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13. ABSTRACT *(Maximum 200)*

Among the growth factor receptors, members of the class I receptor tyrosine kinase family (ErbB) is most frequently implicated in human breast cancers. To delineate the biological function of ErbB-4 receptors in breast cancer, we employed a hammerhead ribozyme strategy to achieve down-regulation of ErbB-4 receptors in various breast cancer cell lines. We observed that down-regulation of ErbB-4 in estrogen receptor positive (ER+) cell lines (MCF-7 and T47D) resulted in a reduction of tumorigenicity both *in vitro* and *in vivo*. However, over time completely down-regulation of ErbB-4 in ER+ cell lines acquired the ability to up-regulate EGFR or ErbB-2 and progressed to a hormone-independent phenotype. These results mimics the clinical observation. Overexpression of EGFR and ErbB-2 is inversely correlated with ER. The expression of ErbB-4 was correlated with ER+ and PgR+ primary breast tumors by immunohistochemistry. These results suggested that ErbB-4 plays different roles in breast cancer progression.

Our data alsosuggested that a complex combination of regulatory mechanisms is involved in this hormone-independent phenotype. ErbB-4 expression is necessary for maintaining ER expression in breast cancer cells and is important for estrogen repression of EGFR and ErbB-2 expression. Down-regulation of ErbB-4 expression disrupts this regulation and exhibits an up-regulation of EGFR and ErbB-2 expression, and ultimately, leads to develop a more aggressive phenotype.

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BACKGROUND AND SIGNIFICANCE:
Clinical significance of ErbB-4 receptor in Breast Cancer

      Breast cancer is one of the most common malignancies and is a leading cause of death in women in the U.S., affecting one out of every nine women. The epidermal growth factor receptor (EGFR/ErbB) family is a group of tyrosine kinases, most frequently overexpressed in a variety of carcinomas (1-3, 9). These receptors include the EGFR, ErbB-2, ErbB-3, and ErbB-4 proteins (4-8). More than a dozen different agonists have been reported for the ErbB family receptors. These growth factors exert their function by binding cell surface receptors with intrinsic protein tyrosine kinase activity and are implicated in the autocrine/paracrine growth of breast epithelial cells. Based on their binding specificity, these EGF-related peptides can be divided into two groups. The first group is represented with epidermal growth factor (EGF); transforming growth factor α (TGFα), amphiregulin (AR) predominantly binds to and activate EGFR (30). These ligands can also activate the other three ErbB-family receptors through ligand-induced receptor hetrodimerization with EGFR (27). Another group of EGF-related peptides binds to multiple ErbB-family receptors. Heparin-binding EGF-like growth factor (HB-EGF) binds to and activates EGFR and ErbB-4 receptors (10, 21). The neu differentiation factors (NDF)/neuregulin (NRG), neuregulin 2 (NRG2), are also known as heregulins, neu differentiation factors, glial growth factors and acetylcholine receptor inducing activity (12-16). In addition, multiple isoforms of NRG and NRG-2 arising from alternative transcriptional splicing are ligands of ErbB-3, ErbB-4 (17-19) and can also transmodulate ErbB-2 and EGFR through heterodimers with ErbB-3 and ErbB-4 (20, 21, 28, 29). Betacellulin (BTC) is a unique member of the EGF-related peptide family due to its ability to bind to and activate EGFR, ErbB-4 and ErbB-2/3 heterodimers (11, 23, 31). High levels of EGFR and HER-2 have been found in 30-40% of breast carcinomas. Expression of these proto-oncogene proteins inversely correlated with estrogen receptor expression and appears to confer a worse prognosis (2). Overexpression of ErbB-4 in NIH 3T3 cells can transform these cells (26). A recent report demonstrated that co-expression of ErbB-2, ErbB-4 and NRG was significantly related to the presence of metastases at diagnosis in human medulloblastoma (25). In addition, the physiological relevance of transmodulation is supported in gene-targeting experiments in transgenic mice. Mice homozygous for disruptions in the ErbB-4 gene die in utero at day 10.5 and lack the trabecular extensions of the developing ventricular myocardium (34). Recent reports demonstrated that frequent coexpression, hetero-dimerization of ErbB-2 and ErbB-4 play a significance role in childhood medulloblastoma (25), as well as in gastric cancer, ErbB-4 mRNA was significantly overexpressed (32).

      Although ectopic expression of recombinant ErbB receptors has provided important information on their signaling properties, the biological function and *in vivo* interplay of these receptors is still poorly understood. Little is known about the biological significance of ErbB-4 in breast cancer. In order to more fully understand the role of ErbB-4 in neoplastic transformation, we employed a hammerhead ribozyme strategy to inactivate ErbB-4 and delineate its role in biological neoplastic transformation. In **7/1/96-6/30/97's annual report**, we have demonstrated that we were able to generate biologically active ribozymes that target the ErbB-4 receptor by using the 32D cells system to study the intracellular enzymatic activity of erbB-4 ribozyme. We demonstrated that the neuregulin-induced mitogenic effect was abolished in ribozyme transfected 32D/ErbB-4 cells, a cell line dependent on signaling through ErbB-4. Inhibition of mitogenesis was proportional to ribozyme-

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mediated down-regulation of ErbB-4 expression. This work has **published in Cancer Research** (30). In the last report (7/1/97-6/30/98), we reported that we have employed these ribozymes to down-regulate endogenous levels of the ErbB-4 receptor in various breast cancer cell lines. We have observed that down-regulation of ErbB-4 in some of the estrogen receptor positive (ER+) breast cancer cell lines expressing relatively high levels of ErbB-4 dramatically reduces the ability of the cells to grow in an anchorage-independent assay. Furthermore, ribozyme-mediated down-regulation of ErbB-4 in these ER+ breast cancer cells exhibited inhibition of tumor formation in athymic nude mice. However, complete down-regulation of ErbB-4 expression in estrogen receptor negative (ER-) breast cancer cell lines expressing low levels of ErbB-4 expression has no effect. These data suggest that ErbB-4 play a proliferative role in cells expressing high levels of ErbB-4. In addition, a pilot study to determine the frequency of ErbB-4 expression in primary breast cancer specimens was conducted by immunohistochemistry. 70% of the primary breast cancer specimens were found to express ErbB-4. High intense immunoreactivity of ErbB-4 was detected in 18% (9 of 50) of these primary breast tumors. Interestingly, expression of ErbB-4 is directly correlated with ER+ and progesterone receptor positive (PR+) human breast carcinomas. These results provide a better understanding of the biological significance of ErbB-4 receptors in breast cancer. Moreover, ribozyme technology provides a useful tool to delineate the role of a particular gene product. These results have submitted to Cancer Research for publication.

In this report, we will discuses our research progress during the last 12 months (7/1/98-6/30/99).

SIGNIFICANCE:
These studies will provide us the important information relating the biological significance of ErbB-4 in human breast cancer. This research will eventually lead to a new direction of more effective therapies for breast cancer.

BODY

Progress Results:

In the previous report (7/1/97-6/30/98), we demonstrated that Ribozyme mediated down-regulation of ErbB-4 in estrogen receptor positive breast cancer cells inhibits proliferation both *in vitro* and *in vivo* (see table 1).

Table 1 Selective growth inhibition with ribozyme-mediated down-regulation of ErbB-4 in breast cancer cells[a]

Relative Expression levels of EGF-family receptors					Effects of down-regulation of ErbB-4	
Cell line	EGFR	ErbB-2	ErbB-3	ErbB-4	ER	% inhibition of colony formation	% inhibition of tumorigenicity
MCF-7	+	++	+++	++++	+	60-80	70
T47D	++	++	+++	++++	+	50-70	50-60
MDA-MB-453	+/-	++++	+++	+	-	0	N/A
MDA-MB-231	++++	+	+/-	-	-	0	N/A

The expression levels of ErbB-family receptors were determined by FACS analysis with specific antibodies against EGFR or ErbB-2 or ErbB-3 or ErbB-4.
N/A: Not evaluated.

In this report, we will discuss that completely down-regulation of ErbB-4 in human breast cancer cell lines.

Down-regulation of ErbB-4 over time promotes up-regulation of other members of EGF-family receptors developing a more aggressive phenotype, which escapes the inhibition of proliferation.

Surprisingly, completely depleted ErbB-4 expression in ribozyme transfected MCF-7 and T47D cells, over time, have developed a more aggressive phenotype. In these late passage (passage ≥15) of MCF-7/Rz (Clone B1 and Clone N101) and T47D/Rz (Pool 20) transfected cells, the expressions of EGFR and ErbB-2 were up-regulated, whereas the ErbB-4 expression remained constant. As depicted in Fig. 1A, T47D/Rz-pool20 at passage 18, the ErbB-2 expression level increased 30% compared with its early passage and T47D/wt cells in the estrogen depleted medium. Nevertheless, the ErbB-4 expression level remained down-regulated. Similar observation was seen in MCF-7/Rz transfected cells. Figure 1B demonstrated that the EGFR expression level was drastically elevated in MCF-7/RzB1 clone compared with MCF-7/wt cells, whereas the ErbB-4 expression was remained almost completely down-regulated (Figure 1A, 1B). However, these phenomena were not seen in the clones were only partially down-regulated ErbB-4 by ribozyme. To avoid clonal variations, we have done two independent

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transfections. The results are identical. These data suggested that due to extensive cross-talk between the ErbB-family receptors, completely down-regulation of ErbB-4 expression in MCF-7 and T47D cells promotes up-regulation of other EGF-family receptors to form alternative growth pathways and escapes the inhibition of proliferation. These data also further proved that ErbB-4 plays a proliferation role in MCF-7 and T47D cells.

Down-regulation of ErbB-4 induced up-regulation of EGFR in MCF-7/Rz transfected cells was due to up-regulation of EGFRmRNA

To further characterize these late passage of MCF-7/Rz ribozyme transfected clones, RNase protection assay was performed to assess whether increasing of EGFR protein expression level was at the transcriptional or translational level. As shown in Figure 2, the mRNA levels of EGFR were dramatically increased in late passages of MCF-7/RzB1 and MCF-7/RzN101 transfectants compared with the MCF-7/wt, the early passage of MCF-7/RzB1 cells as well. These data suggested that elevation of the EGFR protein expression level is likely due to up-regulation of EGFR transcripts.

Down-regulation of ErbB-4 induced up-regulation of ErbB-2 acquisition induction of colony formation and elevation of NRG-induced ErbB-3 phosphorylation

We first assessed the growth of up-regulation of ErbB-2 in MCF-7/RzA4 late passage by anchorage-independent assay. Figure 3 illustrated that up-regulation of ErbB-2 revealed an increasing clony formation. In the early passage (P5), ribozyme-mediated down-regulation of ErbB-4 inhibited colony formation by 65% comparing with wild type cells. However, at passage 18, MCF-7/RzA4 cells acquired more than two folds induction of colony formation comparing with wild type cells. Even more striking that comparing the early and late passage of MCF-7/A4 cells, up-regulation of ErbB-2 resulted nearly ten fold induction of colony formation. Furthermore, NRG induced ErbB-3 phosphorylation was significantly increased in the late passage of MCF-7/A4 cells (data not shown). These data suggested that ErbB-2/ErbB-3 heterodimers might play a role in MCF-7/RzA4 cells progression.

Down-regulation of ErbB-4 induced up-regulation of EGFR acquisition an elevation of EGF-induced EGFR phosphorylation

We also assessed the down-regulation of ErbB-4 mediated up-regulation of EGFR and its affects on the cellular and biochemical actions *in vitro*. The biological activity of EGFR in the late passage of MCF-7/RzB1 cells was evaluated by stimulating the cells with EGF and examining the phosphotyrosine content of the immunoprecipitated EGFR. Compared to the control MCF-7/wt cells, the level of EGF-induced tyrosine phosphorylation of the EGFR was dramatically increased in the late passage of MCF-7/RzB1 cells. Figure 4 illustrated that EGF-induced EGFR phosphorylation was dramatically elevated in the late passage of MCF-7/RzB1 cells. These data also suggested that elevation of EGF-induced EGFR phosphorylation was in accordance with up-regulation of EGFR expression level. Furthermore, these results demonstrated that EGF-induced EGFR is a functional receptor able to couple to downstream signaling molecules.

Down-regulation of ErbB-4 induced up-regulation of EGFR increasing the anti-EGFR antibody sensitivity in late passages of MCF-7/RzB1 cells.

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Reports have shown that antibodies directed against growth factor receptors can potentially block biological functions essential for cell proliferation. Antibodies against EGFR have been shown to have an antitumor effect in a model system that overexpress EGFR. In order to determine whether up-regulation of EGFR expression level in late passage of MCF-7/RzB1 cells plays a proliferative role, we evaluated the ability of EGFR antibody to block the EGF-induced proliferation in late passage of MCF-7/RzB1 cells. As showed in Figure 5A, EGF-induced proliferation was significantly increased and EGFR-antibody was sufficiently blocked EGF-induced proliferation in late passage of MCF-7/RzB1 cells. In contrast, the MCF-7/wt cells display neither of these significant effects neither EGF-induced growth nor growth inhibition by treatment of EGFR-antibody. Furthermore, these MCF-7/RzB1 cells were much sensitive to EGFR-PE (EGF-pseudomonas extoxin) than wild-type MCF-7 cells (Figure 5B). Thus, in late passage of MCF-7/RzB1 cells, EGFR appears to play a proliferative role, whereas in the MCF-7/wt cells, ErbB-4 seems to play a proliferative role. This indicated that down-regulation of ErbB-4 mediated up-regulation of EGFR in late passage of MCF-7/RzB1 cells altered the growth signaling pathways that probably is due to anti-proliferative attribution.

Up-regulation of EGFR in late passage of MCF-7/RzB1 cells acquired a hormone resistant phenotype.

In clinical, overexpression of EGFR has been shown to correlate with poor prognosis and failure of endocrine therapy due to loss of ER ( ). We next directly assessed the growth effect on hormone-responsiveness with late passage of MCF-7/RzB1 cells. We wonder whether complete down-regulation of ErbB-4, which induced up-regulation of EGFR will promote hormone-independent phenotype in late passages of MCF-7/RzB1 cells. When assayed for anchorage-dependent, late passage MCF-7/RzB1 cells were able to growth in the absence of estrogen and in the presence of tamoxifen (Figure 6). As compared to the control cells, MCF-7/wt depends on estrogen for growth and sensitive to tamoxifen treatments. Furthermore, estrogen receptor was lost in late passage MCF-7/RzB1 cells (Data not shown). Many breast tumors appear to follow a predictable clinical pattern. Initially tumors appear to be locally confined and responsive to endocrine therapy and cytotoxic chemotherapy. However, some of the tumors ultimately progress to a more malignant phenotype and no longer response to both endocrine and cytotoxic chemotherapy. These data mimicked the clinical observations that breast cancer progression from hormone-dependent phenotype to hormone-independent phenotype. ErbB-4 may involve in the regulation of EGFR and ErbB-2 gene expression. Down-regulation of ErbB-4 expression altered in the regulation of EGFR and ErbB-2 expression and promoted breast cancer progression from hormone-dependent to hormone-independent phenotype.

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Legends:

Figure 1 A. Down-regulation of ErbB-4 expression induces up-regulation of ErbB-2 expression in T47D cells. The levels of ErbB-2 and ErbB-4 expression in T47D wild-type and ErbB-4 ribozyme transfected T47D were quantitatively measured by flow-cytometry. $1X10^6$ cells were harvested and stained with specific monoclonal antibodies against ErbB-2 or ErbB-4 receptors, in combination with fluorescence-labeled anti-mouse IgG antibody and analyzed by FACScan.

Figure 1B. Down-regulation of ErbB-4 expression induces up-regulation of EGFR expression in MCF-7 cells. The levels of EGFR and ErbB-4 expression in MCF-7 wild-type and ErbB-4 ribozyme transfected MCF-7 cells were quantitatively measured by flow-cytometry. $1X10^6$ cells were harvested and stained with specific monoclonal antibodies against EGFR or ErbB-4 receptors, in combination with fluorescence-labeled anti-mouse IgG antibody and analyzed by FACScan.

Figure 2. Down-regulation of ErbB-4 expression induces up-regulation of EGFR expression in MCF-7/Rz transfectants was due to up-regulation of EGFRmRNA. RNase Protection Assay: Lane 1 and 2 represent the expression level of EGFRmRNA in MCF-7 wild-type cells. Lane 3 and 4 represent the expression level of EGFRmRNA in MCF-7/RzN107 Cells. Lane 5 and 6 represent the expression level of EGFRmRNA in MCF-7/RzB1 Cells. Lane 7 and 8 represent the expression level of EGFRmRNA in MCF-7/RzPB Cells.

Figure 3. Progression of ErbB-4 riobzyme transfected MCF-7 Cells. Anchorage-independent growth assays: A bottom layer of 0.1 ml IMEM containing 0.6% agar and 1% FCS was prepared in 35mm tissue culture dishes. After the bottom layer solidified, cells (10,000 per dish) were then added in a 0.8 ml top layer, containing 0.4% Bacto Agar, and 1% FCS. All samples were prepared in triplicate. The cells were incubated for approximately 12 days at 37°C. Colonies larger than 60μm, 80μm, 100μm, and 120μm were counted by a cell colony counter.

Figure 4. EGF-induced EGFR phosphorylation was significantly increased in late passages of MCF-7/RzB1 cells. Prior lysis the cells, cells were starved in the serum free medium overnight and subsequently treated with 100 ng/ml of EGF for 5 minutes. 1mg of lysates from ErbB-4 ribozyme transfectated MCF-7 cells and the wild-type untransfected MCF-7 cells were immunoprecipitated with a specific anti-EGFR antibody. These precipitated proteins were subsequently subjected to Western blotting with an anti-phosphotyrosine antibody (UBI). Bands were visualized using a chemiluminescence detection system.

Figure 5A. Inhibition of proliferation by EGFR anti-bodies on later passages of MCF-7/Rz B1 cells. Anchorage-dependent growth assays: MCF-7/RzB1 cells were cultured in the presence or absence of EGFR antibody 225 or 528 (100 nM) with EGF stimulation (100 ng/ml).

Figure 5B. Inhibition of Proliferation of later passages of MCF-/RzB1 cells by EGF-PE. Anchorage-dependent growth assays: MCF-7/RzB1 cells were cultured in the presence or absence of EGF-PE with dose-dependent inhibition of growth.

Figure 6. Down-regulation of ErbB-4 expression induces up-regulation of EGFR expression in MCF-7 cells resulted a hormone-insensitive phenotype. Anchorage-independent growth assays: Cells were cultured in the phenol red free medium (IMEM) with 10% CCS to deplete the estrogen (E2) for six days. 1×10^4 cells were then plated on 24 well plates in the presence or absence of E2 or Tamoxifen or ICI. Cells were counted on day 7. Cells were plated in triplicate for each treatment.
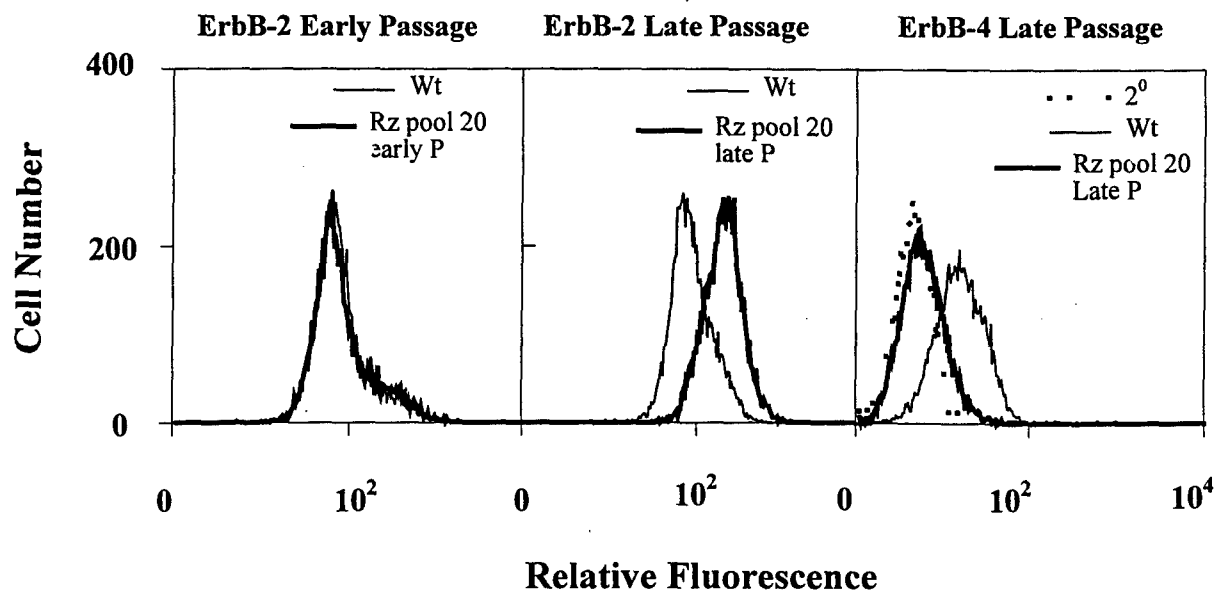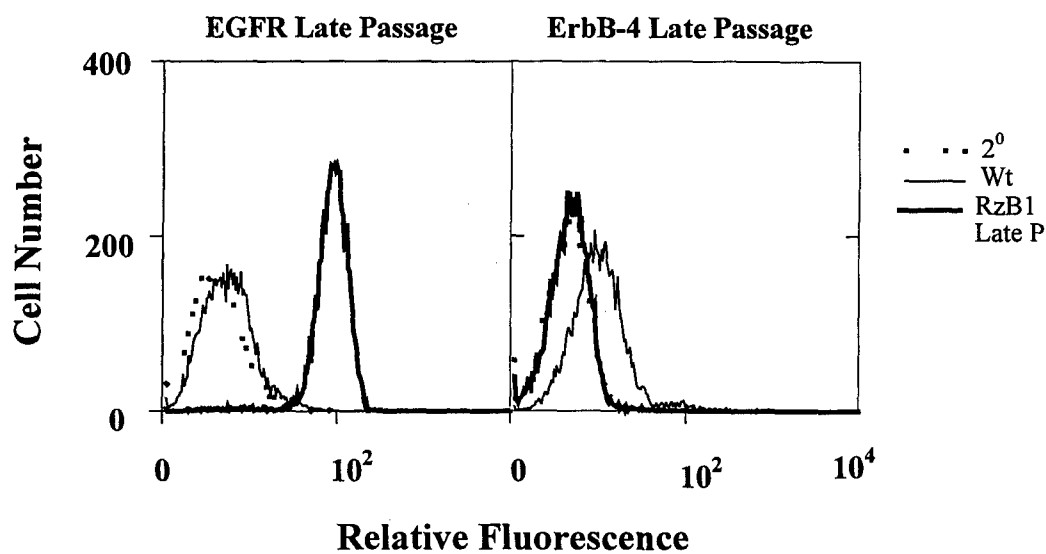
T47D

ErbB-2 Early Passage   ErbB-2 Late Passage   ErbB-4 Late Passage

Relative Fluorescence

MCF-7

EGFR Late Passage   ErbB-4 Late Passage

Relative Fluorescence
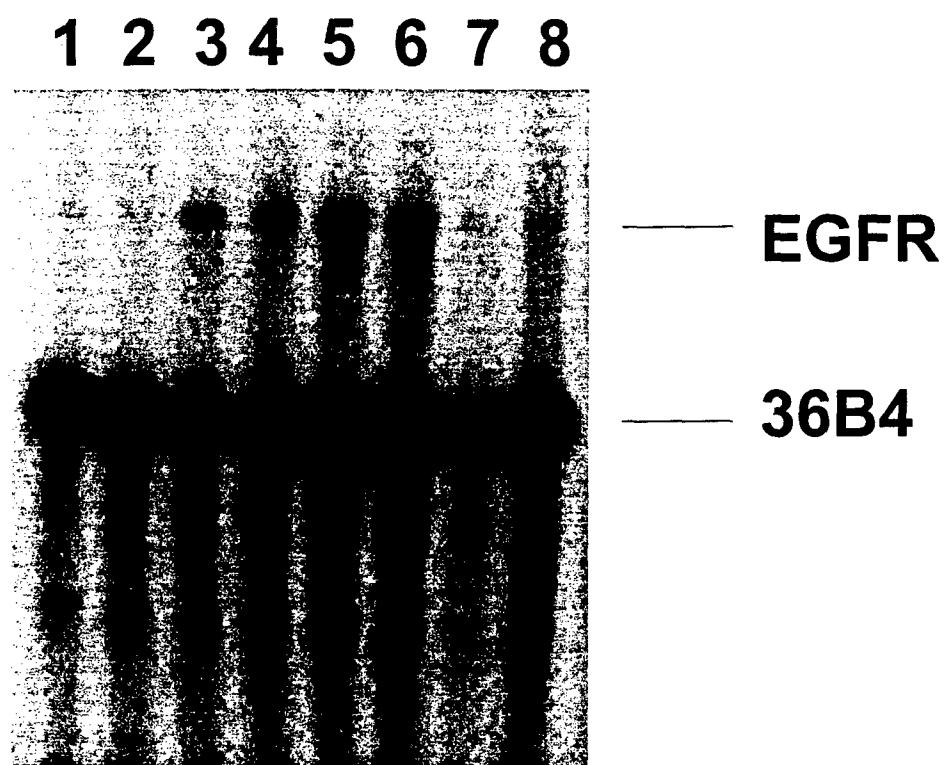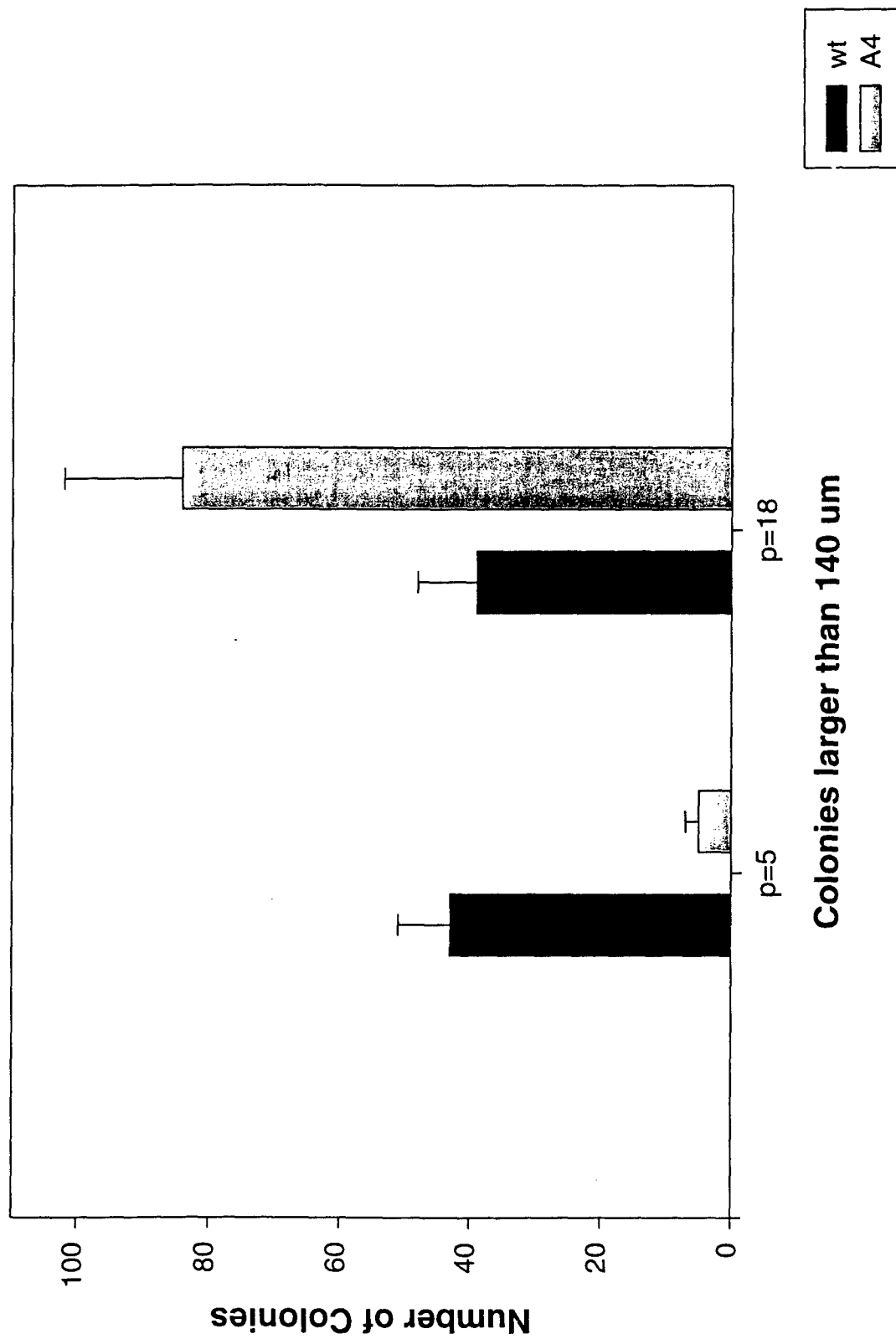
Figure 2

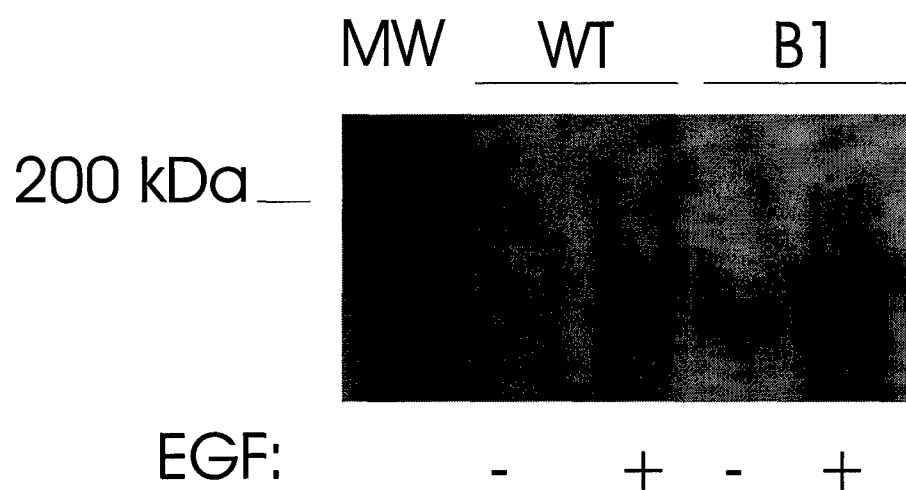# Progression of ErbB-4 Ribozyme Transfected MCF-7 Cells (Anchorage Independent Growth Assay)

Figure 3

Figure 4

Inhibition of proliferation with EGFR-antibodies on later passage of MCF-7/B1 cells
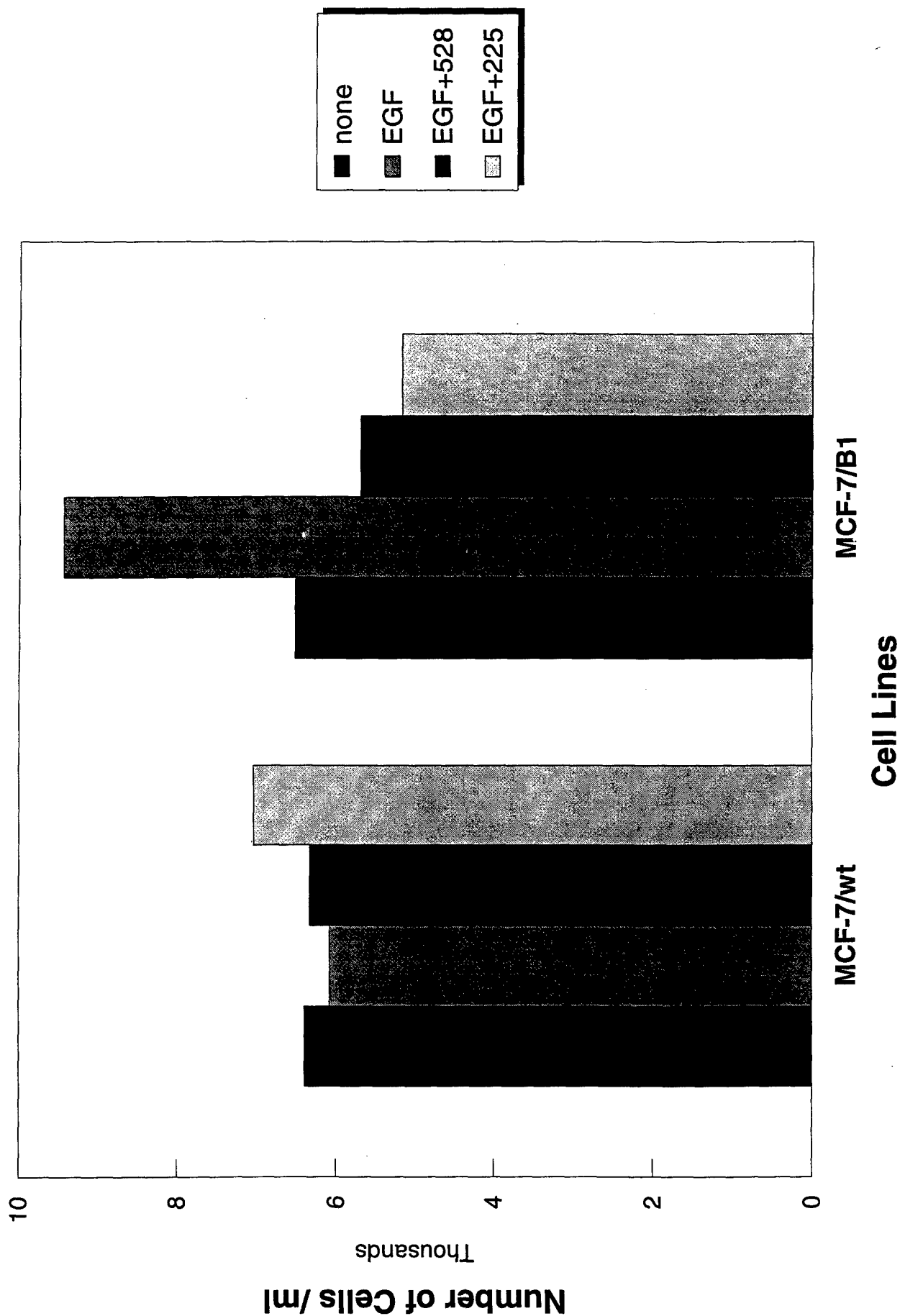
Figure 5A

Inhibition of Proliferation by EGF-PE in MCF-7/B1 Cells

Number of Cells (Thousands)

Cell Lines

MCF-7/wt   MCF-7/B1

none
10-12M
10-10M
10-8M

Figure 5B

Figure 6

Growth Response of MCF-7/ErbB-4 ribozyme Transfecants with Hormone-treatments (Anchorage-Independent Growth Assay)

Legend: none, E2, TAM, TAM+E2, ICI

Y-axis: Number of Colonies (Larger than 60 um)

X-axis: Cell Lines — WT, B5, N103, B1, N107

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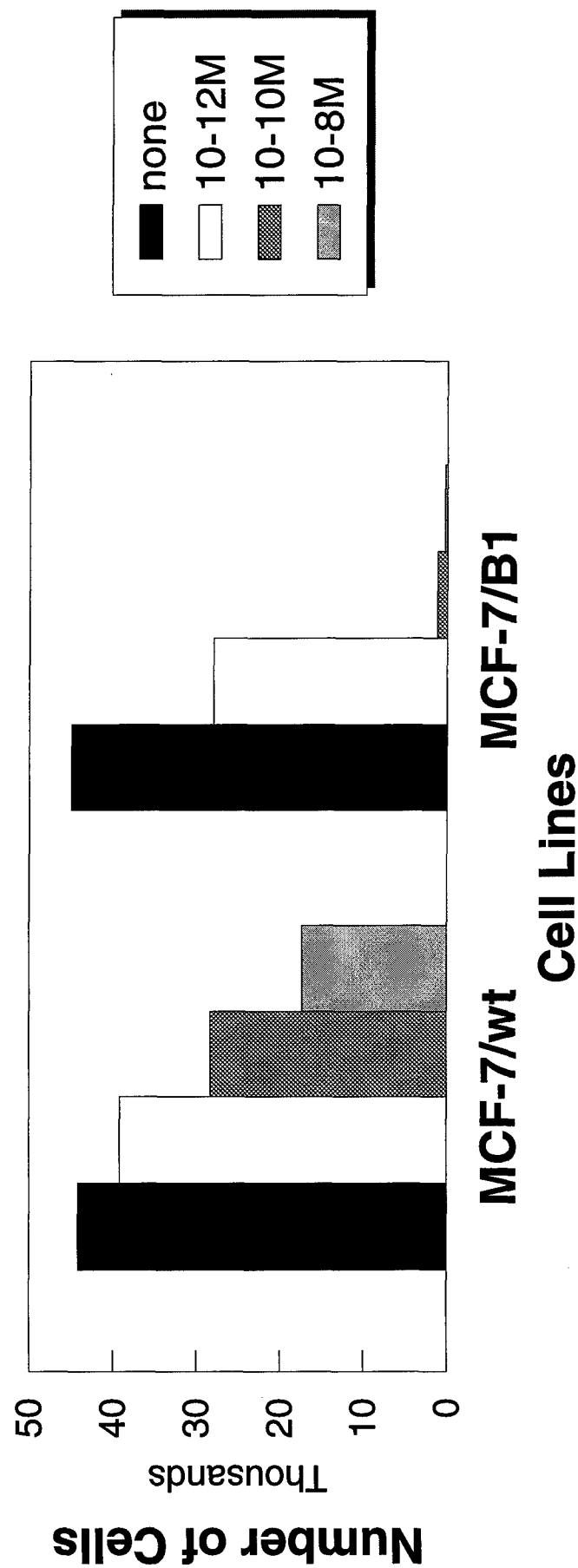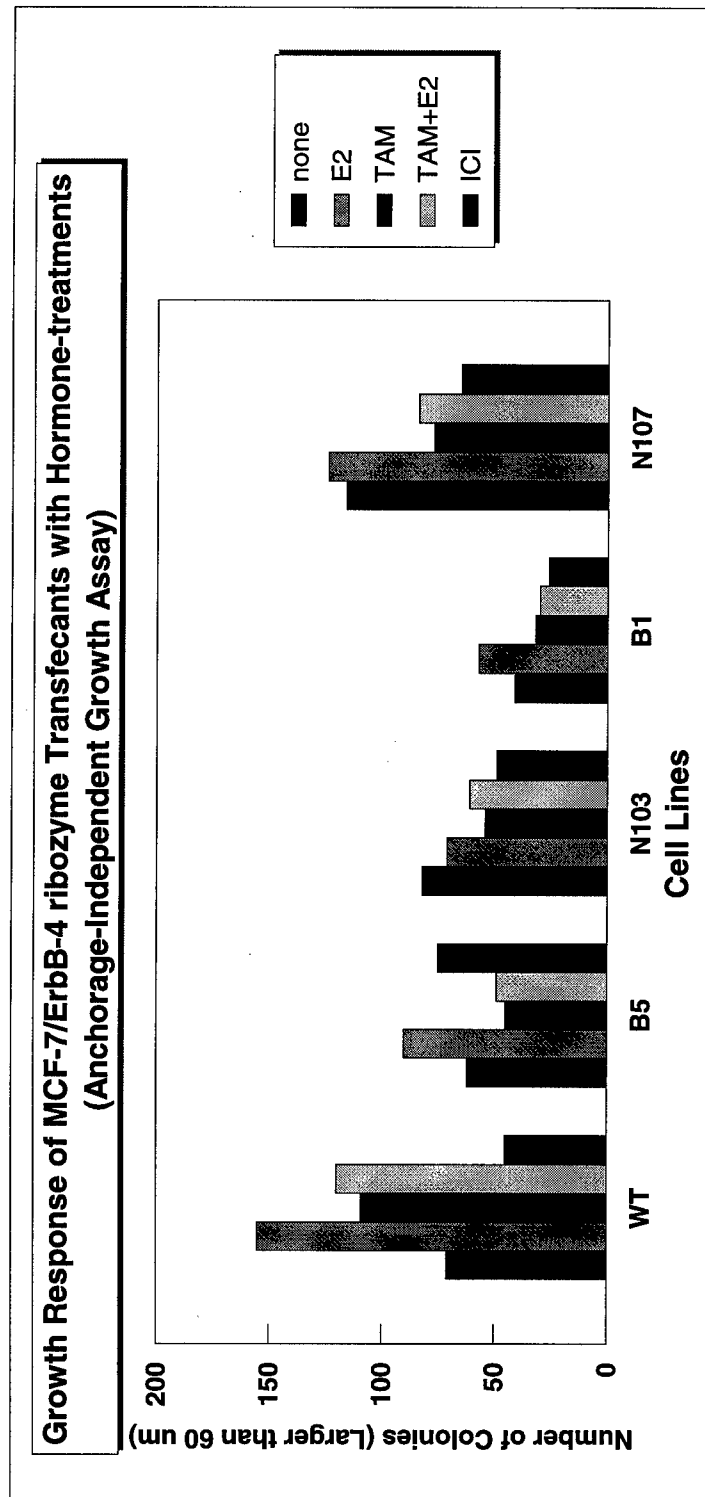2.   Hynes, N. E., and D. F. Stern. The biology of erbB-2/neu/HER-2 and its role in cancer. Biochem. Biophys. Acta., *1198:* 165-184,1994.

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1) Key research accomplishments during the period of 7/1/98-6/30/99.

- ErbB-4 plays different roles in human breast cancer progression.
- ErbB-4 expression correlated with ER+ human breast cancer.
- Down-regulation of ErbB-4 inhibited ER+ breast cancer cell proliferation.
- Completely down-regulation of ErbB-4 induces up-regulation of EGFR or ErbB-2 and progresses to a hormone-independent phenotype.

2) List of reportable outcomes supported by this grant (since 7/1/96).

- **Manuscripts:**
    1) **Tang, C.K.** Goldstein, D.J., Czubayko, F., Alimandi, M., Wang, L-M., Pierce, J.H., & Lippman, M.E. "ErbB-4 ribozymes abolish neuregulin induced mitogenesis" (1998) Cancer Research, 58:3415-3419.
    2) Bianco, C., Kannan S., De Santis M., Seno, M., **Tang, C.K.,** Martinez-Lacaci I., Kim, N., Wallace-Jones B., Lippman, M.E., Ebert, A., Wechselberger C., and Salomon D.S. "Cripto-1 indirectly stimulates the tyrosine phospnorylation of ErbB-4 through a novel receptor" (1999) J.B.C., 274:8624-8629.
    3) **Tang, C.K.** Wu, A.X-Z., Milian, M., Gong, Xiaoqi, Montgomery, E., & Lippman, M.E. "Ribozyme mediated down-regulation of erbB-4 in estrogen receptor positive breast cancer cells inhibits proliferation both *in vitro* and *in vivo*.(1999) Cancer Research, Submitted.

- **Abstract:**
    1) **Tang, C.K.** Milian, M., Oate A., & Lippman, M.E. " The Biological effects of down-regulation of ErbB-4 in human breast cancer cells" (1998) In proceedings of American Association for Cancer Research, Vol. 39, #1415.

- **Presentations:**
    1) "Study of the pathogenic role of ErbB-4 in human breast cancer cells" was selected on platform presentation in the Departmetn of Defense Breast Cancer Research Program "Era of Hope" meeting, Washington D.C., Nov. 1997.
    2) "Biological effects of down-regulation of ErbB-4 in human breast cancer cells" was selected on platform presentation in "Breast Cancer Symposium-Think Tank 8", Tobago, West India, Jan. 1998.

- **Patent:**
  Title: ErbB-4 targeted ribozymes
  Ref: 009/094/SAP
  PCT Patent Appln. For Tang and Lippman
  Data: Oct. 30, 1998

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ErbB-4 Ribozymes Abolish Neuregulin-induced Mitogenesis[1]

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ABSTRACT

The epidermal growth factor-like receptor tyrosine kinase (ErbB) family is frequently overexpressed in a variety of human carcinomas, including breast cancer. To assist in characterizing the role of ErbB-4 in breast cancer, we generated three specific hammerhead ribozymes targeted to the ErbB-4 mRNA. These ribozymes, Rz6, Rz21, and Rz29, efficiently catalyzed the specific cleavage of ErbB-4 message in a cell-free system. We demonstrated that the neuregulin-induced mitogenic effect was abolished in ribozyme Rz29- and Rz6-transfected 32D/ErbB-4 cells. Inhibition of mitogenesis was characterized by ribozyme-mediated down-regulation of ErbB-4 expression. In addition, we provide the first evidence that different threshold levels of ErbB-4 expression and activation correlate with different responses to neuregulin stimulation. High levels of ErbB-4 expression, phosphorylation, and homodimerization are necessary for neuregulin-stimulated, interleukin 3-independent cell proliferation in the 32D/E4 cells. In the case of Rz29-transfected 32D/E4 cells, low levels of ErbB-4 expression allowed neuregulin-induced phosphorylation but were insufficient to couple the activated receptor to cellular signaling. Furthermore, expression of the functional ErbB-4 ribozyme in T47D human breast carcinoma cells led to a down-regulation of endogenous ErbB-4 expression and a reduction of anchorage-independent colony formation. These studies support the use of ErbB-4 ribozymes to define the role of ErbB-4 receptors in human cancers.

INTRODUCTION

The EGFR[3]/ErbB family is a group of tyrosine kinases that is frequently overexpressed in a variety of carcinomas (1–3). This class I subfamily of receptors is composed of four members: EGFR (4); HER2/ErbB-2/neu (5); HER3/ErbB-3 (6, 7); and HER4/ErbB-4 (8). Data from numerous laboratories suggest that the EGFR family members may play a complex role in signaling (9–11). Most human breast cancer cells express more than one of the EGF family receptors, and different combinations of receptors can heterodimerize or homodimerize. These receptor interactions lead to the activation of multiple signaling pathways and contribute to the pathogenicity and tumorigenicity of breast cancer (12). A number of growth factors, classified as EGF-like ligands, have been identified that bind and stimulate the kinase activity of EGF family receptors. EGF, transforming growth factor α, amphiregulin, heparin-binding EGF, and betacellulin have been described as specific for EGFR (13–17). Several differentially spliced variants, named NRG1-α/HRG, or neu differentiation factor (18, 19), acetylcholine-receptor inducing activity

(20), glial growth factor (21), and gp30 (22) were initially identified as candidate neu ligands because of their ability to induce neu tyrosine phosphorylation. However, recent results demonstrate that ErbB-3 and ErbB-4 are primary receptors for neuregulin (23, 24). Activation of ErbB-2 by NRG1-α is thought to occur through transphosphorylation resulting from heterodimerization with either ErbB-3 or ErbB-4 (25–27). Most recently, betacellulin has been shown to activate the ErbB-4 receptor in a Ba/F3 system (28); heparin binding-EGF can bind and activate ErbB-4 as well (29).

Amplification and/or overexpression of EGFR and ErbB-2 are clearly important factors in neoplastic transformation of breast epithelium (30). Elevated ErbB-4 levels have been found in certain breast cancer cell lines (8), but little is known about the expression or the clinical significance of ErbB-4 receptors in the diagnosis and prognosis of human breast cancer. It is therefore imperative that the role of ErbB-4 and its biological significance in breast cancer be defined. To achieve this goal, we used ribozyme technology to disrupt ErbB-4 expression in human breast cancer cells.

Specific gene modulation using oligonucleotides, including triplex DNA, antisense DNA/RNA, and ribozymes, have been used as strategies for suppressing activated oncogenes (31–33). In the present study, we generated three specific hammerhead ribozymes targeted to specific sites within ErbB-4 mRNA. These ErbB-4 ribozymes (Rz6, Rz21, and Rz29) effectively catalyzed the precise cleavage of ErbB-4 mRNA under physiological conditions in a cell-free system. One of these ribozymes, Rz29, down-regulated ErbB-4 receptor expression by as much as 65%, with a corresponding 10-fold decrease in ErbB-4 tyrosine phosphorylation in a 32D cell model system. Furthermore, expression of this functional ErbB-4 ribozyme in T47D human breast carcinoma cells led to a down-regulation of endogenous of ErbB-4 expression and a reduction of anchorage-independent colony formation.

MATERIALS AND METHODS

Cell Lines and Cell Culture. The 32D murine hematopoietic cell line (34) and its derivatives were grown in RPMI (Cellgro) supplemented with 12% FCS (Biofluids) and IL-3 supplied as 6% conditioned medium from the WEHI-3B murine myelomonocytic leukemia cell line.

Generation of ErbB-4 Ribozymes. We used the GCG Package Database program to select the ribozyme sequence: (a) we used this program to predict the optimal and suboptimal secondary structure of ErbB-4 mRNA using the most recent energy minimization method by Zuker and Stiegler (36); (b) we selected ribozyme target sites in the open loop regions with the GUX cleavage site; (c) we then used the same program to predict the secondary structure of the selected regions to see whether these sequences are able to fold into a typical hammerheaded ribozyme three-stem loop structure; and (d) we tested the selected ribozyme sequences for specificity against other known human genes in the GenBank database.

Plasmid Construction. Two synthetic single-stranded ribozyme oligonucleotides were subcloned into the mammalian vector pCR3. The sequence and orientation of the inserts were confirmed by dideoxynucleotide sequencing of the construct using the Sequenase kit, version 2.0 (U. S. Biochemical Corp., Cleveland, OH). ErbB-4 ribozyme sequences were: Rz6, 5'-AAU UCG GCU CAC CCA CUG AGU CCG UGA GGA CGA AAC CCA AAG UCCC-3'; Rz21, 5'-AAU UCG UUG CCC AUC UGA UGA GUC CGU GAG GAC GAA ACA ACC UCA CC-3'; and Rz29, 5'-AAU UCC ACU AAC ACG CUG AUG AGU CCG UGA GGA CGA AAG CCU GUG ACUC-3'.

Ribozyme-mediated mRNA Cleavage *in Vitro*. The substrate ErbB-4 cDNA fragment was derived by reverse transcription-PCR with RNA from MDA-MB-453 cells, which express relatively high levels of ErbB-4. The PCR primers for subcloning of ErbB-4 cDNA were: 5' primer sequence, 5'-AAT TGT CAG CAC GGG ATC TGA GAC-3'; and 3' primer sequence, 5'-GTT TCC TTA AAC AAG ACC AGA TGT-3'. The reverse transcription-PCR products were then cloned into the PCR3 vector. Clones were sequenced to verify that they contained the ErbB-4 cDNA fragment. We then performed *in vitro* run-off transcripts from an ErbB-4 cDNA construct to generate the ErbB-4 ribozyme substrate. Likewise, ribozymes were chemically synthesized as DNA oligonucleotide and subsequently synthesized *in vitro* by using the T7 RNA polymerase. Cleavage reactions were performed in 50 mM Tris-HCl (pH 8.0) and 20 mM MgCl₂. Substrate and ribozyme transcripts were then mixed and incubated at 50°C for 30 min. Reaction products were analyzed on 6% urea polyacrylamide gel, and products were detected by autoradiography.

Transfection by Electroporation. 32D derivative cells (1×10^7) were used for each transfection. Ten μg of plasmid DNA were added to cells resuspended in 300 μl of PBS. Cells were electroporated at 250 V, using a Bio-Rad electroporation system, plated onto 100 mM dishes, and incubated for 24 h. The cells were then selected in growth medium containing 750 μg/ml geneticin (G418-sulfate; Life Technologies, Inc.).

Northern Blot Analysis. Total RNA from cell cultures was isolated using RNasol B (Tel-Test, Inc., Friendwood, TX). Twenty μg of total RNA from each cell line were used to hybridize with an ErbB-4 cDNA probe and autoradiographed for 48 h.

Autophosphorylation of erbB Family Receptors. A total of 2×10^8 32D derivative cells were washed in PBS and resuspended in 50 ml of RPMI supplemented with IL-3 and incubated for 4 h at 37°C. After incubation, cells were washed in PBS and resuspended in 1 ml of PBS with Na₃(VO)₄. Remaining steps were performed on ice. Recombinant HRG-β3 isoform (EGF-like domain) was added at a final concentration of 150 ng/ml. After a 10-min incubation, cells were lysed in a HEPES-lysis buffer, and the cell debris was pelleted by centrifugation (28).

The lysates were then immunoprecipitated with either anti-EGFR (Ab-1; Oncogene Science, Uniondale, NY), anti-ErbB-2 (Ab-3; Oncogene Science), anti-ErbB-3 (C17; Santa Cruz Biotechnology, Santa Cruz, CA), or anti-ErbB-4 (C18; Santa Cruz Biotechnology) in combination with protein A-agarose (Pharmacia, Piscataway, NJ) overnight at 4°C with gentle agitation. For details, see Riese *et al.* (28).

FACS (FACStar) Analysis. Cells (1×10^6) were harvested and then stained for 1 h with either anti-EGFR (Ab-1; Oncogene Science), anti-ErbB-2 (Ab-2; NeoMarker, Fremont, CA), anti-ErbB-3 (Ab-4; NeoMarker), and anti-ErbB-4 monoclonal antibody (Ab-1; NeoMarker); then a secondary FITC-anti-mouse antibody was used, and the ErbB-4 level in each cell was quantitatively measured by flow cytometry.

Anchorage-independent Growth Assay. 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 per dish) were added in a 0.8-ml top layer, 0.4% Bacto Agar and 5% FCS. 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).

Mitogenic Assay. 32D transfected cells were plated at a density of 1×10^4 cells with or without IL-3 supplement or supplemented with 100 ng/ml of NRG1-α in the absence of IL-3. Two days after plating, the cells were labeled with [³H]thymidine for 2 h. [³H]Thymidine incorporation was then analyzed by β-scintillation counter.

In Vitro **Kinase Assay.** 32D/E4, 32D/E4 + V, and 32D/E4 + Rz29 cells were serum starved for 2 h before treatment with or without 100 μg/ml of NRG1-α. Cells then lysed in lysis buffer. Four hundred μg of total protein of each cell line was used to immunoprecipitate with anti-ErbB-4 antibody (C18; Santa Cruz Biotechnology) in combination with protein A-agarose (Pharmacia, Piscataway, NJ). Reactions were carried as described previously (35). Briefly, 50 μl of a solution containing 10 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 10 mM MnCl₂, 10 μCi of [γ-³²P]ATP, and 1 μg of aprotinin were added to the washed beads for 25 min at room temperature. Reactions were terminated by spinning down the Sepharose beads in a microcentrifuge, discarding the supernatant, and resuspending the beads in 50 μl of SDS gel loading buffer. Eluted proteins were analyzed by SDS-PAGE and autoradiography.

RESULTS

Generation and Demonstration of ErbB-4 Ribozyme Efficacy and Specificity in a Cell-free System

To investigate the biological significance of ErbB-4 in human breast cancer cells, we used molecular targeting of the ErbB-4 mRNA by ribozymes. Three ribozymes (Rz6, Rz21, and Rz29) targeted to specific sites within the ErbB-4 mRNA open reading frame were generated. These ribozymes were modeled on the hammerhead structure described previously (36, 37), derived and minimized to the catalytic center portion of 22 nucleotides. The targeted cleavage sites selected for the design of the ribozymes were 60 (Rz6), 210 (Rz21), and 290 (Rz29) nucleotides downstream of the translation initiation site of the ErbB-4 mRNA (Fig. 1). The catalytic activity of these ribozymes was first evaluated in an extracellular system. All three ErbB-4 ribozymes cleaved ErbB-4 mRNA precisely and efficiently under physiological conditions in this cell-free system (Fig. 2A, *Lanes 2–5*). Cleavage was specific because the actual sizes of the cleaved fragments correspond to the expected sizes if cleavage were to occur immediately 3' to the GUN sequence. As a control for specificity, catalytically inactive mutant ribozymes were engineered. Point mutation of G to A in the catalytic domain of either Rz29 or Rz6 (Fig. 2A, *Lanes 6* and *7*) resulted in loss of catalytic activity as predicted by mutational studies of McCall *et al.* (37) reported previously. The specificity of these three ErbB-4 ribozymes was evaluated on a nonspecific mRNA substrate. As expected, no cleavage was observed, following incubation of these ribozymes with ErbB-3 mRNA (Fig. 2B). These results indicate that all three of the GUN sequences chosen in the ErbB-4 mRNA are accessible to ribozyme-mediated cleavage in an extracellular system.

An Intracellular Model System for Evaluating the Specificity and Efficacy of ErbB-4 Ribozymes. We next investigated the catalytic activity of these ribozymes in a model cellular system. Although the ribozyme sensitivity in an extracellular system can be correlated with the predicted secondary structure of the target RNA, the intracellular susceptibility of the target RNAs to ribozymes does not necessarily correlate with their predicted secondary structure. In addition, the complexity of heterodimerization and transphosphorylation between the ErbB family members in breast cancer cells makes it difficult to determine the specificity of ErbB-4 ribozymes. Furthermore, the goal of these ribozymes is to interrupt gene expression. If ErbB-4 is critical for cell proliferation, its down-regulation may be lethal. Thus, an ideal system for screening the intracellular enzymatic activity of these ribozymes requires the following criteria: (*a*) expression of high levels of ErbB-4 receptor; (*b*) no expression of other EGF family receptors; (*c*) nonlethality of ErbB-4 ribozyme introduction; and (*d*) easy detection of ribozyme activity by bioassay. We therefore used the 32D cell system to examine the intracellular efficacy and specificity of the ErbB-4 ribozymes. 32D cells are a murine hematopoietic IL-3-dependent cell line that does not express detectable levels of endogenous EGF family receptors. Studies have shown that IL-3
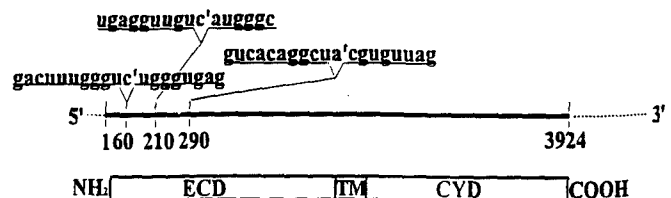

Fig. 1. Targeted sequences and cleavage sites of ErbB-4 ribozymes downstream of the translation initiation site in the ErbB-4 mRNA open reading frame.

A

B

Fig. 2. A. catalytic activity of ErbB-4 ribozyme in an extracellular system. Lane 1, molecular weight markers. Lane 2, ^{32}P-labeled ErbB-4 transcript with an expected size of 622 nucleotides. Lanes 3-5, cleavage products of the three ErbB-4 ribozymes (Rz6: 518, 110 nucleotides; Rz21: 285, 337 nucleotides; Rz29: 232, 390 nucleotides). In Lanes 6 and 7, mutant ribozymes do not cleave ErbB-4 transcript. B: Lane 1, molecular weight marker. Lane 2, ^{32}P-labeled ErbB-3 transcript with an expected size of 698 nucleotides. In Lanes 2-4, ErbB-3 transcript is not cleaved by ErbB-4 ribozymes.
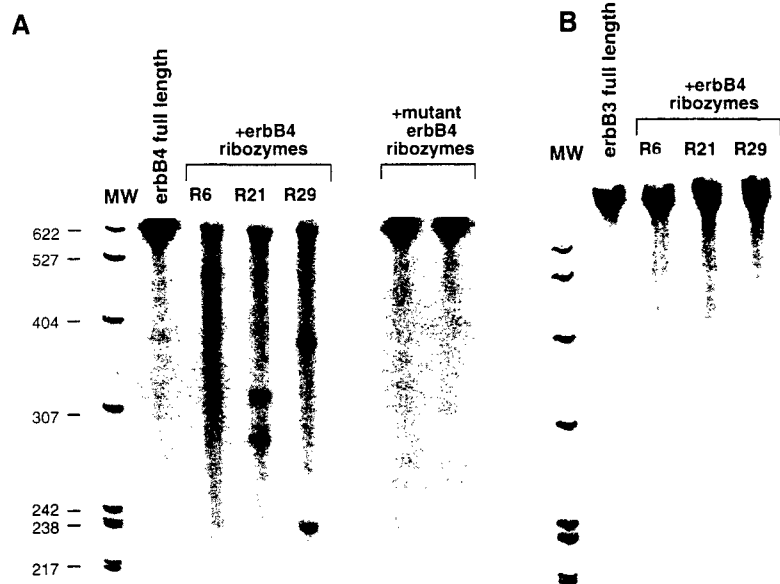
dependence can be abrogated by introduction of foreign growth factor receptor genes, followed by stimulation with the appropriate growth factor (38). The ability of ErbB-4-expressing cells to bypass the IL-3-dependent pathway after NRG1-α activation (34) provides a simple growth assay to determine the biological function of these ribozymes intracellularly.

Biological Function of EGF Family Receptors in 32D Cells. 32D cell transfectants express the EGF receptor family members individually and in pairwise combinations (34). The resultant stably transfected cells were designated as 32D/E1, 32D/E2, 32D/E3, 32D/ E2 + E3, and 32D/E4, where E1, E2, E3, and E4 refer to EGFR, ErbB-2, ErbB-3, and ErbB-4 receptors, respectively. The high levels of receptor expression were confirmed by Western blotting or immunoprecipitation followed by Western blotting (data not shown). No detectable levels of endogenous EGF family receptor expression were found in parental 32D cells. In the absence of cognate ligands, all of the 32D transfected cells remained dependent on IL-3 for survival (39). 32D transfectants were tested for induction of IL-3-independent survival or proliferation. Consistent with previous studies (34, 39), untransfected parental cells did not proliferate or survive after NRG1-α stimulation. Cells transfected with ErbB-4 or coexpressing ErbB-2 and ErbB-3 bypassed the IL-3-dependent pathway in response to NRG1-α stimulation, but cells transfected with ErbB-2 or ErbB-3 alone did not survive and proliferated in an IL-3-dependent manner (Fig. 3). Regulation of tyrosine phosphorylation of each receptor by NRG1-α was evaluated by immunoprecipitating the corresponding receptors and immunoblotting with antiphosphotyrosine. Fig. 4 demonstrates that no autophosphorylation was observed in the parental cells (32D) in the presence of NRG1-α. In both EGFR- and ErbB-4-expressing cells, the receptors were constitutively phosphorylated; however, phosphorylation could be further induced after exposure to its cognate ligands. In 32D/E2 cells, a marginal phosphorylation of ErbB-2 was observed in the absence of NRG1-α, but receptor phosphorylation was not elevated in the presence of NRG1-α (Fig. 4). No phosphorylation was observed in the presence or absence of NRG1-α in 32D/E3 cells. In 32D/E2 + E3 cells, a high basal level of phosphorylated ErbB-3 was observed, and increased phosphorylation was observed after NRG1-α stimulation (Fig. 4). Thus, the 32D cells provide an ideal system to study the specificity and efficacy of the ribozymes targeting the ErbB family receptors.

Demonstration of ErbB-4 Ribozyme Catalytic Activity in 32D Cells

ErbB-4 Ribozymes Abolish NRG1-α-induced IL-3 Independence. All three ErbB-4 ribozymes were cloned into a mammalian expression vector downstream of the cytomegalovirus early promoter. We then transfected the ErbB-4Rz into 32D/E4 cells. We hypothesized that the functional ribozymes would down-regulate ErbB-4 expression and thereby reduce or abolish the NRG1-α-induced, IL-3-independent survival or proliferation. ErbB-4Rz-transfected cells were tested for growth in the presence and absence of NRG1-α. Cell lines expressing one of the ErbB-4 ribozymes (Rz29) failed to respond to NRG1-α and proliferated in an IL-3-dependent manner. In contrast, parental 32D/E4 and vector alone-transfected cells responded to NRG1-α and proliferated in the absence of IL-3. Rz6 partially inhibited the NRG1-α effect. In contrast, Rz21 had no effect on responsiveness to NRG1-α stimulation. Table 1 summarizes the ribozyme effects in these ErbB-4 cells. We next evaluated the specificity of the
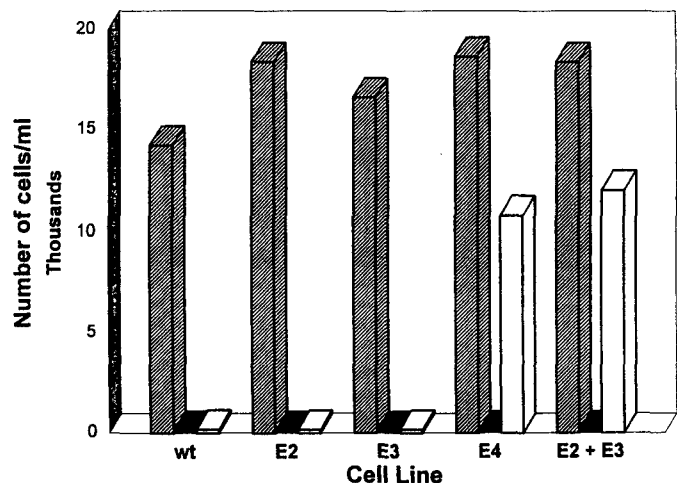
Fig. 3. Growth assay. 32D cells were plated at a density of 1 × 10^4 cells/ml in IL-3-free medium, medium supplemented with IL-3, or in medium lacking IL-3 but supplemented with 100 ng/ml of human recombinant NRG1-α. Viable cells were counted on day 3 after seeding. NRG can induce IL-3-independent growth in 32D/E4 and 32D/E2 + E3 cells. All samples were prepared in triplicate. This assay was repeated more than three times. The SD was within 10%. ▨, IL-3; ■, no IL-3; □, HRG.
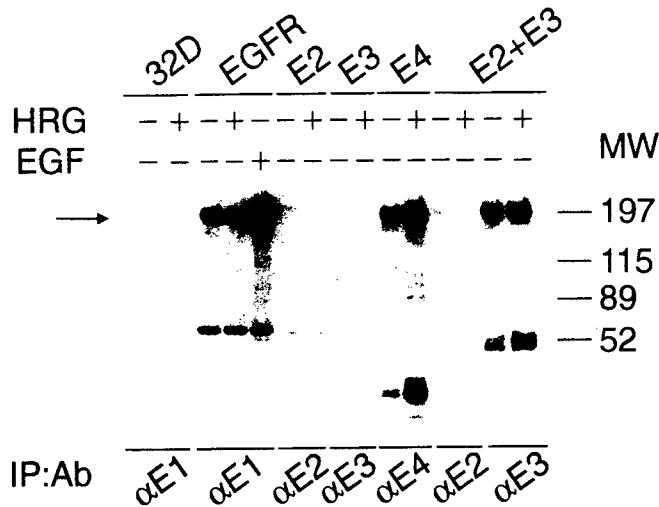
Fig. 4. Regulation of receptor tyrosine phosphorylation by NRG1-α in 32D/E4 and 32D/E2 + E3 cells. Five hundred μg of lysates from untreated or NRG1-α (100 ng/ml for 5 min) treated 32D transfectants (32D/wt, 32D/E2, 32D/E3, 32D/E4, and 32D/E2 + E3) were immunoprecipitated with anti-receptor antibodies (αE2, αE3, and αE4). 32D/EGFR cells (E1) were treated with 100 ng/ml of EGF for 5 min and immunoprecipitated with anti-EGFR antibody (αE1). +, lysates from EGF or NRG1-α-treated cells; −, lysates from untreated cells. The precipitates were then subjected to Western blotting with an anti-phosphotyrosine antibody (UBI). MW, molecular weight; IP, immunoprecipitation.

ErbB-4 ribozymes by expressing all three ErbB-4 ribozymes in 32D/E2 + E3 cells. No effect on the NRG1-α-induced IL-3-independent survival and proliferation was observed. We then evaluated the efficacy of the ribozyme by using an ErbB-2 ribozyme, which has been shown to down-regulate ErbB-2 mRNA specifically in a previous study (40), to target ErbB-4 mRNA. In contrast to the ErbB-4 ri-

Table 1 *Effect of ErbB-4 ribozymes on the density of 32D/E4 cells in response to IL-3 starvation and HRG stimulations*

Cell line	Number of viable cells (×1000 cells/ml)		
	−IL-3	+IL-3	+HRG/−IL-3
E4	1.3	1996	1490
E4/Vector	1	1894	1369
E4/Rz6	1.1	1717	367
E4/Rz21	1	1845	1300
E4/Rz29	1.2	1823	56
E4/ErbB-2 ribozyme	1.2	1798	1279
E2+E3/Rz6	1.1	1869	1307
E2+E3/Rz21	1	1946	1377
E2+E3/Rz29	1.2	1854	1298

bozyme, this ErbB-2 ribozyme did not alter the NRG1-α-induced IL-3 independence of ErbB-4-expressing 32D cells. These data suggest that Rz6 and Rz29 are functional ribozymes, and that the effects of these ErbB-4 ribozymes are highly specific to the ErbB-4 receptor mRNA. Rz29 exhibits a higher level of biological activity compared with Rz6. Rz21 apparently is a nonfunctional ribozyme in 32D cells. The inability of Rz21 to mediate the down-regulation of ErbB-4 may be due to several possibilities. For example, the target site may not be accessible intracellularly, or Rz21 may be unstable in 32D cells.

ErbB-4 Ribozyme Abolishes the NRG1-α Stimulation of Mitogenesis. To confirm the growth-inhibitory activity of the ErbB-4 ribozymes, a mitogenic assay to measure DNA synthesis was performed on ErbB-4Rz-transfected cells. As shown in Fig. 5, all of the 32D-transfected cells exhibited very low levels of [³H]thymidine incorporation in the absence of IL-3. In contrast, all of the 32D-transfected cells exhibited high levels of [³H]thymidine incorporation in the presence of IL-3, as expected. In the 32D/E4 control cells, NRG1-α stimulated high levels of [³H]thymidine incorporation in the absence of IL-3, whereas the [³H]thymidine incorporation was almost completely abolished in the Rz29-transfected cells. [³H]Thymidine incorporation was significantly reduced in Rz6-transfected cells, but to a lesser extent than in Rz29-transfected cells. No significant changes in the Rz21-transfected cells were observed. These results were consistent with the growth assay.

ErbB-4Rz-mediated Down-Regulation of ErbB-4 Expression in 32D/ErbB-4 Cells. To evaluate the intracellular enzymatic cleavage activity of ErbB-4 ribozymes, the ribozyme transfectants were examined for ErbB-4 mRNA levels by Northern blot analysis. Rz6- and Rz29-expressing cells exhibited significantly reduced ErbB-4 mRNA levels relative to control cells or to Rz21-expressing cells (data not shown). Thus, the abolishment of the NRG1-α-induced IL-3 independent biological effect correlates with reduction of ErbB-4 mRNA levels in these cells.

To further characterize the ribozyme effect, we quantitatively examined the ErbB-4 ribozyme-mediated down-regulation of ErbB-4 receptor expression in these ErbB-4Rz-transfected cells by FACS analysis. Consistent with Northern analysis, Rz29- and Rz6-transfected cells expressed significantly less cell surface ErbB-4 receptor relative to the 32D/E4 control cells (65 and 45% less ErbB-4, respectively; Fig. 6). No significant reduction of ErbB-4 expression was detected in Rz21-transfected cells. These data suggest that ErbB-4 Rz29 and Rz6 are biologically functional ribozymes.

Reduction of Autophosphorylation by ErbB-4 Ribozymes. To determine whether the NRG1-α-induced IL-3-independent pheno-
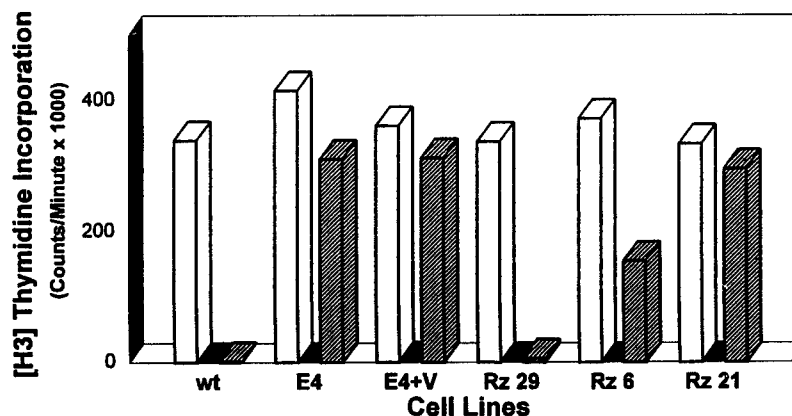
Fig. 5. ErbB-4 ribozyme abolishes NRG1-α-induced mitogenesis. 32D-transfected cells were plated at a density of 1 × 10⁴ cells with or without IL-3 or with 100 ng/ml NRG1-α in the absence of IL-3. Two days after plating, the cells were labeled with [³H]thymidine for two hours. [³H]Thymidine incorporation was then analyzed by scintillation counting. *wt*, parental 32D cells; *E4*, 32D/E4 transfected cells. *E4+V*, empty vector-transfected 32D/E4 cells. Ribozyme-transfected cells are indicated as Rz6, Rz21, and Rz29. Rz29 abolished the NRG1-α-induced IL-3-independent growth. All samples were prepared in triplicate. This assay was repeated three times. The SD was within 10%. □, IL-3; ■, no IL-3; ▨, HRG.

Fig. 6. Rz29 down-regulation of ErbB-4 expression in 32D/ErbB-4 cells. The levels of ErbB-4 in 32D/E4 and Rz29-transfected 32D/E4 cells were quantitatively measured by flow cytometry. Cells (1×10^6) were harvested and stained with an anti-ErbB-4 monoclonal antibody in combination with fluorescence-labeled anti-mouse IgG antibody and analyzed by FACScan. *A.* expression of ErbB-4 in vector-transfected cells (E4/V). *Right-hand curves,* specific staining; *left-hand curves,* nonspecific staining (primary antibody omitted); *ordinates,* relative cell number; *abscissas,* log fluorescence. *B,* Rz29 down-regulates ErbB-4 expression by 50%. *Dotted-line curve,* ErbB-4 expression in ErbB-4/V cells. *Solid-line curve,* ErbB-4 expression in Rz29-transfected cells. In *C,* Rz21 has no effect on ErbB-4 expression. *Dotted-line curve,* ErbB-4 expression in ErbB-4/V cells. *Solid-line curve,* ErbB-4 expression in Rz21-transfected cells. *D,* Rz6 down-regulates ErbB-4 expression by 30%. *Dotted-line curve,* ErbB-4 expression in ErbB-4/V cells. *Solid-line curve,* ErbB-4 expression in Rz6-transfected cells.
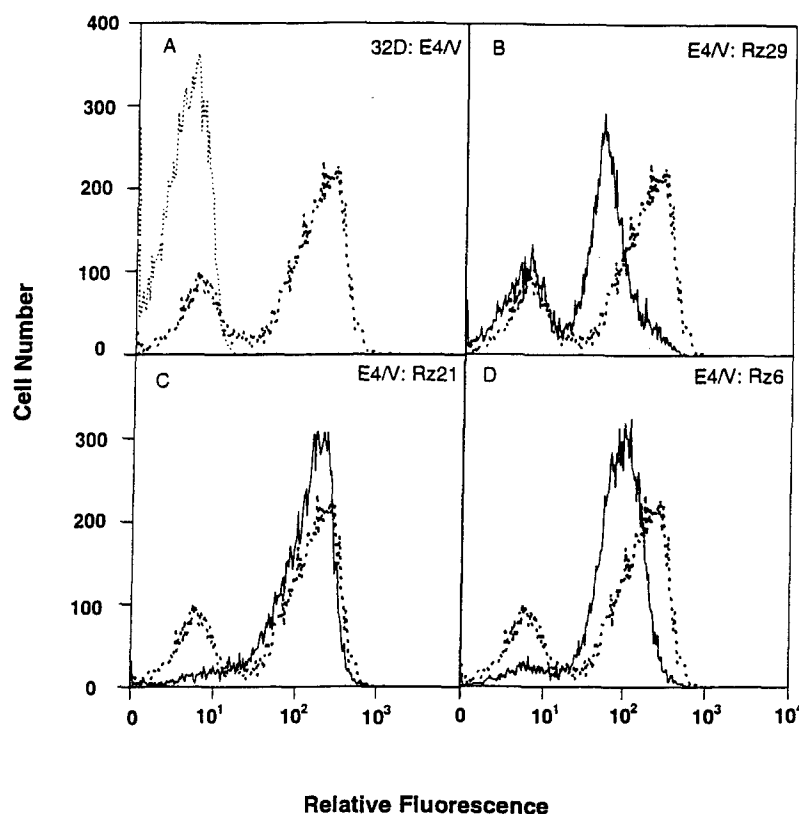
type in ErbB-4 transfectants correlated with an increase in receptor tyrosine phosphorylation, the autophosphorylation of the receptors in these cells was examined by a kinase assay. Fig. 7 demonstrates that the level of ErbB-4 intrinsic tyrosine kinase activity in Rz29-transfected cells was markedly reduced, compared with control transfectants (32D/E4 and 32D/E4/Vector). Because ErbB-4 expression was down-regulated only 65% by Rz29, the cells still express ErbB-4 receptors. NRG1-α was therefore still able to induce the phosphorylation of the remaining ErbB-4 receptors. However, the level of phosphorylation was significantly lower than the 32D/E4 cells or the vector-transfected cells (32D/E4/V). Reduction of phosphorylation correlated with a reduction in expression of ErbB-4. Furthermore, these data also imply that although Rz29 is specifically cleaving its target mRNA, it does not affect the function of those receptors that are expressed. These intracellular experiments demonstrated that the decrease of ErbB-4 protein production, activation, and mRNA expression correlate with the ErbB-4 ribozyme catalytic activity.

Effect of Down-Regulation of ErbB-4 Receptor in Human Breast Cancer Cells

To investigate the biological and biochemical functions of ErbB-4 in human breast cancer, we expressed the ErbB-4 ribozymes in several ErbB-4-positive human breast cancer cell lines. One of the cell lines was T47D, derived from a breast carcinoma. The T47D cells express moderate levels of all of the presently known ErbB receptors. We transfected all three ribozymes (Rz6, Rz29, and Rz21), as well as the empty vector alone and G to A mutants of Rz29 and Rz6. The stably transfected clones were selected by G418. We observed a reduction in G418-resistant colony formation when the Rz6 and Rz29 constructs were transfected. This was evident especially in the Rz29 transfection, and it was extremely difficult to select the stably transfected clones, suggesting that down-regulation of ErbB-4 receptor in T47D cells may be lethal. We partially characterized the pooled population of the

Rz6-transfected cells. We detected 70% down-regulation of the ErbB-4 receptor in these cells by FACS analysis (Fig. 8D), whereas no effect on the level of EGFR, ErbB-2, or ErbB-3 receptors was observed (Fig. 8, *A–C*). Ribozyme mediated down-regulation of ErbB-4 receptor expression in T47D/Rz6 cell was also confirmed by reduction of ErbB-4 mRNA level (Fig. 9). In addition, we observed that anchorage-independent colony formation was significantly reduced (65%) in the ribozyme Rz6-transfected cells (Fig. 10). Furthermore, the nonfunctional ribozyme (Rz21) and the G-to-A mutant
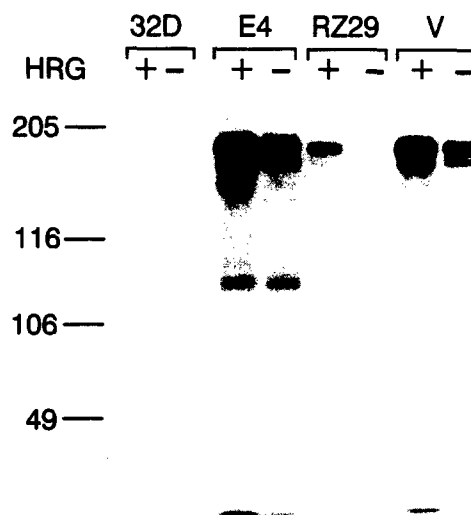
Fig. 7. Reduction of autophosphorylation of ErbB-4 receptor by Rz29 ribozyme. Cells were treated with (+) or without (−) NRG1-α (100 ng/ml) for 5 min prior to lysis, and 400 μg of lysates were immunoprecipitated with specific anti-ErbB-4 antibody. Precipitated proteins were then used for *in vitro* kinase assay as described in "Materials and Methods." Lysates from 32D wild-type and transfected cells are indicated above. *32D,* untransfected cells; *E4,* ErbB-4 transfected cells; *Rz29,* ErbB-4 ribozyme Rz29-expressing 32D/E4 cells; *V,* cells transfected with empty vector. *Left,* molecular weight standards.

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Fig. 8. *A–D*, ErbB-4 ribozyme down-regulation of endogenous ErbB-4 expression in T47D human breast cancer cells. The level of EGF family receptors in T47D/wt and T47D/Rz pool clones were quantitatively measured by flow cytometry. Cells (1×10^6) were harvested and stained with specific monoclonal antibodies against different receptors of the EGF family in combination with fluorescence-labeled anti-mouse IgG antibody and analyzed by FACScan. *Left-hand curve (thin dotted-line curve)*, nonspecific staining (primary antibody omitted). *Bold dotted-line curve*, expression of ErbB family receptors in T47D wild-type cells. *Bold-line curve*, expression of ErbB family receptors in Rz6-transfected T47D cells. *A*, expression of EGFR in T47D/Rz6 cells. *B*, expression of ErbB-2 in T47D/Rz6 cells. *C*, expression of ErbB-3 in T47D/Rz6 cells. *D*, expression of ErbB-4 in T47D/Rz6 cells. *Ordinates*, relative cell number; *abscissas*, log fluorescence. *E* and *F*, no effect on ErbB-4 expression was observed in empty vector or nonfunctional ErbB-4 ribozyme (Rz21)-transfected T47D cells. The level of ErbB-4 receptor in T47D/wt and T47D/V or T47D/Rz21 clones was quantitatively measured by flow cytometry. Cells (1×10^6) were harvested and stained with an anti-ErbB-4 monoclonal antibody in combination with fluorescence-labeled anti-mouse IgG antibody and analyzed by FACScan. *Left-hand curve (thin dotted-line curve)*, nonspecific staining (primary antibody omitted). *Bold dotted-line curve*, expression of ErbB-4 in T47D wild-type cells. *Bold-line curve*, expression of ErbB-4 receptor in T47D/V or T47D/Rz21 cells. *E*, expression of ErbB-4 in empty vector-transfected T47D cells. *F*, expression of ErbB-4 in T47D/Rz21 cells. *Ordinates*, relative cell number; *abscissas*, log fluorescence.
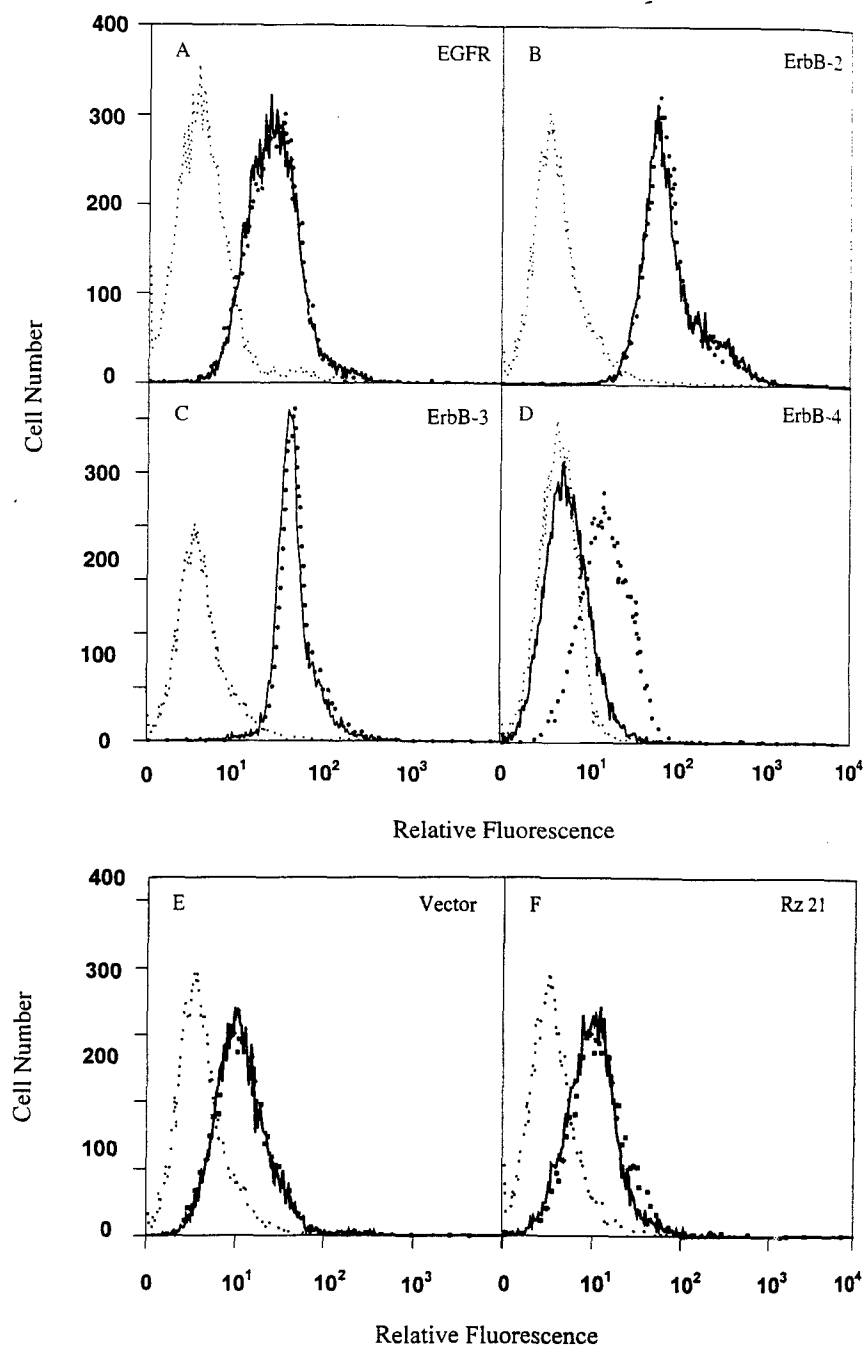
ribozymes have no effect on the level of ErbB-4 and no effect on cell proliferation, as well as the empty vector-transfected cells (Fig. 8, *E* and *F*). These preliminary data suggest that Rz6 is able to down-regulate the endogenous ErbB-4 receptor. The ErbB-4 receptor may therefore play a role in T47D cell proliferation.

DISCUSSION

In this study, we generated three specific hammerhead ribozymes targeted to ErbB-4 mRNA. We have demonstrated that these ErbB-4 ribozymes (Rz6, Rz21, and Rz29) effectively catalyze precise cleavage of ErbB-4 mRNA under physiological conditions in an extracellular system (Fig. 2). Furthermore, we demonstrated that these ribozymes do not cleave mRNA other EGFR family members, despite the high degree of sequence homology shared by these receptors. Point mutation of these ErbB-4 ribozymes in the catalytic domain resulted in loss of catalytic activity and failure to cleave ErbB-4

mRNA. These inactive ribozymes have identical binding arms to the active version but have a mutated catalytic domain. Thus, these mutated versions are capable of binding to the target sequence but are not able to cleave the target mRNA. Taken together, these control experiments demonstrate that the ErbB-4 ribozymes are highly specific for the ErbB-4 mRNA.

Using the 32D cell system to study the intracellular enzymatic activity of ErbB-4 ribozymes, we clearly demonstrated that the ribozymes are specific and effectively down-regulate the EGF receptor family members. In this system, one ErbB-4 ribozyme (Rz29) significantly reduced the ErbB-4 mRNA level and down-regulated ErbB-4 receptor expression (Fig. 6), thereby reversing the NRG1-α-induced IL-3-independent phenotype of 32D/E4 cells (Table 1). Rz6 partially down-regulated the expression of the ErbB-4 receptor and somewhat blocked the IL-3-independent phenotype. In contrast, Rz21 failed to down-regulate the ErbB-4 expression and inhibit the mitogenic response to NRG1-α treatment in 32D/ErbB-4 cells. It is clear from
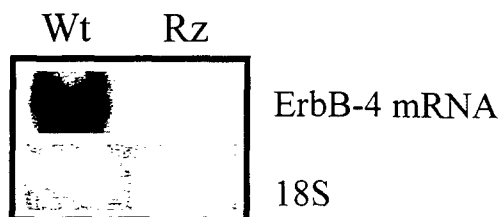
Wt   Rz

ErbB-4 mRNA

18S

Fig. 9. Northern blot analysis of ribozyme-mediated down-regulation of ErbB-4 mRNA in T47D/Rz6 cells. Total RNA was prepared from cultured cells (T47D/wt and T47D/Rz6). Gels were loaded with 20 μg of RNA/lane, transferred to nylon membranes, and hybridized with radiolabeled ErbB-4 probe. 18S RNA is shown as a loading control after the gel was stained with ethidium bromide.

these data that not all of the sites tested are equally amenable to intracellular ribozyme-mediated cleavage. This is in spite of the fact that ribozymes to all of the sites were shown to be catalytically active extracellular biochemical assays. RNA secondary structure or association with cellular proteins may affect target site accessibility. This demonstrates the need for an empirical determination of appropriate target sites. We therefore investigated the specificity and efficacy of these ribozymes in a well-defined cellular system. Two sets of experiments were conducted to control for ribozyme specificity and efficacy intracellularly. Because of the high level of homology between the EGF receptor family members, the intracellular specificity of ErbB-4 ribozymes was demonstrated using 32D cells that ectopically coexpress ErbB-2 and ErbB-3. None of the ErbB-4 ribozymes (Rz6, Rz21, and Rz29) had any effect on the level of ErbB-2 or ErbB-3 expression or the NRG1-α-induced IL-3-independent phenotype in these 32D derivative cells (Table 1). Moreover, an ErbB-2 ribozyme, shown previously to down-regulate the expression of ErbB-2 mRNA, failed to decrease ErbB-4 expression in 32D/ErbB-4 cells. The lack of down-regulation of ErbB-4 expression in these control experiments is evidence that these ErbB-4 ribozymes are highly specific. Furthermore, in the absence of NRG1-α, cells expressing these ribozymes remained strictly dependent on IL-3 for growth. In contrast, two ErbB-4 ribozymes (Rz29 and Rz6) decreased NRG1-α-induced, IL-3-independent proliferation. These phenomena indicate that only the ErbB-4 transcript is directly affected by these ribozymes. Although the ErbB-4 expression was reduced in Rz6- and Rz29-transfected 32D/E4 cells, the remaining ErbB-4 receptors in these cells were still phosphorylated in response to NRG1-α treatment (Fig. 7). This characteristic provides strong support for a cleavage-mediated mechanism of action for the ribozymes. Therefore, the constructed ErbB-4 Rz29 and Rz6 are biologically functional ribozymes and are highly specific for the targeted ErbB-4 mRNA in 32D cells.

To evaluate the effects of the down-regulation of ErbB-4 in an ErbB-4-positive human breast cancer cell line, Rz6 and Rz29 were transfected into T47D cells. We observed a reduction in G418-resistant colony formation in Rz6 transfection. However, we were unable to select clones after Rz29 transfection of T47D cells. These phenomena were not observed in the control ribozyme transfections. The low efficiency of Rz6- and Rz29-expressing, drug-selected clones are unlikely due to nonspecific effects, because all of the ribozymes were cloned into the same vector. One possibility is that if ErbB-4 plays a dominant role in T47D cell proliferation, a complete down-regulation of ErbB-4 may be lethal. Therefore, the inability to select Rz29 clones could be explained by a very efficient ErbB-4 down-regulation and may thus significantly inhibit cell proliferation; isolation of stably transfected T47D cells would be impossible. We were able to select the pooled population of the Rz6-transfected cells. Therefore, we selected Rz6-transfected T47D for further characterization. We observed that the ErbB-4 mRNA was significantly reduced by the ribozyme, and the ErbB-4 receptor expression was down-regulated by 70% in Rz6-transfected T47D cells. Down-regulation of the ErbB-4 receptor in T47D/Rz6 cells resulted in a reduction of colony formation

in an anchorage-independent assay and in transfection efficiency, compared with vector- or Rz21-transfected cells. Furthermore, Rz6 only down-regulated ErbB-4 but not other ErbB-receptor family members. Reduction of colony formation suggests that ErbB-4 expression and mitogenic signaling may be essential for T47D cell survival. To confirm the role of ErbB-4 in cell proliferation, it will be important to extend this study to additional breast cancer cell lines, which express varying levels of ErbB family receptors. Further characterization of ErbB-4 ribozyme-transfected human breast cancer cells will be addressed in a future publication. Presently, we are also conducting these studies using an inducible promoter system. These preliminary findings suggest that a down-regulation of ErbB-4 expression by ErbB-4 ribozyme, as shown by FACS, diminished ErbB-4-mediated intracellular signaling. Because of heterodimerization between the family receptors, down-regulation of the ErbB-4 receptor may also be indirectly interrupting receptor signaling pathways initiated by other family members. This could result in diminished tumorigenicity in T47D cells. These results also show that our ribozyme is active in a human carcinoma cell line.

32D cells are strictly dependent upon IL-3 for survival and proliferation. However, NRG1-α was capable of stimulating its cognate receptors, coupling to cellular signaling pathways in 32D derivatives, and thereby abrogating IL-3 dependence of these cells. Using the ErbB-4 ribozymes in 32D cell system, we provide the first evidence that different threshold levels of ErbB-4 expression and activation correlate with different responses to NRG1-α stimulation. High levels of ErbB-4 expression, phosphorylation, and homodimerization are necessary for NRG1-α-stimulated IL-3-independent cell proliferation in the 32D/E4 cells. Low levels of ErbB-4 expression do allow NRG1-α-induced phosphorylation but are insufficient to couple receptor activation to cellular signaling, particularly in the case of Rz29-transfected 32D/E4 cells. In line with these observations, a recent study using Ba/F3 cell derivatives showed that NRG1-α failed to induce the IL-3-independent pathway in the ErbB-4 transfected cells (28). It is possible that the level of ErbB-4 expression in these Ba/F3/ErbB-4 cells is lower than that of our 32D/E4 cell line. We demonstrate that the IL-3-independent pathway appears to be very sensitive to the amount of ErbB-4 expression, as well as the tyrosine phosphorylation level. The ErbB-4 expression in Rz6-transfected cells was down-regulated by 45% and exhibited a weak response to NRG1-α. On the other hand, the Rz29-transfected cells, the ErbB-4

Cell lines
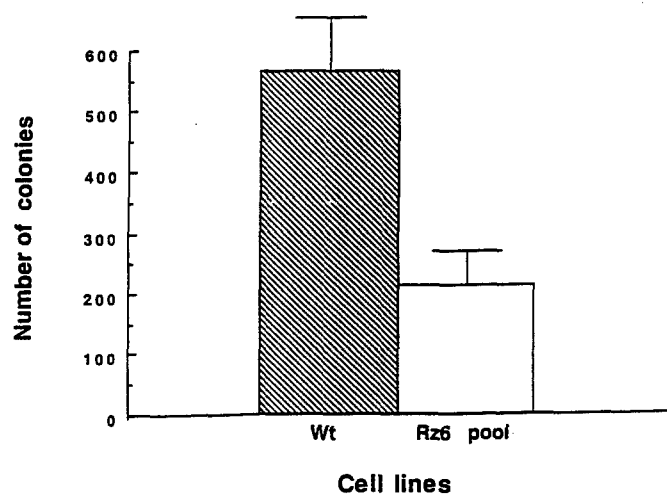
Fig. 10. Anchorage-independent growth assay. The expression of ErbB-4 ribozyme in T47D cells (T47D/Rz6 pool clone) inhibits colony formation by more than 50%. A bottom layer of 0.1 ml Iscove's modified Eagle's medium containing 0.6% agar and 10% FCS was prepared in 35-mm tissue culture dishes. After the bottom layer solidified, cells (10,000 per dish) were added on 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 approximately 12 days at 37°C. Colonies larger than 60 μm were counted in a cell colony counter.

expression level of which was down-regulated by 65%, failed to respond to NRG1-α stimulation. NRG1-α was still able to induce ErbB-4 receptor phosphorylation in these cells, but the level of phosphorylation was much lower than in the 32/E4 cells. This level of phosphorylation is not sufficient to stimulate the cellular response. These results also suggest that homodimers of ErbB-4 can transmit biological signals. This is consistent with a previous report that ErbB-4 homodimers constitute a functional NRG1-α receptor (23). NRG1-α can induce 32D/ErbB-2 + ErbB-3 cells to bypass the IL-3-dependent pathway, presumably due to transphosphorylation and cross-talk between the receptors through heterodimerization of ErbB-2 and ErbB-3. These results are consistent with previous studies concerning ErbB receptor transphosphorylation (34). Although ErbB-3 appears to be a defective tyrosine kinase receptor, it mediates NRG1-α signals through heterodimer formation with either EGFR or ErbB-2 (11). Furthermore, almost all of the breast cancer cell lines express more than one of the EGFR family members. These results imply that interreceptor cross-talk may play an important role in human breast cancer.

Our studies provide strong evidence that ribozymes (Rz6 and Rz29) specifically target ErbB-4 mRNA for degradation extracellularly and intracellularly. These functional ErbB-4 ribozymes should provide important tools for delineating the biological and biochemical consequences of ErbB-4 expression in human breast cancer cells. Furthermore, our study supports the potential for using ribozymes as therapeutic agents for human breast cancer.

ACKNOWLEDGMENTS

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Ribozyme mediated down-regulation of ErbB-4 in estrogen receptor positive breast cancer cells inhibits proliferation both *in vitro* and *in vivo*

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Abstract:

ErbB-4 is a recently discovered member of the class-I receptor tyrosine kinase family (ErbB). Little is known about its expression and its importance in human malignancy. To delineate the biological function of ErbB-4 receptors in breast cancer, we employed a hammerhead ribozyme strategy to achieve down-regulation of ErbB-4 receptors in various breast cancer cell lines. We observed that down-regulation of ErbB-4 in estrogen receptor positive (ER+) human breast cancer cell lines (MCF-7, T47D) which express relatively high levels of ErbB-4, significantly inhibited colony formation. No effects were observed in estrogen receptor negative (ER-) MDA-MB-453 cells, which express low levels of endogenous ErbB4 and high levels of ErbB-2 and ErbB-3. This occurred despite the fact that FACS analysis of these latter cells revealed that the expression of the ErbB-4 receptor was completely abrogated by ribozyme treatment. Furthermore, down-regulation of ErbB-4 in T47D and MCF-7 cells significantly inhibited tumor formation in athymic nude mice ($p < 0.001$ and $p < 0.0003$, respectively). In addition, neuregulin-stimulated phosphorylation of ErbB-4 and NRG induced colony formation was significantly reduced in ribozyme transfected T47D cells. These data provide the first evidence that elevation of ErbB-4 expression plays a role in the proliferation of some ER+ human breast cancer cell lines (T47D, MCF-7), which express high levels of ErbB-4.

We have also investigated the expression of ErbB-4 in human primary breast carcinoma specimens, using immunohistochemical staining with an anti-ErbB-4 monoclonal antibody. ErbB-4 expression was found in 70% of the 50 primary breast tumors examined and high intense immunoreactivity of ErbB-4 was detected in 18% of these primary breast tumors. ErbB-4 receptor expression appeared to correlate with estrogen receptor positive (ER+) and progesterone receptor positive (PR+) primary breast tumors.

These results provide a better understanding of the biological significance of ErbB-4 receptor

in breast cancer. Our data suggest that elevation of ErbB-4 receptor play a role in ER+ breast cancer cells proliferation. Moreover, ribozyme technology provides a useful tool to delineate the role of a particular gene product.

Introduction:

Members of the class I receptor tyrosine kinase family (ErbB) are most frequently implicated in human cancers (1-3). These receptors include the EGFR, ErbB-2, ErbB-3, and ErbB-4 proteins (4-8). ErbB-4 is the most recently discovered member of the ErbB family. More than a dozen different agonists have been reported for the ErbB family receptors. These growth factors exert their function by binding cell surface receptors with intrinsic protein tyrosine kinase activity and are implicated in the autocrine/paracrine growth of breast epithelial cells. The neu differentiation factors (NDF)/neuregulin (NRG), neuregulin 2 (NRG2), (also known as heregulins, neu differentiation factors, glial growth factors, acetylcholine receptor inducing activity) bind to ErbB-3 and ErbB-4, but can only activate ErbB-4 or ErbB-2/ErbB-3 heterodimer and can not activate ErbB-3 (14-18). In addition, multiple isoforms of NRG and NRG-2 arising from alternative transcriptional splicing are ligands of ErbB-3, ErbB-4 (19-21), and can also transmodulate ErbB-2 and EGFR through heterodimers with ErbB-3 and ErbB-4 (22, 23, 31, 32). Recently, it has reported that Heparin-binding EGF-like growth factor (HB-EGF) and Betacellulin (BTC), as well as epidermal growth factor (EGF) can activate ErbB-4 signaling pathways (13, 26, 34). Sequencing of full-length human ErbB-4 cDNAs revealed the existence of two ErbB-4 isoforms (9). The second c-ErbB-4 was found with deletion of 48 bp, which encodes a consensus phosphatidylinositol 3-kinase (PI3K) binding site (9). This implies that the two forms of ErbB-4 might interact with different intacellular signaling pathways (9). Both ErbB-4 transcripts are found to be expressed in normal breast and in most breast cancers (9).

Amplification or over-expression of the ErbB-2 proto-oncogene has been detected in 30% of breast cancers and is associated with a poor prognosis (2). Overexpression of ErbB-4 in NIH 3T3 cells can transform these cells (29). A recent report has indicated that amplification of ErbB-4 was found in 13% of human breast cancer and direct correlated with the tumor size (10). Co-expression of ErbB-2,

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ErbB-4 and NRG is significantly related to the presence of metastases in human medulloblastoma (28). ErbB-4 mRNA was significantly overexpressed in gastric cancer (37). In addition, the physiological relevance of transmodulation is supported in gene-targeting experiments in transgenic mice. Mice that are homozygous for disruptions in the ErbB-4 gene die in utero at day 10.5 and lack trabecular extensions of the developing ventricular myocardium (11).

Although ectopic expression of recombinant ErbB receptors has provided important information on their signaling properties, the biological function and *in vivo* interplay of these receptors is still poorly understood. Little is known about the biological significance of ErbB-4 in breast cancer. In order to more fully understand the role of ErbB-4 in neoplastic transformation, we employed a hammerhead ribozyme strategy to inactivate ErbB-4 and delineate its role in biological neoplastic transformation. In a previous study, it was demonstrated that we were able to generate biologically active ribozymes that target the ErbB-4 receptor. We demonstrated that the neuregulin-induced mitogenic effect was abolished in ribozyme transfected 32D/ErbB-4 cells, a cell line dependent on signaling through ErbB-4. Inhibition of mitogenesis was proportional to ribozyme-mediated down-regulation of ErbB-4 expression (33). In the current study, we have employed these ribozymes to down-regulate endogenous levels of the ErbB-4 receptor in various breast cancer cell lines. We have observed that down-regulation of ErbB-4 in some of the estrogen receptor positive (ER+) breast cancer cell lines expressing relatively high levels of ErbB-4 dramatically reduces the ability of the cells to grow in an anchorage-independent assay. Furthermore, ribozyme-mediated down-regulation of ErbB-4 in these ER+ breast cancer cells exhibited inhibition of tumor formation in athymic nude mice. However, complete down-regulation of ErbB-4 expression in estrogen receptor negative (ER-) breast cancer cell lines expressing low levels of ErbB-4 expression has no effect. These data suggest that ErbB-4 play a proliferative role in cells expressing high levels of ErbB-4. In addition, a pilot study to determine the frequency of ErbB-4 expression in primary breast cancer

specimens was conducted by immunohistochemistry. 70% of the primary breast cancer specimens were found to express ErbB-4. High intense immunoreactivity of ErbB-4 was detected in 18% (9 of 50) of these primary breast tumors. Interestingly, expression of ErbB-4 is directly correlated with ER+ and progesterone receptor positive (PR+) human breast carcinomas. These results provide a better understanding of the biological significance of ErbB-4 receptors in breast cancer. Moreover, ribozyme technology provides a useful tool to delineate the role of a particular gene product.

Materials and Methods:

Cell lines and cell culture: T47D, MCF-7, MDA-MB-453 and MDA-MB-231 breast carcinoma cell lines and their derivatives were maintained in IMEM (Cellgro), supplemented with 10% fetal calf serum (Biofluids).

Transfection: 1 x 10^6 cells and 10-15 μg of plasmid DNA were used for each transfection. Transfections were performed using Calcium Phosphate Transfection System (GibcoBRL Life Technologies, MD), according to the manufacturer's protocol. The cells were then selected in a growth medium containing-appropriated amounts of Geneticin (G418-sulfate, Gibco).

Autophosphorylation of ErbB-family receptors: Prior to cell lysis, the cells were serum starved overnight at 37 °C. Following incubation, cells were then treated with 100 ng/ml of NRG (R & D Systems) or 100 ng/ml of BTC (R & D Systems) for 5 min. at 37 °C. Following a 5 min. incubation, cells were lysed in "Hepes-Lysis buffer" and the cell debris was pelleted by centrifugation (33).

The lysates were then immunoprecipitated with either anti-EGFR (Ab-1, Oncogene Science, Uniondale, NY), anti-ErbB-2 (Ab-3, Oncogene Science, Uniondale, NY), anti-ErbB-3 (C17, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-ErbB-4 (C18, Santa Cruz Biotechnology, Santa Cruz, CA), in combination with protein-A agarose (Pharmacia, Piscataway, NJ) overnight at 4°C with gentle agitation. Immunoprecipitates were then separated by SDS-PAGE, and transferred to nitrocellulose. Bound proteins were immunoblotted with anti-phosphotyrosine monoclonal antibody PY20 (UBI, Lake Placid, New York), followed by blotted with 0.5 μg/ml of secondary antibody linked to horseradish peroxidase. Immunoreactive bands were detected by with an enhanced chemiluminescence reagent (ECL) (Amersham Corp.).

Fluorescence-activated cell sorter (FACStar) analysis: 1 X 10^6 cells were harvested and then stained for one hour with either anti-EGFR (Ab-1), ErbB-2 (Ab-2), ErbB-3 (Ab-4), or anti-ErbB-4

monoclonal antibody (Ab-1, NeoMarker, Fremont) at 4°C. Stained cells were then washed with cold PBS. A secondary FITC-anti-mouse antibody was used and the ErbB-4 level in each cell was quantitatively measured by flow-cytometry.

Anchorage-dependent growth assays: Cells were harvested using trypsin and 1,500 cells/well plated in 24-well plates (Costar). All samples were prepared 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 IMEM containing 0.6% agar and 10% FCS was prepared in 35mm tissue culture dishes. After the bottom layer solidified, cells (10,000 per dish) were then added in a 0.8 ml top layer containing 0.4% Bacto Agar, and 5% FCS. All samples were prepared in triplicate. Cells were incubated for approximately 12 days at 37°C. Colonies larger than 60μm were counted in a cell colony counter (Ommias 3600, Imaging Products Int., Inc. Charley, VA).

In vivo studies: Ovariectomized athymic nude mice were inoculated subcutaneous (s.c.) with either T47D/wt, T47D/poolA, T47D/pool20, or MCF-7/wt, MCF-7/vector, as well as ErbB-4 ribozyme transfected clones, MCF-7/RzB1 and MCF-7/RzA4 in the presence of estrogen source (0.72 mg). The slow-release pellets (60-days release) were implanted s.c. into the cervical scapular space. Tumor growth was monitored twice weekly for 10-12 weeks. Tumor size was measured twice weekly and calculated by measuring tumor volume (length x width x thickness). When tumors reached up to 2 cm in diameter, mice were sacrificed.

Immunohistochemistry of ErbB-4: Paraffin embedded sections of primary breast tumors were deparaffinized in Xylene (Fisher, Pittsburgh, PA) for 5 minutes, dehydrated in reagent alcohol (Fisher) for 5 minutes, air-dried for 5 minutes, dehydrated in reagent alcohol (Fisher) for 5 minutes, and rehydrated in PBS for 10 minutes. Endogenous peroxidase activity was blocked by 5 minutes incubation,

with 3% hydrogen peroxide (Fisher). A monoclonal anti-erbB-4 antibody (1:50 dilution, NeoMarker, Fremont, CA) was incubated with the section for 2 hours at room temperature. After washing with PBS, a horseradish peroxidase-conjugated goat anti-mouse IgG (H+L) secondary antibody (Kirkegaard & Perry Lab, Gaithersburg, MD) was employed at a dilution of 1/100, and incubated for 30 minutes and washed with water. Subsequently, color was developed using diaminobenzidine (sigma) as a substrate. Sections were then counterstained with hematoxylin (Fisher) for 5 minutes. Sections were then placed on a coverslip using Permount. Prior to the analysis of the levels of ErbB-4 staining in clinical breast cancer specimens, the proficiency of the optimized immunocytochemical assay was established by detection of ErbB-4 expression, using a series of breast cancer cell monolayers. MDA-MB-231 cells were used as a negative control and T47D cells were used as a positive control. In addition, assay performance was monitored by the inclusion of a breast cancer positive control section of known immunostaining percentage and intensity.

Results:

Generation of biological active ribozyme targeting ErbB-4 receptors

To investigate the biological significance of ErbB-4 in human breast cancer, we used molecular targeting of the ErbB-4 mRNA by ribozymes. In a previous study, we described that we have generated three ribozymes (Rz6, Rz21, Rz29) targeted to specific sites within the ErbB-4 mRNA open reading frame. We demonstrated that all three ErbB-4 ribozymes cleaved ErbB-4 mRNA precisely and efficiently under physiological conditions in this cell free system (33). Point mutation of G to A in the catalytic domain of these ribozymes resulted in a loss of catalytic activity as expected (25). We also illustrated the intracellular efficacy and specificity of the ErbB-4 ribozymes in a model system (32D-cell system). 32D cells are a murine hematopoietic IL3-dependent cell line that does not express detectable levels of endogenous EGF-family receptors (24). Overexpression of ErbB-4 receptors in 32D cells (32D/ErbB-4) abrogated IL-3-dependence by stimulation with NRG (33). We showed that two of the ErbB-4 ribozymes (Rz6 and Rz29) were able to down-regulate ErbB-4 expression and were capable of abolishing the neuregulin-induced mitogenic effect in 32D/ErbB-4 cells. In contrast, Rz21 had no effect on responsiveness to NRG stimulation (33). These results demonstrated that ribozyme Rz29- and Rz6 are biologically functional ribozymes and that Rz21 is an inactive ribozyme in 32D cells (33).

Ribozyme mediated Down-regulation of endogenous ErbB-4 in Human Breast Cancer Cells

In this study, we use these biologically active ribozymes to down-regulate endogenous levels of ErbB-4 in various human breast cancer cell lines, with different levels of ErbB-4 expression. This was done in order to elucidate the biological significance of ErbB-4 in breast cancer. Four human breast cancer cell lines were selected as recipient cells: T47D, MCF-7, MDA-MB-453 and MDA-MB-231. In estrogen receptor positive (ER+) cell lines (T47D and MCF-7), there is a relatively high level of ErbB-4 receptor expression and a moderate level of other EGF-family receptors, whereas estrogen receptor

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negative (ER-) MDA-MB-453 cells express low endogenous levels of ErbB-4 and high levels of ErbB-2 and ErbB-3. MDA-MB-231 (ER-) expresses a high level of EGFR and a relatively low level of ErbB-2, but does not express detectable level of ErbB-3 or ErbB-4. The functional ErbB-4 ribozymes, as well as a control vector, were introduced into these cell lines by stable transfection. The sublines T47D/Rz, MCF-7/Rz, MDA-MB-453/Rz and MDA-MB-231/Rz as well as empty vector control cell lines were established. We then assessed the ribozyme-mediated down-regulation of ErbB-4 expression by FACS analysis. Figure 1 illustrates that an ErbB-4 ribozyme is capable of down-regulation of endogenous ErbB-4 expression by 30% and 80% in two of the ribozyme transfected T47D pooled population clones (T47D/Rz-poolA and T47D/Rz-pool 20, respectively). We also found that ErbB-4 expression was almost completely down-regulated in some of the ErbB-4 ribozyme transfected MCF-7 cells, such as MCF-7/RzA4 (Figure 2), as well as in ribozyme transfected MDA-MB-453 cells (data not shown). However, no effect was observed on other EGF family receptors in these ErbB-4 ribozyme transfected cells, respectively (Figure 2A-2C, 33). Furthermore, ribozyme-mediated down-regulation of ErbB-4 receptor expression was confirmed by reduction of ErbB-4 mRNA by Northern blot analysis (33).

Reduction of NRG and BTC induced ErbB-4 autophosphorylation in T47D/Rz transfected cells

We next determined whether NRG or BTC-induced ErbB-4 receptor tyrosine phosphorylation was affected by reduction of ErbB-4 expression in ribozyme transfected cells. Phosphorylation experiments were performed on ribozyme transfected clones. Figure 3 demonstrates that the level of ErbB-4 intrinsic tyrosine kinase activity in T47D/Rz-Pool20 transfected cells was markedly reduced when compared with control transfectants (T47D/wt and T47D/Vector) cells. Reduction of phosphorylation correlated with a reduction in ErbB-4 expression level. A similar effect was observed in BTC-induced ErbB-4 tyrosine phosphorylation. These experiments demonstrate that the reduction of ErbB-4 activation correlates with down-regulation of ErbB-4 protein production.

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Down-regulation of ErbB-4 in cell lines expressing relatively high level of ErbB-4 resulted in an inhibition of colony formation

In order to assess the biological significance of ErbB-4 in human breast cancer, we evaluated the *in vitro* growth of ErbB-4 ribozyme transfected T47D, MCF-7, MDA-MB-453 and MDA-MB-231 cells by anchorage-dependent as well as anchorage-independent growth assays. Down-regulation of ErbB-4 expression in ER+ human breast cancer cell lines expressing a relatively high level of ErbB-4 (T47D and MCF-7 cells) resulted in an inhibition of colony formation that was independent of colony size. Figure 4 illustrates that colony formation was almost completely abolished in T47D/Rz-pool20 cells, which had an 80% down-regulation of ErbB-4, indicating a partial reversion of transformation. Down-regulation of ErbB-4 by 30% in T47D/Rz-poolA cells also displayed a 40% reduction in their ability to form colonies in soft agar (data not shown). Furthermore, inhibition of colony formation was independent of threshold colony size. A similar phenotype was observed in ribozyme transfected MCF-7 cells (Figure 5). These data demonstrated that inhibition of growth is correlated with the level of down-regulation of ErbB-4 in these ribozymes transfected cells. However, growth inhibition was not observed in ER- MDA-MB-453/Rz cells, which express low levels of ErbB-4 and high levels of ErbB-2 and ErbB-3. Interestingly, FACS analysis revealed that the expression of the ErbB-4 receptor was completely abrogated by the ErbB-4 ribozyme in these cells as well (data not shown). In a parallel experiment, we verified the specificity and efficacy of the anti-ErbB-4 ribozymes with MDA-MB-231 cells, which do not express detectable level of ErbB-4. Obviously, no effect was observed in ribozyme transfected MDA-MB-231 cells, respectively (data not shown). These data suggest that the biological effect of ErbB-4 receptor expression is dependent upon its relative levels in a given cell line.

The sensitivity of biological responses to different EGF-like ligands is dependent upon the relative levels of ErbB family receptors expression

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The regulation of ErbB receptor family members activation is very complex. A large number of ErbB ligands have been described (38-41). We next compared the effects of EGF-like ligands on ribozyme transfected T47D cells (T47D/Rz) versus T47D/wt. We observed that neuregulin induced colony formation was significantly inhibited in T47D/Rz transfected cells. Down-regulation of ErbB-4 in T47D cells reduced NRG stimulated colony formation by 80%. In contrast, wild type T47D cells exhibited an 11- fold increase in colony formation when treated with neuregulin which appeared to have the most dominant effect among the six of EGF-like ligands tested. *Whereas BTC,* predominantly binds to EGFR and can also activate the ErbB-4 and ErbB-2/ErbB-3 heterodimers, appeared to have the most dominant effect on the induction of colony formation in ErbB-4 depleted T47D cells, when compared with the other EGF-like ligands (Figure 6). These data demonstrate that NRG was significantly more active than other EGF-like ligands in T47D wild type cells, while down-regulation of ErbB-4 in T47D cells revealed almost complete abrogation of the NRG activity, suggesting that NRG signaling occurs primarily through ErbB-4 in T47D cells. Interestingly, BTC was comparable to NRG stimulating colony formation by nearly six folds in T47D wild type cells and is the dominant ligand in ErbB-4 depleted T47D cells. These results suggested that altered the expression of ErbB-family receptors in the cell results in an altering in the biological activities of EGF-related peptides.

Inhibition of tumor formation in nude mice

Down-regulation of ErbB-4 led to dramatic effects on anchorage-dependent and anchorage-independent growth in MCF-7 and T47D cells. We next explored the *in vivo* effects of down-regulation of ErbB-4 in MCF-7 and T47D cells. $5x10^6$ MCF-7 or T47D wild type cells as well as the ribozyme transfected cells were implanted in ovariectomized mice. With estradiol treatments, the T47D wild type cells grew to a mean tumor size of 500 ± 20 mm^3 (Fig. 7; filled circles). In contrast, tumor growth of ribozyme expressing T47D cells was significantly inhibited ($p<0.001$; student's *t* test) with a mean tumor

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size of 80 ± 14 mm^3 (Fig. 7; triangles and squares). Moreover, tumor growth of T47D cells transfected with the catalytically inactive ribozyme (Rz21) was not significantly different from control cells (data not shown). Similar experiments were performed with ribozyme transfected MCF-7 cells. Figure 8 demonstrated that the down-regulation of ErbB-4 expression in MCF-7 cells dramatically reduced the tumor formation. With estradiol treatment, the MCF-7 wild type (MCF-7/wt) and an empty vector transfected MCF-7 cells (MCF-7/vector) grew large tumors with a mean tumor size of 2400 ± 270 mm^3. In contrast, tumor growth of ribozyme expressing MCF-7 cells was drastically inhibited ($p<0.0003$; student's t test) with a mean tumor size of 580 ± 74 mm^3 ($p<0.001$; student's t test). Table 1 summarized the *in vitro* and *in vivo* effects of down-regulation of ErbB-4 in human breast cancer cell lines.

Expression of ErbB-4 in primary breast carcinomas

We conducted a pilot study to investigate the frequency of ErbB-4 expression in breast carcinomas using immunohistochemical analysis with an anti-ErbB-4 monoclonal antibody. The expression of ErbB-4 was analyzed in 5 benign tumors and 50 primary breast carcinomas. No expression was detected in 4 of 5 benign tumors and 1 of 5 these benign tumors was observed with weak ErbB-4 expression. We observed that 70% (35 of 50) of the primary breast carcinomas express ErbB-4. High intense immunoreactivity of ErbB-4 was detected in 18% (9 of 50) of these primary breast tumors. The ErbB-4 immunoreactivity was detected both membrane and cytoplasmic-located in most of the cases. No nuclear staining and negligible of ErbB-4 immunostaining in stromal elements of the tumor specimens was observed in all cases. Figure 9 illustrated a representative breast carcinoma sample stained for ErbB-4 expression. Interestingly, 80%(28 of 35) of the ErbB-4 positive samples were ER+ breast carcinomas and 67% (10 of 15) of the negative or weak ErbB-4 expressions were ER- breast carcinomas (Table 2). It appears that there was a statistically significant ($p= 0.001$) direct correlation between the expression of estrogen receptors and the expression of ErbB-4.

Discussion:

Previous studies from our laboratory have demonstrated that ErbB-4 ribozymes (Rz6 and Rz29) are biologically functional ribozymes and are capable of the down regulation of ErbB-4 in 32D/ErbB-4 cells and the abolishment of NRG induced mitogenesis (33). In this study, we employed ribozyme technology to achieve the functional gene "knockout" strategy, in order to define the role and biological significance of ErbB-4 in human breast cancer. We demonstrated that ErbB-4 ribozymes are capable of down-regulating endogenous ErbB-4 expression in several human breast cancer cell lines. The erbB-4 ribozymes had no effect on other members of the EGF receptor family. In stable mass-transfected T47D cells, ErbB-4 ribozyme expression depleted ErbB-4 mRNA and protein levels by 50-75%. This inhibition is even more remarkable when considering that mass-transfected cells (and not clonal subpopulations) were used. This substantial inhibition enabled us to begin a novel study of the effects of a functional ErbB-4 knockout on *in vitro* and *in vivo* tumor growth of breast cancer cells. We observed that down-regulation of ErbB-4 in ER+ breast cancer cell lines (T47D and MCF-7) which express relatively high levels of ErbB-4 significantly inhibited colony formation. In addition, down-regulation of ErbB-4 in T47D cells significantly impaired NRG-induced ErbB-4 phosphorylation. However, complete depletion of ErbB-4 did not affect the anchorage-dependent and anchorage-independent growth in ER- MDA-MB-453 cells, which express low levels of endogenous ErbB4 and high levels of ErbB-2 and ErbB-3. Furthermore, down-regulation of ErbB-4 in T47D and MCF-7 cells significantly inhibited tumor formation in athymic nude mice with $p < 0.001$, $p < 0.0003$. These data provide the first evidence that an elevation of ErbB-4 expression plays a role in the proliferation of some ER+ human breast cancer cell lines (T47D, MCF-7) both *in vitro* and *in vivo*. These data suggest that there is an inhibition of growth when ***relative high levels of expressed receptors*** are targeted. Furthermore, ErbB receptors undergo extensive heterodimerization. The inactivation or blocking of ErbB-4 signaling may also disrupt and

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diminish the EGFR or ErbB-2 signaling pathways, through heterodimerization with ErbB-4. Expressing low levels of ErbB-4 does not significantly effect breast cancer cell proliferation. A similar conclusion was reported by Hynes and her colleagues (35, 36). Blocking cell surface expression of ErbB-2 and EGFR by intracellular expression of a single-chain antibody specific for ErbB-2 (scFv-5R) and EGFR (scFv-R1R) led to only a slight reduction in colony formation of T47D cells, which express low levels of ErbB-2 and EGFR. However, in MDA-MB-468 cells, scFv-5R and scFvR1R inhibited colony formation by 90% and 94%, respectively. In MDA-MB-468 which express high levels of EGFR and TGFα, treatment with a Mab competes with ligand binding and inhibits cell growth, indicating that these cells are dependent upon an autocrine loop for growth. Despite the fact that these cells have very low levels of ErbB-2, inhibition of colony formation by scFvR suggests that TGFα activated heterodimers of EGFR and ErbB-2 provide the major growth stimulus to these cells (35,36). These data also suggest that depending upon the cellular context, the presence or absence of a specific EGF-family receptors are not the only influence in the nature of cell proliferation, but the actual expression levels of the individual ErbB-family receptors and their cognate ligands contribute a significant role to breast cancer cell proliferation. Overexpression of an ErbB- receptor or co-expression of its cognate ligands plays an essential role in breast cancer proliferation. Conversely, expressing low levels of ErbB-receptors does not significantly contribute to breast cancer cell proliferation. These studies further implied that ErbB-family receptors and their ligands play an important role in breast cancer cell proliferation. In general, the inhibition of growth in breast cancer cells was observed when over expressed receptors were targeted.

Regulation of the activation of ErbB-receptor family members is very complex. ErbB receptors undergo extensive heterodimerization, which makes ligand-induced signaling even more complex. We show that NRG-stimulated phosphorylation of ErbB-4 was significantly reduced and NRG induced colony formation was substantially reduced from 11 fold to only 2.5 fold in ribozyme transfected T47D

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cells (T47D/Rz), indicating that the major NRG signaling was through ErbB-4. It implies that NRG

signaling through ErbB-2/ErbB-3 heterodimers may play a minor role in T47D cells due to their low

expression levels. Furthermore, down-regulation of ErbB-4 expression decreases the spectrum and

potency of EGF-like ligands mediated proliferation. HB-EGF, the ligand for EGFR and ErbB-4,

mediated proliferation were completely abolished. These results suggest that down-regulation of

ErbB-4 expression might interrupt the ErbB-4 heterodimerization which interrupt the other EGF-like

ligands signaling through EGF-family receptors. BTC, a ligand for both EGFR and ErbB-4, as well as

ErbB-2/ErbB-3 heterodimers, exhibited the most dominant effect on induction of colony formation among

the EGF-like ligands in T47D/Rz transfectants (Figure 3). These data indicated that down-regulation of

ErbB-4 only partially affects the BTC signaling. Although, BTC signaling through ErbB-4 may block,

the fact that BTC ability to elicit other ErbB family receptors signaling. These data suggest that the down-

regulation of one of the ErbB-family receptor expressions in the cell result in an alteration of the

biological activities by EGF-related peptides. The sensitivity of a cell line to EGF-like ligands is

correlated with the levels of expression of the ErbB receptors in the cell line. These phenomena

imply that alternative intracellular signaling though ErbB- family receptors and their ligands may

contribute to tuning and diversification of signal transduction thus maintaining their oncogenic potential.

In addition, EGF-related growth factors show distinguishable biological activities, which most likely

depend upon the subsets of ErbB- receptors that become activated. Relative availability of ligands,

receptors and secondary pathways would appear to critically alter the proliferation/differentiation status

of breast cancer.

In addition, we have also investigated the expression of ErbB-4 in primary breast carcinoma,

using immunohistochemical analysis with an anti-ErbB-4 monoclonal antibody. No expression was

detected in majority (4 of 5) of benign tumors and only one case (1 of 5) was observed with very weak

ErbB-4 expression. ErbB-4 expression was found in 70% of the 50 samples examined. High intense

immunoreactivity of ErbB-4 was detected in 18% (9 of 50) of these primary breast tumors. Most of the

staining was found in both cell membrane and cytoplasm. No nuclear staining and negligible of ErbB-4

immunostaining in stromal elements of the tumor specimens was observed in all cases (Figure 9). These

results consistent with other investigator's finds. Expression of ErbB-4 is common in breast cancer cells

and detected as often as in 75% of cases (44, 45). A very recent report has demonstrated that

amplification of ErbB-4 oncogene was detected in 13% of the primary human breast cancer (10). Table

2 summarized the results. Expression of ErbB-4 is correlated with ER+ and PR+ primary breast tumors.

In addition, we also survey the ErbB-4 expression in a panel of human breast cancer cell lines by FACS

analysis. Surprisingly, most of ER+ cell lines express relatively high levels of ErbB-4 and ER- cell lines

express low levels or non-detectable levels of ErbB-4 (data not shown). An inverse correlation was

observed between ErbB-4 and EGFR expression. Taken all together, there may be a suggestion that ErbB-

4 may be a favorable prognostic factor. It is interesting that expression of ErbB-4 is associated with the

prognostically favorable ER phenotype, unlike other EGF-family receptors. As this manuscript was being

prepared for submission, it was reported that a direct association was found between ErbB-3 and ErbB-4

mRNA and ER marker status. Inverse associations were seen between ErbB-3 and ErbB-4 mRNA marker

status and EGFR expression (41). These data are consistent with our findings that ErbB-4 expression is

associated with ER and PR positive breast tumors. Elevated ErbB-4 expression in breast cancer cells,

particularly in ER positive tumors could therefore represent a differentiated feature. In addition, its ligand

NRG-1 has been shown to initiate cellular differentiation in breast cancer cells in vitro (42, 43). It will

be intriguing to define the mechanisms that the expression of the ErbB-4 receptor may utilize in

maintaining the ER expression in human breast cancer.

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In conclusion, elevation of ErbB-4 receptor plays a role in ER+ breast cancer cells proliferation. Furthermore, these results also indicate that the role of ErbB-family receptors in breast cancer cells is not solely dependent on the absolute expression levels of any single ErbB family member, but also on the relative expression levels of all ErbB-family members.

Legends:

Table 1 Selective growth inhibition with ribozyme-mediated down-regulation of ErbB-4 in breast cancer cells[a]

Relative Expression levels of EGF-family receptors						Effects of down-regulation of ErbB-4	
Cell line	EGFR	ErbB-2	ErbB-3	ErbB-4	ER	% inhibition of colony formation	% inhibition of tumorigenicity
MCF-7	+	++	+++	++++	+	60-80	70
T47D	++	++	+++	++++	+	50-70	50-60
MDA-MB-453	+/-	++++	+++	+	-	0	N/A
MDA-MB-231	++++	+	+/-	-	-	0	N/A

The expression levels of ErbB-family receptors were determined by FACS analysis with specific antibodies against EGFR or ErbB-2 or ErbB-3 or ErbB-4.

N/A: Not evaluated.

Table 2 Correlation of ErbB-4 Expression with hormonal receptor status in breast cancer

ErbB-4 expression	ER		PR	
	–	+	–	+
Weak/negative (+/-)	10	5	9	6
Positive (++/+++)	7	28	9	26

N = 50

Figure 1 . ErbB-4 ribozyme-mediated down-regulation of endogenous ErbB-4 expression in T47D human breast cancer cells. The level of EGF family receptors in T47D/wt and T47D/Rz pool clones were quantitatively measured by flow-cytometry. 1×10^6 cells were harvested and stained with specific monoclonal antibodies against ErbB-4 receptor, in combination with fluorescence-labeled anti-mouse IgG antibody and analyzed by FACScan. Left-handed curve (thin dotted-line curve) represents nonspecific staining (primary antibody omitted). The sold thin-line curve represents the expression of ErbB-4 receptor in T47D wild-type cells. The bold-sold-line and bold-dotted-line curves represent the expression of ErbB-4 receptor in two of the ErbB-4-ribozyme transfected T47D pooled clones (T47D/Rz-poolA and T47D/Rz-pool 20). Top left panel of the figure: The expression of EGFR in T47D/wt, T47D/Rz-PoolA and T47D/Rz-pool20 cells. Top right panel of the figure: The expression of ErbB-2 receptor in T47D/wt, T47D/Rz-PoolA and T47D/Rz-pool20 cells. Bottom left panel of the figure: The expression of ErbB-3 receptor in T47D/wt, T47D/Rz-PoolA and T47D/Rz-pool20 cells. No effect was observed that on the levels of EGFR, ErbB-2 and ErbB-3 receptors expression in these ErbB-4-ribozyme transfected T47D pooled clones. Bottom right panel of the figure: The expression of ErbB-4 receptor in T47D/wt, T47D/Rz-PoolA and T47D/Rz-pool20 cells. T47D/ErbB-4 expression was down-regulated by 30% in T47D/Rz-PoolA and 80% in T47D/Rz-pool20 respectively. The ordinates, relative cell number; abscissas, log fluorescence.

Figure 2. ErbB-4 ribozyme-mediated down-regulation of endogenous ErbB-4 expression in MCF-7 human breast cancer cells. The level of EGF family receptors in MCF-7 wild-type (MCF-7/wt), vector transfected only (MCF-7/vector) and two of the ribozyme transfected MCF-7 clones (MCF-7/Rz B1 and MCF-7/RzA4) were quantitatively measured by flow-cytometry. $1X10^6$ cells were harvested and stained with specific monoclonal antibodies against to different receptors of the EGF family in combination with fluorescence-labeled anti-mouse IgG antibody and analyzed by FACScan. Left-handed curve (dotted-line curve) represents nonspecific staining (primary antibody omitted). The solid-line curve represents the expression of ErbB-4. The top left panel of the figure: The expression of ErbB-4 in MCF-7/wt cells. The top right panel of the figure: The expression of ErbB-4 in MCF-7/vector cells. The bottom left panel of the figure: The expression of ErbB-4 in MCF-7/RzB1 cells. The bottom left panel of the figure: The expression of ErbB-4 in MCF-7/RzA4 cells. Ribozyme down-regulated ErbB-4 expression significantly in MCF-7/RzB1 and MCF-7/RzA4 cells. The ordinates, relative cell number; abscissas, log fluorescence.

Figure 3. Reduction of NRG and BTC induced ErbB-4 autophosphorylation in T47D/Rz transfected cells. Cells were treated with or without NRG1-a and BTC (100ng/ml) for 5 minutes prior to lysis, and 400mg of lysates were immunoprecipitated with a specific anti-ErbB-4 antibody. Precipitated proteins were then subjected to Western blotting with an anti-phosphotyrosine antibody (UBI). Lane 1: Molecular weight standards. Lane 2, 5, 8 are untreated samples. Lane 3, 6, 9 are the lysates from T47D/wt, T47D/vector and T47D/Rz-pool20 cells treated with 100 ng/ml of NRG1-a. Lane 4, 7, 10 are the lysates from T47D/wt, T47D/vector and T47D/Rz-pool20 cells treated with 100 ng/ml of BTC. Down-regulation of ErbB-4 in T47D cells dramatically reduced NRG and BTC induced ErbB-4 phosphorylation.

Figure 4. Growth effects of ErbB-4 ribozyme on T47D cells. Expression of the ErbB-4 ribozyme in

T47D cells (T47D/Rz-poolA and T47D/Rz-pool20) inhibits colony formation, independent of colony

size. Anchorage-independent growth assays: A bottom layer of 0.1 ml IMEM containing 0.6% agar and

10% FCS was prepared in 35mm tissue culture dishes. After the bottom layer solidified, cells (10,000 per

dish) were then 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 approximately 12 days at 37°C. Colonies larger than

60μm, 80μm, 100μm, and 120μm were counted by a cell colony counter.

Figure 5. Growth effects of ErbB-4 ribozyme on MCF-7 cells. The degree of reduction of colony formation was correlated with the level of ErbB-4 expression down-regulated by ErbB-4 ribozyme in MCF-7 cells. Clones (MCF-7/RzB1 and MCF-7/Rz A4) exhibited an almost depletion of ErbB-4 expression by ribozyme appeared to reduce colony formation by more than 60% compared with MCF-7/wt and MCF-7/Vector cells. Clones (MCf-7/RzN101 and MCF-7/RzB5) with down-regulation of ErbB-4 expression by 30 –50% inhibited colony formations by 20% to 45% respectively. Anchorage-independent growth assays: A bottom layer of 0.1 ml IMEM containing 0.6% agar and 10% FCS was prepared in 35mm tissue culture dishes. After the bottom layer solidified, cells (10,000 per dish) were then 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 approximately 12 days at 37°C. Colonies larger than 120μm were counted by a cell colony counter. Values indicate the mean of triplicate determinations ± SD.

Figure 6. Down-regulation of endogenous ErbB-4 expression in T47D cells strongly inhibited NRG-induced colony formation. Anchorage-independent growth assays: A bottom layer of 1 ml IMEM, containing 0.6% agar and 10% FCS were prepared in 35mm tissue culture dishes. After the bottom layer solidified, cells (10,000 per dish) were then added on a 0.8 ml top layer containing 0.4% Bacto Agar, 5% FCS and 100 ng/ml of EGF-like ligands. All samples were prepared in triplicate. The cells were incubated for approximately 15 days at 37°C. Colonies larger than 60μm was counted in a cell colony counter. The open bars represent the T47D wild type cells. The solid bars represent the ErbB-4 ribozyme transfected T47D cells (T47D/Rz-pool20). Down-regulation of ErbB-4 expression decreases the spectrum and potency of EGF-like ligands stimulated colony formation. NRG-stimulated colony formation was reduced by 70% and HB-EGF-stimulated colony formation was completely abolished. In the wild type cells, NRG stimulation of colony formation had the most dominant effect of all of the EGF-like ligands. In ribozyme transfected T47D cells (T47D/Rz-pool20), BTC had the dominant effect.

Figure 7. ErbB-4-ribozyme mediated down-regulation of ErbB-4 in T47D cells resulted in reduction of tumor growth *in vivo*. 5×10^6 T47D wild type cells, as well as the ribozyme transfected cells T47D/Rz-poolA and T47D/Rz-pool20) were implanted in ovariectomized mice. With estradiol treatments, the T47D wild type cells grew to a mean tumor size of 500 ± 20 mm^3 (filled circles). In contrast, tumor growth of ribozyme expressing T47D cells was significantly reduced ($p < 0.01$; student's t test) with a mean tumor size of 80 ± 14 mm^3 (triangles and squires).

Figure 8. ErbB-4-ribozyme mediated down-regulation of ErbB-4 in MCF-7 Cells resulted in reduction of tumor growth *in vivo*. 5×10^6 MCF-7 wild type cells, as well as the ribozyme transfected cells were implanted in ovariectomized mice. With estradiol treatments, the MCF-7 wild type cells, as well as the empty vector transfected cells grew large tumors to a mean tumor size of 2000 ± 200 mm^3 (filled & open circles). In contrast, tumor growth of ribozyme expressing MCF-7 cells was significantly inhibited ($p<0.001$; student's *t* test) with a mean tumor size of 600 ± 74 mm^3 (triangles and squires).

Figure 9. Expression of ErbB-4 is correlated with ER+ and PR+ primary breast carcinomas.
Immunohistochemistry staining of ErbB-4 in paraffin sections of human primary breast tumor specimens (Brown). All sections were counterstained with hematoxylin for viewing negatively stained cells (Blue). Top left panel of the figure: The benign ducts do not express ErbB-4. Top right panel of the figure: The infiltrating ductal carcinoma, ER+ tumors express high levels of ErbB-4. Bottom left panel of the figure: The intraductal and infiltrating ductal carcinoma, ER+ tumors express high levels of ErbB-4. Bottom right panel of the figure: The intraductal and infiltrating ductal carcinoma, ER- tumors do not express detectable level of ErbB-4. Expression of ErbB-4 is correlated with ER+ primary breast carcinomas. Stromal cells and the ductal epithelium were negative for ErbB-4 in all cases.

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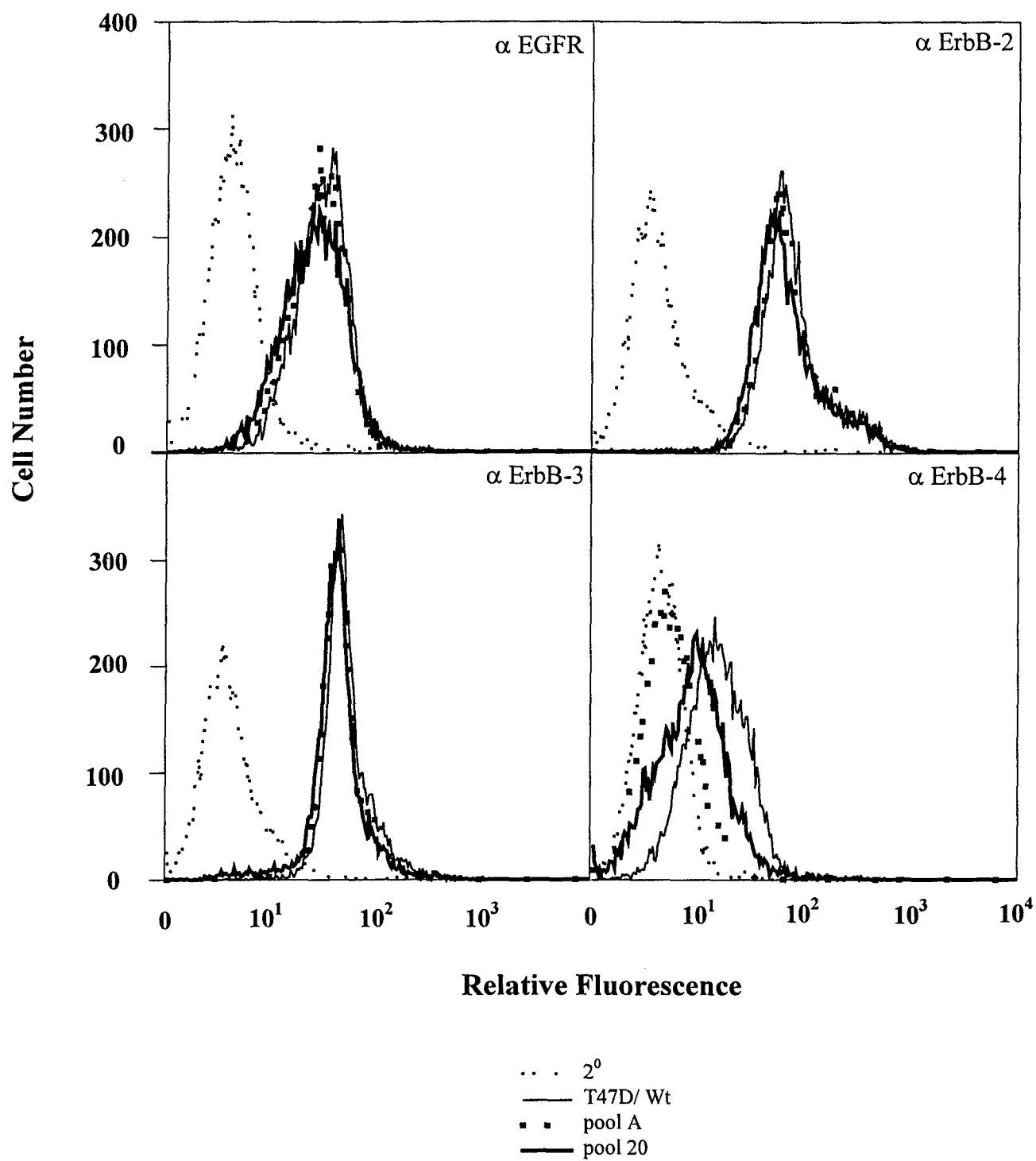   steroid receptors. Am. J. Pathol. 148: 549-558, 1996.

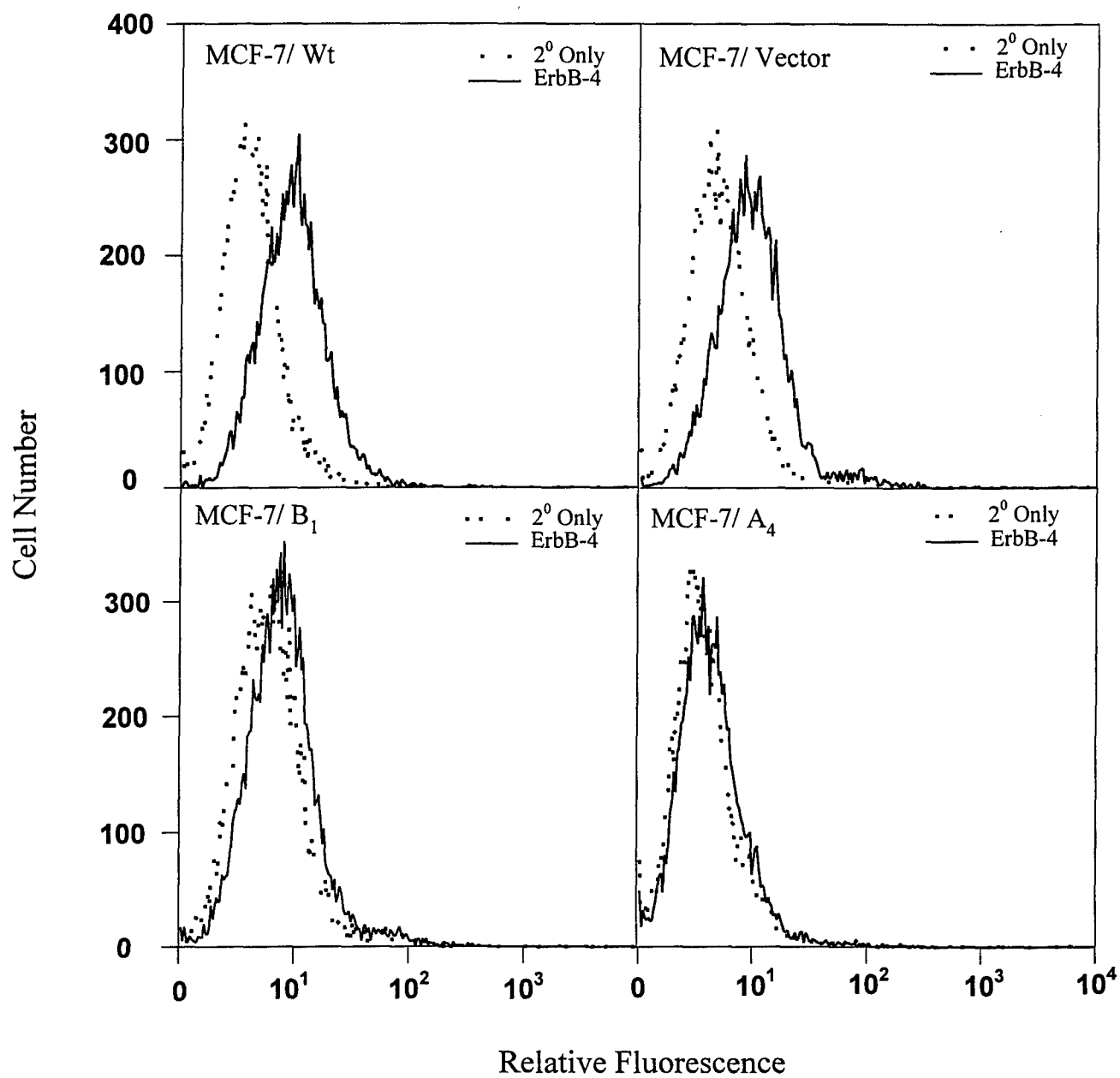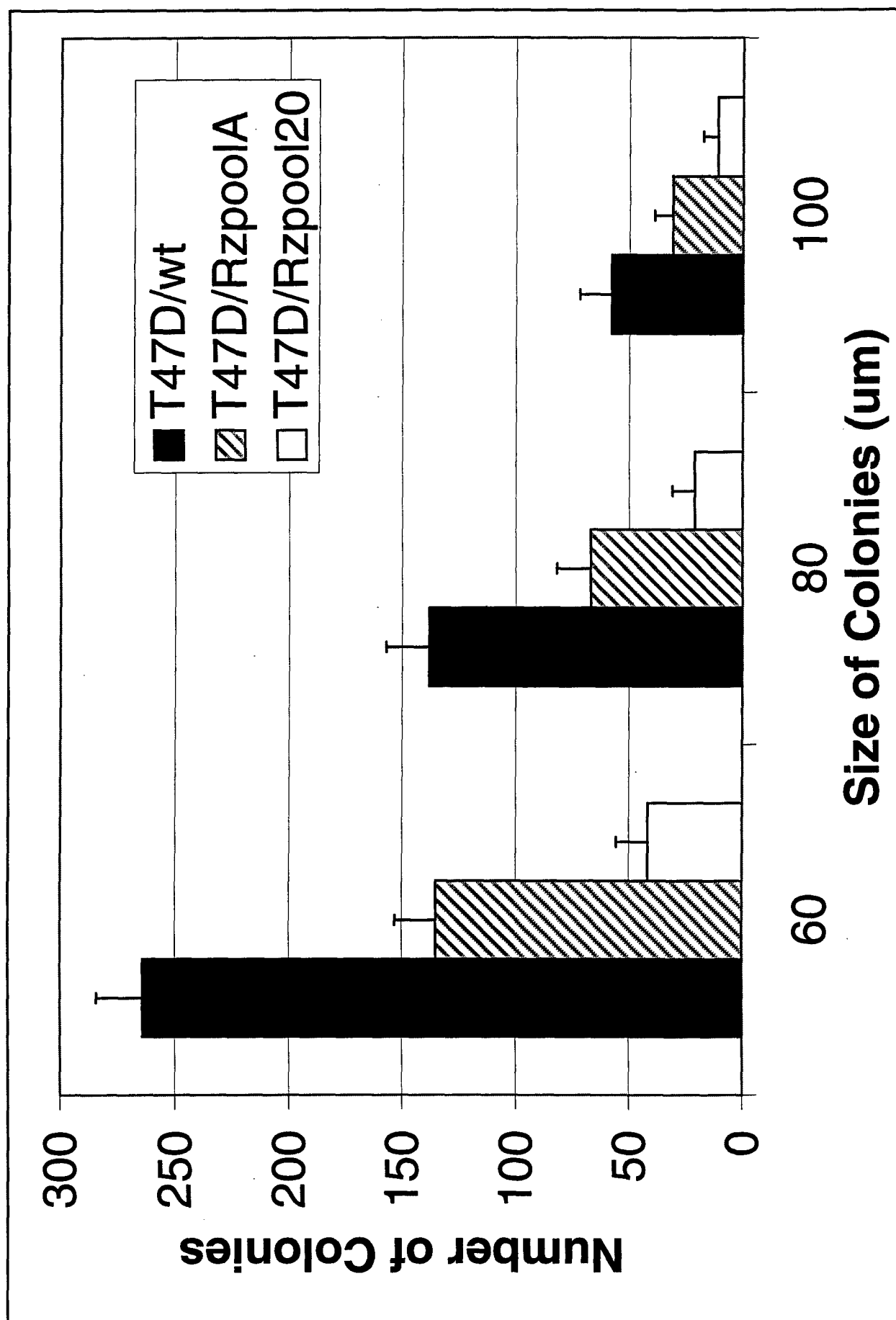Figure 1

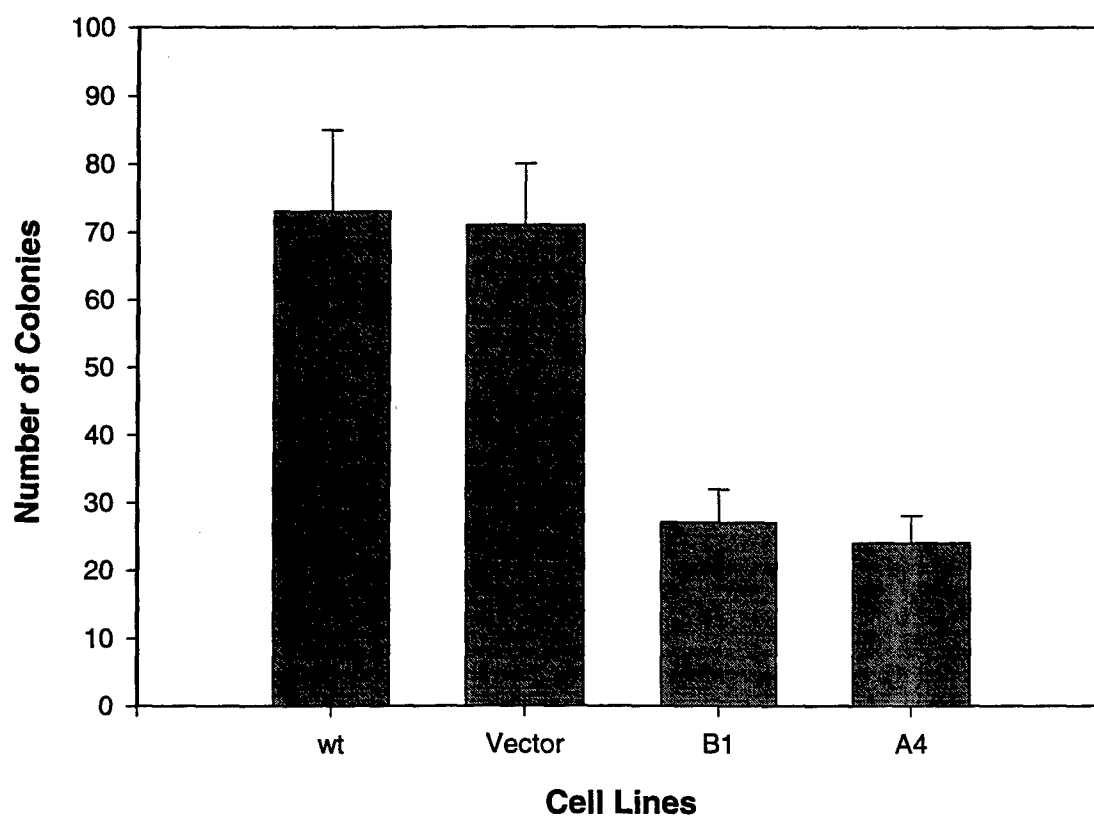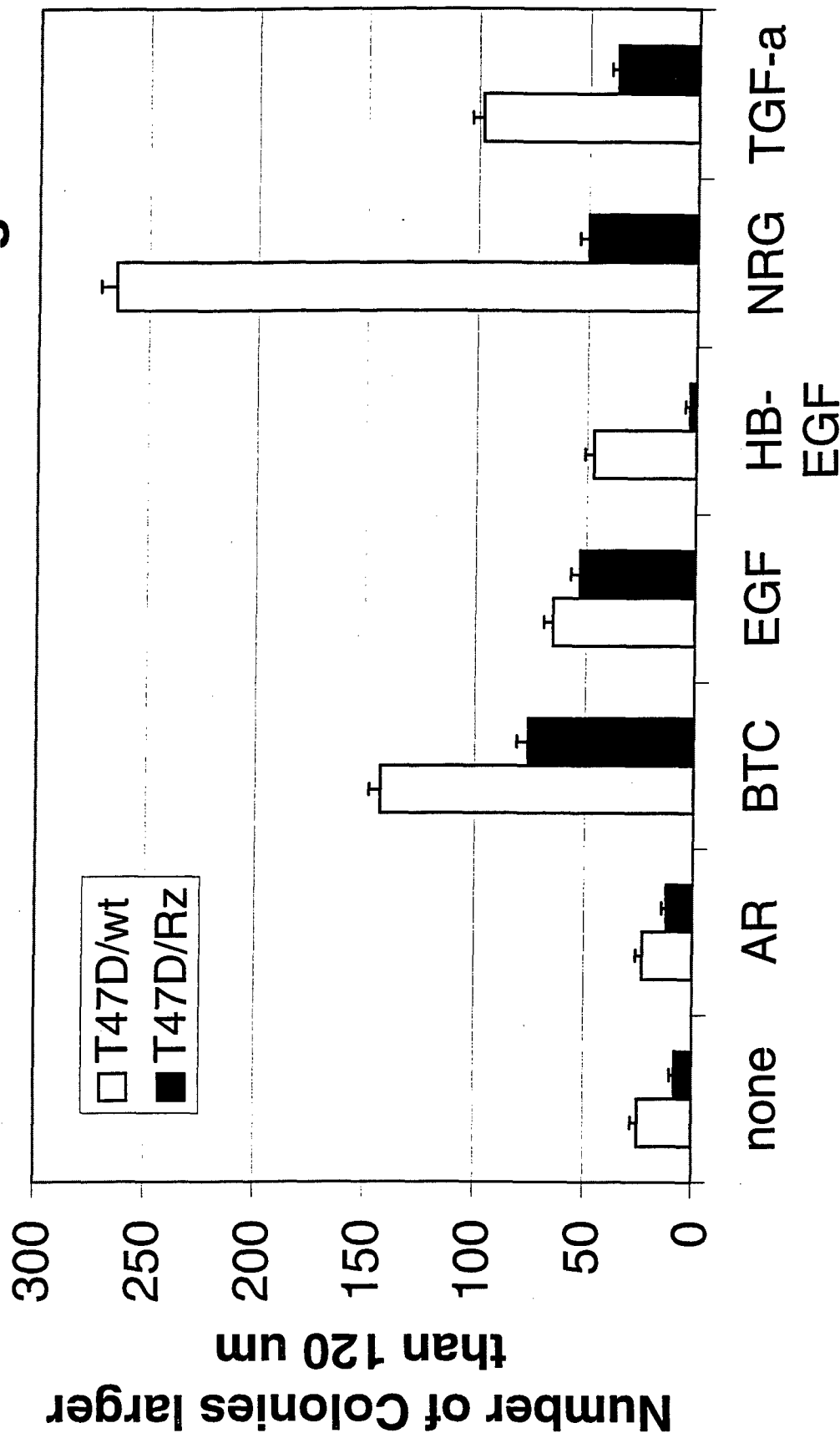Figure 2

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Figure 3

Growth effect on MCF-7/Rz transfectants

Figure 4

The Growth Effect of EGF-like Ligands

Legend:
☐ T47D/wt
■ T47D/Rz

Number of Colonies larger than 120 um

Treatments (100 ng/ml)

none   AR   BTC   EGF   HB-EGF   NRG   TGF-a

Figure 5

Growth of T47Dwt and
Two ErbB-4 Ribozyme Transfectants
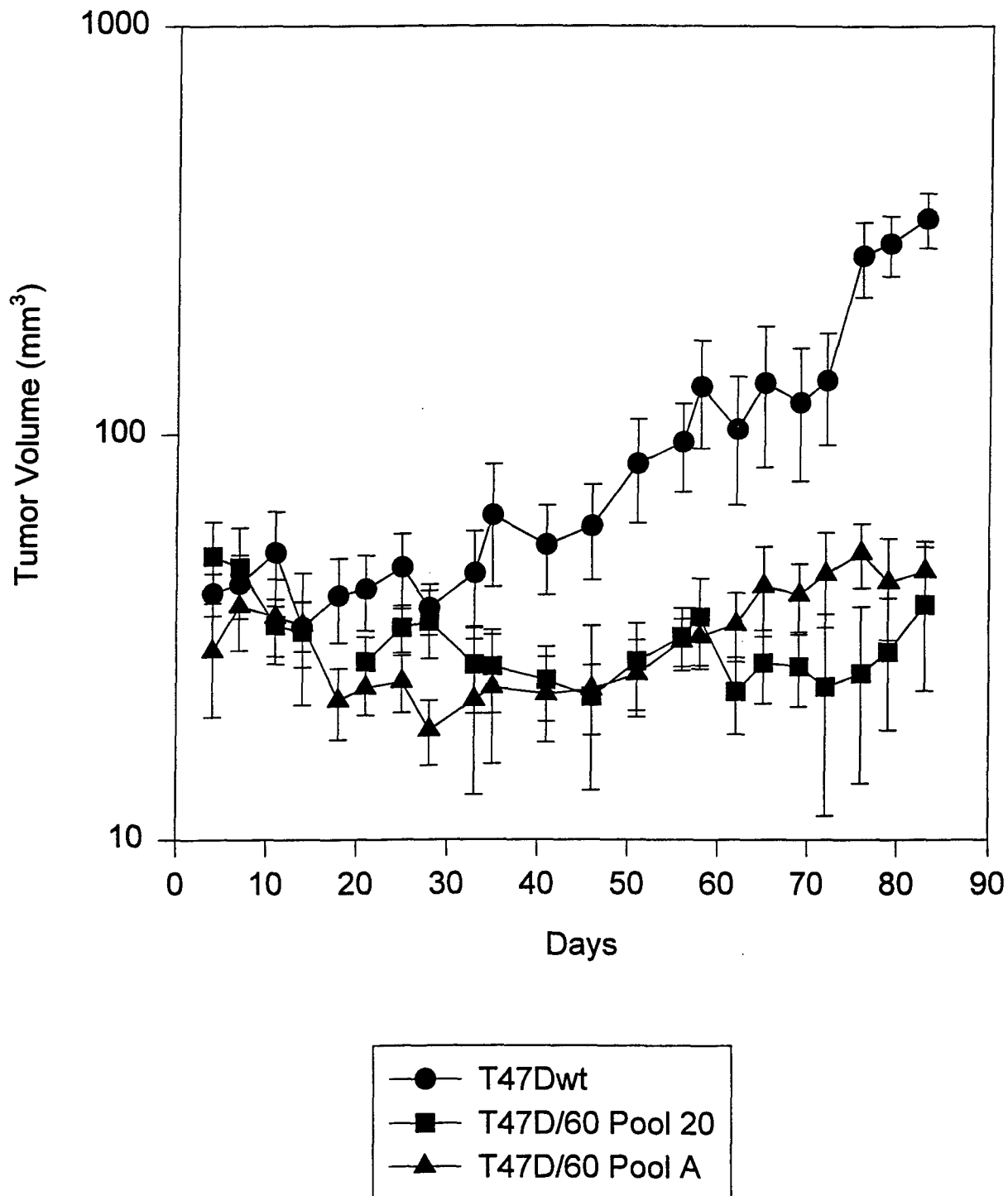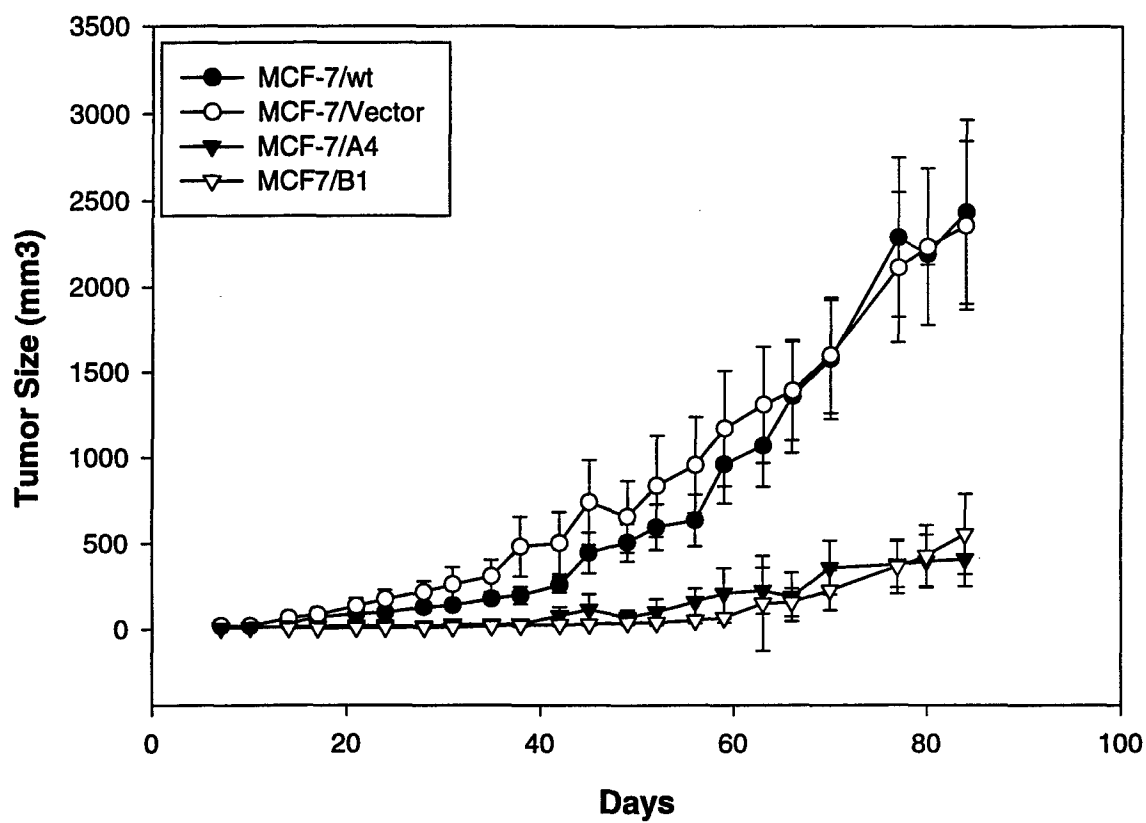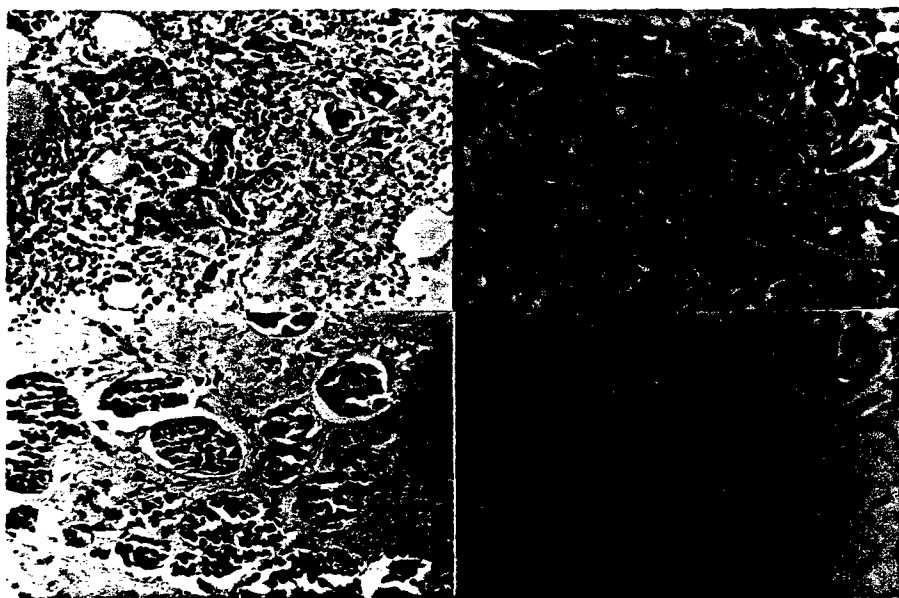## in Athymic Nude Mice

Figure 6

Figure 7

Figure 8

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BIOGRAPHICAL SKETCH

Give the following information for the key personnel and consultants and collaborators. Begin with the principal
investigator/program director. Photocopy this page for each person.

NAME	POSITION TITLE
Careen K. Tang	Assistant Professor

EDUCATION *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
University of Maryland, College Park, MD	B.S	1983	Chemistry
The Johns Hopkins University, Baltimore, MD	Ph.D.	1989	Molecular Biology/Chemistry
NIH/NICHD/CBMB, Bethesda, MD.	**IRTA Fellow,** (Dr. Richard Klausner	1989-1992	Molecular Biology/Cell Biology

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

RESEARCH EXPERIENCE:

8/95 - date　　**Research Assistant professor** Department of Biochemistry/ **Principal Investigator** of Lombardi Cancer Center. (Director: Dr. Marc Lippman) Faculty member of the Tumor Biology Graduate Program.
Georgetown University Medical Center/Lombardi Cancer Center
Washington, D.C.

11/92 - 8/95　　**Research Associate**
Georgetown University Medical Center/Lombardi Cancer Center
Washington, D.C.

HONORS:

1999-2002 **Idea Grant** awarded by DoD Breast Cancer Research Program.

1999-2000　　**Charles and Ella O.Latham Charitable Trust Award.**

1998-1999　　**Susan Komen Breast Cancer Foundation** Grant for basic clinical and translational research Program.

1996-2000　　**"Gold Standard Proposal"** awarded by DoD Breast Cancer Research Program.

1995　　**Outstanding Poster** for 1995 AACR annual meeting

1994　　**Travel Award** by Bristol-Myers Oncology Division for 1994 AACR presenter of outstanding abstract.

1993　　**First Place** for postdoctoral division of the eighth annual research days competition, Georgetown University Medical Center.

1989-1992　　**IRTA Fellowship,** NIH/NICHD/CBMB

1988　　**Sarah and Adolph Roseman Achievement Award** for excellence in teaching and research, The Johns Hopkins University.

1983　　**Academic Honors List,** University of Maryland, College Park, MD.

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REPLY TO
ATTENTION OF:

MCMR-RMI-S   (70-1y)   23 Aug 01

MEMORANDUM FOR Administrator, Defense Technical Information
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Encl   PHYLIS M. RINEHART
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