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Therapeutics, Targets, and Chemical Biology

Elevation of Receptor Tyrosine Kinase EphA2 Mediates Resistance to Trastuzumab Therapy

Guanglei Zhuang[1], Dana M. Brantley-Sieders[2], David Vaught[1], Jian Yu[6], Lu Xie[6], Sam Wells[5], Dowdy Jackson[7], Rebecca Muraoka-Cook[1], Carlos Arteaga[1,2,4], and Jin Chen[1,2,3,4]

Abstract

One arising challenge in the treatment of breast cancer is the development of therapeutic resistance to trastuzumab, an antibody targeting the human epidermal growth factor receptor-2 (HER2), which is frequently amplified in breast cancers. In this study, we provide evidence that elevated level of the receptor tyrosine kinase Eph receptor A2 (EphA2) is an important contributor to trastuzumab resistance. In a screen of a large cohort of human breast cancers, we found that EphA2 overexpression correlated with a decrease in disease-free and overall survival of HER2-overexpressing patients. Trastuzumab-resistant cell lines overexpressed EphA2, whereas inhibiting EphA2 restored sensitivity to trastuzumab treatment *in vivo*. Notably, trastuzumab treatment could promote EphA2 phosphorylation by activating Src kinase, leading in turn to an amplification of phosphoinositide 3-kinase/Akt and mitogen-activated protein kinase signaling in resistant cells. Our findings offer mechanistic insights into the basis for trastuzumab resistance and rationalize strategies to target EphA2 as a tactic to reverse trastuzumab resistance. *Cancer Res; 70(1); 299–308. ©2010 AACR.*

Introduction

Recent advances in the development and application of molecularly targeted therapies for cancer have generated promising new treatments. One such treatment is the recombinant humanized monoclonal anti-HER2 antibody trastuzumab (Herceptin, Genentech). Trastuzumab targets the human epidermal growth factor receptor-2 (HER2/ErbB2) oncoprotein (1), a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases (RTK). HER2 is overexpressed in 25% to 30% of human breast cancers and is associated with poor patient survival (2). Despite the proven benefit of trastuzumab in treating breast cancer (3–5), not all patients with amplified HER2 respond to trastuzumab. Indeed, only one third of women with newly diagnosed HER2-positive breast cancer exhibit tumor regression with trastuzumab monotherapy (5). In addition, the majority of patients who achieve an initial response develop trastuzumab resistance within 1 year (5, 6). Therefore, identifying mechanisms that modulate trastuzumab response and resistance is vital to improving the therapeutic index of this agent.

Eph receptor A2 (EphA2), an Eph-family RTK, has been recently linked to breast tumor initiation and metastatic progression (7–9). Experimentally induced overexpression of EphA2 resulted in malignant transformation of nontransformed MCF10A breast epithelial cells and enhanced malignancy of pancreatic carcinoma cells (10, 11). Conversely, small interfering RNA (siRNA)–mediated inhibition of EphA2 expression impaired the malignant progression of pancreatic, ovarian, and mesothelioma human tumor cell lines, and overexpression of dominant-negative EphA2 constructs suppressed the growth and metastasis of 4T1 mouse mammary adenocarcinoma cells *in vivo* (10, 12–14). EphA2-mediated oncogenesis seems to be ligand independent, and EphA2 often signals through cross talk with other cell surface receptors (15, 16). We recently reported that loss of EphA2 receptor impaired tumor initiation and metastatic progression in mouse mammary tumor virus (MMTV)-Neu mice (17). In human and murine breast carcinoma cells, EphA2 forms a complex with HER2, resulting in enhanced activation of Ras-mitogen-activated protein kinase (MAPK) and RhoA GTPase and increased cell proliferation and motility. These data indicate that EphA2 promotes breast tumor formation and metastatic progression by amplifying HER2 signaling.

In this report, we investigated the role of EphA2 in regulation of breast cancer sensitivity to trastuzumab. We found that high EphA2 levels enhanced both intrinsic and acquired trastuzumab resistance. Elevated EphA2 in resistant cells seems to be activated by trastuzumab treatment–induced Src kinase, and activated EphA2 amplifies signaling through the phosphoinositide 3-kinase (PI3K)/Akt and MAPK pathways in resistant cells. In addition, microarray analysis of a large cohort of human breast cancer specimens revealed that

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high levels of EphA2 expression in HER2-positive patients predict poor prognosis. Thus, these results provide new mechanistic insights into the molecular basis of anti-HER2 resistance, and targeting EphA2 could represent an appealing therapeutic strategy to increase the efficacy of HER2-based treatments in breast cancer.

Materials and Methods

Survival analysis. The van der Vijver database, with microarray profiles of 295 human breast tumors and associated clinical data, was obtained from Rosetta Inpharmics.[8] The first 25% patients that exhibit higher HER2 expression were defined as HER2 positive, as described (18–20). The HER2-positive patients were further stratified into two groups based on the expression levels of EphA2. Kaplan-Meier analyses were computed using R survival package. Statistical differences were determined by log-rank tests.

Cell culture. The MMTV-Neu tumor–derived cell line (21), parental MCF10A cells, and MCF10A cells stably overexpressing HER2 were maintained as described previously (17). Parental and trastuzumab-resistant SK-BR-3 and BT-474 cells were generously provided by Francisco Esteva (The University of Texas M.D. Anderson Cancer Center, Houston, TX; ref. 22) and Carlos Arteaga (Vanderbilt University, Nashville, TN; ref. 22), respectively. Three-dimensional spheroid cultures were established on Matrigel as described (24). Cultures were maintained for 8 d before photodocumentation. Digital images were analyzed and the percentage of Ki67-positive cells was quantified using LSM Image Browser (Zeiss) software. Results were derived from 10 colonies in two independent experiments. Statistical differences among groups were determined by Student's *t* test.

Mice and **in vivo** ***tumor studies.*** Athymic nude female mice, 3 to 4 wk old, were implanted with 1.5-mg, 60-d-release 17β-estradiol pellets s.c. The next day, trastuzumab-resistant BT-474 cells (1.5×10^{7}; HR5) were resuspended in 100 μL PBS/100 μL growth factor–reduced Matrigel and injected into the number 4 inguinal mammary gland fat pad as previously described (22). Tumor engraftment and growth was verified by palpation and tumor volume was measured by a caliper. Two weeks after transplantation, the mice were treated with control IgG (10 mg/kg; clone R347, MedImmune, LLC), anti-EphA2 antibody (10 mg/kg; clone 3F2-3M, MedImmune, LLC), trastuzumab (20 mg/kg), or the combination of anti-EphA2 antibody and trastuzumab by twice-weekly i.p. injections. Tumors were harvested 2 wk after treatment and data were derived from 10 independent animals per treatment group in two independent experiments.

Histologic analyses. Tumors were sectioned by the Vanderbilt University Immunohistochemistry Core Facility. Immunohistochemical staining for EphA2, proliferating cell nuclear antigen (PCNA), and CD31 was done as described previously (25). Proliferation or apoptosis was quantified by calculating the average percentage of PCNA- or terminal
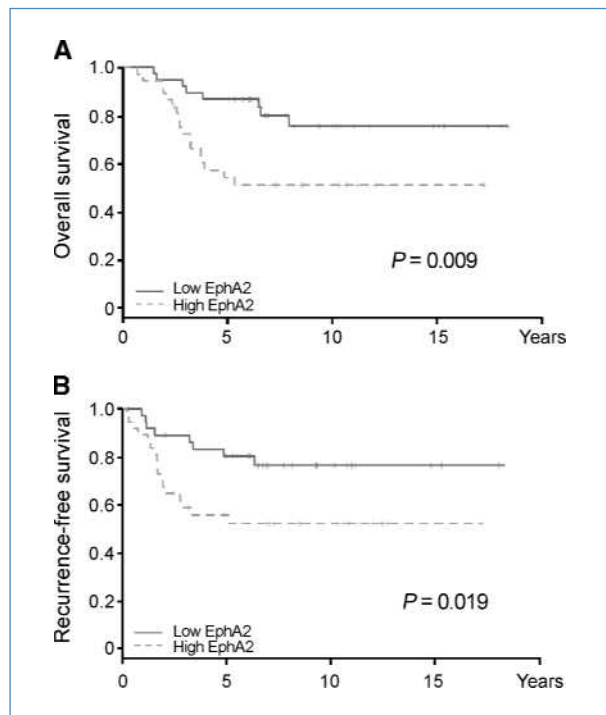
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[8] http://www.rii.com/publications/2002/nejm.html

Figure 1. Overexpression of EphA2 in HER2-positive patients predicts poor prognosis. A previously published microarray data set from the fresh-frozen tissue bank of the Netherlands Cancer Institute for a panel of 295 breast cancer samples was analyzed. The resulting Kaplan-Meier kinetic analyses of survival data revealed that high levels of EphA2 mRNA expression correlated with a decrease in overall survival (*A*; *P* = 0.009) and recurrence-free survival (*B*; *P* = 0.019).

deoxyribonucleotidyl transferase–mediated dUTP nick end labeling (TUNEL)–positive nuclei relative to total nuclei (four random fields of at least four independent tumor samples).

Fluorescence resonance energy transfer analysis of Src biosensor. The MCF7 cells expressing HER2 were transfected with Src biosensor (generously provided by Yingxiao Wang, University of Illinois, Urbana-Champaign, IL) and serum starved for 48 h before being treated with trastuzumab (10 μg/mL). Imaging and fluorescence resonance energy transfer (FRET) analysis were done on an LSM 510 META confocal microscope (Zeiss) using a 40×/1.3 NAPlan-Neofluar objective lens and 458-nm laser excitation for cyan fluorescent protein (CFP) and FRET. Emission from CFP versus yellow fluorescent protein (YFP)/FRET was discriminated using appropriate bandpass emission filters (BP 475-525 for CFP and LP560 for YFP/FRET). The fluorescence intensities of CFP and YFP images were measured using the Zeiss Image Examiner software before being quantified and analyzed by Prism 5 (GraphPad). Quantification was based on 20 cells per time point in two independent experiments. Statistical differences were analyzed using Student's *t* test.

Results

Overexpression of EphA2 in HER2-positive patients predicts poor prognosis. Because our previous investigations in

mouse models suggest that cooperation between HER2 and EphA2 may promote mammary tumor formation, we sought to determine if EphA2 could be an effective therapeutic target for HER2-positive breast cancer patients. To analyze the effect of EphA2 overexpression on the prognosis of HER2-positive breast cancer patients, we examined previously published microarray data for a panel of 295 breast cancer samples (26). Seventy-four HER2-positive samples were examined for EphA2 mRNA expression. The resulting Kaplan-Meier analysis of survival data revealed that high levels of EphA2 expression correlated with a decrease in overall (Fig. 1A) and recurrence-free survival (Fig. 1B) in HER2-positive breast cancer patients. These data indicate that EphA2 overexpression in HER2-positive patients may predict poor prognosis, and elevated EphA2 may enable breast cancer cells to resist anti-HER2 treatment.

EphA2 overexpression confers cellular intrinsic resistance to trastuzumab. To investigate whether EphA2 overexpression is sufficient to confer resistance to trastuzumab, we transduced a constitutively activated (CA-EphA2) or a kinase-dead (KD-EphA2) form of human EphA2 into MCF10A.HER2 cells (ref. 27; Supplementary Fig. S1A). MCF10A.HER2 cells formed large acinar-like structure with a filled lumen and were sensitive to trastuzumab treatment (Fig. 2A; ref. 28). Introduction of CA-EphA2 into in MCF10A.HER2 cells further enhanced cell proliferation, but this increased cell growth in MCF10A.HER2 cells expressing CA-EphA2 was refractory to trastuzumab (Fig. 2A and B). In contrast, expression of catalytically inactive KD-EphA2 in MCF10A.HER2 cells decreased the basal rates of proliferation, which were further decreased on treatment with trastuzumab (Fig. 2A and B). These data are consistent with previous data showing cooperation between HER2 and EphA2 to drive cellular proliferation (17), and further suggest that EphA2 kinase activity is able to promote trastuzumab resistance in HER2-overexpressing breast cells.

Interestingly, MCF10A.HER2 cells express elevated levels of EphA2 protein relative to those in parental MCF10A cells (Supplementary Fig. S1B). To determine if inhibition of EphA2 increases innate sensitivity to trastuzumab,

Figure 2. EphA2 overexpression confers cellular intrinsic resistance to trastuzumab. *A,* constitutively activated (CA-EphA2) or kinase-dead (KD-EphA2) EphA2 receptor were introduced into MCF10A or MCF10A.HER2 cells by retroviral transduction. *tras,* trastuzumab. Pooled G418-resistant cell populations were cultured in three-dimensional Matrigel and stained for Ki67 (*green*) to assess proliferation and counterstained for To-Pro-3 (*red*) to visualize nuclei. Overexpression of CA-EphA2, but not KD-EphA2, desensitizes MCF10A.HER2 cells to trastuzumab. Cell proliferation was quantified in *B.* *, $P < 0.01$, Student's *t* test. *C,* MCF10A or MCF10A.HER2 cells were cultured in three-dimensional Matrigel and treated with antibodies as indicated. Anti-EphA2 antibody inhibited cell growth in MCF10A.HER2 cells. Cell proliferation in *C* is qualified in *D.* *, $P < 0.01$; **, $P < 0.05$, Student's *t* test.
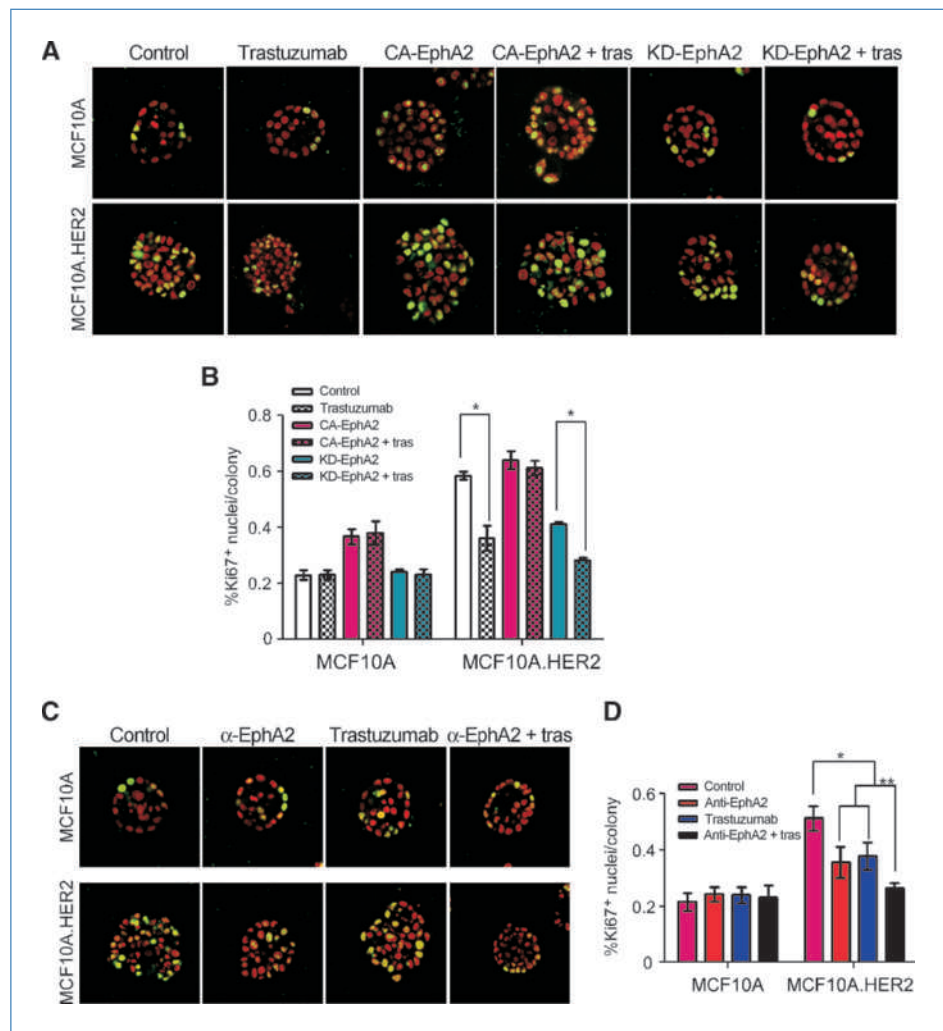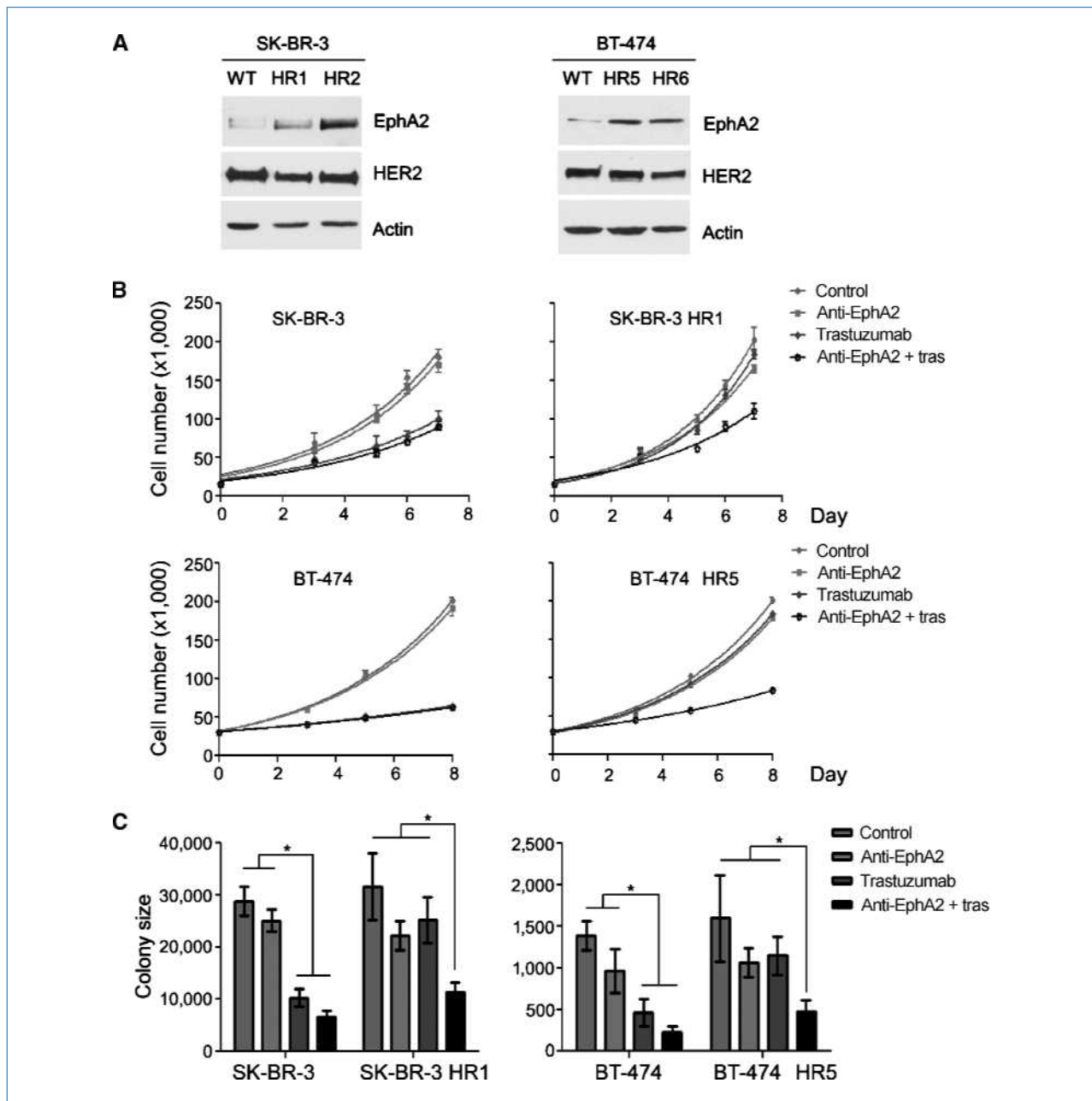
Figure 3. EphA2 elevation contributes to acquired trastuzumab resistance. *A,* trastuzumab-sensitive (*WT*) or trastuzumab-resistant (*HR*) SK-BR-3 or BT-474 cells were subjected to Western blot analysis to assess EphA2 expression levels. *B,* sensitive or resistant SK-BR-3 or BT-474 cells were treated with IgG control, anti-EphA2, trastuzumab, or the combination of anti-EphA2 antibody and trastuzumab. Anti-EphA2 antibody restores cellular sensitivity to trastuzumab. *C,* sensitive or resistant SK-BR-3 or BT-474 cells were cultured in three-dimensional Matrigel. Colonies were photographed at day 7 and colony size was quantified. *, $P < 0.01$, Student's *t* test.

MCF10A.HER2 cells were treated with an antihuman EphA2 antibody, a ligand-mimetic activating antibody that specifically binds to EphA2 and induces receptor internalization and degradation (Supplementary Fig. S1*B*). Whereas the anti-EphA2 antibody had no effect on nontransformed MCF10A cells that express low levels of EphA2, the antibody significantly inhibited cell growth in MCF10A.HER2 cells. More

importantly, the combination of anti-EphA2 antibody and trastuzumab inhibited cell growth with greater potency than either antibody alone (Fig. 2*C* and *D*). Taken together, these data suggest that EphA2 overexpression is one mechanism of intrinsic resistance to trastuzumab.

As an independent approach to determine whether EphA2 expression levels correlate with trastuzumab resistance, we

overexpressed HER2 in a panel of human breast cancer cell lines that express EphA2 protein at low or high levels (Supplementary Fig. S2*A*). BT-474 and SK-BR-3 cells that express high levels of endogenous HER2 but low levels of EphA2 were growth inhibited in response to trastuzumab, and so were MCF7 and T47D that overexpress HER2 (Supplementary Fig. S2*B*). In contrast, HBL100, MDA-468, MDA-231, and BT-549 expressed high levels of EphA2 and were resistant to the growth inhibitory effects of trastuzumab. These data are consistent with a correlation between EphA2 expression and trastuzumab response in HER2-overexpressing human breast cancer cells.

EphA2 elevation contributes to acquired trastuzumab resistance. Genome-wide profiling of gene expression showed that EphA2 and HER2 are not always coexpressed in human breast cancer. We reasoned that on prolonged trastuzumab treatment, a subset of HER2-positive tumors that initially express low levels of EphA2 and respond to trastuzumab may increase EphA2 expression, leading to a decrease in trastuzumab sensitivity. To test this possibility, we analyzed EphA2 expression in two independent trastuzumab-resistant human breast cancer cell lines, SK-BR-3 and BT-474, which were derived from *in vitro* or *in vivo* selection for acquired resistance to trastuzumab, respectively (22, 23). As shown in Fig. 3*A*, EphA2 levels were considerably higher in two independently derived trastuzumab-resistant clones from each cell line relative to their trastuzumab-sensitive parental cells. To test whether this EphA2 overexpression is required to maintain trastuzumab resistance, we treated the parental and the trastuzumab-resistant cells with anti-EphA2 antibody in the presence or absence of trastuzumab. As expected, sensitive SK-BR-3 and BT-474 cells were growth inhibited by trastuzumab whereas resistant cells were not. Anti-EphA2 antibody alone did not significantly affect cell growth in SK-BR-3 or BT-474 cells. However, EphA2 inhibition restored cellular sensitivity to trastuzumab in each resistant cell line, as shown in both two-dimensional cell culture (Fig. 3*B*) and three-dimensional Matrigel culture (Fig. 3*C*). These data suggest that EphA2 is upregulated in treatment-induced, trastuzumab-resistant cells and that high levels of EphA2 in resistant cells contribute to acquired trastuzumab resistance.

Targeting EphA2 inhibits trastuzumab-resistant tumor growth **in vivo.** Having shown the combinatorial activity of anti-EphA2 antibody and trastuzumab for growth inhibition of trastuzumab-resistant cells *in vitro*, we next investigated the therapeutic potential of an anti-EphA2 antibody for the treatment of trastuzumab-resistant tumor growth *in vivo* in an orthotopic xenograft model. Trastuzumab-resistant BT-474 cells were injected into the mammary fat pad of female athymic nude mice. Two weeks after transplantation, when tumor volume reached ∼200 mm^3, mice were treated with either control IgG or antihuman EphA2 antibody (10 mg/kg) in the presence or absence of trastuzumab (20 mg/kg). Consistent with a prior report (22), resistant BT-474 tumors did not respond to trastuzumab treatment as compared with IgG-treated tumors. Anti-EphA2 antibody treatment moderately reduced tumor size

relative to controls. In contrast, coadministration of anti-EphA2 antibody with trastuzumab markedly reduced tumor volume (Fig. 4*A* and *B*).

To examine cellular changes within treated tumors, we analyzed cell proliferation and apoptosis in tissue sections by staining for PCNA and by TUNEL assay, respectively. Quantitation of PCNA-positive nuclei revealed a nearly 2-fold decrease in PCNA staining in tumors treated with the combination of anti-EphA2 antibody versus tumors treated with control IgG ($P < 0.05$; Fig. 4*C*). In contrast, treatment with anti-EphA2 antibody alone or with trastuzumab alone did not significantly alter the proportion of PCNA-positive cells as compared with IgG-treated tumors. Similarly, apoptosis was increased ∼6-fold in tumors treated with the combination of anti-EphA2 antibody and trastuzumab ($P < 0.01$; Fig. 4*C*) but was unaltered in tumors treated with either antibody alone. Taken together, these data suggest that targeting EphA2 may be effective for the suppression of trastuzumab-resistant breast tumor growth.

EphA2 regulates breast cancer cell sensitivity to trastuzumab by modulation of Akt and MAPK activities. Breast cancer resistance to HER2 inhibitors could arise through multiple mechanisms, including activation of alternative growth factor receptors or enhancing downstream signaling pathways. We investigated potential mechanisms by which EphA2 contributes to trastuzumab resistance in HER2-overexpressing breast cancer. We found that elimination of EphA2 by siRNA knockdown or anti-EphA2 antibody reduced phospho-Akt and phospho-extracellular signal-regulated kinase (Erk) levels in trastuzumab-resistant cells (Fig. 5*A* and *B*), suggesting that EphA2 expression and activity are required to maintain signaling through the PI3K-Akt and MAPK signaling pathways.

To determine whether the PI3K-Akt and Ras-MAPK signaling pathways play a causal role in trastuzumab resistance, we treated SK-BR-3 cells with a PI3K inhibitor, LY294002 (Fig. 5*C*), or a mitogen-activated protein/Erk kinase (MEK) inhibitor, U0126 (Fig. 5*D*), and analyzed cell growth in the presence or absence of trastuzumab. In sensitive cells, cell growth is inhibited by trastuzumab, and addition of LY294002 or U0126 did not further affect cell growth significantly. However, although resistant cells do not respond to trastuzumab, they are exquisitely sensitive to the MEK inhibitor (Fig. 5*D*). In fact, resistant cells are more sensitive to U0126 than trastuzumab-sensitive cells, suggesting that trastuzumab-resistant cells are dependent on MAPK signaling. In addition, either PI3K inhibitor or MEK inhibitor significantly restored trastuzumab sensitivity in resistant cells. Together, our data suggest that anti-EphA2 antibody therapy reverses trastuzumab resistance by inhibiting the activation of both Akt and MAPK.

Chronic trastuzumab treatment activates EphA2 through Src kinase. To investigate how EphA2 is activated in trastuzumab-resistant cells, we examined the involvement of Src kinase because prior studies showed that Src directly interacts with HER2 and is activated in HER2-overexpressing cancer cells (29, 30). Coexpression of HER2 and EphA2 in COS7 cells was sufficient to induce tyrosine phosphorylation
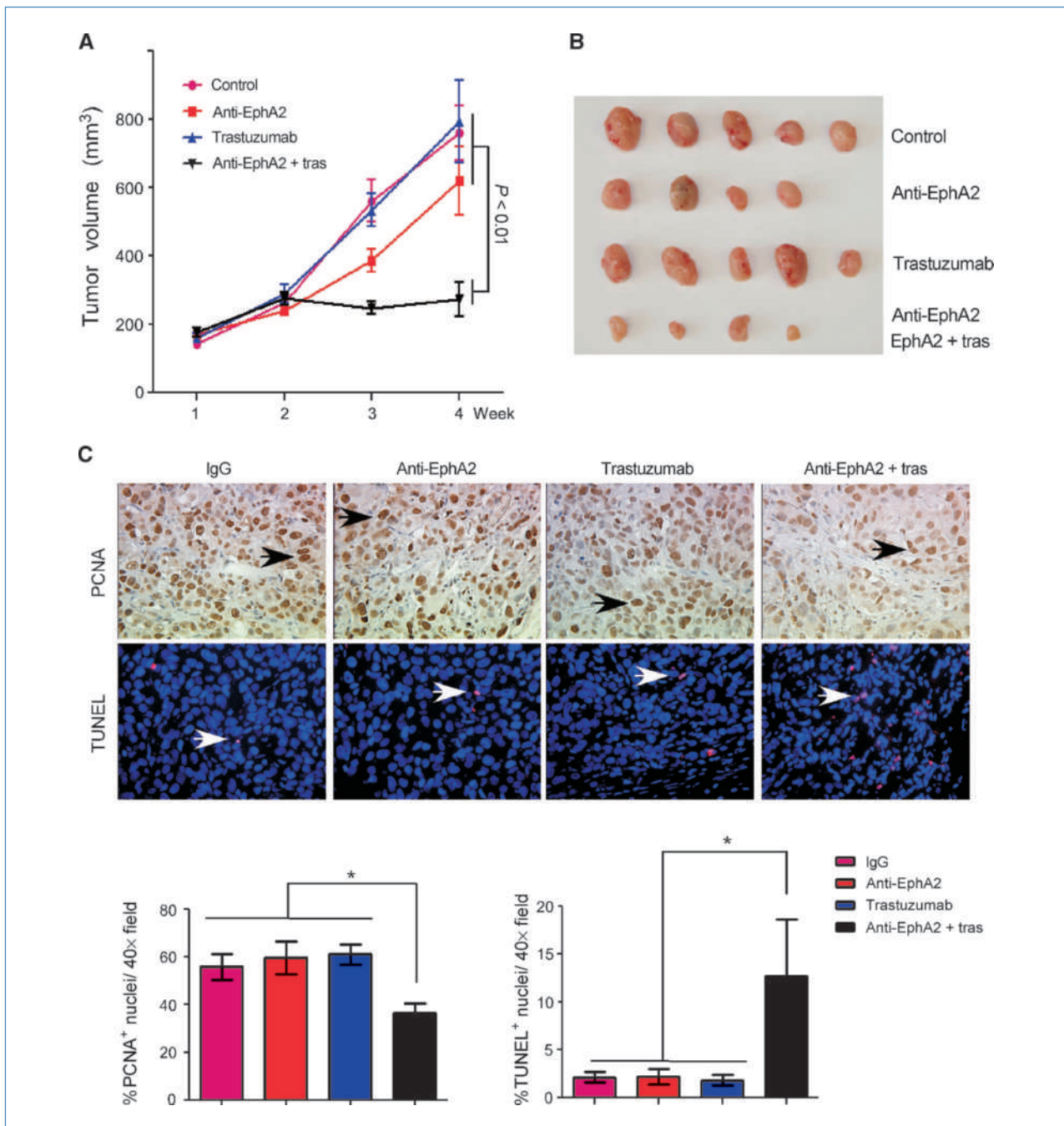
Figure 4. Targeting EphA2 inhibits trastuzumab-resistant tumor growth. *A,* trastuzumab-resistant BT-474 cells were orthotopically transplanted into the mammary glands of nude female mice. Two weeks after transplantation, tumors were treated with control IgG, anti-EphA2 antibody (10 mg/kg), trastuzumab (20 mg/kg), or the combination of anti-EphA2 antibody and trastuzumab twice weekly via i.p. injection. *Points,* mean of 10 mice per treatment group from two independent experiments; *bars,* SEM. *B,* tumors were harvested and photographed. *C,* cell proliferation and apoptosis in tumor sections were evaluated by PCNA immunohistochemistry and TUNEL assay, respectively. *, $P < 0.01$. *Arrowheads,* PCNA- or TUNEL-positive nuclei.

of EphA2, and this process was inhibited by a Src inhibitor, PP2. In addition, constitutively activated v-Src induced phosphorylation of EphA2 independently of HER2 (Supplementary Fig. S3*A*), suggesting that HER2 may modulate EphA2 activity through Src. We next investigated whether Src can

be activated by trastuzumab. A previous study suggested that short exposure to trastuzumab rapidly inhibits Src kinase activity (31). However, we found that longer treatment of SK-BR-3 cells with trastuzumab increased Src phosphorylation at Y416, an indicator of Src activation (Supplementary

Fig. S3*B*). To further determine whether prolonged trastuzumab treatment can activate Src kinase, we used a Src biosensor that enables the visualization of Src activity in live cells with high spatiotemporal resolution by FRET technology (32, 33). Trastuzumab induced a 15% to 25% reduction in Src activity within 1 hour in MCF7.HER2 cells transfected with the Src biosensor, but the decrease in Src activity gradually recovered with prolonged trastuzumab incubation (Fig. 6*A*). After 24 hours of treatment, Src activity increased by 35% in MCF7.HER2 relative to control cells (Fig. 6*B*), whereas EphA2 levels were not changed (Supplementary Fig. S3*C*). These data support the existence of a switch from trastuzumab-induced Src inhibition to activation, which could modulate EphA2 activity in resistant cells. Indeed, EphA2 and Src were highly phosphorylated in trastuzumab-resistant cells. Src inhibitors, PP2 (Fig. 6*C*) or dasatinib (data not shown), inhibited the activities of both Src and EphA2.

To determine whether Src kinase contributes to trastuzumab resistance, we treated SK-BR-3 cells with trastuzumab, dasatinib, or their combination and assessed cell viability. Dasatinib inhibited cell growth in both sensitive and resistant cells. Whereas resistant cells did not respond to trastuzumab, dasatinib partially restored trastuzumab sensitivity in resistant cells (Fig. 6*D*). Together, these results provide a clear link between activation of Src and EphA2 in trastuzumab resistance.

Discussion

In this report, we described a novel mechanism by which HER2-positive breast cancers acquire resistance to trastuzumab. The RTK EphA2 was found to correlate with a poor prognosis in patients with HER2-overexpressing breast cancers and had a greater negative impact on patient survival in HER2-overexpressing breast cancers as compared with other
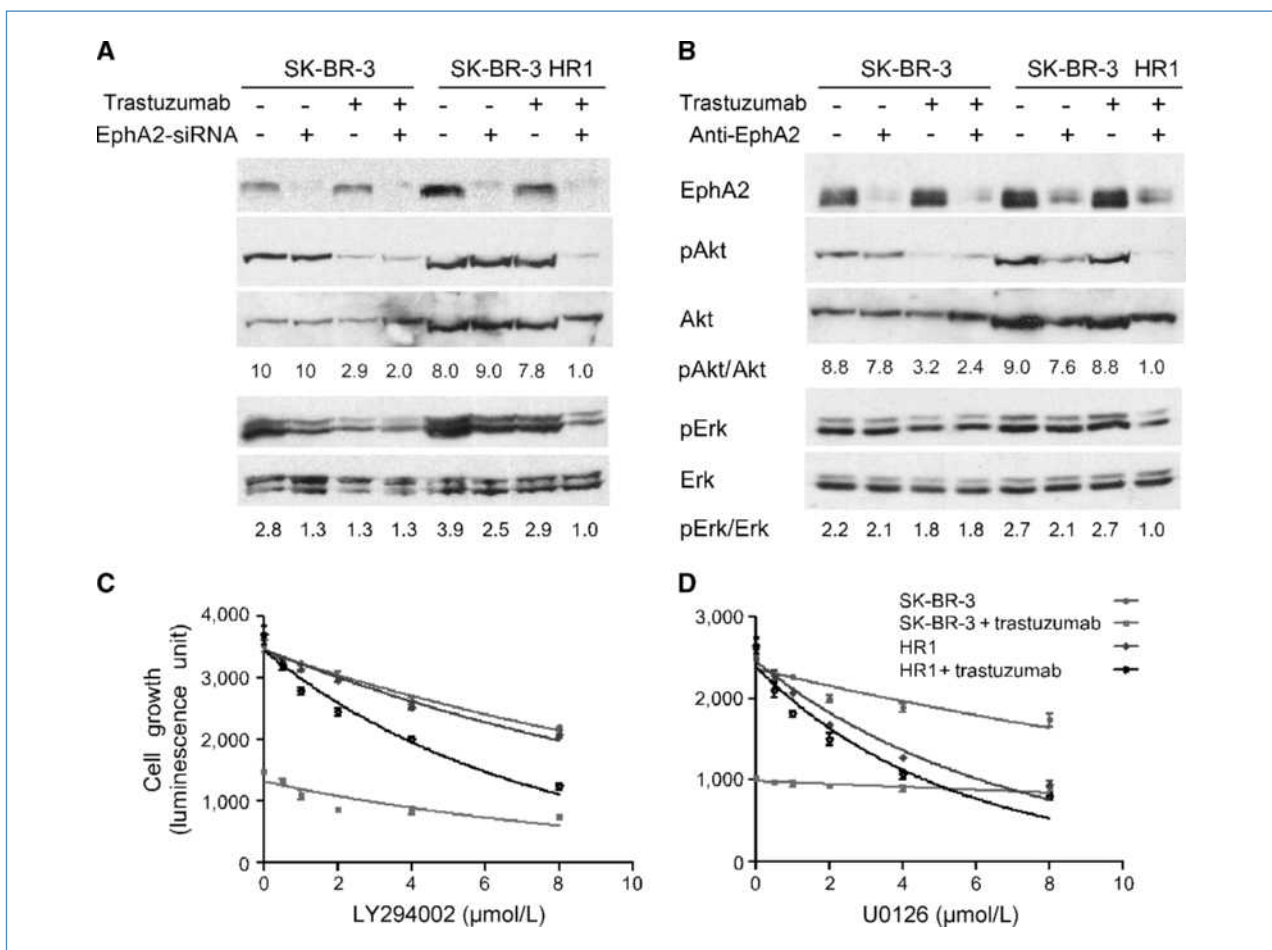

Figure 5. EphA2 regulates breast cancer sensitivity to trastuzumab by modulation of Akt and MAPK activity. *A,* EphA2 was knocked down by siRNA in either parental or trastuzumab-resistant (*HR1*) SK-BR-3 cells. EphA2, phosphor-Akt, and phospho-Erk levels were assessed by Western blot analysis. The ratio of phospho-protein/total protein was determined by densitometry and expressed in arbitrary units. *B,* trastuzumab-sensitive or trastuzumab-resistant cells were treated with control, trastuzumab, anti-EphA2 antibody, or the combination of trastuzumab and anti-EphA2 antibody in the presence of 10% serum. Quantification of phospho-protein/total protein was determined as above. *C* and *D,* trastuzumab-sensitive or trastuzumab-resistant (*HR1*) SK-BR-3 cells were treated with increasing dose of either PI3K inhibitor LY294002 or MEK inhibitor U0126 for 3 d and cell viability was determined.
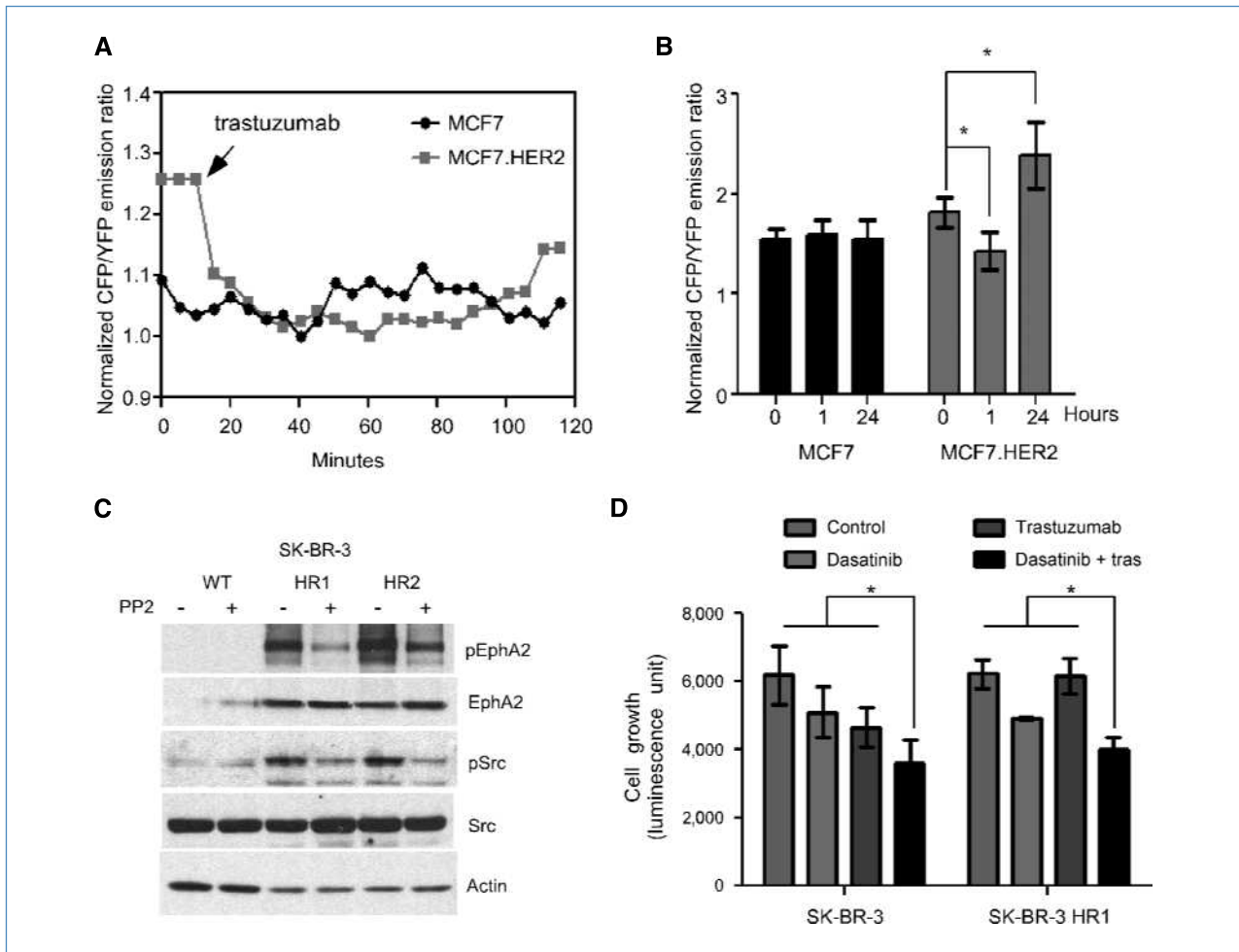
Figure 6. Trastuzumab treatment activates EphA2 through Src kinase. *A,* MCF7.HER2 cells expressing Src reporter were treated with trastuzumab over a time course. The normalized CFP/YFP emission ratio of the Src biosensor over time in response to trastuzumab is shown. *B,* MCF7.HER2 cells expressing Src reporter were treated with trastuzumab for 0, 1, and 24 h. EphA2 levels were not changed during treatment (Supplementary Fig. S3C). Changes in CFP/YFP emission ratio were quantified in 20 cells per experimental group at given time point. *, $P < 0.01$. *C,* sensitive (*WT*) or resistant (*HR*) SK-BR-3 cells were treated with Src inhibitor PP2 or vehicle control. Phospho-Src and phospho-EphA2 levels were assessed by immunoprecipitation and Western blot analysis. *D,* trastuzumab-sensitive or trastuzumab-resistant SK-BR-3 cells were treated with control, dasatinib, trastuzumab, or the combination of dasatinib and trastuzumab for 3 d. Cell growth was determined by luminescent cell viability assay. *, $P < 0.05$, Student's *t* test.

breast cancers. We found that overexpression of EphA2 in HER2-positive breast cancer cells was sufficient to confer innate resistance to trastuzumab. Furthermore, antibody-mediated EphA2 inhibition enhanced tumor response to trastuzumab both in cell culture and *in vivo*. These data suggest that therapeutic inhibition of EphA2 may represent a strategy for improving the clinical response of trastuzumab.

What is the mechanism by which elevated EphA2 confers tumor cell resistance to trastuzumab? Resistance to anti-HER2/ErbB2 agents could arise through multiple mechanisms, including altered receptor-antibody interaction, activation of alternative growth factor receptor signaling pathways, and deregulation of downstream signaling pathways (34, 35). The most common downstream signaling pathway that contributes to trastuzumab resistance is the PI3K-Akt pathway. Persistent activation of PI3K-Akt signaling in resistant cells

could result from multiple mechanisms such as oncogenic mutations of PI3K (36), loss of PTEN (31), or upregulation of insulin-like growth factor-I receptor and EGFR activity (22, 37). In this case, targeting EphA2 inhibited the PI3K-Akt pathway in trastuzumab resistant cells (Fig. 5). In addition to regulating Akt activity, we discovered that EphA2 also modulates phospho-Erk levels in resistant cells. Increased EphA2 expression in resistant cells enhanced phospho-Erk levels, and targeting EphA2 with siRNA or anti-EphA2 antibody inhibited Erk activity (Fig. 5). These data, together with reports from other laboratories (38, 39), suggest that the development of trastuzumab resistance may involve simultaneous activation of multiple parallel signaling cascades including the PI3K-Akt and MAPK pathways (40–42). Indeed, a MEK inhibitor that suppresses phospho-Erk significantly decreased the viability of resistant cells (Fig. 5D). Suppression of MAPK activity by EphA2

antibody was also observed in MCF10A three-dimensional culture (data not shown), as well as in MMTV-Neu cells (Supplementary Fig. S4A), where Erk phosphorylation recovered after prolonged treatment with gefitinib, a dual inhibitor of EGFR and ErbB2/Neu (43). The combination of anti-EphA2 antibody and gefitinib completely abrogated MAPK activity and inhibited tumor growth *in vivo* (Supplementary Fig. S4B). Together, these data suggest that modulation of both Akt and MAPK signaling is a primary mechanism through which EphA2 contributes to trastuzumab resistance.

How is EphA2 receptor activated in trastuzumab-resistant cells? We have previously shown that EphA2 forms a complex with HER2/ErbB2 and can be phosphorylated in the presence of HER2/ErbB2 (17). However, we failed to detect direct EphA2 tyrosine phosphorylation by HER2 in an *in vitro* kinase assay (data not shown), indicating the possibility of involvement of another kinase. One candidate is the non-RTK Src because Src directly interacts with HER2 and is activated in HER2-overexpressing cancer cells (29, 30). Indeed, Src is sufficient to activate EphA2 and is required for the phosphorylation of EphA2 by HER2 (Supplementary Fig. S3A). Although trastuzumab reportedly inhibits Src activity within a short time frame (31), we observed increased Src activity in cells on prolonged exposure to trastuzumab (Supplementary Fig. S3B). Using a FRET-based Src reporter to monitor Src activity in live cells, we found that short-term exposure to trastuzumab inhibits Src kinase activity, consistent with a previous report (31). However, prolonged treatment resulted in increased Src activity (Fig. 6A and B). These results were supported by biochemical studies, in which Src phosphorylation at Y416 was increased with prolonged trastuzumab treatment. The mechanism of switch between trastuzumab-induced Src inhibition and activation is unclear. We speculate that continuous exposure to trastuzumab may cluster HER2 at the plasma membrane and recruit Src into the HER2/EphA2 complex, resulting in activation of Src and phosphorylation of EphA2 receptor.

Our findings that EphA2 coexpresses with HER2 and confers trastuzumab resistance in HER2-positive breast cancers could directly affect the clinical management of these patients. We propose that individuals with EphA2 and HER2-positive breast cancer might benefit from pharmacologic inhibition of EphA2 in combination with anti-HER2 thera-

pies. EphA2 expression may also be used as a prognostic marker to predict trastuzumab resistance and treatment outcome. In patients who initially are negative for EphA2 but subsequently develop resistance to trastuzumab, elevated EphA2 could be one of the mechanisms that confer tumor resistance to HER2 inhibitors. Targeting EphA2 may represent a novel strategy to overcome trastuzumab resistance. In summary, our studies provide new mechanistic insights into the molecular basis of trastuzumab resistance. These studies provide a basis for rational design of combination therapies to overcome tumor resistance to trastuzumab.

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