Published OnlineFirst June 9, 2009; DOI: 10.1158/1535-7163.MCT-09-0140

Molecular Cancer Therapeutics

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Mol Cancer Ther 2009;8:1557-1569. Published OnlineFirst June 9, 2009.

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Rac1 contributes to trastuzumab resistance of breast cancer cells: Rac1 as a potential therapeutic target for the treatment of trastuzumab-resistant breast cancer

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Abstract

Although treatment with trastuzumab improves outcomes for women with ErbB2-positive breast cancer, many patients who achieve an initial response to trastuzumab subsequently acquire resistance within 1 year. Rac1, a Ras-like small GTPase, has been implicated in the control of cell growth and morphology and is believed to be associated with breast cancer progression and metastasis. Here, we show that when parental SKBR3 cells become resistant to trastuzumab, Rac1 activity is increased, leading to altered cell morphology, which is accompanied by significant cytoskeleton disorganization. Furthermore, both trastuzumab-mediated down-regulation of ErbB2 and epidermal growth factor–induced down-regulation of epidermal growth factor receptor are impaired in the trastuzumab-resistant SKBR3 cells, indicating that the endocytic down-regulation of ErbB receptors is compromised in the resistant cells. This results in an aberrant accumulation of ErbB2 on the cell surface and enhanced ErbB2 and extracellular signal-regulated kinase activity in trastuzumab-resistant SKBR3 cells. Additionally, overexpression of constitutively active Rac1G12V in parental SKBR3 cells reduces sensitivity to trastuzumab. After reduction of Rac1 activity by NSC23766, a specific Rac1 inhibitor, trastuzumab-resistant SKBR3 cells display a cellular morphology similar to parental SKBR3 cells. Moreover, we show that NSC23766 restores trastuzumab-mediated endocytic down-regulation of ErbB2 and reduces extracellular signal-regulated kinase activity in resistant SKBR3 cells. Our findings highlight an important role for Rac1 in trastuzumab resistance of human breast cancer cells and identify the impaired trastuzumab-mediated endocytic down-regulation of ErbB2 as a novel mechanism of trastuzumab resistance. The significant effects of NSC23766 on trastuzumab-resistant SKBR3 cells warrant further study of NSC23766 as a potential treatment of trastuzumab-resistant breast cancers. [Mol Cancer Ther 2009;8(6):1557–69]

Received 2/16/09; accepted 3/25/09; published OnlineFirst 6/9/09.

Grant support: U.S. Food and Drug Administration Critical Path Finding for FY2008 and Interagency Oncology Task Force Joint Fellowship Program sponsored by the U.S. Food and Drug Administration and the National Cancer Institute, NIH.

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doi:10.1158/1535-7163.MCT-09-0140

Introduction

Overexpression of ErbB2, a member of the ErbB family of receptor tyrosine kinases, is associated with breast cancer progression, metastasis, poor prognosis, and poor response to therapy (1, 2). Trastuzumab is a humanized monoclonal antibody targeting the ErbB2 receptor extracellular domain. Clinical studies with trastuzumab, either alone or in combination with adjuvant chemotherapy, have shown favorable responses in ErbB2-positive breast cancer patients (3–5). Proposed mechanisms of action for trastuzumab include (*a*) down-regulation of ErbB2 through endocytic uptake of trastuzumab/ErbB2 complex, (*b*) disruption of downstream signaling pathways, (*c*) induction of G1 cell cycle arrest and apoptosis, (*d*) suppression of angiogenesis, (*e*) induction of antibody-dependent cellular cytoxicity, and (*f*) inhibition of ErbB2 extracellular domain proteolysis (1, 2).

Although trastuzumab treatment significantly improves outcomes for women with ErbB2-positive breast cancers, many patients who achieve an initial response to trastuzumab also acquire resistance to trastuzumab (2, 6, 7). Understanding the mechanisms contributing to trastuzumab resistance and identifying novel therapeutic targets is critical for improving the survival of this population of breast cancer patients. Several studies have advanced our understanding of molecular mechanisms of trastuzumab resistance. First, trastuzumab resistance is associated with reduced $p27^{kip1}$, elevated cyclin-dependent kinase 2 activity, and increased heterodimerization of ErbB2 receptor with IGF-IR receptor in trastuzumab-resistant SKBR3 cells (8, 9). Second, in a BT-474 cell line model of trastuzumab resistance, significant increases in the levels of epidermal growth factor (EGF) receptor (EGFR), transforming growth factor α, heparin-binding EGF, and Heregulin occur (10). Third, in a cell line (JIMT-1) established from a patient who failed to respond to trastuzumab and adjuvant chemotherapy, resistance to trastuzumab is associated with heterodimerization of ErbB2 to glycoprotein MUC4, which prevents ErbB2 from binding to trastuzumab (11, 12). Fourth, analysis of clinical samples from trastuzumab responders and nonresponders indicates that loss of PTEN

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tumor suppressor correlates with a significant decrease in response to trastuzumab therapy (13).

Because multiple mechanisms likely contribute to trastuzumab resistance in breast cancer, combining trastuzumab with other therapeutic agents that target either ErbB family receptors or signaling molecules downstream of ErbB receptors may increase the magnitude and duration of trastuzumab response. Lapatinib, which inhibits the tyrosine kinase activity of both EGFR and ErbB2, is currently undergoing clinical testing for patients whose cancers have progressed while receiving trastuzumab (14). Furthermore, synergistic growth inhibition occurs in breast cancer cell lines treated with both Lapatinib and trastuzumab (15). Additionally, IGF-IR–targeting agents have been introduced into pharmaceutical testing and are being assessed in preclinical trastuzumab-resistant models (2).

Rho family GTPases belong to the Ras superfamily of small GTPases that serve as molecular switches. Activation of Rho family GTPases regulates a wide range of cellular functions, including cytoskeletal dynamics, cell polarity, adherens junctions, and member trafficking (16–20). It has been very well documented that Rac1, Cdc42, and RhoA have the ability to control normal cell growth and, when improperly regulated, contribute to tumorigenesis, tumor invasion, and metastasis (17–21). Several lines of evidence indicate that Rac1 and Cdc42 function downstream of ErbB family receptors. For instance, EGF-coupled signaling is sufficient to activate Cdc42 and Rac1, and treatment of cells with Heregulin, an ErbB3 ligand, increases Rac1 activity (16, 22–25). Increased Rac1 activity occurs in MCF10A human mammary epithelial cells overexpressing ErbB2 receptor (26). There is also evidence that Rac1 is overexpressed or hyperactivated in breast cancer tissue (27).

It is becoming more evident that the activities of Rho family GTPases contribute to multiple aspects of oncogenic phenotypes in breast cancer cells, including resistance to chemotherapy (28). Overexpression of Cdc42 and Rac1 has been reported in human breast cancers (29, 30). Up-regulated Rac1 and Cdc42 activities are believed to be associated with breast cancer progression, invasion, and metastasis (29–32). However, it has not been reported whether Rho family GTPases are involved in trastuzumab resistance in breast cancer cells.

SKBR3 breast cancer cells express high levels of ErbB2 and are sensitive to trastuzumab treatment (33). Here, we investigate the mechanisms contributing to trastuzumab resistance using SKBR3 cells and a SKBR3 trastuzumab-resistant derivative clone, known as Clone 3 cells, which were created by chronically exposing parental SKBR3 cells to trastuzumab in culture media (9, 33). We show that Rac1 activity is up-regulated in Clone 3 cells compared with trastuzumab-sensitive parental SKBR3 cells and that, in Clone 3 cells, actin stress fibers are disrupted, resulting in altered cell morphology. Furthermore, trastuzumab-mediated endocytic down-regulation of ErbB2 is impaired in Clone 3 cells. This leads to the accumulation of ErbB2 on the cell surface and increased ErbB2 signaling in Clone 3 cells. Clone 3 cells treated with the Rac1 inhibitor NSC23766 reassemble actin-based stress fibers and display a cellular morphology similar to parental SKBR3 cells. Additionally, inhibition of Rac1 activity by NSC23766 restores endocytotic uptake of the trastuzumab/ErbB2 complex and trastuzumab-mediated ErbB2 degradation. Overexpression of Rac1-activated mutant in parental SKBR3 promotes partial resistance to trastuzumab-induced growth arrest. These findings highlight an important role for Rac1 in trastuzumab resistance of breast cancer cells and provide evidence that targeting Rac1 may be a potential therapeutic approach for the treatment of trastuzumab-resistant breast cancer.

Materials and Methods

Materials

Trastuzumab (Genentech, Inc.) was purchased from the pharmacy at NIH. NSC23766 was obtained from Tocris Cookson, Inc. Antibodies against Cdc42, Rac1, EGFR, and ErbB2 were purchased from BD Transduction Laboratories. Antibodies against p44/42 extracellular signal-regulated kinase 1/2 (ERK1/2), phosphorylated 44/42 ERK1/2, phosphorylated stress-activated protein kinase (SAPK)/c-Jun NH_2 terminal kinase (JNK; Thr^{183}/Tyr^{185}), phosphorylated c-Jun (Ser^{63}) II, phosphorylated ErbB2 (Tyr^{877}), and phosphorylated ErbB2 ($Tyr^{1221/1222}$) were obtained from Cell Signaling Technology. Polyclonal anti-actin antibody was obtained from Sigma. Antibody against EEA1 were obtained from Santa Cruz Biotechnology. Anti-Rho antibody was purchased from Pierce. Monoclonal anti-HA antibody was obtained from Covance. Trypan blue was obtained from Lonza. Rhodamine-conjugated phalloidin and Alexa Fluor 594 donkey anti-mouse antibody were obtained from Invitrogen Molecular Probes. Cy2-conjugated donkey anti-human antibody and rhodamine-conjugated donkey anti-goat secondary antibody were obtained from Jackson ImmunoResearch. EZ-detect Rho activation kit and EZ-detect Rac1 activation kit were obtained from Pierce.

Cell Culture and Transient Transfections

Parental SKBR3 cells (American Type Culture Collection) and Clone 3 cells, which were described previously (33), were obtained from Dr. Esteva of M.D. Anderson Cancer Center and cultured in the standard culture media unless otherwise indicated. The standard culture media for parental SKBR3 cells is DMEM/F12 supplemented with 10% fetal bovine serum. The standard culture media for Clone 3 cells is DMEM/F12 supplemented with 10% fetal bovine serum and trastuzumab (4 μg/mL). JIMT-1 cell line was purchased from DSMZ (German Collection of Microorganisms and Cell Cultures) and described by Tanner and colleagues (12). The JIMT-1 cells are cultured in DMEM supplemented with 10% fetal bovine serum. Transient transfections using electroporation were done according to the manufacturer's protocol (Amaxa, Inc.).

Selection of Stable Cell Lines

After electroporation of SKBR3 cells with plasmid encoding HA-tagged Rac1G12V and pcDNA3.1 vector carrying neomycin resistance gene, the stable cell line selection was achieved using G418 (21).

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Trastuzumab-Mediated Endocytic Down-regulation of ErbB2

Cells were cultured in 60-mm plates for 12 h in the standard culture media. Thereafter, cells were treated with either trastuzumab or trastuzumab plus NSC23766, or left untreated. At the indicated times, cells were harvested and 20 μg whole-cell lysate (WCL) was subjected to SDS-PAGE. ErbB2 protein levels were assessed by Western blot analysis with an anti-ErbB2 antibody.

Cell Growth and Viability Profiles

Cells were plated in either 12-well or 24-well plates and cultured in the standard culture media for 12 h. Cells were either not treated, treated with trastuzumab, or treated with trastuzumab plus NSC23766. At the indicated times, cells were trypsinized and counted. Cell viability was determined using trypan blue staining in a standard hemocytometer setup.

Immunofluorescence Assays

Cells were seeded overnight in chamber slides at 1×10^5 per well and cultured in standard culture media. Cells were then rinsed with PBS, fixed, permeabilized, and stained, as previously described (20).

Trastuzumab Internalization and EGFR Down-regulation Assays

Both parental SKBR3 and Clone 3 cells were cultured in the standard culture media. Parental SKBR3 cells were either treated with trastuzumab or not treated for the indicated times, whereas Clone 3 cells remained in the standard culture media for the indicated times. Internalized or surface-bound trastuzumab was detected by Cy2-conjugated donkey anti-human antibody. EEA1 was detected using a mouse EEA1 monoclonal antibody. For the effects of NSC23766 on trastuzumab internalization, the experimental procedures were essentially the same as that described above, except that NSC23766 was used to treat the cells for the indicated times. For EGFR down-regulation assay, the parental and Clone 3 cells were plated and treated with 100 ng/mL EGF. At the indicated times, cells were harvested and WCL were subjected to SDS-PAGE. The levels of EGFR were assessed by Western blot analysis with anti-EGFR antibody.

GTPase Activity Pull-down Assays

GTPase activity pull-down assays were done using either glutathione *S*-transferase (GST)–p21-binding domain (PBD) of p21-activated kinase or GST-Rho-binding domain (RBD) of Rhotekin, as described previously (20). Briefly, GST-PBD or GST-RBD was immobilized by binding to glutathione-Sepharose beads. The immobilized GST-PBD was used to precipitate activated Cdc42 or Rac1 from WCL. The immobilized GST-RBD was used to precipitate activated Rho from WCL.

Results

Increased Rac1 Activity in Clone 3 Cells Correlates with Actin Cytoskeleton and Cell Morphology

We observed profound differences in cell morphology between parental SKBR3 and Clone 3 cells. Whereas parental SKBR3 cells had a flattened morphology and were translucent by phase-contrast microscopy, Clone 3 cells exhibited a round cell shape with long protrusions extending from the cell body and were refractive on phase-contrast microscopy (Fig. 1A). Clone 3 cells also differed from parental cells, in that Clone 3 cells lacked stress fibers and had strong circular actin staining at the cell edges (Fig. 1A). We then questioned whether the significant cytoskeleton rearrangement displayed by Clone 3 cells was correlated with the changes in the activities of Rho family GTPases. As shown in Fig. 1B, the levels of active Rac1 were increased in Clone 3 cells compared with that in parental SKBR3 cells. Active Cdc42 and Rho proteins were comparable in these two cell lines, and there was no detectable background binding in either of the GST control assays (Fig. 1B). In addition, there were no detectable differences in the protein levels of Rho, Cdc42, and Rac1 between the parental SKBR3 cells and Clone 3 cells in the WCL, which was used for both PBD and RBD pull-down assays (Fig. 1C). Thus, the activity of Rac1 is increased in Clone 3 cells compared with parental SKBR3 cells, which might contribute to the morphologic changes displayed by Clone 3 cells.

Rac1 has been characterized as a downstream effector of ErbB receptors and has recently been shown to associate with ErbB2 in MCF10A cells (16, 22, 26, 36). In light of these data, we investigated whether Rac1 interacted with ErbB2 in Clone 3 cells. Specific binding of ErbB2 to the PBD-Rac1-GTP protein complex was observed in Clone 3 cells, but not in parental SKBR3 cells (Fig. 1D). Binding of ErbB2 to activated Rac1 was further confirmed by transient overexpression of constitutively activated mutants of Rac1 in parental SKBR3 cells. As shown in Fig. 1E, both HA-tagged Rac1F28L, a fast cycling mutant (37), and HA-tagged Rac1Q61L associated with endogenous ErbB2 in parental SKBR3 cells. We next examined whether Rac1 was colocalized with ErbB2 in cells. As shown in Fig. 1F, ErbB2 proteins were mainly localized (closed arrows) at the cell edges in parental SKBR3 cells, whereas Rac1 exhibited a cytosolic staining. No significant colocalization between ErbB2 and Rac1 was detected at the cell edges in parental SKBR3 cells (Fig. 1F). In contrast, both ErbB2 and Rac1 colocalized at the cell edge membrane ring region in Clone 3 cells (Fig. 1F, *open arrows*). These data support idea that the activated Rac1 is associated with ErbB2 in Clone 3 cells.

Trastuzumab-Mediated Endocytic Down-regulation of ErbB2 and EGF-Induced Down-regulation of EGFR Are Impaired in Trastuzumab-Resistant Clone 3 Cells

Trastuzumab, a humanized monoclonal antibody, directly binds to the extracellular domain of ErbB2 receptor to mediate internalization and degradation of ErbB2 receptor (34). As shown in Fig. 2A, after treatment of parental SKBR3 cells with 10 μg/mL trastuzumab for 4 days, trastuzumab was endocytosed as visualized by donkey anti-human Cy2-conjugated antibody (green punctate structures) and partially colocalized with EEA1, an early endosomal marker (*top*), consistent with other reports (34, 35). In contrast, trastuzumab-untreated control SKBR3 cells, for which trastuzumab was only used to recognize ErbB2, did not show any significant colocalization between trastuzumab and EEA1. The

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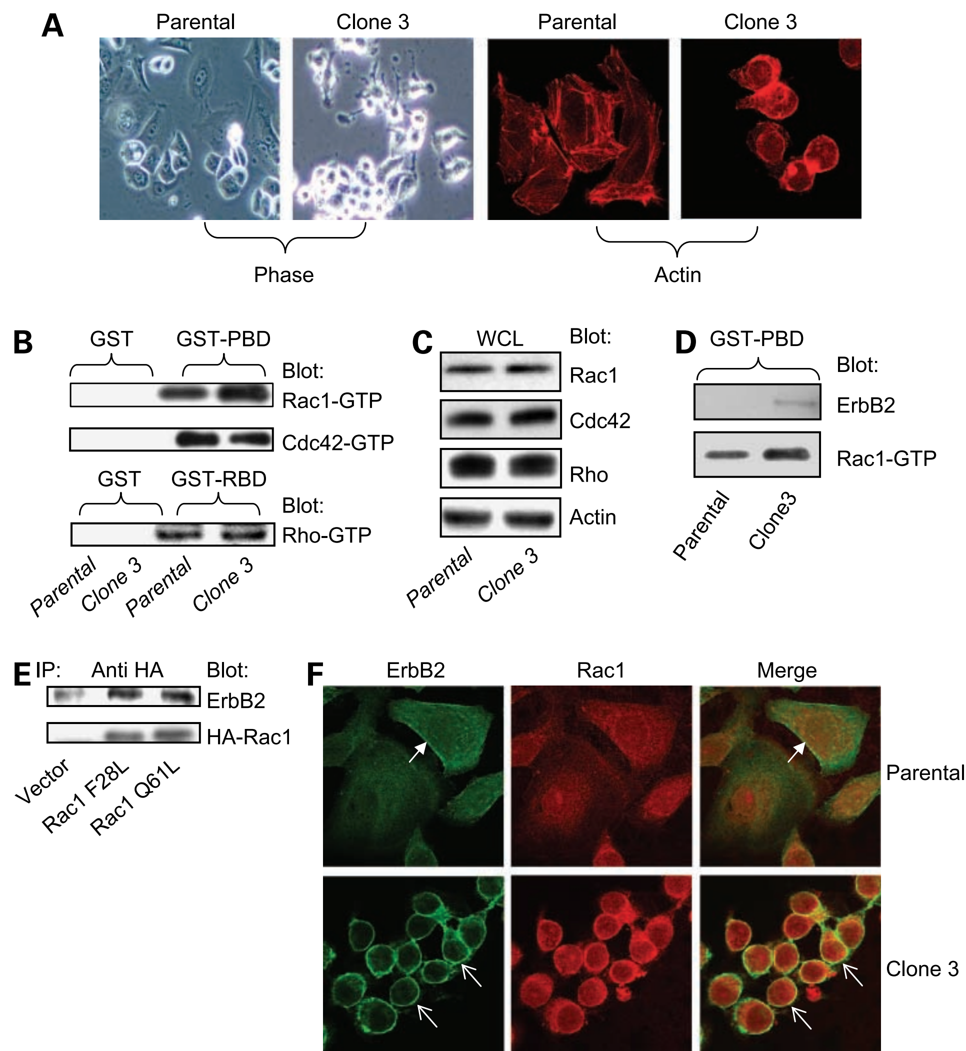

Figure 1. The activity of Rac1 is up-regulated, and the up-regulated Rac1 activity is correlated with the changes in cell morphology and actin cytoskeleton in Clone 3 cells. **A,** phase-contrast micrographs were taken 48 h after plating of parental SKBR3 and Clone 3 cells in 60-mm dishes. Images were acquired using an Olympus CKX41 at a 100× magnification. Pictures were captured and processed using Infinity Capture Imaging Software (Luminera). Actin staining was done with rhodamine-conjugated phalloidin 48 h after plating parental SKBR3 and Clone 3 cells in chamber slides. Microscopy was done using a Bio-Rad 2100 confocal microscope attached to a Nikon E800 microscope. Images were taken using a 60× Plan Apo/numerical aperture (*NA*) 1.4 lens with a 2× zoom. Magnification, 1200×. **B,** Rac1 and Cdc42 activities in parental SKBR3 and Clone 3 cells were assessed in GTPase activity pull-down assays. The PBD of p21-activated kinase was used for GST pull-down of activated Rac1 and Cdc42. GST protein alone was used as a control. Recombinant GST-RBD (RBD of Rhotekin) was used for GST pull-down of activated Rho. The levels of the GTP-bound Rac1, Cdc42, and Rho proteins were assessed by Western blot analysis. **C,** protein levels of total Rho, Cdc42, Rac1, and actin in the WCL of parental SKBR3 and Clone 3 cells were detected by Western blot analysis. The same WCL was used to determine the activity of Rac1, Cdc42, and Rho shown in **B. D,** Rac1 binds to endogenous ErbB2 in Clone 3 cells. GTPase activity pull down assays were done as described in Fig. 1B. The protein levels of ErbB2 and GTP-bound Rac1 were determined by Western blot analysis. The WCL used for this experiment was the same as one used in **C. E,** constitutively activated Rac1 mutants bind to ErbB2 in cells. Parental SKBR3 cells were transiently transfected by electroporation according to manufacturer's instruction with pcDNA3.1 empty vector or pcDNA3.1 vector encoding HA-tagged Rac1F28L or HA-tagged Rac1Q61L. At 48 h after transfection, cells were harvested and WCL were subjected to anti-HA antibody immunoprecipitation. Rac1 and ErbB2 in immunoprecipitates were detected by Western blot analysis with anti-HA to detect Rac1 and anti-ErbB2 to detect ErbB2. **F,** parental SKBR3 and Clone 3 cells were plated in chamber slides and cultured in standard culture media (see Materials and Methods) for 48 h. Cells were then fixed, permeabilized, and stained for Rac1 and ErbB2. ErbB2 staining in parental SKBR3 cells was done using trastuzumab and Cy2-conjugated donkey anti-human secondary antibody (*green*). Rac1 staining was done with mouse anti-Rac1 antibody and Alexa 594–conjugated donkey anti-mouse secondary antibody (*red*). *Yellow,* merge between red (Rac1) and green (ErbB2). *Closed arrow,* ErbB2 localized at the plasma membrane of parental cells; *open arrow,* ErbB2 staining in the plasma membrane of Clone 3 cells.

trastuzumab was mainly located at the cell surface, and no significant green punctate structures were observed in control SKBR3 cells (Fig. 2A, *bottom row*). The enlarged boxed region in Fig. 2B depicts the partial colocalization between the trastuzumab and EEA1. These data indicate that trastuzumab is internalized in parental SKBR3 cells. Under identical trastuzumab treatment conditions, Clone 3 cells differed from parental SKBR3 cells, in that the trastuzumab accumulated at the cell surface, was not detected in the intracellular compartments, and had no colocalization with

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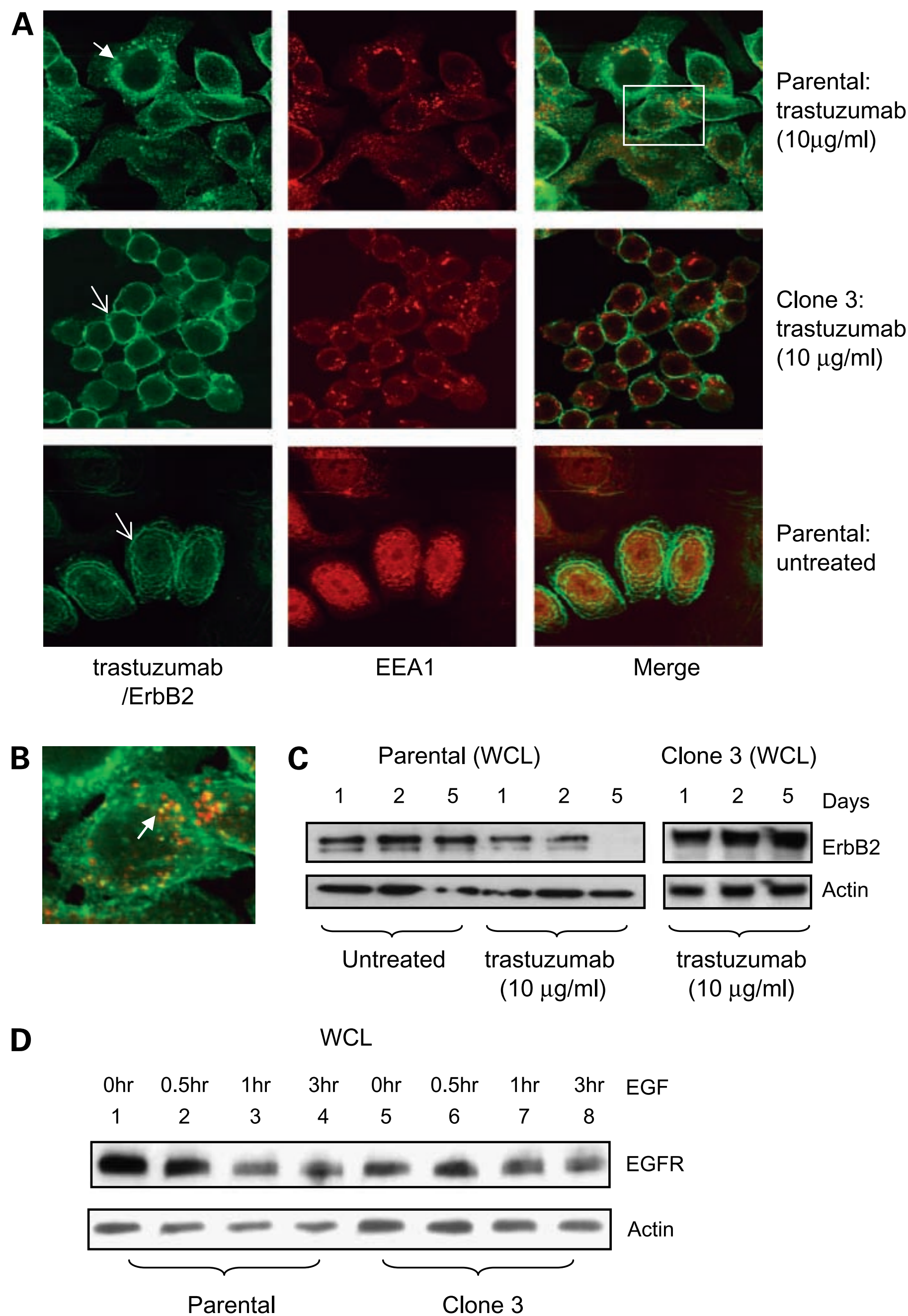

Figure 2. Trastuzumab-mediated endocytic down-regulation of ErbB2 and EGF-induced down-regulation of EGFR are impaired in trastuzumab-resistant Clone 3 cells. **A,** trastuzumab mediates endocytosis of ErbB2 in SKBR3 cells. For pictures in top two rows, parental SKBR3 and Clone 3 cells were seeded in chamber slides overnight and then treated with 10 µg/mL trastuzumab for 4 d. Cells were then fixed and permeabilized. Trastuzumab was visualized with donkey anti-human Cy2-conjugated antibody (*green*). EEA1 staining was done using mouse anti-human EEA1 antibody followed by Alexa Fluor 594–conjugated donkey anti-mouse antibody (*red*). Closed arrow indicates trastuzumab/ErbB2 staining in intracellular compartments in trastuzumab treated parental SKBR3 cells. *Open arrow,* trastuzumab/ErbB2 staining at the plasma membrane in Clone 3 cells. *Yellow,* regions of colocalization between trastuzumab/ErbB2 complex and EEA1. For pictures in the bottom row, parental SKBR3 cells were seeded in chamber slides, cultured in the standard culture media without containing trastuzumab for 2 d, and then fixed and permeabilized. ErbB2 staining was done using trastuzumab followed by anti-human Cy2-conjugated antibody (*green*). EEA1 staining was done using mouse anti-human EEA1 antibody followed by Alexa Fluor 594 (*red*)–conjugated donkey anti-mouse antibody (*bottom*). Immunofluorescent pictures were taken as described in Fig. 1. **B,** enlarged picture, indicated by the rectangle box in Fig. 1A. *Closed arrow,* colocalization between trastuzumab/ErbB2 and EEA1. **C,** trastuzumab-mediated endocytic down-regulation of ErbB2 is impaired in Clone 3 cells. Parental SKBR3 cells and Clone 3 cells were seeded overnight and incubated with or without trastuzumab (10 µg/mL). Cells were harvested at the indicated times, and the total levels of ErbB2 in WCL were detected by Western blot analysis. Actin Western blot analysis was done to confirm equal protein loading. **D,** EGF-induced down-regulation of EGFR is impaired in Clone 3 cells. Parental and Clone 3 cells were seeded in their standard culture media (see Materials and Methods) overnight and then treated with EGF (100 ng/mL). WCL were harvested at the indicated time points and the levels of EGFR probed by Western blot analysis using an anti-EGFR antibody. Actin Western blot analysis was done to confirm equal protein loading.

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EEA1 (Fig. 2A, *second row from top*). We next asked whether Clone 3 cells were capable of down-regulating ErbB2. After being incubated with trastuzumab (10 μg/mL) for 5 days, little or no ErbB2 was detected in parental SKBR3 cells (Fig. 2C, *left*). In contrast, ErbB2 levels were not reduced in Clone 3 cells (Fig. 2C, *right*). Taken together, these data indicate that trastuzumab is able to mediate endocytic down-regulation of ErbB2 in parental SKBR3 cells, and trastuzumab-mediated endocytic down-regulation of ErbB2 is impaired in Clone 3 cells.

The results described in Fig. 2C lead to the question of whether EGF-induced down-regulation of EGFR is also impaired in Clone 3 cells. After the addition of EGF to parental SKBR3 cells, the total receptor levels as detected by Western blotting with an anti-EGFR antibody were significantly reduced over a time period of 0 to 3 hours (Fig. 2D). However, after a 3-hour incubation of EGF with Clone 3 cells, there was no significant reduction in the total levels of EGFR (compare lane 5 with lane 8). These data, when taken together with Fig. 2A and C, suggest that the significant cytoskeleton disorganization described in Fig. 1 may disrupt clathrin-dependent endocytosis and subsequently impair lysosomal degradation of ErbB receptors in Clone 3 cells.

NSC23766 Restores Clone 3 Cell Morphology and Trastuzumab-Mediated ErbB2 Internalization and Degradation

NSC23766 inhibits Rac1 function by preventing Rac1 activation by Tiam 1 and TrioN (38). We used NSC23766 to test whether the changes in cell morphology were associated with the up-regulated Rac1 activity in Clone 3 cells. Cells were cultured in the standard culture media and treated with or without NSC23766 for the indicated times. Cell morphology was assessed using phase-contrast microscopy. As shown in Fig. 3A, NSC23766 treatment of Clone 3 cells for either 2 or 4 days led to significant changes in cell shape, such that cells became flattened and spread out and tended to resemble parental SKBR3 cell morphology. This suggested that the rounded morphology in Clone 3 cells was due to increased Rac1 activity. Moreover, stress fibers were restored in Clone 3 cells treated with NSC23766 (Fig. 3B). Quantification of stress fiber formation indicated a dose-dependent increase in stress fiber restoration (4.6–67.6%) in Clone 3 cells after cells were treated with different concentrations of NSC23766 (Fig. 3B, *graph*). Taken together, these data suggest that the up-regulated Rac1 activity likely contributed to the cytoskeletal and morphologic changes in Clone 3 cells.

We next asked whether NCS23766 had the ability to restore trastuzumab-mediated ErbB2 internalization and degradation in Clone 3 cells. Clone 3 cells were cultured in the standard culture media, which contains trastuzumab, and were then treated with the indicated concentrations of NSC23766 or cultured in the absence of NSC23766 for 2 days. As shown in Fig. 3C, trastuzumab, as detected by Cy2-conjugated donkey anti-human antibody, was internalized and localized to intracellular compartments in NSC23766-treated Clone 3 cells, whereas trastuzumab exhibited cell surface staining in NSC23766-untreated Clone 3 cells. Furthermore, internalization of trastuzumab in Clone 3 cells treated with NSC23766 was increased in a dose-dependent manner (Fig. 3C, *graph*).

We next addressed the question of whether NSC23766 treatment restored trastuzumab-mediated ErbB2 degradation in Clone 3 cells. Clone 3 cells were incubated in the standard culture media and treated with or without NSC23766. ErbB2 levels in WCL were detected by Western blot analysis using an antibody against ErbB2. As shown in Fig. 3D, ErbB2 levels were not changed at day 2 and day 4 when Clone 3 cells were not treated with NSC23766. In contrast, addition of 75 or 100 μmol/L NSC23766 for either 2 or 4 days led to a significant decrease in ErbB2 protein levels. Taken together, these data further support the idea that the increased Rac1 activity in Clone 3 cells leads to significant changes in the actin cytoskeleton, which may, in turn, impair trastuzumab-mediated ErbB2 internalization and degradation in Clone 3 cells. Reduction of Rac1 activity in Clone 3 cells by NSC23766 restores cell morphology and actin cytoskeleton to that similar to parental SKBR3 cells and, therefore, restores ErbB2 internalization and degradation.

NSC23766-Induced Cell Growth Inhibition Is Correlated with the Activity of Rac1 in Cells

Rac1 has been reported to play an essential role in the regulation of cell growth and cancer progression (39, 40). We tested whether NSC23766 was able to affect the growth of parental SKBR3 and Clone 3 cells. Cells were incubated in the standard culture media and treated or not treated with NSC23766 at the indicated concentrations. Although NSC23766 treatment inhibited growth of both parental SKBR3 and Clone 3 cells, our data revealed differential inhibition of cell growth and loss of cell viability between parental SKBR3 cells and Clone 3 cells, such that parental SKBR3 cells were relatively more sensitive to the NSC23766 treatment than Clone 3 cells. As shown in Fig. 4A, when treated with 50 μmol/L NSC23766, Clone 3 cell numbers increased ~4-fold at day 4 (*bottom graph*), whereas the cell number for parental SKBR3 cells was only slightly increased under the same condition (*top graph*). Moreover, 75 μmol/L NSC23766 eliminated the cell growth of parental SKBR3 cells (Fig. 4A, *top graph*). In contrast, numbers for Clone 3 cell doubled by day 4 when grown in media containing 75 μmol/L NSC23766 (Fig. 4A, *bottom graph*). Cell growth for both parental SKBR3 and Clone 3 cells was inhibited when treated with 100 μmol/L NSC23766.

Analysis of cell death by trypan blue exclusion in cells treated with NSC23766 revealed similar trends as those of the growth inhibition shown in Fig. 4A. There was a slight increase in the cell death observed in parental SKBR3 cells compared with Clone 3 cells at days 2 and 4, when cells were treated with 50 μmol/L NSC23766 (Fig. 4B). Whereas the percentage loss in viability for parental SKBR3 cells treated with 75 μmol/L NSC23766 was significantly increased at days 2 and 4, Clone 3 cells exhibited relative resistance to NSC23766 treatment (Fig. 4B). Treatment with 100 μmol/L NSC23766 caused a significant loss in viability in both cell lines at day 4 (Fig. 4B, *bottom graph*). Our data indicate that NSC23766-induced growth inhibition is correlated with the activity of Rac1, such that the higher activity of Rac1 in

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Clone 3 cells may render the cells more resistant to NSC23766 treatment compared with parental SKBR3 cells.

JIMT-1 breast cancer cells are an ErbB2-positive cell line derived from a patient primarily insensitive to trastuzumab therapy (12). We next tested if NSC23766 was able to induce growth inhibition in JIMT-1 cells. Figure 4C shows that NSC23766 treatment caused a marked reduction in cell number in JIMT-1 cells. Taken together, data obtained from JIMT-1 cells may generalize the NSC23766-induced growth inhibition to broader trastuzumab-insensitive cell lines.

Stable Expression of Rac1G12V Mutant Decreases Sensitivity to Trastuzumab, and NSC23766 Enhances Trastuzumab-Mediated Growth Inhibition and ErbB2 Degradation in Parental SKBR3 Cells

As shown in Fig. 5A, after treatment of parental SKBR3 cells with trastuzumab for 48 hours, the cell number was

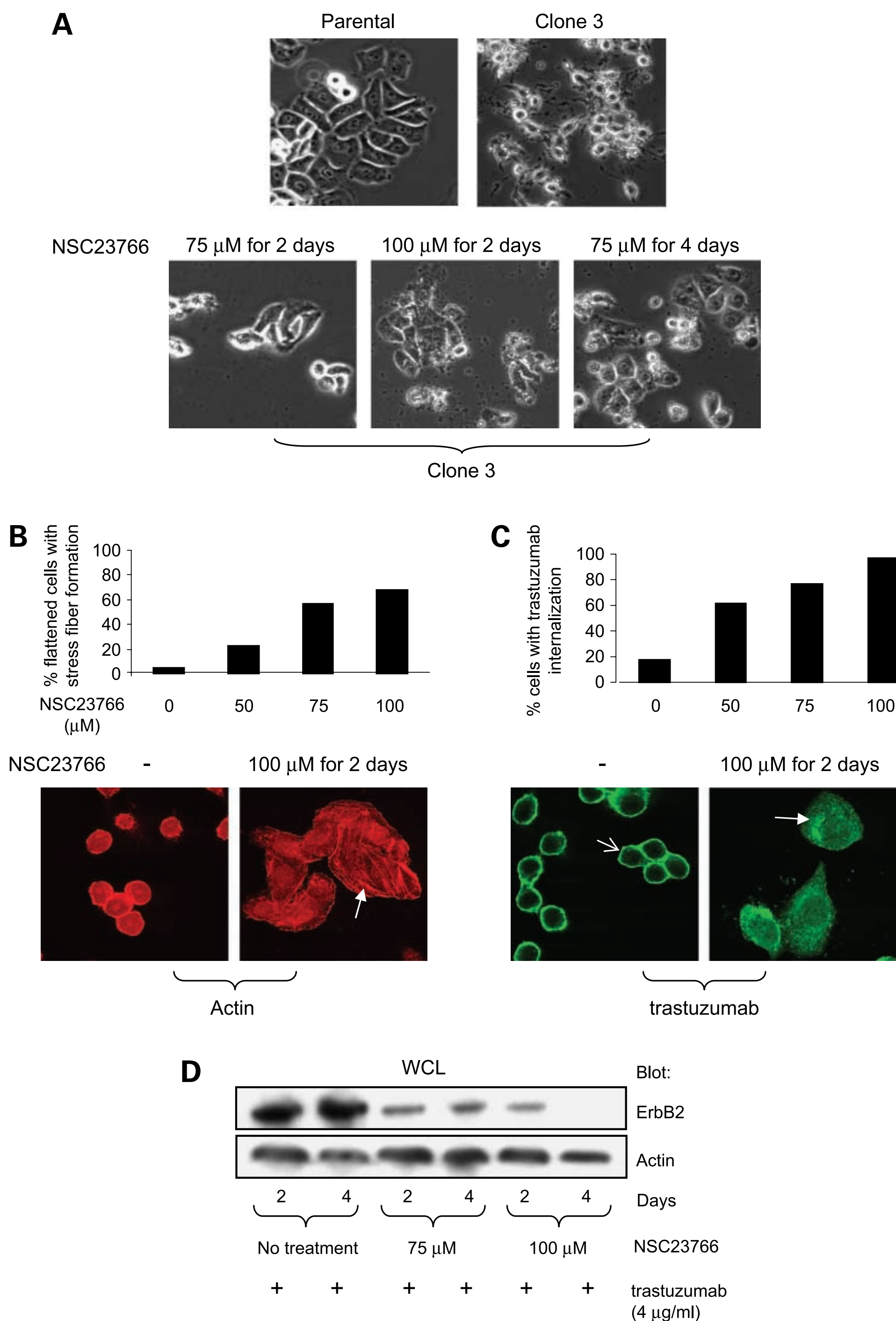

Figure 3. NSC23766 has the ability to restore cell morphology and trastuzumab-mediated endocytic down-regulation of ErbB2 in Clone 3 cells. **A,** parental SKBR3 and Clone 3 cells were cultured overnight in their respective standard culture media (see Materials and Methods). Cells were then left untreated or treated with NSC23766 at the indicated concentrations for 2 or 4 d. Phase-contrast pictures were taken as described in Fig. 1. **B,** Clone 3 cells were cultured in standard culture media and were treated either with or without NSC23766 at the indicted concentrations for 2 d. Actin staining was done with rhodamine-conjugated phalloidin. Immunofluorescent images were taken as described in Fig. 1. Bio-Rad 2100 confocal microscope attached to a Nikon E800 microscope was used to analyze the cell morphology and the presence of the formation of actin-based stress fibers. The graph indicates the percentage of Clone 3 cells that are flattened and have actin-based stress fibers. At least 300 cells per condition were counted for the analysis. *Closed arrow,* actin-based stress fibers in NSC23766 (100 µmol/L)–treated Clone 3 cells. **C,** experimental procedures are essentially the same as that described in **B**, except that trastuzumab was detected using Cy2-conjugated donkey anti-human antibody. *Open arrow,* plasma membrane staining of trastuzumab in untreated Clone 3 cells; *closed arrow,* internalized trastuzumab in NSC23766-treated Clone 3 cells. The diagram indicates the percentage of flattened Clone 3 cells with internalized trastuzumab. At least 300 cells per condition were counted for the analysis. **D,** Clone 3 cells were cultured in the standard culture media, which contains trastuzumab (4 µg/mL) overnight and were then treated with the indicated concentrations of NSC23766 for 2 and 4 d or not treated for 2 and 4 d. The total protein levels of ErbB2 in WCL were determined by Western blot analysis using antibody against ErbB2. Actin Western blot analysis was done to confirm equal protein loading.

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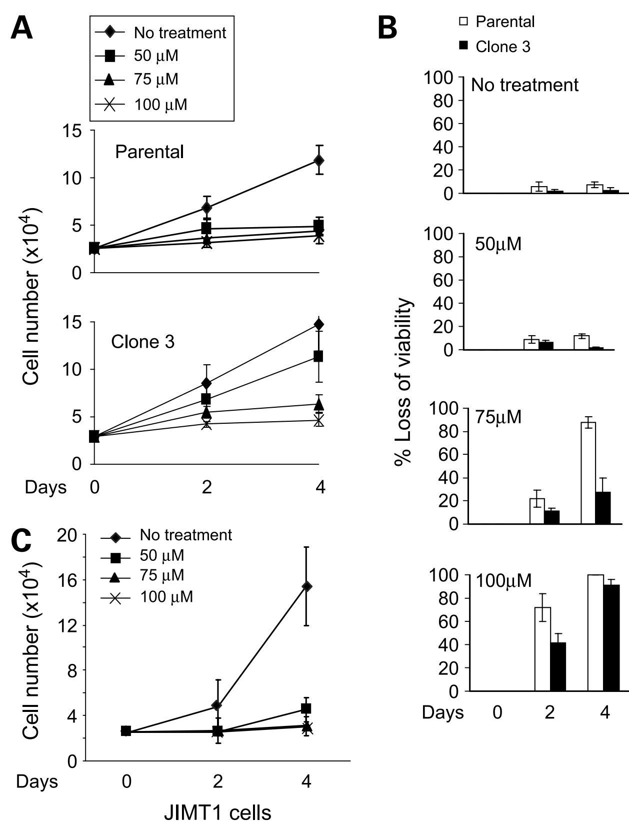

Figure 4. NSC23766 treatment results in the inhibition of cell growth in parental SKBR3, Clone 3 cells, and JIMT1 breast cancer cells. **A,** cell growth profiles of parental SKBR3 and Clone 3 cells after exposure to different concentrations of NSC23766. Parental SKBR3 and Clone 3 cells were plated at 2.5 × 10^4 per well of 24-well plates in triplicates in the standard culture media overnight and then treated with the indicated concentrations of NSC23766. Cells were trypsinized, stained with trypan blue, and counted at the indicted time points. Cells without trypan blue uptake were scored as viable. Cells positive for trypan blue uptake were scored as nonviable. Total cell number represents viable and nonviable cells. **B,** percentage loss of cell viability was calculated by dividing the number of trypan blue–positive cells with the total cell number. *Columns,* mean of triplicates; *bars,* SD. **C,** growth profile for JIMT-1 cells. JIMT-1 cells were plated at 2.5 × 10^4 per well of 12-well plates in triplicates in DMEM supplemented with 10% fetal bovine serum and treated with the indicated concentrations of NSC23766 or not treated. Cells were trypsinized, stained with trypan blue, and counted at the indicted time points. The total cell number, including both trypan blue–positive and trypan blue–negative cells, was used to plot the diagram.

slightly decreased compared with untreated cells, consistent with the previous report (41). Trastuzumab exhibited significant inhibition of cell growth after incubation of cells with trastuzumab for 5 days. If the increased Rac1 activity contributes to trastuzumab resistance of Clone 3 cells, we would expect that stable expression of constitutively active Rac1G12V mutant in parental SKBR3 cells would decrease their sensitivity to the trastuzumab treatment. As shown in Fig. 5B, after the treatment of vector control cells with trastuzumab for 4 days, a 40% reduction in cell number was observed compared with untreated vector control cells. In contrast, after treatment of SKBR3 cells stably expressing constitutively active Rac1G12V (Clone 16-SKBR3 and Clone 18-SKBR3) for 4 days, no significant reduction in cell numbers was observed compared with untreated Rac1G12V expressing cells. Moreover, after trastuzumab treatment for 7 days, there was ~60% decrease in cell number in vector control cells compared with untreated vector control cells, whereas there were 36% and 40% decreases in cell numbers after trastuzumab treatment in Clone 18-SKBR3 cells and Clone 16-SKBR3 cells, respectively, compared with untreated Rac1G12V-expressing cells (Fig. 5B). Figure 5C showed the overexpression levels of Rac1G12V proteins in different clones of SKBR3 cells. Taken together, these data suggest that overexpression of Rac1G12V decreases the sensitivity of parental SKBR3 cells to trastuzumab and provide additional evidence that increased Rac1 activity contributes to trastuzumab resistance in Clone 3 cells.

Based on the results shown in Fig. 5A, 2-day treatment of cells with trastuzumab is an appropriate time point for studying whether trastuzumab-mediated growth inhibition can be enhanced by NSC23766. Parental SKBR3 cells were seeded and cultured in media containing either no trastuzumab or 4 μg/mL trastuzumab and were treated with or without NSC23766 at the indicated concentrations for 2 days. Cells were then examined for growth, viability, and levels of ErbB2 in WCL. A 25% reduction in cell number was observed 48-hour after trastuzumab treatment (Fig. 5D, compare column 2 with column 1). Cells treated with 70 μmol/L NSC23766 for 48 hours in the absence of trastuzumab had ~15% to 20% growth inhibition (compare column 3 with column 1). However, an additive effect on growth inhibition (~50% decrease in cell number) was found when cells were treated with both trastuzumab (4 μg/mL) and 70 μmol/L NSC23766 (compare column 4 with column 1). The same was true when cell viability was quantified in the same experiment (Fig. 5E). We next determined the effect of NSC23766 on trastuzumab-mediated ErbB2 degradation. NSC23766 treatment significantly increased trastuzumab-mediated ErbB2 degradation in the parental SKBR3 cells (Fig. 5F). Whereas the protein levels of ErbB2 were not reduced 48 hours posttreatment with either trastuzumab or NSC23766 (Fig. 5F, *lanes 2, 3,* and *4*), the total ErbB2 level in WCL was significantly reduced when cells were incubated in the culture media containing 70 μmol/L NSC23766 and trastuzumab for 48 hours (*lane 6*), indicating that inhibition of Rac1 activity by NSC23766 enhances trastuzumab-induced down-regulation of ErbB2 in parental SKBR3 cells. Taken together, these data show that trastuzumab-mediated growth inhibition became more effective when the activity of Rac1 was down-regulated by NSC23766.

NSC23766 Treatment Results in Down-regulation of Both ErbB2 and Rac1-Mediated Signaling Pathways in Clone 3 Cells

We next investigated the activity of ErbB2 in both parental SKBR3 cells and Clone 3 cells. At steady-state, the extent of phosphorylation at Tyr^{877} of ErbB2 was elevated in Clone 3 cells compared with parental SKBR3 cells (Fig. 6A). Importantly, steady-state phosphorylation at $Tyr^{1221/2}$ was found in Clone 3 cells, whereas it was almost undetectable in the parental SKBR3 cells (Fig. 6A). These data indicate

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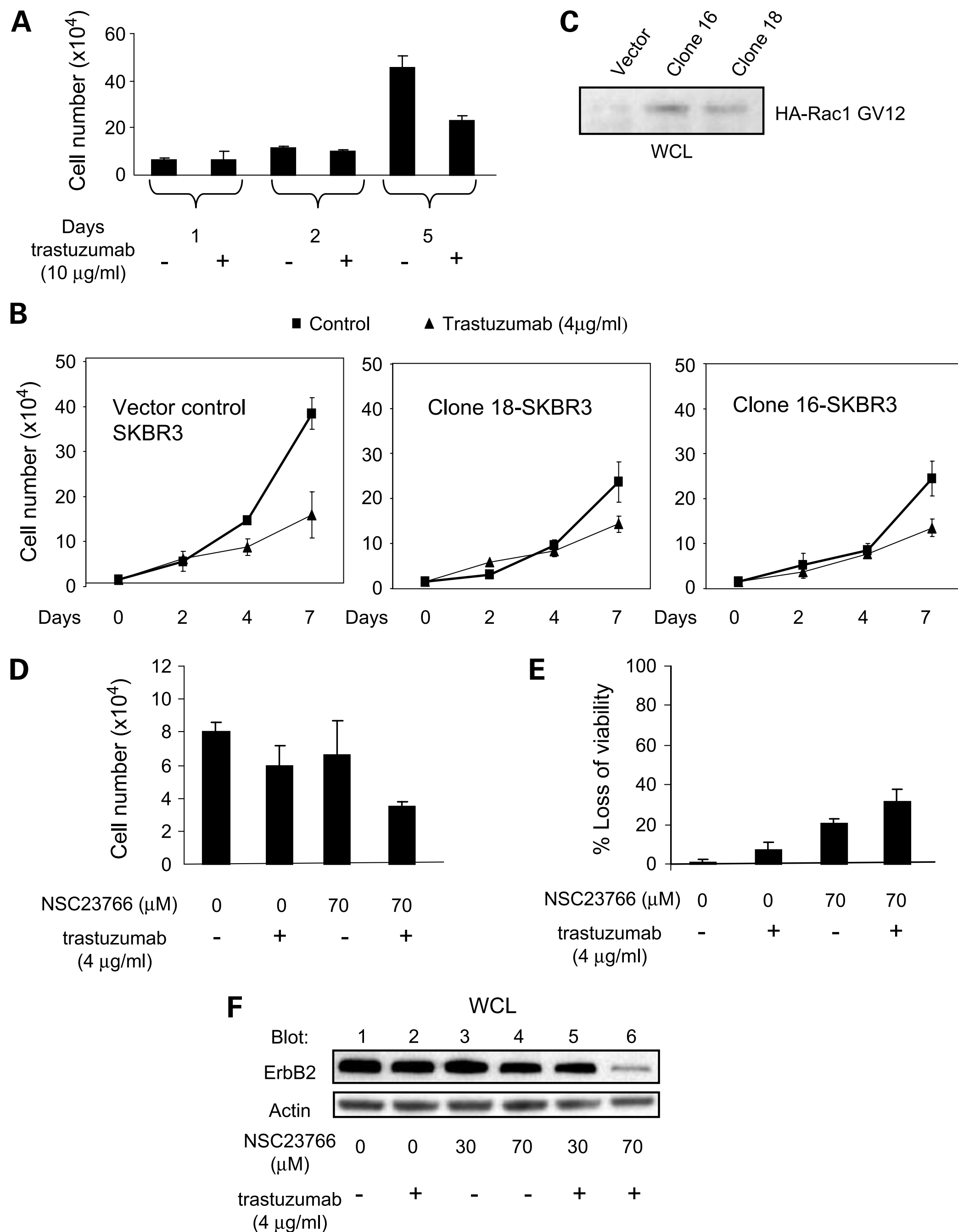

Figure 5. Stable expression of Rac1G12V mutant decreases sensitivity to trastuzumab, whereas NSC23766 enhances trastuzumab-mediated growth inhibition and ErbB2 degradation in parental SKBR3 cells. **A,** parental SKBR3 cells were plated in triplicates at a density of 4.5 × 10^4 per well in a 12-well dish overnight and were then treated with 10 µg/mL trastuzumab for the indicated times. Cells were trypsinized, stained with trypan blue, and counted. Cell number was calculated by counting total cell number (trypan blue–positive and trypan blue–negative cells). *Columns,* mean of three independent data from triplicates; *bars,* SD. **B,** growth profiles for vector control SKBR3 cells, and cells stably overexpressing HA-tagged Rac1G12V. Clone 18-SKBR3 and Clone 16-SKBR3 indicate two clones stably expressing HA-tagged Rac1G12V protein. Cell growth assays were done by plating 1.5 × 10^4 cells per well in a 12-well plate overnight. Cells then were treated with 4 µg/mL trastuzumab for 7 d or untreated. The assays for all indicated SKBR3 clones (vector control, Clone 16 and Clone 18) were done at the same time. Cells were trypsinized, stained with trypan blue, and counted at the indicted time points. Data represent average of three independent counts from triplicates. **C,** the image shows the expression levels of HA-tagged Rac1G12V in different clones. Anti-HA antibody was used to detect the HA-tagged Rac1 G12V. **D,** parental SKBR3 cells (4.5 × 10^4 per well) were seeded in 12-well plate in triplicates for 12 h and were then either not treated or treated with 4 µg/mL trastuzumab alone or in the presence of NSC23766 at the indicated concentrations for 2 d. The rest of the experimental procedures were the same as that in **A. E,** the experimental procedures were the same as that described in Fig. 4B. **F,** parental SKBR3 cells were plated in the standard culture media at a density of 3.5 × 10^5/60-mm plate overnight. Cells then were either not treated or treated with trastuzumab, NSC23766, or trastuzumab plus NSC23766 at the indicated concentrations for 2 d. The cells were then harvested, and the levels of ErbB2 in WCL were assessed by Western blot analysis using an anti-ErbB2 antibody. Actin Western blot analysis was done to control for equal loading.

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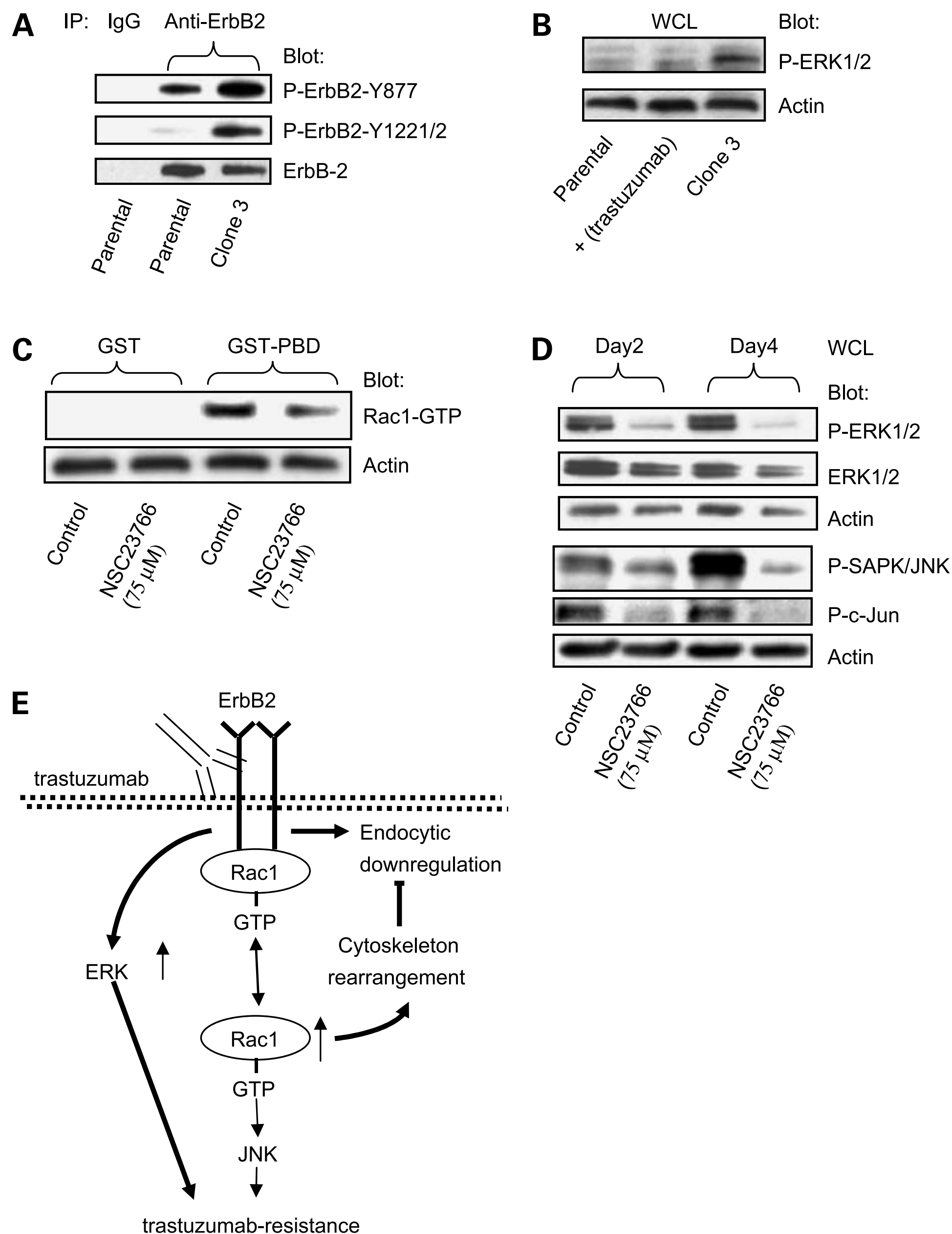

Figure 6. Inhibition of Rac1 by NSC23766 results in the down-regulation of ERK1/2 and SAPK/JNK activity in Clone 3 cells. **A,** parental SKBR3 and Clone 3 cells were cultured in the standard culture media and harvested. The WCL were subjected to immunoprecipitation with trastuzumab or control IgG. The levels of ErbB2 phosphorylation at Y877 or Y1221/2 were detected by Western blot analysis with anti–P-ErbB2-Y877 or P-ErbB2-Y1221/2 antibodies, respectively. The total immunoprecipitated endogenous ErbB2 was detected by Western blot analysis using a mouse anti-ErbB2 antibody. **B,** parental SKBR3 cells and Clone 3 cells were cultured in the standard culture media. Parental SKBR3 cells were then either untreated or treated with trastuzumab at 4 µg/mL for 3 d. The levels of tyrosine-phosphorylated ERK1/2 in WCL were detected by Western blot analysis with an anti–P-ERK1/2 antibody. Actin Western blot analysis was done to control for equal protein loading. **C,** the experimental procedures are essentially the same as in Fig. 1B except that Clone 3 cells were either untreated or treated with 75 µmol/L NSC23766 for 4 d before harvesting. NSC23766 (75 µmol/L) was included in the lysis buffer for harvesting the WCL. Western blot analysis of actin was done on WCL to confirm that the equal amounts of protein were used for the GST-PBD pull-down assay. **D,** clone 3 cells were plated in the standard culture media and were either not treated or treated with NSC23766 (75 µmol/L) for the indicated time points. Cells were harvested, and the levels of total ERK1/2, phosphorylated ERK1/2, phosphorylated SAPK/JNK, and phosphorylated c-Jun in WCL were determined by Western blot analysis. **E,** a model for the role of Rac1 in contributing to trastuzumab resistance in SKBR3 cells. In this scheme, up-regulated Rac1 activity in SKBR3 cells promotes actin cytoskeletal reorganization, leading to morphologic changes. Significant rearrangement of actin cytoskeleton inhibits trastuzumab-mediated endocytic down-regulation of ErbB2. This leads to the accumulation of ErbB2 on the cell surface and the enhanced activation of ErbB2 and ERK in cells, thus contributing to trastuzumab resistance. In this model, we postulate that binding of Rac1 to ErbB2 may stabilize Rac1 activity, which in turn may enhance the activity of Rac1 and provide a positive feed back signal to ErbB2-ERK signaling. Because JNK activity is correlated with the activity of Rac1 in Clone 3 cells, Rac1-mediated JNK signaling may be required for Rac1 to contribute to trastuzumab resistance. When Rac1 activity is reduced by NSC23766, this leads to actin cytoskeletal rearrangement, which is characterized by reassembling the stress fibers, resulting in restoration of cell morphology similar to parental SKBR3 cells and trastuzumab-mediated endocytic down-regulation of ErbB2.

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that Clone 3 cells contain a higher activity of ErbB2 compared with parental SKBR3 cells. Tyr$^{1221/2}$ is one of the major autophosphorylation sites in the ErbB2 receptor and is associated with the activation of Ras-Raf-MAP kinase signal pathway to mediate cell growth (42). We then asked if signaling downstream of ErbB2 was up-regulated in Clone 3 cells. Activated phosphorylated ERK1/2 in WCL was significantly enhanced in Clone 3 cells compared with both untreated and trastuzumab-treated parental SKBR3 cells (Fig. 6B). Consistent with another report (38), NSC23766 treatment reduced Rac1 activity in Clone 3 cells, as assessed by PBD pull-down assay (Fig. 6C). We next questioned whether the up-regulated ERK activity in Clone 3 cells can be reduced by NSC23766. ERK phosphorylation levels in Clone 3 cells were reduced after the addition of 75 μmol/L NSC23766 to the culture media for 2 and 4 days compared with control untreated cells (Fig. 6D, *top*). These data suggest that the ability of NSC23766 to decrease ERK activity may be the consequence of enhanced ErbB2 degradation induced by NSC23766 in Clone 3 cells (Fig. 3D).

It has been reported that SAPK/JNK, when activated, regulates cell growth and survival through its effects on c-Jun (28). Given that NSC23766 is selectively targeting Rac1 activity, we tested whether NSC23766 had the ability to modulate signaling downstream of Rac1 activity. Clone 3 cells were either untreated or treated with NSC23766 for the indicated times. As shown in Fig. 6D, NSC23766 treatment decreased the levels of phosphorylated SAPK/JNK and c-Jun.

These results, when taken together with results shown in Fig. 3, suggest that two mechanisms may be used by NSC23766 to mediate its growth inhibitory effects on Clone 3 cells. First, NSC23766 down-regulation of Rac1 activity results in actin cytoskeleton reorganization that subsequently restores trastuzumab-mediated ErbB2 degradation in Clone 3 cells, leading to the reduction of ERK signaling. Second, by directly targeting Rac1 activity, NSC23766 is capable of reducing JNK signaling in Clone 3 cells.

Discussion

The development of therapeutic resistance to trastuzumab poses a big challenge to the treatment of human ErbB2-positive breast cancer (2, 6). Rac1 has been implicated in the downstream signaling of ErbB receptors and has been reported to be involved in the regulation of breast cancer cell metastasis and invasion (22, 43). Here, we describe a previously unappreciated relationship between increased Rac1 activity and trastuzumab resistance. We show that in Clone 3 cells, Rac1 activity is up-regulated compared with trastuzumab-sensitive parental SKBR3 cells. We also show that Clone 3 cells display a distinctive cell morphology that is accompanied by significant cytoskeleton disorganization and compromised endocytic down-regulation of ErbB receptors. This leads to the aberrant accumulation of ErbB2 on the cell surface, the up-regulated ErbB2 activity, and the enhanced ERK activity in Clone 3 cells, resulting in trastuzumab resistance. When Rac1 activity is inhibited by NSC23766 treatment, however, Clone 3 cells reassemble actin-based stress fibers and have cell morphology similar to parental SKBR3 cells. Moreover, treatment of Clone 3 cells with NSC23766 restores stress fiber formation and trastuzumab-mediated endocytic down-regulation of ErbB2. Because trastuzumab-mediated internalization and degradation of ErbB2 is one of the mechanisms of action for trastuzumab-mediated growth inhibition (3, 34), our findings now highlight an important connection between the impaired endocytic down-regulation of ErbB2 and trastuzumab resistance.

SAPK/JNK activation, which occurs downstream of Rac1, is believed to regulate gene transcription to mediate effects on cell growth and survival (44, 45). Whereas we did not find a difference in JNK activity between parental SKBR3 and Clone 3 cells (data not shown), the activity of JNK is reduced by NSC23766 in trastuzumab-resistant SKBR3 cells, suggesting that Rac1-mediated JNK signaling may be required for Rac1 to contribute to trastuzumab resistance of SKBR3 cells. A model shown in Fig. 6E depicts the role of Rac1 in contributing to trastuzumab resistance in SKBR3 cells.

The activity of Rac1 is correlated with ErbB signaling in many human breast cancers (25, 46). Furthermore, in MCF10A human mammary epithelial cells that overexpress ErbB2, ErbB2-mediated up-regulation of Rac1 activity is blocked by treatment with trastuzumab (26). This suggests that the initial escape from trastuzumab-mediated inhibition of Rac1 activity may be an important mechanism leading to the high levels of Rac1 activity in Clone 3 cells. In support of this model, here, we show that overexpression of Rac1G12V mutant in parental cells reduced the sensitivity to trastuzumab. Whereas we did obtain data indicating that knocking down Rac1 reduced cell growth of parental SKBR3 cells (data not shown), mimicking the growth inhibitory effect of NSC23766 on parental SKBR3 cell, unfortunately, we experienced technical difficulties in silencing the expression of Rac1 in Clone 3 cells, although we attempted several different techniques.

It has been reported that activated Rac1 associates with ErbB2 leading to prolonged activation of Rac1 (36). We also find that activated Rac1 is able to form a stable complex with ErbB2 in Clone 3 cells, but not in parental SKBR3 cells. This may be due to higher levels of activated Rac1 in Clone 3 cells. Binding of Rac1 to ErbB2 may stabilize Rac1 activity, which in turn provides a positive feedback signal to ErbB2-ERK signaling in Clone 3 cells.

Trastuzumab treatment significantly improves outcomes for women with ErbB2 positive breast cancer (2, 7, 47, 48). However, many patients with metastatic breast cancer who initially respond to trastuzumab develop resistance (2). The clinical problem of trastuzumab resistance is becoming increasingly important, as recent studies strongly support a role for trastuzumab in the adjuvant setting for ErbB2 overexpressing early-stage breast cancer (6, 48, 49). Our studies reveal a novel molecular mechanism contributing to trastuzumab resistance and suggest that Rac1 and ERK may be novel therapeutic targets for the treatment of trastuzumab-resistant human breast cancer. Furthermore, whereas primary resistance to cetuximab, a therapeutic monoclonal

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antibody to EGFR, is associated with KRAS mutations of colorectal cancers, increased Rac1 activity may provide a predictive marker for acquired trastuzumab resistance of ErbB2-overexpressing breast cancers (50). Our data show that NSC23766 inhibits Rac1 activity and has the ability to reverse cellular phenotypes of trastuzumab resistance. Further investigations, including preclinical animal models and clinical trials to study the efficacy and toxicity of NSC23766 in trastuzumab-resistant breast cancer patients, are warranted.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed. The information presented in this article reflects the views of the authors and does not necessarily represent the policy of the U.S. Food and Drug Administration.

Acknowledgments

We thank Dr. Francisco J. Esteva for providing parental SKBR3 cells and trastuzumab-resistant SKBR3 cells (Clone 3 cells) and Drs. David Frucht, Sarah Kennett, Wendy Weinberg, and Kathleen Clouse for the critical review of this manuscript.

References

1. Hudis CA. Trastuzumab-mechanism of action and use in clinical practice. N Engl J Med 2007;357:39–51.

2. Nahta R, Yu DH, Hung MC, Hortobagyi GN, Esteva FJ. Mechanisms of disease: understanding resistance to HER2-targeted therapy in human breast cancer. Nat Clin Pract Oncol 2006;3:269–80.

3. Baselga J, Tripathy D, Mendelsohn J, et al. Phase II study of weekly intravenous recombinant humanized anti-p185HER2 monoclonal antibody in patients with HER2/neu-overexpressing metastatic breast cancer. J Clin Oncol 1996;14:737–44.

4. Cobleigh MA, Vogel CL, Tripathy D, et al. Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. J Clin Oncol 1999;17:2639–48.

5. Vogel CL, Cobleigh MA, Tripathy D, et al. Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. J Clin Oncol 2002;20:719–26.

6. Esteva FJ, Valero V, Booser D, et al. Phase II study of weekly docetaxel and trastuzumab for patients with HER-2-overexpressing metastatic breast cancer. J Clin Oncol 2002;20:1800–8.

7. Bender LM, Nahta R. Her2 cross talk and therapeutic resistance in breast cancer. Front Biosci 2008;13:3906–12.

8. Nahta R, Yuan LXH, Zhang B, Kobayashi R, Esteva FJ. Insulin-like growth factor-I receptor/human epidermal growth factor receptor 2 heterodimerization contributes to trastuzumab resistance of breast cancer cells. Cancer Res 2005;65:11118–28.

9. Nahta R, Takahashi T, Ueno NT, Hung MC, Esteva FJ. P27(kip1) down-regulation is associated with trastuzumab resistance in breast cancer cells. Cancer Res 2004;64:3981–6.

10. Ritter CA, Perez-Torres M, Rinehart C, et al. Human breast cancer cells selected for resistance to trastuzumab *in vivo* overexpress epidermal growth factor receptor and ErbB ligands and remain dependent on the ErbB receptor network. Clin Cancer Res 2007;13:4909–19.

11. Nagy P, Friedlander E, Tanner M, et al. Decreased accessibility and lack of activation of ErbB2 in JIMT-1, a Herceptin-resistant, MUC4-expressing breast cancer cell line. Cancer Res 2005;65:473–82.

12. Tanner M, Kapanen AI, Junttila T, et al. Characterization of a novel cell line established from a patient with Herceptin-resistant breast cancer. Mol Cancer Ther 2004;3:1585–92.

13. Nagata Y, Lan KH, Zhou X, et al. PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. Cancer Cell 2004;6:117–27.

14. Burris HA, III, Hurwitz HI, Dees EC, et al. Phase I safety, pharmacokinetics, and clinical activity study of lapatinib (GW572016), a reversible dual inhibitor of epidermal growth factor receptor tyrosine kinases, in heavily pretreated patients with metastatic carcinomas. J Clin Oncol 2005;23:5305–13.

15. Konecny GE, Pegram MD, Venkatesan N, et al. Activity of the dual kinase inhibitor lapatinib (GW572016) against HER-2-overexpressing and trastuzumab-treated breast cancer cells. Cancer Res 2006;66:1630–9.

16. Heasman SJ, Ridley AJ. Mammalian Rho GTPases: new insights into their functions from *in vivo* studies. Nat Rev Mol Cell Biol 2008;9:690–701.

17. Cerione RA. Cdc42: new roads to travel. Trends Cell Biol 2004;14:127–32.

18. Wu WJ, Erickson JW, Lin R, Cerione RA. The γ-subunit of the coatomer complex binds Cdc42 to mediate transformation. Nature 2000;405:800–4.

19. Shen Y, Hirsch DS, Sasiela CA, Wu WJ. Cdc42 regulates E-cadherin ubiquitination and degradation through an epidermal growth factor receptor to Src-mediated pathway. J Biol Chem 2008;283:5127–37.

20. Hirsch DS, Shen Y, Wu WJ. Growth and motility inhibition of breast cancer cells by epidermal growth factor receptor degradation is correlated with inactivation of Cdc42. Cancer Res 2006;66:3523–30.

21. Wu WJ, Tu S, Cerione RA. Activated Cdc42 sequesters c-Cbl and prevents EGF receptor degradation. Cell 2003;114:715–25.

22. Tang Y, Olufemi L, Wang MT, Nie D. Role of Rho GTPases in breast cancer. Front Biosci 2008;13:759–76.

23. Hirsch DS, Wu WJ. Cdc42: an effector and regulator of ErbB1 as a strategic target in breast cancer therapy. Expert Rev Anticancer Ther 2007;7:147–57.

24. Tu S, Wu WJ, Wang J, Cerione RA. Epidermal growth factor-dependent regulation of Cdc42 is mediated by the Src tyrosine kinase. J Biol Chem 2003;278:49293–300.

25. Yang C, Liu Y, Lemmon MA, Kazanietz MG. Essential role for Rac in heregulin β1 mitogenic signaling: a mechanism that involves epidermal growth factor receptor and is independent of ErbB4. Mol Cell Biol 2006;26:831–42.

26. Ueda Y, Wang S, Dumont N, Yi JY, Koh Y, Arteaga CL. Overexpression of HER2 (erbB2) in human breast epithelial cells unmasks transforming growth factor β-induced cell motility. J Biol Chem 2004;279:24505–13.

27. Schnelzer A, Prechtel D, Knaus U, et al. Rac1 in human breast cancer: overexpression, mutation analysis, and characterization of a new isoform, Rac1b. Oncogene 2000;19:3013–20.

28. Fritz G, Kaina B. Rho GTPases: promising cellular targets for novel anticancer drugs. Curr Cancer Drug Targets 2006;6:1–14.

29. Sahai E, Marshall CJ. Differing modes of tumour cell invasion have distinct requirements for Rho/ROCK signaling and extracellular proteolysis. Nat Cell Biol 2003;5:711–9.

30. Fritz G, Brachetti C, Bahlmann F, Schmidt M, Kaina B. Rho GTPases in human breast tumours: expression and mutation analyses and correlation with clinical parameters. Br J Cancer 2002;87:635–44.

31. Chan AY, Coniglio SJ, Chuang YY, et al. Roles of the Rac1 and Rac3 GTPases in human tumor cell invasion. Oncogene 2005;24:7821–9.

32. Baugher PJ, Krishnamoorthy L, Price JE, Dharmawardhane SF. Rac1 and Rac3 isoform activation is involved in the invasive and metastatic phenotype of human breast cancer cells. Breast Cancer Res 2005;7:R965–74.

33. Nahta R, Yuan LXH, Du Y, Esteva FJ. Lapatinib induces apoptosis in trastuzumab-resistant breast cancer cells: effects on insulin-like growth factor I signaling. Mol Cancer Ther 2007;6:667–74.

34. Yarden Y. Biology of HER2 and its importance in breast cancer. Oncology 2001;61 Suppl 2:1–13.

35. Valabrega G, Montemurro F, Sarotto I, et al. TGFα expression impairs trastuzumab-induced HER2 down-regulation. Oncogene 2005;24:3002–10.

36. Wang SE, Shin I, Wu FY, Friedman DB, Arteaga CL. HER2/Neu (ErbB2) signaling to Rac1-1 is temporally and spatially modulated by transforming growth factor β. Cancer Res 2006;66:9591–600.

37. Lin R, Cerione RA, Manor D. Specific contributions of the small GTPases Rho, Rac, and Cdc42 to Dbl transformation. J Biol Chem 1999;274:23633–41.

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Published OnlineFirst June 9, 2009; DOI: 10.1158/1535-7163.MCT-09-0140

38. Gao Y, Dickerson JB, Guo F, Zheng J, Zheng Y. Rational design and characterization of a Rac GTPase-specific small molecule inhibitor. Proc Natl Acad Sci U S A 2004;101:7618–23.

39. Qiu RG, Chen J, Kirn D, McCormick F, Symons M. An essential role for Rac in Ras transformation. Nature 1995;374:457–9.

40. Sahai E, Marshall CJ. RHO-GTPases and cancer. Nat Rev Cancer 2002;2:133–42.

41. Lu Y, Zi X, Zhao Y, Mascarenhas D, Pollak M. Insulin-like growth factor-I receptor signaling and resistance to trastuzumab (Herceptin). J Natl Cancer Inst 2001;93:1852–7.

42. Kwon YK, Bhattacharyya A, Alberta JA, et al. Activation of ErbB2 during wallerian degeneration of sciatic nerve. J Neurosci 1997;17:8293–9.

43. Burbelo P, Wellstein A, Pestell RG. Altered Rho GTPase signaling pathways in breast cancer cells. Breast Cancer Res Treat 2004;84:43–8.

44. Minden A, Lin A, Claret FX, Abo A, Karin M. Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. Cell 1995;81:1147–57.

45. Potapova O, Haghighi A, Bost F, et al. The Jun kinase/stress-activated protein kinase pathway functions to regulate DNA repair and inhibition of the pathway sensitizes tumor cells to cisplatin. J Biol Chem 1997;272:14041–4.

46. Adam L, Vadlamudi R, Kondapaka SB, Chernoff J, Mendelsohn J, Kumar R. Heregulin regulates cytoskeletal reorganization and cell migration through the p21-activated kinase-1 via phosphatidylinositol-3 kinase. J Biol Chem 1998;273:28238–46.

47. Goldenberg MM. Trastuzumab, a recombinant DNA-derived humanized monoclonal antibody, a novel agent for the treatment of metastatic breast cancer. Clin Ther 1999;21:309–18.

48. Pegram MD, Lipton A, Hayes DF, et al. Phase II study of receptor-enhanced chemosensitivity using recombinant humanized anti-p185HER2/neu monoclonal antibody plus cisplatin in patients with HER2/neu-overexpressing metastatic breast cancer refractory to chemotherapy treatment. J Clin Oncol 1998;16:2659–71.

49. Esteva FJ, Cheli CD, Fritsche H, et al. Clinical utility of serum HER2/neu in monitoring and prediction of progression-free survival in metastatic breast cancer patients treated with trastuzumab-based therapies. Breast Cancer Res 2005;7:R436–43.

50. Cappuzzo F, Varella-Garcia M, Finocchiaro G, et al. Primary resistance to cetuximab therapy in EGFR FISH-positive colorectal cancer patients. Br J Cancer 2000;99:83–9.

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