# Targeting ligand-activated ErbB2 signaling inhibits breast and prostate tumor growth

David B. Agus,[1] Robert W. Akita,[2] William D. Fox,[1] Gail D. Lewis,[2] Brian Higgins,[3,5] Paul I. Pisacane,[2] Julie A. Lofgren,[2,6] Charles Tindell,[1] Douglas P. Evans,[1] Krista Maiese,[1] Howard I. Scher,[3] and Mark X. Sliwkowski[2,4]

[1]Cedars-Sinai Prostate Cancer Center, Los Angeles, California 90048
[2]Department of Molecular Oncology, Genentech, South San Francisco, California 94080
[3]Genitourinary Oncology Service, Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, New York 10021
[4]Correspondence: marks@gene.com
[5]Present address: Oncology In Vivo Section, Hoffman La-Roche, Inc., 340 Kingsland Street, Building 123/2314, Nutley, New Jersey 07110.
[6]Present address: Amgen, Inc., Department of Cancer Biology, 1 Amgen Center Drive, Thousand Oaks, California 91320.

## Summary

**ErbB2 is a ligand-less member of the ErbB receptor family that functions as a coreceptor with EGFR, ErbB3, and ErbB4. Here, we describe an approach to target ErbB2's role as a coreceptor using a monoclonal antibody, 2C4, which sterically hinders ErbB2's recruitment into ErbB ligand complexes. Inhibition of ligand-dependent ErbB2 signaling by 2C4 occurs in both low- and high-ErbB2-expressing systems. Since the ErbB3 receptor contains an inactive tyrosine kinase domain, 2C4 is very effective in blocking heregulin-mediated ErbB3-ErbB2 signaling. We demonstrate that the in vitro and in vivo growth of several breast and prostate tumor models is inhibited by 2C4 treatment.**

## Introduction

The ErbB or HER signaling network is a receptor-ligand system that is composed of four receptors and at least eleven ligands (Yarden and Sliwkowski, 2001). In general, receptor expression occurs in the epithelium, whereas ligands are produced in the mesenchyme. Since these ligands are synthesized as membrane bound proforms, ligand processing and release are tightly regulated processes. In any particular tissue the receptors are rarely, if ever, expressed alone, and as a result ErbB2 is frequently activated due to its role as a common coreceptor. An interesting feature of the ErbB system is that it is frequently coopted by various hyperproliferative diseases including cancer (Shawver et al., 2002), psoriasis (Ben-Bassat and Klein, 2000), and vascular restenosis (Kalmes et al., 2000). As a result, two of these receptors, EGFR and ErbB2, are targets for drug development particularly in a number of solid tumors (Arteaga, 2001). One example is trastuzumab (Herceptin), a humanized variant of the 4D5 monoclonal antibody directed against ErbB2, which is overexpressed in a subset of human breast cancers (Cobleigh et al., 1999; Slamon et al., 2001). In these cases, ErbB2 overexpression is the outcome of a specific molecular alteration, i.e., ErbB2 gene amplification that results in excess protein synthesis (Slamon et al., 1987). The accumulation of excess ErbB2 at the plasma membrane results in constitutive receptor activation due to receptor self-association. Constitutive ErbB2 signaling is thought to drive tumor growth. The availability of ErbB2 at the tumor cell surface allows the receptor to be targeted by trastuzumab. Although a number of explanations have been put forward to help explain trastuzumab's clinical benefit, the precise mechanism of action in any particular patient is still not completely defined. At present two primary mechanisms are thought to account for trastuzumab's clinical benefit (Sliwkowski et al., 1999). The first is strictly biological and requires engagement of ErbB2 receptors with the bivalent antibody, which, in turn, results in receptor downmodulation and inhibition of aberrant receptor tyrosine kinase signaling. A second mechanism of action is thought to occur from the interaction of trastuzumab's human Fc region with immune effector cells (Clynes et al., 2000). Regardless of whether either or both of these mechanisms are needed for a meaningful clinical outcome, treatment response is strictly dependent on high ErbB2 expression in the tumor. An

### SIGNIFICANCE

**The ErbB or HER receptor network is frequently dysregulated in a number of solid tumors. Breast cancers containing ErbB2 overexpression can be treated with the ErbB2 antibody, trastuzumab (Herceptin). However, trastuzumab is only effective against high-ErbB2-expressing tumors. In this report, we target ErbB2's role as a coreceptor with other ErbB family members using another monoclonal antibody, 2C4. In contrast to trastuzumab, 2C4 disrupts ErbB2's association with other ErbB receptors and inhibits ligand-stimulated signaling in tumor cells that express lower ErbB2 levels. Potential therapeutic benefit of 2C4 treatment does not require the antibody to contain an intact Fc region and is observed in human breast and prostate cancer models, which do not overexpress ErbB2. Humanization of 2C4 will allow for assessment of its potential clinical utility.**

alternative immunotherapy approach is taken with the closely related EGFR. Here, antibodies are selected to inhibit ligand binding and subsequent downstream receptor signaling (Mendelsohn and Baselga, 2000).

Disruption of EGFR signaling can also be accomplished with compounds that inhibit the intrinsic tyrosine kinase activity of the receptor (Woodburn, 1999). In particular, quinazolines, such as ZD-1839 or CPI-358,774, which compete with one of the kinase substrates, ATP, appear to be promising clinically because they exhibit the requisite specificity for EGFR, are orally bioavailable, and have been generally well-tolerated to date in clinical trials (Baselga and Averbuch, 2000; Hidalgo et al., 2001; Woodburn, 1999).

A number of studies suggest that inappropriate ligand-dependent ErbB2 activation may play a major role in driving tumor growth or survival pathways (Tzahar and Yarden, 1998). To further explore these observations, we utilize an antibody, 2C4, that targets ErbB2 and disrupts ligand activation. The primary focus of this investigation was on breast and prostate cancer lines that do not overtly overexpress ErbB2 and therefore would not be targets for therapeutic agents such as trastuzumab. We demonstrate that 2C4 is effective in suppressing the growth of a number of xenografted tumors that do not overexpress ErbB2.

## Results

### 2C4 blocks the association of ErbB2 with ErbB3

Since ErbB3 lacks intrinsic tyrosine kinase activity, it is incapable of initiating productive signaling without interacting with another receptor tyrosine kinase. In most cases, this receptor is ErbB2 (Graus-Porta et al., 1997; Klapper et al., 1999). We studied whether ErbB3-ErbB2 complexes exist on the plasma membrane in the absence of the ErbB3 ligand, HRG. To answer this question, cell lines expressing ErbB3 and ErbB2 were treated with HRG or control buffer. Cells were then lysed and immunoprecipitations were performed with an anti-ErbB2 antibody that recognizes the intracellular domain of ErbB2. The immunoprecipitates were then subjected to SDS-PAGE and transferred to a nitrocellulose membrane for Western blotting using an anti-ErbB3 monoclonal antibody. The cell lines used for these experiments were MCF7, which expresses low or normal levels of ErbB2, and SK-BR-3, which overexpresses ErbB2. As shown Figure 1A, ErbB3 is found in ErbB2 immunoprecipitates only when MCF7 cells are treated with the ErbB3 ligand HRG. A similar result is obtained when immunoprecipitates are prepared from the high-ErbB2-expressing cell line SK-BR3 (Figure 1A). These data suggest that the recruitment of ErbB2 to ErbB3 occurs in a HRG-dependent manner. Moreover, even in cell lines that overexpress ErbB2, such as SK-BR3, little or no preformed complexes can be detected. Thus, the formation of the ErbB3-HRG-ErbB2 complex occurs sequentially, i.e., ErbB3 binds HRG and then, in a second reaction, ErbB2 is added to the complex. Alternatively, ErbB2 and ErbB3 may form a transient complex in the absence of HRG but the association may be too weak to allow detection of the receptor complex. HRG binding may serve to stabilize the ErbB2-ErbB3 complex once it has formed.

We then studied whether antibodies to the extracellular domain of ErbB2 were capable of disrupting the formation of ErbB3-HRG-ErbB2 complexes. Two antibodies to ErbB2 were used in these studies, trastuzumab, which has been shown to

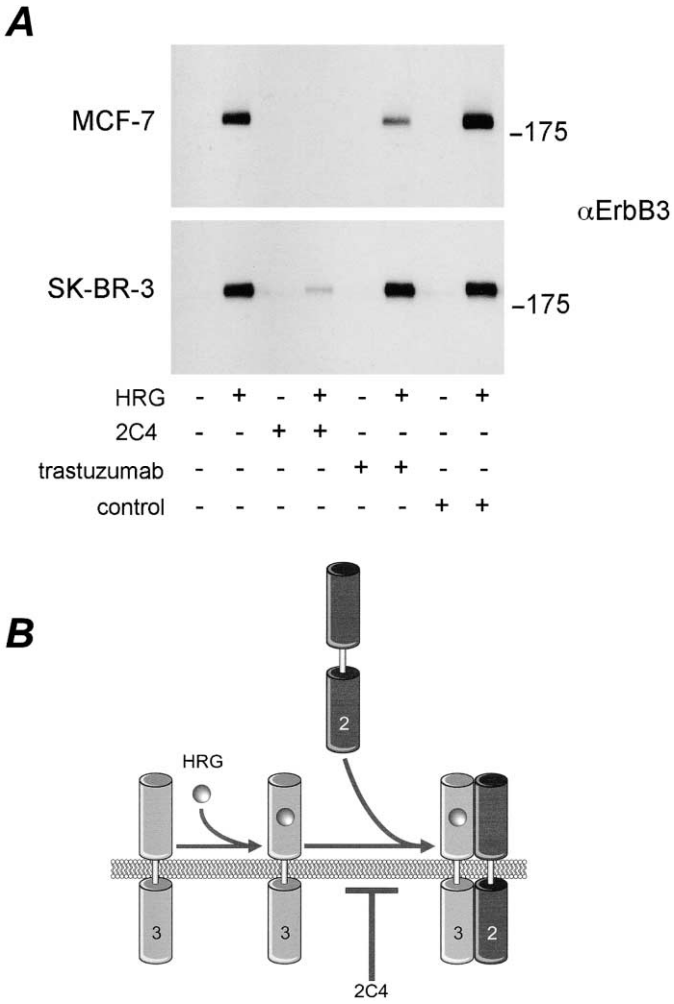


**Figure 1.** 2C4, but not trastuzumab, blocks the association of ErbB2 with ErbB3 in breast cancer cell lines that express normal/low and high ErbB2 levels

**A:** MCF7 (top), which expresses ErbB3 and low or normal levels of ErbB2, and SK-BR-3 (bottom), which expresses ErbB3 and overexpresses ErbB2 at high levels, were treated with HRG or control buffer. ErbB3 was found in ErbB2 immunoprecipitates only when MCF7 cells are treated with the ErbB3 ligand HRG. A similar result is obtained when immunoprecipitates are prepared from the high ErbB2-expressing cell line SK-BR-3. **B:** A model for the proposed mechanism of 2C4 activity.

be therapeutically active in the treatment of metastatic breast cancer patients whose tumors overexpress ErbB2, and 2C4, which has been shown to ablate HRG-mediated signaling (Lewis et al., 1996; Sliwkowski et al., 1994). Crossblocking studies, as well as epitope-mapping studies, have shown that trastuzumab and 2C4 bind to distinct epitopes on the extracellular domain of the ErbB2 receptor (Fendly et al., 1990). More recently, it was demonstrated that trastuzumab inhibits ErbB2 ectodomain cleavage by matrix metalloproteases, whereas 2C4 does not (Molina et al., 2001). As shown in Figure 1A, 2C4 is far more effective in disrupting ligand-mediated ErbB3-ErbB2 complex formation than trastuzumab or a control antibody. It is noteworthy that the 2C4-mediated disruption of this receptor complex is independent of ErbB2 expression levels, in that it is effective in blocking receptor complex formation in both low- and high-

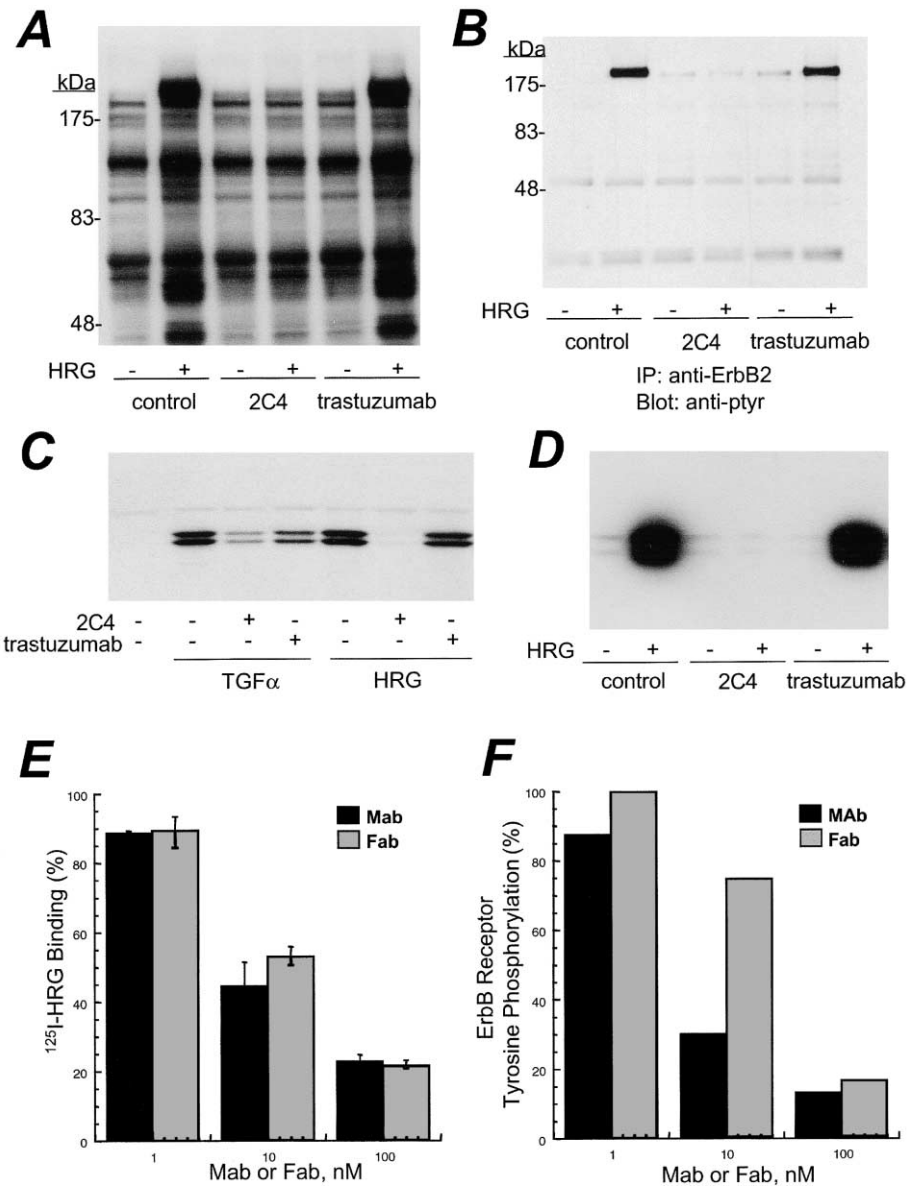


**Figure 2.** 2C4 inhibits ligand activation of ErbB2 in the breast cancer cell line MCF7
**A:** The monoclonal antibody 2C4, but not trastuzumab or control antibody, blocks HRG-induced tyrosine phosphorylation in whole cell lysates. **B:** Whole cell lysates were immunoprecipitated as described in Figure 1 and then probed for anti-phosphotyrosine. **C:** HRG- or TGFα-induced MAPK activation are inhibited by 2C4 but not by trastuzumab. **D:** HRG-dependent Akt activation is inhibited by 2C4 and not trastuzumab. **E:** Inhibition of $^{125}$I-HRG binding by 2C4 or 2C4-Fab. **F:** Inhibition of ErbB3-ErbB2 tyrosine phosphorylation by 2C4 or 2C4-Fab. The phosphorylation signal at 180 kDa was quantified by densitometry as described previously (Lewis et al., 1996).

ErbB2-expressing cell lines. Based on the data presented, a model for the disruption of ligand-dependent ErbB2 signaling by 2C4 is proposed in Figure 1B.

### 2C4 diminishes ligand-activated ErbB2 signaling in breast cancer cell lines

Since ErbB3 requires the association with ErbB2 to initiate signal transduction, we determined the effect of anti-ErbB2 monoclonal antibodies on receptor activation. The MCF7 breast cancer cell line was treated with HRG in the presence or absence of anti-ErbB2 monoclonal antibodies. As shown in Figure 2A, a number of tyrosine phosphorylated proteins are detected in whole-cell lysates of HRG-treated MCF7 cells. The prominent band at ~180 kDa is known to be a mixture of ErbB3 and ErbB2 (Sadick et al., 1996; Sliwkowski et al., 1994). The phosphorylation of these bands is blocked by 2C4 treatment. In contrast, little if any decrease was observed in cells treated with trastuzumab. To verify the identity of ErbB2, MCF7 cell lysates were immunoprecipitated with antibodies directed to a cytoplasmic epitope of ErbB2, and immunoblots were probed for phosphotyrosine (Figure 2B). Consistent with the results shown in Figure 2A, 2C4 treatment blocks the appearance of a HRG-dependent phosphorylation signal of ErbB2, whereas trastuzumab does not.

A downstream target of ErbB2 activation is MAPK (Erk 1 and Erk 2). To assess the effect of anti-ErbB2 MAbs on MAPK activation, MCF7 cells were treated, as described above, and MAPK activation was measured using a phospho-specific MAPK antibody (Figure 2C). Cells treated with HRG or TGFα show strong activation of MAPK. This activation is diminished with 2C4 treatment and to a much lesser degree with trastuzumab. These data suggest that ErbB2 association with either ErbB3 or EGFR is critical for complete activation of signal transduction to MAPK. This conclusion is in accordance with previous

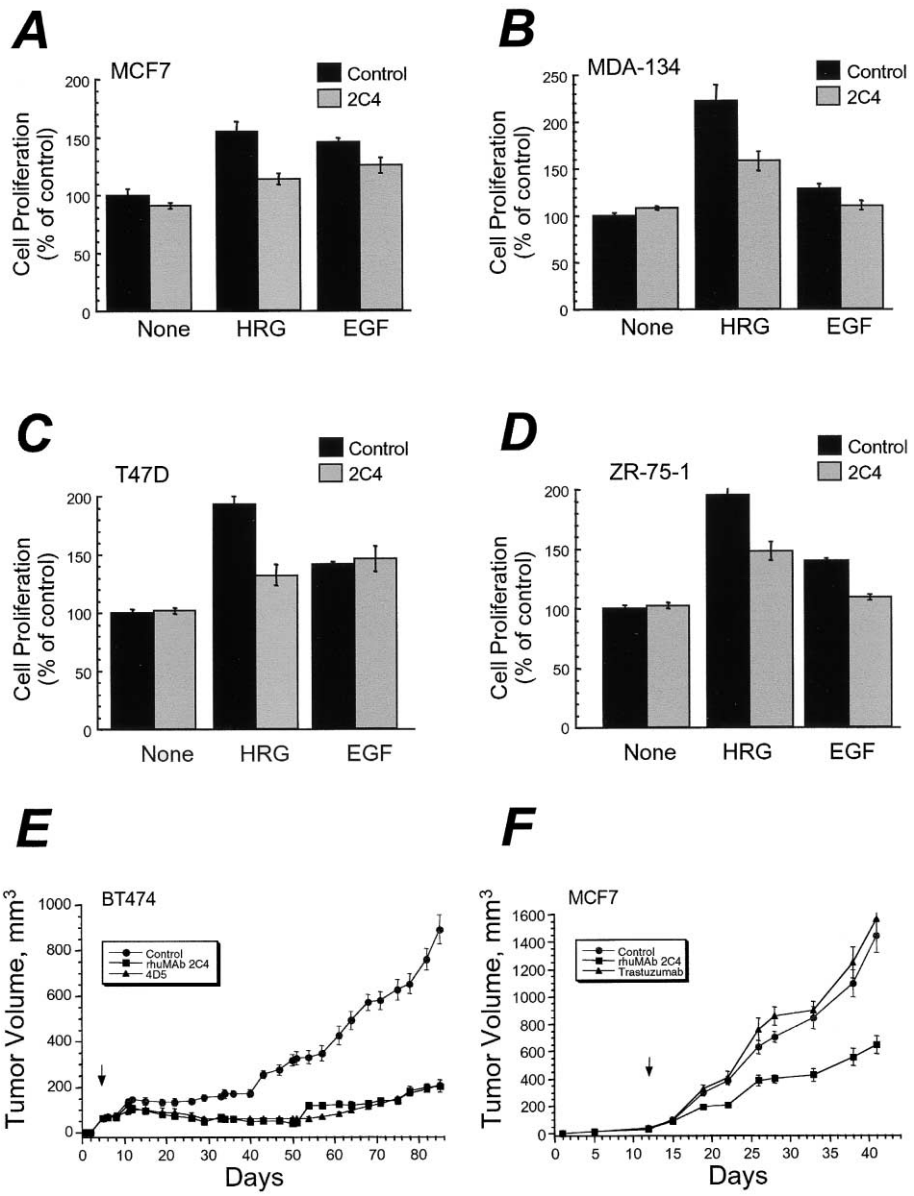


**Figure 3.** Effect of 2C4 on breast tumor cell growth in vitro and in vivo

**A–D:** The response of (**A**) MCF7, (**B**) MDA-134, (**C**) T47D, and (**D**) ZR-75-1 monolayer cell culture growth to the addition of 2C4 with and without the ligands HRG and EGF. Results are shown as percentage of control cell proliferation. **E** and **F:** The response of (**E**) BT474 and (**F**) MCF7 breast cancer xenograft tumors to 2C4 (■), trastuzumab (▲), and control (●) are shown. Arrow indicates the initiation of therapy. Results are given as mean tumor volume ± SE.

reports demonstrating that ErbB2 is a potent activator of the Ras/Raf/MAPK pathway (Marte et al., 1995; Pinkas-Kramarski et al., 1996a).

Due to the presence of six YXXM motifs, ErbB3 is a very potent activator of the PI3 kinase cascade (Soltoff et al., 1994). To address the effect of ErbB2 MAbs on PI3 kinase activation, we measured the activity of the downstream kinase Akt using a synthetic fusion protein derived from GSK3$\alpha/\beta$. As seen in Figure 2D, treatment of MCF7 cells with the ErbB3 ligand, HRG, results in activation of Akt that is inhibited by the pretreatment with 2C4, but not by pretreatment with trastuzumab.

To determine whether 2C4 must be bivalent in order to prevent ErbB2's recruitment into HRG-ErbB3 complexes, we assessed the properties of a monovalent Fab version of 2C4. A characteristic of an ErbB3-ErbB2 complex is the formation of a high-affinity HRG binding site (Fitzpatrick et al., 1998; Sliwkowski et al., 1994). As shown in Figure 2E, we examined the effect of intact 2C4 or a Fab version of 2C4 on $^{125}$I-HRG binding to MCF7 cells (Lewis et al., 1996). Both versions of 2C4 are effective in blocking high-affinity $^{125}$I-HRG binding. To further characterize the requirement for bivalency, we measured the ErbB2-ErbB3 tyrosine phosphorylation levels in the presence or absence of 2C4 or the 2C4-Fab. As shown in Figure 2F, both 2C4 and the 2C4-Fab block the HRG-stimulated phosphorylation of the ErbB2-ErbB3 complex. In this assay format, the 2C4-Fab is less potent than the intact antibody. A possible explanation for this effect may be the increased avidity expected for the intact, bivalent antibody in comparison to the monovalent Fab fragment.

### 2C4 inhibits the growth of human breast cancer cell lines only in the presence of ligand

The ability of 2C4 to inhibit the in vitro proliferation of low-ErbB2-expressing breast tumor cells was studied. The expression of

ErbB2 and other ErbB receptors in these breast cancer cell lines was reported by deFazio et al. (2000). As demonstrated in Figure 3, 2C4 does not directly inhibit growth of MCF7 (Figure 3A), MDA-MB-134 (Figure 3B), T-47D (Figure 3C), or ZR-75-1 (Figure 3D) cells compared to untreated cells. However, when the cells are treated with the ErbB ligand HRG, there is significant growth stimulation (between 155% and 230% of control growth), which is inhibited in all cases by treatment with 2C4. Exposure of the cells to EGF resulted in modest growth induction, which was also inhibited by 2C4 in the MCF7, MDA-MB-134, and ZR-75-1 breast tumor cells.

**2C4 inhibits the growth of low- and high-ErbB2-expressing human breast cancer xenograft tumors**

To further examine the effect of 2C4 treatment on tumor cell growth, animals with established BT474 (high-ErbB2) or MCF7 (low-ErbB2) breast cancer xenograft tumors were administered anti-ErbB2 antibodies. Growth inhibition was demonstrated in the high-ErbB2 xenografts with both trastuzumab and 2C4 compared with control-treated tumors (trastuzumab, 77% growth inhibition, $p = 0.004$, $n = 6$; 2C4, 80% growth inhibition, $p = 0.001$, $n = 10$; Figure 3E). The results with trastuzumab are similar to those reported previously (Baselga et al., 1998). In experiments not shown here, 2C4 also inhibits the growth of several ErbB2 overexpressing tumor xenograft models including MCF7 cells transfected to overexpress ErbB2, MDA-MB-361 (which displays moderate or 2+ ErbB2 overexpression), and the lung adenocarcinoma cell line, CALU-3. We conclude that 2C4 shares trastuzumab's (or 4D5's) inhibitory activity toward models in which tumor growth is driven by ligand-independent ErbB2 activation. As expected (Pegram et al., 1999), trastuzumab did not inhibit growth of the low-ErbB2 tumors compared with control-treated tumors, but in this study there was significant growth inhibition by 2C4 in this treatment group (59% growth inhibition, $p < 0.001$, compared with controls or trastuzumab-treated groups, $n = 12$; Figure 3F).

**2C4 diminishes ligand-activated ErbB2 signaling in both androgen-dependent and -independent prostate cancer cell lines**

A number of studies suggest that ErbB receptor activation may be important in the growth and survival of both androgen-dependent and androgen-independent prostate cancer (Agus et al., 2000; Kim et al., 1999). Therefore, 2C4 was evaluated in both in vitro and in vivo prostate cancer models. Similar to the experiments using breast cancer cell lines, HRG-activated receptor phosphorylation levels were significantly decreased in prostate cancer cells that were pretreated with 2C4 in both the androgen-dependent (22v1) and androgen-independent (22Rv1) prostate cancer cell lines (data not shown). Neither of these cell lines overexpress ErbB2. The effect of 2C4 treatment on MAPK activation in both the androgen-dependent and androgen-independent prostate cancer cells was studied (Figure 4A). Cells treated with HRG show strong activation of MAPK. This activation is diminished with 2C4 treatment in both the androgen-dependent and androgen-independent strains. These data suggest that ErbB2 association with ErbB3 is critical for complete activation of signal transduction to MAPK in prostate cancer cells, similar to the data described in the breast cancer experiments. The effect of ErbB2 MAbs on PI3 kinase activation was measured by studying the activity of the downstream kinase Akt. As seen in Figure 4A, similar to the MCF7 cells, treatment of both 22v1 and 22Rv1 cells with the ErbB3 ligand, HRG, results in activation of Akt that is inhibited by treatment with 2C4.

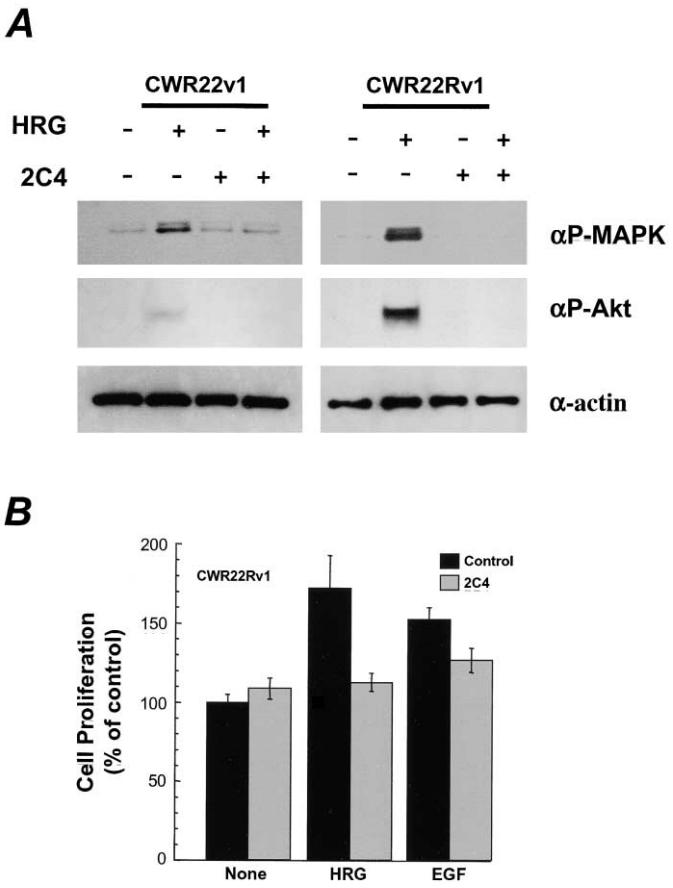


**Figure 4.** 2C4 inhibits HRG activation of MAPK or Akt in androgen-dependent (CWR22v1) and androgen-independent (CWR22Rv1) prostate cancer cell lines

**A:** HRG-induced MAPK activation and HRG-dependent Akt activation is inhibited by rhuMAb 2C4. **B:** The response of CWR22Rv1 monolayer cell culture growth to the addition of 2C4 with and without rHRGβ1 or EGF is shown. Results are shown as percentage of control cell proliferation.

The ability of 2C4 to inhibit the in vitro proliferation of the 22Rv1 (androgen-independent) cell line was studied. As demonstrated in Figure 4B, 2C4 does not inhibit growth of 22Rv1 cells compared with untreated cells ($p = 0.86$). However, when cells are treated with HRG or EGF, there is significant growth induction (172% and 153% of control for HRG and EGF, respectively) that can be inhibited by treatment with 2C4.

**2C4 inhibits the growth of both androgen-dependent and androgen-independent human prostate cancer xenografts**

To determine if our in vitro observations translated into tumor growth inhibition, we evaluated the efficacy of 2C4 on the growth of the androgen-independent prostate cancer xenograft CWR22R. As shown in Figure 5A, three different versions of 2C4 were tested including the murine version (muMAb 2C4) and a fully humanized variant (rhuMAb 2C4) (C. Adams, D. Allison, J. Clark, B. Fan, T. Breece, C. Schmelzer, S. Brignoli, K. Totpal, G. Phil-

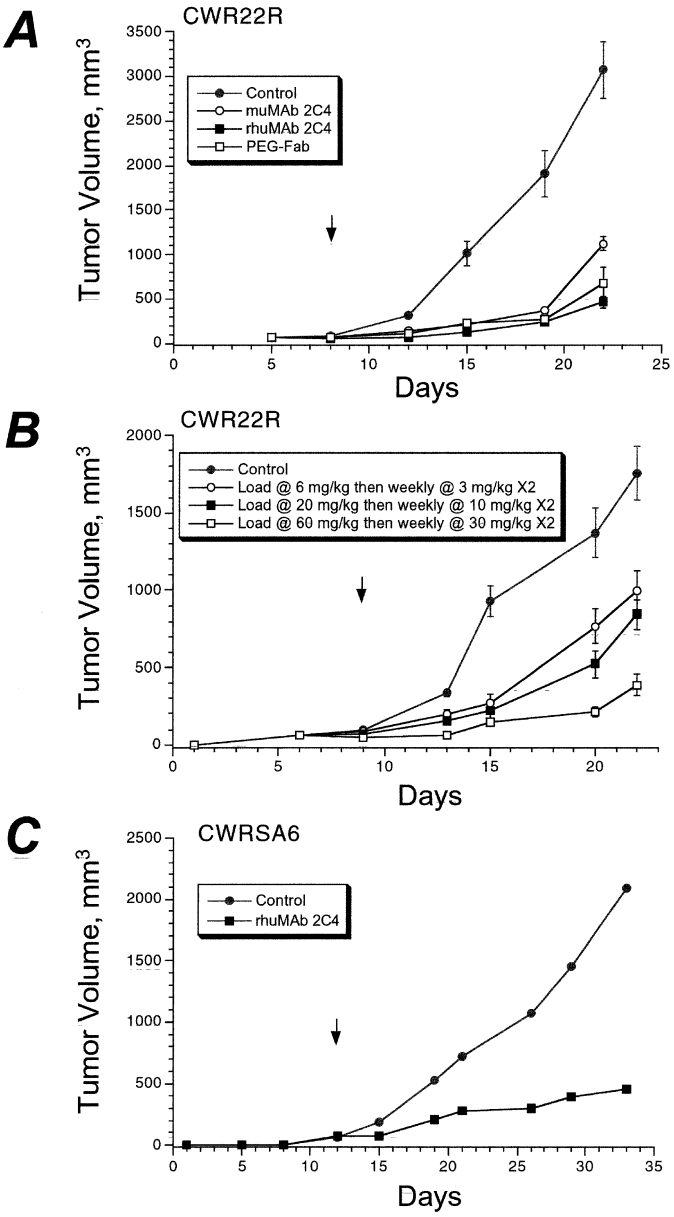


**Figure 5.** Effect of 2C4 on androgen-independent prostate tumor xenografts

**A:** The response of CWR22R tumors to murine 2C4 (muMAb 2C4) (○), human 2C4 (rhuMAb 2C4) (■), a polyethylene glycol derivative of the human 2C4 Fab (▲), or control Mab (●). Arrow indicates the initiation of therapy. **B:** Dose response of rhuMAb 2C4 treatment using the CWR22R model. Tumor-bearing animals were treated with an isotype control antibody (●); 6 mg/kg of rhuMAb 2C4 followed by two weekly injections at 3 mg/kg (○); 20 mg/kg of rhuMAb 2C4 followed by two weekly injections at 10 mg/kg (■); or 60 mg/kg of rhuMAb 2C4 followed by two weekly injections at 30 mg/kg (▲). **C:** CWRSA6 treated with rhuMAb 2C4 or trastuzumab, each administered at 20 mg/kg, twice per week.

lips, L. Presta, and M.X.S. unpublished data). In addition, we also tested a Fab version of rhuMAb 2C4 that was derivatized with polyethylene glycol (PEG-Fab) to allow for a longer serum half-life. In contrast to previous experiences with trastuzumab (Kelley et al., 1992), all versions of 2C4, including the monovalent Fab version, suppressed the growth of the androgen-independent tumor. We conclude that the 2C4-mediated growth inhibition of the tumors was not through an immunologic mechanism, as intact Fc region was not required for tumor growth inhibition. The dose response relationship for CWR22R tumor growth inhibition by rhuMAb 2C4 is shown in Figure 5B. In this study, animals were administered a loading dose to more rapidly sustain rhuMAb 2C4 blood levels. Maximum growth inhibition was achieved with a loading dose of 60 mg/kg followed by weekly doses of 30 mg/kg. This dose response relationship is similar to that reported with trastuzumab and the ErbB2-transfected MCF7 cell line (Pietras et al., 1998). The growth of a second androgen-independent model, CWRSA6 (Agus et al., 1999b), was also examined for sensitivity to rhuMAb 2C4 (Figure 5C). As reported previously (Agus et al., 1999c), trastuzumab had no effect on CWRSA6 tumor growth when compared to control (data not shown, $p = 0.63$, $n = 10$). In contrast, rhuMAb 2C4 demonstrated significant tumor growth inhibition relative to control-treated animals (CWRSA6, 82% growth inhibition, $p = 0.0047$, $n = 10$). To determine whether rhuMAb 2C4 also inhibited the growth of androgen-dependent prostate tumors, three different models were tested (Figure 6). rhuMAb 2C4 demonstrated tumor growth inhibition in the androgen-dependent models of prostate cancer. At the doses and schedules tested, the degree of inhibition for trastuzumab (Agus et al., 1999c) and rhuMAb 2C4 was similar. For trastuzumab (data not shown), growth inhibition observed with CWR22 was 68% ($p = 0.003$, $n = 12$), LNCaP was 89% growth inhibition ($p = 0.002$, $n = 12$), and LuCaP35 was 78% ($p = 0.002$, $n = 12$). rhuMAb 2C4 treatment demonstrated similar growth inhibition. With the CWR22 xenograft (Figure 6A), 93% growth inhibition was observed ($p = 0.0001$); with LNCaP (Figure 6B), there was 83% growth inhibition ($p = 0.001$); and with LuCaP35 (Figure 6C), there was 90% growth inhibition ($p = 0.001$). None of the prostate cancer models used in this study overexpress ErbB2 in comparison to breast tumors with known ErbB2 gene amplification or breast cancer cell lines such as BT-474 or SK-BR-3 (Agus et al., 1999c).

### Expression of ErbB receptors and ligands in androgen-dependent and -independent tumor explants

Since the ErbB pathway is thought to be activated in the progression from androgen dependence to androgen independence (Craft et al., 1999; Scher et al., 1995), we examined relative ErbB receptor and ligand expression in the androgen-dependent and androgen-independent tumors. Since the genetic background of the CWR tumors is similar (Nagabhushan et al., 1996), the ratio of gene expression in CWR22R to the parental CWR22 and CWRSA6 to CWR22 was measured. A summary of this analysis was made for the four ErbB receptors and four ligands (Figure 7). Interestingly, the androgen-independent tumors appear to express higher levels of three EGFR ligands. Specifically, CWR22R expresses ~19-fold and ~9-fold higher levels of HB-EGF and TGFα, respectively, relative to CWR22. In contrast, CWRSA6 expresses ~9-fold and ~5-fold levels of HB-EGF and EGF, respectively.

### Discussion

In previous reports, we investigated the role of ligand-activated ErbB2 on the in vitro growth of various breast cancer cell lines (Lewis et al., 1996; Schaefer et al., 1997). The present study

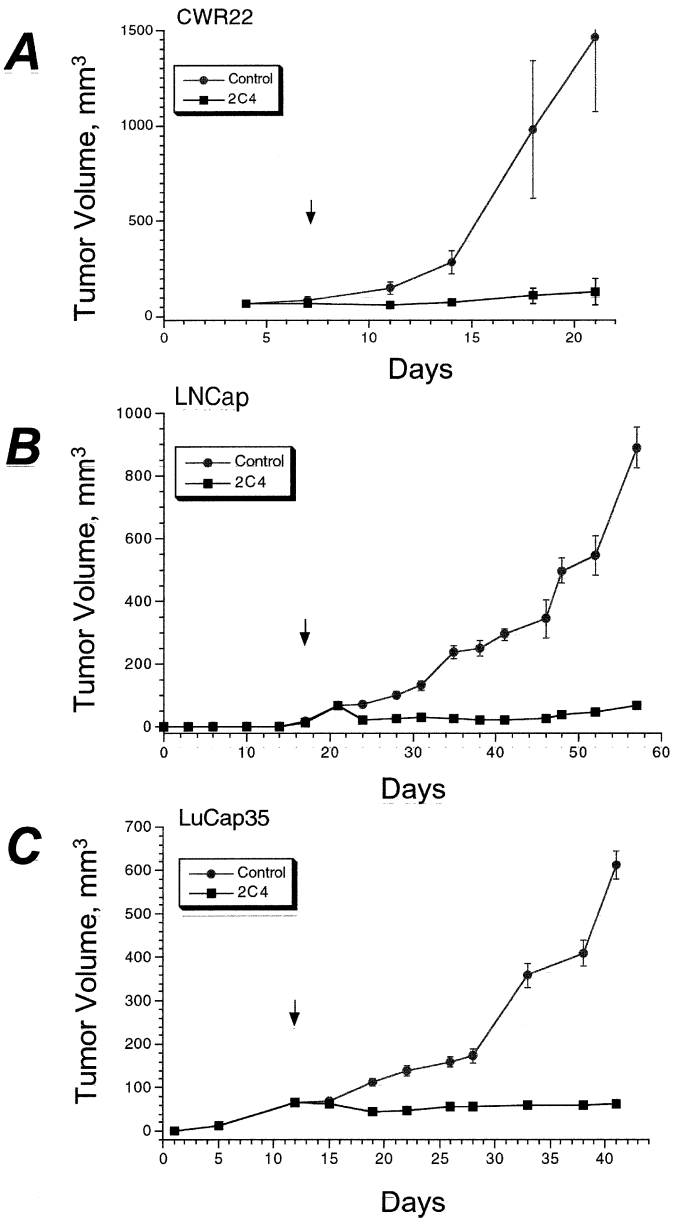


**Figure 6.** Effect of 2C4 on androgen-dependent prostate tumor xenografts

The response of CWR22 (**A**), LNCaP (**B**), or LuCap 35 (**C**) tumors to rhuMAb 2C4 (■) or isotype control antibody (●), each administered at 20 mg/kg twice per week. Arrow indicates the initiation of therapy. Results are given as mean tumor volume (n = 8) ± SE.

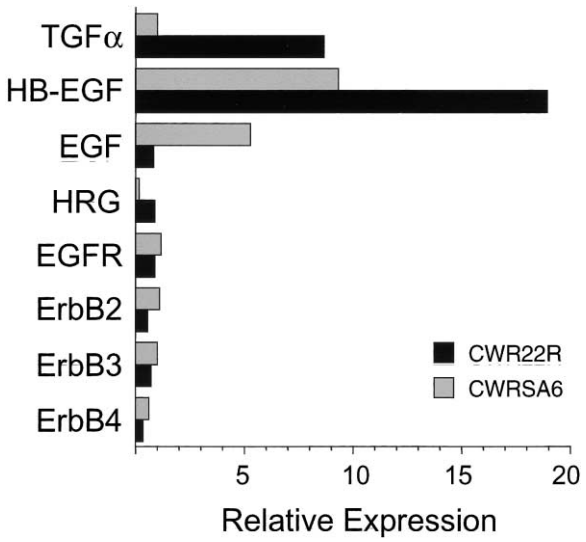


**Figure 7.** Real-time quantitative reverse-transcription PCR analysis of expression of ErbB receptors and ligands

Gene expression is plotted for the ratio of CWR22R/CWR22 and CWR22SA6/CWR22. Assays were performed in triplicate and the average is shown.

extends these observations and further examines the role of ligand-activated ErbB2 signaling in breast and prostate tumor models. The developing and adult prostate is regulated by androgen, a feature shared by most early forms of prostate cancer. Disease progression is the result of the emergence of an androgen-independent phenotype (Grossmann et al., 2001). Although these tumor cells still express androgen receptor, the responsiveness of the receptor to agonists or antagonists is frequently altered. A number of recent studies have demonstrated that androgen receptor activation can also occur as a result of peptide growth factor activation (Wells, 1999). Several reports suggest that activation of the ErbB kinase axis results in androgen receptor activation (Kim et al., 1999). Some of the molecular details defining these signal transduction pathways are beginning to emerge. For example, ErbB2 activation can lead to androgen target gene expression through activation of the MAPK pathway (Yeh et al., 1999). More recently it has been reported that a constitutively active mutant form of ErbB2 leads to Akt activation and the androgen receptor is a substrate for Akt (Wen et al., 2000). ErbB2 activation through Akt to the androgen receptor is proposed as a major pathway that promotes androgen-independent prostate cancer growth and survival. At present the mechanisms by which ErbB2 becomes activated in prostate cancer are unclear. Several reports suggested that ErbB2 gene amplification was common in prostate cancer (Kallakury et al., 1998; Ross et al., 1997). However, a number of recent studies have failed to confirm these initial findings (Oxley et al., 2002; Reese et al., 2001; Savinainen et al., 2002). An alternative scenario has suggested that excess ErbB2 protein synthesis or decreased protein degradation may take place in the absence of gene amplification. Moreover, the onset of ErbB2 protein overexpression may coincide with the emergence of androgen independence. Experimental evidence for this possibility has been demonstrated by Craft et al. (1999) with the prostate cancer cell line LNCaP. Recently it was reported that a similar phenomenon may occur in prostate cancer patients (Osman et al., 2001; Shi et al., 2001; Signoretti et al., 2000).

In the present study, we adopted an alternative strategy to targeting ErbB receptors that is geared toward disrupting the recruitment of ErbB2 into ErbB receptor-ligand complexes. The ramifications of our approach are dependent upon the particular repertoire of ErbB receptors expressed by the tumor. In the case of EGFR, which is fully functional as a tyrosine kinase, blocking the recruitment of ErbB2 into EGFR-ligand complexes does not ablate signaling but dampens the diversity, intensity,

and duration of signaling (Beerli and Hynes, 1996; Graus-Porta et al., 1997; Karunagaran et al., 1996). A number of biochemical and biological explanations have been put forward to help integrate the role that ErbB2 plays in complex with EGFR. Thus, in comparison to when EGFR is expressed alone, the EGFR-ErbB2 complex exhibits higher ligand affinities (Jones et al., 1999; Karunagaran et al., 1996), a more complex and diverse initiation of signal transduction pathways (Olayioye et al., 1998; Pinkas-Kramarski et al., 1996b), decreased rate of endocytosis (Worthylake et al., 1999), and altered receptor complex trafficking (Lenferink et al., 1998). EGFR-ligand complexes containing ErbB2 exhibit a longer duration of signaling due to the fact that the complexes remain at the cell surface longer. Although a role for ErbB4 in most solid tumors is ill defined, it is likely those ramifications of ErbB2 in complex with ErbB4 are similar to that observed with EGFR.

In contrast, the inhibition of association of ErbB2 with ErbB3 results in the near complete absence of receptor complex signaling (Beerli et al., 1994, 1995; Guy et al., 1994). The analysis of clinical specimens derived from human tumors indicates that the dynamic range of ErbB3 expression seems to be narrower than that observed for either EGFR or ErbB2. A teleological reason for this observation may be ErbB3's lack of tyrosine kinase activity, which, if upregulated, would not convey a growth advantage to the tumor because it lacks the ability to signal autonomously. Instead, we hypothesize that inappropriate ligand activation of ErbB3-ErbB2 signaling may play an important role in tumor growth. Support for this hypothesis lies in the observation that ErbB3 expressed together with ErbB2 is the most potent ErbB complex with regard to receptor signaling (Pinkas-Kramarski et al., 1996a). Moreover, the transformation potential of ErbB2-ErbB3 is also far greater than that observed for any other ErbB receptor complex (Alimandi et al., 1995; Wallasch et al., 1995).

Since ErbB2 functions as a coreceptor with other ErbB family members, overexpression of the receptor is not required for signaling if ErbB ligands are available to drive biological responses. Here we tested this notion directly using an antibody directed against ErbB2 that appears to sterically block the association of ErbB2 with other ErbB family members. Using this approach, we demonstrate that ErbB2 activation may play a critical role in driving the growth and survival of human breast and prostate tumor xenografts. At present, we are uncertain about several details regarding ErbB activation. Our failure to detect 2C4 effects, in the absence of exogenous ligand addition, on low-ErbB2-expressing cell lines derived from our tumor models suggests that the growth of these cell lines in vitro is not driven by autocrine production of ErbB ligands. A likely source of ligand production is the tumor stroma. Stroma-epithelial interactions have been studied extensively with regard to breast and prostate development (Chung, 1993; Cullen and Lippman, 1992; Cunha, 1994). Several ErbB ligands are known to be expressed in prostate and may be differentially expressed in prostate tumors (Scher et al., 1995; Adam et al., 1999; Duque et al., 2001; Torring et al., 2000). We surmise that disrupting ErbB signaling by 2C4 may account for the inhibition of tumor growth.

In conclusion, we demonstrate that an antibody, 2C4, directed against ErbB2, disrupts its role as a coreceptor and exhibits promising activity in models of breast and prostate cancer. The observations that monovalent versions of 2C4 are also effective in blocking ErbB2 function in vitro and tumor growth in vivo suggest that biological activity is not dependent on bivalency or the presence of an Fc region. To facilitate clinical investigations in cancer patients, the pharmacokinetic and toxicological properties of an intact, humanized variant of 2C4 was examined in nonhuman primates (C. Adams, D. Allison, J. Clark, B. Fan, T. Breece, C. Schmelzer, S. Brignoli, K. Totpal, G. Phillips, L. Presta, and M.X.S., unpublished data). The results of these preclinical studies suggest that rhuMAb 2C4 behaves very similar to trastuzumab with regard to pharmacokinetic properties. Disrupting ligand-activated ErbB2 may complement other strategies devised to target ErbB signaling in human neoplasms.

## Experimental procedures

### Cell and tumor lines

The 22v1 and 22Rv1 cell strains were obtained by mincing CWR22/CWR22R xenografts, placing the cells into 6-well matrigel basement membrane matrix coated dishes (Biocoat cell environments, Becton Dickinson, Franklin Lakes, NJ) containing Dulbecco's modified Eagle's medium, supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (GIBCO-BRL, Rockville, MD) and incubating at 37°C, 5% $CO_2$, in a humidified atmosphere for 3–4 days to allow for cell attachment. Following the initial incubation, the media was replaced with minimal essential medium containing D-val (MEM D-val, ATCC, Manassas, VA) supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (GIBCO-BRL). The CWR22/CWR22R cells were then allowed to grow for 4 weeks or until only the epithelial cells remained. The MCF7, MDA-MB-134, T47D, and ZR-75-1 breast tumor cell lines and 22Rv1 prostate carcinoma line were obtained from the American Type Culture Collection (Manassas, VA) and used for proliferation studies. Cells were maintained in high-glucose DMEM:Ham's F-12 (50:50) supplemented with 10% heat-inactivated FBS and 2 mM l-glutamine. For proliferation assays, cells were seeded into 96-well microtiter plates at a density of $10^4$ cells per well and allowed to adhere overnight. The media were then replaced with low-serum media (1% FBS) ± rhuMAb 2C4 at a concentration of 100-fold molar excess over ligand concentration (30 nM rhuMAb 2C4 for 22Rv1 and 300 nM for MCF7, MDA-134, T47D, and ZR-75-1). The cells were allowed to sit at room temperature for 2 hr, after which the ErbB ligands HRG-β1 (0.3 nM) or EGF (3 nM for MCF7, 0.3 nM for 22Rv1) were added. Following a 3 day incubation, cell monolayers were washed with PBS and stained with crystal violet dye for determination of relative cell proliferation as previously described (Lewis et al., 1996).

The xenograft studies were performed as previously described (Agus et al., 1999b, 1999c). Four- to six-week-old nude athymic BALB/c male and female mice were obtained from the National Cancer Institute-Frederick Cancer Center and maintained in pressurized ventilated caging at the Cedars-Sinai Medical Center vivarium. Male animals were inoculated subcutaneously with $1 \times 10^7$ LNCaP cells or minced tumor tissue from the androgen-dependent CWR22 (gift from T. Pretlow) and LuCaP (gift from R. Vessela), and females received the androgen-independent sublines CWR22R, CWRSA1, or CWRSA6, which were obtained by selecting tumors for regrowth and increased serum PSA after androgen withdrawal (Agus et al., 1999a). Female mice were inoculated with $1 \times 10^7$ MCF7 cells. All lines were injected together with reconstituted basement membrane (Matrigel; Collaborative Research, Bedford, MA), as described previously (Nagabhushan et al., 1996; Wainstein et al., 1994). To maintain serum testosterone or estrogen (for the MCF-7 line) levels, mice were implanted with 12.5 mg sustained release testosterone pellets or 0.72 mg sustained release 17 β-estradiol pellets (Innovative Research of America, Sarasota, FL) subcutaneously before receiving the tumor cell inoculation. Treatments consisted of twice weekly intraperitoneal injection of 20 mg/kg trastuzumab (Herceptin®, rhuMAb ErbB2, trastuzumab, Genentech, Inc., South San Francisco, CA) or 2C4 (Genentech) in PBS for no less than 3 weeks. Control mice were given vehicle alone. Tumors were measured every 3–4 days with vernier calipers, and tumor volumes were calculated by the formula: $\pi/6 \times$ larger diameter $\times$ (smaller diameter)$^2$. Animals with palpably established tumors of at least 65 $mm^3$ in volume were designated to treatment groups.

**MAP kinase (MAPK) and Akt assays**

To assess MAPK phosphorylation, cells ($10^5$/well) were plated in serum-containing media in 12-well culture plates. The next day, media were removed and fresh media containing 0.1% serum were added to each well. This procedure was then repeated the following day, and prior to assay the media were replaced with serum-free binding buffer (Jones et al., 1998). Cells were allowed to equilibrate to room temperature and then incubated for 30 min with 0.5 ml of 200 nM trastuzumab or 2C4. Cells were then treated with 1 nM EGF or TGFα or 0.2 nM rHRGβ$1_{177–244}$ (rHRGβ1) for 15 min. The reaction was stopped by aspirating the cell medium and then adding 0.2 ml SDS-PAGE sample buffer containing 1% DTT. MAPK activation was assessed by Western blotting using an anti-active MAPK antibody (Promega, Madison, WI) as described previously (Jones et al., 1998).

For Akt activation, 10 cm dishes seeded the night before with $3 \times 10^6$ cells/dish were incubated for 3 hr in serum-free media. The cells were then incubated an additional hour in serum-free media containing either 2C4 or trastuzumab at a concentration of 100 nM, or in serum-free media without additions (control). Ligand stimulation was carried out using 1 nM rHRGβ1 for 12 min. Akt activity was determined with an assay kit (Cell Signaling Technology) based on phosphorylation of GSK-3α/β. GSK-3 is a natural substrate of the Akt kinase. Cell lysates were prepared and processed according to the kit manufacturer's directions. Briefly, immunoprecipitations were performed on the cell lysates using an immobilized anti-Akt affinity gel. The washed immunoprecipitates were then resuspended in a kinase buffer and incubated with a recombinant fusion protein that contains a portion of the GSK-3α/β sequence. Supernatants from these incubations were diluted in SDS-sample buffer, and phosphorylation of the fusion protein was assessed by Western blot using an antibody specific for phosphorylation of GSK-3α at Ser21 or GSK-3β at Ser9.

**Immunoprecipitation**

The ability of ErbB3 to associate with ErbB2 was tested in a coimmunoprecipitation experiment. MCF-7 or SKBR-3 cells ($10^6$ cell/well) were seeded in 6-well tissue culture plates in 50:50 DMEM/Ham's F12 medium containing 10% fetal bovine serum and 10 mM HEPES (pH 7.2) (growth medium) and allowed to attach overnight. The cells were starved for 2 hr in growth medium without serum prior to beginning the experiment. The cells were washed briefly with PBS and then incubated with either 100 nM of the indicated antibody diluted in 0.2% w/v BSA, RPMI medium, with 10 mM HEPES (pH 7.2) (binding buffer), or with binding buffer alone (control). After 1 hr at room temperature, rHRGβ1 was added to a final concentration of 5 nM to half the wells and a similar volume of binding buffer was added to the other wells. The incubation was continued for approximately 10 min. Supernatants were removed by aspiration, and the cells were lysed in RPMI, 10 mM HEPES (pH 7.2), 1.0% v/v Triton X-100, and 1.0% w/v CHAPS (lysis buffer) containing 0.2 mM PMSF, 10 μg/ml leupeptin, and 10 U/ml aprotinin. The lysates were cleared of insoluble material by centrifugation.

ErbB2 was immunoprecipitated using a monoclonal antibody covalently coupled to an affinity gel (Affi-Prep 10, Bio-Rad, Hercules, CA). This antibody (Ab-3, Oncogene Sciences, Boston, MA) recognizes a cytoplasmic domain epitope. Immunoprecipitation was performed by adding 10 μl of gel slurry containing approximately 8.5 μg of immobilized antibody to each lysate, and the samples were allowed to mix at room temperature for 2 hr. The gels were then collected by centrifugation. The gels were washed batch-wise three times with lysis buffer to remove unbound material. SDS sample buffer was then added and the samples were heated briefly in a boiling water bath.

Supernatants were run on 4%–12% polyacrylamide gels and electroblotted onto nitrocellulose membranes. The presence of ErbB3 was assessed by probing the blots with a polyclonal antibody against a cytoplasmic domain epitope (c-17, Santa Cruz Biotechnology, Santa Cruz, CA). The blots were visualized using a chemiluminescent substrate (ECL, Amersham Pharmacia Biotech, Piscataway, NJ).

**HRG binding assays**

Assays to assess specific binding of $^{125}$I-HRG were performed as described previously (Lewis et al., 1996).

**Real-time quantitative PCR**

RNA was prepared using *RNeasy* kits obtained from Qiagen (either maxi 75161 or midi 75163, Qiagen, Valencia, CA). Gene expression was quantified using real-time quantitative PCR (TaqMan, ABI PRISM 7700, Applied Biosystems, Foster City, CA) technique, as previously described (Gibson et al., 1996; Heid et al., 1996). The sequences of the primer/probe sets used for this analysis are as follows. F and R are the forward and reverse primers, respectively, and P is the fluorescent-labeled probe. EGFR: (F) 5′-TTCCTGTGGATCCAGAGGA-3′, (R) 5′-AGCGTAATCCCAAGGATGT-3′, (P) 5′-FAM-AGGACGGACCTCCATGCCTTTGAGAA-TAMARA-p-3′; ErbB2/*neu*: (F) 5′-TCTGGACGTGCCAGTGTGAA-3′, (R) 5′-TGCTCCCTGAGGACA CATCA-3′, (P) 5′-FAM- CAGAAGGCCAAGTCCGCAGAAGCC-TAMARA-p-3′; ErbB3: (F) 5′-TTCTCTACTCTACCATTGCCCAAC-3′, (R) 5′-CACCAC TATCTCAGCATCTCGGTC-3′, (P) 5′-FAM-ACACCAACTCCAGCCACGCT CTGC-TAMARA-p-3′; ErbB4: (F) 5′-GAGATAACCAGCATTGAGCACAAC-3′, (R) 5′-AGAGGCAGGTAACGAAACTGATTA-3′, (P) 5′-FAM-CCTCTCC TTCCTGCGGTCTGTTCGA-TAMARA-p-3′; EGF: (F) 5′-AGCTAACCCAT TATGGCAACA-3′, (R) 5′-AGTTTTCACTGAGTCAGCTCCAT-3′, (P) 5′-FAM-AGGGCCCTGGACCCACCAC-TAMARA-p-3′; TGFα: (F) 5′-GGACAGCACT GCCAGAGA-3′, (R) 5′-CAGGTGATTACAGGCCAAGTAG-3′, (P) 5′-FAM-CCTGGGTGTGCCACAGACCTTC-TAMARA-p-3′; HRG: (F) 5′-TGGCTGAC AGCAGGACTAAC-3′, (R) 5′-CTGGCCTGGATTTCTTC-3′, (P) 5′-FAM-CAG CAGGCCGCTTCTCGACAC-TAMARA-p-3′; HB-EGF: (F) 5′-GAAAGACTTC CATCTAGTCACAAAGA-3′, (R) 5′-GGGAGGCCCAATCCTAGA-3′, (P) 5′-FAM-TCCTTCGTCCCCAGTTGCCG-TAMARA-p-3′.

Ribosomal protein 19 (RPL19) was used as a housekeeping gene for EGFR family receptor genes. Primer/probe sets for RPL19 are (F) 5′-ATG TATCACAGCCTGTACCTG-3′, (R) 5′-TTCTTGGTCTCTTCCTCCTTG-3′, and (P) 5′-FAM-AGGTCTAAGACCAAGGAAGCACGCAA-TAMRA-p-3′.

β-actin was used as a housekeeping gene for the EGFR family ligands. Primer/probe sets for β-actin, listed here, were obtained from PE Applied Biosystems (Foster City, CA): (F) 5′-TCACCCACACTGTGCCCATCTACGA-3′, (R) 5′-CAGCGGAACCGCTCATTGCCAATGG-3′, and (P) 5′-FAM-ATGCCCX (TAMARA)CCCCCATGCCATC-p-3′.

TaqMan analysis was performed in a standard 96-well plate format. Standard curves were constructed using 3.9–1000 ng of total mRNA prepared from cultured cells of T47D for EGF and receptor genes and MDA-MB-231 for all other ligands and 31.25–500 ng for RPL19. Each dilution was run in duplicate. All samples were run in triplicate using 100 ng of mRNA for each reaction.

**Statistical analysis of the xenograft experiments**

Pairwise differences between the tumor volumes of the treatment groups were compared over time using a permutation test. The null hypothesis for this test is that treatment has no differential effect on the tumor volumes over time. The statistic (SS_Dev) used to test the hypothesis was the sum of the squared differences between mean tumor volume summed over all time points. This statistic reflects the amount by which the trajectories of average tumor volume of the two treatment groups are different (Agus et al., 1999c).

**Acknowledgments**

We thank Drs. Camellia Adams and Len Presta for the generation of rhuMAb 2C4 and Dr. Charlie Schmelzer, Tim Breece, and Kurt Schroeder for antibody production. Allison Bruce and David Wood are thanked for assistance with graphics. H.I.S. is supported by CaPCURE and the PepsiCo Foundation. D.B.A. is supported by CaPCURE and the Eleanor and Paul Stephens Foundation.

Received: June 14, 2002
Revised: July 22, 2002

**References**

Adam, R.M., Borer, J.G., Williams, J., Eastham, J.A., Loughlin, K.R., and Freeman, M.R. (1999). Amphiregulin is coordinately expressed with heparin-binding epidermal growth factor-like growth factor in the interstitial smooth muscle of the human prostate. Endocrinology *140*, 5866–5875.

Agus, D.B., Cordon-Cardo, C., Fox, W., Drobnjak, M., Koff, A., Golde, D., and Scher, H.I. (1999a). Prostate cancer cell cycle regulators: response to

androgen withdrawl and development of androgen independence. J. Natl. Cancer Inst. *91*, 1869–1876.

Agus, D.B., Cordon-Cardo, C., Fox, W., Drobnjak, M., Koff, A., Golde, D.W., and Scher, H.I. (1999b). Prostate cancer cell cycle regulators: response to androgen withdrawal and development of androgen independence. J. Natl. Cancer Inst. *91*, 1869–1876.

Agus, D.B., Scher, H.I., Higgins, B., Fox, W.D., Heller, G., Fazzari, M., Cordon-Cardo, C., and Golde, D.W. (1999c). Response of prostate cancer to anti-Her-2/neu antibody in androgen-dependent and -independent human xenograft models. Cancer Res. *59*, 4761–4764.

Agus, D.B., Akita, R.W., Fox, W.D., Lofgren, J.A., Higgins, B., Maiese, K., Scher, H.I., and Sliwkowski, M.X. (2000). A potential role for activated HER-2 in prostate cancer. Semin. Oncol. *27*, 76–83.

Alimandi, M., Romano, A., Curia, M.C., Muraro, R., Fedi, P., Aaronson, S.A., Di Fiore, P.P., and Kraus, M.H. (1995). Cooperative signaling of ErbB3 and ErbB2 in neoplastic transformation and human mammary carcinomas. Oncogene *10*, 1813–1821.

Arteaga, C.L. (2001). The epidermal growth factor receptor: from mutant oncogene in nonhuman cancers to therapeutic target in human neoplasia. J. Clin. Oncol. *19*, 32S–40S.

Baselga, J., and Averbuch, S.D. (2000). ZD1839 ('Iressa') as an anticancer agent. Drugs *60*, 33–40.

Baselga, J., Norton, L., Albanell, J., Kim, Y.-M., and Mendelsohn, J. (1998). Recombinant humanized anti-HER2 antibody (Herceptin™) enhances the antitumor activity of paclitaxel and doxorubicin against HER2/neu overexpressing human breast cancer xenografts. Cancer Res. *58*, 2825–2831.

Beerli, R.R., and Hynes, N.E. (1996). Epidermal growth factor-related peptide activate distinct subsets of ErbB receptors and differ in their biological activities. J. Biol. Chem. *271*, 6071–6076.

Beerli, R.R., Wels, W., and Hynes, N.E. (1994). Intracellular expression of single chain antibodies reverts ErbB-2 transformation. J. Biol. Chem. *269*, 23931–23936.

Beerli, R., Graus-Porta, D., Woods-Cook, K., Chen, X., Yarden, Y., and Hynes, N.E. (1995). Neu differentiation factor activation of ErbB-3 and ErbB-4 is cell specific and displays a differential requirement for ErbB-2. Mol. Cell. Biol. *15*, 6496–6505.

Ben-Bassat, H., and Klein, B.Y. (2000). Inhibitors of tyrosine kinases in the treatment of psoriasis. Curr. Pharm. Des. *6*, 933–942.

Chung, L.W. (1993). Implications of stromal-epithelial interaction in human prostate cancer growth, progression and differentiation. Semin. Cancer Biol. *4*, 183–192.

Clynes, R.A., Towers, T.L., Presta, L.G., and Ravetch, J.V. (2000). Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. Nat. Med. *6*, 443–446.

Cobleigh, M.A., Vogel, C.L., Tripathy, D., Robert, N.J., Scholl, S., Fehrenbacher, L., Wolter, J., Paton, V., Shak, S., Lieberman, G., and Slamon, D.J. (1999). 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. *17*, 2639–2648.

Craft, N., Shostak, Y., Carey, M., and Sawyers, C.L. (1999). A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the HER-2/neu tyrosine kinase. Nat. Med. *5*, 280–285.

Cullen, K.J., and Lippman, M.E. (1992). Stromal-epithelial interactions in breast cancer. Cancer Treat. Res. *61*, 413–431.

Cunha, G.R. (1994). Role of mesenchymal-epithelial interactions in normal and abnormal development of the mammary gland and prostate. Cancer *74*, 1030–1044.

deFazio, A., Chiew, Y.E., Sini, R.L., Janes, P.W., and Sutherland, R.L. (2000). Expression of c-erbB receptors, heregulin and oestrogen receptor in human breast cell lines. Int. J. Cancer *87*, 487–498.

Duque, J.L., Adam, R.M., Mullen, J.S., Lin, J., Richie, J.P., and Freeman, M.R. (2001). Heparin-binding epidermal growth factor-like growth factor is an autocrine mediator of human prostate stromal cell growth in vitro. J. Urol. *165*, 284–288.

Fendly, B.M., Winget, M., Hudziak, R.M., Lipari, M.T., Napier, M.A., and Ullrich, A. (1990). Characterization of murine monoclonal antibodies reactive to either the human epidermal growth factor receptor or HER2/neu gene product. Cancer Res. *50*, 1550–1558.

Fitzpatrick, V.D., Pisacane, P.I., Vandlen, R.L., and Sliwkowski, M.X. (1998). Formation of a high affinity heregulin binding site using the soluble extracellular domains of ErbB2 with ErbB3 or ErbB4. FEBS Lett. *431*, 102–106.

Gibson, U.E.M., Heid, C.A., and Williams, P.M. (1996). A novel method for real time quantitative PCR. Genome Res. *6*, 986–994.

Graus-Porta, D., Beerli, R.R., Daly, J.M., and Hynes, N.E. (1997). ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. EMBO J. *16*, 1647–1655.

Grossmann, M.E., Huang, H., and Tindall, D.J. (2001). Androgen receptor signaling in androgen-refractory prostate cancer. J. Natl. Cancer Inst. *93*, 1687–1697.

Guy, P.M., Platko, J.V., Cantley, L.C., Cerione, R.A., and Carraway, K.L., III. (1994). Insect cell-expressed p180erbB3 possesses an impaired tyrosine kinase activity. Proc. Natl. Acad. Sci. USA *91*, 8132–8136.

Heid, C.A., Stevens, J., Livak, K.J., and Williams, P.M. (1996). Real time quantitative PCR. Genome. Res. *6*, 986–994.

Hidalgo, M., Siu, L.L., Nemunaitis, J., Rizzo, J., Hammond, L.A., Takimoto, C., Eckhardt, S.G., Tolcher, A., Britten, C.D., Denis, L., et al. (2001). Phase I and pharmacologic study of OSI-774, an epidermal growth factor receptor tyrosine kinase inhibitor, in patients with advanced solid malignancies. J. Clin. Oncol. *19*, 3267–3279.

Jones, J.T., Ballinger, M.D., Pisacane, P.I., Lofgren, J.A., Fairbrother, W.J., Wells, J.A., and Sliwkowski, M.X. (1998). Binding interaction of the heregulinβ *EGF*-domain with ErbB3 and ErbB4 receptors assessed by alanine scanning mutagenesis. J. Biol. Chem. *273*, 11667–11674.

Jones, J.T., Akita, R.W., and Sliwkowski, M.X. (1999). Binding specificities and affinities of *egf* domains for ErbB receptors. FEBS Lett. *447*, 227–231.

Kallakury, B.V., Sheehan, C.E., Ambros, R.A., Fisher, H.A., Ronald, P., Kaufman, J., Muraca, P.J., and Ross, J.S. (1998). Correlation of $p34^{cdc2}$ cyclin-dependent kinase overexpression, CD44s downregulation, and HER-2/neu oncogen amplification with recurrence in prostatic adenocarcinomas. J. Clin. Oncol. *16*, 1302–1309.

Kalmes, A., Vesti, B., Daum, G., Abraham, J.A., and Clowes, A.W. (2000). Heparin blockade of thrombin-induced smooth muscle cell migration involves inhibition of epidermal growth factor (EGF) receptor transactivation by heparin-binding EGF-like growth factor. Circ. Res. *87*, 92–98.

Karunagaran, D., Tzahar, E., Beerli, R.R., Chen, X., Graus-Porta, D., Ratzkin, B.J., Seger, R., Hynes, N.E., and Yarden, Y. (1996). ErbB-2 is a common auxiliary subunit of NDF and EGF receptors: implications for breast cancer. EMBO J. *15*, 254–264.

Kelley, R.F., O'Connell, M.P., Carter, P., Presta, L., Eigenbrot, C., Covarrubias, M., Snedecor, B., Bourell, J.H., and Vetterlein, D. (1992). Antigen binding thermodynamics and antiproliferatvie effects of chimeric and humanized anti-p185HER2 antibody Fab fragments. Biochemisty *31*, 5434–5441.

Kim, H.-G., Kassis, J., Souto, J.C., Turner, T., and Wells, A. (1999). EGF receptor signaling in prostate morphogenisis and tumorigenesis. Histol. Histopathol. *14*, 1175–1182.

Klapper, L.N., Glathe, S., Vaisman, N., Hynes, N.E., Andrews, G.C., Sela, M., and Yarden, Y. (1999). The ErbB-2/HER2 oncoprotein of human carcinomas may function solely as a shared coreceptor for multiple stroma-derived growth factors. Proc. Natl. Acad. Sci. USA *96*, 4995–5000.

Lenferink, A.E., Pinkas-Kramarski, R., van de Poll, M.L., van Vugt, M.J., Klapper, L.N., Tzahar, E., Waterman, H., Sela, M., van Zoelen, E.J., and Yarden, Y. (1998). Differential endocytic routing of homo- and hetero-dimeric ErbB tyrosine kinases confers signaling superiority to receptor heterodimers. EMBO J. *17*, 3385–3397.

Lewis, G.D., Lofgren, J.A., McMurtrey, A.E., Nuijens, A., Fendly, B.M., Bauer,

K.D., and Sliwkowski, M.X. (1996). Growth regulation of human breast and ovarian tumor cells by heregulin: evidence for the requirement of ErbB2 as a critical component in mediating heregulin responsiveness. Cancer Res. *56*, 1457–1465.

Marte, B.M., Graus-Porta, D., Jeschke, M., Fabbro, D., Hynes, N.E., and Taverna, D. (1995). NDF/heregulin activates MAP kinase and p70/p85 S6 kinase during proliferation or differentiation of mammary epithelial cells. Oncogene *10*, 167–175.

Mendelsohn, J., and Baselga, J. (2000). The EGF receptor family as targets for cancer therapy. Oncogene *19*, 6550–6565.

Molina, M.A., Codony-Servat, J., Albanell, J., Rojo, F., Arribas, J., and Baselga, J. (2001). Trastuzumab (herceptin), a humanized anti-Her2 receptor monoclonal antibody, inhibits basal and activated Her2 ectodomain cleavage in breast cancer cells. Cancer Res. *61*, 4744–4749.

Nagabhushan, M., Miller, C.M., Pretlow, T.P., Giaconia, J.M., Edgehouse, N.L., Schwartz, S., Kung, H.J., de Vere White, R.W., Gumerlock, P.H., Resnick, M.I., et al. (1996). CWR22: the first human prostate cancer xenograft with strongly androgen-dependent and relapsed strains both in vivo and in soft agar. Cancer Res. *56*, 3042–3046.

Olayioye, M.A., Graus-Porta, D., Beerli, R.R., Rohrer, J., Gay, B., and Hynes, N.E. (1998). ErbB-1 and ErbB-2 acquire distinct signaling properties dependent upon their dimerization partner. Mol. Cell. Biol. *18*, 5042–5051.

Osman, I., Scher, H.I., Drobnjak, M., Verbel, D., Morris, M., Agus, D., Ross, J.S., and Cordon-Cardo, C. (2001). HER-2/neu (p185neu) protein expression in the natural or treated history of prostate cancer. Clin. Cancer Res. *7*, 2643–2647.

Oxley, J.D., Winkler, M.H., Gillatt, D.A., and Peat, D.S. (2002). Her-2/neu oncogene amplification in clinically localised prostate cancer. J. Clin. Pathol. *55*, 118–120.

Pegram, M., Hsu, S., Lewis, G., Pietras, R., Beryt, M., Sliwkowski, M., Coombs, D., Baly, D., Kabbinavar, F., and Slamon, D. (1999). Inhibitory effects of combinations of HER-2/neu antibody and chemotherapeutic agents used for treatment of human breast cancers. Oncogene *18*, 2241–2251.

Pietras, R.J., Pegram, M.D., Finn, R.S., Maneval, D.A., and Slamon, D.J. (1998). Remission of human breast cancer xenografts on therapy with humanized monoclonal antibody to HER-2 receptor and DNA-reactive drugs. Oncogene *17*, 2235–2249.

Pinkas-Kramarski, R., Soussan, L., Waterman, H., Levkowitz, G., Alroy, I., Klapper, L., Lavi, S., Seger, R., Ratzkin, B.J., Sela, M., and Yarden, Y. (1996a). Diversification of Neu differentiation factor and epidermal growth factor signaling by combinatorial receptor interactions. EMBO J. *15*, 2452–2467.

Pinkas-Kramarski, R., Soussan, L., Waterman, H., Levkowitz, G., Alroy, I., Klapper, L., Lavi, S., Seger, R., Ratzkin, B.J., Sela, M., and Yarden, Y. (1996b). Diversification of Neu differentiation factor and epidermal growth factor signaling by combinatorial receptor interactions. EMBO J. *15*, 2452–2467.

Reese, D.M., Small, E.J., Magrane, G., Waldman, F.M., Chew, K., and Sudilovsky, D. (2001). HER2 protein expression and gene amplification in androgen-independent prostate cancer. Am. J. Clin. Pathol. *116*, 234–239.

Ross, J.S., Sheehan, C., Hayner-Buchan, A.M., Ambros, R.A., Kallakury, B.V., Kaufman, R., Fisher, H.A., and Muraca, P.J. (1997). HER-2/neu gene amplification status in prostate cancer by fluorescence in situ hybridization. Hum. Pathol. *28*, 827–833.

Sadick, M.D., Sliwkowski, M.X., Nuijens, A., Bald, L., Chiang, N., Lofgren, J.A., and Wong, W.L.T. (1996). Analysis of heregulin-induced ErbB2 phosphorylation with a high throughput "Kinase Receptor Activation" (KIRA) ELISA. Anal. Biochem. *235*, 207–214.

Savinainen, K.J., Saramaki, O.R., Linja, M.J., Bratt, O., Tammela, T.L., Isola, J.J., and Visakorpi, T. (2002). Expression and gene copy number analysis of ERBB2 oncogene in prostate cancer. Am. J. Pathol. *160*, 339–345.

Schaefer, G., Fitzpatrick, V.D., and Sliwkowski, M.X. (1997). γ-heregulin: a novel heregulin isoform that is an autocrine growth factor for the human breast tumor cell line, MDA-MB-175. Oncogene *15*, 1385–1394.

Scher, H.I., Sarkis, A., Reuter, V., Cohen, D., Netto, G., Petrylak, D., Lianes, P., Fuks, Z., Mendelsohn, J., and Cordon-Cardo, C. (1995). Changing pattern of expression of the epidermal growth factor receptor and transforming growth factor alpha in the progression of prostatic neoplasms. Clin. Cancer Res. *1*, 545–550.

Shawver, L., Slamon, D., and Ullrich, A. (2002). Smart drugs: tyrosine kinase inhibitors in cancer therapy. Cancer Cell *1*, 117–123.

Shi, Y.A.N., Brands, F.H., Chatterjee, S., Feng, A.-C., Groshen, S., Schewe, J., Lieskovsky, G., and Cote, R.J. (2001). Her-2/neu expression in prostate cancer: high level of expression associated with exposure to hormone therapy and androgen independent disease. J. Urol. *166*, 1514–1519.

Signoretti, S., Montironi, R., Manola, J., Altimari, A., Tam, C., Bubley, G., Balk, S., Thomas, G., Kaplan, I., Hlatky, L., et al. (2000). Her-2-neu expression and progression toward androgen independence in human prostate cancer. J. Natl. Cancer Inst. *92*, 1918–1925.

Slamon, D.J., Clark, G.M., Wong, S.G., Levin, W.J., Ullrich, A., and McGuire, W.L. (1987). Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science *235*, 177–182.

Slamon, D.J., Leyland-Jones, B., Shak, S., Fuchs, H., Paton, V., Bajamonde, A., Fleming, T., Eiermann, W., Wolter, J., Pegram, M., et al. (2001). Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N. Engl. J. Med. *344*, 783–792.

Sliwkowski, M.X., Schaefer, G., Akita, R.W., Lofgren, J.A., Fitzpatrick, V.D., Nuijens, A., Fendly, B.M., Cerione, R.A., Vandlen, R.L., and Carraway, K.L., III. (1994). Coexpression of erbB2 and erbB3 proteins reconstitutes a high affinity receptor for heregulin. J. Biol. Chem. *269*, 14661–14665.

Sliwkowski, M.X., Lofgren, J., Lewis, G.D., Hotaling, T.E., Fendly, B.M., and Fox, J.A. (1999). Nonclinical studies addressing the mechanism of action of trastuzumab (herceptin). Semin. Oncol. *26, Suppl 12*, 60–70.

Soltoff, S.P., Carraway, K.L., III, Prigent, S.A., Gullick, W.G., and Cantley, L.C. (1994). ErbB3 is involved in activation of phosphatidylinositol 3-kinase by epidermal growth factor. Mol. Cell. Biol. *14*, 3550–3558.

Torring, N., Jorgensen, P.E., Sorensen, B.S., and Nexo, E. (2000). Increased expression of heparin binding EGF (HB-EGF), amphiregulin, TGF alpha and epiregulin in androgen-independent prostate cancer cell lines. Anticancer Res. *20*, 91–95.

Tzahar, E., and Yarden, Y. (1998). The ErbB-2/HER2 oncogenic receptor of adenocarcinomas: from orphanhood to multiple stromal ligands. Biochim. Biophys. Acta *1377*, M25–37.

Wainstein, M.A., He, F., Robinson, D., Kung, H.J., Schwartz, S., Giaconia, J.M., Edgehouse, N.L., Pretlow, T.P., Bodner, D.R., Kursh, E.D., et al. (1994). CWR22: androgen-dependent xenograft model derived from a primary human prostatic carcinoma. Cancer Res. *54*, 6049–6052.

Wallasch, C., Weiss, F.U., Niederfellner, G., Jallal, B., Issing, W., and Ullrich, A. (1995). Heregulin-dependent regulation of HER2/neu oncogenic signaling by heterodimerization with HER3. EMBO J. *14*, 4267–4275.

Wells, A. (1999). EGF receptor. Int. J. Biochem. Cell Biol. *31*, 637–643.

Wen, Y., Hu, M.C., Makino, K., Spohn, B., Bartholomeusz, G., Yan, D.H., and Hung, M.C. (2000). HER-2/neu promotes androgen-independent survival and growth of prostate cancer cells through the Akt pathway. Cancer Res. *60*, 6841–6845.

Woodburn, J.R. (1999). The epidermal growth factor receptor and its inhibition in cancer therapy. Pharmacol. Ther. *82*, 241–250.

Worthylake, R., Opresko, L.K., and Wiley, H.S. (1999). ErbB-2 amplification inhibits down-regulation and induces constitutive activation of both ErbB2 and epidermal growth factor receptors. J. Biol. Chem. *274*, 8865–8874.

Yarden, Y., and Sliwkowski, M.X. (2001). Untangling the ErbB signalling network. Nat. Rev. Mol. Cell Biol. *2*, 127–137.

Yeh, S., Lin, H.-K., Kang, H.-Y., Thin, T.H., Lin, M.-F., and Chang, C. (1999). From HER2/Neu signal cascade to androgen receptor and its coactivators: a novel pathway by induction of androgen target genes through MAP kinase in prostate cancer cells. Proc. Natl. Acad. Sci. USA *96*, 5458–5463.