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TITLE: Tissue Specific Activation and Inactivation of the Neu Proto-Oncogene in Transgenic Mice Using Cre Recombinase

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_____ Kathleen H. Moore 7 Feb 01

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13. ABSTRACT ***(Maximum 200 Words)***
In human breast cancer, 20 to 30 % of human breast cancers amplify and overexpress the Neu proto-oncogene. To fully elucidate the role of this gene in normal development and breast cancer, we have designed and implemented experiments to test the gain and loss of function of Neu in the mouse mammary epithelium. Since the knockout of Neu results in embryonic lethality, the null mutation is not easily examined. To examine mammary development in the absence of Neu, we have knocked Neu out specifically in the mammary epithelium through the use of Cre Recombinase. The preliminary analysis has not revealed a phenotype, however we have not yet examined lactation and regression. To address the role of Neu in mammary tumorigenesis, I have conditionally placed NeuNT under the control of the endogenous promoter specifically in the mammary gland. These mice express NeuNT during mammary gland development, resulting in abnormal mammary gland development, characterized by numerous side branches and lobuloalveolar side buds. Tumorigenesis in this strain occurs in 50% of the afflicted female by 13.52 months and is associated with both membrane and cytoplasmic immunoreactivity for NeuNT. Interestingly, there is both amplification and overexpression of the NeuNT allele in this tumor model.

14. SUBJECT TERMS
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Introduction

The original research proposal outlined several experiments to elucidate the role of the Neu (HER2, c-erbB-2) proto-oncogene (1-5) in mammary gland development and tumorigenesis. Given that 20-30% of human breast cancers amplify and overexpress Neu (6-10), I sought to elucidate the role of Neu in the normal development of the mammary gland. However, since the knockout of this receptor tyrosine kinase results in embryonic lethality (11), I have engineered a tissue specific knockout approach using Cre / loxP technology. Thus, by circumventing the lethality associated with the knockout, we are able to determine the role of neu in the development of the mammary gland. At this point, preliminary analysis has not revealed a major role for Neu in mammary development. However, we have yet to examine lactation and regression in detail. The role of Neu in tumorigeneis has been previously examined in various transgenic models (12-14). When the oncogenic Neu allele was expressed under control of the Mouse Mammary Tumor Viral (MMTV) Promoter, mammary tumors were noted in 50% of female mice by 89 days (14). Further, when this was repeated using the wild type allele, tumors were detected by 205 days in 50% of the female mice (13). While instructive, these studies relied on a viral promoter which is of questionable relevance to the human condition and which is subject to hormonal regulation. Thus, to address the hormonal aspect of Neu mediated tumorigenesis, I have generated mice which express the activated Neu allele under the control of the endogenous promoter specifically in the mammary gland. This mammary directed expression resulted in focal tumorigenesis and included both amplification and overexpression of the activated Neu allele. I have characterized tumorigenesis in this strain and am now poised to examine the hormonal regulation of this process.

Research Accomplishments

1. The Mammary Specific Inactivation of Neu

To examine the role of Neu in normal mammary gland development, lactation and involution the study of a null mutation is essential. However, since the knockout of Neu was embronically lethal (11), a tissue specific approach will be employed. To generate a mammary specific inactivation of the Neu proto-oncogene we have generated two strains of mice. In the first, we have replaced exon 1 of the endogenous neu allele with a loxP flanked neu cDNA followed by a selectable marker (Figure One). This construct was electroporated into Embryonic Stem (ES) Cells and mice were generated in the SV129 / Balbc background. To facilitate the removal of this loxP flanked sequence, we have also generated mice expressing Cre recombinase under the control of the MMTV promoter in the FVB background (Figure Two, A). Several lines were then screened for Cre expression in the mammary gland and mice expressing Cre were then screened for Cre expression in a variety of tissues. Of the various lines, Cre-7 had the highest expression which was restricted fairly tightly to the mammary gland. These two lines were then interbred to generate a mammary specific inactivation of the Neu proto-oncogene due to the remaining marker and the loss of exon one. However, upon interbreeding these lines we discovered that we lost expression of the Cre transgene with the introduction of the SV129 / Balbc background (Figure Three). The loss of expression is likely due to methylation which has

previously been noted in these strains. Thus, instead of immediately addressing the specific aims in Task 1, I have backcrossed the loxP flanked Neu strain into the FVB background to alleviate the loss of Cre expression. At this point I have now generated mice that are at F9 into the FVB background, 99.8 % FVB assuming random assortment. For the final cross I have interbred the F9 loxP flanked Neu mice with the FVB MMTV-Cre mice. This should ensure that we have maximal Cre expression and I am now preparing to determine whether these mice have a mammary phenotype by wholemount and histological methods. A preliminary analysis has not revealed a major role for ErbB-2 in mammary gland development. These mice appear to lactate normally and nurse their offspring. Further, a limited series of wholemount analyses has failed to reveal any significant differences between wild type and ErbB2 null mammary epithelium at 12 weeks of development. To determine that Cre is efficiently mediating excision in the mammary epithelium we have initiated a collaboration with Hal Moses' lab who crossed our MMTV-Cre mice to a B-gal reporter strain. Additionally, since the mammary phenotype may be difficult to discern in these mice, we have recruited a technician who is preparing to examine the mammary epithelium in vitro on a complex basement membrane. In this manner we can examine the secondary structures that form for their response to various hormones and growth factors. As a result of these experiments, we should clearly define the role of ErbB2 in the development of the mammary gland.

2. Tumorigeneis in Mice with a Tissue Specific Activation of the Oncogenic Neu Allele

Previous experiments have addressed the role of Neu in tumorigenesis under MMTV control (12-14), while these experiments were extremely instructive they are of questionable relevance to the human condition. Thus, another major goal of the proposal was to examine the effect of the activated neu allele on mammary development and tumorigenesis under the control of the endogenous promoter. Since it was possible that simply replacing the endogenous allele with the activated allele would result in embryonic lethality, we have employed a tissue specific approach. As described in the original proposal, the mice have been created so that a loxP flanked neomycin cassette has been inserted 5' to the NeuNT cDNA, which has replaced exon 1 of the endogenous neu allele (Figure Four). Upon Cre mediated recombination, the activated allele is then placed under the control of the intact promoter. Excision of the neomycin cassette has occurred in the salivary gland, spleen and mammary gland (Figure Five), and by phosphoimager imager analysis appears to be approximately 30 to 40 % complete. Thus, we have created mice which control expression of the NeuNT oncogene using the endogenous promoter, specifically in the mammary gland. Using a wholemount and histological analysis we examined the MMTV-Cre transgenics, the unactivated knock-in mice and the bigenics. While no differences were noted in either the MMTV-Cre or the knock-in mice, when the conditionally activated bigenics were examined, a significant deviation was noted. In the wholemount analysis of the bigenics there was more extensive side branching and numerous lobuloalveolar side buds in comparison to the normal mammary epithelial structure of the wild type controls. (Figure Six, A and B). Ultimately, these conditionally activated mice went on to develop tumors which expressed both membrane and cytoplamic immunoreactivity for Neu. In comparison to the MMTV-Neu induced tumors which exhibit only membrane immunoreactivity, this cytoplasmic localization suggests rapid internalization of neu after constitutive dimerization (Figure Six). Interestingly, the average onset of tumorigenesis in this strain of mice was 411 days (Figure

Seven), a significant difference from the MMTV-NeuNT model where tumorigenesis occurred on average at 89 days. Consistent with the immunoreactivity exhibited in the tumors, an RNase protection analysis revealed that NeuNT expression was elevated in the tumor samples (Figure Eight). To extend this analysis, the tumors and adjacent glands were examined through a western analysis revealing overexpression of Neu in the tumors (Figure Nine, Panel A). Additionally, elevated levels of tyrosine phosphorylated Neu was detected in the tumors (Figure Nine, Panel B). Other EGFR family members were examined, however only ErbB-3 levels were elevated in the tumors(Figure Nine, Panel C, EGFR and ErbB-4 results not shown). Since the human condition has both amplification and overexpression of the neu proto-oncogene, we tested the mammary adenocarcinomas for amplification. Strikingly, all tumors tested exhibit amplification of the recombinant allele. After phosphoimager analysis, there were found to be on average 8 copies of the recombinant allele for every copy of the wild type allele (Figure Ten, Panels A and B). Thus, tumorigenesis in this strain of mice has been found to be associated with the amplification of the knock-in allele and is coupled with overexpression of the oncogene at the RNA and protein levels.

In an effort to reduce the tumor latency and increase the utility of this model, we have attempted to engineer mice so that the wild type allele would be replaced by an activated allele. Theoretically, by offering two targets at which amplification could occur, we should reduce the latency. Further, there is evidence that the wild type allele may squelch the activity of an activated allele. To create mice without a WT allele in the mammary epithelium, we microinjected a Chicken B-actin Cre plasmid into LoxP flanked Neomycin NeuNT zygotes. Excision occurred at the two cell stage, as determined by Southern analysis, and the resulting heterozygous mice were viable (Figure 11). However, when the NeuNT / WT mice were interbred, we did not detect any NeuNT homozygotes, indicating that a homozygous knock-in of the activated allele was embryonically lethal. Unfortunately for our studies of mammary tumorigenesis, when these mice were crossed to the conditionally activated mice, we did not observe any bigenics. Essentially, the NeuNT knock-in / Flox neo NeuNT knockout mice were embryonically lethal and we were not able to observe tumorigenesis in this strain of mice. However, in collaboration with a postdoctoral fellow in our lab we plan to examine the NeuNT / NeuNT embryos. A preliminary examination has revealed that lethality is occurring at 13 days post coitum, two days after lethality due to the previously published knockout. Thus, this experiment was not able to decrease the latency of mammary tumor onset due to the associated embryonic lethality.

3. Hormonal Regulation of Neu Mediated Tumorigenesis

Since previous mouse models of Neu mediated tumorigenesis have relied on the MMTV promoter to direct expression of the proto-oncogene they do not lend themselves to the study of hormonal regulation of tumorigenesis. However, since the loxP flanked neomycin NeuNT MMTV-Cre model places control of NeuNT under the control of the endogenous promoter after Cre mediated recombination, it provides a unique opportunity to study the hormonal aspect of tumor development. The initial observations of tumor formation in this strain were made in a mixed genetic background and preliminary results indicate that interbreeding to the FVB background may result in an acceleration of tumorigenesis. Accordingly, I have backcrossed the Flox neomycin NeuNT strain to the FVB background and am currently at the F8 generation, over

99% FVB, assuming random assortment. Further, I have crossed these mice to the FVB MMTV-Cre mice and have generated bigenic mice. As an initial experiment, we will examine the effect of removing the hormonal signals originating in the ovary. Also, by implanting the pituitary gland into the kidney we will increase the hormonal signals received by the mammary gland. To facilitate this study, I have learned the ovariectomy procedure. The removal of ovarian hormonal signals should have the effect of increasing the latency before tumor onset. Additionally, I have learned how to harvest the pituitary gland and am now attempting to master the surgery where the pituitary gland is implanted under the kidney capsule. With the additional hormonal signals, we expect that tumor latency should be dramatically shortened in these mice. These two experiments will provide a valuable insight into the hormonal aspect of Neu mediated tumorigenesis.

Training Accomplishments

To achieve the research goals outlined in the original proposal I have learned several surgical techniques in the past year. Perhaps one of the most valuable skills that I mastered was the creation of transgenic mice. A senior member of the lab instructed me all aspects involved in creating transgenics, from preparing the injection fragment to the reimplatation surgery. This set of skills will serve me well in the future, not only in the creation of transgenics, but with the associated surgeries. Indeed, I have also learned how to ovarectimize mice, and given the similarities to the reimplantation surgery, this technique was relatively easy to learn. In addition to these surgical procedures, under the tutelage of Dr. R. Cardiff, a prominent pathologist with which our lab collaborates, I have learned how to clear the mammary fat pad of endogenous mammary epithelium and implant either mammary epithelial cells or tissue fragments. This technique will be extremely useful in the dissection of the slight mammary phenotype in the conditional inactivation of Neu. Further, this will allow us to address any stromal / epithelial interactions required for tumorigenesis in the conditionally activated mice.

In addition to these surgical skills, I have learned several new procedures involved in the analysis of tumors. These skills range widely, from the initial tumor palpation and dissection to the histological and molecular analyses to round out the study. I have been privileged to learn many aspects of the histological analysis from Dr. Cardiff, both at our lab and at a workshop he conducted at The Jackson Labs in Bar Harbor, Maine.

Key Research Accomplishments

- Generation of the conditionally activated NeuNT mediated mouse mammary tumor model, including characterization of the amplification and overexpression of NeuNT in the tumors. This study was published in March, 2000.

- Backcrossed loxP flanked Neu to the FVB background after loss of Cre expression in the SV129 / Balbc background. I have generated mice to be examined immediately for any effect of ablation of Neu in the mammary gland.

- Backcrossed the conditionally activated NeuNT tumor model to the FVB background and am generating mice to be used in the study of the hormonal regulation of Neu mediated tumorigenesis.

- Generation of embryonically lethal mice where expression of the NeuNT oncogene is controlled by the endogenous promoter in all tissues as a result of an effort to decrease the latency of tumor onset in the conditionally activated mice.

Reportable Outcomes

Papers Published

Amplification of the neu/erbB-2 oncogene in a mouse model of mammary tumorigenesis. Andrechek ER, Hardy WR, Siegel PM, Rudnicki MA, Cardiff RD, Muller WJ.
Proc Natl Acad Sci U S A. 2000 Mar. 28;97(7):3444-9.

Tyrosine kinase signaling in breast cancer: Tyrosine kinase-mediated signal transduction in transgenic mouse models of human breast cancer.
Eran R Andrechek, William J Muller
Breast Cancer Res. 2000 2:211-216.

Accelerated mammary tumor development in mutant polyomavirus middle T transgenic mice expressing elevated levels of either the Shc or Grb2 adapter protein.
Rauh M.J., Blackmore V., Andrechek E.R., Tortorice C.G., Daly R., Lai V.K., Pawson T., Cardiff R.D., Siegel P.M., Muller W.J.
Mol. Cell Biol. 1999 Dec. 19;(12):8169-79

Seminars Given

Insights from Mouse Models of Neu Mediated Mammary Tumorigenesis, Modeling Human Mammary Cancer in Mice, Jackson Labs, October 5-8, 1999.

Posters

Amplification and Overexpression of Neu / ErbB-2 in an Inducible Mouse Model of Mammary Tumorigenesis, 16th Annual Meeting on Oncogenes, June 22-25, 2000.

1st Prize, A Neu Tumor Model of Mammary Cancer, Biology Poster Day, McMaster University, March 3, 2000.

Conclusions

In the last year of funding, several key experiments have revealed important insights into our mouse model of mammary carcinogenesis. Notably, placing the oncogenic neu allele under the control of the endogenous mouse erbB-2 promoter resulted in mammary tumorigenesis. Interestingly, tumor formation in this strain of mice required both the amplification and the overexpression of the oncogene. Further, the normal development of the mammary gland was altered, resulting in an epithelial structure characterized by numerous lobuloalveolar side buds and extensive branching. An interesting facet of this tumor model is that we have escaped the normal hormonal regulation of the MMTV promoter by using the endogenous promoter in a tissue specific fashion. Thus, this model lends itself to the study of the hormonal regulation of neu induced tumorigenesis. Accordingly, I have backcrossed these mice to an FVB background to accelerate tumor formation and have learned the surgeries required to ablate and increase the hormonal signals received by the mammary epithelium.

Given the frequent involvement of neu in human tumor formation, the role of this gene in normal mammary gland development is also being examined. To examine neu ablation in mammary development, we have employed a tissue specific approach using Cre recombinase. Unfortunately, when the MMTV-Cre mice were interbred with the loxP flanked Neu knock-in mice, expression of the Cre transgene was lost, likely due to methylation of the transgene. To address this problem I have now interbred the loxP flanked Neu mice into the FVB background and am poised to examine the role of neu in mammary gland development.

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Appendix One

Figures One to Eleven

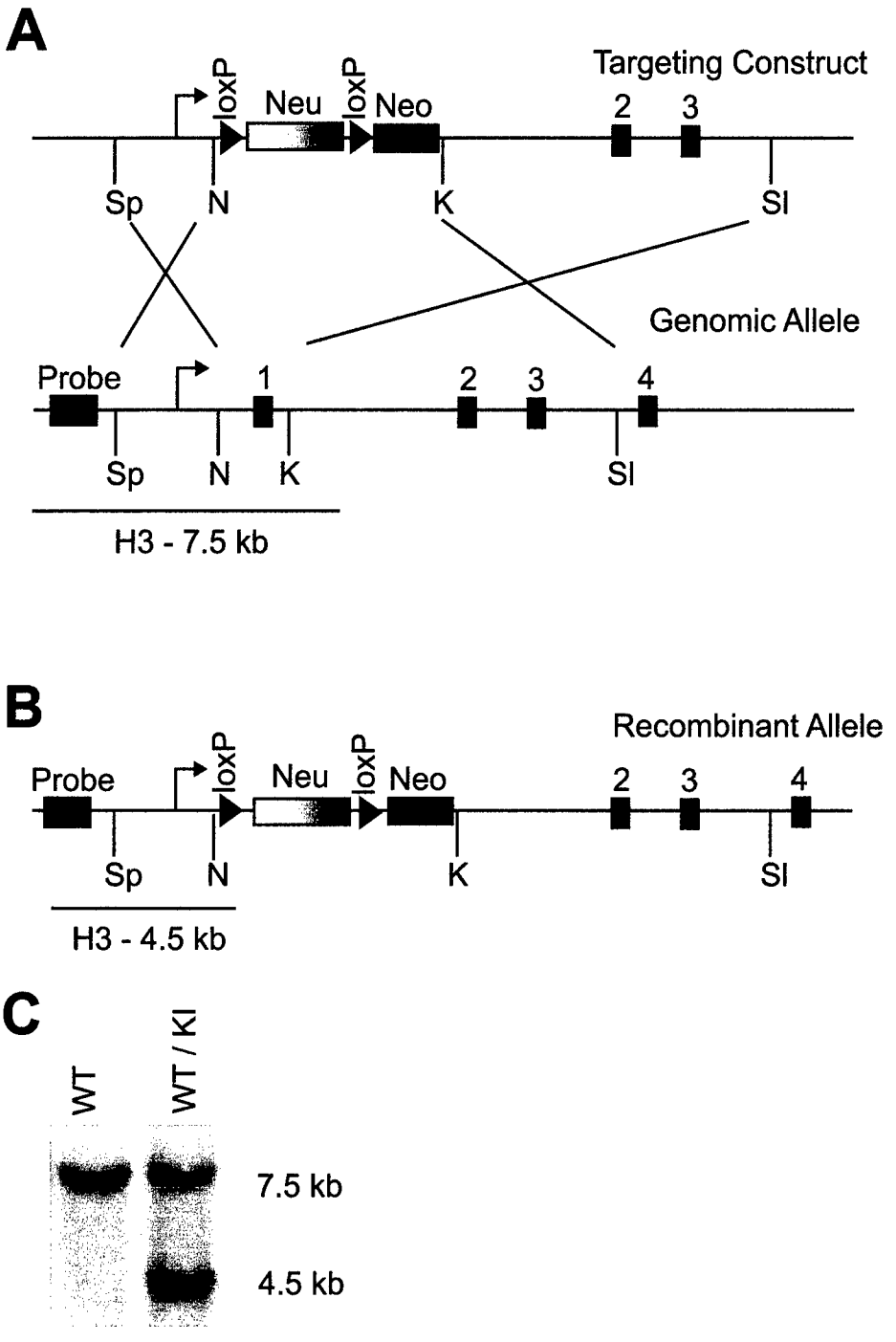


Figure One - Targeting Strategy to Replace Exon 1 of ErbB-2 with a LoxP flanked Neu cDNA
(A) The loxP flanked Neu cDNA and neomycin selectable marker are illustrated. Also shown are the 5' and 3' arms of homology (5' - SP to N, 3' K to Sl) for a total of 10.5 kb of homology. The proposed targeted homologous recombination is shown by the crossed lines. (B) The targeted allele is shown where the loxP flanked Neu cDNA and neo cassette have replaced Exon 1 of the endogenous allele. (C) After digesting the ES cell DNA with Hind III (H3) and using a probe external to the arms of homologous recombination a Southern analysis was conducted. The presence of a 4.5 kb band indicates the presence of the recombinant allele.

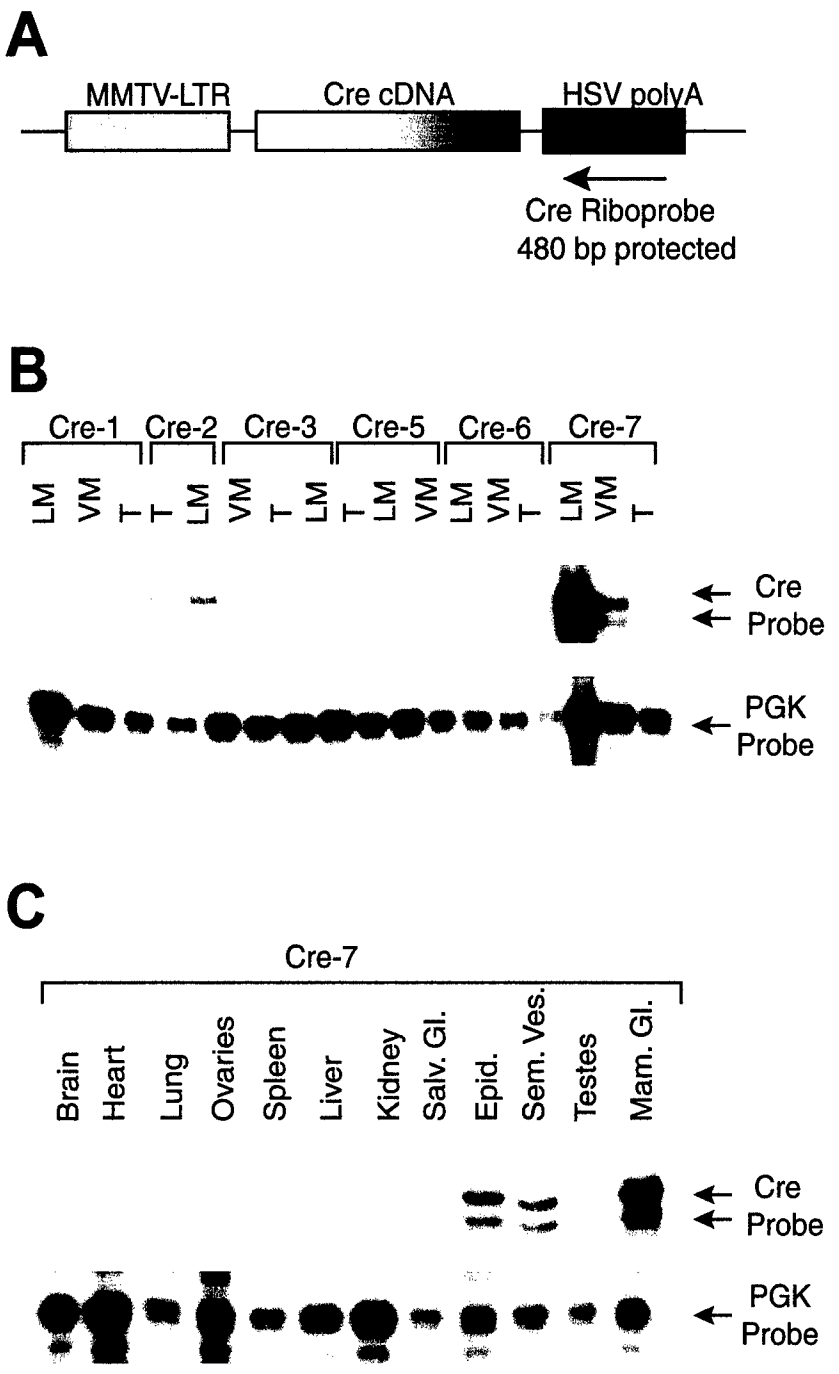


Figure Two - Characterization of MMTV-Cre Transgenics
(A) The schematic indicating how the cDNA for Cre recombinase has been placed under the control of the Mouse Mammary Tumor Virus promoter. Also shown is the probe used in Cre RNase protection experiments. (B) An RNase expression on the 6 lines of MMTV-Cre transgenics is shown. Note the high expression in MMTV-Cre7 mammary glands. (C) An RNase protection on various tissues from the MMTV-Cre7 line is shown. Expression is primarily limited to the mammary epithelium. PGK was included as a loading control.

UNPUBLISHED DATA

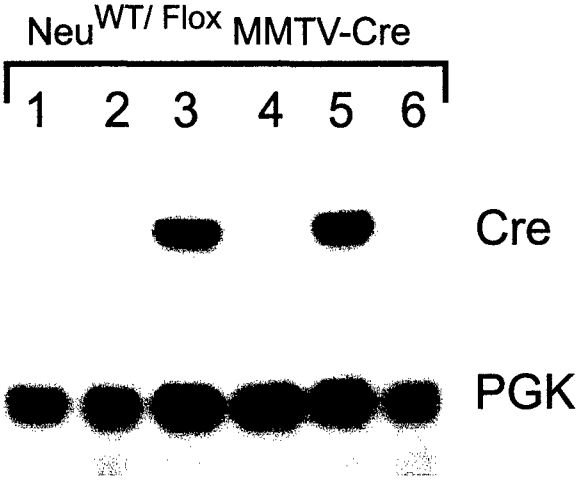


Figure Three - Loss of Cre expression in the SV129 / Balbc Background
An RNase protection illustrating the loss of Cre expression as the mice are introduced into the SV129 / Balbc background. This representative RNase protection was performed on mice that were heterozygous for the recombinant allele, however similar results were obtained with wild type MMTV-Cre and homozygous knock-in MMTV-Cre mice.

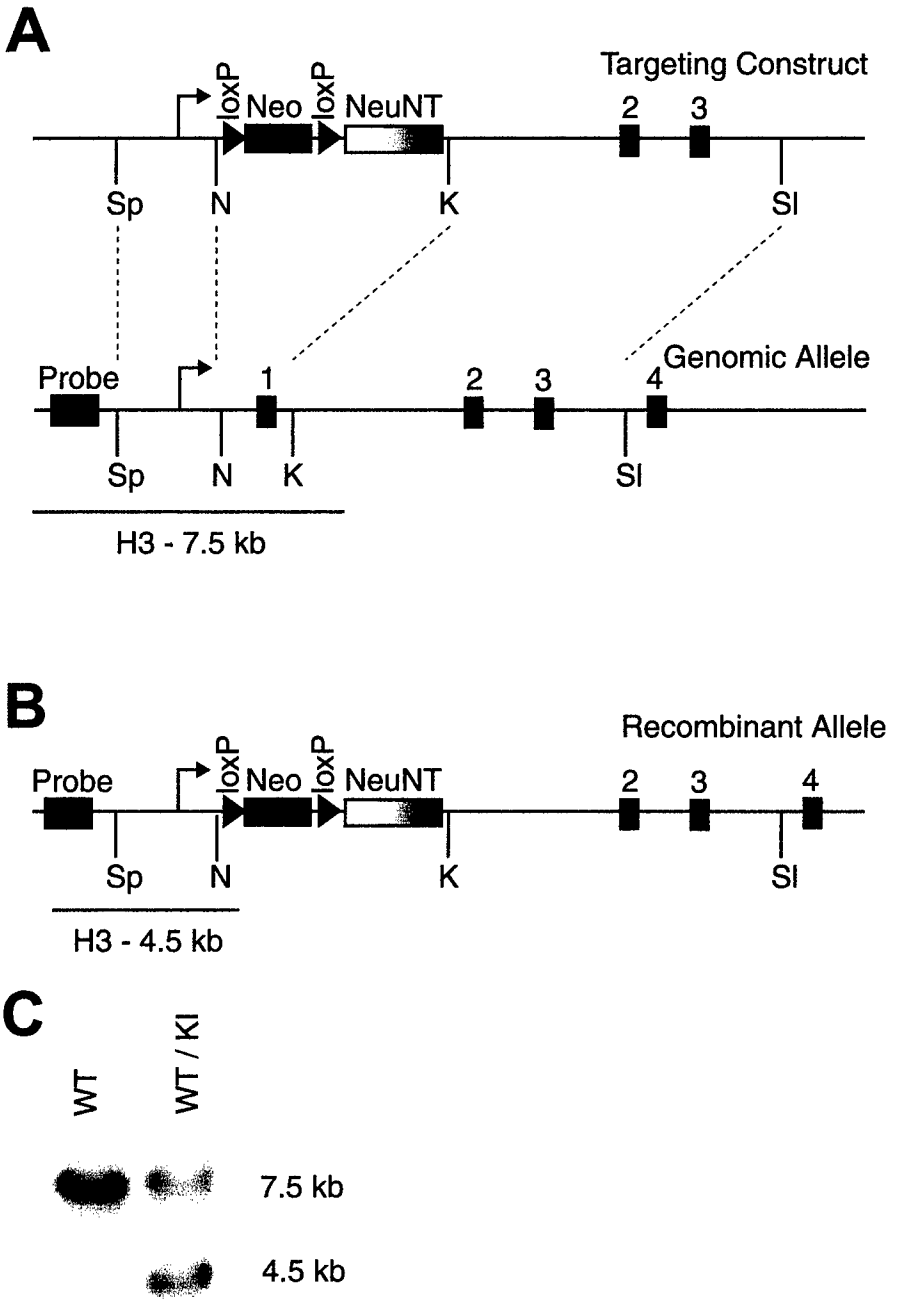


Figure Four - Targeting of the Conditionally Activated Allele
(A) The targeting construct is shown where the loxP flanked neomycin cassette is 5' to the oncogenic Neu allele. This loxP flanked neo NeuNT sequence is then targeted to replace exon one of the endogenous ErbB-2 allele through the arms of homology (5' - SP to N, 3' - K to Sl). The Hind III (H3) fragment detected with the external probe is also shown. (B) The recombinant allele is illustrated after the proposed homologous recombination event. The H3 fragment for the recombinant allele is shown below the recombinant allele. (C) A representative Southern analysis is also shown illustrating the recombination event.

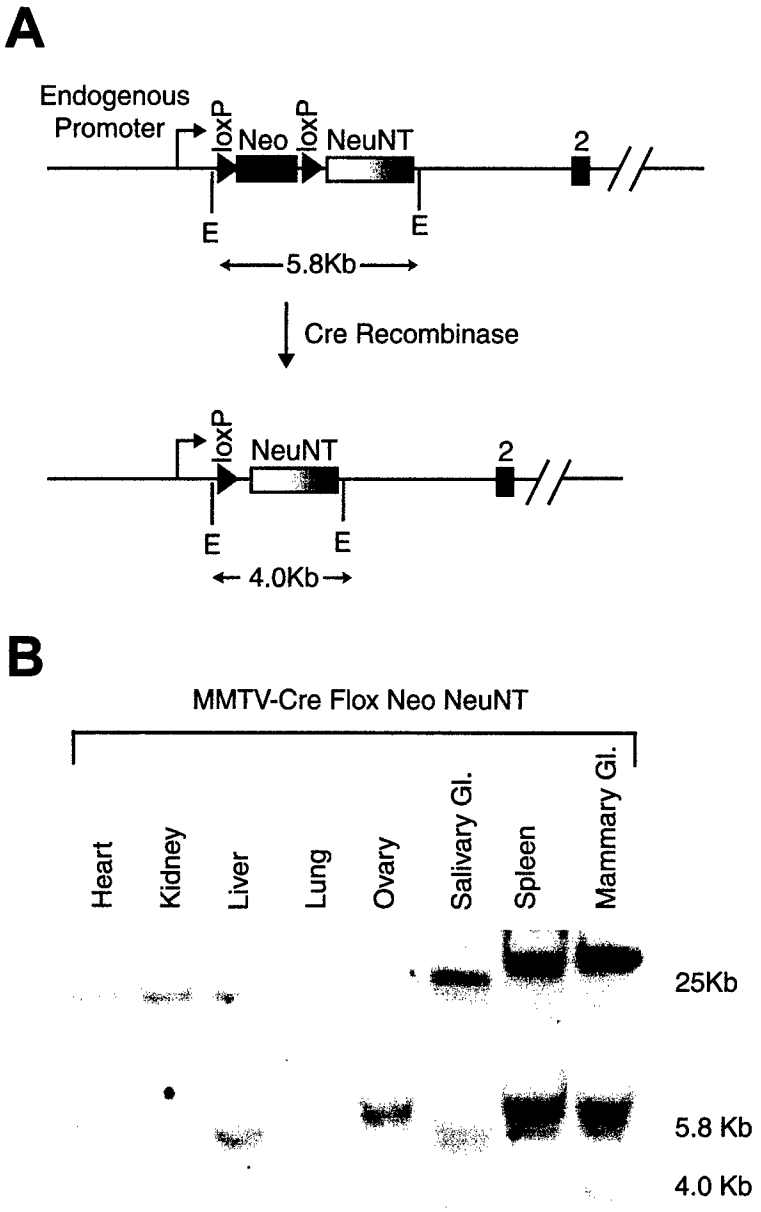


Figure Five - Excision of the loxP flanked Neomycin Cassette
(A) The recombinant allele is shown prior to and following Cre mediated excision of the loxP flanked neomycin cassette. Using the NeuNT cDNA as a probe in a Southern analysis should result in the illustrated sizes following the EcoR1 digest. (B) Excision of the loxP flanked neomycin cassette, resulting the in 4kb band is found in the spleen, salivary and mammary glands. Importantly, this excision has placed NeuNT under the control of the endogenous promoter.

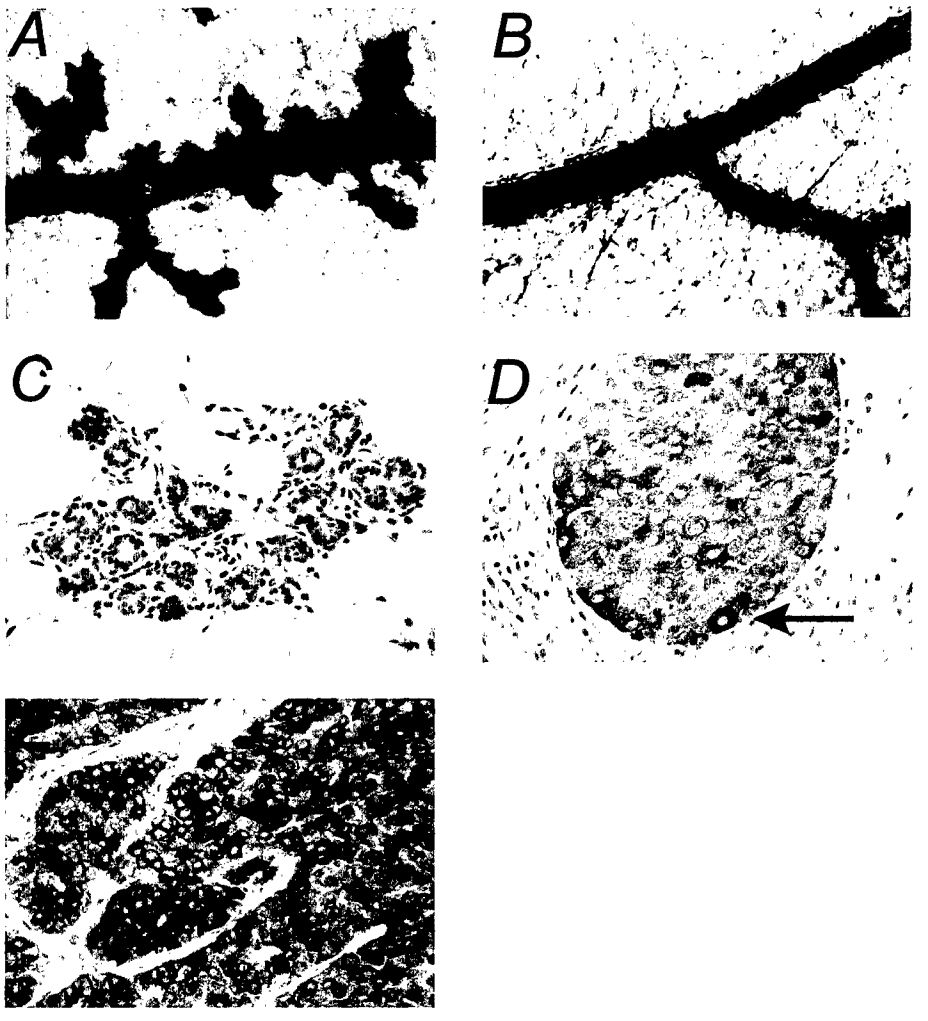


Figure Six - Digitized micrographic images of the MMTV-Cre FIneo NeuNT mammary gland and tumors

(A) Whole mount of a virgin MMTV-Cre FIneo NeuNT mammary gland at 9 months. The extensive side branching terminating in lobuolalveolar units should be noted. (B) Whole mount of a virgin wild-type control mammary gland at a similar stage of development illustrating a normal duct with few side branches. (C) Immunohistochemical analysis of the same virgin gland shown in A. Note the absence of anti-Neu staining in the acinar structures, which are not dysplastic. Immunohistochemical analysis on the bigenic (D) and a control MMTV-Neu (E) induced tumor shows high levels of Neu expression in these tumors. The contrast in the cytoplasmic and membrane localization of the stain indicated by the arrows in D and E, respectively should be noted. (Magnifications: A and B, 3 40; C–E, 3320.)

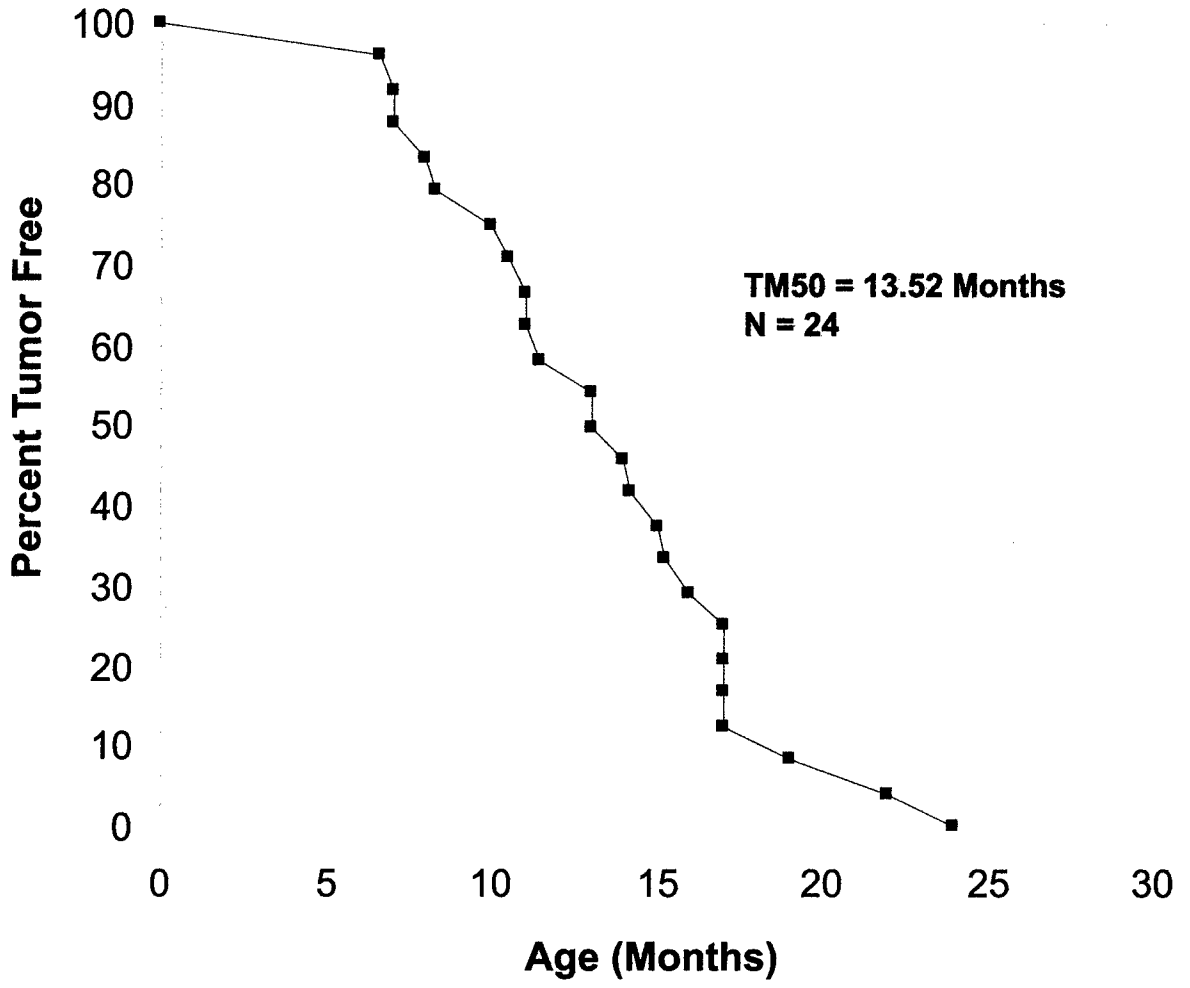


Figure Seven - Tumor Kinetics in the Conditionally Activated Tumor Mice
As a percentage of all mice with tumors, the tumor kinetics curve is shown above. For the 24 mice examined to this point, the time at which half of the females bore tumors was on average, 13.52 months.

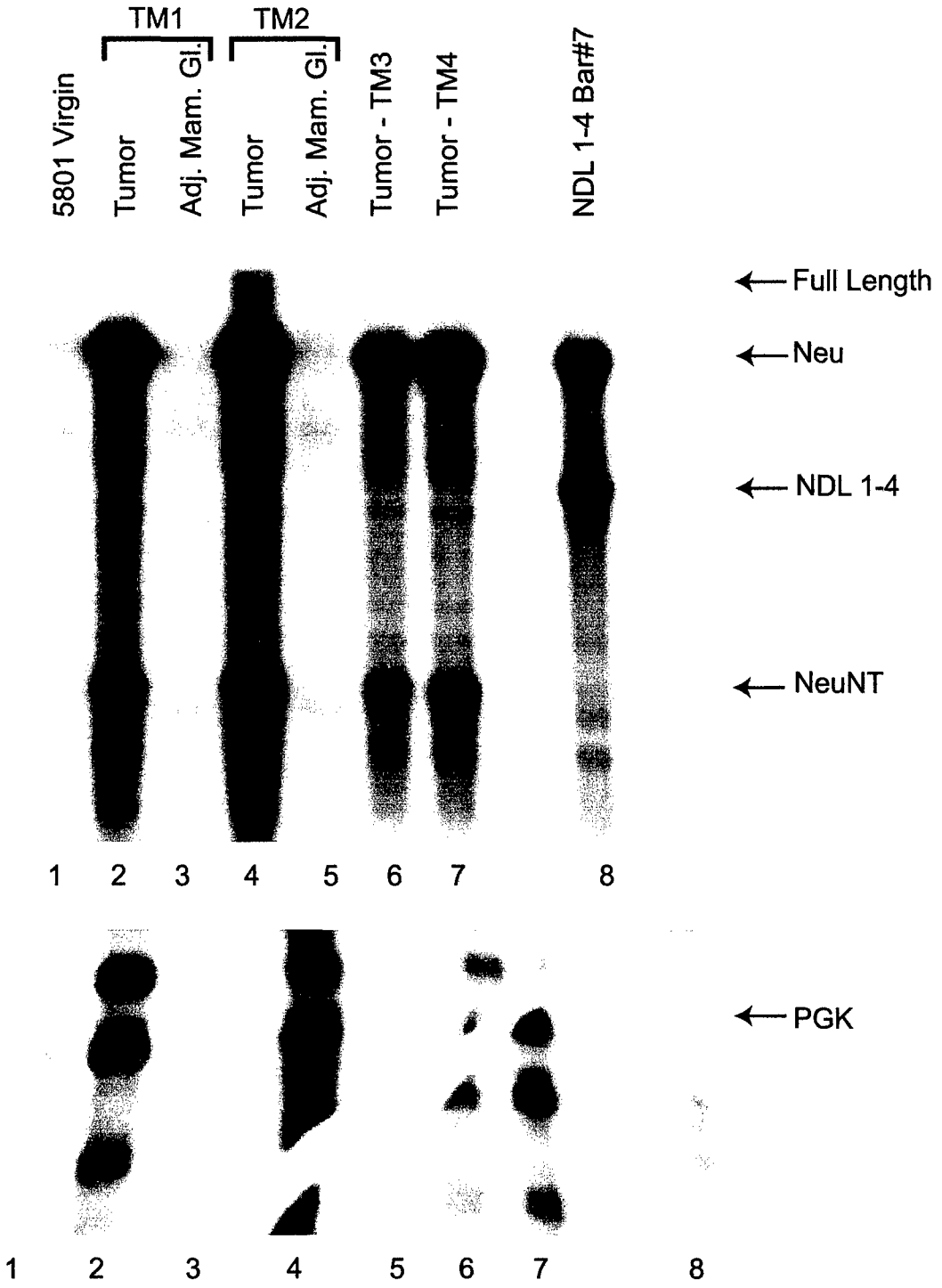


Figure Eight - Tumors Overexpress the Activated Neu Allele
An RNase protection on tumor tissue and the adjacent mammary glands reveals that the tumors overexpress the activated allele. The NDL samples was included as a control for complete digestion, which would be indicated by a single band. However, since digestion was not complete, the tumor samples were likely overexpressing only the activated allele.

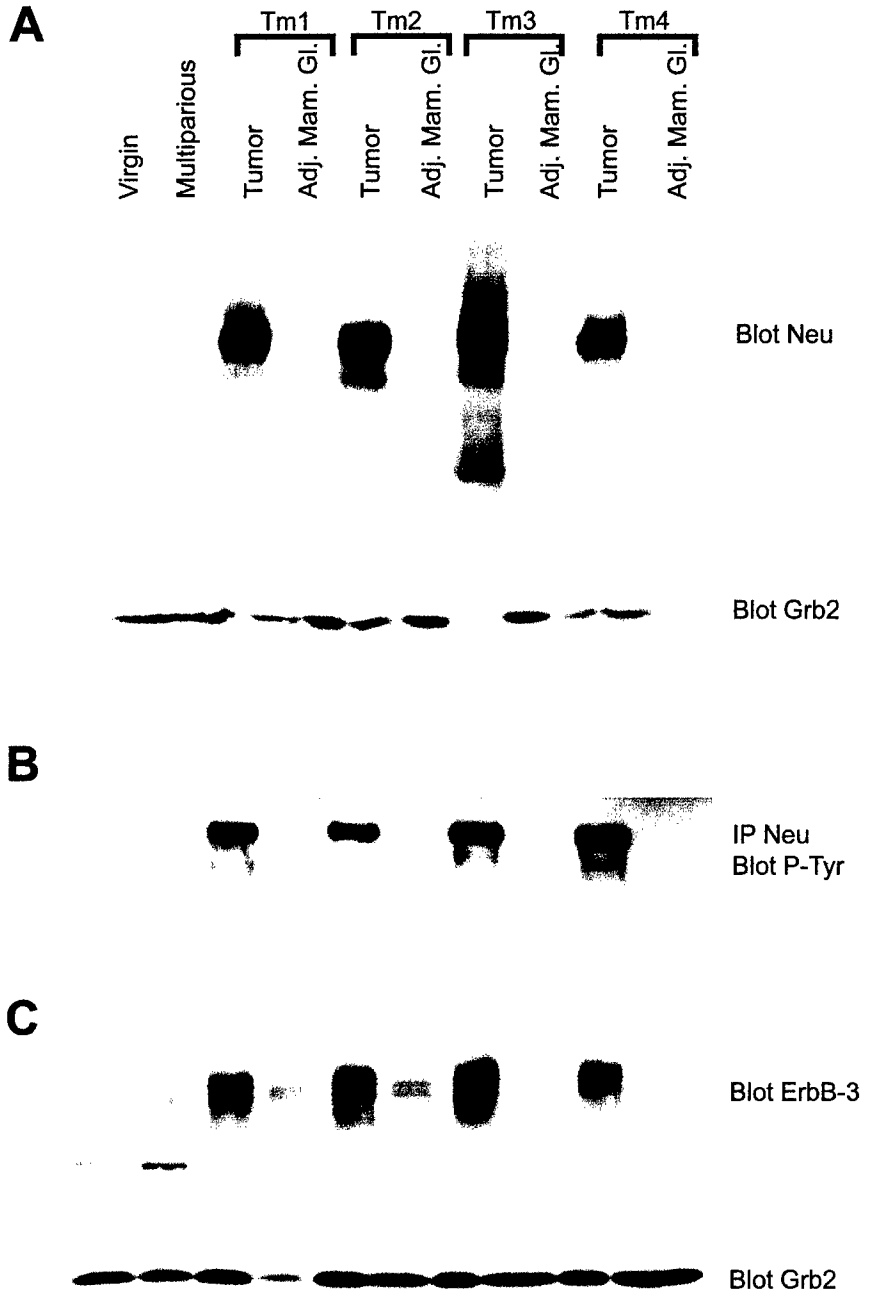
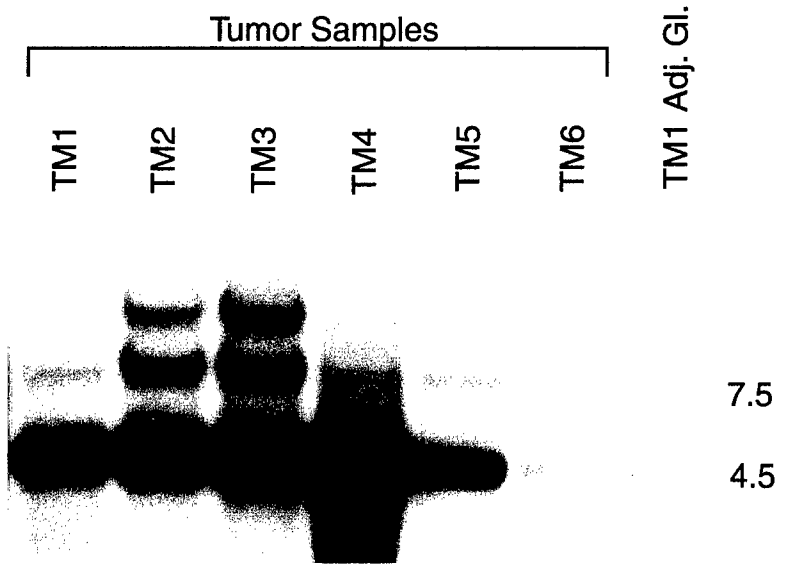


Figure Nine - Overexpression of Neu Protein

(A) Blotting for Neu reveals that only the tumors have elevated levels of Neu. Grb-2 was used to control for equal loading of the 120 mg of protein in each sample. (B) After immunoprecipitating for Neu, a blot was probed for phosphorylated tyrosine (P-Tyr), which demonstrated that catalytically active Neu was overexpressed in the tumors. 1.3 mg of protein was used in this immunoprecipitation. (C) Blotting for ErbB3 reveals that the tumors are also overexpressing ErbB3 in comparison to the adjacent gland.

A



B

Sample	# of Copies Relative to WT	Age at Tumor Detection	Pariety
TM1	8.7	8	MP
TM2	6.0	16	MP
TM3	6.1	15.3	V
TM4	21.6	15	V
TM5	4.8	13	MP
TM6	1.7	14	V
TM7	NC	17	V
TM8	NC	17	V
TM9	NC	17.3	V
Average	8.0	14.7	NA

Figure Ten - Amplification of the NeuNT Knock-in Allele
(A) DNA from the tumor samples was subjected to a Southern analysis as outlined in Figure Four Panel C. Interestingly, the knock-in allele at 4.5 kb is amplified when it is compared to the wild type allele. Also, a new band has appeared in some smples just above the wild type band, like an effect of the amplification event. (B) Using the Adjacent gland as a control, the samples were analysed by phosphoimager analysis. The average number of copies relative to wild type is shown, as is the pairity status of each mouse. MP - multiparious, V - Virgin, NC - Not completed.

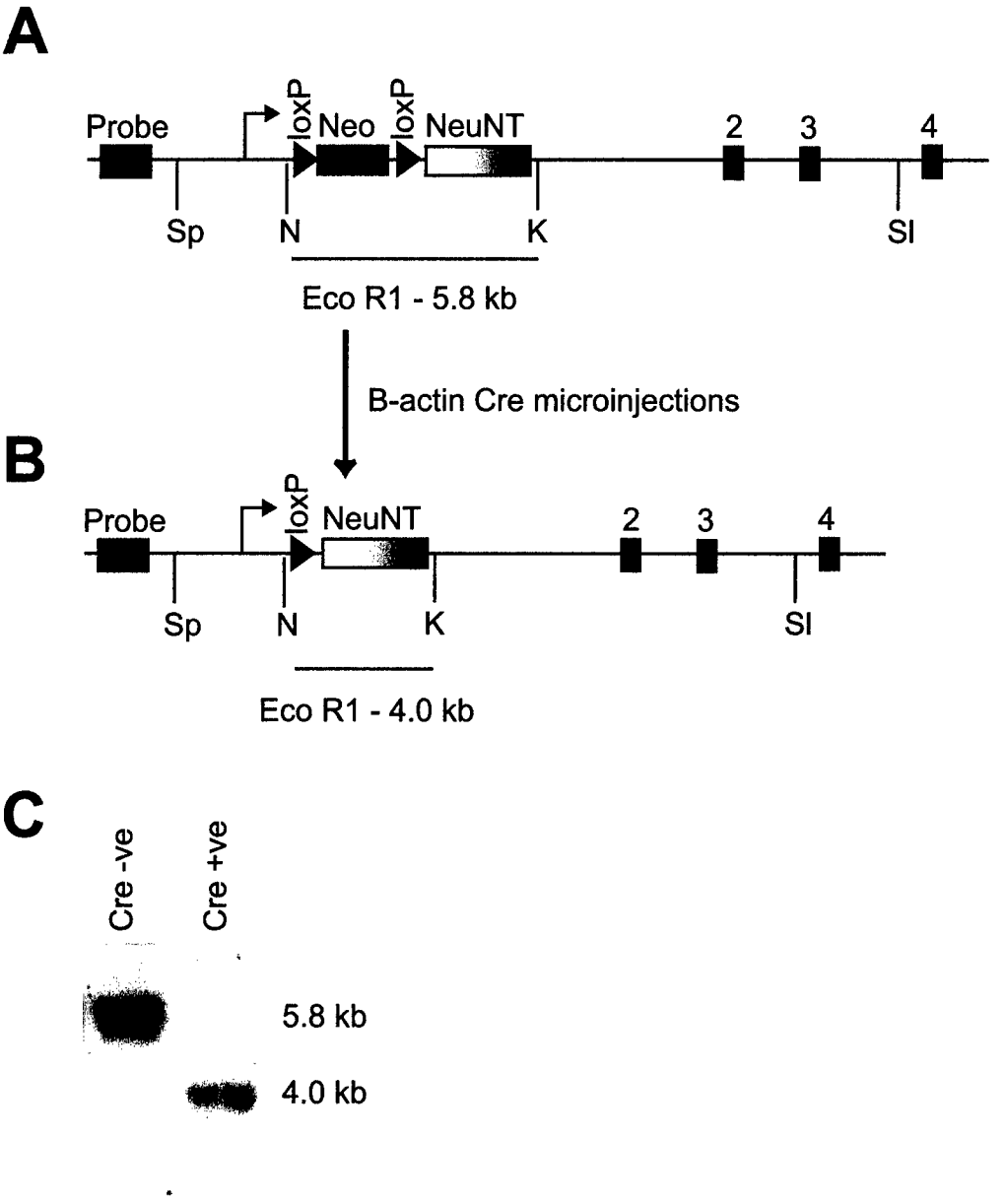


Figure Eleven - Creation of NeuNT knock-in Mice
(A) The original construct is illustrated where the endogenous promoter is not able to control transcription of the oncogenic Neu allele. To decrease the latency of mammary tumorigenesis, A Chicken B-actin Cre plasmid was microinjected, resulting in the allele shown in (B). The endogenous promoter now regulates NeuNT expression at all time points in development. (C) A representative Southern analysis showning that excision of the loxP flanked neomycin cassette is complete, using the same method as shown in Figure Five.

Appendix Two

Reportable Outcomes

Seminar Abstract for the Modelling Human Mammary Cancer in Mice Conference, The Jackson Lab, Maine

Insights from Mouse Models of Neu Mediated Mammary Tumorigenesis

Eran R. Andrechek[1], Peter M. Siegel[1], Robert D. Cardiff[2] and William J. Muller[1]
1 -McMaster University, 2 - University of California at Davis

Amplification and the consequent overexpression of the neu / erbB-2 receptor tyrosine kinase has been observed in 20-30% of human breast cancers. Consistent with the human data, transgenic mice overexpressing wild type neu developed focal mammary adenocarcinomas. Interestingly, a portion of these tumors exhibited deletions affecting conserved cystiene residues in the juxtatransmembrane region, resulting in homodimerization of the receptor. To directly establish the importance of the activated forms of neu, transgenic mice expressing these alleles were created. In contrast to wild type neu expressing strains, which develop focal mammary tumors after a long latency, these activated transgenics rapidly developed multifocal mammary tumors. Observations suggest that activation of intrinsic tyrosine kinase activity is an important step in neu induced tumorigeneisis. However, neu expression in these mice is driven by a strong viral promoter of questionable relevance to the human disease. To ascertain whether expression of activated neu under the control of the endogenous promoter in the mammary gland was sufficient to induce mammary tumors we have generated mice that conditionally express activated neu under the transcriptional control of the intact endogenous neu promoter. Expression of oncogenic neu in the mammary gland resulted in accelerated lobulo-alveolar development and formation of focal mammary tumors after a long latency period. However, expression of activated neu under the normal transcriptional control of the endogenous promoter was not sufficient for the initiation of mammary carcinogenesis as all mammary tumors bore amplified copies of the activated neu allele relative to the wild type allele. Consequently, these tumors express highly elevated levels of neu transcript and protein. This, like human erbB-2 positive breast tumors, mammary tumorigenesis in this mouse model requires the amplification and commensurate elevated expression of the neu gene.

Abstract for the 16th Annual Meeting of Oncogenes

Amplification and Overexpression of Neu / ErbB-2 in an Inducible Mouse Model of Mammary Tumorigenesis

Eran R. Andrechek[1], William R. Hardy[1], Peter M. Siegel[1], Michael A. Rudnicki[1], Robert D. Cardiff[2] and William J. Muller[1]
McMaster University[1], University of California at Davis[2]

The *neu* (*c-erbB-2, Her-2*) proto-oncogene is amplified and overexpressed in 20-30% of human breast cancers. Previous mouse models have clearly demonstrated a role for Neu in the induction of mammary tumors. However, to drive expression of Neu, these mouse models relied upon a strong promoter of questionable relevence to the human disease. To determine whether expression of activated Neu under normal transcriptional control is sufficient to induce tumorigenesis, we have conditionally placed activated Neu under the control of the endogenous promoter. To limit neu expression to tissues of interest, a loxP flanked neomycin cassette was placed between the endogenous promoter and the cDNA for the activated allele. Mammary specific excision of the loxP flanked sequence was achieved using mice expressing Cre recombinase under the control of the MMTV promoter. Expression of the oncogenic form of neu in the mammary gland initially resulted in altered ductal development characterized by numerous side branches terminating in lobulo-alveolar units. After a long latency period (13.2 months), focal mammary tumors developed in these mice. However, expression of activated Neu under the control of the endogenous promoter was not sufficient for the induction of mammary tumorigenesis. Interestingly, all mammary tumors contain an amplification of the activated Neu allele (2-22 copies) relative to the wild type allele and express highly elevated levels of *neu* transcript and protein. Thus, like human *erbB-2*-positive breast tumors, mammary tumorigenesis in this mouse model requires the amplification and commensurate elevated expression of the *neu* gene.

A Neu Tumor Model of Mammary Cancer

Biology Poster Day, March 3, 2000

Eran R. Andrechek, William R. Hardy, Peter M. Siegel, Robert D. Cardiff and William J. Muller

The *neu* (*c-erbB-2*, *Her-2*) proto-oncogene is amplified and overexpressed in 20 to 30% of human breast cancers. While transgenic mouse models have illustrated the role of Neu in the induction of mammary tumors, a strong viral promoter drives the expression of Neu in these models, leading to questions about their relevance to the human disease. In order to determine whether expression of activated Neu under the control of the endogenous promoter in the mammary gland could induce mammary tumors we have generated mice that conditionally express activated Neu under the transcriptional control of the intact endogenous Neu promoter. Expression of oncogenic *neu* in the mammary gland resulted in accelerated lobulo-alveolar development and formation of focal mammary tumors after a long latency period. However, expression of activated Neu under the normal transcriptional control of the endogenous promoter was not sufficient for the initiation of mammary carcinogenesis. Strikingly, all mammary tumors bear amplified copies (2-22 copies) of the activated *neu* allele relative to the wild type allele and express highly elevated levels of *neu* transcript and protein. Thus, like human *erbB-2*-positive breast tumors, mammary tumorigenesis in this mouse model requires the amplification and commensurate elevated expression of the *neu* gene.

Amplification of the *neu/erbB-2* oncogene in a mouse model of mammary tumorigenesis

Eran R. Andrechek*, William R. Hardy*, Peter M. Siegel*, Michael A. Rudnicki*†, Robert D. Cardiff‡, and William J. Muller*†§

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Edited by Philip Leder, Harvard Medical School, Boston, MA, and approved January 6, 2000 (received for review September 23, 1999)

The *neu* (*c-erbB-2, Her-2*) protooncogene is amplified and overexpressed in 20–30% of human breast cancers. Although transgenic mouse models have illustrated the role of Neu in the induction of mammary tumors, Neu expression in these models is driven by a strong viral promoter of questionable relevance to the human disease. To ascertain whether expression of activated Neu under the control of the endogenous promoter in the mammary gland could induce mammary tumors we have generated mice that conditionally express activated Neu under the transcriptional control of the intact endogenous Neu promoter. Expression of oncogenic *neu* in the mammary gland resulted in accelerated lobuloalveolar development and formation of focal mammary tumors after a long latency period. However, expression of activated Neu under the normal transcriptional control of the endogenous promoter was not sufficient for the initiation of mammary carcinogenesis. Strikingly, all mammary tumors bear amplified copies (2–22 copies) of the activated *neu* allele relative to the wild-type allele and express highly elevated levels of *neu* transcript and protein. Thus, like human *erbB-2*-positive breast tumors, mammary tumorigenesis in this mouse model requires the amplification and commensurate elevated expression of the *neu* gene.

The *neu* (*c-erbB2, Her-2*) protooncogene is a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases (1, 2). This family is comprised of several members including; EGFR (ErbB-1, HER) (3), Neu(ErbB-2, HER2) (4–8), ErbB-3 (HER3) (9) and ErbB-4 (HER4) (10). Enhanced expression of members of the EGFR family have been implicated in human breast cancer. In particular, amplification and consequent overexpression of *neu* has been observed in a significant proportion of human breast cancers (11–15). Consistent with these clinical observations, expression of the *neu* oncogene under the transcriptional control of the mouse mammary tumor virus (MMTV) long terminal repeat results in the rapid induction of multifocal mammary tumors (16–18). In contrast to the rapid tumor progression observed with these activated *neu* strains, mammary epithelial specific expression of the *neu* protooncogene in transgenic mice results in the induction of focal mammary tumors that occur only after a long latency period (19). Tumor progression in these strains further correlates with the occurrence of activating mutations in the juxta-transmembrane region of Neu (20). These alterations within Neu involve a set of cysteine residues and result in constitutive disulfide-mediated dimerization of the Neu receptor (20). Although comparable mutations have not yet been observed in Neu-induced human breast cancer, a splice variant of *neu* that closely resembles these sporadic *neu* transgene mutations has been demonstrated to be oncogenic because of its capacity to undergo disulfide-mediated dimerization (21, 22). Taken together, these observations suggest that oncogenic activation of *neu* may be a critical step in mammary tumor progression.

One limitation of these transgenic studies is that activated *neu* expression is driven by a strong viral promoter that is of questionable physiological relevance to human breast cancer. In addition, because the MMTV promoter activity is modulated by steroid hormones such as progesterone and estrogen, the use of these MMTV-based transgenic breast cancer models in elucidating the role of these sex hormones in tumor progression is problematic. One possible solution to these limitations is to generate transgenic mice that carry the activated *neu* oncogene under the transcriptional control of the endogenous *neu* promoter. However, given the importance of *neu* in embryonic development (23), inappropriate expression of activated *neu* may have deleterious consequences. Indeed expression of the *neu* oncogene in organs such as the kidney and lung has been documented to result in perinatal lethality (24).

To circumvent these limitations, we used a gene targeting strategy that replaced the first exon of endogenous *neu* with a Cre-inducible activated *neu* cDNA. To induce expression of this activated *neu* allele in the mammary gland, these targeted knock-in strains of mice subsequently were interbred with transgenic mice expressing Cre recombinase under the transcriptional control of the MMTV promoter enhancer. Mammary-specific expression of activated *neu*, mediated by Cre recombinase, initially resulted in accelerated mammary epithelial development. Subsequently, focal mammary tumors arose in these mice after a long latency period. Further, tumorigenesis in this model was associated with selective amplification of the activated *neu* allele (2–22 copies) that correlated with elevated levels of *neu* transcript and protein. These observations suggest that like human breast cancer, mammary tumorigenesis in this transgenic mouse model requires the concerted amplification and overexpression of the Neu protein.

Materials and Methods

Generation of MMTV-Cre Transgenics. The cDNA encoding Cre recombinase was subcloned into pMMTV-SV40Pa (p206) (25). This construct was prepared for and injected as described (20). Transgenic progeny were identified by Southern analysis (20) using the *Bam*HI–*Cla*I (840–1209) fragment of the Cre cDNA (26) as a probe.

Gene Targeting. ErbB-2 genomic sequences were isolated from a 129/SvJ genomic library. A 2.5-kb 5′ arm of homology (*Sph*I to *Nar*1) 5′ to exon one and a 8-kb 3′ arm of homology (*Kpn*I to *Sal*I) including exons 2 and 3 were designed. A loxP-flanked phosphoglycerate kinase(PGK)-neomycin-herpes simplex virus (HSV) poly(A) cassette was placed 5′ of the *neuNT* cDNA, which was flanked at the 3′ end by a simian virus 40 poly(A) sequence.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: MMTV, mouse mammary tumor virus; HSV, herpes simplex virus; PGK, phosphoglycerate kinase.

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This loxP-flanked Neo NeuNT sequence replaced exon 1 of the genomic allele. The construct was linearized by a *Not*I site 5′ to the 5′ arm of homology before R1 embryonic stem (ES) cell electroporation, and G418 selection. After screening 288 G418-resistant colonies by Southern analysis using a probe 5′ to the *Sph*I site, six positive cell lines were identified. Six- to 8-week-old BALB/c females were used to generate blastocysts that were harvested 3.5 days postcoitum. ES cells were injected into the blastocoel cavity, and the blastocysts then were reimplanted into pseudopregnant CD1 females. The chimeras were identified on the basis of coat color and were interbred with BALB/c mice. The genotype of the mice was determined by Southern analysis on tail DNA using a *Hin*dIII digest and the 600-bp external probe (*Sma*I fragment). All techniques were performed as described (27).

Whole-Mount and Immunohistochemical Analysis. A whole-mount analysis was completed on the #4 mammary gland of mature virgin females as described (28, 29) because of the ease of examination and the presence of a lymph node, which can be used to quantitate the epithelial penetration of the fat pad. Immunohistochemistry was performed according to standard methods using a rabbit polyclonal antibody (800–874-8667) against Neu (Ciba Corning) as described (30).

RNA Analysis. RNA was isolated and RNase protections were carried out as described (20). The antisense *neu* riboprobe was the *Sma*I–*Xba*I fragment of *neu* cDNA including the transmembrane domain (22). The antisense *cre* riboprobe was directed against 480 bp of the HSV pA sequence. The control *pgk* riboprobe was prepared as described (22).

Immunoprecipitation and Immunoblotting. Protein lysates from tissues were prepared and quantified by using conventional methods (20). Neu was detected by a mouse mAb (AB-3, Oncogene Research Products). Immunoblotting for ErbB-3 (C-17) and Grb-2 (C-23) was performed by using rabbit polyclonal antibodies (Santa Cruz Biotechnology). Immunoprecipitations were completed in accordance with standard protocols using a mouse mAb for Neu (7.16.4) and were probed by using an antiphosphotyrosine antibody (PY20, Transduction Laboratories, Lexington, KY) (22).

Quantification of Amplification. To determine the extent of amplification, a Southern analysis was conducted on samples that were restricted with *Hin*dIII and probed by using the external probe using methods described above. The intensity of the wild-type and recombinant alleles was measured and compared by PhosphorImager analysis (Molecular Dynamics).

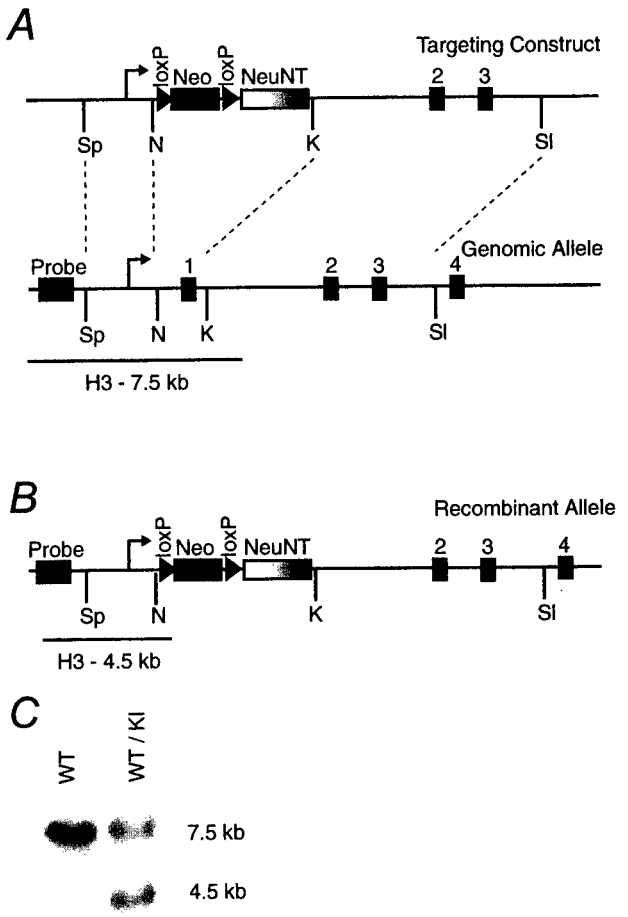

Fig. 1. Targeting of the conditionally activated *neu* allele. (*A*) A schematic representation of the targeting construct and the genomic allele. The 2.5-kb 5′ arm of homology (*Sph*I to *Nar*1) and the 8-kb 3′ arm of homology (*Kpn*I to *Sal*I) were used to direct the homologous recombination to the wild-type allele, illustrated by the dashed lines. Exon 1 of the endogenous allele was replaced by a loxP (triangle)-flanked PGK-neomycin-HSV poly(A) (Neo) cassette, followed by the *neuNT* cDNA and a simian virus 40 poly(A) (NeuNT). The loxP-flanked Neo NeuNT cDNA has replaced exon 1 contained within the *Nar*1/*Kpn*I fragment. The size of the genomic *Hin*dIII (H3)-restricted fragment when detected by a probe 5′ to the site of homologous recombination also is depicted. Sp, *Sph*I; N, *Nar*1; K, *Kpn*I; Sl, *Sal*I. (*B*) The recombinant allele containing the loxP-flanked Neo NeuNT cassette in place of exon 1 and the corresponding size of the *Hin*dIII restriction fragment detected by the external probe are shown. (*C*) A representative Southern blot of tail DNA from mice that are wild type (WT) and heterozygous for the knock-in (KI) allele.

Results

Generation and Characterization of Transgenic Mice that Conditionally Expressed Activated *neu* cDNA. To place an oncogenic *neu* allele under the transcriptional control of the endogenous promoter in the mammary gland, we have used a strategy where the first coding exon of the endogenous *neu* gene was replaced by an oncogenic *neu* cDNA containing an activating point mutation in the transmembrane domain. We previously have demonstrated that replacement of the first coding exon of *neu* with the wild-type *neu* cDNA can completely rescue the embryonic lethality associated with the germ-line inactivation of *neu* (W.R.H., R. Chan, M. A. Laing, and W.J.M., unpublished observations). To circumvent the potential deleterious consequences of expressing an activated *neu* oncogene, we designed a targeting vector in which a loxP-flanked neomycin cassette was placed upstream of the oncogenic *neu* cDNA, preventing activated *neu* expression in the absence of Cre recombinase (Fig. 1*A*). We previously have shown that this loxP neo cassette can block *neu* expression from the Moloney murine leukemia viral enhancer (E.R.A., W.R.H., and W.J.M., unpublished observations). Electroporation of this targeting vector resulted in the generation of several independent embryonic stem cell lines carrying the expected targeted allele (Fig. 1*B*). After germ-line transmission of this recombinant allele (Fig. 1*C*), interbreeding of mice carrying this activated *neu* allele generated only wild-type and heterozygous pups, indicating that the nonexcised recombinant *neu* allele was indeed acting as a null allele, resulting in embryonic lethality.

Induction of Activated *neu* Expression in the Mammary Epithelium. To induce expression of the activated *neu* allele in the mammary epithelium, transgenic mice expressing Cre recombinase in the mammary epithelium were generated by placing the *Cre* cDNA under the control of the MMTV promoter (Fig. 2*A*). Seven transgenic founders were identified, of which six transmitted the

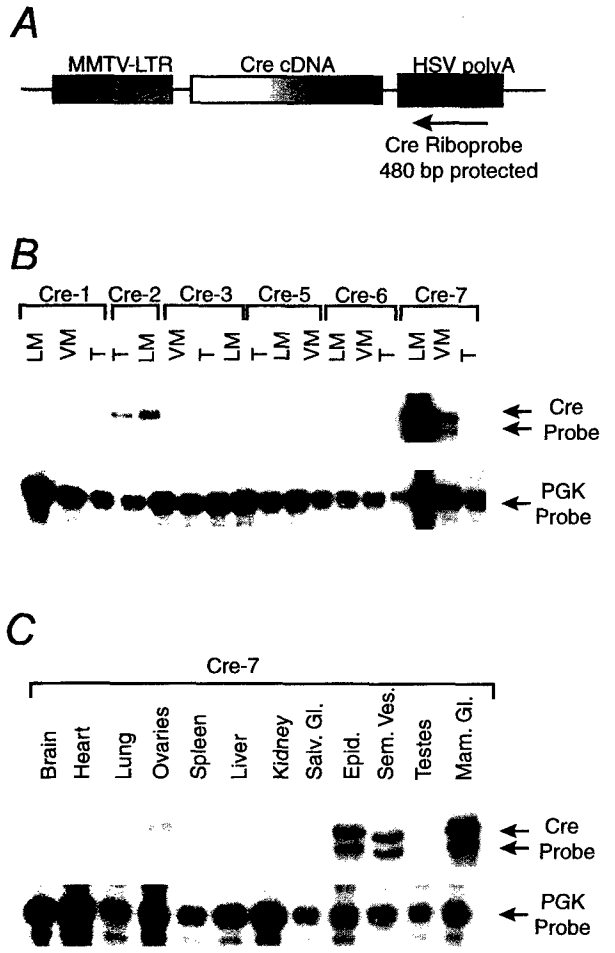

Fig. 2. Generation of MMTV-Cre transgenics. (*A*) A schematic of the transgenic construct is shown. The MMTV-long terminal repeat (MMTV-LTR) (gray) was used to drive expression of the *cre* cDNA (gradient fill). The *cre* cDNA was followed by the HSV polyadenylation [HSV poly(A)] sequence (black). The 480-bp antisense riboprobe is depicted by the arrow and is directed against the HSV poly(A). (*B*) The RNase protection on the lactating mammary gland (L), virgin mammary gland (V), and testes (T) for the six lines that passed the transgene shows that expression was occurring in the Cre-2, Cre-3, and Cre-7 lines. (*C*) An RNase protection screening major organs of the Cre-7 line. Note the expression primarily limited to the mammary gland and male accessory reproductive organs. The *pgk* riboprobe was included as an internal control for equal RNA loading.

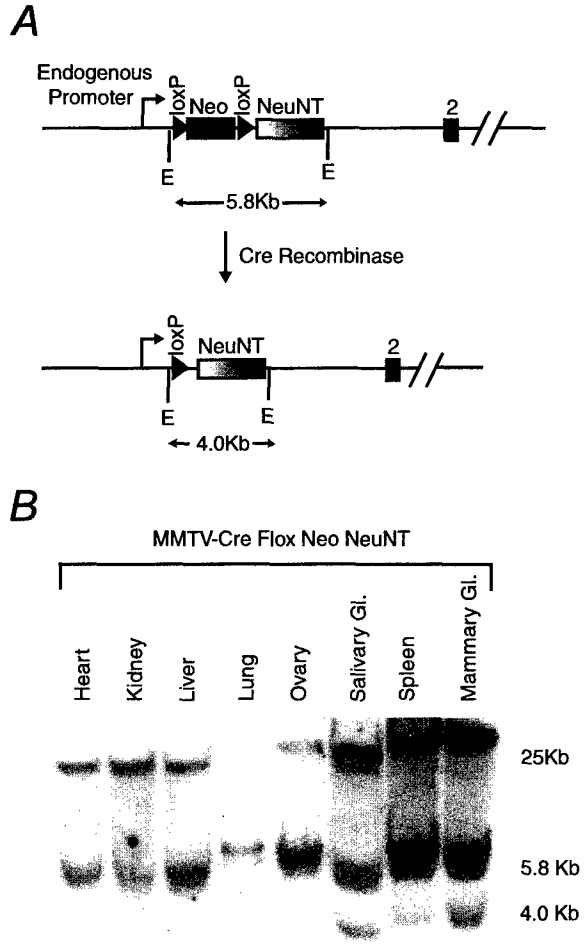

Fig. 3. Excision of the loxP- flanked neomycin cassette. (*A*) The recombinant allele is shown before and after Cre recombinase-mediated excision. Before excision the endogenous promoter will not regulate *neuNT* expression because of the poly(A) in the neomycin cassette. To detect removal of the neomycin cassette, the *neuNT* cDNA was used as a probe in an *Eco*RI (E) digest. The size of this fragment is depicted in both the excised and nonexcised forms. (*B*) A Southern blot shows that excision of the loxP-flanked Neo is limited to the spleen, salivary, and mammary glands by the presence of a 4-kb band in those samples. The wild-type allele also is detected by the *neuNT* cDNA probe, resulting in a faint band of 6.0 kb and a stronger band of 25 kb.

transgene to their progeny. An RNase protection assay on RNA isolated from testes, and mammary glands revealed three lines that expressed *Cre* in the mammary gland (Fig. 2*B*). Because leaky Cre expression in other organs could potentially result in *neu*-induced lethality, we performed a further RNase protection analyses on RNA harvested from major organs in these three lines. These analyses revealed that the Cre-2 and Cre-3 lines were unsuitable for our experiments because of expression in the brain and heart, respectively. Importantly, the RNase protection analysis, which surveyed major organs from the Cre-7 line, revealed that there was a high level of expression in the mammary gland with expression in other tissues primarily limited to the male accessory reproductive organs (Fig. 2*C*).

Mating the MMTV-Cre-7 mice with the mice harboring the inducible activated *neu* allele resulted in bigenic mice at the expected Mendelian ratio. Consistent with the RNase protection results, Southern blot analyses of DNA from all major organs revealed that excision of the loxP-flanked neomycin cassette was restricted primarily to the mammary gland, although spleen and salivary excision also were noted (Fig. 3). Quantitative PhosphorImager analyses of the Southern blots demonstrated that excision of the loxP-flanked neomycin cassette was 30–40% complete in the mammary gland. Although excision of the neomycin cassette was noted in the spleen and salivary glands, examination of these organs at the gross or histological level failed to reveal any abnormalities.

Whole-Mount and Immunohistochemical Analysis of Mammary Glands and Tumors. Using whole-mount analysis to examine the mammary epithelium, no significant deviation from the wild-type mammary structure was observed in either the Cre-7 or Flneo NeuNT mice (data not shown). However, when virgin bigenic mammary glands were examined, an abnormal mammary structure was observed (Fig. 4*A*). In contrast to the normal pattern of lobuloalveolar development (Fig. 4*B*), numerous lobular side buds were observed in the mammary glands of mice carrying the activated *neu* allele (Fig. 4*A*). High magnification histology of this abnormal structure revealed that the lobular side buds have formed acinar structures and are not solid dysplasias (Fig. 4*C*).

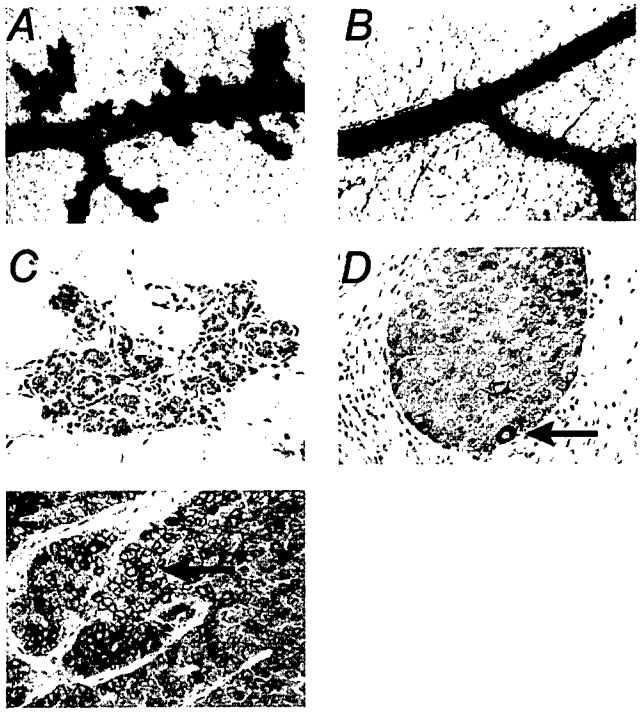

Fig. 4. Digitized micrographic images of whole-mount (*A* and *B*) and immunohistochemical (*C–E*) analysis of the MMTV-Cre Flneo NeuNT mammary gland and tumors. (*A*) Whole mount of a virgin MMTV-Cre Flneo NeuNT mammary gland at 9 months. The extensive side branching terminating in lobuolalveolar units should be noted. (*B*) Whole mount of a virgin wild-type control mammary gland at a similar stage of development illustrating a normal duct with few side branches. (*C*) Immunohistochemical analysis of the same virgin gland shown in *A*. Note the absence of anti-Neu staining in the acinar structures, which are not dysplastic. Immunohistochemical analysis on the bigenic (*D*) and a control MMTV-Neu (*E*) induced tumor shows high levels of Neu expression in these tumors. The contrast in the cytoplasmic and membrane localization of the stain indicated by the arrows in *D* and *E*, respectively should be noted. (Magnifications: *A* and *B*, × 40; *C–E*, ×320.)

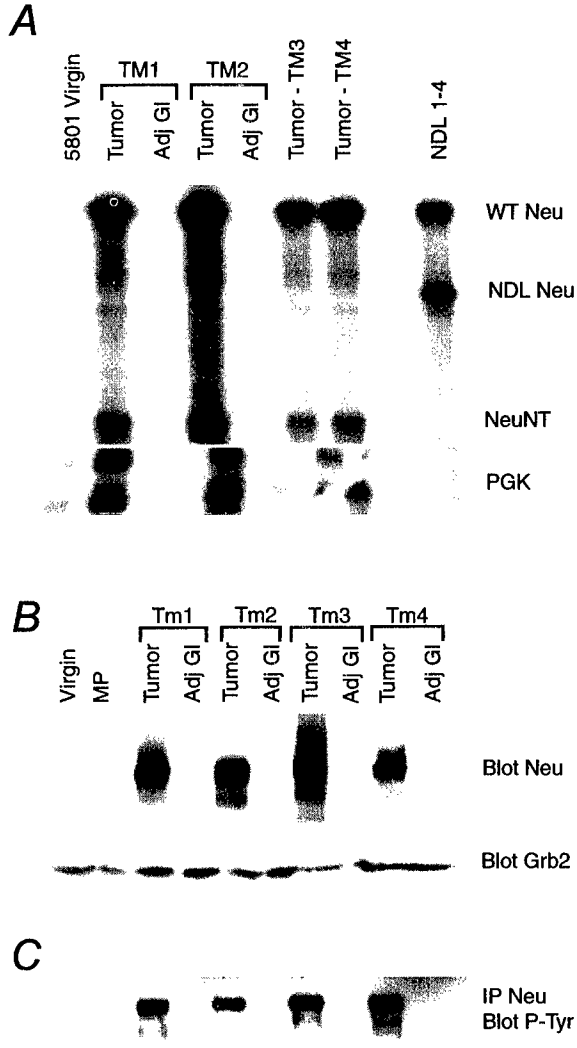

Fig. 5. Overexpression of activated NeuNT in mammary tumors. (*A*) Using a probe that spans the transmembrane domain of Neu, an RNase protection reveals that MMTV-Cre Flneo NeuNT tumors (TM1–4) overexpress the activated form of *neu*. The mammary gland from a virgin MMTV-Cre Flneo NeuNT mouse (5801) was included, as was a positive control known to overexpress an activated form of *neu* (NDL 1–4). The full-length protected fragment is shown at the band labeled WT Neu. The mutant alleles are labeled NDL Neu and NeuNT, corresponding to their activating mutations. Thirty micrograms of RNA was used, except in the control NDL 1–4 tumor sample, where 20 μg was used. *pgk* was included as an internal control for equal loading of the samples. Adj. Gl., adjacent mammary gland. (*B*) Blotting for Neu reveals that only the tumors have elevated levels of Neu. Grb-2 was used to control for equal loading of the 120 μg of protein in each sample. MP, multiparious. (*C*) After immunoprecipitating for Neu, a blot was probed for phosphorylated tyrosine (P-Tyr), which demonstrated that catalytically active Neu was overexpressed in the tumors. Protein (1.3 mg) was used in this immunoprecipitation.

Interestingly, these early lesions have no detectable staining for Neu. Although the initial mammary phenotype observed was limited to enhanced branching, older female mice developed focal comedo-adenocarcinomas (n = 9, 45% penetrant in mice over 1 year of age). Immunohistochemical examination of these tumors expressing activated Neu revealed both membrane and cytoplasmic immunoreactivity for Neu (Fig. 4*D*), in contrast to the MMTV-Neu induced tumors where the staining for Neu is distinctly membrane specific (Fig. 4*E*). Importantly, this MMTV-Neu transgenic control is expressing a wild-type *neu* allele, which does not force the constitutive dimerization associated with the activated allele. Despite the difference in localization of Neu, the mammary tumors from these strains were histologically identical. The apparent difference in localization likely reflects the rapid endocytosis of activated Neu, which is known to undergo constitutive dimerization (31).

Induction of Mammary Tumors Involves Elevated Expression and Amplification of the Activated *neu* cDNA. To ascertain whether tumor progression in these strains involved the up-regulation of activated *neu* allele, we determined the levels of *neu* transcript and protein in mammary tumors and adjacent normal mammary gland. To assess the levels of *neu* transcript in these tissues we performed RNase protection analyses on RNA from these tissues with a probe complementary to the transmembrane domain of Neu. In contrast to the normal mammary epithelium, elevated levels of protected fragment corresponding to the expression of the oncogenic *neu* allele were detected in mammary tumors (Fig. 5*A*, NeuNT band). Given that the NDL 1–4 control does not express wild-type *neu*, the presence of the protected fragment corresponding to the wild-type allele in these tumor samples likely reflects the nonstringent hybridization and digestion conditions used in these analyses. Further, because the activated rat *neu* cDNA differs from the endogenous mouse cDNA in its *Bam*HI restriction profile, we recently have shown by reverse transcriptase–PCR analyses that the majority of *neu* transcripts from the tumor RNA are derived from the activated rat allele (data not shown). Consistent with the RNase protec-

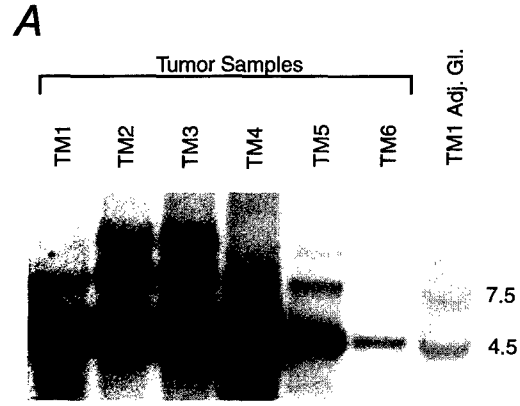

Fig. 6. Amplification of *neu* in mammary tumors. Southern analysis showing the amplification of the recombinant allele (4.5 kb) in respect to the wild-type allele (7.5 kb) in the MMTV-Cre FIneo NeuNT tumors (TM1–6). The TM1 adjacent gland (TM1 Adj. Gl.) also is shown, illustrating that detectable amplification has not occurred in the normal mammary gland. The samples were restricted with *Hin*dIII and probed with the external probe using the same strategy described in Fig. 1. Interestingly, a new band has appeared above the wild-type band in TM2, TM3, and TM5, likely a result of the amplification process. This Southern analysis was subjected to a quantitative PhosphorImager analysis where the increase in *neuNT* copy number was determined relative to the wild-type allele. The results are shown in Table 1.

tion analyses, immunoprecipitations and immunoblots with Neu and phosphotyrosine-specific antibodies revealed elevated levels of both Neu and tyrosine-phosphorylated Neu protein in the tumor tissues compared with matched adjacent mammary glands. (Fig. 5 *B* and *C*).

Given the elevated expression of *neu* in the mouse mammary tumor samples, they were examined for amplification of the recombinant and wild-type alleles. A Southern blot analyses of genomic DNA from tumor tissues revealed that in all tumors examined ($n = 6$), there was a selective amplification of the recombinant *neu* allele relative to the wild-type *neu* allele (Fig. 6). Additionally, a third band above the wild-type and recombinant bands appeared with the same number of copies as the wild-type allele in half of the surveyed tumors, likely a result of the amplification process. Quantitative PhosphorImager analyses revealed a 2- to 22-fold amplification of this activated *neu* allele relative to the wild-type allele (Table 1), suggesting that mammary tumorigenesis in this strain requires both the amplification and elevated expression of the *neu* oncogene and does not appear to correlate with pariety status.

Table 1. Amplification of NeuNT

Sample	# of copies relative to wild type	Age at tumor detection, months	Pariety
TM1	8.7	8	MP
TM2	6.0	16	MP
TM3	6.1	15.3	V
TM4	21.6	15	V
TM5	4.8	13	MP
TM6	1.7	14	V
TM7	NC	17	V
TM8	NC	17	V
TM9	NC	17.3	V
Average	8.0	14.7	

Differences in detection of different-sized fragments by Southern analysis were negated by standardizing to the adjacent gland control. MP, multiparious; V, virgin; NC, not completed.

Discussion

We have demonstrated that mammary epithelial-specific expression of oncogenic *neu* under the transcriptional control of its physiological relevant promoter is capable of inducing abnormal mammary development and ultimately mammary tumors. To achieve mammary-specific induction of activated *neu* expression, we have taken advantage of a transgenic mice expressing Cre recombinase under the transcriptional control of the MMTV promoter enhancer. A previous report that suggested that MMTV-directed Cre expression resulted in widespread excision in many different tissues (32). In contrast to that report, our studies have revealed that induction of activated *neu* expression by the Cre recombinase was limited to relatively narrow set of tissues, including the mammary gland, spleen, and salivary gland (Fig. 3). The discordance between studies likely reflects differences in the tissue specificity of Cre transgene expression. Indeed, more recently, mammary-specific excision of the BRCA1 gene has achieved by crossing loxP-flanked BRCA1 strains with a separate MMTV/Cre transgenic strain (33). Future studies with these MMTV/Cre strains should prove useful in creating mammary-specific activation or deletion of genes critical for normal development.

In contrast to the rapid tumor progression observed in transgenic mice carrying MMTV-driven activated *neu* alleles, expression of activated *neu* under transcriptional control of its physiological promoter is not sufficient for induction of mammary tumors. Rather, these transgenic mice have accelerated lobuloalveolar development that correlates with the low levels of activated *neu* expression. However, female mice that express *neu* from the endogenous promoter eventually develop mammary tumors after a long latency period. Tumor progression in these strains was correlated with a dramatic elevation of both *neu* transcript and protein when compared with adjacent gland controls. One possible explanation for the elevated level of *neu* transcripts is that the selective amplification of the recombinant *neu* allele during tumor progression has occurred, analogous to the amplification and overexpression of *erbB-2* observed in human breast cancer (11–15). Indeed, when the tumors were analyzed for amplification, they were found to have between two and 22 additional copies of the recombinant *neu* allele. The strong selection for amplification of the activated *neu* allele in this model may reflect the requirement of a critical threshold level of Neu for oncogenic conversion of the mammary epithelial cell. However, because the levels of activated *neu* do not precisely correspond to the extent of *neu* overexpression, there may be other molecular mechanisms involved in the observed elevated expression of the activated *neu* allele. In this regard, it has been reported that a certain percentage of *neu*-expressing human breast carcinomas do not exhibit evidence of *neu* amplification (13). Taken together, these observations suggest that amplification and the consequent elevated expression of activated *neu* is required for efficient transformation of the mammary epithelial cell.

In addition to closely mimicking the human disease, another important feature of this model is that activated *neu* expression ultimately is controlled by transcription factors that regulate the endogenous *neu* promoter sequence. Given the importance of number of transcription factors such as c-myc (34) and the estrogen receptor (35) in the progression of breast cancer, future studies with these mice should provide insight into the role that these critical transcription factors play in *neu*-induced breast cancer. In addition to breast cancer, a number of recent studies have suggested that *neu* may be involved in the induction of ovarian, lung, gastric, and prostate cancers (2, 36, 37). Given the Cre-inducible nature of this activated *neu* allele, future studies with tissue-specific expression of Cre should allow investigators to directly assess the role of activated *neu* in these tissue sites.

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Accelerated Mammary Tumor Development in Mutant Polyomavirus Middle T Transgenic Mice Expressing Elevated Levels of Either the Shc or Grb2 Adapter Protein

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The Grb2 and Shc adapter proteins play critical roles in coupling activated growth factor receptors to several cellular signaling pathways. To assess the role of these molecules in mammary epithelial development and tumorigenesis, we have generated transgenic mice which individually express the Grb2 and Shc proteins in the mammary epithelium. Although mammary epithelial cell-specific expression of Grb2 or Shc accelerated ductal morphogenesis, mammary tumors were rarely observed in these strains. To explore the potential role of these adapter proteins in mammary tumorigenesis, mice coexpressing either Shc or Grb2 and a mutant form of polyomavirus middle T (PyV mT) antigen in the mammary epithelium were generated. Coexpression of either Shc or Grb2 with the mutant PyV mT antigen resulted in a dramatic acceleration of mammary tumorigenesis compared to parental mutant PyV mT strain. The increased rate of tumor formation observed in these mice was correlated with activation of the epidermal growth factor receptor family and mitogen-activated protein kinase pathway. These observations suggest that elevated levels of the Grb2 or Shc adapter protein can accelerate mammary tumor progression by sensitizing the mammary epithelial cell to growth factor receptor signaling.

The murine mammary gland represents a unique system to study the responsiveness of cells to diverse signals stimulating cell death, survival, proliferation, and differentiation. The control of mammary epithelial proliferation and differentiation is ultimately regulated by hormonal and peptide factors that exert their biological action through a variety of receptor molecules. Elevated expression of growth factors or their cognate receptors can result in deregulated mammary epithelial cell proliferation, which can ultimately progress to the malignant phenotype. For example, elevated expression of the ErbB-2/Neu receptor tyrosine kinase has been implicated in the genesis of a large proportion of human breast cancers (39, 40). Consistent with these observations, mammary epithelial expression of ErbB-2 in transgenic mice results in the efficient induction of mammary tumors (4, 14, 16, 25, 35). Whereas it is clear that oncogenes such as *erbB-2* induce malignancy, the precise molecular mechanism by which this occurs is unclear.

One potential mechanism by which receptor tyrosine kinases (RTKs) can induce proliferation is through interaction with a number of Src homology 2 (SH2)- or protein tyrosine binding domain (PTB)-containing adapter proteins (27). Although adapter proteins such as Shc (Src homology and collagen) and Grb2 (growth factor receptor-bound protein 2) lack intrinsic enzymatic activity, they play an important role in connecting growth factors to specific signaling pathways (23, 28, 29, 32). Grb2 is a 25-kDa protein which contains a central SH2 domain flanked by two SH3 domains. Activation of RTKs can result in the direct recruitment of Grb2 via its SH2 domain to specific tyrosine-phosphorylated residues within the receptor. Subsequent recruitment of the guanine nucleotide exchange factor Sos to the plasma membrane via interaction with the SH3 domain of Grb2 results in nucleotide exchange on Ras and activation of the Ras/mitogen-activated protein kinase (MAPK) pathway (23, 32).

Another mechanism by which Grb2 can be indirectly recruited to RTKs is through its specific association with the Shc adapter protein. The human *Shc* gene is localized on chromosome 1q21 and encodes three distinct Shc isoforms. The p52 and p46 forms of Shc, which result from the use of distinct start translation sites, possess a conserved N-terminal PTB domain, a central collagen homology (CH-1) domain, and a C-terminal SH2 domain (3). The p66 form of Shc is generated by alternative splicing and encodes an additional N-terminal CH-2 domain. The association between Shc and Grb2 is mediated through the interaction of the Grb2 SH2 domain with two tyrosine phosphorylation sites present with the central CH-1 domain of Shc (tyrosines 239 and 240 and tyrosine 317) (13, 17, 32, 38, 45). Shc in turn can bind activated RTKs through a PTB domain that recognizes specific NPXY motifs within the receptor (1, 2, 5, 44). In addition to the PTB domain, Shc also possesses an SH2 domain, which is capable of interacting with a number of phosphotyrosine-containing proteins (12, 28).

There is considerable evidence implicating the Shc and Grb2 adapter proteins as critical functional components of oncogene-mediated signal transduction pathways. Indeed, elevated levels of Grb2 can be detected in a large percentage of human breast cancers and their derived cell lines (8, 46). Interestingly, in a subset of these breast tumors, the chromosomal regions

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encoding the *Grb2* gene are amplified (8, 46). Moreover, the fact that Shc is constitutively phosphorylated in a high percentage of human breast tumors and breast cancer cell lines (30, 42) suggests that it is functionally involved in coupling RTKs to the Ras signaling pathway. Direct evidence for the involvement of the Grb2 adapter protein in mammary tumorigenesis has been derived from the recent observation that polyomavirus middle T (PyV mT) oncogene-expressing transgenic mice heterozygous for a *Grb2* null allele demonstrate a significant delay in the onset of mammary tumors (6). Consistent with these observations, transgenic mice expressing a mutant PyV mT oncogene decoupled from the Shc and Grb2 adapter proteins in mammary epithelium exhibit a significant delay in the onset of mammary tumors compared to mice expressing the wild-type PyV mT oncogene (48). Taken together, these observations suggest that activation of the Shc and Grb2 adapter proteins plays a critical role in the induction of mammary tumors.

To further explore the role of the Shc and Grb2 adapter proteins in mammary tumor progression, transgenic mice expressing the *Grb2* or *Shc* cDNA under the transcriptional control of the mouse mammary tumor virus (MMTV) promoter were generated. Female transgenic mice expressing these adapter proteins in the mammary epithelium were capable of nursing their litters. Whole-mount analyses of virgin mammary glands revealed that both the MMTV/Grb2 and MMTV/Shc strains exhibited evidence of enhanced tertiary branching. Specifically, the MMTV/Shc strains exhibited an increased number of terminal end buds, whereas the MMTV/Grb2 strains displayed enhanced side branching. Despite these mammary epithelial abnormalities, the MMTV/Shc mice rarely developed mammary tumors, while the MMTV/Grb2 strains failed to develop tumors during the observation period. To assess the importance of Grb2 and Shc in mammary tumorigenesis, these strains were interbred with transgenic mice expressing a mutant PyV mT antigen incapable of directly coupling to the Shc pathway. The results of these studies demonstrated that coexpression of either Grb2 or Shc with this mutant PyV mT oncogene resulted in accelerated tumor development, which was correlated with activation of the MAPK pathway.

MATERIALS AND METHODS

DNA constructions. All transgene constructs were previously derived by inserting the appropriate cDNAs into the MMTV long terminal repeat (LTR) expression vector, p206 (25). The MMTV LTR component of p206 was derived from plasmid pA9 (20), and the simian virus 40 (SV40) transcriptional processing signals 3′ to the cDNA were derived from plasmid CDM8 (33). MTY250F was constructed by standard M13 mutagenesis of PyV mT and cloned into the *Hin*dIII and *Eco*RI sites of p206 to generate MMTV/MTY250F (48). MMTV/p52 Shc (herein referred to as MMTV/Shc) was generated by cloning the mouse p52 Shc cDNA into the *Hin*dIII and *Eco*RI sites of p206 and was provided by Venus Ka-Man Lai and Tony Pawson (Mount Sinai Hospital, Toronto, Ontario, Canada). Finally, MMTV/Grb2 was provided by R. Daly (Garvan Institute of Medical Research, Sidney, New South Wales, Australia) and was constructed by inserting into p206 the *Eco*RI fragment of the human Grb2 cDNA. To aid in the identification of tissue specificity of transgene expression, plasmid pASV was used to generate an antisense riboprobe (25). A phosphoglycerate kinase 1 internal control was obtained from M. Rudnicki (McMaster University) and contains the *Acc*I/*Pst*I fragment of phosphoglycerate kinase 1 in the appropriate sites of pSP64 (Promega).

Generation and identification of transgenic mice. DNA was prepared for microinjection by digestion of pMMTV/Shc and MMTV/Grb2 with 4 U of *Sal*I and *Spe*I per μg for 1.5 h. The DNA was subsequently electrophoresed through a 1% agarose gel, and the resultant fragment was purified as previously described (36). The night prior to injection, superovulated FVB/N mice were mated with FVB/N males (Taconic Farms, Germantown, N.Y.). Fertilized, one-cell embryos were isolated, and the pronuclei of the zygotes were injected with 0.5 to 1 pl of DNA (5 μg/ml). This was followed by oviduct transfer of viable embryos to pseudopregnant Swiss-Webster mice (Taconic Farms). The MMTV/MTY250F 5a strain was established as described previously (48).

To identify potential transgenic founders, DNA was extracted from 1.5-cm tail clippings of the progeny as previously described by Muller et al. (25). Briefly, tail clippings were digested overnight in PK (proteinase K) buffer (10 mM Tris [pH 8.0], 100 mM NaCl, 10 mM EDTA [pH 8.0], 0.5% sodium dodecyl sulfate [SDS], 0.2 mg of PK [Canadian Life Technologies, Burlington, Ontario, Canada] per ml). DNA was isolated by several buffer-saturated phenol-chloroform extractions and precipitated in 2 volumes of absolute ethanol–0.1 volume of 3 N sodium acetate. The nucleic acid pellet was resuspended in 50 μl of Tris-EDTA buffer (10 mM Tris [pH 8.0], 1 mM EDTA, RNase A [20 μg/ml]) to an approximate concentration of 1 mg/ml. A volume of 15 μl of the solution was digested with 30 U of *Bam*HI at 37°C for 1.5 h. Digested samples were subsequently electrophoresed through 1.0% agarose gels. Gels were then denatured for 45 min to 1 h with 600 mM NaCl–200 mM NaOH followed by an equal duration of neutralization in 600 mM NaCl–1 M Tris (pH 7.5) under constant shaking at room temperature. Gels were then transferred to GeneScreen (Dupont) according to the method of Southern (41) and cross-linked to the filters by using a UV Stratalinker device (Stratagene, La Jolla, Calif.). Filters were prehybridized at 60°C for several hours in 0.1 mg of sheared salmon sperm DNA per ml, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.5% SDS, and 5× Denhardt's reagent. A [α-^{32}P]dCTP-labelled SPA (SV40 polyadenylation) DNA probe was prepared by random priming (10) using the gel-purified 750-bp *Bam*HI/*Eco*RI fragment from pSPA. Filters were hybridized with the SPA probe overnight. The next day, filters were washed with 150 mM sodium phosphate buffer–1% SDS once for 20 min at room temperature and once for 20 min at 60°C. After blotting dry, tail DNA restriction fragments hybridizing with the SPA probe were detected by autoradiography using Kodak X-Omat AR film (Kodak, Rochester, N.Y.).

RNA analyses. To determine the tissue specificity of transgene expression and to select several lines that demonstrated a high degree of expression in the mammary gland, RNase protection analysis (24) was performed on mouse tissues. Briefly, RNA was isolated from various tissues by the method of Chirgwin et al. (7). To generate the antisense SPA riboprobe, pASV was linearized by *Hin*dIII digestion, and the gel-purified fragment (Geneclean; Biocan) was in vitro transcribed. The gel was dried, and tissues which expressed SV40-hybridizing transcripts were detected by autoradiography using Kodak X-Omat AR film. RNA was purified by phenol-chloroform extraction followed by precipitation in 2 volumes of absolute ethanol–0.1 volume of 3 N sodium acetate (pH 5.2). Finally, RNA was resuspended in 100:1 diethyl pyrocarbonate-water, and yield was determined by UV absorption at 260 nm (Uvikon).

Northern blot analysis of RNA was performed as follows. Thirty micrograms of total RNA in a volume of 6.75 μl was incubated with 3 μl of 10× MOPS (morpholinepropanesulfonic acid) buffer (200 mM MOPS, 50 mM sodium acetate, 10 mM EDTA), 5.25 μl of formaldehyde (37%), and 15 μl of deionized formamide for 15 min at 55°C. Samples were chilled on ice, and 3.3 μl of loading dye (50% glycerol, 1 mM EDTA, 1 mg of xylene cyanol FF per ml, 1 mg of bromophenol blue per ml) was added. Samples were electrophoresed on formaldehyde gels (1% agarose, 1× MOPS, 0.7% formaldehyde, 0.5 μg of ethidium bromide per ml) at 100 V in 1× MOPS buffer for approximately 2 h. Following electrophoresis, gels were washed twice for 5 min in distilled water, followed by two incubations in 20× SSC (3.0 M NaCl, 0.34 M sodium citrate). Gels were transferred overnight to Hybond-N membranes (Dupont) according to the method of Southern (41). Random-primed DNA probes were prepared according to the method of Feinberg and Vogelstein (10). [α-^{32}P]CTP-labeled probes were added to 3 ml of hybridization buffer and incubated with the Hybond-N membranes overnight. Membranes were then blotted dry, and DNA-RNA hybrids were detected by autoradiography using Kodak X-Omat AR film.

Antibodies. Antibodies used include a mouse monoclonal antibody to Shc, PG-797 (Santa Cruz product no. sc-967), and a rabbit polyclonal Shc antibody, S14630 (Transduction Laboratories). Antibodies used to detect Grb2 include a mouse monoclonal antibody, G16720 (Transduction Laboratories), and a rabbit polyclonal antibody, C-23 (Santa Cruz product no. sc-255). Tyrosine-phosphorylated proteins were specifically detected by using either a mouse monoclonal antibody, PY20 (Transduction Laboratories), or a rabbit polyclonal antibody, P11230 (Transduction Laboratories). Rabbit polyclonal antibodies specific to phosphorylated p44/42 MAPK (Thr202/Tyr204) and the unphosphorylated forms were obtained from New England Biolabs (product no. 9101S and 9102, respectively). ErbB-2 was detected by using a rabbit polyclonal antibody (Upstate Biotechnology), while the rabbit polyclonal antibody C-17 (Santa Cruz product no. sc-285) was used to identify ErbB-3. The epithelial cell-specific marker, cytokeratin-8 (34), was detected by using a 1:10 dilution of the rat hybridoma tissue culture supernatant containing TROMA-1 (generous gift from M. Rudnicki). ^{125}I-conjugated goat anti-rat and goat anti-rabbit secondary antibodies were received from Dupont.

Protein extract preparation. Mouse mammary gland tissues were flash frozen in liquid nitrogen and ground to a fine powder with a chilled mortar and pestle. Cells were lysed in 2 ml of either CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate} lysis buffer (0.7% CHAPS, 50 mM Tris [pH 8.0], 50 mM NaCl) or 2 ml of radioimmunoprecipitation assay (RIPA) lysis buffer (1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 10 mM sodium phosphate buffer [pH 7.2], 150 mM NaCl, 2 mM EDTA, 50 mM NaF) (where indicated) for 20 min on ice with agitation. Tyrosine phosphatase inhibitor (1 mM Na_3VO_4) and protease inhibitors (aprotinin [10 μg/ml] and leupeptin [10 μg/ml]) were freshly added to both CHAPS and RIPA lysis buffers before use, while serine/threonine phosphatase inhibitors (5 mM sodium pyrophosphate and 40 mM

glycerophosphate) were freshly added to RIPA buffer only. Lysates were cleared twice by centrifugation at 13,000 rpm for 10 min at 4°C. When RIPA was used as the lysis buffer, lysates were first sheared with a 21-gauge needle before being cleared by centrifugation. Supernatants were then decanted, and protein concentration was determined by using a Bio-Rad Bradford assay kit.

Immunoblot analysis. A total of 60 μg of total protein lysate was used for immunoblot analysis. To each lysate was added an equal volume of 2× SDS-polyacrylamide gel electrophoresis (PAGE) sample load buffer (62.5 mM Tris [pH 6.8], 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.02% bromophenol blue), and samples were incubated at 95°C for 10 min. Proteins were electrophoresed first through a 1 mM SDS–polyacrylamide stacking gel (4.93% acrylamide, 0.017% bisacrylamide, 0.125 M Tris, 0.1% SDS, 0.1% ammonium persulfate, 0.1% *N,N,N′,N′*-tetramethylethylene diamine [TEMED] [pH 6.8]) followed by a 1-mm SDS-polyacrylamide resolving gel (8.7% acrylamide, 0.3% bisacrylamide, 0.375 M Tris, 0.1% SDS, 0.1% ammonium persulfate, 0.1% TEMED [pH 8.8]) at a constant voltage of 60 V. Proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore) for 2 h at 0.6 mA, using a Bio-Rad wet transfer apparatus and the appropriate buffer (20% methanol, 0.025 M Tris, 0.2 M glycine). Membranes were incubated overnight at 4°C or for 1 h at room temperature in 3% powdered skim milk in Tris-buffered saline (TBS; 20 mM Tris [pH 7.5], 150 mM NaCl, 5 mM KCl). Subsequently, membranes were incubated overnight at 4°C or for 2 h at room temperature in 3% milk–TBS containing primary antibody at 1:1,000 dilution (except P11230, which was used at 1:500). Membranes were then washed twice for 5 min in TBS–0.01% Tween 20 and once for 5 min in TBS. The appropriate ^{125}I-conjugated secondary antibody (100 μCi) was then added to 50 ml of 3% milk–TBS and incubated with the membranes for 1 h at room temperature. The proteins of interest were then detected by autoradiography using Kodak X-Omat AR film or detected and quantitated by PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.) analysis.

Immunoprecipitations. Immunoprecipitations were performed by first preincubating the specific monoclonal antibody (2 μg of antibody per mg of total protein) with 20 μl of protein G-Sepharose Fast Flow (Pharmacia) in 800 μl of phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4) for 2 h at 4°C on an end-over-end rotator. Bound antibodies were washed once with 1 ml of PBS and once with 1 ml of lysis buffer. Two milligrams of total protein lysate was added, and the volume was brought to 600 μl with lysis buffer. Beads were then washed three times in lysis buffer and resuspended in 60 μl of 2× SDS-PAGE sample load buffer. After incubating at 95°C for 10 min, samples were split 1/3 for Shc analysis and 2/3 for phosphotyrosine analysis.

Histological and whole-mount evaluation. Necropsies were performed as described by Muller et al. (25), with both gross and microscopic examination being conducted. Upper left mammary fat pad tissues (3L) were removed from CO_2-euthanized female mice and fixed in 4% paraformaldehyde (in PBS) overnight at 4°C. Tissues were transferred to and stored (4°C) in 70% ethanol the next day. Specimens were then blocked in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin by Pathology Research Services, McMaster University Medical Centre. Lungs of tumor-bearing mice were prepared and examined in an identical fashion.

Mammary glands were also analyzed by whole-mount preparation using the upper right mammary fat pad tissue (3R) (47). Briefly, glands were spread out on glass slides and allowed to air-dry overnight at room temperature. The next day, mammary glands were defatted overnight in acetone. The following day, glands were pressed between two glass slides and again transferred to fresh acetone to enhance the defatting process. This was followed by overnight staining in Harris's modified hematoxylin (Fisher Scientific, Ottawa, Ontario, Canada). Glands were then destained in successive changes of acid-alcohol destain solution (1% concentrated HCl, 75% ethanol) until the epithelial component was seen in sharp contrast to the background of the fat pad. The stain was fixed for 1 min in 0.02% ammonium hydroxide; the specimens were placed in 75% ethanol for 1 h and transferred to 100% ethanol for several hours. The slides were then placed in xylenes, and the glands were cleared overnight. Finally, the glands were mounted in Permount (Fisher Scientific), and a coverslip was placed over the slide.

The development of mammary tumors was monitored in MMTV/MTY250F, MMTV/Shc/MTY250F, and MMTV/Grb2/MTY250F virgin female mice by regular palpation of all mammary fat pads. Necropsies were performed 2 months after the first detection of tumors by palpation. Histological and whole-mount analyses were conducted on mammary tumors, and lungs were histologically examined for the presence of metastases.

RESULTS

Generation and characterization of the MMTV/Grb2 and MMTV/Shc strains. To derive transgenic mice expressing elevated levels of *Grb2* and *Shc*, cDNAs encoding the 52-kDa form of Shc and 25-kDa form of Grb2 were placed under the transcriptional control of the MMTV promoter/enhancer and microinjected into one-cell mouse zygotes (Fig. 1). A total of 9 MMTV/Shc and 10 MMTV/Grb2 transgenic founder animals

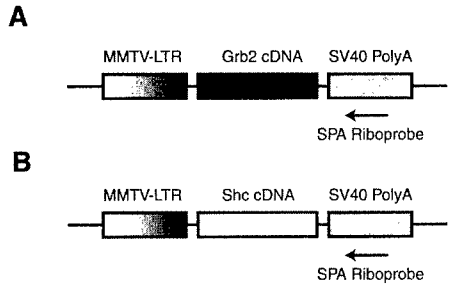

FIG. 1. Schematic representations of the MMTV/Grb2 (A) and MMTV/Shc (B) transgenes. The shaded region represents the MMTV LTR. The solid region displays the cDNA encoding the human Grb2 protein. The gray region represents the SV40 splicing and polyadenylation signals. Also shown is the SPA riboprobe used to assess the tissue-specific pattern of transgene expression.

were generated (Table 1). To assess the tissue-specific pattern of expression of the transgene, 20 μg of total RNA was isolated from a variety of tissues and subjected to RNase protection analyses with an antisense riboprobe complementary to the SV40 component of the transgene. The results of these analyses revealed that two MMTV/Shc and five MMTV/Grb2 strains expressed the transgene in the mammary epithelium. In addition to these tissue sites, transgene specific expression was detected in salivary glands and the male reproductive tract (Table 1). Given the elevated levels of transgene expression observed in the Shc-3 and Grb2-6 strains, these strains were subjected to further molecular analyses.

To gauge the extent of transgene expression, RNA and protein analyses were conducted on mammary gland tissues derived from female FVB/N, MMTV/Grb2-6, and MMTV/Shc-3 mice. To avoid differences in epithelial content, age-matched samples were harvested from lactating females and subjected to Northern blot analyses with either *Grb2*- or *Shc*-specific probes. The results of these analyses revealed a moderate but variable upregulation of *Shc*-specific transcripts in the mammary tissues of the MMTV/Shc mice (Fig. 2A, lanes 8 to 11). In addition to transcript comigrating with the endogenous *Shc* transcript, we have consistently observed a slower-migrating transcript in these tissues. Although the origin of the transcript is unclear, it likely derives from initiation of transcription from the MMTV-directed transcription start site. Interestingly, upregulation of endogenous *Shc* transcripts was also noted in the mammary epithelium of the MMTV/Grb2 strains (Fig. 2A, Shc, lanes 1 to 3). The observed differences in transcript levels in these samples were not due to differences in RNA loading since these samples displayed similar levels of 28S rRNA. Comparable Northern blot analyses revealed that elevated levels of *Grb2*-specific transcript could be detected in the mammary tissues derived from the MMTV/Grb2 animals (Fig. 2A, Grb2, lanes 1 to 3). In contrast, very low levels of endogenous *Grb2* transcript could be detected in the mammary tissues of either FVB or MMTV/Shc transgenic mice (Fig. 2A, Grb2, lanes 4 to 11).

To confirm that the observed upregulation of Grb2 and Shc transcripts corresponded to comparable levels of Grb2 and Shc protein, the protein lysates from the same samples were subjected to immunoblot analyses with Grb2- and Shc-specific antibodies. Immunoprecipitation of protein lysates with Shc-specific antisera followed by immunoblot analyses with Shc antibodies revealed that MMTV/Shc lysates expressed moderately elevated levels of the p52 isoform of Shc compared to either the MMTV/Grb2 or FVB protein lysates (Fig. 2B; compare lanes 1 to 5 to lanes 6 and 7). However, the levels of the 46-kDa form of Shc were comparable in all samples tested.

TABLE 1. Transgene expression and tumor phenotype in MMTV/Shc and MMTV/Grb2 mice

Line	Expression[a]					Tumor phenotype (% penetrance)	Avg time (days) of tumor onset (days) ± SD[b]
	V.M.gl.	Sal.gl	Ovary	Testis	SV		
Shc-1	−	−	−	−	−		
Shc-2	−	−	−	−	−		
Shc-3	++	+/−	−	−	−	Focal papillary m.gl. adenocarcinoma (7%)	403 ± 151 (n = 28)
Shc-4	−	−	−	−	−		
Shc-5	−	−	−	−	−		
Shc-6	−	−	−	−	−		
Shc-7	−	−	−	−	−		
Shc-8	−	−	−	−	−		
Shc-9	+	−	−	−	−		
Grb2-1	+++	+++	−	+	+++		
Grb2-2	−	−		ND	ND		
Grb2-3	++	++	−	ND	ND		
Grb2-4	+	+	ND	ND	++		
Grb2-5	−	−	−	ND	ND		
Grb2-6	+++	+++	−	−	+++		
Grb2-8	−	−	−	ND	ND		
Grb2-9	+	+	−	ND	ND		
Grb2-10	+	+	−	ND	ND		

[a] RNase protection analysis was performed on 20 μg of total RNA isolated from a variety of organs in MMTV/Shc and MMTV/Grb2 transgenic strains as described in Materials and Methods. Relative levels of transgene expression are indicated by − (not detected), +/− (very low), + (low), ++ (intermediate), and +++ (high). V.M.gl., virgin mammary gland; Sal.gl., salivary gland; SV, seminal vesicle; m.gl., mammary gland; ND, not determined.

[b] Multiparous transgene carriers were monitored regularly for the appearance of tumors by physical palpation of mammary glands.

Given that the MMTV transgene encodes the 52-kDa isoform of Shc, these observations are consistent with observed upregulation of Shc transgene transcripts observed in this strain.

Immunoblot analyses of the levels of Grb2 protein revealed that MMTV/Grb2 samples possessed dramatically elevated levels of Grb2 protein in the mammary epithelium compared to either the FVB or MMTV/Shc transgenic tissues (Fig. 2C; compare lanes 1 and 2 to lanes 3 to 9). The observed differences in Grb2 or Shc could not be attributed to differences in epithelial content since the protein lysates expressed comparable levels of the epithelial marker cytokeratin-8. Despite the elevated levels of Shc transcript observed in the MMTV/Grb2 strains, a concomitant increase in Shc protein was not observed. These observations indicate that the MMTV/Grb2 strains expressed dramatically elevated levels of Grb2 whereas the MMTV/Shc animals only moderately overexpressed Shc.

Aberrant mammary ductal morphogenesis in MMTV/Shc and MMTV/Grb2 transgenic mice. To ascertain whether elevated expression of either Shc or Grb2 resulted in abnormal mammary gland development, whole-mount analyses were conducted on virgin mammary glands. Virgin female mammary glands from MMTV/Shc and MMTV/Grb2 transgenic mice, along with nontransgenic FVB controls, were examined after necropsy by whole-mount and histological analyses at the end of puberty (Fig. 3). Mammary epithelial cell-specific expression of Shc in the mammary gland resulted in aberrant pubertal mammary ductal morphogenesis. In contrast to the wild-type FVB mammary glands, which displayed normal development, the mammary whole-mount preparations from the MMTV/Shc mice exhibited extensive side branching (compare Fig. 3A and B with Fig. 3C and D). This enhanced branching phenotype was also noted in whole-mount preparations derived from the MMTV/Grb2 strains (Fig. 3E and F). However, there are also subtle but distinct differences between the MMTV/Grb2 and MMTV/Shc mammary phenotypes. In particular, the mammary epithelium derived from the MMTV/Grb2 strains exhibited more extensive lobuloalveolar development than was observed in the MMTV/Shc strains. The differences between these strains were also evident at 14 weeks of age, when normal mammary gland ductal extension and branching has mostly ceased in the female mouse (19). Taken together, these data indicate that mammary epithelial cell-specific expression of either Grb2 or Shc can perturb the normal development of the murine mammary gland.

Although mammary epithelial expression of either Grb2 or Shc had pronounced effects on normal gland development, long-term monitoring of these strains revealed that induction of mammary tumors is extremely rare in either the MMTV/Grb2 or MMTV/Shc strain. Two of 28 multiparous MMTV/Shc females developed focal mammary tumors at 296 and 510 days, whereas none of the 15 Grb2 females had developed mammary tumors (Table 1). These data suggest that elevated expression of Grb2 or Shc is not sufficient to induce mammary tumors in these transgenic mice.

Elevated expression of Shc and Grb2 adapter proteins can accelerate tumor progression in transgenic mice expressing a mutant PyV mT oncogene. The striking branching phenotype exhibited in the virgin female mammary glands suggested that elevated expression of Grb2 and Shc may influence neoplastic transformation. To further explore the role of Grb2 and Shc in tumor progression, we mated the MMTV/Grb2-6 and MMTV/Shc-3 lines with transgenic mice expressing a mutant PyV mT oncogene decoupled from the Shc signaling pathway (MTY250F-5a strain) (48). Unlike the wild-type PyV mT strains, which rapidly develop metastastic tumors, female carriers from the MTY250F-5a line develop focal mammary tumors with delayed kinetics (48). In addition, given that this model is also defective in Shc/Grb2 signaling, it presents a unique model system to examine the interaction of Shc and Grb2 adapter proteins with endogenously activated growth factor receptors. Indeed, we have previously demonstrated that tumor progression in the MTY250F strains is associated with increased expression of the ErbB-2 and ErbB-3 RTKs (48).

To assess whether either Grb2 or Shc could influence mammary tumor progression in the MTY250F strain, we initially performed whole-mount and histological analyses of 14-week-old monogenic MMTV/MTY250F (Fig. 4A and D) and bigenic MMTV/Shc/MTY250F (Fig. 4B and E) and MMTV/Grb2/

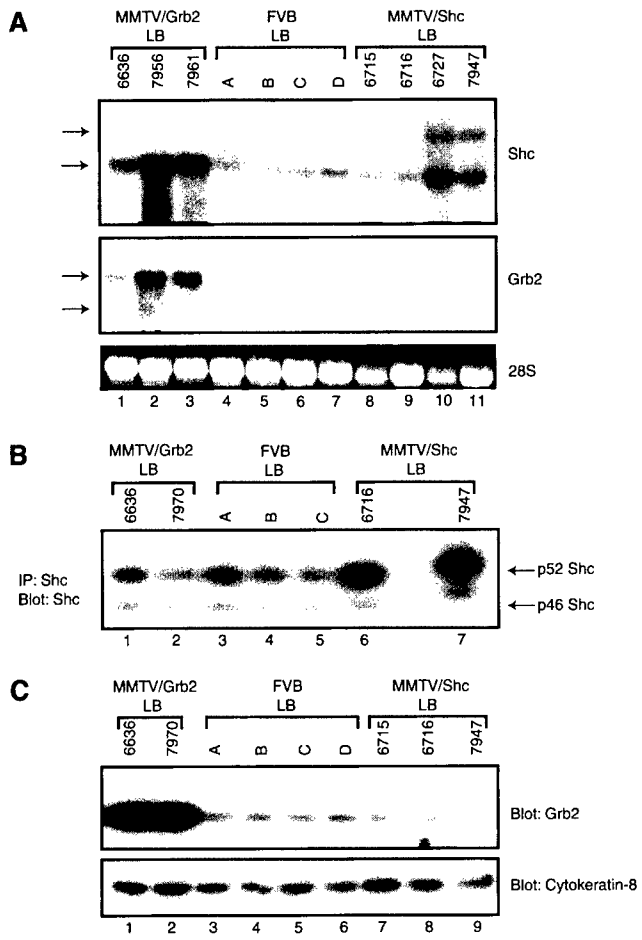

FIG. 2. Expression of the MMTV/Shc-3 and MMTV/Grb2-6 strains. (A) Northern blot analyses of *Shc* and *Grb2* mRNA levels. Total RNA (30 μg) was isolated from lactating mammary glands (LB) of MMTV/Grb2 (lanes 1 to 3), FVB (lanes 4 to 7), and MMTV/Shc (lanes 8 to 11) female mice. The gels were probed with Shc and Grb2 cDNAs, as indicated. Also shown is the 28S ribosomal marker. (B) Immunoblot (Blot)-immunoprecipitation (IP) analyses with Shc-specific antibodies. Immunoprecipitations were performed with 700 μg of total protein obtained from lactating glands from either MMTV/Grb2 (lanes 1 and 2), and FVB (lanes 3 to 5), or MMTV/Shc (lanes 6 and 7) females and immunoblotted with Shc-specific antisera. (C) Immunoblot analyses of Grb2 protein from lactating glands of MMTV/Grb2 (lanes 1 and 2), FVB (lanes 3 to 6), and MMTV/Shc (lanes 7 to 9) mice. The lower panel was probed with antibodies specific to cytokeratin-8.

MTY250F (Fig. 4C and 4F) virgin female mice. Although all three genotypes possess atypical epithelial and cystic alveolar hyperplasia, there was a dramatic difference in the degree of epithelial hyperplasia exhibited by the different genotypic combinations. In contrast to monogenic MMTV/MTY250F glands, MMTV/Shc/MTY250F hyperplasias exhibited a profound increase in lobuloalveolar development (Fig. 4C). In addition, a significant amount of inflammation and fibrosis was observed around the ducts (Fig. 4D). Detailed histological analysis of the MMTV/Shc/MTY250F epithelial hyperplasias also revealed larger nucleoli and more open chromatin. Although the MMTV/Grb2/MTY250F epithelial hyperplasias were cytologically similar to those in the Shc bigenic mice, the extent of lobuloalveolar development observed was dramatic. Indeed, in contrast to the other two genotypes, the alveoli from the MMTV/Grb2/MTY250F virtually filled the entire fat pad (Fig. 4E).

To assess whether the onset of tumors in these bigenic strains was affected by mammary epithelial expression of these adapter proteins, bigenic and monogenic virgin females were monitored for the onset of mammary tumors by physical palpation. Although coexpression of Shc or Grb2 with this mutant PyV mT oncogene did not recapitulate the phenotype exhibited by transgenic mice expressing wild-type PyV mT (15), ectopic expression of the adapter protein Shc or Grb2 significantly accelerated tumor onset in MTY250F transgenic females (Fig. 5). In contrast to the MMTV/MTY250F strain, which developed mammary tumors with an average latency of 111 days, the MMTV/Shc/MTY250F and MMTV/Grb2/MTY250F mice developed mammary tumors with average latencies of 94 and 80 days, respectively (Fig. 5).

Biochemical characterization of mammary tumors expressing Shc and Grb2 adapter proteins. To confirm that the accelerated tumor onset observed in these crosses was due to coexpression of the mutant PyV mT oncogene and the respective adapter protein, both RNA and protein analyses were conducted on the hyperplastic and tumor tissues from these mice. Analyses of *Shc* transcript levels in epithelial hyperplasias derived from the various genotypes revealed that the MMTV/Shc/MTY250F hyperplasias expressed slightly elevated levels of *Shc* transcript compared to the parental MMTV/MTY250F samples (Fig. 6, Shc; compare lanes 8 to 10 and lanes 4 to 7). Interestingly, the MMTV/Grb2/MTY250F samples possessed a further threefold elevation of the endogenous *Shc* transcripts (Fig. 6, Shc, lanes 1 to 3). Consistent with previous Northern blot analyses (Fig. 2), analyses of the levels of *Grb2* transcript revealed that the MMTV/Grb2/MTY250F samples expressed dramatically elevated levels of *Grb2* transcript compared to either MMTV/Shc/MTY250F or MMTV/MTY250F tissues (Fig. 6, Grb2; compare lanes 1 to 3 to lanes 4 to 10). The observed differences in *Grb2* and *Shc* transcript did not reflect differences in RNA loading since equal levels of 28S rRNA were observed in all samples (Fig. 6, 28S, lanes 1 to 10). Despite the differences in the levels in *Grb2* and *Shc* transcripts in the various samples, all of the mammary hyperplasias expressed comparable levels of mutant PyV mT transcripts. Therefore, these observations suggest that the phenotypic differences observed between the various hyperplastic tissues (Fig. 3) do not reflect differences in the levels of mutant PyV mT transcripts but rather are a result of differences in the levels of expression of the Grb2 and Shc adapter molecules.

Another possible explanation for the accelerated tumor phenotype observed in the bigenic mice is through the indirect activation of growth factor receptor signaling. Indeed, we have previously demonstrated that tumor progression in the MMTV/MTY250F strain is associated with the induction of elevated levels of ErbB-2 and ErbB-3 growth factor receptors (48). To test this possibility, protein extracts from MMTV/Grb2/MTY250F, MMTV/Shc/MTY250F, and MMTV/MTY250F tumors were subjected to quantitative ^{125}I immunoblot analyses with ErbB-2- and ErbB-3-specific antisera (Fig. 7). To control for variations in epithelial content, the same samples were also probed with a cytokeratin-8-specific antibody. After controlling for variations in epithelial content, quantitative comparison of the levels of ErbB-2 and ErbB-3 revealed that tumor samples from the various genotypic combinations possessed similar elevated levels of ErbB-2 and ErbB-3 proteins per epithelial cell. As for the MMTV/MTY250F strains, we observe a comparable increase in ErbB-2 and ErbB-3 levels during tumor progression in these bigenic mice (30a). Thus, the observed differences in tumor latency are not due to alteration in the levels of these activated growth factor receptors but rather reflect the elevated levels of Grb2 and Shc.

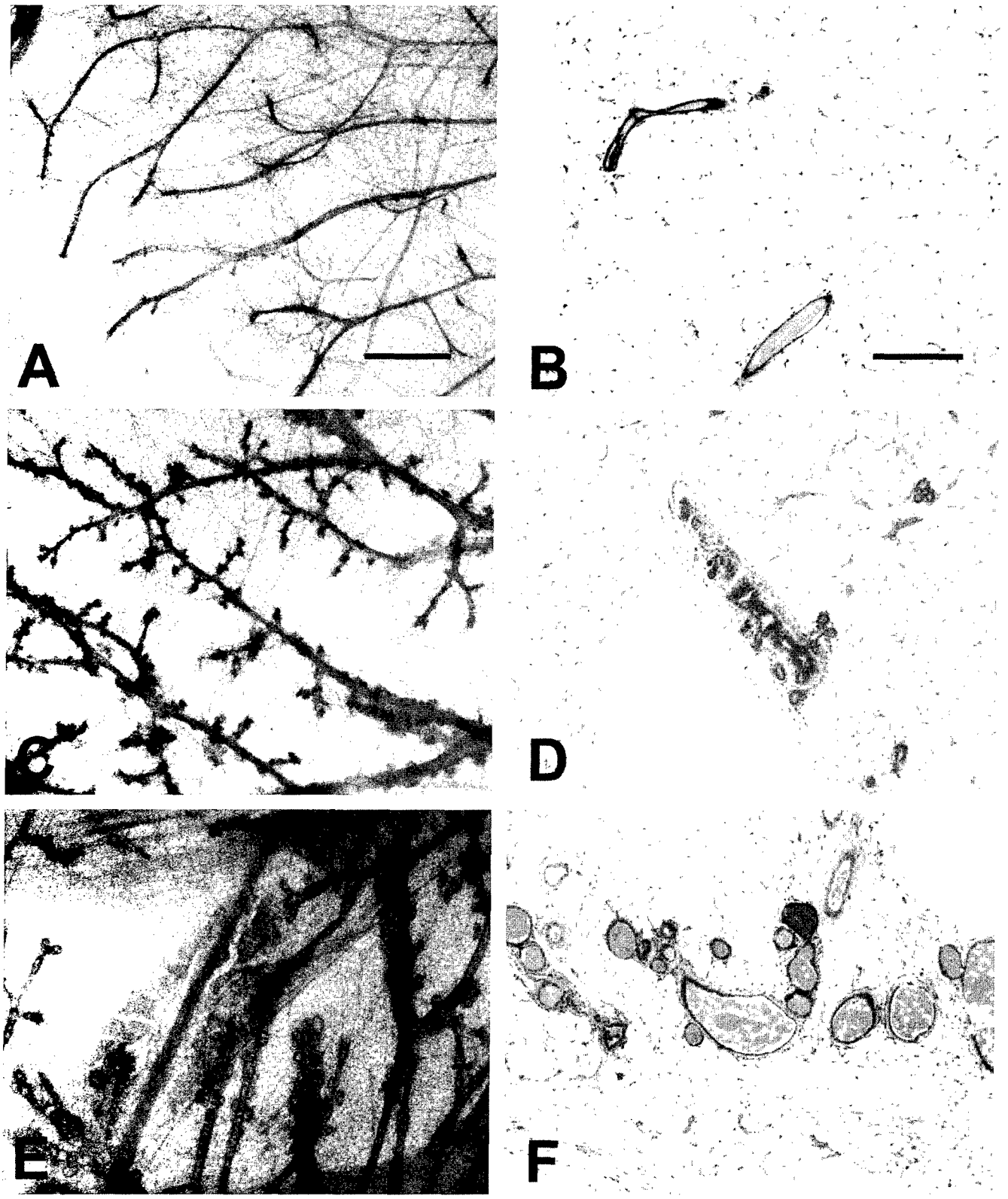

FIG. 3. Histological analyses of virgin mammary glands derived from FVB, MMTV/Shc, and MMTV/Grb2 female mice. Photoimages of whole mounts (A, C, and E) and histological sections (B, D, and F) show mammary development in virgin FVB wild-type (A and B), Shc-3 (C and D), and Grb2-6 (E and F) females at 8.5 weeks after birth. Note the side branching and alveolar development in Shc-3 mammary gland (C and D) compared to the straight ductal structure of the wild-type gland (A and B). Note also that the Grb2-6 mammary gland has a more complex structure with more extensive lobuloalveolar development (E and F). The scale bars represent 0.5 mm (A, C, and E) and 200 μm (B, D, and F).

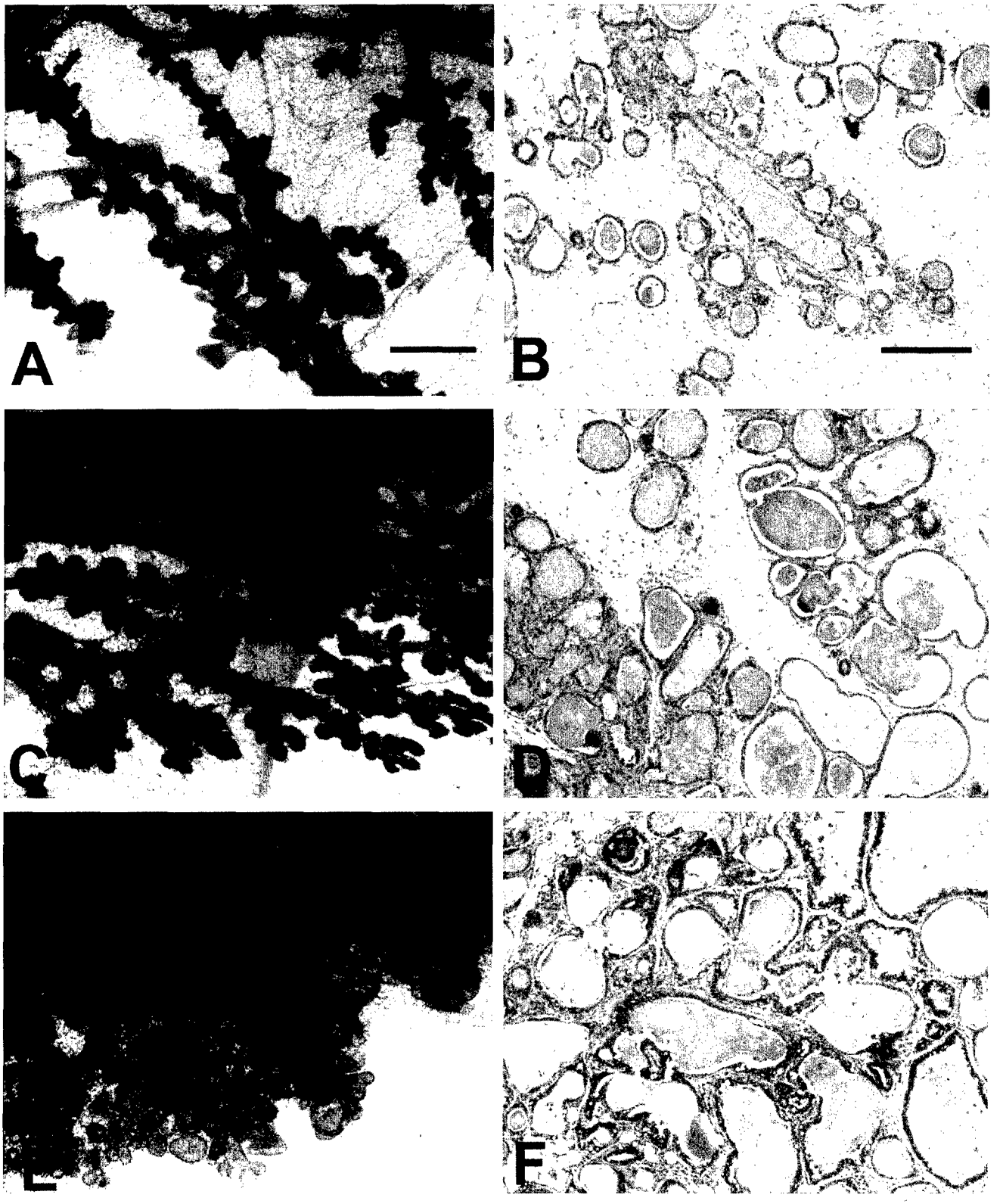

FIG. 4. Histological analyses of virgin mammary glands derived from MMTV/MTY250F, MTY250F/Shc, and MTY250F/Grb2 female mice. Photoimages of whole mounts (A, C, and E) and histological sections (B, D, and F) show mammary development in virgin MMTV/MTY250F (A and B), MTY250F/Shc (C and D), and MTY250F/Grb2 (E and F) females 14 weeks after birth. Note the extensive side branching and lobuloalveolar development in the dual transgene carriers compared to the MMTV/MTY250F strain (A and B). The degree of lobuloalveolar development is greater in the MTY250F/Shc mammary gland (C and D) than in the monogenic MTY250F gland (A and B). However, the degree of lobuloalveolar development is the greatest in the MTY250F/Grb2 gland, virtually filling the fat pad with alveoli. The histological analysis reveals that all three transgenic glands have various degrees of fibrosis as well as lobuloalveolar development. At 14 weeks, cytological changes are present. The scale bars represent 0.5 mm (A and C) and 200 μm (B, D, and E).

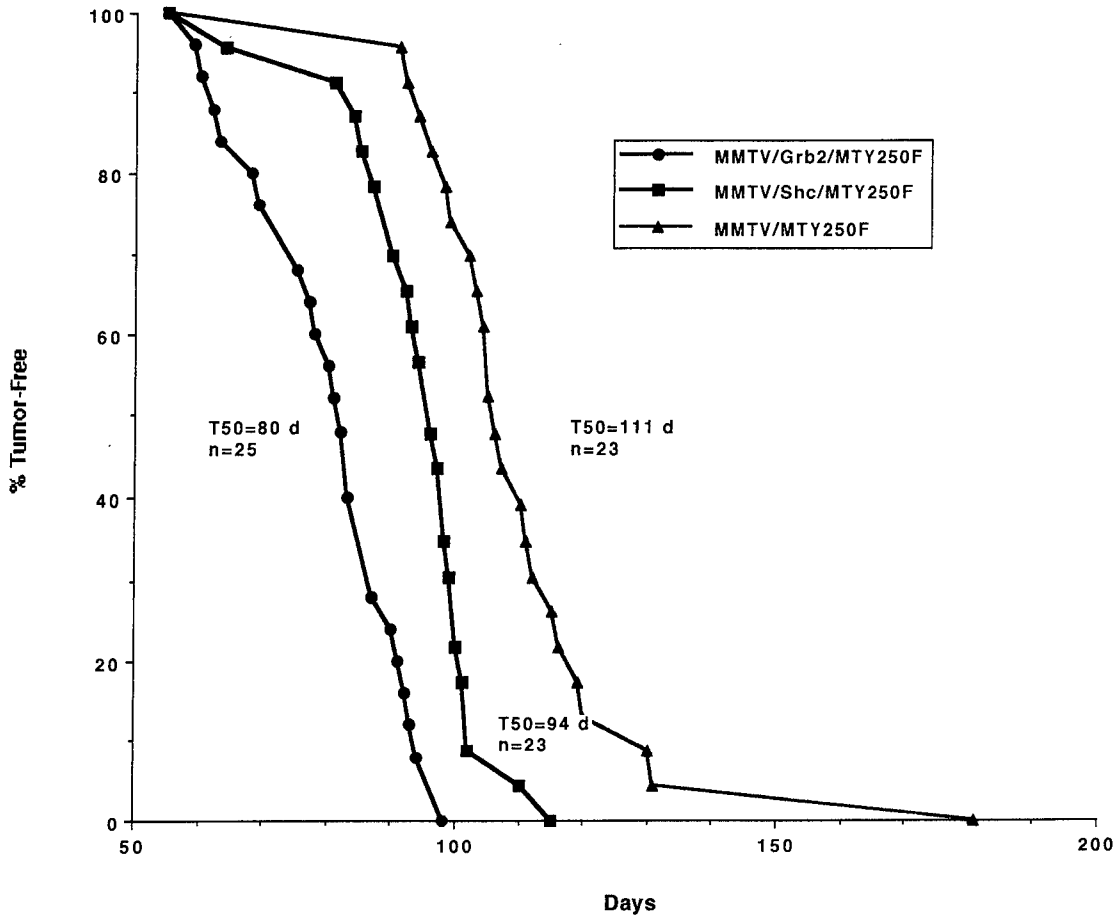

FIG. 5. Kinetics of tumor onset in the MMTV/MTY250F, MTY250F/Shc, and MTY250F/Grb2 strains. The number of animals analyzed for each strain (n) and the median age (days [d]) at which tumors were first palpable (T50) are indicated.

Given the ability of both Grb2 and Shc adapter molecules to couple ErbB-2 and ErbB-3 growth factor receptors to the Ras signaling pathway, we were also interested in assessing whether there was evidence of enhanced Ras signaling in tumor material coexpressing these adapter proteins. One measure of Ras activation is stimulation of the MAPK signaling pathway. To explore this possibility further, we performed quantitative ^{125}I immunoblot analyses on these tumor samples with antisera specific to Shc, Grb2, p42 MAPK, phosphospecific p42 MAPK, and cytokeratin-8 antibodies. Consistent with Northern blot analyses (Fig. 6), dramatically elevated levels of Grb2 protein were noted in tumor samples derived from the MMTV/Grb2/MTY250F samples compared to either MMTV/Shc/MTY250F or parental MMTV/MTY250F samples (Fig. 8, Grb2; compare lanes 1 and 2 to lanes 3 to 8). Given the lower levels of expression of the Shc transgene, inspection of the levels of Shc protein failed to reveal significant differences in the levels of Shc protein expression. Quantitative analyses of the ratio of phosphospecific MAPK to the levels of MAPK protein revealed that MMTV/Grb2/MTY250F tumors had a twofold increase in the specific activity of MAPK compared to the parental MMTV/MTY250F tumor samples. In contrast, the MMTV/Shc/MTY250F samples possessed only slightly elevated levels of MAPK activity compared to the parental tumors. Taken together, these observations suggest that elevated levels of Grb2 in these tumors result in stimulation of the Ras signaling pathway.

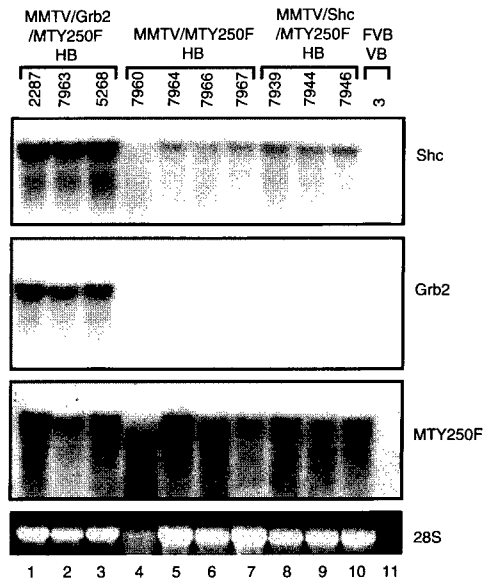

FIG. 6. Northern blot analyses of Shc, Grb2, and MTY250F mRNA levels in hyperplastic mammary glands. Total cellular RNA was isolated from 14-week virgin Grb2/MTY250F (lanes 1 to 3), MTY250F (lanes 4 to 7), and Shc/MTY250F (lanes 8 to 10) hyperplastic mammary tissue (HB). Also included were virgin mammary glands (VB) obtained from an FVB female (lane 11). The gels were probed with Shc, Grb2, and MTY250F radiolabeled probes, as indicated at the right. Also shown for each sample is the 28S ribosomal species.

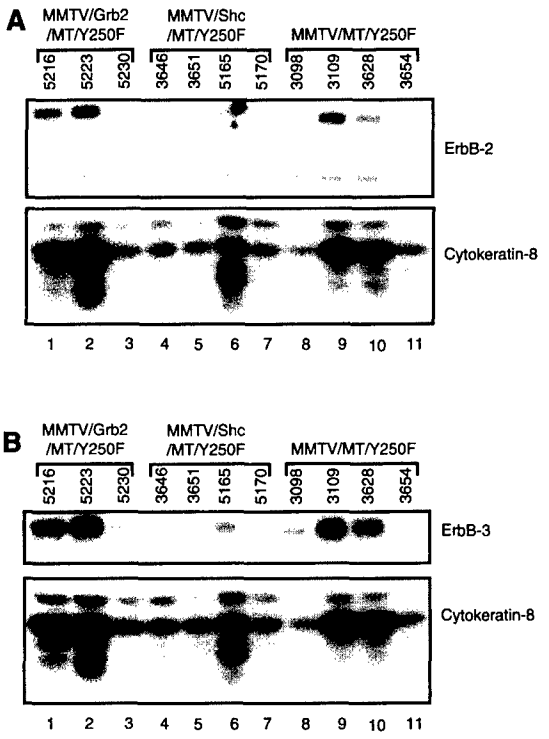

FIG. 7. Expression of the EGFR family is not altered in the different intercrosses. ErbB-2 (A) and ErbB-3 (B) levels in mammary tumors derived from the MMTV/Grb2/MTY250F (lanes 1 to 3), MMTV/Shc/MTY250F (lanes 4 to 7), and MMTV/MTY250F (lanes 8 to 11) strains were measured by immunoblot analyses. Cytokeratin-8 levels in the samples (bottom) were determined to normalize for epithelial content.

DISCUSSION

The Grb2 and Shc adapter proteins play critical roles in regulating the response of mammalian cells to a variety of proliferative and differentiation stimuli. To assess the importance of these adapter proteins in mammary gland development and tumorigenesis, we have generated separate transgenic strains expressing Shc or Grb2 under the transcriptional control of the MMTV promoter. Although female MMTV/Shc and MMTV/Grb2 mice appear to nurse their offspring normally, whole-mount analyses revealed that virgin glands exhibit enhanced ductal branching and lobuloalveolar development that also correlated with expression of either *Shc* or *Grb2* transcript and encoded proteins. We have further demonstrated that mammary epithelial cell-specific expression of the Grb2 and Shc adapter proteins can accelerate mammary tumor development in transgenic mice expressing a mutant PyV mT oncogene decoupled from the Shc adapter protein. Tumor progression in mice coexpressing Grb2 and the mutant PyV mT oncogene was further correlated with a modest activation of the MAPK pathway. These observations argue that the levels of Grb2 and Shc adapter proteins can modulate the response of the mammary epithelial cell to growth factor and oncogenic stimuli.

The observation that elevated mammary epithelial cell-specific expression of Grb2 and Shc in transgenic mice can result in altered mammary epithelial morphogenesis has important implications in understanding the biological function of these adapter proteins in mammary gland development. Whole-mount analyses of virgin mammary glands isolated from MMTV/Grb2 and MMTV/Shc females have revealed that both strains display enhanced tertiary branching and lobuloalveolar development (Fig. 3). However, careful histological analyses of these strains revealed that the MMTV/Grb2 and MMTV/Shc strains display subtle differences in the nature of epithelial hyperplasias. Although both the MMTV/Shc and MMTV/Grb2 female mammary glands possess enhanced branching and lobuloalveolar development, the extent of lobuloalveolar development in the MMTV/Grb2 glands was more extensive (Fig. 3). Interestingly, the mammary phenotype exhibited by the transgenic strains closely resembles epithelial hyperplasias seen in the MMTV/heregulin strains (22). Given that members of the heregulin family of growth factors are ligands for the epidermal growth factor receptor (EGFR) family members, elevated expression of Shc and Grb2 may potentiate the action of the endogenous EGFR family during normal mammary gland development. In this regard, it has recently been demonstrated that stimulation of mammary organ culture systems with heregulins causes induction of alveolar structures resembling those seen within the MMTV/Grb2 and MMTV/Shc mice (26). These investigators further showed that the heregulin-induced alveolar phenotype was dependent on the activation of the MEK/MAPK (26). Given the importance of Shc and Grb2 in coupling the ErbB-2 and ErbB-3 receptors to the MAPK pathway, it is conceivable that the elevated levels of Shc sensitize the mammary epithelial cell to heregulin-mediated signals. Future crosses between the MMTV/adapter strains and MMTV/heregulin strains should allow this hypothesis to be tested.

Although mammary epithelial expression of either Shc or Grb2 was capable of altering normal mammary gland development, the occurrence of mammary tumors was extremely rare. Only 7% of MMTV/Shc female mice and none of the female MMTV/Grb2 strains developed mammary tumors (Table 1). In this regard, previous studies have demonstrated that elevated expression of Shc is capable of transforming fibro-

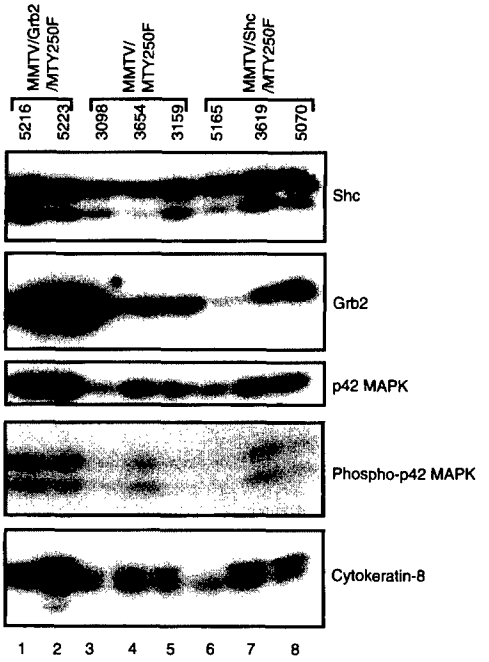

FIG. 8. Grb2 and Shc protein expression in hyperplastic and neoplastic mammary tissues. Total protein lysates prepared from virgin MMTV/Grb2/MTY250F (lanes 1 and 2), MMTV/MTY250F (lanes 3 to 5), and MMTV/Shc/MTY250F (lanes 6 to 8) hyperplasias were subjected to immunoblot analyses with Shc, Grb2, p42 MAPK, phospho specific p42 MAPK, and cytokeratin-8 antisera, as indicated at the right.

blasts (29), whereas comparable levels of Grb2 are incapable of mediating oncogenic transformation (43). Consistent with these observations, only the MMTV/Shc mice developed tumors despite the comparatively low levels of Shc expression observed in the mammary glands of these transgenic strains.

To further explore the role of these adapter proteins in mammary tumorigenesis, we have crossed the separate MMTV/Grb2 and MMTV/Shc strains with transgenic mice expressing a mutant form of PyV mT oncogene decoupled from the Shc adapter protein (MTY250F strain). Consistent with the effect of these adapter proteins on normal mammary gland development, mammary epithelial expression of either Grb2 or Shc adapter had a dramatic effect on tumor progression in the MTY250F strain. Indeed, whole-mount analyses of these bitransgenic strains revealed that elevated expression of Grb2 and Shc profoundly enhanced the abnormal lobuloalveolar hyperplasias induced by the MTY250F oncogene (Fig. 4). Moreover, comparison of the average times of onset of tumors revealed that elevated expression of these adapter proteins could significantly decrease the latency period required for tumor development (Fig. 5).

One potential explanation for accelerated tumor development in these strains is that an elevated level of either Grb2 or Shc sensitizes the mammary epithelial cell to endogenous growth factor receptor signaling pathways. For example, we have previously demonstrated that tumor progression in the mutant PyV mT strains involves upregulation of EGFR family members ErbB-2 and ErbB-3 (48). Consistent with these findings, mammary tumors derived from transgenic mice coexpressing the adapter proteins and mutant PyV mT strains also express elevated levels of these EGFR family members (Fig. 7). Although the levels of ErbB-2 and ErbB-3 protein were elevated in each of these tumors, quantitative PhosphorImager analyses of these blots revealed no significant differences between the various mutant samples. Therefore, the dramatic changes observed in the mammary glands of bigenic mice cannot simply be ascribed to elevated expression of these growth factor receptors but rather reflect increased expression of Grb2 and Shc.

Given the ability of ErbB-2 and ErbB-3 receptors to bind either Grb2 or Shc protein (9, 21, 30), the accelerated rates of tumor development may reflect an increased sensitivity of the mammary epithelial cell expressing these adapter proteins to growth factor stimulation. Consistent with this hypothesis, we have demonstrated that the tissues coexpressing Grb2 and mutant PyV mT oncogene possess elevated MAPK activity (Fig. 8). The marginal activation of MAPK activity observed in the samples coexpressing Shc and mutant PyV mT oncogene likely reflects the comparatively low levels of Shc observed in these samples. Whereas the levels of Shc are slightly elevated, we have observed a fourfold increase in the levels of tyrosine-phosphorylated Shc during tumor progression (30a). Another possible explanation for differences in MAPK activation between the Shc and Grb2 strains is that Grb2-coupled growth factor receptor may be more efficient in activating the MAPK pathway. Indeed, it has recently been reported that EGF stimulation of the MAPK pathway does not require Shc but is absolutely dependent on a functional Grb2 protein (18).

Further evidence supporting a role for these adapter proteins stems from the observation that a 50% reduction of Grb2 levels can have a profound effect on the induction of mammary tumors in transgenic mice expressing the wild-type PyV mT oncogene (6). Interestingly, whole-mount analyses of the mice carrying one of the knockout Grb2 alleles exhibit a defect in ductal morphogenesis compared to their wild-type siblings (6). These observations indicated that reduction of Grb2 dosage can also affect the ability of the mammary epithelial cell to respond to endogenous levels of growth factor stimulation.

Consistent with these observations related to transgenic mice, elevated expression of the Grb2 adapter protein is frequently observed in primary human breast cancers and their derived cell lines (8). In a more recent study, 50% of primary breast cancers exhibited a greater than twofold upregulation of Grb2 mRNA relative to normal breast epithelial cells (49). Interestingly, low-EGFR-expressing tumors expressed significantly higher Grb2 mRNA levels than high-EGFR-expressing tumors (49). Together these observations suggest that modulation of the dosage of Grb2 in breast cancer may play an important role in signal amplification from activated growth factor receptors. Further support for the importance of Shc and Grb2 adapter proteins in modulating the response to growth factor receptor stimulation stems from observations made in established cell lines. For example, elevated expression of Grb2 enhances both activation of Ras and MAPK in response to EGF (11, 43). In a similar fashion, elevated Grb2 expression results in an enhanced MAPK response to insulin (37). The increased activation of MAPK was further correlated with an increase in Grb2-Sos complex formation (37). Indeed, increased Grb2-Sos complex formation has also been noted in breast cancer cell lines expressing elevated Grb2 levels (8). Taken together, these observations suggest that the Grb2 and Shc adapter proteins could serve as important therapeutic targets in the treatment of human breast cancer.

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