Transforming Growth Factor β Induces Clustering of HER2 and Integrins by Activating Src-Focal Adhesion Kinase and Receptor Association to the Cytoskeleton

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Abstract

It has been proposed that cross talk between integrin and growth factor receptor signaling such as ErbB2 (HER2) is required for activation of downstream effectors and ErbB2-mediated mammary tumorigenesis. Here we show that transforming growth factor β (TGF-β) induced focal adhesion kinase (FAK)–dependent clustering of HER2 and integrins α_6, β_1, and β_4 in HER2-overexpressing mammary epithelial cells without altering the total and surface levels of HER2 receptors. This effect was mediated by ligand-induced epidermal growth factor receptor (EGFR) activation and the subsequent phosphorylation of Src and FAK. We have previously reported that TGF-β up-regulates EGFR ligand shedding through a mechanism involving the phosphorylation of tumor necrosis factor-α–converting enzyme (TACE/ADAM17). Knockdown of TACE, FAK, or integrin α_6 by siRNA or inhibition of EGFR or Src by specific inhibitors abrogated TGF-β–induced receptor clustering and signaling to phosphatidylinositol 3-kinase-Akt. Finally, inhibition of Src-FAK reversed TGF-β–induced resistance to the therapeutic HER2 inhibitor trastuzumab in HER2-overexpressing breast cancer cells. Taken together, these data suggest that, by activating Src-FAK, TGF-β integrates ErbB receptor and integrin signaling to induce cell migration and survival during breast cancer progression. [Cancer Res 2009;69(2):475–82]

Introduction

The integrin family of transmembrane receptors of the extracellular matrix (ECM) mediates a wide variety of cellular behaviors including cell adhesion, migration, proliferation, and survival (1, 2). In addition to transducing signals in the ECM to the cell membrane and initiating intracellular signaling through integrin-associated proteins such as focal adhesion kinase (FAK), integrins are also involved in growth factor signaling through coclustering with the growth factor receptor tyrosine kinases (RTK) such as the epidermal growth factor receptor (EGFR), HER2 (ErbB2), and platelet-derived growth factor receptor (PDGFR; refs. 3, 4). Phosphorylation of integrins by RTKs and non-RTKs such as FAK and Src may, in turn, regulate "inside-out" integrin signaling by altering their affinity and avidity to ECM ligands. The membrane-proximal clustering of integrin and RTK requires FAK as the scaffold that interacts with both RTK and integrin receptors through different domains (3).

HER2 gene amplification or overexpression of HER2 is observed in ~30% of invasive breast cancers, where it correlates with poor patient prognosis. Several integrin-associated signaling molecules, including both integrins β_1 and β_4, integrin-linked kinase, and FAK, have been implicated in the initiation and progression of mammary tumors driven by oncogenes such as Neu (the rat homologue of HER2) and the polyomavirus middle T (PyVMT; refs. 4–7). In the transgenic mouse model of mouse mammary tumor virus (MMTV)/Neu, targeted deletion of β_4 integrin was shown to suppress mammary tumor onset and invasive growth (4).

Transforming growth factor β (TGF-β), a multitasking cytokine involved in development, differentiation, tissue regeneration, and immune response, has also been shown to synergize with Neu/ErbB2 (8–11). Overexpression of active TGF-β1 or active mutants of the type I TGF-β receptor (Alk5) in the mammary gland of bitransgenic mice also expressing MMTV/Neu accelerates metastases from Neu-induced mammary cancers (8–10). In the MCF10A human mammary epithelial cells that were engineered to overexpress HER2 (MCF10A/HER2), TGF-β induces cell migration and invasion (12, 13). Inhibition of HER2 with the HER2-neutralizing antibody trastuzumab blocked the promigratory effect of TGF-β on HER2-overexpressing mammary epithelial cells (12), suggesting that the proto-oncogene is required for the transforming effect of TGF-β in HER2-overexpressing transformed cells.

We have previously shown that TGF-β induces HER2 translocalization to the lamellipodia through a phosphatidylinositol 3-kinase (PI3K)–dependent mechanism that involves activation of Rac1 and Rak1 and reorganization of actin cytoskeleton (13). Moreover, blockade of integrin signaling by an inhibitory antibody against β_1 integrin abrogates the function of TGF-β to induce motility in MCF10A/HER2 cells (12). Because TGF-β is known to influence various integrin functions such as adhesion and migration, we focused in this study on the role of integrin signaling in the cross talk between TGF-β and HER2 in breast cancer progression.

Materials and Methods

Cells, reagents, and viruses. MCF10A/HER2 cells were generated and maintained as described previously (12, 14). Human breast cancer cell lines MDA-MB-453 and BT-474 were purchased from the American Type Tissue Culture Collection and maintained in IMEM (Cellgro) containing 10% fetal

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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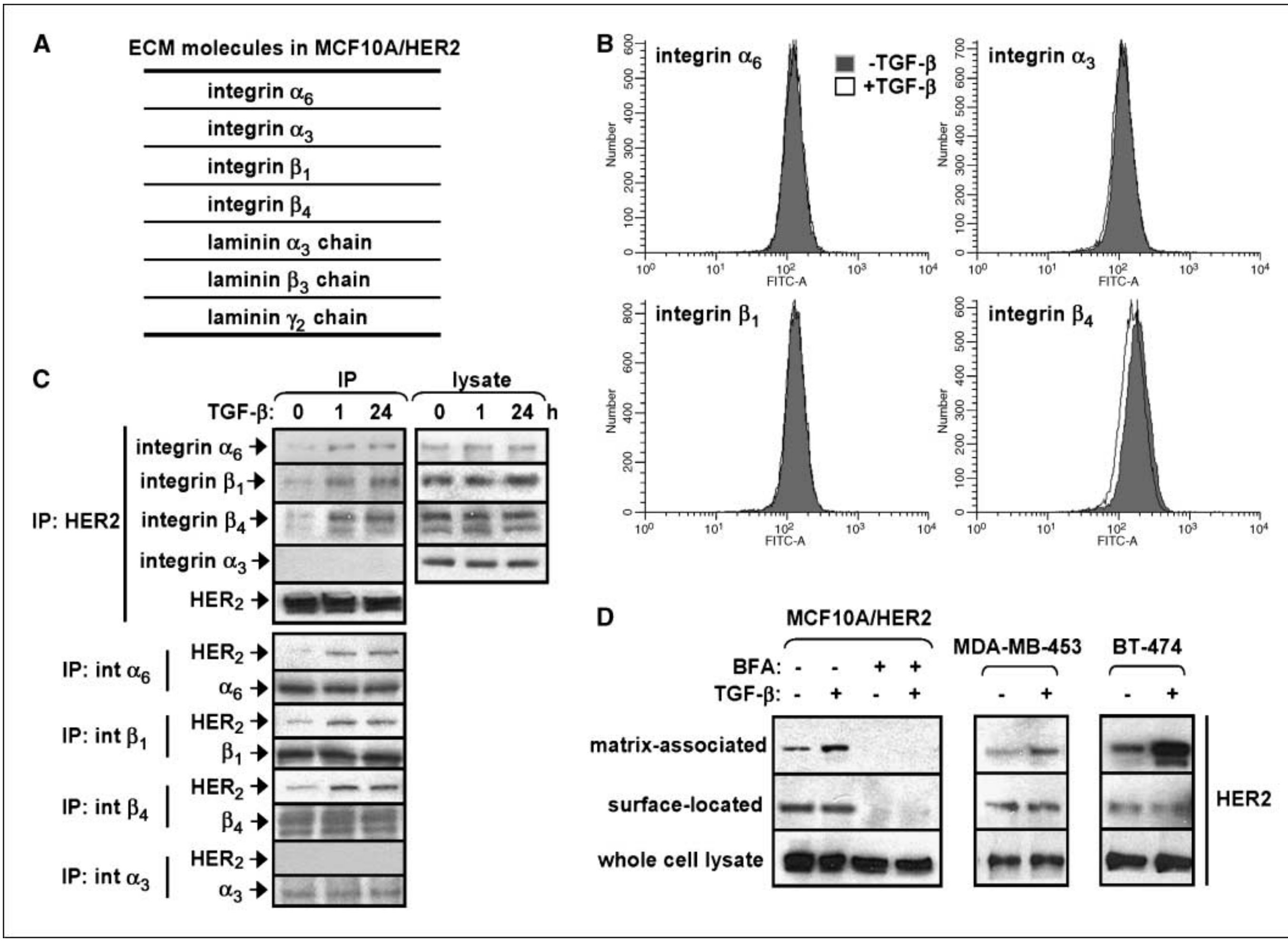

Figure 1. TGF-β induces clustering of HER2 and integrins α_6, β_1, and β_4 at the cell membrane. *A,* matrix and matrix-associated proteins were prepared from MCF10A/HER2 cells grown on a 100-mm culture dish as described in ref. 17. The preparations were separated by SDS-PAGE and the identities of the proteins were determined by peptide mass mapping and single-stage MS as indicated in Materials and Methods. *B,* MCF10A/HER2 cells grown on culture dishes were treated with TGF-β (2 ng/mL) or equal amount of PBS (vehicle control) for 24 h in growth medium. At the end of treatment, cells were harvested by trypsinization and stained with antibodies against integrins α_6, α_3, β_1, and β_4, followed by a secondary FITC-conjugated antibody. Stained cells were then analyzed by flow cytometry for the level of FITC. *C,* MCF10A/HER2 cells were serum starved for 16 h before being treated with TGF-β (2 ng/mL) for 1 or 24 h. At the end of the treatment, cells were lysed for immunoprecipitation with antibodies against HER2 and integrin α_6, β_1, or β_4. The pull-downs were subjected to immunoblot with the indicated antibodies. *D,* MCF10A/HER2, MDA-MB-453, and BT-474 cells were serum starved for 16 h and treated with TGF-β or PBS (vehicle control) for 1 h before being subjected to matrix preparation or cell-surface biotinylation as described in Materials and Methods. Prepared matrix proteins, the streptavidin pull-down for surface-located proteins, and the whole cell lysate were subjected to immunoblot with HER2 antibody. Brefeldin A (10 μg/mL) was added to cells 1 h before TGF-β in the indicated lanes.

bovine serum (Hyclone) in a humidified 5% CO_2 incubator at 37°C. Recombinant human TGF-β1 and TGF-α were purchased from R&D Systems. Brefeldin A, protein phosphatase 2 (PP2), and cytochalasin D were purchased from Sigma. Trastuzumab and cetuximab were purchased at the Vanderbilt University Medical Center Pharmacy. Lapatinib ditosylate (GW-572016) was from LC Laboratories. Adenoviruses encoding dominant negative p85 (AxΔp85) were described previously (15). The plasmid encoding the hemagglutinin-tagged full-length mouse TACE [TACE(HA)] was described previously (16).

Matrix preparation and mass spectrometry. Preparation of deposited ECM and matrix-associated proteins was done as described (17). Briefly, confluent MCF10A/HER2 cells were lysed on plate by incubating in 20 mmol/L sterile NH_4OH for 5 min at room temperature, followed by gentle scraping and extensive washing. The remaining matrix preparation was directly scraped into reducing Laemmli sample buffer, boiled, and applied to SDS-PAGE. The gel was stained with Coomassie blue. Excised protein bands were subjected to in-gel digestion as described elsewhere (13).

Cell-surface biotinylation, immunoprecipitation, and immunoblot. Cells grown in 100-mm dishes were washed in cold PBS (pH 8.0) thrice before being incubated with freshly prepared Sulfo-NHS-Biotin reagent (2 mmol/L; Pierce) 30 min at 4°C. The reaction was quenched with 100 mmol/L glycine in PBS and the cells were lysed in NP40 lysis buffer (20 mmol/L Tris, 150 mmol/L NaCl, 1% NP40, 0.1 mmol/L EDTA, plus protease and phosphatase inhibitors). After sonication for 10 s and centrifugation (14,000 rpm), protein concentration in the supernatants was measured using the bicinchoninic acid protein assay reagent (Pierce). Equal amounts of protein extracts (500 μg) were subjected to precipitation using Streptavidin Magnetic Spheres (Promega) followed by SDS-PAGE and HER2 immunoblot. For immunoprecipitation, cells were washed twice with cold PBS and lysed in NP40 lysis buffer. Immunoprecipitation and immunoblotting were done as described (14) using horseradish peroxidase–conjugated secondary antibodies (Promega). Primary antibodies include integrins α_6, α_3, β_1, and β_4 and TACE (Chemicon); EGFR and HER2/ErbB2 (NeoMarkers); actin (Sigma); FAK and Src (Santa Cruz Biotechnology); P-EGFR(Y1068), P-HER2(Y1248), P-Src(Y416), P-FAK(Y397), P-Akt(S473), and Akt (Cell Signaling); and p85 (Upstate Biotechnology). Bands were quantified using ImageJ.

Fluorescence-activated cell sorting analysis. Cells were harvested by trypsinization, washed with PBS, and exposed to monoclonal antibodies of

the appropriate integrin (1:50 in DMEM) followed by a FITC-coupled rabbit anti-mouse secondary IgG (1:50). Flow cytometry was done with a FACScan instrument (Becton Dickinson).

Cell viability, motility, and invasion assays. For viability assays, cells growing on six-well plates were serum starved for 48 h and collected for trypan blue staining as described elsewhere (18). Trypan blue–positive cells were counted manually and their percentage was calculated over the total cell input. Transwell motility assays were done as described previously (19). Invasion assay was done using laminin- or collagen-coated transwells (BD Biosciences) according to the manufacturer's protocol. Cells were seeded at 2.5×10^4 per well on coated transwells and allowed to invade toward serum-free medium ± TGF-β. At 16 h, cells that invaded through the transwell filters were stained and counted as described (19).

Three-dimensional cell culture. Cells were seeded on growth factor–reduced Matrigel (BD Biosciences) in eight-well chamber slides following the protocol described by Debnath and colleagues (20). Inhibitors were added into the medium 12 h after cell seeding and were replenished every 3 d. Phase-contrast images were taken every 3 d. For cell number counting, cultures growing on Matrigel were trypsinized and cell numbers were measured in a Coulter counter.

RNA interference studies. Silencer siRNAs against human TACE were obtained from Qiagen in a FlexiTube format. Four species of siRNAs against different TACE target sequences (TCCCATGAAGAACACGTGTAA, CTGCAGTAAACAATCAATCTA, CAGGATGTAATTGAACGATTT, and AAGAAACAGAGTGCTAATTTA) were mixed in equal amount to generate a TACE siRNA pool (siTACE). SiRNAs against FAK target sequences (CACCTGGGTACTGGTATGGAA, AATCACACACCAAATTCGAGT, CCGGTCGAATGATAAGGTGTA, and AACAATTTATGTTCACATTAA) and integrin α_6 target sequences (CAGGGTAATAAACTTAGGTAA, CC-GGCCTGTGATTAATATTCA, AAGGATGGGTGGCAAGATATA, and AACCCTGTGGCTACAGGATAA; Qiagen) were used. SiRNAs were transfected into cell lines using HiPerFect transfection reagent (Qiagen) according to the manufacturer's procedures. In a six-well plate format, a total of 150 ng of siRNAs and 12 μL of HiPerFect reagent were used for each transfection. Mismatched siRNAs were used as negative control (siCTRL).

Results

TGF-β induces clustering of HER2 and integrins α_6, β_1, and β_4 at the cell membrane. We first determined the ECM deposited by MCF10A/HER2 cells. Cells growing on tissue culture dishes were lysed as described in Materials and Methods; proteins attached to the plate were harvested and subjected to mass spectrometry (MS). Laminin-5, which consists of the laminin α_3, β_3, and γ_2 chains, as well as integrins α_6, α_3, β_1, and β_4, were identified in the matrix deposited by 10A/HER2 cells by MS (Fig. 1*A*). We also analyzed the ECM composition in cells treated with TGF-β for 24 hours using the same approach and did not find different protein species between treated and untreated cells. As determined by flow cytometry using specific antibodies,

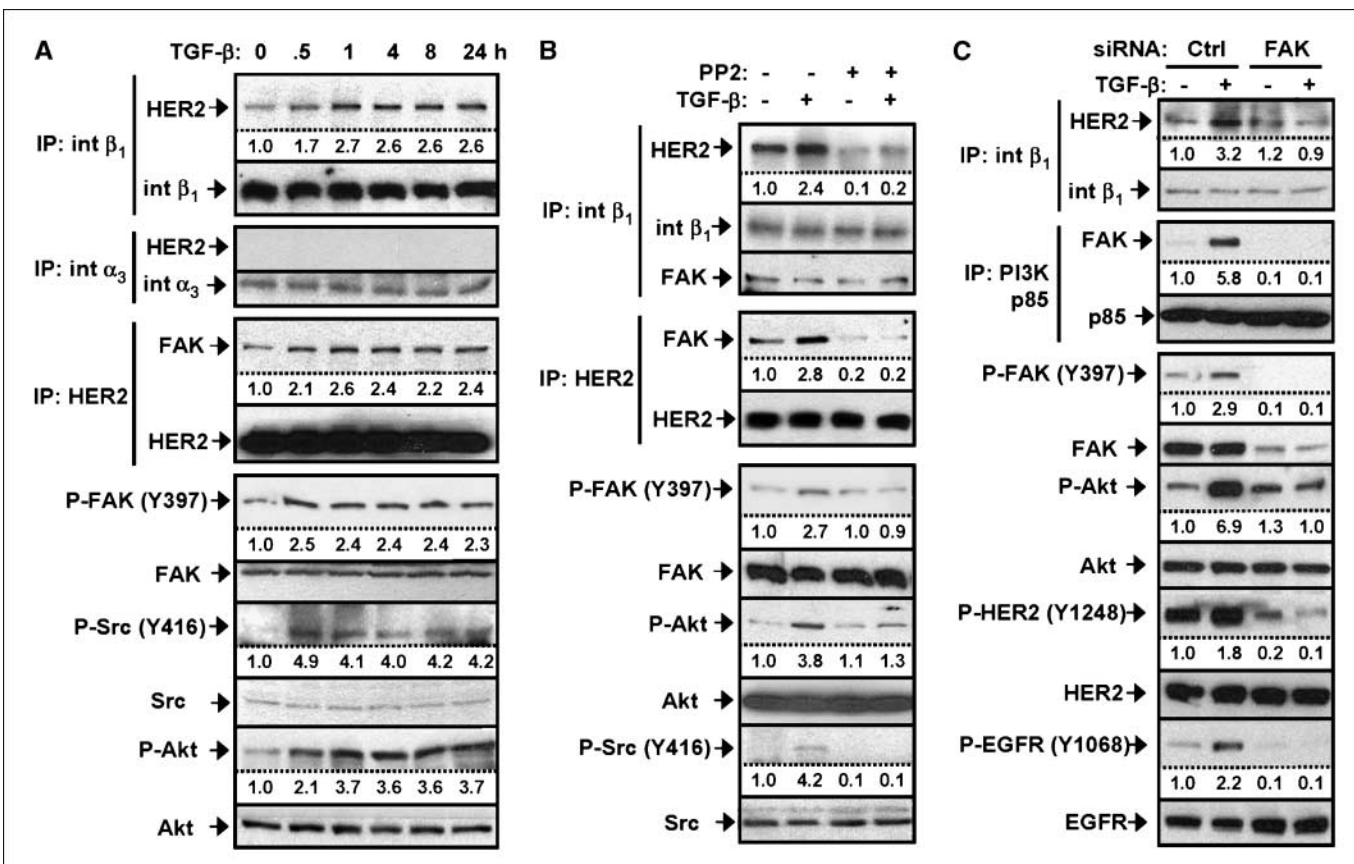

Figure 2. TGF-β activates Src-FAK, leading to receptor clustering and activation of PI3K-Akt. *A,* MCF10A/HER2 cells were serum starved for 16 h and treated with TGF-β for the indicated time before being subjected to immunoprecipitation with antibodies against integrin β_1 and HER2. The pull-downs and whole cell lysate were subjected to immunoblot with the indicated antibodies. *B,* MCF10A/HER2 cells were serum starved for 16 h and pretreated with PP2 (5 μmol/L) or DMSO (vehicle control) for 1 h before being treated with TGF-β or PBS (vehicle control) for 1 h and subjected to immunoprecipitation and immunoblot. *C,* MCF10A/HER2 cells grown on six-well plates were transiently transfected with siRNA oligonucleotides targeting FAK or a control sequence. Seventy-two hours after transfection, cells were serum starved for 16 h and treated with TGF-β or PBS (vehicle control) for 1 h before being subjected to immunoprecipitation and immunoblot. Bands were quantified using ImageJ, with untreated cell samples (*first lanes*) set as 1.0.

treatment with TGF-β for 24 hours did not affect the abundance of integrins α_6, α_3, and β_1 on the cell surface while slightly decreasing the surface levels of integrin β_4 (Fig. 1*B*). By imunoprecipitation and immunoblot analysis with HER2 and integrin antibodies, TGF-β induced the association between HER2 and integrins α_6, β_1, and β_4 at 1 hour after treatment without affecting their total levels. Although expression of integrin α_3 was detected in these cells by immunoblot, HER2 did not associate with this integrin in the absence or presence of TGF-β (Fig. 1*C*). In 10A/HER2 and in HER2-overexpressing MDA-MB-453 and BT474 breast cancer cells, HER2 was detected in ECM-associated complexes, and this association was markedly increased by TGF-β (Fig. 1*D*). The level of cell surface–localized HER2, as determined by surface biotinylation followed by pull-down with streptavidin beads and HER2 immunoblot, was not affected by TGF-β in all three cell lines. Brefeldin A, an inhibitor of intracellular protein transport, abrogated the cell-surface localization of HER2 and its association with the ECM (Fig. 1*D*).

TGF-β activates Src-FAK, leading to receptor clustering and activation of PI3K-Akt. It has been shown that the membrane-proximal clustering of integrin and RTK requires the scaffolding function of FAK (3). Thus, to determine the role of the FAK in TGF-β–modulated clustering of HER2 and integrin, we performed immunoprecipitation with HER2 and integrin β_1 antibodies followed by FAK immunoblot. FAK associated with both HER2 and integrin β_1, and this association was increased by the addition of TGF-β in the same time course as it induced HER2:integrin clustering (Fig. 2*A*). Concurrently, tyrosine phosphorylation of FAK (site Y397) and Src (Y416) was also induced by TGF-β. Phosphorylation of the Y397 Src-binding site of FAK is required for p85 binding and PI3K activation (21). In line with this report, activation of Akt was also detected along with FAK phosphorylation during the time course following TGF-β treatment (Fig. 2*A*). Inhibition of Src kinase activity with PP2 abolished both basal and TGF-β–induced association of HER2 with FAK and integrin β_1 and the TGF-β–induced activation of FAK and Akt (Fig. 2*B*). Finally, knockdown of FAK expression using specific siRNA abolished the ability of TGF-β to induce receptor clustering and FAK-mediated activation of Akt and severely impaired both basal and TGF-β–induced phosphorylation of HER2 and EGFR as detected with site-specific phospho-antibodies (Fig. 2*C*). Control immunoprecipitation with anti–integrin α_3 was also done in all experiments and showed negative results (data not shown).

TGF-β induces Src-FAK through EGFR activation. In a recent study (22), we have reported that TGF-β induces the shedding of ErbB ligands TGF-α, amphiregulin, and heregulin through a TACE-dependent mechanism. To explore whether this is responsible for the activation of the Src-FAK pathway by TGF-β, we used the EGFR antibody cetuximab to block ligand binding to EGFR or siRNA to knock down the expression of TACE. Both cetuximab and RNAi of TACE abrogated TGF-β–induced FAK activation and HER2 clustering with integrin (Fig. 3*A* and *B*). Transfection of a full-length mouse TACE construct [TACE(HA)] reconstituted TGF-β–induced effects in cells transfected with human TACE siRNA

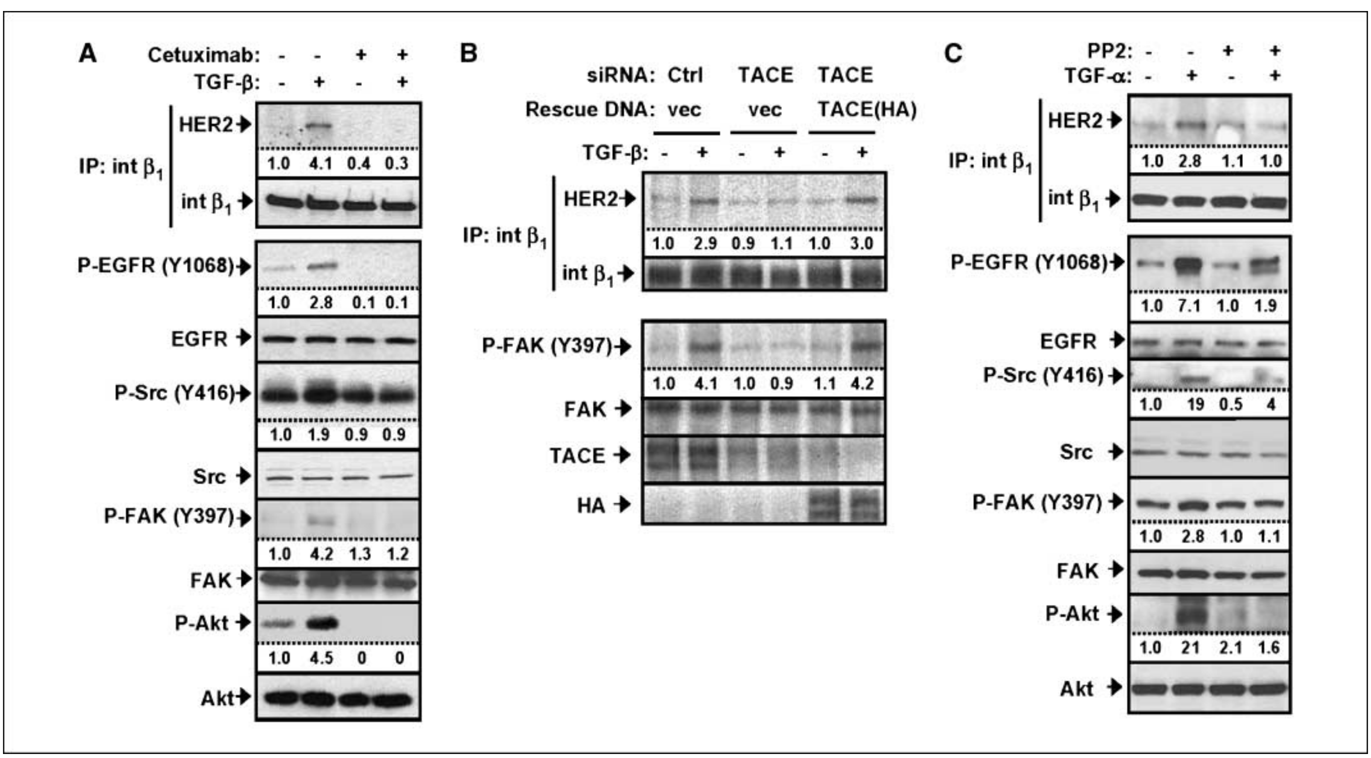

Figure 3. TGF-β induces Src-FAK through EGFR activation. *A,* MCF10A/HER2 cells were serum starved for 16 h and pretreated with cetuximab (10 μg/mL) or PBS (vehicle control) for 1 h before being treated with TGF-β or PBS (vehicle control) for 1 h and subjected to immunoprecipitation and immunoblot. *B,* MCF10A/HER2 cells grown on six-well plates were transfected by siRNA oligonucleotides targeting human TACE or a control sequence. To rescue TACE expression, a plasmid encoding the full-length mouse TACE [*TACE(HA)*] or empty vector (*vec*) was cotransfected with the siRNA oligonucleotides. Seventy-two hours after transfection, cells were serum starved for 16 h and treated with TGF-β or PBS (vehicle control) for 1 h before being subjected to immunoprecipitation and immunoblot. *C,* MCF10A/HER2 cells were serum starved for 16 h and pretreated with PP2 or DMSO (vehicle control) for 1 h before being treated with TGF-α (10 ng/mL) or PBS (vehicle control) for 1 h and subjected to immunoprecipitation and immunoblot. Bands were quantified using ImageJ, with untreated cell samples (*first lanes*) set as 1.0.

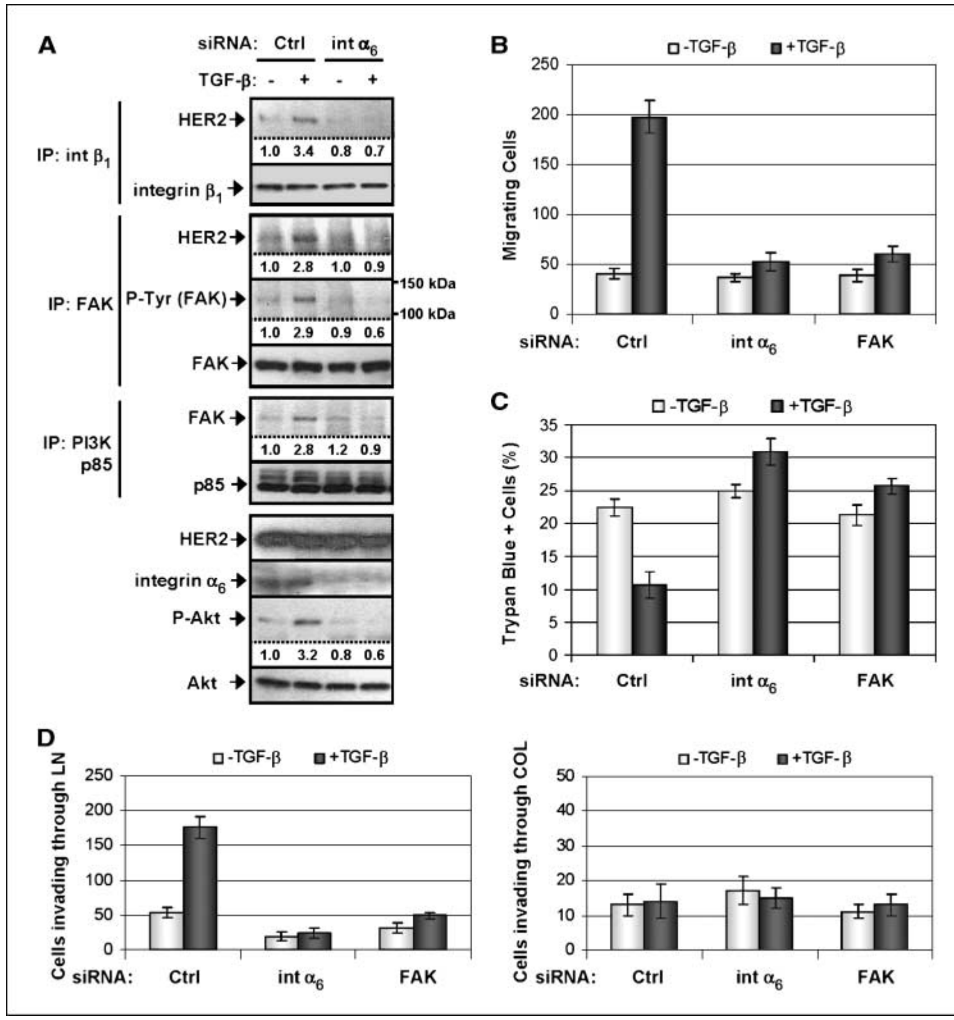

Figure 4. Integrin α_6 is essential for the clustering of HER2, integrin β_1, and FAK. *A,* MCF10A/HER2 cells grown on six-well plates were transiently transfected with siRNA oligonucleotides targeting integrin α_6 or a control sequence. Seventy-two hours after transfection, cells were serum starved for 16 h and treated with TGF-β or PBS (vehicle control) for 1 h before being subjected to immunoprecipitation and immunoblot. *B,* MCF10A/HER2 cells were transiently transfected with siRNA targeting integrin α_6 or the control sequence and grown on six-well plates for 2 d to 100% confluence. Cell monolayers were serum starved for 16 h before wounding and the addition of 2 ng/mL TGF-β. Wound closure was captured at 24 h. *Bar,* 50 μm. *C,* MCF10A/HER2 cells that had been transfected with siRNA targeting integrin α_6, FAK, or the control sequence for 48 h were changed to serum-free medium +/− TGF-β. After 48 h, cells were harvested for trypan blue staining. *Points,* mean of three wells; *bars,* SD. *D,* MCF10A/HER2 cells that had been transfected with siRNA targeting integrin α_6, FAK, or the control sequence for 48 h were seeded at 2.5×10^4 per well on laminin (*LN*)- or collagen (*COL*)-coated transwells and allowed to invade toward serum-free medium +/− TGF-β. At 16 h, the cells that invaded through transwell filters were counted. *Columns,* mean of three wells; *bars,* SD. Bands were quantified using ImageJ, with untreated cell samples (*first lanes*) set as 1.0.

(Fig. 3*B*). Further, EGFR activation by its ligand TGF-α was sufficient to induce activation of the Src-FAK pathway as well as receptor clustering. This was impaired by the addition of the Src kinase inhibitor PP2 (Fig. 3*C*). In HER2-overexpressing BT-474 breast cancer cells, TGF-β also induced Src activation and HER2 clustering with integrin; both effects were abolished by TACE siRNA oligonucleotides or the addition of PP2 (Supplementary Fig. S1*A* and *B*).

Integrin α_6 is essential for the clustering of HER2, integrin β_1, and FAK. Clustering between HER2 and integrin has only been observed with integrins $\alpha_6\beta_1$ and $\alpha_6\beta_4$, suggesting a unique role of α_6 integrin in HER2 signaling. We further evaluated the function of this integrin in HER2 signaling and its cross talk with the TGF-β network using RNA interference. Knockdown of α_6 expression abolished the formation of the HER2:FAK:integrin complex and impaired FAK-mediated PI3K signaling to Akt (Fig. 4*A*). In addition, the ability of TGF-β to enhance migration and survival in MCF10A/HER2 cells in serum-free medium was lost on depletion of α_6 integrin or FAK (Fig. 4*B* and *C*). TGF-β induced invasion of 10A/HER2 cells on laminin but not collagen; this effect was also impaired when α_6 integrin or FAK was knocked down with siRNA oligonucleotides (Fig. 4*D*).

TGF-β induces association of HER2 and integrins with the cytoskeleton. We have previously shown that TGF-β spatially regulates HER2 signaling by inducing its association with the actin cytoskeleton and relocalization to lamellipodia in a PI3K-dependent manner (13). Integrin receptors are also anchored to the cytoskeleton through adaptor proteins such as FAK (23). In this study, TGF-β induced association of actin with both HER2 and integrin α_6; this association was impaired by the HER2 tyrosine kinase inhibitor lapatinib ditosylate and the Src kinase inhibitor PP2 (Fig. 5*A*). Expression of dominant-negative p85 also abolished the association of integrin β_1 with actin and HER2:FAK:integrin complex formation (Fig. 5*B*). Treatment with cytochalasin D, a potent inhibitor of actin polymerization, impaired FAK phosphorylation and receptor clustering on TGF-β treatment and diminished HER2 signaling in both basal and TGF-β–treated conditions (Fig. 5*C*). Basal Akt phosphorylation was increased as a result of cytochalasin D treatment; the mechanism of this is unknown, but we speculate that it may reflect a compensatory cellular response to the stress from drug treatment.

Inhibition of Src-FAK reverses TGF-β–induced resistance to trastuzumab in HER2-overexpressing breast cancer cells. In a recent work, we have shown that TGF-β up-regulates PI3K/Akt and, in turn, desensitizes HER2-overexpressing cells to trastuzumab (22). Here we further found that FAK was phosphorylated at Y397 and associated with PI3K p85 on TGF-β

stimulation; knockdown of FAK using siRNA oligonucleotides abolished TGF-β–mediated Akt activation (Fig. 2*C*). Therefore, we speculated that inhibition of Src-FAK might reverse TGF-β–induced resistance to trastuzumab. To explore this, we cultured HER2-overexpressing BT-474 breast cancer cells on Matrigel in the absence or presence of TGF-β and PP2 and examined their responses to trastuzumab. Addition of PP2 blocked the protective effect of TGF-β and restored trastuzumab action in BT-474 cells (Fig. 6), consistent with the ability of the Src inhibitor to inhibit P-Akt (shown in Figs. 2 and 3).

Discussion

TGF-β is known to play dual roles in tumor progression. Whereas it functions as a tumor suppressor in normal cells or during early stages of tumorigenesis, it is often found as a prometastatic factor in established or invasive tumors through a combination of autocrine and paracrine mechanisms (24, 25). Coexpression of activated oncogenes is a cue to the protumorigenic functions of TGF-β. In cancer models that depend on Neu (ErbB2), PyVMT, or mutant Ras, TGF-β synergizes with these oncogenes to accelerate cancer progression and metastasis (8, 10, 26–28). Recent work (22) and data shown herein indicate that in HER2 (ErbB2)-overexpressing cells, TGF-β enhances ErbB-initiated signal transduction by increasing ErbB ligand shedding, HER2-containing heterodimers, and their cross talk with integrins (Fig. 5*D*). The latter is mediated by the activation of Src-FAK; inhibition of this kinase complex not only abolishes the ability of TGF-β to facilitate cancer progression but also reverses TGF-β–mediated resistance to trastuzumab. Interestingly, RNA interference of integrin α_6 or FAK completely abrogated the prosurvival and promigratory functions of TGF-β in HER2-overexpressing cells

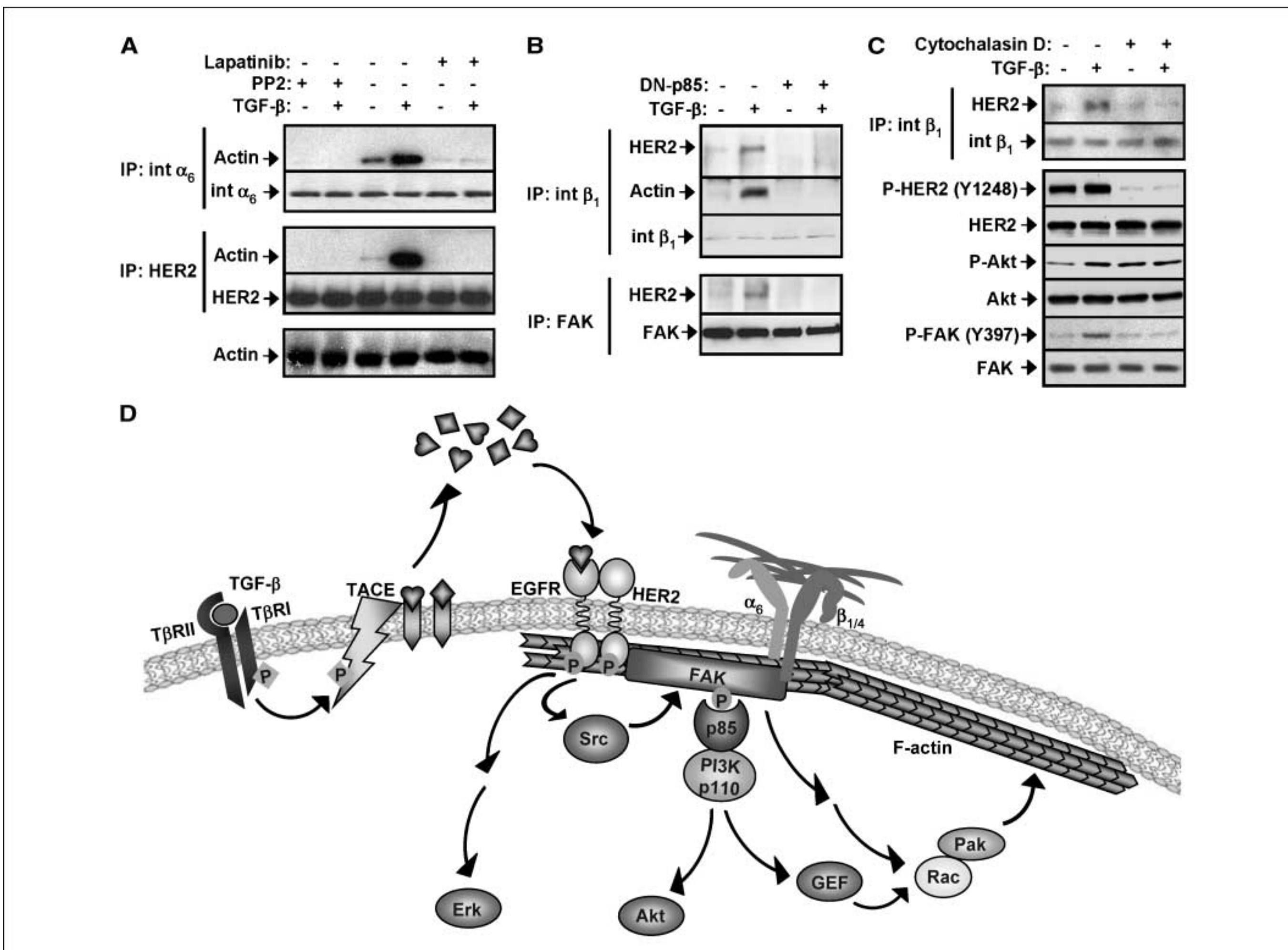

Figure 5. TGF-β induces association of HER2 and integrin with the cytoskeleton. *A,* MCF10A/HER2 cells were serum starved for 16 h and pretreated with PP2 or lapatinib (1 μmol/L) or DMSO (vehicle control) for 1 h before being treated with TGF-β or PBS (vehicle control) for 1 h and subjected to immunoprecipitation and immunoblot. *B,* MCF10A/HER2 cells grown on six-well plates were infected by adenoviruses encoding the dominant negative (*DN*) PI3K p85 subunit or control viruses expressing β-gal (multiplicity of infection, 1:5). Sixteen hours after infection, cells were serum starved for 16 h and treated with TGF-β or PBS (vehicle control) for 1 h before being subjected to immunoprecipitation and immunoblot. *C,* MCF10A/HER2 cells were serum starved for 16 h and pretreated with cytochalasin D (5 μmol/L) or DMSO (vehicle control) for 1 h before being treated with TGF-β or PBS (vehicle control) for 1 h and subjected to immunoprecipitation and immunoblot. *D,* TGF-β induces membrane-proximal clustering of HER2 and integrins by activating Src-FAK and receptor association to cytoskeleton. Signals from extracellular TGF-β are transduced into cells through TGF-β receptors. Activated type I TGF-β receptor (*TβRI*) induces phosphorylation of TACE, resulting in its translocation to the cell surface, where TACE cleaves EGFR proligands. EGFR ligands will initiate autocrine and paracrine EGFR signaling, which is amplified in HER2-overexpressing cells, leading to Src-FAK activation and cytoskeleton rearrangement; both are required for the membrane-proximal clustering of HER2 and integrin.

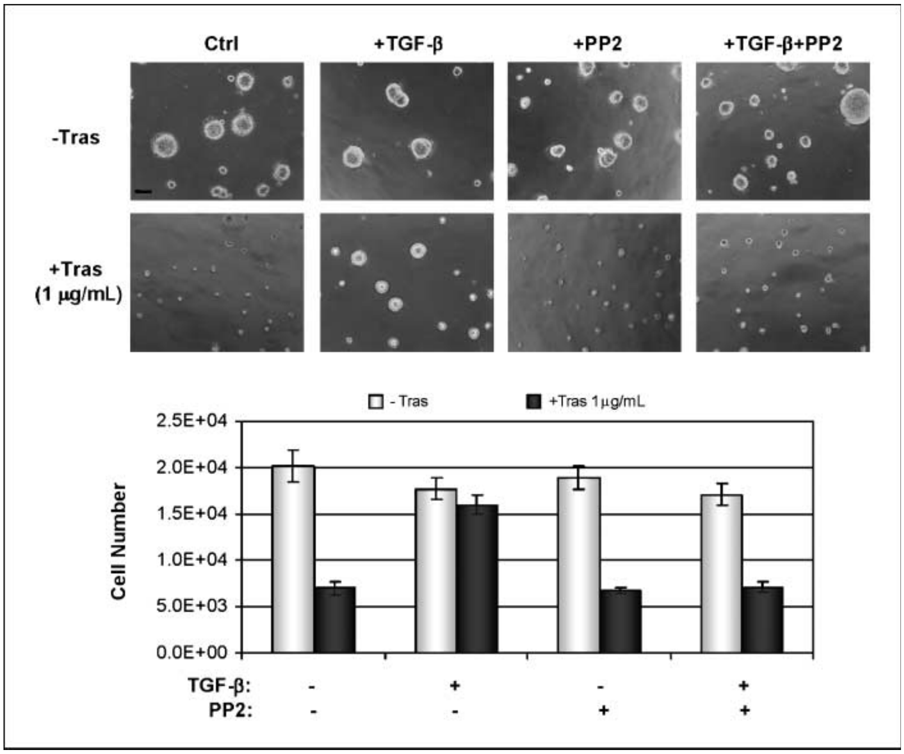

Figure 6. Inhibition of Src-FAK reverses TGF-β–induced resistance to trastuzumab. BT-474 cells were plated in Matrigel in eight-well chambers and allowed to grow in the absence or presence of TGF-β (2 ng/mL) and PP2 (1 μmol/L) as indicated. Trastuzumab was added to the top medium 12 h after cell seeding. The inhibitors were replenished every 3 d. Phase-contrast images shown were recorded 9 d after the initial seeding of cells. *Bar,* 50 μm. *Bottom,* 9-d acini were trypsinized and total cell number was determined. *Columns,* mean of four wells; *bars,* SD.

(Fig. 4*C*), suggesting that integrin-regulated interactions of HER2 with the actin cytoskeleton, and not the total levels of HER2 protein, can specify the qualitative response to TGF-β.

In a previous study, we have examined effects of TGF-β on the cellular distribution and signaling of HER2 using MCF10A/HER2 and breast cancer lines that naturally overexpress the proto-oncogene. In these cells, treatment with TGF-β activates PI3K and activates the Vav2 guanine nucleotide exchange factor, which then activates Rac1 and its effector Pak1, resulting in colocalization of HER2 with these molecules as well as actin cytoskeleton components at cell protrusions (13). Here we show that FAK is required for PI3K/Akt activation by TGF-β. In addition to activating PI3K/Akt, FAK is also known to activate CAS-Crk-Rac signaling and regulate the assembly and turnover of focal adhesions at the leading lamellipodia of migrating cells. This occurs through the regulation of integrin-mediated cell adhesion and cytoskeleton rearrangement (Fig. 5*D*).

The dual kinase Src/FAK complex has an established role in human cancers (29). In cancers, both growth factor receptor and FAK signaling are up-regulated. Autophosphorylation of FAK on Y397 creates a SH2 domain binding site that can recruit and activate Src. FAK phosphorylated at this site has been found in various tumors including ovarian carcinoma, cervical carcinoma, and acute myeloid leukemia (reviewed in ref. 29). Phosphorylation of this site is also responsible for the direct association of FAK with the regulatory subunit of PI3K, p85, as well as the activation of PI3K by ligand-bound PDGFR (21). Thus, FAK integrates both growth factor RTK and integrin networks and serves as an effector for these two signaling systems. Indeed, cells lacking FAK are refractory to EGF- or PDGF-induced tyrosine phosphorylation of EGFR and PDGFR, respectively, and cell motility (3). Consistently, FAK RNAi suppressed both basal and TGF-β–induced tyrosine phosphorylation of HER2 and EGFR (Fig. 2*C*), suggesting that FAK-mediated cross talk between RTKs and integrins is required for receptor activation.

Integrin signaling has been shown to play a crucial role in ErbB2-mediated mammary tumorigenesis. Integrin β_4 amplifies ErbB2 signaling to promote mammary tumor onset and invasion in transgenic mice (4). Moreover, deletion of the β_4 signaling domain enhances the efficacy of gefitinib, a small-molecule inhibitor of the EGFR tyrosine kinase (4). The role of the Src-FAK complex on regulating the transforming role of integrins suggests that it can be targeted therapeutically in human cancers. Inhibitors of the Src kinase are currently in early clinical development for the treatment of solid tumors including breast cancer. In addition, amplified TGF-β signaling has been shown to up-regulate PI3K and desensitize the antitumor effect of HER2 antagonist trastuzumab (22). Our data herein further suggest that (*a*) Src-FAK is essential for the activation of PI3K that results from the interaction between HER2 and TGF-β, and (*b*) inhibition of Src-FAK reverses TGF-β–induced resistance to HER2 antagonist trastuzumab.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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