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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Breast cancer is a prevalent, yet poorly understood disease. Of the women afflicted with breast cancer, 20-30 % of cases contain amplified and overexpressed ErbB2 (Neu, HER2). In order to examine the role of ErbB2 in mammary gland development and tumorigenesis, I have sought to both create mice containing a mammary specific knockout of ErbB2 and a mammary specific activation of the oncogenic form of ErbB2. To achieve these goals, a line of transgenic mice expressing Cre Recombinase in the mammary epithelium was created. These mice have been interbred with mice containing a loxP flanked *erbB2* cDNA in place of exon one at the genomic *erbB2* locus. This resulted in a delay in ductal outgrowth in the early stages of mammary gland development. However, by 6 weeks of development, the mammary glands lacking ErbB2 are indistinguishable from wild type glands and appear to lactate normally. To examine the role of activated ErbB2 in mammary adenocarcinomas, I created a line of mice that would express the activated allele under the control of the endogenous promoter in the mammary gland. The results mimic the human condition where amplification and overexpression of ErbB2 is observed in the resulting tumors.

14. SUBJECT TERMS
breast cancer, ErbB2, transgenic mice, Cre recombinase

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Introduction

The research objectives outlined in the initial proposal were twofold, to determine the role of ErbB2 (Neu, HER2) in normal mammary gland development and to elucidate the role of ErbB2 in mammary tumorigenesis. This work was initially based on the observation that 20-30 % of human breast cancers contain amplified copy numbers of HER2 and overexpress the proto-oncogene(1-4). Unfortunately, due to the early lethality of the mouse ErbB2 knockout, the role in mammary gland development in these mice could not be addressed (5). Further experiments revealed that the mammary gland expressed ErbB2 but did not suggest how ErbB2 was involved in mammary gland development (6). While the role of ErbB2 in mammary gland development has not been elucidated, the role in tumorigenesis has been examined. Expression of the activated form of ErbB2 under the control of the Mouse Mammary Tumor Virus Promoter / Enhancer (MMTV-ErbB2) resulted in mammary tumorigenesis by 89 days in 50% of female mice (7-9). Interestingly, when the same experiment was repeated using the wild type allele, tumorigenesis was observed in 50% of female mice by 205 days. While defining a role for ErbB2 in mammary tumorigenesis, these results were based on expression of ErbB2 under the control of a viral promoter of questionable relevance to the human condition. Accordingly, I have sought to create mouse models where ErbB2 is specifically deleted in the mammary gland and where activated ErbB2 is expressed under the control of the endogenous promoter in the mammary gland. In the mammary specific deletion of ErbB2 I observed defects in early ductal elongation. I have also observed tumorigenesis, amplification and overexpression of ErbB2 when the activated allele is expressed under the endogenous promoter in the mammary gland (10). Further analysis of these tumors has revealed that they are more differentiated with respect to the MMTV-ErbB2 mediated tumors and that tumor formation does not occur when the same construct is subjected to excision in the germline of the mice.

Research Accomplishments

1. Mammary Specific Expression of Activated ErbB2 under the control of the endogenous promoter results in tumorigenesis.

One of the two major goals outlined in the initial proposal was the expression of an activated form of ErbB2 in the mammary gland under the control of the endogenous promoter. It was hoped that this would result in a better mouse model of ErbB2 mediated tumorigenesis since it did not rely upon the hormonally driven MMTV promoter. I have achieved the goal of expressing activated ErbB2 under the endogenous promoter specifically in the mammary gland and have observed alterations in mammary gland development. Previous annual summaries and published data have illustrated that these mammary glands contain amplified copies of activated ErbB2, overexpress activated ErbB2 and that they develop tumors by 16 months in 50 % of female mice (10). Additionally, there are characteristic karyotypic alterations that include a deletion in chromosome four and double minute chromosomes that contain the activated *erbB2* cDNA (11). Recent results also show that flank tumor formation still can occur after hydryoxyurea treatment to remove these double minute chromosomes from cell lines established from the primary tumors. Currently I am also attempting to ascertain the role of Grb7 in tumor formation since it is also overexpressed and amplified. Through an antisense approach, I have specifically knocked down the level of the 51 kDa isoform of Grb7 and am examining the invasive properties of this cell line.

Given the long latency of tumor formation, I have also recently attempted to create a mouse line that would contain only the activated *erbB2* allele. By microinjecting a circular

plasmid containing a chicken B-actin promoter driving Cre expression, we have excised the neomycin cassette in the germline (Figure 1). Excision is complete and the activated allele is passed on to the progeny. Thus, to decrease the latency of tumor formation, mice harboring the activated *erbB2* allele in a heterozygous state were interbred to generate mice carrying only activated *erbB2* alleles. This resulted in embryonic lethality (Figure 2) at 12.5 days post coitum due to trabecular defects in the heart and neurological defects. However, since the heterozygous mice were viable, their mammary glands were examined as they were expected to mimic the conditional activation of ErbB2. Surprisingly, the mammary glands of the germline excision and the mammary specific excision were distinctly different (Figure 3). Indeed, mammary gland from mice with the germline excision resembled the wild type glands. Further, the mice harboring this germline excision did not develop tumors (Figure 4). These mammary glands should be identical, but for the time of excision, and given the differences were subjected to a comparison by Affymetrix chip analysis. This anaylsis revealed several interesting targets from the hyperplastic glands that were previously described as tumor markers (Figure 5). Several targets were then confirmed though quantitative RT-PCR (Figure 6).

In summary, the initial aim for the conditional activation of ErbB2 in the mammary gland has been met and extended to both Grb7 analysis and to an examination of germline expression of the activated allele.

2. Inactivation of ErbB2 in the mammary gland.

The second major goal defined in the initial proposal was to examine mammary gland development in the absence of ErbB2. To achieve this goal, I have placed loxP sites on either side of the ErbB2 cDNA and this loxP flanked cDNA, followed by a selectable marker has replaced exon 1 one of the wild type allele. The recombinant alleles ($ErbB2^{flox/flox}$) function as wild type alleles until Cre mediated recombination occurs. At that point, the recombinant alleles are excised and null alleles are created. Upon interbreeding the MMTV-Cre transgenic mice with the $ErbB2^{flox/flox}$ mice, I have previously observed a loss of Cre expression in the mammary gland. This initially occurred in the mixed background and subsequently occurred in a 98 % FVB background. To exclude the possibility of methylation induced transgene silencing, I have now interbred the $ErbB2^{flox/flox}$ mice 12 generations into the FVB background. I have now generated MMTV-Cre $ErbB2^{flox/flox}$ mice in the FVB background and observe excision of *erbB2*, resulting in a mammary specific deletion for ErbB2 (Figure 7). These mice have been observed to lactate normally and have no difficulty in rearing multiple litters. However, when I examined early development of the *erbB2* null mammary glands I noted a delay in the ductal elongation. At three weeks of development, the *erbB2* null gland is roughly one week behind the wild type controls. This persists to approximately 6 weeks of development when the mammary glands become essentially indistinguishable.

While the specific aims have been met, I am currently interbreeding these mice with another strain that require an amplification of *erbB2* to form metastatic tumors. These experiments will help define the role of ErbB2 in those mammary tumors and forms a natural extension of this work.

3. Hormonal Regulation of ErbB2 mediated tumorigenesis.

Although not extensively described in the scope of the main report, a minor goal addressed in the original statement of work was the examination of hormonal regulation of ErbB2 mediated tumorigenesis. Initial ovarectamy experiments did not yield results dictating that the project should move forward in the removal of hormonal regulation. However, to further explore the role of hormonal regulation of ErbB2 mediated tumorigenesis, we will examine the effect of interbreeding the conditionally activated ErbB2 mice described in section one with transgenic mice overexpressing an activated form of the Estrogen Receptor in the mammary gland. To this end, I cloned the activated estrogen receptor cDNA into the Mouse Mammary Tumor Virus Promoter / LTR plasmid and we have created several lines of transgenic mice that will shortly be screened for expression of the cDNA.

Training Accomplishments

Over the three years of this training grant I have acquired numerous skills. In addition to basic laboratory skills that are standard in molecular biology, I have learned, practiced and taught several specialized techniques. I have become proficient in the technique of microinjection to create transgenic mice and have created several strains of mice. I have gained experience in the creation of cell lines derived from primary tumors. Indeed, I have now identified three cell lines that overexpress ErbB2 at high levels. Further, I have gained experience in the purification of epithelial cells and their culture on Matrigel. In collaboration with Frank Graham's laboratory, I have also now acquired the training and skills required to purify recombinant adenovirus and infect cells with an adenovirus containing Cre recombinase to mediate excision of the loxP flanked constructs.

Academically, I have now completed all course work required for my Ph.D. degree and have completed my comprehensive examinations, receiving a designation of "With Distinction" on both the written and the oral examination. Finally, since my Ph.D. should be complete early next year, I have applied for and received interviews for postdoctoral positions. In order to further my career in breast cancer research, I have accepted a position with Dr. Joe Nevins at Duke University starting September 2003 to examine the role of E2F transcription factors in mammary tumorigenesis.

Key Research Accomplishments

- **Generation of a mouse model of mammary tumorigenesis with remarkable similarity to the human condition exhibiting amplification and overexpression of ErbB2 and similar karyotypic events.**
- **Demonstration of mammary epithelium escaping the confines of a normal mammary gland by the conditional activation of ErbB2.**
- **Generation of mice that have an activated erbB2 allele in place of one of the wild type alleles. The homozygous mice suffer from embryonic lethality and although the heterozygous mice express the activated erbB2 allele in the mammary gland, they fail to develop tumors.**
- **Generation of cell lines from mammary tumors with amplification and overexpression of ErbB2.**
- **Generation and characterization of mice lacking ErbB2 specifically in the mammary gland. The major phenotype observed in these mice is a delay in ductal outgrowth.**

- **Demonstration that Cre mediated excision to result in ErbB2 null cells is possible by interbreeding with transgenic mice and by adenoviral infection.**

Reportable Outcomes

Papers Published

Centrosome abnormalities, recurring deletions of chromosome 4, and genomic amplification of HER2/neu define mouse mammary gland adenocarcinomas induced by mutant HER2/neu.
Montagna C, Andrechek ER, Padilla-Nash H, Muller WJ, Ried T.
Oncogene. 2002 Jan 31;21(6):890-8.

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

Manuscripts in Preparation

Gene expression profiling and the role of Grb7 in mouse models of HER2 / Neu mediated tumorigenesis.
Eran R. Andrechek, Michael A. Laing, Adele A. Girgis-Gabardo, Peter M. Siegel and William J. Muller
Based largely on the work presented in the previous annual summary and the current Grb7 antisense work.

Seminars Given

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

Posters Presented

The Role of ErbB2 in Mammary Gland Development and Tumorigenesis, Era of Hope Meeting, Orlando, September 25-28, 2002.

Mammary Gland Development and Tumorigenesis; Neu Models, Biology Poster Day, McMaster University, March 2002.

Signalling in Normal and Cancer Cells, Banff, March 2-6, 2001

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.

Awards Received

The Lee Nelson Roth Award, an annual award to a doctoral student working in the area of cancer research at McMaster University based on the conditional activation of ErbB2 in the mouse mammary gland.

Positions Accepted

Based on the work supported by this grant, I interviewed with five principle investigators and have accepted a position in the lab of Dr. Joe Nevins at Duke University. This postdoctoral position will be focused on the role of the E2F transcription factors in mammary tumorigenesis and is to start in September of 2003.

Conclusion

Funding was provided for this project in order to address the role of ErbB2 in mammary gland development and tumorigenesis. After three years of funding, the most important result is the development of a mouse model of mammary carcinogenesis that mimics the human condition in several facets. Expression of an activated ErbB2 allele under the control of the endogenous promoter specifically in the mammary gland resulted in mammary adenocarcinomas that had amplified and overexpressed ErbB2. Expression of the activated ErbB2 allele also results in mammary epithelium that escapes from the confines of the fat pad. This work suggests that deleting ErbB2 in the mammary gland may impact the outgrowth of the ductal network. Indeed, after creating a mammary gland lacking ErbB2 it was observed that this hypothesis was correct and a delay in ductal outgrowth was noted. The major goals have been met and have led to interesting new questions about mammary gland development and tumorigenesis that I am currently pursuing before starting my postdoctoral position in September of 2003.

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3) Venter, D.J. et al, *Lancet* **2**, 69-72, 1987.
4) Zeillinger, R et al, *Oncogene* **4**;109-114, 1989.
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10) Andrechek E.R. et al, *PNAS* **97**;3444-3449, 2000.
11) Montagna C. et al, *Oncogene*. **21**;890-8, 2002.

Appendix One
Figures 1-8

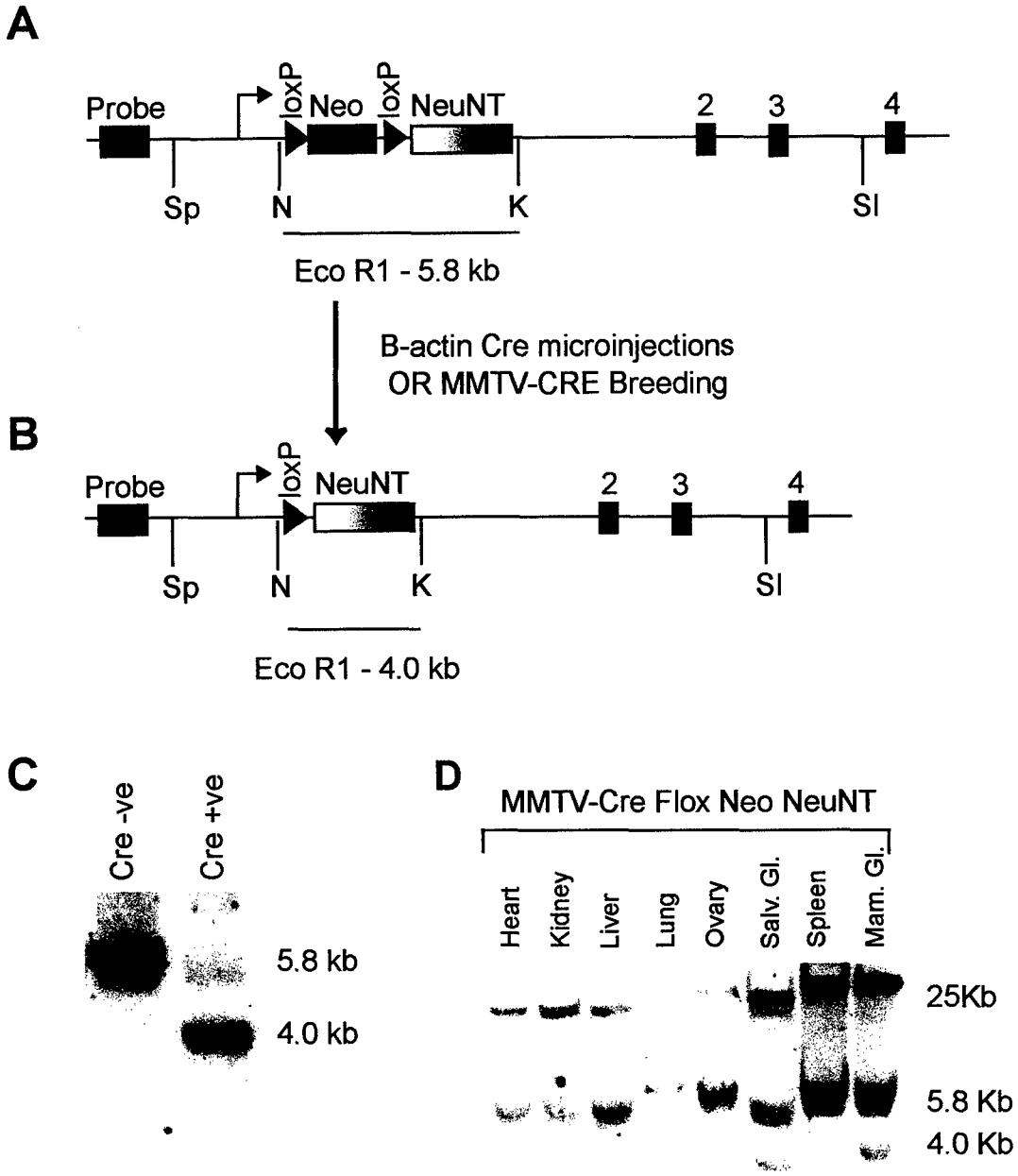


Figure One - Expression of the Activated ErbB2 allele in the germline and in the mammary gland. An activated erbB2 allele (NeuNT) was placed after a neomycin cassette (A) under the control of the endogenous promoter. After Cre mediated excision at either the two cell stage or in the mammary gland, the activated allele is placed under control of the endogenous promoter (B). Upon micro-injection of the B-actin Cre plasmid, excision was noted in tail DNA by southern analysis (C). These mice are therefore harboring the allele shown in panel B. Interbreeding mice containing the allele shown in panel A with MMTV-Cre transgenics results in excision in the mammary gland, salivary gland and the spleen (D). These mice are therefore expressing the activated allele only in the mammary gland. The B-actin mice are referred to as the germline activation and the MMTV-Cre mice are noted as somatic or conditionally activated.

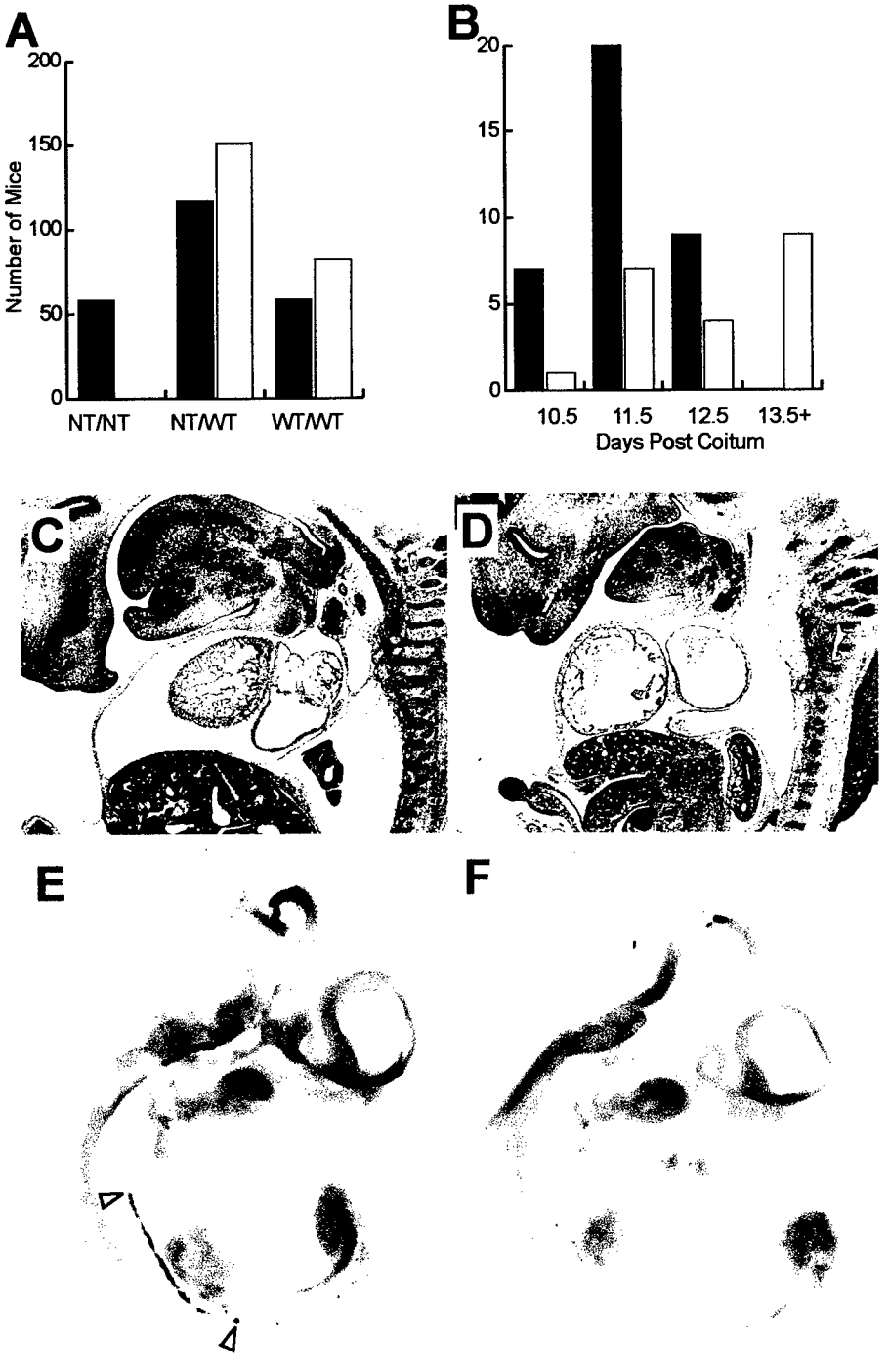


Figure Two - Embryonic Lethality in Mice carrying two activated ErbB2 alleles
When genotyping potential homozygous mice for the activated erbB2 allele (NT / NT), the expected Mendellian ratio (black bars) was not observed (A). Rather, only heterozygous (NT / WT) and wild type (WT / WT) mice were noted. To determine the point of embryonic lethality, embryos from timed matings were examined (B). The black bars illustrate the viable homozygous embryos and the grey bars denote the homozygous embryos that were dead or dying. H & E stained histology of heterozygous (C) and the homozygous (D) mice revealed defects in cardiac trabeculation. Further, *in situ* staining for *phox*, a neural marker at 11.5 dpc revealed that the homozygous mice also have neurological defects.

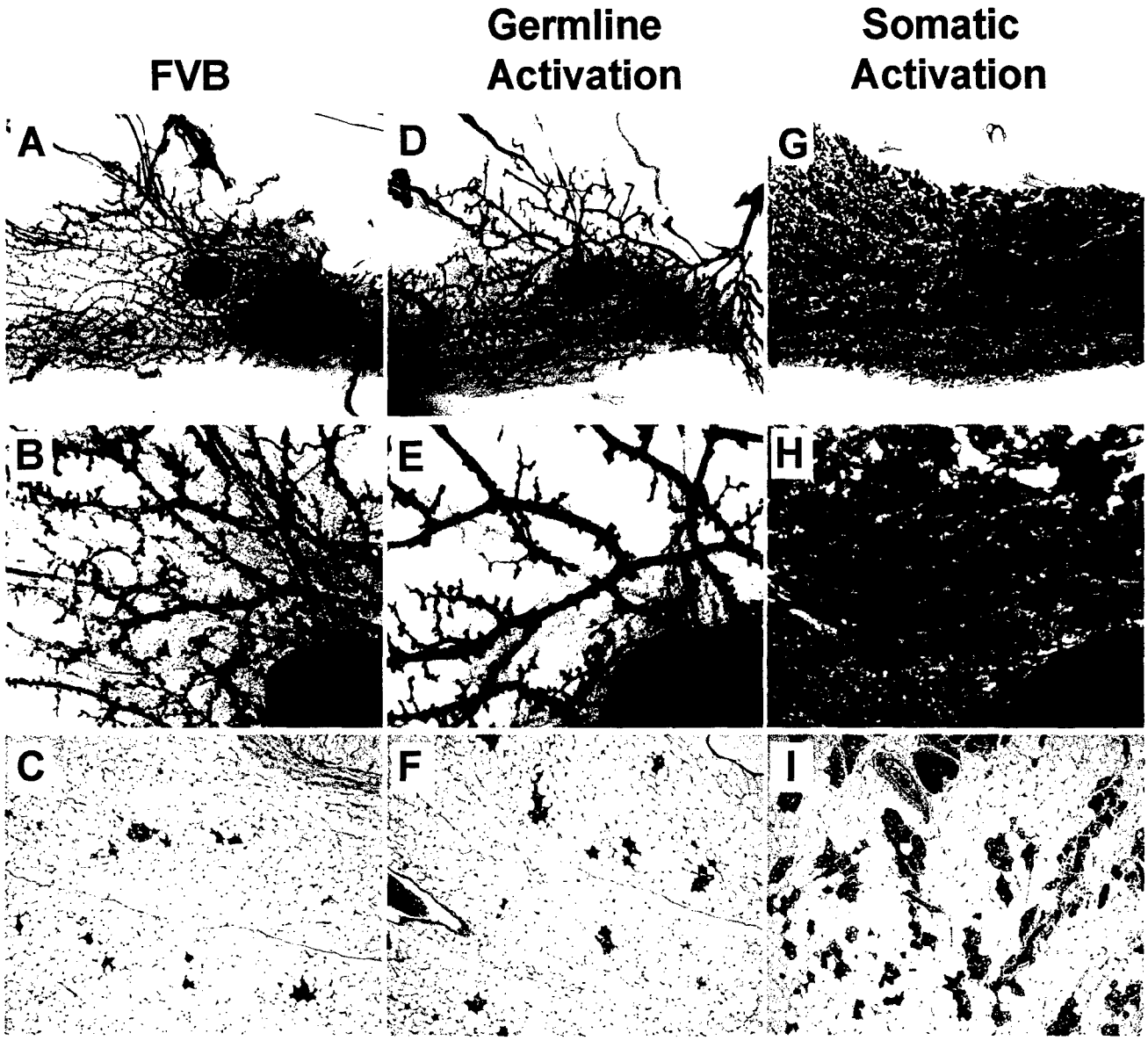


Figure Three - Comparison of germline and mammary specific activated ErbB2 expression
Wild type mammary glands at 10 months of age (A-C) were compared against mammary glands from mice harboring the activated erbB2 allele expressed under the control of the endogenous promoter in either the germline (D-F) or in a mammary specific fashion (G-I). Clearly, the germline expression of an activated erbB2 allele is not significantly different from the wild type mammary gland. When excision occurs in the mammary gland (Figure 1, D) instead of the germline (Figure 1, C), the effect is striking (G-I). Clearly, when the oncogene is expressed in the germline, the effect is not as pronounced as expression solely in the mammary gland.

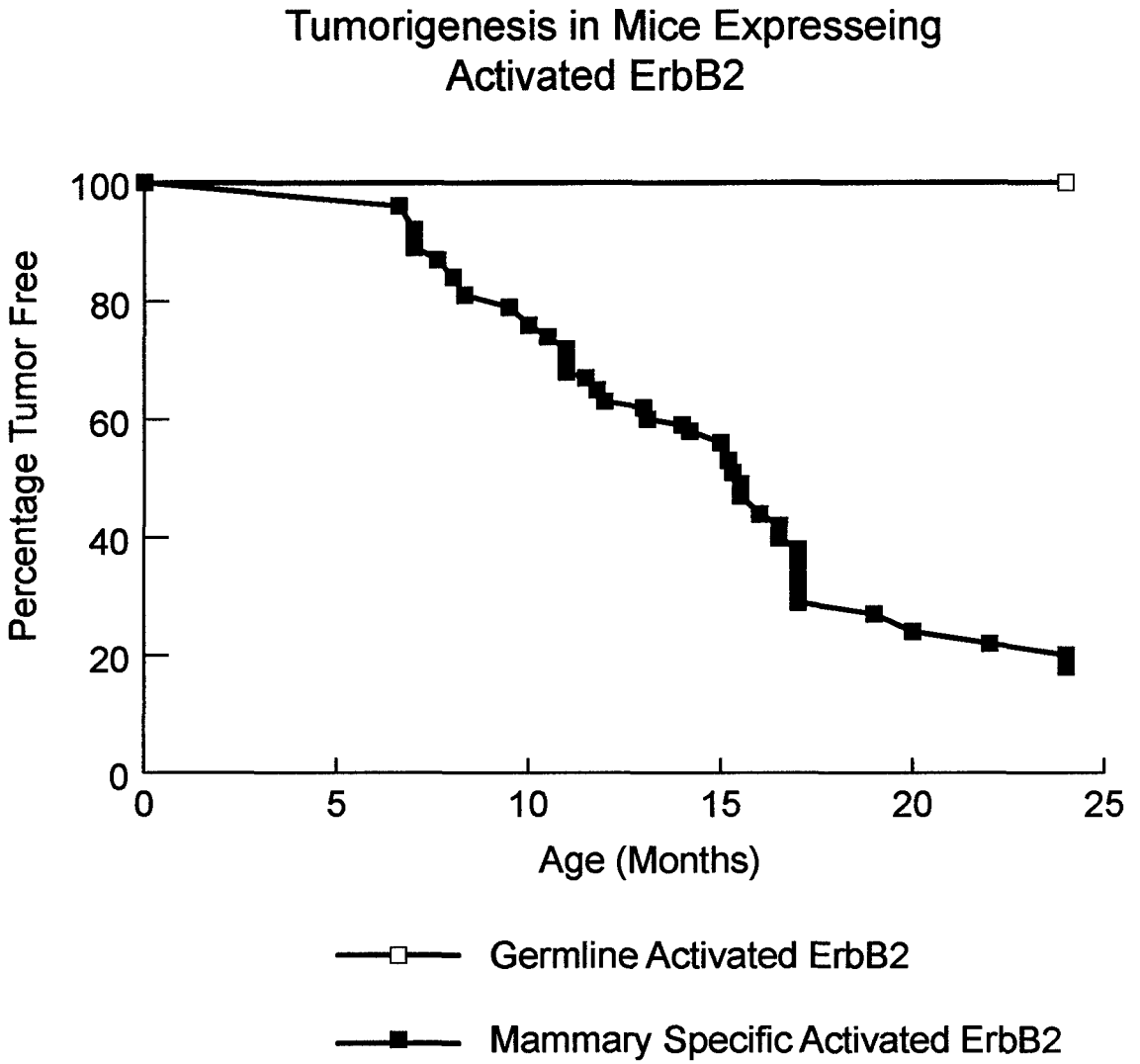


Figure Four - Kaplan-Meier Plots for Germline and Mammary Specific Tumor Formation
The incidence of tumor formation is shown for the germline and mammary specific expression of the oncogenic erbB2 allele. Of the 41 mice with mammary specific expression, the average onset of mammary adenocarcinomas is 16 months for 50 % of the females. Surprisingly, the 25 mice with the germline expression of the oncogenic erbB2 allele have remained tumor free for over 2 years. Not only are these mice free from mammary tumors, but they are free from any carcinomas.

A

Fold Change	Gene Name	Notes
21.1	Epsilon Casein	Stim by hormone
11.7	Fatty Acid Binding Protein	Differentiated mam. gl. marker
11.3	WAP	
8.9	Glycam1	Elevated in tumor model!
8.6	Glycoprotein	
8.3	Connexin-30	Differentiation Marker
4.8	Connexin-26	Expressed in Pregnancy
4.3	alpha-lactalbumin	Stim by hormone
3.2	Butyrophilin	Milk Protein
8.3	MRP8	Elevated in breast cancer PMID: 11591886
8.0	Cea10 related tag	Maybe - some Cea members dereg in tumors
5.3	Cea10	Maybe - some Cea members dereg in tumors
4.9	Glycerol kinase	Raf induces expression in MCF10a PMID: 11316792
4.6	WDNM1	Elevated in ErbB2 and Ras tumors PMID: 7970700
4.4	lactotransferrin	Elevated in ErbB2 and Ras tumors PMID: 7970700
4.3	CAII	Inhibitors have anti-tumor properties
3.7	Ceruloplasmin	Copper transporter, some Breast cancer evidence
3.2	MRP14	See MRP8
3.0	Kappa-casein	Elevated in ErbB2 and Ras tumors PMID: 7970700
2.8	CRBPI	Elevated in ErbB2 and Ras tumors PMID: 7970700
7.0	CAB1	co-amp with B2 - small tumor in set #1?
3.0	MAT-8	Elevated in ErbB2 and Ras tumors PMID: 7970700

B

Fold Change	Gene Name	Notes
-3.9	EBI-1	G-protein coupled receptor - enhance anti-tumor immunity PMID:11267967
-3.4	Neuronatin	Identified as a tumor supressor PMID: 10623765
-3.1	Similar to 53BP1	Similar to a p53 binding protein (a tumor supressor)
-4.9	retinal oxidase	Absent in MCF7 PMID: 11585737
-4.1	ALDR	induced by retinoic acid
-8.9	Reelin	Upreg is esophogeal cancer PMID : 11880184
-6.7	Adrenergic receptor	
-4.6	Similar to PKC	
-4.4	S3-12	Adipocyte secreted or cell surface protein
-4.3	MUP V	Urinary protein
-4.1	Mesenchyme homeobox 2	
-4.0	Slfn1	Growth regulatory genes
-3.6	matrilin-2	

Figure Five - Affymetrix chip comparison of mammary glands from Germline and Mammary Specific Expression of the Oncogenic ErbB2 Allele

Genes with higher expression in the mammary specific mammary gland are shown (A), as are the genes with lower expression (B). Of note are the many genes reflecting a more differentiated mammary gland (A) and the presence of numerous neoplastic markers such as WDNM1.

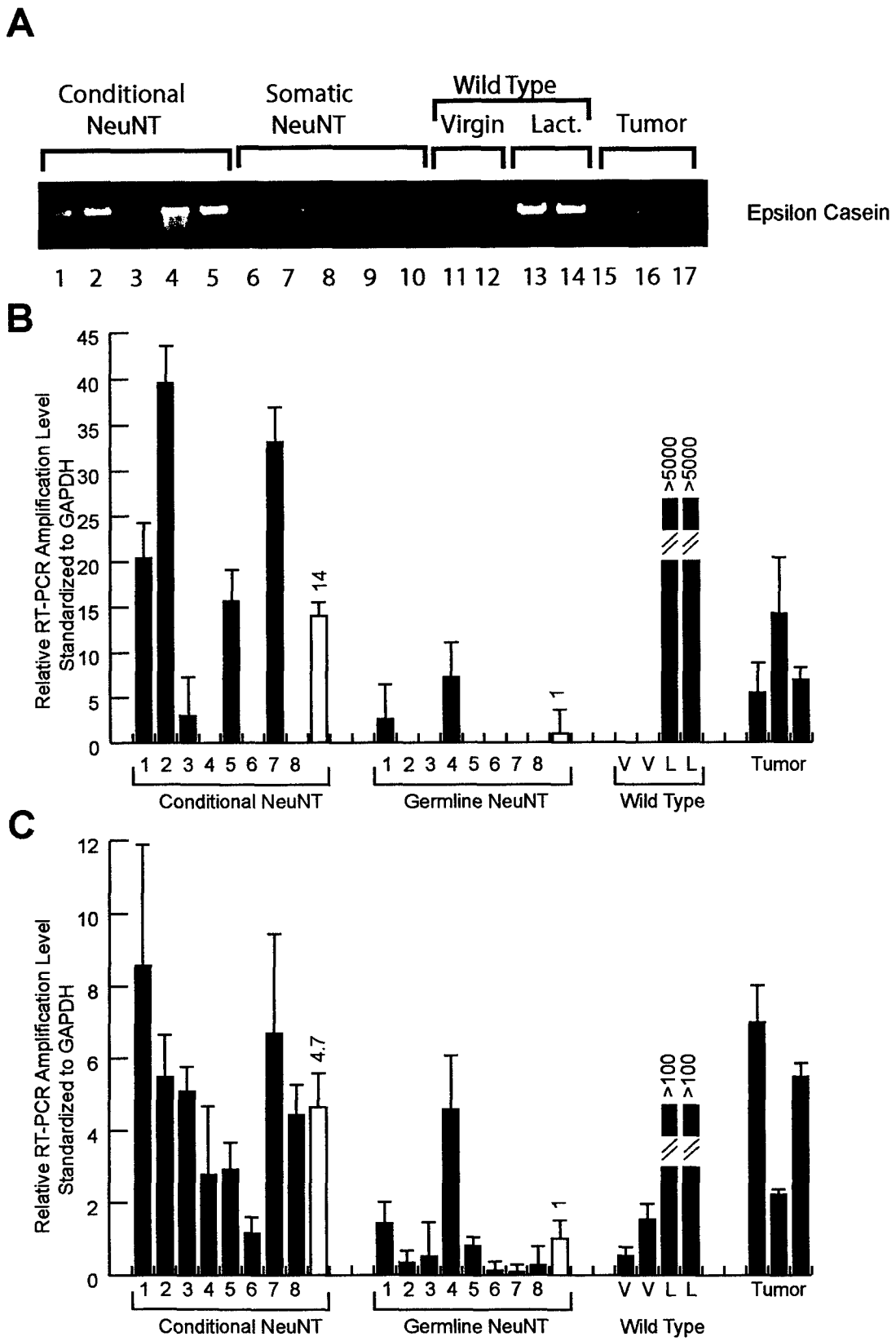
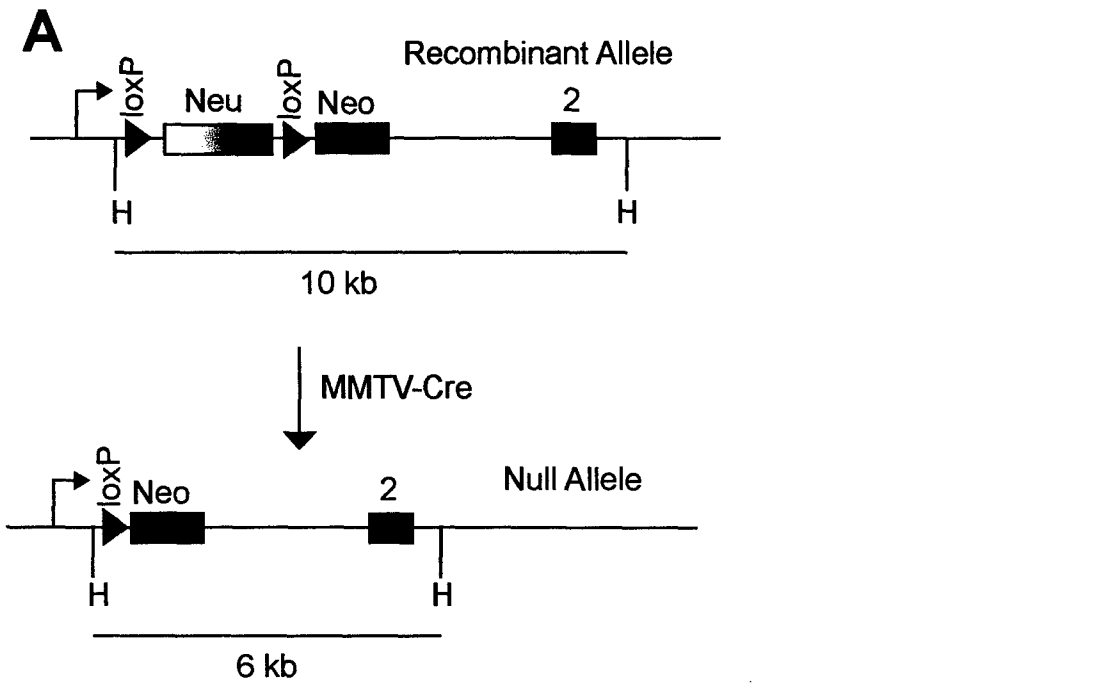
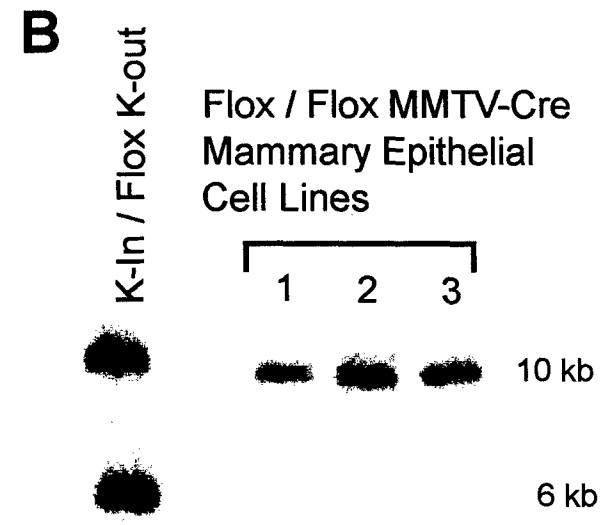


Figure Six - Confirmation of the Affymetrix Chip Data
Lightcycler quantitative RT-PCR was performed for epsilon casein (A-B) and WDNM1 (C) to determine if the chip data (Figure 5) was accurate. Although there is a large variance between the tumor samples, the averages (open bars in B and C) are in agreement with the chip data. Interestingly, WDNM1 is also highly expressed in the lactating mammary gland where its role remains unknown.





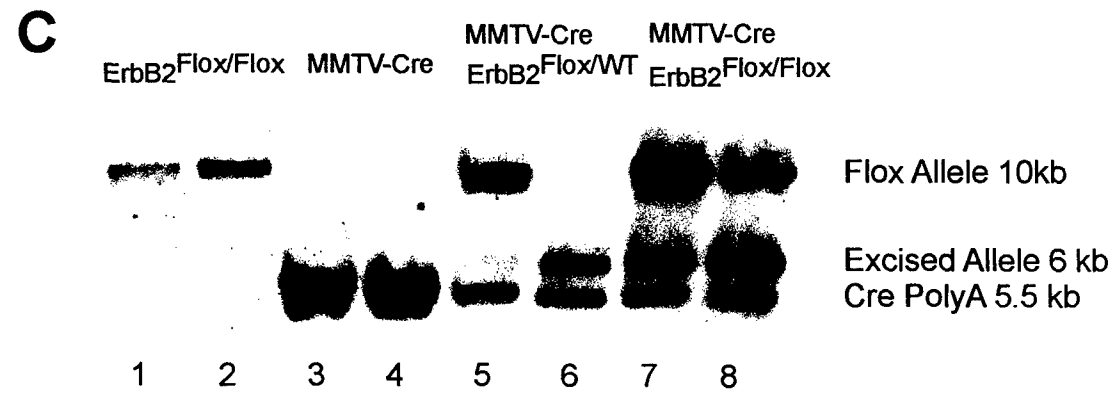


Figure 7 - Mammary specific deletion of ErbB2
An ErbB2 cDNA (Neu) was flanked with loxP sites and was targeted to replace exon one of the genomic erbB2 allele (A). Upon Cre mediated recombination, a null allele is created (A). However, when the mammary glands and mammary epithelial cells from the ErbB2 Flox / Flox MMTV-Cre mice were examined, excision was not noted (B). After 12 generations of backcrossing into the FVB background, excision was noted in the heterozygous and homozygous mice (C - lanes 5-8).

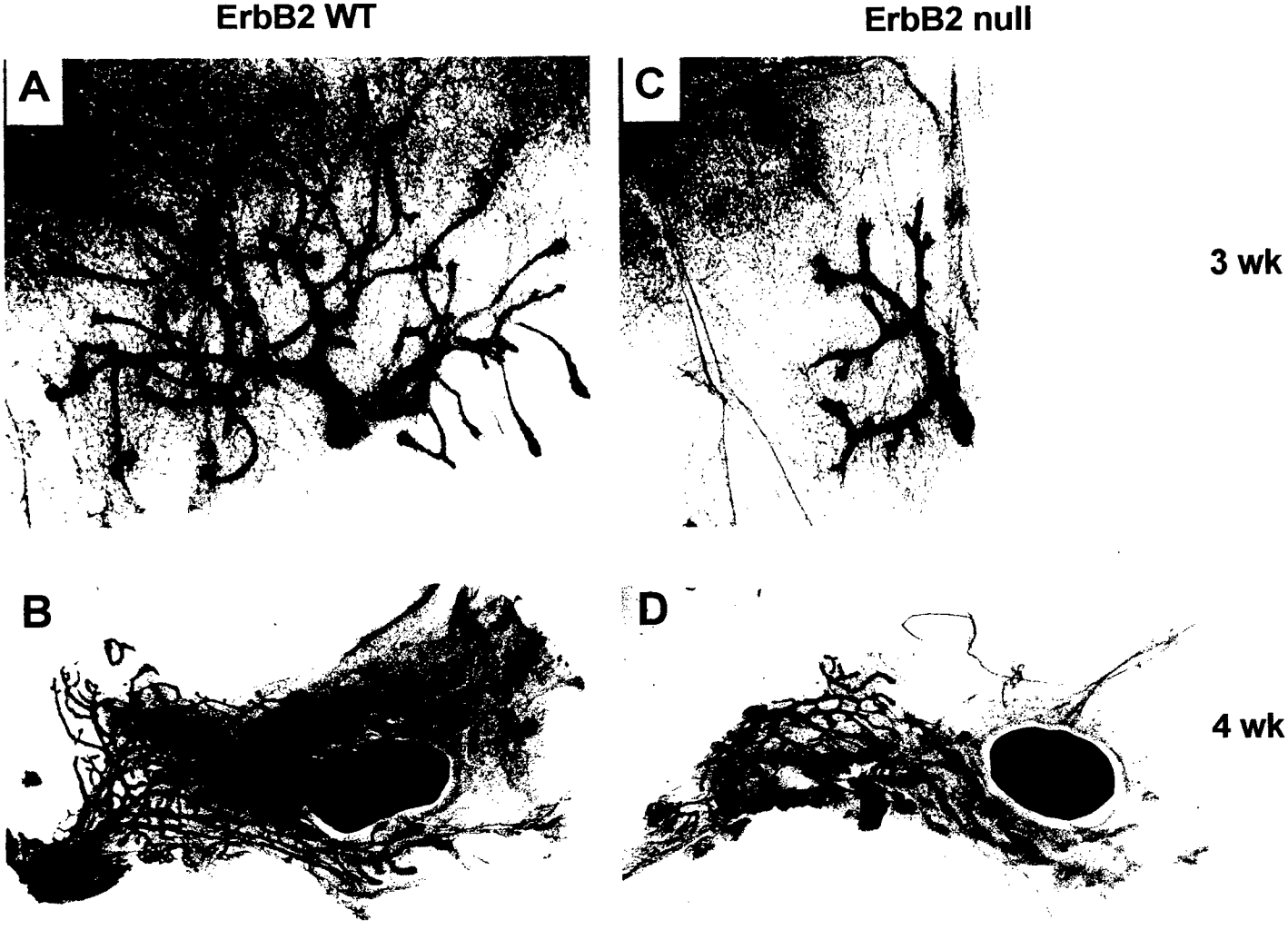


Figure Eight - Mammary effects of loss of ErbB2
Wild Type (A-B) and ErbB2 null (C-D) mammary glands are shown at 3 weeks (A,C) and 4 weeks (B,D) of development. Clearly, the deletion of ErbB2 from the mammary epithelium results in a delay in ductal elongation. Not shown are the wholemounts at 6 weeks of age where the erbB2 null mammary glands are essentially indistinguishable from the wild type controls.

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MCMR-RMI-S (70-1y) 28 July 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

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