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A Novel Interaction Between HER2/neu and Cyclin E in Breast Cancer

Elizabeth A. Mittendorf, M.D., Yanna Liu, M.S., Susan L. Tucker, Ph.D., Tamra McKenzie, M.D., Na Qiao, Ph.D., Said Akli, Ph.D., Anna Biernacka, M.D., Yan Liu, M.S., Laurent Meijer, Ph.D., Khandan Keyomarsi, Ph.D.* , and Kelly K. Hunt, M.D.*

Departments of Surgical Oncology (EAM, Yanna Liu, TM, NQ, KKH), Bioinformatics and Computational Biology (SLT), and Experimental Radiation Oncology (Yan Liu, SA, AB, KK), M. D. Anderson Cancer Center, Houston, TX and Protein Phosphorylation and Disease (LM), Centre National de la Recherche Scientifique, Station Biologique, Roscoff, France.

Abstract

HER2/neu (HER2) and cyclin E are important prognostic indicators in breast cancer. Since both are involved in cell cycle regulation we investigated whether there was a direct interaction between the two. HER2 and cyclin E expression levels were determined in 395 breast cancer patients. Patients with HER2-overexpression and high levels of cyclin E had decreased 5-year disease-specific survival compared with low levels of cyclin E (14% versus 89%, $P < .0001$) In vitro studies were performed in which HER2-mediated activity in HER2-overexpressing breast cancer cell lines was downregulated by transfection with HER2 siRNA or treatment with trastuzumab. Cyclin E expression levels were determined, and functional effects investigated using kinase assays, MTT assays to assess cell viability as a marker of proliferation, and FACS analysis to determine cell cycle profiles. Decreased HER2-mediated signaling resulted in decreased expression of cyclin E, particularly the low molecular weight (LMW) isoforms. Decreased HER2 and LMW cyclin E expression had functional effects, including decreased cyclin E-associated kinase activity and decreased proliferation, due to increased apoptosis and an increased accumulation of cells in the G1 phase. In vivo studies performed in a HER2-overexpressing breast cancer xenograft model confirmed the effects of trastuzumab on cyclin E expression. Given the relationship between HER2 and cyclin E, in vitro clonogenic assays were performed to assess combination therapy targeting both proteins. Isobologram analysis showed a synergistic interaction between the two agents (trastuzumab targeting HER2 and roscovitine targeting cyclin E). Taken together, these studies demonstrate that HER2-mediated signaling effects LMW cyclin E expression, which in turn effects cell cycle regulation. LMW cyclin E has

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Correspondence: Kelly K. Hunt, M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030 (phone: 713-792-7216; fax: 713-792-4689; khunt@mdanderson.org) and Khandan Keyomarsi, M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030 (phone: 713-792-4845; fax: 713-794-5369; kkeyomar@mdanderson.org).

*These authors contributed equally to this work

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Conflict of Interest

Dr. L. Meijer is a coinventor on the patent on roscovitine licensed to Cyclacel.

prognostic and predictive roles in HER2-overexpressing breast cancer, warranting further study of its potential as a therapeutic target.

Keywords

Breast cancer; HER2/neu; cell cycle regulation; cyclin E

INTRODUCTION

Improved understanding of pathways controlling cancer cell growth has led to refinements in risk stratification and identification of therapeutic targets. An example of this paradigm in breast cancer is HER2/neu (HER2) overexpression and use of the monoclonal antibody trastuzumab in patients with HER2-overexpressing tumors (Slamon et al., 1987; Slamon et al., 1989). As a single agent, trastuzumab was effective in approximately 35% of patients (Cobleigh et al., 1999; Vogel et al., 2002). Thus, identifying HER2 overexpression alone does not ensure response to targeted therapy and there is a need to identify additional markers that can predict response and serve as targets for novel therapeutics. Because HER2 mediates signal transduction pathways affecting cell cycle regulation, we examined the interplay between HER2 and cyclin E, a G1 cell cycle regulator.

When overexpressed in breast cancer, HER2 promotes growth and proliferation, and increases invasive and metastatic capabilities (Yarden & Sliwkowski, 2001; Yu & Hung, 2000). HER2-overexpressing breast cancer patients have poorly differentiated tumors with high proliferative rates and an increased risk of recurrence and death (Gusterson et al., 1992; McCann et al., 1991; Slamon et al., 1987; Slamon et al., 1989; Wright et al., 1989). The oncogenic effect of HER2 occurs through several mechanisms, including cell cycle perturbation. Specifically, activation of HER2 signal transduction promotes cell proliferation by shortening the G1 phase (Timms et al., 2002).

Cyclin E, a crucial regulator maintaining the G1/S transition (Ohtsubo & Roberts, 1993; Ohtsubo et al., 1995) is believed to promote tumorigenesis through shortening G1, promoting faster G1/S transition, increased cyclin E-associated kinase activity, and genomic instability (Akli & Keyomarsi, 2003; Akli et al., 2004; Dulic et al., 1993; Spruck et al., 1999). In a study of 395 breast cancer patients, cyclin E overexpression was the most powerful predictor of overall and disease-free survival (Keyomarsi et al., 2002).

The principal mode of cyclin E deregulation is at the protein level with some breast cancer cell lines and human breast cancers expressing up to five low molecular weight (LMW) isoforms (Keyomarsi & Herliczek, 1997; Keyomarsi et al., 1994). These LMW forms activate CDK2 and phosphorylate substrates more efficiently than the full-length form. They are more resistant to the CDK inhibitors p21 and p27 and to anti-estrogens, and they induce genomic instability (Akli et al., 2004; Porter et al., 2001; Wingate et al., 2005). In a transgenic mouse model, mice expressing LMW cyclin E had an increased incidence of mammary tumor formation and distant metastasis indicating LMW isoforms add metastatic potential (Akli et al., 2007).

In this study, we examined the relationship between HER2 and cyclin E in breast cancer. We found that patients with HER2-overexpressing tumors and high cyclin E expression have a significantly decreased 5-year disease-specific survival (DSS) compared with patients whose tumors have HER2-overexpression but low levels of cyclin E. Having established this clinically relevant relationship, we investigated consequences of HER2 overexpression and downregulation in the context of LMW cyclin E in breast cancer cell lines and xenografts. We found that the functions of HER2 and cyclin E are interlinked, suggesting that treatment strategies targeting both may be better than targeting either one alone.

RESULTS

Patients with HER2-Overexpressing Tumors and High Levels of Cyclin E Have Worse 5-Year DSS

To determine if a relationship exists between HER2 and cyclin E, we analyzed data from a cohort of 395 breast cancer patients that were originally studied to determine the relationship between total (full-length + LMW) cyclin E expression and survival (Keyomarsi et al., 2002). No patients in this cohort received trastuzumab therapy. Five-year DSS rates were significantly worse in patients with HER2-positive tumors ($P < .0001$) (Figure 1a). We stratified patients with HER2-positive tumors ($n = 117$) by total cyclin E levels and found those with high total cyclin E ($n = 59$) had a 5-year DSS of 14% compared to 89% for those with low total cyclin E ($n = 58$; $P < .0001$; Figure 1b). When stratified by LMW cyclin E levels, patients with high LMW cyclin E ($n = 50$) had a 5-year DSS of 10%; compared with 82% for patients with low LMW cyclin E ($n = 67$; $P < .0001$; Figure 1c). These data demonstrate that overexpression of both HER2 and cyclin E, particularly LMW cyclin E, contributes to an aggressive phenotype of breast cancer.

HER2 Expression Alters Cyclin E Expression

Activation of HER2 signaling promotes cellular proliferation by shortening the G1 phase of the cell cycle (Timms et al., 2002). Similarly, overexpression of cyclin E shortens the length of the G1 phase. Therefore, we hypothesized that HER2 overexpression may modulate cyclin E expression or activity. To this end, we transfected the HER2-overexpressing breast cancer cell line MCF-7-HER-18, with HER2 siRNA. MCF-7-HER-18 has exogenous HER2 overexpression established by stable transduction of the parental cell line, MCF7. MCF-7-HER-18 expresses both full-length and LMW cyclin E (Figure 2a). The HER2 siRNA was effective in knocking down HER2 expression (Figure 2b) and this was accompanied by a decrease in cyclin E expression (Figure 2c).

We repeated the transfections using a second HER2 siRNA and an additional cell line, SKBr3, a breast cancer cell line with endogenous HER2 overexpression that also expresses full-length and LMW cyclin E (Figure 2a). Immunofluorescence confocal microscopy revealed that transfection with HER2 siRNA decreased HER2 (Figure 2d) and cyclin E (Figure 2e) expression. Furthermore, western blot analysis confirmed a decrease in HER2 expression and revealed the decrease in cyclin E was primarily due to decreased expression of LMW isoforms (Figure 2f).

To determine if a feedback loop exists between HER2 and cyclin E, we investigated HER2 expression in MCF-7 cells engineered to overexpress full-length or LMW cyclin E. There were no changes in HER2 expression or its phosphorylated form, suggesting that cyclin E lies downstream of HER2 (Supplementary Figure 1).

HER2 Downregulation Results in Decreased Cyclin E Activity and Proliferation

To assess the effect of HER2 signaling on cyclin E activity, we altered HER2 levels using siRNA then examined cyclin E-associated kinase activity. HER2 downregulation decreased cyclin E-associated kinase activity (Figure 3a) which was more pronounced in SKBr3 cells (64% decrease in cyclin E-associated kinase activity) than in MCF-7-HER-18 cells which have higher baseline levels of LMW cyclin E (51% decrease). These data suggest that the extent of decrease in cyclin E-associated kinase activity is related to baseline levels of LMW cyclin E expression.

Since the functionality of cyclin E is crucial in cell cycle progression, we explored the effect of HER2 downregulation and subsequent decrease in LMW cyclin E expression on proliferation. MTT assays were used to assess cell viability as a marker of proliferation (Figure 3b). After transfection with HER2 siRNA, we noted a pronounced decrease in proliferation. Because decreased proliferation can be due to an increase in apoptosis or cell cycle arrest, we assessed both of these. As demonstrated in figure 3c, part of the decrease in proliferation was due to an increase in apoptosis as evidenced by an increased percentage of cells in the sub-G1 phase (Figure 3c). In three duplicate experiments, the average percent \pm SEM in sub-G1 in MCF-7-HER-18 cells was $1.69\% \pm 0.58$ in control cells versus $22.08\% \pm 3.95$ in HER2 siRNA transfected cells ($11.65\% \pm 0.33$ in HER2 siRNA #2 transfected cells). In SKBr3 the average percent \pm SEM in sub-G1 was $3.11\% \pm 0.17$ in control cells versus $12.48\% \pm 1.17$ in HER2 siRNA transfected cells ($12.45\% \pm 0.10$ in HER2 siRNA #2 transfected cells). The increase in apoptosis after transfection with HER2 siRNA was confirmed using an annexin V assay (Supplementary Figure 2). Cell cycle analysis demonstrated a concomitant increase in the percentage of cells in G1 phase of the cell cycle in MCF-7-HER-18 cells using both siRNA sequences. In SKBr3 cells, a significant increase was seen in the average percent in G1 using HER2 siRNA #1 but not using siRNA #2 (Figure 3d). Taken together, these data suggest that the decrease in proliferation seen after HER2 downregulation is multifactorial with both an increase in apoptosis and alterations in cell cycle distribution contributing with the increase in apoptosis being the more consistent, pronounced effect.

Effect of HER2 on Cyclin E Expression is Post-Transcriptional

To determine if HER2 siRNA-mediated downregulation of cyclin E is transcriptionally regulated, RNA extracted from cells transfected with HER2 siRNA was subjected to qRT-PCR, after which comparative quantitation analysis was performed. In three duplicate experiments performed on MCF-7-HER-18 cells, the mean cyclin E/ β -actin mRNA ratios were .006, .009 ($P = .18$), and .013 ($P = .18$) for controls, cells transfected with random sequence siRNA, and cells transfected with HER2 siRNA, respectively (Supplementary Table 1a). Experiments were repeated using SKBr3 cells, and mean cyclin E/ β -actin ratios were .157, .288 ($P = .46$), and .128 ($P = .24$) (Supplementary Table 1b). These data suggest

that HER2 does not regulate cyclin E transcription. This supports our finding that the effect of HER2 is greatest on LMW isoforms, which are the result of post-translational modification of the full-length protein.

Decreased HER2-Mediated Signaling Results in Decreased Cyclin E Expression and Altered Expression and Localization of G1 Regulators

Based on these results, we hypothesized that cyclin E lies downstream of HER2-mediated signaling and that decreased activity through these pathways effects cyclin E expression and activity. Using immunofluorescence confocal microscopy to assess cyclin E expression, we found a dose-dependent decrease in cyclin E expression in MCF-7-HER-18 and SKBr3 cells treated with different doses of trastuzumab. In particular, cyclin E expression in MCF-7-HER-18 and SKBr3 cells treated with 20 $\mu\text{g}/\text{mL}$ trastuzumab decreased by 86.5% and 86.8%, respectively, compared to untreated cells (Figure 4a). Western blot confirmed the decrease was primarily in the LMW isoforms (Figure 4b). Similar to the effect seen after transfection with HER2 siRNA, decreased HER2-mediated signaling due to trastuzumab treatment resulted in decreased cyclin E-associated kinase activity (Figure 4c). Consistent with results from the kinase assays performed on HER2 siRNA-transfected cell lysates (Figure 3a), this effect was most pronounced in the SKBr3 cell line (60% decrease versus 26% decrease in MCF-7-HER-18 cells).

To better determine the effects of decreased HER2-mediated signaling on the G1 checkpoint, we assessed the effects of trastuzumab on cyclin D1, and the CDK inhibitors p21 and p27. Consistent with other reports (Lee et al., 2000) that cyclin D1 expression increases when cells are transfected with HER2, we found that trastuzumab treatment decreased cyclin D1 levels (Figure 4b). We did not find appreciable change in p21 or p27 expression (Figure 4b); however, confocal immunofluorescence microscopy revealed increased nuclear localization of both (Figure 4d).

Trastuzumab Therapy Alters Proliferation and Cell Cycle Profiles

Because one proposed mechanism of action of trastuzumab is decreased proliferation (Bacus et al., 1992; Brockhoff et al., 2007;), we assessed cell viability as a marker of proliferation using MTT assays in cells treated with trastuzumab and found that proliferation decreased in a dose-dependent manner. The number of viable MCF-7-HER-18 cells decreased by 56.8% after treatment with 20 $\mu\text{g}/\text{mL}$ of trastuzumab, compared to untreated cells, and the number of viable SKBr3 cells decreased by 67% (Supplemental Figure 3a). Concomitant with the decreased proliferation was an increased percentage of cells in G1 phase (Supplementary Figure 3b). The mean percentage increase was greater in SKBr3 cells, which have endogenous HER2 overexpression. These data suggest that breast cancer cells with endogenous HER2 overexpression depend on mitogenic signaling through HER2 pathways to increase cellular proliferation. In contrast, MCF-7-HER-18 cells that were transfected to exogenously express HER2 may have additional oncogenic pathways affecting their rate of cellular proliferation.

Having demonstrated decreased proliferation after trastuzumab treatment, we investigated the effect of overexpression of cyclin E on this therapy. HER2-overexpressing SKBr3 cells

were infected with adenoviruses overexpressing full-length cyclin E and the T1 LMW isoform. Cells were treated with trastuzumab and proliferation assessed. Expression of both full-length and LMW cyclin E resulted in inhibition of the anti-proliferative effect of trastuzumab treatment (Supplementary Figure 3c). In uninfected cells, growth was inhibited by trastuzumab by 22% compared to untreated cells ($P=.03$). In full-length cyclin E and cyclin E-T1 overexpressing cells, trastuzumab did not inhibit cell proliferation compared to untreated cells (8.4% increase in proliferation in full-length infected cells $P=.23$; 3.2% increase in T1 infected cells $P=.65$). These results demonstrate that trastuzumab-induced growth inhibition can be overcome by cyclin E overexpression.

These data suggested potential utility in targeting cyclin E and HER2. We therefore investigated the effects of combining trastuzumab with roscovitine, an olomucine-related purine that preferentially inhibits CDK1 and CDK2. High-throughput clonogenic assays were used to compare cytotoxic effects of trastuzumab alone, roscovitine alone, or the combination in SKBr3 and BT474 (breast cancer cell line with endogenous HER2 overexpression) cells. When given individually, both agents showed a dose-dependent reduction in cell viability (Figure 5a). With the combination, there was significantly decreased cell viability compared with either agent alone. The combination index showed synergistic cytotoxicity between the two agents (Figure 5b).

Inhibition of HER2-Mediated Signaling Results In Decreased Cyclin E Expression In Vivo

To evaluate effects of HER2 on cyclin E expression in vivo, we created a HER2-overexpressing breast cancer xenograft model by injecting MCF-7-HER-18 breast cancer cells into the mammary fat pads of nude mice. After tumors reached 100 mm³, mice received intraperitoneal injections of trastuzumab or PBS twice weekly for 3 weeks.

Immunohistochemical analyses of tumors showed that trastuzumab-treated mice had lower levels of phosphorylated HER2 expression, confirming a treatment effect (percentage of pHER2 positive membranes: control group $41.7 \pm 3.3\%$ vs. treated group $22.4 \pm 1.7\%$, $p<0.01$). In addition, there was a decrease in cyclin E expression (percentage of cyclin E positive nuclei: control group $57.1 \pm 12.3\%$ vs. treated group $17.5 \pm 4.1\%$, $p<0.01$) (Figure 6a). Western blot for cyclin E on lysates from these tumors demonstrated that the decrease in cyclin E was primarily due to a decrease in the LMW forms (Figure 6b). These data provide in vivo confirmation of the effects of decreased HER2-mediated signaling on cyclin E expression.

DISCUSSION

In this article, we report a novel interaction between HER2 and cyclin E in breast cancer. Downregulation of HER2 using siRNA and decreased HER2-mediated signaling using trastuzumab both resulted in decreased expression of cyclin E, particularly the LMW isoforms. Decreased LMW cyclin E expression led to reduced cyclin E-associated kinase activity and decreased proliferation due to induction of apoptosis as well as increased percentage of cells in G1 phase of the cell cycle. Our clinical data provide evidence that HER2-overexpressing breast cancers that also overexpress cyclin E are a more aggressive

phenotype. Effective treatment for patients whose tumors overexpress both proteins may require targeting HER2 and cyclin E, particularly the LMW isoforms.

Our data suggest that the effect of HER2 on G1 phase may be mediated in part by its impact on cyclin E. We and others have demonstrated a linkage between tumorigenesis and cyclin E by correlating the altered expression of cyclin E to the loss of growth control in breast cancer (Buckley et al., 1993; Keyomarsi & Herliczek, 1997; Keyomarsi & Pardee, 1993). Le et al. demonstrated that treatment of SKBr3 cells with the anti-HER2 antibody 4D5 caused decreased cyclin E-associated kinase activity (Le et al., 2006). One question this study raised is whether the decrease in cyclin E-associated kinase activity was due to changes in full-length or LMW cyclin E expression. Our data suggests that the decrease is due to changes in LMW cyclin E.

Elastase, a serine protease that cleaves full-length cyclin E at two sites in the amino terminus (Porter et al., 2001), mediates generation of these tumor-specific LMW isoforms through post-translational processing. Consistent with these findings, we found no significant change in cyclin E gene expression between HER2 siRNA-transfected cells and control cells using qRT-PCR. This suggests that the effects of HER2 on cyclin E are post-transcriptional. We are currently investigating expression and activity of elastase as a likely downstream target of HER2-mediated signaling.

Besides effecting cyclin E, a positive regulator of the cell-cycle, HER2 also effects the CDK inhibitors p21 and p27 which are negative regulators (Yang et al., 2000; Zhou et al., 2001). This effect is primarily due to altered localization of these proteins (Zhou et al., 2001; Yang et al., 2000)). Our data confirm that HER2 effects the localization of p21 and p27, as treatment with trastuzumab increased nuclear localization. This finding is particularly important for HER2-overexpressing tumors that also overexpress LMW cyclin E, in that our group has previously demonstrated the LMW isoforms are resistant to growth inhibition by p21 and p27 (Akli et al., 2004; Wingate et al., 2005). Treatment with trastuzumab decreases the extent to which the resistant LMW cyclin E isoforms are expressed and may increase the amount of nuclear p21 and p27 above a threshold level needed to effectively inhibit the activity of the remaining LMW cyclin E.

In addition to its effect on the G1 phase of the cell cycle, our data show that decreasing HER2 expression by siRNA knockdown may mediate cytotoxicity by increasing apoptosis. This is consistent with data published by Roh et al. who demonstrated increased activation of apoptotic pathways in BT474 cells transfected with HER2 antisense oligonucleotides (Roh et al., 2000). Interestingly, in vitro studies using trastuzumab, which has minimal effects on HER2 expression, have not consistently demonstrated an increase in apoptosis. An early report by Yakes and colleagues reported increased apoptosis following treatment with trastuzumab in SKBr3 cells but not BT474 cells (Yakes et al., 2002). A more recent publication by Brockhoff et al. found that treatment with trastuzumab did not result in appreciable amounts of apoptosis in either SKBr3 or BT474 cells (Brockhoff et al., 2007) Together with the findings from the current study, this suggests that there may be utility in further studying the regulation of HER2 expression either post-transcriptionally or at the

level of protein stability in order to identify additional therapeutic strategies that might enhance apoptosis in HER2 overexpressing breast cancer.

Data from our study reveals a subtype of HER2-overexpressing breast cancer with high levels of LMW cyclin E that is a particularly aggressive phenotype. A previous report from Potemski et al. showed that cyclin E expression was more often seen in HER2-positive tumors but they did not report an impact on survival (Potemski et al., 2006). This group did not look at the LMW isoforms. Importantly, the clinical data in the current study and previous reports from our group, suggest a prognostic role for LMW cyclin E (Keyomarsi et al., 2002). Our findings suggest that tumors overexpressing HER2 and LMW cyclin E may respond to HER2 targeted-therapy in part because HER2 downregulation results in decreased cyclin E-associated kinase activity and an increased percentage of cells in G1, which decreases proliferation. We have also shown that overexpression of full-length or LMW cyclin E may abrogate the growth inhibitory effects of trastuzumab. This suggests that combination therapy using trastuzumab and roscovitine which targets CDK2/cyclin E may have therapeutic efficacy. Such a strategy is supported by our data demonstrating synergism between these two agents. It is possible this synergism is due to the activity of both agents on the cyclin E-CDK2 complex. Another possibility is that roscovitine, which also targets CDK1 is inducing apoptosis allowing for synergism with trastuzumab which is acting primarily on the G1 phase. Collectively, these findings suggest that routine assessment of LMW cyclin E in HER2 -overexpressing breast tumors may have clinical utility.

In conclusion, we have identified an interaction between HER2 and cyclin E that contributes to the existing knowledge regarding effects of HER2 overexpression on regulation of the G1 checkpoint and cellular proliferation. We show that HER2 acts post-transcriptionally to effect the tumorigenic LMW cyclin E isoforms. These data suggest that LMW cyclin E overexpression in HER2-overexpressing breast cancer has prognostic and predictive roles and that LMW cyclin E may serve as an additional therapeutic target. Further studies investigating the mechanism by which HER2 effects formation of LMW cyclin E may lead to the design of new therapeutic strategies.

MATERIALS AND METHODS

Cell Lines

MCF-7, SKBr3, and BT474 breast cancer cells were obtained from American Type Culture Collection (Manassas, VA). MCF-7-HER-18 was a gift from Dr. Mien-Chie Hung (M. D. Anderson Cancer Center). Cells were passaged in culture less than 6 weeks in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mg streptomycin (Gibco; Invitrogen Corp, Grand Island, NY) in 5% CO₂ at 37°C. Media for MCF-7-HER-18 included 0.5 mg/ml G418.

Plasmids and siRNA

FLAG-tagged constructs for full-length, N-terminal truncated and LMW cyclin E isoforms were generated as previously described (Harwell et al., 2000). These included cyclin EL (EL1), T1 (EL2/EL3), and T2 (EL5/6) isoforms stably overexpressed in MCF-7 cells.

Validated HER2/neu siRNA (ID#540 and ID#42836) and Silencer negative control #1 siRNA were purchased from Ambion (Austin, TX).

siRNA Transfections

Transfections with HER2 siRNA were performed with $5 \times 10^4 - 1 \times 10^5$ cells/well in 6-well plates at a concentration of 100 nM. Briefly, 12.5 μ L of 50 μ M HER2 siRNA was added to 500 μ L of opti-MEM media (Gibco; Invitrogen, Carlsbad, CA) and 7.5 μ L of X-treme GENE transfection reagent (Roche Applied Science, Basel, Germany) was added to 500 μ L of opti-MEM. Mixtures were combined and 200 μ L added to wells. After 4 hours, media was added to ensure a final siRNA concentration of 100 nM.

Adenovirus transduction

Cyclin E adenoviruses were constructed using the AdEasy XL adenoviral vector system (Stratagene; La Jolla, CA). Tumor cells were infected at a multiplicity of infection selected to ensure > 70% transduction efficiency.

Treatment With Trastuzumab

Trastuzumab (Herceptin, Genentech, San Francisco, CA) reconstituted in normal saline (21 mg/mL) was diluted in media to concentrations of 10 and 20 μ g/mL. Twenty-four hours after plating cells, media were changed to low-serum and 24 hours later, cells were treated with trastuzumab for 24 to 72 hours prior to harvesting.

Confocal Immunofluorescence Microscopy

Cells were plated at 4×10^5 cells/well overnight before treatment. Cells were washed with PBS, permeabilized with .2% Triton X-100 for 20 minutes at 4 °C, blocked with 1% normal goat serum for 1 hour, and incubated with primary antibody overnight at 4 °C {(monoclonal HER2 antibody (Cell Signaling, Danvers, MA) and polyclonal cyclin E, p21, and p27 antibodies (Santa Cruz Biotechnology Inc, Santa Cruz, CA)}. Cells were incubated with secondary antibody: FITC-conjugated goat anti-mouse IgG for monoclonal antibodies, and rhodamine-conjugated goat anti-mouse IgG for polyclonal antibodies. Cells were washed, and TO-PRO-3-iodide added for nuclear staining. Cells were visualized using the confocal immunofluorescence microscope (Olympus FV 500 confocal microscope, Melville, NY) with a 40 \times oil immersion lens. The multi-line argon laser was used to stimulate green and red fluorescence, after which images were obtained by merging green and red channels.

Western Blot Analysis and Immunoprecipitation Kinase Assays

Cell lysates were prepared for western blot as previously described (Rao *et al.*, 1998). Primary antibodies were HER2 (Cell Signaling; Danvers, MA), cyclin E and cyclin D1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), p21 (Oncogene Research Products, Boston, MA), and p27 (Transduction Laboratories, Lexington, KY). Lysates were run on SDS-page gels then transferred to polyvinylidene fluoride membranes. Blots were washed and incubated with secondary antibodies then developed and signals detected using chemiluminescence detection reagents (Amersham Biosciences, Buckinghamshire, England).

Kinase assays using cyclin E polyclonal antibody were performed as previously described (Porter *et al.*, 2001). For quantitation of relative kinase activity, bands corresponding to histone H1 were analyzed on a phosphoimager Typhoon 9400 machine (Amersham Biosciences, Sunnyvale, CA).

Cell Proliferation Assays

Cell viability was determined by MTT assays (Sigma, St. Louis, MO). Cells were transfected with HER2 siRNA or treated with trastuzumab, then fixed with dimethyl sulfoxide and stained with MTT solution. Absorbance was read with a spectrophotometer (EL808 Ultramicroplate reader; Bio-Tek instruments, Inc., Winooski, VT) at 570 nm. Values were normalized and plotted as percentage change relative to control cells (mean \pm SEM). The MTT assay was modified for use as a high-throughput clonogenic assay (HTCA) to determine cell viability (Lambert *et al.*, 2008). Briefly, cells were treated with media, trastuzumab (10 or 20 $\mu\text{g}/\text{ml}$), roscovitine (5 to 15 $\mu\text{mol}/\text{L}$) (provided by Dr. Laurent Meijer, Centre national de la Recherche Scientifique, Roscoff, France), or a combination. Media was replaced every 48 hours and MTT solution added ten days after plating. Resultant crystals were solubilized and absorbance read at 590 nm. Drug interactions were assessed using CalcuSyn software (Biosoft, Inc.; Ferguson, MO).

RNA Extraction and cDNA Synthesis by Reverse Transcription

Total cellular RNA was extracted isolated using Qiagen RNeasy kits (Qiagen Inc., Valencia, CA). Briefly, cells were lysed then homogenized in guanidine-thiocyanate-containing buffer to inactivate RNases. Ethanol was added to provide appropriate binding conditions and samples were applied to an RNeasy spin column. RNA was eluted and quantified. cDNA was synthesized from 1 μg of total RNA using the Roche Transcriptor First Strand cDNA Synthesis kit (Roche Applied Science, Indianapolis, IN). All reverse transcriptase reactions were carried out with anchored oligo(dT)18 primers to target transcription of polyadenylated mRNA and generate full-length cDNAs.

Quantitative Real-Time Polymerase Chain Reaction

RT-PCR reactions were performed on a Rotor-Gene 2000 Real-Time cyler (Corbett Research, Sydney, Australia). The primer sequences for cyclin E (forward primer 5'-TTCTTGAGCAACACCCTCTTCTGCAGCC-3', reverse primer 5'-TCGCCATATACCGGTCAAAGAAATCTTGTGCC-3') yielded a 138-bp product. The primer sequences for β -actin, an endogenous control, (forward primer 5'-TCACCCACACTGTGCCCCATCTACGA-3', reverse primer 5'-TGAGGTAGTCAGTCAGGTCCCG-3') yielded a 155-bp product (obtained from Integrated DNA Technologies, Inc., Coralville, IA). PCR products were detected using SYBR Green Jumpstart Taq Ready Mix (Sigma, St. Louis, MO). Reactions were performed in triplicate and data analyzed using Rotor-Gene Analysis software, version 5.0 (Corbett Research).

Animal Studies

Nude mice obtained from Charles River Laboratories (Wilmington, MA) were injected with 0.5-mg estrogen pellets. 5×10^6 MCF-7-HER-18 cells were injected into the mammary fat pad. When tumors reached 100 mm³, mice were divided into groups receiving intraperitoneal treatments twice weekly for 3 weeks: group 1 110 µg trastuzumab; group 2 PBS. Animals were cared for and euthanized according to institutional guidelines.

Tumors were divided and processed for western blot or immunohistochemistry (IHC). For IHC, rabbit polyclonal antibodies to cyclin E (Santa Cruz Biotechnology) and p-HER2/ ErbB2 (Cell Signaling) were diluted 1:500 and 1:300 respectively in 1% goat serum.

Study Patients

Clinical data from 395 breast cancer patients were previously reported (Keyomarsi *et al.*, 2002). Full-length and LMW cyclin E were evaluated by western blot analysis of tumor tissue lysates. Protein levels were measured by densitometry and LMW and total (full-length plus LMW) cyclin E scored as low (the level of protein found in normal breast epithelium) or high (>normal-cell controls).

Statistical Analysis

DSS was calculated from date of surgery to date of death or last follow-up. Patients dying from causes other than breast cancer were censored at time of death. DSS survival curves were computed by the Kaplan-Meier method. Univariate analyses of DSS survival according to levels of HER2, total and LMW cyclin E were performed with a two-sided log-rank test. Continuous data obtained from experiments analyzing cell cycle profiles and qRT-PCR reactions was compared using a Student t-test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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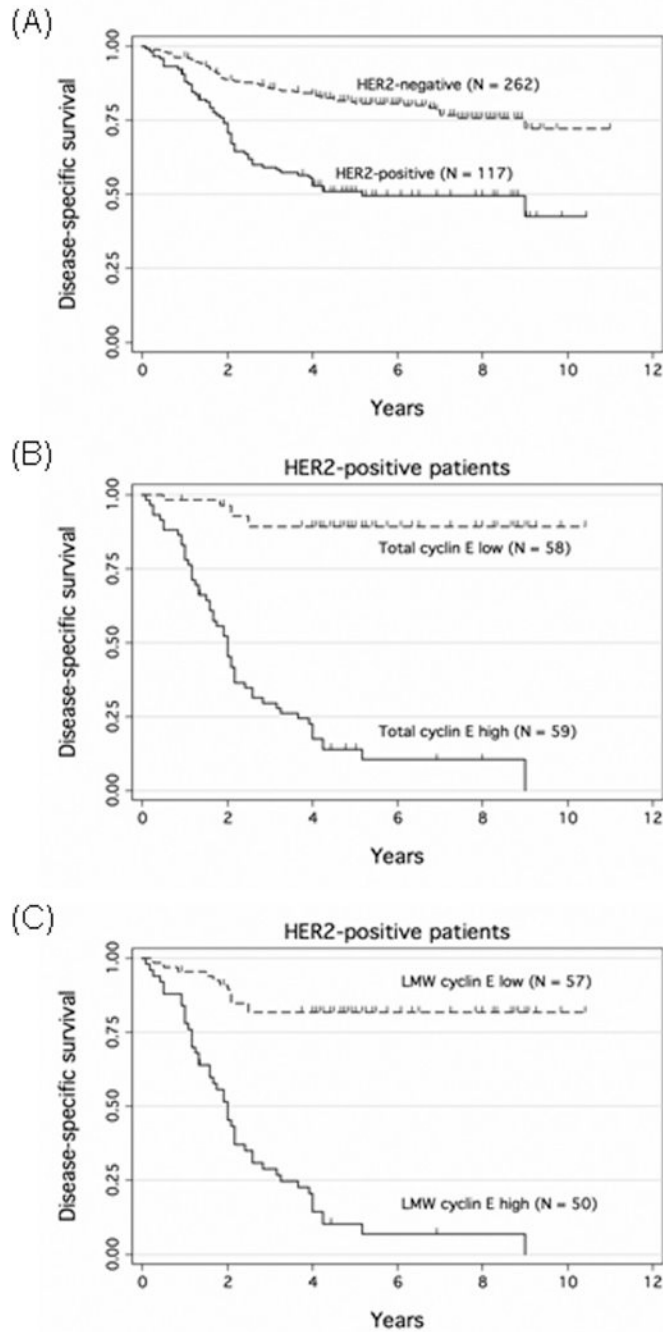


Figure 1.

Relationship between HER2 and cyclin E in breast cancer. Tumors from 395 patients (Keyomarsi et al., 2002) were assessed for HER2 and cyclin E levels by western blot. **A)** Disease-specific survival (DSS) was higher in patients with HER2-negative (n = 262) tumors ($P < .0001$). **B)** HER2-overexpressing tumors were stratified by total cyclin E expression. Patients with high total cyclin E had markedly decreased DSS (median DSS, 2 years) ($P < .0001$). **C)** Stratification by LMW cyclin E demonstrates decreased median DSS

in patients with high LMW cyclin E (median DSS, 2 years) ($P < .0001$). (Total cyclin E = full-length + LMW cyclin E).

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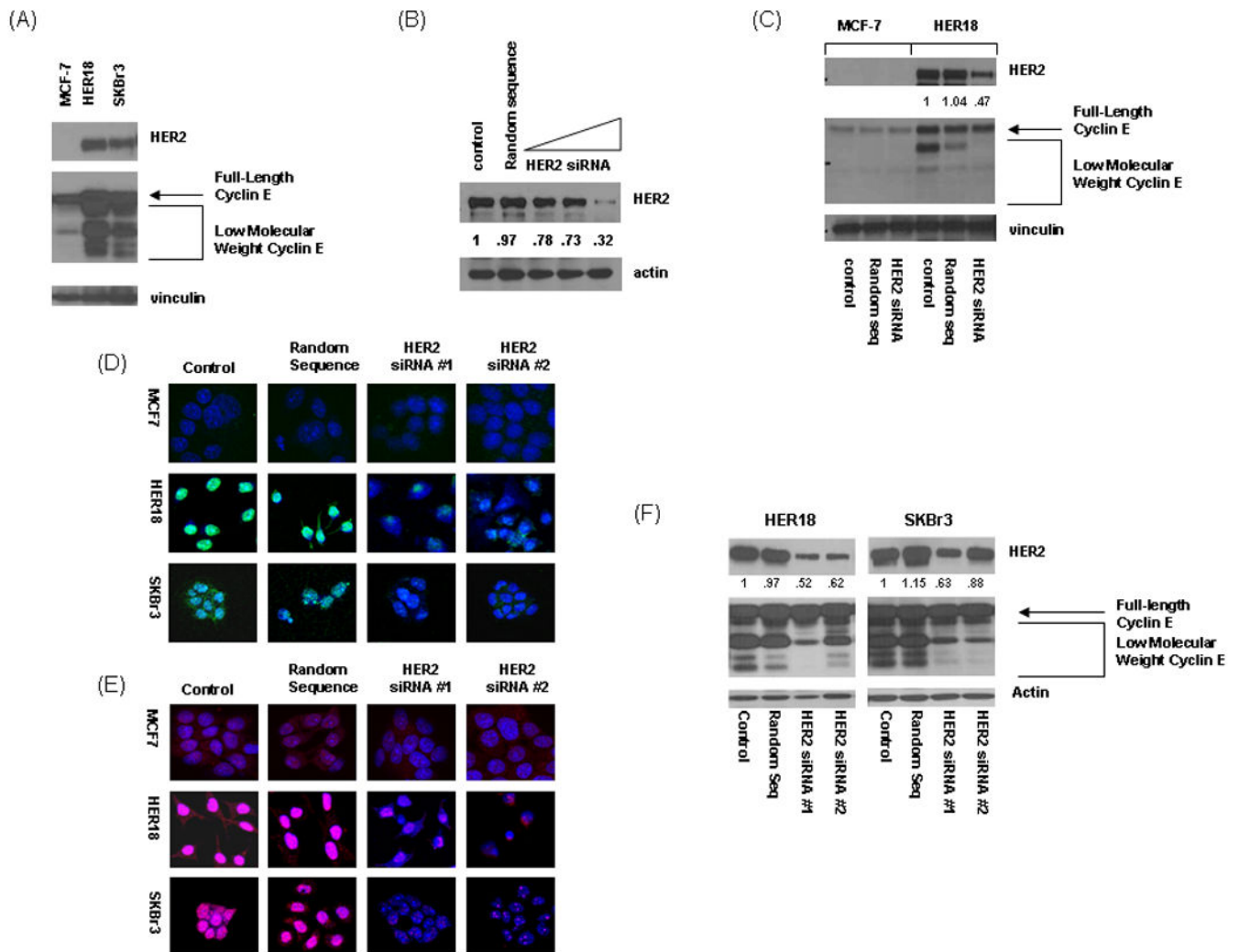
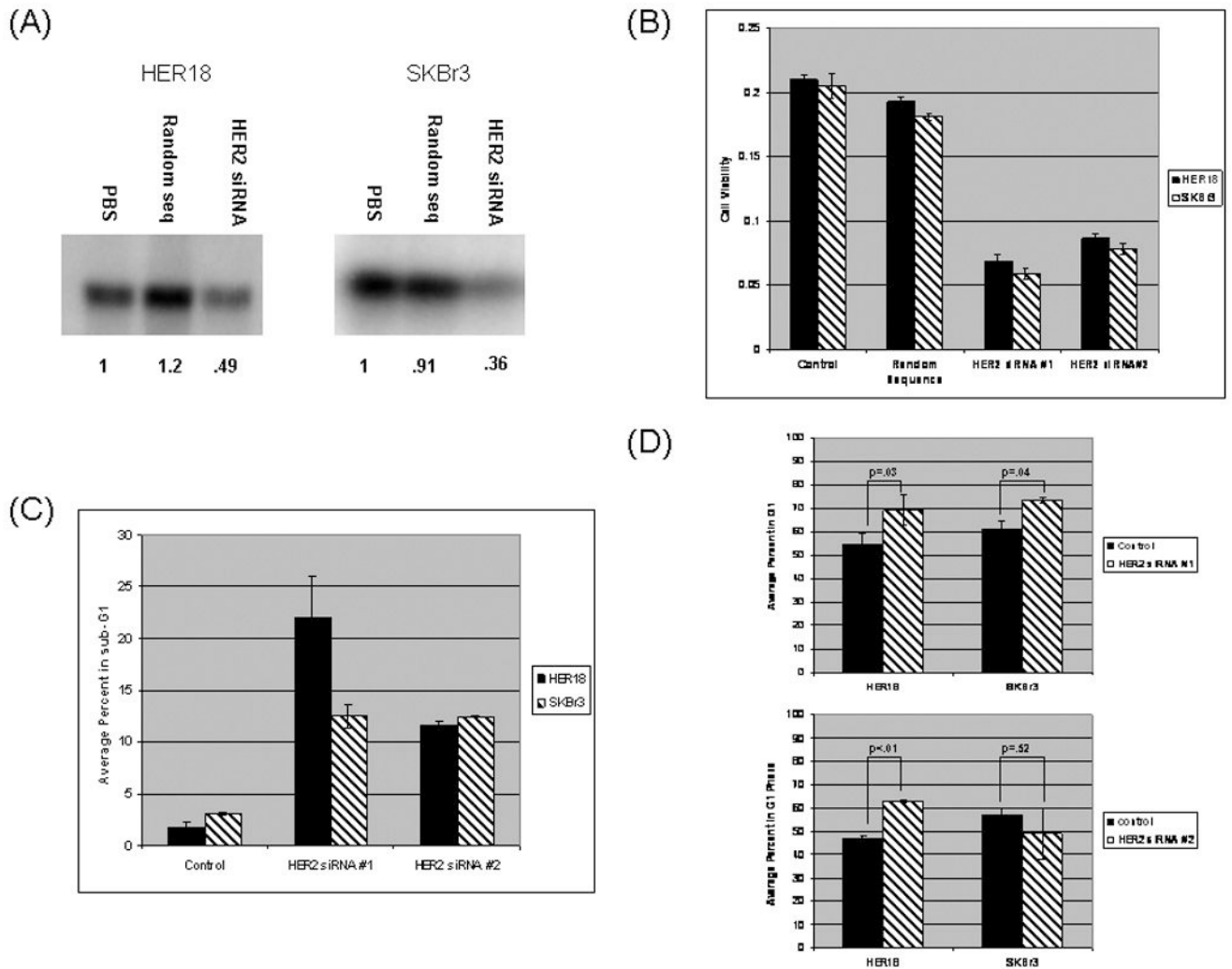


Figure 2.

Effect of HER2 downregulation on cyclin E expression. **A)** HER2-overexpressing MCF-7-HER-18 and SKBr3 breast cancer cells were grown exponentially (MCF-7 cells were used as a HER2 negative control). Lysates from these cells were used to perform western blot analysis probing for HER2 and cyclin E. Western blots were performed using a 7% (HER2) or 10% (cyclin E) SDS-PAGE gel. For a given experiment, gels for HER2 and cyclin E were run using lysates that had been prepared as a single sample that was then aliquoted to the two gels that were run concurrently. **B)** HER2-overexpressing MCF-7-HER-18 cells were transfected with increasing concentrations of HER2 siRNA. Cells were harvested 48 hours after transfection and western blots performed on lysates obtained from these cells demonstrated decreased HER2 expression which was quantitated using densitometry. Mock transfected (control) and cells transfected with a random sequence, negative silencer siRNA were used as controls. **C)** After optimization of HER2 siRNA transfection conditions, HER2-overexpressing MCF-7-HER-18 cells were again transfected with HER2 siRNA. Lysates harvested from these cells were used to perform western blot analysis probing for HER2 and cyclin E. Western blot demonstrated a decrease in HER2 expression with a

concomitant decrease in cyclin E expression, primarily a decrease in the LMW forms. MCF-7 was used as a HER2-negative control. **D)** Confocal immunofluorescence microscopy demonstrated knockdown of HER2 (green) in cells transfected with HER2 siRNA, compared to mock transfected or random sequence siRNA transfected controls. (TO-PRO-3-iodide (blue) used for nuclear staining). **E)** Confocal immunofluorescence microscopy using C-19 antibody against cyclin E (red) showed decreased total cyclin E expression in HER2-overexpressing cells that had decreased HER2 expression after transfection with HER2 siRNA. **F)** Western blot confirmed HER2 knockdown after transfection with two different HER2 siRNA in MCF-7-HER18 and SKBr3 breast cancer cell lines. Densitometry was performed to quantitate HER2 expression. Western blot with HE-12 antibody against cyclin E revealed decreased LMW cyclin E in HER2 siRNA transfected cells compared to controls confirming the differential effect on full-length versus LMW cyclin E.

**Figure 3.**

Effect of HER2 downregulation on cyclin E-associated kinase activity and cell cycle profiles. **A)** HER2-overexpressing cells were transfected with HER2 siRNA, random sequence siRNA or mock transfected. Protein lysates (250 μ g) were immunoprecipitated with anti-cyclin E antibody and protein G-sepharose beads using Histone H1 as a substrate. Bands corresponding to Histone H1 phosphorylation were quantitated through phosphoimaging. Cells transfected with HER2 siRNA had decreased cyclin E-associated kinase activity compared to controls. This was more pronounced in SKBr3 cells which have lower levels of LMW cyclin E than MCF-7-HER-18 cells. **B)** Cell viability was assessed with MTT assay. Cells were transfected with HER2 siRNA, random sequence siRNA or mock transfected. Following transfection with HER2 siRNA the number of tumor cells was decreased compared to control cells, suggesting that HER2 knockdown resulted in decreased proliferation. Error bars represent standard deviation. **C)** Propidium iodide staining was performed to determine the percentage of cells undergoing apoptosis following transfection with HER2 siRNA. Experiments were repeated in triplicate, and mean percentage of cells in the sub-G1 phase for mock transfected controls versus siRNA-transfected MCF-7-HER-18

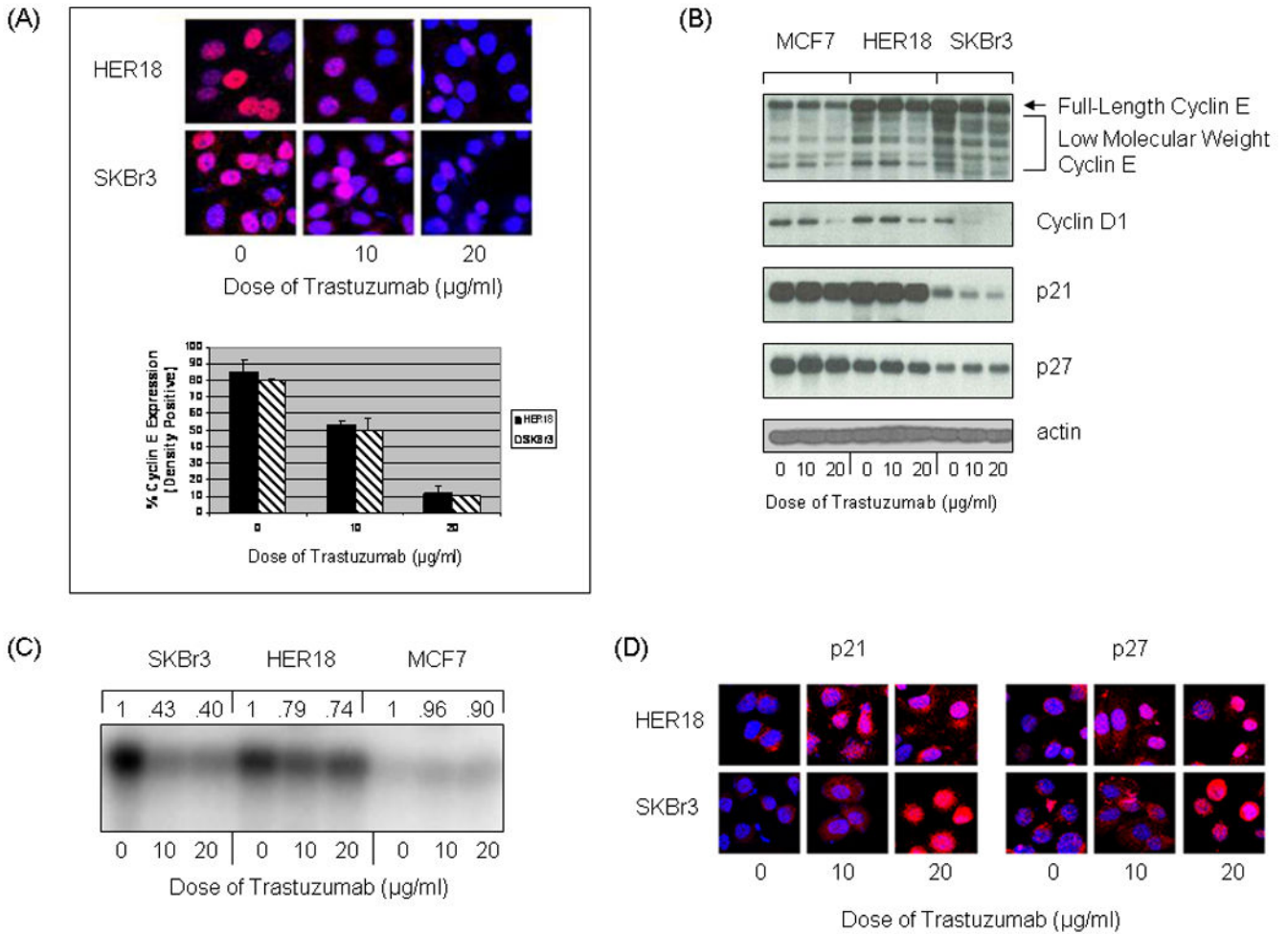
and SKBr3 cells is shown. Error bars represent the standard error of the mean. There was a significant increase in the percentage of cells in sub-G1 phase after transfection with HER2 siRNA consistent with HER2 knockdown causing an increase in apoptosis. D) . Cell cycle profiles were determined using FACS analysis. Experiments were repeated in triplicate, and the mean percentage of cells in the G1 phase for mock transfected controls versus siRNA-transfected MCF-7-HER-18 and SKBr3 cells is shown. Error bars represent the standard error of the mean. There was a significant increase in the percentage of MCF-7-HER-18 cells in G1 phase after transfection with both HER2 siRNA sequences. For SKBr3 cells, there was a significant increase in the percentage of cells in G1 phase after transfection with HER2 siRNA #1.

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**Figure 4.**

Effect of decreased HER2-mediated cell signaling after trastuzumab treatment. **A)** HER2-overexpressing cells were treated with trastuzumab for 48 hours. Confocal immunofluorescence microscopy revealed decreased total cyclin E (red) expression as quantified by Image-Pro Plus Software (TO-PRO-3-iodide (blue) used for nuclear staining). Error bars represent the standard deviation. **B)** Western blot performed following trastuzumab treatment showed decreased LMW cyclin E and cyclin D1 expression in trastuzumab-treated cells. There was no difference in p21 or p27 expression. Western blots were performed using separate 10% SDS-PAGE gels for each antibody. All gels were run concurrently using lysates that were prepared as a single sample and aliquoted to the individual gels. **C)** Cyclin E-associated kinase activity was decreased in trastuzumab-treated cells compared to controls. This was more pronounced in SKBr3 cells (MCF-7 used as HER2 negative control). **D)** Following trastuzumab treatment for 48 hours, confocal immunofluorescence microscopy revealed increased nuclear localization of both p21 (red, left) and p27 (red, right) (TO-PRO-3-iodide (blue) used for nuclear staining).

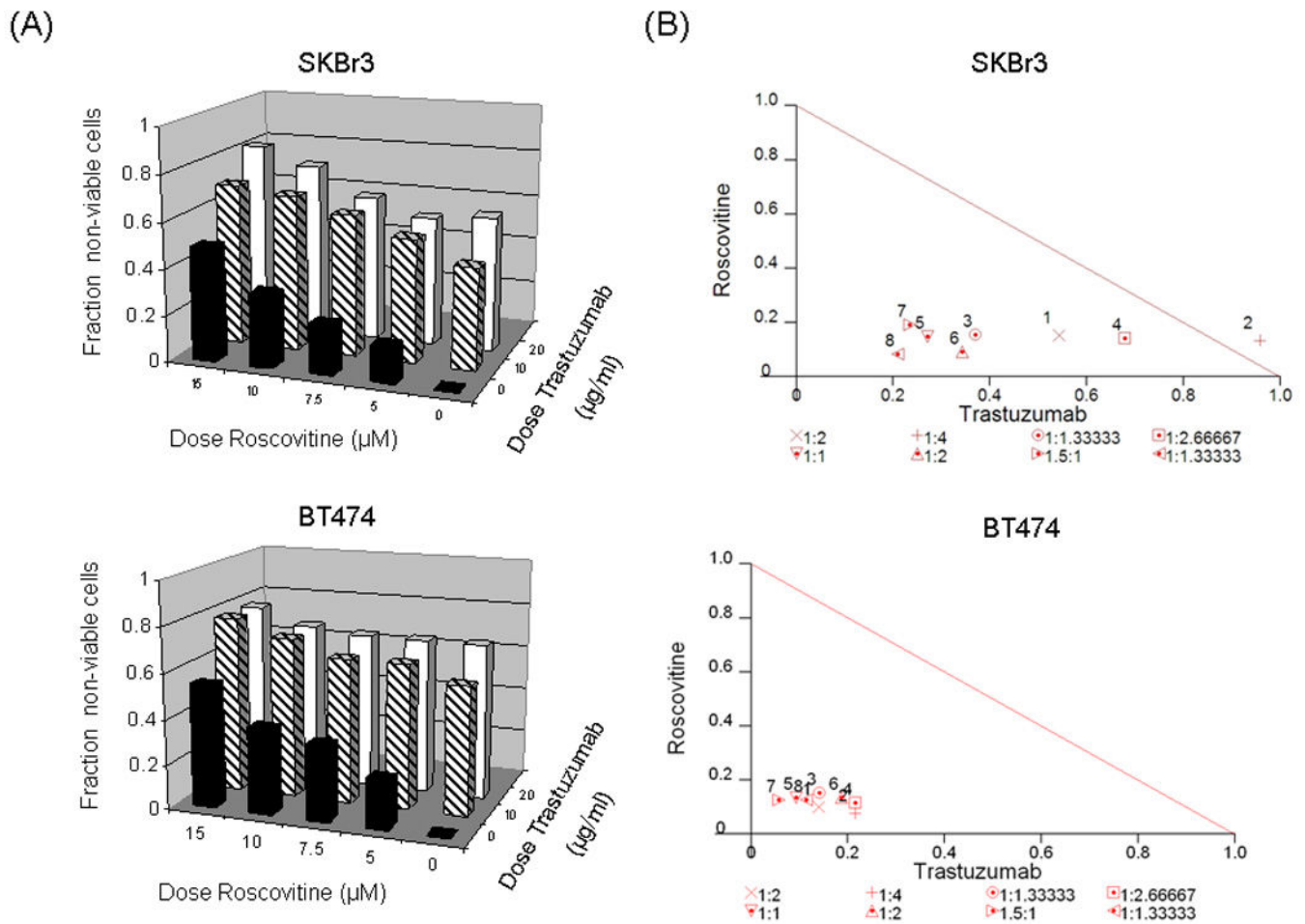


Figure 5. Synergistic effect of trastuzumab and roscovitine in breast cancer cell lines overexpressing HER2 and cyclin E. **A)** High throughput clonogenic assays were used to compare the cytotoxic effects of trastuzumab alone, roscovitine alone, and the combination in SKBr3 and BT474 breast cancer cells (X-axis: roscovitine μM ; Y-axis: trastuzumab $\mu\text{g/ml}$; Z-axis: fraction non-viable cells). **B)** Isobologram analysis showed a synergistic interaction between the two agents. Isobologram analysis and graphs were obtained using *CalcuSyn* software, which performs drug dose-effect calculation using the median effect method. Experiments were performed in triplicate and representative data are shown.

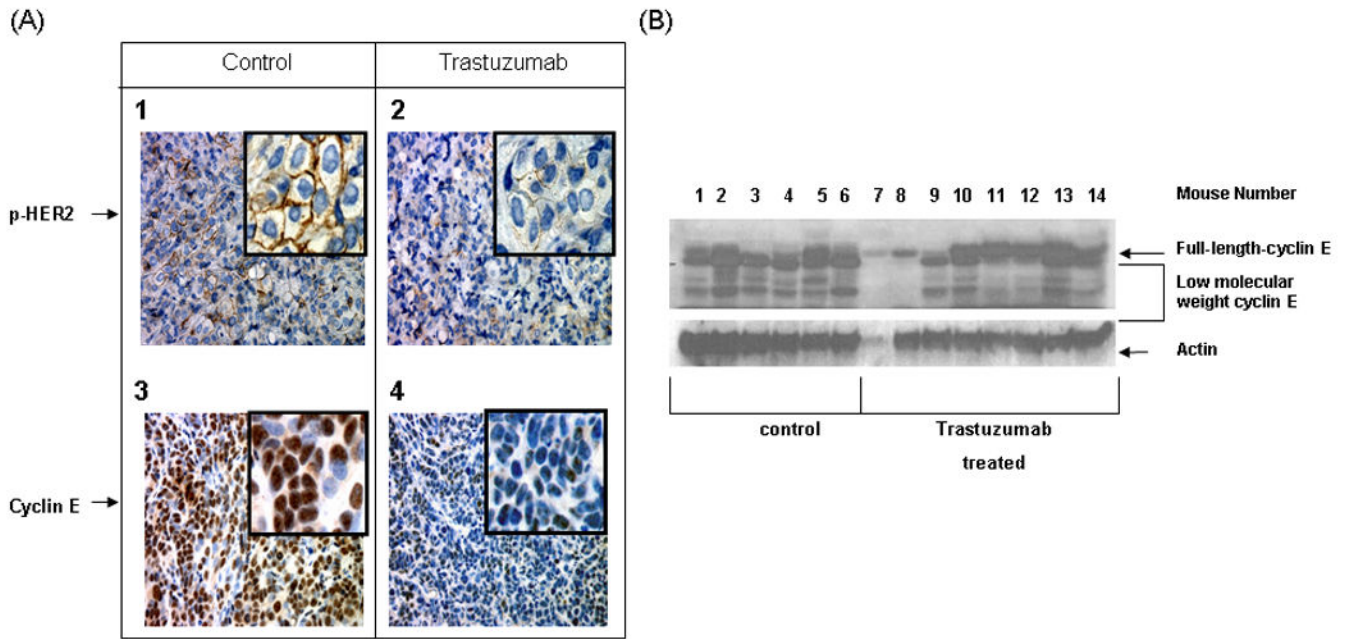


Figure 6.

In vivo effects of trastuzumab therapy. MCF-7-HER-18 cells were injected into the mammary fat pads of nude mice. When tumors reached 100 mm³, intraperitoneal injections of trastuzumab or PBS were given twice weekly for 3 weeks. **A)** Tumors from trastuzumab-treated mice showed decreased p-HER2 staining (**2** versus **1**) and a concomitant decrease in cyclin E expression (**4** versus **3**). **B)** Lysates were obtained from tumors from 13 mice (6 treated with PBS and 7 treated with trastuzumab). Lane 7 contains lysate that was likely subcutaneous tissue, not tumor, as evidenced by the low actin expression. For the remaining tumors, western blot for cyclin E expression revealed that those obtained from trastuzumab-treated mice demonstrated decreased expression of cyclin E, particularly the LMW forms when compared to PBS-treated control mice.