Germ-line expression of an oncogenic *erbB2* allele confers resistance to *erbB2*-induced mammary tumorigenesis

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We have previously shown that mammary epithelial specific expression of the activated *erbB2* allele under the control of the endogenous promoter in mice resulted in the formation of mammary adenocarcinomas. To assess whether mammary tumorigenesis in this model is influenced by the developmental window of expression, we generated mice that expressed the activated *erbB2* allele in the germ line. Although we were able to derive viable transgenic mice that were heterozygous for the activated *erbB2* allele, mice homozygous for the activated *erbB2* allele died at 12.5 days of embryogenesis. These two separate lines of mice expressed activated *erbB2* at equal levels in the mammary gland. Surprisingly, unlike the tumor-prone mice expressing activated ErbB2 in the mammary epithelium, mice with the germ-line *erbB2* allele failed to develop tumors. Gene expression analysis of the preneoplastic mammary glands revealed that there were a number of luminal epithelial markers expressed at higher levels in the tumor-prone mice. These data suggest either an expansion of a susceptible population in the tumor-prone mice or the loss of this population in the tumor-resistant mice. Taken together, these observations suggest that the temporal pattern of expression of activated ErbB2 is a critical determinant in mammary tumorigenesis. These results strongly suggest that there are feedback mechanisms present that can compensate for the expression of a potent oncogene.

Amplification and overexpression of the Neu (ErbB2, HER2) protooncogene has been observed in 20–30% of human breast cancers and correlates with a poor prognosis for the patient (1, 2). Direct evidence supporting a role for the various epidermal growth factor receptor family members and their ligands in mammary tumorigenesis is derived from observations made with transgenic mice (3). For example, mammary-specific expression of activated ErbB2 results in the rapid induction of mammary tumors (4–6). Although mammary epithelial expression of the activated *erbB2* oncogene is capable of efficiently inducing multifocal mammary tumors, no comparable activating mutations have been detected in the transmembrane domain of human ErbB2 (7). Thus, the primary mechanism by which ErbB2 induces mammary tumorigenesis in human breast cancer is through overexpression of the wild-type receptor. Interestingly, focal mammary tumors arose in mice expressing the wild-type receptor under the control of the mouse mammary tumor virus (MMTV) promoter after a long latency period (8). Tumor progression in these mice is associated with the activation of ErbB2 tyrosine kinase activity caused by somatic activating mutations in the transgene in 70% of the mammary tumors analyzed (9–11). These mutations are confined to the cysteine-rich region of the receptor located in juxtatransmembrane domain (9). Further analyses revealed that these cysteine alterations promote the formation of intermolecular cysteine bridges between ErbB2 monomers, resulting in receptor dimerization and activation (11). Although these transgenic studies suggested that activation of *erbB2* is a critical step in tumor progression, they relied on a strong viral promoter for transgene expression. In an attempt to more closely mimic the events involved in human ErbB2-induced mammary tumor progression, we derived transgenic mice that carried a Cre inducible activated *erbB2* allele under the transcriptional control of the endogenous *erbB2* promoter (12). In contrast to the rapid tumor progression observed in the MMTV strains, focal mammary tumors arose only after an extended latency period. Tumor progression was associated with a dramatic elevation of both ErbB2 protein and transcript. Remarkably, the elevated expression of ErbB2 was correlated with genomic amplification of the activated *erbB2* allele (12). Thus, like human breast cancers, amplification of *erbB2* appears to be a critical event in mammary tumor progression in this unique mouse model.

Although these studies suggested that somatic activation of ErbB2 could predispose these mice to development of mammary tumors, one important issue that remained to be addressed was whether the developmental timing of activated ErbB2 expression could perturb the kinetics of mammary tumor induction. To assess the effect of introducing a potent gain-of-function mutant of *erbB2* in the germ line of mice on both tumorigenesis and development, we derived mice that carried activated *erbB2* in the germ line. Surprisingly, viable mice heterozygous for the germ-line-activated *erbB2* were obtained at the expected ratio. However, mice homozygous for the germ-line-activated allele died at 12.5 days postcoitum (dpc) due to defects in both cardiac and neural development. Although heterozygous mice bearing either the germ-line- or mammary-specific activation of ErbB2 expressed comparable levels of *erbB2* transcript in their mammary glands, mice bearing germ-line activation of the identical *erbB2* allele were completely resistant to mammary tumor development, in contrast to the mammary tumor-prone phenotype of the conditional activated strains (12). These observations suggest that developmental window of expression of *erbB2* is a critical factor in mammary tumor development.

Materials and Methods

Generation and Characterization of Mice. The derivation of the conditionally activated mice has been described in detail (12). To generate mice that expressed NeuNT under the control of the endogenous promoter in the germ line, a construct expressing Cre recombinase under the control of the chicken β-actin promoter was microinjected without linearization into embryos derived from mice bearing the loxP-neo-loxP-NeuNT allele controlled by the endogenous ErbB2 promoter. The resulting mice were examined for the presence of the excised recombinant allele through Southern analysis. Mammary gland analysis was completed as described (12). For embryo analysis, the extra-

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Abbreviation: dpc, days post coitum.

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embryonic tissue surrounding the embryos was dissected free, and the visceral yolk sac was retained for genotyping. The embryos were either fixed or flash frozen for protein or RNA extraction. The *in situ* analysis for Phox2a was completed as published (13).

Immunoblotting. Western blotting was conducted as described (12). To detect ErbB2, the AB3 antibody (Oncogene Science) was used.

Array Analysis. To compare the conditional and germ-line activation of ErbB2, RNA was obtained from the mammary glands of 10 virgin mice for each genotype through a standard CsCl gradient method. The RNA was pooled into two sets from five mice each and was compared through an Affymetrix array. Data were the analyzed by using the Affymetrix MICROARRAY SUITE and the DATA MINING TOOL to discard genes with marginal results and low *P* values. Final results were filtered for fold changes using a 3-fold difference as a cutoff point, and selected results are shown.

Quantitative RT-PCR. For quantitative RT-PCR, the reverse transcription, PCR, and quantification were carried out in a single capillary by using the LightCycler RNA Amplification Kit SYBR Green (Roche Diagnostics). The suggested protocol was followed in the RT-PCR reactions by using 200 ng of total RNA. The primers used to amplify *WDNM1* were as follows; 5′-TCT TTG TTC TGG TAG CTT TGA TTT-3′ and 5′-GTT TGC AGG CAT GAC CAC AG-3′. The primers used to amplify *ε casein* were 5′-CTT TTG GCC GTT GCT CTT G-3′ and 5′-TTG CTG TAT CGT TTC ATT TTG TTC-3′. The primers used to amplify *cea10* were 5′-TGG TAC AAG GGA AAC AGT GG-3′ and 5′-CAA GGA GGG TAA AAG TGA GG-3′. The primers used to amplify *erbB2* were 5′-CCC AGA TCT CCA CTG GCT CC-3′ and 5′-TTC AGG GTT CTC CAC AGC ACC-3′ for both mouse and rat and 5′-AAC CAC GTC AAG ATT ACA GAT-3′ and 5′-AAA TCA GGG ATC TCC CGG-3′ for rat specific transcripts. The PCR was stopped while all samples were in the log-linear phase of amplification, and the product was subject to a melting curve analysis. To standardize the level of RNA in these samples, RT-PCR was also completed for *GAPDH* by using the following primers; 5′-TCA TGA CCA CAG TGG ATG CC-3′ and 5′-GGA GTT GCT GTT GAA GTC GC-3′.

Results

Transgenic Mice Homozygous for the Germ-Line-Activated *erbB2* Allele Die at 12.5 Days of Embryogenesis. To explore whether expression of an activated *erbB2* allele could be tolerated during embryogenesis, single-cell embryos with one copy of the Cre inducible *erbB2* recombinant allele (Fig. 1*A*) were microinjected with a plasmid containing Cre Recombinase under the control of the chicken β-actin promoter. The β-actin promoter is expressed at the two-cell phase in the mouse embryo and should direct excision in the germ line to place activated *erbB2* under the control of the endogenous promoter in all tissues in which it is normally expressed (Fig. 1*B*) (14). In several of the Cre-injected progeny, there was complete excision of the loxP flanked sequence in a Southern analysis (Fig. 1*C*). Subsequent breeding of these mice confirmed that one copy of the activated *erbB2* allele could be passed through the germ line without any obvious phenotypic abnormality.

To assess whether mice carrying two copies of the activated erbB2 allele were compatible with viability, the heterozygous germ-line-activated ErbB2 mice ($ErbB2^{WT/NT}$) were interbred to generate homozygous offspring. The results showed that mice homozygous for the germ-line-activated *erbB2* allele ($ErbB2^{NT/NT}$) were not detected (Fig. 2*A*). To determine when the $ErbB2^{NT/NT}$ mice were succumbing to embryonic lethality, embryos were harvested from 10.5 to 13.5 dpc. This revealed that we were able to

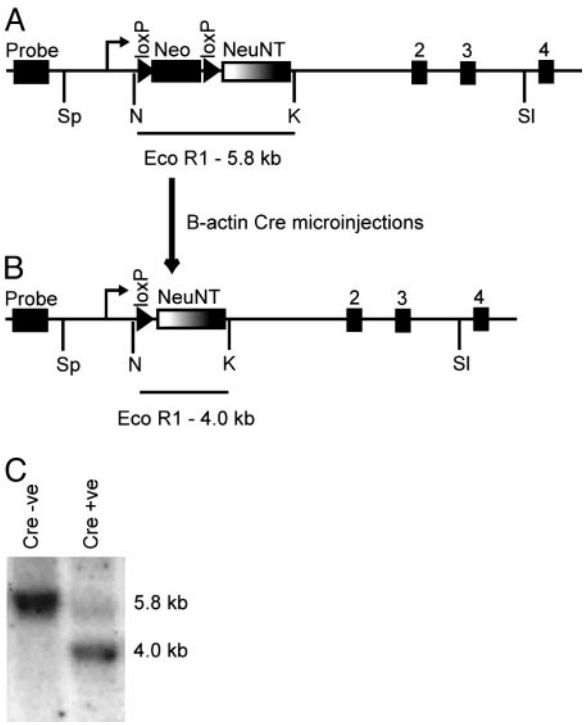

Fig. 1. Generation of mice expressing NeuNT under the control of the endogenous promoter in the germ line. To create mice expressing the activated *erbBB2* allele under the control of the endogenous promoter, mice containing a loxP neomycin loxP NeuNT sequence in place of exon 1 of the endogenous erbB2 allele (*A*) were interbred (12). The single-cell embryos from this cross were then microinjected with a circular β-actin Cre plasmid to excise the loxP flanked sequence resulting in the germ-line NeuNT allele ($ErbB2^{WT/NT}$) (*B*). The excision of the neomycin cassette can be detected through a shift in size of the EcoR1 fragment detected in a Southern analysis using the activated *erbB2* cDNA as a probe (*C*). Without the addition of Cre recombinase, there is a single band at 5.8 kb. However, after Cre-mediated excision, this band shifts to 4.0 kb, placing the activated *erbB2* cDNA under the control of the endogenous promoter.

generate the expected number of viable $ErbB2^{NT/NT}$ embryos at 10.5 dpc (Fig. 2*B*), but that by 11.5 and 12.5 dpc, a proportion of these embryos were dying. After 12.5 dpc, we were unable to detect viable $ErbB2^{NT/NT}$ embryos (Fig. 2*B*).

To investigate the cause of the embryonic lethality observed in the $ErbB2^{NT/NT}$ embryos, embryos were harvested at 12.5 dpc. Although the $ErbB2^{WT/WT}$ and $ErbB2^{WT/NT}$ embryos had normal cardiac trabeculation (Fig. 2*C*), the $ErbB2^{NT/NT}$ embryos had a reduction in the amount of trabeculation (Fig. 2*D*). In many respects, this phenotype resembled the trabecular defects observed in *erbB2* null mice (15). However, unlike the germ-line ablation, which lacked trabeculae entirely, the $ErbB2^{NT/NT}$ embryos have small trabeculae, which likely allowed the embryos to survive for 2 days longer than their null counterparts. We also examined whether this strain exhibited defects in the peripheral nervous system. Using expression of *phox2a* as a marker of neural development, we performed *in situ* hybridization on 11.5-dpc embryos with *phox2a*-specific probes. In both the $ErbB2^{WT/WT}$ and $ErbB2^{WT/NT}$ embryos, the presence of *phox2a* expression in the developing sympathetic chain ganglia was clearly present (Fig. 2*E*). However, the presence of *phox2a* expression could not be detected in the $ErbB2^{NT/NT}$ embryos (Fig. 2*F*). These results suggest that the expression of an activated *erbB2* allele under the control of the endogenous promoter results in both cardiac and neurological defects reminiscent of the *erbB2* null mice. To exclude the possibility that these phenotypes reflected loss of expression of activated erbB2 allele, we

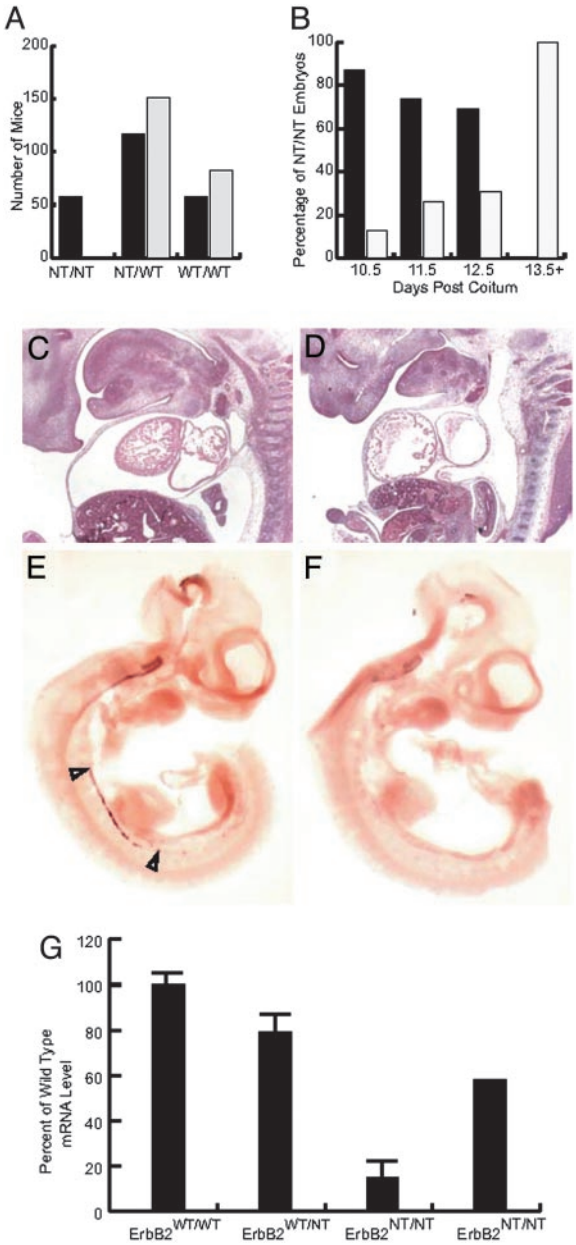

Fig. 2. Embryonic lethality in ErbB2$^{NT/NT}$ mice at 12.5 dpc due to cardiac and neural defects. After interbreeding ErbB2$^{WT/NT}$ mice, the progeny were genotyped, and the expected number of each genotype is shown with black bars. The observed genotypes are shown (gray bars), indicating that no viable ErbB2$^{NT/NT}$ mice were detected (*A*). On genotyping embryos from timed matings, we noted that no viable ErbB2$^{NT/NT}$ embryos were observed after 12.5 dpc. Examining only the ErbB2$^{NT/NT}$ genotype, the percentage of viable and dead or dying embryos is shown. The percentage of viable ErbB2$^{NT/NT}$ embryos (black bars) and the number of ErbB2$^{NT/NT}$ embryos that showed signs of being resorbed (gray bars) are shown from 10.5 to 13.5 dpc (*B*) ($n = 58$). Sections of the ErbB2$^{WT/NT}$ (*C*) and ErbB2$^{NT/NT}$ (*D*) embryos were examined at 12.5 dpc and illustrated that there was a defect in cardiac trabeculation in the ErbB2$^{NT/NT}$ embryos (*D*). Moreover, when the sympathetic chain ganglia was examined through a *phox2a in situ* analysis, it was clear that the heterozygous control was developing normally (*E*), whereas the ErbB2$^{NT/NT}$ embryos lacked proper development (*F*). The level of expression of the *erbB2* transcript was examined in 10.5-dpc embryos through a quantitative RT-PCR analysis (*G*). When compared against the ErbB2$^{WT/WT}$ control, it was observed that the level of both ErbB2$^{WT/NT}$ and ErB2$^{NT/NT}$ transcript was reduced. Moreover, when the wild-type and heterozygous embryos were compared, an expected transcript level for the ErbB2$^{NT/NT}$ was generated that was far higher than the observed levels. Error bars denote standard deviation measured on four samples per genotype repeated three times and standardized to a *GAPDH* control.

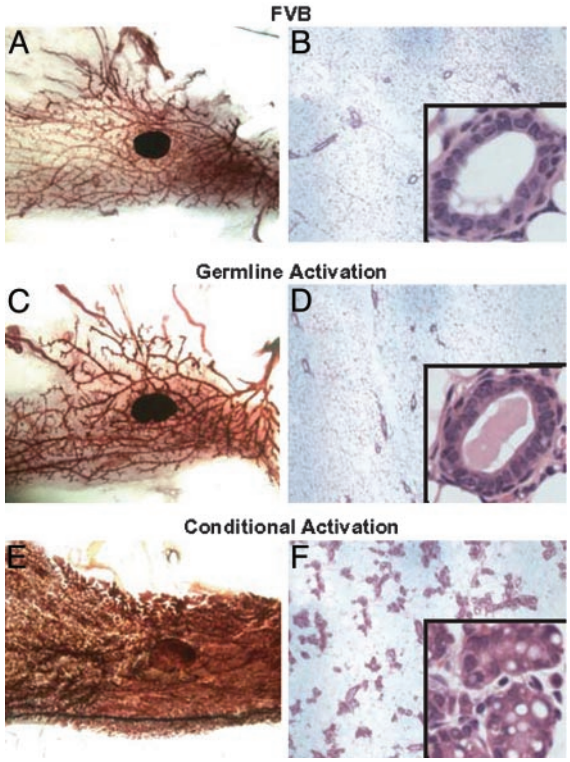

Fig. 3. Effect of NeuNT expression on mammary gland development. Mammary glands from adult mice were compared to assess the effect of activated ErbB2 expression under the control of the endogenous promoter through both whole mounts and hematoxylin/eosin-stained histology. Wild-type (*A* and *B*), germ-line activation (*C* and *D*), and conditional activation (*E* and *F*) models were compared in this analysis. The wild-type and germ-line activation mammary glands are similar, whereas the conditional activation of ErbB2 results in a hyperplastic gland. The hematoxylin/eosin-stained sections of these whole mounts reinforce the differences in ductal density between the samples. Higher magnification (*Insets*) of these sections reveals no striking differences in ductal architecture between the wild-type and germ-line activation, whereas the hyperplasia in the conditional activation is readily observed. Whole-mount photomicrographs were taken at ×1.4, low-magnification histology at ×50, and *Insets* at ×400.

measured the levels of activated *erbB2* transcript in 11.5-dpc embryos. The results revealed that transcripts specific to activated *erbB2* could be detected, albeit at lower-than-expected levels (Fig. 2*G*). Taken together, these observations suggest that germline expression of activated *erbB2* can be tolerated only in mice heterozygous for the activated *erbB2* allele.

The Developmental Window of Activated *erbB2* Expression Determines Mammary Tumor Susceptibility. One expected phenotype of the ErbB2$^{WT/NT}$ mice was the development of mammary epithelial hyperplasias and tumors, because mammary-specific activation of the same activated *erbB2* allele resulted in development of tumors (12). To examine whether the ErbB2$^{WT/NT}$ mice exhibited mammary epithelial abnormalities, we compared the mammary gland morphology of mice carrying either the germline *erbB2* allele or mice carrying mammary-specific activation of the same *erbB2* allele. In contrast to the precocious lobualveolar hyperplasias observed in mice expressing the conditional activated *erbB2* allele in the mammary epithelium (Fig. 3 *E* and *F*), age-matched mammary glands derived from ErbB2$^{WT/NT}$ mammary glands (Fig. 3 *C* and *D*) exhibited morphologies indistinguishable from wild type Freund leukemia virus/B strain mice (Fig. 3 *A* and *B*). Given that the same recombinant allele expressing activated *erbB2* under the control of the endogenous

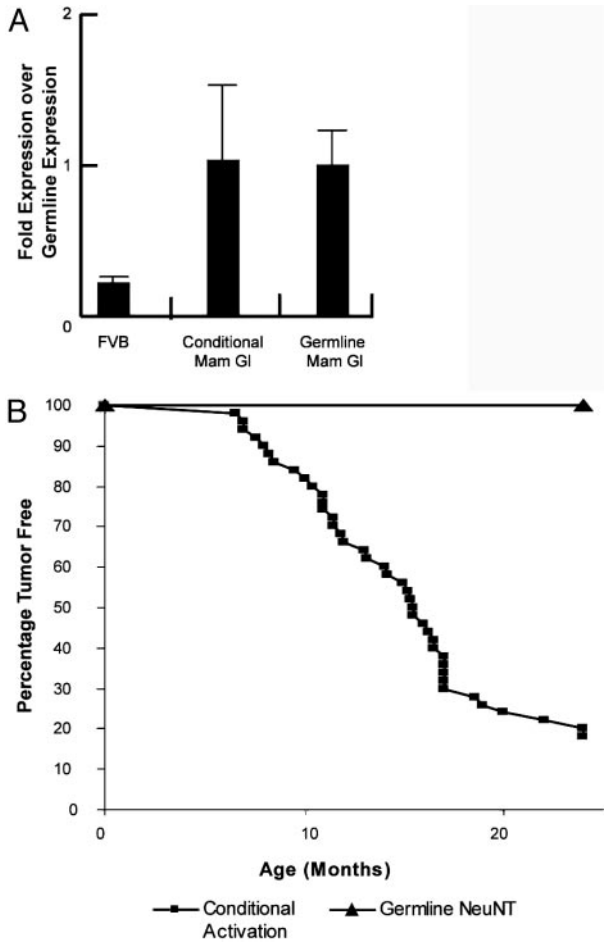

Fig. 4. Lack of tumors in ErbB2$^{WT/NT}$ mice. Given the differences in mammary gland morphology between the germ-line and conditional models, the level of *erbB2* transcripts were measured by quantitative RT-PCR with primers specific for the activated *erbB2* allele. After setting the level of the germ-line expression to 1, it was clear that the conditional and germ-line models expressed the activated *erbB2* allele at identical levels (*A*). Error bars represent standard deviation measured in four samples after repeating the analysis three times and standardizing to *GAPDH*. To determine whether the ErbB2$^{WT/NT}$ mice were susceptible to tumors, 25 mice were observed for >2 years and remained tumor free in all tissues (*B*). This is in stark contrast to the conditional model, where the tumor latency is 15.9 months for 50% of female mice.

promoter was present in both strains, these results indicate that differences in either the temporal or spatial expression patterns are capable of causing striking changes in the susceptibility of the mammary gland to hyperplasias. Although it is possible that developmental abnormalities could have arisen in the germ-line ErbB2$^{WT/NT}$ mice, it was noted that these mice were fully capable of lactating, suggesting that the mammary gland differentiated normally.

To preclude the possibility that the difference in hyperplasia susceptibility between these strains was due to differences in the levels of expression, we measured the levels of activated *erbB2* transcript with activated *erbB2* allele specific primers. The results showed that the mammary gland derived from either conditional or germ-line ErbB2$^{WT/NT}$ mice expressed equivalent levels of activated *erbB2* transcript (Fig. 4*A*), illustrating that the dramatic difference in the mammary phenotype cannot be accounted for by different levels of activated *erbB2* transcript.

To explore whether the expression pattern impacted the ability of these strains to develop mammary tumors, virgin female mice with either the conditionally activated *erbB2* allele or the germ-line ErbB2$^{WT/NT}$ were monitored for mammary tumors. Mice with the mammary-specific activation developed mammary tumors with an average latency period of 15.9 months with >90% affected by 2 years (Fig. 4*B*). In contrast, over a 2-year observation period, mice carrying the germ-line ErbB2$^{WT/NT}$ allele failed to develop tumors. Moreover, because ErbB2 has been implicated in other cancers, these mice were observed for tumor formation in all tissues for 2 years and were found to be completely tumor free.

Gene Expression Profiling Reveals a Distinctive Tumor-Prone Signature. To explore the molecular basis for the differential response of the mammary epithelium to activated *erbB2* in these strains, we compared the gene expression profiles of RNA derived from mammary gland the conditionally activated *erbB2* mice to the ErbB2$^{WT/NT}$ strain. Ten virgin mammary glands from each line were compared through a set of Affymetrix gene chip analyses. In Table 1, the genes that are expressed at a higher level in the conditional model in both repeats of the gene chip experiment are shown with the average fold increase in a positive value. Accordingly, the genes expressed at a higher level in the ErbB2$^{NT/WT}$ mammary gland are shown as a negative average fold increase.

One category of genes that were consistently up-regulated in mammary glands derived from the conditionally activated model are markers of mammary gland differentiation and lactation that are observed includes genes such as ε *casein* (21-fold increase), *wap* (11-fold increase) *connexin 26, connexin 30*, and α-*lactalbumin* (4-fold increase). Included in this list of differentiation markers is *glycam1* (9-fold increase), which was previously observed to be elevated in tumors arising in the conditional activation of *neu* (16). The preneoplastic markers are another class consistently up-regulated in the conditional activated mammary epithelium and are also frequently up-regulated in Ras or Neu initiated mammary tumors (17) and include *WDNM*1 (5.1-fold increase), *lactotransferrin* (4.4-fold increase), κ-*casein* (3.0-fold increase), and *CRBP1* (2.8-fold increase) (Table 1).

To confirm the accuracy of the gene expression data, quantitative RT-PCR was performed for various samples from the conditional and germ-line activation models. In addition, ErbB2-induced tumors and wild-type virgin and lactating mammary glands were included as controls. The quantification for ε *casein*, *WDNM1*, and *cea10* is shown, illustrating that the trends revealed by the gene chip were accurate (Fig. 5). Elevated levels of these transcripts were also observed in ErbB2 tumors and lactating mammary tissue. Significantly, these markers were expressed at elevated levels in mammary epithelium before overt tumor formation, suggesting that the expression of these genes in virgin epithelium represents an expansion of cell type targeted by ErbB2.

Discussion

The ability of an activated oncogene to induce tumors depends on multiple factors. Here we demonstrate that the temporal timing of expression of an activated oncogene is a critical parameter in tumor induction. Previous studies with activation of an identical activated *erbB2* allele in the mammary epithelium revealed that these mice developed focal mammary carcinomas with a long latency period (12). To explore whether timing of expression of the *erbB2* oncogene would affect tumorigenesis, we induced activation of an identical activated *erbB2* allele in single-cell embryos. Surprisingly, mice heterozygous for the activated *erbB2* had no obvious phenotype, but we were not able to derive mice that were homozygous for the activated *erbB2* allele (Figs. 1 and 2). Examination of the ErbB2$^{NT/NT}$ embryos revealed that embryonic lethality was occurring at 12.5 dpc because of defects in cardiac trabeculation and development of the nervous system. Interestingly, expression of the activated *erbB2* allele allowed the ErbB2$^{NT/NT}$ embryos to proceed past the

Table 1. Expression profile of germ-line and conditionally activated erbB2 mammary glands

Fold change	Gene name	Ontology	GenBank accession no.
Differentation			
21.1	ε Casein	Stimulated by hormones	V00740
11.7	Fatty Acid B.Protein	Differentiated mammary marker	X14961
11.3	WAP		V00856
8.9	Glycaml	Elevated in tumor model	M93428
8.6	Glycoprotein		Z22552
8.3	Connexin-30	Differentiation marker	Z70023
4.8	Connexin-26	Expressed in pregnancy	M81445
4.3	α-lactalbumin	Stimulated by hormones	M87863
3.2	Butyrophilin	Milk protein	U67065
Potential neoplastic markers			
8.3	MRP8	Elevated in breast cancer (24)	M83218
8.0	Cea10 related tag	Some cea proteins deregulated in tumors	AV381191
5.3	Cea10	Some cea proteins deregulated in tumors	D38422
4.9	Glycerol kinase	Raf induces expression in MCF10a	U48403
5.1	WDNMI	Elevated in ErbB2 and Ras tumors (17)	X93037
4.4	lactotransferrin	Elevated in ErbB2 and Ras tumors (17)	J03298
4.3	CAII	Inhibitors have antitumor properties	M25944
3.7	Ceruloplasmin	Copper transporter, some breast cancer evidence	U49430
9.2	MRP14	See MRP8	M83219
3.0	κ-casein	Elevated in ErbB2 and Ras tumors (17)	M10114
2.8	CRBPI	Elevated in ErbB2 and Ras tumors (17)	X60367
7.0	CAB1	Coamplified with ErbB2	X82457
3.0	Mat8	Elevated in ErbB2 and Ras tumors (17)	
Tumor suppressors			
−3.9	EBI-1	Enhances antitumor immunity	L31580
−3.4	Neuronatin	Identified as a tumor supressor	X83569
−3.1	Similar to 53BP1	Similar to a p53-binding protein	A1593047
Others			
4.9	β-1-globin		V00722
3.7	Arginase II		AF032466
−4.0	Slfn1	Growth regulatory genes	AF099972
−4.1	ALDR	Induced by retinoic acid	Z48670
−4.3	MUP V	Urinary protein	M16360
−4.6	Similar to PKC		AV336804
−4.9	Retinal oxidase	Absent in MCF7 cells	AB017482
−6.7	Adrenergic receptor		X72862
−8.9	Reelin	Up-regulated in esophageal cancer	U24703

Mammary glands from both the conditional and germ-line NeuNT mice were compared through Affymetrix chip analysis. A portion of the results of this analysis is shown after using a 3-fold change as the cutoff. Genes that were expressed at a higher level in the conditional model are shown as a positive-fold elevation, whereas genes expressed at higher levels in the germ-line model are shown as a negative-fold elevation. The fold change in gene expression, gene name, a brief ontology, and the accession number are shown.

point of embryonic lethality observed in the ErbB2 null mice but was not sufficient to rescue embryonic lethality. Importantly, when the wild-type *erbB2* cDNA was used, the resulting homozygous mice were viable (13). These results suggest that normal signaling from ErbB2 is required for development of the mouse. Further, when the expected level of *erbB2* mRNA was calculated, the level of observed *erbB2* expression was far lower than expected in the homozygous sample (Fig. 2*G*). Previous results using the same targeting strategy for several alternate *erbB2* alleles resulted in unchanged levels of *erbB2* mRNA, although a reduction in protein levels was noted (13). The reduction of *erbb2* mRNA in the ErbB2$^{NT/NT}$ model suggests that expression of activated *erbB2* has triggered a feedback loop that regulates the promoter activity of *erbB2*.

Although two copies of the activated *erbB2* allele were not tolerated during embryonic development, ErbB2$^{WT/NT}$ mice exhibited no immediate phenotype. Given that the mammary-specific activation of the same activated *erbB2* allele resulted in the induction of mammary carcinomas (12), we anticipated that germ-line transmission of activated *erbB2* allele would result in a similar phenotype in the ErbB2$^{WT/NT}$ mice. However, both histological and phenotypic analyses revealed that these animals failed to develop mammary hyperplasias or tumors (Figs. 3 and 4). The dramatic difference in tumor phenotype between the two strains was not due to changes in activated *erbB2* transcript, because both mammary samples expressed equivalent levels of *erbB2* (Fig. 4*A*).

One possible explanation for the absence of tumors in the germ-line-activated *erbB2* strains is that, because of its expression in the embryonic state, there is adaptation of mammary epithelium to constitutive *erbB2* signaling. These results are reminiscent of experiments using Rous sarcoma virus (RSV) (18). Infection of newly hatched chicks with RSV resulted in a sarcoma at the site of injection and was associated with expression of active *v-src*. However, with an *in ovo* infection, there was no corresponding sarcoma development (18, 19). Interestingly, in the chicks infected *in ovo*, *v-src* was detected to be both expressed and active despite the lack of sarcoma formation (19). Given that the germ-line expression of activated *erbB2* occurs in the embryonic state and that conditional activation would result

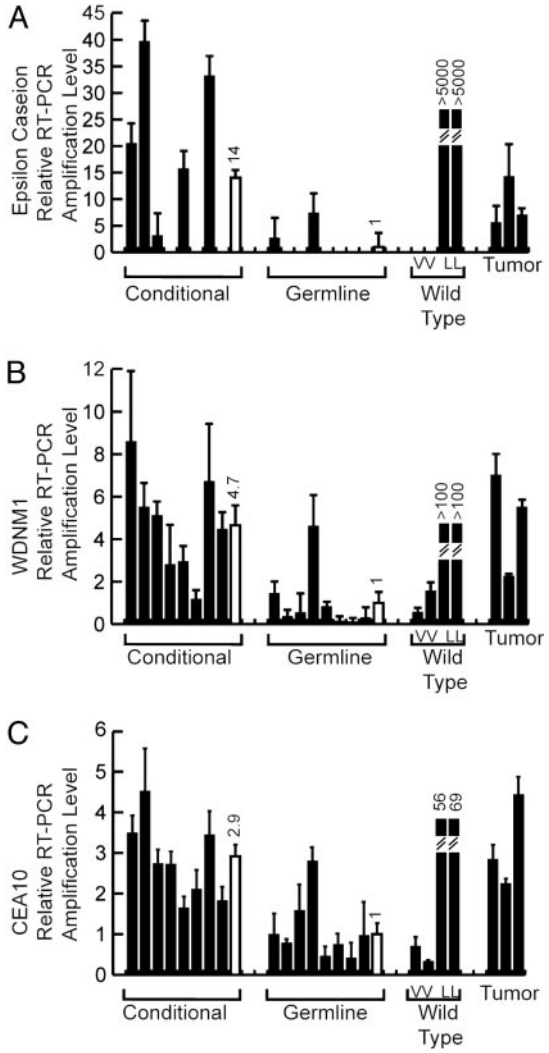

Fig. 5. Confirmation of the expression analysis. To confirm the gene expression data, quantitative RT-PCR was performed for three target genes. *ε casein* (*A*), *WDNM1* (*B*), and *CEA10* (*C*) were tested for expression, and the results of three repeats standardized to *GAPDH* are shown for each of eight samples for both the conditional and germ-line expression of the activated *erbB2* allele (black bars). The average of these samples is shown with the open bar and is stated above the bar. These data are also compared to the virgin (V) and lactating (L) wild-type controls in addition to the conditional tumor control.

in excision and activation of activated *erbB2* in the postnatal mouse, there are striking similarities in these results. Further support for this view stems from observations with patients carrying germ-line activated fibroblast growth factor receptor mutations. Although these patients exhibit a variety of developmental defects, they do not show a greater predisposition to develop cancer (20). These observations strongly argue that the developmental window of expression of an activated oncogene can profoundly affect its oncogenic potential.

Another possible mechanism by which embryonic expression of *erbB2* may influence tumor development is by indirectly affecting the epithelial target population for *erbB2*-mediated transformation. Comparison of gene expression profiles of virgin mammary glands between the mammary-specific and germ-line-activated ErbB2$^{WT/NT}$ revealed substantial differences in the expression of a number of genes. In particular, the expression of a number of luminal epithelial markers such *WMND1* and *κ casein* is dramatically down-regulated in the germ-line strains (Table 1 and Fig. 5). Significantly, elevated expression of these markers is frequently observed in Ras- and ErbB2-initiated tumors and during luminal differentiation (17), suggesting that there is a deficiency in the luminal cell type that is targeted for transformation by activated *erbB2* in the ErbB2$^{WT/NT}$ strain. Consistent with these observations, the ErbB2$^{WT/NT}$ mice fail to exhibit the precocious lobular hyperplasias characteristic of the conditional activated erbB2 strain (Fig. 3). These data suggest that germ-line expression of activated erbB2 may have altered the normal course of mammary epithelial differentiation. The future elucidation of the precise mechanism by which germ-line expression of *erbB2* confers resistance to tumor induction will provide important insight into the molecular mechanism of *erbB2*-induced tumorigenesis. Interestingly, ovexpression of the ErbB2 by various keratin promoters in transgenic mice has resulted in developmental abnormalities in both the skin and hair follicles (21), skin hyperplasia (22), and squamous cell carcinomas (23). The lack of tumors in the mammary gland, skin, or any of the other tissues that would normally express ErbB2 suggests there is a general resistance in these mice to ErbB2-mediated tumorigenesis. In the future, modulation of this pathway would have clear benefits for treatment of ErbB2 positive tumors.

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