Elevated expression of activated forms of Neu/ErbB-2 and ErbB-3 are involved in the induction of mammary tumors in transgenic mice: implications for human breast cancer

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To assess the importance of Neu activation during mammary tumorigenesis, altered receptors harboring in-frame deletions within the extracellular domain were expressed in transgenic mice. Females from several independent lines develop multiple mammary tumors that frequently metastasize to the lung. Tumor progression in these strains was associated with elevated levels of tyrosine-phosphorylated Neu and ErbB-3. Consistent with these observations, a survey of primary human breast tumors revealed frequent co-expression of both *erbB-2* and *erbB-3* transcripts. The ability of altered Neu receptors to induce mammary tumorigenesis in transgenic mice prompted us to examine whether similar mutations occurred in ErbB-2 during human breast cancer progression. Interestingly, an alternatively spliced form of *erbB-2*, closely resembling spontaneous activated forms of *neu*, was detected in human breast tumors. The ErbB-2 receptor encoded by this novel transcript harbors an in-frame deletion of 16 amino acids in the extracellular domain and can transform Rat-1 fibroblasts. Together, these observations argue that co-expression of ErbB-2 and ErbB-3 may play a critical role in the induction of human breast tumors, and raise the possibility that activating mutations in the ErbB-2 receptor may also contribute to this process.

Keywords: activating mutations/ErbB receptors/
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Introduction

The type I subclass of receptor tyrosine kinases has been associated with several types of human cancers, particularly those involving the breast (reviewed in Rajkumar and Gullick, 1994). Currently, this family of proteins consists of the epidermal growth factor receptor (EGFR/ErbB-1/HER), Neu (ErbB-2/HER2), ErbB-3 (HER3) and ErbB-4 (HER4) (Ullrich *et al.*, 1984; Coussens *et al.*, 1985; Bargmann *et al.*, 1986a; Yamamoto *et al.*, 1986; Kraus *et al.*, 1989; Plowman *et al.*, 1990, 1993). For the sake of clarity, the term *neu* is used when referring to the rat gene or cDNA, whereas the human and mouse homologs are designated *erbB-2*. The *neu* oncogene was first identified in transfection experiments using genomic

DNA isolated from chemically induced rat neuroblastomas (Shih *et al.*, 1981; Padhy *et al.*, 1982). Oncogenic activation of Neu occurs as the result of a single point mutation in the transmembrane domain, converting a valine residue to glutamic acid (Bargmann *et al.*, 1986a,b). The presence of this transmembrane mutation causes an increase in the tyrosine kinase activity of Neu (Bargmann and Weinberg, 1988; Stern *et al.*, 1988; Weiner *et al.*, 1989a) by inducing ligand-independent receptor oligomerization (Weiner *et al.*, 1989b).

Numerous studies estimate that *erbB-2* is amplified and overexpressed in 20–30% of primary breast cancers, which correlates with poor patient prognosis (Slamon *et al.*, 1987, 1989; Gullick *et al.*, 1991; Paterson *et al.*, 1991; Press *et al.*, 1993; Andrulis *et al.*, 1998). Further support for the involvement of *neu/erbB-2* in the initiation and progression of breast cancer comes from the generation and analysis of transgenic mice. In several of these transgenic strains, mammary gland-specific expression of an oncogenic form of *neu* (Bargmann *et al.*, 1986b) results in the rapid induction of multifocal mammary tumors (Muller *et al.*, 1988; Bouchard *et al.*, 1989; Lucchini *et al.*, 1992; Guy *et al.*, 1996). While these transgenic studies demonstrate that expression of the *neu* oncogene induces mammary tumorigenesis in mice, the same activating mutation has not yet been identified in human breast tumors (Slamon *et al.*, 1989; Lemoine *et al.*, 1990; Zoll *et al.*, 1992). Therefore, overexpression of the human *erbB-2* proto-oncogene appears to be the primary mechanism by which *erbB-2* induces breast cancers.

To test directly whether overexpression of wild-type *neu* is sufficient to induce mammary tumors, transgenic mice were engineered to overexpress the *neu* proto-oncogene under the control of the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) (Guy *et al.*, 1992a). In contrast to the rapid onset of multifocal mammary tumors observed in the majority of activated *neu* transgenic mice, these wild-type *neu*-expressing strains develop focal mammary tumors that arise next to hyperplastic mammary tissue after a long latency period. Although comparable levels of Neu protein were detected in both normal and tumor tissues from many of these animals, catalytically active Neu was detected solely in tumor tissue. Further characterization of MMTV/wild-type *neu* mice revealed that at least 65% of the mammary tumors that arose in these strains carried somatic mutations in the transgene (Siegel *et al.*, 1994). These alterations, which are confined to a small region in the extracellular domain of Neu, promote the transforming activity of this protein via the formation of intermolecular disulfide bonds (Siegel *et al.*, 1994; Siegel and Muller, 1996). Given that the majority of tumors possess such activating mutations, they are likely to represent a critical rate-limiting step during tumor formation in this model system.

To assess directly whether these activating mutations are responsible for inducing the mammary tumors observed in MMTV/wild-type *neu* animals, transgenic mice have been generated expressing two different *neu* deletion mutants (NDL) under the control of the MMTV-LTR. Two independent lines for each transgenic strain (NDL1 and NDL2) developed mammary tumors faster than observed in MMTV/wild-type *neu* mice. Biochemical characterization of these mammary tumors revealed the presence of Neu receptor dimers that were constitutively tyrosine phosphorylated. Tumor progression in these strains was also associated with a 10- to 15-fold increase in tyrosine-phosphorylated ErbB-3 protein. However, because similar levels of *erbB-3* transcript could be detected in both tumor and adjacent mammary epithelium, the elevated levels of ErbB-3 protein probably result from enhanced translation or prolonged receptor stability. Consistent with these transgenic results, a survey of human breast tumor RNA samples revealed frequent co-expression of both *erbB-2* and *erbB-3* transcripts. Given that expression of altered Neu receptors could induce mammary gland transformation in transgenic mice, we sought to identify comparable mutations in ErbB-2. Interestingly, we have detected an alternatively spliced form of *erbB-2* in human breast tumor samples which contains an in-frame deletion located in the same region previously identified in *neu* (Siegel *et al.*, 1994) and encodes a receptor capable of transforming cells *in vitro*. Taken together, these observations raise the possibility that activated forms of ErbB-2 may collaborate with ErbB-3 to promote the development of breast cancer in humans.

Results

Generation and characterization of MMTV/*neu* deletion mice

To examine the ability of altered Neu receptors to transform mammary epithelium *in vivo*, two *neu* cDNAs possessing activating mutations were placed under the transcriptional control of the MMTV-LTR (Figure 1). The NDL1 and NDL2 transgenes contain in-frame deletions previously designated as *neu*8142 and *neu*8342, respectively (Siegel *et al.*, 1994). A total of four founder animals carrying the MMTV/NDL1 transgene, and six founder animals harboring the MMTV/NDL2 transgene were generated by pro-nuclear injection of fertilized one-cell mouse zygotes. To determine the tissue specificity of transgene expression, total RNA derived from various tissues was hybridized to an antisense riboprobe complementary to the SV40 polyadenylation signal of the transgene (SPA; Figure 1) and subjected to RNase protection analyses. To ensure equivalent amounts of RNA in each hybridization reaction, an internal control riboprobe specific for the phosphoglycerate kinase-1 gene (*PGK-1*) was also included. Quantitative phosphorimager analyses revealed that the major sites of transgene expression in female carriers of both strains included the mammary and salivary glands, while lower levels were also observed in the lungs and ovaries (Table I). In addition, high levels of lung-specific expression of the transgene were evident in those animals that developed metastatic lesions. In male carriers, transgene expression was detected in salivary gland and reproductive organs, such as the epididymis, seminal vesicles and testes.

Variable transgene expression was also observed in the mammary gland and lungs. To confirm that the transcripts originating from each transgene possessed the expected deletions, selected samples were subjected to RNase protection analyses with a riboprobe directed to the region of *neu* harboring these mutations (Siegel *et al.*, 1994). These analyses confirmed that the transgene transcripts possessed the expected deletions in the region encoding the extracellular domain of Neu (unpublished data). Based on these expression analyses, further detailed characterization of MMTV/*neu* deletion mice was restricted to two independent strains for each of the activated *neu* alleles (NDL1-2, NDL1-4, and NDL2-1, NDL2-5, respectively).

Consistent with previous studies examining mammary epithelial expression of *neu* in transgenic mice (Muller *et al.*, 1988; Bouchard *et al.*, 1989; Lucchini *et al.*, 1992; Guy *et al.*, 1992a, 1996), females from all four MMTV/NDL lines developed mammary adenocarcinomas (Table II). The penetrance of this phenotype was incomplete, with ~80% of transgene carriers developing mammary tumors prior to 1 year of age. Animals failing to develop tumors within this time frame were considered tumor free. Given that pregnancy and lactation are known to increase expression from the MMTV-LTR (Stewart *et al.*, 1984; Kitsberg and Leder, 1996), both virgin and multiparous females were monitored for mammary tumor formation. Although not required, multiple pregnancies did accelerate tumor onset in each of the NDL lines examined when compared with MMTV/wild-type *neu* mice (N202) (Table II).

In addition to the accelerated rates of tumor development relative to those strains expressing the *neu* proto-oncogene, expression of these activated *neu* alleles resulted in a significant increase in the number of mammary tumors present within each fat pad (Figure 2). Histologically, these tumors are similar to those described for both activated (Muller *et al.*, 1988; Cardiff *et al.*, 1991; Guy *et al.*, 1996) and wild-type *neu*-expressing mice (Guy *et al.*, 1992a), appearing as nodular masses amongst hyperplastic and dysplastic tissue (Figure 3). Transplantation of tumor explants, derived from NDL1-2 and NDL1-4 females, into sub-cutaneous sites of syngeneic mice confirmed the neoplastic nature of these mammary tumors (unpublished data).

Another important phenotype displayed by these activated *neu* strains is the frequent occurrence of metastatic lesions. Inspection of the lungs from MMTV/NDL females revealed the presence of lung metastases in all four lines (Table II). Although the same phenotype was observed in mice expressing a wild-type *neu* transgene (Guy *et al.*, 1992a), a direct comparison cannot readily be made with the transgenic lines derived in this study. Metastases were scored in MMTV/NDL mice after 30–60 days following first tumor palpation (Table II), whereas metastasis was assessed in wild-type *neu*-expressing animals that were tumor-bearing for at least 90 days (Guy *et al.*, 1992a). RNase protection analysis, using β -casein as a marker for mammary epithelium, confirmed that those tumor cells discovered in the lungs originated from the mammary gland (unpublished data). Taken together, these observations suggest that mammary epithelial-specific expression of these activated forms of *neu* results in the efficient induction of metastatic mammary adenocarcinomas.

Fig. 1. Structure of *neu* deletion (NDL) transgenes. The unshaded region indicates vector sequences (pBluescript KS), the striped region represents the MMTV-LTR, filled regions correspond to the *neu* cDNA which harbors the deletion, and the gray portion indicates polyadenylation signals derived from the SV40 early transcription unit. The amino acid sequence of each deletion mutation encoded by the two transgenes is compared with the wild-type sequence and is outlined in single-letter code above the transgene schematic. The phenylalanine (F) residue at the end of the indicated sequence is directly adjacent to the start of the transmembrane domain (TM), which is denoted by the black box. The restriction sites used to isolate the injection fragment are indicated above the diagram, and the SpA (SV40 polyadenylation) riboprobe used for the RNase protection analyses is also shown.

Table I. Transgene expression in MMTV/*neu* deletion mice^a

Line No.	Ear tag No.	Sex	Expression											
			Brain	Epid.	Heart	Kidney	Liver	Lung	A.M.gl.	Ovary	Sal.gl.	SV	Spleen	Testes
NDL1-1	6542 (F)	F	—	—	—	—	—	—	—	—	—	—	—	—
NDL1-2	7985	F	—	—	—	—	—	+	+++	+	++	—	—	—
	7991	M	—	+++	—	—	—	+	++	—	+++	+++	—	+
NDL1-3	7270 (F)	F	—	—	—	—	—	+++	+++	—	—	—	—	—
NDL1-4	8203	F	—	—	—	—	—	+	++	—	+++	—	—	—
	9768	M	—	ND	—	—	—	+	++	—	++	+	—	++
NDL2-1	8398	F	—	—	+	—	—	+++	+++	+	+	—	—	—
	1189	M	—	+	—	—	—	+	—	—	+	—	—	+
NDL2-2	7216 (F)	M	—	—	—	—	—	—	—	—	—	—	—	—
NDL2-3	7626 (F)	M	—	—	—	—	—	—	—	—	—	—	—	—
NDL2-4	2902 (F)	F	—	—	—	—	—	—	—	—	—	—	—	—
NDL2-5	4465	F	—	—	—	—	—	+	+++	—	++	—	—	—
	2916 (F)	M	—	++	—	—	—	+	—	—	++	+++	—	+
NDL2-6	2918 (F)	M	—	—	—	—	—	—	—	—	—	—	—	—

^aRNase protection analysis was performed on 20 µg of total RNA isolated from a variety of organs in the MMTV/*neu* deletion strains as described in Materials and methods. Relative levels of transgene expression are indicated by: — (not detected), + (low), ++ (intermediate) and high (+++). Epid., epididymis; A.M.gl., adjacent mammary gland; Sal.gl., salivary gland; SV, seminal vesicle; (F), founder animal; ND, not determined.

To confirm that these altered transcripts encoded activated Neu receptors, immunoprecipitates from tumor lysates were subjected to parallel immunoblots using Neu- and phosphotyrosine-specific antibodies. The results showed that mammary tumors derived from virgin females expressed Neu protein that was constitutively tyrosine phosphorylated (Figure 4A and B). We have demonstrated previously that activation of these altered forms of Neu occurs through the formation of disulfide bond-stabilized dimers (Siegel and Muller, 1996). To examine whether the presence of tyrosine-phosphorylated Neu detected in these tumors was due to the same mechanism, immunoprecipitates were separated under non-reducing and reducing electrophoresis conditions and subjected to immunoblot analysis with Neu-specific antibodies. These results demonstrate that tyrosine phosphorylation of altered Neu receptors expressed in MMTV/NDL tumors reflects their ability to undergo intermolecular dimerization (Figure 4C and D).

ErbB-3 is expressed at elevated levels during tumor progression in the activated neu strains

While our observations suggest that Neu homodimers are involved in the induction of these mammary tumors, it is conceivable that heterodimerization between the activated forms of Neu and other EGFR family members may also be involved in tumor progression. Indeed, it has been demonstrated previously that co-expression of Neu/ErbB-2 with other EGFR family members can act synergistically to transform established fibroblast lines *in vitro* (Kokai *et al.*, 1989; Alimandi *et al.*, 1995; Wallasch *et al.*, 1995; Cohen *et al.*, 1996; Zhang *et al.*, 1996). To explore this possibility, mammary tumor extracts from both *neu* deletion strains were subjected to immunoblot analyses with EGFR-, ErbB-3- and ErbB-4-specific antisera. In contrast to low and variable levels of EGFR and ErbB-4, elevated levels of endogenous ErbB-3 were detected consistently in every tumor lysate examined ($n = 14$) (Figure 5). The observed expression patterns did not

Fig. 2. Whole-mount preparations of hematoxylin-stained mammary glands comparing the morphology of non-transgenic FVB/N (A) with transgenic wild-type *neu* (B), NDL1-2 (C) and NDL2-5 (D) females at 6 months of age. Note that both NDL1-2 and NDL2-5 mammary glands (C and D) exhibit multifocal, solid proliferating tumors, whereas the mammary gland derived from the wild-type *neu* female displays only diffuse cystic hyperplasia, lacking focal proliferative lesions (B).

Table II. Onset of tumor formation in MMTV/*neu* deletion mice^a

Line	Sex	Virgin animals		Multiparous animals		Phenotypes (% penetrance ^d)
		Average onset of mammary tumor formation, days ^b	% of animals with lung metastases ^c	Average onset of mammary tumor formation, days ^b	% of animals with lung metastases ^c	
N202	F	228 ± 47 (<i>n</i> = 21)	ND	192 ± 40 (<i>n</i> = 41)*	ND	M.gl. adenocarcinoma (81%)
NDL1-2	F	143 ± 37 (<i>n</i> = 35)	67 (6/9)	135 ± 37 (<i>n</i> = 7)	57 (4/7)	M.gl. adenocarcinoma (82%) Harderian gland hyperplasia
NDL1-4	F	182 ± 50 (<i>n</i> = 22)	67 (6/9)	151 ± 21 (<i>n</i> = 14)	29 (2/7)	M. gl. adenocarcinoma (79%) Harderian gland hyperplasia
NDL2-1	F	236 ± 26 (<i>n</i> = 24)	33 (2/6)	172 ± 31 (<i>n</i> = 21)	38 (3/8)	M. gl. adenocarcinoma (80%)
NDL2-5	F	161 ± 10 (<i>n</i> = 8)	67 (4/6)	122 ± 26 (<i>n</i> = 10)	60 (3/5)	M. gl. adenocarcinoma (81%)

^aTransgene carriers were monitored regularly for the appearance of tumors by physical palpation of mammary glands. ND, not determined; *n*, number of animals analyzed; M. gl., mammary gland. *Data obtained from animals monitored in a previous study (Guy *et al.*, 1992a).

^bValues represent the average time (days) that mammary tumors were first palpated ± SD.

^cPercentage of animals possessing lung metastases that were tumor bearing for 30–60 days.

^dPercentage of transgene carriers that develop the indicated phenotype. Values listed are representative of both virgin and multiparous animals.

reflect variations of total protein between samples since immunoblot analyses revealed comparable levels of Grb-2 in each lysate (Figure 5).

To ensure that elevated ErbB-3 levels in mammary tumors were not due to increased epithelial content, we also measured the levels of the epithelial marker cytokeratin-8 in these samples. To accomplish this, quantita-

ative ¹²⁵I immunoblots were performed on both tumor and adjacent tissue from NDL1-2 and NDL2-1 animals using either ErbB-3- or cytokeratin-8-specific antisera (Figure 6A and B). Quantitative phosphorimager analysis of the ErbB-3/cytokeratin-8 ratio revealed a 10- to 15-fold increase of ErbB-3 protein in the tumor compared with adjacent mammary tissue in these activated Neu

Fig. 3. The characteristic subgross (A–D) and histological (E–H) patterns found at the terminal ends of 6-month-old nulliparous wild-type FVB/N (A and E) mammary glands as compared with animals carrying the cNeu (B and F) and NDL (C, D, G and H) transgenes. Note that the terminal ends of all three transgenic mice contain more fluid and cells than the FVB/N control. The cNeu termini are generally hollow cysts, filled with fluid (B and F), while the NDL termini have more cysts with cells (C and G) or entirely filled with cells displaying nuclear pleomorphism (D and H) and a high rate of DNA synthesis. Whole-mount (A–D) and histological (E–H) images were taken at $\times 10$ and $\times 20$ magnification, respectively.

strains (Figure 6D). A similar analysis of an MMTV/wild-type *neu* transgenic line (N202) revealed an ~ 10 -fold increase of endogenous ErbB-3 in tumor versus adjacent tissue (Figure 6C and D). Because ErbB-3 lacks intrinsic tyrosine kinase activity (Guy *et al.*, 1994), activation of this receptor is dependent on transactivation by other members of the EGFR family.

To test whether ErbB-3 is transphosphorylated in these Neu-induced mammary tumors, ErbB-3 immunoprecipitates were subjected to immunoblot analysis using anti-phosphotyrosine-specific antibodies. The results showed that ErbB-3 is constitutively tyrosine phosphorylated in mammary tumors arising in MMTV/*neu* deletion mice (Figure 7). The tyrosine-phosphorylated ErbB-3 observed in these tumors does not result from stable complex formation between Neu and ErbB-3 since repeated attempts to detect an association between the two receptors by reciprocal immunoprecipitation and immunoblot ana-

Fig. 4. Altered Neu receptors encoded by both MMTV/*neu* deletion transgenes (NDL1 and NDL2) are constitutively tyrosine phosphorylated and form dimers *in vivo*. (A) Neu was immunoprecipitated (IP) from 1 mg of mammary tumor lysates (BT) derived from each of four different transgenic lines expressing Neu deletion mutants (lanes 2–9). Tumor tissue was collected from two separate transgenic strains (NDL1 and NDL2) and two independent lines were examined from each strain (NDL1-2, NDL1-4, and NDL2-1, NDL2-5). One-third of the immunoprecipitate was electrophoresed on an SDS-9.0% polyacrylamide gel, transferred to a PVDF membrane and subjected to immunoblot analysis (BLOT) with a Neu-specific antibody. The position of Neu is indicated by the arrow. (B) The remaining two-thirds of the immunoprecipitate was subjected to immunoblot analysis for anti-phosphotyrosine (P-Tyr) as described in (A). The position of tyrosine-phosphorylated Neu is indicated by the arrow. (C and D) Neu was immunoprecipitated, in duplicate, from 600 μ g of mammary tumor lysates using one representative sample for each line (lanes 2–5). A non-transgenic mammary tissue lysate (FVB Mam. Gl.; lane 1) was included for control for endogenous Neu expression in this organ. The immunoprecipitates were then electrophoresed through 4–12% gradient SDS-polyacrylamide gels under non-reducing (C) and reducing (D) conditions. Neu dimers are indicated by the solid arrow, while the position of the monomers is marked by the open arrows (D and M, respectively).

Fig. 5. The expression of endogenous ErbB-3 receptors is significantly elevated in tumors derived from both NDL1 and NDL2 transgenic animals. (A) Equivalent amounts of total protein (50 μ g) from mammary tumor lysates (BT) were electrophoresed on a 4–12% gradient SDS–polyacrylamide gel and transferred to a PVDF membrane (lanes 2–9). The membrane was cut, and the upper half of the blot was probed with EGFR-specific mouse monoclonal antibodies, while the lower portion of the membrane was blotted with Grb-2-specific rabbit polyclonal antisera. Similarly, immunoblots were performed for both ErbB-3 (B) and ErbB-4 (C) using the same tumor lysates. As in (A), Grb-2 immunoblots were used to confirm that equivalent amounts of protein were present in each lane. Lysates from non-transgenic mammary tissue (FVB Mam. Gl.) were included in each panel to control for endogenous levels of each ErbB receptor expressed in the normal mammary gland (lane 1). The positions of EGFR, ErbB-3, ErbB-4 and Grb-2 are indicated by arrows.

lysis have been unsuccessful (unpublished observations). However, such negative data do not disprove a transient physical association between these two receptors. Taken together, these observations argue that co-expression of activated Neu and wild-type ErbB-3 may play an important role in tumor progression in these strains.

One potential explanation for the elevated levels of

ErbB-3 protein in these tumors is that signaling by activated Neu receptors increases transcription of the *erbB-3* gene. To address this possibility, we subjected RNA samples from both adjacent and tumor tissues to RNase protection analysis using a riboprobe specific for *erbB-3*. To control for variation in epithelial content, the same RNA samples were hybridized with a riboprobe specific to cytokeratin-8 (Figure 8A). A quantitative comparison of the levels of *erbB-3* RNA from tumor tissue with that derived from adjacent mammary epithelium showed a <3-fold difference between these tissues (Figure 8B). This modest elevation in *erbB-3* message cannot alone account for the 10- to 15-fold increase in the levels of ErbB-3 protein. These data argue that the elevated ErbB-3 levels observed during tumor progression in these strains result from either increased protein translation or prolonged stability of the receptor.

The accumulation of tyrosine-phosphorylated ErbB-3 in the mammary tumors of several *neu*-expressing transgenic strains suggests a functional role for this receptor in Neu-mediated transformation. Indeed, wild-type ErbB-2 and ErbB-3 previously have been shown to cooperate in transformation of fibroblasts (Alimandi *et al.*, 1995; Wallasch *et al.*, 1995; Zhang *et al.*, 1996). To determine whether ErbB-3 and one of the altered Neu receptors used in this study could synergize to transform cells in culture, expression plasmids encoding ErbB-3 and Neu8142 (NDL1) were co-transfected into Rat-1 fibroblasts and the number of foci scored. The results showed that co-expression of Neu8142 with ErbB-3 consistently resulted in a 50% increase in the number of foci when compared with Neu8142 alone (Table III). These observations argue that activated *neu* alleles can cooperate with *erbB-3* to promote oncogenic transformation.

An alternatively spliced erbB-2 isoform, closely resembling transforming neu alleles, is co-expressed with erbB-3 in primary human breast tumors

The transforming potential demonstrated by Neu and ErbB-3 in cell culture systems suggests that this receptor combination may also be involved in the genesis of human breast cancer. Indeed, several studies have examined ErbB-3 expression in human breast tumor samples (Lemoine *et al.*, 1992; Bacus *et al.*, 1994; Gasparini *et al.*, 1994; Quinn *et al.*, 1994; Bodey *et al.*, 1997), and a subset of these noted co-expression of ErbB-2 and ErbB-3 in the same tumors (Quinn *et al.*, 1994; Bodey *et al.*, 1997). To extend further our observations with these transgenic strains to human breast cancers, a limited number of RNA samples from several *erbB-2*-expressing breast tumors were subjected to RNase protection analysis with an *erbB-3*-specific riboprobe. The results of this analysis revealed that the majority of *erbB-2*-expressing tumors also possessed *erbB-3* message (Figure 9).

These observations suggest that mammary gland transformation in MMTV/*neu* deletion mice involves similar events that occur during human breast tumor progression. Given the frequent occurrence of activated *neu* alleles within mammary tumors of wild-type *neu*-expressing animals, and their ability to induce tumorigenesis when expressed in the mammary gland of transgenic mice, we sought to identify similar activating events in *erbB-2*.

Fig. 6. The levels of ErbB-3 protein are increased in tumor tissue versus adjacent mammary epithelium. Total protein (50 μ g) was isolated from mammary tumor tissue (BT) and adjacent mammary epithelium (Adj. M. Gl.) of three transgenic mouse strains; NDL1-2 (A), NDL2-1 (B) and N202 (C). Protein lysates were separated through SDS-9.0% polyacrylamide gels, transferred to PVDF membranes and probed with antibodies specific to ErbB-3 (B-3) or cytokeratin-8 (CK-8). 125 I-conjugated secondary antibodies were used for quantitative immunoblot analysis. (D) The fold increase of ErbB-3 protein in mammary tumors versus adjacent epithelium is indicated for each strain. Phosphorimager values for ErbB-3 were first divided by those obtained for cytokeratin-8 in order to account for the epithelial content within each sample. These ratios were then used to determine the fold increase of ErbB-3 protein in mammary tumors versus adjacent epithelium. The numbers of samples analyzed are indicated (n).

Fig. 7. Endogenously expressed ErbB-3 is tyrosine phosphorylated. (A) ErbB-3 was immunoprecipitated (IP) from 4 mg of mammary tumor lysates (BT) derived from NDL transgenic animals (NDL1 and NDL2) (lanes 2-9). A lysate from non-transgenic mammary tissue (FVB Mam. Gl.) was also included as a negative control (lane 1). One-half of each immunoprecipitate was electrophoresed on an SDS-9.0% polyacrylamide gel, transferred to a PVDF membrane and subjected to immunoblot (BLOT) analysis with an ErbB-3-specific antibody. The position of ErbB-3 is indicated by the arrow. (B) The remaining half of the immunoprecipitate was subjected to immunoblot analyses with anti-phosphotyrosine (P-Tyr)-specific antibodies, as described in (A). The position of tyrosine-phosphorylated ErbB-3 is indicated by the arrow.

Recent studies have revealed that alternative splicing may provide a unique mechanism for the generation of novel transcripts encoding activated growth factor receptors. Indeed, several reports document the existence of splice variants which encode Met, Ron and fibroblast growth factor receptor 2 (FGFR2) receptor tyrosine kinases

harboring deletions within their extracellular domains (Rodrigues *et al.*, 1991; Li *et al.*, 1995; Collesi *et al.*, 1996; Meyers *et al.*, 1996). In this regard, inspection of the intron-exon structure of the human *erbB-2* gene revealed that removal of a coding exon immediately preceding the transmembrane domain could result in

Fig. 8. The levels of *erbB-3* transcript are not substantially increased in tumor versus adjacent mammary gland tissue. (A) Total RNA was isolated from mammary tumor tissue (BT) and adjacent mammary epithelium (Adj. M. Gl.) of three transgenic mouse strains (NDL1-2, NDL2-1 and N202). The 375 nucleotide protected fragment corresponding to the *erbB-3* (*B-3*) message is indicated. An antisense control probe, directed towards cytokeratin-8, was included to account for epithelial content of the RNA sample. The 142 nucleotide protected fragment is indicated by *CK-8*. (B) The fold increase of *erbB-3* transcript in mammary tumors versus adjacent epithelium is indicated for each strain. Phosphorimager values for the *B-3* protected fragment were first divided by those obtained for the *CK-8* protected fragment to account for the epithelial content within each sample. These ratios were then used to determine the fold increase of *erbB-3* message in the mammary tumor versus adjacent epithelium. The numbers of samples analyzed are indicated (n).

Table III. Transformation of Rat-1 fibroblasts by co-expression of Neu and ErbB-3 receptors

Expression plasmid ^a	Focus assay #1 Average no. of foci/plate ^b	Focus assay #2 Average no. of foci/plate ^b	Focus assay #3 Average no. of foci/plate ^b	Experimental average ^c
pJ4QB3	0	0	0	0
pJ4QN	0	0	0	0
pJ4Q8142	208 ± 12	164 ± 21	112 ± 10	161 ± 48
pJ4QN/B3	0	0	0	0
pJ4Q8142/B3	309 ± 15	256 ± 12	175 ± 12	247 ± 67

Three independent focus assays were performed by lipofectamine transfection using established Rat-1 fibroblasts.

^aAll cDNAs placed under control of the MMLV-LTR within the pJ4Q expression vector. B3, *erbB-3* cDNA; N, wild-type *neu* cDNA; 8142, altered *neu* cDNA corresponding to *neu* deletion 1 (NDL1, Figure 1). The designation N/B3 or 8142/B3 indicates co-expression of *neu* and *erbB-3* cDNAs.

^bValues listed represent the mean number of foci counted on six plates ± SD.

^cValues listed represent the mean number of foci obtained over all three experiments ± SD.

an alteration closely resembling the transgene mutations observed in both MMTV/wild-type *neu* and MMTV/NDL transgenic mice (Figure 10A and B). To determine whether this putative splice form could be detected in human breast tumors, total RNA derived from both normal human breast tissue and tumor samples was subjected to RT-PCR with oligonucleotide primers flanking the region of interest. These results revealed that, in addition to the expected wild-type *erbB-2* message, both normal and tumor tissue expressed the spliced transcript (Figure 10C). Direct sequence analyses confirmed that the upper RT-PCR product represented wild-type *erbB-2*, whereas the lower fragment contained the sequence expected from the alternative splicing event (Figure 10B). Quantitative analyses of the PCR products by phosphorimager analysis revealed that the spliced form represented ~2–5% of the wild-type *erbB-2* transcript present within each sample.

Given the resemblance of this spliced form of *erbB-2* to the oncogenic *neu* alleles that arise during mammary tumorigenesis in MMTV/wild-type *neu* mice, the transforming activity of the protein encoded by the splice variant

was compared with two previously characterized oncogenic ErbB-2 receptors. To accomplish this, expression plasmids bearing the wild-type *erbB-2* cDNA (WT), a transforming *erbB-2* allele possessing a point mutation in the region encoding the transmembrane domain (VE), a previously characterized sporadic deletion discovered in the *neu* proto-oncogene (Siegel et al., 1994) that was engineered in *erbB-2* (ECD DEL), or the spliced form of *erbB-2* (SPICE) were electroporated into Rat-1 cells and assessed for their capacity to induce foci (Figure 11A). Whereas expression of wild-type *erbB-2* failed to transform Rat-1 fibroblasts, expression of the spliced form and of the two previously characterized transforming alleles resulted in the induction of foci (Figure 11B and Table IV). The spliced form of *erbB-2* transformed at 26% the level observed with the transmembrane mutant of *erbB-2* (VE) and possessed transforming activity similar to that of the previously characterized sporadic deletion (ECD DEL) (Table IV).

To determine whether the oncogenic potential of the spliced form of *erbB-2* was related to activation of the receptor's catalytic activity, established Rat-1 cells

Fig. 9. Primary human breast tumors frequently express both *erbB-2* and *erbB-3* transcripts. (A) RNase protection analysis of total RNA (20 μ g) from primary human breast tumors hybridized to an antisense *erbB-2* riboprobe (lanes 3–11). The 473 nucleotide *erbB-2* protected fragment is indicated. (B) The same RNA samples (20 μ g) were hybridized to an antisense *erbB-3* riboprobe. The 720 nucleotide *erbB-3* protected fragment is indicated. An antisense riboprobe, directed against human β -actin, was used to monitor the RNA integrity of each sample. The β -actin probe protects a 212 nucleotide fragment as indicated. The migration pattern of each undigested riboprobe is indicated (A and B, lane 1). In addition to the tumor RNA, 20 μ g of tRNA was included in both sets of hybridizations as a negative control (lane 2). Migration of labeled DNA markers (ϕ X174, Gibco-BRL) is indicated on the left in nucleotides.

expressing the various *erbB-2* alleles were generated. To assess the tyrosine phosphorylation status of these receptors, protein extracts derived from the various cell lines were immunoprecipitated with ErbB-2-specific antibodies and subjected to immunoblot analyses with anti-phosphotyrosine antibodies. As shown in Figure 12A, tyrosine-phosphorylated ErbB-2 could be detected easily in transformed cells expressing the oncogenic alleles of *erbB-2* but was barely detectable in morphologically normal cell lines expressing elevated levels of wild-type *erbB-2*. A parallel immunoblot for ErbB-2 revealed that each established cell line expressed similar levels of the receptor (Figure 12B). To determine whether ErbB-2 receptors encoded by the novel splice variant were activated by the formation of disulfide bond-stabilized dimers, ErbB-2 immunoprecipitates were resolved under non-reducing and reducing electrophoresis conditions. ErbB-2 immunoblot analyses revealed that the oncogenic potential and constitutive phosphorylation status of these activated ErbB-2 receptors reflect their ability to undergo disulfide bond-stabilized dimerization (Figure 12C and D). These

Fig. 10. Alternative mRNA splicing results in an *erbB-2* transcript harboring an in-frame deletion. (A) Schematic representation of a small region of the human *erbB-2* genomic locus indicating the exon–intron boundaries. The values indicated above the schematic represent nucleotide numbers corresponding to the cDNA. Upper case letters designate coding sequences, whereas nucleotides within the introns are in lower case letters. The aberrant splicing event leading to the deletion of an exon (gray) is indicated by the dashed lines. (B) An alignment of the wild-type and alternatively spliced *erbB-2* messages indicating the sequences removed by the aberrant splicing event (gray box). (C) RT-PCR analysis of both normal breast and tumor tissue. The negative control represents an RT-PCR performed in the absence of RNA (distilled water). The PCR was conducted in the presence of [α - 32 P]dCTP and the products resolved on a 5% polyacrylamide gel, dried and exposed to film. The positions of the wild-type and spliced products are indicated.

observations demonstrate that the alternatively spliced *erbB-2* transcript encodes an oncogenic receptor that frequently is co-expressed with *erbB-3* in primary human breast tumors.

Discussion

Oncogenic mutations within the extracellular domain of the Neu receptor previously have been associated with mammary tumorigenesis in transgenic mice (Siegel *et al.*, 1994). In the present study, we directly demonstrate that mammary epithelial expression of these activated Neu receptors results in the rapid induction of metastatic mammary carcinomas (Table II). Although the transforming potential of these activated *neu* alleles in the mammary gland is clearly greater than that of the wild-type *neu* proto-oncogene, their expression is not sufficient to transform the entire mammary epithelium. Indeed, hyperplastic and dysplastic lesions are often detected in the

Fig. 11. Expression of altered ErbB-2 receptors results in morphological transformation. **(A)** Partial amino acid sequence alignment of the wild-type and altered ErbB-2 receptors tested in Rat-1 focus assays. ErbB-2 (WT) represents the wild-type receptor, ErbB-2 (VE) possesses a valine to glutamic acid point mutation in the transmembrane domain, ErbB-2 (ECD DEL) harbors a deletion which mimics a previously characterized mutation in the Neu receptor (Neu8142; Siegel *et al.*, 1994; Siegel and Muller, 1996), and ErbB-2 (SPLICE) contains the deletion encoded by the alternatively spliced *erbB-2* message. A portion of the transmembrane domain is indicated by the open box and designated TM. The asterisk indicates the position of the valine to glutamic acid substitution in ErbB-2 (VE). **(B)** Shown are representative plates from focus assay #1 (see Table IV) illustrating the relative transforming abilities of the various forms of ErbB-2 indicated in (A).

Table IV. Transformation of Rat-1 cells by altered ErbB-2 receptors

Expression plasmid ^a	Focus assay #1		Focus assay #2		Focus assay #3		Relative transforming ability ^d (%)
	Average no. of foci/plate ^b	% Transformation of pJ4QB2(VE) ^c	Average no. of foci/plate ^b	% Transformation of pJ4QB2(VE) ^c	Average no. of foci/plate ^b	% Transformation of pJ4QB2(VE) ^c	
pJ4QB2 (WT)	0	0	0	0	0	0	0
pJ4QB2 (VE)	284 ± 15	100	523 ± 28	100	672 ± 13	100	100 ± 0
pJ4QB2 (ECD DEL)	63 ± 6	22	130 ± 16	25	195 ± 14	29	25 ± 4
pJ4QB2 (SPLICE)	73 ± 8	26	127 ± 15	24	194 ± 14	29	26 ± 3

Three independent focus assays were performed with established Rat-1 fibroblasts. The first two experiments were conducted with plasmid DNA from the same large-scale preparation, while the third experiment was performed with DNA obtained from an independent plasmid preparation. The Rat-1 fibroblasts were maintained in a monolayer for 14 days following electroporation at which time they were stained with Geimsa.

^aAll cDNAs placed under the transcriptional control of the MMLV-LTR.

^bValues listed represent the mean number of foci counted on eight plates ± SD.

^cValues represent the ratio of the mean number of foci obtained for each construct with respect to pJ4QB2(VE).

^dValues listed represent the mean transforming abilities from all three experiments ± SD.

mammary glands of these strains before overt mammary carcinomas appear (Figure 3). These results suggest that, while necessary, the altered Neu receptors utilized in this study are not sufficient for mammary tumor formation.

One possible explanation for these observations is that in addition to Neu activation, the expression of another EGFR family member is required for efficient tumor induction. In this regard, elevated levels of endogenous ErbB-3 are observed consistently in Neu-induced mammary tumors (Figure 5B). Although the levels of ErbB-3 protein were dramatically elevated in tumors (10- to 15-fold, Figure 6), a comparable increase in the levels of

erbB-3 transcript was not observed between adjacent and tumor tissue (Figure 8). These observations argue that the elevated ErbB-3 protein levels are due to increased protein translation or prolonged receptor half-life. Indeed, the phosphatidylinositol (PI) 3-kinase/Akt pathway recently has been implicated in the phosphorylation and inactivation of 4E-BP1, a member of a family of translational repressor proteins (Gingras *et al.*, 1998). Given that ErbB-3 can bind and activate PI 3-kinase directly (Fedi *et al.*, 1994; Kim *et al.*, 1994; Prigent and Gullick, 1994; Soltoff *et al.*, 1994), inactivation of 4E-BP1 may, in part, account for the elevated protein levels of ErbB-3 in these tumors.

Fig. 12. Altered ErbB-2 receptors are constitutively tyrosine phosphorylated and undergo disulfide bond-stabilized dimerization when expressed in Rat-1 cells. (A) Lysates (600 μ g) from stable cell lines expressing wild-type ErbB-2 (WT) (lanes 2 and 3) or the three altered receptors (VE, ECD and SPLICE) (lanes 4–11) were immunoprecipitated for ErbB-2 and the immunoprecipitates subjected to immunoblot analysis with phosphotyrosine-specific antibodies. (B) Lysates (600 μ g) from stable cell lines expressing the various ErbB-2 receptors were immunoprecipitated for ErbB-2 and the immunoprecipitates subjected to immunoblot analysis with ErbB-2/Neu-specific antibodies. In each panel, Rat-1 indicates the parental cell line which does not contain any *erbB-2* expression plasmid (A and B, lane 1). The position of ErbB-2 is indicated in each panel by the arrow. (C and D) ErbB-2 was immunoprecipitated, in duplicate, from 500 μ g of protein lysate derived from established Rat-1 cell lines expressing wild-type (WT, lane 2), an engineered mutant (ECD, lane 3) or the ErbB-2 receptors encoded by the splice variant (SPLICE, lanes 4 and 5). The parental Rat-1 cell line lacking exogenous ErbB-2 receptor expression was also included (Rat-1, lane 1). The immunoprecipitates were then electrophoresed under non-reducing (C) and reducing (D) conditions. ErbB-2 dimers are indicated by the solid arrow, while the position of the monomers is marked by the open arrows (D and M, respectively).

While such a pathway would sustain high levels of ErbB-2 protein, the factor(s) resulting in its initial accumulation are unclear.

The results of these studies suggest that co-expression of activated Neu and wild-type ErbB-3 may play a critical role in mammary tumor progression. The observation that ErbB-3 is tyrosine phosphorylated in these tumors and enhances transformation induced by one of the altered Neu receptors utilized in this study suggests that ErbB-3 expression is functionally involved in Neu-mediated transformation. Given that ErbB-3 possesses impaired tyrosine kinase activity and fails to become phosphorylated in the absence of other ErbB receptors (Guy *et al.*, 1994; Pinkas-Kramarski *et al.*, 1996), it seems likely that the observed ErbB-3 phosphorylation results from transmodulation by another family member. Indeed, the obvious candidate is Neu, since elevated levels of the activated receptor are present in these tumor tissues. However, stable dimers between these two receptors could not be detected *in vivo* (unpublished data). The inability to detect Neu–ErbB-3 heterodimers may reflect the transient nature of this interaction, which may be affected further by conformational changes present within the extracellular domain of altered Neu receptors. In addition, previous data have demonstrated that interactions between members of the EGFR family are notoriously difficult to detect *in vivo* (Muller *et al.*, 1996). Despite the lack of detectable Neu–ErbB-3 heterodimers, elevated levels of tyrosine-phosphorylated ErbB-3 may collaborate with Neu to transduce a strong proliferative signal, leading to tumor formation in *neu*-expressing transgenic mice. Consistent with these transgenic results, expression of ErbB-3 has been shown both to enhance ErbB-2-mediated transformation of NIH 3T3 cells *in vitro* (Alimandi *et al.*, 1995; Wallasch *et al.*, 1995; Zhang *et al.*, 1996) and to promote the tumorigenic growth of these cells in soft agar and nude mice (Cohen *et al.*, 1996). Moreover, of the possible interactions that occur between members of the ErbB family (Riese *et al.*, 1995; Karunakaran *et al.*, 1996; Pinkas-Kramarski *et al.*, 1996; Tzahar *et al.*, 1996), the ErbB-2–ErbB-3 heterodimer is the preferred receptor combination capable of delivering strong proliferative signals (Pinkas-Kramarski *et al.*, 1996; Tzahar *et al.*, 1996).

Another potential role for ErbB-3 in mammary tumor progression in these transgenic strains is to provide an anti-apoptotic signal through its ability to couple preferentially with the PI 3-kinase pathway. Indeed, ErbB-3 possesses seven consensus SH2 domain-binding sites for PI 3-kinase and is thought to recruit this signaling molecule to other members of the EGFR family through heterodimerization (Fedi *et al.*, 1994; Kim *et al.*, 1994; Prigent and Gullick, 1994; Soltoff *et al.*, 1994). Activation of the PI 3-kinase pathway is known to protect numerous cell types from apoptosis induced by serum deprivation, removal of survival factors, detachment of cells from the extracellular matrix, UV radiation and c-myc expression under serum-free conditions (Yao and Cooper, 1995; Dudek *et al.*, 1997; Kaufmann-Zeh *et al.*, 1997; Kennedy *et al.*, 1997; Khwaja *et al.*, 1997; Kulik *et al.*, 1997). More recently, the importance of PI 3-kinase in mammary tumorigenesis has been demonstrated in transgenic mice expressing a mutant polyomavirus (PyV) middle T (mT) antigen uncoupled from the PI 3-kinase signaling pathway. In contrast to the potent transforming activity of the wild-type PyVmT antigen, transgenic mice expressing the

mutant PyVmT transgene develop highly apoptotic epithelial hyperplasias. Interestingly, in the focal mammary tumors that evolve from these hyperplasias, there is a dramatic elevation of both endogenous ErbB-2 and ErbB-3 protein levels (Webster *et al.*, 1998). Taken together, these observations suggest that co-expression of ErbB-2 and ErbB-3 may be a common event in mammary tumor progression.

The importance of activated ErbB-2 and ErbB-3 receptors in mammary tumorigenesis is supported further by analyses of primary human breast cancers. We have demonstrated that, in a limited subset of human breast cancers, a majority co-express both *erbB-2* and *erbB-3* transcripts (Figure 9). Consistent with these observations, other studies have noted co-expression of both *erbB-2* and *erbB-3* in human breast tumor samples (Quinn *et al.*, 1994; Bodey *et al.*, 1997). Another important similarity between our transgenic models and human breast cancer is the discovery of an alternatively spliced form of *erbB-2* which closely resembles activated *neu* alleles (Siegel *et al.*, 1994). It is particularly striking that the majority of the tumors that arise in the MMTV/wild-type *neu* strains contain deletions in the same region of *erbB-2* which undergoes this alternative splicing event. One potential explanation for the frequent occurrence of somatic mutations observed in this strain is the fact that the transgene harbors the *neu* cDNA which is incapable of splicing. Therefore, the only mechanism available to mimic the novel *erbB-2* spliced isoform is the generation of somatic activating mutations. Given the strong biological selection for the occurrence of such oncogenic mutations in the mammary tumors of wild-type *neu*-expressing animals, the analogous spliced *erbB-2* transcript described in this study may also be involved in breast cancer progression in humans.

Interestingly, two of the 16 amino acids removed by the proposed splicing event are cysteine residues. In this respect, the *erbB-2* splice variant differs from the mutations identified in the *neu* proto-oncogene, which affected only one cysteine residue (Siegel *et al.*, 1994). Based on the disulfide bond structure of the EGFR extracellular domain (Abe *et al.*, 1998), the protein encoded by the novel *erbB-2* splice variant would possess two unpaired cysteine residues that potentially could form an aberrant intramolecular disulfide bond rather than an intermolecular cysteine bridge. However, our results indicate that the enhanced oncogenic properties (Figure 11 and Table IV) and constitutive tyrosine phosphorylation of these altered ErbB-2 receptors (Figure 12A and B) result from the formation of disulfide-stabilized dimers, which is reminiscent of the mechanism identified for activated Neu receptors (Figure 4; Siegel and Muller, 1996).

The levels of *erbB-2* spliced message are 2–5% that observed for the wild-type *erbB-2* transcript present in these mammary tissues. Although expressed in morphologically normal mammary epithelium, it is conceivable that the absolute levels of the spliced transcript are higher in tumor tissue. Once a critical threshold of this activated ErbB-2 receptor has been reached, malignant transformation of the mammary epithelial cell would result. Future studies with this spliced *erbB-2* isoform should allow this hypothesis to be tested.

The observation that alternative splicing can lead to

activation of growth factor receptors is supported by several recent studies. For example, mutations in FGFR2 were identified in patients with Crouzon and Pfeiffer syndrome that result in the use of a novel splice donor site, causing a 51 bp (17 amino acid) deletion in the extracellular domain (Li *et al.*, 1995; Meyers *et al.*, 1996). A spliced form of *Ron* has also been described which encodes an activated receptor lacking 49 amino acids in the extracellular domain (Collesi *et al.*, 1996). Like the *erbB-2* spliced isoform, the deletion in *Ron* results from the removal of an entire exon, which leads to the loss of a cysteine residue in the resulting protein. Constitutive disulfide bond-stabilized dimers can be detected in cells expressing the activated *Ron* receptor, and these cells display an invasive phenotype *in vitro* (Collesi *et al.*, 1996). Furthermore, activation of many receptor families can occur through mechanisms other than alternative splicing. Deletions in EGFR (Sugawa *et al.*, 1990; Wong *et al.*, 1992) and TrkA (Coulier *et al.*, 1990), as well as point mutations in the erythropoietin receptor (Watowich *et al.*, 1992), RET (Mulligan *et al.*, 1993) and three of the four FGFRs (Webster and Donoghue, 1997) have been reported. In many instances, the observed mutations affect cysteine residues situated in the extracellular domain and serve to activate these receptors (Nishikawa *et al.*, 1994; Asai *et al.*, 1995; Santoro *et al.*, 1995; Galvin *et al.*, 1996; Robertson *et al.*, 1998). Together, these observations suggest that activation of growth factor receptors through alternative splicing or mutation plays a critical role in the induction of both developmental disorders and human cancers.

Materials and methods

DNA constructions

To generate the MMTV/NDL expression plasmids, cDNAs possessing two deletions that correspond to *neu*8142 and *neu*8342 (Siegel *et al.*, 1994; Siegel and Muller, 1996) were excised from pJ4 Ω (Morganstern and Land, 1990) as *Hind*III–*Eco*RI fragments and inserted into the corresponding sites of pMMTV-SV40 (p206) (Guy *et al.*, 1992b). The *erbB-3* expression plasmid contains the human cDNA inserted into pJ4 Ω as a *Sal*I fragment, whereas construction of pJ4 Ω /*neu*8142 and pJ4 Ω /*neu*8142 has been described previously (Siegel *et al.*, 1994). Expression plasmids containing various human *erbB-2* cDNAs were constructed by inserting each cDNA as a *Hind*III fragment into the corresponding site of pJ4 Ω . The cDNAs encoding both wild-type and activated *erbB-2* (valine to glutamic acid substitution in the transmembrane domain) were generous gifts of Nancy E.Hynes (Friedrich Miescher Institute, Basel, Switzerland). The *erbB-2* (ECD DEL) mutant was generated by oligonucleotide-directed mutagenesis, and the *erbB-2* (SPLICE) mutation was amplified from human breast tumor RNA. The PCR products were sequenced to ensure that only the desired mutations were present.

A plasmid containing the puromycin resistance gene under the transcriptional control of the *PGK* promoter was provided by Michael A.Rudnicki (McMaster University, Hamilton, Ontario, Canada). The plasmid used to generate the SV40 polyadenylation-specific riboprobe (SPA) has been described previously (pASV; Muller *et al.*, 1988). The internal control riboprobe used to detect *PGK-1* was obtained from Michael A.Rudnicki and has been described elsewhere (Siegel *et al.*, 1994). The template plasmid used to produce the murine *erbB-3* riboprobe was generated by inserting a 375 bp fragment, corresponding to nucleotides 2489–2864 of the human cDNA (Plowman *et al.*, 1990), into the *Eco*RI–*Bam*HI sites of pSL301 (Invitrogen). A pSL301 vector containing a 142 bp fragment of the murine cytokeratin-8 cDNA (nucleotides 552–694; Morita *et al.*, 1988) was used to generate the antisense riboprobe. The *erbB-2* riboprobe was constructed by inserting a 473 bp fragment, corresponding to nucleotides 2018–2491 of the human cDNA (Coussens *et al.*, 1985), into the *Sph*I–*Nde*I sites of pSL301. A 720 bp fragment of human *erbB-3* (nucleotides 1661–2381;

Plowman *et al.*, 1990) was inserted into *EagI*–*Bam*HI sites of pSL301 to construct the riboprobe template plasmid. An internal control plasmid (pGem-1) containing a 212 bp *Bam*HI–*Sac*I fragment of human β -actin (nucleotides 1371–1583; Ng *et al.*, 1985) was kindly provided by Ann-Marie Mes-Mason (University of Montreal, Montreal, Quebec, Canada).

Generation and identification of transgenic mice

DNA was prepared for microinjection by digestion of the MMTV/*neu* deletion plasmids with *Sa*II and *Spe*I. The injection fragment was recovered by electroelution, subjected to sequential phenol–chloroform/chloroform extractions, ethanol precipitated and resuspended in distilled water. Six-week-old FVB/N female mice (Taconic Farms, Germantown, PA) were superovulated by an intraperitoneal (i.p.) injection with pregnant mare's serum [PMS; 5 IU in 0.1 ml of 1× phosphate-buffered saline (PBS)] followed 46–48 h later by an i.p. injection of human chorionic gonadotropin (hCG; 5 IU in 0.1 ml of distilled water). Superovulated FVB/N females were mated with FVB/N males the night before injection, and Swiss-Webster females (Taconic Farms), to be used as foster mothers, were mated with vasectomized Swiss-Webster males. The next day, FVB/N females possessing a copulation plug were sacrificed, and fertilized one-cell mouse embryos were isolated from the oviducts. The pronuclei of these zygotes were injected with 0.5–1.0 pl of the prepared injection fragment (5 μ g/ml DNA solution). Following microinjection, viable eggs were transferred into the oviducts of pseudo-pregnant Swiss-Webster mice.

Transgenic progeny were identified as previously described (Webster *et al.*, 1998). The filters were hybridized to a 794 bp fragment of the *neu* cDNA (nucleotides 1380–2174; Bargmann *et al.*, 1986a) that was radiolabeled with [α -³²P]dCTP by random priming. After identification of founder animals, routine colony maintenance was performed by PCR genotyping.

RNA analysis

RNA was isolated from mouse tissues by guanidinium thiocyanate extraction followed by cesium chloride sedimentation gradient centrifugation as previously described (Webster *et al.*, 1998). The RNA pellet was resuspended in 100 μ l of tissue resuspension buffer [5 mM EDTA (pH 8.0), 0.5% *N*-lauroylsarcosine, 0.7 M 2-mercaptoethanol] followed by 300 μ l of sterile water that had been treated with diethyl pyrocarbonate. Following one phenol–chloroform extraction, the RNA was ethanol precipitated and the yield determined.

RNase protection assays were performed essentially as described by Webster *et al.* (1998) with minor modifications. Following overnight hybridization in a 40 μ l volume, 300 μ l of digestion buffer [300 mM NaCl, 10 mM Tris (pH 7.4), 5 mM EDTA (pH 8.0), 200 U of RNase T1, 30 μ g/ml RNase A) was added to each sample and allowed to incubate at 37°C for 30 min. Upon addition of 20 μ l of 10% SDS and 10 μ l of proteinase K (10 μ g/ μ l), the samples were again incubated at 37°C for 20 min. Following extraction with phenol–chloroform, the samples were ethanol precipitated in the presence of 20 μ g of RNase-free tRNA and dried for 20 min in a heating block. The remaining steps were performed as outlined (Webster *et al.*, 1998).

The reverse transcription reaction was performed as previously described (Siegel *et al.*, 1994). After incubation at 37°C for 2 h, 1.0 μ l of the reverse transcription mix was added to the following: 0.5 μ l of each oligonucleotide primer (10 μ M), 2.0 μ l of 10× PCR buffer [300 mM Tris–HCl (pH 8.4), 500 mM KCl], 1.0 μ l of 50 mM MgCl₂, 1.0 μ l of deoxynucleotide triphosphate (dNTP) mix (10 mM each dATP, dCTP, dGTP and dTTP), 1.0 μ l of [α -³²P]dCTP (10 μ Ci) and distilled water to bring the final volume to 20.0 μ l. The reaction mixture was overlaid with 10.0 μ l of paraffin oil and heated to 95°C for 5 min. The temperature was dropped to 80°C for 2 min and 5.0 μ l of the following mixture was added to each sample: 0.1 μ l of *Taq* DNA polymerase (Gibco-BRL), 0.5 μ l of 10× PCR buffer and 4.4 μ l of distilled water. PCR amplification was performed for 30 cycles of 1 min at 94°C, 30 s at 55°C and 1 min at 72°C. Both the wild-type and alternatively spliced form of *erbB-2* were amplified with the following primers; AB12904 (TTTCCAGATGAGGAGGGC) and AB12903 (CGGAATTCCTGTCCACGACTG-CACCGT), which amplify the region corresponding to nucleotides 1996–2544 of the human *erbB-2* cDNA (Coussens *et al.*, 1985).

Cell culture and focus assays

Rat-1 fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin, streptomycin and fungizone (Gibco-BRL). Stable cell lines were derived by electroporation as described previously (Siegel *et al.*, 1994). DNA was introduced into the Rat-1 cells at a 50:1 ratio

of expression plasmid:puromycin resistance plasmid (PGK-puro), and resistant colonies were selected for 10 days in media containing puromycin (3.0 μ g/ml) prior to deriving clonal cell lines.

The *neu/erbB-3* focus assays were conducted by lipofectamine transfection (Gibco-BRL) following the manufacturer's instructions. One μ g of each pJ4 Ω expression plasmid was used for each co-transfection experiment. In those instances when only one plasmid was transfected, sheared salmon sperm DNA was included to ensure that the same amount of DNA (2 μ g) was used for each condition. A total of 2×10⁵ cells/well were seeded in 6-well plates the day prior to transfection. Once transfected, the cells were allowed to reach confluence and were then split into two 10 cm plates. The cells were maintained in culture for 12 days after reaching a monolayer. Medium (DMEM supplemented with 2% FBS, penicillin, streptomycin and fungizone) was changed every 3 days during the course of the experiment. The *erbB-2* focus assays were performed essentially as described (Siegel *et al.*, 1994), with the exception that monolayers were maintained for 12 days in DMEM supplemented with 2% FBS, penicillin, streptomycin and fungizone. Five micrograms of each pJ4 Ω expression plasmid was electroporated per 1×10⁶ cells.

Immunoprecipitation and immunoblotting

Tissue samples were ground to a powder under liquid nitrogen with a chilled mortar and pestle and lysed for 20 min on ice in TNE lysis buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 1% NP-40, 2 mM EDTA (pH 8.0), 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin]. Lysates from established cell lines were prepared from 10 cm tissue culture plates once the cells had reached confluence. The cells were washed twice in ice-cold 1× PBS (140 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄) and lysed for 20 min on ice in TNE lysis buffer. Lysates were cleared by centrifugation for 10 min at 4°C and the protein concentration determined by Bradford assay (Bio-Rad).

Immunoprecipitations of lysates from mammary tissues and stable cell lines (see figure legends for the amount of total protein used in each experiment) were performed as described (Muthuswamy *et al.*, 1994). The mouse monoclonal antibodies used to immunoprecipitate Neu (Ab-4) and ErbB-2 (Ab-5) were purchased from Oncogene Research Products, Inc. ErbB-3 was immunoprecipitated with a rabbit polyclonal antibody (C17) obtained from Santa Cruz Biotechnology, Inc.

Protocols for immunoblot analysis have been described in detail elsewhere (Muthuswamy *et al.*, 1994). Total blots were performed using 50 μ g of protein lysate. Neu was detected with a mouse monoclonal antibody (Ab-3) obtained from Oncogene Research Products, Inc. (1:1000). The EGFR was detected with a mouse monoclonal antibody (E12020) purchased from Transduction Laboratories (1:1000). Immunoblots for ErbB-3 (C-17) and ErbB-4 (C-18) were performed with rabbit polyclonal antibodies sold by Santa Cruz Biotechnology, Inc. (1:1000). Grb-2 was detected with a rabbit polyclonal antibody (C-23) from Santa Cruz Biotechnology, Inc. (1:1000). Tissue culture supernatant from a rat monoclonal antibody (TROMA-1; Brulet *et al.*, 1980; Kemler *et al.*, 1981) was used to detect cytokeratin-8 (1:50). Phosphotyrosine blots were probed with mouse anti-phosphotyrosine antibodies (PY20) obtained from Transduction Laboratories (1:1000). In each case, horseradish peroxidase-conjugated anti-mouse, anti-rabbit or anti-rat secondary antibodies (1:2500) (Jackson Laboratories) were used. Quantitative immunoblot analysis was performed using ¹²⁵I-conjugated secondary antibodies.

Detection of Neu dimer formation in tumors derived from MMTV/NDL transgenic mice and ErbB-2 dimer formation in established Rat-1 cell lines was performed as described previously (Siegel and Muller, 1996). Lysates from NDL mammary tumors were incubated with anti-Neu antibodies (Ab-4) while lysates from Rat-1 stable cell lines were immunoprecipitated with anti-ErbB-2 antibodies (Ab-5). Protein G–Sepharose was then added and immunoprecipitations were carried out for 3 h at 4°C followed by 3–4 washes in TNE lysis buffer. Duplicate immunoprecipitates were separated by electrophoresis through 4–12% gradient SDS–polyacrylamide gels, in the presence or absence of 2-mercaptoethanol. Immunoblotting was performed with rabbit polyclonal anti-Neu antibodies (06-562) from Upstate Biotechnology.

Histological evaluation

Complete autopsies were performed, and both gross and microscopic examinations were conducted. Virgin female animals from both wild-type FVB/N and each transgenic strain were sacrificed at 6 months of age to examine the mammary tumors histologically. Tissues were fixed overnight in 4% paraformaldehyde and transferred to 70% ethanol the

next day. Samples were then blocked in paraffin, sectioned at 4 μ m, mounted on glass slides and stained with hematoxylin and eosin (Anatomical Pathology, McMaster University). Whole-mount analyses were also performed as described (Vonderhaar and Geco, 1979), with the exception that number three mammary fat pads were used instead of the number four glands.

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Kwong and Hung (1998) have recently reported a similar splice variant of HER2.