Androgen-dependent mammary carcinogenesis in rats transgenic for the Neu proto-oncogene

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Summary

Transgenic rats were created with overexpression of the Neu proto-oncogene in the mammary gland of both sexes, yet only males developed mammary cancer in an androgen-dependent fashion. Transgenic females only developed mammary cancer if treated with androgens. These tumors were positive for androgen receptor (AR), but negative for estrogen and progesterone receptors. Extensive analysis failed to detect mutations anywhere within the *neu* transgene from mammary carcinomas. Established mammary carcinomas eventually escaped their dependency on androgens. Transgenic long-term gonadectomized rats did not develop mammary cancer, but Neu overexpression stimulated the growth of their mammary glands. Our results suggest crosstalk between the Neu proto-oncogene and AR signaling pathways in the growth of both the normal and cancerous mammary epithelium.

Introduction

Neu (also known as HER-2/ErbB-2) is one of four members in a family of tyrosine kinase growth factor receptors that also includes HER-1/EGFR/ErbB-1, HER-3/ErbB-3, and HER-4/ErbB-4. Comprehensive in vitro studies in cell lines have established that these four receptors form a complex array of homodimers and heterodimers in response to ligand binding, with Neu serving as the preferred heterodimer binding partner for the other three members. In particular, Neu stabilizes ErbB heterodimers, leading to enhanced mitogenic signaling (Yarden, 2001). These in vitro experiments have solidified the importance of Neu heterodimer formation in regulation of the ErbB signaling network. In contrast, mutant-activated *neu* oncogenes predominantly form Neu homodimers (Siegel et al., 1994; Siegel and Muller, 1996; Yarden, 1990).

The ErbB family has been associated with many different human cancers. The connection between Neu and breast cancer has received the most attention. Slamon was the first to show that *neu* gene amplification occurred in 25%–30% of human breast cancers and that this correlated with a poor patient prognosis (Slamon et al., 1987). Subsequent studies revealed that the Neu protein also was overexpressed in the breast carcinomas, correlating with gene amplification (Berger et al., 1988). Numerous clinical studies since then have consistently found the same association between Neu overexpression and breast cancer. Recently, there have been advances in the development of monoclonal antibody therapies directed specifically against Neu-overexpressing breast cancers (Cobleigh et al., 1999; Slamon et al., 2001; Vogel et al., 2001). However, not all women respond to these treatments, and the precise role that Neu plays in the etiology of breast cancer remains poorly understood. A critical question that remains unknown is whether Neu is involved directly in the initiation of breast cancer or rather instead only helps to drive cancer progression.

Several rodent models have been developed to investigate the role of Neu in breast cancer. The first transgenic (TG) mice developed to study Neu utilized the mouse mammary tumor virus long terminal repeat (MMTV LTR) to direct expression of a mutated rat *neu* oncogene to the mammary gland (Bouchard et al., 1989; Muller et al., 1988). The same mutated *neu* oncogene also has been delivered to the mammary epithelium of rats via infusion of recombinant retroviral vectors directly into the mammary duct in situ (Wang et al., 1991a). The *neu* oncogene used in these studies contains a single point mutation in the transmembrane domain (Bargmann et al., 1986), which results in its constitutive dimerization and activation of the intrinsic tyrosine kinase activity (Bargmann and Weinberg, 1988; Weiner et al., 1989). In all of these rodent models, the activated *neu* oncogene induced multiple mammary carcinomas. However, in human breast cancer, activating mutations of *neu* have not been found (Lemoine et al., 1990). The finding that human breast cancer is associated with overexpression of only the nonmutated Neu proto-oncogene led to the development of additional TG rodent models. The human *neu* proto-oncogene under the control of the MMTV was used to generate both TG mice (Suda

SIGNIFICANCE

The contribution of Neu to the etiology of breast cancer is poorly understood. The novel transgenic rat model that we describe here is a rodent model of the *neu* proto-oncogene that does not require somatic mutational activation of Neu to induce mammary carcinomas. This finding is pertinent to human breast cancer, where Neu mutations are nonexistent. This report also shows an absolute requirement for androgens in Neu-mediated mammary carcinogenesis. Our results potentially could lead to a greater understanding of the mechanistic pathways that drive mammary carcinogenesis associated with Neu overexpression in both men and postmenopausal women, for whom elevated testosterone is a strong risk factor for the disease.

et al., 1990) and rats (Davies et al., 1998). However, in neither case were mammary carcinomas induced by the transgene. In the case of the TG rats, there was at most only a very low expression of the transgene (Davies et al., 1998). The rat *neu* proto-oncogene regulated by MMTV also has been used to create TG mice (Guy et al., 1992). These female TG mice developed mammary carcinomas, but the total tumor burden was much reduced and the latency was considerably longer compared to mammary carcinogenesis in the activated *neu* TG mice (Muller et al., 1988). A subsequent study, however, revealed that at least 65% of the mammary carcinomas in the *neu* proto-oncogene TG mice contained somatic deletion mutations within the extracellular region of the *neu* transgene (Siegel et al., 1994). The net effect of these mutations was constitutive homodimerization and activation of Neu (Siegel and Muller, 1996), which is functionally analogous to the transmembrane point mutation studied in earlier models. Therefore, it remained to be shown in an experimental rodent model whether the Neu proto-oncogene, when not associated with mutational activation, could induce mammary cancer. Thus, we chose the rat to further explore the possible role of the Neu proto-oncogene in the etiology of mammary cancer. Rat mammary cancers, similar to human cancers, display a wide spectrum of responses to hormonal therapy (Wang et al., 1992). In addition, both rat and human mammary cancers frequently are hormone dependent, while murine mammary tumors are virtually all hormone independent (Nandi et al., 1995). In this report, we describe the generation and characterization of TG rats overexpressing the rat Neu proto-oncogene in the mammary gland and the effects of androgens in mediating mammary carcinogenesis.

Results

Generation of *neu* proto-oncogene TG rats and distribution of neu mRNA expression

We generated TG Sprague-Dawley rats using a construct containing the rat *neu* proto-oncogene cDNA under the control of the MMTV. A real-time RT-PCR assay based on TaqMan chemistry was used to quantitatively assess the level of neu mRNA expression in a variety of tissues from TG rats or their nontransgenic (NTG) littermates. After normalization of the neu levels to 18S rRNA, the mean neu mRNA expression for each group was compared between TG and NTG tissues. neu mRNA levels were determined first in mammary glands from young (78–82 days) or old (419–431 days) adult male and female rats (Figure 1A). Both NTG males and females had little neu mRNA expression in the mammary gland at either age. Compared to their NTG littermates, young TG males and females demonstrated 4.9-fold ($p = 0.004$) and 1.4-fold ($p = 0.147$) neu mRNA overexpression, respectively, in the mammary gland. This overexpression of neu mRNA was increased in the old TG rats, with males and females expressing 10.9-fold ($p = 0.007$) and 7.6-fold ($p = 0.008$) higher levels of neu, respectively, compared to age-matched NTG rats. In the old TG mammary gland, neu mRNA was expressed at equivalent levels in the male and female ($p = 0.219$).

We next evaluated whether neu mRNA was overexpressed in any other tissues of the TG male (Figure 1B). At a threshold of $p < 0.01$, there was neu mRNA overexpression in TG males relative to NTG males in the lung (2.0-fold), dorsolateral prostate (1.5-fold), young ventral prostate (2.3-fold), and old ventral prostate (4.7-fold). With aging, neu mRNA expression in the TG ventral prostate increased 2.2-fold ($p = 0.048$). In all other tissues, there was no statistical difference in neu mRNA expression between TG and NTG males at a level of significance $p < 0.01$.

In addition to evaluating the female mammary gland for neu mRNA expression, we also analyzed neu mRNA levels in the ovary of the young female rat. There was no difference in ovarian neu mRNA expression between TG and NTG females ($p = 0.070$).

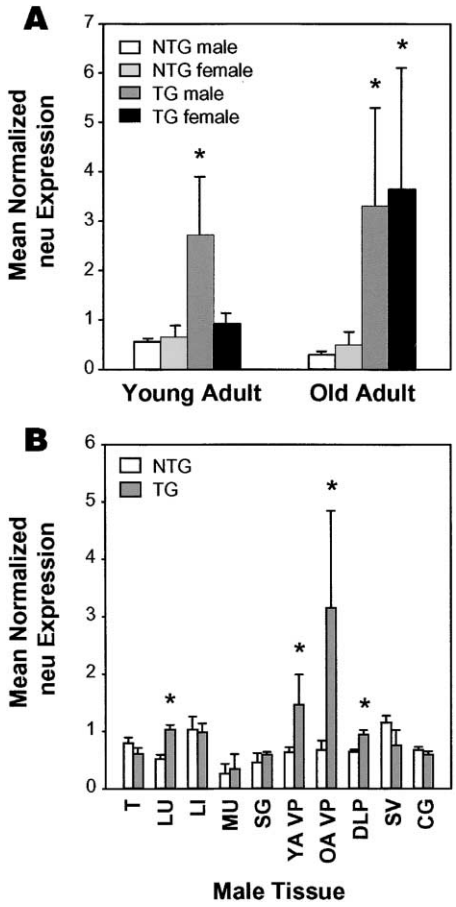

Figure 1. Determination of neu mRNA expression in a variety of tissues from TG and NTG rats

A: Mammary glands were collected from young (78–82 days) or old (419–431 days) adult NTG or TG rats. Total RNA was isolated and the level of neu mRNA was determined from 4–6 rats/group following normalization to 18S rRNA using quantitative RT-PCR. The data is presented as the mean normalized neu expression for each group plus SD. Data was compared between NTG and TG rats for each sex and age group, with an asterisk indicating higher expression in TG rats ($p < 0.01$). **B:** Testis (T), lung (LU), liver (LI), skeletal muscle (MU), and submaxillary salivary gland (SG) were collected from young (78–81 days) adult males. Dorsolateral prostate (DLP), seminal vesicle (SV), and coagulating gland (CG) were collected from old (419–425) adult males. Ventral prostate (VP) was collected from both young (YA) and old (OA) adult males. Total RNA was isolated and the level of neu mRNA in each tissue was determined following normalization to 18S rRNA using quantitative RT-PCR from 2–3 rats/group (CG) or 3–4 rats/group (all other tissues). The data is presented as the mean normalized neu expression for each group plus SD. Data was compared between NTG and TG rats for each tissue, with an asterisk indicating higher expression in TG rats ($p < 0.01$).

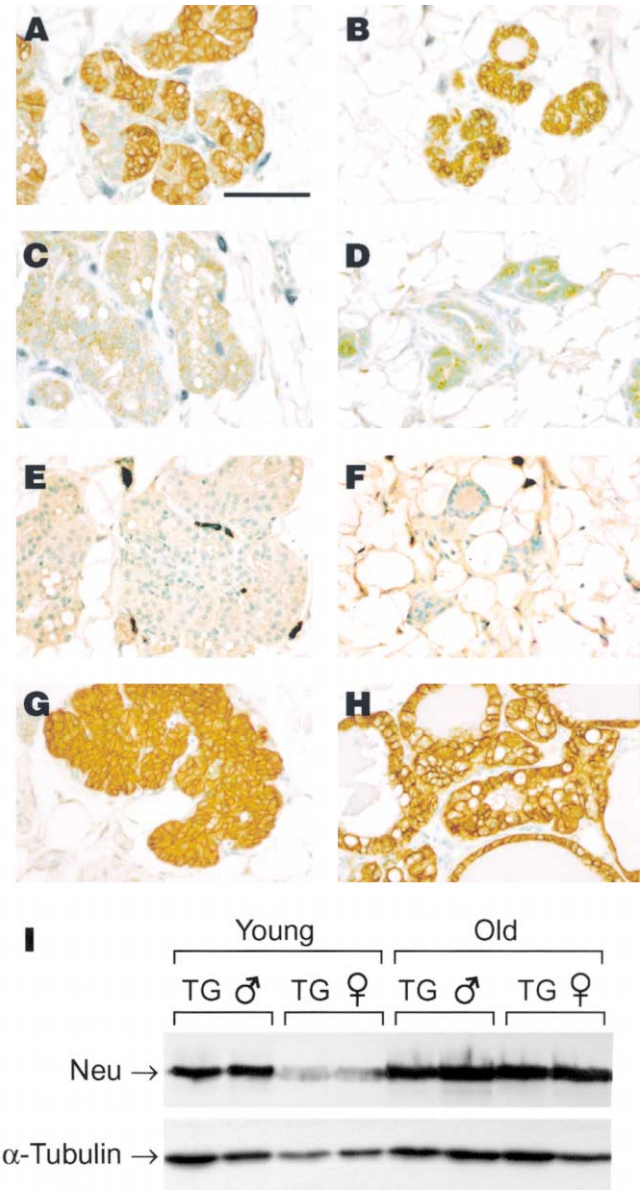

Figure 2. Neu protein is overexpressed in the mammary gland of the *neu* TG rat

Mammary gland sections were stained with a mouse monoclonal anti-Neu (**A**–**D** and **G**–**H**) or nonspecific mouse IgG (**E** and **F**). Samples were from young (78–82 days, **A**–**F**) or old (419–431 days, **G** and **H**) adult rats. For TG and NTG rats, sections from 3–5 and 2 rats/group, respectively, were stained. Representative results are shown from TG male (**A**, **E**, **G**), TG female (**B**, **F**, **H**), NTG male (**C**), and NTG female (**D**). The bar in **A** is 100 μm and the same scale was used for all panels. **I:** Whole cell lysates of TG rat mammary glands were immunoblotted with a mouse monoclonal anti-Neu (top) or α-tubulin (bottom).

The TG rat mammary gland overexpresses Neu protein

The mammary glands of TG and NTG rats also were compared utilizing immunohistochemistry with a mouse monoclonal antibody directed against Neu. In both young TG males and females, there was clear Neu overexpression at the plasma membrane of epithelial cells compared to the NTG (compare Figures 2A versus 2C and 2B versus 2D). Stromal cells displayed little to no Neu expression. Cells expressing high levels of Neu were clustered together as multicellular patches distributed heterogeneously throughout the gland. Among the young TG glands, there was an apparent higher percentage of epithelial cells overexpressing Neu in the male, compared to the female. However, there was no difference in the intensity of Neu immunostaining between the male and female glands. As a control, TG male and female mammary glands were incubated with nonspecific mouse IgG that demonstrated lack of any staining (Figures 2E and 2F). Old TG males and females continued to express high levels of Neu in the mammary epithelium (Figures 2G and 2H), while old NTG rats had barely detectable amounts of the protein (data not shown). Overall, there was an increase in the relative ratio of mammary epithelial cells overexpressing Neu in the old TG rats compared to the young animals. Western blotting for Neu of whole-cell mammary gland lysates from TG rats confirmed expression of the protein (Figure 2I).

In contrast to the large overexpression of Neu within the TG female mammary gland, the ovary, uterus, and cervix of TG females did not overexpress Neu (data not shown).

Mammary carcinogenesis in the *neu* TG rat is androgen dependent

Having demonstrated that the mammary glands of both TG males and females produced elevated levels of Neu, we next determined if long-term Neu overexpression would lead to mammary cancer (Table 1). Untreated TG and NTG males and females were palpated for the presence of mammary carcinomas beginning at approximately 9 months of age. All females were maintained as virgins. By 419–434 days of age, two cohorts of TG males demonstrated a mammary carcinoma incidence of 78% and 100%. Multiple mammary carcinomas arose in the afflicted rats, with a mean of 3.0 and 4.9 mammary glands/rat involved with tumor. By 496–587 days of age, all TG males had developed multiple mammary carcinomas in approximately 6 glands/rat. Two TG male rats had gross metastatic lesions, occurring in the lung, liver, kidney, intestines, and skull. For male and female rats, complete necropsies were performed. Interestingly, despite the overexpression of neu within the TG prostate, no tumors were observed within this gland in either intact or androgen-supplemented rats. In contrast to the TG male, all TG females remained free of mammary cancer out to 539 days of age. In addition, TG females did not develop cancers of the ovary, uterus, or cervix, irrespective of their hormonal status. Neither NTG males nor females developed mammary carcinomas. Finally, there were no other gross or histological pathologies in any of the rats due to the presence of the transgene. Importantly, the mammary tumor induction in TG males was not associated with coincident pituitary tumors.

We next evaluated the role of the hormonal environment in controlling susceptibility to mammary carcinogenesis in the *neu* TG rat (Table 1). Young adult TG males and females underwent bilateral gonadectomy. NTG littermates were similarly treated. All of the rats were sacrificed between 421 and 468 days of age, approximately 1 year after gonadectomy. Orchidectomy completely prevented the development of mammary carcinomas in all of the TG males, while ovariectomy of the TG female did not result in mammary cancer. All NTG rats were cancer-free. To verify that the absence of androgens was responsible for the inhibition of carcinogenesis, we reconstituted an androgenic environment in gonadectomized TG and NTG males and fe-

Table 1. Mammary carcinogenesis in the *neu* TG rat is androgen dependent

Genotype	Sex	Hormonal status	Age at necropsy (days)	n	% rats with mammary carcinomas	Mean age to palpable carcinoma (days)	Mean number glands with carcinomas
TG	male	intact	419–434	9	78	371 ± 40.4	3.0 ± 2.7
				7	100	361 ± 43.5	4.9 ± 2.8
		intact	496–587	3	100	427 ± 45.2	6.0 ± 0.0
				17	100	nd	6.7 ± 2.8
TG	female	intact	425–430	17	0	-	-
			530–539	4	0	-	-
NTG	male	intact	420–433	17	0	-	-
			623–624	3	0	-	-
NTG	female	intact	425–449	17	0	-	-
			530–539	6	0	-	-
TG	male	orch	422–424	7	0	-	-
TG	female	ovx	425–468	8	0	-	-
NTG	male	orch	421–425	7	0	-	-
NTG	female	ovx	427–431	8	0	-	-
TG	male	orch+DHT	397–438	10	100	338 ± 55.2	7.3 ± 2.0
TG	male	orch+T	390–416	9	100	291 ± 20.6	8.9 ± 1.4
TG	female	ovx+DHT	463–466	10	80	407 ± 50.8	3.7 ± 2.5
TG	female	ovx+T	400–469	8	75	**	3.4 ± 2.6
NTG	male	orch+DHT	432–450	10	0	-	-
NTG	male	orch+T	435–464	9	0	-	-
NTG	female	ovx+DHT	464–467	11	0	-	-
NTG	female	ovx+T	468–471	10	0	-	-
TG	female	intact (biparous)	430–446	8	0	-	-
NTG	female	intact (biparous)	427–446	13	0	-	-

TG and NTG male and female rats were randomly divided into various treatment groups at 47–57 days of age. Females were kept as virgins or consecutively mated twice as young adults (biparous). Mammary carcinomas were scored as palpable when they reach 3 mm in diameter. Double asterisk indicates palpable tumors could not be accurately assessed due to the formation of numerous milk cysts. Mammary carcinoma data was collected at necropsy. The data is presented as the group mean ± SD. Abbreviations: orch, orchidectomy; ovx, ovariectomy; nd, not determined.

males by long-term treatment with either testosterone (T) or the nonaromatizable androgen 5α-dihydrotestosterone (DHT). We did not examine the effects of androgen treatment on mammary carcinogenesis in intact female rats, because androgens induce numerous changes to the rat ovary, including atrophy, cyst formation, and arrest of estrous (Burrows, 1949). These effects would confound interpretation of the results. The rats were maintained continuously on androgens until sacrifice at 390–471 days of age. While androgen treatment did not induce mammary tumors in any of the NTG rats, multiple mammary carcinomas did develop in almost all of the TG rats. The incidence of carcinomas among the males was 100%, irrespective of which androgen was used. For the females, 75% and 80% of those treated with T and DHT, respectively, developed mammary carcinomas. Radioimmunoassays of serum androgen levels at necropsy from a subset of the rats showed that T implants resulted in a 2- to 3-fold elevation of T and a 2- to 4-fold elevation of DHT compared to the untreated male. DHT implants resulted in 12-fold elevated DHT levels, while T was undetectable ($<$0.1 ng/ml, data not shown). Androgen-treated gonadectomized male and female rats had serum estradiol levels equal to that of the untreated male, and there were no appreciable differences in serum levels of T or DHT among similarly treated males and females (data not shown).

Finally, we asked whether mammary carcinogenesis in the TG female could be driven without androgen supplementation if neu expression was elevated to levels above that normally found in the untreated TG female. We were able to show that pregnancy stimulated neu mRNA expression in the TG female mammary gland. At days 10–14 of pregnancy, neu mRNA levels were elevated by 2.5-fold compared to the young virgin TG female (data not shown). Therefore, we placed young females through two consecutive full-term pregnancies and then sacrificed them approximately 235 days after the second pregnancy when they were an average of 439 days of age (Table 1). Despite having a 4.5-fold higher level of neu mRNA expression compared to the intact TG male, TG biparous females remained cancer-free. The effect of additional pregnancies beyond two on mammary carcinogenesis was not investigated, due to our experience that lactating Sprague-Dawley rats are susceptible to developing ulcerating milk-filled lesions, which necessitates sacrifice. These lesions are more likely to occur with increasing number of pregnancies. However, it should be pointed out that an additional cohort of biparous TG females ($n = 4$) were observed for approximately 13 months after the first pregnancy. Therefore, this cohort experienced elevated neu expression levels for a period of time equivalent to 13-month-old TG males, in whom the mammary cancer incidence was approximately 86% with numerous, large carcinomas in multiple glands. When this group of biparous TG females was sacrificed at 558 days of age, there was only a single small mammary carcinoma in a single rat. However, spontaneous mammary cancers can be observed in normal female rats of advanced age.

The *neu* transgene is amplified in mammary carcinomas

Fluorescence in situ hybridization (FISH) was used to map the transgene in lines 6500 and 2477. Metaphase chromosomal

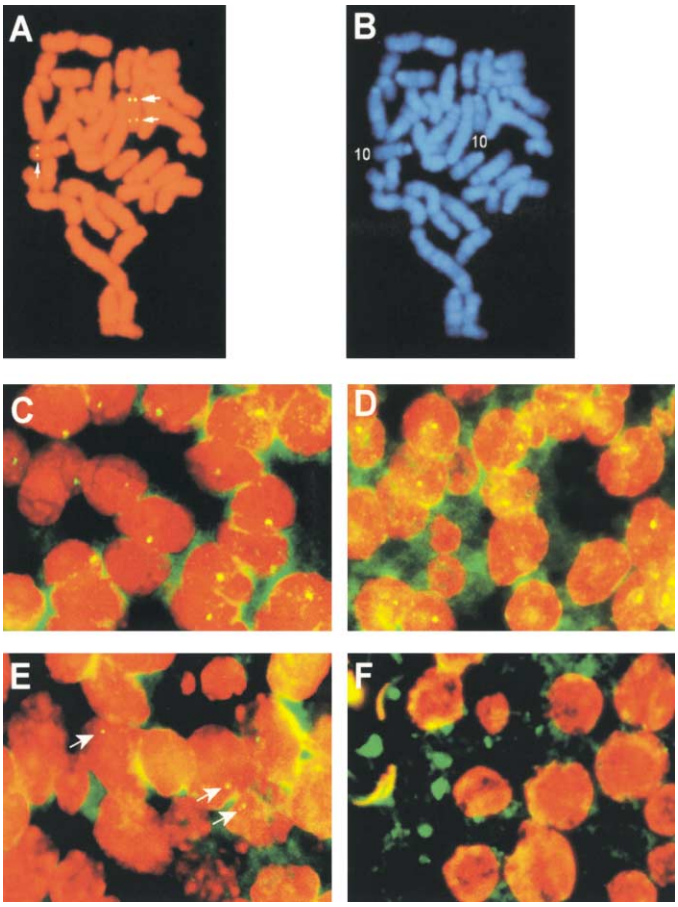

Figure 3. Chromosomal localization of the *neu* transgene and amplification in mammary carcinomas

Metaphase FISH was performed on spleen lymphocytes from TG males (**A**). The endogenous *neu* gene is shown on the telomeric end of chromosome 10 (small arrow) (Koelsch, 1998), while the transgene of line 6500 was integrated into the centromeric region of chromosome 10 (large arrow). DAPI banding patterns are shown from the same metaphase preparation (**B**). Interphase FISH was conducted on paraffin-embedded tissue sections from mammary carcinomas (**C** and **D**), adjacent normal mammary epithelium (**E**), and testis (**F**) of TG males. The transgene signal was amplified in carcinoma compared to normal gland (**E**, arrows). No signal was detected in testis.

preparations from spleen lymphocytes of heterozygous TG males at approximately 1 year of age were hybridized with a probe containing the entire transgene. The probe was able to detect the endogenous *neu* gene on the telomeric region of chromosome 10 (Figure 3A, small arrow). The signal intensity of the endogenous *neu* gene was used to estimate the copy number of the *neu* transgene. Line 6500 contained 3–6 copies of the transgene on chromosome 10q12 (Figure 3A, large arrow), while line 2477 had 1–2 transgene copies on the telomeric region of chromosome 5 (data not shown). DAPI banding patterns identified the specific rat chromosomes containing positive integrated FISH signals (Figure 3B).

We next determined if mammary carcinomas in heterozygous TG males displayed amplification of the *neu* transgene. Interphase FISH was performed on two paraffin-embedded carcinomas from two untreated 14-month-old males using the complete transgene as a probe. A single strong signal was detected in 25%–40% of mammary carcinoma nuclei (Figures 3C and 3D). In comparison, normal mammary epithelium surrounding the carcinoma displayed only a weak FISH signal in 20%–30% of the nuclei (Figure 3E, arrows). Hybridization with the probe did not occur in mammary stromal fibroblasts and vascular cells within the same sections. Also, testis from the same cohort of TG males did not have detectable FISH signals (Figure 3F). The endogenous *neu* gene was not detected in interphase chromosomes, unlike in the lymphocyte metaphase chromosomal preparation.

Mammary carcinogenesis is not associated with somatic mutational activation of the *neu* proto-oncogene in the *neu* TG rat

To assess if the mammary carcinomas arose due to somatic mutational activation of the *neu* proto-oncogene as was found in the *neu* TG mouse, the entire coding region of the *neu* transgene was sequenced from five carcinomas from five individual untreated TG males. Similarly, the complete *neu* transgene was sequenced from three mammary carcinomas arising in orchidectomized plus DHT-treated TG males and three carcinomas from ovariectomized plus DHT-treated TG females. These carcinomas from DHT-treated rats were selected from three different individual males and females. All of the mammary carcinomas analyzed were completely free of mutations within the entire *neu* coding region, regardless of sex or hormonal treatment.

Because the *neu* transgene was amplified in the carcinomas, rare *neu* copies with mutations might remain undetected by sequencing the pooled PCR-amplified transgene. To address this, we therefore performed an extensive clonal sequence analysis of an approximately 750 bp region of the *neu* transgene spanning the transmembrane and adjacent extracellular domain, which is a hotspot for mutations in the *neu* TG mouse. This region was PCR amplified using DNA from the same six mammary carcinomas arising in DHT-treated gonadectomized TG males and females (three from each sex) that were completely sequenced earlier. Because random mutations induced by DNA polymerase were expected, we set criteria that a mutation would only be considered "relevant" if it appeared in two separate PCR reactions from the same carcinoma. Consequently, for each carcinoma, two independent PCR reactions were used, and the resulting PCR products were cloned. A total of 54–66 individual clones from each PCR reaction (108–114 clones per carcinoma) was sequenced throughout the 750 bp *neu* fragment. A total of 465,038 bases was sequenced from 660 clones. There was a total of 125 mutated bases, representing 0.0269% of the total sequenced. This extremely small error rate is well within the expected error rate due to misincorporations by DNA polymerase during PCR. No clones contained the previously described activating point mutations within the transmembrane and adjacent extracellular domains that are associated with mammary carcinomas in the *neu* TG mice models. Of the mutations that were found, only one (a C → T change of nucleotide 2137) was present in clones derived from both PCR reactions of the same tumor. However, this mutation did not result in a change of the encoding amino acid.

We next investigated whether the somatic *neu* mutants found in the TG mouse were capable of inducing mammary cancer in the rat if introduced into the gland exogenously. This experiment was conducted to address the possibility that *neu*

mutations might be arising spontaneously in the untreated female gland but not leading to the formation of mammary carcinomas. Therefore, we subcloned the activated *neu* oncogenes 8142 and 8567 into a retroviral expression vector and introduced retrovirus directly into the mammary gland ducts of 13–14 NTG female rats. We could not test these retroviral constructs in the male rat mammary gland, as the lack of nipples in the male rat precludes ductal infusion. *neu* 8142 and 8567 are examples of somatic constitutively active *neu* mutants containing deletions in the extracellular domain that are responsible for mammary carcinogenesis in the *neu* proto-oncogene TG mice (Siegel et al., 1994). These deletion mutants were compared against the previously characterized *neu* T, which contains a single activating point mutation in the transmembrane domain (Wang et al., 1991a). As with *neu* T, both *neu* 8142 and *neu* 8567 served as powerful oncogenes in the rat, rapidly transforming nearly all of the infused mammary glands in 100% of the animals.

TG mammary carcinomas express Neu and AR

Mammary carcinomas from TG males and females were compared histologically. All mammary carcinomas, irrespective of sex or androgen treatment, had a uniform morphology consisting of compact epithelium with a minimal amount of intratumoral stroma (Figures 4A and 4B). Occasionally, there was extensive invasion across the stromal capsule or into adjacent skeletal muscle. Abundant levels of Neu with clear membrane localization were confirmed in both male and female carcinomas from lines 6500 and 2477 by immunohistochemistry (Figures 4C–4E). Normal mammary gland adjacent to the carcinomas was always observed to have high Neu expression by immunohistochemistry. Quantitative comparison of neu mRNA levels in the intact, untreated TG male between mammary carcinoma ($n = 6$) and normal mammary gland ($n = 4$) revealed 12.6-fold higher neu expression in the carcinomas ($p = 0.0003$).

Expression of steroid hormone receptors in carcinomas from untreated or orchidectomized DHT-treated males and ovariectomized DHT-treated females were next determined by immunohistochemistry. All analyzed carcinomas were completely negative for both estrogen and progesterone receptor (ER, PR; data not shown). In contrast, epithelial expression of AR was observed in 100% of the evaluated male carcinomas from both TG lines (Figure 4F and data not shown). Carcinomas incubated with nonspecific rabbit IgG demonstrated lack of nuclear staining (Figure 4G). The status of AR also was assessed in carcinomas from both males and females treated with DHT. In both sexes, there was strong AR expression within the epithelial cells of the carcinomas (Figures 4H and 4I). Collectively, the mammary carcinomas in the *neu* transgenic rat expressed levels of AR close to that seen in the rat ventral prostate (Figure 4J).

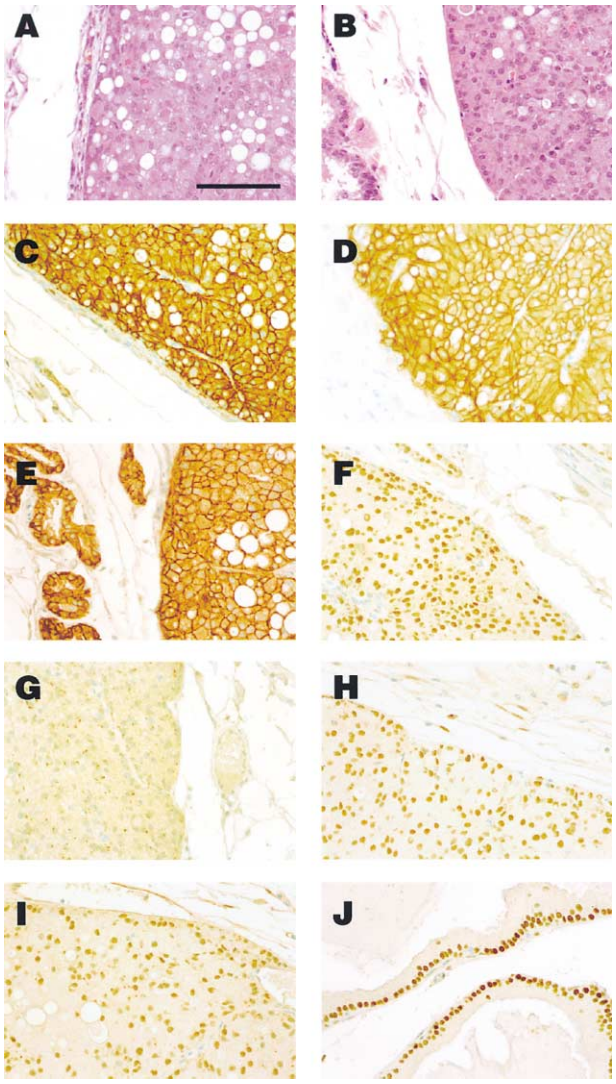

Figure 4. Mammary carcinomas in the *neu* TG rat express both Neu and AR

Mammary carcinoma sections from TG rats were stained with H&E (**A** and **B**), mouse monoclonal anti-Neu (**C**–**E**), rabbit polyclonal anti-AR (**F**, **H**, **I**), or nonspecific rabbit IgG (**G**). Rat ventral prostate from an old adult NTG male was stained for AR (**J**). Representative results are shown from multiple stained sections. Mammary carcinomas displayed are from TG male of line 6500 that was untreated (**A**, **C**, **F**, **G**) or orch + DHT (**H**), untreated TG male of line 2477 (**D**), and TG female of line 6500 that was ovx + DHT (**B**, **E**, **I**). The bar in **A** is 100 μm and the same scale was used for all panels. Abbreviations: orch, orchidectomy; ovx, ovariectomy.

Physiological levels of androgens are not necessary for neu transgene expression

We next addressed whether an intact hormonal environment was necessary for expression of the *neu* transgene regulated by the MMTV. This was done to determine if the absence of mammary carcinomas in the gonadectomized TG rats was due to loss of neu expression. Real-time RT-PCR was used to quantify the level of neu mRNA from mammary glands of old intact and long-term gonadectomized rats. The mean neu mRNA expression from each group was plotted against the mean number of mammary glands with carcinomas (Figure 5). As expected, serum levels of T were undetectable in both long-term gonadectomized TG males and females (<0.1 ng/ml, data not shown). Despite the absence of T, mammary gland expression of neu mRNA in gonadectomized TG males and females was not statistically different from that of the intact, untreated TG male. This suggests that other factors, such as glucocorticoids, are sufficient to maintain high neu expression levels in the absence of androgens. In contrast to neu mRNA levels being unaffected by gonadectomy alone, gonadectomized TG rats given exogenous DHT demonstrated increased neu mRNA expression in the mammary gland. Compared to their intact, untreated TG controls, DHT-treated males had 2.7-fold ($p = 0.021$) and DHT-

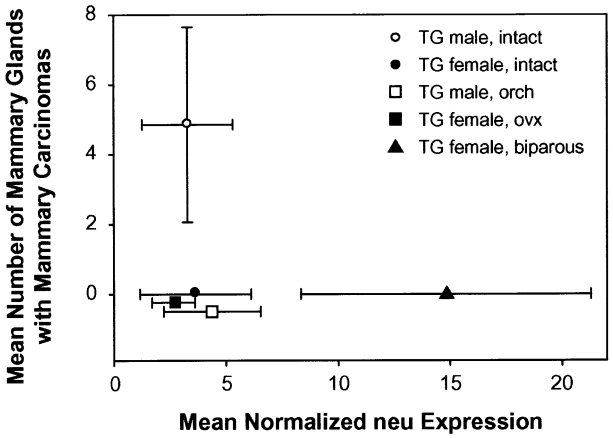

Figure 5. Physiological levels of androgens are not necessary for neu transgene expression

Mammary glands were collected from old (419–468 days) TG rats. Females were kept as virgins or consecutively mated twice as young adults (biparous), after which they remained untreated until the termination of the experiment. Rats were kept intact or underwent bilateral gonadectomy 1 year prior to necropsy. Total RNA was isolated and the level of neu mRNA was determined from 4–6 rats/group following normalization to 18S rRNA using quantitative RT-PCR. The mean neu mRNA expression per group is plotted against the mean number of mammary glands with carcinomas at the time of necropsy. Horizontal and vertical error bars represent SD for the mean neu mRNA expression and the mean number of mammary glands with carcinomas, respectively. Note that female (intact), female (ovx), and male (orch) are all actually at the zero point for the *y* axis. They are shown separated for clarity purposes only. Abbreviations: orch, orchidectomy; ovx, ovariectomy.

treated females had 3.6-fold ($p = 0.035$) higher neu mRNA expression.

We also determined levels of neu mRNA in the biparous TG female mammary gland approximately 235 days after the cessation of pregnancy. Compared to the intact, untreated TG male, neu mRNA expression in the biparous TG female was 4.5-fold higher ($p = 0.033$). This elevated level of neu mRNA expression was similar to that of the DHT-treated female, yet cancer did not arise in these biparous females. Overall, there was no correlation between elevated levels of neu mRNA in the various treatment groups and mammary carcinogenesis.

Neu overexpression substitutes for androgens in the maintenance of the male rat mammary gland morphology

Through histological analysis, we found that the morphology of the rat mammary gland is sexually dimorphic. The male mammary gland contains a high density of alveoli with solid nests of epithelium devoid of a lumen (Figure 6A). In contrast, the female mammary gland is made up of sparse alveoli with a single layer of epithelium arranged around a central lumen (Figure 6B). Mammary gland morphology did not differ between intact TG and NTG rats. Orchidectomy of young male rats resulted in dramatic morphological changes to the mammary gland in both TG and NTG males (Figure 6C). The mammary epithelial content was markedly reduced. This was accompanied by a loss of central epithelium from the alveoli and the development of a lumen. These morphological changes were established by 1 month after orchidectomy. These changes in the mammary epithelium were proven to be due to androgen ablation, as reconstitution of the androgenic environment with DHT completely restored the mammary gland morphology to that of the intact male (Figure 6D). Furthermore, females maintained on DHT also gained a mammary morphology similar to that of the intact male

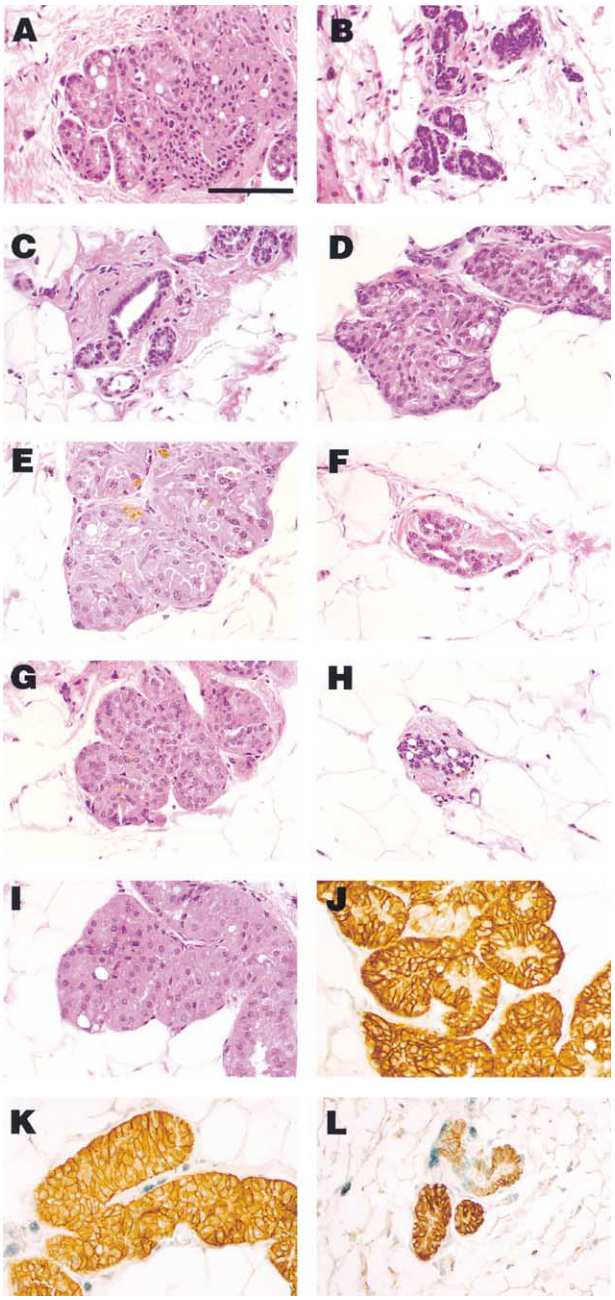

Figure 6. Neu overexpression substitutes for androgens in maintenance of the male rat mammary gland morphology

Mammary gland sections from young (78–82 days; **A**–**C**, **L**) or old (419–467 days; **D**–**K**) adult rats were stained with H&E (**A**–**I**) or mouse monoclonal anti-Neu (**J**–**L**). Sections from at least four rats were evaluated for each group. Representative results are shown from NTG intact male (**A**), NTG intact female (**B**), TG orch (for 1 month) male (**C**, **L**), NTG orch + DHT (for 1 year) male (**D**), NTG ovx + DHT (for 1 year) female (**E**), NTG orch (for 1 year) male (**F**), TG orch (for 1 year) male (**G**, **J**), NTG ovx (for 1 year) female (**H**), and TG ovx (for 1 year) female (**I**, **K**). The bar in **A** is 100 μm and the same scale was used for all panels. Abbreviations: orch, orchidectomy; ovx, ovariectomy.

(Figure 6E). Both TG and NTG mammary glands were restored by DHT treatment.

We next evaluated the effect of neu overexpression on mammary gland morphology in the long-term gonadectomized TG rats without androgen supplementation compared to age- and treatment-matched NTG controls. One year after orchidectomy, the mammary epithelium of all NTG male rats had persisted in a severe, atrophied state (Figure 6F). However, in striking contrast, the epithelial morphology of orchidectomized TG males had been restored to that of the intact male (Figure 6G). Morphological changes also were apparent in TG females. One year after ovariectomy, the NTG female gland consisted largely of atrophied epithelium (Figure 6H). However, 63% (5/8) of the ovariectomized TG females displayed partial conversion of the mammary epithelium to that of an intact male (Figure 6I). In order to determine if these changes to the mammary epithelium were due to Neu overexpression, we performed immunohistochemistry on mammary gland sections from the long-term gonadectomized rats. While the atrophic epithelium of the NTG rats was negative for Neu expression (data not shown), the intact male-like mammary epithelium of the gonadectomized TG males (Figure 6J) and females (Figure 6K) expressed abundant Neu. We then evaluated Neu expression in the young TG males and females 1 month after gonadectomy (Figure 6L and data not shown). Although the epithelium was atrophied at this early point after gonadectomy, in both sexes there was a small subpopulation of epithelial cells overexpressing Neu. The stimulation of mammary epithelial growth in the long-term gonadectomized TG males and females was independent of androgens, as all of the gonadectomized mammary glands were negative for AR expression (data not shown).

Mammary carcinomas in the *neu* TG rat acquire the ability to grow in an androgen-depleted environment

Two additional questions of interest were whether established mammary carcinomas would display a strict androgen dependence for growth and whether Neu overexpression could promote the emergence of mammary cancer in an androgen-depleted environment. To address these issues, untreated TG males with established mammary carcinomas underwent orchidectomy at 423–430 days of age. The rats were monitored by palpation to ascertain the effects of orchidectomy on tumor maintenance (Figure 7A). Complete regression was defined as the point at which there were no palpable tumors. By 69 days after orchidectomy, 75% of the rats had undergone complete regression with a mean time of 46 days. However, after a period of time averaging 96 days without palpable lesions, tumors began to reemerge. By the time the rats were 598–605 days of age, which was approximately 6 months after orchidectomy, the mammary carcinoma incidence was 88%, involving an average of 1.4 mammary glands/rat.

To verify that tumor regression was not simply a consequence of loss of neu, we performed real-time RT-PCR to measure the level of neu mRNA in carcinomas from an additional cohort of rats 10 days following orchidectomy. By this time, serum testosterone levels were undetectable ($<$0.1 ng/ml, data not shown), and many of the tumors were actively regressing. The level of neu mRNA in these carcinomas was compared with those present 6 months after orchidectomy as well as carcinomas in intact, untreated males (Figure 7B). Orchidectomy had no significant effect on neu mRNA expression within the carcinomas. We further extended these findings by using immunohistochemistry to stain carcinomas for Neu expression 10 days (Figure 7C) or 6 months (Figure 7E) after orchidectomy. These experiments confirmed that the carcinomas continued to express high levels of Neu in an androgen-depleted hormonal environment. We also assessed the AR status of the carcinomas present 6 months after orchidectomy and all were found to be negative (Figure 7F). The histological morphology of these carcinomas did not differ from those present in intact, untreated males (Figure 7D).

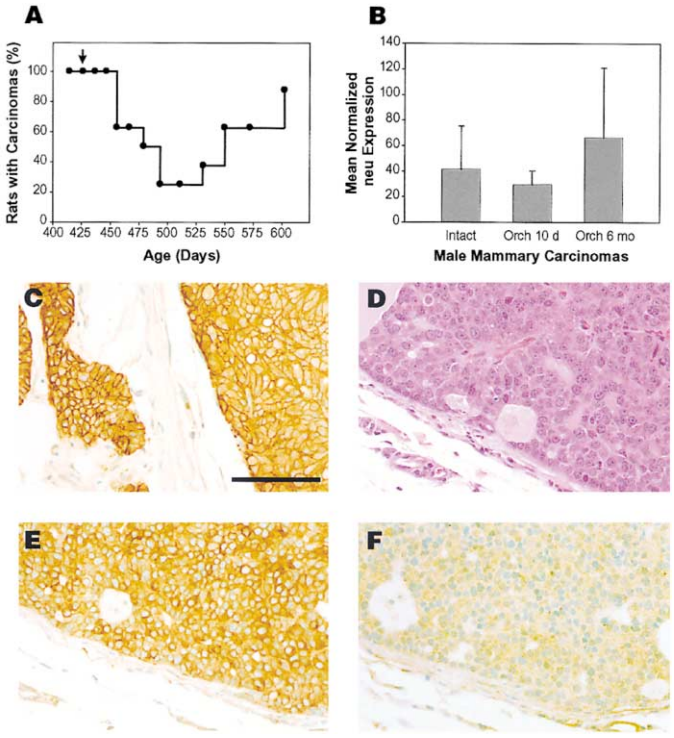

Figure 7. Mammary carcinomas in the *neu* TG rat acquire the ability to grow in an androgen-depleted environment

A: Old TG males bearing at least one mammary carcinoma measuring 10 mm in diameter underwent bilateral orch (at age indicated by the arrow) and were regularly palpated for mammary carcinomas ≥3 mm in diameter until necropsy at 602 days of age (n = 8). The percentage of rats bearing at least one carcinoma at each palpation time point was plotted against the age of the rats in days. **B:** Total RNA was isolated from mammary carcinomas of old TG males and the level of neu mRNA was determined after normalization to 18S rRNA by quantitative RT-PCR. Carcinomas were collected from males that were untreated (intact), or orch for either 10 days (orch, 10 dy) or 6 months (orch, 6 mo). Five to six carcinomas from each group were analyzed. The data is presented as the mean neu mRNA expression for each group + SD. **C–F:** Mammary carcinoma sections from TG males were stained with mouse monoclonal anti-Neu (**C** and **E**), H&E (**D**), or rabbit polyclonal anti-AR (**F**). Representative results are shown from at least 3–4 rats/group for orch, 10 dy (**C**) or orch, 6 mo (**D–F**). The bar in **C** is 100 μm and the same scale was used for all panels. Abbreviation: orch, orchidectomy.

Discussion

In this report, we describe a TG rodent model in which mammary cancer arises due to overexpression of the nonmutated Neu proto-oncogene. This contrasts with an earlier TG rat model of the human *neu* proto-oncogene described by Davies et al., in

which almost no mammary cancers arise in TG females. In addition, virgin female TG rats described by Davies et al. had no transgene expression in the mammary gland (Davies et al., 1999). This is a key difference with our *neu* TG females described here, which display high Neu overexpression within the virgin mammary gland. Davies et al. did not evaluate the mammary glands of male TG rats, so it is not possible to compare our findings in *neu* TG males with those of theirs. However, the absence of mammary cancers in the rats described by Davies et al. could likely be explained by their very low neu expression in the mammary gland. In agreement with this hypothesis, we have not observed mammary cancers in male or female TG rats from several *neu* proto-oncogene TG lines that display very low to nondetectable levels of neu in the mammary gland. These observations imply that there might be a threshold level of neu overexpression required to induce mammary cancer.

We thoroughly searched for mutations within the *neu* transgene from mammary carcinomas from both untreated TG males and rats of both sexes treated with DHT. Sequencing of the entire *neu* coding region from 11 carcinomas failed to find any mutations in pooled PCR products. Because we found the *neu* transgene to be moderately amplified in mammary carcinomas, we also analyzed 660 individual clones containing a fragment of *neu* cloned from both TG male and female mammary carcinomas for mutations. This region of *neu* almost always contains oncogenic mutations in mammary carcinomas from the *neu* proto-oncogene TG mouse (Siegel et al., 1994; Siegel and Muller, 1996). Despite our extensive searching, we did not find any significant mutations.

In addition, we considered the possibility that activating mutations might be arising in female rats but not leading to mammary cancer. Therefore, activating *neu* deletion mutants (Siegel et al., 1994) were introduced into the female rat mammary gland via retroviral infusion to ask whether these *neu* mutants could serve as oncogenes in the rat. Mammary cancer was readily induced in 100% of the infused females, confirming the potent oncogenicity of these Neu mutants in the rat mammary gland. Therefore, since no mammary cancer spontaneously develops in the untreated *neu* proto-oncogene TG female rat, we infer that these somatic activating deletion mutations are not arising. Hence, our model shares an important attribute with human breast cancers, which are not associated with mutational activation of the *neu* proto-oncogene (Lemoine et al., 1990).

Although we used the same transgene construct to create our TG rats as that utilized by Muller and colleagues for production of *neu* TG mice, we nevertheless observed striking phenotypic differences from the mouse model. In the *neu* TG rat, mammary carcinogenesis was androgen dependent and arose spontaneously only in the male. Significantly, carcinogenesis could be induced in ovariectomized TG females, but only if they were treated with androgens. This is in complete contrast to the *neu* TG mice, in which mammary carcinomas develop only in females and males have no lesions (Guy et al., 1992). The male rat mammary cancer phenotype has been demonstrated in two independent TG lines (6500 and 2477), and in both lines these cancers displayed expression of Neu and AR. Therefore, the novel phenotype that we describe here is not likely to be simply a byproduct of a unique integration site for the transgene.

To exclude the possibility of sex differences in transgene expression accounting for the male-specific cancers, we used a real-time RT-PCR assay to quantify the levels of neu mRNA in the male and female mammary glands under various hormonal states. This analysis revealed that intact TG males and females expressed equivalent amounts of neu mRNA at 419–434 days of age, a time when nearly 100% of the males had multiple mammary carcinomas. Furthermore, neu mRNA expression was maintained at the same level as the intact male in long-term gonadectomized rats of both sexes. In addition, the biparous female displayed a level of neu mRNA expression 4.5-fold higher than even the intact TG male. These important findings led to two conclusions. First, neu levels per se do not determine the susceptibility of the mammary gland to carcinogenesis. Secondly, physiological levels of androgens are not needed to maintain expression of the *neu* transgene. An implication from these experiments is that androgens serve a necessary function for Neu-mediated mammary carcinogenesis apart from transcriptional regulation of the MMTV LTR.

Consistent with this hypothesis, all of the mammary carcinomas analyzed from males and females were AR positive but were negative for both ER and PR. This makes it unlikely that either ER or PR is making a major contribution to mammary carcinogenesis in the *neu* TG rat. This is consistent with human breast cancers, for which there is an inverse correlation between ER, PR, and Neu overexpression (Berns et al., 1992; Ito et al., 1995; Quenel et al., 1995; Zeillinger et al., 1989). Indeed, in breast cancer cell lines, ER and Neu downregulate each other (Pietras et al., 1995; Read et al., 1990). These observations have led to the general idea that Neu is preferentially associated with hormone-independent breast cancers. However, the potential role of AR in breast carcinogenesis has received little attention compared to ER and PR. This is despite the fact that AR expression has been demonstrated in 31%–85% of breast cancers using a variety of methodologies (Allegra et al., 1979; Hall et al., 1998; Isola, 1993; Lea et al., 1989; Miller et al., 1985). Few reports have evaluated associations between Neu and AR in breast cancer (Isola, 1993; Kollara et al., 2001; Zeillinger et al., 1989). None of these studies found a positive correlation between the two proteins. It is, however, difficult to draw firm conclusion from these reports as all consisted of relatively small sample sizes.

Nevertheless, we present evidence here that, in the *neu* TG rat, there are interactions between the AR and Neu signaling pathways that regulate growth of both the normal and cancerous gland. In the normal, NTG rat, the male mammary gland was found to be strictly dependent on physiological levels of androgens to maintain its normal morphology. In addition, the normal female rat mammary epithelium underwent morphological transition to that of a male rat in response to long-term androgen treatment. These observations underscore the critical role that androgens serve in the normal rat mammary gland. An intriguing finding was that Neu overexpression in the TG male rat was able to rescue mammary morphology after long-term orchidectomy. This restoration occurred despite almost complete androgen ablation. Even more surprising was the female-to-male morphological conversion in the mammary glands of ovariectomized TG females. Neu overexpression was not able to prevent initial gonadectomy-induced mammary gland atrophy in the transgenic rats. However, the clear presence of a subpopulation of epithelial cells overexpressing Neu in the young, gonadectomized TG gland strongly suggests that these cells were imparted with a selective growth advantage, allowing for eventual mor-

phological restoration with a minimal level of androgens. The cancerous mammary epithelium also benefited from Neu overexpression in an androgen-depleted environment. Although most of the mammary carcinomas were initially androgen dependent and regressed in response to orchidectomy, mammary cancers eventually reemerged several months after androgen deprivation.

In both the normal gland and the mammary cancers after long-term gonadectomy, there was no detectable expression of AR. Given the requirement for androgens in maintenance of the epithelium following gonadectomy, these results suggest that these growth-stimulatory effects of Neu are mediated in part through the AR pathway in a process that is independent of the receptor itself. As residual amounts of androgens remain even after long-term orchidectomy, it is not possible to determine from our data whether these are truly androgen-independent mammary epithelial cells or rather are hypersensitive to the low levels of androgens that remain. However, the fact that all of these mammary glands were negative for AR would suggest the former, although more sensitive protein assays would be needed to completely rule out the presence of AR.

Our report shows interplay between the Neu and AR signaling pathways within the mammary gland. Recently, it was reported that Neu and AR crosstalk in the prostate. Androgen-independent xenografts spontaneously developed elevated Neu (Craft et al., 1999). In addition, forced overexpression of Neu in the androgen-dependent prostate cancer cell line LNCaP resulted in the acquisition of androgen-independent transactivation of AR target genes and growth (Craft et al., 1999; Yeh et al., 1999). Furthermore, this Neu-mediated AR transactivation was dependent upon a functional MAP kinase pathway (Yeh et al., 1999), which is a known downstream effector of Neu (Pinkas-Kramarski et al., 1996). Another group subsequently indicated Akt was also critical for Neu-AR crosstalk (Wen et al., 2000). Several clinical studies have corroborated the earlier in vitro experiments. The percentage of prostate cancers overexpressing Neu increased with clinical progression to androgen independence (Osman et al., 2001; Shi et al., 2001; Signoretti et al., 2000). One report, however, failed to find such a relationship (Reese et al., 2001). Overall, the evidence to date strongly supports the hypothesis that Neu is a key mediator in the evolution of androgen-independent prostate cancer. With the addition of our findings here, we extend those observations first established in prostate cancer and suggest that interactions between Neu and AR may be important in the etiology of a subset of breast cancers, and in particular with escape from androgen dependency.

Besides possible revelations into the mechanisms underlying Neu-mediated mammary carcinogenesis, the TG rats that we describe here could have more general applications in furthering the understanding of breast cancer. It may be a useful model for male breast cancer, for which there are no well-characterized models. Although only 1% of breast cancers occur in men (Ravandi-Kashani and Hayes, 1998), the disease generally results in a less favorable outcome compared to its female counterpart and is even less well understood. The fact that Neu also is overexpressed in 17%–50% of male breast cancers provides a further clinical connection between our model and the human male disease (Bruce et al., 1996; Dawson et al., 1992; Joshi et al., 1996; Rayson et al., 1998; Shpitz et al., 2000; Temmim et al., 2001; Willsher et al., 1997). The observation that ovariectomized TG female rats treated with androgens developed mammary cancer at high incidence means that this model could also help to clarify the role of testosterone in postmenopausal female breast cancer. Several prospective clinical studies have shown that an elevated level of testosterone is a major risk factor for breast cancer in postmenopausal women (Berrino et al., 1996; Cauley et al., 1999; Dorgan et al., 1996; Thomas et al., 1997). Additionally, treatment of both male (Liao et al., 1998) and female (Xie et al., 1999) Noble rats with a combined regimen of testosterone and estrogen induced a high incidence of mammary cancer. Our model, therefore, provides an additional tool to study the role of androgens in breast cancer with the further advantage that coincident estrogen treatment is not necessary for mammary carcinogenesis.

In summary, the work that we present here could lead to a greater understanding of the role of androgens in mediating breast cancers in both men and women. In particular, it suggests the need to extend these findings to a study of breast cancer patients. It will be important in this study to examine the correlation of serum androgen levels with the overexpression of Neu in postmenopausal women with breast cancer.

Experimental procedures

Generation of TG rats

Linearized plasmid encoding the rat *neu* proto-oncogene under the MMTV (pMMTVneuN) (Guy et al., 1992) was microinjected into the pronuclei of Sprague-Dawley single-cell zygotes. The technical aspects of generating TG rats have been described (Charreau et al., 1996). Microinjections were performed by the Transgenic Animal Facility at the University of Wisconsin. Of 12 founder lines generated, 10 displayed Mendelian inheritance. To date, four lines have been evaluated. Lines 4311 and 6490 did not express the transgene in either sex and were without phenotype. Two lines (6500, 2477) overexpressed Neu in the male and female mammary gland, with development of male mammary cancer. Line 6500 was further characterized. All experiments are from line 6500 unless otherwise specified. TG rats used in all experiments were heterozygous for the transgene. Screening for the transgene was performed by PCR using primers directed against the SV40 region of the transgene or *neu*. These primers did not amplify endogenous *neu*. SV40 primers were 5′-ACTCCACACAGGCATAGAGTGTCTGC-3′ and 5′-AGGACACAGAGGAGCTTCCTGGGGAT-3′. *neu* primers were 5′-CGG AACCCACATCAGGCC-3′ and 5′-TTTCCTGCAGCAGCCTACGC-3′.

Animal surgeries and hormone treatment

All protocols involving animals were approved by the Animal Care Committee at the University of Wisconsin-Madison. Rats were gonadectomized by standard surgical techniques. For androgen treatment, two 4 cm Dow Corning Laboratory Grade Silastic tubes, i.d. 1.96 mm, o.d. 3.18 mm, (Fisher, Pittsburgh, PA) were filled with either T or 5α-DHT (Sigma, St. Louis, MO) and sealed with Silicone Medical Adhesive (Factor II, Lakeside, AZ). The Silastic tubes were implanted s.c. and replaced after 8 months.

Real-time RT-PCR

Frozen tissues were homogenized with a Polytron and total RNA was isolated using the TRI Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's protocol. After treatment of the RNA with DNA-free DNase I (Ambion, Austin, TX), two-step real-time quantitative RT-PCR was performed on the ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA) using components from Applied Biosystems. A multiplex TaqMan assay was run with simultaneous amplification of *neu* and 18S rRNA. For *neu*, the forward primer was 5′-GCTGCTGCAGGAAACT GAGTTA-3′ and the reverse primer was 5′-CTGATCCAAGCACCTTCAC CTT-3′. The *neu* TaqMan probe was 5′-6FAM-ATGCCCAACCAGGCTCAG ATGCG-TAMRA-3′ (Applied Biosystems). Primers and probes for 18S rRNA were from the Ribosomal RNA Control Reagents (Applied Biosystems). Both *neu* primers were used at a final concentration of 500 nM, while the *neu*

probe was used at 125 nM. Each 18S rRNA primer was used at 50 nM and the 18S rRNA probe was at 200 nM. A cDNA standard curve was prepared from rat kidney total RNA (Ambion). The relative level of neu mRNA expression was determined after normalization to 18S rRNA.

Histology and immunohistochemistry

Tissues were fixed in 10% neutral buffered formalin and routinely processed for histological analysis. After deparaffinization and rehydration, antigen unmasking was done by microwaving in Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA) for Neu, ER, and PR or 5 mM EDTA for AR. Endogenous peroxidases were quenched by soaking the sections in 3% H_2O_2, followed by blocking in 10% horse or goat serum. Primary antibodies and final concentrations used were mouse anti-Neu (Ab-17, NeoMarkers, Fremont, CA) at 0.5 μg/ml, mouse anti-ER (Ab-14, NeoMarkers) at 1:200, mouse anti-PR (Ab-10, NeoMarkers) at 5 μg/ml, and rabbit anti-AR (N-20, Santa Cruz Biotechnology, Santa Cruz, CA) at 1 μg/ml. Positive control sections for ER were human breast carcinoma (NeoMarkers) and rat uterus and for PR were rat mammary carcinoma (NeoMarkers) and rat uterus. As a negative control, normal mouse or rabbit IgG was substituted for the primary antibody at an equal concentration for all experimental samples (Santa Cruz). Secondary antibodies and ABC Reagent were from the Vectastain Elite ABC-Peroxidase Kit and used according to the manufacturer's protocol (Vector Laboratories). Sections were developed using the DAB Substrate Kit followed by counterstaining with VECTOR Methyl Green (Vector Laboratories). The slides were dehydrated and coverslips mounted with VectaMount mounting media (Vector Laboratories).

SDS-PAGE and Western blotting

Frozen mammary tissue was homogenized with a Polytron in 2 ml of ice-cold homogenization buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 2 mM EDTA, 10 mM Na_2HPO_4, 10 mM $Na_4P_2O_7 \cdot 10\ H_2O$, 1% Triton X-100, 0.1% SDS, 0.5% Na deoxycholate, 100 μg/ml soybean trypsin inhibitor [Roche Molecular Biochemicals, Indianapolis, IN], 1% Protease Inhibitor Cocktail Set III [Calbiochem-Novabiochem, San Diego, CA], 1% Phosphatase Inhibitor Cocktail Set II [Calbiochem-Novabiochem]). Protein concentration of the lysate was determined by the BCA assay (Pierce, Rockford, IL). Fifty micrograms of lysate was run in a 7.5% SDS-PAGE using the Mini-PROTEAN 3 Cell (Bio-Rad, Hercules, CA). Separated proteins were transferred to Immobilon-P PVDF membranes (Millipore Corporation, Bedford, MA) using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) in an overnight transfer. The membrane was blocked with TBST + 5% dry milk followed by incubation with primary antibody for 2 hr at room temperature. Primary antibodies and final concentrations used were mouse anti-Neu (Ab-17, NeoMarkers) at 1 μg/ml and mouse anti-α-tubulin (Santa Cruz) at 0.5 μg/ml. Goat anti-mouse, peroxidase-conjugated secondary antibody (Pierce) was used at 0.04 μg/ml. Signal was detected with the SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce) and developed on film.

FISH

All FISH experiments were conducted by SeeDNA Biotech (Windsor, Ontario, Canada). Paraffin-embedded tissues were treated with Proteinase K, dehydrated, and then hybridized with biotinylated transgene cDNA probe. The methods for FISH and DAPI banding identification have been previously described (Heng et al., 1992; Heng and Tsui, 1993).

Sequencing of the *neu* transgene

DNA was isolated from frozen mammary carcinomas. All PCR reactions were performed with Platinum Pfx DNA Polymerase (Invitrogen, Carlsbad, CA). For sequencing of the entire *neu* coding region, primers were used that flanked this area. The forward and reverse primers were 5′-CAT AGTGTTTTGGGGGTTGG-3′ and 5′-GTTCCTGGTAGAGCCGTCTG-3′, respectively. The 4.3 kb PCR product was then sequenced using the following primers: 5′-ACCGCCACAGACATGAAGTT-3′, 5′-GGTGAGAGTCCGGAA GACTG-3′, 5′-CCTGAGCAGCTCCAAGTGTT-3′, 5′-CTTTGGATCGGAGGCT GAT-3′, 5′-GTATGTGATGGCTGGTGTGG-3′, 5′-TACCTCAGCCTCCAATC TGC-3′, 5′-TGACCTCAGCCCTCTACAGC-3′, and 5′-CAAAACTCCTCCCT TCAGGA-3′. For clonal sequence analysis, a 1.3 kb fragment of *neu* was PCR amplified using forward and reverse primers 5′-CCTGAGCAGCTC CAAGTGTT-3′ and 5′-CGTCCTCCAGGTAGCTCATC-3′, respectively. PCR products were cloned using the Zero Blunt PCR Cloning Kit (Invitrogen). Approximately 750 bp of the *neu* insert was sequenced from multiple individual clones using the primer 5′-CTTTGGATCGGAGGCTGAT-3′.

Ductal infusion of retroviral *neu* mutant vectors

neu 8142 and *neu* 8567 from pJ4Ωneu8142 and pJ4Ωneu8567 (Siegel et al., 1994) were subcloned into the retroviral expression vector pJR (Wang et al., 1991b). The construct JR/*neu* T was generated previously and reported as JR/*neu* (Wang et al., 1991a). The preparation of concentrated retroviral stocks and ductal infusion into the rat mammary gland has been described in detail (Thompson and Gould, 2000). All retroviral *neu* constructs were infused at a titer of 1×10^7 CFU/ml.

Statistics

The level of neu gene expression was modeled using analysis of variance. Factorial models were fit to various subsets of the data. Likelihood ratio tests were used to eliminate predictor variables that did not significantly improve the fit of the models. For significant main effects and interactions with more than two levels, tests were conducted to assess pairwise differences. The response was transformed to the log scale before analysis to obtain approximately normally distributed residuals.

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