary antibody, one receptor was labeled with Texas red (red)–conjugated secondary antibody, and another one was labeled with FITC (green)–conjugated secondary antibody. In both SKBR3 and BT474 cells, the red and green colors were separately located. In the resistant lines, however, the colors merged, giving a yellow membranous signal (Supplementary Fig. S1), suggesting that any two of the three receptors colocalized in trastuzumab-resistant cells.

erbB2, erbB3, and IGF-IR form heterotrimers in trastuzumab-resistant breast cancer cells. The aforementioned data suggest two possibilities about the associations of the three RTKs: (*a*) all three receptors may interact to form one unique heterotrimer and (*b*) three heterodimers of erbB2/erbB3, IGF-IR/erbB2, and erbB3/IGF-IR may coexist in the trastuzumab-resistant breast cancer cells. To distinguish these two possibilities, total cell lysates generated from pool2 and HR20 cells were subjected to immunodepletion with an anti–IGF-IR antibody. After binding with protein A–agarose, the samples were centrifuged, and then the supernatant was further immunoprecipitated with an anti-erbB3 antibody and followed by Western blot analysis with an anti-erbB2 antibody. For a control experiment, the total cell lysates of pool2 and HR20 cells were directly immunoprecipitated with the anti-erbB3 antibody without immunodepletion and followed by Western blot analysis with the anti-erbB2 antibody. A strong interaction between erbB3 and erbB2 was observed in both pool2 and HR20 cells (Fig. 2A, lanes 5 and 6), which was consistent with our previous data (Fig. 1D). Importantly, after immunodepletion with the anti–IGF-IR antibody, there was no erbB3-associated erbB2 remaining in the supernatant, indicating that the anti–IGF-IR antibody also depleted erbB3/erbB2 complexes, although some free erbB3 protein might still remain in the supernatant (Fig. 2A, lanes 3 and 4). These results indicate that the three RTKs exist within a multiprotein complex that is immunodepleted by the anti–IGF-IR antibody. These data also

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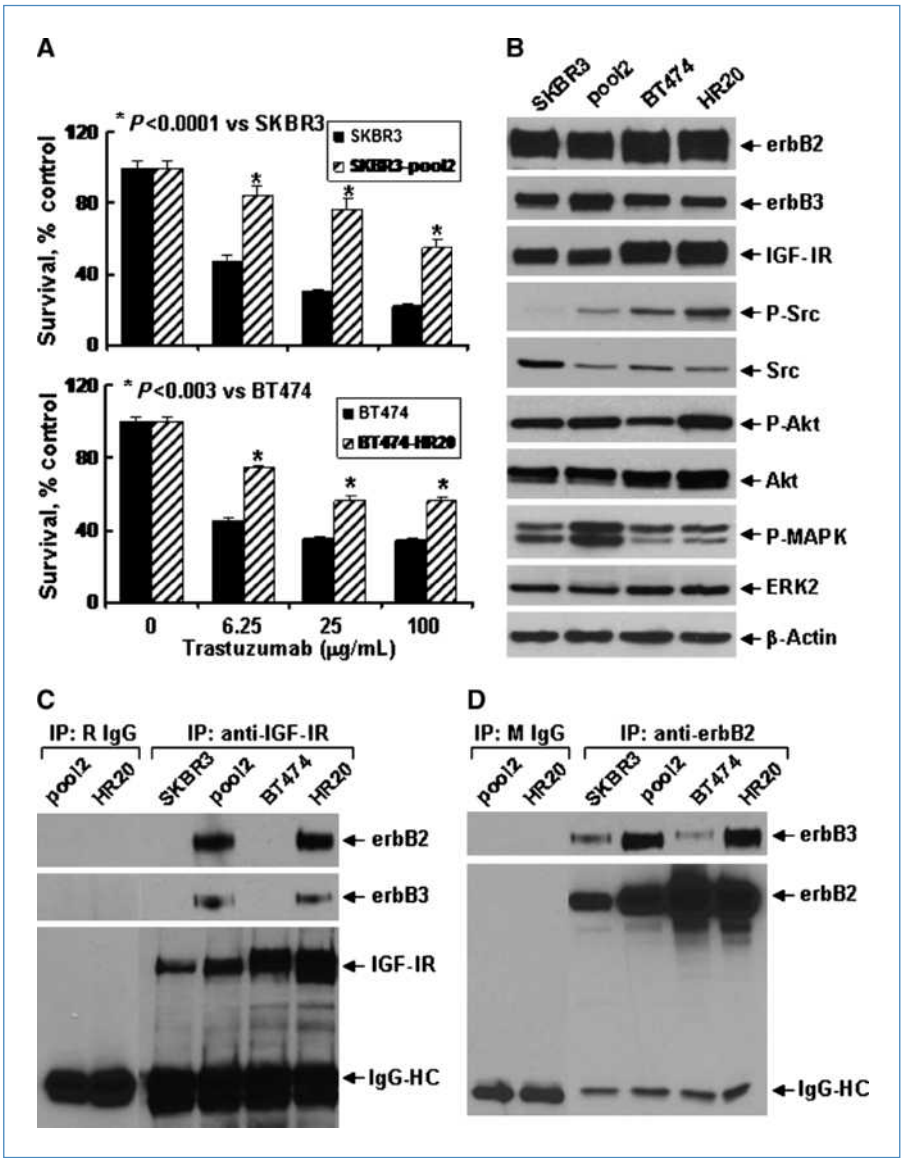

Figure 1. Coexpression of erbB2, erbB3, IGF-IR, and their interactions in trastuzumab-sensitive and trastuzumab-resistant breast cancer cells. A, the indicated cells were plated onto 96-well plates and followed by cell proliferation assays. Bars, SD. Data show a representative of three independent experiments. B, equal amount of cell lysates from indicated cells was subjected to Western blot analyses of erbB2, erbB3, IGF-IR, P-Src, Src, P-Akt, Akt, P-MAPK, MAPK, or β-actin. C and D, coimmunoprecipitation assays were performed to detect the protein-protein interactions. C, equal amount of cell lysates was subjected to immunoprecipitation (*IP*) with a rabbit IgG (*R IgG*) or rabbit anti–IGF-IR antibody and followed by Western blot analyses of erbB2, erbB3, or IGF-IR. D, similarly, immunoprecipitation with a mouse IgG (*M IgG*) or mouse anti-erbB2 antibody and followed by Western blot analyses of erbB3 or erbB2.

argue against the existence of erbB2/erbB3, IGF-IR/erbB2, and erbB3/IGF-IR heterodimers because erbB3 and erbB2 associations were no longer detectable after immunodepletion with the anti–IGF-IR antibody (Fig. 2A). To the best of our knowledge, this was the first study showing that the three receptors participated in a multiprotein complex in trastuzumab-resistant breast cancer cells.

To provide direct evidence showing that the three RTKs may form heterotrimers in trastuzumab-resistant cells, we performed native PAGE, which would maintain the multiprotein complex intact. Western blot analyses with erbB2, erbB3, or IGF-IR antibody following native PAGE revealed that a major signal right below the 500 kDa marker was clearly observed in both pool2 and HR20 but not SKBR3 and BT474 cells (Fig. 2B–D). By calculation of their molecular weight, a heterotrimer consisting of all three RTKs would be 460 kDa = 180 kDa (erbB3) + 185 kDa (erbB2) + 95 kDa (IGF-IR). These data strongly indicated that the three RTKs actually formed heterotrimers in the resistant cells. In addition, both erbB2 and erbB3 antibodies recognized the dimerization signals of the RTKs, 365 kDa = 180 kDa (erbB3) + 185 kDa (erbB2), mainly in SKBR3 and BT474 cells. The erbB2, but not erbB3, antibody also recognized monomers in SKBR3, BT474, and HR20 cells, suggesting that these cells may produce more erbB2 than erbB3. Hence, some erbB2 molecules seem to exist as monomers, whereas the majority of erbB3 molecules exist as either heterodimers (SKBR3 and BT474 cells) or heterotrimers (pool2 and HR20 cells). The strong monomer signals observed in BT474 and HR20 cells with anti–IGF-IR antibody might be due to the higher IGF-IR expression levels in these two cell lines than SKBR3 and pool2 cells (Fig. 1B).

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Disruption of heterotrimerization by specific knockdown of either erbB3 or IGF-IR expression abrogates trastuzumab resistance. To determine whether the heterotrimerization of erbB3/erbB2/IGF-IR causally results in trastuzumab resistance, we investigated if specific knockdown of either erbB3 or IGF-IR would overcome the resistant phenotype. We used vector-based shRNAs in a lentiviral system to ensure specific and stable gene silencing. The *erbB3* shRNA was able to specifically and efficiently knock down erbB3 expression without affecting erbB2 or IGF-IR expressions. Specific knockdown of erbB3 significantly decreased levels of P-erbB3 and P-Akt in both pool2 and HR20 cells, produced a minor reduction of P-Src in pool2 cells, and did not change levels of P-MAPK (Fig. 3A). There was no significant effect on erbB2/IGF-IR associations on knockdown of erbB3 (Fig. 3B), suggesting that erbB2 was directly interacting with IGF-IR in the resistant cells. Although the combinations of erbB3 knockdown and trastuzumab did not further decrease the levels of P-Akt and P-Src, they were able to reduce P-erbB3 and P-MAPK and induce $p27^{kip1}$ expression (Fig. 3C). More importantly, the resistant cells infected with the lentivirus containing *erbB3* shRNA, but not control shRNA, became significantly sensitive to trastuzumab-mediated growth inhibition (Fig. 4D).

Similar results were obtained with IGF-IR. Specific knockdown of IGF-IR by the IGF-IR shRNAs had no effect on the expression levels of erbB2 and erbB3 (Fig. 4A) and did not alter the erbB2/erbB3 associations (Fig. 4B), suggesting a direct interaction between erbB2 and erbB3 in pool2 and HR20 cells. Knockdown of IGF-IR significantly resensitized the resistant cells to trastuzumab-mediated growth inhibition (Fig. 4D). Interestingly, the changes in the downstream signaling resulting from specific knockdown of IGF-IR were different from that of erbB3 knockdown. Knockdown of IGF-IR resulted in a clear reduction in the levels of P-Src, a minor decrease in P-MAPK, and no effect on P-erbB3 and P-Akt (Fig. 4A); however, it dramatically enhanced trastuzumab-mediated inhibitory effects on P-erbB3 and P-Akt (Fig. 4C). The combinations of IGF-IR knockdown and trastuzumab induced upregulation of $p27^{kip1}$ (Fig. 4C). Collectively, these data strongly show that disruption of the heterotrimerization of the three RTKs via specific knockdown of either erbB3 or IGF-IR abrogates trastuzumab resistance, and it is the heterotrimer of erbB3/erbB2/IGF-IR, not the heterodimers of IGF-IR/erbB2 or erbB2/erbB3, that plays a critical role in causing trastuzumab resistance.

Specific knockdown of erbB2 results in apoptosis of trastuzumab-resistant cells. To explore whether the activation of downstream signaling pathways is dependent of erbB2 receptor, and to study if erbB3 is directly interacting with IGF-IR in heterotrimer, we performed additional knockdown experiments using two shRNA sequences. Knockdown of

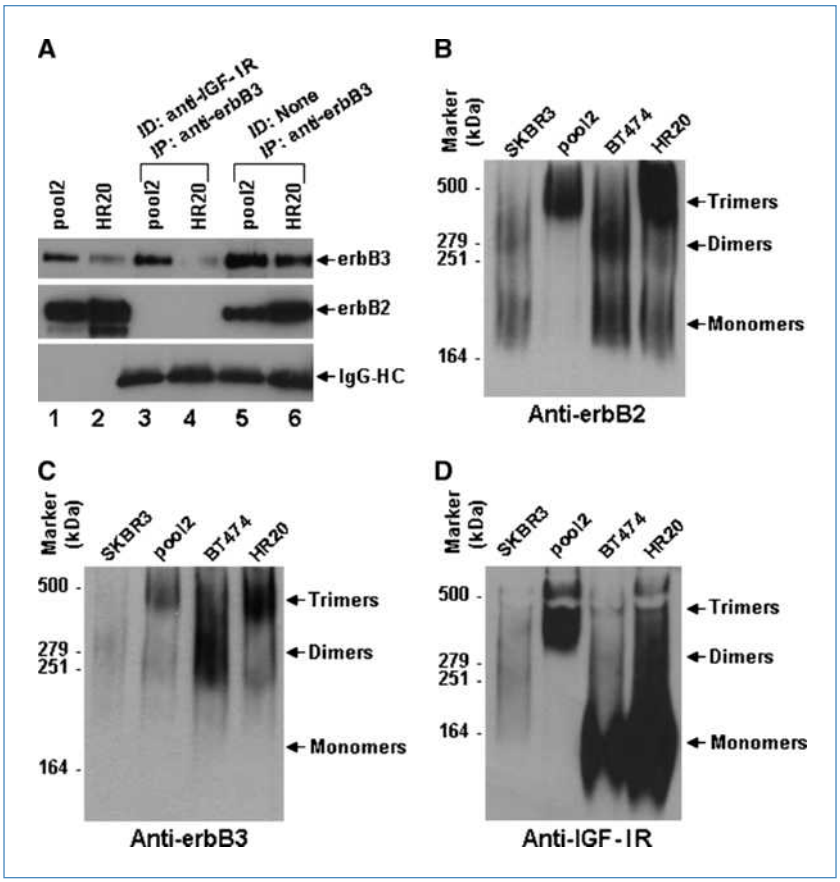

Figure 2. erbB2, erbB3, and IGF-IR form a heterotrimer complex in trastuzumab-resistant breast cancer cells. A, equal amount of cell lysates was subjected to immunodepletion (*ID*) with an anti–IGF-IR antibody. Lanes 3 and 4, after binding with protein A–agarose and centrifugation, the remaining supernatant was subjected to immunoprecipitation with an anti-erbB3 antibody. Lanes 5 and 6, same cell lysates were directly immunoprecipitated with the anti-erbB3 antibody. Lanes 1 and 2, total cell lysates were used for controls. All samples were separated by SDS-PAGE and followed by Western blots of erbB3 or erbB2. B to D, equal amount of total cell lysates was separated by native PAGE and followed by Western blots of erbB2 (B), erbB3 (C), or IGF-IR (D). Strong signals just below the 500-kDa marker recognized by all three antibodies indicate that the heterotrimer complex existed only in the resistant cells. The heterodimers or monomers of the receptors were also observed.

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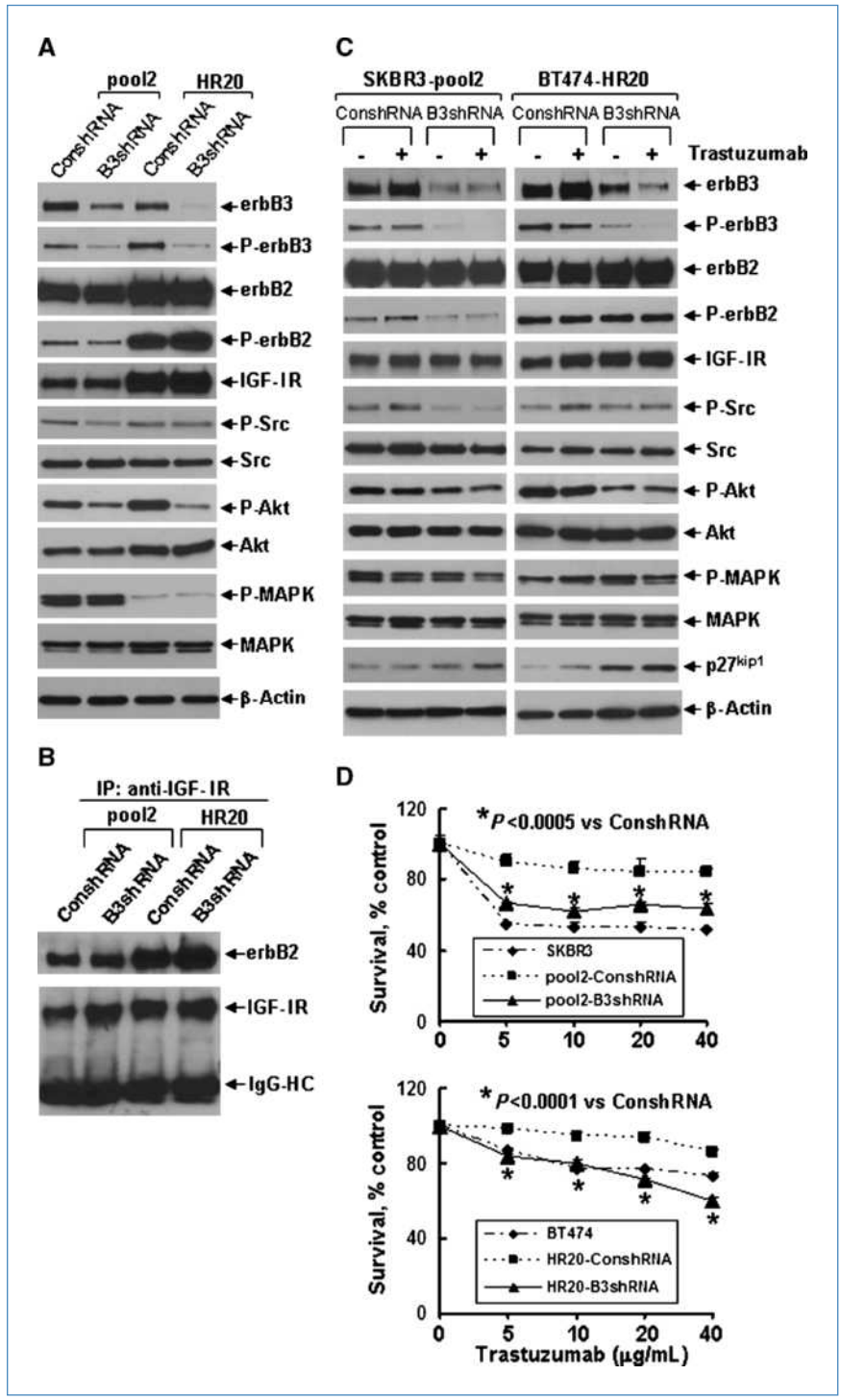

Figure 3. Specific knockdown of erbB3 does not alter the interactions between IGF-IR and erbB2 but resensitizes the resistant cells to trastuzumab-mediated growth inhibition. A, lentivirus containing either control shRNA (*ConshRNA*) or specific *erbB3* shRNA (*B3shRNA*) was used to infect pool2 and HR20 cells. After puromycin selection, the cells were collected, lysed, and followed by Western blot analyses of erbB3, P-erbB3, erbB2, P-erbB2, IGF-IR, P-Src, Src, P-Akt, Akt, P-MAPK, MAPK, or β-actin. B, equal amount of cell lysates from pool2 or HR20 cells infected with lentivirus containing either ConshRNA or B3shRNA was subjected to immunoprecipitation with an anti–IGF-IR antibody and followed by Western blot analyses of erbB2 or IGF-IR. C, SKBR3-pool2 or BT474-HR20 cells infected with lentivirus containing either ConshRNA or B3shRNA were cultured in the presence or absence of trastuzumab (20 μg/mL) for 24 h. The cells were then collected and subjected to Western blot analyses of erbB3, P-erbB3, erbB2, P-erbB2, IGF-IR, P-Src, Src, P-Akt, Akt, P-MAPK, MAPK, $p27^{kip1}$, or β-actin. D, pool2 (top) and HR20 (bottom) cells infected with lentivirus containing either ConshRNA or B3shRNA were plated onto 96-well plates and followed by cell proliferation assays. SKBR3 and BT474 cells were used as positive controls. Bars, SD. Data show a representative of three independent experiments.

erbB2 by the *erbB2* shRNAs did not significantly affect the levels of erbB3, IGF-IR, P-Src, and P-MAPK (Fig. 5A), suggesting that the *erbB2* shRNAs were specific and efficient, and the activation of Src and MAPK kinases was independent of erbB2 action. Nonetheless, the levels of P-erbB2, P-erbB3, and P-Akt were significantly reduced on erbB2 knockdown, and their reduction rates were clearly correlated with the effectiveness of shRNAs on downregulating erbB2 expression (Fig. 5A). Moreover, specific knockdown of erbB2 promoted both pool2 and HR20 cells undergoing apoptosis, evidenced by increased cleavages of PARP, caspase-8, caspase-9, and caspase-3 (Fig. 5C) and by apoptotic ELISA (Fig. 5D). The induction of

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apoptosis was not observed in the cells with specific knockdown of either erbB3 or IGF-IR (data not shown). These results indicated that erbB2 RTK played an absolutely critical role in activation of erbB3-mediated PI3K/Akt signaling to maintain survival of the resistant cells. Similar to the results obtained from the studies on erbB3 and IGF-IR, specific knockdown of erbB2 expression did not affect the erbB3/IGF-IR associations (Fig. 5B). Thus, any two of the three RTKs exhibited direct interactions in both pool2 and HR20 cells. Whereas specific knockdown of IGF-IR expression showed a major inhibitory effect on Src and MAPK signaling (Fig. 4A), both erbB3 and erbB2 knockdown led to a significant impairment mainly on PI3K/Akt signaling (Figs. 3A and 5A).

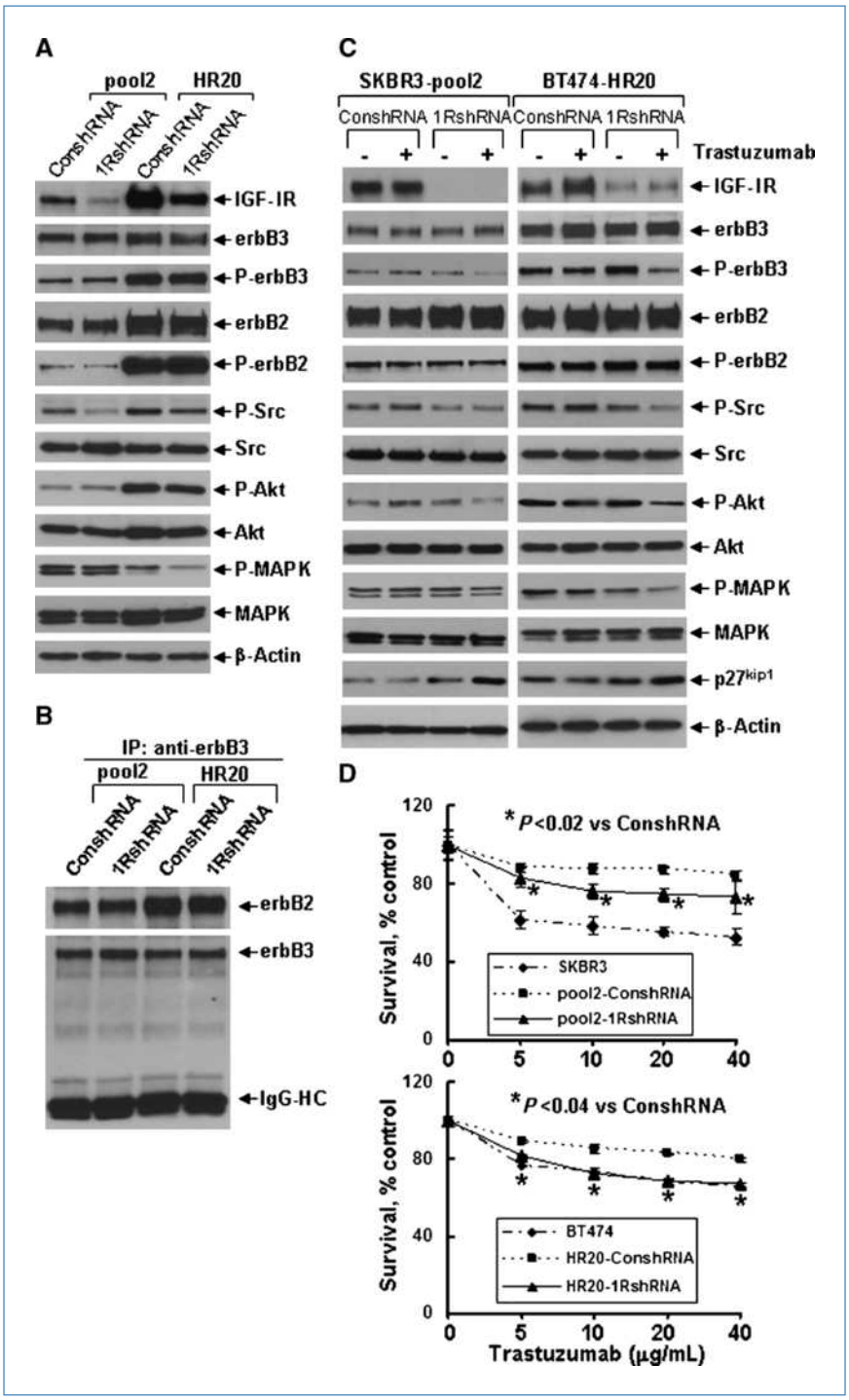

Figure 4. Specific knockdown of IGF-IR does not alter the interactions between erbB3 and erbB2 but resensitizes the resistant cells to trastuzumab-mediated growth inhibition. A, lentivirus containing either ConshRNA or specific IGF-IR shRNA (*1RshRNA*) was used to infect pool2 and HR20 cells. After puromycin selection, the cells were collected, lysed, and followed by Western blot analyses of IGF-IR, erbB3, P-erbB3, erbB2, P-erbB2, P-Src, Src, P-Akt, Akt, P-MAPK, MAPK, or β-actin. B, equal amount of cell lysates from pool2 or HR20 cells infected with lentivirus containing either ConshRNA or 1RshRNA was subjected to immunoprecipitation with an anti-erbB3 antibody and followed by Western blot analyses of erbB2 or erbB3. C, SKBR3-pool2 or BT474-HR20 cells infected with lentivirus containing either ConshRNA or 1RshRNA were cultured in the presence or absence of trastuzumab (20 μg/mL) for 24 h. The cells were collected and subjected to Western blot analyses of IGF-IR, erbB3, P-erbB3, erbB2, P-erbB2, P-Src, Src, P-Akt, Akt, P-MAPK, MAPK, $p27^{kip1}$, or β-actin. D, pool2 (top) and HR20 (bottom) cells infected with lentivirus containing either ConshRNA or 1RshRNA were plated onto 96-well plates and followed by cell proliferation assays. SKBR3 and BT474 cells were used as positive controls. Bars, SD. Data show a representative of three independent experiments.

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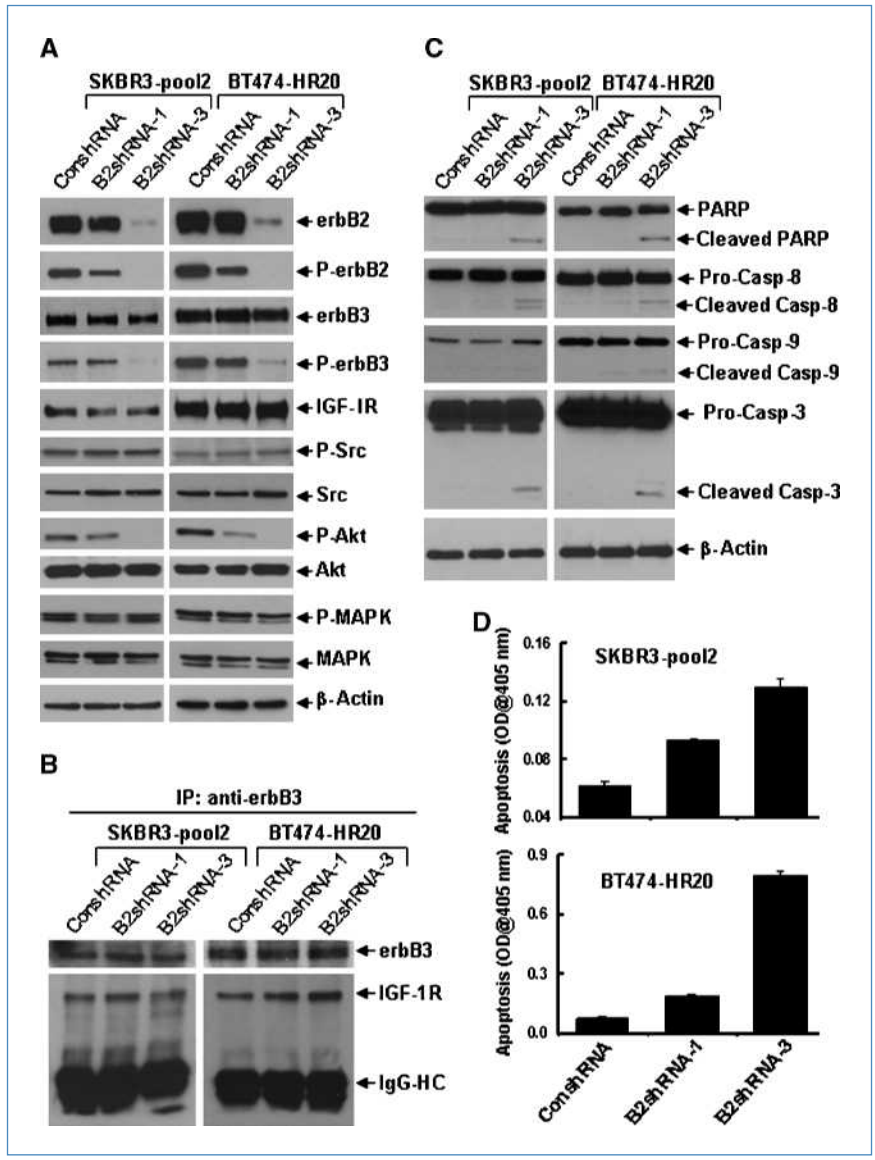

Figure 5. Specific knockdown of erbB2 expression results in apoptosis of trastuzumab-resistant cells without effect on interactions between erbB3 and IGF-IR. A, lentivirus containing either ConshRNA or specific *erbB2* shRNAs (*B2shRNA-1* or *B2shRNA-3*) was used to infect SKBR3-pool2 and BT474-HR20 cells. After puromycin selection, the cells were collected, lysed, and followed by Western blot analyses of erbB2, P-erbB2, erbB3, P-erbB3, IGF-IR, P-Src, Src, P-Akt, Akt, P-MAPK, MAPK, or β-actin. B, equal amount of cell lysates from the cells infected with lentivirus containing either ConshRNA or B2shRNA-1 or B2shRNA-3 was subjected to immunoprecipitation with an anti-erbB3 antibody and followed by Western blot analyses of erbB3 or IGF-IR. C and D, the cells infected with lentivirus containing either ConshRNA or B2shRNA-1 or B2shRNA-3 were collected and subjected to Western blot analyses of PARP, caspase-8, caspase-9, caspase-3, or β-actin (C) or apoptosis ELISA (D).

Discussion

Trastuzumab resistance currently represents a significant clinical problem in breast cancer treatment. Elucidating the molecular mechanisms underlying trastuzumab resistance is critical to improve the survival of breast cancer patients whose tumors overexpress erbB2. Several studies have revealed the importance of IGF-I/IGF-IR signaling, including IGF-IR/erbB2 association, in causing trastuzumab resistance (27, 29, 30); however, the role of erbB3 in the cross-talk of IGF-IR and erbB2 remains unknown. Our current studies indicate that erbB3 plays as an important role as IGF-IR, and both erbB3 and IGF-IR work cooperatively with erbB2 in trastuzumab-resistant breast cancer cells. Although dimerization between erbB2 and another RTK, such as erbB3, EGFR, or IGF-IR, has been the focus of many research laboratories in exploring the mechanisms of trastuzumab resistance, here we provide compelling evidence to support a novel hypothesis—heterotrimer formation of erbB3/erbB2/IGF-IR leads to trastuzumab resistance. Our data indicate that any two of these three RTKs interact directly and lead to activation of unique downstream signaling pathways in trastuzumab-resistant breast cancer cells (Fig. 6). Although specific knockdown of any one of the three receptors does not interfere with the associations of the remaining two, each receptor exhibits distinct roles in maintaining activation of the multiple signaling pathways. Knockdown of erbB3 has no effect on P-MAPK but induces a dramatic decrease of P-Akt and a minor reduction in P-Src (Fig. 3A). In contrast, knockdown of IGF-IR reduces the levels of P-Src and P-MAPK but not P-Akt; however, it does render the cells to trastuzumab-mediated inactivation of Akt (Fig. 4A and C).

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These data are consistent with previous reports that PI3K/Akt signaling plays an important role in trastuzumab resistance (7, 20, 21). Another potential molecular mechanism of trastuzumab resistance may be due to changes of erbB2 conformation on heterotrimerization with erbB3 and IGF-IR, which hinder trastuzumab binding. As depicted in Fig. 6, on knockdown of either erbB3 or IGF-IR, the conformation of erbB2 in monomers or heterodimers (erbB2/IGF-IR or erbB2/erbB3) may render trastuzumab binding effectively, and subsequently attenuates the downstream signaling and upregulates the CDK inhibitor $p27^{kip1}$. It will be very interesting to determine if the conformation of erbB2 differs in heterotrimers and heterodimers and if distinct domains mediate erbB2 interactions in the heterotrimerization and heterodimerization of the RTKs.

Interestingly, a recent report reveals that another erbB2 splice variant, erbB2Δ16, which was detected in erbB2-overexpressing breast cancer cells (38, 43, 44), promotes trastuzumab resistance via direct coupling with Src tyrosine kinase (45). We have found that specific knockdown of either erbB3 or IGF-IR reduces the levels of P-Src and resensitizes the resistant cells to trastuzumab-mediated growth inhibition in

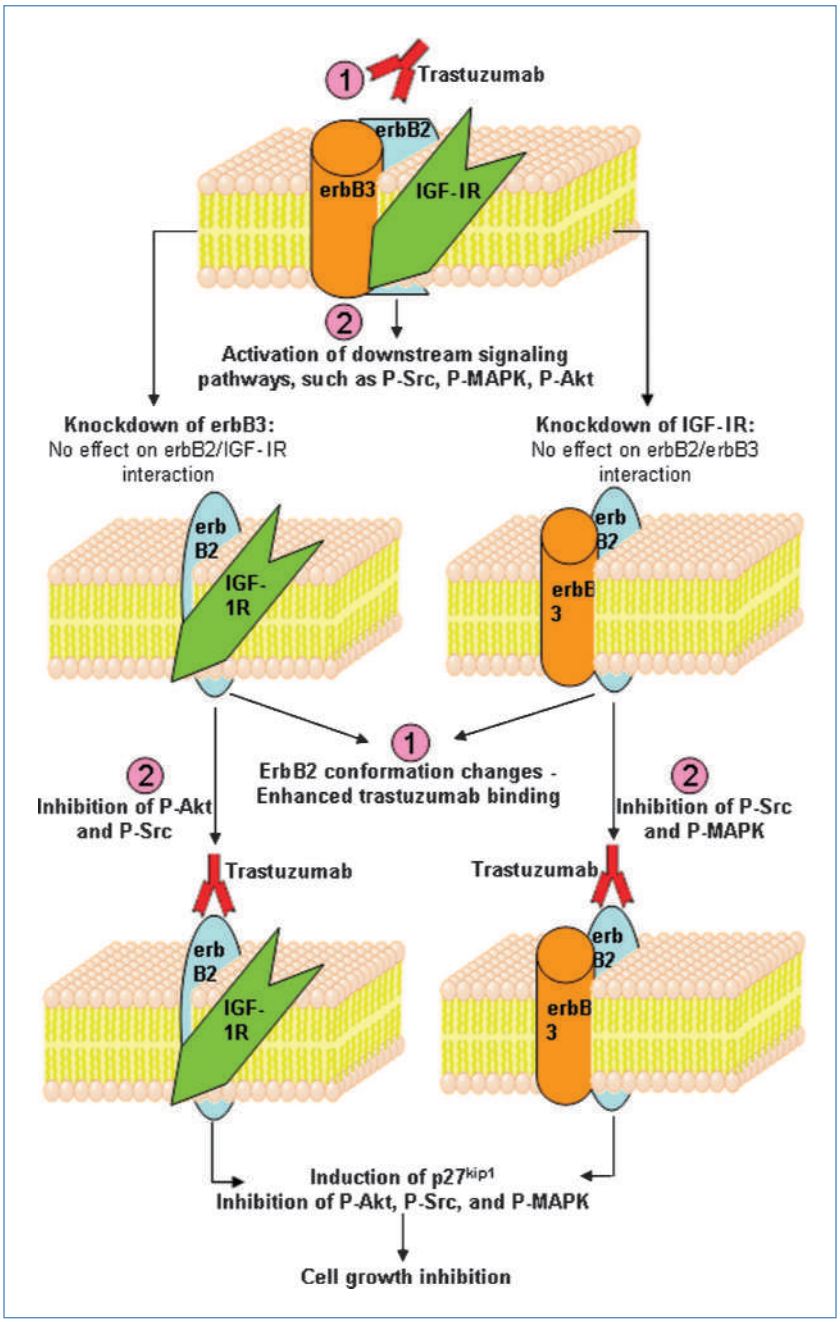

Figure 6. Proposed model of erbB3/erbB2/IGF-IR heterotrimerization resulting in trastuzumab resistance. Our studies show that any two of the three receptors directly interact with each other to form a heterotrimer complex, which may prevent/inhibit trastuzumab binding effectively to erbB2 (1) and activate the unique downstream signaling (2). Disruption of the heterotrimerization by specific knockdown of either erbB3 or IGF-IR not only enhances the binding efficiency of trastuzumab due to erbB2 receptor conformation changes but also inhibits kinase activities of Src, Akt, or MAPK and therefore abrogates trastuzumab resistance via inactivation of the downstream signaling pathways and upregulation of $p27^{kip1}$.

both pool2 and HR20 cells. Because of the importance of PI3K/Akt signaling and Src in trastuzumab resistance, we sought to define the role of Akt and Src activation in erbB3/erbB2/IGF-IR heterotrimer-mediated trastuzumab resistance. Specific inhibitors were used to explore whether inhibition of Akt or Src may reverse the resistant phenotype in pool2 and HR20 cells. P-Src was significantly reduced on dasatinib treatment in both pool2 and HR20 cells (Supplementary Fig. S2A). The PI3K inhibitor LY294002 alone inhibited Akt activation only in HR20 cells; however, the combinations of LY294002 and trastuzumab dramatically decreased the P-Akt levels in both pool2 and HR20 cells (Supplementary Fig. S2B). Importantly, both dasatinib and LY294002 were able to significantly enhance trastuzumab-mediated growth inhibition (Supplementary Fig. S2C). These data suggest that activation of both PI3K/Akt signaling and Src contributes to erbB3/erbB2/IGF-IR heterotrimer-mediated trastuzumab resistance in pool2 and HR20 cells.

It is not clear, at this moment, how the three RTKs form heterotrimers in the resistant cells. It has been reported that the ligands for erbB3 and IGF-IR, secreted (soluble) heregulin and IGF-I, attenuate the inhibitory effect of trastuzumab in erbB2-overexpressing breast cancer cells (14, 29). Moreover, *in vivo* selected trastuzumab-resistant lines from BT474 xenograft tumors overexpress EGFR and multiple erbB ligands, including heregulin (15). A recent article show that activation of erbB3 signaling by ADAM17, which is the major sheddase for heregulin (46), is required for transforming growth factor β–induced trastuzumab resistance in erbB2-overexpressing breast cancer cells (47). If we can confirm the hypothesis that both IGF-I (or IGF-II) and heregulin may be upregulated in pool2 and HR20 cells by the long-term selection of trastuzumab, then approaches to block ligands binding to their corresponding receptors may be novel strategies to overcome the resistant phenotype in breast cancer cells.

We believe this is the first report showing a heterotrimerization of erbB3/erbB2/IGF-IR in trastuzumab-resistant breast cancer cells, which can activate multiple downstream signaling pathways resulting in trastuzumab resistance. The data we show have significant clinical implications. Nonetheless, the confirmation of our hypothesis with clinical samples is urgently needed. We are collaborating with physician scientist to determine whether these three receptors can also form a heterotrimer complex in tumor tissues obtained from those breast cancer patients who fail from trastuzumab-based therapy. Our studies may change the view of cancer biologists about the protein-protein interactions among the three RTKs. erbB2, erbB3, and IGF-IR can form not only heterodimers but also heterotrimers in trastuzumab-resistant cells, suggesting that novel approaches simultaneously targeting these three receptors may ultimately benefit breast cancer patients whose tumors overexpress erbB2. For a subset of trastuzumab-resistant patients, anti-erbB3 and anti–IGF-IR blocking antibodies may work synergistically to overcome trastuzumab resistance and enhance its therapeutic efficacy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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