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Original Articles

A Mouse Model for Breast Cancer Induced by Amplification and Overexpression of the *neu* Promoter and Transgene

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Abstract

Background: ErbB-2 is a critical oncogenic marker in human breast cancer. Its appearance correlates with poor prognosis and it is, therefore, an important target for physiologic investigation and therapeutic intervention. With this in mind, we have created and characterized two mouse breast cancer models that express rat wild type *neu,* the homologue of *ErbB-2,* and rat mutant *neu* under the control of the normal mouse *neu* promoter. These models in which the copy number of the neu gene is moderately amplified should more closely parallel the expression pattern of ErbB-2 seen in some cases of human breast cancer.

Materials and Methods: Transgenic mouse models were constructed by injecting one of the two pronuclei of a fertilized FVB/n egg and implanting it into a pseudopregnant Swiss /Webster mouse. Tissue expression was analyzed through the use of reverse transcription polymerase chain reaction and mammary histopathology examined by fixing, staining and mounting of the entire gland.

Results: In the former wild type model, we show that low level, long term expression of *neu* leads to abnormal lobuloalveolar development in virginal glands and incomplete regression in multiparous glands. Malignant foci form following multiple rounds of pregnancy and regression. In the latter model, a similarly directed transgene carrying the constitutively activated, mutant form of the rat neu gene, a stronger but similar phenotype is displayed.

Conclusion: Evidently minor perturbations in amplified neu expression are sufficient to alter mammary development and induce malignant transformation.

Introduction

The neu oncogene was first isolated from DNA of adrenal neuroblastomas of neonatal rats (1). The human homologue of this gene was later identified (2–4) and named *c-erbB-2* in reference to its homology to the *c-erb-B* proto-oncogene. Since its discovery, *c-erbB-2* amplification and over-expression have been associated with a number of human malignancies, including breast, colon, lung and ovarian (5–7). Amplification of this gene in breast cancer is seen in 17.5% to 34% of tumors examined, with copy numbers ranging from 4 to 50 (8,9). Amplification of *c-erbB-2* correlates with a higher risk for metastatic disease (10,11), as well as shortened disease-free and overall survival periods (12). This is most likely due to the fact that amplification is closely correlated to over-expression of *c-erbB-2* (13,14).

The transforming potential of *erbB-2/neu* has been demonstrated in a number of ways. In

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vitro transformation assays have been utilized to show that high levels of expression of *c-erbB-2* mediate transformation in NIH3T3 cells (15), as determined by tumor formation after injection into athymic mice and in anchorage-independent growth assays. The level of expression has been shown to be critical for transformation. Mouse mammary tumor virus (MMTV)–based expression vectors with *neu* were proven to achieve transformation in the NIH3T3 cell line; whereas, SV40–driven *neu,* at a 20-fold lower level of expression, failed to do so (16,17). The effect of over-expression in murine mammary epithelia was analyzed through construction of MMTV/*neu* transgenic mice (18). Multiparous female mice with the oncogenic rat *neu* (MMTV/*neu**) develop mammary adenocarcinomas at an average age of three months. MMTV promoter–based overexpression of the proto-oncogenic form of rat *neu* (MMTV/*neu*) also results in focal breast tumors but with a longer latency (19).

In order to further understanding of cellular *neu*'s in vivo regulation, the 5' upstream region of the transcriptional start site of the mouse gene was cloned and characterized (20). Chloramphenicol acetyltransferase (CAT) assays were used to determine functional variation in transcriptional activity among various lengths of the upstream region in NIH3T3 cells. One such construct containing 1.2 kb 5' flanking sequence was found to have CAT levels at thirteen fold higher than that of the full length 4.5 kb promoter.

Most mouse models developed to study the effects of over-expression of an oncogene in the mammary gland utilize extremely strong promoters that allow for high levels of transcriptional activity (21). Although these animals have proven useful in the study of signal transduction in the transformed tissue, the promoters they utilize are—of necessity—nonphysiologic. Not only are artificially elevated levels of oncogene expression allowed by these promoters, but, they are also not necessarily spatially nor temporally regulated in a physiologic manner. Although most human breast cancers are not associated with current pregnancy, most of the promoters that are widely used in mouse models of mammary gland tumorigenesis require the induction of pregnancy to reach high levels of transcriptional activity (22,23). *Neu* is expressed in the virgin, pregnant, lactating and regressing mammary gland of the mouse (24), making it difficult for transgene promoters, such as those of MMTV or the whey acidic protein (WAP), to accurately model the consequence of amplification of the *neu* gene throughout the course of murine development. We desired to create a model that involves overexpression of *neu* in a manner more consistent with that seen in human breast cancer. By constructing transgenic mice containing the first 1.2 kb upstream sequence of the mouse *neu* gene fused to either the cellular or transformed (Val664Glu) rat *neu* transgene, we designed a system that attempts to mimic cases of human breast cancer involving amplification of the *erbB-2* locus in expression and regulation.

Materials and Methods

Cloning the Murine neu *Promoter*

A 544 base pair fragment of the *neu* promoter was amplified by polymerase chain reaction (PCR) from mouse genomic DNA using the primers 5'-GATATCCCAGAGAGTCTT-3' and 5'-TCAGGCTGGACCAGGCTGCG-3', and ligated into pCR (Invitrogen, Carlsbad, CA). This was then used as a probe to screen a 129 mouse genomic library (Genome Systems, St. Louis, MO). The clones were digested with *Nco I* and *Xba I* in order to isolate the 1.2 kb fragment and blunt end cloned into the *pSV2IgH/neu* or *pSV2IgH/neu** vector from which the IG promoter/enhancer had been excised by *Hind III* digest.

Mammary Gland Whole Mounts

The inguinal mammary gland fat pad was excised with scissors and forceps and spread on a glass slide. It was allowed to air dry for 1 min before overnight incubation in Tellyesniczky's fixative (70% ethanol:formaldehyde:glacial acetic acid at 20:1:1 ratio) at room temperature. Tissue was rinsed with running water for 1 hr followed by three 6-hour incubations in acetone. Mammary glands were then rehydrated by successive washings in 100%, 95% and 70% ethanol, followed by 30 min in running water. Tissues were then stained overnight in Carmine Red Stain [1 gram carmine red dye (Sigma, St. Louis, MO), 2.5 g potassium alum in 500 ml water]. After washing tissues in running water for 2 hr they were dehydrated in successive washes of 50%, 70%, 95% and 100% ethanol, followed by two overnight incubations in xylene.

Histologic Evaluation

Upon necropsy, tissue samples were preserved in Optimal Fix (American Master Tech Scien-

tific, Inc., Lodi, CA). 4 μm sections were cut and stained with hematoxylin and eosin by the Transgenic Core Pathology Laboratory at the University of California at Davis, CA.

Transgene Injection

The pNcN plasmid was linearized with *Pvu I.* Approximately 10 μg of DNA was run on a 1% agarose gel. The appropriate sized band was excised and the DNA was purified on a Qiagen column. The DNA was diluted in injection buffer (0.15 M KCl, 5 mM NaCl, 10 mM PIPES) at a concentration of 1–10 ng/μl. The DNA was then injected into one of the two pronuclei of a fertilized FVB/n egg and implanted into the oviduct of a pseudopregnant Swiss/Webster mouse.

Identification of Transgenic Animals

DNA was prepared from tail samples by incubating tails overnight at 50°C in 500 μl Tail Buffer (17 mM Tris [pH 7.5], 17 mM EDTA, 170 mM NaCl, 0.85% SDS and 0.2% proteinase K added immediately prior to use). After proteins were pelleted in 250 μl 6M NaCl and centrifuged, DNA was precipitated in 95% ethanol and resuspended in 100 μl water. The DNA was electrophoresed through a 0.8% agarose gel after complete digestion with *BamHI* restriction enzyme. The gel was blotted overnight in 0.4 N sodium hydroxide onto a Genescreen Plus membrane (NEN Research Products, Boston, MA). The filter was then rinsed for 10 min in 2× SSC (0.30 M sodium chloride, 0.030 M sodium citrate) and crosslinked by ultraviolet light. Filters were prehybridized for at least 1 hr at 42°C in hybridization solution (50% formamide, 5× SSC, 5% dextran sulfate, 20 mM sodium phosphate, 1× Denhardt's reagent, 0.5% sodium dodecyl sulfate and 20 μg/ml sonicated herring sperm DNA) before probe was added. An 800-basepair *Pst I* fragment of SV40 polyA vector (18) was labeled with [$\propto^{32}$P]dCTP using random priming (Stratagene, La Jolla, CA) and hybridized to the membrane overnight at 42°C. Filters were washed at room temperature twice for 15 min in 2 × SSC/0.1% SDS and once for 10 min in 0.1 × SSC/0.1% SDS before exposing to film.

Tumor Lysates and Western Blot Analysis

Frozen tumor was homogenized in 2 ml NP-40 lysis buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1% NP-40, 2 mM EDTA, 1 mM sodium orthovanadate, 10 mM NaF plus one Complete tablet (Roche Diagnostics, Chicago IL)] for 3 min on ice and incubated at 4°C for 30 min. The lysates were spun at maximum speed in a microcentrifuge at 4°C for 20 min and the supernatant was transferred into a clean Eppendorf tube. Equal concentrations of protein, as determined using the BioRad dye-binding microassay with bovine serum album as a standard, were electrophoresed through 8% SDS-PAGE gels. Bound proteins were then transferred to a PVDF membrane (Millipore, Bedford MA) and blocked overnight in blocking buffer (1× TBS with 0.1% Tween-20 and 5% nonfat dried milk). Immunodetection was performed using anti-*neu* Ab-3 (Oncogene Research Products, Cambridge, MA) at a dilution of 1:200. Signal development was performed using an enhanced chemiluminescence method (Amersham, Piscataway, NJ).

Transfections into MCF-7 Cells

CalPhos Maximizer (Clontech, Palo Alto, CA) was used to transfect MCF-7 cells with DNA at a concentration of 5.0 μg of the construct of interest and 0.5 μg of marker in 35 mm plates (Corning, Acton, MA). Cells were incubated 4.5 hours at 37°C and 5% CO_2 in DMEM (Gibco-BRL, Gaithersburg, MD) supplemented with 10% FBS (Sigma), 2% L-glutamate (Gibco-BRL) and 1% penicillin/streptomycin (Gibco-BRL). The cells were washed twice with 1× PBS and incubated overnight in supplemented DMEM, and expanded into 15 cm plates and allowed to grow for 24 hr. Geneticin (Gibco-BRL) was added to DMEM at a concentration of 800 μg/ml. After allowing 3 days in selection media for non-transfected cells to die, clones were picked and expanded.

RT-PCR/Southern

Organs were excised, frozen on dry ice and stored at −70°C. Reverse transcription was performed on 2 μg of total RNA isolated using RNA STAT-60 (Tel-Test, Inc, Friendswood, TX). cDNA was generated using Superscript II reverse transcriptase (Gibco-BRL) following the manufacturer's protocol. The forward and reverse primers used for amplification of the SV40 polyA region were 5'-CCTTACTTCTGTGGTGTGAC-3' and 5'-CCTCTACAAATGTGGTATGG-3', respectively. β-actin primers were utilized for the purpose of loading control: 5'-GCCGCCAGCTCACCATGG-3' and 5'-GGAGTACTTGCGCTCAGG-3'. Cycle conditions were: hot start, 94°C for 3 min; de-

naturation, 94°C for 1 min; annealing, 52°C for 1 min; and extension, 68°C for 2 min. All PCR reactions underwent 30 cycles, unless otherwise noted. Amplified products were electrophoresed through 1% agarose gels and transferred overnight in 0.4 N NaOH to Genescreen Plus membrane (NEN Research Products). The filters were then prehybridized and probed as described above. All cDNAs were individually tested in parallel reactions lacking reverse transcriptase to confirm a lack of contaminating genomic DNA.

Results

Expression in Mammary Epithelial Cells

We first sought to determine if a fragment of the *neu* promoter would be active in mammary epithelial cells. The 1.2 kb region 5' of the neu transcriptional start site was previously shown to induce high levels of CAT activity when transfected into NIH3T3 cells (20). This 1.2 kb promoter was isolated from a mouse 129 genomic library and blunt end cloned into the *pSv2IgH/neu* vector (25) from which the immunoglobulin (IG) promoter/enhancer had been excised. The human mammary epithelial cell line MCF-7 was then transfected with this construct, termed *pNcN,* or vector alone, and multiple clonal populations were picked. Reverse transcription polymerase chain reaction (RT-PCR) was then performed using primers for the SV40 polyA region. The RT-PCR was also performed on a cell line, termed 980NF, established from a mammary gland tumor excised from an MMTV/*neu** transgenic mouse (18), which served as a positive control. Multiple clones of cells transfected with the *neu* promoter/*neu* transgene construct do transcribe the transgene from the murine promoter (Fig. 1A), showing that the promoter is active in mammary cells. RT-PCR was performed using human β-actin primers to control for loading (Fig. 1B). No bands were identified in the cell line with vector alone nor in any of the control reactions lacking reverse transcriptase.

Construction of Transgenic Mice

We next created transgenic mice overexpressing the *neu* promoter/*neu* transgene construct. Recombinant plasmid was microinjected into the male pronucleus of a one cell mouse embryo and three transgenic founder animals were created. One founder died at approximately 1 month of age and one was incapable of passing the transgene, but the third founder

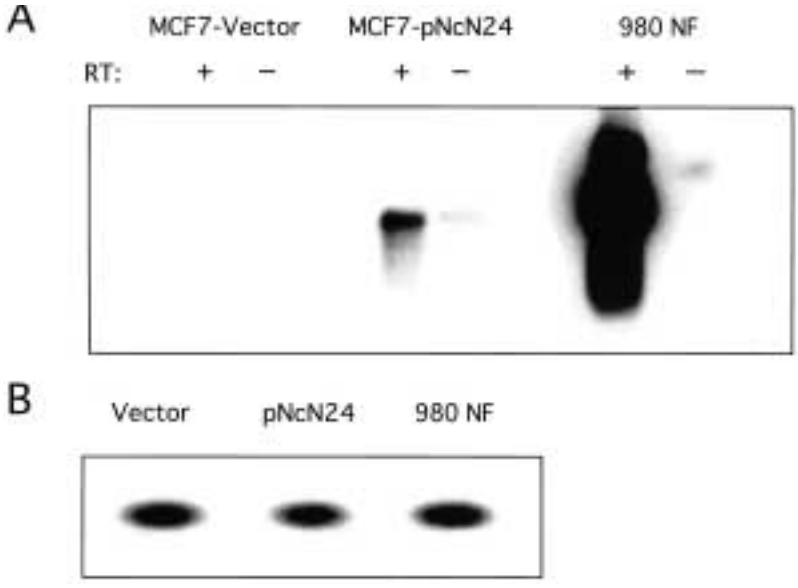

Fig. 1. Analysis of transgenic *neu* promoter activity in MCF-7 cells. (A) RT-PCR of MCF-7 cells transfected with pNcN construct or vector alone. The mammary cell line 980NF was derived from an MMTV-*neu**–induced tumor and serves as positive control. Reverse transcription was performed with either Superscript II RT or water and cDNA was amplified using SV40 polyA primers. (B) β-actin is amplified from the cDNAs to show consistent loading.

did survive to adulthood and passed the transgene to progeny in a Mendelian fashion as determined by Southern blot analysis with an SV40 polyA probe (Fig. 2A). This line, named NN60, has been fully characterized and will be described herein.

In order to determine the plasmid insertion number, restriction enzyme digests were performed on genomic DNA from wild type and transgenic mice (Fig. 2B). When digested with *BamHI*, DNA from transgenic animals showed an additional band when hybridized with a probe for *neu.* This probe recognizes both the endogenous murine gene as well as the transgenic rat *neu.* When quantified by phosphoimager densitometry analysis, it can be seen that the NN60 line of mice contains only a single additional copy of the murine *neu* promoter, followed by the rat transgene (Fig. 2C).

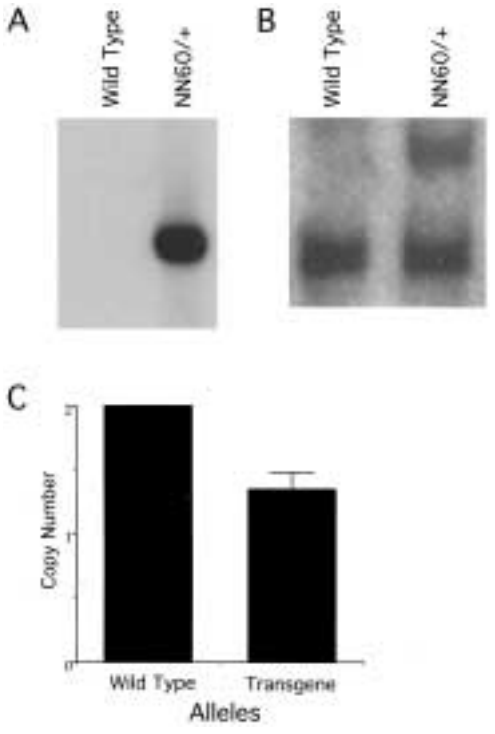

Fig. 2. Southern blot analysis of NN60 transgenic mice. Genomic tail DNA was digested with *BamHI.* (A) Hybridization with SV40 polyA probe. (B) Hybridization with a NN60-3 probe (corresponding to rat nucleotides 2384-2770). (C) Copy number of wild type and transgenic neu was calculated by comparing volume of wild type band with volume of transgenic band and normalizing the wild type value at 2. Calculations performed using phosphoimager densitometry.

Expression of the Transgene in the Mouse

Endogenous *neu* expression varies, depending on the organism and the stage of development investigated. Inconsistencies can be found throughout the literature, presumably due to the method of detection utilized (26). We wanted to determine which organs were expressing the transgene and to confirm that the promoter was indeed active in the mammary gland. Through use of RT-PCR with primers to the SV40 polyA region, we found that the transgenic promoter was active in the ovary, lung, heart and testis (Fig. 3A). The liver and kidney, however, failed to express the transgene. In all cases, a control RT-PCR was performed with water substituted for the reverse transcriptase enzyme to ensure that amplification was occurring from cDNA and not genomic DNA. All of the control lanes were negative.

The same primer pairs were used to elucidate the transgene activity in the various stages of mammary gland development (Fig. 3B). Amplification of the targeted cDNA was clearly seen in the lanes representing the virgin, 7-day pregnant and 9-day regressing mammary gland. The transcript of the transgene did not appear to be present in the lactating animal. A wild type virgin female was used as a control for specificity of the primers. The lane in Figure 3B representing this animal contains no signal, nor do the lanes representing RT-PCR reactions with water substituted for reverse transcriptase.

Effects of an Additional Copy of neu in the Mammary Gland

We decided to study the consequence of transgene expression in the mammary gland by examining ductal morphogenesis. Our expression analysis showed that the transgene was produced in the mammary gland even before the animal was mated. Therefore, we sought to determine if there were any long-term developmental abnormalities associated with low level transgene expression in the virgin mammary glands. Mammary gland whole mounts stained with carmine red alum were prepared in order to study the growth of the ductal tree (27).

At the onset of puberty, in the first 3–4 weeks of development, end buds appear at the mammary ductal tips in female wild type mice.

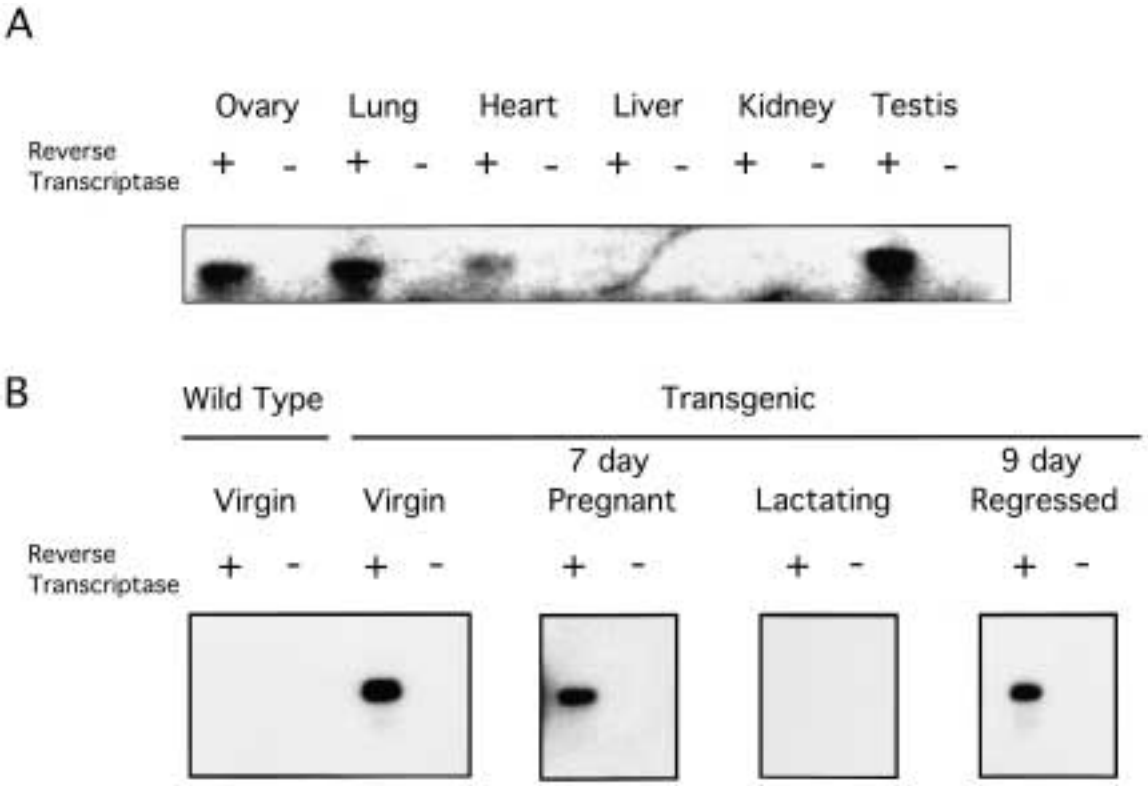

Fig. 3. Expression of the pNcN transgene in transgenic mice. (A) RT-PCR was performed on ovary, lung, heart, liver and kidney of a virgin female and testis of a male NN60 transgenic mouse using SV40 polyA primers. (B) RT-PCR with SV40 polyA primers used on female mammary gland tissue from virgin 7-day pregnant, lactating and 9-day regressed transgenic mice. A wild type virgin is also shown for control of primer specificity. These samples underwent 40 rounds of PCR amplification.

Terminal end buds drive morphogenesis within the gland by supplying differentiated ductal and myoepithelial cells during elongation of the ducts (28). The pattern of growth into the periphery continues in the virgin until the ducts reach the limits of the mammary fat pad, at which time the end buds regress. With the exception of some activity associated with the estrous cycle, the gland remains mitotically quiescent until pregnancy occurs. A clear zone of the fat pad remains between the ducts of the mature virgin, which will eventually be occupied by lobules during pregnancy (29). This quiescent nature of the adult virgin mammary gland was not seen in the transgenic animal. Although additional ductal branching did not seem to occur, epithelial cells were seen to fill the interductal spaces (Figs. 4A and 4B). This was reminiscent of a gland in early stages of pregnancy. With continuing age, lobuloalveolar structures were formed and multiplied and some degree of ductal enlargement occurred (Figs. 4C and 4D). Precocious lobular development and alveolar hyperplasia were confirmed by hematoxylin and eosin staining of the virgin mammary gland sections. A majority of the alveoli showed evidence of secretory activity and small droplets of fat could be seen throughout the cytoplasm of the epithelial cells (Figs. 4E and 4F). In addition, the epithelial secretions showed a punctate pattern of yellow staining, indicative of lactoferrin, a protein product seen in lactating or regressing glands (30,31).

A more severe phenotype of this mouse could be seen in the mammary gland after two rounds of pregnancy and regression. In a wild type mammary gland there was a brief phase of secretory engorgement after weaning, followed by absorption of the milk proteins and collapse of the alveoli in a wild type mammary gland (32; Figs. 5A and 5C). In contrast, the transgenic mammary gland showed a failure to fully

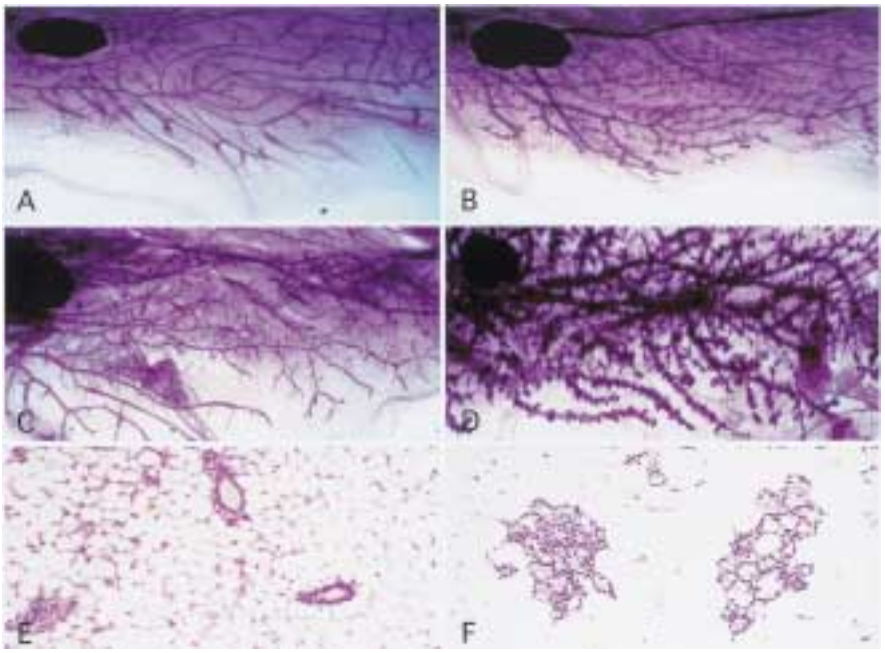

Fig. 4. Whole mounts and hematoxylin/eosin stained sections of wild type and NN60 transgenic virgin mammary glands. Virgin mammary gland from 9-month-old (A) wild type female and (B) NN60 transgenic littermate. Whole mounts were spread on slides, fixed and stained with carmine red in order to elucidate ductal structure. Mammary gland from 19-month virgin (C) wild type compared was that of (D) NN60 transgenic littermate. Hematoxylin/eosin stained sections viewed at 200× highlight the proliferation of alveoli and production of lipids and lactoferrin in the (F) transgenic gland in contrast to the (E) wild type gland.

involute (Figs. 5B and 5D), with lobuloalveolar structures branching off of the primary and secondary ducts persisting months after weaning. This occurred after the first pregnancy, but became increasingly more pronounced after multiple rounds of lactation and weaning. The ducts also appeared to be slightly dilated. However, as in the case of the virgin transgenic animals, the overall ductal structure was largely preserved.

Even after continuous mating for over a year and a half, these transgenic animals carrying the *neu* promoter/*neu* transgene failed to develop palpable mammary tumors. Upon dissection, however, small foci could be seen throughout the fourth inguinal and third thoracic glands. Many of these lesions were keratoacanthomas, composed largely of squamous epithelium producing large quantities of laminar keratin (Figs. 5E and 5F). Small areas of glandular differentiation were seen at the margins of some mammary glands (Fig. 5E), which were delineated by fibrous connective tissue. In some cases, there were nests of dysplastic squamous cells producing extensive laminar keratin (Fig. 5G) and displaying high mitotic rates. All of these tumors appeared to be surrounded by dense fibrous stroma and confined to the mammary gland tissue. No primary tumors were found in any other tissue and metastases were never noted.

Effect of Expression of Oncogenic neu *when Transcribed from the* neu *Promoter*

In addition to the construction of the NN60 transgenic line, we also created a transgenic mouse over-expressing the oncogenic form of *neu* (Val664Glu) transcribed by the murine *neu* promoter. This mouse, termed TNT for "transforming *neu* transgene," contained the same transgene that was found in the NN60 mouse, except for the point mutation in the transmembrane domain, which rendered the protein constitutively active. We had multiple lines of this mouse with the same phenotype, which was extremely similar to that of the NN60 animals. In the adult virgin mammary gland, there was pre-

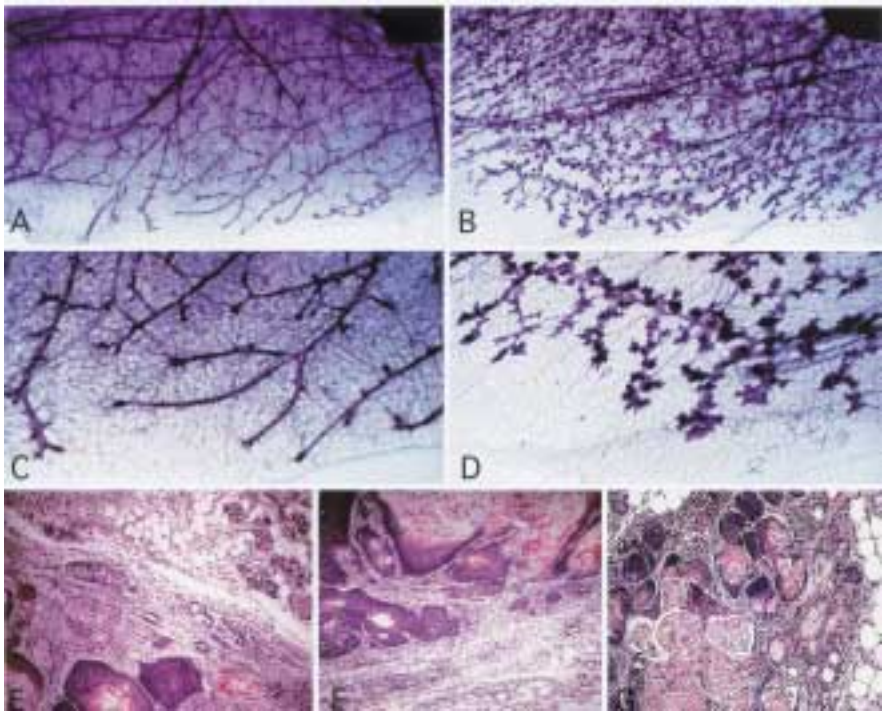

Fig. 5. Multiparous mammary glands of wild type and NN60 transgenic mice. Mammary gland whole mounts taken from wild type (A and C) and transgenic (B and D) animals 17 days after weaning of their second litter. At the lower portion of 10× magnification (A and B), proliferation of lobuloalveoar structures can be seen throughout the transgenic gland. Higher magnification of 30× (C and D) reveals slightly dilated ducts and numerous clusters on each branching duct of the NN60 mouse. (E-G) Hematoxylin and eosin staining of sections of small tumor foci found in the third thoracic mammary gland as seen at a magnification of 200×.

cocious lobuloalveolar development throughout the tissue, as well as dilation of the primary and secondary ducts (Figs. 6A and 6B) with some regions showing more extensive proliferation. Similar to the virgin gland containing the *neu* promoter and the cellular *neu* transgene, the overall architecture of the ducts appeared to be intact.

The phenotype of the multiparous animal was even more striking. After three rounds of pregnancy, the mammary gland of the *neu* promoter/transforming *neu* transgene animal showed an inability to involute when compared with the wild type control (Figs. 6C and 6D). Although some residual acinar structure usually remained in an FVB/n female after many pregnancies, the level seen in these transgenics was extraordinarily high. Hematoxylin and eosin stained slides revealed that the mammary gland appeared to be in a stage of early regression (Fig. 6G), presenting with copious amounts of both lipid secretions and lactoferrin. In addition, when the fourth inguinal mammary gland was examined by whole mount, one could see an average of four to five small tumor foci spread throughout the gland. Several multiparous females also developed palpable tumors, with an average age of onset of 14.5 months. Hematoxylin and eosin stained slides of these tumor sections revealed tumors with multiple morphologies. Several tumors were composed of solid nests of cells (Fig. 6E), that, as in the case with the nodules from the mammary gland of the *neu* promoter/cellular *neu* transgene animal, appeared to be producing noticeable quantities of laminar keratin. Masses consisting of dysplastic glands and squamous epithelium were also noted. A tumor not usually noted in MMTV/*neu* mice was also seen, in which nests of micropapillary neoplasm were formed and surrounded by a dense connective tissue (Fig. 6F), with necrosis occurring within the centers.

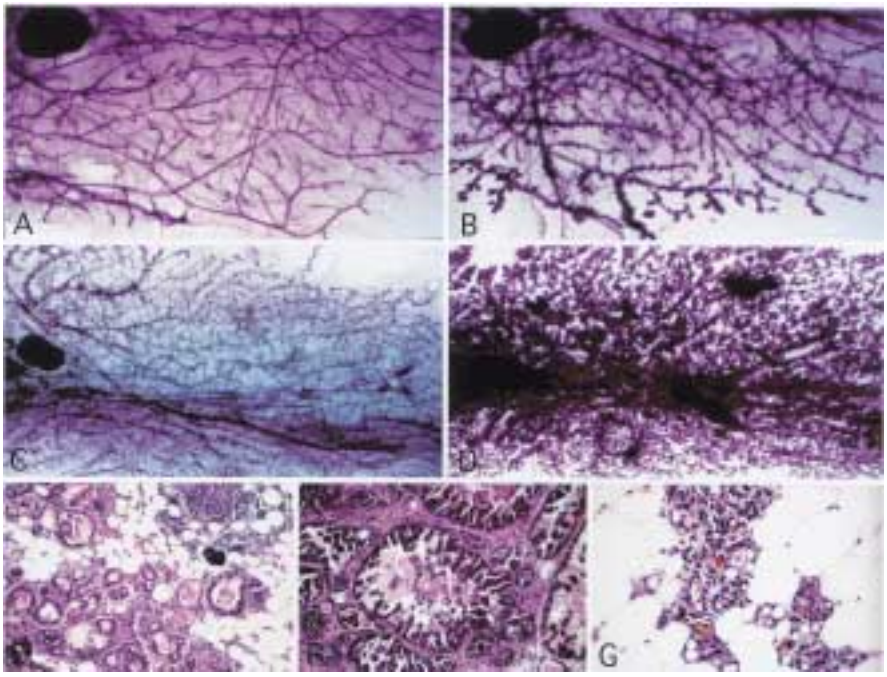

Fig. 6. Mammary gland whole mounts and tumor histopathology of the TNT transgenic mouse. (A) One year virgin mammary whole mount compared with (B), its TNT transgenic virgin littermate. Both mammary glands display normal ductal structure; whereas, the transgenic shows aberrant proliferation of lobuloeolar structures along the ducts. (C) Wild type adult multiparous mammary gland has undergone complete regression and appears similar to the virgin animal; whereas, the TNT transgenic (D) failed to regress even after several months. Small foci can be distinguished throughout the transgenic mammary gland. Magnification at 200× of hematoxylin and eosin stained sections of activated *neu* induced (E) solid nests of anaplastic cells, (F) micropapillary adenocarcinoma or, (G) hyperplastic mammary gland producing lactoferrin and lipid droplets.

Expression of Transforming neu *in Mammary Gland Tumors*

The phenotype of the TNT transgenic mice was considerably more subtle than that of the MMTV/activated neu line, which developed mammary gland tumors in multiparous animals within 3 to 4 months of life. Since the transgene was the same in both mouse models, we hypothesized that differences may exist in expression levels. Lysates were prepared from several tumors of the TNT mice and analyzed by western blot with anti-ErbB-2 antibodies (Fig. 7). Levels of the transgene varied among TNT tumor samples, but all tumors showed elevated levels of expression when compared with wild type mammary tissue. In addition, all of the lysates examined showed considerably lower expression levels than those of the MMTV/*neu* tumors.

Discussion

The correlation between over-expression and amplification of the *c-erbB-2* gene and human cancer is persuasive and well documented (33,34). Equilibrium between monomeric and dimeric *erbB-2* protein may play a role in explaining the importance of gene over-expression in carcinomas. As gene amplification and over-expression occurs, the ratio of protein shifts to the dimerized state, which leads to higher levels of receptor phosphorylation and activation of cellular substrates. It therefore follows, and has been shown in vitro (16,17), that high levels of expression of *neu* will transform cells in culture; whereas, lower levels transcribed from weaker promoters may not. Although not able to transform cells over a short period of time, we hypothesized that a weak promoter driving low levels of *neu* throughout the life of the mouse would have a pathological effect on the mammary gland. To create a mouse model for human breast cancer, we amplified *neu* by adding a transgene of the *neu* promoter directing *neu* cDNA expression. This promoter should have expression levels more closely related to those seen in patients than

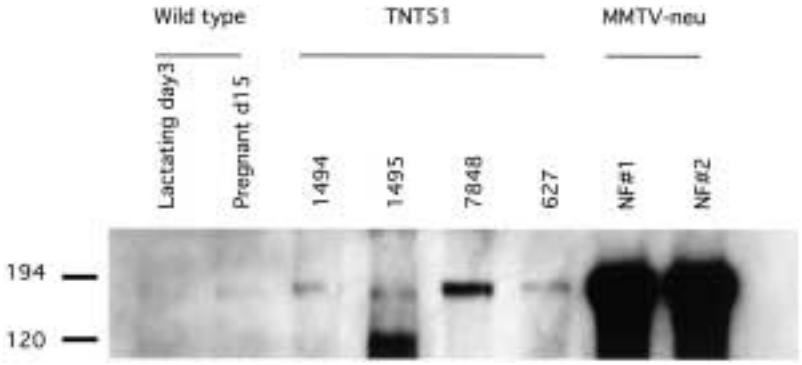

Fig. 7. Western blot analysis of TNT tumor lysates. Wild type mammary glands from day 3 lactation and day 15 pregnancy, TNT tumors and MMTV-*neu** tumors were homogenized and run on an 8% SDS-PAGE gel. Band of approximately 185 kDa elucidated using anti-neu (Ab-3) from Oncogene Research Products, Cambridge, MA.

those achieved in past models. Furthermore, the *neu* promoter should regulate temporal and spatial transcription of *neu* in a way that physiologically parallels cases of human cancer involving *neu* amplification and overexpression. Examination of expression levels in MCF-7 cells, a human mammary epithelial cell line, helped to fortify this belief. The promoter was found to be transcriptionally active, but at a lower level than that observed with the MMTV promoter.

The transgenic mouse line created with a single inserted copy of this construct, called NN60, does show elevated levels of *neu* in the mammary gland. The extremely sensitive method of RT-PCR is needed to show transcription of the transgene. Since this protocol is not quantitative, we cannot compare the level of transgene in the various stages of mammary gland development nor compare it to the levels of endogenous *neu.* It is clear, however, that RNA from the transgene is present in the virgin, pregnant and regressed mammary gland, while absent or below the level of detection in the lactating gland. It should be noted that an identical pattern of expression was seen by others when examining endogenous *neu* through use of RT-PCR (24). In addition, the promoter proved to be active in the ovary, testis, lung, and heart of the adult mouse, while inactive in the liver and kidney. Most of these findings confirm previous expression studies (26). Of notable exception was the study that showed *neu* expression in the adult rat kidney (35), leading one to expect expression in the mouse as well. In addition, although there have been no rodent studies documenting the presence of *neu* in the testis, one report of immunostaining for erbB-2 indicated that the testes were negative in adult human tissue (36). Whether these are species-specific differences or the result of upstream enhancer elements missing from the transgenic promoter remains to be evaluated. Perhaps a more interesting facet concerning tissue expression is that not all tissues over-expressing *neu* show a pathological phenotype. The transgenic animals have a normal lifespan and, although there are clearly phenotypes in the female mammary gland, organs such as the heart, ovary and lung appear normal. It is not clear, however, whether this difference in organ response to the transgene is due to levels of expression within the organ or the need for additional, tissue-specific factors to influence the activity of *neu.*

The NN60 transgenic mouse displays a significant mammary phenotype, despite having only a single additional copy of the *neu* promoter/transgene. With only moderate over-expression, the virgin mammary tree undergoes lobuloalveolar hyperplasia throughout the gland. This effect is enhanced with time. Whole mounts of a 19-month-old virgin transgenic mouse appeared similar to that of a mouse in an early stage of pregnancy. As previously noted, earlier mouse models for the study of mammary gland carcinoma development utilized promoters that were basically inactive until pregnancy was induced in the animal. The *neu* promoter, however, does show a uniform effect in the virgin mammary gland and

may be useful in the study of the effect of other oncogenes on the virgin gland.

The proliferative phenotype is enhanced once the mouse has undergone multiple rounds of pregnancy and regression. Rather than reverting to a near virgin state, the gland continues to produce milk proteins and lipids long after weaning. These results present an interesting contrast to those obtained from the MMTV/*neu* transgenic mouse (19), wherein mammary gland function and development was normal until the appearance of focal tumors. This may be due to the fact that *neu,* under the direction of the transgenic *neu* promoter, appears to be expressed evenly throughout the gland, as can be seen by observing its proliferative effects in a mammary gland whole mount. The MMTV promoter, however, tends to be active in some regions of the mammary gland, while inactive in others. This model also raises interesting questions concerning the protective effect of pregnancy against breast cancer. It has long been established that a woman's risk of developing breast cancer decreases if she has undergone full-term pregnancy before the age of twenty (37). This model suggests, however, that in cases where the *neu* gene is amplified, there is a proliferative effect associated with pregnancy, as can be seen in the whole mounts of the multiparous mammary glands.

Oncogenic forms of *neu* transgenes have facilitated the study of the transforming potential of *neu* in mice, although their physiologic relevance has been questionable. An activating point mutation in the transmembrane *ErbB-2,* similar to the rat Val664Glu has never been identified in human breast cancer patients. Recent work, however, revealed somatic mutations within the transgene of MMTV/*neu* mice over-expressing the non-transforming proto-oncogene, which lead to elevated levels of ErbB-2 tyrosine phosphorylation (38). Our mice carrying the *neu* promoter/transforming *neu* transgene recapitulate the phenotype seen in the NN60 line and develop palpable mammary gland tumors at approximately 14.5 months of age. These tumors not only have a much longer latency than those of the MMTV/activated *neu* mice, but show significantly lower levels of transgenic protein.

The mouse models described here show that even a small perturbation in *neu* expression can lead to a clear phenotype in the mammary gland. This reinforces the strong correlation between *neu* amplification and breast cancer. In addition, these models demonstrate the usefulness of the *neu* promoter in the study of oncogenes in the virgin mammary gland.

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