

Activation of ErbB2 by Overexpression or by Transmembrane Neuregulin Results in Differential Signaling and Sensitivity to Herceptin

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Abstract

The ligands of the epidermal growth factor family and their receptors, the ErbB proteins, have been linked to the development of different types of cancer. Particular attention has focused on ErbB2, whose activation may occur by receptor overexpression or by ligand-induced oligomerization with other ErbB receptors. Whether these two modes of ErbB2 activation cause the same biological responses is unknown. Here, we uncovered important differences in the signaling, proliferation rates, and the response to anti-ErbB2 antibodies when comparing MCF7 cells expressing the ligand neuregulin, to MCF7 cells overexpressing ErbB2. Expression of neuregulin caused higher proliferation than ErbB2 overexpression. Transmembrane neuregulin expression was accompanied by constitutive activation of ErbB2, ErbB3, and ErbB4 receptors. ErbB2 overexpression caused tyrosine phosphorylation of ErbB2, whereas ErbB3 and ErbB4 were only slightly tyrosine phosphorylated. Autocrine transmembrane neuregulin also caused constitutive activation of several signaling pathways, such as the Erk1/2, Erk5, and Akt routes, which have been linked to breast cancer cell proliferation. Interestingly, expression of neuregulin increased p21 levels and this was required for the proliferation of MCF7 cells. Treatment with the anti-ErbB2 receptor antibody Herceptin had an inhibitory effect on proliferation only in cells expressing neuregulin but not on cells overexpressing ErbB2, and its inhibitory activity was accompanied by a decrease in p21. These results suggest that Herceptin may also be of help in the treatment of tumors in which neuregulin feeds the tumoral tissue. (Cancer Res 2005; 65(15): 6801-10)

Introduction

The ligands of the epidermal growth factor (EGF) family and their receptors, the ErbB proteins, comprise an important group of biologically active proteins that have been implicated in several physiologic and pathologic processes (1–3). In mammals, four receptor tyrosine kinases of the ErbB family, termed EGF receptor (HER1 or ErbB1), ErbB2 (HER2 or neu), ErbB3 (HER3), and ErbB4 (HER4), have been identified. With respect to the ligands and including the splice variants of neuregulins (NRGs), around two dozens of different EGF family ligands have been described in mammals (4–6).

Increased function or expression of the ErbB receptors or their ligands has been associated with the development of various types

of cancer (7, 8). Particular interest has been centered in breast cancer, where ErbB2 is overexpressed in about 25% of patients, and its presence correlates with a poor prognosis (9) and resistance to antineoplastic treatments (3). Several studies have supported a role for ErbB2 in deregulated cell proliferation. In immortalized breast epithelial cells, expression of ErbB2 has been reported to cause premalignant alterations of the mammary acini, including invasion of the lumen and increased proliferation of the epithelial cells (10). In mice, targeted overexpression of ErbB2 to the mammary gland results in the development of mammary tumors, and repression of ErbB2 expression by the use of a regulated transgene causes tumor shrinkage in early phases of tumor growth (11). The identification of ErbB2 as an important regulator of breast cancer cell proliferation in these different models led to the development of strategies aimed at reducing ErbB2 receptor levels or activity (3). One such strategy, that has reached the clinic, is based in the use of Herceptin, an antibody directed to the ectodomain of ErbB2, which is effective in the treatment of some ErbB2-positive breast cancer patients (3). However, whereas these and other studies have supported a role of ErbB2 in breast cancer initiation/progression (3), a number of questions still remain to be answered. Thus, whereas *in vitro* analyses using different cell lines have confirmed the oncogenic potential of ErbB2 receptor overexpression (12), others have shown that overexpression of ErbB2 is insufficient to drive breast cancer cell proliferation (13). Furthermore, Herceptin has shown little or no activity in a number of ErbB2-positive patients (14) and failed to inhibit proliferation of certain cell lines overexpressing ErbB2 (15). Therefore, it is presumably that other factors, besides the ErbB2 status, may be required for breast cancer cell proliferation and for the antiproliferative action of Herceptin.

The role of EGF ligands in breast cancer development has been less well studied, particularly when compared with ErbB2. Studies in mice have shown that overexpression of transforming growth factor- α causes hyperplasia of the mammary gland and pancreatic carcinomas (16, 17), and that overexpression of NRG in the mammary tissue results in the generation of adenocarcinomas (18) and favors the metastatic spread of breast cancer cells *in vivo* (19). In addition, reduction of NRG availability by the use of antisense oligonucleotides has been reported to reduce tumorigenesis and metastasis in breast cancer cells (20). Increasing evidence indicates that NRGs may also play an important role in human breast cancer. Thus, NRG has been shown to be expressed in a significant proportion of human breast cancer biopsies (21). Furthermore, cytogenetic analyses indicate that a subgroup of breast cancer patients has breaks in the *NRG1* gene, and this correlates with poor histopathologic grade (22). Interestingly, this set of patients was reported to have normal ErbB2 levels. *In vitro*, NRG induces a strong mitogenic response in cancer cell lines that express moderate levels of ErbB receptors (23), indicating that these

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factors may have a role in tumor progression if available from the stroma or the tumor itself.

Given the important role of ErbB2 and NRG in cancer, a question that needs to be addressed is whether ErbB2 activation by overexpression causes the same biological responses than ErbB2 activation by NRG. To address this question, we did a side-by-side comparison of these two situations. This comparative study has allowed us to uncover profound differences in cell proliferation, signaling, and response to anti-ErbB2 treatments in cells overexpressing ErbB2 with respect to cells expressing NRG. Expression of the latter caused resting activation of ErbB2, ErbB3, and ErbB4, whereas overexpression of ErbB2 resulted in activation of ErbB2, with minimal activation of ErbB3 and ErbB4. Expression of NRG caused a small but sustained increase in the phosphorylation state of several signaling molecules. In contrast, and although ErbB2 was strongly tyrosine phosphorylated in ErbB2-overexpressing cells, it did not cause a major stimulation of the phosphorylation of these signaling proteins. Proliferation studies indicated that expression of NRG strongly stimulated cell proliferation, whereas ErbB2 overexpression was less efficient to stimulate cell proliferation. The fact that Herceptin inhibited the proliferation of NRG-expressing cells but not the proliferation of ErbB2-overexpressing cells raises the important clinical question of whether the spectrum of cancer patients susceptible to treatment with Herceptin should include those with expression of NRGs.

Materials and Methods

Reagents and immunochemicals. Culture medium, sera, and G418 were purchased from Life Technologies (Gaithersburg, MD). Protein A-Sepharose was from Amersham-Pharmacia (Piscataway, NJ). Immobilon-P membranes were from Millipore Corp. (Bedford, MA). PD98059 and LY294002 were from Calbiochem (La Jolla, CA). Other generic chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), Roche Biochemicals, or Merck (Darmstadt, Germany). GST-Rb was from Santa Cruz Biotechnology (Santa Cruz, CA).

The anti-proNRG α 2c has been described (24). The monoclonal anti-HA antibody was from Roche Biochemicals (Barcelona, Spain). The mouse monoclonal anti-phosphotyrosine, anti-p21, and anti-phospho-Erk1/2 antibodies, the rabbit polyclonal anti-Erk, anti-p27, anti-ErbB3 and anti-ErbB4 antibodies used for immunoprecipitation were from Santa Cruz Biotechnology. The Ab3 anti-ErbB2 antibody used for Western blotting was from Oncogene Science (Uniondale, NY). The antibodies to pAkt, Akt, cyclin D1, Rb, CDK2, CDK4, and p21 were from BD Biosciences (Palo Alto, CA). The anti-pY1248 and the anti-pY1221/1222 that recognize ErbB2 phosphorylated at these tyrosines were from Cell Signalling (Beverly, MA). The anti-pY1139 anti-pErbB2 was from Biosource International (Camarillo, CA). The monoclonal anti-HER2 ectodomain antibody Herceptin and NRG were generously provided by Dr. Mark X. Sliwkowski (Genentech, San Francisco, CA). The Cy3- or Cy2-conjugated secondary antibodies were from Jackson Immuno-Research (West Grove, PA). Horseradish peroxidase conjugates of anti-rabbit and anti-mouse IgG were from Bio-Rad Laboratories (Cambridge, MA).

Cell culture and transfections. The conditions for the culture of MCF7 cells have been described (25). Transfections in MCF7 cells were done by calcium phosphate, and clones selected by G418. Single clones were analyzed for their content of transfected proteins by Western blotting with the anti-proNRG antibody or the Ab3 antibody.

To generate MCF7 cell lines that expressed HA-proNRG α 2c in a regulated manner, we used the tetracycline transactivator system. MCF7^{TetOff} cells (BD Biosciences) were transfected with pTRE2-HA-proNRG α 2c, or with pTRE2, together with pBabe-Puro, using LipofectAMINE (Invitrogen, San Diego, CA). Clones were then selected with 3 μ g/mL of puromycin, and the expression of the proteins analyzed by Western blotting, comparing the amount of HA-proNRG α 2c in the absence or presence of 10 ng/mL doxycycline.

Plasmids. The human ErbB2 cDNA (provided by Dr. M. Kraus, Istituto Europeo di Oncologia, Milan, Italy) was subcloned into the *Xho*I site of pCDNA3 to create the pCDNA3-ErbB2 mammalian expression plasmid. To generate inducible clones of MCF7 cells, HA-proNRG α 2c was subcloned into *Bam*HI/*Not*I sites of the pTRE2 vector (BD Clontech).

Immunoprecipitation, immunofluorescence, and Western blotting. The Immunoprecipitation, Western blotting, and confocal immunofluorescence microscopy procedures have been described (25). For the immunoprecipitation of ErbB3 and ErbB4 following treatment with Herceptin, the lysates were subjected to seven rounds of preclearing of Herceptin by incubating with protein A-Sepharose. The depletion of Herceptin was followed by Western blotting of the precipitates with the Ab3 anti-ErbB2 antibody.

p21 antisense experiments. MCF7 cells at a concentration of 30,000 cells/mL were plated in 24-well plates or in 60-mm dishes. Twenty-four hours later, cells were transfected with phosphorothioate oligodeoxynucleotides (250 nmol/L) using LipofectAMINE reagent (Invitrogen) following the manufacturer's instructions. Eighteen to 24 hours post-transfection, the medium was replaced with DMEM + 10% fetal bovine serum (FBS), and 3 days later, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was done to assess proliferation. In parallel, the cells in the 60-mm dishes were lysed and p21 content was analyzed by Western blotting. The p21 antisense oligo and control oligo were purchased from MWG Biotech (Ebersberg, Germany). The antisense oligo, 5'-CCAACTCAT-CCCGGCCTCGC-3', is complementary to the coding region of p21, and the control oligo, 5'-AACCCCTCCGGCCCTCAG-3', corresponds to a scrambled sequence.

Cell proliferation and cell cycle analyses. Conditions for the analysis of the proliferation of MCF7 cells have been reported (25). To measure the proliferation of the MCF7-NRG α 2c^{TetOff} clone, cells were plated at 10,000 cells per well and cultured overnight in DMEM + 10% FBS with or without 10 ng/mL doxycycline. The next day (day 1 of culture), an MTT assay was done and considered the starting point. Five days after plating, another MTT uptake was carried out and parallel cultures of cells were shifted to DMEM + 0.1% FBS, containing or not doxycycline and 10 nmol/L Herceptin. Four (day 9 of culture) and 6 (day 11 of culture) days after the addition of Herceptin, the MTT uptake assay was done as above. In parallel, cells being treated or not with doxycycline were lysed at the different times at which the MTT assays were done, and the content of HA-proNRG α 2c analyzed by Western blotting. Cell proliferation curves done by measurement of cell numbers indicated a high correlation between the data obtained by using MTT measurements or by cell counting (data not shown).

For cell cycle analyses, cells were cultured in 100-mm dishes, grown to 50% to 70% confluence and serum starved for 48 hours. Monolayers were then trypsinized and resuspended in 1 mL of PBS. After three washes with PBS, the cellular pellets were resuspended in 70% ethanol and incubated for 30 minutes at 4°C. Cells were then pelleted by centrifugation and the pellet was then resuspended in 1 mL of PBS containing 50 μ g/mL of propidium iodide in 0.5% Tween 20 and 0.1 μ g/mL RNase A. DNA content and cell cycle analysis were done on a fluorescence-activated cell sorting machine using CellQuest software (BD Biosciences).

In vitro kinase assay. MCF7, MCF7-ErbB2, and MCF7-NRG α 2c cells were plated in 100-mm dishes and 24 hours later were starved for 24 hours. Cells were then treated with or without NRG (10 nmol/L) for 24 hours and washed twice with PBS, lysed in 1 mL of ice-cold lysis buffer, and centrifuged at 10,000 \times g at 4°C for 10 minutes. The protein concentration of the supernatant was determined, and 600 μ g of lysate protein were immunoprecipitated with 0.8 μ g of anti-CDK4 antibody, 0.8 μ g of anti-p27 antibody, or 1 μ L of anti-p21 antiserum, and 60 μ L of protein A-Sepharose for 2 hours at 4°C. The immune complexes were recovered by a short centrifugation followed by three washes with 1 mL of cold lysis buffer and two washes with 1 mL of kinase buffer [20 mmol/L Tris-HCl (pH 7.4), 7.5 mmol/L MgCl₂, and 1 mmol/L DTT] and incubated for 30 minutes at 30°C in 25 μ L of kinase buffer containing 30 μ mol/L ATP, 2 μ Ci of (γ -³²P)-ATP and 1 μ g of GST-Rb (Santa Cruz Biotechnology). The reactions were terminated by addition of an equal volume of

2× sample buffer and run in a 10% SDS-PAGE. The gels were then exposed to a Fuji IIIIS sensitive screen and radioactivity in gels digitally analyzed using a BAS1500 apparatus.

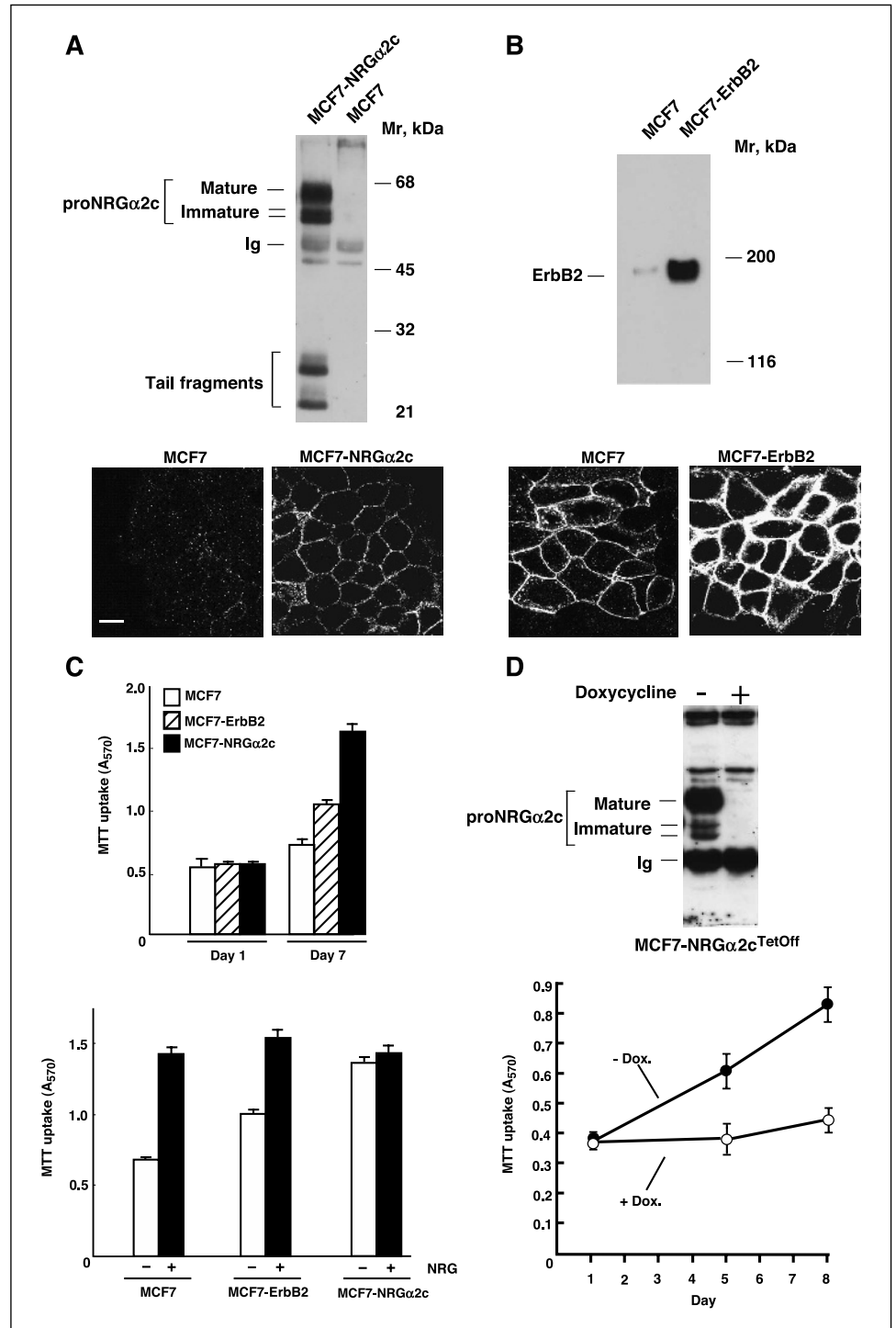
Results

To compare the contribution of ErbB2 and NRG to the proliferation of breast cancer cells, we used the MCF7 cell line. This cell line contains normal to low levels of ErbB2 and other ErbB receptors (26) and does not express NRG (Fig. 1A; data not shown).

In addition, this cell line responds mitogenically to the addition of soluble NRG (27). Based on this cellular model, we created MCF7 cells overexpressing ErbB2, or expressing the NRG isoform proNRGα2c.

Transfection of the cDNA coding for proNRGα2c into MCF7 cells resulted in the isolation of several G418-resistant clones whose expression of proNRGα2c was analyzed by Western blotting with an antibody that identifies several molecular forms of proNRGα2c in MCF7-NRGα2c cells (Fig. 1A, top). The slower migrating form

Figure 1. Proliferation of MCF7-NRGα2c cells and MCF7-ErbB2 cells. *A*, expression of proNRGα2c in MCF7 cells transfected with empty vector (MCF7) or with the same vector containing the proNRGα2c cDNA. *Top*, ProNRGα2c was immunoprecipitated from cell lysates with the anti-proNRG antiserum, and the blot was probed with the same antiserum. Position of the *M_r* markers (*right*). *Ig*, immunoglobulin heavy chain. *Bottom*, immunofluorescence analysis of proNRGα2c expression. Cells were prepared for immunofluorescence as indicated in Materials and Methods and incubated with the anti-proNRGα2c antibody followed by a Cy3-conjugated anti-rabbit antibody. Bar, 20 μm. *B*, *top*, ErbB2 was analyzed by Western blotting of immunoprecipitates from 1 mg of MCF7 and a clone of MCF7 cells transfected with the cDNA coding for ErbB2 (MCF7-ErbB2). *Bottom*, immunofluorescence pictures of MCF7 and MCF7-ErbB2 cells. Cells were stained with the anti-ErbB2 ectodomain antibody. *C*, *top*, comparative proliferation of MCF7 cells, MCF7 cells expressing proNRGα2c, or overexpressing ErbB2. Cells from the different types were plated in complete medium at identical densities in 24-well plates, and 12 hours later, placed in DMEM without serum. MTT uptake was measured at the indicated times. *Columns*, means of quadruplicates of an experiment that was repeated four times; *bars*, ±SD. *Bottom*, effect of exogenously added NRG on the proliferation of MCF7, MCF7-NRGα2c, and MCF7-ErbB2. Cells were plated in the presence of serum for 12 hours and switched to serum-free medium with or without 10 nmol/L NRG. MTT uptake was measured 7 days later. *D*, *top*, regulated expression of proNRGα2c in a MCF7^{TetOff} cell line. MCF7-NRGα2c^{TetOff} cells were cultured in the presence or absence of doxycycline (10 ng/mL), and expression of proNRGα2c was analyzed by Western blotting as above. *Bottom*, proliferation of MCF7-NRGα2c^{TetOff} cells. Cells were plated in 24-well plates and incubated with or without doxycycline (10 ng/mL) in the absence of serum. MTT uptake was measured at the indicated points. *Points*, mean of quadruplicates of an experiment that was repeated twice; *bars*, ±SD.



corresponds to mature proNRG α 2c, a form that is expressed at the cell surface. The antibody also recognized two faster migrating forms that correspond to immature proNRG α 2c (ref. 24; data not shown). Finally, the antibody detected several cell-bound tail fragments that are devoid of the extracellular domain of proNRG α 2c. Immunofluorescence experiments indicated that proNRG α 2c and the tail fragments accumulated at the plasma membrane, and in some intracellular sites (Fig. 1A, bottom).

We created ErbB2-overexpressing MCF7 cells by transfection of ErbB2 and selection of G418-resistant clones followed by analysis of ErbB2 expression by Western blotting. This resulted in the identification of several clones that expressed ErbB2 at different levels above those present in wild-type MCF7 cells (Fig. 1B, top;

data not shown). Immunofluorescence analysis with the Herceptin antibody revealed a significantly higher cell surface ErbB2 staining of MCF7-ErbB2 cells compared with parental MCF7 cells (Fig. 1B, bottom).

Comparison of the growth properties of MCF7, MCF7-ErbB2, and MCF7-NRG α 2c cells indicated that cells expressing proNRG α 2c grew to higher densities than parental or ErbB2-overexpressing MCF7 cells (Fig. 1C, top). These effects were evidenced when cells were cultured at low serum concentrations (0%, 0.1%, or 1% FBS; Fig. 1C, top; data not shown). At higher serum concentrations (10% FBS) the three cell types grew analogously (data not shown). As expected, NRG did not significantly increase the proliferation of MCF7 cells already expressing proNRG α 2c (Fig. 1C, bottom). In

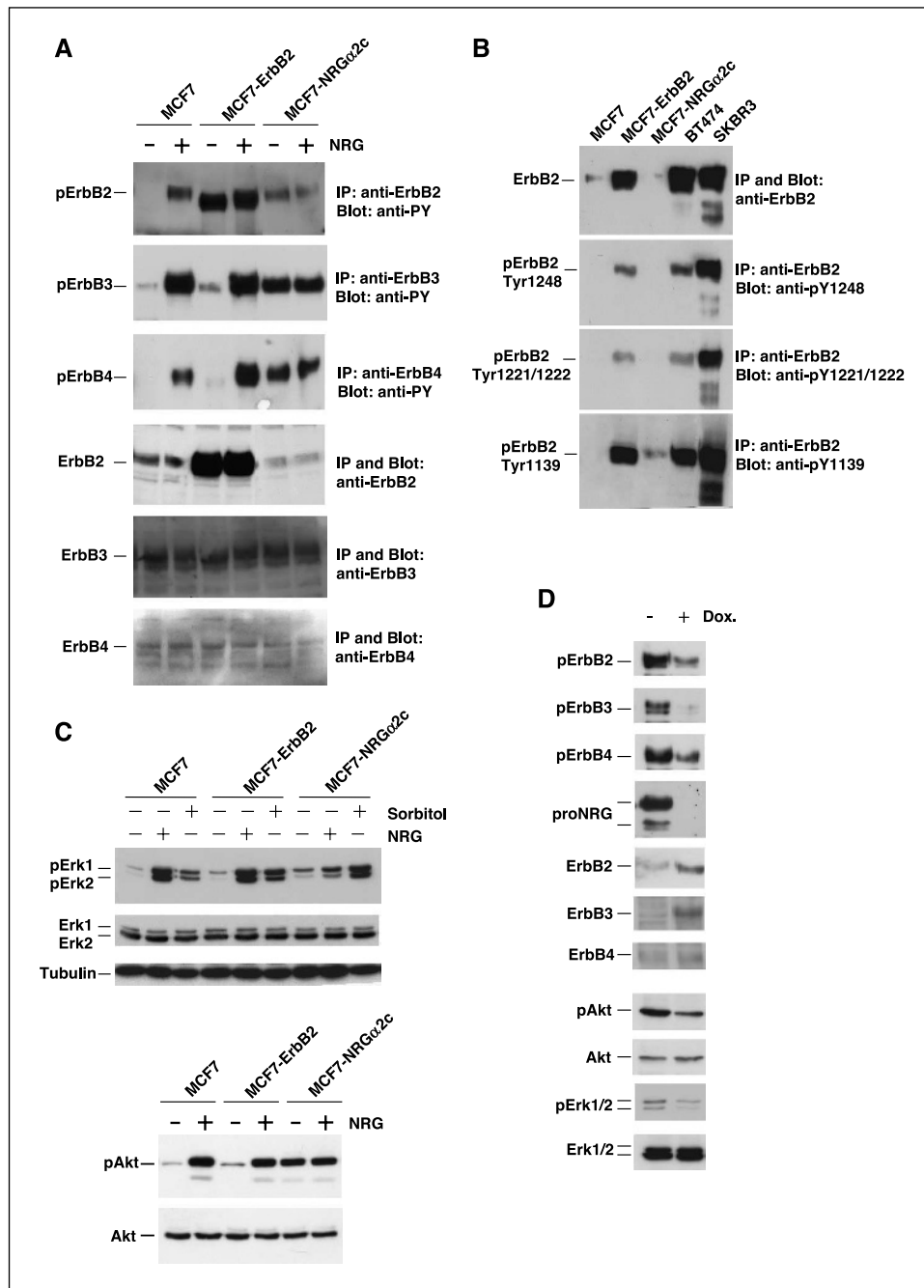


Figure 2. Analysis of early cell signaling events in MCF7, MCF7-NRG α 2c, and MCF7-ErbB2 cells. **A**, cells were serum starved for 12 hours and treated where indicated with 10 nmol/L NRG for 15 minutes, lysed, and immunoprecipitated with anti-ErbB2, anti-ErbB3, or anti-ErbB4 antibodies. One half of the precipitate was run on a 6% gel, and blots were probed with anti-phosphotyrosine antibodies. The other part of the immunoprecipitate was also run on a 6% gel and blotted with the respective anti-receptor antibodies. **B**, phosphorylation of different tyrosine residues in ErbB2. One milligram of cell extracts from the different cell lines was prepared and immunoprecipitated with anti-ErbB2 antibodies, followed by Western blotting with antibodies specific for pTyr¹²⁴⁸, pTyr^{1221/1222}, pTyr¹¹³⁹, or ErbB2. **C**, Erk1/2 and Akt activation state in MCF7, MCF7-NRG α 2c, and MCF7-ErbB2 analyzed by Western blotting of cell lysates. NRG (10 nmol/L, 15 minutes), or sorbitol (0.7 mol/L, 30 minutes) was added where indicated. Erk1/2 dual phosphorylation was analyzed with an anti-phosphoErk1/2 antibody. As controls for loading of the anti-pErk blot, blots of total Erk1/2 and tubulin are shown. pAkt was analyzed in cell lysates from cells treated or not with NRG (10 nmol/L) by using an anti-pAkt antibody. Levels of Akt (bottom). **D**, status of different signal transduction molecules in MCF7-NRG α 2c^{TetOff} cells. Cells were cultured with doxycycline where indicated (10 ng/mL), and the levels and activation of different signaling molecules analyzed as indicated above.

contrast, both wild-type and ErbB2-overexpressing MCF7 cells were strongly stimulated to grow when NRG was added to the culture medium. Interestingly, the MTT uptake values reached in the presence of the soluble growth factor were of a magnitude analogous to that observed in MCF7-NRG α 2c cells. Similar results were obtained with two different ErbB2-overexpressing clones and with an MCF7-ErbB2-overexpressing clone obtained from another laboratory, indicating that the results obtained in MCF7-ErbB2 represented the response of MCF7 cells to ErbB2 overexpression. To further verify that the expression of proNRG α 2c positively stimulated MCF7 cell proliferation and exclude any clonal differences, we expressed proNRG α 2c in MCF7 cells under the control of the tetracycline transactivator system. As shown in Fig. 1D, proNRG α 2c expression could be strongly repressed by doxycycline in MCF7-NRG α 2c^{TetOff} cells. In the absence of doxycycline, these cells proliferated over time, and by 8 days the MTT uptake had doubled the values obtained at the start of the experiment (Fig. 1D, bottom). Repression of NRG production strongly reduced MTT uptake.

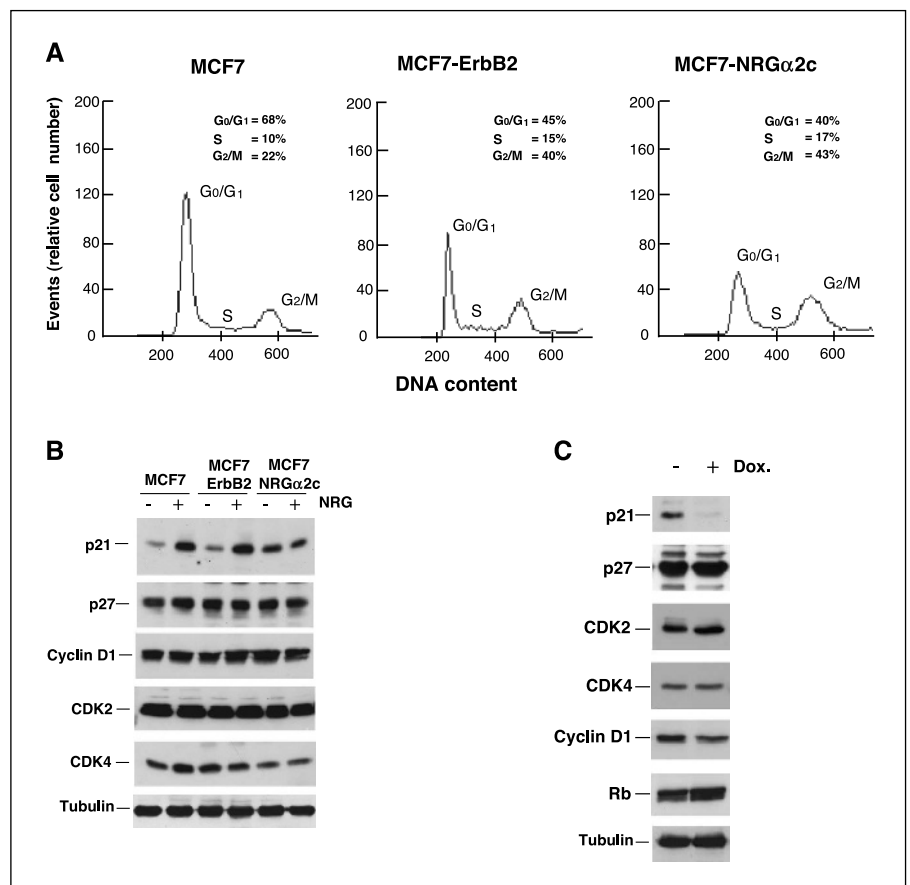
Differential activation of neuregulin receptors in MCF7, MCF7-ErbB2, and MCF7-NRG α 2c cells. To search for possible differences in signaling routes that may explain the distinct proliferation rates observed in these cell lines, we started analyzing the tyrosine phosphorylation state of ErbB2, ErbB3, and ErbB4. Under resting conditions, tyrosine phosphorylation of these receptors was very low to undetectable in parental MCF7 cells (Fig. 2A). ErbB2 was highly tyrosine phosphorylated in MCF7-ErbB2 cells, whereas ErbB3 and ErbB4 tyrosine phosphorylation was much lower. In MCF7-NRG α 2c cells, ErbB2, ErbB3, and ErbB4

were tyrosine phosphorylated under resting conditions, indicating that in these cells expression of proNRG α 2c caused permanent resting activation of ErbB2, ErbB3, and ErbB4 receptors.

Soluble NRG caused ErbB2, ErbB3, and ErbB4 tyrosine phosphorylation in MCF7 cells, and of ErbB3 and ErbB4 in MCF7-ErbB2 cells (Fig. 2A). Addition of soluble NRG to MCF7-NRG α 2c cells did not substantially affect tyrosine phosphorylation of ErbB receptors. Analysis of the levels of ErbB receptors indicated that the amounts of ErbB3 and ErbB4 were identical in the different cell lines. ErbB2 levels were higher in MCF7-ErbB2 cells than in MCF7 cells, that in turn had a higher level of expression than MCF7-NRG α 2c cells. We also investigated the phosphorylation of distinct phosphotyrosine residues of ErbB2 in MCF7-ErbB2 and MCF7-NRG α 2c cells. In these experiments, we also used as controls BT474 and SKBR3 cells that are widely used models of ErbB2 overexpressor cell lines. Western blotting with antibodies specific to pTyr¹²⁴⁸, pTyr^{1221/1222}, and pTyr¹¹³⁹ of ErbB2 indicated that ErbB2 overexpression was associated with phosphorylation at all these residues (Fig. 2B), very much resembling the phosphorylation state of these sites in BT474 cells. In SKBR3 cells, the level of phosphorylation of these individual phosphotyrosine sites was higher. In MCF7-NRG α 2c, tyrosine phosphorylation of residues 1248 and 1221/1222 was very hard to detect. However, tyrosine phosphorylation at residue 1139 was detected in these cells.

Signaling by MCF7, MCF7-ErbB2, and MCF7-NRG α 2c cells. The differences found in the state of ErbB receptors in the three cell lines analyzed prompted us to study the downstream signaling routes that link these receptors to proliferative responses in breast

Figure 3. Cell cycle regulatory proteins in MCF7, MCF7-NRG α 2c, and MCF7-ErbB2 cells. A, cell cycle analysis of the three different cell lines. Exponentially growing cells were trypsinized and cell cycle profiles analyzed by propidium iodide staining and fluorescence-activated cell sorting as described in Materials and Methods. Calculated percentage of cells in each phase of the cell cycle. B, MCF7, MCF7-NRG α 2c, and MCF7-ErbB2 cells were treated, where indicated, with NRG (10 nmol/L, 24 hours in serum-free medium), and the levels of p21, p27, cyclin D1, CDK2, and CDK4 analyzed by Western blotting. As a control for the loading of the samples, a blot evaluating the levels of tubulin was done. C, expression of cell cycle proteins in MCF7-NRG α 2c^{TetOff} cells. Doxycycline (10 ng/mL) was added, where indicated, for 4 days in serum-free medium, and then lysates prepared. Western blotting of the indicated proteins was done with the antibodies shown.



cancer cells. Figure 2C shows the results of the analyses done on the Erk1/2 and the Akt routes. Western blotting with antibodies that recognize activated forms of Erk1/2 indicated that the resting levels of pErk1/2 were higher in MCF7-NRGα2c cells than in MCF7 or in MCF7-ErbB2 cells (Fig. 2C). Erk1/2 dual phosphorylation was strongly induced by NRG in both MCF7 and MCF7-ErbB2 cells. In contrast, exogenous NRG caused a small increase in the amount of pErk1/2 in MCF7-NRGα2c cells. That this failure was not due to a refractoriness of Erk1/2 to be phosphorylated in MCF7-NRGα2c cells was indicated by the stimulation with sorbitol (Fig. 2C). We also analyzed Erk5 activation state because this mitogen-activated protein kinase has been linked to the regulation of cell proliferation (25). Erk5 was partially activated in MCF7-NRGα2c cells, whereas in wild-type cells most of the Erk5 was in the inactive state (data not shown).

Akt phosphorylation was also up-regulated in MCF7-NRGα2c cells with respect to MCF7 or MCF7-ErbB2 cells (Fig. 2C). Addition of NRG increased Akt phosphorylation in MCF7 and MCF7-ErbB2 cells, but in MCF7-NRGα2c cells, the soluble growth factor was unable to increase Akt phosphorylation above resting levels. The effect of proNRGα2c on the activation state of these signaling pathways was confirmed in MCF7-NRGα2c^{TetOff} cells (Fig. 2D). Interestingly, reduction of NRG expression was followed by up-regulation of ErbB2.

p21 increases in neuregulin-expressing cells and participates in the proliferation of MCF7 cells. We also analyzed the effect of NRG expression and ErbB2 overexpression on the cell cycle profile and the expression of several proteins involved in cell cycle regulation. As shown in Fig. 3A, a decrease in G₀-G₁ phases of the cell cycle and a concomitant increase in G₂-M was observed in both MCF7-ErbB2 and MCF7-NRGα2c, when compared with wild-type MCF7 cells. Analyses of several proteins implicated in cell cycle progression indicated no major changes in CDK4, CDK2, cyclin D1, and p27 (Fig. 3B). However, comparison of the level of p21 between MCF7, MCF7-ErbB2, and MCF7-NRGα2c cells indicated that the latter had a higher resting level of p21 (Fig. 3B). Furthermore, addition of exogenous NRG induced an increase in p21 in MCF7 and MCF7-ErbB2 cells. NRG did not substantially affect the levels of p21 in MCF7-NRGα2c cells. That NRG expression caused a sustained increase in the level of p21 was further verified in MCF7-NRGα2c^{TetOff} cells (Fig. 3C).

Analyses of p21- or p27-associated CDK4 indicated that this kinase, as well as cyclin D1, equally associated to p27 in MCF7, MCF7-ErbB2, and MCF7-NRGα2c cells (Fig. 4A). This association was independent of the presence of exogenously added NRG. Resting p21-associated cyclin D1 and CDK4 levels were much higher in MCF7-NRGα2c cells than in MCF7 or MCF7-ErbB2 cells. The latter showed a slightly higher resting level of p21-associated

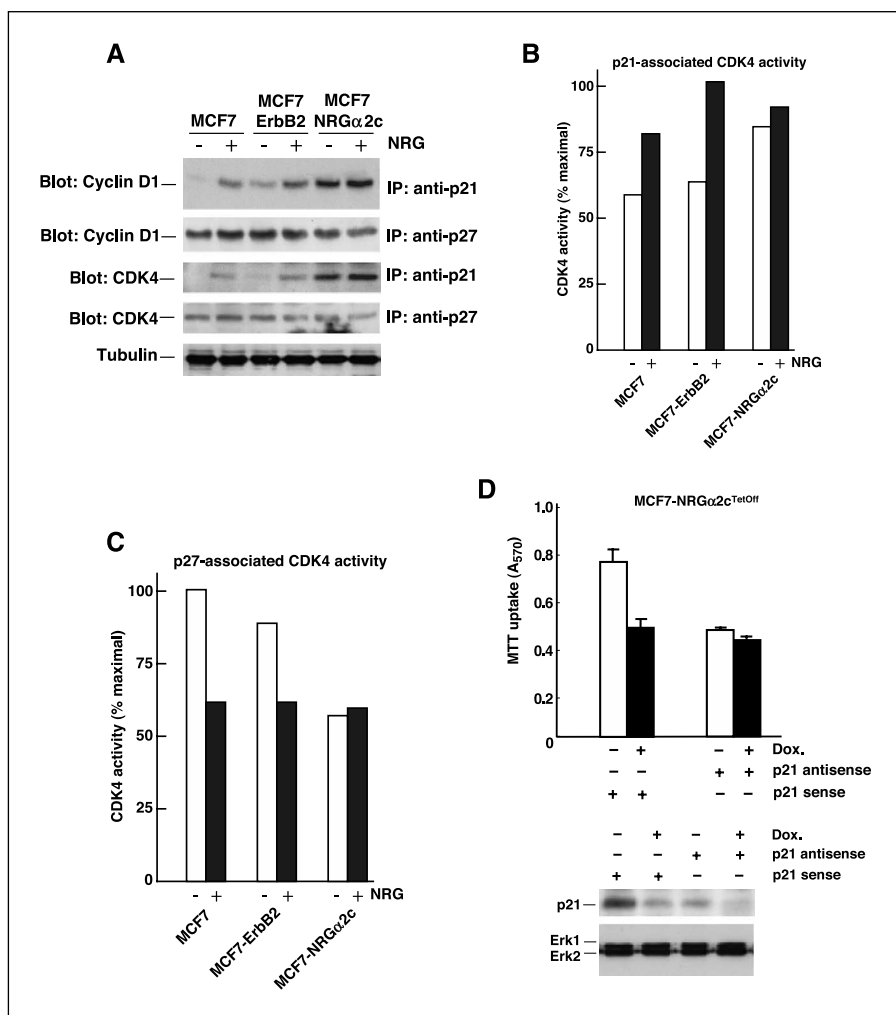


Figure 4. Positive regulatory role of p21 on CDK activity and cell proliferation in MCF7 cells. **A**, MCF7, MCF7-NRGα2c, and MCF7-ErbB2 cells were incubated with NRG for 24 hours in serum-free medium, and then lysates prepared to be immunoprecipitated with anti-p21 or anti-p27 antibodies. Blots were probed with anti-cyclin D1 or anti-CDK4 antibodies. Coimmunoprecipitations were carried out as above. As a control for the loading of the samples, a blot evaluating the levels of tubulin before the immunoprecipitations was done. p21-associated (**B**) and p27-associated (**C**) CDK4 activity. Where indicated, MCF7, MCF7-NRGα2c, and MCF7-ErbB2 cells were incubated with NRG for 24 hours, and then p21 or p27-associated CDK4 activity measured by an *in vitro* kinase assay using GST-Rb as a substrate. Dried gels were subjected to digital analysis of radioactivity and the results represented. Data are from an experiment that was done thrice. **D**, cells were transfected with the sense or antisense p21 oligonucleotides. Cell proliferation was analyzed 3 days later. *Columns*, mean of quadruplicates; *bars*, ±SD. In parallel, cell lysates were obtained under the same conditions, and the levels of p21 were analyzed by Western blotting. Doxycycline was added at 10 ng/mL where indicated. Erk1/2 was used as loading control.

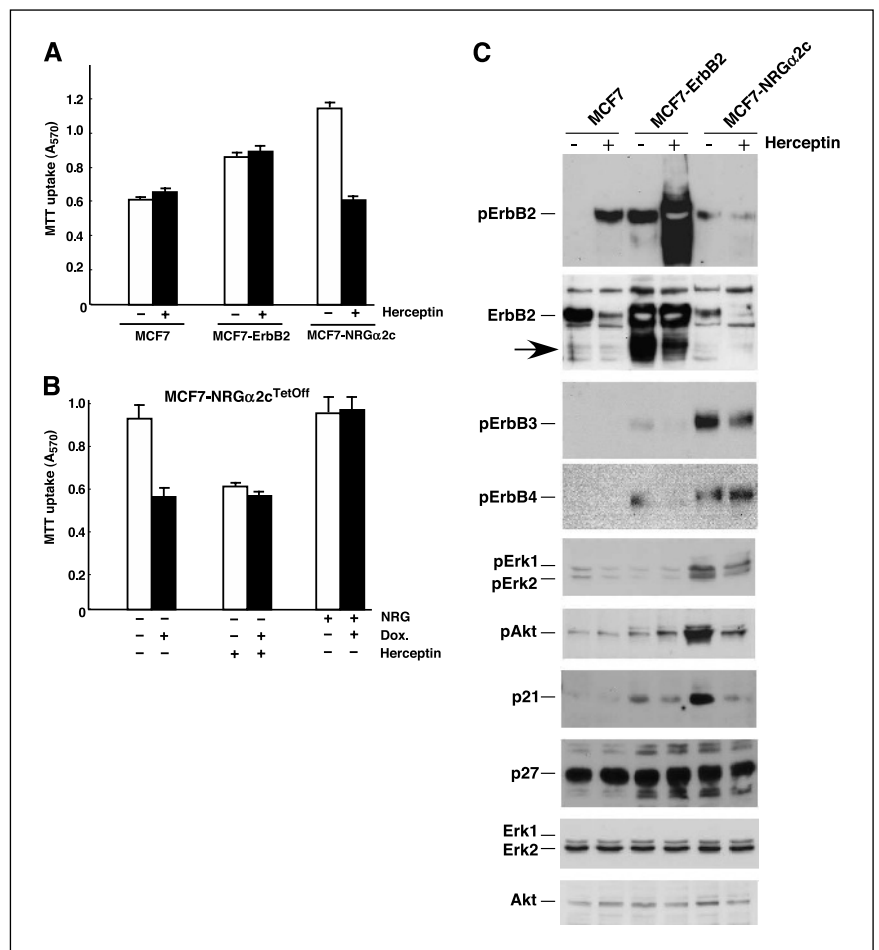
cyclin D1 than wild-type MCF7 cells. Addition of exogenous NRG increased the amounts of CDK4 and cyclin D1 associated with p21 in MCF7 and MCF7-ErbB2 cells but had no effect on the association of p21 with these molecules in MCF7-NRG α 2c cells. p21-associated CDK4 activity was up-regulated in MCF7 and MCF7-ErbB2 cells treated with NRG with respect to untreated cells but was only slightly increased in MCF7-NRG α 2c cells (Fig. 4B). Interestingly, the resting level of p21-associated CDK4 activity in MCF7-NRG α 2c cells was higher than that present in MCF7 or MCF7-ErbB2 cells. In contrast, p27-associated CDK4 activity was significantly decreased in MCF7 and MCF7-ErbB2 cells treated with NRG, whereas it did not substantially change in MCF7-NRG α 2c cells (Fig. 4C). In the latter, the resting level of p27-associated CDK4 was analogous to the values found in MCF7 and MCF7-ErbB2 cells treated with NRG.

Because of the clear positive regulation of p21 levels in MCF7 cells by NRG, we explored to which extent proliferation of these cells depends on p21. To this end, MCF7-NRG α 2c^{TetOff} cells were transfected with an antisense oligodeoxynucleotide previously used to decrease p21 levels in MCF7 cells (28). A significant decrease in the proliferation of these cells was observed when they were transfected with the antisense oligodeoxynucleotide compared with the proliferation of cells transfected with the sense oligodeoxynucleotide that was used as a control (Fig. 4D). Western blotting of p21 verified that the antisense oligodeoxynucleotide did in fact decrease the endogenous p21 levels (Fig. 4D).

Anti-ErbB2 antibodies prevent the proliferation of MCF7-NRG α 2c cells and down-regulate p21. The differences in signaling between MCF7-ErbB2 and MCF7-NRG α 2c cells led us to investigate whether they also showed a distinct behavior in their response to Herceptin. As shown in Fig. 5A, treatment with the antibody completely blunted the effect that on proliferation had the expression of proNRG α 2c. Cells treated with the antibody grew to a similar extent than MCF7 parental cells (Fig. 5A) or MCF7-NRG α 2c^{TetOff} cells treated with doxycycline (Fig. 5B). Doxycycline did not affect NRG responsiveness as indicated by the fact that addition of exogenous NRG to MCF7-NRG α 2c^{TetOff} cells caused proliferation of these cells to levels analogous to cells allowed to express transfected NRG (Fig. 5B). The proliferation of MCF7, and more interestingly that of the MCF7-ErbB2 cells, was not decreased by treatment with the antibody (Fig. 5A).

We analyzed the effect of the Herceptin treatment on ErbB2-mediated signaling in the three cellular conditions. Treatment with Herceptin increased tyrosine phosphorylation of ErbB2 in wild-type MCF7 and in MCF7-ErbB2 cells (Fig. 5C). The effect was sustained as it was observed upon 48 hours of treatment with the antibody. In MCF7-NRG α 2c cells, the antibody slightly decreased ErbB2 tyrosine phosphorylation. Although the antibody increased the tyrosine phosphorylation of ErbB2 in MCF7 cells, the antibody decreased the content of this receptor tyrosine kinase. The antibody also decreased the amount of ErbB2 in MCF7-NRG α 2c cells, although the continuous presence of NRG decreased the

Figure 5. Action of the anti-ErbB2 antibody Herceptin on proliferation and signaling of MCF7, MCF7-NRG α 2c, and MCF7-ErbB2 cells. **A**, MCF7, MCF7-NRG α 2c, and MCF7-ErbB2 cells were plated at identical densities in 24-well dishes, and serum-containing medium replaced 1 day later by serum-free medium with the addition, where indicated, of 50 nmol/L Herceptin. Cell proliferation was measured 4 days later. *Columns*, mean of quadruplicates of an experiment that was repeated four times; *bars*, \pm SD. **B**, action of Herceptin on the proliferation of MCF7-NRG α 2c^{TetOff} cells. Cells were plated in the presence or absence of the indicated compounds (NRG 10 nmol/L, Herceptin 50 nmol/L, doxycycline 10 ng/mL) and cell proliferation was measured 3 days later. **C**, action of Herceptin (50 nmol/L, 48 hours) on the levels and phosphorylation state of several signaling proteins in MCF7, MCF7-NRG α 2c, and MCF7-ErbB2 cells. The levels and phosphorylation state of the analyzed proteins was carried out by Western blotting of cell extracts, with the exception of pErbB2, ErbB2, pErbB3, and pErbB4, which were analyzed after immunoprecipitation. The invariant levels of Erk1/2 were taken as an indication of equal protein loading.



steady-state level of ErbB2. In MCF7-ErbB2 cells, the Herceptin antibody was unable to substantially decrease the amount of ErbB2 even at high (50 nmol/L) antibody concentrations. A small decrease in a truncated form of ErbB2, often present in ErbB2-overexpressing cells (29), was observed (Fig. 5C, *arrow*). Treatment with Herceptin did not affect tyrosine phosphorylation of ErbB3 or ErbB4 in MCF7 cells. In MCF7-ErbB2 cells, that presented a small degree of ErbB3 and ErbB4 tyrosine phosphorylation, Herceptin decreased the amount of tyrosine phosphorylation of these receptors. In MCF7-NRG α 2c cells, ErbB3 tyrosine phosphorylation was slightly decreased by treatment with Herceptin, whereas the level of ErbB4 tyrosine phosphorylation was not substantially modified by the antibody. Analysis of the phosphorylation status of Erk1/2 and Akt indicated that the antibody decreased the amount of these phosphorylated proteins in MCF7-NRG α 2c cells, especially that of Akt, without affecting their total amount. A strong decrease in the amount of p21 was also observed in MCF7-NRG α 2c cells, and a more moderate decrease occurred in MCF7-ErbB2 cells treated with Herceptin. However, the level of p27 did not significantly change in the presence of Herceptin in any of the different cell lines.

Discussion

In this work, the side-by-side comparison of breast cancer cells overexpressing ErbB2 or expressing membrane NRG, has allowed us to uncover important differences in the signaling and proliferation in these two cellular situations. In addition, the differential sensitivity of both cell types to Herceptin may have clinical implications, considering the potential extension of the use of this antibody to cases in which NRG acts feeding the tumoral tissue, even in the absence of ErbB2 overexpression.

We initiated the present study with the purpose of comparing the proliferative potential of breast cancer cells overexpressing ErbB2, to those producing the ErbB ligand NRG under conditions of low to normal ErbB2 expression. The main reason that moved us to carry out this study was the absence of such a comparative study. Significant differences were found when proliferation of wild-type MCF7 cells was compared with MCF7-NRG α 2c and MCF7-ErbB2 cells. MCF7-NRG α 2c proliferated at higher rates than MCF7-ErbB2 cells that in turn proliferated better than MCF7 cells. The level of proliferation of MCF7-NRG α 2c was unaffected by the addition of exogenous NRG. However, addition of this soluble factor caused MCF7 and MCF7-ErbB2 cells to proliferate to values analogous to those of MCF7-NRG α 2c. This indicates that the proliferation potential of the three cell lines in response to NRG was identical and that availability of the ligand was the critical factor, rather than overexpression of ErbB2. This also indicates that availability of the ligand by a tumoral cell may be more advantageous to its proliferation than overexpression of the ErbB2 protein, especially in regions of the tumoral tissue where nutrient supply is limited. In fact, when the proliferation of these three cell types was analyzed in the presence of high serum concentrations, their growth was identical, suggesting that factors present in the serum may substitute the growth-supporting properties conferred by NRG availability or ErbB2 overexpression. Furthermore, when the three cell lines were analyzed for their sensitivity to Herceptin under conditions of optimal serum concentrations, the antibody slightly but significantly reduced their proliferation potential, indicating that some of the factors present in the serum may correspond to ErbB ligands with capacity to activate ErbB2.¹

An important difference between the distinct cell types was the tyrosine phosphorylation levels of ErbB proteins. MCF7 cells had low or undetectable levels of ErbB2, ErbB3, or ErbB4 tyrosine phosphorylation, and their phosphorylation was increased by treatment with NRG. In contrast, MCF7-NRG α 2c had a resting level of ErbB2, ErbB3, and ErbB4 phosphorylation, and the degree of phosphorylation of these receptors did not substantially change upon NRG addition. In the case of MCF7-ErbB2 cells, overexpression of ErbB2 was accompanied by a high degree of resting tyrosine phosphorylation of ErbB2, and this was not substantially increased by treatment with NRG. The degree of tyrosine phosphorylation was even higher than that observed in MCF7 cells treated with NRG or MCF7-NRG α 2c cells.

The differences in the tyrosine phosphorylation status of ErbB receptors in MCF7-ErbB2 versus MCF7-NRG α 2c cells in relation to the proliferation of these two cell types merit some comments. It is unclear why a high level of ErbB2 tyrosine phosphorylation did not correlate with a strong proliferation response, particularly because overexpression of ErbB2 has been shown to cause oncogenic transformation in other cell lines (12). A possibility that may explain the dissociation between ErbB2 tyrosine phosphorylation and cell proliferation is ErbB2 phosphorylation at tyrosine sites that are unable to drive a proliferation signal. An indirect indication for such idea is the observation that stimulation with NRG caused the ErbB2 receptor to migrate with an apparently higher M_r than overexpressed and phosphorylated ErbB2, as shown in Fig. 2. This distinct mobility could be due to NRG-induced phosphorylation of ErbB2 in different or more tyrosine residues, or in other residues important in the mitogenic effect of ErbB2. Five major tyrosine phosphorylation sites have been studied for their implications in the transforming activity of ErbB2 (1). Tyr¹¹³⁹, Tyr¹¹⁹⁶, Tyr^{1221/1222}, and Tyr¹²⁴⁸ seem to independently mediate transforming signals, whereas phosphorylation of Tyr¹⁰²³ acts negatively, impairing neu-dependent transformation (30). Analyses by phosphorylation state antibodies indicated that overexpression of ErbB2 in MCF7 cells was accompanied by a strong increase in the tyrosine phosphorylation of Tyr¹¹³⁹, Tyr^{1221/1222}, and Tyr¹²⁴⁸. In MCF7-NRG α 2c cells, the only residue of the three analyzed that was clearly phosphorylated was Tyr¹¹³⁹. Therefore, whereas differences exist between the tyrosine phosphorylation status of ErbB in MCF7-ErbB2 with respect to MCF7-NRG α 2c cells, the preferential phosphorylation of ErbB2 at the sites analyzed fails to explain why MCF7-ErbB2 cells grew less than MCF7-NRG α 2c cells.

An alternative situation that may explain the relatively poor coupling of ErbB2 overexpression in MCF7 cells to proliferation could be inefficient lateral signaling from ErbB2 to other ErbB receptors (31). In fact, overexpression of ErbB2 was not followed by tyrosine phosphorylation of ErbB3 and ErbB4. On the other side, expression of NRG in MCF7 cells resulted in resting tyrosine phosphorylation of ErbB2, ErbB3, and ErbB4 that correlated with efficient proliferation responses. Therefore, it seems that effective proliferation signaling from ErbB2 requires interaction of ErbB2 with other ErbB receptors, and the sole increase in tyrosine phosphorylation of ErbB2 may not be a sufficiently strong proliferative stimulus, at least in our cellular model. In line with this, a recent study done in several cell lines overexpressing ErbB2 has concluded that overexpression of ErbB2 alone is not sufficient

¹ L. Yuste and A. Pandiella, unpublished observations.

and that ErbB3 is required for efficient proliferation (13). Our findings complement this concept indicating that coexpression of ErbB3 with ErbB2 overexpression is insufficient to drive proliferation, unless both receptors are activated.

Downstream signaling differences were also found among the three cell types. MCF7-NRG α 2c had higher resting levels of active forms of Erk1/2, Erk5, and Akt than the other two cell lines. These signaling pathways could easily be activated in MCF7 and MCF7-ErbB2 by exogenous NRG to higher levels than those present in MCF7-NRG α 2c. In addition, in the latter, exogenous NRG did not have a major effect on the activation of Akt and caused only a small increase in the levels of pErk1/2 that however never reached the levels obtained by acute stimulation of MCF7 or MCF7-ErbB2 cells with NRG. The interesting conclusion in this respect is that stimulation of proliferation probably does not require a high degree of sustained activation of proliferative signaling pathways. In fact, a small but tonic stimulation of signaling molecules may be sufficient to trigger an optimal proliferative response.

Our findings on p21 fall in line with the concept of p21 acting as a "modulator" (32) rather than merely an "inhibitor" of MCF7 proliferation. Biochemical analyses of cell cycle regulatory proteins showed that the amounts of CDK4, CDK2, p27, and cyclin D1 did not significantly change among the three cell lines. However, a substantial difference was observed when analyzing the amount of p21. The level of this protein was high in MCF7-NRG α 2c cells when compared with MCF7 and MCF7-ErbB2 cells. In addition, prolonged stimulation of the latter two cell lines with NRG provoked a substantial increase in p21. This was found to be unexpected given the cell cycle inhibitory role proposed for p21 (33). However, evidence indicates that the presence of p21 is also required for proper assembly of cyclin-CDK complexes and normal cell cycle physiology (34). Furthermore, studies carried out in MCF7 cells have indicated that p21 up-regulation is required for serum (28) or insulin-like growth factor-I-induced proliferation (35). In line with these findings were our results in MCF7 cells. Here, increased levels of p21 were accompanied by augmentation of p21-associated cyclin D1, CDK4, and CDK2. In contrast, the levels of p27 associated with cyclin D1 and CDK4 did not substantially change in any of the cell lines studied and irrespective of whether they were treated or not with NRG. However, we did detect a differential p27-associated CDK4 activity. Thus, in MCF7 and MCF7-ErbB2 cells, treatment with NRG caused a decrease in p27-associated CDK4 activity to levels analogous to the resting CDK4 activity of MCF7-NRG α 2c cells. In contrast, p21-associated CDK4 activity was increased by NRG in MCF7 and MCF7-ErbB2 cells but not in MCF7-NRG α 2c. Therefore, p21 may act as a modulator of cell cycle entry in MCF7 cells by positively up-regulating CDK4 activity. In agreement with a role of p21 in cell cycle progression in MCF7 cells are the results obtained by using the antisense p21 oligonucleotide. These experiments indicated that p21 reduction by antisense treatment caused a significant decrease in MCF7 cell number in either MCF7² or MCF7-NRG α 2c^{TetOff} cells.

One of the most interesting findings of our work was the differential response of the three cell types to Herceptin. Biochemically, Herceptin increased ErbB2 tyrosine phosphorylation in MCF7 and MCF7-ErbB2 cells but decreased the resting level of ErbB2 tyrosine phosphorylation in MCF7-NRG α 2c cells

and decreased ErbB2 levels in the three cell types. Interestingly, although the antibody did increase ErbB2 tyrosine phosphorylation in MCF7 and especially in MCF7-ErbB2 cells, it did not significantly increase the proliferation of these cells. This is in line with the comments above on the dissociation between ErbB2 tyrosine phosphorylation and cell proliferation and further supports that the sole induction of ErbB2 phosphorylation is inefficient to stimulate cell duplication in MCF7 cells. Rather, a partnership with other ErbB receptors seems required for ErbB2 to act as an efficient stimulator of proliferation. In this respect, it is interesting that treatment with Herceptin decreased pErbB3, analogously to the effect of the antibody on pErbB3 in BT474 cells (36), and did not affect pErbB4 tyrosine phosphorylation levels in MCF7-NRG α 2c cells. These results, when complemented with the strong inhibition of cell proliferation by Herceptin in these cells, further support the concept that ErbB2 is required for proliferation of MCF7 cells when ErbB3/4 are activated by NRG and also indicates that the sole activation of ErbB3 or ErbB4 is insufficient to drive a proliferation signal in MCF7 cells when NRG is available. In MCF7-NRG α 2c cells, Herceptin significantly decreased the phosphorylation of pAkt, pErk1/2, and the levels of p21, in concordance with the role of these molecules in the regulation of MCF7 cell proliferation by NRG. Whereas other reports have indicated that the antiproliferative action of Herceptin is accompanied by an increase in p27 levels (15, 36), we did not observe major changes in p27 upon antibody treatment of MCF7 cells. However, it should be commented that these studies used different cell types to the ones used here, a condition that may explain such differences. It is also possible that Herceptin may affect p27 function without affecting its levels. In fact, Akt-dependent phosphorylation of p27 at Thr¹⁵⁷ has been shown to control its nucleocytoplasmic distribution and function (37, 38). Because Akt phosphorylation is decreased in MCF7-NRG α 2c cells upon Heregulin treatment, it is possible that this may be followed by p27 relocalization. Further work will be required to investigate this possibility. Whatever the reason, our studies complement those reported before (15, 36), indicating that Heregulin may act through p27-dependent and p27-independent mechanisms in distinct breast cancer cell types.

The inhibition of signaling in MCF7-NRG α 2c cells by Herceptin is apparently in contrast to previous reports that indicated that NRG-induced signaling was unaffected by this antibody but was sensitive to pertuzumab, a distinct anti-ErbB2 antibody (26). In fact, when MCF7 cells were treated with soluble NRG, signaling and proliferation were insensitive to Herceptin.³ This indicates that the interaction of soluble NRG with ErbB receptors may somehow be distinct from that of transmembrane NRG, and opens the interesting question of how ErbB receptors may be activated by the transmembrane ligand. A study trying to address the differences in signaling and proliferation between cells expressing soluble or membrane-bound NRG is required.

In our experimental model, Herceptin did not affect the proliferation of wild-type or ErbB2-overexpressing MCF7 cells. In contrast, the antibody abrogated the proliferation of MCF7-NRG α 2c cells. In the presence of Herceptin, the proliferation rate of NRG-expressing MCF7 cells was analogous to that of wild-type MCF7 cells. Several interesting conclusions can be extracted

² A. Pandiella and J.C. Montero, data not shown.

³ J.C. Montero and A. Pandiella, in preparation.

from such data. The high efficiency of Herceptin in preventing proliferation of breast cancer cells expressing normal levels of the ErbB receptors, but having access to NRG (autocrinely produced, or provided by the surrounding stroma), provokes the question of whether expression of NRG should be assessed in order for treatments with anti-ErbB2 to be more efficient. Furthermore, expression of NRG1 has been found in 30% of breast cancers (20), and more studies are required to fully understand how often expression of NRGs from other genes is present (21). In this respect, whereas data on the expression of transmembrane NRGs in breast pathology is missing, preliminary studies indicate that proNRG is present in a number of pretumoral and neoplastic breast lesions.⁴ At present, we are retrospectively evaluating the response to Herceptin treatment in breast cancer patients with known content of proNRG and ErbB2. In view of such a situation, it seems quite reasonable that

correct molecular pathologic analysis of breast carcinomas should include the detection of NRGs, ErbBs, and active forms of these receptors and signaling intermediates.

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⁴ A. Pandiella, unpublished data.

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