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## Introduction

ErbB4 is a member of the ErbB family of receptor tyrosine kinases, a family that also includes the Epidermal Growth Factor Receptor (EGFR/ErbB1), ErbB2/HER2/Neu, and ErbB3/HER3. EGFR and ErbB2 are frequently overexpressed in prostate tumor samples, and overexpression and constitutive signaling by these receptors is thought to contribute to prostate malignancy. In contrast, ErbB4 expression and or signaling in many tumor types correlates with a differentiated, less aggressive phenotype and loss of ErbB4 expression and/or signaling correlates with tumorigenesis and increased tumor aggression [Bacus, *et al.*, 1995; Graber, *et al.*, 1999; Kew, *et al.*, 2000; Pawlowski, *et al.*, 2000]. Particularly intriguing is that whereas ErbB4 is expressed in benign basal and luminal prostate tissue, ErbB4 expression is not observed in the vast majority of a small number of prostate tumor specimens [Robinson, *et al.*, 1996; Grasso, *et al.*, 1997; Lyne, *et al.*, 1997]. This suggests that ErbB4 signaling may suppress tumorigenesis or the aggressive behavior of tumor cells. *Thus, our hypothesis is that ErbB4 is a prostate tumor suppressor that is coupled to reduced prostate tumor cell aggressiveness.*

## Body

The approved statement of work contained four tasks. In this section we will describe our progress in addressing these aims.

*1. Generate LnCAP, PC-3, DU-145, and MDA-Pca-2b prostate cancer cell lines that express constitutively-active ErbB4 mutants.* Last year we reported the generation of three constitutively-active ErbB4 mutants (see Figures 1 and 2 of Penington, *et al.*, 2002). We also reported that we had generated LnCAP, PC-3, DU-145, and MDA-Pca-2b cell lines that stably express the constitutively-active H647C and A648C ErbB4 mutants but that we had failed to generate cell lines that stably express the ErbB4 Q646C mutant. The mechanism of this failure appeared to be that infection with the recombinant retrovirus encoding the Q646C ErbB4 mutant (and a neomycin resistance gene) resulted in much fewer G418-resistant colonies of DU-145 and PC-3 cells than did infection with the other retroviruses (see Figures 1 and 2 of Williams, *et al.*, 2003). Indeed, last year we reported that the Q646C ErbB4 mutant specifically inhibited drug-resistant colony formation by the PC-3 and DU-145 cells by 75 to 90% respectively (see Table 1 and the associated text of Williams, *et al.*, 2003). These data indicating that ErbB4 inhibits colony formation on plastic by human prostate tumor cell lines suggest that ErbB4 is a prostate tumor suppressor. Thus, while we have failed to complete the task, we developed an alternative strategy to evaluate our hypothesis and this strategy was successful in generating data in support of our hypothesis.

*2. Identify the biological effects of ErbB4 signaling on the four prostate cancer cell lines.* Since we are unable to generate prostate tumor cell lines that express the Q646C mutant, we have opted to assay the effects of ErbB4 signaling on prostate tumor cell lines using the assay for inhibition of drug-resistant colony formation [Williams, *et al.*, 2003]. Again, these data support our hypothesis that ErbB4 is a prostate tumor suppressor.

Currently we are assessing whether signaling by the Q646C mutant inhibits drug-resistant colony formation by PC-3 and DU-145 cells by inducing apoptosis or by causing growth arrest. Analogous experiments have been performed using the MCF-10A human mammary epithelial cell line. MCF-10A cells were seeded at low density in a 60 mm culture dish. The following

day, twenty isolated cells were marked by circling their location on the underside of the dish using an indelible marker. The cells were then infected with the ErbB4 Q646C retrovirus at a multiplicity of infection of 10. Cells were maintained in drug-free medium

for 5-7 days, after which the marked cells were examined and photographed. Control infections were performed in parallel using the C127 fibroblast cell line and a recombinant retrovirus that expresses wild-type ErbB4. The constitutively-active ErbB4 Q646C mutant inhibited colony formation on plastic by MCF-10A cells (Figure 1). Moreover, MCF-10A cells that express the ErbB4 Q646C mutant remained as single cells rather than disappeared (Table 1). This suggests that the ErbB4 Q646C mutant induces growth arrest rather than apoptosis. Similar experiments are underway using the DU-145 and PC-3 human prostate tumor cell lines to evaluate whether the ErbB4 Q646C mutant inhibits colony formation on plastic by inducing growth arrest or by inducing apoptosis. Flow cytometry experiments are also underway to evaluate whether growth arrest caused by the ErbB4 Q646C mutant is cell cycle specific. Thus, while we have largely completed this task, several experiments remain to be completed in this last year of the project.

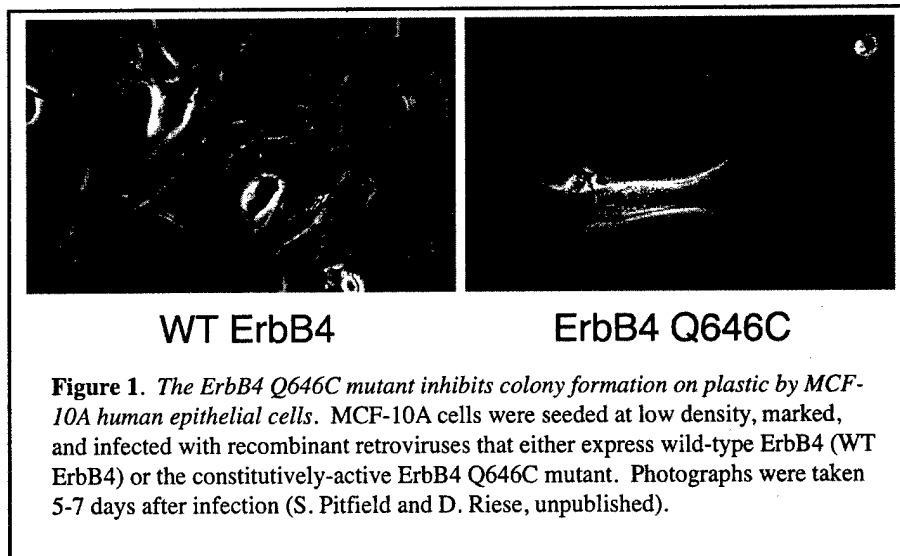


**Figure 1.** The ErbB4 Q646C mutant inhibits colony formation on plastic by MCF-10A human epithelial cells. MCF-10A cells were seeded at low density, marked, and infected with recombinant retroviruses that either express wild-type ErbB4 (WT ErbB4) or the constitutively-active ErbB4 Q646C mutant. Photographs were taken 5-7 days after infection (S. Pitfield and D. Riese, unpublished).

| Cell Line | Virus          | Cell Fate Following Infection |        |         |
|-----------|----------------|-------------------------------|--------|---------|
|           |                | Present                       | Absent | Colony  |
| C127      | Mock           | 3.0                           | 0.0    | 17.0    |
|           | ErbB4          | 5.0                           | 0.5    | 14.5    |
|           | ErbB4 Q646C    | 5.5                           | 0.0    | 14.5    |
|           | ErbB4 vs Q646C | p=0.841                       |        | p=0.978 |
| MCF 10A   | Mock           | 6.0                           | 2.3    | 11.8    |
|           | ErbB4          | 4.5                           | 2.8    | 12.8    |
|           | ErbB4 Q646C    | 13.3                          | 1.3    | 5.5     |
|           | ErbB4 vs Q646C | p=0.003                       |        | p=0.001 |

**Table 1.** The ErbB4 Q646C mutant induces growth arrest of MCF-10A human epithelial cells. MCF-10A cells were seeded at low density, marked, and infected with recombinant retroviruses that either express wild-type ErbB4 (WT ErbB4) or the constitutively-active ErbB4 Q646C mutant. The marked infected cells were photographed and scored as being present as a single cell, absent, or present as a colony of cells. The data shown represent the average of three independent experiments performed using twenty cells per each infection (S. Pitfield and D. Riese, unpublished).

**3. Determine whether ErbB4 tyrosine kinase activity is required to couple ErbB4 to biological responses.** Last year we reported that we had introduced a K751M mutation into the ErbB4 Q646C background. Data reported last year indicated that this Q646C/K751M double mutant exhibits reduced ligand-independent tyrosine phosphorylation. Furthermore, data

reported last year indicated that this ErbB4 double mutant fails to inhibit drug-resistant colony formation by DU-145 and PC-3 cells (Table 2). This suggests that ErbB4 kinase activity is required to couple ErbB4 to tumor suppression. Thus, we have completed this task.

4. Identify the sites of ErbB4 tyrosine phosphorylation that are sufficient to couple ErbB4 to biological responses. We have mutated the ten putative sites of ErbB4 tyrosine phosphorylation (Y984, Y1022, Y1056, Y1150, Y1162, Y1188, Y1202, Y1242, Y1258, and Y1284) to phenylalanine and introduced

these mutations into the background of the ErbB4 Q646C mutant. In Table 2 we describe several of these mutants and their activity with respect to inhibition of colony formation. Preliminary data indicate that mutating eight tyrosine residues (Y1022, Y1162, Y1188, Y1202, Y1242, Y1258, Y1284) to phenylalanine does not reduce inhibition of colony formation by the Q646C mutant (Q646C/Chg8 mutant). However, preliminary data indicate that mutating either Y984 or Y1056 in either the context of the Chg8 mutant or in the context of the nine other putative phosphorylation sites significantly abrogates inhibition of colony formation (Q646C Chg9 and Chg1 mutants). In sum, these preliminary data indicate that phosphorylation of ErbB4 Tyr984 and Tyr1056 are together necessary to couple ErbB4 to inhibition of colony formation. Moreover, these data suggest that ErbB4 Tyr984 and Tyr1056 together are sufficient to couple ErbB4 to inhibition of colony formation. As was discussed earlier, a kinase-deficient Q646C mutant (Q646C/K751M) exhibits minimal inhibition of colony formation. However, preliminary data indicate that the substitution of a glutamic acid residue (which mimics a phosphotyrosine residue) for Tyr1056 in the context of the Q646C/K751M mutant partially restores function. This suggests that phosphorylation of Tyr1056 is solely sufficient to couple ErbB4 to inhibition of colony formation and that phosphorylation of Tyr984 regulates Tyr1056, possibly by positively regulating its phosphorylation. We are pursuing experiments to test this hypothesis and we are continuing to assay the activity of the ErbB4 phosphorylation site mutants. Thus, while we have largely completed the experiments of this task, we are continuing experiments to confirm these preliminary observations and we are pursuing additional experiments that build on these studies.

| Mutants: | Q646C | Q646C<br>Chg8<br>Y0984/Y1056 | Q646C<br>Chg9<br>Y0984 | Q646C<br>Chg9<br>Y1056 | Q646C<br>Chg1<br>Y0984F | Q646C<br>Chg1<br>Y1056F | Q646C<br>K751M | Q646C<br>K751M/<br>Y1056E |
|----------|-------|------------------------------|------------------------|------------------------|-------------------------|-------------------------|----------------|---------------------------|
| Lys0751  | Lys   | Lys                          | Lys                    | Lys                    | Lys                     | Lys                     | Met            | Met                       |
| Tyr0984  | Tyr   | Tyr                          | Tyr                    | Phe                    | Phe                     | Tyr                     | Tyr            | Tyr                       |
| Tyr1022  | Tyr   | Phe                          | Phe                    | Phe                    | Tyr                     | Tyr                     | Tyr            | Tyr                       |
| Tyr1056  | Tyr   | Tyr                          | Phe                    | Tyr                    | Tyr                     | Phe                     | Tyr            | Glu                       |
| Tyr1150  | Tyr   | Phe                          | Phe                    | Phe                    | Tyr                     | Tyr                     | Tyr            | Tyr                       |
| Tyr1162  | Tyr   | Phe                          | Phe                    | Phe                    | Tyr                     | Tyr                     | Tyr            | Tyr                       |
| Tyr1188  | Tyr   | Phe                          | Phe                    | Phe                    | Tyr                     | Tyr                     | Tyr            | Tyr                       |
| Tyr1202  | Tyr   | Phe                          | Phe                    | Phe                    | Tyr                     | Tyr                     | Tyr            | Tyr                       |
| Tyr1242  | Tyr   | Phe                          | Phe                    | Phe                    | Tyr                     | Tyr                     | Tyr            | Tyr                       |
| Tyr1258  | Tyr   | Phe                          | Phe                    | Phe                    | Tyr                     | Tyr                     | Tyr            | Tyr                       |
| Tyr1284  | Tyr   | Phe                          | Phe                    | Phe                    | Tyr                     | Tyr                     | Tyr            | Tyr                       |

| <i>Inhibition of Colony Formation:</i> |       |       |      |      |      |      |       |       |
|----------------------------------------|-------|-------|------|------|------|------|-------|-------|
| MCF-10A                                | 80.0% | 89.3% | < 1% | < 1% | < 1% | < 1% | 02.0% | ND    |
| DU-145                                 | 98.1% | 95.9% | < 1% | ND   | ND   | < 1% | < 1%  | 31.5% |
| PC-3                                   | 89.9% | 89.8% | < 1% | ND   | ND   | < 1% | < 1%  | 48.5% |

**Table 2.** Construction and analysis of additional ErbB4 mutants. We constructed the indicated ErbB4 mutants and subcloned them into a recombinant retroviral expression vector. MCF-10A, DU-145, and PC-3 cell lines were infected with the resulting recombinant amphotropic retroviruses. Mouse C127 cells were infected as a control for viral titers. Infected cells were selected using G418. Colonies of drug resistant cells appeared approximately 14 days after infection and were stained and counted. Viral titers were calculated for each combination of cell line and retrovirus. Using these viral titers, inhibition of colony formation by each retrovirus was calculated according to published procedures [Williams, *et al.*, 2003]. (R. Gallo, S. Pitfield, I. Bryant, and D. Riese, unpublished).

### Key Research Accomplishments

1. Generated three constitutively-active ErbB4 mutants (Q646C, H647C, A648C).
2. Determined that the ErbB4 Q646C mutant inhibits drug-resistant colony formation *in vitro* by the PC-3 and DU-145 human prostate tumor cell lines. Generated preliminary data suggesting that the ErbB4 Q646C mutant may inhibit colony formation by coupling to growth arrest.
3. Generated a kinase-deficient version of the constitutively-active ErbB4 Q646C mutant (Q646C/K751M). Demonstrated that the Q646C/K751M mutant displays less tyrosine phosphorylation than does Q646C. Also demonstrated that the Q646C/K751M mutant fails to inhibit drug-resistant colony formation *in vitro* by DU-145 and PC-3 human prostate tumor cell lines. These data indicate that ErbB4 tyrosine kinase activity is required for ErbB4 coupling to biological responses.
4. Generated versions of the constitutively-active Q646C ErbB4 mutant that lack putative tyrosine phosphorylation site mutants. Generated preliminary data that suggest that Tyr984 and Tyr1056 play critical roles in coupling ErbB4 to inhibition of colony formation.

### Reportable Outcomes

#### Publications

Penington, D.J., I. Bryant, and D.J. Riese II. "Constitutively active ErbB4 and ErbB2 mutants exhibit distinct biological activities." *Cell Growth and Differentiation* **13**: 247-256 (2002).

Williams, E.E., L.J. Trout, R.M. Gallo, S.E. Pitfield, I. Bryant, D.J. Penington, and D.J. Riese II. "A constitutively-active ErbB4 mutant inhibits drug-resistant colony formation by the DU-145 and PC-3 human prostate tumor cell lines." *Cancer Letters* **192**: 67-74 (2003).

#### Degrees

Eric E. Williams, Doctor of Pharmacy (Pharm.D.), Purdue University, May 2003.

#### Presentations

"Identification of ErbB4 Phosphorylation Sites That Couple a Constitutively-Active Mutant to Inhibition of Drug-Resistant Colony Formation by Human Breast and Prostate Cell Lines." Nineteenth Annual Meeting on Oncogenes, Hood College, Frederick, MD, June 2004.

"Genetic and Biochemical Analysis of Tyrosine Phosphorylation Sites that Couple ErbB4 to Breast and Prostate Tumor Suppression." Amelia Project Meeting, Indiana University Cancer Center, Indianapolis, IN, February 2004.

## Conclusions

Our data clearly indicate that constitutive ErbB4 signaling is linked to inhibition of drug-resistant colony formation by the PC-3 and DU-145 human prostate tumor cell lines. Thus, the data suggest that ErbB4 is indeed a prostate tumor suppressor. The inhibition of drug-resistant colony formation appears to be the result of growth arrest rather than apoptosis. Experiments are underway to examine whether the growth arrest is cell cycle specific. The inhibition of drug-resistant colony formation appears to require ErbB4 kinase activity as well as specific sites of ErbB4 tyrosine phosphorylation. These results provide important clues to the identity of the signaling events and molecules that lie downstream of ErbB4.

The relevance of these studies is that they support the hypothesis that ErbB4 is a tumor suppressor and that loss of ErbB4 signaling plays a critical role in prostate tumorigenesis. Thus, levels of ErbB4 expression or signaling in hyperplastic and malignant prostate specimens may be of predictive value. Furthermore, inherited hypomorphic mutations in ErbB4 may contribute to an increased risk of developing aggressive prostate tumors.

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**Appendices**

Penington, D.J., I. Bryant, and D.J. Riese II. "Constitutively active ErbB4 and ErbB2 mutants exhibit distinct biological activities." *Cell Growth and Differentiation* **13**: 247-256 (2002).

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# Constitutively Active ErbB4 and ErbB2 Mutants Exhibit Distinct Biological Activities<sup>1</sup>

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## Abstract

ErbB4 is a member of the epidermal growth factor receptor (EGFR) family of tyrosine kinases, which includes EGFR/ErbB1, ErbB2/HER2/Neu, and ErbB3/HER3. These receptors play important roles both in normal development and in neoplasia. For example, deregulated signaling by ErbB1 and ErbB2 is observed in many human malignancies. In contrast, the roles that ErbB4 plays in tumorigenesis and normal biological processes have not been clearly defined. To identify the biological responses that are coupled to ErbB4, we have constructed three constitutively active ErbB4 mutants. Unlike a constitutively active ErbB2 mutant, the ErbB4 mutants are not coupled to increased cell proliferation, loss of contact inhibition, or anchorage independence in a rodent fibroblast cell line. This suggests that ErbB2 and ErbB4 may play distinct roles in tumorigenesis *in vivo*.

## Introduction

ErbB4 (HER4/p180<sup>erbB4</sup>) is a member of the EGFR<sup>3</sup> (EGFR/ErbB) family of receptor tyrosine kinases. These receptors play important roles in the embryonic development of heart, lung, and nervous tissues (1–4), and they have been implicated in the progression of metastatic disease. For example, EGFR/ErbB1 is overexpressed, amplified, or mutated in a number of human malignancies including breast, ovary, prostate, and lung cancers (5–7). ErbB2 overexpression cor-

relates with tumor aggressiveness and poor prognosis in node-positive breast cancer patients (reviewed in Ref. 8). Finally, ErbB3 overexpression is observed in a subset of human mammary and gastric cancers (9, 10).

Some reports indicate that increased ErbB4 expression or signaling is associated with tumorigenesis. ErbB4 overexpression has been observed in a variety of cancers, including tumors of the thyroid, breast, and gastrointestinal tract (11–14). However, the prognostic significance of ErbB4 expression in tumors may also depend on which ErbB family members are coexpressed with ErbB4. In the case of childhood medulloblastoma (one of the most common solid tumors of childhood), patients with tumors overexpressing both ErbB2 and ErbB4 have a significantly worse prognosis than patients with tumors that overexpress either receptor alone (15).

Other reports indicate that increased ErbB4 expression or signaling correlates with tumor cell differentiation and reduced aggressiveness. ErbB4 overexpression in breast tumors is associated with progesterone receptor and estrogen receptor expression and a more favorable prognosis (16–17). In contrast, ErbB2 overexpression varies inversely with progesterone receptor and estrogen receptor levels and indicates tumors that are more likely to be metastatic and fatal (18). In one survey of common solid human cancers, the loss of ErbB4 expression is seen in a significant percentage of breast, prostate, and head and neck malignancies (19). These findings raise the intriguing possibility that ErbB4 is unique to the ErbB family of receptors in that ErbB4 expression and signaling may couple to reduced tumorigenesis or tumor cell proliferation. However, in the face of the conflicting evidence we have summarized here, it remains unclear what general or specific roles ErbB4 plays in differentiation, tumor suppression, or proliferation.

Efforts to elucidate ErbB4 function have been hampered by many factors. There are no known agonists or antagonists specific to the ErbB4 receptor. All of the peptide hormones of the EGF family that are capable of binding ErbB4 also bind at least one other ErbB family member. For example, epiregulin and betacellulin bind and activate both ErbB1 and ErbB4 (20, 21). Furthermore, ligands that do not bind an ErbB family receptor can still activate signaling by that receptor in *trans* through ligand-induced receptor heterodimerization (reviewed in Refs. 22, 23). For example, EGF stimulates ErbB2 tyrosine phosphorylation when ErbB2 is coexpressed with ErbB1, whereas EGF will not stimulate ErbB2 tyrosine phosphorylation in the absence of ErbB1 (24). Consequently, ligands that bind and directly activate ErbB4 (neuregulin, betacellulin, and epiregulin) also stimulate ErbB1, ErbB2, and ErbB3 signaling (Refs. 20, 21, 25, 26; reviewed in Refs. 22, 23). Therefore, in most contexts it is virtually impossible to use an EGF family hormone to study the functional consequences of ErbB4 signaling.

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<sup>3</sup> The abbreviations used are: EGFR, epidermal growth factor receptor; cfu, colony-forming unit(s); FR3T3, Fischer rat 3T3; LMP, low melting point; LTR, long terminal repeat.

To study ErbB4 function, we have opted to generate ErbB4 mutants that contain a cysteine substitution in the extracellular domain. This is predicted to result in constitutively dimerized and constitutively active ErbB4 mutants. Introducing cysteine residues to form covalently linked, dimeric, constitutively active receptor tyrosine kinases is not novel. This strategy has been used to generate dimeric, constitutively active mutants of EGFR/ErbB1 and ErbB2 (27, 28). Cysteine substitutions also lead to constitutively active mutants of the fibroblast growth factor receptors 2 and 3 (29, 30).

Here we report the generation and characterization of three constitutively active ErbB4 mutants. These mutants were generated through the introduction of a cysteine residue in the extracellular region of ErbB4. These mutants exhibit increased ligand-independent ErbB4 tyrosine phosphorylation, dimerization, and kinase activity. However, these constitutively active ErbB4 mutants do not induce increased proliferation, loss of contact inhibition, or anchorage-independent growth in FR3T3 fibroblasts. In contrast, a constitutively active ErbB2 mutant does induce increased proliferation, loss of contact inhibition, and anchorage-independent growth in FR3T3 fibroblasts. These results suggest that ErbB4 and ErbB2 couple to different signaling pathways and biological responses. These results also suggest that ErbB4 and ErbB2 may play distinct roles in tumorigenesis *in vivo*.

## Results

**ErbB4 Mutants Are Constitutively Tyrosine Phosphorylated.** We substituted a single cysteine for amino acids Pro-645, Gln-646, His-647, Ala-648, and Arg-649 in the juxtamembrane region of the ErbB4 extracellular domain. These ErbB4 mutants (P645C, Q646C, H647C, A648C, and R649C) were generated in the context of the pLXSN-ErbB4 recombinant retroviral expression vector (26). Because these cysteine substitutions might cause inappropriate protein folding and decreased protein stability, we assayed the ErbB4 mutants for stable expression. We transfected the recombinant retroviral vectors containing the ErbB4 mutant constructs into the  $\Psi$ 2 ecotropic retrovirus packaging cell line, selected for stable transformants, and generated pooled cell lines. We harvested low-titer ecotropic retrovirus stocks from these cell lines, and we analyzed the expression and tyrosine phosphorylation of the ErbB4 mutants in these cell lines. Three ErbB4 mutants (Q646C, H647C, and A648C) exhibit abundant expression and ligand-independent tyrosine phosphorylation (data not shown). However, the R649C ErbB4 mutant is not efficiently expressed, and the P645C mutant does not display ligand-independent tyrosine phosphorylation (data not shown).

Previous studies indicate that transfection and subsequent overexpression of ErbB family receptors lead to ligand-independent receptor tyrosine phosphorylation (31–33). Consequently, we were concerned that the ligand-independent phosphorylation of the Q646C, H647C, and A648C ErbB4 mutants in the transfected  $\Psi$ 2 cells was a consequence of overexpression. Therefore, we infected the PA317 amphotropic retrovirus packaging cell line with the ErbB4 mutant recombinant ecotropic retroviruses at low multiplicities of infection ( $<0.1$ ), selected for infected cells, and generated

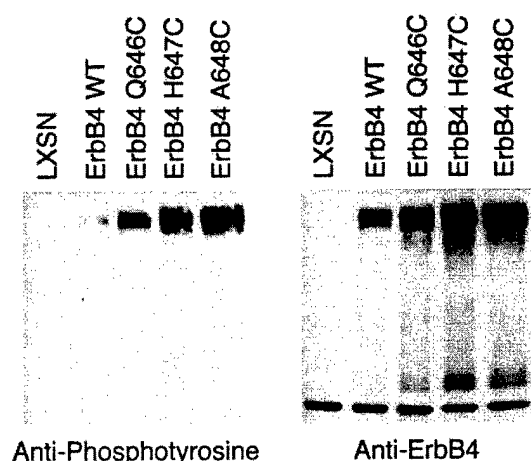


Fig. 1. ErbB4 mutants are constitutively tyrosine phosphorylated. ErbB4 expression and tyrosine phosphorylation were assayed in PA317 cells infected with retroviruses that direct the expression of wild-type ErbB4 or the ErbB4 mutants. Cells infected with the LXSN recombinant retrovirus vector control served as the negative control. Lysates were prepared from each of the cell lines, and ErbB4 was immunoprecipitated from 1000  $\mu$ g of each lysate. Samples were resolved by SDS-PAGE, electroblotted to nitrocellulose, and immunoblotted with an anti-phosphotyrosine antibody (left panel). The blot was then stripped and probed with an anti-ErbB4 rabbit polyclonal antibody (right panel). The band at the top of the blots represents ErbB4.

pooled cell lines. Because these cell lines were generated by infection at low multiplicities of infection, it is likely that each cell contains only one or two copies of the ErbB4 expression construct. This reduces the likelihood of ErbB4 overexpression in these cell lines.

We analyzed ErbB4 expression and tyrosine phosphorylation in the PA317 cell lines by anti-ErbB4 immunoprecipitation and either anti-ErbB4 (Fig. 1, right panel) or anti-phosphotyrosine (Fig. 1, left panel) immunoblotting. As expected, cells infected with the LXSN vector control retrovirus do not exhibit ErbB4 expression (Fig. 1, right panel) or tyrosine phosphorylation (Fig. 1, left panel). Cells infected with the wild-type or mutant ErbB4 retroviruses exhibit ErbB4 expression (Fig. 1, right panel). However, cells infected with the mutant ErbB4 retroviruses exhibit abundant ErbB4 tyrosine phosphorylation, whereas cells infected with the wild-type ErbB4 retrovirus exhibit minimal ErbB4 tyrosine phosphorylation (Fig. 1, left panel).

Quantification of the chemilumigrams shown in Fig. 1 suggests that the expression levels of the three ErbB4 mutants is less than three times greater than the amount of wild-type ErbB4 expression (Table 1). In contrast, the amounts of tyrosine phosphorylation of the three ErbB4 mutants appear to be much greater than the amount of wild-type ErbB4 tyrosine phosphorylation. Moreover, the ratios of ErbB4 tyrosine phosphorylation to ErbB4 expression for the three ErbB4 mutants appear to be at least four times greater than the ratio for wild-type ErbB4. These data suggest that the three ErbB4 mutants exhibit greater amounts of tyrosine phosphorylation on a per-molecule basis than does wild-type ErbB4. Consequently, these data indicate that the Q646C, H647C, and A648C ErbB4 mutants are constitutively active for signaling.

**Table 1** The Q646C, H647C, and A648C ErbB4 mutants exhibit increased normalized tyrosine phosphorylation

| Cell line       | ErbB4 tyrosine phosphorylation | ErbB4 expression | Ratio |
|-----------------|--------------------------------|------------------|-------|
| Wild-type ErbB4 | 210000                         | 1800000          | 0.12  |
| ErbB4 Q646C     | 1900000                        | 3300000          | 0.58  |
| ErbB4 H647C     | 2900000                        | 4700000          | 0.62  |
| ErbB4 A648C     | 4000000                        | 4500000          | 0.89  |

#### ErbB4 Mutants Have Increased *in Vitro* Kinase Activity.

Next, we assessed whether the increased tyrosine phosphorylation of the three ErbB4 mutants correlates with increased kinase activity. Equal amounts of the same lysates used for the experiments described in Fig. 1 were immunoprecipitated with an anti-ErbB4 polyclonal antibody. Kinase reactions were performed on the immunoprecipitates in the presence of [ $\gamma$ - $^{32}$ P]ATP. The reaction products were resolved by SDS-PAGE on a 7.5% acrylamide gel. The gel was dried, and the reaction products were visualized by autoradiography.

In Fig. 2, we show that PA317 cells infected with the LXSN vector control retrovirus lack detectable ErbB4 kinase activity. Moreover, PA317 cells that express the three constitutively active ErbB4 mutants exhibit greater ErbB4 tyrosine kinase activity than cells that express wild-type ErbB4. Quantification of the bands on the autoradiogram indicates that the Q646C and H647C ErbB4 mutants exhibit approximately five times more kinase activity than does wild-type ErbB4, whereas the A648C ErbB4 mutant exhibits approximately nine times more kinase activity than does wild-type ErbB4. Given that the expression of the ErbB4 mutants (in these same lysates) is somewhat greater than the expression of wild-type ErbB4 (Fig. 1 and Table 1), it appears that the intrinsic kinase activity of the three ErbB4 mutants is three to four times greater than the intrinsic kinase activity of wild-type ErbB4.

**Constitutively Active ErbB4 Mutants Do Not Induce a Loss of Contact Inhibition.** Once we determined that the Q646C, H647C, and A648C ErbB4 mutants are constitutively active for signaling, we performed experiments using these mutants to identify the biological events coupled to ErbB4 signaling. A common assay for genes that encode growth control or signaling proteins involves introducing the gene into an established rodent fibroblast cell line and assaying for foci of piled-up cells. These foci indicate a loss of contact inhibition, a common attribute of malignant cells. Thus, this gene transfer assay is commonly used to identify genes that encode proteins that are coupled to malignant growth transformation.

Conflicting results have been obtained from assays for growth transformation by ErbB4. Transfection and consequent overexpression of ErbB4 induces foci (loss of contact inhibition) in NIH 3T3 clone 7 cells in the absence of ligand. Moreover, in these cells focus formation was stimulated by the ErbB4 ligand neuregulin  $\beta$ . In contrast, NIH 3T3 clone 7d cells (which lack EGFR expression) transfected with wild-type ErbB4 did not form foci in the presence or absence of neuregulin  $\beta$ ; however, ErbB4 cotransfected with EGFR/ErbB1 or ErbB2 does induce foci in these cells (32, 33). One

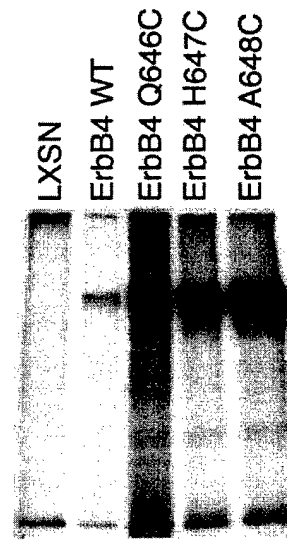


**Fig. 2.** Q646C, H647C, and A648C mutants exhibit increased *in vitro* kinase activity. Equal amounts of protein lysates (1000  $\mu$ g) from PA317 cells that stably express wild-type ErbB4 or the ErbB4 mutants (Q646C, H647C, and A648C) were immunoprecipitated with an anti-ErbB4 rabbit polyclonal antibody. Lysates from PA317 cells that express the LXSN vector served as the negative control. Kinase reactions were performed on the immunoprecipitates in the presence of [ $\gamma$ - $^{32}$ P]ATP. The products were resolved by SDS-PAGE. The gel was dried overnight and exposed to X-ray film for ~20 h to visualize the products of the kinase reactions.

possible explanation is that ErbB4 lacks intrinsic transforming activity but does permit EGFR/ErbB1 or ErbB2 signaling and coupling to growth transformation in the presence of an ErbB4 ligand.

To test whether ErbB4 signaling is sufficient to transform the growth of cultured rodent fibroblasts, FR3T3 fibroblasts were infected with 200 cfu of the ErbB4 mutant recombinant ecotropic retrovirus stocks and assayed for focus formation. Cells infected with 200 cfu of the LXSN vector control recombinant ecotropic retrovirus and with 200 cfu of the wild-type ErbB4 recombinant ecotropic retrovirus served as negative controls. Cells infected with 200 cfu of the constitutively active (V664E transmembrane domain) mutant ErbB2\* retrovirus served as a positive control.

FR3T3 cells infected with the ErbB2\* retrovirus had formed foci within 9 days after infection, whereas cells infected with the vector control retrovirus had not (Fig. 3). Furthermore, cells infected with the wild-type or mutant ErbB4 retroviruses had not formed foci within 9 days after infection. Within 18 days after infection, the foci arising from FR3T3 cells infected with the ErbB2\* retrovirus had completely covered the surface of the tissue culture plate and had begun to detach from the surface of the plate (data not shown). Within 18 days after infection, FR3T3 cells infected with the mutant ErbB4 retroviruses had formed relatively high-density clumps (data not shown). These high-density clumps did not exhibit the overlapping cell processes characteristic of foci (data not shown). The cells comprising these clumps were cloned and expanded into cell lines, as were cells from less dense regions of the cell monolayers. The cells from the clumps are morphologically indistinguishable from cells derived from the

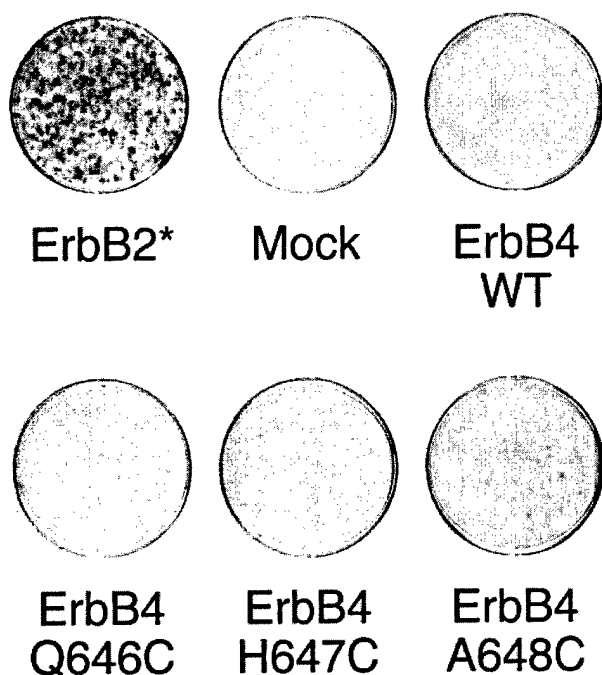


Fig. 3. Constitutively active ErbB4 receptors do not induce a loss of contact inhibition. FR3T3 fibroblasts infected with the LXSN (vector control) retrovirus, the wild-type ErbB4 retrovirus, the constitutively active ErbB2\* retrovirus, or the constitutively active ErbB4 mutant retroviruses were assayed for loss of contact inhibition (focus formation).

less dense regions of the plates and are morphologically indistinguishable from cells that express wild-type ErbB4 or cells infected with the vector control retrovirus (data not shown). Again, this suggests that the constitutively active ErbB4 mutants do not transform the growth of FR3T3 fibroblasts.

We were concerned that the apparent failure of the constitutively active ErbB4 mutants to transform the growth of FR3T3 fibroblasts might be specific to this cell type. Consequently, we performed similar experiments with mouse C127 fibroblasts. Infection with the ErbB2\* retrovirus resulted in numerous foci, whereas infection with the constitutively active ErbB4 mutant retroviruses did not (data not shown). Thus, again, whereas the constitutively active ErbB2\* mutant readily induces foci in fibroblasts, the constitutively active ErbB4 mutants do not. This suggests that ErbB2 and ErbB4 couple to distinct cellular signaling pathways and biological events.

**Constitutively Active ErbB4 Mutants Do Not Induce Anchorage-independent Growth.** Next, we assayed FR3T3 cells that express the constitutively active ErbB4 mutants for growth while suspended in semisolid medium. Because anchorage-independent growth is another characteristic attribute of tumor cells *in vivo*, this assay is another way to determine whether ErbB4 signaling is coupled to malignant growth transformation.

FR3T3 cells were infected with the ErbB4 mutant recombinant ecotropic retroviruses at a low multiplicity of infection, and infected cells were selected using G418. Drug-resistant

colonies of cells were pooled and expanded into cell lines. Control cell lines were generated through infection of FR3T3 cells with the wild-type ErbB4 retrovirus, the constitutively active ErbB2 retrovirus, and with the LXSN vector control retrovirus. These cell lines were seeded at a density of  $2 \times 10^4$  cells/ml in 60-mm dishes in semisolid medium containing 0.3% LMP-agarose. Fresh medium containing LMP-agarose was added every 3 days. Photographs were taken of representative fields after 10 days.

FR3T3 cells that express the constitutively active ErbB2\* mutant exhibit anchorage-independent growth (Fig. 4). In contrast, cells that were infected with the LXSN recombinant retroviral vector control and cells that express wild-type ErbB4 or the ErbB4 mutants do not exhibit anchorage-independent growth. The results of this assay are consistent with the results of the focus formation assay; both assays indicate that ErbB4 signaling is distinct from ErbB2 signaling in that ErbB4 signaling is not coupled to malignant growth transformation in FR3T3 fibroblasts.

**Constitutively Active ErbB4 Mutants Do Not Increase the Growth Rate or Saturation Density.** Another characteristic of malignantly transformed fibroblasts is that their growth rates and saturation densities are higher than those of their nontransformed counterparts. Indeed, constitutive ErbB2 signaling is coupled to increased growth rates (reviewed in Ref. 8). Thus, we assessed whether the constitutively active ErbB4 mutants affected the growth rate or saturation density of FR3T3 fibroblasts. The FR3T3 cell lines described earlier were seeded in 60-mm dishes at a density of  $2 \times 10^4$  cells/dish ( $700 \text{ cells/cm}^2$ ). Cells were incubated for 10 days to permit proliferation. During this period, cells were counted every 24 h.

The growth rate of the cells that express ErbB2\* is slightly greater than the growth rates of the other cell lines (Fig. 5). Note that the growth rates of the cells that express the constitutively active ErbB4 mutants are indistinguishable from the growth rates of cell lines that express wild-type ErbB4 or the vector control. The growth curves in Fig. 5 were used to determine the saturation densities for the six cell lines (Table 2). Note that the saturation density of the cell line that expresses ErbB2\* is higher than the saturation densities of the other cell lines. Moreover, the saturation densities of the cell lines that express the ErbB4 mutants are not markedly higher than the saturation densities of the vector control cell line or the cell line that expresses wild-type ErbB4. Once again, these data suggest that constitutive ErbB4 signaling is not coupled to malignant growth transformation in fibroblasts. Thus, the signaling pathways and biological responses that are coupled to ErbB4 are distinct from those that are coupled to ErbB2.

**The Constitutively Active ErbB4 Mutants Are Expressed and Are Constitutively Tyrosine Phosphorylated in FR3T3 Cells.** We were concerned that the apparent failure of the constitutively active ErbB4 mutants to transform the growth of FR3T3 fibroblasts might be attributable to the absence of ErbB4 expression or constitutive tyrosine phosphorylation in these cells. In parallel with the infections described in Fig. 3, we infected FR3T3 cells with 200 cfu of the constitutively active mutant ErbB4 recombinant retroviruses

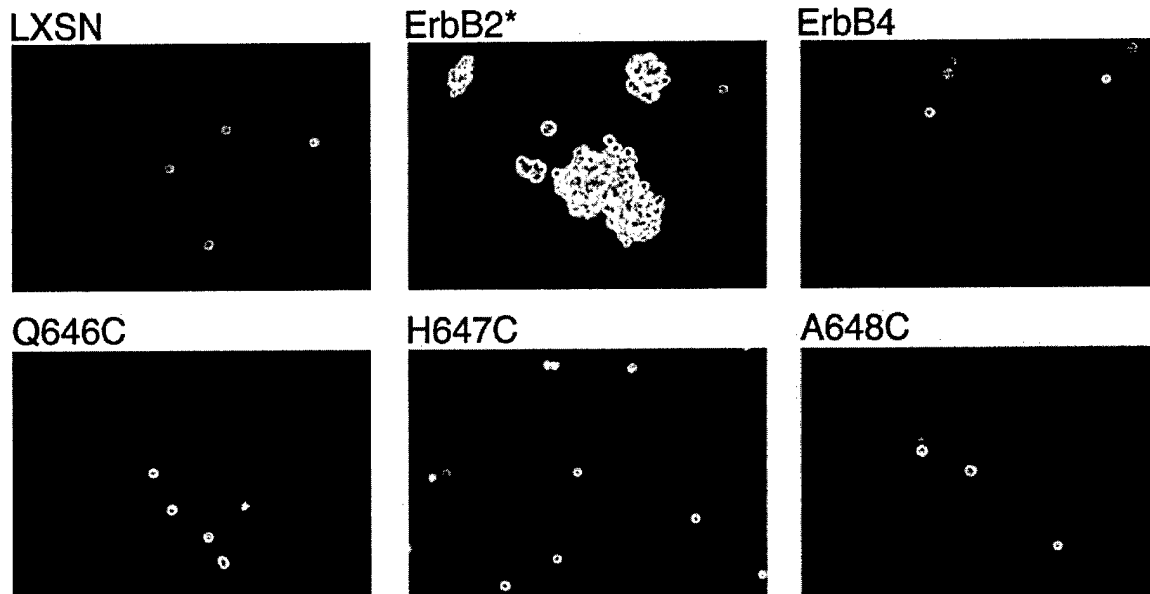


**Fig. 4.** Constitutively active ErbB4 receptors do not induce growth in semisolid medium. FR3T3 cells that stably express the LXSN vector control, the constitutively active ErbB2 mutant (ErbB2\*), wild-type ErbB4, or the constitutively active ErbB4 mutants (Q646C, H647C, and A648C) were seeded in semisolid medium at a density of  $2 \times 10^4$  cells/ml in 60-mm dishes. The cells were incubated for 10 days, after which images were recorded by photomicroscopy. Images shown are representative of those obtained in three independent experiments.

#### FR3T3 Growth Curves

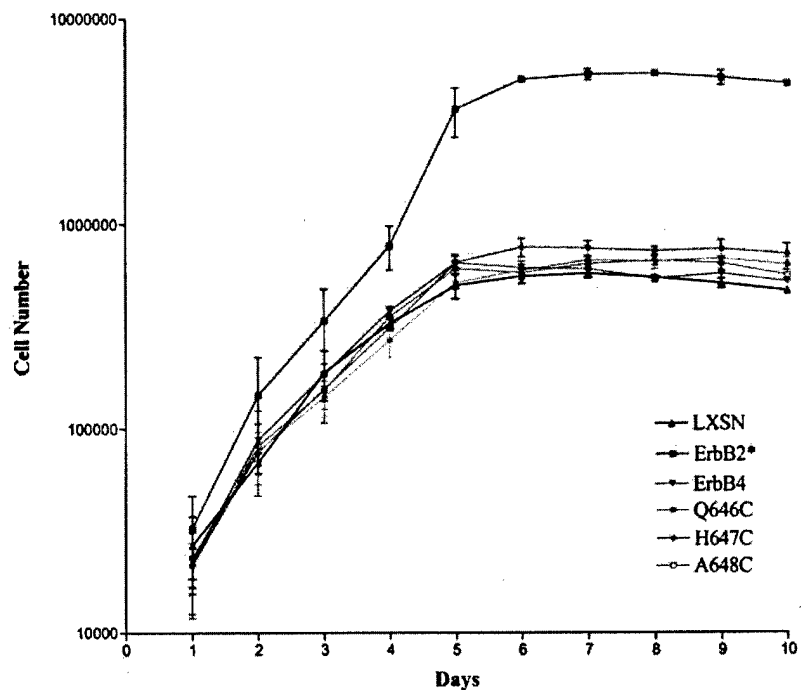


**Fig. 5.** Constitutively active ErbB4 mutants do not increase the growth rate of FR3T3 fibroblasts. FR3T3 cells that express the LXSN vector control, the constitutively active ErbB2\* mutant, wild-type ErbB4, or the constitutively active ErbB4 mutants (Q646C, H647C, and A648C) were plated at a density of  $2 \times 10^4$  cells in 60-mm dishes ( $700 \text{ cells/cm}^2$ ) and were incubated for 1–10 days. Cells were counted daily to assess growth rates and saturation densities. The means for three independent experiments; bars, SE.

and selected for stable infection using G418. As controls, we also infected FR3T3 cells with 200 cfu of the vector control retrovirus, 200 cfu of the ErbB2\* retrovirus, and with 200 cfu of the wild-type ErbB4 retrovirus. Drug-resistant colonies

were pooled and expanded into stable cell lines. The cell lines were starved of serum in the presence of  $500 \mu\text{M}$   $\text{Na}_3\text{VO}_4$  (34) to decrease the background level of tyrosine phosphorylation and to increase the phosphorylation of the

Table 2 Constitutively active ErbB4 mutants do not increase the saturation density of FR3T3 fibroblasts

| Saturation Densities |                           |
|----------------------|---------------------------|
| LXSN                 | $5.8 \pm 0.3 \times 10^5$ |
| ErbB2*               | $5.4 \pm 0.1 \times 10^6$ |
| ErbB4                | $6.1 \pm 0.5 \times 10^5$ |
| Q646C                | $6.6 \pm 0.6 \times 10^5$ |
| H647C                | $7.6 \pm 0.7 \times 10^5$ |
| A648C                | $6.6 \pm 0.4 \times 10^5$ |

constitutively active ErbB4 mutants. We prepared lysates and analyzed ErbB4 expression and tyrosine phosphorylation by precipitation with an anti-ErbB4 antibody and sequential anti-phosphotyrosine and anti-ErbB4 immunoblotting.

In Fig. 6, lower panel, we show that ErbB4 expression is detectable in the FR3T3 cell lines infected with the wild-type ErbB4 retrovirus or the constitutively active ErbB4 mutant retroviruses. However, ErbB4 tyrosine phosphorylation is observed only in the FR3T3 cell lines infected with the constitutively active ErbB4 mutant retroviruses (Fig. 6, upper panel). The amount of phosphorylation exhibited by the ErbB4 mutants is less than the amount of phosphorylation exhibited by the constitutively active ErbB2 mutant. Furthermore, the expression of wild-type ErbB4 appears to be less than the expression of the ErbB4 mutants. Nonetheless, these data suggest that the apparent failure of the constitutively active ErbB4 mutants to transform the growth of FR3T3 fibroblasts is not attributable to an absence of expression and tyrosine phosphorylation of these mutants in these cells.

## Discussion

In this report, we describe the construction and initial characterization of three constitutively active ErbB4 mutants. These mutants display increased dimerization (data not shown) and ligand-independent tyrosine phosphorylation and kinase activity. In these respects, the ErbB4 mutants resemble constitutively active mutants of ErbB2 or EGFR. However, unlike constitutively active ErbB2 mutants, these mutants are not coupled to malignant growth transformation in FR3T3 fibroblasts; they do not induce foci, anchorage-independent growth, or increases in the growth rate or saturation density. These data suggest that ErbB2 and ErbB4 play distinct roles in tumorigenesis *in vivo*. This conclusion is supported by the observation that NIH3T3 clone 7d cells do not form foci after ErbB4 transfection and treatment with the ErbB4 ligand neuregulin but do form foci after ErbB2 and ErbB4 cotransfection and neuregulin treatment (32, 33).

Of course, another potential explanation is that the amounts of tyrosine phosphorylation displayed by the three constitutively active ErbB4 mutants are insufficient to couple to malignant growth transformation in fibroblasts. This is consistent with the observation that the three constitutively active ErbB4 mutants are less phosphorylated than the constitutively active ErbB2 mutant (Fig. 6). However, anti-phosphotyrosine immunoblotting is not a sensitive method for assessing ErbB family receptor signaling and coupling to biological responses. Indeed, the neuregulin concentration

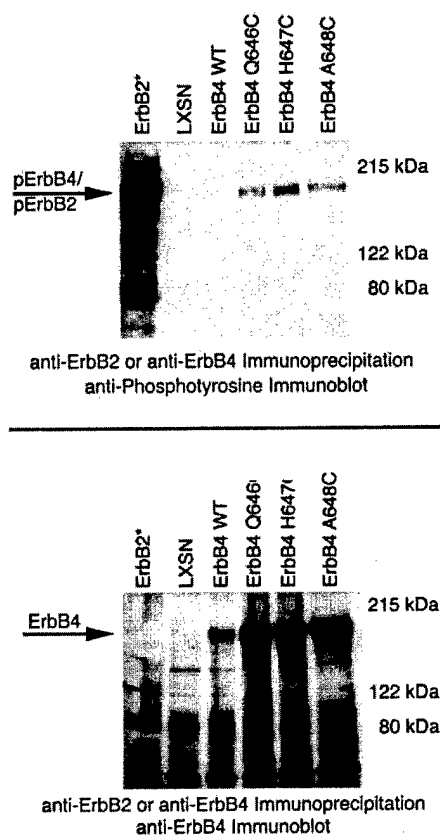


Fig. 6. Constitutively active ErbB4 mutants are expressed and are constitutively tyrosine phosphorylated in FR3T3 cells. ErbB4 expression and tyrosine phosphorylation were assayed in FR3T3 cells infected with retroviruses that direct the expression of wild-type ErbB4 or the ErbB4 mutants. Cells infected with the LXSN recombinant retrovirus vector control or with the ErbB2\* retrovirus served as controls. Lysates were prepared from each of the cell lines, and ErbB receptors were precipitated from 1.5 mg of each lysate using protein A-Sepharose and either an anti-ErbB4 rabbit polyclonal antibody or an anti-ErbB2 rabbit polyclonal antibody. Samples were resolved by SDS-PAGE, electroblotted to nitrocellulose, and immunoblotted with an anti-phosphotyrosine antibody (upper panel). The blot was then stripped and probed with an anti-ErbB4 rabbit polyclonal antibody (lower panel). Arrows, positions of ErbB2 and ErbB4 on the blots.

required for maximal ErbB4 tyrosine phosphorylation is ~10-fold greater than the neuregulin concentration sufficient for maximal ErbB family receptor coupling to biological responses. Furthermore, the neuregulin concentration sufficient for maximal ErbB family receptor coupling to biological responses stimulates, at most, only modest amounts of ErbB4 tyrosine phosphorylation (26). Thus, it is not likely that the failure of the constitutively active ErbB4 mutants to couple to malignant growth transformation in fibroblasts is attributable to insufficient ErbB4 tyrosine phosphorylation.

Clearly, additional work is necessary to define the roles that ErbB4 plays in tumorigenesis and in regulating cellular functions *in vivo*. However, important clues have emerged to guide these future studies. In a significant percentage of breast tumor samples, ErbB4 expression correlates with estrogen receptor expression, which indicates a favorable prognosis (16–17). Furthermore, ErbB4 expression is fre-

quently lost in tumors of the breast and prostate (19). Finally, ligands for ErbB4 can induce terminal differentiation and growth arrest of some mammary tumor cell lines (35–37). These data indicate that ErbB4 signaling may be coupled to differentiation, growth arrest, and tumor suppression. The ErbB4 mutants described in this study will enable us to evaluate this hypothesis. Indeed, preliminary data from our laboratory indicate that the Q646C ErbB4 mutant causes reduced colony formation in plastic dishes by a number of cultured human breast and prostate tumor cell lines.

We will also perform additional studies to characterize the biochemistry of signaling by the three ErbB4 mutants. Whereas these mutants exhibit greater ligand-independent tyrosine phosphorylation and autokinase activity than the wild-type receptor, it is unclear whether this is attributable to increased intrinsic kinase activity or attributable to increased availability of the substrate. Additional experiments are warranted to distinguish between these two possibilities.

Another area of future study will focus on identifying the mechanisms by which ErbB4 is coupled to biological responses. Initial studies will identify the sites of ErbB4 tyrosine phosphorylation for these mutants. If our preliminary studies indicating that the Q646C ErbB4 mutant is coupled to prostate and mammary tumor cell growth arrest hold true, then we will use genetic strategies to identify the sites of ErbB4 tyrosine phosphorylation that are sufficient and necessary to couple the Q646C ErbB4 mutant to this biological response. A similar strategy has been used to identify the sites of ErbB2 and platelet-derived growth factor receptor tyrosine phosphorylation that are critical for coupling these receptors to biological responses (38, 39).

Once we have identified the site(s) of tyrosine phosphorylation that is sufficient for coupling to biological responses, we will identify signaling proteins that bind this phosphorylation site and couple it to biological responses. Using this strategy, we will begin to characterize the ErbB4 signaling pathway. Our prediction is that the three constitutively active ErbB4 mutants are phosphorylated on different tyrosine residues and that these mutants differentially couple to biological responses. We have shown previously that different ErbB4 ligands cause phosphorylation on different sites on ErbB4 and differential coupling to biological responses (40). Moreover, one cysteine substitution mutation in the rat ErbB2 extracellular domain (V656C) results in low amounts of constitutive receptor tyrosine phosphorylation and efficient coupling to malignant growth transformation in rodent fibroblasts. In contrast, another rat ErbB2 extracellular domain cysteine substitution mutant (T657C) exhibits very high levels of constitutive receptor tyrosine phosphorylation but a relatively low amount of coupling to malignant growth transformation in rodent fibroblasts (28).

We were somewhat surprised to discover that the three constitutively active ErbB4 mutants failed to couple to malignant growth transformation in a rodent fibroblast cell line. Nonetheless, these mutants will enable us to assess ErbB4 function in a wide variety of cell, tissue, and organismal contexts. Given that ErbB4 appears to regulate diverse functions in a number of distinct contexts, much work remains to complete this story.

## Materials and Methods

**Cell Lines, Cell Culture, and Antibodies.** The  $\Psi$ 2, PA317, C127, and FR3T3 cell lines were generous gifts from Daniel DiMaio (Yale University New Haven, CT). All cell lines were propagated in DMEM supplemented with 10% FBS, 50 IU/ml penicillin, 50  $\mu$ g/ml streptomycin (Mediatech), and 0.25  $\mu$ g/ml Fungizone (Amphotericin B; Life Technologies, Inc.). Recombinant cell lines generated in the course of the experiments described in this report were propagated in the medium described above supplemented with 200  $\mu$ g/ml G418 (Mediatech).

The anti-ErbB4 mouse monoclonal (SC-8050), anti-ErbB4 rabbit polyclonal (SC-283), and anti-ErbB2 rabbit polyclonal (C-18) antibodies were purchased from Santa Cruz Biotechnology. Goat antimouse and goat antirabbit horseradish peroxidase-conjugated antibodies were purchased from Pierce. Enhanced chemiluminescence (ECL) Western blotting reagents, Redivue adenosine 5'-[ $\gamma$ - $^{32}$ P]triphosphate, and Protein-A Sepharose (CL-4B) were purchased from Amersham Pharmacia Biotech. The 4G10 anti-phosphotyrosine mouse monoclonal antibody was purchased from Upstate Biotechnology.

**Plasmids.** The recombinant retroviral vector pLXSN (41) was obtained from Daniel DiMaio (Yale University). This construct contains two recombinant LTRs derived from the Maloney murine leukemia virus and the Maloney murine sarcoma virus. These LTRs flank the  $\Psi$  packaging signal and the aminoglycoside 3'-phosphotransferase (*Neo<sup>R</sup>*) gene under the transcriptional control of the SV40 early promoter. The *Neo<sup>R</sup>* gene confers resistance to the aminoglycoside antibiotic G418 (geneticin; Life Technologies, Inc.).

The recombinant retroviral construct pLXSN-ErbB4 (26) was generated by subcloning the human ErbB4 cDNA into pLXSN. In this construct, the ErbB4 cDNA is under the transcriptional control of the upstream LTR. The recombinant retroviral construct pLXSN-ErbB2\* (42) was a gift of Lisa Petti (Albany Medical College, Albany, NY). It was generated by subcloning the cDNA encoding the constitutively active rat ErbB2 mutant (V664E transmembrane domain mutant, ErbB2\*) into pLXSN. In this construct, the ErbB2\* cDNA is under the transcriptional control of the upstream LTR.

**ErbB4 Mutagenesis.** The plasmid pLXSN-ErbB4 was used as the template for site-directed mutagenesis (QuikChange Site Directed Mutagenesis kit; Stratagene) to construct the putative constitutively active ErbB4 mutants. The mutants were constructed by introducing mutations that substitute a cysteine residue for proline 645, glutamine 646, histidine 647, alanine 648, or arginine 649 in the ErbB4 extracellular juxtamembrane domain. These mutants are denoted as follows: P645C, Q646C, H647C, A648C, and R649C. A new restriction enzyme site was also engineered in each mutant to facilitate the identification of the mutants. The following primers were used for mutagenesis. "T" denotes the upper primer, whereas "B" denotes the lower primer. The novel cysteine codons and anticodons are indicated by bold type, the point mutations that create the novel cysteine residues are double underlined, and the novel restriction enzyme sites are singly underlined.

P645CT:5'-ATTTACTACCCATGGACCGGTCATTCCACTT  
 TATGCCAACATGCTAGAACTCCC-3'  
 P645CB:5'-GGGAGTTCTAGCATGTTGGCATAAAGTGG  
 ATGACCGGTCCATGGGTAGTAAAT-3'  
 Q646CT:5'-TACTACCCATGGACCGGTCATTCCACTTTAC  
 CATGCCCATGCTAGAACTCCCCTG-3'  
 Q646CB:5'-CAGGGGAGTTCTAGCATGGCATGGTAAAGT  
 GGAATGACCGGTCCATGGGTAGTA-3'  
 H647CT:5'-CATTTACTACCCATGGACCGGTCATTCCACT  
 TTACCACAAATGTGCTAGAACTCCCCT-3'  
 H647CB:5'-AGGGGAGTTCTAGCACAATTGGTAAAGTG  
 GAATGACCGGTCCATGGGTAGTAAATG-3'  
 A648CT:5'-TCCACTTTACCACAACATTGTAGAACTCCTC  
 TGATTGCAGCTGGA-3'  
 A648CB:5'-TCCAGCTGCAATCAGAGGAGTTCTACAATG  
 TTGTGGTAAAGTGA-3'  
 R649CT:5'-ACTTTACCACAACATGCTTGCACTCCTCTGA  
 TTGCAGCTGGA-3'  
 R649CB:5'-TCCAGCTGCAATCAGAGGAGTGCAAGCATG  
 TTGTGGTAAAGT-3'

The site-directed mutagenesis reactions were performed according to the manufacturer's instructions. Standard techniques (43) were used for bacterial transformations, small-scale plasmid DNA preparations, restriction enzyme analysis of the clones, and large-scale plasmid DNA preparations. Positive clones were sequenced by the University of Wisconsin-Madison Biotechnology Center to confirm their identity.

**Production of Recombinant Retroviruses and Retroviral Infections.** The ErbB4 mutant constructs were transfected using standard techniques (44, 45) into the  $\psi$ 2 ecotropic retrovirus packaging cell line (46) to generate cell lines that express the ErbB4 mutants and to package the constructs into low-titer ecotropic retrovirus particles (44, 45).  $\psi$ 2 cells were transfected with the pLXSN vector control plasmid, pLXSN-ErbB4, and pLXSN-ErbB2\* to generate control cell lines and recombinant ecotropic retroviruses. The PA317 amphotropic packaging cell line (47) and the FR3T3 rat fibroblast cell line were infected with the ecotropic recombinant retroviruses using standard techniques (44, 45) to generate additional cell lines that express the ErbB4 mutants.

**Immunoblot Assays for Receptor Tyrosine Phosphorylation and Expression.** The analysis of ErbB4 and ErbB2 tyrosine phosphorylation by immunoprecipitation and antiphosphotyrosine immunoblotting has been described previously (21, 26). Briefly, cell lysates were generated, and protein content was quantified using a Coomassie Protein Assay Reagent (Ref. 48; Pierce Chemical). ErbB2 or ErbB4 was immunoprecipitated from equal amounts of protein using specific antibodies. The immunoprecipitates were resolved by SDS-PAGE on a 7.5% acrylamide gel and were electrotransferred onto nitrocellulose. The blots were probed with the anti-phosphotyrosine monoclonal antibody 4G10. Antibody binding was detected and visualized using a goat antimouse horseradish peroxidase-coupled antibody and enhanced chemiluminescence. The blots were then stripped and probed with the anti-ErbB4 polyclonal antibody to as-

sess ErbB4 expression levels. Antibody binding was detected and visualized using a goat antimouse horseradish peroxidase-coupled antibody and enhanced chemiluminescence.

The amounts of receptor tyrosine phosphorylation and expression were quantified by digitizing the chemilumigrams using a Linotype-Hell Jade two-dimensional scanning densitometer set at 600-dpi resolution. The bands on the images were quantified using NIH Image for Macintosh v1.6 software. Values are expressed as arbitrary units. Background levels were computed using the vector control lanes and were subtracted from the gross values to produce net receptor expression and tyrosine phosphorylation values. The digitized images were also cropped and annotated using Adobe Photoshop for Macintosh v3.0.5 software.

**In Vitro Kinase Assay.** ErbB2 and ErbB4 were immunoprecipitated from protein extracts from PA317 cells as described previously (26). Immune complex kinase reactions were performed as described previously (31). Briefly, 35  $\mu$ l of protein A-Sepharose and 5  $\mu$ l of anti-ErbB2 or anti-ErbB4 rabbit polyclonal antibodies were used to immunoprecipitate the receptors from lysates containing the same amount of protein (1000  $\mu$ g). Immunoprecipitates were washed five times in 500  $\mu$ l of kinase buffer [20 mM Tris-HCl (pH 7.4), 5 mM MgCl<sub>2</sub>, and 3 mM MnCl<sub>2</sub>]. After the last wash, the samples were resuspended in 100  $\mu$ l of kinase buffer supplemented with 10  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP and were incubated for 10 min at room temperature to permit the kinase reaction to occur. The beads were then washed two times in NET-N buffer (49) and boiled for 5 min in SDS-PAGE protein sample buffer. The samples were resolved by SDS-PAGE on a 7.5% acrylamide gel. The gels were dried overnight and exposed to X-ray film for ~20 h. The autoradiograms were digitized using a Linotype-Hell Jade two-dimensional scanning densitometer set at 600-dpi resolution. The bands on the images were quantified using NIH Image for Macintosh v1.6 software. Values are expressed as arbitrary units. Background levels were computed using the vector control lanes and were subtracted from the gross values to produce net kinase activity values. The digitized images were also cropped and annotated using Adobe Photoshop for Macintosh v3.0.5 software.

**Focus Formation Assay for Loss of Contact Inhibition.** FR3T3 and C127 cells were infected with recombinant ecotropic retroviruses as described earlier and in reports published previously (44, 45). Briefly, 60-mm dishes of cells at ~70% confluence were infected with ecotropic retrovirus stocks. Approximately 24 h after infection, cells were passaged into three 60-mm dishes. Cells were maintained in DMEM supplemented with 10% FBS until foci appeared. During this period, the medium was changed every 3 days. Once robust foci appeared, cells were fixed in 100% methanol and stained with Giemsa (Fisher) to visualize the foci. The plates were digitized using a Linotype-Hell Jade two-dimensional scanning densitometer set at 600-dpi resolution. The digitized images were cropped and annotated using Adobe Photoshop for Macintosh v3.0.5 software.

**Assay for Anchorage Independence.** FR3T3 cells were seeded at a density of  $2 \times 10^4$  cells in 60-mm dishes containing 2.5 ml of 0.3% LMP-agarose (Life Technologies, Inc.)

as described previously (50). Every 3 days, DMEM supplemented with 10% FBS and 0.3% LMP-agarose was added to each plate. The cells were incubated at 37°C for 10 days, and fields were photographed with an Olympus OM-10 camera attached to an Olympus CK-2 phase-contrast inverted microscope. The images were digitized by the photofinisher. These images were cropped and annotated using Adobe Photoshop for Macintosh v3.0.5 software. Images are representative of three independent experiments.

**Growth Rate/Saturation Density Assay.** Stable FR3T3 cell lines expressing the wild-type ErbB4 receptor, ErbB2\*, or the ErbB4 mutants (Q646C, H647C, and A648C) were plated in 10 60-mm dishes at a density of  $2 \times 10^4$  cells/dish. Cells were incubated from 1 to 10 days at 37°C. Cells were counted (Coulter Counter ZM) each day for a total of 10 days. The mean and SE are representative of three independent experiments.

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## A constitutively active ErbB4 mutant inhibits drug-resistant colony formation by the DU-145 and PC-3 human prostate tumor cell lines

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### Abstract

ErbB4 (HER4) is a member of the ErbB family of receptor tyrosine kinases, a family that also includes the Epidermal Growth Factor Receptor (EGFR/ErbB1/HER1), Neu/ErbB2/HER2, and ErbB3/HER3. Several groups have hypothesized that signal transduction by the ErbB4 receptor tyrosine kinase is coupled to differentiation, growth arrest, and tumor suppression in mammary and prostate epithelial cells. In this report we demonstrate that a constitutively active ErbB4 mutant inhibits the formation of drug-resistant colonies by the DU-145 and PC-3 human prostate tumor cell lines. This is consistent with our hypothesis that ErbB4 signaling is growth inhibitory and may be coupled to tumor suppression in prostate cells.

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**Keywords:** ErbB4; Receptor tyrosine kinase; Growth inhibition; Tumor suppression; Prostate cancer

### 1. Introduction

ErbB4 is a member of the ErbB family of receptor tyrosine kinases, a family that also includes the epidermal growth factor (EGF) receptor (EGFR/ErbB1/HER1), ErbB2/HER2/Neu, and ErbB3/HER3 [1–3]. The agonists for these receptors are members of the EGF family of peptide hormones, which includes more than 20 different growth factors (reviewed in [2–4]). The signaling network comprised of these hormones and receptors regulates cell proliferation and differentiation, as well as other

cellular functions. Moreover, deregulated signaling by this network, typically due to inappropriate receptor or ligand (over)expression, plays a significant role in many human tumors [3,5–7]. For example, EGFR or ErbB2 overexpression is detected in a significant percentage of human breast tumors and this overexpression correlates with increased metastatic potential, chemoresistance, and poorer patient prognosis.

In contrast, relatively little is known about the roles that ErbB4 plays in tumorigenesis. ErbB4 overexpression is much less common in mammary tumor samples than is EGFR or ErbB2 overexpression. Moreover, ErbB4 overexpression in mammary tumor samples correlates with a more favorable prognosis,

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not a less favorable prognosis [8–11]. The expression of ErbB4 and its ligands in the developing mouse mammary epithelium is highest late in pregnancy and during lactation, and corresponds with a period of terminal differentiation of the mammary epithelium and only limited proliferation [12,13]. Finally, the normal human prostate epithelium exhibits abundant ErbB4 expression; in contrast, ErbB4 expression has not been detected in any cultured human prostate tumor cell line studied to date [14,15]. These data have led investigators to hypothesize that ErbB4 signaling is coupled to terminal differentiation, growth arrest, and tumor suppression in the mammary and prostate epithelia.

A typical strategy for studying the function of a given ErbB family receptor involves assessing the effect of an EGF family hormone that binds to the ErbB family receptor of interest. These studies can be done either in cells that endogenously express the receptor of interest or in cells that overexpress the appropriate receptor. However, EGF family hormones stimulate heterodimerization of the cognate (binding) ErbB family receptor with any other ErbB family receptor present. This results in tyrosine phosphorylation and signaling by both the cognate ErbB family receptor as well as any other ErbB receptor. Thus, in human breast and prostate tumor cell lines, which frequently express EGFR, ErbB2, and ErbB3, ligands for ErbB4 stimulate not only ErbB4 signaling, but signaling by the other ErbB family receptors as well. Consequently, stimulation with ErbB4 ligands has been of limited value in studying ErbB4 function. Nonetheless, the ErbB4 ligand Neuregulin1beta (NRG1 $\beta$ ) stimulates differentiation of mammary epithelium to lobuloalveoli *in vivo* [16] and stimulates *in vitro* differentiation of the AU-565 human tumor cell line [17,18]. Furthermore, ErbB4 expression in the SUM102 human mammary tumor cell lines permits the induction of differentiation and growth inhibition by NRG1 $\beta$  [19]. However, efforts by our laboratory to extend these results to other human breast tumor cell lines and to prostate tumor cell lines have failed.

In response, we have embarked on a genetic strategy to study ErbB4 function. We have previously reported the construction of three constitutively active human ErbB4 mutants. These mutants are the result of a single cysteine substitution for Gln646, His647, or

Ala648 of the ErbB4 extracellular, juxtamembrane domain. Our initial analyses of these mutants revealed that these mutants, unlike a constitutively active ErbB2 mutant, fail to malignantly transform the growth of rodent fibroblast cell lines [20]. In this report we show that one of these mutants inhibits drug-resistant colony formation by two human prostate tumor cell lines. These data suggest that ErbB4 may indeed be coupled to differentiation, growth arrest, and tumor suppression in the prostate epithelium.

## 2. Materials and methods

### 2.1. Cell lines and cell culture

Mouse C127 fibroblasts and the  $\psi$ 2 and PA317 recombinant retrovirus packaging cell lines are generous gifts of Dr Daniel DiMaio (Yale University, New Haven, Connecticut, USA). These cells were cultured essentially as described previously [21,22]. PC-3 and DU-145 human prostate tumor cell lines were obtained from American Type Culture Collection and were cultured in accordance with vendor recommendations. Cell culture media and supplements were obtained from GIBCO/BRL/Life Technologies. Fetal bovine serum and G418 were obtained from Gemini Bioproducts. Plasticware and Giemsa stain were obtained from Fisher Scientific.

### 2.2. Retrovirus infections and drug-resistant colony formation assays

Recombinant amphotropic retroviruses were produced essentially as described earlier [22]. Briefly, the recombinant retroviral constructs pLXSN (vector) [23], pLXSN-ErbB4 (ErbB4 WT) [24], pLXSN-ErbB2 V664E (ErbB2\*) [25], pLXSN-ErbB4 Q646C, pLXSN-ErbB4 H647C, and pLXSN-ErbB4 A648C [20] were transfected into the  $\psi$ 2 ecotropic retrovirus packaging cell line [26]. Transfected cells were selected using G418 and drug-resistant colonies were pooled and expanded into stable cell lines. Recombinant ecotropic retroviruses were recovered from the conditioned media of the recombinant  $\psi$ 2 cell lines. These stocks were used to infect the PA317 amphotropic retrovirus packaging cell line [27].

Infected cells were selected using G418 and drug-resistant colonies were pooled and expanded into stable cell lines. Recombinant amphotropic retroviruses were recovered from the conditioned media of the recombinant PA317 cell lines. pLXSN is a generous gift of Dr Daniel DiMaio (Yale University, New Haven, Connecticut, USA). pLXSN-ErbB2\* is a generous gift of Dr Lisa Petti (Albany Medical College, Albany, New York, USA).

C127, DU-145, and PC-3 infections with the recombinant amphotropic retroviruses were performed essentially as described earlier [20–22]. Infected cells were selected using G418. Approximately 12 days after infection, drug-resistant colonies were stained using Giemsa. The tissue culture plates were digitized using an Epson flatbed scanner set for 600 dpi. The digital images were cropped, annotated and combined into composite images. The contrast of the images was enhanced and the background was minimized to maximize the signal–noise ratio. Manipulations of the digital images were performed using Adobe Photoshop.

Drug-resistant colonies were counted manually and the retrovirus titer for each combination of retrovirus and cell line was determined by dividing the number of colonies by the volume of retrovirus used in the infection. The average viral titers were calculated from at least ten independent sets of infections. The efficiency of drug-resistant colony formation was calculated for each retrovirus stock in the DU-145 cell line by dividing the retroviral titers in the DU-145 cells by the corresponding retroviral titers in the C127 cells. These values are expressed as mean percentages calculated from at least ten independent sets of infections. The standard error was also calculated for each mean percentage. Analogous calculations were performed to calculate the efficiency of drug-resistant colony formation for each retrovirus stock in the PC-3 cell lines.

### 2.3. Immunoprecipitation and immunoblotting

Anti-ErbB4 immunoprecipitations and anti-phosphotyrosine immunoblotting were performed essentially as described earlier [20]. Briefly, C127 cells were starved overnight in serum-free medium, then lysed using an ice-cold isotonic lysis buffer supplemented with the non-ionic detergent NP-40

(Sigma). Nuclei and cellular debris were cleared from the lysates by centrifugation. The protein content of the lysate supernatants was determined using a modified Bradford protein assay (Pierce). ErbB4 was immunoprecipitated from equal amounts of lysate using protein A sepharose (Amersham/Pharmacia) and an anti-ErbB4 rabbit polyclonal antibody (Santa Cruz Biotechnology). The precipitates were washed with an isotonic lysis buffer and the proteins were released from the sepharose beads by boiling in a reducing SDS sample buffer. The samples were resolved by SDS-PAGE using a 7.5% acrylamide gel and were electroblotted onto nitrocellulose. The resulting blot was probed with an anti-phosphotyrosine mouse monoclonal antibody (Upstate Biotechnology). Primary antibody binding was detected and visualized using a goat anti-mouse antibody conjugated to horseradish peroxidase (Pierce) and enhanced chemiluminescence (Amersham/Pharmacia). The chemilumigram was digitized using an Epson flatbed scanner set for 600 dpi resolution. The digital images were cropped and annotated using Adobe Photoshop.

## 3. Results

### 3.1. The ErbB4 Q646C mutant inhibits drug-resistant colony formation by the DU-145 human prostate tumor cell line

We previously described the construction and packaging of recombinant retroviral vectors that express the neomycin resistance gene as well as the constitutively active ErbB4 mutants [20]. We infected DU-145 cells with these retroviruses and selected for drug-resistant colonies using G418 to assess whether any of the constitutively active ErbB4 mutants inhibits drug-resistant colony formation. As controls we also infected DU-145 cells with recombinant retroviruses that carry only the neomycin resistance gene (Vector), with recombinant retroviruses that express a constitutively active (V664E) mutant of the rat ErbB2 gene (ErbB2\*) [25], and with recombinant retroviruses that express the wild-type ErbB4 gene. To control for differences in absolute viral titers, we infected C127 mouse fibroblasts in parallel and

assayed the formation of drug-resistant colonies of infected cells.

As shown in Fig. 1, DU-145 cells infected with the recombinant retrovirus that expresses the ErbB4 Q646C mutant form fewer drug-resistant colonies than do DU-145 cells infected with the other recombinant retroviruses. Furthermore, the titer of the ErbB4 Q646C recombinant retrovirus in the DU-145 cells is less than the titers of the other recombinant retroviruses (Table 1). However, the titer of the ErbB4 Q646C recombinant retrovirus in C127 fibroblasts is not less than the titer of most of the other recombinant retroviruses (Table 1). Thus, the

ratio of the ErbB4 Q646C retroviral titers in DU-145 and C127 cells is much less than the corresponding ratios of the other retrovirus titers (Table 1). Indeed, it appears that the ErbB4 Q646C mutant inhibits drug-resistant colony formation by DU-145 cells by approximately 90%.

### 3.2. The ErbB4 Q646C mutant inhibits drug-resistant colony formation by the PC-3 human prostate tumor cell line

We infected PC-3 cells in parallel with the DU-145 and C127 infections. The results of these infections

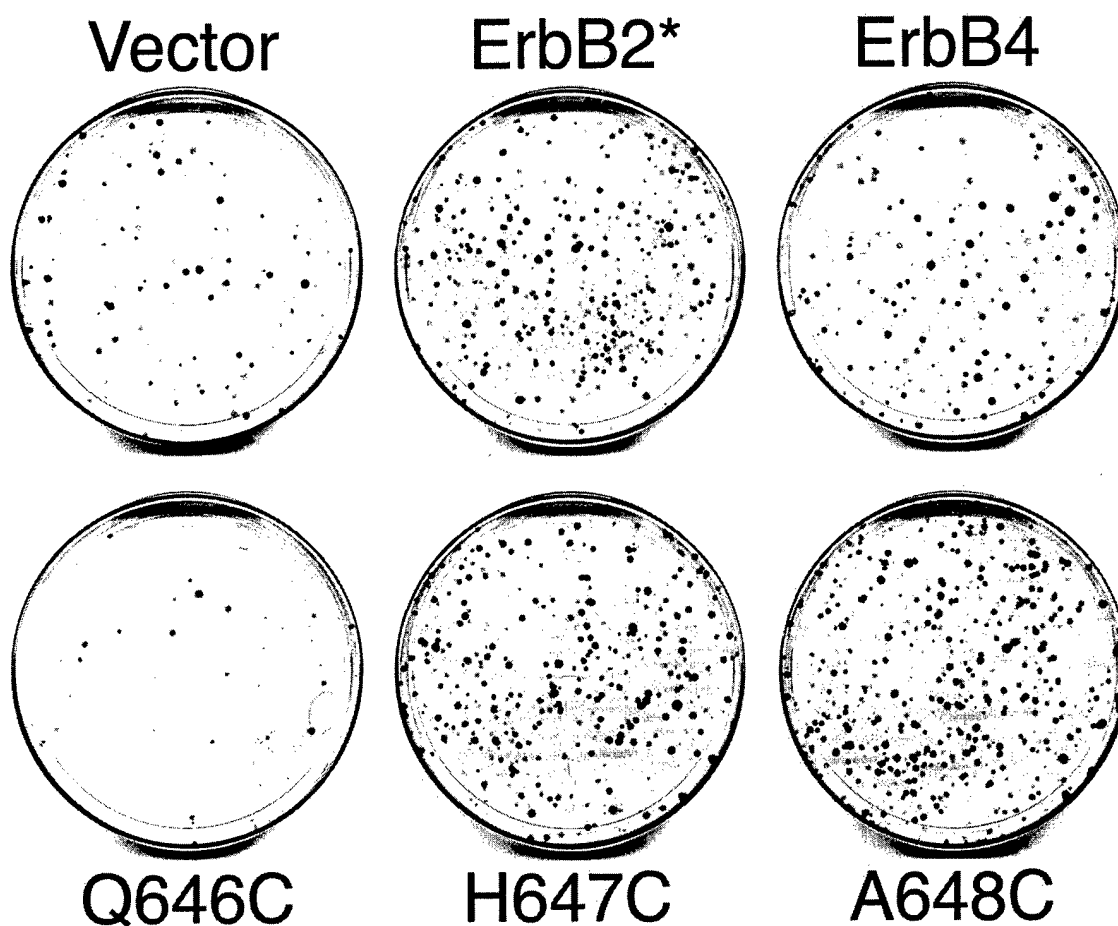


Fig. 1. The ErbB4 Q646C mutant inhibits drug-resistant colony formation by the DU-145 human prostate tumor cell line. DU-145 human prostate cells were infected with recombinant amphotropic retroviruses that carry the neomycin resistance gene (Vector) or with retroviruses that carry the neomycin resistance gene along with a constitutively active ErbB2 mutant (ErbB2\*), wild-type ErbB4 (ErbB4), or constitutively active ErbB4 mutants (Q646C, H647C, A648C). Infected cells were selected using 600  $\mu$ g/ml G418. Colonies of drug-resistant cells were stained using Giemsa and counted.

Table 1  
The ErbB4 Q646C mutant specifically inhibits drug-resistant colony formation by the DU-145 and PC-3 human prostate tumor cell lines<sup>a</sup>

| Virus    | Viral titers |            |             | Colony formation efficiency |            |
|----------|--------------|------------|-------------|-----------------------------|------------|
|          | Cell line    |            |             | Ratios                      |            |
| Stock    | C127         | DU-145     | PC-3        | DU-145/C127                 | PC-3/C127  |
| Vector   | 1.14E + 06   | 7.88E + 04 | 1.21 E + 05 | 10.7 ± 2.7                  | 19.4 ± 5.7 |
| ErbB2*   | 2.92E + 05   | 3.23E + 04 | 3.09E + 04  | 11.9 ± 1.8                  | 15.6 ± 3.9 |
| ErbB4 WT | 1.55E + 05   | 1.44E + 04 | 2.27E + 04  | 12.0 ± 3.1                  | 25.3 ± 7.5 |
| Q646C    | 6.17E + 05   | 3.42E + 03 | 1.56E + 04  | 0.6 ± 0.1                   | 3.1 ± 0.8  |
| H647C    | 8.65E + 05   | 4.59E + 04 | 6.27E + 04  | 7.2 ± 1.2                   | 17.3 ± 6.3 |
| A648C    | 1.49E + 05   | 1.46E + 04 | 1.67E + 04  | 11.8 ± 2.1                  | 15.0 ± 2.8 |

<sup>a</sup> We counted the number of colonies on each plate of infected DU-145, PC-3, and C127 cells and divided by the volume of retrovirus used to infect the cells to determine the titer of each retrovirus stock in each of the three cell lines. To compare the relative efficiency of each retrovirus stock at inducing drug-resistant colony formation in the DU-145 cell line, we divided the titer of each retrovirus stock in the DU-145 cell line by the titer of the same retrovirus stock in the C127 cell line. This value is expressed as a mean percentage calculated from at least ten independent sets of infections. The standard error for each mean was calculated and is reported. We performed analogous calculations to determine the efficiency of drug-resistant colony formation of each retrovirus stock in the PC-3 cell lines.

are similar to the results of the DU-145 infections. PC-3 cells infected with the recombinant retrovirus that expresses the ErbB4 Q646C mutant form fewer drug-resistant colonies than do PC-3 cells infected with the recombinant retroviruses that express the other ErbB4 constructs (Fig. 2). Furthermore, the titer of the ErbB4 Q646C recombinant retrovirus in the PC-3 cells is less than the titers of the other recombinant retroviruses (Table 1). Finally, the ratio of the ErbB4 Q646C retroviral titers in PC-3 and C127 cells is much less than the corresponding ratios of the other retrovirus titers (Table 1). Indeed, it appears that the ErbB4 Q646C mutant inhibits drug-resistant colony formation by PC-3 cells by approximately 75%.

### 3.3. The constitutively active ErbB4 mutants are expressed and tyrosine phosphorylated in the mouse C127 fibroblast cell line

We were concerned that the failure of the ErbB4 H647C and A648C mutants to inhibit drug-resistant colony formation by the DU-145 and PC-3 human prostate tumor cell lines may be due to an absence of expression and/or tyrosine phosphorylation of these ErbB4 mutants. Consequently, we pooled drug-resistant colonies that resulted from infections of C127 cells and generated stable cell lines. We assayed ErbB4 expression and tyrosine phosphorylation in

these cell lines by ErbB4 immunoprecipitation and anti-phosphotyrosine immunoblotting.

In Fig. 3 we show that all three constitutively active ErbB4 mutants are expressed and display ligand-independent tyrosine phosphorylation in the appropriate C127 cell lines. Indeed, it appears that the ErbB4 Q646C mutant exhibits less tyrosine phosphorylation than the ErbB4 H647C and A648C mutants. This suggests that the failure of the ErbB4 H647C and A648C mutants to inhibit drug-resistant colony formation by the DU-145 and PC-3 cell lines is not due to an absence of expression and/or tyrosine phosphorylation of these ErbB4 mutants.

## 4. Discussion

Here we demonstrate that the Q646C constitutively active ErbB4 mutant inhibits drug-resistant colony formation by the DU-145 and PC-3 human prostate tumor cell lines. This suggests that ErbB4 signaling is coupled to prostate cell growth arrest and tumor suppression. Several issues remain to be resolved in future experiments.

The phenotype that underlies ErbB4 coupling to inhibition of drug resistant colony formation has yet to be determined. For example, it is possible that ErbB4 couples to specific cell cycle arrest. However, it is also possible that ErbB4 is coupling to apoptosis rather than

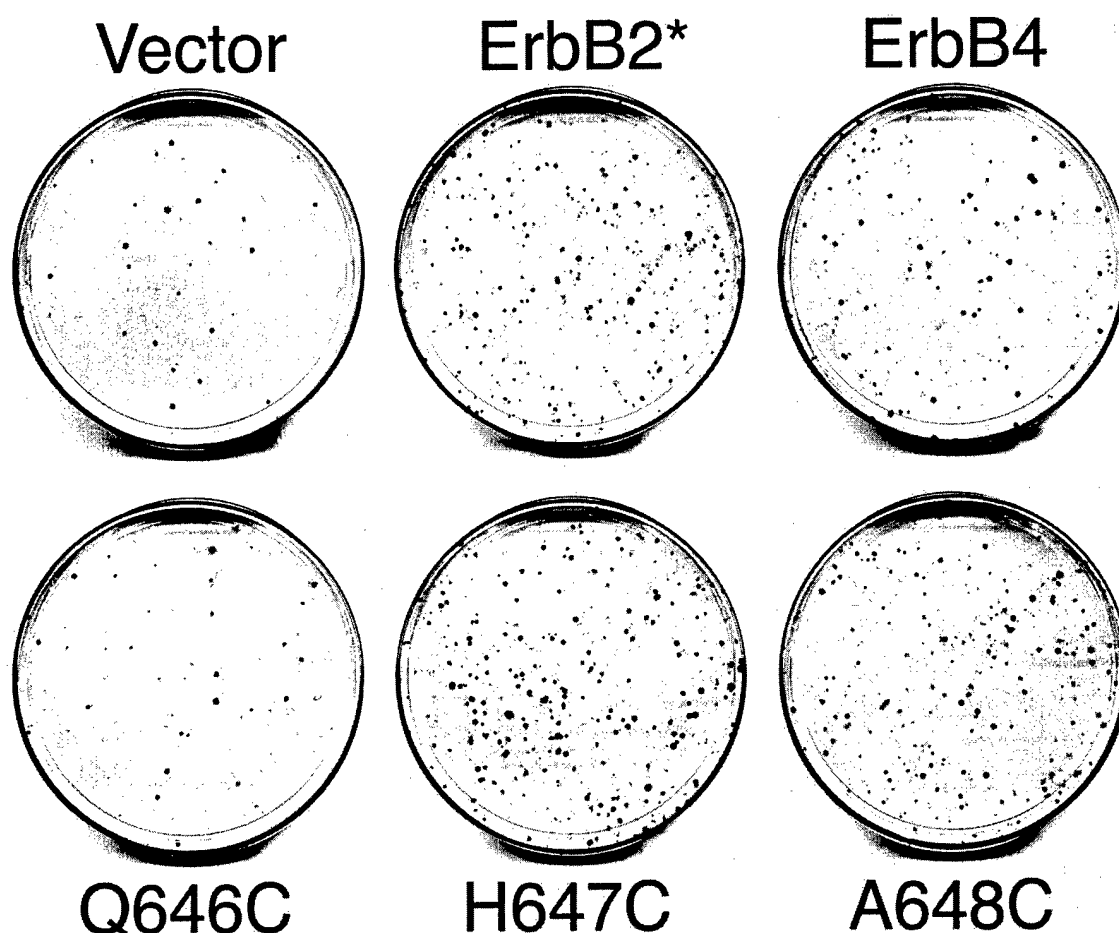


Fig. 2. The ErbB4 Q646C mutant inhibits drug-resistant colony formation by the PC-3 human prostate tumor cell line. PC-3 human prostate cells were infected with recombinant amphotropic retroviruses that carry the neomycin resistance gene (Vector) or with retroviruses that carry the neomycin resistance gene along with a constitutively active ErbB2 mutant (ErbB2\*), wild-type ErbB4 (ErbB4), or constitutively active ErbB4 mutants (Q646C, H647C, A648C). Infected cells were selected using 600  $\mu$ g/ml G418. Colonies of drug-resistant cells were stained using Giemsa and counted.

growth arrest. Since it is impossible to evaluate these hypotheses with the experimental system described in this report, we are developing a conditional expression system that should enable us to determine whether ErbB4 signaling is coupled to cell cycle arrest, apoptosis, or non-specific growth arrest.

Another goal for future experiments is to determine why the Q646C ErbB4 mutant is coupled to inhibition of drug-resistant colony formation by prostate tumor cell lines, whereas the H647C and A648C ErbB4 mutants are not. The differential coupling of these ErbB4 mutants is analogous to the differential coupling of constitutively phosphorylated rat ErbB2

mutants to growth transformation of rodent fibroblasts [28]. It is also analogous to the differential coupling of mutants of the bovine papillomavirus (BPV) E5 protein to malignant growth transformation of rodent fibroblasts. This differential coupling is in marked contrast to the fact that several of these BPV E5 mutants stimulate abundant platelet-derived growth factor receptor tyrosine phosphorylation [29,30]. In both of these examples, it is believed that the constitutively phosphorylated receptor tyrosine kinases are phosphorylated on different individual tyrosine residues, resulting in differential coupling to downstream signaling proteins and biological

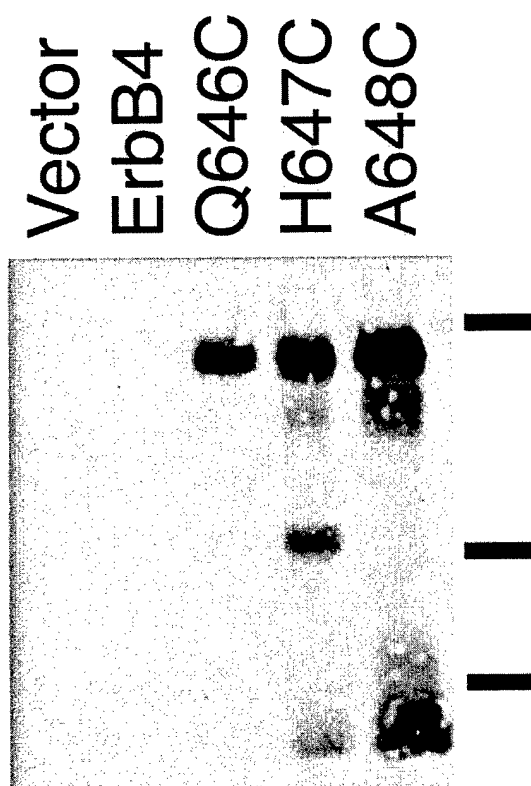


Fig. 3. The constitutively active ErbB4 mutants are expressed and tyrosine phosphorylated in the mouse C127 fibroblast cell line. C127 fibroblasts were infected with recombinant amphotropic retroviruses that express the neomycin resistance gene (Vector) or with retroviruses that express the neomycin resistance gene along with wild-type ErbB4 (ErbB4) or constitutively active ErbB4 mutants (Q646C, H647C, A648C). Infected cells were selected using 1000  $\mu$ g/ml G418. Colonies of drug-resistant cells were pooled and expanded into stable cell lines. Confluent 100 mm plates of each cell line were incubated in serum-free medium for 24 h, after which cells were lysed. ErbB4 was precipitated using specific antibodies and the precipitates were resolved by SDS-PAGE and electroblotted onto nitrocellulose. The blot was probed with an anti-phosphotyrosine mouse monoclonal antibody. Antibody binding was detected and visualized using a goat anti-mouse secondary antibody coupled to horseradish peroxidase and enhanced chemiluminescence. Bars indicate the positions of the molecular weight markers (198 kDa, 115 kDa, and 93 kDa). Tyrosine phosphorylated ErbB4 is represented by the band with apparent mobility of approximately 190 kDa.

responses. Indeed, different ErbB4 ligands cause different patterns of ErbB4 phosphorylation and differential coupling to downstream signaling effectors and biological responses [31]. Thus, we hypoth-

esize that the functional differences between the ErbB4 Q646C mutant and the other constitutively active ErbB4 mutants are due to phosphorylation on different ErbB4 tyrosine residues. Mapping the sites of ErbB4 tyrosine phosphorylation for the three constitutively active ErbB4 mutants and genetic studies to identify the sites of ErbB4 tyrosine phosphorylation that couple ErbB4 to inhibition of drug resistant colony formation will enable us to formally address this hypothesis.

Finally, additional experiments will be necessary to formally test the hypothesis that ErbB4 is a prostate tumor suppressor. Male transgenic mice that exhibit tissue specific ectopic expression of the Q646C ErbB4 mutant in the prostate gland would be an appropriate *in vivo* model system for assessing whether constitutive ErbB4 signaling is sufficient to suppress prostate tumorigenesis.

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