

## PEA3 in Mouse Mammary Gland Development and Tumorigenesis

THE ROLE OF PEA3 IN MAMMARY GLAND DEVELOPMENT AND  
TUMORIGENESIS

By  
LESLEY MACNEIL B.Sc.

A Thesis  
Submitted to the School of Graduate Studies  
in Partial Fulfillment of the Requirements  
for the Degree  
Master of Science

McMaster University  
Copyright by Lesley MacNeil, September 1999

MASTER OF SCIENCE (1999)  
(Biology)

McMaster University  
Hamilton, Ontario

TITLE:               The Role of PEA3 in Mammary Gland Development and  
                          Tumorigenesis

AUTHOR:             Lesley MacNeil, B.Sc. (University of Guelph)

SUPERVISOR:        Professor John A. Hassell

NUMBER OF PAGES:xi, 132

## ABSTRACT

PEA3 is a member of the ets family of transcription factors. It is expressed throughout embryonic development and in mouse mammary adenocarcinomas induced by expression of the receptor tyrosine kinase Neu. Mice lacking PEA3 due to a targeted disruption of the gene, develop normally, however, male mice fail to mate for yet undetermined reasons. To further understand the role of PEA3 in mammary gland development and tumorigenesis, the effects of loss of function of PEA3 were examined in tumor formation and in mammary gland development.

Analysis of tumor formation in PEA3  $+/+$  and PEA3  $-/-$  animals failed to show a statistically significant difference in tumor onset. Loss of PEA3 did not affect the tumor morphology, nor did it inhibit metastasis of these tumors to the lung. These data indicate that PEA3 is not required for tumor formation or metastasis.

PEA3 deficient animals displayed defects in branching morphogenesis in the mammary gland. Decreased ductal branching was observed in virgin and pregnant females. Mice with decreased levels of PEA3 expression also exhibited defects in branching morphogenesis, indicating a dosage effect. PEA3 is expressed in the myoepithelial cells during puberty and pregnancy. It is also expressed in the highly proliferative cap cell layer of the terminal end bud. In the embryonic mammary gland, PEA3 is expressed as early as 10.5 days in the mammary epithelium and continues late in embryogenesis. Expression in the male mammary gland is lost at approximately embryonic day 16.



## **ACKNOWLEDGEMENTS**

I never could have made it without the help and friendship of everyone in the Hassell, Muller and Rudnicki labs. I would like to thank John Hassell for the opportunity to work in his lab and for many arguments that raised my blood pressure, but always made me think. I would like to thank Bill Muller and Roger Jacobs for serving on my committee. I would also like to thank Bill Muller for making me an honorary member of the lab and for his friendship through the years. Thanks to Sophie Perron, Michael Cox, Harold Kim, Jane Barrett and Laura Hastings for their encouragement, understanding and for always making me laugh. Sophie and Michael, thank you for always being there until the wee hours, I don't know how I'll survive without you. Most of all, I would like to thank Rod Hardy for teaching me everything I know about science and for always encouraging me. I couldn't have done it without you Rod!

# TABLE OF CONTENTS

Abstract.....	iii
Acknowledgements.....	iv
List of Tables.....	ix
List of Figures.....	x
List of Abbreviations.....	xii

## Chapter 1. Introduction

1.1. The ets Family of Transcription Factors .....	1
1.2. Ets Proteins in Development.....	5
1.3. The PEA3 Subfamily of Transcription Factors.....	7
1.4. The Role of Ets Family Transcription Factors in Tumor Formation...	9
1.5. The Role of PEA3 in Tumor Formation.....	13
1.6. Neu-Mediated Tumorigenesis.....	14
1.7. Mammary Gland Development.....	16
1.8. Cell Populations in the Mammary Gland.....	18
1.9. Experimental Rationale.....	19

## Chapter 2. Materials and Methods

2.1. Transgenic and ES Cell Derived Mice.....	29
2.2. Collection of Mice.....	29
2.3. Mammary Gland Wholemount Analysis.....	30
2.4. Histology.....	30
2.5. $\beta$ -galactosidase Staining.....	31
2.6. RNA Isolation.....	32

2.7.	Generation of RNase Protection Probes.....	32
2.8.	RNase Protection Analysis.....	33
2.9.	Immunohistochemistry.....	34
2.10.	Genotyping of Animals.....	34
2.11.	Synthesis of Radiolabelled Probes.....	34
2.12.	Southern Blot Analysis.....	35
2.13.	Embryo Collection.....	36

### **Chapter 3. Results**

3.1.	PEA3 Expression in MMTV-Neu Induced Tumors.....	41
3.2.	The Effect of Loss of PEA3 on Tumor Formation.....	47
3.3.	PEA3 in Lung Metastasis.....	57
3.4.	PEA3 in Normal Mammary Gland Development.....	60
3.5.	Decreased Expression of PEA3 Results in a Branching Defect.....	67
3.6.	PEA3 in Ductal Branching During Pregnancy.....	72
3.7.	Analysis of PEA3 Knockout Mice During Lactation.....	75
3.8.	PEA3 <sup>-/-</sup> Mammary Glands During Involution.....	82
3.9.	PEA3 Expression in the Mammary Gland.....	82
3.10.	PEA3 Expression in the Embryonic Mammary Gland.....	87

### **Chapter 4. Discussion**

4.1.	PEA3 is Overexpressed in Adjacent and Tumor Tissues in Mice Expressing Activated Neu.....	95
4.2.	The Role of PEA3 in Tumor Formation.....	97
4.3.	The Role of PEA3 in Metastasis.....	98

4.4.	The Role of PEA3 in Virgin Mammary Gland Development.....	99
4.5.	The Role of PEA3 in Alveolar Development.....	104
4.6.	PEA3 Expression in the Developing Mammary Gland.....	105
4.7.	Functional Redundancy in the PEA3 Sub-Family.....	107
4.8.	Feedback Mechanisms in the PEA3 Sub-Family.....	108
4.9.	Future Directions.....	108
	References.....	111
	Appendix 1.....	131

## LIST OF TABLES

Table 1.1.	Phenotypes of Mice Lacking Specified ets Proteins	22
Table 1.2.	Mammary Phenotypes Resulting from Loss of Function of Specified Genes	26
Table 1.3.	Summary of Transgenic Mice Having Defects in Mammary Gland Development	28

## LIST OF FIGURES

Figure 1.1.	Diagrammatic Representation of Mammary Gland Development	24
Figure 2.1.	Structure of Transgenes expressed in MMTV-Neu mice	38
Figure 2.3.	Structure of Targeted PEA3 Alleles	40
Figure 3.1.	Expression of PEA3 Subfamily Members in NDL2-5 Tissues	44
Figure 3.2.	Quantitation of Expression of PEA3 Subfamily Members in NDL2-5 Tissues	46
Figure 3.3.	Expression of Transgenes in a Mixed Genetic Background	49
Figure 3.4.	Tumor Onset in Mice Expressing Activated Neu	53
Figure 3.5.	Histology From PEA3 $-/-$ and PEA3 $+/+$ Tumors	56
Figure 3.6.	RNase Protection Analysis of ER81 and ERM Expression in PEA3 $+/+$ and $-/-$ Tumors	59
Figure 3.7.	Wholemout Analysis of Two Week Old Virgin Mammary Glands	62
Figure 3.8.	Wholemout Analysis of Mammary Glands During Puberty	64
Figure 3.9.	Number of TEB and Progression Through the Fat Pad of Five and Six Week Old Siblings	66
Figure 3.10.	Wholemout Analysis of Six Month Old Virgin Mammary Glands	69
Figure 3.11.	Wholemout Analysis of PEA3-3'UTR Mammary Glands	71
Figure 3.12.	Wholemout Analysis of Mammary Glands During Pregnancy	74
Figure 3.13.	Hematoxylin and Eosin Stained Sections of Mammary Glands During Pregnancy	77

Figure 3.14.	Hematoxylin and Eosin Stained Sections of Mammary Glands During Lactation	79
Figure 3.15.	PCNA Immunostaining of PEA3 <sup>-/-</sup> Lactating Mammary Glands	81
Figure 3.16.	Hematoxylin and Eosin Stained Sections of Mammary Glands at Three Days of Involution	84
Figure 3.17.	Hematoxylin and Eosin Stained Sections of Mammary Glands at Ten Days of Involution	86
Figure 3.18.	$\beta$ -galactosidase Staining in the Virgin Mammary Gland of PEA3-EX6-NL LacZ mice	89
Figure 3.19.	$\beta$ -galactosidase Staining in the Mammary Glands of Pregnant PEA3-EX6-NL LacZ mice	91
Figure 3.20.	$\beta$ -galactosidase Staining in the Embryonic Mammary Glands of PEA3-EX6-NL LacZ mice	94

## LIST OF ABBREVIATIONS

DCIS	Ductal Carcinoma in situ
EGFR	Epidermal Growth Factor Receptor
ERK	Extracellular Regulated Kinase
Her-2	Human EGF Receptor 2
HGF	Hepatocyte Growth Factor
IRES	Internal Ribosome Entry Site
K14	Keratin 14
MAPK	Mitogen Activated Protein Kinase
MMTV LTR	Mouse Mammary Tumor Virus Long Terminal Repeat
NDF	Neu Differentiation Factor
NDL	Neu Deletion
PCNA	Proliferating Cell Nuclear Antigen
PEA3	Polyomavirus Enhancer Activator 3
PR	Progesterone Receptor
RT-PCR	Reverse Transcriptase PCR
SRF	Serum Response Factor
TEB	Terminal End Bud
WAP	Whey Acidic Protein



# Chapter 1

## Introduction

### 1.1. The ets Family of Transcription Factors

The ets family of transcription factors is comprised of a number of proteins containing a conserved 85 amino acid (aa) DNA binding domain, termed the ets domain (reviewed in Janknecht and Nordheim, 1993). The first ets protein identified, v-ets, was found as part of a fusion protein, along with gag and myb, in the oncogenic E26 (E-Twenty Six) retrovirus (Nunn et al., 1983). This retrovirus produces myeloid and erythroid leukemias in chicken mediated by this fusion protein (Metz et al., 1991). Subsequently, greater than 30 genes sharing homology in the DNA binding domain have been identified in wide ranging species including *C. elegans*, *Drosophila* and human. Members of the Ets family bind to DNA sequences having purine rich cores (GGAA/T) with additional specificity being conferred by surrounding DNA sequences (Karim et al., 1990). Once bound to DNA, Ets family proteins can activate or repress transcription. Ets proteins are grouped into sub-families based on homology within the ets domain (Reviewed in Wasylyk et al., 1993).

Ets DNA binding sites have been identified in numerous transcriptional promoters and enhancers. Some of these sites are promiscuous and capable of binding multiple ets proteins. The polyoma enhancer, for example, is capable of binding PEA3, Ets-1 and Ets-2. Similarly, Erg3, PU.1 and Fli are capable of binding a single site in the Immunoglobulin Heavy Chain enhancer (Rivera et al.,

1993). Although not all ets binding sites are capable of binding multiple Ets proteins, additional specificity is conferred by other mechanisms including tissue specific expression of Ets proteins, post-translational regulation of these proteins and interaction or cooperation with other factors.

Ets proteins can bind DNA as monomers, but most have weak transactivation domains (reviewed in Crepieux et al., 1994). To enhance their transcriptional abilities, many ets family members have been shown to cooperate with other transcription factors. This co-operation can occur through physical interaction or functional cooperation. Physical interactions have been described for a number of Ets proteins. The ets proteins, Elk-1 and Sap1 have been reported to interact with serum response factor (SRF) to activate the serum response element in the c-fos promoter (Shore et al., 1994). These proteins, called Ternary Complex Factors (TCFs) contain an N-terminal ets domain, a B-box, which mediates interaction with SRF, and a C-terminal activation domain phosphorylated by MAPKs (Reviewed in Treisman et al., 1994). Phosphorylation of Elk-1 following ras activation has been shown to increase the ability of this complex to form and to activate transcription (Gille et al., 1995). ERG acts synergistically with the transcription factor, AP-1 to activate transcription from the collagenase 1 promoter. AP-1 is a heterodimeric complex of fos and Jun that acts as a transcriptional activator. ERG interacts physically with AP-1, and although the nature of this interaction is unknown, activation of this promoter by ERG requires AP-1 DNA binding (Buttice et al., 1996). Similarly, ERM acts synergistically with AP-1 to activate transcription. ERM has been shown to physically interact with the AP-1 component, Jun (Nakae et al., 1995).

Protein-protein interactions have been shown to permit or enhance DNA binding of Ets proteins. This binding can increase their affinity for DNA, or block inhibitory domains that interfere with DNA binding. For example, ets-1 and ets-2 contain auto-inhibitory domains that impair DNA binding. Ets-1 physically interacts with AML through this domain to transactivate the T cell receptor (TCR)  $\beta$  chain promoter (Kim et al., 1999). Through this interaction, inhibition of DNA binding is relieved. Ets-1 DNA binding is also increased through its interaction with Pax-2 or Pax-5 on the B cell specific mb-1 promoter or with CBF on the TCR  $\beta$  chain promoter (Fitzsimmons et al., 1996; Wheat et al., 1999; Wotton et al., 1994). Although the nature of these interactions is not known, it seems likely that they also relieve inhibition of DNA binding through interaction with this domain.

In addition to being regulated by DNA binding, some ets proteins can regulate DNA binding of other factors. The Ets protein GABP $\alpha$  forms a tetrameric complex with GABP $\beta$ , this interaction both increases DNA binding of GABP $\alpha$ , and allows GABP $\beta$  interaction with DNA (Thompson et al., 1991). The action of PU.1 on the Immunoglobulin  $\kappa$  3' enhancer is another example of this function. Binding of the transcription factor NF-EM5 to this enhancer requires interaction with PU.1 (Pongubala et al., 1992).

Interaction with other proteins may act to negatively regulate Ets function. Negative regulation of the Ets protein TEL has been reported by interaction with the ubiquitin-conjugating enzyme UBC9 (Chakrabarti et al., 1999). Interaction with UBC9 does not force Tel degradation but does block transcriptional repression by Tel (Chakrabarti et al., 1999). Similarly, interaction of Elf-1 with Rb inhibits Elf-1 activation of the GM-CSF promoter (Wang et al., 1993). This interaction is dependent on the phosphorylation status of Rb. Elf-1 binds

specifically to the underphosphorylated form of Rb and is released following phosphorylation of Rb.

Activity of Ets proteins is highly dependent on promoter context. Synergism has been observed between Ets proteins and other transcription factors for which a physical interaction has not been observed. Ets-1, Elf-1, and PEA3 have been shown to cooperate with AP-1 in order to activate transcription from specific promoters (Nerlov et al., 1992, Wang et al., 1994, Higashino et al., 1995). Ets-1 has also been shown to act synergistically with SP1 to activate transcription from the human T cell lymphotropic virus type I LTR and from the PTHrP promoter (Gegonne et al., 1993; Dittmer et al., 1997). PEA3 has recently been shown to act synergistically with  $\beta$ -catenin and LEF-1 to activate transcription from the matrilysin promoter. ERG and Fli-1 can cooperate with the helix loop helix (HLH) protein E12, to activate transcription of the Immunoglobulin heavy chain promoter (Rivera et al., 1993).

Most Ets proteins act as either activators or repressors, although both functions have been reported for ets-1, PU.1, Fli-1 and Tel (Gold berg et al., 1994, Borrás et al., 1995, Kelmsz et al., 1990, Tamir et al., 1999). Repression by Ets proteins may act by competing with activators for binding sites, by interaction with activation domains or through active repression. ERF is the only ets protein reported to have an active repression domain (Sgouras et al., 1995). The drosophila protein Yan represses Pointed target genes, likely by competing for the same binding sites. Fli-1 is reported to repress Rb expression, although a competing activator has not been identified (Tamir et al., 1999). PU.1 is reported to mediate repression of GATA-1 transcriptional activity through direct interaction with GATA-1 at the enhancer site (Rekhtman et al., 1999). Ets-1 has been

reported to repress AP-1 driven transcription from the polyoma virus enhancer (Goldberg et al., 1994).

A number of ets proteins are regulated by post-translational modification. The Elk1/SAP1/Net subfamily, Ets1, Ets-2, ERF and the drosophila pointed P2 and Yan are regulated through phosphorylation following activation of ras (Reviewed in Wasylyk et al., 1998). Ets-1, Ets-2, Elk-1 and pointed P2 are positively regulated by ras while the repressive function of ERF and Yan is inhibited by phosphorylation (O'Neill et al., 1994, Sgouras et al., 1995).

## 1.2. Ets Proteins in Development

The expression of ets genes has been reported during a number of developmental processes including hematopoiesis, feather development, mammary gland development and oocyte maturation (Scott et al., 1994, Yamamoto et al., 1998, Turque et al., 1997, Chen et al., 1990).

Mutations in ets genes have been described in *C. elegans* and *Drosophila*. In *C. elegans*, Lin-1, which is most similar to the Elk-1/Sap-1/Net subfamily, regulates vulval development (Beitel et al., 1995). Loss of Lin-1 function results in the inappropriate adaptation of vulval fate by vulval precursor cells (Beitel et al., 1995). Normally, three of six vulval precursors cells (VPC) adopt vulval fates. However, in Lin-1 mutants, almost all VPCs adopted vulval fates resulting in the appearance of one to three pseudovulvae.

The drosophila proteins Pointed P2, Pointed P1 and Yan regulate development of the eye. Mutations in the *pointed* gene, which codes for Pointed P2 and Pointed P1, and in the *yan* gene result in abnormal numbers of photoreceptors (O'Neill et al., 1994; Lai et al., 1992). Pointed and Yan work

antagonistically to regulate photoreceptor development. Loss of *yan* results in supernumerary photoreceptors while loss of *pointed* results in a decreased number of photoreceptors.

Mutations in the *pointed* gene also result in defects of the tracheal and central nervous systems (CNS). Tracheal development in *Drosophila* takes place by the migration and fusion of cells to form three levels of branching structures. Loss of *pointed* results in a loss of secondary and tertiary branching of the tracheal system. Central nervous system defects are the result of improper differentiation of glial cells resulting in fusion of anterior and posterior commissures as glial cells fail to migrate to the dorsal side of the CNS (Klambt 1993; Klaes et al., 1994).

Mutations in *Elg*, the *Drosophila* homologue of  $GABP\alpha$ , result in defects in abdominal segmentation and oogenesis (Schultz et al., 1993). Severe mutations in *Elg-1* result in a lethal phenotype. Mutants die late in pupal development and lack certain abdominal segments. Less severe mutations result in female sterility (Shulz et al., 1993). Analysis of mutant eggs revealed that dorsoventral polarity of follicle cells in the egg is disrupted and border cells fail to migrate properly (Gajewski and Schulz, 1995). Border cells are specialized follicle cells that are required for proper formation of the micropyle, the site of sperm entry into the egg. The fertility defect observed is therefore likely the result of the inability of these eggs to be fertilized.

*Ets-2* is required for proper oogenesis in *Xenopus*. Amphibian oocytes can exist for years in the diplotene phase of meiosis. Maturation of the oocytes into mature ovum, requires resumption of meiosis which is characterized by the breakdown of the oocyte nucleus, the germinal vesicle. Expression of *ets-2* anti-

sence mRNA in oocytes blocks germinal vesicle breakdown thereby halting oogenesis (Chen et al., 1990).

Specific mutations in ets genes involved in human congenital abnormalities have not been described. However, duplication or loss of ets containing loci is sometimes a consequence of gross chromosomal rearrangements. In Down's syndrome there is amplification of the ets-2 gene. The skeletal abnormalities observed in Down's syndrome are similar to those observed in a transgenic animal overexpressing ets-2 from the metallothionein promoter (Sumarsono et al., 1996). Loss of function of a number of ets proteins has been accomplished through targeted mutation in mice. The phenotypes of these mice, summarized in table 1.1, underline the role of ets proteins in normal development.

### **1.3. The PEA3 Subfamily of Transcription Factors**

PEA3 (polyomavirus enhancer activator 3) was first identified as a protein binding to the polyomavirus enhancer to activate transcription from this site. It was subsequently isolated from an FM3A cDNA library and shown to be a member of the ets family of transcription factors (Xin et al., 1992). Two additional PEA3 subfamily members, ER81 and ERM, were identified by virtue of their similarity to the Ets family or to PEA3, respectively. ER81 was cloned from a mouse day 9.5 embryonic cDNA library by PCR using degenerate primers for the ets domain (Brown and McKnight, 1992). ERM/ETV5 was isolated from a human testes cDNA library using the PEA3 ets domain as a probe (Monte et al., 1996). These three proteins are reported to be activators of transcription (Chen and Wright, 1991; Chen et al., 1996; DeLaunoit et al., 1998)

PEA3, ERM and ER81 share greater than 95% identity in the DNA binding domain and greater than 50% identity overall at the amino acid level. These three proteins share distinct but overlapping expression patterns in adult animals. ERM is expressed in most adult tissues whereas expression of PEA3 and ER81 is more restricted. PEA3 is most highly expressed in the brain and the epididymis and ER81 expression is observed in the heart, kidney, brain, lung and colon (Xin et al., 1992; Brown and McKnight, 1992; Monte et al., 1996). PEA3 and ER81 are expressed in motor neuron pools and subsets of muscle sensory afferents (Lin et al., 1998). The expression pattern of these proteins in motor pools matches the expression pattern in the sensory afferent from the same muscle. Motor neuron pools and subsets of sensory afferents can therefore be defined by PEA3 and ER81 expression.

PEA3, ERM and ER81 are expressed throughout embryonic development in a number of developing systems including the nervous system, the eye, the heart, the kidney, the mammary gland and the lung (Chotteau-Lelievre et al., 1997). In the mouse, PEA3 family members exhibit unique, but overlapping, expression patterns during development. Embryonic expression patterns for PEA3 and ER81 have also been described in Zebrafish and *Xenopus* respectively (Brown et al., 1998, Chen et al., 1999). In *Xenopus*, ER81 expression was detected in all embryonic stages tested, including the unfertilized egg. As in the mouse, ER81 expression is observed in the fore mid and hind brain, in the lens, the branchial arches, tail tip and pronephros (Chen et al., 1999). Like ER81, PEA3 was detected early in zebrafish embryogenesis, with expression being observed at 4.3 hours. Expression of PEA3 is observed in



developing somites, in the fore, hind and mid brain, in neural crest cells and in neurons (Brown et al., 1998).

The PEA3 subfamily may also play a role in developmental and homeostatic processes in the adult. In mice, all three family members are expressed in the developing mammary gland, with expression being highest during puberty and in early pregnancy (Sheppard and Hassell, unpublished). PEA3 has also been shown to play a role in muscle cell differentiation. In cell culture, overexpression of PEA3 forces differentiation of myogenic precursors (Taylor et al., 1997). Expression of a truncated, inhibitory PEA3 protein encoding only the DNA binding domain, delays this differentiation.

Although PEA3 is expressed throughout embryonic development, PEA3 null animals are viable (Laing et al., in preparation). PEA3 null adult males fail to mate although sperm can fertilize eggs in-vitro. The defect observed likely reflects erectile dysfunction or an inability to ejaculate. Like PEA3, ER81 is expressed throughout embryonic development. Consistent with its role in motor neuron specification, animals deficient in ER81 are severely runted, display neurological defects, and die between four and six weeks of age from unknown causes.

#### **1.4. The Role of Ets Family Transcription Factors in Tumor Formation**

Ets proteins are downstream targets of ras signaling, are sometimes translocated in human cancers and are found in oncogenic viruses. These finding may indicate that they are involved in tumor formation. Consistent with this notion, overexpression of Ets proteins has been reported in a variety of tumors and tumor cell lines. Ets-1 was shown to be overexpressed in pancreatic

and gastric carcinomas (Nakayama et al., 1996; Ito et al., 1998). ESX is overexpressed in human ductal carcinoma in situ (DCIS) also overexpressing the receptor tyrosine kinase Her-2 (Chang et al., 1997). PU.1 is overexpressed in a number of myeloid leukemia cell lines suggesting that its expression may be correlated with transformation or immortalization of myeloid cell types. Ets-2, although not expressed in epithelial tumor cells, has been shown to be overexpressed by fibroblasts adjacent to neoplastic cells (Wernert et al., 1994). Thus, Ets proteins may also be involved in stromal regulation of tumor cells.

Cancer-associated translocations have been reported for a number of ets genes. In mice, mutant forms of fli-1 and spi1/PU.1 resulting from viral DNA integration have been observed in erythroleukemias and erythroid tumors respectively (Ben-David et al., 1991). In human tumors, amplification of ets-1 has been observed in myelodysplastic syndrome, a clonal stem cell disorder characterized by defective haematopoiesis resulting in an increased risk of myeloblastic leukemia (Ohyashiki et al., 1990). Translocations of Fli-1, ETV-1/ER81, ERG and E1A-F with EWS, have been observed in Ewings sarcoma (Delattre et al., 1992; Zucman et al., 1993; Jeon et al., 1995; Urano et al., 1996). These translocations result in the fusion of EWS, a ubiquitously expressed protein, to the DNA binding domains of ets proteins. The exact function of EWS is currently unknown, although it has been demonstrated to bind hsRPB7, a subunit of RNA polymerase II, and EWS-Fli-1 can activate transcription (Petermann et al., 1998).

Cancers are frequently characterized by a loss of cellular proliferative control and a concomitant block in differentiation. Thus, overexpression of ets proteins may contribute to tumor formation by blocking differentiation. Consistent

with this idea, high levels of Fli-1 expression can block Epo mediated differentiation of erythroid cells (Tamir et al., 1999). Similarly, down-regulation of PU-1 is required for terminal differentiation of murine erythroleukemia cells (Rao et al., 1997). Expression of ets-1 may block differentiation of B cells. Terminal differentiation of B cells is reportedly increased in the absence of ets-1 (Bories et al., 1995).

Activating mutations of ras are among the most frequent mutations observed in human cancers. Ras is a membrane localized GTPase which links growth factor signaling with activation of downstream signals. Ets proteins are among these downstream signals and are therefore believed to play a role in ras mediated transformation. Activation of ras ultimately results in activation of MAPK and ERK cascades. These cascades positively regulate the activities of ets-1, ets-2, elk-1 and Sap1. Activation of ras also results in increased transcription from promoters having ras responsive elements (RRE). RREs contain an ets binding site and deletion mutants of PU.1, ets-1 and ets-2, bearing the DNA binding domain alone can repress ras activated transactivation from these elements.

A causative role of ets proteins in cancer formation is suggested by the observation that some ets proteins can transform cells in culture. v-ets, the prototype of the ets family, is expressed as a fusion protein with the oncogenic v-myb and causes transformation in cell culture. Transformation by the cellular ets proteins, ets-1 and ets-2, has been reported in NIH 3T3 cells (Seth and Papas, 1990; Seth et al., 1990). Ets-1 transformed cells have been shown to display anchorage independent growth, colony formation in soft agar and growth in low serum (Seth and Papas, 1990). Furthermore, transplanting of ets-1 transformed

cells results in rapid formation of solid tumors in nude mice (Seth and Papas, 1990). Although *ets-2* is reported to transform in culture, it has also been reported to block transformation mediated by *ras*. Overexpression of full length *ets-2*, of *ets-2* DNA binding domain alone, or of *ets-2* fused to the VP16 activation domain, blocked *ras*-mediated transformation of NIH3T3 cells (Foos et al., 1998).

The most convincing evidence for a causative role for *ets* proteins in tumor formation comes from studies of the erythroid-specific PU.1. In cell culture, overexpression of PU.1 is sufficient for immortalization of erythroblasts (Schuetze et al., 1993). Furthermore, dominant negative PU.1, containing the DNA binding domain alone, is capable of blocking colony formation in soft agar, growth in low serum and tumor formation in nude mice by *K-ras* transformed cells (Wasylyk et al., 1994). Consistent with these observations, transgenic mice overexpressing PU.1 in erythroid cells develop erythroleukemias (Moreau-Gachelin et al., 1996).

Loss of *ets* function may also have a role in tumor formation. TEL is frequently rearranged or deleted in leukemias of myeloid and lymphoid origin (Bishop et al., 1970). Some Tel rearrangements have reported functions. For example, fusion with the PDGF receptor results in dimerization of the receptor and fusion with AML is reported to block transactivation of AML target genes (Reviewed in Dittmer and Nordheim, 1998). In addition to contributing to numerous translocations, TEL is sometimes lost by chromosomal rearrangement, this has led investigators to suggest TEL may act as a repressor and that leukemias may result from deregulation of TEL repressed genes (Wang et al., 1997). ERF, a transcriptional repressor, has been shown to suppress transformation by the *gag-myb-ets* fusion protein (Sgouras et al., 1995). Loss of

ERF expression has not been reported in human tumors. Loss or rearrangement of ELF5 has been reported in a number of carcinoma cell lines (Zhou et al., 1998). Furthermore, a locus containing the ELF5 gene is frequently lost in human tumors. Ets proteins may block tumor formation by inducing apoptosis. Expression of the p42 splice variant of ets-1 results in apoptosis of colon carcinoma cells following the upregulation of ICE/caspase-1 (Huang et al., 1997; Li et al., 1999). The contribution of this splice variant in human tumors is however unknown.

### **1.5. The Role of PEA3 in Tumor formation**

Overexpression of PEA3 has been observed in mouse mammary tumors and lung metastases induced by overexpression of the receptor tyrosine kinase Neu, the rat form of the human Her-2 (Trimble et al., 1993). Examination of PEA3 RNA expression in human cancers revealed that PEA3 was overexpressed in 85% of Her-2 positive tumors (Benz et al., 1996). Furthermore, the PEA3 family of proteins is overexpressed in a number of breast cancer cell lines (Baert, 1997). Overexpression of PEA3 fails to transform NIH 3T3 cells indicating that PEA3 is not sufficient for cell transformation (Crnac MSc. thesis, 1997). The role of PEA3 in tumor formation is currently unknown.

PEA3 is believed to play a role in metastasis. Overexpression of E1A-F in MCF-7 cells, a non-invasive human breast cancer cell line, resulted in invasive behaviour in a matrigel assay system (Kaya et al., 1996). Expression in a mouse fibrosarcoma cell line resulted in increased metastatic potential following injection of these cells into syngeneic mice (Habelhah et al., 1999). E1AF is believed to increase metastatic potential by increasing the expression of matrix degrading

proteins. Transcriptional activity from the promoters of three matrix metalloproteases, stromelysin (MMP-3), collagenase 1 (MMP-1) and gelatinase B (MMP-9), is upregulated in response to E1AF expression in an osteosarcoma cell line (Higashino et al., 1995). Similarly, expression of MT-MMP1 is reported to increase in response to E1A-F in fibrosarcoma cells (Habelhah et al., 1999).

The matrix metalloproteases, potential targets genes of PEA3, have an established role in tumor invasion, angiogenesis and metastasis. The involvement of these proteins in tumor initiation has recently been shown. Overexpression of stromelysin-1 using the whey acidic promoter (WAP) results in the formation of premalignant and malignant lesions in the mammary gland (Sternlicht et al., 1999). Loss of matrilysin expression has also been shown to decrease tumor formation in mice carrying a mutation in the APC gene (Wilson et al., 1997). Loss of either gelatinase B or stromelysin 1 slows the development of squamous cell carcinomas induced by human papilloma virus 16 (Sternlicht et al., 1999). Overexpression of collagenase 1 also resulted in enhanced skin carcinogenesis following DMBA treatment (D'Armiento et al., 1995).

### **1.6. Neu-Mediated Tumorigenesis**

Neu/ErbB2/Her-2 is a receptor tyrosine kinase first identified in rat neuroectodermal tumors initiated by treatment of pregnant rats with ethylnitrosourea (Shin et al., 1981). It is a member of the Epidermal Growth Factor Receptor (EGFR) family, which includes EGFR/ErbB1, ErbB-3 and ErbB4. These receptors are activated by homo and hetero-dimerization following ligand binding. Dimerization of receptors results in activation of kinase activity followed by tyrosine autophosphorylation. Once phosphorylated, these tyrosines serve as

docking sites for Src, Shc, Grb2, PI3' Kinase and PLC $\gamma$  which couple EGFR family members to downstream signal transduction pathways. EGF (Epidermal Growth Factor) and TGF- $\alpha$  (Tumor Growth Factor  $\alpha$ ) are the ligands for the ERGR, neuregulin is the ligand for both ErbB3 and ErbB4. A Ligand for Her2/Neu has not been reported.

Overexpression or amplification of Her2 has been reported in a number of human tumors (Reviewed in Hynes & Stern, 1994). It has also been reported to correlate with poor prognosis in breast cancer. Overexpression of Neu in breast cancers is most often observed in large cell comedo-type ductal carcinoma in situ (DCIS). DCIS is characterized by hyperplasia of the luminal cells of the mammary duct without invasion through the basement membrane. Comedo-carcinomas are rapidly growing carcinomas often exhibiting necrosis and calcification. DCIS is the most common lesion identified in human breast tissues adjacent to invasive carcinomas and is therefore believed to be a precursor to invasive carcinoma.

In cell culture, overexpression of wild type Neu is sufficient to transform NIH 3T3 cells (Shih et al., 1981). Mice overexpressing the rat Neu driven by the MMTV-LTR develop focal mammary adenocarcinomas which metastasize to the lungs (Guy et al., 1992). These tumors display activating mutations in the Neu transgene (Siegel et al., 1994). Overexpression of these activated forms of Neu result in increased focus formation and decreased rates of tumor formation when compared to wildtype Neu (Siegel et al., 1996). The focal nature of tumors resulting from overexpression of activated Neu demonstrates that Neu-mediated tumor formation is a multistep phenomenon requiring additional mutations for tumor progression. Analysis of chromosomal markers in Neu induced tumors

revealed frequent loss of heterozygosity (LOH) on chromosomes 3 and 4 (Ritland et al., 1997). Furthermore, chromosome 4 was frequently lost. These chromosomes may harbour tumor suppressor genes whose loss of function is another vital step in tumor formation.

### **1.7. Mammary Gland Development**

Little development of the mammary gland occurs before birth. In the day 10-11 embryo, the mammary gland is present as a single layer of ectoderm called the mammary streak (Sakakura, 1991). This streak extends from the anterior limb bud to the posterior limb bud. Between days 11 to 16 of gestation, there is slow growth as the mammary streak thickens in five areas to form five distinct mammary buds. The mammary line between these areas slowly disappears as cells migrate to one of the early mammary buds. These buds grow into bulb-shaped structures and grow toward the embryonic fat pad.

Sexual determination of the mammary gland begins between day 13 and 14 of gestation. In male mice, the mammary bud will become surrounded by the dense mesenchyme and decrease in volume in response to androgens. The epithelial stalk that connects the mammary gland to the epidermis subsequently becomes narrow and ruptures following the condensation of the dense mesenchyme. Growth of the mammary tree is halted at this point.

The mammary fat pad first appears at embryonic day 14 as undifferentiated tissue below the mammary rudiment which differentiates before birth. In female mice, the growing epithelium moves into the fat pad at embryonic day 18. Before birth, canalization of the ducts occur as cells differentiate into myoepithelial and luminal epithelial cells. The myoepithelial cells, also called the



basal layer, are in contact with the basement membrane, while luminal epithelial cells line the ductal lumen.

At birth, animals have a rudimentary branching structure which grows slowly in the first three weeks. When puberty begins, between 3 and 6 weeks of age, terminal end buds (TEB) appear and mammary development is accelerated. Terminal end buds are large, multicellular structures which mediate branching during puberty. When mammary ducts reach the edge of the fat pad at eight to ten weeks of age, the terminal end buds regress and the period of accelerated growth ends. Spurts of growth occur in the adult mammary gland during estrous. This contributes to the increased branching seen in older animals. Mammary gland development is summarized in Figure 1.1.

During pregnancy, the mammary gland prepares itself for lactation. The first few days of pregnancy are accompanied by increased branching. Development of alveolar structures begins during mid-pregnancy and continues until parturition. Production of milk proteins,  $\beta$ -casein and WAP, begins in mid-pregnancy. Milk droplets are retained in alveolar cells during pregnancy and are released into the lumen during lactation. Alveoli are composed of alveolar cells, that synthesize milk and myoepithelial cells and form a basket like structure around the alveolar cells and function to squeeze milk into the ducts.

Following lactation, there is a restructuring of the gland to a virgin-like state, this restructuring process is called involution. Once suckling stops, the build up of milk in the ducts causes a decrease in prolactin levels which initiates involution of the gland (Traver et al., 1996). First, milk is evacuated from the ducts by myoepithelial cells which contract in response to oxytocin to squeeze milk out of the ducts. Milk synthesizing cells, which are no longer required

undergo apoptosis within the first few days. Endothelial cells also undergo apoptosis as the capillary bed rapidly shrinks during the first week of involution (Walker et al., 1989). The second phase of apoptosis is accompanied by upregulation of matrix metalloproteases that function to restructure the gland (Lund et al., 1996).

Altering expression of many proteins through targeted mutation or through overexpression results in defects of mammary gland development. These phenotypes are summarized in tables 1.2 and 1.3.

### **1.8. Cell Populations in the Mammary Gland**

The mammary gland is composed of a number of specialized cells that interact to ultimately form a lactation competent gland. It has been reported that the entire mammary epithelium can be reconstituted from a single cell (Kordon and Smith, 1998). The mammary gland thus contains a number of multi-potent stem cells. Identification and isolation of this population of cells is of interest to development as well as cancer biology. It is believed that these stem cells may be the cells of origin of mammary carcinoma.

The TEB, present during puberty, is a highly proliferative structure that is believed to contain a stem cell population. These cells, the cap cells, are present at the advancing edge of the TEB. Following puberty, the TEBs regress, but the mammary gland retains a stem cell population. This stem cell population has not been unambiguously identified. Until recently, the stem cell population was believed to be within the myoepithelial layer. However, recent studies by Pechoux and others (1999) have shown that at least a subset of luminal epithelial

cells are capable of differentiating into myoepithelial cells, suggesting that the luminal cells may contain the precursors of myoepithelial cells.

A number of subpopulations of luminal and myoepithelial cells have been identified based on morphology and expression of cellular markers. One of these populations may represent the stem cell population. EM techniques have been used to analyze cell morphology and ultrastructure. Cells at the advancing edge of lateral buds are morphologically similar to cap cells (Ormerod and Rudland, 1984). Russo and others (1983) analyzed epithelial cell types in normal mammary gland and DMBA induced tumors, they identified three types of luminal cells: light, intermediate and dark. The intermediate cells represent a small percentage of the cells in the normal duct, but represent 90 % of tumor cells. They also represented greater than 75% of the neoplastic population. Rama 25, a cell line which may represent a stem cell like population, is morphologically equivalent to an intermediate cell.

### **1.9. Experimental Rationale**

Breast cancer results from mutations that alter or disrupt the normal function of proteins. In mice, PEA3 is overexpressed in mammary adenocarcinomas induced by overexpression of Neu (Trimble et al., 1993). It is also expressed in 85% of Her-2 positive tumors. PEA3 is expressed throughout mammary gland development during periods of increased proliferation. These observations suggest that PEA3 is involved in normal mammary gland development and neoplasia. To explore these possibilities, the consequences of loss of PEA3 expression on mammary gland development and tumor formation were examined.

Mice expressing Neu from the MMTV promoter were crossed with mice deficient in PEA3, in order to generate MMTV-Neu mice with and without PEA3. These mice were examined for onset of tumor formation and occurrence of lung metastasis. Expression of ERM and ER81 was examined in tumors of PEA3<sup>+/+</sup> and PEA3<sup>-/-</sup> animals in order to determine whether loss of PEA3 expression affected expression of the other subfamily members.

Development of the mammary gland in PEA3 deficient animals was analyzed during puberty, pregnancy, lactation and involution. Wholemound and histological analyses were used to determine whether the structure of the mammary gland was disturbed. In order to further understand the role of PEA3 in mammary gland development, the expression of PEA3 in the mammary gland was examined using mice carrying a  $\beta$ -galactosidase marker driven by the PEA3 promoter. Cell type expression was determined during puberty and during pregnancy.

Table 1.2. Phenotypes of mice lacking specified Ets proteins. \*Ets 1<sup>-/-</sup> ES cells were injected into Rag 2<sup>-/-</sup> blastocysts. Mature B and T cells cannot develop in the absence of RAG 2, all B and T cell lineages are therefore derived from Ets 1<sup>-/-</sup> ES cells. \*\* Ets 1<sup>-/-</sup> ES cells were injected into wild type blastocysts to produce chimeric mice, thereby rescuing the embryonic lethality.

Ets Gene Knockout	Phenotype	Reference
Tel	Defective yolk sac angiogenesis. Apoptosis of mesenchymal and neural cells	Wang et al., 1997
Ets-1	Skeletal abnormalities	Sumarsono et al., 1996
RAG 2 <sup>-/-</sup> Ets-1 <sup>-/-</sup> Complementation*	Decreased number of mature thymocytes and peripheral T cells, T cell proliferation defect	Muthusamy et al., 1995
Fli-1	Defect in thymus development, reduced number of lymphocytes	Melet et al., 1996
PU.1	Embryonic Lethality. Loss of lymphocyte and monocyte precursor cells	Scott et al., 1994
SpiB	Abnormal B cell function, increased B cell apoptosis, abnormal T cell antigenic responses	Su et al., 1997
Ets-2	Defective trophoblast, early embryonic lethal	Yamamoto et al., 1998
Chimeric rescue Ets-2**	Abnormal Hair follicles	Yamamoto et al., 1998
PEA3	Males do not produce offspring, sperm is fertile	Laing et al., submitted
ER81	Neurologic Defect, mice die 4-6 weeks of age	Arbre et al., unpublished

Figure 1.1. Diagrammatic representation of mammary gland development. Progression of growth through the fat pad is shown in the virgin between two and twelve weeks of age. Diagrams represent the number four inguinal mammary gland. Alveolar development is shown during pregnancy and lactation. The appearance of ducts following involution is also shown.



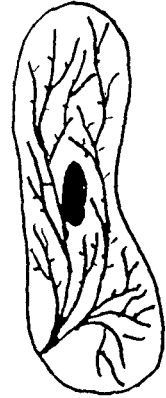
2 Weeks



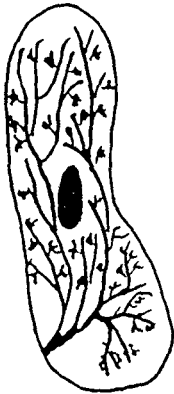
5 Weeks



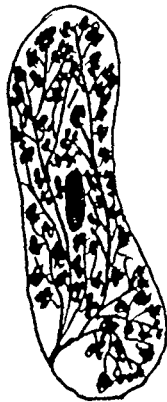
8 Weeks



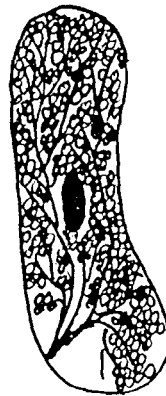
12 Weeks



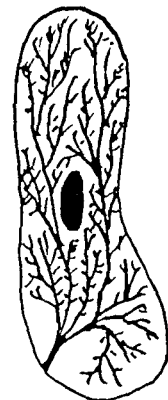
4d Pregnant



18d Pregnant



Lactating



Involuted



Table 1.2. Mammary phenotypes resulting from loss of function of specified genes. NA= Not applicable, NR= Not reported. \*PTHrP  $-/-$  lethality was rescued with a transgene expressing PTHrP in chondrocytes. \* \*Naturally occurring mutants, not generated by targeted recombination. \*\*\* Mammary targeted mutant derived by crossing Floxed BRCA1 mice and WAP-Cre mice. References: (1) Robinson et al., 1998. (2) Ormandy et al., 1997. (3) Lubahn et al., 1993. (4) Lydon et al., 1995. (5) Xu et al., 1999. (6) Wysolmerski et al., 1998. (7) Radice et al., 1997. (8) Fisher et al., 1998. (9) Pollard et al., 1994. (10) Horseman et al., 1997. (11) Liu et al., 1997. (12) Wagner et al., 1997. (13) Labosky et al., 1997. (14) Chen et al., 1999. (15) Toscani et al., 1997. (16) Fowler et al., 1995. (17) Zhao et al., 1999. (18) van Genderen et al., 1994. (19) Fantl et al., 1995.

Gene	Virgin	Pregnant	Lactating
C/EBP $\beta$ (1)	Reduced branching, enlarged ducts	Reduced alveolar development, limited lateral branching	Little $\beta$ -casein, no WAP detected, mice unable to nurse
Inhibin $\beta$ B (2)	Retarded growth through the fat pad, disorganized end buds	Reduced alveolar development, abnormal end buds persist	Mice unable to nurse
Estrogen Receptor (3)	Loss of TEBs	Can't sustain pregnancy	NA
Progesterone Receptor (4)	Reduced Branching	Can't sustain pregnancy	NA
BRCA1 (5)	Reduced branching	Decreased alveolar development	Mice able to nurse
PTHrP* (6)	No mammary epithelium.	NA	NA
P-Cadherin (7)	Alveolar development, mammary hyperplasia	NR	Mice able to nurse
Aromatase cytochrome p450 (cyp19) (8)	Mammary glands are pre-pubertal in appearance	Can't sustain pregnancy	NA
CSF-1** (9)	Reduced branching	Alveoli formed	Cells fail to differentiate into lactation competent cells
Prolactin (10)	Normal	No alveolar development	Mice unable to nurse
Stat5a (11)	Normal	Small alveoli, small alveolar lumen	Reduced milk production
Oxytocin (12)	Normal	Normal	Milk not ejected, premature involution
Myf3 (13)	Normal	Normal	Milk not ejected, premature involution
Hoxa9, Hoxb9, Hoxd9 (14)	Normal	Reduced growth, lobuloalveolar development restricted to ends of bud	Abnormal alveolar morphology, mice unable to nurse
A-myb (15)	Normal	Reduced alveolar development	Mice unable to nurse
EGF** (waved-2) (16)	NR	NR	Impaired lactation
Relaxin (17)	NR	NR	Milk not ejected, mice unable to nurse
LEF-1 (18)	Lack mammary glands	NA	NA
Cyclin D1 (19)	Normal	No functional alveoli	Mice unable to nurse

Table 1.3. Summary of transgenic mice having defects in mammary gland development. MMTV= Mouse mammary tumor virus LTR, WAP = Whey Acidic Protein, K14= Keratin 14, BLG= $\beta$ -lactoglobulin. NR=Not reported. References: (1) Pierce et al., 1993. (2) Sympson et al., 1994. (3) Krane and Leder, 1996. (4) Matsui et al., 1990. (5) Gorska et al., 1998. (6) Li et al., 1994. (7) Jhappan et al., 1992. (8) Hadsell et al., 1996. (9) Humphreys et al., 1996, Jager et al., 1997. (10) Li et al., 1996 (11), Jhappa et al., 1993. (12) Lundgren et al., 1997. (13) Shyamala et al., 1998. (14) Wysolmerski et al., 1995.

Transgene	Virgin	Pregnant	Lactating/ Involuting
MMTV-TGF $\beta$ (1)	Reduced branching	Normal	Normal
MMTV-stromelysin (2)	Increased branching, alveolar development	Reduced WAP and $\beta$ -casein expression	Small alveoli, small alveolar lumen
MMTV-NDF (3)	Persistence of TEBs, adenocarcinomas	NR	NR
MMTV-TGF $\alpha$ (4)	Alveolar development, hyperplasia	NR	NR
MMTV Dominant negative TGF $\beta$ type II receptor (5)	Alveolar development, hyperplasia	NR	NR
WAP-p53(172A-L) (6)	Some animals have non-uniform ductal diameter	Reduced lobuloalveolar development	Reduced milk production
MMTV-int-3 (7)	No TEBs, incomplete ductal penetration into FP	Incomplete lobuloalveolar development	Mice unable to nurse
WAP-IGF-1 (8)	NR	NR	Delay in involution, decreased apoptosis
WAP-Bcl-2 (9)	Disrupted organization of TEBs	NR	Apoptosis inhibited during involution
WAP-SV40Tag (10)	NR	Apoptosis of alveolar cells	No milk produced
WAP-TGF $\beta$ 1 (11)	NR	No lobuloalveolar development	Mice unable to nurse
BLG-MDM2 (12)	NR	Fewer alveolar structures, abnormal ductal and alveolar cells	Mice unable to nurse
GAL4 PR-A x CMVGAL4 (13)	Increased lateral branching, ductal hyperplasia	NR	NR
K14-PTHrP (14)	Reduced branching	Defect in induction of terminal ducts	Reduced secretory alveoli

## **CHAPTER 2**

### **MATERIALS AND METHODS**

#### **2.1. Transgenic and ES Cell Derived Mice**

MMTV-Neu (N202) and MMTV activated Neu (NDL1-2, NDL1-4 and NDL2-5) mice were provided by William Muller, McMaster University (Guy et al., 1992, Siegel et al., 1999). These mice were generated in the FVB mouse strain. These transgenes are shown in Figure 2.1. PEA3 knockout mice expressing a nuclear localized  $\beta$ -galactosidase (EX6-NL-LacZ) and PEA3 knock-in mice, with a Tau-LacZ fusion inserted into the PEA3 3' UTR (3'UTR) were provided by Tom Jessell, Columbia University, New York, New York. These mice were generated in the C57/Sv129 mouse strain. PEA3 knockout mice (DD1 mice) were generated as described (Laing et al., submitted). These mice were generated in a BalbC/Sv129 background. The structure of the targeted alleles is shown in Figure 2.2 .

#### **2.2. Collection of Mice**

Mice were mated after 8 weeks of age. Mice used for pregnant time points were timed based on the presence of vaginal plugs. Mice were then examined when collected for the presence of embryos. Lactating glands were collected 14 days postpartum, one to two hours after separation from the pups to allow glands to accumulate milk. For involuting mammary glands, mice were allowed to lactate for 14 days and were then separated from their pups for 3 or

10 days. Mice were genotyped by Southern blot and euthanized by CO<sub>2</sub> asphyxiation.

Mice used to generate tumor curves were palpated weekly, tumor onset was recorded as the number of days after which a tumor was first palpable. When possible, animals were kept for two months after detection of a tumor, after which time mice were sacrificed and lungs were visually examined for the presence of lung metastasis.

### **2.3. Mammary Gland Wholemount Analysis**

Inguinal (#4) mammary glands were isolated, spread onto glass slides and dried. The following day, fat was removed by incubation overnight in acetone. Tissues were then stained overnight in Harris' modified hematoxylin (Fisher Scientific). Excess stain was removed by washing in three changes of 1% HCl in 70% ethanol until the mammary tree was readily visible against the fat pad. The stain was then fixed in 0.02% ammonium hydroxide for one minute. Glands were dehydrated first in 70% ethanol and then in 100% ethanol each for several hours. Tissues were cleared in 100% xylenes overnight. Glands were subsequently mounted with Permount (Fisher Scientific) and photographed.

### **2.4. Histology**

Tissue samples were fixed overnight at 4°C in 4% paraformaldehyde (BDH Laboratory supplies) and transferred to 70% ethanol for embedding. Further processing and embedding into paraffin wax was carried out by anatomical pathology, McMaster Hospital. Sections for hematoxylin and eosin

staining were sectioned at 4  $\mu\text{m}$  and stained by anatomical pathology, McMaster Hospital.

## 2.5. $\beta$ -galactosidase Staining

Mammary glands and embryos for  $\beta$ -galactosidase staining were fixed, at room temperature, in 2% paraformaldehyde, 0.25% glutaraldehyde for one hour. Following fixation, tissues were washed twice for 30 minutes in 0.01% Na desoxycholate, 0.2% NP40 (Sigma), 2mM  $\text{MgCl}_2$  in 0.05M  $\text{Na}_2\text{HPO}_4$  buffer. The glands were then washed twice for 30 minutes in decreased Na-desoxycholate (0.01%). Tissues were incubated overnight at room temperature in X-gal staining solution (2mg/mL X-gal (Gibco-BRL), 30mM  $\text{K}_4\text{Fe}(\text{CN})_6$ , 30 mM  $\text{K}_3\text{Fe}(\text{CN})_6\text{-H}_2\text{O}$ , 2 mM  $\text{MgCl}_2$ , 0.01% Na-desoxycholate, 0.02% NP-40). Tissues were subsequently washed twice for 5 minutes in phosphate buffered saline (PBS). Wildtype glands and embryos were stained to control for endogenous  $\beta$ -galactosidase. No endogenous activity was detected.

Mammary glands to be used for wholemount analysis were defatted for 4 hours in acetone, dehydrated in 70% ethanol, then 100% ethanol, each for several hours and cleared in xylenes for 4 hours. Mammary glands were then mounted with Permount (Fisher Scientific).

Tissues and embryos were embedded in paraffin and sectioned at 8  $\mu\text{m}$ . Sections were dehydrated overnight at 37°C and counterstained with eosin Y (Sigma) using standard procedures (Ausubel et al., 1992).

## 2.6. RNA Isolation

Tissues were isolated and immediately homogenized in guanididium Isothiocyanate containing 0.5% 2-mercaptoethanol. Total RNA was then isolated by CsCl centrifugation (Sambrook, 1989). RNA was resuspended in Diethyl pyrocarbonate (DEPC) (Sigma) treated water and quantitated by OD<sub>260</sub>. Tissues labelled 'adjacent' are those directly adjacent to the tumor, whereas 'contralateral' refers to tumor free mammary glands from from the same animal.

## 2.7. Generation of RNase Protection Probes

In order to synthesize anti-sense RNA, 0.5 µg of linearized plasmid DNA was mixed with 10 mM ATP, GTP, CTP (Gibco, BRL), 1 µL RNA guard (Gibco BRL), 5 µL enzyme buffer, 5 µL [ $\alpha$ -<sup>32</sup>P] UTP (Amersham) and 20 Units (U) RNA polymerase to a final volume of 50 µL. The reaction mix was then incubated for 45 minutes at 37°C. One µl 10 mM unlabelled UTP was then added to complete unfinished product, and incubated for an additional 10 minutes before DNase treatment. In order to digest the template DNA, 2 µL of DNase I (Gibco BRL) and 1 µL 0.5M MgCl<sub>2</sub> were added before incubation for 15 minutes at 37°C. DNase I was removed by phenol-chloroform extraction and riboprobes were precipitated by the addition of 20 µg yeast tRNA, two volumes of 2.5M NH<sub>4</sub>OAc pH 5.2 and 7.5 volumes ethanol. Pellets were dried and resuspended in 50 µL DEPC (sigma) treated H<sub>2</sub>O. Probes were counted in a scintillation counter by the addition of 1 µL of probe to 1 mL scintillation fluid (Beckman).

Riboprobes were generated from linearized plasmids. The PEA3 riboprobe was synthesized using SP6 RNA polymerase to generate a 350 bp anti-sence message corresponding to the 3' end of PEA3. Antisense ERM was



generated using T3 RNA polymerase (Boehringer Mannheim) to synthesize a 280 nucleotide product corresponding to an EcoRI to PstI fragment of the mouse cDNA. The ER81 riboprobe was synthesized using T7 RNA polymerase (Boehringer Mannheim) to generate a 250 nucleotide antisense product corresponding to a BamHI/HindIII fragment of ER81. L32 was synthesized using T3 RNA polymerase to generate a protected fragment of 195 bp.

## **2.8. RNase Protection Analysis**

Anti-sense riboprobes were synthesized as described above. 10 µg of total RNA was mixed with  $1 \times 10^5$  CPM (counts per minute) probe and denatured at 85 °C for 5 minutes. RNA samples were then hybridized overnight at 50°C. Yeast tRNA was hybridized as a negative control. The following day, samples were quick chilled on ice and 15 µg RNase A (Pharmacia) , 0.45 µL RNase T1 (Boehringer Mannheim) was used to digest samples for 30 minutes at 37°C in 300mM NaCl, 10 mM Tris-HCl, 5 mM EDTA pH 7.4. The digestion reaction was stopped by adding 20 µL 10% SDS, and 50 µg Proteinase K (Boehringer Mannheim) per sample and incubated for 20 minutes at 37°C. Proteinase K was removed by phenol-chloroform extraction and RNA samples were precipitated by the addition of 40 µg yeast tRNA and 2 volumes of ethanol. Samples were precipitated at -80°C for 30 minutes and then centrifugated at 13000 rpm in a microcentrifuge for 30 minutes. Pellets were resuspended in 5 µL RNA loading buffer (80% formamide, 1 mM EDTA, .1% xylene cyanol, .1% bromophenol blue) and heated for 5 minutes at 85°C before loading onto a 6% acrylamide gel containing 7M Urea in TBE (Sambrook et al., 1997). Gels were pre-run for 2

hours at 50V and run for approximately 2.5 hours after loading samples. Gels were then dried for 2 hours in a gel dryer and exposed for 1-3 days at -80°C.

## **2.9. Immunohistochemistry**

Tissues were fixed overnight at 4°C in 4% paraformaldehyde and subsequently transferred to 70% ethanol. Immunohistochemistry was performed on paraffin embedded sections using the Mouse on Mouse peroxidase kit (Dyna) as per manufacturers instructions. Anti-proliferating cell nuclear antigen (PCNA) antibody (Dako) was used at a 1/100 dilution and hybridized at room temperature for 1 hour. In order to visualize the peroxidase labelled antibodies, sections were incubated in 3-amino-9-ethyl carbazole (AEC) containing 0.3% H<sub>2</sub>O<sub>2</sub> in acetate buffer (0.05 M acetic acid, 0.5M sodium acetate, pH5.0). Slides were mounted using gellitol mounting media (Zymed).

## **2.10. Genotyping of Animals**

Animals were ear tagged and a one centimeter piece of the tail was cut for genotyping. Tail clips was digested overnight at 50°C with 10 µg/mL Proteinase K in 500 µl tail digestion buffer (0.5% SDS, 1 mM Tris, pH 8, 0.1M NaCl, 10 mM EDTA). Undigested tissue was removed by centrifugation for 5 minutes at 13000 rpm. DNA was then precipitated with two volumes of isopropanol and resuspended in 30 µL TE.

## **2.11. Synthesis of Radiolabelled Probes**

Probes were synthesized by the random priming protocol of Feinberg and Vogelstein (1983). DNA templates were excised from the plasmids and

separated on low melting point (LMP) agarose. Reactions were carried out in LMP agarose. Random hexamers were obtained from Boehringer-Mannheim, deoxyribonucleotides from Pharmacia and [ $\alpha$ - $^{32}$ P]dCTP from Amersham. Labelling reactions were done using the Large Fragment of DNA Polymerase I (Gibco-BRL). The template used for PEA3 Southern blots was a Pst/SmaI fragment of the PEA3 cDNA corresponding to the 3' end. The wildtype allele produced a band of approximately 6.3 Kb, while the knockout allele produced a band of 6.8 Kb. To detect the Neu transgene, DNA was digested overnight with 50U BamHI and blots were probed with a probe synthesized from the SV40 polyadenylation cassette. A 1.6 kb fragment results in mice carrying the transgene, no band is present in negative samples.

## 2.12. Southern Blot Analysis

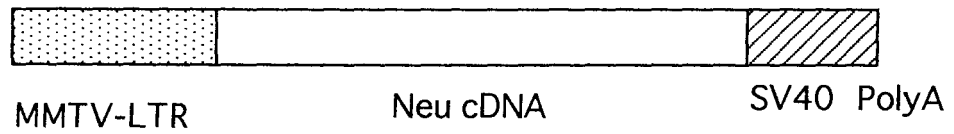
Southern blots were performed by standard procedures (Sanbrook et al., 1989). To detect the Neu transgene, 4  $\mu$ L of DNA was digested overnight with 50 U BamHI (Gibco-BRL). For PEA3 southern blots, 4  $\mu$ L of DNA was digested overnight with 50U EcoRI (Gibco-BRL). DNA was transferred to Gene Screen nylon membranes and fixed to the membrane using the stratagene uv crosslinker. Radiolabelled probes were synthesized as described above and hybridized at 65°C overnight. Membranes were then washed three times for 10 minutes in 2XSSC, 0.5% SDS, and once for 45 minutes in 0.2X SSC 0.5% SDS at 65°C.

### **2.13. Embryo Collection**

Embryos were collected based on the presence of vaginal plugs. Embryos were dissected away from the uterus and extraembryonic tissues and the yolk sac was collected for genotyping by the same method described above for tail clips.

Figure 2.1. Structure of Transgenes expressed in MMTV-Neu mice. A. N202 transgene, expressing the wildtype rat cDNA from the MMTV promoter. B. NDL-1 and NDL-2 transgenes, expressing Neu deletion mutants resulting in activation of the receptor. Deletions in the Neu cDNA are shown compared to the wildtype sequence (top amino acid sequence). SV40 polyA = SV40 polyadenylation signal.

A



B

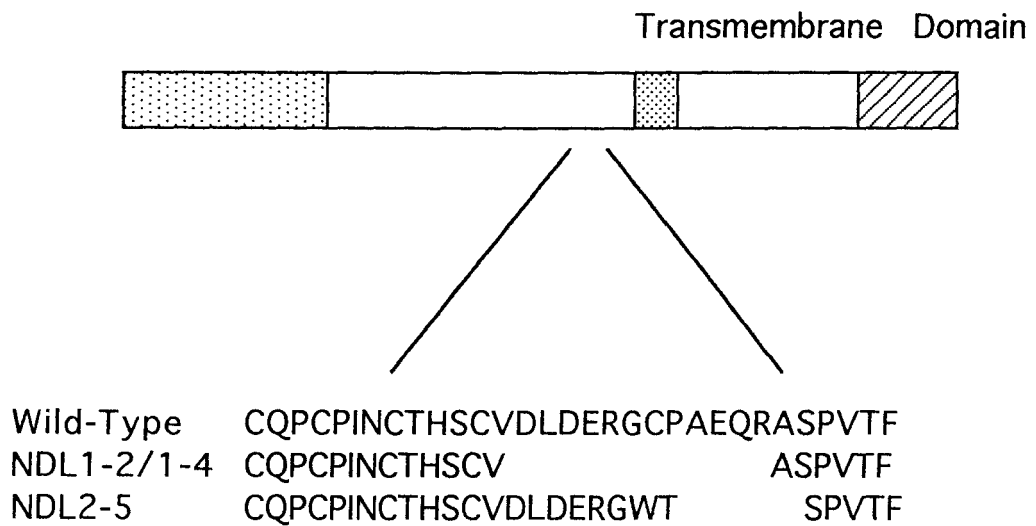
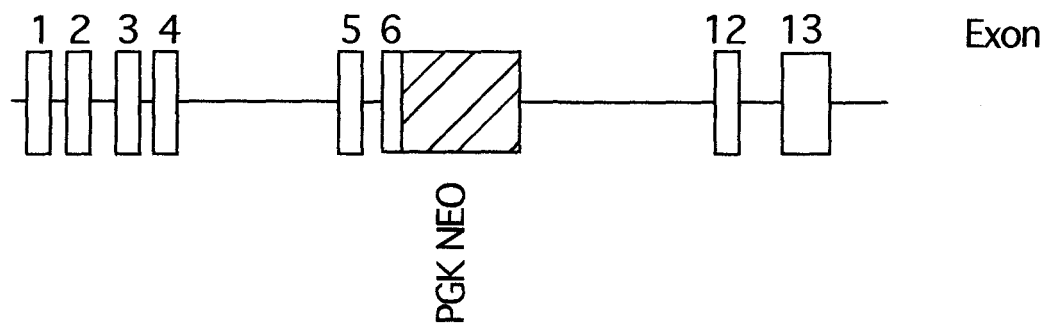
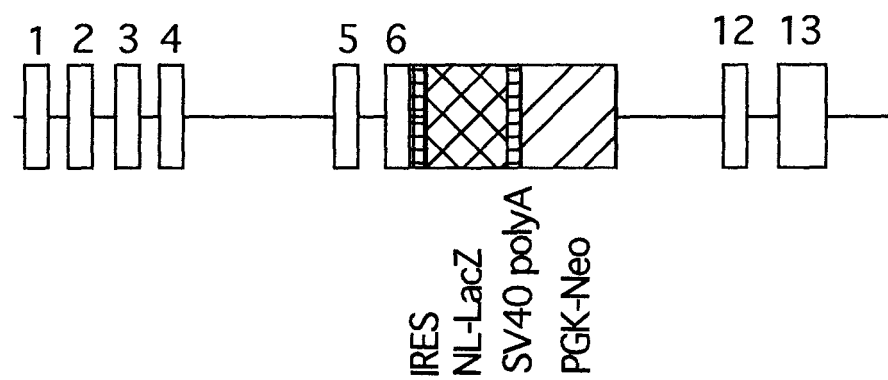


Figure 2.2. Structure of the targeted PEA3 alleles. (A) PEA3 (BalbC/Sv129) targeted allele. A PGK-Neo cassette is inserted into exon 6 of PEA3, removing a portion of exon 6, and the entirety of exons 7 to 11 (B). PEA3-EX6 NL LacZ targeted allele. Targeting construct is inserted in exon 6, as with A. Additionally, the targeting construct includes an IRES-nuclear localized LacZ (NL-LacZ). (C) PEA3 3'UTR targeted allele. Targeting construct is inserted into the 3' untranslated region of PEA3. A Tau- $\beta$ -galactosidase fusion protein is expressed from the PEA3 promoter. IRES=Internal ribosome entry site. PGK-Neo mediates Neomycin resistance.

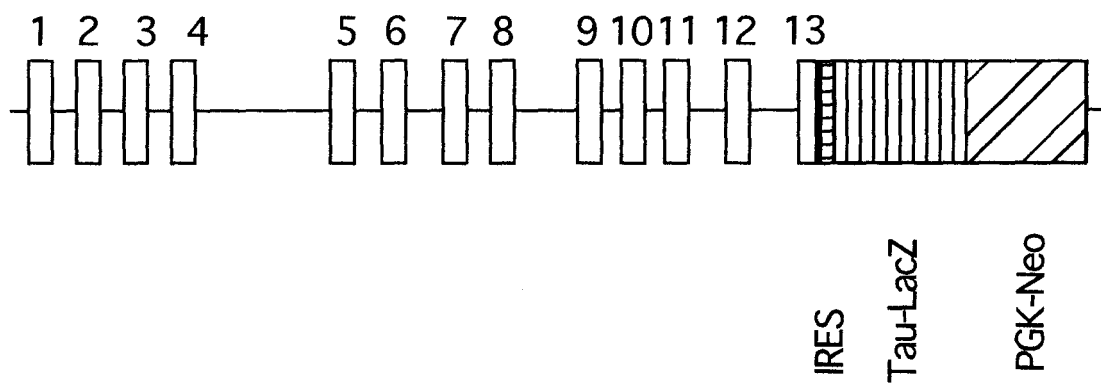
A



B



C





## **CHAPTER 3**

### **RESULTS**

#### **3.1. PEA3 Expression in MMTV-Neu Induced Tumors**

Her-2/Neu is a receptor tyrosine kinase that, when activated, can transform cultured cells. The Her-2 gene is amplified and overexpressed in a significant proportion of human breast tumors (Reviewed in Hynes & Stern, 1994). The expression of Neu has been correlated with poor prognosis in human breast cancer (Slamon et al., 1989). Overexpression of the wildtype Neu in the mouse mammary gland resulted in focal tumors that metastasized to the lung (Guy et al., 1992). Analysis of PEA3 RNA expression in these animals revealed that PEA3 is overexpressed in tumors and lung metastases compared to mammary tissues adjacent to the tumor and to non-transgenic mammary gland, suggesting that PEA3 may play a role in tumorigenesis and metastasis (Trimble et al., 1993).

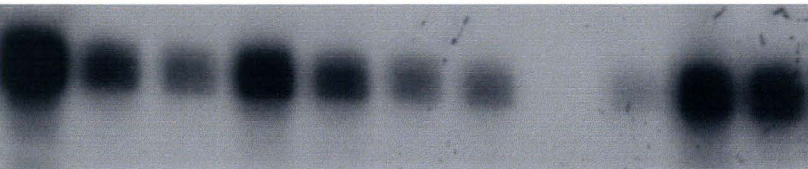
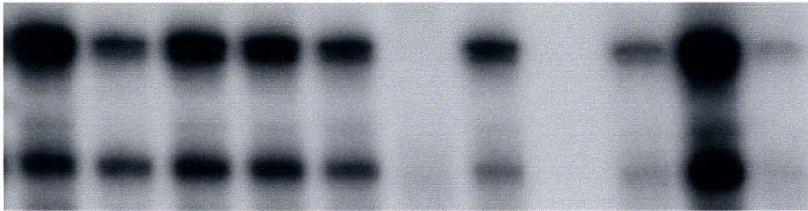
Following the discovery that overexpression of Neu could induce tumor formation, analysis of Neu-induced tumor RNA revealed a number of mutations and deletions in the Neu transgene (Siegel et al., 1996). These mutations, clustered in the extracellular domain, resulted in activation of the receptor as measured by autophosphorylation and dimerization of the Neu receptors. Two of these mutant alleles (NDL alleles) were expressed using the MMTV promoter in transgenic mice and resulted in accelerated tumorigenesis compared to mice expressing the wild type Neu. These mice also displayed obvious hyperplasia in

tumor free glands (Siegel et al., 1999). To further assess their possible role in tumor formation, expression of PEA3 subfamily members was examined in tumor bearing NDL2-5 mice.

For tumor analysis, mice were sacrificed two months after palpation of a tumor or when the tumor size exceeded 10% of the animal's mass, whichever occurred first. Tumors from animals with visible lung metastasis (Lanes 5-10) as well as tumors from animals that did not display metastasis were examined (Lanes 1-4). When possible, mammary tissue immediately adjacent to the tumor was isolated (adjacent tissue). If the adjacent tissue did not yield sufficient RNA for analysis, RNA from a tumor free mammary gland from the same mouse was used (contralateral). RNase protection analysis of PEA3 subfamily expression in tumor and adjacent tissues of mice overexpressing the activated Neu (NDL2-5), revealed that PEA3, ERM and ER81 were overexpressed both in the tumor and in the adjacent mammary gland, compared to virgin mammary gland (Figure 3.1). Expression was quantitated by phosphorimager analysis and normalized to expression of L32, a ribosomal gene. Expression was slightly higher, on average, in adjacent, or contralateral (tumor free gland) tissues compared to tumor tissues (Figure 3.2). Expression of ERM and ER81 was not detected in one of the tumors examined (Figure 3.1, lane 6). The average difference in PEA3 expression between the tumor and adjacent tissues was a three fold decrease in expression in the tumor tissues. The difference observed may be increased if adjusted for epithelial content. Since the majority of cells in these tumors are epithelial, epithelial content would be much higher than in adjacent tissues which still contain a great deal of stroma. This suggests that expression of the PEA3 subfamily is induced by activation of Neu and that this induction is

Figure 3.1. RNase Protection assay showing expression of PEA3 subfamily members, PEA3, ER81 and ERM, in NDL2-5 tumor and adjacent tissues. L32, a ribosomal protein gene, was used to control for loading. 10  $\mu$ g of total RNA was used. ADJ=Adjacent, TUM=Tumor, Con=Contralateral, VMG=8 wk virgin mammary gland.

ADJ  
TUM  
CON  
TUM  
ADJ  
TUM  
ADJ  
TUM  
ADJ  
TUM  
VMG



1 2 3 4 5 6 7 8 9 10 11

PEA3

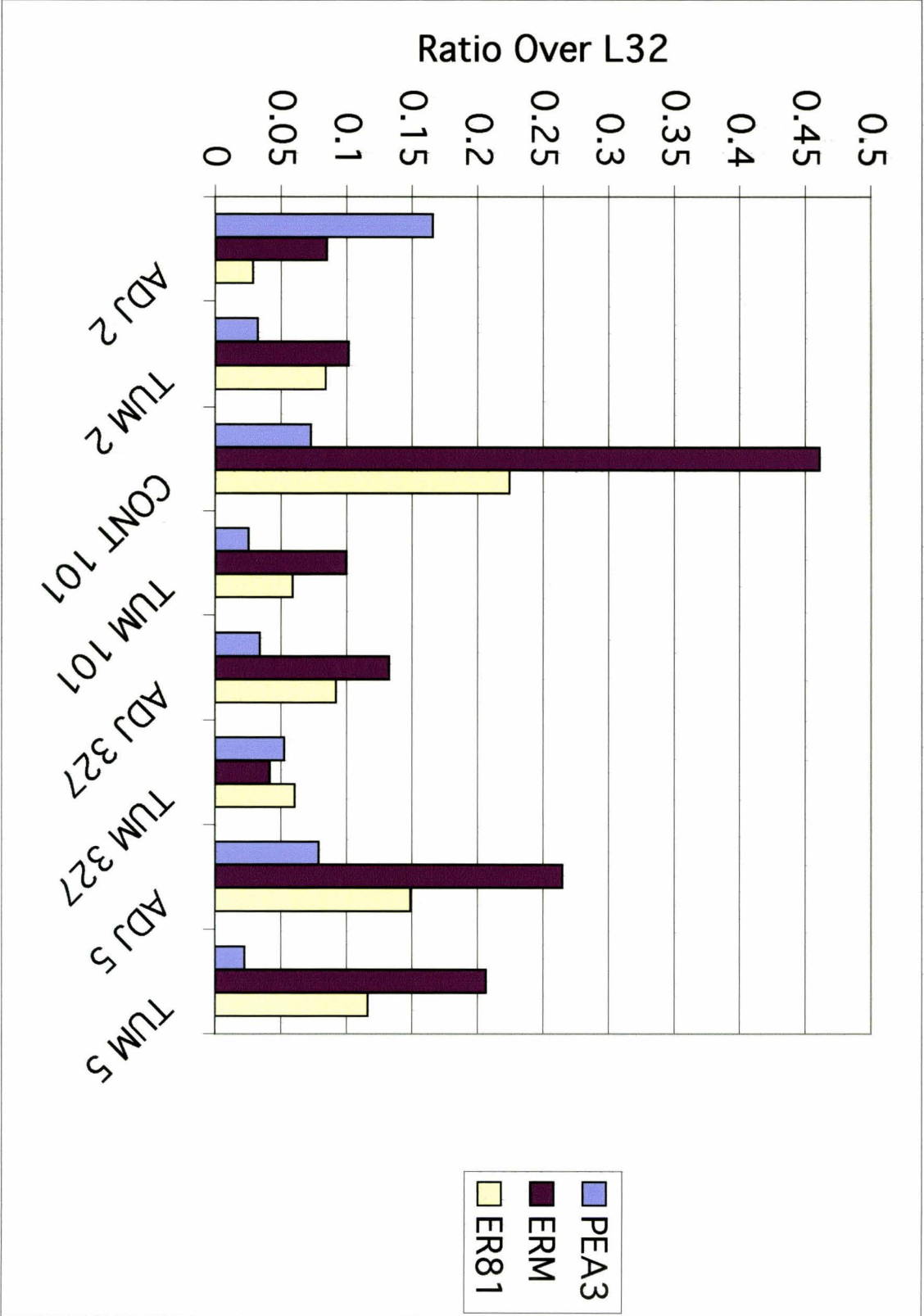
ERM

ER81

L32

Lane

Figure 3.2. Quantitation of PEA3 subfamily expression in NDL2-5 tumors. RNase protection shown in Figure 3.1 was quantitated by phosphorimager analysis. Levels of ERM, PEA3 and ER81 are expressed as a ratio over L32 expression. Lanes 7 and 8 (from figure 3.2) were not included, due to the absence of RNA in lane 8. ADJ= Adjacent; TUM=Tumor, CONT=Contralateral. Numbers represent ear tag numbers of the animals used.



reduced as tumorigenesis progresses.

### **3.2. The Effect of Loss of PEA3 on Tumor Formation**

The correlation between PEA3 overexpression and Neu activation suggests that PEA3 may play a role in Neu induced tumorigenesis. To test this possibility, PEA3 null animals (BalbC/Sv129) were crossed with three lines of mice expressing activated forms of Neu (NDL1-2, NDL1-4, NDL2-5) and one line of mice expressing normal Neu (N202) under the MMTV promoter. Because transgene integration is random, the position of the inserted transgene in the genome often affects transgene expression. This occurs presumably through methylation of some chromosomal regions. The pattern of methylation differs between strains and therefore expression or lack of expression of transgenes can be strain specific (Weng et al., 1995; Allen et al., 1990). In order to verify that the transgenes were expressed in this mixed genetic background (BalbC/Sv129/FVB), mammary glands were isolated from the F1 generation of mice, and assayed for transgene expression by RNase protection. N202 and NDL2-5 transgenes were expressed in the mixed genetic background while NDL1-4 and NDL1-2 were not (Figure 3.3).

To analyze tumor progression in the presence and absence of PEA3, PEA3 knockout animals were bred with mice carrying the MMTV-N202 and MMTV NDL2-5 transgenes. F1 generation animals were crossed to generate animals carrying the Neu transgenes in the presence and absence of PEA3. This was done in order to generate PEA3  $-/-$  and  $+/+$  animals from the same parents. This analysis of siblings was done to minimize the effects of strain specific modifier genes. Modifier genes have been reported to affect tumor

Figure 3.3. Neu Transgene expression in the F1 generation of mice (BalbC/Sv129/FVB) resulting from crosses of PEA3  $-/-$  (BalbC/Sv129) animals with transgenic animals of the NDL1-2, NDL1-4, NDL2-5 and N202 lines (FVB). Expression of transgenes was assayed at 12 weeks of age from virgin mammary gland. A riboprobe representing the SV40 poly adenylation cassette was used to detect transgene expression, PGK was used to control for total RNA content.



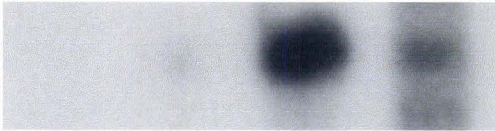
NDL1-2

NDL1-4

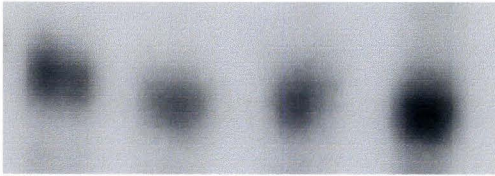
NDL2-5

N202

Transgene



SV40 polyA



PGK

formation in different mouse strains. Tumor onset in MMTV-N202 transgenic animals has been analyzed in FVB and C57/B6 strains of mice (Rowse et al., 1998). C57/B6 animals displayed decreased rates of tumor formation compared to FVB animals, independent of transgene levels. Tumor initiation in both N202 and NDL2-5 animals was slowed in the mixed genetic background compared to FVB transgenics. This study concluded that approximately three independent modifier genes were involved in tumor latency in the C57/B6 background.

Tumor onset occurred more slowly in the N202 and NDL2-5 mice of the mixed genetic background (BalbC/Sv129/FVB) than in the FVB strain. While the average tumor onset in FVB N202 mice was approximately seven months (Guy et al., 1992), the average tumor onset of PEA3 +/+ N202 mice in the mixed genetic background was approximately 13 months. Similarly, tumor onset in the NDL2-5 line was approximately five to six months in the FVB strain (Siegel et al., 1999), and ten months in the mixed background (BalbC/Sv129/FVB). The decreased onset of tumor formation observed was likely due to the effect of modifier genes. Transgene levels in these two genetic backgrounds were not compared, differences in transgene levels, therefore, cannot be discounted.

Data obtained for tumor onset in the N202 mice was complicated by the fact that many N202 (BalbC/Sv129/FVB) mice died before tumors occurred. A number of N202 animals from the mixed background had to be euthanized due to the presence of uterine and ovarian tumors (n=3; 1-/-, 1+/, 1+/-). These tumors were not observed in N202 females of the FVB strain. Additional animals were sacrificed due to wasting of the hind limb muscles and partial hindlimb paralysis which occurred with age (n=4; 2+/-, 2+/+). A further set of mice (n=6; 3-/-, 1+/, 2+/-) were found dead. Some of these animals may have died through

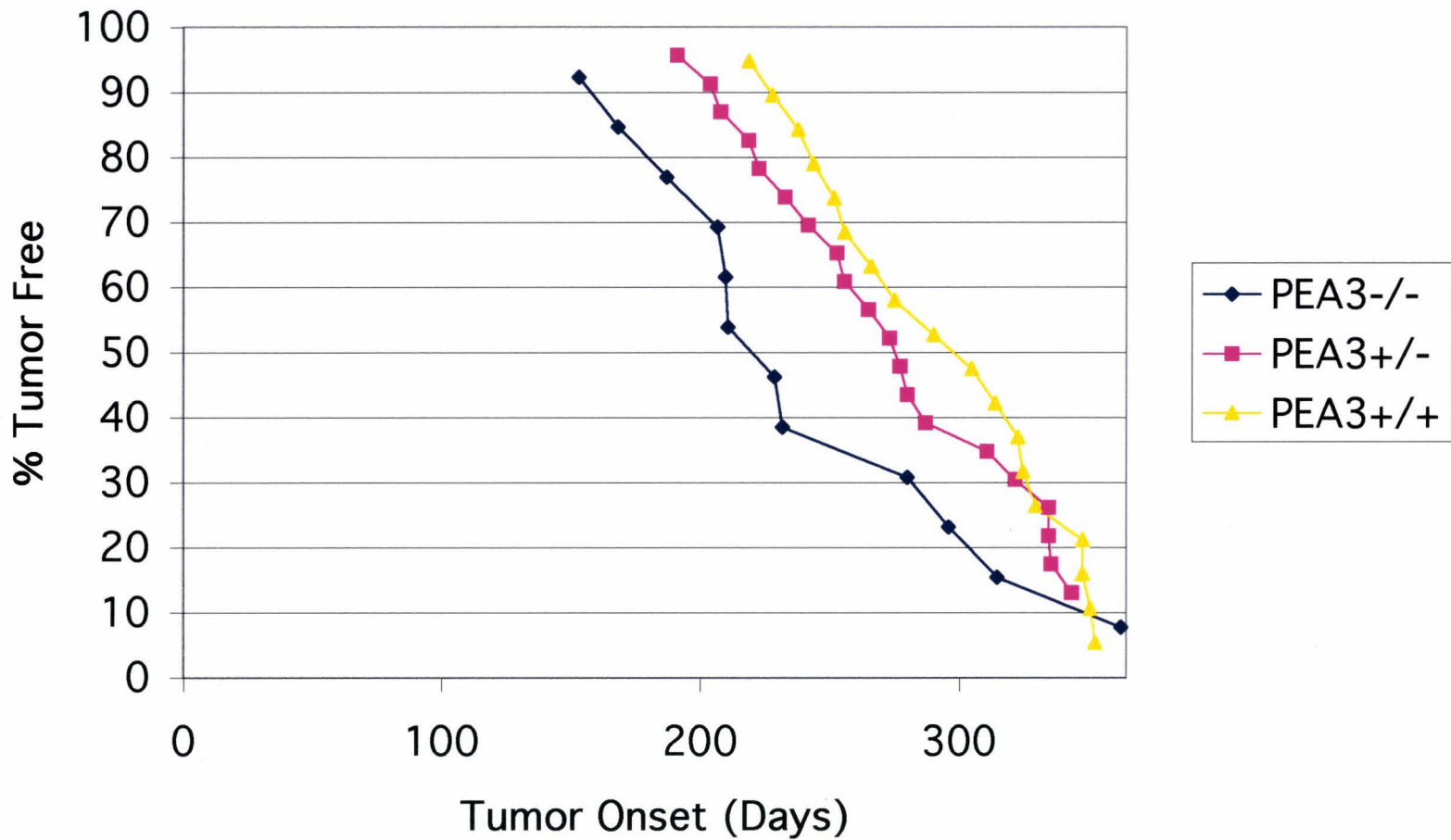
complications associated with uterine or ovarian tumors, however, tissue degradation did not permit autopsies. Interestingly, one animal (PEA3<sup>+/-</sup>) having a uterine tumor displayed metastasis to the liver. In mice that survived and developed mammary tumors, the average time of tumor onset was 361 days (n=8) for knockout females, 391 days for heterozygotes (n=6) and 370 days for wildtype females (n=13). The range of tumor onset between mice was very large in the mixed background (BalbC/Sv129/FVB). Tumors occurred between 249 and 550 days for the knockout animals, between 315 and 461 days for the heterozygotes and between 306 and 537 days for the wild type animals. The reported tumor onset in the FVB N202 mice was between 150 and 300 days, however, analysis was not continued beyond 300 days, at which time, 25% of the animals did not display tumors (Guy et al., 1992). Extending this analysis over a longer time period would likely increase this range.

Mice of the MMTV-NDL2-5 line (BalbC/Sv129/FVB) were palpated weekly for mammary tumors over a one year period. During this time, ovarian and uterine tumors were not observed and very few animals died before tumor onset. This data is represented in Figure 3.4 as the percent animals tumor free over time. 50% of the NDL2-5 animals were tumor bearing at 230 days for the <sup>-/-</sup> animals, 270 days for the <sup>+/-</sup> animals and 300 days for the <sup>+/+</sup> animals. Only four animals in this study did not display palpable tumors after 365 days, one <sup>+/+</sup>, one <sup>-/-</sup> and two <sup>+/-</sup> animals. At seven months, 40% (6/13) of the PEA3 <sup>-/-</sup> animals displayed palpable tumors, while only 6% (1/18) of the wild type animals had tumors at this time point. Tumor onset in the NDL2-5 mice (BalbC/Sv129/FVB) ranged from 158 to 363 days for the PEA3 <sup>-/-</sup> animals, 191 to 344 days for PEA3 <sup>+/-</sup> animals and 219 to 353 days for the PEA3 <sup>+/+</sup> animals. The range of tumor

Figure 3.4. Onset of mammary tumors in mice expressing activated Neu (NDL2-5). Tumor onset represents the number of days at which a tumor was first palpated. Data is expressed as the percent of animals tumor free over time.

50% of the animals were tumor free at 230 days for the PEA3  $-/-$  animals, at 270 days for the PEA3  $+/-$  animals and at 300 days for the PEA3  $+/+$  animals. Total number of animals: PEA3  $-/-$   $n=13$ , PEA3  $+/-$   $n=20$ , PEA3  $+/+$   $n=18$ .

# Tumor Onset in NDL2-5 Mice



formation in the NDL2-5 (FVB) animals was not reported (Siegel et al., 1999).

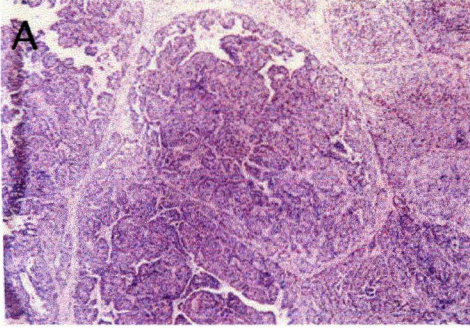
Tumor onset data for the NDL2-5 mice (BalbC/Sv129/FVB) was analyzed using the log-rank test (Shown in Appendix 1) This test is used to compare the occurrence of an event over time between two exposure groups. Here the incident is tumor formation and the exposure groups are plus or minus PEA3. The observed differences in tumor formation between knockout and wild type animals were not statistically significant ( $p < .05$ ), suggesting that the effects observed are minor and that a greater number of animals may be required to achieve statistical significance.

A number of proteins influence tumor morphology. ets-1 expression, for example, has been associated with epithelial to mesenchymal (EMT) transitions. To examine whether the loss of PEA3 affected tumor morphology, histological analysis of NDL2-5 tumors was accomplished. There was no detectable difference in morphology between tumors of PEA3 null and wild type animals (Figure 3.5). Tumors of both genotypes displayed a duct-like organization consistent with mammary adenocarcinoma (Figure 3.5, panels A&B). Tumors displayed areas of dense cell growth where cells were typically large with hyperchromatic nuclei. Areas of necrosis were also observed in both genotypes. This morphology is consistent with large cell comedocarcinomas.

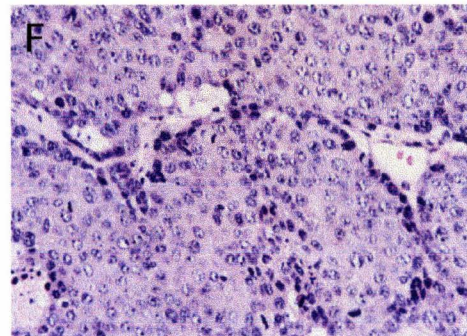
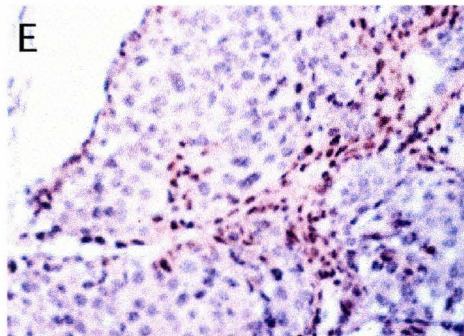
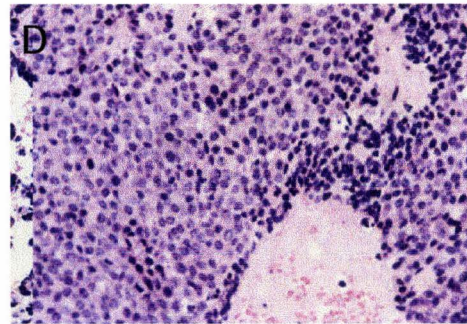
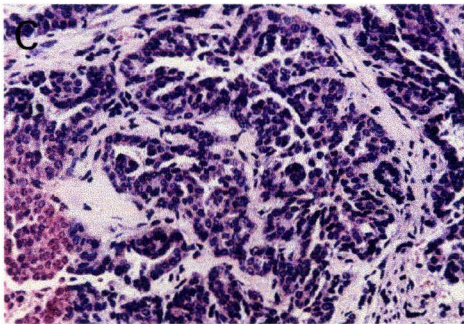
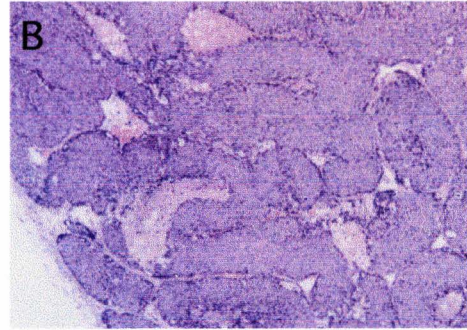
PEA3, ERM and ER81 share greater than 95% homology (amino acid level) in their DNA binding domains. The high homology in this domain suggests that these three proteins are capable of binding to the same target sequences. These proteins may regulate common targets and expression of all three proteins may be commonly regulated. Expression of ERM and ER81 in tumors from PEA3<sup>+/+</sup> and PEA3<sup>-/-</sup> mice was examined in order to determine whether loss of

Figure 3.5. Histology of PEA3 +/+ (A,C,E) and PEA3 -/- (B,D,F) NDL2-5 tumors. Tumors display gland-like organization (A & B), typical of adenocarcinoma. (Original magnification of A & B 50 x). Cells are typically large and tumors of each genotype display areas of dense growth (C&D) as well as areas undergoing cell death (E&F). (Original magnification of C-F 200x)

$+/+$



$-/-$



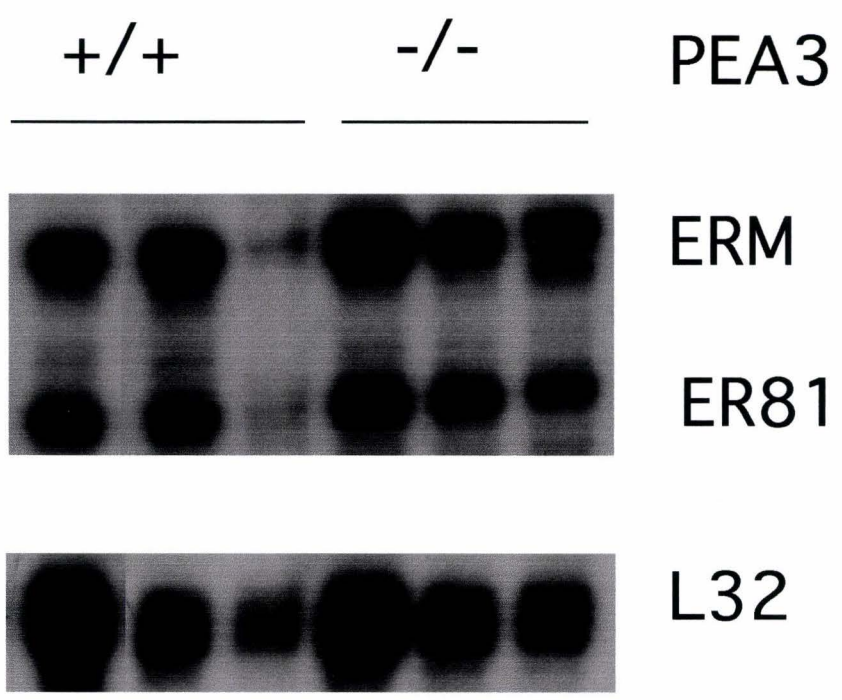


PEA3 expression affected expression of the other subfamily members. Expression of ERM and ER81 was accomplished by RNase protection and quantitated by phosphorimager. Loss of PEA3 did not affect the expression of ERM or ER81 in these tumors (Figure 3.6).

### **3.3. PEA3 in Lung Metastasis**

PEA3 is also overexpressed in lung metastases and has been shown to upregulate expression of matrix metalloproteases in a cell culture system (Trimble et al., 1993; Kaya et al., 1996; Habelhah et al., 1999). Furthermore, overexpression of E1A-F, the human homologue of PEA3, induced invasive behaviour of a non-invasive cell line, MCF-7 (Kaya et al., 1996). These data suggest that PEA3 plays a role in metastasis. To this hypothesis, the occurrence of lung metastasis in PEA3  $-/-$  and PEA3  $+/+$  mice was examined. Following the identification of tumors, mice were kept for two months, unless their tumor masses exceeded 10% of their body weight (approximated by tumor size) at which time mice were euthanized for humane reasons. Eight knockout animals (of 12), eight heterozygote animals (of 18) and nine wildtype animals (of 17) survived two months tumor bearing and were examined for visible lung metastasis. Animals of all three genotypes acquired lung metastases. 63% (5/8) of the knockout animals, 63% of the heterozygotes (5/8) and 44% (4/9) of the wildtype animals displayed visible lung metastasis. This data indicates that PEA3 is not required for metastasis.

Figure 3.6. A. RNase protection analysis of ER81 and ERM expression in PEA3 +/+ and -/- NDL2-5 tumors. Bands representing ERM and ER81 are as indicated. L32 was used as an internal control. (Image was cut to remove lanes)



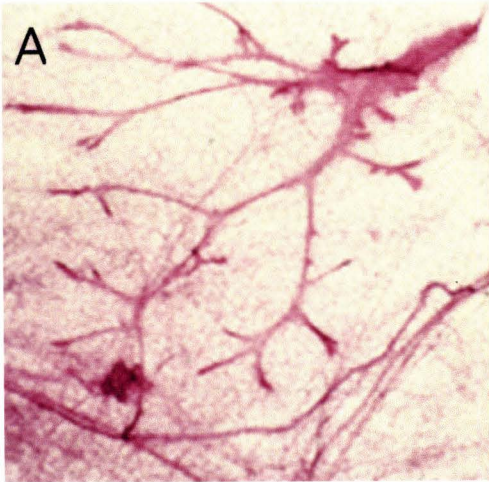
### 3.4. PEA3 in Normal Mammary Gland Development

Many proteins that play a role in mammary tumor formation also play a role in the normal development of the mammary gland. To test the possibility that PEA3 plays a role in normal mammary gland development, whole mount and histological techniques were used to examine the mammary glands of PEA3 knockout females during mammary gland development. Whole mount analysis of PEA3-null (BalbC/Sv129) mammary glands revealed an obvious defect in the normal development of the ductal system. This defect was obvious in mice as early as two weeks of age (Figure 3.7). At this time, mice have not entered puberty and only a rudimentary branching structure is present. The number of branches in this rudimentary tree was reduced in PEA3  $-/-$  animals when compared to wild type animals

Puberty begins in the female mouse between four and five weeks of age. At this time, terminal end buds (TEB) form and growth of the mammary gland is accelerated as ducts proliferate to fill the mammary fat pad. At five weeks of age, there is a reduction in the branching of the primary ducts in PEA3  $-/-$  (BalbC/Sv129) mice as compared to PEA3  $+/+$  mice (Figure 3.8). At eight weeks of age, wild type ducts have reached the edge of the fat pad and regression of terminal end buds occurs. More terminal end buds were obvious in knockout animals than in wild type animals at this time point, suggesting that progression through the duct may be slowed, resulting in delayed regression of TEBs. Analysis of five and six week old virgin females revealed that mammary glands from PEA3 $-/-$  mice were typically delayed in their movement through the fat pad and had fewer terminal end buds compared to wildtype siblings (Figure 3.9). Although this was not always the case, this was the general trend. Individual

Figure 3.7. Wholemout analysis of mammary glands from PEA3 +/+ (A) and PEA3 -/- (B) 2 week old virgin females. Branching in the mammary gland is reduced at two weeks of age in a PEA3 -/- animal compared to a wildtype sibling. (Original Magnification 50x)

$+/+$



$-/-$

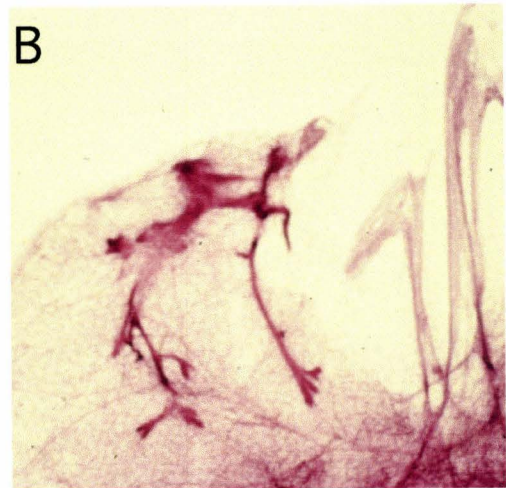


Figure 3.8. Wholemout analysis of mammary glands during puberty from PEA3 +/+ (A,D,G), +/- (B, E, H) and -/- (C,F,I) virgin animals at 5 weeks (A-C), 8 weeks (D-F) and 12 weeks (G-I) of age. Reduced branching in the mammary gland is observed in PEA3 -/- and +/- animals compared to PEA3+/+ animals. (Original Magnification 6.4 x)



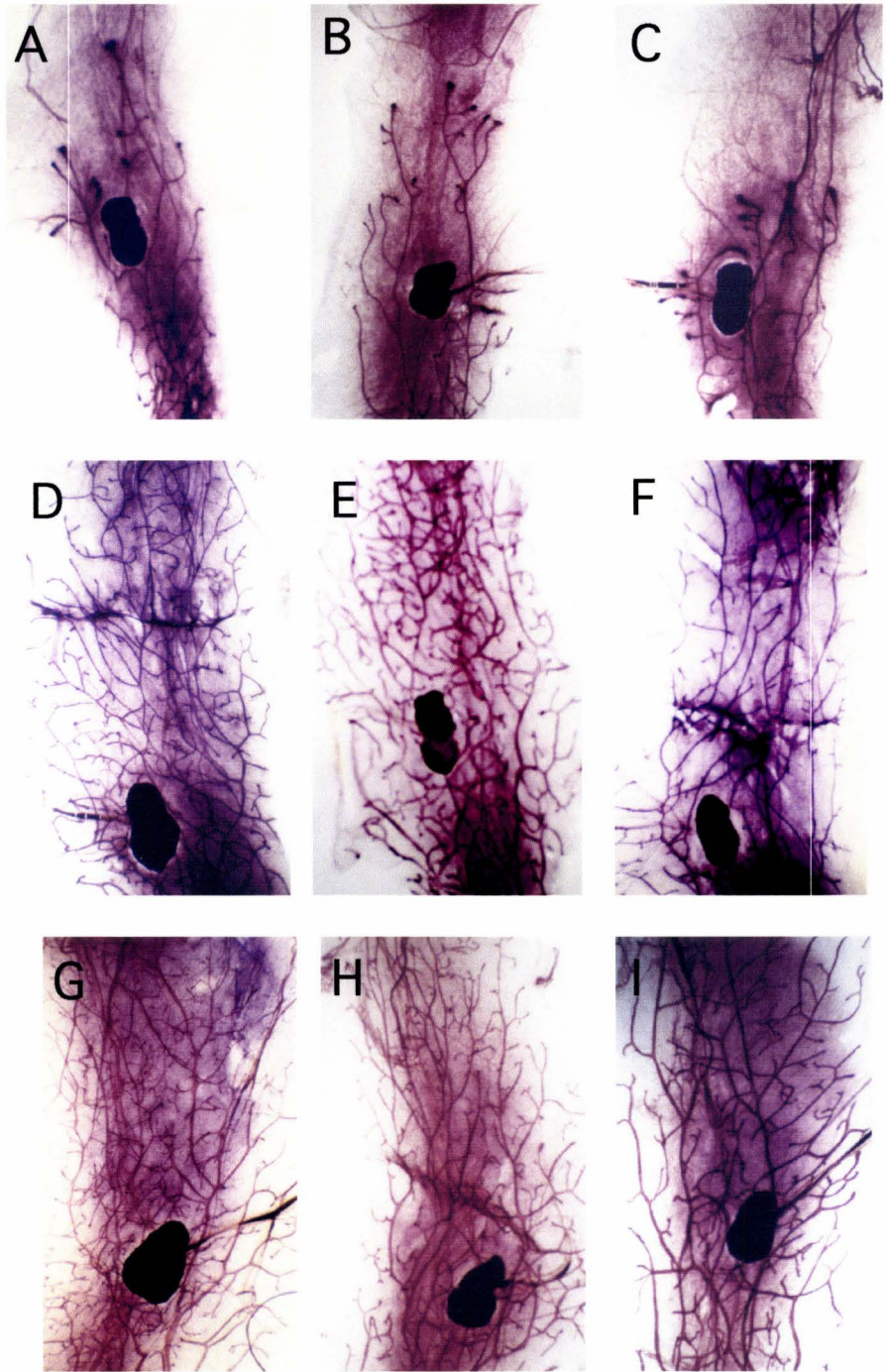


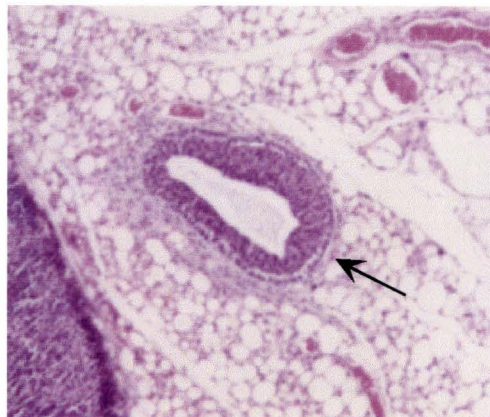


Figure 3.9. A. The number of terminal end buds was counted and the distance branching structures had moved through the mammary fat pad were measured in five sets of siblings between five and six weeks of age. Each box represents one group of siblings, each row one mouse. Data was recorded by visual examination of hematoxylin stained mammary wholemounts. B. Structure of terminal end bud in 5 week old virgin PEA3<sup>-/-</sup> mouse. TEB shows organized cap cell layer (arrow) and discontinuity of fibroblasts at the advancing edge of the terminal end bud. This is consistent with normal TEB morphology (Original Magnification 400x).

A.

Age	Genotype	#Terminal End Buds	Distance Past Lymphnode (mm)
5 Weeks	+/+	25	4
	+/-	19	5
	-/-	11	2
	-/-	14	0
5 Weeks	+/+	24	4
	+/+	9	.1
	+/-	21	4
6 Weeks	+/+	14	3.5
	+/-	13	3
	-/-	17	2.5
6 Weeks	+/+	17	2
	+/-	11	2
6 Weeks	+/+	20	1.25
	+/+	15	2.75
	+/-	4	0
	-/-	14	1.5

B



mice likely differed slightly in the onset of puberty and rate of growth, contributing to differences observed between siblings in the rate of development of the mammary gland.

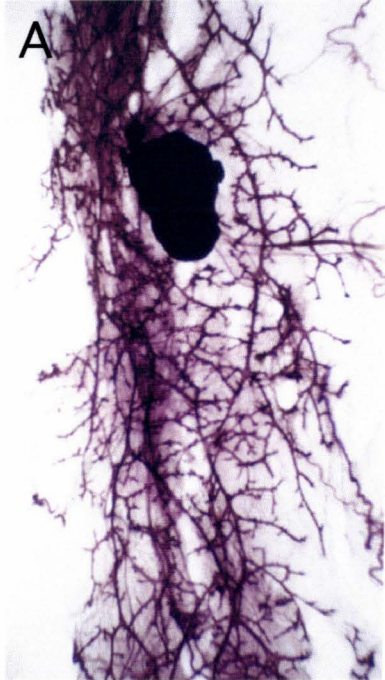
At eight and twelve weeks of age, a marked reduction in secondary and tertiary branching in the virgin animals was observed (Figure 3.8). In 6 month old virgin animals, lateral buds were virtually absent on the mammary ducts of PEA3<sup>-/-</sup> animals, while they were obvious in wild type animals (Figure 3.9). Additionally, a number of post-pubertal PEA3-null virgin animals exhibited widened ducts when compared to wild type controls (4/12 over 8 weeks old). Ducts of PEA3<sup>-/-</sup> virgin animals were often reversed in direction and ducts appeared more tortuous than those of wildtype animals.

### **3.5. Decreased Expression of PEA3 Results in a Branching Defect**

Levels of PEA3 expression appeared to be important for proper development of the mammary gland. PEA3<sup>+/-</sup> females displayed a decrease in branching that was intermediate between wildtype and knockout animals (Figure 3.8). Because these mice carry a targeted mutation in exon seven, a protein encoding exons one to six could still be produced. This protein could potentially act as a dominant negative molecule. However, mice carrying a targeted interruption of the 3' untranslated region, but expressing full length PEA3, also exhibited reduced branching in the mammary gland. Wholemound analysis of mammary glands from animals 8, 12 and 16 weeks of age revealed that these mice displayed a defect in branching morphogenesis similar to that observed in the PEA3<sup>-/-</sup> and <sup>+/-</sup> animals (Figure 3.11). Although PEA3 protein levels in the mammary glands of these mice were not examined, it seems likely that

Figure 3.10. Wholemout analysis of mammary glands from PEA3  $-/-$  (B & D) and PEA3  $+/+$  (A & C) six month old virgin females. Note the loss of lateral branching in the PEA3  $-/-$  animal (D) compared to the wildtype animal (C). A & B original magnification 6.4 x, C & D original magnification 25x.

**+/+**



**-/-**

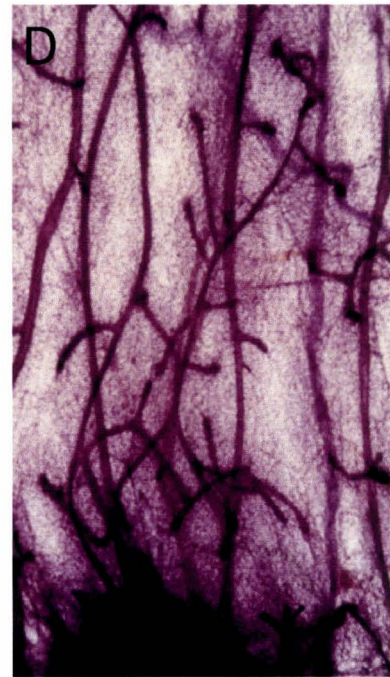
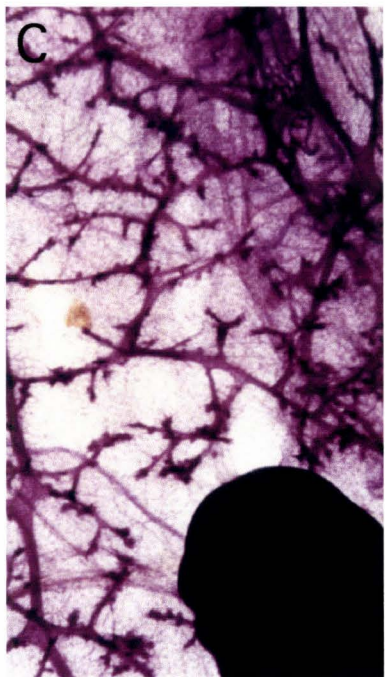
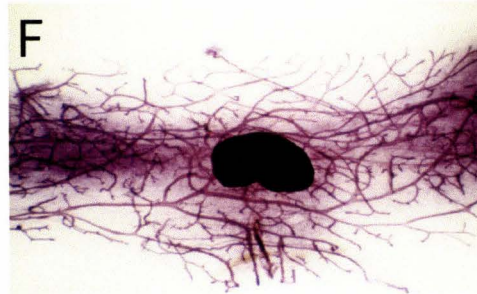
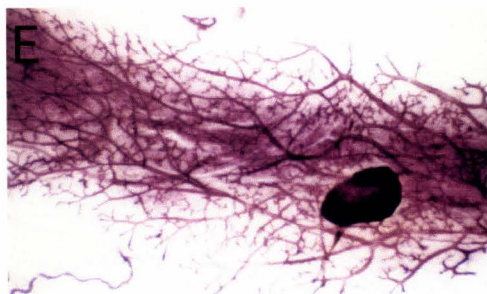
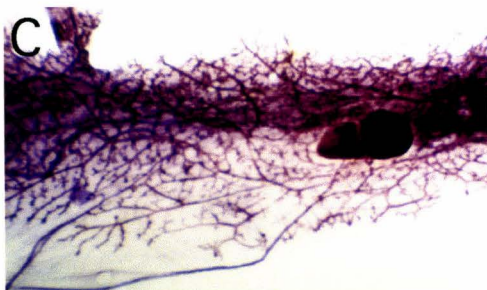
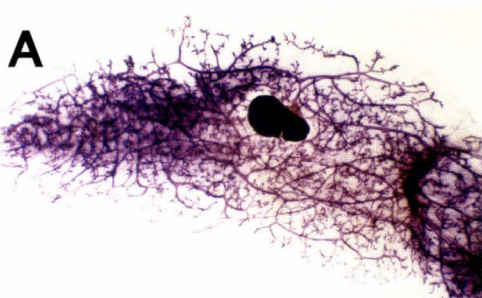


Figure 3.11. Wholemount analysis of PEA3-3'UTR (B,D,F) and C57/Sv129 wildtype mammary glands (A,C,E) at 8 (A&B), 12 (C&D) and 16 (E&F) weeks of age. Branching in the PEA3 3'UTR animals is reduced compared to wildtype animals. (Original magnification 6.4 x)



PEA3 +/+

PEA3-3'UTR



interruption the 3' untranslated region decreased RNA stability resulting in reduced protein levels. The phenotype observed in the PEA3 +/- (BalbC/Sv129) animals suggests that levels of PEA3 expression are important for proper mammary gland development. This is further supported by the finding that 3'UTR mice display reduced branching. Furthermore, the presence of a phenotype in these mice suggests that the phenotype observed in the PEA3-/- and +/- (BalbC/Sv129) mice was not due to a dominant negative effect of the truncated PEA3 protein expressed in these mice.

### **3.6. PEA3 in Ductal Branching During Pregnancy**

During pregnancy, the female mammary gland prepares itself for milk production. The first few days of pregnancy are accompanied by increased proliferation followed by alveolar development during mid-pregnancy. PEA3 -/- females displayed reduced branching during pregnancy compared to wild type animals (Figure 3.12). The degree of branching observed varied greatly in the PEA3 -/- females at 4 days of pregnancy. Some animals showed the appearance of large, bulb-like structures (shown in Figure 3.12 Panel C), others showed a slight increase in branching compared to virgin animals, while others appeared as virgin glands. A noticeable increase in branching was observed at ten days of pregnancy in all PEA3 -/- mice compared to virgin PEA3 -/- animals, although, again, it was not equivalent to that observed in the wild type animals. The phenotype observed in the heterozygous animals at four and ten days pregnant varied between animals. Some animals appeared intermediate in branching while others were indistinguishable from wild type animals.

While fewer alveoli may have been present at ten days of pregnancy, the

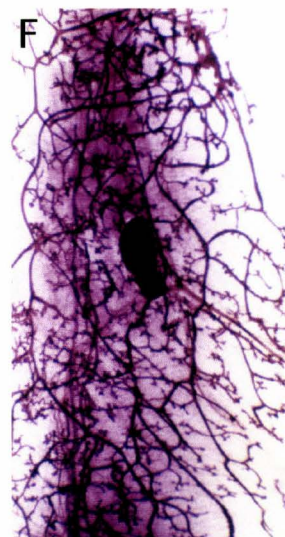
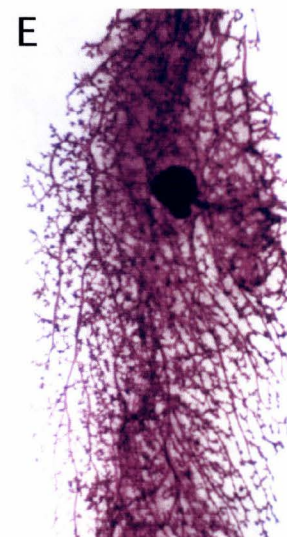
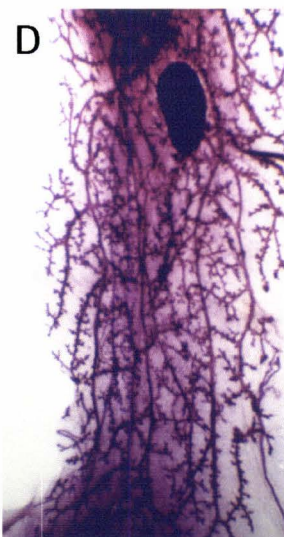
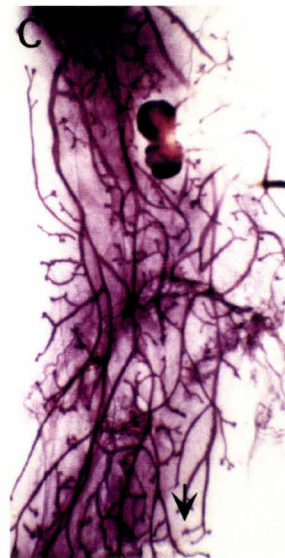
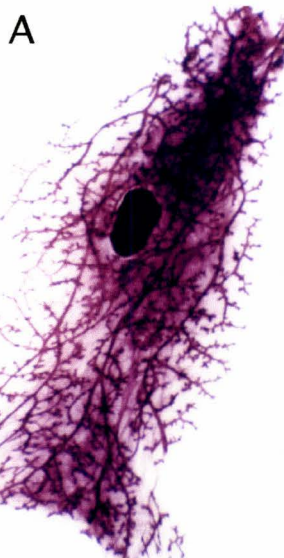


Figure 3.12. Mammary gland development during pregnancy. Wholemout analysis of PEA3 +/+ (A,D), +/- (B,E) and -/- (C,F) mice at 4 (A-C) and 10 days of pregnancy (D-F). Note the bulb-like structures present at 4 days in the PEA3 -/- gland (arrow). Also note the ducts showing reversed direction in this gland. (Original magnification 6.4 x)

+/+

+/-

-/-

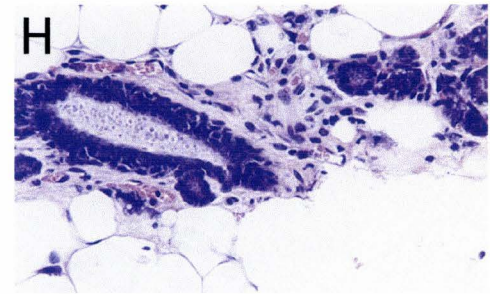
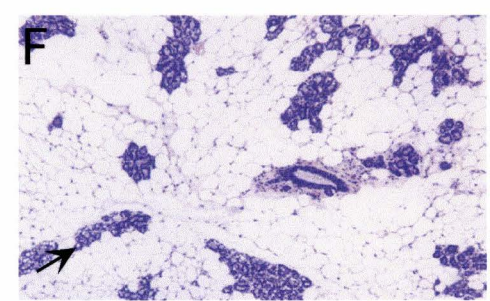
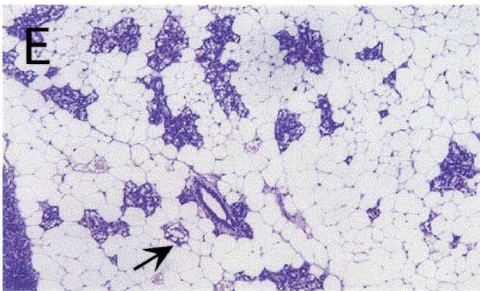
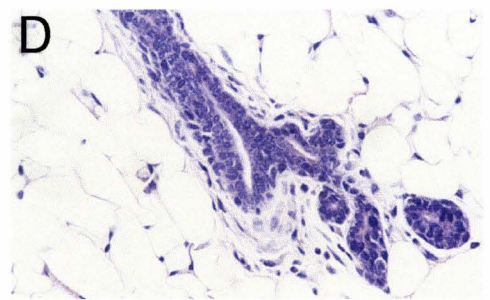
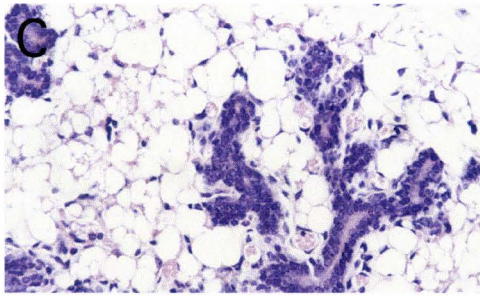
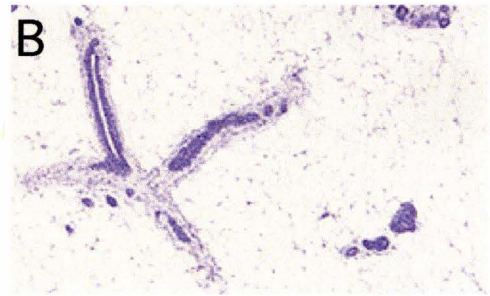
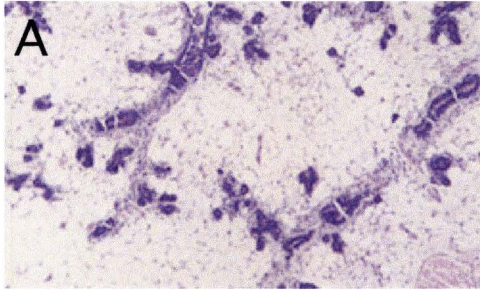


structure of ducts and alveoli in PEA3  $-/-$  animals appeared normal (Figure 3.13). Alveolar development was obvious at ten days, as expected. At 18 days, lipid droplets, indicative of milk production, were obvious in the alveolar cells of both wild type and knockout animals, indicating that glands had developed for lactation.

### **3.7. Analysis of PEA3 Knockout Mice During Lactation**

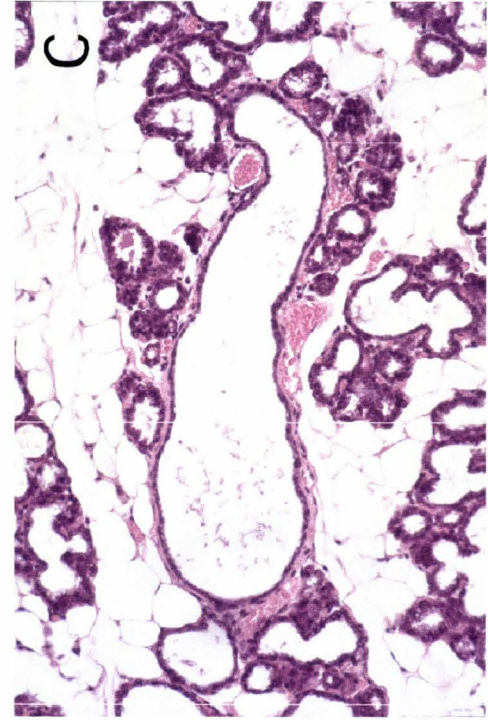
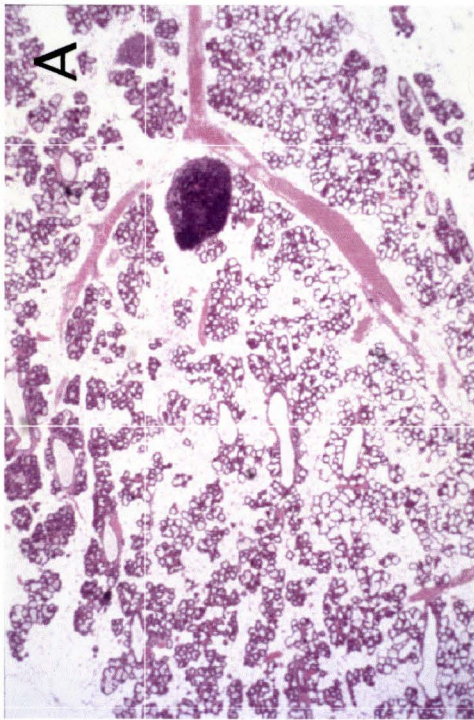
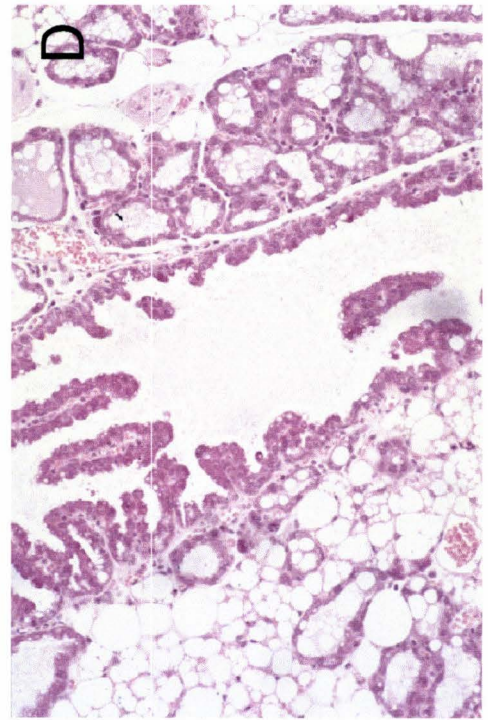
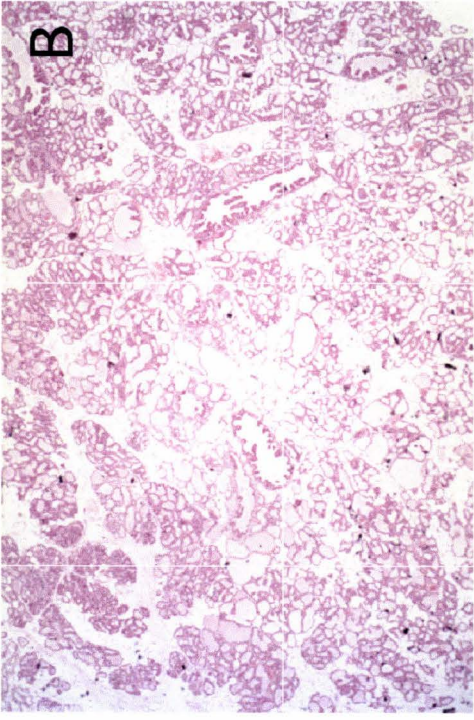
A number of transgenic and knockout mice that display branching defects in the virgin animal also display defects in alveolar development. In order to determine whether alveolar development was affected in PEA3 $-/-$  mice, mammary glands were analyzed during lactation. PEA3 knockout mice developed functional alveoli despite the branching defect observed in virgin animals. The fat pads of these animals filled completely with alveoli, a decrease in alveolar units was not observed. Analysis of lactating glands revealed an unexpected phenotype. PEA3 null animals displayed irregular margins of the ductal lumen (Figure 3.14). Furthermore, islands of cells were present within the lumens of the ducts. PCNA immunostaining revealed that these structures were not proliferative (Figure 3.15). The irregular margins observed were therefore not likely due to increased proliferation of this cell layer but of a folding or collapsing of the duct. The luminal epithelial cells lining the ducts appeared abnormal. PEA3  $-/-$  animals displayed the presence of lipid droplets in luminal epithelial cells of the ducts, this was not observed in wildtype animals. This may indicate that in PEA3  $-/-$  females milk is produced by cells of the duct, in addition to cells of the alveoli.

Figure 3.13. Hematoxylin and Eosin stained sections of PEA3<sup>+/+</sup> (A,C,E,G) and PEA3<sup>-/-</sup> (B,D,F,H) mammary glands during pregnancy. Although the number of alveoli may be reduced, ducts appear normal in PEA3<sup>-/-</sup> mice at 10 days of pregnancy (A-D). Alveoli in glands of both PEA3<sup>-/-</sup> and PEA3<sup>+/+</sup> animals contain lipid droplets at 18 days pregnant (E-H) (arrow). Original magnification 50x (A,B,E,F) or 200x (C,D,G,H).



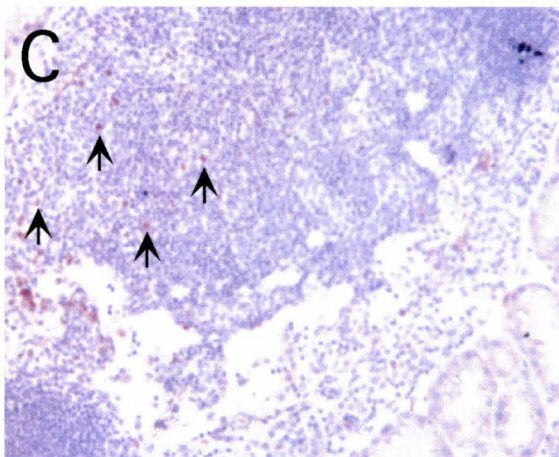
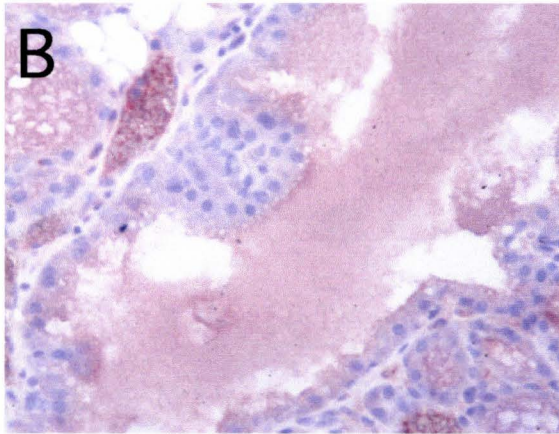
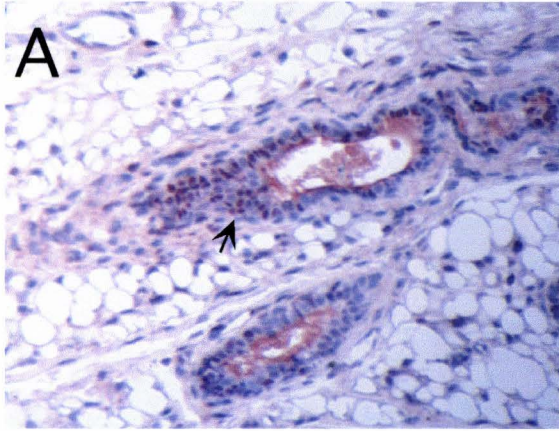


3.14. Hematoxylin and eosin staining of PEA3  $-/-$  (B & D) and  $+/+$  (A & C) lactating mammary glands. PEA3  $-/-$  glands display folding of the ducts not seen in wildtype animals. PEA3  $-/-$  animals contain milk droplets in the ductal cells (Arrows). Few ductal cells in the PEA3  $+/+$  mice contain milk droplets. (Original magnification of A & B, 25 x, C&D, 200x)



3.15. Immunohistochemical analysis of PCNA expression, counterstained with hematoxylin. Positive staining nuclei are brown, negative nuclei are blue. A. 5 week virgin wild type animal was used as a positive control for proliferating cells (200x). B. PEA3 <sup>-/-</sup> lactating mammary gland showing that ductal cells are not proliferating (200x). C. Lymph node from PEA3<sup>-/-</sup> lactating animal, showing positive staining cells within the gland (arrows) (100x).





### 3.8. PEA3 <sup>-/-</sup> Mammary Glands During Involution

The process of involution involves apoptosis and tissue remodeling. This remodeling is accompanied by an upregulation of matrix degrading proteins, proteins that are believed to be targets of PEA3 regulation. Mammary glands of PEA3 <sup>-/-</sup> (BalbC/Sv129) and PEA3 <sup>+/+</sup> animals were therefore examined at three and ten days of involution. The margins of ducts at three days of involution also appeared irregular compared to wild type animals (Figure 3.16). Luminal cells of PEA3 <sup>-/-</sup> ducts (n=2) contained lipid droplets, whereas wildtype animals (n=2) did not. Apoptotic bodies were evident in ducts of both genotypes, indicating that involution had been initiated.

At ten days of involution, the PEA3 <sup>-/-</sup> animals still displayed milk within the ducts. Apoptotic bodies were also observed at this time in the PEA3 <sup>-/-</sup>, but not the PEA3 <sup>+/+</sup> mammary glands. The presence of milk at this time point suggests that apoptosis is delayed in these animals. Ducts of the PEA3 <sup>-/-</sup> animals show varying degrees of abnormalities. Some ducts appeared to lack a cell layer (Figure 3.17 E), while ductal structure in other ducts in the same animal appeared relatively normal. The absence of a cell layer in the PEA3 <sup>-/-</sup> ducts may indicate elevated apoptosis of ductal cells.

### 3.9. PEA3 Expression in the Mammary Gland

To further understand the role of PEA3 in mammary gland development, analysis of PEA3 expression in the developing mammary gland was undertaken. The cell type specific expression pattern was determined using mice carrying a targeted interruption in exon six of PEA3 (EX6-NL LacZ mice). These mice carry an IRES-nuclear localized LacZ construct that directs  $\beta$ -galactosidase expression

3.16. Hematoxylin and Eosin stained sections of mammary gland from 3 day involuting mice. The PEA3 +/+ (A) mammary gland displays a greater ratio of fat:alveoli than the wild type mammary gland (B), suggesting involution may be slowed (Original Magnification 50x). Ductal margin appears disorganized in the PEA3 -/- mammary gland (D) compared to the wildtype mammary gland (C) (Original magnification 200 x). Ductal cells of the PEA3 -/- mammary glands appear to contain milk droplets (arrow). Apoptotic bodies are seen in the ducts (arrowhead).

$+/+$

$-/-$

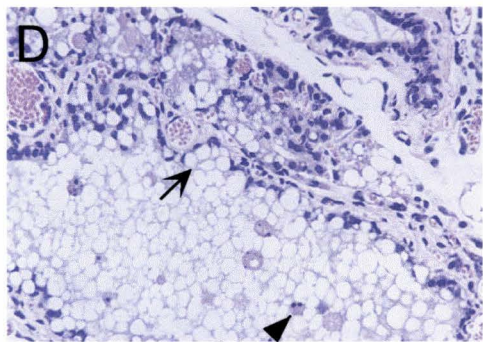
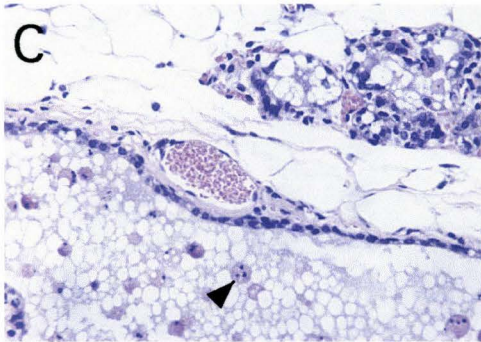
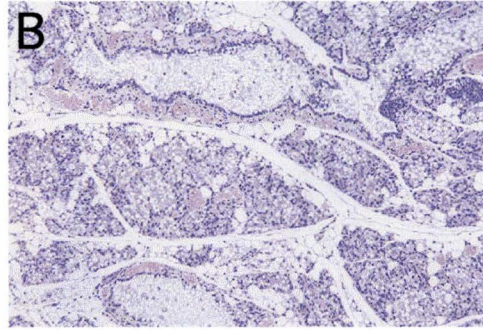
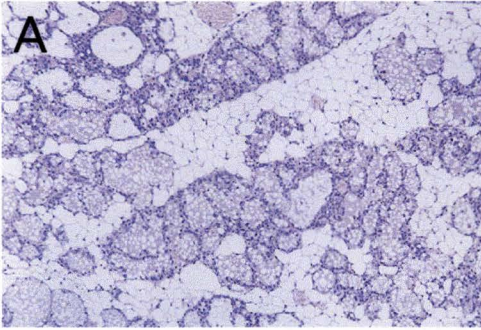
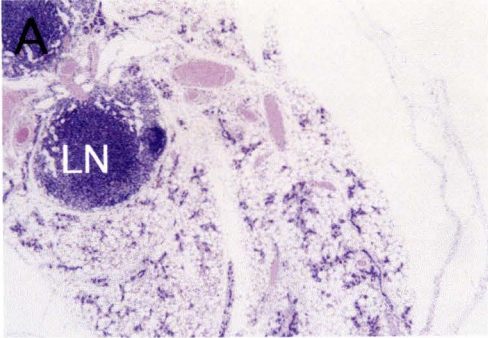


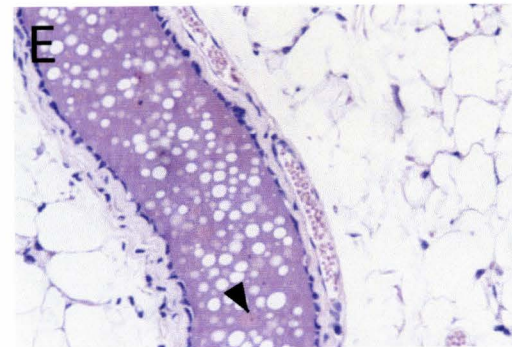
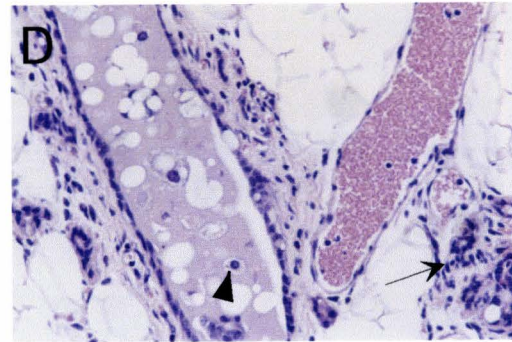
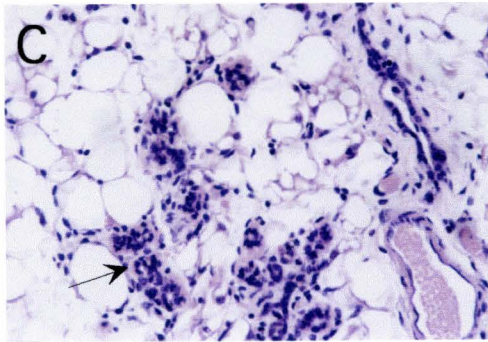
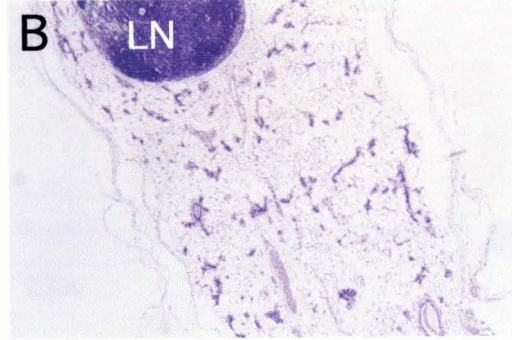
Figure 3.17. Hematoxylin and Eosin stained sections of 10 day involuting mammary glands from PEA3 +/+ (A,C) and PEA3 -/- (B,D,E) animals. Alveoli are collapsed in both PEA3-/- and PEA3 +/+ animals (arrow). Ducts of PEA3 -/- animals contain lipid droplets and apoptotic bodies (arrowhead). Figure E shows a severe phenotype, with ducts appearing to lack a cell layer. (Original magnification A & B 50 x, C-E 200 x)



**+/+**



**-/-**



from the PEA3 promoter.  $\beta$ -galactosidase staining of whole mammary glands revealed that PEA3 is expressed during puberty and pregnancy. Between five and 12 weeks of age,  $\beta$ -galactosidase staining was seen throughout the branching structure. During puberty,  $\beta$ -galactosidase staining was most intense in the terminal end buds (Figure 3.18). Sections of  $\beta$ -galactosidase stained mammary glands revealed that PEA3 expression was localized to the highly proliferative cap cells as well as to myoepithelial cells of the ducts (Figure 3.18). There was no difference observed in the staining pattern in knockout and heterozygous mice. Wholemout analysis revealed that the branching phenotype observed in the PEA3<sup>-/-</sup> (BalbC/Sv129) mice, is also detected in the PEA3-EX6-NL LacZ (C57/Sv129) mice. Thus, this phenotype is conserved in these two genetic backgrounds.

On wholemout analysis,  $\beta$ -galactosidase staining was seen throughout the branching structures at 4, 10 and 18 days of pregnancy (Figure 3.19). Staining was less intense at 18 days. This may reflect decreased expression levels, however, penetration of substrate is a greater problem at this time point due to the increased size of the gland. Analysis of sections of the mammary glands during pregnancy revealed that the staining observed in the wholemout analysis corresponded to the myoepithelial cells of the ducts as well as the myoepithelial cells surrounding the alveoli (Figure 3.19). Expression in luminal epithelial cells, fibroblasts and adipocytes was not observed at any time point.

### **3.10. PEA3 Expression in the Embryonic Mammary Gland**

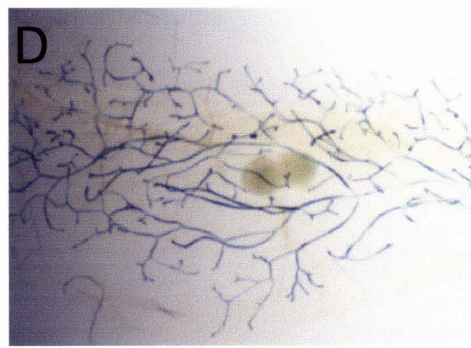
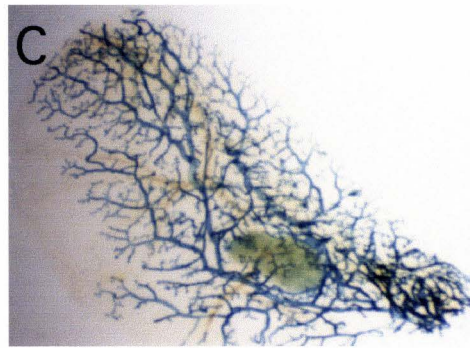
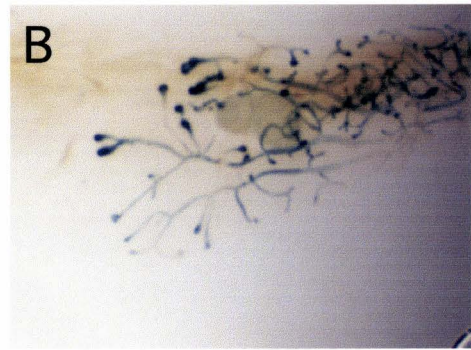
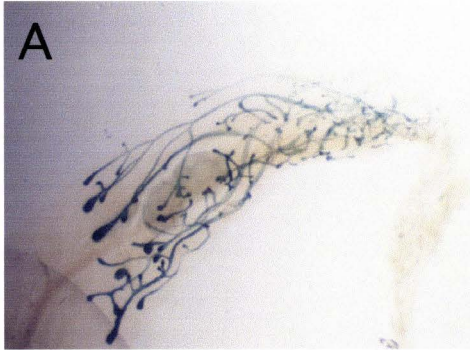
Mammary gland development begins in the embryo at day 10.5. Expression of PEA3, as measured by  $\beta$ -galactosidase staining, in the embryonic

3.18.  $\beta$ -galactosidase staining in the virgin mammary gland. At five weeks, staining is seen in the ducts of PEA3 +/- (A) and -/- (B) animals. staining is most intense in the terminal end buds (arrow). At 12 weeks of age (C & D) staining is observed throughout the mammary tree in PEA3 +/- (C) and -/- (D) animals. Cap cells in the terminal end bud express PEA3 (arrow) as well as myoepithelial cells of the duct (E) (PEA3 -/- animal shown in E). A-D Original magnification 6.4 x. E-original magnification 200 x.



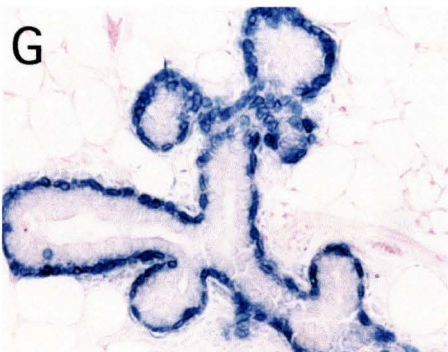
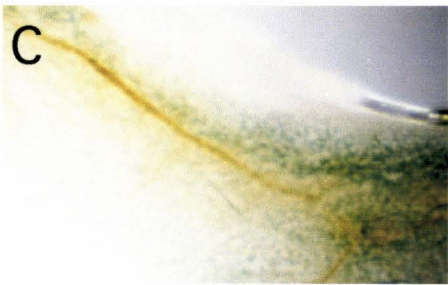
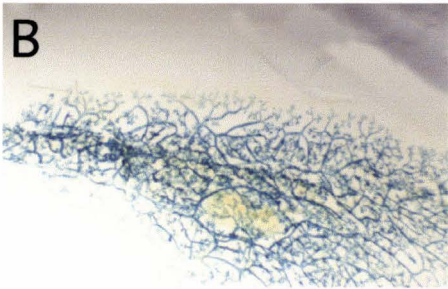
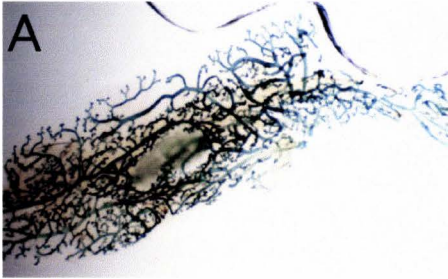
+/-

-/-

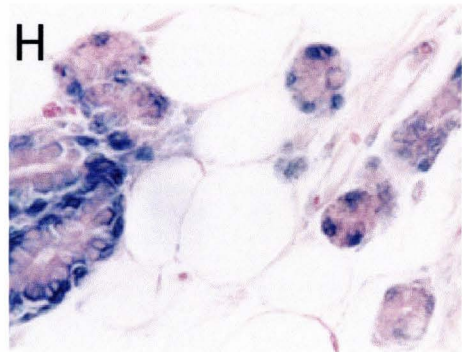
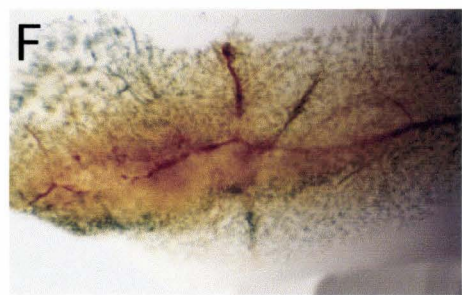
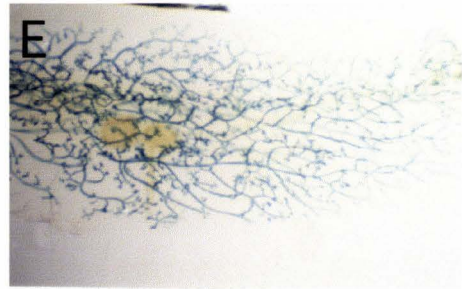
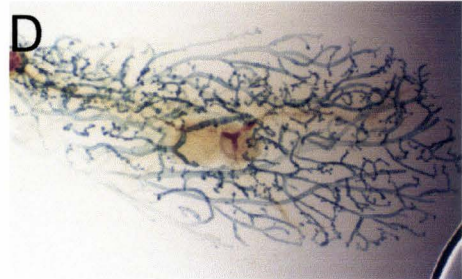


3.19.  $\beta$ -galactosidase staining in the pregnant mammary gland of PEA3-NL-LacZ +/- (A,C,E) and -/- (B,D,F) females. Staining is seen throughout the mammary tree at 4 days (A&B), 10 days (C&D) and 18 days (E&F) of pregnancy.  $\beta$ -galactosidase expression is seen in the myoepithelial cells of the duct at 4 days (G) and in the myoepithelial cells of the duct and surrounding the alveoli at 10 days pregnant (H). Sections in G & H are from PEA3 -/- animals. (Original magnification of A-F, 6.4 x; G & H 200 x)

+/-



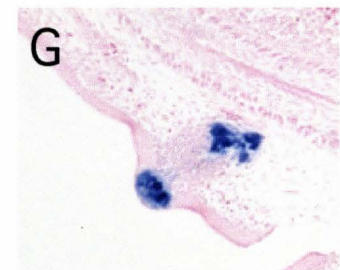
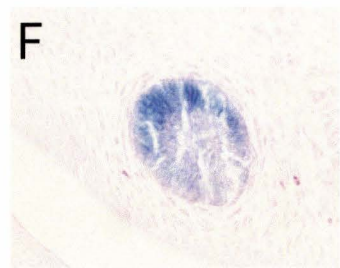
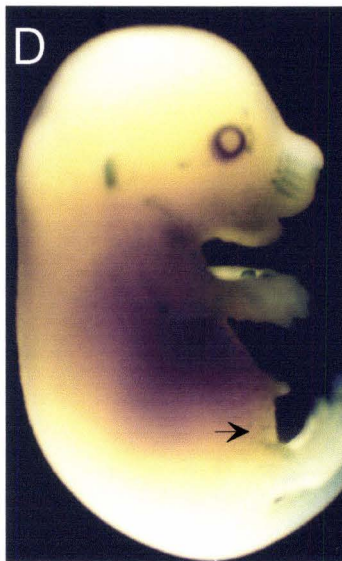
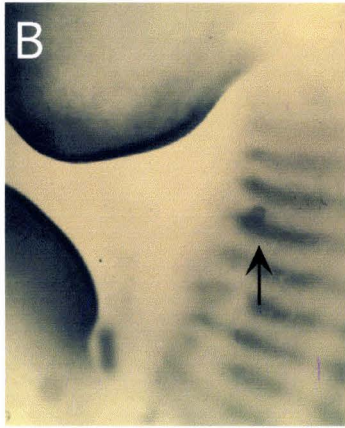
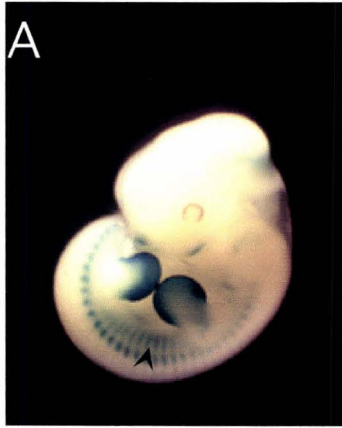
-/-



mammary gland is first observed at day 11.5 (Figure 3.20). Expression could not be observed at day 10.5, when the mammary gland is present as the mammary streak. However, detection of staining at this time point is complicated by  $\beta$ -galactosidase staining of underlying somites. Staining of this single layer of cells may therefore have been obscured by the intense staining of underlying somites. Expression continues in the mammary epithelium in all embryos until sometime after day 15.5, when expression was lost in approximately 50% of the embryos. These embryos were male, suggesting that PEA3 expression is lost when development of the mammary gland stops. Expression was also seen in the nipple of female embryos at embryonic day 15.5.



3.20.  $\beta$ -galactosidase staining in PEA3-NL-LacZ embryos at 11.5 days (A&B), 14.5 days (C&F) and 15.5 days (D, E, G). The earliest visible expression of PEA3 occurs at 11.5 days, where the presence of individual mammary buds is seen. At 14.5 days, expression is seen in the mammary epithelium (C&F). Expression of PEA3 in the mammary gland continues until approximately day 15.5 when expression is lost in male embryos (D). Female embryos retain expression (E). Expression in the early mammary gland is in the epithelial bud (F), and expression is seen in later embryos (E15.5) in the nipple (G). (Magnification of embryos: A-10 x, B-24x, C-E 6.4 x, F-G 200x).



## **CHAPTER 4 DISCUSSION**

### **4.1. PEA3 is Overexpressed in Adjacent and Tumor Tissues in Mice Expressing Activated Neu**

Previously, overexpression of PEA3 was reported in tumors induced by the receptor tyrosine kinase Neu. Sequence analysis of Neu cDNA in tumors of transgenic animals revealed activating mutations in the extracellular domain of Neu (Siegel et al., 1994). The NDL2-5 mice express one of these mutant forms of Neu from the MMTV promoter. Analysis of PEA3 expression in these animals revealed that PEA3 family members are overexpressed in the tumors as well as in the adjacent mammary gland, when compared to normal FVB animals. This is in contrast to mice overexpressing the wild type Neu cDNA, in which overexpression is seen in the tumor but not in the adjacent mammary gland (Trimble et al., 1993). This pattern of expression suggests that the activation of Neu results in an increased transcription of PEA3 family members. This may furthermore suggest that activation of Neu or the occurrence of hyperplasia may force PEA3 expression in additional cell types. Interestingly, expression of PEA3 was slightly higher in adjacent tissues than in tumors of the NDL2-5 mice. This analysis did not compensate for epithelial content, which would likely increase the difference observed since epithelial content is much higher in tumors than in the adjacent tissues. Expression should be re-evaluated using a control for epithelial content, such as a cytokeratin, to determine the true extent of overexpression.

PEA3 in the normal mammary gland is expressed in myoepithelial cells. These cells are tumor suppressive in nature and are lost during tumor progression (Shao et al., 1998). Increased expression of PEA3 in adjacent tissues of the NDL2-5 mice, may reflect expression of PEA3 in an additional cell type. This is supported by the fact that PEA3 is not normally expressed in cells reported to express MMTV. In situ work has demonstrated the expression of MMTV driven transgenes in luminal epithelial cells but not in myoepithelial cells (Faraldo et al., 1998). Furthermore, the main transcriptional activator of MMTV is the progesterone receptor (PR) (Bradham and Bolander, 1989). Expression of PR by in situ hybridization, is observed in luminal epithelial cells but not in myoepithelial cells (Russo et al., 1999). Given the high levels of PEA3 expression in tissues adjacent to tumors in the NDL2-5 line, it seems likely that expression of PEA3 is extended to the luminal epithelial cells following expression of the activated receptor. This should be confirmed using in situ techniques on tumor-free mice.

PEA3 expression may be an early indicator of ductal hyperplasia. The difference in PEA3 expression in adjacent mammary glands of N202 versus NDL2-5 animals may be the result of two factors, the status of Neu and the nature of these two tissues. Adjacent mammary gland in the N202 mice expresses the unactivated form of Neu and displays normal histology and low levels of PEA3 expression, while that of the NDL2-5 mice expresses the activated form of Neu, displays hyperplastic growth and increased PEA3 expression (Guy et al., 1992; Trimble et al., 1993; Siegel et al., 1999). PEA3 expression may thus be a good clinical indicator of activation of Neu or of DCIS in human tissues.



## 4.2. The Role of PEA3 in Tumor Formation

Loss of PEA3 expression did not result in decreased tumor onset. The average time of tumor onset in the NDL2-5 mice is 230 days for PEA3<sup>-/-</sup> mice and 300 days for PEA3<sup>+/+</sup> animals. 42% (6/13) of the PEA3<sup>-/-</sup> mice displayed detectable tumors before seven months, while only 6% (1/18) of PEA3<sup>+/+</sup> mouse had palpable tumors at this time point. Analysis of this data by the log-rank test failed to demonstrate a statistically significant difference between these two sets of data. Lack of statistical significance does not however indicate that tumor formation in PEA3 knockout animals is equivalent to that of wildtype animals. This test concludes that the hypothesis that tumor formation is accelerated in PEA3<sup>-/-</sup> mice, cannot be supported with 95% certainty. Rejection of the null hypothesis in this test does not imply that tumor formation is equal in both groups. Statistical significance in these studies is difficult to achieve due to the wide range over which animals develop tumors. A larger number of animals may be required in order to achieve statistical significance. The use of inbred strains may also aid by decreasing the variability in tumor onset observed.

A complication of tumor studies carried out in mixed genetic backgrounds is the presence of modifier genes. These genes differ between mouse strains and can have strain-specific effects on tumor formation. Because the mice analyzed were not of a pure genetic background, there exists the possibility of modifier genes influencing tumor onset. In order to minimize the effect of modifiers, crosses were set up using F1 heterozygous parents in order to generate sibling wild type and knockout animals for analysis. By restricting analyses in this way, there is no selection for modifiers. Furthermore,

segregation of modifiers would be random unless linked to PEA3. The problem of modifiers could be circumvented by carrying out the analysis in a single genetic background. To this effect, PEA3<sup>-/-</sup> mice were backcrossed into the FVB strain.

There is little evidence to support the theory that PEA3 contributes to the development of mammary tumors. Although it has been shown to be overexpressed in tumors, a number of tumor suppressor proteins are also overexpressed in tumors compared to normal tissues (Elledge and Allred, 1994). Expression alone, therefore does not indicate an oncogenic role. PEA3 does not transform cells in culture, nor has it been reported to cooperate with other proteins to enhance transformation.

#### **4.3. The Role of PEA3 in Metastasis**

Previously reported data from cell culture experiments suggest a causal role for PEA3 in metastasis (Kaya et al., 1996; Habelhah, 1999). In contrast, PEA3 knockout and wildtype animals appear to develop lung metastasis with approximately the same frequency. Five of eight PEA3<sup>-/-</sup> and four of nine PEA3<sup>+/+</sup> animals displayed lung metastasis two months after tumor formation. Loss of PEA3, therefore, did not hinder metastasis. This finding indicates that PEA3 plays a minor role in this process.

The ability of cells expressing PEA3 to induce metastasis in cultured cells cannot be paralleled to this animal model. Overexpression of MMP-1, 3 and 9 induced by PEA3 in cell culture does not reflect the *in vivo* situation. Expression of these metalloproteases in breast tumors has been reported in fibroblasts but not in the epithelial tumor cells (Heppner et al., 1996). Furthermore, expression

of these three metalloproteases in tumors induced by the NDL2-5 transgene is not higher than in the normal mammary gland (Peter Siegel, PhD thesis). Other modifications present in the cell line examined may be required for the effect of PEA3 described.

#### **4.4. The Role of PEA3 in Virgin Mammary Gland Development**

PEA3 is required for proper development of the mammary gland. Loss of PEA3 in the virgin mammary gland results in decreased branching at 2, 5, 8, 12 and 24 weeks. Indeed, analysis of mice at 24 weeks of age revealed that this was a loss of branching structures and not a delay in their formation. Moreover, lateral buds, the buds that form on secondary and tertiary branches in older animals, obvious in wild type animals, were virtually absent in the knockout animals. The intermediate phenotype observed in the PEA3 heterozygote animals reflects the importance of levels of PEA3 expression. Loss of one functional allele results in decreased branching compared to wild type animals. Although the PEA3 knockout animals still produce the N-terminal portion of PEA3, it is unlikely that this protein acts as a dominant negative. This notion is supported by two observations. First, the male infertility phenotype observed in knockout animals is not observed in heterozygous animals, secondly, a phenotype similar to that observed in the heterozygous animals is observed in the PEA3-3'UTR mice which express the full length coding region of PEA3. These mice likely have decreased protein levels due to destabilization of the PEA3 RNA message resulting from interruption of the 3' untranslated region.

Reduced ductal branching such as that observed in PEA3-deficient virgin females has been reported in transgenic mice overexpressing parathyroid

hormone-related protein (Wysolmerski et al., 1995), activated TGF- $\beta$ , (Pierce et al., 1993), and in mice deficient in the  $\beta$ B inhibin subunit, CSF-1, estrogen receptor, progesterone receptor or C/EBP $\beta$  (Robinson & Hennighausen, 1997; Pollard et al., 1994; Lydon et al., 1995). The phenotype of the PEA3 null animals is however distinct from these previously reported phenotypes . With the exception of the MMTV-TGF- $\beta$  overexpressing mice, these mice also suffer from defects in lobulo-alveolar development resulting in an inability to lactate. Loss of PEA3 does not affect lobuloalveolar development.

Reduced branching in the virgin mammary gland may reflect defects of cell proliferation, migration or differentiation. PEA3 may act as a regulator of one of these pathways. Cell proliferation in the mammary gland is regulated by both mitogenic and growth suppressive signals (Reviewed in Hennighausen and Robinson, 1998). Apoptosis is required in concert with proliferation for proper formation of terminal end buds and branching structures. Decreased proliferation of cells in the mammary gland may result from the inability of cells to respond to growth inducing stimuli, through loss of these stimuli, or through increased synthesis of growth suppressive proteins. Loss of PEA3 expression may result in deregulation of these pathways by altering expression of cell cycle proteins, mitogenic proteins, apoptotic proteins or growth suppressive proteins.

In addition to proliferative signals, the mammary gland must respond to signals to form branching structures. HGF has been reported to stimulate branching in the mammary gland (Niemann et al., 1996). The phenotype observed might thus be a reflection of the glands decreased ability to respond to signals initiating branching. This may suggest that PEA3 is a mediator of the Met pathway. If branching was not signaled, but proliferation remained normal,

proliferating cells would be added to existing branches rather than contributing to forming new ones. This may explain the widened ducts observed in the PEA3  $-/-$  mice. Widened ducts were also observed in C/EBP $\beta$  deficient mice although no mechanism has been reported (Robinson & Henninghausen, 1997)

Formation of the mammary gland requires cells to organize themselves into complex, branching structures. The requirement for cell migration in mammary gland development has not been investigated. Migration during branching morphogenesis has been described for tracheal branching in *Drosophila* (Samakovlis et al., 1996). This system differs from the branching system seen in the mammary gland in that no cellular division occurs once branching has begun, organization into primary, secondary and tertiary branches occurs solely through the migration, and organization of cells already present. This process is reported to require the ets protein Pointed. Organization of cells into branching structures in the mammary gland occurs as the cells proliferate. Movement of ducts is in part regulated by the availability of space in the fat pad. Mammary ducts will not cross one another, when a duct encounter another duct, it redirects itself or stops growth. Direction of growth is therefore likely regulated, at least in part, by this inhibitory effect. Decreased arborization of the gland may decrease this inhibition and result in the reversed direction of ducts observed. This phenotype may thus be a result of decreased arborization of the gland rather than a defect in migration.

Proper organization and maintenance of cells into ductal structures requires complex interactions between adhesion molecules and the extracellular matrix (ECM) (Sakakura, 1991; Robinson and Henninghausen, 1997). Cells express a variety of surface molecules that control interactions with the ECM and

adjacent cells. Expression of these molecules determines cell polarity, allowing them to organize into complex cell layers and structures. Mis-regulation of adhesion molecules can interfere with normal development of mammary gland. For example, loss of P-cadherin, a cellular adhesion molecule, results in alveolar development in virgin animals (Radice et al., 1997). ERM has been reported to activate the ICAM promoter. PEA3 may thus also regulate cellular adhesion molecules. Decreased integrity of the ducts may explain the folding of ducts observed during lactation and the abnormal appearance of ductal cells during involution.

Regulation by the extracellular matrix is important for proper development of the mammary epithelium. ECM can influence cell adhesion, migration, proliferation and differentiation (Reviewed in Matrisian and Hogan, 1990). Remodeling of the extracellular matrix occurs during ductal development, alveolar development and reorganization of the gland during involution. Maintenance of the ECM is required to maintain the integrity of ducts. In the mammary gland, degradation of ECM ahead of branching structures is required for proper growth. This is demonstrated by the discontinuity of the ECM around the TEB, where primary branching occurs. Misregulation of the ECM can result in defects of the branching structure. For example, the decreased branching observed following overexpression of TGF- $\beta$  was attributed to increased synthesis and deposition of extracellular matrix components (Daniel et al., 1989).

Expression of the matrix metalloproteases, enzymes which degrade the ECM, has been demonstrated to have a critical role in the normal development of the mammary gland (Sympson et al., 1994, Lefebvre et al., 1992). For example, overexpression of stromelysin 1 in the mammary gland results in increased

branching (Witty et al., 1995). This effect is likely due to decreased inhibition of growth by ECM as well as by release of growth factors from the ECM. Molecules contained in the ECM can form complexes with growth factors, allowing their retention within the extracellular matrix. Degradation of ECM can therefore release these factors allowing rapid stimulation of downstream pathways. For example, fibronectin can bind to TGF- $\beta$  without causing its inactivation (Silberstein et al., 1992). In the normal mammary gland, expression of MMPs, with the exception of matrilysin, is restricted to the fibroblasts surrounding the duct (Witty et al., 1995, Lefebvre et al., 1992). Matrilysin expression has been reported in the myoepithelial cells and is reported to be upregulated by PEA3. It is however unlikely that decreased matrilysin expression results in the phenotype observed. Firstly, overexpression of matrilysin had no effect on branching, while overexpression of PEA3 results in increased branching (Sheppard and Hassell unpublished data). Secondly, during periods of highest PEA3 expression, matrilysin expression is low and only detectable by RT-PCR. Taken together, these findings suggest that while matrilysin expression may contribute to some of the phenotypes observed, it is likely not the sole factor.

The mammary stroma includes fibroblasts and fat cells. The action of some growth factors in the mammary gland is mediated through the stroma. For example, cleared mammary fat pad experiments demonstrate that the decreased branching observed in mice lacking Inhibin  $\beta$ b is due to defects in the stroma (Robinson and Henninghausen, 1997). When wild type mammary epithelial cells were transplanted into the cleared fat pads of these mice, they failed to form normal branching structures. Mammary gland development cannot take place in the absence of growth hormone. It is required for development of terminal end

buds and alveolar structures. The growth stimulatory effects of growth hormone however, are not mediated through the epithelium but through the fat pad (Walden et al., 1998). PEA3 expression was not observed in stromal cells, but the possibility of stromal effects nevertheless needs to be addressed.

PEA3 may be required for the proper differentiation of particular cell types in the mammary gland. Overexpression of PEA3 has previously been shown to accelerate differentiation of myoblasts (Taylor et al., 1997). The role of PEA3 in differentiation of other cell lineages has not yet been investigated. Improper differentiation of myoepithelial cells may also explain the phenotypes observed. These cells may be reduced in their ability to respond to stimuli to form branching structures and may respond abnormally to signals for contraction and relaxation in the lactating duct.

#### **4.5. The Role of PEA3 in Alveolar Development**

While loss of PEA3 results in decreased branching in virgin females, alveolar structures form and mice are able to lactate normally. The normal increase in lateral branching initiated at the onset of pregnancy is reduced in the PEA3 knockout animals. Some PEA3 knockout animals display large bulbous structures at 4 d preg, which are similar in appearance, on wholemount analysis, to TEBs. This may however, be due to the lack of lateral branching. Increased branching would thus be derived from larger branches resulting in larger structures being formed. Branching was reduced in PEA3  $-/-$  animals at 10 days of pregnancy compared to wildtype animals. As observed in wild type females, alveolar development was initiated by 10 days of pregnancy in the PEA3  $-/-$  animals and milk droplets were present in alveolar cells at 18 days. Measuring



expression of the milk proteins whey acidic protein and  $\beta$ -casein during pregnancy should be undertaken to determine whether or not alveolar development is delayed.

In the lactating mammary gland, PEA3  $-/-$  animals display irregular ductal margins. This phenotype appears to be due to folding of the ducts and has not been previously reported in other animals. The phenotype observed may be due to decreased integrity of the ducts or possibly a defect of the myoepithelial cells. Alternatively, the phenotype observed may result from ductal cells acting as alveolar cells. In ducts of the PEA3  $-/-$  animals, there was an increased number of milk droplets in the luminal epithelial cells compared to a wild type duct. The adjacent myoepithelial cells may thereby act as alveolar myoepithelial cells and contract in response to milk production in adjacent tissues, resulting in folding of the ducts. The production of milk by these cells would further explain the observed phenotypes during involution. If these cells act as milk secreting cells during lactation, they may apoptose during involution, resulting in the apparent loss of a cell layer in some ducts observed at 10 days of involution. However, not all ducts in any animal displayed this dramatic phenotype, other ducts appeared to have more than one cell layer in the ductal wall. This may result if a proliferative response has compensated for apoptosed cells. Less severely affected ducts would thus represent an intermediate between the apoptotic response and the compensatory proliferative response.

#### **4.6. PEA3 Expression in the Developing Mammary Gland**

PEA3 is expressed in the branching epithelium during puberty and pregnancy. These are periods of increased cellular proliferation, suggesting that

it may play a role in proliferation. Expression of PEA3 in all myoepithelial cells, during puberty and during pregnancy, suggests that PEA3 expression is not restricted to undifferentiated cells nor is it restricted to actively dividing cells.

Expression in the embryonic mammary gland and in the cap cell layer may indicate that PEA3 is expressed in the stem cell population of the mammary gland. PEA3 over expression in mammary tumors supports this theory because these cells represent undifferentiated cell types. Expression of PEA3 in all myoepithelial cells during puberty and during pregnancy demonstrates that PEA3 is not sufficient to retain cells in an undifferentiated state.

Loss of PEA3 expression in mammary glands of male embryos likely reflects loss of cell growth or acquisition of a terminally differentiated phenotype. Loss of expression in the male buds is not likely due to hormonal regulation of PEA3 expression, since it is the dense mesenchyme which responds to androgen by halting mammary gland development in male animals (Sakakura, 1982). Furthermore, sexual determination in the mammary gland occurs between embryonic day 13 and 14, while loss of PEA3 expression occurred around day 16.

Expression of PEA3 in myoepithelial cells has several implications. Firstly, formation of lateral buds is believed to occur through de-differentiation of myoepithelial cells. The loss of branching observed in the PEA3 knockout animals may thus be due to the inability of these cells to de-differentiate. The implications for expression of PEA3 in tumor formation are less clear. Myoepithelial cells are believed to play a tumor suppressive role. These cells are typically lost during tumor formation. Furthermore, conditioned media from

myoepithelial cell lines has been shown to display anti-proliferative effects on a series of mammary epithelial cell lines (Shao et al., 1998).

#### **4.7. Functional Redundancy in the PEA3 Sub-Family**

While PEA3 is expressed throughout embryonic development, mice are viable and develop normally. This suggests a redundant role of PEA3 subfamily members during development. PEA3, ERM and ER81 share greater than 95% homology in the DNA binding domain. It is therefore assumed that these proteins bind to the same DNA sequence, and exhibit some degree of functional redundancy. The mammary gland phenotype may be the first indication that these proteins have distinct roles. It is interesting that although ERM and ER81 are expressed throughout mammary gland development, they are not able to compensate for loss of PEA3. Although the cell types expressing ERM and ER81 are unknown, it is possible that expression of each is restricted to a different compartment. ERM and ER81 would thus be unable to rescue the PEA3 defect due to cell type expression. However, it seems more likely, given their co-ordinate upregulation in normal mammary gland development and in tumors, that expression of all three is upregulated in response to the same stimuli. Activities of these three proteins may be regulated by interaction with other proteins. This could confer distinct roles on each of the family members. If all three members are expressed in the myoepithelial cells, this would suggest that they are not functionally redundant and that they may play distinct roles in the mammary gland and possibly in other systems.

#### **4.8. Feedback Mechanisms in the PEA3 subfamily**

PEA3, ERM and ER81 share greater than 95% homology in the DNA binding domain. It is therefore assumed that some degree of functional redundancy exists between these proteins, with specificity being conferred by differential expression of the proteins. In the mammary gland and in Neu induced tumors, expression of all three proteins seems to be coordinately upregulated. Expression of these proteins is therefore likely regulated by a common signaling mechanism. The possibility exists for complex regulation within this family, whereby expression of one family member affects the expression of the other subfamily members.

Analysis of ERM and ER81 expression in PEA3  $-/-$  and PEA3 $+/+$  NDL2-5 tumors, indicated that there was no upregulation of either gene in response to loss of PEA3 expression. This suggests that no feedback mechanism exists whereby expression of one family member is upregulated to compensate for lost PEA3 function.

#### **4.9. Future Directions**

Future experiments should address the nature of the defect observed in the PEA3 null animals. BrdU incorporation or PCNA immunostaining could be used to test whether or not there is decreased proliferation in the mammary gland. In situ apoptotic assays should be done to determine whether apoptosis is increased in virgin mice, and whether involution is slowed in the PEA3 null mice. Expression of myoepithelial markers could be undertaken to address the issue of differentiation.

Cell culture assays could be used in order to gain insight into the role of PEA3 in mammary gland development. A number of mammary epithelial cell lines are available, transfection or retroviral infection of these cells with PEA3 should be undertaken to determine the effects of PEA3 expression in these cells. EpH4 cells can form branching structures in culture following stimulation with HGF (Niemann et al., 1998). Expression of PEA3 should be assayed in these cells to determine whether PEA3 alone can induce branching. Primary myoepithelial cells have also been shown to form branching structures in collagen gels following stimulation with HGF. Isolation of PEA3<sup>-/-</sup> and wild type myoepithelial cells should be undertaken to compare their branching in culture.

Although PEA3 expression was only detected in the epithelial component, cleared fat pad experiments should be undertaken to ensure that the observed phenotype is due to an epithelial defect and not a defect of the stroma. Cell type expression of ERM and ER81 should be determined in order to address the possibility of functional redundancy. If these proteins are all expressed in myoepithelial cells, it would suggest that their functions are not redundant and that PEA3 has a unique role in the mammary gland.

Further experiments into the role of PEA3 in tumor formation should address the effect of PEA3 on tumor initiation. Focus assays, or colony forming assays should be performed to compare the transforming effect of activated Neu alone or in combination with PEA3. RNA collected from tumors of PEA3<sup>-/-</sup> and PEA3<sup>+/+</sup> mice should be used to examine expression of proteins known to affect Neu mediated tumor formation, including Rb, p53 and BRCA1. RNA should also be collected from NDL2-5 mice before tumor formation. Analysis of potential

PEA3 target genes may be more informative in these tissues, since they have undergone fewer genetic alterations than tumor tissues.

Expression of PEA3 family members should be examined in mice overexpressing activated Neu before tumor formation in order to address PEA3 overexpression in hyperplastic tissues. Cell type expression of PEA3 in the NDL2-5 mice should be undertaken in order to determine whether increased PEA3 expression in adjacent and contralateral tissues is due to upregulation in myoepithelial cells, or due to PEA3 expression in an additional cell type, the luminal epithelial cells. Experiments using the MMTV-PEA3 and MMTV-dominant negative PEA3 should address the importance of PEA3 expression in luminal epithelial cells, since MMTV does not direct expression in myoepithelial cells.

Additional models of hyperplasia, independent of Neu, should be examined to determine whether PEA3 is overexpressed in those hyperplasias. Treatment of mammary glands with DMBA is reported to result in mammary hyperplasia. If overexpression of PEA3 can be correlated with hyperplasia, expression of PEA3 could be used as a diagnostic test to detect hyperplasias before the appearance of solid tumors. Currently, there is no diagnostic test for mammary hyperplasias and they are not detected until a solid tumor is formed. PEA3 expression could potentially identify hyperplasias and DCIS before their transformation into malignant tumors.

Allred, D. C., O'Connell, P., Fuqua, S. A., and Osborne, C. K. (1994). Immunohistochemical studies of early breast cancer evolution. *Breast Cancer Res Treat* 32, 13-8.

Anderson, M. K., Hernandez-Hoyos, G., Diamond, R. A., and Rothenberg, E. V. (1999). Precise developmental regulation of Ets family transcription factors during specification and commitment to the T cell lineage. *Development* 126, 3131-48.

Athanasiou, M., Mavrothalassitis, G. J., Yuan, C. C., and Blair, D. G. (1996). The Gag-Myb-Ets fusion oncogene alters the apoptotic response and growth factor dependence of interleukin-3 dependent murine cells. *Oncogene* 12, 337-44.

Baert, J. L., Monte, D., Musgrove, E. A., Albagli, O., Sutherland, R. L., and de Launoit, Y. (1997). Expression of the PEA3 group of ETS-related transcription factors in human breast-cancer cells. *Int J Cancer* 70, 590-7.

Baik, M. G., Lee, M. J., and Choi, Y. J. (1998). Gene expression during involution of mammary gland (review). *Int J Mol Med* 2, 39-44.

Bassuk, A. G., and Leiden, J. M. (1997). The role of Ets transcription factors in the development and function of the mammalian immune system. *Life Sci* 60, 1605-12.

Behre, G., Whitmarsh, A. J., Coghlan, M. P., Hoang, T., Carpenter, C. L., Zhang, D. E., Davis, R. J., and Tenen, D. G. (1999). c-Jun is a JNK-independent coactivator of the PU.1 transcription factor. *J Biol Chem* 274, 4939-46.

Beitel, G. J., Tuck, S., Greenwald, I., and Horvitz, H. R. (1995). The *Caenorhabditis elegans* gene *lin-1* encodes an ETS-domain protein and defines a branch of the vulval induction pathway. *Genes Dev* 9, 3149-62.

Bemark, M., Martensson, A., Liberg, D., and Leanderson, T. (1999). Spi-C, a novel Ets protein that is temporally regulated during B lymphocyte development. *J Biol Chem* 274, 10259-67.

Ben-David, Y., Giddens, E. B., Letwin, K., and Bernstein, A. (1991). Erythroleukemia induction by Friend murine leukemia virus: insertional activation of a new member of the ets gene family, Fli-1, closely linked to c-ets-1. *Chromosoma* 100, 395-409.

Bergelson, S., and Daniel, V. (1994). Cooperative interaction between Ets and AP-1 transcription factors regulates induction of glutathione S-transferase Ya gene expression. *Biochem Biophys Res Commun* 200, 290-7.

Bories, J. C., Willerford, D. M., Grevin, D., Davidson, L., Camus, A., Martin, P., Stehelin, D., and Alt, F. W. (1995). Increased T-cell apoptosis and terminal B-cell differentiation induced by inactivation of the Ets-1 proto-oncogene. *Nature* 377, 635-8.

Borras, F. E., Lloberas, J., Maki, R. A., and Celada, A. (1995). Repression of I-A beta gene expression by the transcription factor PU.1. *J Biol Chem* 270, 24385-91.

Bottinger, E. P., Jakubczak, J. L., Haines, D. C., Bagnall, K., and Wakefield, L. M. (1997). Transgenic mice overexpressing a dominant-negative mutant type II transforming growth factor beta receptor show enhanced tumorigenesis in the mammary gland and lung in response to the carcinogen 7,12- dimethylbenz-[a]-anthracene. *Cancer Res* 57, 5564-70.

Bradham, B.M. and Bolander, F.F. (1989). The role of sex steroids in the expression of MMTV in the normal mouse mammary gland. *Biochem Res Commun* 159, 1020-5.

Brown, L. A., Amores, A., Schilling, T. F., Jowett, T., Baert, J. L., de Launoit, Y., and Sharrocks, A. D. (1998). Molecular characterization of the zebrafish PEA3 ETS-domain transcription factor. *Oncogene* 17, 93-104.

Brown, T. A., and McKnight, S. L. (1992). Specificities of protein-protein and protein-DNA interaction of GABP alpha and two newly defined ets-related proteins. *Genes Dev* 6, 2502-12.

Brunner, D., Ducker, K., Oellers, N., Hafen, E., Scholz, H., and Klambt, C. (1994). The ETS domain protein pointed-P2 is a target of MAP kinase in the sevenless signal transduction pathway. *Nature* 370, 386-9.

Buijs, A., Sherr, S., van Baal, S., van Bezouw, S., van der Plas, D., Geurts van Kessel, A., Riegman, P., Lekanne Deprez, R., Zwarthoff, E., Hagemeijer, A., and et al. (1995). Translocation (12;22) (p13;q11) in myeloproliferative disorders results in fusion of the ETS-like TEL gene on 12p13 to the MN1 gene on 22q11. *Oncogene* 10, 1511-9.



Buttice, G., Duterque-Coquillaud, M., Basuyaux, J. P., Carrere, S., Kurkinen, M., and Stehelin, D. (1996). Erg, an Ets-family member, differentially regulates human collagenase1 (MMP1) and stromelysin1 (MMP3) gene expression by physically interacting with the Fos/Jun complex. *Oncogene* *13*, 2297-306.

Carrere, S., Verger, A., Flourens, A., Stehelin, D., and Duterque-Coquillaud, M. (1998). Erg proteins, transcription factors of the Ets family, form homo, heterodimers and ternary complexes via two distinct domains. *Oncogene* *16*, 3261-8.

Carroll, M., Tomasson, M. H., Barker, G. F., Golub, T. R., and Gilliland, D. G. (1996). The TEL/platelet-derived growth factor beta receptor (PDGF beta R) fusion in chronic myelomonocytic leukemia is a transforming protein that self-associates and activates PDGF beta R kinase-dependent signaling pathways. *Proc Natl Acad Sci U S A* *93*, 14845-50.

Celada, A., Borrás, F. E., Soler, C., Lloberas, J., Klemsz, M., van Beveren, C., McKercher, S., and Maki, R. A. (1996). The transcription factor PU.1 is involved in macrophage proliferation. *J Exp Med* *184*, 61-9.

Chakrabarti, S. R., Sood, R., Ganguly, S., Bohlander, S., Shen, Z., and Nucifora, G. (1999). Modulation of TEL transcription activity by interaction with the ubiquitin-conjugating enzyme UBC9. *Proc Natl Acad Sci U S A* *96*, 7467-72.

Chang, C. H., Scott, G. K., Kuo, W. L., Xiong, X., Suzdaltseva, Y., Park, J. W., Sayre, P., Erny, K., Collins, C., Gray, J. W., and Benz, C. C. (1997). ESX: a structurally unique Ets overexpressed early during human breast tumorigenesis. *Oncogene* *14*, 1617-22.

Chen, F., and Capecchi, M. R. (1999). Paralogous mouse Hox genes, Hoxa9, Hoxb9, and Hoxd9, function together to control development of the mammary gland in response to pregnancy. *Proc Natl Acad Sci U S A* *96*, 541-6.

Chen, H. M., and Boxer, L. M. (1995). Pi 1 binding sites are negative regulators of bcl-2 expression in pre-B cells. *Mol Cell Biol* *15*, 3840-7.

Chen, J. H., Vercamer, C., Li, Z., Paulin, D., Vandebunder, B., and Stehelin, D. (1996). PEA3 transactivates vimentin promoter in mammary epithelial and tumor cells. *Oncogene* *13*, 1667-75.

- Chen, J. H., and Wright, C. D. (1993). PEA3, Oct 1 and Oct 2 positively regulate the human ETS1 promoter. *Oncogene* 8, 3375-83.
- Chen, Y., Hollemann, T., Grunz, H., and Pieler, T. (1999). Characterization of the Ets-type protein ER81 in *Xenopus* embryos. *Mech Dev* 80, 67-76.
- Chen, Z. Q., Burdett, L. A., Seth, A. K., Lautenberger, J. A., and Papas, T. S. (1990). Requirement of ets-2 expression for *Xenopus* oocyte maturation. *Science* 250, 1416-8.
- Choi, S. G., Yi, Y., Kim, Y. S., Kato, M., Chang, J., Chung, H. W., Hahm, K. B., Yang, H. K., Rhee, H. H., Bang, Y. J., and Kim, S. J. (1998). A novel ets-related transcription factor, ERT/ESX/ESE-1, regulates expression of the transforming growth factor-beta type II receptor. *J Biol Chem* 273, 110-7.
- Chotteau-Lelievre, A., Desbiens, X., Pelczar, H., Defosse, P. A., and de Launoit, Y. (1997). Differential expression patterns of the PEA3 group transcription factors through murine embryonic development. *Oncogene* 15, 937-52.
- Coffer, P., de Jonge, M., Mettouchi, A., Binetruy, B., Ghysdael, J., and Kruijer, W. (1994). junB promoter regulation: Ras mediated transactivation by c-Ets-1 and c-Ets-2. *Oncogene* 9, 911-21.
- Crepieux, P., Coll, J., and Stehelin, D. (1994). The Ets family of proteins: weak modulators of gene expression in quest for transcriptional partners. *Crit Rev Oncog* 5, 615-38.
- Crnac, D. Construction and Characterization of a Chimeric Transcription Factor: EWS-PEA3. MSc. Thesis, McMaster University, Hamilton, Ontario.
- Crocoll, A., Zhu, C. C., Cato, A. C., and Blum, M. (1998). Expression of androgen receptor mRNA during mouse embryogenesis. *Mech Dev* 72, 175-8.
- D'Armiento, J., DiColandrea, T., Dalal, S. S., Okada, Y., Huang, M. T., Conney, A. H., and Chada, K. (1995). Collagenase expression in transgenic mouse skin causes hyperkeratosis and acanthosis and increases susceptibility to tumorigenesis. *Mol Cell Biol* 15, 5732-9.
- Daniel, C. W., Silberstein, G. B., Van Horn, K., Strickland, P., and Robinson, S. (1989). TGF-beta 1-induced inhibition of mouse mammary ductal growth: developmental specificity and characterization. *Dev Biol* 135, 20-30.

- Daniel, C. W., Strickland, P., and Friedmann, Y. (1995). Expression and functional role of E- and P-cadherins in mouse mammary ductal morphogenesis and growth. *Dev Biol* 169, 511-9.
- Day, R. N., Liu, J., Sundmark, V., Kawecky, M., Berry, D., and Elsholtz, H. P. (1998). Selective inhibition of prolactin gene transcription by the ETS-2 repressor factor. *J Biol Chem* 273, 31909-15.
- de Launoit, Y., Audette, M., Pelczar, H., Plaza, S., and Baert, J. L. (1998). The transcription of the intercellular adhesion molecule-1 is regulated by Ets transcription factors. *Oncogene* 16, 2065-73.
- Defosse, P. A., Baert, J. L., Monnot, M., and de Launoit, Y. (1997). The ETS family member ERM contains an alpha-helical acidic activation domain that contacts TAFII60. *Nucleic Acids Res* 25, 4455-63.
- Degnan, B. M., Degnan, S. M., Naganuma, T., and Morse, D. E. (1993). The ets multigene family is conserved throughout the Metazoa. *Nucleic Acids Res* 21, 3479-84.
- Delattre, O., Zucman, J., Plougastel, B., Desmazes, C., Melot, T., Peter, M., Kovar, H., Joubert, I., de Jong, P., Rouleau, G., and et al. (1992). Gene fusion with an ETS DNA-binding domain caused by chromosome translocation in human tumours. *Nature* 359, 162-5.
- Dickinson, L. A., Trauger, J. W., Baird, E. E., Dervan, P. B., Graves, B. J., and Gottesfeld, J. M. (1999). Inhibition of Ets-1 DNA binding and ternary complex formation between Ets-1, NF-kappaB, and DNA by a designed DNA-binding ligand. *J Biol Chem* 274, 12765-73.
- Dittmer, J., Gegonne, A., Gitlin, S. D., Ghysdael, J., and Brady, J. N. (1994). Regulation of parathyroid hormone-related protein (PTHrP) gene expression. Sp1 binds through an inverted CACCC motif and regulates promoter activity in cooperation with Ets1. *J Biol Chem* 269, 21428-34.
- Dittmer, J., Pise-Masison, C. A., Clemens, K. E., Choi, K. S., and Brady, J. N. (1997). Interaction of human T-cell lymphotropic virus type I Tax, Ets1, and Sp1 in transactivation of the PTHrP P2 promoter. *J Biol Chem* 272, 4953-8.
- Fantl, V., Edwards, P. A., Steel, J. H., Vonderhaar, B. K., and Dickson, C. (1999). Impaired mammary gland development in *Cyl-1(-/-)* mice during pregnancy and lactation is epithelial cell autonomous. *Dev Biol* 212, 1-11.

Fantl, V., Stamp, G., Andrews, A., Rosewell, I., and Dickson, C. (1995). Mice lacking cyclin D1 are small and show defects in eye and mammary gland development. *Genes Dev* 9, 2364-72.

Faraldo, M. M., Deugnier, M. A., Lukashev, M., Thiery, J. P., and Glukhova, M. A. (1998). Perturbation of beta1-integrin function alters the development of murine mammary gland. *Embo J* 17, 2139-47.

Feinberg, A.P. and Vogelstein, B. (1983). A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Analytical Biochem* 137, 266-267.

Fisher, C. R., Graves, K. H., Parlow, A. F., and Simpson, E. R. (1998). Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the *cyp19* gene. *Proc Natl Acad Sci U S A* 95, 6965-70.

Fitzsimmons, D., Hodsdon, W., Wheat, W., Maira, S. M., Wasyluk, B., and Hagman, J. (1996). Pax-5 (BSAP) recruits Ets proto-oncogene family proteins to form functional ternary complexes on a B-cell-specific promoter. *Genes Dev* 10, 2198-211.

Foos, G., Garcia-Ramirez, J. J., Galang, C. K., and Hauser, C. A. (1998). Elevated expression of Ets2 or distinct portions of Ets2 can reverse Ras-mediated cellular transformation. *J Biol Chem* 273, 18871-80.

Gajewski, K. M., and Schulz, R. A. (1995). Requirement of the ETS domain transcription factor D-ELG for egg chamber patterning and development during *Drosophila* oogenesis. *Oncogene* 11, 1033-40.

Gegonne, A., Bosselut, R., Bailly, R. A., and Ghysdael, J. (1993). Synergistic activation of the HTLV1 LTR Ets-responsive region by transcription factors Ets1 and Sp1. *Embo J* 12, 1169-78.

Gille, H., Kortenjann, M., Thomae, O., Moomaw, C., Slaughter, C., Cobb, M. H., and Shaw, P. E. (1995). ERK phosphorylation potentiates Elk-1-mediated ternary complex formation and transactivation. *Embo J* 14, 951-62.

Giovannini, M., Biegel, J. A., Serra, M., Wang, J. Y., Wei, Y. H., Nycum, L., Emanuel, B. S., and Evans, G. A. (1994). EWS-erg and EWS-Fli1 fusion transcripts in Ewing's sarcoma and primitive neuroectodermal tumors with variant translocations. *J Clin Invest* 94, 489-96.

Goldberg, Y., Treier, M., Ghysdael, J., and Bohmann, D. (1994). Repression of AP-1-stimulated transcription by c-Ets-1. *J Biol Chem* 269, 16566-73.

Golub, T. R., Barker, G. F., Bohlander, S. K., Hiebert, S. W., Ward, D. C., Bray-Ward, P., Morgan, E., Raimondi, S. C., Rowley, J. D., and Gilliland, D. G. (1995). Fusion of the TEL gene on 12p13 to the AML1 gene on 21q22 in acute lymphoblastic leukemia. *Proc Natl Acad Sci U S A* 92, 4917-21.

Gorska, A. E., Joseph, H., Derynck, R., Moses, H. L., and Serra, R. (1998). Dominant-negative interference of the transforming growth factor beta type II receptor in mammary gland epithelium results in alveolar hyperplasia and differentiation in virgin mice. *Cell Growth Differ* 9, 229-38.

Guy, C. T., Webster, M. A., Schaller, M., Parsons, T. J., Cardiff, R. D., and Muller, W. J. (1992). Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. *Proc Natl Acad Sci U S A* 89, 10578-82.

Habelhah, H., Okada, F., Kobayashi, M., Nakai, K., Choi, S., Hamada, J., Moriuchi, T., Kaya, M., Yoshida, K., Fujinaga, K., and Hosokawa, M. (1999). Increased E1AF expression in mouse fibrosarcoma promotes metastasis through induction of MT1-MMP expression. *Oncogene* 18, 1771-6.

Hagemeier, C., Bannister, A. J., Cook, A., and Kouzarides, T. (1993). The activation domain of transcription factor PU.1 binds the retinoblastoma (RB) protein and the transcription factor TFIID in vitro: RB shows sequence similarity to TFIID and TFIIB. *Proc Natl Acad Sci U S A* 90, 1580-4.

Hennighausen, L., and Robinson, G. W. (1998). Think globally, act locally: the making of a mouse mammary gland. *Genes Dev* 12, 449-55.

Higashino, F., Yoshida, K., Noumi, T., Seiki, M., and Fujinaga, K. (1995). Ets-related protein E1A-F can activate three different matrix metalloproteinase gene promoters. *Oncogene* 10, 1461-3.

Huang, C. C., Papas, T. S., and Bhat, N. K. (1997). A variant form of ETS1 induces apoptosis in human colon cancer cells. *Oncogene* 15, 851-6.

Humphreys, R. C., Krajewska, M., Krnacik, S., Jaeger, R., Weiher, H., Krajewski, S., Reed, J. C., and Rosen, J. M. (1996). Apoptosis in the terminal endbud of the

Hynes, N. E., and Stern, D. F. (1994). The biology of erbB-2/neu/HER-2 and its role in cancer. *Biochim Biophys Acta* 1198, 165-84.

Ito, T., Nakayama, T., Ito, M., Naito, S., Kanematsu, T., and Sekine, I. (1998). Expression of the ets-1 proto-oncogene in human pancreatic carcinoma. *Mod Pathol* 11, 209-15.

Jager, R., Herzer, U., Schenkel, J., and Weiher, H. (1997). Overexpression of Bcl-2 inhibits alveolar cell apoptosis during involution and accelerates c-myc-induced tumorigenesis of the mammary gland in transgenic mice. *Oncogene* 15, 1787-95.

Janknecht, R., and Nordheim, A. (1993). Gene regulation by Ets proteins. *Biochim Biophys Acta* 1155, 346-56.

Jeon, I. S., Davis, J. N., Braun, B. S., Sublett, J. E., Roussel, M. F., Denny, C. T., and Shapiro, D. N. (1995). A variant Ewing's sarcoma translocation (7;22) fuses the EWS gene to the ETS gene ETV1. *Oncogene* 10, 1229-34.

Jhappan, C., Gallahan, D., Stahle, C., Chu, E., Smith, G. H., Merlino, G., and Callahan, R. (1992). Expression of an activated Notch-related int-3 transgene interferes with cell differentiation and induces neoplastic transformation in mammary and salivary glands. *Genes Dev* 6, 345-55.

Karim, F. D., Urness, L. D., Thummel, C. S., Klemsz, M. J., McKercher, S. R., Celada, A., Van Beveren, C., Maki, R. A., Gunther, C. V., Nye, J. A., and et al. (1990). The ETS-domain: a new DNA-binding motif that recognizes a purine-rich core DNA sequence [letter]. *Embo J* 9, 3137-44.

Kaya, M., Yoshida, K., Higashino, F., Mitaka, T., Ishii, S., and Fujinaga, K. (1996). A single ets-related transcription factor, E1AF, confers invasive phenotype on human cancer cells. *Oncogene* 12, 221-7.

Kim, W. Y., Sieweke, M., Ogawa, E., Wee, H. J., Englmeier, U., Graf, T., and Ito, Y. (1999). Mutual activation of Ets-1 and AML1 DNA binding by direct interaction of their autoinhibitory domains. *Embo J* 18, 1609-20.

Klaes, A., Menne, T., Stollewerk, A., Scholz, H., and Klambt, C. (1994). The Ets transcription factors encoded by the *Drosophila* gene pointed direct glial cell differentiation in the embryonic CNS. *Cell* 78, 149-60.

- Klamt, C. (1993). The *Drosophila* gene pointed encodes two ETS-like proteins which are involved in the development of the midline glial cells. *Development* 117, 163-76.
- Klemsz, M. J., McKercher, S. R., Celada, A., Van Beveren, C., and Maki, R. A. (1990). The macrophage and B cell-specific transcription factor PU.1 is related to the *ets* oncogene [see comments]. *Proc Natl Acad Sci U S A* 87, 3723-7.
- Krane, I. M., and Leder, P. (1996). NDF/hereregulin induces persistence of terminal end buds and adenocarcinomas in the mammary glands of transgenic mice. *J Clin Invest* 97, 2225-32.
- Lai, Z. C., and Rubin, G. M. (1992). Negative control of photoreceptor development in *Drosophila* by the product of the *yan* gene, an ETS domain protein. *Cell* 70, 609-20.
- Lefebvre, O., Wolf, C., Limacher, J. M., Hutin, P., Wendling, C., LeMeur, M., Basset, P., and Rio, M. C. (1992). The breast cancer-associated stromelysin-3 gene is expressed during mouse mammary gland apoptosis. *J Cell Biol* 119, 997-1002.
- Li, B., Greenberg, N., Stephens, L. C., Meyn, R., Medina, D., and Rosen, J. M. (1994). Preferential overexpression of a 172Arg-->Leu mutant p53 in the mammary gland of transgenic mice results in altered lobuloalveolar development. *Cell Growth Differ* 5, 711-21.
- Li, M., Hu, J., Heermeier, K., Hennighausen, L., and Furth, P. A. (1996). Expression of a viral oncoprotein during mammary gland development alters cell fate and function: induction of p53-independent apoptosis is followed by impaired milk protein production in surviving cells. *Cell Growth Differ* 7, 3-11.
- Li, R., Pei, H., and Papas, T. (1999). The p42 variant of ETS1 protein rescues defective Fas-induced apoptosis in colon carcinoma cells. *Proc Natl Acad Sci U S A* 96, 3876-81.
- Lin, J. H., Saito, T., Anderson, D. J., Lance-Jones, C., Jessell, T. M., and Arber, S. (1998). Functionally related motor neuron pool and muscle sensory afferent subtypes defined by coordinate ETS gene expression. *Cell* 95, 393-407.
- Liu, X., Robinson, G. W., Wagner, K. U., Garrett, L., Wynshaw-Boris, A., and Hennighausen, L. (1997). Stat5a is mandatory for adult mammary gland development and lactogenesis. *Genes Dev* 11, 179-86.

Lubahn, D. B., Moyer, J. S., Golding, T. S., Couse, J. F., Korach, K. S., and Smithies, O. (1993). Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. *Proc Natl Acad Sci U S A* *90*, 11162-6.

Lund, L. R., Romer, J., Thomasset, N., Solberg, H., Pyke, C., Bissell, M. J., Dano, K., and Werb, Z. (1996). Two distinct phases of apoptosis in mammary gland involution: proteinase-independent and -dependent pathways. *Development* *122*, 181-93.

Lundgren, K., Montes de Oca Luna, R., McNeill, Y. B., Emerick, E. P., Spencer, B., Barfield, C. R., Lozano, G., Rosenberg, M. P., and Finlay, C. A. (1997). Targeted expression of MDM2 uncouples S phase from mitosis and inhibits mammary gland development independent of p53. *Genes Dev* *11*, 714-25.

Lydon, J. P., DeMayo, F. J., Funk, C. R., Mani, S. K., Hughes, A. R., Montgomery, C. A., Jr., Shyamala, G., Conneely, O. M., and O'Malley, B. W. (1995). Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. *Genes Dev* *9*, 2266-78.

Marais, R., Wynne, J., and Treisman, R. (1993). The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. *Cell* *73*, 381-93

Matrisian, L. M., and Hogan, B. L. (1990). Growth factor-regulated proteases and extracellular matrix remodeling during mammalian development. *Curr Top Dev Biol* *24*, 219-59 1S223/225.

Melet, F., Motro, B., Rossi, D. J., Zhang, L., and Bernstein, A. (1996). Generation of a novel Fli-1 protein by gene targeting leads to a defect in thymus development and a delay in Friend virus-induced erythroleukemia. *Mol Cell Biol* *16*, 2708-18.

Metz, T., and Graf, T. (1991). Fusion of the nuclear oncoproteins v-Myb and v-Ets is required for the leukemogenicity of E26 virus. *Cell* *66*, 95-105.

Monte, D., Coutte, L., Dewitte, F., Defosse, P. A., Le Coniat, M., Stehelin, D., Berger, R., and de Launoit, Y. (1996). Genomic organization of the human ERM (ETV5) gene, a PEA3 group member of ETS transcription factors. *Genomics* *35*, 236-40.



Moreau-Gachelin, F., Wendling, F., Molina, T., Denis, N., Titeux, M., Grimber, G., Briand, P., Vainchenker, W., and Tavittian, A. (1996). Spi-1/PU.1 transgenic mice develop multistep erythroleukemias. *Mol Cell Biol* 16, 2453-63.

Muthusamy, N., Barton, K., and Leiden, J. M. (1995). Defective activation and survival of T cells lacking the Ets-1 transcription factor. *Nature* 377, 639-42.

Nakae, K., Nakajima, K., Inazawa, J., Kitaoka, T., and Hirano, T. (1995). ERM, a PEA3 subfamily of Ets transcription factors, can cooperate with c-Jun. *J Biol Chem* 270, 23795-800.

Nakayama, T., Ito, M., Ohtsuru, A., Naito, S., Nakashima, M., Fagin, J. A., Yamashita, S., and Sekine, I. (1996). Expression of the Ets-1 proto-oncogene in human gastric carcinoma: correlation with tumor invasion. *Am J Pathol* 149, 1931-9.

Nerlov, C., De Cesare, D., Pergola, F., Caracciolo, A., Blasi, F., Johnsen, M., and Verde, P. (1992). A regulatory element that mediates co-operation between a PEA3-AP-1 element and an AP-1 site is required for phorbol ester induction of urokinase enhancer activity in HepG2 hepatoma cells. *Embo J* 11, 4573-82.

Neuenschwander, S., Schwartz, A., Wood, T. L., Roberts, C. T., Jr., Henninghausen, L., and LeRoith, D. (1996). Involution of the lactating mammary gland is inhibited by the IGF system in a transgenic mouse model. *J Clin Invest* 97, 2225-32.

Niemann, C., Brinkmann, V., Spitzer, E., Hartmann, G., Sachs, M., Naundorf, H., and Birchmeier, W. (1998). Reconstitution of mammary gland development in vitro: requirement of c- met and c-erbB2 signaling for branching and alveolar morphogenesis. *J Cell Biol* 143, 533-45.

Nunn, M. F., Seeburg, P. H., Moscovici, C., and Duesberg, P. H. (1983). Tripartite structure of the avian erythroblastosis virus E26 transforming gene. *Nature* 306, 391-5.

O'Neill, E. M., Rebay, I., Tjian, R., and Rubin, G. M. (1994). The activities of two Ets-related transcription factors required for *Drosophila* eye development are modulated by the Ras/MAPK pathway. *Cell* 78, 137-47.

Ohyashiki, K., Ohyashiki, J. H., Tauchi, T., Iwabuchi, H., Iwabuchi, A., and Toyama, K. (1990). ETS1 gene in myelodysplastic syndrome with chromosome change at 11q23. *Cancer Genet Cytogenet* 45, 73-80.

Papadopoulos, P., Ridge, S. A., Boucher, C. A., Stocking, C., and Wiedemann, L. M. (1995). The novel activation of ABL by fusion to an ets-related gene, TEL. *Cancer Res* 55, 34-8.

Pechoux, C., Gudjonsson, T., Ronnov-Jessen, L., Bissell, M. J., and Petersen, O. W. (1999). Human mammary luminal epithelial cells contain progenitors to myoepithelial cells. *Dev Biol* 206, 88-93.

Pereira, R., Quang, C. T., Lesault, I., Dolznig, H., Beug, H., and Ghysdael, J. (1999). FLI-1 inhibits differentiation and induces proliferation of primary erythroblasts. *Oncogene* 18, 1597-608.

Petermann, R., Mossier, B. M., Aryee, D. N., Khazak, V., Golemis, E. A., and Kovar, H. (1998). Oncogenic EWS-Fli1 interacts with hsRPB7, a subunit of human RNA polymerase II. *Oncogene* 17, 603-10.

Pierce, D. F., Jr., Johnson, M. D., Matsui, Y., Robinson, S. D., Gold, L. I., Purchio, A. F., Daniel, C. W., Hogan, B. L., and Moses, H. L. (1993). Inhibition of mammary duct development but not alveolar outgrowth during pregnancy in transgenic mice expressing active TGF-beta 1. *Genes Dev* 7, 2308-17.

Pollard, J. W., and Hennighausen, L. (1994). Colony stimulating factor 1 is required for mammary gland development during pregnancy. *Proc Natl Acad Sci U S A* 91, 9312-6.

Pongubala, J. M., Nagulapalli, S., Klemsz, M. J., McKercher, S. R., Maki, R. A., and Atchison, M. L. (1992). PU.1 recruits a second nuclear factor to a site important for immunoglobulin kappa 3' enhancer activity. *Mol Cell Biol* 12, 368-78.

Radice, G. L., Ferreira-Cornwell, M. C., Robinson, S. D., Rayburn, H., Chodosh, L. A., Takeichi, M., and Hynes, R. O. (1997). Precocious mammary gland development in P-cadherin-deficient mice. *J Cell Biol* 139, 1025-32.

Rao, E., Dang, W., Tian, G., and Sen, R. (1997). A three-protein-DNA complex on a B cell-specific domain of the immunoglobulin mu heavy chain gene enhancer. *J Biol Chem* 272, 6722-32.

Rekhtman, N., Radparvar, F., Evans, T., and Skoultchi, A. I. (1999). Direct interaction of hematopoietic transcription factors PU.1 and GATA-1: functional antagonism in erythroid cells. *Genes Dev* 13, 1398-411.

Ritland, S. R., Rowse, G. J., Chang, Y., and Gendler, S. J. (1997). Loss of heterozygosity analysis in primary mammary tumors and lung metastases of MMTV-MTA<sub>g</sub> and MMTV-neu transgenic mice. *Cancer Res* 57, 3520-5.

Rivera, R. R., Stuiver, M. H., Steenbergen, R., and Murre, C. (1993). Ets proteins: new factors that regulate immunoglobulin heavy-chain gene expression. *Mol Cell Biol* 13, 7163-9.

Robinson, G. W., and Hennighausen, L. (1997). Inhibins and activins regulate mammary epithelial cell differentiation through mesenchymal-epithelial interactions. *Development* 124, 2701-8.

Robinson, G. W., and Hennighausen, L. (1997). Inhibins and activins regulate mammary epithelial cell differentiation through mesenchymal-epithelial interactions. *Development* 124, 2701-8.

Robinson, G. W., Johnson, P. F., Hennighausen, L., and Sterneck, E. (1998). The C/EBP $\beta$  transcription factor regulates epithelial cell proliferation and differentiation in the mammary gland. *Genes Dev* 12, 1907-16.

Rowse, G.J., Ritland, S.R. and Gendler, S.J. (1998). Genetic modulation of neu proto-oncogene-induced mammary tumorigenesis. *Cancer Res*. 58, 2675-9.

Russo, J., Ao, X. Grill, C. and Russo, I.H. (1999). Pattern of distribution of cells positive for estrogen receptor alpha and progesterone receptor in relation to proliferating cells in the mammary gland. *Breast Canc. Res. Treat.* 53, 217-227.

Russo, J., Tait, L., and Russo, I. H. (1983). Susceptibility of the mammary gland to carcinogenesis. III. The cell of origin of rat mammary carcinoma. *Am J Pathol* 113, 50-66.

Sakakura, T. (1991). New aspects of stroma-parenchyma relations in mammary gland differentiation. *Int Rev Cytol* 125, 165-202.

Sakakura, T., Sakagami, Y., and Nishizuka, Y. (1982). Dual origin of mesenchymal tissues participating in mouse mammary gland embryogenesis. *Dev Biol* 91, 202-7.

Samakovlis, C., Hacohen, N., Manning, G., Sutherland, D. C., Guillemin, K., and Krasnow, M. A. (1996). Development of the *Drosophila* tracheal system occurs

by a series of morphologically distinct but genetically coupled branching events. *Development* 122, 1395-407.

Sambrook, J., Fritsch, E.F. and T. Maniatis. (1989). *Molecular Cloning: A Laboratory Manual* (3 volumes). Second Edition. Cold Spring Harbor Laboratory Press.

Schuetze, S., Stenberg, P. E., and Kabat, D. (1993). The Ets-related transcription factor PU.1 immortalizes erythroblasts. *Mol Cell Biol* 13, 5670-8.

Schulz, R. A., The, S. M., Hogue, D. A., Galewsky, S., and Guo, Q. (1993). Ets oncogene-related gene *Elg* functions in *Drosophila* oogenesis. *Proc Natl Acad Sci U S A* 90, 10076-80.

Scott, E. W., Simon, M. C., Anastasi, J., and Singh, H. (1994). Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science* 265, 1573-7.

Seth, A., and Papas, T. S. (1990). The *c-ets-1* proto-oncogene has oncogenic activity and is positively autoregulated. *Science* 250, 1416-8.

Seth, A., Watson, D. K., Blair, D. G., and Papas, T. S. (1990). *c-ets-2* protooncogene has mitogenic and oncogenic activity. *Crit Rev Oncog* 1, 409-36.

Sgouras, D. N., Athanasiou, M. A., Beal, G. J., Jr., Fisher, R. J., Blair, D. G., and Mavrothalassitis, G. J. (1995). ERF: an ETS domain protein with strong transcriptional repressor activity, can suppress *ets*-associated tumorigenesis and is regulated by phosphorylation during cell cycle and mitogenic stimulation. *Embo J* 14, 4781-93.

Shao, Z. M., Nguyen, M., Alpaugh, M. L., O'Connell, J. T., and Barsky, S. H. (1998). The human myoepithelial cell exerts antiproliferative effects on breast carcinoma cells characterized by p21WAF1/CIP1 induction, G2/M arrest, and apoptosis. *Exp Cell Res* 241, 394-403.

Shih, C., Padhy, L. C., Murray, M., and Weinberg, R. A. (1981). Transforming genes of carcinomas and neuroblastomas introduced into mouse fibroblasts. *Nature* 290, 261-4.

Shore, P., and Sharrocks, A. D. (1994). The transcription factors Elk-1 and serum response factor interact by direct protein-protein contacts mediated by a short region of Elk-1. *Mol Cell Biol* 14, 3283-91.

- Siegel, P. M., Dankort, D. L., Hardy, W. R., and Muller, W. J. (1994). Novel activating mutations in the neu proto-oncogene involved in induction of mammary tumors. *Mol Cell Biol* 14, 7068-77.
- Siegel, P. M., and Muller, W. J. (1996). Mutations affecting conserved cysteine residues within the extracellular domain of Neu promote receptor dimerization and activation. *Proc Natl Acad Sci U S A* 93, 8878-83.
- Siegel, P. M., Ryan, E. D., Cardiff, R. D., and Muller, W. J. (1999). Elevated expression of activated forms of Neu/ErbB-2 and ErbB-3 are involved in the induction of mammary tumors in transgenic mice: implications for human breast cancer. *Embo J* 18, 2149-64.
- Silberstein, G. B., Flanders, K. C., Roberts, A. B., and Daniel, C. W. (1992). Regulation of mammary morphogenesis: evidence for extracellular matrix-mediated inhibition of ductal budding by transforming growth factor- beta 1. *Dev Biol* 152, 354-62.
- Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ullrich, A., and et al. (1989). Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* 244, 707-12 Institute, Bethesda, Maryland.
- Sternlicht, M. D., Lochter, A., Sympson, C. J., Huey, B., Rougier, J. P., Gray, J. W., Pinkel, D., Bissell, M. J., and Werb, Z. (1999). The stromal proteinase MMP3/stromelysin-1 promotes mammary carcinogenesis. *Cell* 98, 137-46.
- Su, G. H., Chen, H. M., Muthusamy, N., Garrett-Sinha, L. A., Baunoch, D., Tenen, D. G., and Simon, M. C. (1997). Defective B cell receptor-mediated responses in mice lacking the Ets protein, Spi-B. *Embo J* 16, 7118-29.
- Sumarsono, S. H., Wilson, T. J., Tymms, M. J., Venter, D. J., Corrick, C. M., Kola, R., Lahoud, M. H., Papas, T. S., Seth, A., and Kola, I. (1996). Down's syndrome-like skeletal abnormalities in Ets2 transgenic mice. *Nature* 379, 534-7.
- Sympson, C. J., Talhouk, R. S., Alexander, C. M., Chin, J. R., Clift, S. M., Bissell, M. J., and Werb, Z. (1994). Targeted expression of stromelysin-1 in mammary gland provides evidence for a role of proteinases in branching morphogenesis and the requirement for an intact basement membrane for tissue-specific gene expression. *J Cell Biol* 125, 681-93.

Tamir, A., Howard, J., Higgins, R. R., Li, Y. J., Berger, L., Zacksenhaus, E., Reis, M., and Ben-David, Y. (1999). Fli-1, an Ets-related transcription factor, regulates erythropoietin- induced erythroid proliferation and differentiation: evidence for direct transcriptional repression of the Rb gene during differentiation. *Mol Cell Biol* 19, 4452-64.

Taylor, J. M., Dupont-Versteegden, E. E., Davies, J. D., Hassell, J. A., Houle, J. D., Gurley, C. M., and Peterson, C. A. (1997). A role for the ETS domain transcription factor PEA3 in myogenic differentiation. *Mol Cell Biol* 17, 5550-8.

Thompson, C. C., Brown, T. A., and McKnight, S. L. (1991). Convergence of Ets- and notch-related structural motifs in a heteromeric DNA binding complex [see comments]. *Science* 253, 789-92.

Toscani, A., Mettus, R. V., Coupland, R., Simpkins, H., Litvin, J., Orth, J., Hatton, K. S., and Reddy, E. P. (1997). Arrest of spermatogenesis and defective breast development in mice lacking A-myb. *Mol Cell Biol* 17, 3155-63.

Travers, M. T., Barber, M. C., Tonner, E., Quarrie, L., Wilde, C. J., and Flint, D. J. (1996). The role of prolactin and growth hormone in the regulation of casein gene expression and mammary cell survival: relationships to milk synthesis and secretion. *Endocrinology* 137, 1530-9.

Treisman, R. (1994). Ternary complex factors: growth factor regulated transcriptional activators. *Curr Opin Genet Dev* 4, 96-101.

Trimble, M. S., Xin, J. H., Guy, C. T., Muller, W. J., and Hassell, J. A. (1993). PEA3 is overexpressed in mouse metastatic mammary adenocarcinomas. *Oncogene* 8, 3037-42.

Turque, N., Buttice, G., Beuscart, A., Stehelin, D., Crepieux, P., and Desbiens, X. (1997). Hydrocortisone modulates the expression of c-ets-1 and 72 kDa type IV collagenase in chicken dermis during early feather morphogenesis. *Int J Dev Biol* 41, 103-9.

Urano, F., Umezawa, A., Hong, W., Kikuchi, H., and Hata, J. (1996). A novel chimera gene between EWS and E1A-F, encoding the adenovirus E1A enhancer-binding protein, in extraosseous Ewing's sarcoma. *Biochem Biophys Res Commun* 219, 608-12.

Wagner, K. U., Young, W. S., 3rd, Liu, X., Ginns, E. I., Li, M., Furth, P. A., and Hennighausen, L. (1997). Oxytocin and milk removal are required for post-partum mammary-gland development. *Genes Funct* 1, 233-44.

Walden, P. D., Ruan, W., Feldman, M., and Kleinberg, D. L. (1998). Evidence that the mammary fat pad mediates the action of growth hormone in mammary gland development. *Endocrinology* 139, 659-62.

Walker, N. I., Bennett, R. E., and Kerr, J. F. (1989). Cell death by apoptosis during involution of the lactating breast in mice and rats. *Am J Anat* 185, 19-32.

Wang, C. Y., Bassuk, A. G., Boise, L. H., Thompson, C. B., Bravo, R., and Leiden, J. M. (1994). Activation of the granulocyte-macrophage colony-stimulating factor promoter in T cells requires cooperative binding of Elf-1 and AP-1 transcription factors. *Mol Cell Biol* 14, 1153-9.

Wang, C. Y., Petryniak, B., Thompson, C. B., Kaelin, W. G., and Leiden, J. M. (1993). Regulation of the Ets-related transcription factor Elf-1 by binding to the retinoblastoma protein. *Science* 260, 1330-5.

Wang, L. C., Kuo, F., Fujiwara, Y., Gilliland, D. G., Golub, T. R., and Orkin, S. H. (1997). Yolk sac angiogenic defect and intra-embryonic apoptosis in mice lacking the Ets-related factor TEL. *Embo J* 16, 4374-83.

Wasylyk, B., Hagman, J., and Gutierrez-Hartmann, A. (1998). Ets transcription factors: nuclear effectors of the Ras-MAP-kinase signaling pathway. *Trends Biochem Sci* 23, 213-6.

Wasylyk, B., Hahn, S. L., and Giovane, A. (1993). The Ets family of transcription factors. *Eur J Biochem* 211, 7-18.

Wasylyk, C., Maira, SM., Sobieszczuk, P., and Wasylyk, B. (1994). - Reversion of Ras transformed cells by Ets transdominant mutants. *Oncogene* 9, 3665-73.

Wasylyk, B., Wasylyk, C., Flores, P., Begue, A., Leprince, D., and Stehelin, D. (1990). The c-ets proto-oncogenes encode transcription factors that cooperate with c-Fos and c-Jun for transcriptional activation. *Nature* 346, 191-3.

Wasylyk, C., Gutman, A., Nicholson, R., and Wasylyk, B. (1991). The c-Ets oncoprotein activates the stromelysin promoter through the same elements as several non-nuclear oncoproteins. *Embo J* 10, 1127-34.

- Wasylyk, C., Kerckaert, J. P., and Wasylyk, B. (1992). A novel modulator domain of Ets transcription factors. *Genes Dev* 6, 965-74.
- Wasylyk, C., Maira, S. M., Sobieszczuk, P., and Wasylyk, B. (1994). Reversion of Ras transformed cells by Ets transdominant mutants. *Oncogene* 9, 3665-73.
- Wasylyk, C., and Wasylyk, B. (1992). Oncogenic conversion alters the transcriptional properties of ets. *Cell Growth Differ* 3, 617-25.
- Wasylyk, C., and Wasylyk, B. (1993). Oncogenic conversion of Ets affects redox regulation in-vivo and in- vitro. *Nucleic Acids Res* 21, 523-9.
- Weng, A., Magnuson, T and Storb, U. (1995). Strain-specific transgene methylation occurs early in mouse development and can be recapitulated in embryonic stem cells. *Development* 121, 2853-9.
- Wernert, N., Gilles, F., Fafeur, V., Bouali, F., Raes, M. B., Pyke, C., Dupressoir, T., Seitz, G., Vandenbunder, B., and Stehelin, D. (1994). Stromal expression of c-Ets1 transcription factor correlates with tumor invasion. *Cancer Res* 54, 5683-8.
- Wheat, W., Fitzsimmons, D., Lennox, H., Krautkramer, S. R., Gentile, L. N., McIntosh, L. P., and Hagman, J. (1999). The highly conserved beta-hairpin of the paired DNA-binding domain is required for assembly of Pax-Ets ternary complexes. *Mol Cell Biol* 19, 2231-41.
- Wilson, C. L., Heppner, K. J., Labosky, P. A., Hogan, B. L., and Matrisian, L. M. (1997). Intestinal tumorigenesis is suppressed in mice lacking the metalloproteinase matrilysin. *Proc Natl Acad Sci U S A* 94, 1402-7.
- Witty, J. P., McDonnell, S., Newell, K. J., Cannon, P., Navre, M., Tressler, R. J., and Matrisian, L. M. (1994). Modulation of matrilysin levels in colon carcinoma cell lines affects tumorigenicity in vivo. *Cancer Res* 54, 4805-12.
- Witty, J. P., Wright, J. H., and Matrisian, L. M. (1995). Matrix metalloproteinases are expressed during ductal and alveolar mammary morphogenesis, and misregulation of stromelysin-1 in transgenic mice induces unscheduled alveolar development. *Mol Biol Cell* 6, 1287-303.



- Wotton, D., Ghysdael, J., Wang, S., Speck, N. A., and Owen, M. J. (1994). Cooperative binding of Ets-1 and core binding factor to DNA. *Mol Cell Biol* *14*, 840-50.
- Wysolmerski, J. J., McCaughern-Carucci, J. F., Daifotis, A. G., Broadus, A. E., and Philbrick, W. M. (1995). Overexpression of parathyroid hormone-related protein or parathyroid hormone in transgenic mice impairs branching morphogenesis during mammary gland development. *Development* *121*, 3539-47.
- Xin, J. H., Cowie, A., Lachance, P., and Hassell, J. A. (1992). Molecular cloning and characterization of PEA3, a new member of the Ets oncogene family that is differentially expressed in mouse embryonic cells. *Proc Natl Acad Sci U S A* *89*, 2105-9.
- Yamada, T., Kihara-Negishi, F., Yamamoto, H., Yamamoto, M., Hashimoto, Y., and Oikawa, T. (1998). Reduction of DNA binding activity of the GATA-1 transcription factor in the apoptotic process induced by overexpression of PU.1 in murine erythroleukemia cells. *Exp Cell Res* *245*, 186-94.
- Yamamoto, H., Flannery, M. L., Kupriyanov, S., Pearce, J., McKercher, S. R., Henkel, G. W., Maki, R. A., Werb, Z., and Oshima, R. G. (1998). Defective trophoblast function in mice with a targeted mutation of Ets2. *Genes Dev* *12*, 1315-26.
- Yamamoto, H., Kihara-Negishi, F., Yamada, T., Hashimoto, Y., and Oikawa, T. (1999). Physical and functional interactions between the transcription factor PU.1 and the coactivator CBP. *Oncogene* *18*, 1495-501.
- Yang, B. S., Hauser, C. A., Henkel, G., Colman, M. S., Van Beveren, C., Stacey, K. J., Hume, D. A., Maki, R. A., and Ostrowski, M. C. (1996). Ras-mediated phosphorylation of a conserved threonine residue enhances the transactivation activities of c-Ets1 and c-Ets2. *Mol Cell Biol* *16*, 538-47.
- Yang, C., Shapiro, L. H., Rivera, M., Kumar, A., and Brindle, P. K. (1998). A role for CREB binding protein and p300 transcriptional coactivators in Ets-1 transactivation functions. *Mol Cell Biol* *18*, 2218-29.
- Zhang, L., Eddy, A., Teng, Y. T., Fritzler, M., Kluppel, M., Melet, F., and Bernstein, A. (1995). An immunological renal disease in transgenic mice that overexpress Fli- 1, a member of the ets family of transcription factor genes. *Mol Cell Biol* *15*, 6961-70.

Zhang, L., Lemarchandel, V., Romeo, P. H., Ben-David, Y., Greer, P., and Bernstein, A. (1993). The Fli-1 proto-oncogene, involved in erythroleukemia and Ewing's sarcoma, encodes a transcriptional activator with DNA-binding specificities distinct from other Ets family members. *Oncogene* *8*, 1621-30.

Zhang, P., Behre, G., Pan, J., Iwama, A., Wara-Aswapati, N., Radomska, H. S., Auron, P. E., Tenen, D. G., and Sun, Z. (1999). Negative cross-talk between hematopoietic regulators: GATA proteins repress PU.1. *Proc Natl Acad Sci U S A* *96*, 8705-10.

Zhao, L., Roche, P. J., Gunnensen, J. M., Hammond, V. E., Tregear, G. W., Wintour, E. M., and Beck, F. (1999). Mice without a functional relaxin gene are unable to deliver milk to their pups. *Endocrinology* *140*, 445-53.

Zhou, J., Ng, A. Y., Tymms, M. J., Jermiin, L. S., Seth, A. K., Thomas, R. S., and Kola, I. (1998). A novel transcription factor, ELF5, belongs to the ELF subfamily of ETS genes and maps to human chromosome 11p13-15, a region subject to LOH and rearrangement in human carcinoma cell lines. *Oncogene* *17*, 2719-32.

Time Period(days)	Tumor	Tumor Free	Total	$E_i$	$V_i$
150-170	0.00	18.00	18.00	1.2	2.46405229
	2.00	11.00	13.00		
	2.00	29.00	30.00		
171-190	0.00	18.00	18.00	0.62068966	1.00653595
	1.00	10.00	11.00		
	1.00	28.00	29.00		
191-220	1.00	17.00	18.00	2.57142857	3.1372549
	3.00	7.00	10.00		
	4.00	24.00	28.00		
221-241	2.00	15.00	17.00	2.83333333	2.05882353
	2.00	5.00	7.00		
	4.00	20.00	24.00		
241-260	3.00	12.00	15.00	2.25	1.21428571
	0.00	5.00	5.00		
	3.00	17.00	20.00		
261-280	2.00	10.00	12.00	2.11764706	1.59090909
	1.00	4.00	5.00		
	3.00	14.00	17.00		
281-300	1.00	9.00	10.00	1.42857143	1.06666667
	1.00	3.00	4.00		
	2.00	12.00	14.00		
301-320	2.00	7.00	9.00	2.25	1.125
	1.00	2.00	3.00		
	3.00	9.00	12.00		
321-340	2.00	5.00	7.00	1.55555556	0.66666667
	0.00	2.00	2.00		
	2.00	7.00	9.00		
341-360	4.00	1.00	5.00	2.85714286	1.2
	0.00	2.00	2.00		
	4.00	3.00	7.00		
361-380	1.00	1.00	2.00	1.00	4.00
	1.00	1.00	2.00		
	2.00	2.00	4.00		
Sum				20.6843685	19.5301948

$X^2_{LR}$	0.24782139
$X^2_{1, .95}$	3.84

$X^2_{LR} < X^2_{1, .95}$	Reject Hypothesis
---------------------------	-------------------

APPENDIX 1

Log-Rank Test For Statistical Significance in Tumor Formation  
Between PEA3 -/- and PEA3 +/+ Mice

Hypothesis: There is a statistical difference in tumor onset in these two groups		
IF:	$X^2_{LR} < \text{or} = X^2_{1, .95}$	Reject
IF:	$X^2_{LR} > X^2_{1, .95}$	Accept

Terms:

- $n_{i1}$             Number of +/+ Animals not having tumors at the beginning of the time interval
- $n_{i2}$             Number of -/- Animals not having tumors at the beginning of the time interval
- $a_i$              Number of +/+ Animals having tumors
- $b_i$              Number of -/- Animals not having tumors during time interval

Formulae

$$V = \sum_{i=1}^k V_i = \sum_{i=1}^k \frac{(a_i + b_i)(c_i + d_i)(a_i + c_i)(b_i + d_i)}{N_i^2(N_i - 1)}$$

$$O = \sum_{i=1}^k a_i$$

$$E = \sum_{i=1}^k E_i = \sum_{i=1}^k \frac{(a_i + b_i)(a_i + c_i)}{N_i}$$

$$X^2_{LR} = \frac{(|O - E| - .5)^2}{V}$$

Organization of Tables:

Time Period	Tumor	Tumor Free	Total	$E_i$	$V_i$
+/+	$a_i$	$b_i$	$n_{i1}$		
-/-	$c_i$	$d_i$	$n_{i2}$		
	$a_i + c_i$	$b_i + d_i$	$N_i$		