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Epidermal Growth Factor Receptor vIII Enhances Tumorigenicity in Human Breast Cancer[1]

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ABSTRACT

Epidermal growth factor receptor vIII (EGFRvIII) is a tumor-specific, ligand-independent, constitutively active variant of the EGFR. Its expression has been detected in gliomas and various other human malignancies. To more fully characterize the function and potential biological role of EGFRvIII in regulating cell proliferation and in tumorigenesis, we transfected EGFRvIII cDNA into a nontumorigenic, interleukin 3 (IL-3)-dependent murine hematopoietic cell line (32D cells). We observed 32D cells expressing high levels of EGFRvIII (32D/EGFRvIII P5) to be capable of abrogating the IL-3-dependent pathway in the absence of ligands. In contrast, the parental cells, 32D/EGFR, 32D/ErbB-4, and 32D/ErbB-2+ErbB-3 cells, all depended on IL-3 or EGF-like ligands for growth. 32D/EGFRvIII P5 cells subjected to long-term culture conditions in the absence of IL-3 revealed further elevation of EGFRvIII expression levels. These results suggested that the IL-3-independent phenotype is mediated by EGFRvIII. The level of expression is a critical driving force for the IL-3-independent phenotype. Dose-response analysis revealed 32D/EGFRvIII cells to require 500-fold higher concentrations (50 ng/ml) of EGF to further stimulate the EGF-mediated proliferation than in the 32D/EGFR cells (100 pg/ml). Similar effects were also observed in betacellulin-mediated proliferation. Moreover, 32D cells expressing high levels of EGFRvIII formed large tumors in nude mice, even when no exogenous EGF ligand was administered. In contrast, no tumors grew in mice injected with 32D/EGFR, 32D/ErbB-4, and 32D/ErbB-2+ErbB-3 cells or low-expressing clone 32D/EGFRvIII C2 cells or the parental 32D cells. The changes of the ligand specificity support the notion for an altered conformation of EGFRvIII to reveal an activated ligand-independent oncoprotein with tumorigenic activity analogous to v-erbB. These studies clearly demonstrate that EGFRvIII is capable of transforming a nontumorigenic, IL-3-dependent murine hematopoietic cell line (32D cells) into an IL-3-independent and ligand-independent malignant phenotype *in vitro* and *in vivo*.

To delineate the biological significance of EGFRvIII in human breast cancer, we expressed EGFRvIII in the MCF-7 human breast cancer cell line. Expression of EGFRvIII in MCF-7 cells produced a constitutively activated EGFRvIII receptor. Expression of EGFRvIII in MCF-7 cells also elevated ErbB-2 phosphorylation, presumably through heterodimerization and cross-talk. These MCF-7/EGFRvIII transfectants exhibited an ~3-fold increase in colony formation in 1% serum with no significant effect observed at higher percentages of serum. A similar result was also seen in anchorage-dependent assays. Furthermore, EGFRvIII expression significantly enhanced tumorigenicity of MCF-7 cells in athymic nude mice with $P < 0.001$. Collectively, these results provide the first evidence that EGFRvIII could play a pivotal role in human breast cancer progression.

INTRODUCTION

Overexpression of tyrosine kinase receptors has a deleterious effect on normal cell growth, leading to the induction of transformation (1). The EGFR[3] is an activated oncogene associated with human tumor biology (2, 3). Enhanced expression of EGFR is frequently detected in a variety of carcinomas, including breast, lung, and head and neck, as well as glioblastoma (4–8). High levels of EGFR and HER-2 have been found in 30–40% of breast carcinomas and correlated with poor prognosis (9). A number of agonists can bind to and activate EGFR, including EGF, TGF-α, amphiregulin, BTC, and heparin-binding EGF-like growth factors (HB-EGF; Ref. 10). Overexpression of EGFR in human malignancy has been extensively studied, where it has become increasingly apparent that alterations of the *EGFR* gene may be as important as amplification toward the oncogenic effects (11, 12). A spontaneous rearrangement within the *EGFR* gene termed EGFRvIII was first identified in primary human glioblastoma tumors (13–16). This type III EGF deletion-mutant receptor (EGFRvIII) is characterized by the deletion of exons 2–7 in the EGFR mRNA and correspond to cDNA nucleotides 275-1075, which encode amino acids 6–276, presumably through alternative splicing or rearrangements (13–15). Deletion of 801 bp within the extracellular domain of the *EGFR* gene causes an in-frame truncation of the normal EGFR protein, resulting in a 145-kDa receptor. A number of functional differences between EGFRvIII and normal EGFR have been characterized. The EGFRvIII molecule appears to be unregulated by EGF or TGF-α (17–19). However, EGFRvIII is constitutively activated. Overexpression of EGFRvIII in NIH3T3 and NR6 cells results in transformed morphology, enhanced growth, and tumorigenicity in athymic mice (17, 20). Recent reports demonstrated that the EGFRvIII is also frequently detected in other human cancers, including breast, ovarian, lung, and medulloblastoma tumors (21, 22), but has not been detected in normal adult tissue (21, 23). The frequent expression of this EGFRvIII in various tumors types suggests a strong selective advantage conferred upon tumor cells *in vivo* (24, 25). However, the tumorigenic potential of EGFRvIII in breast cancer cells has not yet been explored. Understanding the function and biology of EGFRvIII will have important implications in the prognosis and treatment of breast cancer.

In this report, we describe the biological consequences resulting from the expression of EGFRvIII in a murine, hematopoietic, IL-3-dependent cell line lacking endogenous EGF-family receptors, as well as in a breast cancer cell line (MCF-7). In this comprehensive study, our data provide evidence that overexpression of EGFRvIII is capable of transforming a nontumorigenic, IL-3-dependent murine hematopoietic cell line (32D) into an IL-3-independent, ligand-independent, and highly tumorigenic phenotype in athymic nude mice. Such profound transforming activity has not been observed in any homo- or heterodimers of wild-type ErbB-family receptors in this system. Transformation ability of EGFRvIII is dependent upon the level of EGFRvIII expression. We also demonstrated clearly that expressing a relatively low level of EGFRvIII in human breast cancer cells significantly enhanced its tumorigenicity compared with untransfected

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[3] The abbreviations used are: EGFR, epidermal growth factor receptor; TGF, transforming growth factor; BTC, betacellulin; IMEM, Iscove's modified Eagle's medium; IL, interleukin; FACS, fluorescence-activated cell sorter.

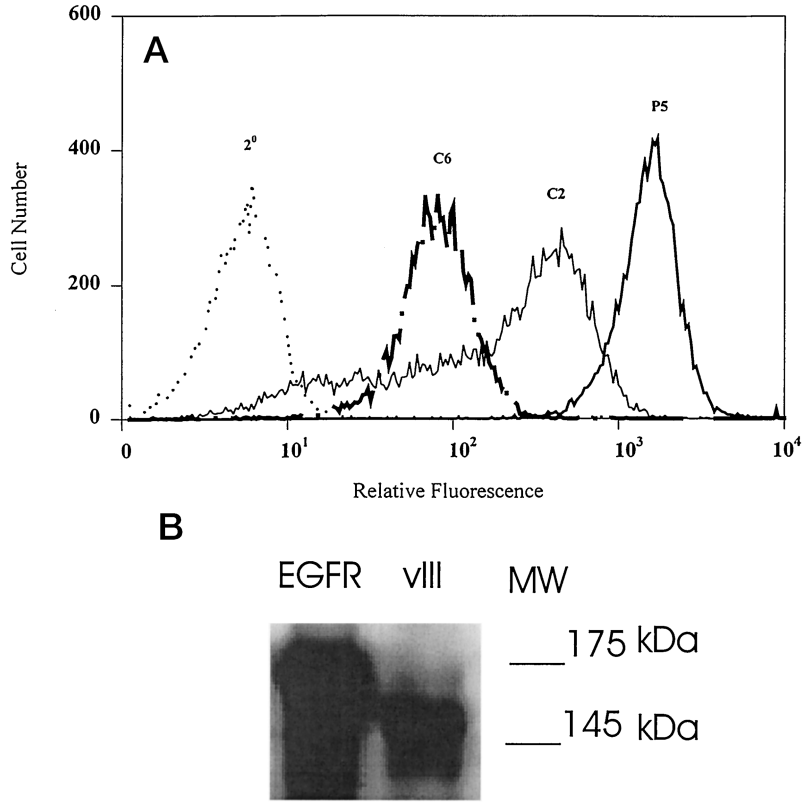

Fig. 1. Analysis of EGFRvIII expression in 32D/EGFRvIII transfectants. *A*, the levels of EGFRvIII in the 32D/EGFRvIII transfectants were quantitatively measured by flow cytometry. The *leftmost curve* (*thin dotted-line curve*) represents nonspecific staining (primary antibody omitted). The other line curves represent the expression of EGFRvIII receptor in 32D/EGFRvIII transfectants. *Bold dotted line*, 32D/EGFRvIII C6 pool clone; *thin solid line*, 32D/EGFRvIII C2 pool clone; *bold solid line*, 32D/EGFRvIII P5 pool clone. The 32D/EGFRvIII C6 expresses the lowest levels of EGFRvIII among these transfectants. The 32D/EGFRvIII C2 expresses medium levels of EGFRvIII, whereas 32D/EGFRvIII P5 expresses the highest level of EGFRvIII among these transfectants. *B*, Western immunoblot analysis of the molecular weight of EGFRvIII. Total cell lysates (30 μg) from 32D/EGFR and 32D/EGFRvIII P5 cells were electrophoresed on SDS-PAGE and transferred onto nitrocellulose membranes. Bands were visualized using monoclonal EGFR antibody, which recognizes both wild-type EGFR and the mutant form of EGFR (EGFRvIII), and a chemiluminescence detection system. 32D/EGFR expressed a 175-kDa protein, and 32D/EGFRvIII expressed a 145-kDa protein, respectively.

cells. These results are agreement with those observed with NIH3T3, NR6 cells, and U87 MG where EGFRvIII is a potent oncoprotein with ligand-independent transforming activity (18, 20, 24). Our results provide the evidence for the pivotal role of EGFRvIII in human breast cancer progression.

MATERIALS AND METHODS

Cell Lines and Tissue Culture. The 32D mouse pro-B-lymphocyte cell line derivatives were grown in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% FCS (Biofluids) and IL-3 supplied as 10% conditioned medium from the WEHI-3B mouse myelomonocytic leukemia cell line (26). MCF-7 breast carcinoma cell lines and their derivatives were maintained in IMEM (Cellgro) supplemented with 10% FCS (Biofluids).

Transfection. 32D derivative cells (1×10^7) were used for transfection. Ten μg of the full-length EGFRvIII cDNA were electroporated into 32D cells at 250 V, using a Bio-Rad electroporation system. Derivatives transfected with constructs expressing EGFRvIII receptors were selected by supplementation with 750 μg of G418 in the medium.

For MCF-7 Cells, 1×10^6 cells and 10–15 μg of plasmid DNA were used for each transfection. Transfections were performed using the Calcium Phosphate Transfection System (Life Technologies, Rockville, MD), according to the manufacturer's protocol. The cells were then selected in a growth medium containing appropriate amounts of Geneticin (G418 sulfate; Life Technologies, Inc.).

Autophosphorylation of ErbB-Family Receptors. Prior to cell lysis, the cells were serum starved overnight at 37°C. After incubation, cells were then treated with 100 ng/ml of neuregulin (R & D Systems) or 100 ng/ml of BTC (R & D Systems) for 5 min at 37°C. After a 5-min incubation, cells were lysed in a HEPES-lysis buffer, and the cell debris was pelleted by centrifugation (27).

The lysates were immunoprecipitated with either anti-EGFR (Ab-1; NeoMarkers, Union City, CA) or anti-ErbB-2 (Ab-3; Oncogene Science, Uniondale, NY) overnight at 4°C with gentle agitation. Protein A-Sepharose (50 μl; Pharmacia, Piscataway, NJ) was then added to the immunocomplex and incubated at 4°C for 1 h. Immunoprecipitates were then separated by SDS-PAGE and transferred to nitrocellulose. Bound proteins were immunoblotted with anti-phosphotyrosine monoclonal antibody PY20 (UBI, Lake Placid, NY), followed by blotting with 0.5 μg/ml of secondary antibody linked to horseradish peroxidase. Immunoreactive bands were detected by an enhanced chemiluminescence reagent (ECL; Amersham Corp.).

Fluorescence-activated Cell Sorter (FACStar) Analysis. Cells (1×10^6) cells were harvested and then stained for 1 h with anti-EGFRvIII antibody at 4°C. Stained cells were then washed with cold PBS. A secondary FITC-antirabbit antibody, was added and the EGFRvIII level was quantitated by flow cytometry.

Anchorage-dependent Growth Assays. Cells were harvested using trypsin, and 1500 cells/well were plated in 24-well plates (Costar). Three independent assays were performed in triplicate. Cells were counted in a Coulter Counter (Coulter Electronics Ltd., Hialeah, FL) on day 1 (the following day), day 3, and day 7. Values indicate the mean of triplicate determinations ± SD.

Anchorage-independent Growth Assays. A bottom layer of 1 ml of IMEM containing 0.6% agar and 10% FCS was prepared in 35-mm tissue culture dishes. After the bottom layer solidified, cells (10,000/dish) were then added in a 0.8-ml top layer containing 0.4% Bacto Agar and 5% FCS. Three independent assays were performed, and all samples were prepared in triplicate. Cells were incubated for ~12 days at 37°C. Colonies larger than 60 μm were counted in a cell colony counter (Ommias 3600; Imaging Products International, Inc., Charley, VA).

***In Vivo* Studies.** Ovariectomized athymic nude mice were inoculated s.c. with either 32D/wt, 32D/EGFR, 32D/EGFRvIII P5, or MCF-7/wt, MCF-7/vector, as well as EGFRvIII transfected clone, MCF-7/EGFRvIII C4 in the presence of estrogen source (0.72 mg). The slow-release pellets (60-day release) were implanted s.c. into the cervical scapular space. Tumor growth was monitored twice weekly for 3–6 weeks. Tumor size was measured twice weekly and calculated by measuring tumor volume (length × width × thickness). When tumors reached up to 2 cm in diameter, mice were sacrificed.

RESULTS

Elucidation of the Potential Biology and Function of EGFRvIII in a Cellular Model System

Generation of EGFRvIII-expressing 32D Cells. To elucidate the potential biological functions of EGFRvIII, we expressed EGFRvIII

in a specific cellular model system (32D cell). 32D cells are a murine hematopoietic, nontumorigenic, IL-3-dependent cell line. These cells offer the advantage of receptor analysis in the absence of cross-talk, because parental 32D cells express no known ErbB family members. In addition, the survival and proliferation of the cells are tightly regulated by an exogenous growth factor (IL-3). We, as well as other groups, have shown previously that the IL-3-dependent pathway can be abrogated by introduction of ErbB-family receptor genes, followed by stimulation with the appropriate growth factor (12, 26, 28). This unique model system is suitable for evaluating mitogenic potential of EGFRvIII.

We first transfected EGFRvIII cDNA into 32D cells. The resulting transfectants were designated 32D/EGFRvIII. A number of the 32D/EGFRvIII clones were selected for biochemical and biological characterization. The expressions of the cell surface EGFRvIII receptors in these transfectants were quantitatively measured by flow cytometric analysis (FACS) with an EGFRvIII-specific antibody. Three pooled populations, 32D/EGFRvIII P5, 32D/EGFRvIII C2, and 32D/EGFRvIII C6, which express relatively high, medium, and low levels of EGFRvIII, respectively, were selected for further characterization (Fig. 1*A*). The appropriate molecular mass of the EGFRvIII was confirmed by Western blot analysis with a specific antibody, which recognizes the intracellular domain of either the wild-type EGFR or EGFRvIII receptors. Fig. 1*B* shows the appropriate 145-kDa protein expressed by 32D/EGFRvIII cells, whereas 32D/EGFR (wt) cells expressed a 170-kDa protein. The EGFR transfected 32D cells are denoted as 32D/EGFR.

EGFRvIII Activated a Ligand-independent, IL-3-independent Pathway in 32D Cells. We next determined the ability of 32D/EGFRvIII cells to proliferate in the absence of IL-3. The high-expressing 32D/EGFRvIII P5 transfectant was capable of proliferating in the absence of IL-3 (Fig. 2*A*). The addition of exogenous EGF and BTC both further enhanced their growth (Fig. 2*A*). However, TGF-α, HB-EGF, and amphiregulin had no significant effect in these EGFRvIII transfectants. In contrast, the parental 32D cells absolutely required IL-3 for growth and were unresponsive to any of the EGF-like ligand treatment. The wild-type 32D/EGFR cells required either IL-3 or ligands for EGFR for growth (Fig. 2*A*). In the absence of IL-3, 32D/EGFR cells did not survive, despite the fact that FACS analysis revealed that the EGF receptor expression level was similar to EGFRvIII in 32D/EGFRvIII P5. However, the intermediate-expressing transfectant (32D/EGFRvIII C2) sustained cell survival in the absence of IL-3 but not growth, whereas EGF and BTC stimulated low levels of mitogenic activity. In the low-expressing clone 32D/EGFRvIII C6, EGF had no significant biological effect (Fig. 2*A*). Apparently, overexpression of EGFRvIII was able to abrogate the IL-3 requirement for 32D cells in the absence of ligand. In contrast, neither homo- nor heterodimers of the wild-type EGF-family receptors have the same ability to grow in the absence of ligand supplementation (29–32). Furthermore, dose-response analysis revealed 32D/EGFR cells to require 100 pg/ml of EGF to stimulate a maximal level of proliferation, whereas 32D/EGFRvIII cells required a 500-fold higher concentration (50 ng/ml) of EGF to further stimulate the EGF-mediated proliferation (Fig. 2*B*). Similar effects were also observed in BTC-mediated proliferation.

To further evaluate whether 32D/EGFRvIII P5 cells were capable of continuously proliferating independently of IL-3, 32D/EGFRvIII P5 cells were cultured in the absence of IL-3. The growth rate of 32D/EGFRvIII cells was initially reduced in the medium lacking IL-3. After an adaptation period of 10 days, these cells could be cultured infinitely, independent of IL-3, and possessed a similar doubling time as the transfectant propagated in IL-3 (Fig. 3*A*). We further examined growth rate differences between the short-term and long-term culture in the absence of IL-3, which correlated with expression levels of EGFRvIII. FACS analysis revealed a higher expression level of EGFRvIII in the long-term, IL-3-withdrawn culture than the short-term, IL-3-withdrawn culture (Fig. 3*B*). These results support the argument for an IL-3-independent phenotype, which depends upon levels of EGFRvIII expression. The differences between the EGFRvIII and the wild-type EGF-family receptors indicated that EGFRvIII is a more potent mitogenic oncoprotein.

Overexpression of EGFRvIII Not Only Abrogated the IL-3 Growth Factor Requirement of 32D Cells But Also Caused Them to Become Tumorigenic in Nude Mice. We also characterized the tumorigenicity of EGFRvIII on 32D cells *in vivo* in comparison with 32D parental cells and 32D/EGFR transfectant. 32D/EGFRvIII cells grew large tumors with a mean tumor size of 4500 ± 804 mm^3 within 3 weeks (Table 1). Although the 32D/EGFR cells exhibited high levels of functional EGFR, the parental 32D cells and 32D/EGFR

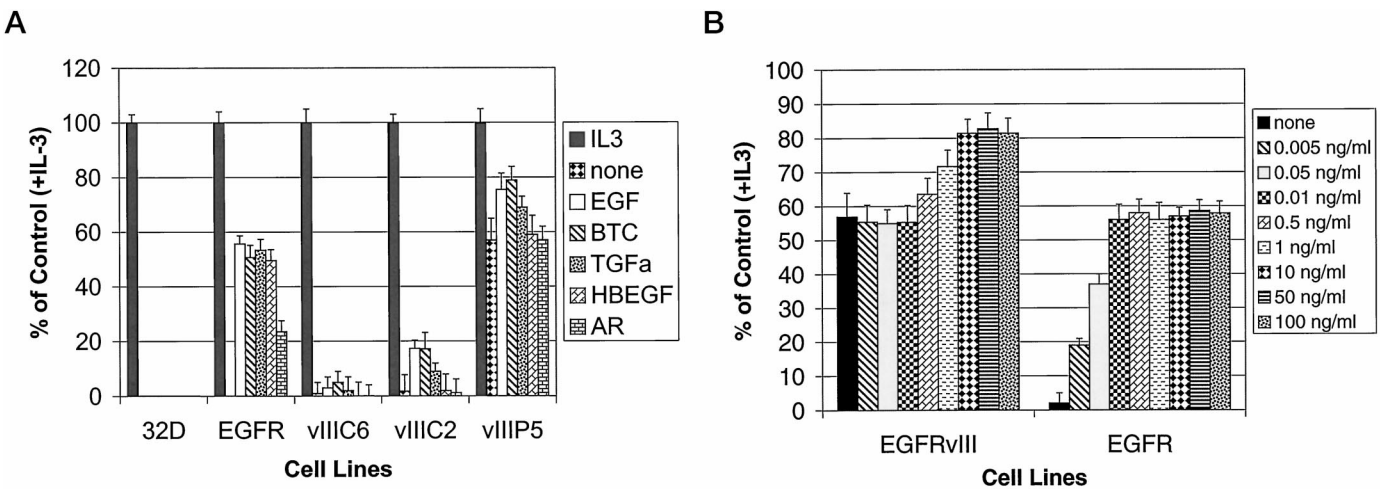

Fig. 2. EGFRvIII-mediated IL-3-independent phenotype in 32D/EGFRvIII P5 cells. *A*, 32D, 32D/EGFR, 32D/EGFRvIII, C6, C2, and P5 cells were plated at a density of 5×10^4 cells/ml in IL-3-free medium, medium supplemented with IL-3, or medium lacking IL-3 but supplemented with 100 ng/ml of various EGF-like ligands as indicated. Viable cells were counted on day 3 after seeding. All samples were prepared in triplicate. This assay was repeated more than three times. *Bars*, SD. *B*, comparisons of EGF-induced proliferation in wild-type EGFR and EGFRvIII P5-transfected 32D cells. Five $\times 10^4$ cells/ml of these transfectants were treated with IL-3 (control) or untreated (none) or with various concentrations of EGF as indicated. A 500-fold higher concentration of EGF was required to stimulate proliferation in 32D/EGFRvIII P5 cells when compared with 32D/EGFR cells. *Bars*, SD.

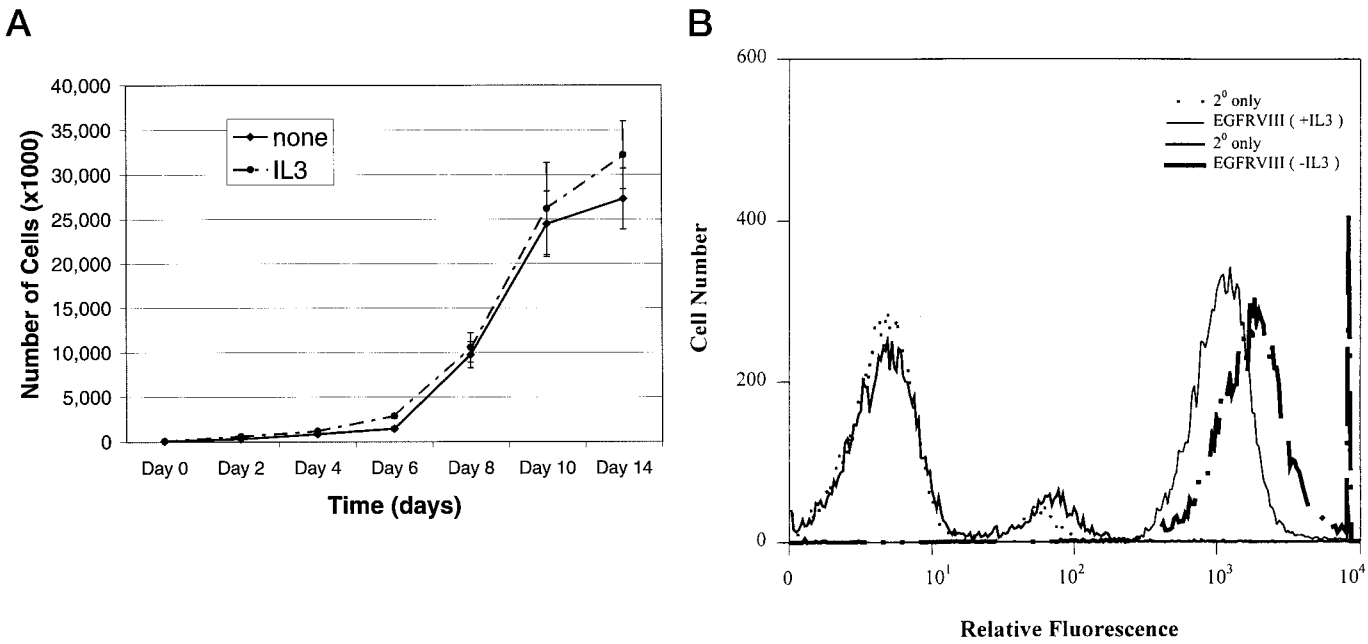

Fig. 3. High levels of EGFRvIII expression in 32D cells (32D/EGFRvIII P5) completely abolished IL-3-dependent phenotype and grew as well as in the absence of IL-3. *A*, prior to the growth assay, 32D/EGFRvIII P5 cells were depleted of IL-3 for 10 days. Five $\times$ 10^4 cells/ml of 32D/EGFRvIII cells were then treated with IL-3 (dotted growth curve) or untreated (none, solid growth curve). Viable cells were then counted on days 2, 4, 6, 8, 10, and 14 in triplicate for each experimental point. No differences in growth rates in the presence or absence of IL-3 were observed in 32D/EGFRvIII P5 cells. *B*, analysis of EGFRvIII expression levels in 32D/EGFRvIII P5 in the presence or absence of IL-3. Prior to the FACS analysis, the 32D/EGFRvIII P5 cells were grown in the presence or absence of IL-3 for 7 days. *Curves* on the *left*, nonspecific staining (primary antibody omitted, *2° only*). *Thin-line curve* on the *right*, cells that grew in the presence of IL-3-containing medium. *Bold-line curve* on the *right*, cells that grew in the absence of IL-3 prior to the FACS analysis. Higher EGFRvIII expression was observed in the cells grown in the absence of IL-3 than in the presence of IL-3.

cells remained nontumorigenic during a 2-month observation period. Furthermore, none of the EGFR family receptors, ectopically expressed singly or in pairwise combinations in 32D cells, had a similar ability (Table 1). A dose-dependent experiment was conducted to assess the minimal number of 32D/EGFRvIII P5 cells that would induce tumor growth in nude mice. Strikingly, 100% of these mice produced tumors independent of the number of cells injected (Table 2). A distinguishing feature of this experiment was that mice given 1×10^6 cells required elicited a longer latency period for tumor formation (10 days) as compared with the one receiving 5×10^6 cells.

Biological Effect of EGFRvIII in Human Breast Cancer Cells

Ligand-independent Constitutive Autophosphorylated EGFRvIII in EGFRvIII-transfected MCF-7 Cells. To assess the biological significance of EGFRvIII in human breast cancer, we expressed EGFRvIII in MCF-7 cells. The expression of EGFRvIII was evaluated by FACS analysis (Fig. 4). MCF-7/EGFRvIII transfectants from single clones and pooled population clones appeared to exhibit similar expression levels of EGFRvIII. We then evaluated the activation of EGFRvIII in MCF-7/EGFRvIII-transfected cells. Autophosphorylation of EGFRvIII was assessed by immunoprecipitation with an anti-EGFRvIII-specific antibody and Western blot with an anti-phosphotyrosine-specific antibody. Fig. 5 shows that EGFRvIII was constitutively activated in a ligand-independent manner in MCF-7/EGFRvIII cells. In addition, ErbB-2 phosphorylation was enhanced in EGFRvIII-transfected MCF-7 cells. These results indicated that EGFRvIII could activate ErbB-2 kinase activity.

Effects of EGFRvIII on Human Breast Cancer Cell Growth *in Vitro*. We next evaluated the influence of EGFRvIII on the cellular growth rate in MCF-7/EGFRvIII cells by anchorage-dependent as well as anchorage-independent growth assays. There was no significant growth effect in medium containing 10% FBS (data not shown). However, at 1% FBS, overexpression of EGFRvIII resulted in a significant induction of proliferation in an anchorage-dependent growth assay and 3-fold induction of colony formation in an anchorage-independent growth assay (Fig. 6). Induction of colony formation was independent of threshold colony size. Moreover, EGF-like ligands increased proliferation substantially (Fig. 7). These data suggest that EGFRvIII could play an important role in breast cancer progression. Constitutively autophosphorylated EGFRvIII may contribute to enhanced proliferation of MCF-7/EGFRvIII cells. Expression of EGFRvIII increases the spectrum and potency of ligand-mediated proliferation *in vitro*.

EGFRvIII Enhances Tumorigenicity in Human Breast Cancer Cells *in Vivo*. Finally, we explored the potential tumorigenicity of EGFRvIII in human breast cancer cells *in vivo*. Five $\times$ 10^6 MCF-7/EGFRvIII cells and MCF-7 parental cells, as well as MCF-7/vector

Table 1 *Effect of ErbB family receptors and EGFRvIII on 32D cell tumorgenicity in s.c. implantation*

	Size of tumors (mm^3)				
Cell lines	Day 7	Day 10	Day 14	Day 19	Incidence of tumors
32D/EGFR	0	0	0	0	0/10
32D/EGFRvIII P5	199.36 ± 27.45	560.04 ± 99.96	2070.20 ± 521.8	6472.50 ± 1159.5	10/10
32D/ErbB2+ErbB3	0	0	0	0	0/10
32D/ErbB-4	0	0	0	0	0/10

Tumor size was quantitated at the indicated times. For 32D/EGFRvIII, when tumors reached up to 2 cm in diameter, mice were sacrificed on day 20. However, no tumors were formed within a 2-month period with other 32D cell transfectants.

Table 2 *Dose dependence of tumor formation with 32D/EGFRvIII (P5) cells*

Cell dose	Size of tumors (mm^3)				Incidence of tumors
	Day 7	Day 10	Day 14	Day 19	
1×10^6	0	0	58.63 ± 12.18	384.40 ± 139.71	10/10
3×10^6	15.30 ± 9.24	373.73 ± 140.48	792.93 ± 210.81	2972.47 ± 525.50	10/10
5×10^6	199.36 ± 27.45	560.04 ± 99.96	2070.20 ± 521.80	6472.50 ± 1159.5	10/10

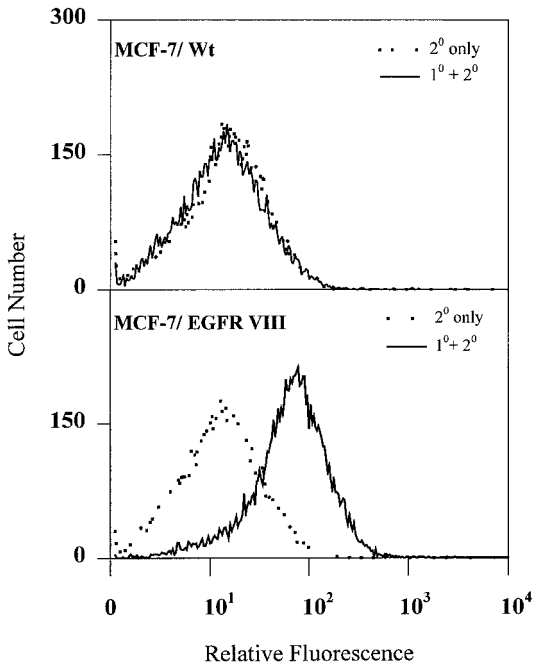

Fig. 4. Analysis of EGFRvIII expression in MCF-7/EGFRvIII transfectants. The levels of EGFRvIII in the MCF-7/EGFRvIII transfectants were quantitatively measured by flow cytometry. *Dotted-line curve*, nonspecific staining (primary antibody omitted, *2° only*). *Solid-line curve*, expression of EGFRvIII receptor (*1° + 2°*). *Top panel*, MCF-7 wild-type cells do not express detectable levels of EGFRvIII. *Bottom panel*, EGFRvIII expression in MCF-7/EGFRvIII transfectants.

cells, were injected s.c. in athymic nude mice with estrogen supplement. As illustrated in Fig. 8, MCF-7/EGFRvIII cells grew large tumors with a mean tumor size of 600 ± 125 mm^3 within 6 weeks, whereas tumors derived from the parental MCF-7 cells were smaller (80 ± 40 mm^3). Tumors developed by MCF-7/EGFRvIII cells were more than seven times larger than the parental MCF-7 cells. Thus, EGFRvIII significantly enhances tumorigenicity in breast cancer cells.

DISCUSSION

Previous reports have demonstrated the introduction of EGFRvIII into NIH3T3 and NR6 murine fibroblast cell lines to induce colony formation *in vitro* and enhance tumor formation *in vivo* as compared with untransfected cells. However, the parental cell lines are immortalized fibroblast cell lines, which are capable of forming colonies in soft agar and forming tumors at high dosage in nude mice (17, 19, 20). Thus, the ability of EGFRvIII to transform a cell from a nontumorigenic to a tumorigenic phenotype has not yet been demonstrated. Our study provides the first evidence that EGFRvIII is capable of transforming an IL-3-dependent, nontumorigenic murine hematopoietic cell line (32D cells) into an IL-3-independent and ligand-independent malignant phenotype *in vitro* and *in vivo*. In this model system, we demonstrated that high level expression of EGFRvIII in 32D cells (32D/EGFRvIII P5) makes the cells capable of abrogating the IL-3-dependent pathway in the absence of ligands, whereas the parental cells, as well as 32D/EGFR cells, depend on IL-3 or EGFR ligands for growth (Fig. 2*A*). 32D/EGFRvIII P5 cells subject to long-term culture conditions in the absence of IL-3 revealed further elevation of EGFRvIII expression levels (Fig. 3). These data further indicated that the IL-3-independent phenotype is mediated by EGFRvIII. The level of expression is a critical driving force for the IL-3-independent phenotype. Moreover, 32D/EGFRvIII P5 formed large tumors in nude mice, even when no exogenous EGF ligand was administered (Table 1). These tumors progressed rapidly, necessitating sacrifice of all animals within 1 month. In contrast, no tumors grew in the mice injected with 32D/EGFR cells, with low-expressing clone 32D/EGFRvIII C2 cells, or with parental 32D cells after 2 months (Table 1). Cytological analysis of 32D/EGFRvIII P5 xenografts showed these EGFRvIII tumors were characteristic of high-grade tumors. High percentages of the tumor cells appeared to be in mitosis (data not shown). This degree of mitogenic activity and highly tumorigenic property of the EGFRvIII-transfected cells has not been observed in any of the homo- or heterodimers EGF-family receptors. These results suggest that the EGFRvIII is an extremely potent oncoprotein and is capable of contributing to malignant transformation, which is substantially greater than any of the wild-type EGF-family receptors.

A recent report has demonstrated that 78% (21 of 27) of primary poorly differentiated breast carcinomas express EGFRvIII (21). EGFRvIII expression has not been detected in normal breast tissues or in benign breast neoplasms (21, 23). Similar results were

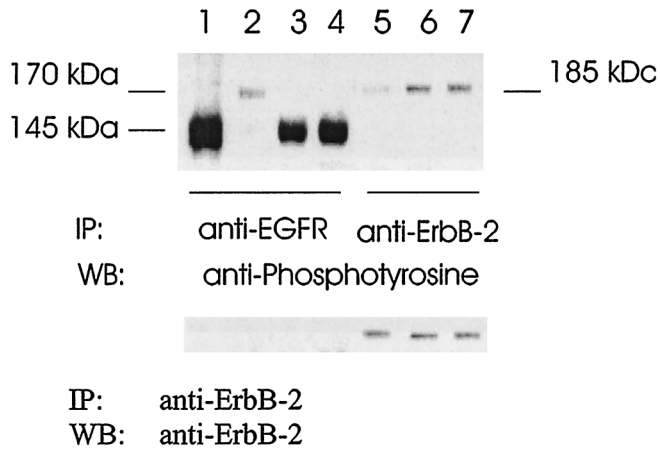

Fig. 5. The expression of EGFRvIII induces autophosphorylation of EGFRvIII and ErbB-2 in MCF-7 cells. One mg of lysates from various EGFRvIII-transfected MCF-7 cells and the wild-type untransfected MCF-7 cells as well as EGFRvIII-transfected NIH3T3 cells (control) were immunoprecipitated with a specific anti-EGFR antibody, which recognizes both the wild-type and the mutant form (EGFRvIII) of EGFR. The MCF-7 wild-type and MCF-7 transfectants were also immunoprecipitated with a specific anti-ErbB-2 antibody. These precipitated proteins were subsequently subjected to Western blotting with an anti-phosphotyrosine antibody (UBI). Bands were visualized using a chemiluminescence detection system. *Lane 1*, EGFRvIII-transfected NIH3T3 cell lysates were immunoprecipitated with anti-EGFR antibody. *Lane 2*, MCF-7 wild-type cell lysates were immunoprecipitated with anti-EGFR antibody. *Lanes 3* and *4*, EGFRvIII-transfected MCF-7 cell lysates were immunoprecipitated with anti-EGFR antibody. *Lane 5*, MCF-7 wild-type cell lysates were immunoprecipitated with anti-ErbB-2 antibody. *Lane 6*, lysate from the pooled population MCF-7/EGFRvIII transfectant. *Lane 7*, lysate from a single clone of MCF-7/EGFRvIII transfectant immunoprecipitated with anti-ErbB-2 antibody. *IP*, immunoprecipitation; *WB*, Western Blot.

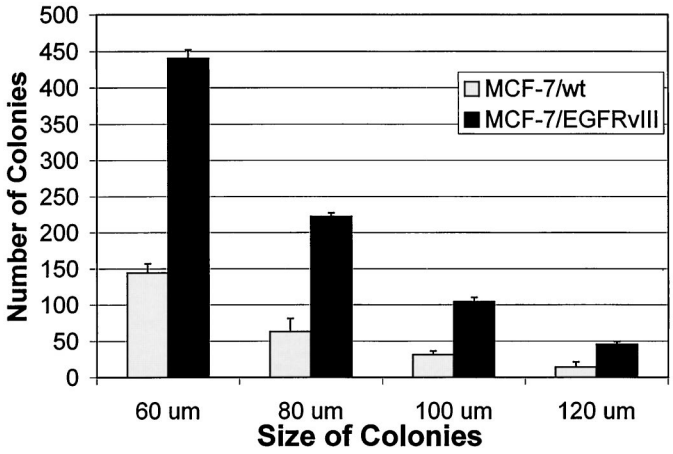

Fig. 6. Growth effects of EGFRvIII on MCF-7 cells. Expression of EGFRvIII in MCF-7 cells induces colony formation, independent of colony size. For anchorage-independent growth assays, a bottom layer of 0.1 ml of IMEM containing 0.6% agar and 10% FCS was prepared in 35-mm tissue culture dishes. After the bottom layer solidified, cells (10,000/dish) were added in a 0.8-ml top layer, containing 0.4% Bacto Agar and 5% FCS. All samples were prepared in triplicate. The cells were incubated for ~12 days at 37°C. Colonies larger than 60, 80, 100, and 120 μm were counted by a cell colony counter. *Bars,* SD.

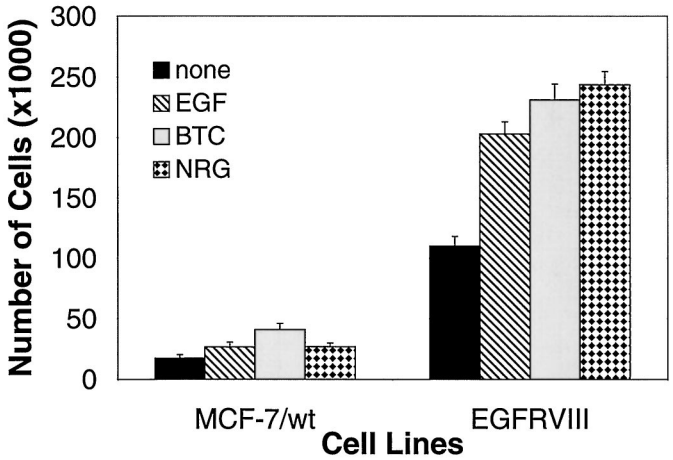

Fig. 7. Expression of EGFRvIII in MCF-7 cells increases the spectrum and potency of EGF-like ligand-mediated proliferation in an anchorage-dependent assay. Cells were plated on 24-well plates in IMEM with 1% of FBS and untreated (control) or treated with 100 ng/ml of EGF, BTC, or neuregulin (*NRG*). Cells were then counted on days 2, 4, and 7. The histogram represents the results from day 7. All samples were prepared triplicate.

obtained in our tissue array experiments (data not shown). However, the tumorigenicity of EGFRvIII in human breast cancer cells has not yet been explored. The understanding of the function and biology of EGFRvIII is important in the prognosis and treatment of breast cancer. In this study, we explored the potential biological role of EGFRvIII in breast cancer by transfecting the EGFRvIII cDNA into the MCF-7 breast cancer cell line. These MCF-7/EGFRvIII cells exhibited a 3-fold increase in colony formation in 1% serum but no significant effect at higher percentages of serum (Fig. 6). The dependency of colony formation on serum concentration and the reduction of serum requirement for proliferation in MCF-7/EGFRvIII cells characterized EGFRvIII-mediated enhancement of proliferation as ligand independent. In addition, expression of EGFRvIII elevated ErbB-2 phosphorylation (Fig. 5), presumably through heterodimerization and cross-talk. These results suggested the possibility for EGFRvIII to heterodimerize with other EGF-family receptors and to diversify its signaling pathways. Furthermore, EGFRvIII expression dramatically enhanced tumorigenicity of MCF-7/EGFRvIII cells with estrogen supplement (Fig. 8), although the expression levels of EGFRvIII was relatively low. It suggested that EGFRvIII is a potent oncoprotein. These results provide the first evidence that EGFRvIII could play a role in human breast cancer progression.

Studies have shown EGFRvIII to be unregulated by extracellular ligands (17, 18, 20). The deletions in EGFRvIII correspond to subdomains I and II of the EGFR (27, 33–36). Although the ligand-binding domain of the EGFR subdomain III (32) is preserved in the EGFRvIII oncoprotein, EGFRvIII fails to bind ligand in NIH3T3 cells (17) and has minimal low-affinity ligand binding in some other system (20). Although TGF-α cannot bind to EGFRvIII when expressed in Chinese hamster ovary cells, these cells had increased levels of DNA synthesis and the receptor was constitutively phosphorylated (19). Similar observations were seen in our EGFRvIII-transfected 32D cell system. Dosage-dependent studies demonstrated requirement for a 500-fold increase of EGF to stimulate the EGF-mediated proliferation in 32D/EGFRvIII P5 cells than in the 32D/EGFR cells (Fig. 2*B*).

Our results confirm and extend the observations in NIH3T3 and U87 MG and Chinese hamster ovary cells, where cells expressing EGFRvIII proved to be ligand independent and highly tumorigenic (19, 23, 24, 37). The extreme aggressiveness of EGFRvIII *in vivo* suggested the potential role for its altered conformation to include the possibility to recruit different downstream effectors and to contribute to the potent mitogenic activities and tumorigenicity *in vivo*. It is also possible that EGFRvIII is able to interact with unknown ligands or dimerize with other receptor members in the family, thereby eliciting an amplification of biological effects. This could explain why expression of EGFRvIII, even at relatively low levels in MCF-7 cells, influences their phenotype *in vivo.* Moreover, the high prevalence of the EGFRvIII in a wide variety of human tumors suggests a preferential selection during the process of tumorigenesis attributable to some alteration or enhancement of the mitogenic properties of the EGFR. Taken together, the altered EGFRvIII conformation might be the essential feature responsible for its extremely potent transforming ability.

Two model systems (32D and MCF-7 cells), which we have reported here, indicate that transformation to a malignant phenotype *in vivo* requires high levels of EGFRvIII expression or coexpression with

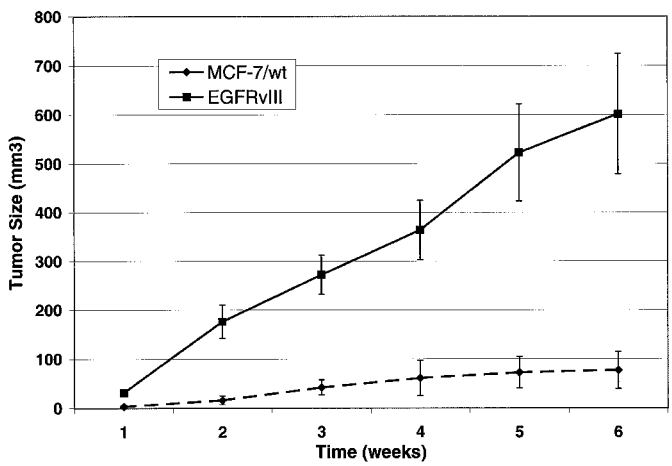

Fig. 8. EGFRvIII enhances tumorigenicity in human breast cancer cells in athymic nude mice. Five $\times 10^6$ of MCF-7/EGFRvIII cells or MCF-7 parental cells were injected s.c. in athymic nude mice with estrogen supplement. Five mice for each cell line were used for this experiment, and each mouse received injections at both left and right mammary fat pads. MCF-7/EGFRvIII cells grew large tumors with a mean tumor size of 600 $\pm$ 125 mm^3 within 6 weeks, whereas tumors that grew in the parental MCF-7 cells were smaller (80 $\pm$ 40 mm^3). One hundred percent of the mice produced tumors. *Bars,* SD.

other family members, such as low levels of ErbB-2. Given the potency of EGFRvIII and detection of EGFRvIII expression in a high percentage of poorly differentiated breast carcinomas, it is possible for low levels of EGFRvIII expression to play a role in human breast cancer evolution.

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