Insulin-Like Growth Factor-1 Receptor Signaling Increases the Invasive Potential of Human Epidermal Growth Factor Receptor 2–Overexpressing Breast Cancer Cells via Src-Focal Adhesion Kinase and Forkhead Box Protein M1

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Received August 12, 2014; accepted November 12, 2014

ABSTRACT

Resistance to the human epidermal growth factor receptor (HER2)-targeted antibody trastuzumab is a major clinical concern in the treatment of HER2-positive metastatic breast cancer. Increased expression or signaling from the insulin-like growth factor-1 receptor (IGF-1R) has been reported to be associated with trastuzumab resistance. However, the specific molecular and biologic mechanisms through which IGF-1R promotes resistance or disease progression remain poorly defined. In this study, we found that the major biologic effect promoted by IGF-1R was invasion, which was mediated by both Src-focal adhesion kinase (FAK) signaling and Forkhead box protein M1 (FoxM1). Cotargeting IGF-1R and HER2 using either IGF-1R antibodies or IGF-1R short hairpin RNA in combination with trastuzumab resulted in significant but modest growth inhibition. Reduced invasion was the most significant biologic effect achieved by cotargeting IGF-1R and HER2 in trastuzumab-resistant cells. Constitutively active Src blocked the anti-invasive effect of IGF-1R/HER2 cotargeted therapy. Furthermore, knockdown of FoxM1 blocked IGF-1-mediated invasion, and dual targeting of IGF-1R and HER2 reduced expression of FoxM1. Re-expression of FoxM1 restored the invasive potential of IGF-1R knockdown cells treated with trastuzumab. Overall, our results strongly indicate that therapeutic combinations that cotarget IGF-1R and HER2 may reduce the invasive potential of cancer cells that are resistant to trastuzumab through mechanisms that depend in part on Src and FoxM1.

Introduction

Breast cancer is the most commonly diagnosed cancer among women in the United States (Siegel et al., 2014). Multiple subtypes of breast cancer have been identified through gene profiling studies (Perou et al., 2000). Breast cancers that show amplification and overexpression of the human epidermal growth factor receptor 2 (her2) gene represent approximately 20%–30% of metastatic cases (Slamon et al., 1987). Overexpression of the HER2 receptor tyrosine kinase is associated with poor prognosis, reduced overall survival, and the development of resistance to some types of chemotherapy (Slamon et al., 1987). The specific overexpression of HER2 in breast cancers serves as a selective target for anticancer drugs. Trastuzumab (Herceptin; Genentech, South San Francisco, CA), a humanized monoclonal antibody against an extracellular epitope on domain IV of HER2 (Carter et al., 1998),
Trastuzumab plus chemotherapy improves overall response rates, time to progression, and the overall survival of patients with HER2-positive metastatic breast cancer beyond that achieved with chemotherapy alone (Slamon et al., 2001). However, clinical trials demonstrate that the median duration of single-agent trastuzumab or trastuzumab-containing chemotherapy regimens is less than 1 year (Cobleigh et al., 1999; Seidman et al., 2001; Slamon et al., 2001; Esteva et al., 2002). Furthermore, single-agent trastuzumab achieves moderate response rates among HER2-overexpressing metastatic breast cancers (Cobleigh et al., 1999). These data indicate that acquired resistance and primary resistance to trastuzumab are clinical concerns in the treatment of HER2-positive metastatic breast cancer.

Multiple mechanisms of trastuzumab resistance have been described in the literature. Constitutive activation of downstream phosphoinositide 3-kinase/Akt signaling through phosphatase and tensin homolog (PTEN) downregulation or PI3KCA hyperactivating mutations has been reported to significantly abrogate response to trastuzumab (Nagata et al., 2001; Berns et al., 2007). In addition, the lack of an effective ADCC immune response has been shown to result in trastuzumab resistance (Clynes et al., 2000; Arnould et al., 2006; Varchetta et al., 2007). Increased expression or compensatory signaling through other receptor tyrosine kinases, including insulin-like growth factor-1 receptor (IGF-1R), epidermal growth factor receptor, or HER3, and/or crosstalk of receptor kinases to HER2 have also been reported as mechanisms of acquired resistance to trastuzumab (Lu et al., 2001; Nahta et al., 2005; Ritter et al., 2007; Junttila et al., 2009; Dua et al., 2010; Huang et al., 2010).

The first study to implicate IGF-1R in trastuzumab resistance showed that stable overexpression of IGF-1R reduces the ability of trastuzumab to induce G1 arrest and growth inhibition of HER2-overexpressing breast cancer cell lines (Lu et al., 2001). Furthermore, among cases of HER2-overexpressing breast cancer, high IGF-1R expression or phosphorylation correlates with worse response to preoperative trastuzumab and chemotherapy (Harris et al., 2007) and reduced progression-free survival (Gallardo et al., 2010). We and others have reported that crosstalk from IGF-1R to HER2 results in sustained HER2 phosphorylation in the presence of trastuzumab (Nahta et al., 2005; Chakraborty et al., 2008; Huang et al., 2010). However, the specific mechanisms through which IGF-1R activates HER2 and the major downstream molecular and biologic effects remain poorly defined.

In this study, we found that Src activity maintained HER2 phosphorylation in trastuzumab-resistant cells. Furthermore, we showed that the major biologic effect promoted by IGF-1R was cellular invasion mediated by both Src-focal adhesion kinase (FAK) and HER2-Forkhead box protein M1 (FoxM1) signaling. Cotargeting IGF-1R and HER2 suppressed the invasiveness of trastuzumab-resistant cells and appeared to depend in part on FoxM1 and Src inhibition, because overexpression or activation of these molecules blocked the antiinvasive effect of IGF-1R/HER2 cotargeting. These results suggest that therapeutic combinations that block IGF-1R and HER2 may reduce the invasive potential of cancer cells that are resistant to trastuzumab.

Materials and Methods

Trastuzumab (Genentech) was obtained from the Emory Winship Cancer Institute pharmacy (Atlanta, GA) and dissolved in sterile water to a stock concentration of 20 mg/ml. Lapatinib ditosylate (Santa Cruz Biotechnology, Dallas, TX) was dissolved in dimethylsulfoxide (DMSO) to a final concentration of 10 mM. The IGF-1R antibody α-IR3 (Calbiochem, San Diego, CA) was provided at a stock concentration of 1 mg/ml. The IGF-1R antibody IGF-IR-56-81 was developed by Dr. Pravin Kaumaya (The Ohio State University, Columbus, OH) (Foy et al., 2014). Briefly, rabbits were immunized with 1 mg of IGF-1R peptide, Ac-LLFRVAGLESLDFPLNLTIRGWKL-NH2; antibodies were purified from rabbit sera by affinity chromatography using protein A/G columns. IGF-1 ligand (Sigma-Aldrich, St. Louis, MO) was dissolved in sterile water at a stock concentration of 1 mg/ml. The IGF-1R kinase inhibitor NVPAEW541 (7-icis-3-(1-azetidinylmethyl)cyclobutyl)-5-[3-(phenylmethoxy)phenyl]-1H-pyrrolo[2,3-d]pyrimidin-4-amine, dihydrochloride; Cayman Chemical, Ann Arbor, MI) was dissolved in DMSO to a final concentration of 10 mM. In-solution Src kinase inhibitor PP2 (4-amino-5-(4-chlorophenyl)-7-(dimethylamino)pyrazolo[3,4-d]pyrimidine; Calbiochem) was provided at a stock concentration of 10 mM in DMSO. FAK Inhibitor II (PF573228 (3,4-dihydro-6-[4-[[3-(methylsulfonyl)phenyl]methyl]amino]-5-(trifluoromethyl)-2-pyrimidinyl)amine-2H-chloride); Santa Cruz Biotechnology) was dissolved in DMSO to a stock concentration of 20 mM. The pLKO.1-IGF-1R-αβ short hairpin RNA (shRNA) plasmid and pLKO.1 empty vector plasmid (negative control) were purchased from Open Biosystems (Huntsville, AL). FoxM1 small interfering RNA (siRNA) (sc-270048) and control siRNA (sc-37007) (Santa Cruz Biotechnology) were resuspended in RNase-free water. FoxM1 expression plasmid was purchased from OriGene (Rockville, MD).

Cell Culture. JIMT-1 cells were purchased from DSMZ (Braunschweig, Germany); all other cell lines were purchased from American Type Culture Collection (Manassas, VA). HCC1954 cells were maintained in RPMI 1640 with glutamine (Corning, Manassas, VA), and supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. JIMT-1 and HCC1954 cells have premetastatic neotissue (CM) which can support cell survival under conditions of cell-cell contact. CM is generated by passage of JIMT-1 cells on collagene (from Collagen Corp) and confluent in vitro cultures of mouse NIH-3T3 and human MCF-7 cells. Medium containing CM can be filtered. JIMT-1 cells were seeded at subconfluent densities and infected with lentiviral vectors (1:20 dilution) in fresh culture media. Viral stocks were harvested from culture media and filtered. JIMT-1 cells were seeded at subconfluent densities and infected with lentiviral vectors (1:20 dilution) in fresh culture media. Viral stocks were harvested from culture media and filtered. JIMT-1 cells were seeded at subconfluent densities and infected with lentiviral vectors (1:20 dilution) in fresh culture media. Viral stocks were harvested from culture media and filtered.
Stimulation Experiments. Cells were plated and serum starved for 24 hours. During serum starvation, cells were either untreated or treated with 500 nM PF573228 (FAK inhibitor), 100 nM lapatinib, varying concentrations of NVPAEWS41, or vehicle control. Cells were then either lysed for protein or stimulated with vehicle control or 100 ng/ml IGF-1 for varying time points. Experiments were repeated at least three times with reproducible results.

Trypan Blue Exclusion. For growth inhibition assays, cells were plated in complete DMEM at 2 x 10^4 cells per well in 12-well plates. The next day, media were aspirated and replaced with media containing control mouse IgG, α-IR3 (0.25 μg/ml), trastuzumab (20 μg/ml), or α-IR3 plus trastuzumab in triplicate. After 72 hours, viable cells were counted under a light microscope by trypan blue exclusion. Assays were repeated at least three times with reproducible results.

Anchorage-Independent Cell Culture Growth. Cells were plated in matrigel (BD Biosciences, Franklin Lakes, NJ) at a 1:1 dilution (media/matrigel). The matrigel-cell suspension was allowed to solidify, and media containing control mouse IgG, α-IR3 (0.25 μg/ml), trastuzumab (20 μg/ml), or α-IR3 plus trastuzumab were added to cells in triplicate cultures. Media and drugs were renewed twice a week for 3 to 4 weeks. Photographs were taken with an Olympus IX50 inverted microscope at x4 magnification (Olympus, Tokyo, Japan). Matrigel was digested using Dispase (BD Biosciences), and viable cells were counted by trypan blue exclusion. The average cell viability of triplicates and the S.D. were calculated. Experiments were performed at least twice with reproducible results.

Transfection. Cells were plated in antibiotic-free media at a concentration of 2 x 10^5 cells/ml. The next day, cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with 10 μg/ml of one of the following plasmids (kind gifts from Dr. Sumin Kang, Emory University, Atlanta, GA): constitutively active Src, kinase-dead Src, wild-type Src, or pcDNA3 empty vector control. Media were changed after 6 hours of transfection and replaced with complete media; cells were harvested after 48 hours.

Spheroid Migration Assays. JIMT-1 (4.0 x 10^4) cells were untreated or were suspended in complete media containing one of the following treatments: control IgG, 0.25 μg/ml α-IR3, 20 μg/ml trastuzumab, or α-IR3 plus trastuzumab. Cells were seeded on a 96-well plate and cultured for 4 hours in a humidified atmosphere containing 5% CO2 at 37°C. Immune competent spheroids were transferred to a 96-well plate and cultured in complete media containing respective inhibitors or control vehicle for 48 hours. Spheroids and migrated cells were fixed with 100% methanol, stained with 0.05% crystal violet, and observed using a normal light microscope (x20) and an Olympus DP-30BW digital camera. Experiments were repeated three times with reproducible results, representative images are shown for all groups.

Invasion Chamber Assays. Cells were plated in serum-free media in BD BioCoat Matrigel Invasion Chambers (BD Biosciences) (1 x 10^5 cells/ml) with 0.75 ml of chemoattractant (culture media containing 10% FBS) in the wells. Depending on the experiment, cells were pretreated with 500 nM FAK inhibitor or 10 μM P2P2 for 24 hours or were transfected with control siRNA, FoxM1 siRNA, empty vector, or FoxM1 expression plasmid overnight prior to placing cells in invasion chambers, at which point they were treated with control or IGF-1 (100 ng/ml) for 24 hours. In other experiments, control mouse IgG, α-IR3 (0.25 μg/ml), IGF-1R-56-81 (400 μg/ml), trastuzumab (20 μg/ml), α-IR3 plus trastuzumab, IGF-1R-56-81 plus trastuzumab, or IGF-1 (100 ng/ml) was added. Treatments were added directly to chambers in all experiments. Noninvading cells were removed from the interior surface of the membrane by scrubbing gently with a dry cotton-tipped swab. Each insert was then transferred into 100% methanol for 10 minutes followed by crystal violet staining for 20 minutes. Membranes were washed in water and allowed to air dry completely before being separated from the chamber. Membranes were mounted on slides with Permount permanent mounting medium (Fisher Scientific, Waltham, MA). Multiple photographs of each sample were taken at ≥20 magnification, with triplicates performed per treatment group. The number of cells was counted in each field; the sum total of the fields was calculated for each sample. Experiments were performed at least twice with reproducible results.

Cell Cycle Analysis. Cells were treated with control mouse IgG, α-IR3 (0.25 μg/ml), trastuzumab (20 μg/ml), or α-IR3 plus trastuzumab for 48 hours. Cells were harvested, washed twice with Dulbecco's phosphate-buffered saline (DPBS) plus 10% FBS, fixed in ice-cold 80% ethanol, and stored at -20°C for at least 24 hours. Fixed cells were incubated in 50 μl of propidium iodide (PI) buffer (20 μg/ml PI (Sigma-Aldrich), 0.1% Triton-X 100, and 200 μg/ml RNaseA (Promega) in DPBS) for 30 minutes in the dark. The cells were then resuspended in 400 μl DPBS for flow cytometric analysis. Samples were analyzed using a BD FACS Canto II cytometer (BD Biosciences, San Jose, CA) and BD FACS Diva software; experiments were performed in triplicate and repeated twice with reproducible results.

Western Blot Analyses. Cells were lysed in radioimmunoprecipitation assay buffer (Cell Signaling Technology, Danvers, MA) supplemented with protease and phosphatase inhibitors (Sigma-Aldrich). Total protein extracts were run on SDS-PAGE and blotted onto nitrocellulose. Blots were probed overnight. The following antibodies were purchased from Cell Signaling Technology: rabbit anti-phospho-IGF-1R (Y1135/1136), rabbit anti-IGF-1Rβ (Y1131; no. 3021, 1:200), rabbit anti-IGF-1Rβ (Y3018, 1:250), rabbit anti-phospho-FAK (Y927; 8556, 1:250), rabbit anti-FAK (3285), rabbit anti-phospho-p44/p42 mitogen-activated protein kinase (MAPK; extracellular signal-regulated kinase 1/2 [Erk1/2] (Thr202/Tyr204; no. 9101, 1:1000), rabbit anti-p44/42 MAPK (Erk1/2; no. 9102, 1:1000), rabbit anti-phospho-Src (Y416; 2101, 1:1000), rabbit anti-Src (2123, 1:1000), and rabbit anti-FoxM1 (no. 5436, 1:200). The following antibodies were purchased from AbCam (Cambridge, MA): rabbit anti-phospho-erythroblastic leukemia viral oncogene homolog 2 (erbB2/Erbb2 (Y877; no. ab47262, 1:200), rabbit anti-phospho-ErbB2 (Y877; no. ab108371, 1:200), and mouse anti-ErbB2 (no. ab16901, 1:200). Mouse anti-β-actin was purchased from Sigma-Aldrich (AC-15, 1:15,000). All primary antibodies were diluted in 5% bovine serum albumin/Tris-buffered saline and Tween 20. Goat anti-mouse secondary IRDye 800 antibody (no. 926-32210, 1:1,000) was purchased from Li-Cor Biosciences (Lincoln, NE). Goat anti-rabbit Alexa Fluor 680 secondary antibody (no. 1027681, 1:1,000) was purchased from Invitrogen (Grand Island, NY). Protein bands were detected using the Odyssey Imaging System (Li-Cor Biosciences). All blots were repeated at least three times with reproducible results.

ADCC Assays. The ADCC assay was performed as previously described (Kaumaya et al., 2009) using effector peripheral blood mononuclear cells, which were obtained from normal human donors and isolated by density-gradient centrifugation in Ficoll-Hypaque (Pharmacia Biotech, Piscataway, NJ). The cells were washed twice in RPMI 1640 with 5% fetal calf serum and then serially diluted in 96-well plates to give effector/target ratios of 100:1, 20:1, and 4:1. The following day, 1 x 10^5 target cells (JIMT-1) were treated with trastuzumab, IGF-1R antibody, combination, normal rabbit IgG (control for IGF-1R antibody), normal human IgG (control for trastuzumab), or a combination of control IgGs. Cells were incubated for 2–4 hours at 37°C, after which cell death was measured with a nonradioactive assay using the aCella-TOX reagent kit according to the manufacturer's instructions. Experiments were performed in triplicate.

Statistical Analyses. P values were determined for experimental versus control treatments by two-tailed t tests.

Results

IGF-1 Stimulates Crosstalk from IGF-1R to HER2. The JIMT-1 cell line represents a model of HER2-overexpressing breast cancer that exhibits intrinsic resistance to trastuzumab (Tanner et al., 2004). Cells were serum starved overnight and stimulated with IGF-1 at intervals
ranging from 0 to 60 minutes. IGF-1 not only induced phosphorylation of IGF-1R but also promoted phosphorylation of HER2 (Fig. 1A). Phosphorylation of Src, FAK, and ERK1/2 was also induced by IGF-1 stimulation. Pretreatment of JIMT-1 cells with the IGF-1R tyrosine kinase inhibitor NVPAEW541 abrogated IGF-1–mediated phosphorylation of HER2 in a concentration-dependent manner (Fig. 1B). These results indicate that IGF-1 stimulates phosphorylation of HER2 through IGF-1R kinase activation.

 Src Kinase Regulates Phosphorylation of HER2 in Resistant Cells. Increased Src kinase activity has been linked to trastuzumab resistance; furthermore, Src induces phosphorylation of receptor tyrosine kinases, including epidermal growth factor receptor and HER2 (Zhang et al., 2011). We found that Src phosphorylation was increased in response to IGF-1 stimulation in trastuzumab-resistant cells (Fig. 1). Transfection of wild-type or constitutively active Src constructs resulted in increased levels of phosphorylated HER2 and FAK in JIMT-1 cells, in contrast to transfection of kinase-dead Src (Fig. 2A). Furthermore, pharmacological inhibition of Src with the Src kinase inhibitor PP2 showed a dose-dependent decrease in HER2 phosphorylation (Fig. 2B). These results suggest that Src activity regulates HER2 phosphorylation in trastuzumab-resistant breast cancer cells.

 Effects of Pharmacological Inhibition of IGF-1R Plus Trastuzumab on Cell Growth. Next, we examined the effects of cotargeting IGF-1R and HER2 in trastuzumab-resistant cells. Fluorescent-activated cell sorting analysis of PI-stained cells indicated that combination treatment with the IGF-1R–targeted antibody α-IR3 plus trastuzumab did not have major effects on the cell cycle distribution of JIMT-1 cells after 48 hours (Supplemental Fig. 1). Trypan blue exclusion demonstrated modest, statistically significant reductions in the growth of trastuzumab-resistant JIMT-1 and HCC1954 cells in response to the combination of α-IR3 and trastuzumab when administered for a slightly longer treatment time period than that used in the fluorescent-activated cell sorting assays (Fig. 3A). Approximately 20% fewer cells were present in the treatment groups after 72 hours. Longer-term (2 to 3 weeks), matrigel-based cultures of JIMT-1 cells showed higher levels of growth inhibition in response to the combination treatment, with approximately 50% growth inhibition (Fig. 3B). As a single agent, trastuzumab reduced anchorage-independent growth of JIMT-1 cells by approximately 30% compared with a complete lack of growth inhibition by trastuzumab in anchorage-dependent cultures. This is likely due to prolonged exposure of matrigel cultures to treatment. These results suggest that JIMT-1 cells retain a low level of trastuzumab sensitivity, although they are relatively resistant compared with accepted models of sensitivity, such as BT474 and SKBR3 cells (not shown). In contrast with JIMT-1, long-term, matrigel-based cultures of HCC1954 cells showed a similar level of growth inhibition as that observed in trypan blue exclusion assays, with approximately 20% fewer cells in the combination group compared with controls, and no inhibition by single agents. Overall, these results indicate that the combination of α-IR3 and trastuzumab modestly reduces the growth of intrinsically resistant HER2-positive breast cancer cells to a greater extent than achieved with single-agent α-IR3 or trastuzumab.

 Pharmacological Inhibition of IGF-1R in Combination with Trastuzumab Suppresses Invasion of Resistant Cells. In contrast with effects on cell growth, the combination of α-IR3 and trastuzumab showed dramatic effects on the invasive potential of JIMT-1 and HCC1954 cells. Although neither of the antibodies reduced invasion when administered as single agents, the combination of IGF-1R and HER2 antibodies almost completely suppressed the abilities of JIMT-1 (Fig. 4A) and HCC1954 (Fig. 4B) to invade across matrigel-coated Boyden chambers. In contrast, the combination of α-IR3 and trastuzumab

![Figure 1](molpharm.aspetjournals.org)
did not reduce the invasiveness of IGF-1R-expressing MDA231 breast cancer cells (Supplemental Fig. 2), which lack over-expression of HER2. These results reduce the likelihood that off-target effects mediate the anti-invasive effect of this antibody combination and suggest that endogenous HER2 overexpression may be required to elicit this effect. Similar to the Boyden assays, the combination of α-IR3 and trastuzumab reduced the migration of JIMT-1 cells in spheroid assays (Fig. 4C). These results indicate that the suppression of invasion is a major effect of cotargeting IGF-1R and HER2 in trastuzumab-resistant cells.

To gain additional evidence that cotargeting IGF-1R and HER2 suppresses invasion, we treated JIMT-1 cells with a different IGF-1R antibody, IGF-IR-56-81 (Foy et al., 2014); this antibody is directed against a different epitope of IGF-1R than α-IR3. Similar to α-IR3 plus trastuzumab, the combination of IGF-IR-56-81 plus trastuzumab significantly reduced JIMT-1 cell invasion, whereas neither antibody alone affected invasion (Fig. 5A). In addition, the combination of IGF-1R and HER2 antibodies induced significant ADCC of JIMT-1 cells compared with controls and compared with either of the single agents (Fig. 5B). These data provide further evidence that targeting IGF-1R improves response to trastuzumab. Furthermore, these data suggest that blockade of invasion and induction of ADCC are two major biologic effects of cotargeting IGF-1R and HER2 with selective antibodies.

**Combination Knockdown of IGF-1R Plus Trastuzumab Reduces Growth and Invasion.** In addition to pharmacological inhibition of IGF-1R, we examined effects of knocking down IGF-1R by stably infecting JIMT-1 cells with lentiviral shRNA against IGF-1R versus control shRNA. Knockdown of IGF-1R improved the sensitivity of cells to trastuzumab, as demonstrated by reduced cell counts in a trypan blue exclusion assay.
kinase activity was required for IGF-1 blocked IGF-1 for IGF-1 Fig. 3), suggesting that crosstalk to HER2 may not be necessary lapatinib. Lapatinib blocked HER2 phosphorylation in JIMT-1 cells Src and FAK, we treated cells with the HER2 kinase inhibitor signaling is required for IGF-1 Trastuzumab-Resistant Cells. Because FoxM1 functions are known to promote cancer cell invasion, we examined the role of FoxM1 in IGF-1-mediated invasion in resistant cells, similar to FAK inhibition (Fig. 8). These results suggest that IGF-1-mediated invasion in trastuzumab-resistant breast cancer cells depends in part on Src-FAK signaling. Furthermore, these data indicate that Src inhibition may be essential to achieve an anti-invasive effect with a combination approach that cotargets IGF-1R and HER2.

**FoxM1 Contributes to IGF-1-Stimulated Invasion of Trastuzumab-Resistant Cells.** To determine whether HER2 signaling is required for IGF-1-stimulated phosphorylation of Src and FAK, we treated cells with the HER2 kinase inhibitor lapatinib. Lapatinib blocked HER2 phosphorylation in JIMT-1 cells but did not reduce IGF-1 signaling to Src or FAK (Supplemental Fig. 3), suggesting that crosstalk to HER2 may not be necessary for IGF-1-stimulated Src-FAK signaling. However, lapatinib blocked IGF-1-mediated invasion (Fig. 9), indicating that HER2 kinase activity was required for IGF-1-stimulated invasion in JIMT-1 cells.

We previously showed that the transcription factor FoxM1 promotes resistance to lapatinib via mitogen-activated protein kinase kinase (MEK) signaling in JIMT-1 cells, whereas knockdown of FoxM1 improves lapatinib sensitivity (Gayle et al., 2013). Because FoxM1 functions are known to promote cancer cell invasion, we examined the role of FoxM1 in IGF-1-mediated invasion of JIMT-1. Importantly, knockdown of FoxM1 blocked the ability of IGF-1 to promote cellular invasion (Fig. 9). Furthermore, stable IGF-1R knockdown plus trastuzumab treatment downregulated FoxM1 expression and reduced Erk1/2 phosphorylation, whereas IGF-1R knockdown alone did not (Fig. 10A). These results suggest that IGF-1R and HER2 may cooperatively regulate the expression of FoxM1 in JIMT-1 cells. Importantly, re-expression of FoxM1 restored invasion to stable IGF-1R knockdown cells treated with trastuzumab (Fig. 10, B and C). Thus, FoxM1 expression blocked the anti-invasive effect of combination IGF-1R knockdown plus trastuzumab. Together with the FoxM1 knockdown results (Fig. 9), these data suggest that FoxM1 expression affects the anti-invasive effect of IGF-1R/HER2 cotargeting, such that FoxM1 suppression may be necessary for this approach to be effective.

**Discussion**

Trastuzumab remains the primary first-line treatment administered for HER2-overexpressing metastatic breast cancer. Primary resistance and acquired resistance to trastuzumab occur in many patients; thus, a clear understanding of molecular mechanisms that drive resistance are required to improve therapeutic approaches for resistant tumors. We previously demonstrated that IGF-1R and HER2 form a unique receptor complex and demonstrate crosstalk in models of acquired resistance (Nahta et al., 2005). This finding was further corroborated by another study showing that IGF-1R and HER2 form a unique receptor complex and demonstrate crosstalk in models of acquired resistance (Nahta et al., 2005). This finding was further corroborated by another study showing that IGF-1R and HER2 form a unique receptor complex and demonstrate crosstalk in models of acquired resistance (Nahta et al., 2005). This finding was further corroborated by another study showing that IGF-1R and HER2 form a unique receptor complex and demonstrate crosstalk in models of acquired resistance (Nahta et al., 2005). This finding was further corroborated by another study showing that IGF-1R and HER2 form a unique receptor complex and demonstrate crosstalk in models of acquired resistance (Nahta et al., 2005). This finding was further corroborated by another study showing that IGF-1R and HER2 form a unique receptor complex and demonstrate crosstalk in models of acquired resistance (Nahta et al., 2005). This finding was further corroborated by another study showing that IGF-1R and HER2 form a unique receptor complex and demonstrate crosstalk in models of acquired resistance (Nahta et al., 2005). This finding was further corroborated by another study showing that IGF-1R and HER2 form a unique receptor complex and demonstrate crosstalk in models of acquired resistance (Nahta et al., 2005). This finding was further corroborated by another study showing that IGF-1R and HER2 form a unique receptor complex and demonstrate crosstalk in models of acquired resistance (Nahta et al., 2005). This finding was further corroborated by another study showing that IGF-1R and HER2 form a unique receptor complex and demonstrate crosstalk in models of acquired resistance (Nahta et al., 2005).
was also activated by IGF-1 and appeared to be critical for maintaining HER2 phosphorylation, because a small-molecule Src kinase inhibitor achieved dose-dependent inhibition of HER2 phosphorylation in the HER2-overexpressing JIMT-1 cell line, which exhibits primary resistance to trastuzumab. Furthermore, wild-type and constitutively active Src induced phosphorylation of HER2 and FAK, indicating that Src regulates baseline phosphorylation of HER2 and FAK in resistant cells. In addition to regulating HER2 phosphorylation status, Src proved to be an important mediator of invasion in resistant cells. Src kinase inhibition blocked IGF-1-mediated invasion, and constitutively active Src overcame the anti-invasive effect of the trastuzumab/a-IR3 combination.

Src has previously been shown to have multiple important roles in the development of resistance. For example, although Src is inhibited by trastuzumab in sensitive cells (Nagata et al., 2004), resistant cells show increased activation of Src (Zhang et al., 2011). Inhibition of Src normally results in PTEN dephosphorylation with subsequent membrane relocalization and phosphatase activation of PTEN (Nagata et al., 2004); in contrast, Src activity in resistant cells blocks PTEN activity and increases phosphoinositide 3-kinase signaling (Zhang et al., 2011). Src activation has been reported to occur downstream of multiple mechanisms of trastuzumab resistance, including increased signaling from growth factors and receptors, such as transforming growth factor-β (Wang et al., 2009), ephrin type-A receptor 2 (Zhuang et al., 2010), and growth differentiation factor-15 (Joshi et al., 2011). As a result, Src inhibition has been shown to improve trastuzumab response in multiple models (Zhang et al., 2011; Han et al., 2014; Peiró et al., 2014). Thus, our data that Src contributes to the regulation of HER2 phosphorylation and the invasive potential of resistant cells are consistent with previous reports supporting a central role for Src in trastuzumab resistance.

The contribution of Src to the invasive potential of resistant cells may be partially due to the activation of FAK, because...
Src kinase activation increased FAK phosphorylation, and small-molecule inhibitors of both Src and FAK reduced IGF-1-stimulated invasion. The role of FAK in the invasiveness of HER2-overexpressing breast cancer and trastuzumab resistance is supported by previous studies. Recruitment of FAK to HER2 has been reported to occur in response to heregulin stimulation (Vadlamudi et al., 2003); furthermore, phosphorylation levels of Src, FAK, and HER2 correlate in

Fig. 6. IGF-1R knockdown combined with trastuzumab treatment reduces growth and invasion. (A) JIMT-1 shPLKO.1 or shIGF-1R cells were treated with trastuzumab (20 µg/ml) or vehicle control. After 72 hours, cells were counted by trypan blue exclusion. Data are reported as a percentage of the control group. Results represent the average of triplicate cultures per group (t test, *P < 0.05); error bars represent the S.D. Western blots of total protein lysates were performed on the remaining cells for total IGF-1R to confirm knockdown; experiments were repeated at least three times. (B) JIMT-1 control shRNA stables (shPLKO.1) or IGF-1R shRNA stables (shIGF1R) were seeded and treated with control or 20 µg/ml trastuzumab in Boyden chambers in serum-free media. Media containing 10% FBS were placed in the well as the chemotactic agent. After 24 hours of invasion, photos were taken, and the number of invaded cells was counted in 12 random fields and added together; results represent the average of triplicate cultures per group. Representative photos are shown. The experiment was performed at least twice with reproducible results (t test, *P < 0.05); error bars represent the S.D. Western blots of total protein lysates were performed on the remaining cells for total IGF-1R to confirm knockdown. Tras, trastuzumab.

Fig. 7. Constitutively active Src blocks the anti-invasive effect of IGF-1R/HER2 cotargeting. JIMT-1 cells were transfected for 24 hours with empty vector pcDNA3.1 or constitutively active Src. (A) Western blots of total protein lysates were performed for p-Tyr416 Src, total Src, or actin. (B and C) Transfected cells were seeded in Boyden chambers in serum-free media with 10% FBS in the chamber as the chemotactic agent plus indicated treatments with control IgG, 20 µg/ml trastuzumab, 0.25 µg/ml α-IR3, or α-IR3 plus trastuzumab. After another 24 hours, photos were taken; representative photos are shown in (B). (C) The number of invaded cells was counted in 10 random fields and added together; results represent the average of triplicate cultures per group. The experiment was performed twice with similar results (t test, *P ≤ 0.05); error bars represent the S.D. aIR3, α-IR3; CA, constitutively active; Tras, trastuzumab.
clinical breast cancer samples (Schmitz et al., 2005). Similar to our results with IGF-1 stimulation, transforming growth factor-β has been shown to induce FAK phosphorylation downstream of Src (Wang et al., 2009). In addition, a phase I investigation of the Src kinase inhibitor, saracatinib, in patients with advanced solid tumors, including 13 metastatic breast cancers, showed that FAK phosphorylation is a useful surrogate marker for Src activity (Baselga et al., 2010). FAK inhibitors, including a dual IGF-1R/FAK inhibitor, have been shown to induce apoptosis in models of HER2-overexpressing breast cancers. Our data suggest that these agents may have additional utility in the setting of IGF-1–driven trastuzumab resistance.

Fig. 8. Inhibition of Src or FAK suppresses IGF-1–stimulated invasion of resistant cells. JIMT-1 cells were pretreated for 24 hours with DMSO, 10 μM PP2, or 500 nM FAK Inhibitor II (PF573228) in serum-free media. Cells were then seeded in Boyden chambers in serum-free media with 10% FBS in the chamber as the chemoattractant. Drug treatment was continued, and IGF-1 ligand was added to the chambers of treatment groups where indicated. (A) After 24 hours of invasion, photos were taken; representative photos are shown. (B) Western blots of total protein lysates were performed for p-Tyr416 Src, total Src, p-Tyr397 FAK, or total FAK to ensure inhibition of the target; representative blots are shown. (C) The number of invaded cells was counted in 12 random fields and added together; results represent the average of triplicate cultures per group. The experiment was performed twice with reproducible results (t test, *P ≤ 0.05); error bars represent the S.D. FAKi, FAK inhibitor.

Fig. 9. HER2 kinase and FoxM1 contribute to IGF-1–stimulated invasion. (A) JIMT-1 cells were pretreated for 24 hours with DMSO or 100 nM lapatinib in serum-free media or were transfected with 100 nM control siRNA or FoxM1 siRNA. Cells were then seeded in Boyden chambers in serum-free media with 10% FBS in the well as the chemoattractant. Drug treatment was continued, and IGF-1 ligand was added to the chambers of treatment groups where indicated. Western blots of total protein lysates were performed for FoxM1, p-Tyr877 HER2, and total HER2 to ensure inhibition of the target; representative blots are shown. After 24 hours of invasion, photos were taken; representative photos are shown. (B) The number of invaded cells was counted in 12 random fields and added together; results represent the average of triplicate cultures per group. Black bars, no IGF-1 stimulation; gray bars, plus IGF-1 stimulation. The experiment was performed twice with reproducible results (t test, *P ≤ 0.05); error bars represent the S.D. siFoxM1, FoxM1 siRNA.
The recruitment and activation of intracellular kinases, such as FAK, by IGF-1R have been shown to occur through integrins in some cell systems (Desgrosellier and Cheresh, 2010). Thus, the possibility that IGF-1 promotes Src-FAK signaling and invasion through integrins in the context of trastuzumab resistance should be considered in future studies. For example, HER2 function and resistance to HER2-targeted therapies were previously associated with integrin-mediated adhesion to the extracellular protein laminin-5 in association with increased FAK signaling (Yang et al., 2010). Overexpression of β1 integrin has also been shown to mediate trastuzumab resistance (Lesniak et al., 2009). Furthermore, the erbB growth factor heregulin has been shown to regulate αvβ3 integrin levels in invasive breast cancers to affect downstream MAPK signaling (Vellon et al., 2005). The potential importance of integrins to resistance is further reflected by the finding that cancer cells that overexpress both HER2 and the integrin receptor αvβ4 exhibit a highly aggressive and malignant phenotype (Falcioni et al., 1997). Thus, there is a clear body of literature supporting a link between integrins, HER2 signaling, and resistance. The role of IGF-1 in this context is supported by the finding that IGF-1 stimulation disrupts the αv integrin/E-cadherin/IGF-1R ternary complex, leading to integrin redistribution to focal contact sites and increased invasion (Canonica et al., 2008). Thus, future studies should examine the role that integrins play in IGF-1–mediated trastuzumab resistance and the effect of integrin signaling on the efficacy of IGF-1R/HER2 combination approaches, particularly as they relate to invasion. Another important mediator of invasion activated downstream of Src and FoxM1 downstream of HER2. Coinhibition of IGF-1R and HER2 is required to overcome the proinvasive effects of Src-FAK signaling and FoxM1 in trastuzumab-resistant cells. Ab, antibody; TKI, tyrosine kinase inhibitor; Tras, trastuzumab.

The recruitment and activation of intracellular kinases, such as FAK, by IGF-1R have been shown to occur through integrins in some cell systems (Desgrosellier and Cheresh, 2010). Thus, the possibility that IGF-1 promotes Src-FAK signaling and invasion through integrins in the context of trastuzumab resistance should be considered in future studies. For example, HER2 function and resistance to HER2-targeted therapies were previously associated with integrin-mediated adhesion to the extracellular protein laminin-5 in association with increased FAK signaling (Yang et al., 2010). Overexpression of β1 integrin has also been shown to mediate trastuzumab resistance (Lesniak et al., 2009). Furthermore, the erbB growth factor heregulin has been shown to regulate αvβ3 integrin levels in invasive breast cancers to affect downstream MAPK signaling (Vellon et al., 2005). The potential importance of integrins to resistance is further reflected by the finding that cancer cells that overexpress both HER2 and the integrin receptor αvβ4 exhibit a highly aggressive and malignant phenotype (Falcioni et al., 1997). Thus, there is a clear body of literature supporting a link between integrins, HER2 signaling, and resistance. The role of IGF-1 in this context is supported by the finding that IGF-1 stimulation disrupts the αv integrin/E-cadherin/IGF-1R ternary complex, leading to integrin redistribution to focal contact sites and increased invasion (Canonica et al., 2008). Thus, future studies should examine the role that integrins play in IGF-1–mediated trastuzumab resistance and the effect of integrin signaling on the efficacy of IGF-1R/HER2 combination approaches, particularly as they relate to invasion. Another important mediator of invasion activated downstream of HER2 is the FoxM1 transcription factor; expression of FoxM1 correlates with poor prognosis and HER2 overexpression in breast cancer (Bektas et al., 2008; Francis et al., 2009; Carr et al., 2010). We previously reported that FoxM1 expression levels and cellular localization are heavily regulated by MEK signaling in trastuzumab-resistant cells, including JIMT1 cells (Gayle et al., 2013). Coinhibition of HER2 and MEK downregulated FoxM1 expression and blocked the growth of trastuzumab-resistant cancer cell xenografts (Gayle et al., 2013). In addition to our previous results showing that HER2-MEK signaling regulates FoxM1 expression, the results of this study indicate that FoxM1 expression is codependent on IGF-1R and HER2 in resistant cells. Stable knockdown of IGF-1R alone did not alter FoxM1 expression; however, IGF-1R knockdown plus trastuzumab reduced Erk1/2 phosphorylation, downregulated FoxM1
expression, and reduced the invasive potential of resistant cells. This is likely due to the codependence of these cells on IGF-1R and HER2, such that both receptors must be inhibited to achieve meaningful downstream signaling blockade. Re-expression of FoxM1 restored the invasive ability of resistant cells in the context of IGF-1R knockdown plus trastuzumab treatment. Furthermore, we found that FoxM1 was an important mediator of the invasive potential of resistant cells, such that knockdown of FoxM1 blocked IGF-1–mediated invasion. IGF-1R and HER2 signaling coregulated FoxM1 expression in resistant cells, such that coinhibition of both receptor kinases was required to reduce FoxM1 expression. These results support an important function for FoxM1 in IGF-1–mediated resistance, and suggest that reduced expression of FoxM1 may be necessary to achieve the anti-invasive effect of cotargeted IGF-1R/HER2 therapy.

Past studies have shown somewhat conflicting results regarding the association between overall IGF-1R expression levels and response to trastuzumab (Smith et al., 2004; Köstler et al., 2006; Harris et al., 2007; Gallardo et al., 2012; Yerushalmi et al., 2012). However, there is strong evidence to suggest that cotargeting IGF-1R and HER2 has increased benefit against HER2-positive breast cancers. Blockade of IGF-1R signaling with antibodies (Nahta et al., 2005), tyrosine kinase inhibitors (Nahta et al., 2005; Chakraborty et al., 2008; Esparis-Ogando et al., 2008), genetic knockdown (Huang et al., 2010), or expression of IGF-1–sequestering proteins (Lu et al., 2001; Jerome et al., 2006) has been shown to improve sensitivity to trastuzumab in multiple models of trastuzumab resistance; sensitivity was primarily assessed by proliferation, apoptosis, and xenograft tumor growth in these reports. An important finding of our study was that cotargeting IGF-1R and HER2 had modest, although significant, effects on the growth inhibition of cells with primary trastuzumab resistance, but almost completely suppressed cellular invasion. FoxM1 downregulation appeared to be an essential downstream mediator of the anti-invasive effect of cotargeting IGF-1R and HER2. In addition to blocking invasion, coinhibition of IGF-1R and HER2 induced ADCC of resistant cells, which is believed to be a major mechanism through which antibody-based therapies promote tumor regression. These results support strategies to simultaneously block both signaling pathways and support FoxM1 as a fundamental regulator of cellular invasion in trastuzumab-resistant cancers.

Overall, our results indicate that the invasiveness of resistant cells is codependent on IGF-1R and HER2 signaling, such that coinhibition of both receptors is required to suppress invasion and overcome downstream signaling (Fig. 10D). Constitutively active Src and FoxM1 overexpression overcame the anti-invasive effects of dual IGF-1R/HER2 inhibition. These results lend additional support to the growing concept that Src represents a potential therapeutic target in trastuzumab-resistant breast cancer, and demonstrate that FoxM1 is an important target worthy of further investigation, particularly in the context of cancers that coexpress IGF-1R and HER2. Future experiments will investigate the effects of cotargeting IGF-1R and HER2 on the local invasion and metastasis of resistant tumors in vivo, and the overall contributions of Src-FAK and FoxM1 to the in vivo progression of HER2-positive breast cancers.

Acknowledgments

The authors thank the Winship Cancer Institute Cell Imaging and Microscopy Center for assistance with microscopy.

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Wrote or contributed to the writing of the manuscript: Sanabria-Figueroa, Donnelly, Foy, Buss, Castellino, Paplomata, Taliaferro-Smith, Kaumaya, Nahta.

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