THE ROLE OF THE *PEA3* SUBFAMILY *ETS* GENES IN MAMMARY TUMORIGENESIS: USE OF TRANSGENIC MOUSE MODELS OF BREAST CANCER

By

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ABSTRACT

The *ets* genes encode DNA-binding proteins capable of regulating the expression of target genes that contain ETS sites within their promoter sequences. The *ets* gene *PEA3* is overexpressed in a majority of human breast tumour samples, as well as in the mammary tumours and lung metastases of several transgenic mouse models of this disease. The *Pea3* subfamily genes, which include *Pea3*, *Erm*, and *Er81*, are expressed during mouse mammary gland development. However, in mammary tumours induced by the HER2/Neu receptor tyrosine kinase, the *Pea3* subfamily genes were upregulated by five- to twenty-fold as compared to normal mammary tissue of wild-type control mice. To understand the role of the PEA3 subfamily proteins in mammary tumorigenesis, transgenic mice were generated which overexpress either PEA3 or a dominant-negative mutant of PEA3 (ΔNPEA3En) under the transcriptional control of the mouse mammary tumour virus (MMTV) promoter/enhancer. ΔNPEA3En was able to inhibit the function of all three PEA3 subfamily proteins and reduce oncogenic Neu-induced transformation of mouse fibroblasts in cell culture-based assays. ΔNPEA3En was able to delay tumour formation as well as reduce the number and the size of tumours that developed in MMTV-*neu*/ΔNPEA3En bi-transgenic mice as compared to MMTV-*neu* transgenic female mice. The tumours that did form in bi-transgenic mice generally lacked ΔNPEA3En expression indicating that PEA3 subfamily function is required for tumour progression. The mammary glands of MMTV-PEA3 transgenic female mice exhibited increased ductal branching and side bud formation in virgin mice, and decreased lobulo-alveolar differentiation during pregnancy. Surprisingly, MMTV-*neu*/PEA3 bi-transgenic

mice had a longer latency in mammary tumour formation than did MMTV-*neu* female mice, similar to what was observed in MMTV-*neu*/ΔNPEA3En bi-transgenic mice. These results implicate PEA3 subfamily protein function during mammary gland development and in Neu-induced mammary tumorigenesis. Thus, inhibiting PEA3 subfamily protein function, or that of one or more specific target gene products, may complement other current therapies in the treatment of human breast cancer.

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LIST OF ABBREVIATIONS

Abl	Abelson kinase
ALCAM	activated leukocyte cell adhesion molecule
AML	acute myeloid leukemia
APC	adenomatous polyposis coli
ATP	adenosine 5'-triphosphate
β-gal	beta-galactosidase
BRCA1	breast cancer associated gene 1
CCND1	cyclin D1
cDNA	complementary DNA
CTP	cytidine 5'-triphosphate
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
ΔNPEA3En	dominant-negative PEA3
ΔNPU.1	dominant-negative PU.1
dNTP	2'-deoxynucleotide 5'-triphosphate
E1A-F	adenovirus E1A enhancer-binding protein
EDTA	ethylene-diamine tetra-acetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ER	estrogen receptor
ER81	ets-related protein 81

ERBB	erythroblastosis oncogene B
ERF	Ets2 repressor factor
ERG	ets-related gene
ERK	extracellular-regulated kinase
ERKO	estrogen receptor-knockout
ERM	Ets related molecule PEA3-like
ESE	epithelial-specific ets
ETS	avian retrovirus E26 oncogene
ETV	ets translocation variant
EWS	Ewing's sarcoma
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FLI-1	Friend leukemia integration-1
GABPα	GA-binding protein alpha
Grb	growth factor receptor bound protein
GSK3-β	glycogen synthase kinase-3 beta
GTP	guanosine 5'-triphosphate
HB-EGF	heparin-binding epidermal growth factor
HER	human epidermal growth factor receptor
HGF/SF	hepatocyte growth factor/scatter factor
HLH	helix-loop-helix
ICAM-1	intercellular adhesion molecule-1

IRES	internal ribosome-entry site
Jak	Janus kinase
JNK	Jun N-terminal kinase
lacZ	beta-galactosidase gene
LEF1	lymphoid enhancer factor-1
luc	luciferase gene
MAPK	mitogen-activated protein kinase
MMP	matrix metalloproteinase
MMTV	mouse mammary tumour virus
MMTV-LTR	mouse mammary tumour virus-long terminal repeat
mRNA	messenger ribonucleic acid
MT-MMP	membrane-type matrix metalloproteinase
NDF	neu differentiation factor
NDL	neu deletion
NeuNT	oncogenic Neu (V659E point mutation)
nls	nuclear localization signal
OPN	osteopontin
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGFR	platelet-derived growth factor receptor
PEA3	polyoma virus enhancer A binding protein 3
PNT	Pointed domain

PR	progesterone receptor
PRKO	progesterone receptor-knockout
PRL	prolactin
PRLR	prolactin receptor
PyVMT	polyoma virus middle T antigen
RNA	ribonucleic acid
RRE	Ras responsive element
RT-PCR	reverse transcriptase-polymerase chain reaction
RTK	receptor tyrosine kinase
rtTA	reverse tetracycline transactivator
SDS	sodium dodecyl sulphate
SPA	simian virus 40 polyadenylation cassette
SRE	serum response element
SRF	serum response factor
SSC	sodium chloride/sodium citrate
STAT	signal transducers and activators of transcription
SV40	simian virus 40
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TBS-T	Tris-buffered saline-Tween 20
TCF	T cell factor
tetO	tetracycline operator

TGFα	transforming growth factor-alpha
TGFβ	transforming growth factor-beta
TIMP-1	tissue inhibitor of metalloproteinases
TPE	Tris-phosphate-EDTA
uPA	urokinase plasminogen activator
VEGF	vascular endothelial growth factor
VP16	herpes simplex viral protein 16
WAP	whey acidic protein

CHAPTER ONE

INTRODUCTION

1.1 The molecular etiology of human breast cancer

Breast cancer is the most prevalent form of cancer diagnosed, and is second only to lung cancer as the leading cause of death due to cancer, among women (National Cancer Institute of Canada, 1997). There is no one direct cause of the disease, but in almost all cases it is a result of a complex interplay of both environmental and endogenous factors. Since those individuals with a family history are at the greatest risk of developing breast cancer this implies the existence of an underlying genetic predisposition for the disease (Knudson, 1993). Indeed, several different tumour suppressor genes and oncogenes have been identified as having potential causative roles in the initiation and malignant progression of breast cancer (Bieche and Lidereau, 1995).

1.1.1 Tumour suppressor genes

BRCA1 and BRCA2 Analyses of families with a predisposition for early-onset breast cancer, and in some cases an accompanying ovarian cancer, revealed genetic loci that may be involved. The first of these was the chromosomal region 17q21 of which 50-70% of families with high prevalence of breast and ovarian cancers were linked to this locus (Easton et al., 1993; Hall et al., 1990). Eventually the putative tumour suppressor gene was cloned and identified as breast cancer associated (*BRCA*)1 gene (Castilla et al., 1994;

Miki et al., 1994). The *BRCA1* gene encodes a zinc-finger containing protein originally implying that it may act as a transcription factor; however, it is also involved in regulating apoptosis, the cell cycle, and DNA repair due to protein-protein interactions with several different protein partners (Deng and Brodie, 2000; Irminger-Finger et al., 1999). Due to the size of the *BRCA1 gene*, 100 kbp comprising 24 exons, and the heterogeneity of mutations identified to date, it has been difficult to accurately screen and identify all the potential genetic lesions in *BRCA1* that associate with these families (Bieche and Lidereau, 1995).

Subsequent to the identification of *BRCA1* and its correlation with familial breast cancer, a second gene, *BRCA2*, was identified which appears to be more specific to families with risk for both breast and ovarian cancers as well as breast cancer in males (Wooster et al., 1994). Loss of heterozygosity at 13q12-13 is observed in these tumours implicating *BRCA2* as a tumour suppressor gene (Collins et al., 1995). Although unrelated to BRCA1 in sequence, BRCA2 functions in DNA damage checkpoint and repair similar to BRCA1 (Baer and Lee, 1998).

p53 Germline mutations in the *p53* tumour suppressor gene have been identified and give rise to a variety of cancers known generally as Li-Fraumeni syndrome (Malkin et al., 1990). Individuals bearing these mutations potentially develop a variety of different cancers, one of which is early-onset breast cancer. The p53 gene encodes a transcription factor critically involved in DNA damage checkpoint and repair, and in apoptosis (Ryan et al., 2001). Although the implication of mutant *p53* gene in the development of cancer, including that of the mammary gland, has been illustrated in transgenic mouse models of

the disease (Donehower et al., 1992), evidence for mutations in *p53* in sporadic human breast cancers have been quite rare (Birch et al., 1994).

It should also be noted that there are an entire host of putative tumour suppressor genes that have not been identified but can be implied by the observed preponderance of specific chromosomal deletions in various breast tumour samples (Bieche and Lidereau, 1995).

1.1.2 Oncogenes

Putative oncogenes implicated in the initiation and progression of breast cancer were initially identified on the basis of genomic DNA amplification. The most prevalent of these amplicons are at chromosomal regions 17q21, 8q24, and 11q13. The specific oncogenes involved at each of these respective loci have been identified: *HER2*, *MYC*, and *CCND1*.

HER2 Amplification and/or overexpression of the *HER2* gene is observed in 20-30% of all human breast cancers (Slamon et al., 1987; van de Vijver et al., 1988), and has been correlated with distant metastasis to the lymph nodes and poor prognosis in node-negative patients. In most cases the *HER2* gene is overexpressed in addition to its amplification implying transcriptional upregulation as well (van de Vijver et al., 1988). Indeed, several transcription factors, including Ets proteins are elevated in breast cancers, and may be involved in the transcriptional upregulation of *HER2* in these tumours (Benz et al., 1997; Scott et al., 2000; Scott et al., 1994). The most convincing data implicating a

direct functional role of *HER2* in breast cancer has been via studies involving transgenic mice (Siegel et al., 2000). For example, overexpression of the rat homologue of *HER2*, *neu*, in the mouse mammary epithelium results in the appearance of stochastic, focal mammary tumours that frequently metastasize to the lung (Guy et al., 1992). This latter phenotype is noteworthy since most transgenic mouse models of breast cancer rarely metastasize. These mouse studies, along with the correlation of *HER2* amplification and overexpression with lymph node metastases in humans, clearly demonstrate a causative role for *HER2* in the malignant progression of breast cancer.

Breast cancer progression mediated by HER2 likely involves other members of the HER family of receptor tyrosine kinases, including the epidermal growth factor (EGF) receptor *EGFR* (*HER1, ERBB1*), *HER3/ERBB3* and *HER4/ERBB4*. These related genes are commonly overexpressed in breast cancers as well (Knowlden et al., 1998; Lemoine et al., 1992; Naidu et al., 1998). Receptor dimerization is initiated in response to binding of one of several different ligands, such as EGF, transforming growth factor-alpha (TGFα), heregulin, or neu differentiation factor (NDF), which leads to increased kinase activity, tyrosine phosphorylation of the receptors, and activation of downstream signalling molecules (Hynes and Stern, 1994). However, activation of these receptors may occur simply through overexpression, or by mutations that enhance ligand-independent receptor dimerization (Siegel and Muller, 1996; Siegel et al., 1999).

MYC The *MYC* proto-oncogene has been deregulated in many different cancers due to either amplification or chromosomal translocation. Involvement of the *MYC* gene in breast cancer was originally identified due to its amplification in a subset of human breast

tumour cells (Kozbor and Croce, 1984). The *MYC* gene is located at 8q24 and is amplified in approximately 15% of human breast cancers (Berns et al., 1992; Escot et al., 1986; Varley et al., 1987). Like the *HER2* gene, in many cases *MYC* is overexpressed as well as amplified, and in some tumours where amplification is not present the level of Myc expression is still elevated (Guerin et al., 1988).

Evidence for a causal role of Myc in breast cancer came with the derivation of transgenic mice whereby the c-*myc* proto-oncogene was overexpressed in the mammary epithelium by the MMTV-LTR (Stewart et al., 1984). Female MMTV-*myc* mice eventually developed mammary tumours after an extremely long latency. However, when these mice were mated with MMTV-*Ha-ras* transgenic mice that develop mammary tumours with a slightly higher penetrance and shorter latency period, the MMTV-*myc*/MMTV-*Ha-ras* bitransgenic females developed mammary tumours with much more rapid kinetics (Sinn et al., 1987). This was the first evidence in a mouse model of breast cancer for the synergistic action of oncogenes in the development and progression of the disease.

The Myc protein contains helix-loop-helix and leucine zipper domains and functions as a transcription factor by dimerizing with the related protein Max to activate target gene expression (Amati and Land, 1994). Myc-Max functions in regulating cell growth and differentiation as well as apoptosis (Dang et al., 1999). Other dimerization partners are known to inhibit the function of Myc (Amati and Land, 1994); however,

overexpression of *MYC* in breast cancer most likely overrides this negative regulation and allows for cell growth to go unchecked.

CCND1 Amplification at the chromosomal location 11q21 has been observed in a subset of breast tumours (Lidereau et al., 1988; Schuuring et al., 1992) and the gene localized to this region was *CYCLIN D1* (*CCND1*) (Motokura et al., 1991). Originally termed *PRAD1* (Schuuring et al., 1992), this gene is amplified in approximately 15-20% of breast cancers, as well as in several other cancers. However, the *CCND1* gene, like *MYC* and *HER2*, is overexpressed in about half of human breast tumours (Gillett et al., 1994), the majority of which display no amplification of 11q21.

The implication of *CCND1* in breast cancer has been strengthened by the studies using MMTV-*cyclinD1* transgenic mice that display mammary epithelial hyperplasia and eventually develop tumours (Wang et al., 1994). Even more remarkable is that *CyclinD1*-null female mice bearing either the MMTV-*Ha-ras* or MMTV-*neu* oncogenic transgenes do not develop mammary tumours (Yu et al., 2001). The *CyclinD1*-null females do however develop mammary tumours induced by MMTV-*Wnt1* and MMTV-*myc* with identical kinetics to their wildtype littermates, implying that CyclinD1 is an obligate D-type cyclin required for progression of Ras and Neu-induced mammary tumours. The *CCND1* gene is normally regulated at the mRNA level during the cell cycle, and the CyclinD1 protein acts to regulate the activity of the cyclin dependent kinases CDK4 and CDK6 for progression from G0 to G1 in the cell cycle (Jacks and Weinberg, 1998; Sherr, 1996). Thus, amplification and overexpression of *CCND1* in

breast cancer can lead to deregulation of the cell division cycle and allow for the aberrant cell proliferation of the tumour cell.

The genes encoding fibroblast growth factor-3 (*FGF3)* and *FGF4* are co-amplified with *CCND1* (Schuuring et al., 1992). The *FGF* genes have been implicated in breast cancer by the observation that MMTV provirus integration occurs at these loci to upregulate their expression and cause mouse mammary tumours (Shackleford et al., 1993).

Although *HER2*, *MYC* and *CCND1* represent the most common amplified and overexpressed genes in breast cancer, others have been observed, such as the FGF receptor (*FGFR*)1 and *FGFR2* genes (Adnane et al., 1991). In addition, duplication of entire chromosome arms have also been observed, especially the long arm of chromosome 1 (Bieche et al., 1995), but the identification of the one or more genes within such a large region which may be involved in human breast cancer has not been achieved.

1.2 Mouse models of human breast cancer

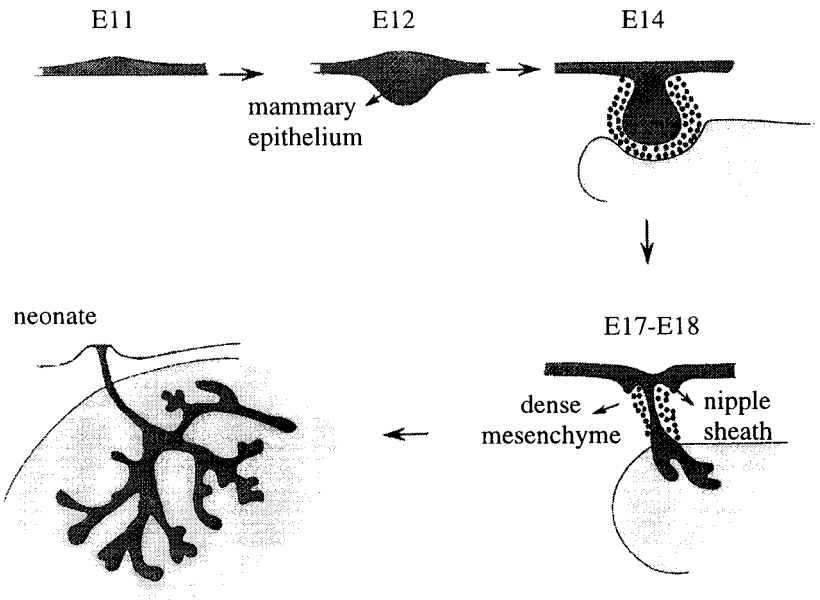
1.2.1 Mouse mammary gland development

Although the majority of mammary gland development and differentiation occurs in the postnatal mouse, the primordial mammary gland develops in the embryo beginning at approximately embryonic day 9.5 (Sakakura, 1991) (Figure 1.1). In the absence of cell proliferation the ectodermal cells comprising the mammary streak migrate into the dense

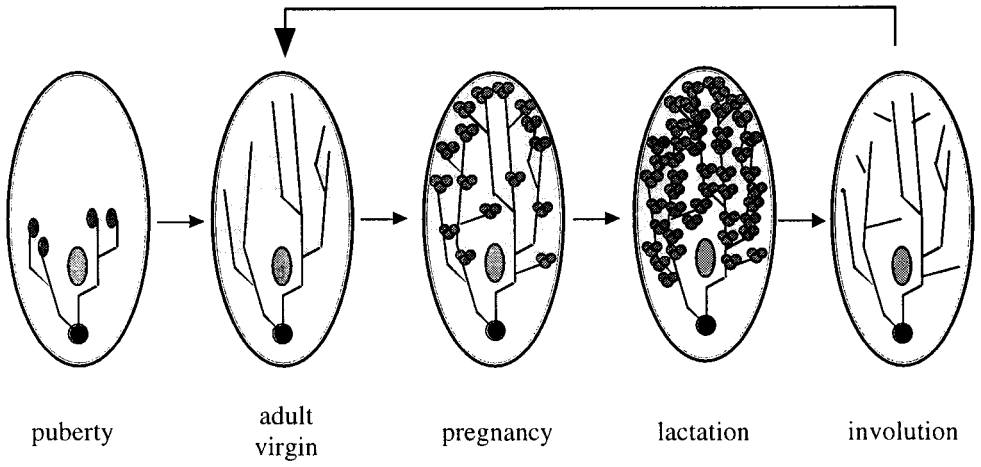
Figure 1.1 Stages of mammary gland development in the embryo and postnatal mouse.

(*a*) Development of the mammary anlagen in the mouse embryo. Early development of the mouse mammary gland occurs primarily during the second half of gestation (embryonic days E10-E19) with the proliferation and migration of epithelium (red) from the ectoderm into the underlying dense mesenchyme (small dots). A rudimentary mammary anlagen of about 15-20 primary and secondary ducts is present in the fat pad (yellow) just prior to birth and remains at this stage until the onset of puberty (~4 weeks of age) (adapted from Sakakura, 1991). (*b*) Stages of postnatal mouse mammary gland development. Rapid cell proliferation in terminal end buds (TEBs, blue) during puberty results in filling of the mammary fat pad (yellow) with primary and secondary ducts. Pregnancy results in more ductal branching and the development and differentiation of milk-producing lobulo-alveoli (red). After lactation, cell death by apoptosis and extensive remodeling of the mammary gland occurs by the process of involution. The mammary gland then returns to a state that is similar, yet not identical, to the adult nulliparous female. The mammary gland cycles through the same stages with each subsequent pregnancy (adapted from Hennighausen and Robinson, 1998).

a


E11
E12
E14
mammary
epithelium
neonate
E17-E18
dense
mesenchyme
nipple
sheath

b


puberty
adult
virgin
pregnancy
lactation
involution

mesenchymal tissue underlying the dermis. This occurs in five pairs along the two mammary streaks to give rise to six thoracic and four inguinal mammary buds. These epithelial buds are connected to the nipple by a nipple sheath and after progression into the stroma of the mammary fat pads they develop approximately 15-20 primary ducts within a few days prior to birth. In male mice, high circulating levels of androgens stimulate androgen receptors present in the dense mesenchyme surrounding the mammary epithelial buds and cause complete regression of the mammary gland. Thus, similar to the development of the female reproductive system, early development of the female mammary gland can be considered a default pathway that is controlled by negative regulation in males.

Up until about four to five weeks after birth, the mouse mammary gland consists of a rudimentary mammary anlage with five pairs of glands, three of which are thoracic and two are inguinal (Daniel and Silberstein, 1987). At the onset of puberty, systemic hormones and locally acting growth factors stimulate growth from structures called terminal end buds (TEBs; Figure 1). TEBs consist of several layers of different cell types, including the undifferentated cap cell layer, the cuboidal epithelium, and the luminal epithelium. The highest levels of cell proliferation are observed in TEBs, which proliferate and branch to fill the fat pad, producing primary and secondary ducts. The cap cells give rise to myoepithelial cells that surround the ducts in a continuous layer, separating luminal epithelial cells from the basal lamina and stromal fat pad. It is believed that this separation may be critical for maintaining structure and inhibiting growth, since breakdown is observed at the onset of lateral branch formation (Silberstein

et al., 1992), as well as in alveolar development (Witty et al., 1995). The ovarian hormones necessary to stimulate ductal elongation at puberty are estrogen and progesterone, and the pituitary hormones are growth hormone and prolactin (Daniel and Silberstein, 1987; Hennighausen and Robinson, 1998). Locally acting growth factors, including EGF (Daniel and Silberstein, 1987) and TGFα stimulate mammary epithelial growth, while TGFβ inhibits mammary epithelial growth (Silberstein and Daniel, 1987; Silberstein et al., 1992). The extracellular matrix (ECM) surrounding the ducts serves as a reservoir containing these growth factors (Sakakura, 1991), providing another example of how ECM integrity is important in maintaining mammary gland structure.

Further ductal growth, and eventually terminal differentiation, occurs during pregnancy and lactation. Lateral branching increases the size of the mammary ductal tree and lobulo-alveolar development gives rise to specialized secretory epithelial cells. Milk protein production occurs in the alveoli beginning at mid-pregnancy and continues until one-to-two days post-weaning (Rosen, 1987). The myoepithelial cells that encapsulate the secretory epithelium of the alveoli have contractile function and serve in milk ejection (Daniel and Silberstein, 1987). After weaning, involution takes place. This process involves programmed cell death and extensive remodelling of the mammary gland (Lund et al., 1996).

Within twenty-four hours post-weaning, apoptosis occurs in the secretory epithelium thereby reducing milk production levels (Lund et al., 1996). Subsequently, remodelling of the ECM surrounding the alveoli restores the mammary gland to a resting

state, similar to its state in a virgin animal (Lund et al., 1996). The mammary gland will go through these phases again with each round of pregnancy and lactation. Therefore, the mouse mammary gland undergoes extensive organ development in the postnatal mouse, and provides a powerful model for studying growth regulation and tumorigenesis.

The fate of mammary epithelium is directly affected by integrity of the mammary mesenchymal tissue. This characteristic interaction is manifest at the earliest stages of mammary gland development in the growing embryo (Sakakura, 1991). The surrounding dense mesenchyme is responsible for establishing the mammary anlagen in female embryos as well as in the regression of the same primordial epithelial tissue in male embryos. In the postnatal mouse, development and differentiation of the mammary epithelium into a complete ductal network capable of producing milk is also dependent on signals from the stroma immediately surrounding it (Cunha and Hom, 1996). This was best illustrated with the development of mammary epithelial transplants into different host tissues (DeOme et al., 1959). For example, transplantation of mammary epithelium into the salivary gland resulted in branching structures resembling those of the mammary gland, but having a functional differentiation gene expression profile of the salivary gland. This overt requirement for the proper stromal environment is also illustrated by the inability of mammary epithelial cells to successfully grow and differentiate in a number of tissue types.

Intercellular communication between cells of the mesenchyme, principally the fibroblasts and adipocytes, and those of the mammary epithelium typically occurs across the basal lamina. The basal lamina, or basement membrane, forms a continuous sheath of

matrix around the mammary ducts containing various growth factors and signalling molecules (Daniel and Silberstein, 1987). Breakdown of the basal lamina through the action of various proteases expressed by either the stroma or epithelium is important in the growth of terminal end buds during puberty and in the development of new secondary and tertiary ducts during estrus cycling and pregnancy. Loss of the basal lamina is best observed at the leading edge of TEBs where the pluripotent cap cells are in direct contact with mesenchyme of the fat pad (Daniel and Silberstein, 1987). It is believed this direct stromal-epithelial interaction is essential for proper growth and differentiation of the mammary gland ductal network (Cunha and Hom, 1996). It should be noted that integrity of the basement membrane is compromised in invasive breast cancers thereby leading to the progression of tumours to full malignancy.

The technology of targeted gene disruption in mice has allowed for analysis of many genes during mammary gland development. The ovarian hormone estrogen is necessary during puberty to allow for ductal arborization of the fat pad (Daniel and Silberstein, 1987). The estrogen receptor (ER) is expressed in both the mammary epithelium and the surrounding stroma. However, reciprocal mammary gland transplantation studies involving ER-knockout (ERKO) female mice revealed that the action of estrogen on ductal outgrowth resides in the stroma (Cunha et al., 1997; Korach et al., 1996).

Both the ovarian hormone progesterone and pituitary-derived prolactin (PRL) are involved in lobuloalveolar development and differentiation of the mammary gland during pregnancy. Again through the use of progesterone receptor-knockout (PRKO) mice,

progesterone appears to act in both the epithelium and the stroma during pregnancy (Humphreys et al., 1997; Lydon et al., 1995). The PR is required in the mammary epithelium for lobuloalveolar development (Lydon et al., 1995), but may also be required in the stroma for ductal outgrowth (Humphreys et al., 1997).

Studies involving PRL and the downstream components of its signalling pathway have been performed using various targeted gene knockout mice. Disruption of the *Prl* gene results in impaired ovulation in female mice. Consequently, these mice fail to become pregnant and lactate (Horseman et al., 1997). Prolactin may also have a role in mammary gland morphogenesis during puberty since virgin female *Prl*-knockout mice have reduced ductal outgrowth. Interestingly, disruption of a single allele of the Prl receptor (*PrlR*) gene in mice resulted in the inability of females to undergo sufficient lobuloalveolar development and differentiation to produce milk during their first pregnancy (Ormandy et al., 1997). Upon subsequent pregnancies, sufficient lobuloalveoli had formed and the females were able to nurse their young. Analyses of pregnant and lactating PRLR-knockout female mice could not be performed since embryos failed to implant.

Biochemical studies of PRL receptor signalling have revealed the importance of a JAK-Stat pathway (Hennighausen and Robinson, 1998). Activation of the PRLR by binding of PRL recruits Jak2 to the receptor. Jak2, in turn, phosphorylates Stat5a and Stat5b which heterodimerize and translocate to the nucleus to activate gene expression (Hynes et al., 1997). The requirement for Stat5a in mediating this PRL-induced

signalling cascade was observed in *Stat5a*-knockout mice where females were unable to produce milk due to absence of lobuloalveoli (Liu et al., 1997). However, female *Stat5b*-knockout mice had no apparent defect in mammary gland development and differentiation (Teglund et al., 1998).

1.2.2 Transgenic mouse models of breast cancer

Transgenic mice have provided important insights into the molecular mechanisms influencing the progression of mammary tumorigenesis (Siegel et al, 2000; Webster and Muller, 1994). Directing specific gene expression to the mouse mammary gland is typically achieved by using either the mouse mammary tumour virus-long terminal repeat (MMTV-LTR), or the whey acidic protein (*Wap*) gene promoter. The mouse mammary tumour virus itself is a retrovirus which when integrated into several specific loci of the mouse genome is able to induce mammary tumours. The proto-oncogenes located at these sites were identified as *int-1* (*Wnt-1*), *int-2* (*Fgf3*), and *int-3* (*Notch4*) (Gallahan and Callahan, 1987; Gallahan et al., 1987; Peters et al., 1984). The MMTV-LTR has been shown to confer high levels of transgene expression in the female mouse mammary gland, and is highly induced by several hormonal stimuli. The MMTV-LTR is expressed in the epithelium throughout all of mouse mammary development (Pattengale et al., 1989), but its spatial and temporal expression can be quite promiscuous in the mouse (Cardiff, 1996).

The *Wap* gene is tightly regulated at the transcriptional level. It is induced at mid-pregnancy and abruptly reduced post-weaning (Rosen, 1987). The promoter of this gene

has also been utilized to direct transgene expression to the mouse mammary gland (Webster and Muller, 1994). Although leaky expression has been observed to allow for transgene expression in virgin female mice, usually pregnancy is required to induce high levels of transgene expression (Cardiff, 1996).

The MMTV-LTR and the *Wap* promoter are useful tools whereby function of a gene in mammary gland development and tumorigenesis can be addressed. However, transgene expression is quite promiscuous both in the target organs and tissues in which they are expressed as well as in their temporal regulation (Cardiff, 1996). Transgenes driven by the MMTV-LTR can be expressed in the salivary glands, Harderian glands, several male reproductive organs, lungs, spleen, and haematopoietic cells of the lymphoid lineage (Webster and Muller, 1994). Although the *Wap* promoter appears to be more tissue-specific, leaky expression has been observed in other organs in specific mouse strains. This is most likely the direct result of different, random integration sites into the genome in each mouse strain generated, in addition to variations in transgene copy number (Cardiff, 1996).

As mentioned above, integration of the MMTV retrovirus into the mouse genome can result in the development of mammary tumours by activation of *Wnt-1*, *Fgf-3*, and *Notch-4*. However, neither the mutational activation nor the overexpression of these genes has been observed in human breast cancers (Bieche and Lidereau, 1995). Transgenic mouse technology has allowed for the direct assessment of putative oncogenes and tumour suppressor genes on the development of the mouse mammary gland and on tumorigenesis (Webster and Muller, 1994). Several genes have been

studied in this manner, including activated *Ha-ras* (Andres et al., 1987; Andres et al., 1988; Sinn et al., 1987), *myc* (Andres et al., 1988; Leder et al., 1986; Sinn et al., 1987; Stewart et al., 1984), *neu* (Guy et al., 1992; Muller et al., 1996; Suda et al., 1990), activated *neu* (*neuNT*) (Bouchard et al., 1989; Guy et al., 1996; Muller et al., 1988), polyoma virus middle tumour antigen (PyVMT) (Guy et al., 1992; Guy et al., 1994; Rauh et al., 1999), activated *src* (Webster et al., 1995), mutant *p53* (Li et al., 1997), *Cyclin D1* (Wang et al., 1994), *TGF-α* (Matsui et al., 1990; Muller et al., 1996), *TGF-β* (Pierce et al., 1993), *stromelysin-1* (Sympson et al., 1995; Sympson et al., 1994), matrilysin (Rudolph-Owen et al., 1998; Rudolph-Owen et al., 1998), NDF/heregulin (Krane and Leder, 1996). The result of transgene expression in each case is quite distinct, reflective of the differences in the oncogenic pathways involved. Specific morphological alterations of normal mammary gland development has also been noted and depends on the transgene involved. The PyVMT and Src tyrosine kinases induce focal hyperplasias, which in some cases represent neoplastic lesions due to their ability to form solid tumours when implanted into the mammary gland of recipient mice (Guy et al., 1992; Webster et al., 1995). On the other hand, the Neu receptor tyrosine kinase causes increased development of acinar structures in nulliparous female mice (Guy et al., 1992). Virgin TGFα transgenic mice develop large cystic structures resembling lobuloalveoli during late pregnancy or lactation (Matsui et al., 1990). Although the proteins encoded by each of these transgenes is thought to activate similar and overlapping signal transduction pathways in cells, it is obvious that the overall phenotypic outcome illustrates distinct functions.

The tumours that arise in these different transgenic mice are also quite distinct. The best comparisons have been made among the MMTV-*Ha-ras*, MMTV-*myc*, and MMTV-*neu* strains of mice (Cardiff et al., 1991). MMTV-*Ha-ras* tumours are comprised of small light-staining cells with small round-to-oval nuclei. Tumour cells induced by MMTV-*myc* are dark-staining, contain large pleomorphic nuclei, and form pseudoacini. In contrast, MMTV-*neu* tumour cells are intermediate-staining as compared with the two tumour types described above, are nodular and contain more uniform nuclei. This observation that the phenotype of a tumour cell may be indicative of the specific oncogenic lesion(s) has clear implications in the possibility of more specific diagnosis and treatment of breast cancer in humans.

Most of these transgenes induce mammary tumours stochastically and with variable latency, indicating that secondary events are required. This may involve the activation of other oncogenes, deregulated expression of the transgene itself, or even selection of transgene mutations conferring elevated gene function. For example, increased levels of Src kinase activity (Guy et al., 1992), as well as ErbB3 protein expression and heterodimerization with Neu (Siegel et al., 1999), are observed in the tumours of MMTV-*neu* mice.

The stochastic nature of tumour formation and long latency may be due to relatively low levels of transgene expression in most transgenic mouse strains (Cardiff, 1996). Most tumours that arise have elevated transgene expression in the tumour proper but low expression in the surrounding normal mammary epithelium. Recently, binary systems have been used to control oncogene transcription in response to an inducible

transactivator that is expressed from the MMTV-LTR (D'Cruz et al., 2001). These tightly-regulated systems can allow for greater transgene expression which shortens tumour latency (D'Cruz et al., 2001) (Chodosh, 2001, personal communication). Double transgenic mice have been used to study the multistep progression of breast cancer. MMTV-*Ha-ras*/MMTV-*myc* mice show an increased rate of tumour progression as compared to either transgene alone (Sinn et al., 1987). Neu and TGF-α synergize in mammary tumourigenesis when transgenic mice of both strains are crossed (Matsui et al., 1990). MMTV-neu transgenic female mice develop mammary tumours with a much shorter latency when crossed with transgenic mice overexpressing the 172Arg-His p53 mutant (Li et al., 1997).

The requirement for specific gene function in tumour development can also be studied in gene knockout mice. Since *CCND1* overexpression has been implicated in human breast cancer, mice bearing targeted inactivation of the *CyclinD1* gene have been crossed with four different transgenic oncogene mice (Yu et al., 2001). Surprisingly, *CyclinD1* is absolutely required for neoplastic transformation of the mammary epithelium mediated by Ras and Neu. In contrast, tumours of MMTV-*Wnt-1* and MMTV-*myc* female mice arose with similar kinetics between wildtype and *CyclinD1* deficient mice. This oncogene specificity in *CyclinD1* requirement appeared to be a result of the expression of only CyclinD1 among the D-type cyclins in Ras and Neu-induced tumours, whereas the expression of CyclinD2 and D3 in Wnt1 and Myc mammary tumours served a redundant function. Interestingly, this preferential role for CyclinD1 downstream of

Ras and Neu was specific to mammary epithelial cells, since *CyclinD1*-null embryonic fibroblasts were transformable by Ras and Neu oncogenes.

MMTV-PyVMT transgenic mice have been mated with *ets2+/-* heterozygous animals. Tumours arising in MMTV-PyVMT *ets2+/-* females are reduced in size as compared with those in wildtype *ets2* mice (Neznanov et al., 1999). Apparently this decrease in tumour size is not due to a lower cell division rate (Neznanov et al., 1999). The mechanism by which a reduction in functional Ets2 affects tumorigenesis in this model is still forthcoming.

1.2.3 MMTV-*neu* transgenic mice

As mentioned above transgenic mice bearing the rat *neu* gene under the transcriptional control of the MMTV-LTR have been generated. The resultant female mice develop focal mammary adenocarcinomas stochastically and after long latency (Guy et al., 1992). Since there was a long delay before onset of tumour formation it seemed likely that secondary mutations were necessary for mammary tumorigenesis. Indeed, analyses of the MMTV-*neu* transgene revealed that in approximately 65% of mammary tumours deletions occurred in the *neu* cDNA comprising sequences in the juxtamembrane extracellular domain (Siegel et al., 1994). This results in a change in the number of cysteine residues, an enhancement of receptor dimerization, and ultimately stimulation of downstream signalling pathways (Siegel and Muller, 1996).

Transgenic mice have also been generated which bear several of these deletion mutants of *neu* targeted to the mouse mammary gland by the MMTV-LTR. Although

these female transgenic mice develop mammary tumours with a slightly earlier onset than that of the original MMTV-*neu* female mice, it is obvious from these studies that tumour development is still dependent on cooperating stochastic events (Siegel et al., 1999). It should be noted that a splice-variant of the human *HER2* mRNA has been identified in several breast tumour specimens and in normal human breast tissue which resembles the tumour derived deletion mutants of MMTV-*neu* transgenic mice (Siegel et al., 1999). Elevated levels of the related c-ErbB3 receptor tyrosine kinase have been correlated with mammary tumorigenesis both in these mice (Siegel et al., 1999) as well as in human tumours (Lemoine et al., 1992; Naidu et al., 1998). The c-ErbB3 receptor, due to a mutation of a highly conserved amino acid residue in the ATP-binding site of the kinase domain, does not have any intrinsic kinase activity (Sierke et al., 1997). However, overexpression of both HER2/Neu and ErbB3 results in heterodimerization and transphosphorylation of ErbB3 by HER2/Neu, thereby facilitating stimulation of ErbB3-dependent signalling pathways. It should be noted that HER2/Neu is considered to be the preferred heterodimerization partner among the c-ErbB receptor tyrosine kinases (Hynes and Stern, 1994).

An oncogenic mutant of the rat *neu* gene has been identified in chemically-induced neuroblastomas in rats (Shih et al., 1981). A single point mutation of Val664Glu (NeuNT) within the transmembrane domain of the receptor confers constitutive homodimerization, tyrosine kinase activity, and stimulation of downstream signalling pathways (Bargmann et al., 1986). Several strains of MMTV-*neuNT* mice have been generated and in most instances these mice develop multifocal mammary tumours with

what appear to be single-step kinetics (Guy et al., 1996; Muller et al., 1988). In one other study the female mice develop mammary carcinomas with a lengthened latency and are focal in nature (Bouchard et al., 1989). Although these early studies with a highly oncogenic mutant of the HER2/Neu receptor tyrosine kinase revealed its potential to induce mammary tumours, a similar point mutation has never been observed in human tumours (Hynes and Stern, 1994).

Recently, a transgenic/knockin mouse model that more closely mimics the human disease has been generated (Andrechek et al., 2000). The activated *neuNT* cDNA was placed into the endogenous mouse c-*erbB2* locus by homologous recombination. This insertion allele was designed so that expression of the NeuNT was under control of the endogenous promoter only after intervening sequences flanked by *loxP* sites were removed by expression of Cre recombinase. When these mice were mated with MMTV-Cre mice, mammary tumours that closely mimicked the histopathology of human breast tumours appeared after a long latency. Close examination of the modified c-*erbB2* locus revealed amplification of this genomic region had occurred in the mouse mammary tumours, similar to what is observed in 20-30% of human breast tumour studied. Closely linked genes that are present in this amplicon in humans (Kishi et al., 1997) are also amplified in the mouse lesions which may illustrate cooperativity of multiple gene products within the amplicon. One such gene, *GRB7*, encodes an SH2-containing adaptor protein that may function in binding to phosphorylated tyrosines on activated ErbB3 or ErbB4 receptors and thereby recruit and activate effectors of the downstream signalling cascade (Fiddes et al., 1998).

1.3 The *ets* gene family of transcription factors

1.3.1 Ets protein structure and function

The *ets* gene family of transcription factors comprises 30 members in humans and is found in all metazoans studied to date (Lautenberger et al., 1992). The characteristic hallmark of these genes is the ETS motif, a DNA-binding domain of ~85 amino acids which recognizes a core element of 5'-GGA, A/T- 3' found in the promoters of various genes involved in proliferation, development, differentiation, and in disease (Graves and Petersen, 1998; Macleod et al., 1992). The founding member is *v-ets*, isolated as an oncogenic component of the avian retrovirus E-twenty-six (Leprince et al., 1983). Soon after description of *v-ets*, its cellular counterpart *Ets-1* was isolated (Watson et al., 1985). The *Ets-2* gene was subsequently identified and is most homologous in sequence to *Ets-1* among all the *ets* genes known to date (Boulukos et al., 1988). In the past 15 years, many other *ets* genes have been identified with varying degrees of similarity within the ETS DNA-binding domain and within other putative functional domains (Graves and Petersen, 1998). The *ets* gene family has been subdivided based on the similarity of ETS domain sequences and the presence of specific structural domains (Laudet et al., 1999). This has allowed for the phylogenetic analysis of the *ets* gene family and an understanding of the origin and divergence in the numerous family members present to date.

The discovery and initial characterization of *ets* genes revealed the capacity of their protein products to bind DNA and influence gene transcription. Most Ets proteins bind to almost any -GGA, A/T- containing element at least to some degree. Studies involving several Ets proteins have revealed that several nucleotides flanking this core element influence DNA binding. In fact, the affinity for specific DNA binding sites of up to 10 basepairs appears to follow the same *ets* subfamily classification system prepared by ETS domain amino acid sequence. The structure of the ETS domain is that of a winged-helix-turn-helix (Donaldson et al., 1994; Donaldson et al., 1996; Kodandapani et al., 1996) with negative regulatory domains immediately flanking it (Graves and Petersen, 1998). Sequences both amino- and carboxyl-terminal to the ETS domain are required to negatively regulate its DNA binding and it appears that an alpha helix within the amino-terminal portion unfolds to allow access to the DNA element (Graves and Petersen, 1998).

Almost all Ets proteins act as transcriptional activators, potentially by interacting with coactivators via transactivation domains, synergizing with other transcription factors, or influencing the general transcription machinery directly (Graves and Petersen, 1998; Macleod et al., 1992). Most ETS sites found in the promoters of target genes are in the vicinity of other transcription factors known to synergize with Ets proteins. For example, Ras-responsive elements comprise juxtaposed ETS and AP1 sites found in the gene promoters of many matrix metalloproteinases (Matrisian, 1994), the serine protease

urokinase plasminogen activator (*uPA*) (D'Orazio et al., 1997), tissue inhibitor of metalloproteinases-1 (*Timp-1*) (Logan et al., 1996), and heparin-binding epidermal growth factor (*HB-EGF*) (McCarthy et al., 1997). Ets proteins have been shown to regulate gene expression from these promoters and cooperate with the AP1 complex to activate transcription. The ternary complex, consisting of the Ets protein Elk-1 and serum response factor (SRF), binds to the serum response element (SRE) in the c-*fos* promoter to elicit growth factor induced gene activation (Hill et al., 1993). Several Ets proteins contain a motif termed the Pointed (PNT) domain (Graves and Petersen, 1998). Due to studies illustrating the apparent self-association of TEL proteins, the PNT domain was thought to comprise a helix-loop-helix (HLH) motif that would facilitate oligomerization of Ets proteins (Jousset et al., 1997). However, the true structure of the PNT domain is that of 4 to 5 helices with no presence of a characteristic HLH motif (Graves and Petersen, 1998). In addition, it has been shown that almost all Ets proteins, with the exception of GABPα, bind to DNA as monomers. However, the ability of the PNT domain of TEL to facilitate dimerization may still be manifest, since in various translocations involving TEL the aberrant chimaeric fusion proteins contain the PNT domain fused to the kinase domain of various non-nuclear signalling molecules (Golub et al., 1994; Golub et al., 1996; McLean et al., 1996). The dimerization of these fusion proteins via the PNT domain allows for the deregulated kinase activity and oncogenic progression in these various human leukaemias.

Only a few Ets proteins have been identified as repressors to date. ERF has been shown to be a strong transcriptional repressor (Sgouras et al., 1995). In *Drosophila*, two repressor Ets proteins, Yan, and an isoform of E74, act as repressors to negatively regulate the activity of genes that are upregulated by activator Ets proteins (Fletcher and Thummel, 1995; Lai and Rubin, 1992).

1.3.2 *ets* genes in cancer

Several *ets* genes have been implicated in human cancers. These genes are either activated through chromosomal translocations that yield mutant fusion proteins, or are overexpressed in the pathologically affected tissue (Dittmer and Nordheim, 1998).

Ewing's sarcoma and related cancers involve a translocation between the *EWS* gene and one of several *ets* genes: *FLI-1*, *ERG*, *ETV1*, *PEA3*, or *FEV* (Arvand and Denny, 2001). In fact, the vast majority of Ewing's sarcomas possess either an EWS-FLI-1 or EWS-ERG fusion protein (95%). In all cases, however, the amino terminus of the EWS protein is juxtaposed to the ETS DNA-binding domain of the respective Ets protein. Studies of EWS-FLI-1 and EWS-PEA3 revealed that the mutant fusion protein has a higher transcriptional activity than the wildtype protein (Crnac, 1997). The increased transcriptional activity may lead to the capacity of EWS-ETS proteins to be oncogenic. Indeed it has been shown that overexpression of EWS-FLI-1 and EWS-ETV1 leads to the oncogenic transformation of NIH 3T3 cells (Thompson et al., 1999). To date, few EWS-ETS specific target genes have been isolated. Interestingly it has been

observed that EWS-FLI-1 represses expression of the TGFβ type II receptor gene promoter (Hahm et al., 1999; Im et al., 2000). Downregulation of this gene may be important in bypassing the growth inhibition and pro-apoptotic functions of TGFβ signalling.

Other translocations in human cancers involve the *ets* gene *TEL*. In most of these situations however it is the amino-terminus of TEL, containing the putative protein-protein interaction PNT domain, that is fused with other gene products, those typically harbouring kinase activity. These include the platelet-derived growth factor receptor (PDGFR), the Abelson kinase (Abl), the Janus kinase 2 (JAK2), and the AML1 proteins (Golub et al., 1994; Golub et al., 1996; Lacronique et al., 1997; McLean et al., 1996). The TEL-PDGFR, TEL-Abl, and TEL-Jak2 fusion proteins possess enhanced tyrosine kinase activity thereby leading to aberrant stimulation of downstream signalling pathways. The translocations yielding TEL-PDGFR and TEL-Abl fusions have been isolated in chronic myelogenous leukemias and the TEL-Jak2 fusion proteins in acute lymphoblastic leukemias.

Overexpression of various *ets* genes has been observed in several human malignancies. *ETS-1* overexpression occurs in a high proportion of hepatocellular carcinomas (Ito et al., 2000). *ETS-1* is also overexpressed in approximately 30% of pancreatic cancers (Ito et al., 2000). Overexpression of an *ets* gene may ultimately lead to dysregulation of one or more target genes. Overexpression of *ETS*-1 has been correlated with upregulation of the urokinase plasminogen activator (*uPA*) gene, which encodes a

serine protease that may be important in invasive and metastatic properties of the tumour itself, as well as in neovascularization (Kitange et al., 2000; Takanami et al., 2001).

Overexpression of several *ets* genes has been observed in carcinomas of the breast and prostate gland. *PEA3* is overexpressed in 76% of human tumour samples as evidenced by *in situ* hybridization, and *PEA3* is overexpressed in 93% of those that are *HER2*-positive (Benz et al., 1997). This high positive coorelation between the HER2 receptor tyrosine kinase and PEA3 implicates the *PEA3* gene as a putative direct target of HER2 signalling. It should also be noted that an ETS DNA-binding site present in the proximal *HER2* promoter is required for efficient expression of the *HER2* gene (Scott et al., 1994). PEA3, Elf-1 and ESX have been shown to bind the *HER2* promoter at this site and affect expression (Scott et al., 2000). In addition, small molecules mimicking the amino acids of the ETS domain involved in DNA-binding are capable of binding to the ETS site in the *HER2* promoter and are able to block gene expression (Chiang et al., 2000). The epithelium-specific *ets* gene, *ESX* (also known as *ESE-1* and *JEN*) is upregulated early in human breast oncogenesis (Chang et al., 1997).

Members of the *PEA3* subfamily *ets* genes—*PEA3*, *ERM* (*ETV5*), and *ER81* (*ETV1*)—are expressed in the vast majority of human breast tumour cell lines and in human colon carcinomas and cell lines (Baert et al., 1997; Crawford et al., 2001). Upregulation of these gene products can lead to their ability to cooperate with Jun, β-catenin and TCF/Lef-1 to stimulate the expression of the target gene matrilysin (MMP-7)

(Crawford et al., 2001). In turn, MMP-7, among other MMPs, may lead to increased tumour cell invasiveness and metastasis.

1.4 The *PEA3* subfamily of *ets* genes

1.4.1 PEA3 subfamily protein structure and function

PEA3 is a member of the Ets family of transcription factors whose members share sequence homology in an ~85 amino acid region encompassing the ETS DNA-binding domain. PEA3 was originally identified as a polyoma virus enhancer binding activity (Martin et al., 1988). Subsequently, the full-length mouse *Pea3* cDNA was cloned from an FM3A mammary adenocarcinoma cell line expression library via its ability to bind to the 5'-AGGAAG-3' polyoma virus enhancer element (Xin et al., 1992). PEA3 is able to transactivate reporter gene expression when several consensus PEA3 motifs are placed upstream of a minimal promoter (Bojovic and Hassell, 2001; Xin et al., 1992). PEA3 binding sites have been observed in the promoters of many genes including growth factors and their receptors (Scott et al., 1994), and proteases and their inhibitors (Matrisian, 1994), and PEA3 is able to transactivate many of these promoters (Benz et al., 1997; Crawford et al., 2001; Higashino et al., 1995). A common theme is the juxtaposition of candidate PEA3 elements with AP-1 transcription factor binding sites in these promoters, and cooperativity both in DNA-binding and transactivation is often observed (Crawford et al., 2001; D'Orazio et al., 1997).

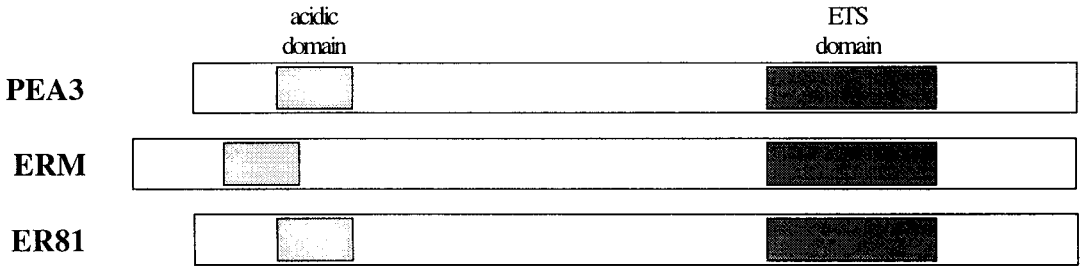
Pea3 is the founding member of the *Pea3* subfamily of *ets* genes, which includes *Er81* (Brown and McKnight, 1992; Monte et al., 1995) and *Erm* (Monte et al., 1994; Monte et al., 1996). The three PEA3 subfamily proteins have greater than 95% homology within their ETS domains, 80% similarity in their acidic domains, and ~50% amino acid sequence identity overall (de Launoit et al., 2000) (Figure 1.2). The human *PEA3* subfamily genes have very similar genomic architecture illustrating that they most likely originated from a single common ancestor (Figure 1.3). It should be noted, however, that after the duplication events occurred, each of the *PEA3* subfamily genes have diverged and are found on different chromosomes in humans (Jeon and Shapiro, 1998).

Each of the PEA3 subfamily proteins functions as a transcriptional activator. The transactivation domain in each is composed of an approximately 40 amino acid stretch of acidic residues. This may form an alpha-helical structure capable of interacting directly with TAFII160 of the transcriptional machinery (Defossez et al., 1997). Since the PEA3 subfamily proteins share greater than 95% amino acid sequence identity within their ETS DNA-binding domains, they more than likely bind to similar promoter elements with similar binding affinity. Ets proteins have been known to cooperate with several other transcription factors in the transcriptional activation of target genes (Crawford et al., 2001; D'Orazio et al., 1997). Both the DNA-binding and transactivation capacities of PEA3 subfamily proteins are negatively regulated by domains which flank the ETS

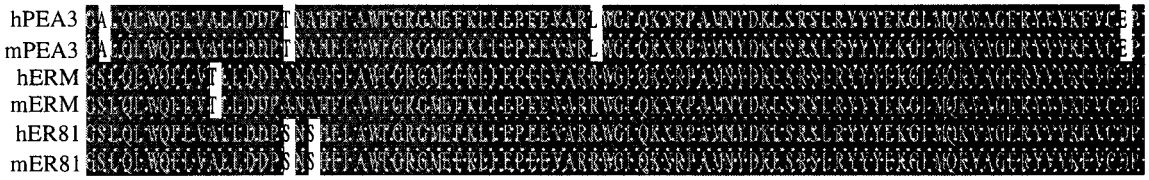
Figure 1.2 Alignment of the PEA3 subfamily proteins.

(***a***) Schematic diagram of PEA3, ERM, and ER81 proteins illustrating the relative size of each translated full-length gene product and common location of the ETS DNA-binding domain (red) and acidic transcriptional activation domain (yellow). The PEA3 subfamily proteins share approximately 50% amino acid identity overall. (***b***) Amino acid alignment of the ETS domain among human and mouse PEA3 subfamily proteins. There is greater than 95% amino acid homology among the three proteins within this domain implying similar specificity in DNA-binding to ETS sites in the promoters of target genes. (***c***) Amino acid alignment of the transcriptional activation domain among human and mouse PEA3 subfamily proteins. There is greater than 85% amino acid homology among the three proteins within this domain implying that similar mechanisms in activating transcription may be utilized. Alignments were derived using the DNAstar MegAlign software program.

a


acidic
domain
ETS
domain
PEA3
ERM
ER81

b


hPEA3
mPEA3
hERM
mERM
hER81
mER81

c

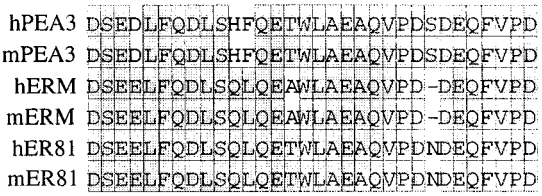
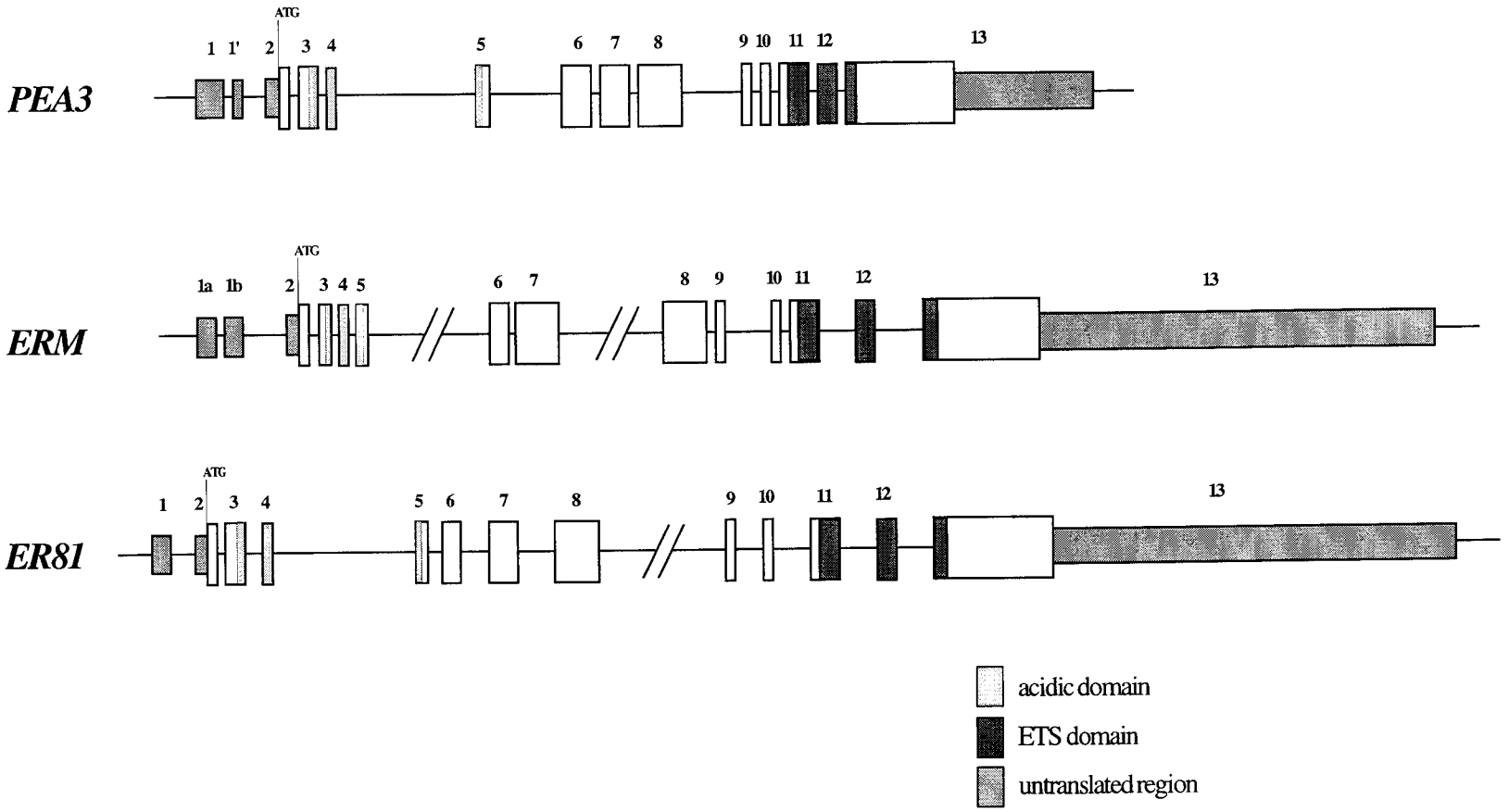

hPEA3 DSEDLFQDLSHFQETWLAEAQVPDSDEQFVPD
mPEA3 DSEDLFQDLSHFQETWLAEAQVPDSDEQFVPD
hERM DSEELFQDLSQLQEAWLAEAQVPD-DEQFVPD
mERM DSEELFQDLSQLQEAWLAEAQVPD-DEQFVPD
hER81 DSEELFQDLSQLQETWLAEAQVPDNDEQFVPD
mER81 DSEELFQDLSQLQETWLAEAQVPDNDEQFVPD

Figure 1.3 The *PEA3* subfamily *ets* genes share a common genomic architecture.

The genomic architecture of the genes for human *PEA3*, *ERM* and *ER81* is illustrated with the approximate relative sizes of the exons (boxes) and introns (lines). Thirteen exons comprise each of the transcribed mRNA species with two different transcriptional start sites present in the *PEA3* and *ERM* genes leading to alternative exon 1 usage. The initiation codon (ATG) is present in exon 2 of each gene with the ETS DNA-binding domain (red) encoded within exons 11 through 13 and the acidic transactivation domain (yellow) within exons 3 through 5. The 5' and 3' untranslated regions are illustrated in gray. It should be noted that the *PEA3* gene is relatively much smaller in size versus *ERM* and *ER81* due to differences in intron length among the *PEA3* subfamily genes, as well as in the size of the 3' untranslated region of exon 13. However, exon size and spacing has been maintained which ultimately yields protein products of similar size and structure (see Figure 1.2). (adapted from de Launoit et al., 2000)


PEA3
ATG
1 1' 2 3 4 5 6 7 8 9 10 11 12 13
ERM
ATG
1a 1b 2 3 4 5 6 7 8 9 10 11 12 13
ER81
ATG
1 2 3 4 5 6 7 8 9 10 11 12 13
acidic domain
ETS domain
untranslated region

DNA-binding and acidic transactivation domains, respectively (Bojovic and Hassell, 2001; Janknecht, 1996; Janknecht et al., 1996). Negative regulation of DNA-binding has been identified in other Ets proteins, and may involve conformational changes in the amino- and carboxyl-terminal helices flanking the ETS DNA-binding domain (Graves and Petersen, 1998). The mechanism by which this negative regulation of transactivation by the PEA3 subfamily proteins is alleviated is unknown as of yet.

Many transcription factors are regulated at the post-translational level by phosphorylation. Ets proteins, such as Ets-2 and Elk-1, may be regulated in this manner by cytoplasmic signalling components (Wasylyk et al., 1998). There is also some evidence that PEA3 subfamily proteins may be downstream targets of cytoplasmic signalling pathways involved in regulating their transactivation capacity. PEA3 is a phosphoprotein and is phosphorylated primarily on serine residues (O'Hagan et al., 1996), with 50% of its total phosphorylation comprising only three proline-directed serine residues, putative sites for MAP kinase phosphorylation (R. Tozer and J. A. Hassell, unpublished). *In vitro* phosphorylation of GST-PEA3 by recombinant ERKs and JNKs results in phosphopeptide maps resembling transient expression of PEA3 *in vivo* (R. Tozer and J. A. Hassell, unpublished). So too, are both ERM and ER81 proteins phosphorylated by MAP kinases and protein kinase A (PKA) (Janknecht, 1996; Janknecht et al., 1996) and their potential for transcriptional activation is increased by PKA-mediated phosphorylation (Baert et al., 2002).

1.4.2 *Pea3* subfamily gene expression

Of the three *PEA3* subfamily genes, *ERM* has the most widespread expression pattern, with expression in most adult mouse and human tissues analyzed (Monte et al., 1994). Alternatively, *PEA3* and *ER81* show more limited mRNA expression in these organisms (Monte et al., 1994; Monte et al., 1995; Xin et al., 1992). *Pea3* is expressed primarily in the adult mouse brain and male epididymis, with lower expression in the mammary gland. Each *Pea3* subfamily member is expressed in developing mouse embryos, with *Pea3* and *Erm* showing overlapping expression patterns early in development, suggesting redundancy during these stages (Chotteau-Lelievre et al., 1997; Chotteau-Lelievre et al., 2001)(M. Laing and J. A. Hassell, unpublished). However, *Pea3* and *Erm* expression patterns diverge later in embryogenesis. *Er81* on the other hand has an entirely different expression pattern indicating more divergent function during these stages (Chotteau-Lelievre et al., 1997)(M. Laing and J. A. Hassell, unpublished). Expression of *Pea3* and *Erm* occurs in many epithelial tissues, especially those which involve branching morphogenesis, such as the lung, salivary gland, mammary gland and kidney (Chotteau-Lelievre et al., 1997). These genes are also expressed in developing migratory neurons. The possible involvement of *Pea3* and *Erm* genes in the invasion and migration of cells is strengthened by the observation that the PEA3 subfamily proteins can regulate many genes involved in these processes (de Launoit et al., 2000).

1.4.3 Targeted disruption of the *Pea3* subfamily genes in mice

Although *Pea3-null* mice are viable, male *Pea3*$^{-/-}$ mice are unable to mate (Laing et al., 2000). It appears that PEA3 may function in the stimulation of the smooth muscle required for normal penile function in mating, since the male reproductive organs are morphologically normal and spermatogenesis occurs to yield sperm capable of *in vitro* fertilization. Virgin female *Pea3*$^{-/-}$ mice have reduced ductal branching of their mammary glands although this does not inhibit the ability of mothers to successfully nurse their offspring (MacNeil, 1999). Analyses of *Erm*- and *Er81-null* animals is ongoing, however *ER81*$^{-/-}$ mice do not live longer than a few weeks of age (Arber et al., 2000), and *Erm*$^{-/-}$ mice are embryonic lethal (N. Kurpios, S. Arber and J. A. Hassell, unpublished).

1.4.4 The *PEA3* subfamily in breast cancer

Pea3 mRNA is overexpressed in mammary adenocarinomas induced by HER2/Neu and PyVMT, as well as in the resultant lung metastases (Trimble et al., 1993). *PEA3* is also overexpressed in a majority of human breast tumour samples as evidenced by *in situ* hybridization (Benz et al., 1997). In addition, there is a strong positive correlation (greater than 90%) between *HER2* overexpression and that of *PEA3* in these samples. PEA3 itself may be involved in the regulation of the *HER2* gene since a candidate ETS site is required for efficient expression of *HER2*, and the ability of PEA3, among other Ets proteins, to bind to and transactivate the *HER2* promoter requires this

site (Benz et al., 1997; Scott et al., 1994). Interestingly, PEA3 has been shown to repress the *HER2* promoter in cell culture, as well as reduce the tumorigenic potential of HER2/Neu in cell culture and nude mice (Xing et al., 2000). This contradicts most published data that PEA3 functions to activate transcription (Benz et al., 1997; Bojovic and Hassell, 2001; Crawford et al., 2001; Xin et al., 1992), in addition to its high prevalence for overexpression in *HER2*-positive breast tumours in mice and humans (Benz et al., 1997; Trimble et al., 1993). Expression of the *PEA3* subfamily has also been observed in human mammary epithelial cell lines and in a majority of human breast tumour cell lines (Baert et al., 1997). Strikingly, ectopic expression of PEA3 in the nonmetastatic MCF7 human breast cancer cell line renders these cells invasive (Kaya et al., 1996) via the induction of several matrix metalloproteinases involved in extracellular matrix breakdown and remodelling (Higashino et al., 1995). Upregulation of one or more of the *PEA3* subfamily members and their subsequent deregulation of target genes may be important in the invasive and metastatic properties of breast cancer cells.

1.5 Objectives and experimental rationale

A. Construct and characterize various putative dominant-negative mutants of the Ets transcription factor PEA3

B. Generate and characterize lines of transgenic mice that overexpress PEA3, dominant-negative PEA3, and a putative oncogenic variant of PEA3, EWSΔN268PEA3, in the mammary gland of female mice

C. Generate bitransgenic females by mating MMTV-PEA3, and MMTV-ΔNPEA3En, transgenic mice with MMTV-*neu* transgenic mice to study the role of *Pea3*, and its related *ets* subfamily genes, *Erm* and *Er81* in mammary tumorigenesis

D. Assess the expression of *Pea3*, *Erm*, and *Er81* during normal mouse mammary gland development and in mammary tumours of MMTV-*neu* transgenic female mice

To meet these objectives, the first step entailed deriving various putative dominant-inhibitory mutants of the PEA3 protein. PEA3, as do most Ets transcription factors, binds to DNA at specific ETS sites within the promoters of target genes to positively modulate gene expression. To verify whether the putative dominant-negative PEA3 mutants could block the function of PEA3, and its related *ets* subfamily members ERM and ER81, analyses were performed in a standard cell culture setting using transient transfection techniques and specific PEA3-responsive luciferase reporter plasmids. In addition, focus forming assays were performed to test the role of PEA3 in cellular

transformation mediated by an oncogenic variant of the Neu receptor tyrosine kinase, NeuNT.

These assays were extended *in vivo* via the generation of various lines of transgenic mice by utilizing the mammary epithelial specific promoter and enhancer elements of the mouse mammary tumour virus. The MMTV-LTR has been widely used as a well-characterized means of targeting transgene expression to the mammary gland of female mice. Targeting the expression of PEA3, dominant-negative PEA3, and an oncogenic variant of PEA3 (EWSΔN268PEA3) to the mammary epithelium of female FVB/N mice would enable a better characterization of the function of PEA3 subfamily proteins in mammary gland development and differentiation.

Since *Pea3* is overexpressed in mammary adenocarcinomas and lung metastases of MMTV-*neu* and MMTV-PyVMT transgenic female mice, a more extensive analysis of *ets* gene expression during all stages of normal mammary gland development and in several MMTV-*neu*-induced mammary tumours was undertaken. Again, to better understand the role of the *Pea3* subfamily in mammary tumorigenesis, MMTV-PEA3 and MMTV-ΔNPEA3En transgenic mice were used in crosses with MMTV-*neu* transgenics and analyses of tumour onset, tumour burden, and frequency of lung metastasis were scored. The combination of cell culture techniques and the *in vivo* MMTV-based transgenic mouse model system has extended our understanding of the role of the *Pea3* subfamily genes in the normal developmental processes and in the oncogenically transformed state of the female mammary gland.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Plasmid DNA constructs

The pCANmyc/ΔNPEA3 expression plasmid was made by PCR amplification of the 3' end of the PEA3 open reading frame using the primers 5'-CGG GAT CCC GGG GTG CCT TAC AA-3' and 5'-CCC TCG AGC TAG TAA GAA TAT CCA CC-3' and pGEM7/PEA3 as a template (Laing, 1998). The resultant PCR product was digested with *Bam*HI and *Xho*I (sites are underlined in primer sequences) and ligated to *Bam*HI-, *Xho*I-digested pCANmyc1 vector (provided by Dr. Paul Polakis; ONYX Pharmaceuticals, San Francisco) to yield the pCANmyc/ΔNPEA3 expression plasmid.

The pCANmyc/PEA3En expression plasmid was made by PCR amplification of the 3' end of the PEA3 open reading frame using the primers 5'-CGC CCT GAT CAA ACA GGA GCG CAC-3' and 5'-CCC GAA TTC GTA AGA ATA TCC ACC-3' and pCANmyc/PEA3 (Bojovic and Hassell, 2001) as a template. The resultant PCR product was digested with *Bcl*I and *Eco*RI. The 5' end of the PEA3 open reading frame was prepared by digesting pCANmyc/PEA3 (isolated from *dam*$^-$/*dcm*$^-$ SCS110 bacterial cells; Stratagene) with *Hin*dIII and *Bcl*I. The 3' PEA3 *Bcl*I-*Eco*RI PCR fragment and the 5' PEA3 *Hin*dIII-*Bcl*I fragment were ligated simultaneously into *Hin*dIII-, *Eco*RI-digested pCANmyc/En plasmid to yield the pCANmyc/PEA3En expression plasmid.

The pCANmyc/ΔNPEA3En expression plasmid was generated by PCR amplification of the 3' end of the mouse PEA3 open reading frame using the primers 5'-CGG GAT CCC GGG GTG CCT TAC AA-3' and 5'-CCC GAA TTC GTA AGA ATA TCC ACC-3' and the pGEM7/PEA3 plasmid as a template. The *Drosophila* En repression domain sequence was generated by PCR amplification using the primers 5'-CCC GAA TTC GCC CTG GAG GAT CGC TGC-3' and 5'-CCG CTC GAG CTA GGA TCC CAG AGC AGA TTT CTC AGG-3' and the pSCDMS/MEnT plasmid (provided by Dr. Ali Fattaey) (Badiani et al., 1994) as a template. The ΔNPEA3 PCR product was digested with *Bam*HI and *Eco*RI (sites in primers are underlined) and ligated into *Bam*HI-, *Eco*RI-digested pCANmyc1 vector to generate pCANmyc/ΔNPEA3. This construct differs from the one described above since it does not possess a stop codon after the PEA3-encoding sequence. The En PCR product was digested with *Eco*RI and *Xho*I (sites in primers are underlined) and ligated into *Eco*RI-, *Xho*I-digested pCANmyc/ΔNPEA3 vector to generate pCANmyc/ΔNPEA3En.

The pCANmyc/En expression plasmid was generated by PCR amplification of the En repression domain sequence using the primers 5'-CCC GAA TTC GCC CTG GAG GAT CGC TGC-3' and 5'-CCG CTC GAG CTA GGA TCC CAG AGC AGA TTT CTC AGG-3' and the pSCDMS/MEnT plasmid as a template. The resultant PCR product was digested with *Eco*RI and *Xho*I (sites in primers are underlined) and ligated into *Eco*RI-, *Xho*I-digested pCANmyc1 vector to yield pCANmyc/En.

The pCANmyc/ΔPU.1 expression vector was generated by PCR amplification of the 3' end of the PU.1 open reading reading frame using the primers 5'-CCC GGA TCC

ACA GGC AGC AAG AAA AAG ATT CGC-3' and 5'-CCC GAA TTC GTG GGG CGG GAG GCG CCG CTC-3' and the pGEX/PU.1 plasmid (generated by S. Bowman and J. A. Hassell, unpublished) as a template. The PU.1 PCR product was digested with *Bam*HI and *Eco*RI (sites in primers are underlined) and ligated to *Bam*HI-, *Eco*RI-digested pCANmyc1 plasmid to yield pCANmyc/ΔPU.1.

The pCANmyc/ΔNPU.1En expression vector was generated by PCR amplification of the 3' end of the PU.1 open reading frame using the primers 5'-CCC GGA TCC ACA GGC AGC AAG AAA AAG ATT CGC-3' and 5'-CCC GAA TTC GTG GGG CGG GAG GCG CCG CTC-3' and the pGEX/PU.1 plasmid as a template. The sequence of this PCR product lacks a stop codon after the codon representing the carboxyl-terminal amino acid of PU.1. This PU.1 PCR product was digested with *Bam*HI and *Eco*RI (sites in primers are underlined) and ligated to *Bam*HI-, *Eco*RI-digested pCANmyc1 to generate pCANmyc/ΔPU.1. This pCANmyc/ΔPU.1 plasmid was subsequently digested with *Hind*III and *Eco*RI and ligated to *Hind*III-, *Eco*RI-digested pCANmyc/En to generate the pCANmyc/ΔPU.1En expression vector.

Construction of the pCANmyc/PEA3 expression vector has been described previously (Bojovic and Hassell, 2001). The pCANmyc/ERM expression vector was generated by reverse transcription of MMTV-*neu* tumour RNA using random hexamers followed by PCR amplification of the mouse ERM cDNA encoding the open reading frame using the primers 5'-CCC AAG CTT GGA AGC ACC ATG GAT GGG-3' and 5'-CGG GAT CCG CTA GCT CTA GGG TGT G-3'. The ERM cDNA PCR product was digested with *Cla*I and *Xba*I (sites in primers are underlined) and ligated into *Cla*I-, *Xba*I-

digested pBluescript II KS plasmid (Stratagene). This plasmid was then digested with *Cla*I and *Xba*I and ligated into *Cla*I-, *Xba*I-digested pCANmyc1 plasmid to generate pCANmyc/ERM. The pCANmyc/ERM expression plasmid was generated by Natasza Kurpios, a graduate student in the lab of Dr. J. A. Hassell. The pCANmyc/ER81 expression vector was generated by PCR amplification of the open reading frame of mouse ER81 using the primers 5'-CGG AAT TCG GCG GAG ATG GAT TTA TGA CCA GCA A-3' and 5'- CGC TCG AGC TTG ACG GGT TAC TCA TGT TA-3' and the pBS/ER81 plasmid (provided by Dr. Tom Jessell, Columbia University, New York) as a template. The resultant ER81 PCR product was digested with *Eco*RI (site in primer is underlined) and *Bam*HI (internal restriction site) and this fragment encompassing the 5' end of the ER81 open reading frame was ligated into *Eco*RI-, *Bam*HI-digested pBluescript II KS plasmid (pBS/ER81-5'). The *Bam*HI-*Xba*I fragment representing the 3' end of the ER81 open reading frame was isolated from pBS/ER81 and ligated into *Bam*HI-, *Xba*I-digested pBS/ER81-5' plasmid (pBS/ER81-5'-3'). The *Eco*RI-*Eco*RI fragment representing the complete open reading frame of ER81 was isolated from pBS/ER81-5'-3' and ligated to *Eco*RI-digested pCANmyc1 plasmid, and correct orientation of ER81 was subsequently verified by *Bam*HI digestion.

Generation of the pJ4Ω/NeuNT expression vector has been described previously (Siegel et al., 1994).

The MMTV-PEA3-SV40pA transgene plasmid was generated by first digesting pGEM7/PEA3 with *Hin*dIII and *Sac*I and ligating the full-length PEA3 cDNA into *Hin*dIII-, *Sac*I-digested pGEM4 plasmid (Promega). The pGEM4/PEA3 plasmid was

then digested with *Hin*dIII and *Eco*RI and the PEA3 cDNA fragment was ligated into *Hin*dIII-, *Eco*RI-digested MMTV-SV40 plasmid (Guy et al., 1992) to yield MMTV-PEA3-SV40pA.

The MMTV-ΔNPEA3En-SV40pA expression vector was generated by digestion of pCANmyc/ΔNPEA3En with *Xho*I, filling in the 3'-OH recessed end using Klenow DNA polymerase, and then digesting with *Hin*dIII. The MMTV-SV40 vector was digested with *Eco*RI, the 3'-OH recessed end was filled using Klenow, and then digested with *Hin*dIII. The ΔNPEA3En DNA fragment was ligated into the digested MMTV vector to generate MMTV-ΔNPEA3En-SV40pA.

Construction of the MMTV-EWSΔN268PEA3-SV40pA expression vector has been described previously (Crnac, 1997).

The 3xPEA3-*luc* reporter plasmid was generated by isolating three of the four PEA3 elements and AdMLP-TATA from 4xPEA3-CAT (Xin et al., 1992) by digestion with *Bam*HI and *Eco*RI. This fragment was cloned into *Bam*HI-, *Eco*RI-digested pBluescript II KS, then subsequently digested with *Bam*HI and *Hin*dIII and cloned into *Bgl*II-, *Hin*dIII-digested pGL3-Basic (Promega) to generate 3xPEA3-*luc*.

The *uPA-luc* reporter plasmid has been described previously (provided by Dr. Craig Hauser) (Stacey et al., 1995). This reporter contains the enhancer element of the *uPA* gene comprising the PEA3-AP1 composite site juxtaposed to the proximal *uPA* promoter upstream of the luciferase gene in the pGL2-Basic vector.

The 5xPEA3-*luc* reporter plasmid has been described previously (Bojovic and Hassell, 2001). This reporter contains 5 copies of the optimal PEA3 binding site (termed 4_4; S. Bowman and J. A. Hassell, unpublished) upstream of the AdMLP-TATA sequence and the luciferase gene of the pGL3-Basic vector.

The 5xGAL4-*luc* reporter plasmid and the pSG424-GAL4-VP16 expression vector have been described previously (Bojovic and Hassell, 2001).

The MMTV-*luc* reporter plasmid was generated by digesting the pGL3-Basic vector with *Hin*dIII and *Xba*I to initially isolate the luciferase gene. The *Hin*dIII-*Xba*I luciferase DNA fragment was first cloned into the pSL301 cloning vector (Stratagene), subsequently digested with *Hin*dIII and *Eco*RI, and the *Hin*dIII-*Eco*RI DNA fragment was ligated to the *Hin*dIII-, *Eco*RI-digested MMTV-SV40 plasmid to yield MMTV-*luc*.

The *Pea3* riboprobe template was generated by isolating the *Dra*I-*Pvu*II fragment from pGEM7/PEA3.9 (Xin et al., 1992) encompassing nucleotides 1445-1941 of mouse *Pea3* and cloning it into *Eco*RV-digested pBluescript II KS (pBS/PEA3DP). The pBS/PEA3DP plasmid was linearized with *Bam*HI to generate an antisense *Pea3* probe using T3 RNA polymerase. The *Erm* riboprobe template was generated by isolating the *Eco*RI-*Pst*I fragment from pCRII/ERM (provided by Dr. Yves de Launoit) (Chotteau-Lelievre et al., 1997) encompassing nucleotides 162-438 of mouse *Erm* and cloning it into *Eco*RI-, *Pst*I-digested pSL301 (pSL/ERM280). The pSL/ERM280 plasmid was linearized with *Hin*dIII to generate an antisense *Erm* probe using T3 RNA polymerase. The *Er81* riboprobe template was generated by isolating the *Hin*dIII-*Bam*HI fragment from pBS/ER81 encompassing nucleotides 945-1197 of mouse *Er81* and cloning it into

pBluescript II KS (pBS/ER81250). The pBS/ER81250 plasmid was linearized with *Hin*dIII to generate an antisense *Er81* probe using T7 RNA polymerase.

The *ets-1* riboprobe template was generated by PCR amplification using the primers 5'-CCC AAG CTT CCG TCG ATC TCA AGC CGA CTC-3' and 5'-CCC AAG CTT TGT CTG CAA GGT GTC TGT CTG G-3' and cDNA reverse transcribed from mouse thymus tissue RNA. The PCR product encompassing nucleotides 135-787 of mouse *Ets-1* was cloned into pCR2.1 vector (Invitrogen) then digested with *Hin*dIII and cloned into pBluescript II KS (Stratagene). The plasmid was linearized with *Xho*I to generate an antisense *Ets-1* probe using T7 RNA polymerase. The *Ets-1* riboprobe plasmid was generated by Michelle Szrajber, a former undergraduate student in the lab of Dr. J. A. Hassell. The *Ets-2* riboprobe template (provided by Dr. Robert Oshima) has been described previously (Yamamoto et al., 1998). The plasmid was linearized with *Eco*RI to generate an antisense *Ets-2* probe using SP6 RNA polymerase. The *Gabpα* riboprobe template was generated by PCR amplification using the primers 5'-CCC AAG CTT GCT GAA TGT GTA AGC CAG GCC-3' and 5'-CCC AAG CTT TCT GTA GCC TTC CAG TGC AGC-3' and cDNA reverse transcribed from virgin FVB mouse mammary gland RNA. The PCR product encompassing nucleotides 690-975 of mouse *Gabpα* was cloned into pCR2.1 vector then digested with *Hin*dIII and cloned into *Hin*dIII-digested pBluescript II KS. The plasmid was linearized with *Bam*HI to generate an antisense *Gabpα* probe using T3 RNA polymerase. The *Gabpα* riboprobe plasmid was generated by Michelle Szrajber.

The *WAP* and *β-casein* riboprobe plasmids (provided by Dr. Bill Muller) have been described previously (Guy et al., 1992; Robinson et al., 1995). The mouse *WAP* riboprobe plasmid was linearized by digestion with *Hin*dIII to generate an antisense *WAP* probe using T7 RNA polymerase. The mouse *β-casein* riboprobe plasmid was linearized with *Bam*HI to generate an antisense *β-casein* probe using SP6 RNA polymerase.

The *rpL32* riboprobe template has been described previously (Trimble et al., 1993). The rpL32 27-2-3 plasmid was linearized with *Xba*I to generate an antisense *rpL32* probe using T3 RNA polymerase.

The *neu* riboprobe template has been described previously (Siegel et al., 1994). The pSL/neu plasmid was linearized with *Sma*I to generate an antisense *neu* probe using T7 RNA polymerase.

The SPA riboprobe template has been described previously (Guy et al., 1992). The pGEM/SPA plasmid was linearized with *Hin*dIII to generate an antisense SPA probe using SP6 RNA polymerase. This plasmid was also used to generate the SPA probe used in Southern blotting analysis of transgenic mouse genomic DNA.

The pBS/PEA3 3'UTR plasmid was generated by digesting the pGEM7/PEA3.9 plasmid with *Sac*I and *Eco*RI to isolate the 3' end of the *Pea3* cDNA sequence. The *Sac*I-*Eco*RI fragment of *Pea3* was ligated into *Sac*I-, *Eco*RI-digested pBluescript II KS to generate pBS/PEA3 3'UTR. pBS/PEA3 3'UTR was used to generate the *Pea3* probe used in Southern blotting analysis of *Pea3*-null mouse genomic DNA.

2.2 Reverse transcription (RT) and polymerase chain reaction (PCR)

Reverse transcription reactions were carried out using SUPERSCRIPT™ II reverse transcriptase (GibcoBRL/Life Technologies) as per manufacturer's instructions. One μg of total RNA that had been previously passed through a column using the RNeasy Mini Kit (Qiagen) was incubated at 65°C for 5 minutes with 50 ng of Random Hexamers (Roche Biochemicals) and 1 μL of 10 mM dNTP mix (Amersham Pharmacia) in a total volume of 12 μL. After a brief chill on ice, 4 μL of 5x First Strand Buffer (GibcoBRL/Life Technologies), 2 μL of 0.1 M DTT, and 40 U of RNaseOUT Ribonuclease Inhibitor (GibcoBRL/Life Technologies) were added, the sample was incubated at 42°C for 2 minutes, 200 U of SUPERSCRIPT™ II (GibcoBRL/Life Technologies) was added, and the reaction was allowed to proceed for 50 minutes at 42°C. The reactions were terminated by incubation at 70°C for 15 minutes then used in subsequent PCR rections.

PCR reactions were performed using Taq DNA polymerase (GibcoBRL/Life Technologies) as per manufacturer's instructions. Approximately 50 ng of plasmid DNA template, or 1 μL of reverse transcription reaction, was PCR amplified in a reaction volume of 100 μL containing 1x PCR Reaction Buffer (GibcoBRL/Life Technologies), 1 mM dNTP mix (Amersham Pharmacia), 1 μM of each primer, and 1 U of recombinant Taq DNA polymerase (GibcoBRL/Life Technologies). Several different concentrations of $MgCl_2$ were used, ranging between 1 and 3 mM, depending on the requirements of the primer pair used in amplification. All PCR reactions were performed using a Perkin Elmer GeneAmp PCR System 9600. Samples were incubated at 95°C for 30 s, followed

by 30 to 35 cycles of PCR (melting for 30 s at 95°C, annealing for 30 s at 55 to 60°C, and extension for 30 s to 1 minute at 72°C). The annealing temperature varied depending upon the requirements for each primer pair. A portion (~10%) of the PCR products were electrophoresed on an ethidium bromide stained agarose gel and visualized on a UV transilluminator. Products that were to be used in cloning were phenol:chloroform extracted, precipitated with 2 volumes of absolute ethanol/0.1 volume 3 M sodium acetate pH 5.4, and resuspended in TE pH 8.

Oligonucleotides used as primers in PCR amplification reactions were generated in the MOBIX Central Facility by Dinsdale Gooden. Lyophilized oligonucleotides were resuspended in sterile Millipore-H_20 to a concentration of 100 μM and stored at –20°C until use.

All DNA sequences generated by PCR amplification were verified by PCR-based automated DNA sequencing carried out in the MOBIX Central Facility by Brian Allore.

2.3 DNA ligations and bacterial transformations

All cloning steps were performed using an in-gel ligation protocol (Struhl, 1985). Briefly, restriction endonuclease-digested DNA was electrophoresed on a low-melting point agarose/1xTAE gel and the appropriate fragments were excised using a sterile scalpel. Ligations were performed at room temperature overnight in a total reaction volume of 40 μL. The total amount of gel per ligation reaction was restricted to a maximum of 15 μL to prevent solidification of the sample at room temperature. The

following day the ligations were heated at 65°C for 10 minutes after which 80 µL of ice-cold TCM buffer (10 mM Tris-Cl pH 7.5, 10 mM $CaCl_2$, 10 mM $MgCl_2$) was added.

Transformations were performed using 20 µL of ligation reaction per 100 µL of commercially available competent bacteria, or 1 µL of ligation reaction per 40 µL of freshly prepared electrocompetent bacteria. Transformations using commercially available competent bacteria (XL2-Blues Ultracompetent Cells, Stratagene; Maximum Efficiency DH5α Competent Cells, GibcoBRL/Life Technologies) were performed as per manufacturer's instructions. Electrocompetent bacteria were prepared by inoculating 100 mL of LB with an actively growing overnight culture of XL2-Blues. When the absorbance at 595 nm reached between 0.4 and 0.5, the bacteria were pelleted by centrifugation at 2 500 rpm for 10 minutes at 4°C. The cells were resuspended gently in 100 mL ice-cold sterile Millipore-H_20, incubated on ice for 5 minutes, then centrifuged at 2 500 rpm for 10 minutes at 4°C. The cells were resuspended gently in 50 mL of ice-cold sterile Millipore-H_20, incubated on ice for 5 minutes, then centrifuged at 2 500 rpm for 10 minutes at 4°C. The cells were resuspended in 20 mL of ice-cold 10% sterile glycerol, incubated on ice for 5 minutes, then centrifuged at 2 500 rpm for 10 minutes at 4°C. The supernatant was carefully decanted and the remaining glycerol in the tube (~500 µL) was used to resuspend the cells. 40 µL of bacteria was added to 1 µL of ligation reaction and placed into a chilled *E. coli* electroporation cuvette (Biorad) which was kept at 4°C until electroporation was performed. Electroporations were performed using a Biorad Gene Pulser™ and Pulse Controller set at 2.25 kV, 25 µF, and 200 Ω with a time constant of approximately 4.5. After the pulse, 960 µL of ice-cold LB was added to the cuvette, the

contents were decanted into a microcentrifuge tube, and placed at 37°C for one hour. The bacteria were centrifuged at 13 000 rpm for 20 seconds, approximately 900 µL of LB was removed, and the bacteria were carefully resuspended in the remaining LB. The transformations were plated onto 100 mm LB-agar plates containing 100 µg/mL ampicillin and placed at 37°C overnight to allow growth of transformed bacteria. The resultant bacterial colonies were screened by alkaline lysis miniprep of plasmid DNA which was analyzed by standard restriction endonuclease digestions.

2.4 Plasmid DNA isolation

For alkaline lysis minipreparation of plasmid DNA, single bacterial colonies were inoculated into 5 mL of LB media containing 100 µg/mL ampicillin and allowed to grow overnight at 37°C while shaking at 225 rpm. Approximately 1.5 mL of culture was decanted into a microcentrifuge tube and centrifuged at 13 000 rpm for 20 seconds. The supernatant was aspirated and the bacterial pellets were resuspended in 100 µL of ice-cold GTE solution (50 mM glucose, 25 mM Tris-Cl pH 8, 10 mM EDTA pH 8) containing 100 µg/mL RNase A by vortexing. 200 µL of 200 mM NaOH/1% SDS solution was added and the tubes were gently inverted several times then placed on ice for 5 minutes. 150 µL of 3 M potassium acetate solution was added, the tubes were inverted several times, and again placed on ice for 5 minutes. The samples were then microcentrifuged for 5 minutes at 13 000 rpm and each supernatant (approximately 400 µL) carefully transferred to a fresh microcentrifuge tube. 800 µL of 95% ethanol was added to each supernatant and the tubes were shaken vigorously for several seconds.

After incubation at room temperature for 2-to-3 minutes the samples were microcentrifuged for 2 minutes at 13-14 000 rpm. The supernatants were aspirated completely and DNA pellets resuspended in 50 µL of TE pH 8.0 buffer. 5 µL of each miniprep sample was analyzed by standard restriction endonuclease digestion in a 20 µL reaction volume followed by agarose gel electrophoresis and visualization of ethidium bromide staining over a long-wave ultraviolet transilluminator.

For large-scale preparation of plasmid DNA, 500 mL of LB containing ampicillin (100 µg/mL) was inoculated with a single transformed bacterial colony and allowed to grow overnight at 37°C with shaking. The next day the bacteria were pelleted by centrifugation at 4 000 rpm for 10 minutes and resuspended in 10 mL of ice-cold GTE solution. 20 mL of 200 mM NaOH/1% SDS solution was added and the samples were gently inverted several times then placed on ice for 10 minutes. 15 mL of ice-cold 3 M potassium/5 M acetate solution was added, the samples were inverted several times and placed on ice for 10 minutes. The samples were centrifuged at 4 500 rpm for 20 minutes, the supernatant transferred to a separate tube, and 0.7 volumes of isopropanol was added. The samples were mixed vigorously and left at room temperature for approximately 30 minutes, after which the samples were centrifuged for 30 minutes at 4 500 rpm. The supernatant was aspirated and the pellets were allowed to dry briefly, after which they were resuspended in 8 mL TE pH 8.0. 9.6 g of cesium chloride was added to each sample, loaded into a Beckman ultracentrifuge tube with 200 µL of 5 mg/mL ethidium bromide, and centrifuged overnight at 55 000 rpm and 16°C under vacuum using a Beckman V Ti 65.1 rotor and H class Beckmann ultracentrifuge. The following day the

tubes were carefully removed from the rotor and the ethidium bromide-stained supercoiled plasmid DNA bands were extracted using a 3 mL syringe and 18 gauge needle. The ethidium bromide was removed from the DNA by several extractions using 1 M NaCl-saturated butanol until no visible staining persisted. The volume was increased to 4 mL with TE pH 8.0 and 400 µL of 3 M sodium acetate and 8 mL of absolute ethanol were added to precipitate the DNA. The samples were centrifuged for 30 minutes at 7 500 rpm and 4°C, the pellets were washed once with 70% ethanol, and allowed to dry briefly. The pellets were resuspended in 8 mL of TE pH 8.0 and prepared for a second cesium chloride centrifugation as described above. The final DNA pellets were resuspended in 0.5-1.0 mL of TE pH 8.0 and their concentration was determined by measuring absorbance at 260 nm using a Beckmann spectrophotometer. Each plasmid prep was verified by standard restriction endonuclease digests followed by agarose gel electrophoresis and visualization of ethidium bromide staining over a long-wavelength UV transilluminator.

For preparation of large-scale plasmid DNA that was not to be used in transfections or embryo microinjections, DNA isolation was performed using QIAfilter™ Plasmid Maxi Kit (Qiagen) as per manufacturer's instructions.

2.5 RNA isolation

Total RNA extraction from mouse tissues was performed as described previously (Chirwigin et al., 1979). Mouse tissues were homogenized immediately after dissection in Tissue Guanidinium Solution (5 M guanidinium isothiocyanate, 50 mM Tris-Cl pH 7.5, 10 mM EDTA pH 8, 0.72 M β-mercaptoethanol) by two 10-second pulses at setting 4 on

a Brinkman Teflon homogenizer. The homogenized tissue samples were ultracentrifuged overnight through a DEPC-treated 5.7 M cesium chloride/10 mM EDTA cushion at 32 000 rpm and 20°C under vacuum using a Beckman SW-41.Ti rotor and Beckman H class ultracentrifuge. RNA pellets were resuspended in Tissue Resuspension Buffer (5 mM EDTA, 0.5% Sarkosyl, 0.72 M β-mercaptoethanol), extracted with phenol:chloroform, then chloroform, and precipitated with 2 volumes absolute ethanol/0.1 volume DEPC-treated 3 M sodium acetate. The resultant RNA pellet was resuspended in DEPC-treated Millipore-H_20 and stored at –80°C until use.

For cultured cells, RNA was extracted from five 100 mm dishes of 90-95% confluent cells with TRIZOL® Reagent (GibcoBRL/Life Technologies) as per manufacturer's instructions.

2.6 Ribonuclease (RNase) protection assays

RNase protection assays were performed as described previously (Siegel et al., 1994). Riboprobe templates were prepared by digestion of 10 μg plasmid DNA with the appropriate restriction endonuclease (see section 2.1). Digested DNA was then extracted in phenol:chloroform extraction, precipitated with 2 volumes absolute ethanol/0.1 volume sodium acetate, washed in 70% ethanol, and resuspended in 20 μL DEPC-treated Millipore-H_20. *In vitro* transcription was carried out at 37°C using 0.5 μg of DNA template, 0.4 mM each of ATP, CTP, and GTP, 0.04 mM UTP, 50 μCi α^{32}P-UTP, 40 U RNaseOUT RNase inhibitor (Gibco/BRL), and 20 U of the appropriate RNA polymerase. After 45 minutes an additional 10 nmol of UTP was added to the reaction. The DNA

template was subsequently digested with 20 U of RNase-free DNase I (Roche Biochemicals) for 15 minutes, 10 µg of yeast tRNA added, phenol:chloroform extracted, and precipitated with 7.5 volumes absolute ethanol/2 volumes DEPC-treated 2.5 M ammonium acetate. The radiolabeled riboprobes were purified by electrophoresis on a 6% polyacrylamide/7 M urea/1xTBE gel followed by excision of the gel slice and elution overnight at 37°C in elution buffer (500 mM sodium acetate, 1 mM EDTA, 0.1% SDS). The riboprobes were then precipitated from the eluate through addition of 10 µg yeast tRNA and 2.5 volumes absolute ethanol, followed by a wash with 70% ethanol/0.1 M sodium acetate and resuspension in hybridization buffer (5:1 formamide:10x Pipes buffer, 0.4 M Pipes pH 6.4, 4 M NaCl, 10 mM EDTA). Riboprobes were quantified using a scintillation counter and 50 000 cpm of each probe diluted in hybridization buffer was added to the RNA samples, to a final reaction volume of 40 µL (50% formamide, 1x Pipes buffer, final concentrations). Hybridization was carried out overnight at 50°C. RNase digestion of the hybridization reactions was performed using 15 µg RNase A/4.5 U RNase T1 (Roche Biochemicals) diluted in digestion buffer (300 mM NaCl, 10 mM Tris-Cl pH 7.4, 5 mM EDTA) for 30 minutes at 37°C. The reaction was stopped using 50 µg Proteinase K/0.5% SDS followed by phenol:chloroform extraction. The RNA was precipitated with 500 µL absolute ethanol and 20 µg yeast tRNA and the RNA pellets were dried and resuspended in loading buffer (80% formamide, 1 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol). After incubation at 85°C for 5 minutes the samples were electrophoresed for approximately 2.5 hours on a 6% polyacrylamide/7 M urea/1xTBE gel. The gel was dried and exposed to X-ray film (Kodak Imaging Systems)

for subsequent autoradiography, as well as quantified by PhosphorImager analysis using ImageQuant 3.3 software (Molecular Dynamics).

2.7 Cell lines and culture conditions

FM3A mouse adenocarcinoma cells, COS-1 african green monkey kidney cells, NDL mouse mammary tumour cells, and Rat-1 rat fibroblast cells were grown in 90% DMEM, 10% fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin (GibcoBRL/Life Technologies). NIH 3T3 mouse fibroblast cells were grown in 90% DMEM, 10% calf serum, 100 units/mL penicillin and 100 μg/mL streptomycin. For selection of transfected cells in colony formation assays using NIH 3T3 cells, GENETICIN™ (GibcoBRL/Life Technologies) was added at 400 μg/mL to the medium. All cells were maintained at 37°C in a humidified 5% CO_2 environment.

2.8 Transient transfection of DNA into cultured cells

Transient transfections were carried out using LipofectAMINE Reagent™ (Gibco BRL/Life Technologies) as per manufacturer's instructions. In transfections using FM3A cells, cells were seeded at $5x10^5$ cells per 35 mm well the day before transfection. The total amount of DNA was maintained at 2 μg using sheared calf thymus DNA and diluted in a total of 100 μL DMEM. 6 μL of LipofectAMINE Reagent™ diluted in a total volume of 100 μL DMEM was added to the DNA mixture. After a 30-minute incubation, the mixtures were quenched with 800 μL DMEM and added to the cells that were washed once with DMEM prior to application of the transfection mixture. The transfection

proceeded for 5 hours after which time 1 mL of 80% DMEM, 20% fetal bovine serum was added to the cells. The cells were maintained in antibiotic-free media for the duration of the experiment.

COS-1 transfections were performed similarly to those described above except for the following modifications. $1.25x10^5$ cells were seeded the day before transfection. The amounts of both total DNA and LipofectAMINE Reagent™ were the same as those described above. After the 5-hour transfection, cells were washed once with DMEM, once with 1xPBS, and then normal growth media was added to the cells.

Transient transfections of DNA into NDL cells were performed similar to those using COS-1 cells except $2.5x10^5$ cells were seeded per 35 mm well the day prior to transfection.

2.9 Luciferase assays

Assays for luciferase reporter gene activity using the pGL2- and pGL3-based reporter plasmids were performed as per manufacturer's instructions (Promega). Transfected cells cultured in 35 mm wells were washed twice in 1xPBS and lysed in 1x Reporter Lysis Buffer (Promega) for 15 minutes at room temperature in the culture dish. The lysed cells were then scraped into a microcentrifuge tube, centrifuged at room temperature for 20 s at 13 000 rpm, and the supernatant was transferred to a separate tube. 20 µL of lysate was assayed in a Sarstedt 12x75 mm polystyrene tube using 100 µL of Luciferase Assay Reagent (Promega) and a Lumat luminometer (Berthold). Relative light units as measured by the luminometer were corrected for total protein for each

sample. Total protein values were determined by standard Bradford assay using 5 μL of lysate in 1 mL of diluted Protein Assay Reagent (Biorad). Absorbance was measured at 595 nm using a Beckman spectrophotometer.

2.10 Focus formation assays

Transfection of NIH 3T3 cells was performed similarly to those described above except for the following modifications. $1.0x10^5$ cells were seeded the day before transfection. Transfections using LipofectAMINE Reagent™ were allowed to proceed for only one hour, after which time the cells were washed once with DMEM, once with 1xPBS, and then normal growth media was added back to the cells. The next day, cells were washed with 1xPBS, trypsinized, then transferred from each 35 mm well to a 100 mm dish. Media was changed every 2 to 3 days until foci were apparent on the dishes (approximately 2 weeks post-transfection). Cells were then washed with 1xPBS, fixed with 10% phosphate-buffered formalin for one hour, and stained with 4% Giemsa/1xPBS overnight. The stain was rinsed off with distilled H_20 and allowed to dry before foci were scored.

2.11 Colony formation assays

Transfections were performed as described above for focus assays. Cells were treated with the appropriate antibiotic for selection of stably transfected cells and maintained in the selection media for the duration of the experiment. Media was changed every 2 to 3 days until recognizable colonies formed (approximately 2 to 3 weeks) at which time the cells were fixed and stained as described above.

2.12 Protein isolation from cultured cells

For total protein isolation from COS-1 and NDL cells, the transfected cells were washed twice with 1xPBS, then scraped into 500 µL of ice-cold 1xPBS and transferred to a microcentrifuge tube. The cells were pelleted by centrifugation at 1 500 rpm for 5 minutes at 4°C. The supernatant was carefully removed, 100 µL of ice-cold lysis buffer (1% Nonidet-P40/IGEPAL, 50 mM Tris-Cl pH 7.4, 5 mM EDTA, 400 mM NaCl, 10 µg/mL leupeptin, 20 µg/mL aprotinin, 200 µg/mL PMSF, 10 µg/mL pepstatin, 10 mM NaF) was added, and the samples were placed on ice for 20 minutes. The samples were centrifuged at 13 000 for 5 minutes at 4°C, after which the supernatant was carefully removed and transferred to a fresh microcentrifuge tube. The protein concentration of each sample was determined by Bradford assays using Biorad Protein Assay Reagent and measuring the absorbance at 595 nm using a Beckman spectrophotometer.

For total protein isolation from FM3A cells the following modifications of the procedure outlined above were performed. The cell pellets were resuspended in lysis buffer containing 150 mM NaCl and left on ice for 5 minutes, after which time the NaCl concentration was increased to 400 mM, and the samples were incubated for an additional 15 minutes on ice. The remainder of the protein isolation was carried out as described above. This modification allowed for sufficient lysis of the nucleus and subsequent identification of the transiently transfected dominant-negative PEA3 mutants by Western immuno-blotting analyses.

2.13 Western immuno-blotting

Total protein cell lysates were boiled for 5 minutes in 1x SDS loading buffer (50 mM Tris-Cl pH 6.8, 10% glycerol, 2% SDS, 0.29 M β-mercaptoethanol, 0.0005% bromophenol blue) then electrophoresed on a discontinuous 10% polyacrylamide-0.1% SDS gel for several hours at 120 V or overnight at 60V. The protein was transferred to an Immobilon®-P membrane (Millipore) (the membrane was rinsed in methanol and distilled H_20 prior to transfer) using transfer buffer (20 mM Tris-Cl pH 8, 150 mM glycine, 20% methanol) at 60 V for 5 hours or overnight at 25 V. The membrane was removed carefully, rinsed briefly in TBS-T (10 mM Tris-Cl pH 7.3, 150 mM NaCl, 0.05% Tween-20), air dried briefly, then incubated in blocking buffer (5% skim milk in TBS-T) for 2 to 4 hours at room temperature or overnight at 4°C with rotation. The blot was incubated for one hour at room temperature with the anti-9E10 c-*myc* monoclonal primary antibody (ONYX Pharmaceuticals, San Francisco) diluted at 1:1000 in blocking buffer. The blot was washed 3 times in TBS-T with agitation, then subsequently incubated for one hour at room temperature with goat anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (KPL; 474-1806) diluted at 1:3000 in blocking buffer. The blot was washed twice with TBS-T and once with TBS, each for 10 minutes at room temperature with agitation. Excess TBS wash buffer was allowed to drip off, after which the blot was treated with the chemiluminescent substrate (Boehringer Mannheim; NEN) for one minute. The excess substrate was allowed to drip off and the blot was sealed between two plastic acetate sheets. The blot was exposed to Kodak X-ray film for differing lengths of time depending on the strength of the signal.

2.14 Generation of transgenic mice

Generation of the MMTV-*neu* and MMTV-NDL2-5 lines of transgenic mice have been described previously (Guy et al., 1992; Siegel et al., 1999).

Generation of mice bearing the $Pea3^{nlslacZ}$ allele has been described previously (MacNeil, 1999).

Generation of MMTV-PEA3, MMTV-ΔNPEA3En and MMTV-EWSΔN268PEA3 transgenic mice was performed as described previously (Guy et al., 1992). The transgene DNA was linearized and purified prior to embryo microinjection as previously described (Guy et al., 1992). 50 μg of MMTV-ΔNPEA3En-SV40pA plasmid purified by 2x cesium chloride centrifugation was digested with *Sph*I and *Spe*I, and electrophoresed on a 0.8% agarose/1xTAE gel to separate the transgene from the pBluescript vector backbone. The excised gel slice containing the transgene DNA fragment was placed into freshly prepared dialysis tubing and the DNA was electroeluted. The eluted DNA was extracted 5 times using phenol:chloroform:isoamyl alcohol, followed by 3 extractions using chloroform:isoamyl alcohol. The DNA was precipitated with absolute ethanol, washed with 70% ethanol, then resuspended in sterile Millipore-H_20 to a final concentration of 5 μg/mL.

The MMTV-PEA3-SV40pA and MMTV-EWSΔN268PEA3-SV40pA constructs were prepared as described above with the exception of the MMTV-EWSΔN268PEA3-SV40pA construct that was digested with *Pvu*II.

FVB/N female mice were injected intraperitoneally with pregnant mare serum then set up to mate with age-matched FVB/N male mice. The next day, females bearing copulatory plugs were sacrificed and the one-cell embryos removed from their oviducts. Approximately 0.5 to 1 pg of prepared DNA was microinjected into the male pronucleus of the embryos which were subsequently implanted into the oviducts of pseudopregnant Swiss-Webster female mice. Linda Wei, a former technician in the lab of Dr. W. J. Muller, performed the embryo microinjections and implantations.

2.15 Genomic DNA isolation from mouse tail biopsies

Genomic DNA was isolated from mouse tail biopsies as described previously (Laird et al., 1991). Briefly, 0.5-1.0 cm tail clippings were lysed overnight at 55°C in 500 µL of mouse tail lysis buffer (100 mM Tris-Cl pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, 100 µg/mL Proteinase K). The lysates were vortexed briefly then microcentrifuged for 10 minutes at 13 000 rpm. The supernatants were decanted into a fresh tube and 500 µL of isopropanol was added to precipitate the DNA by vigorous shaking for several seconds. Again the samples were microcentrifuged for 10 minutes at 13 000 rpm, after which the supernatant was aspirated completely. The DNA pellets were resuspended in 100 µL sterile Millipore-H_20 and stored at –20°C until use in Southern analyses.

2.16 Southern analyses

The pGEM/SPA plasmid was digested with *Hin*dIII and *Bam*HI for several hours then

electrophoresed on a low-melting point agarose gel. The fragment representing the SV40 polyadenylation cassette (~900 bp) was excised from the gel using a sterile scalpel, carefully removing excess gel from the slice. The gel slice was melted by incubation at 65°C for approximately 10 minutes, vortexed, then 5 µL were immediately removed for use in DNA probe synthesis. For preparation of the *Pea3* probe template, the pBS/PEA3-3'UTR plasmid was digested with *Hin*dIII and *Sac*I, electrophoresed on a low-melting point agarose gel and the *Pea3* cDNA fragment (~600 bp) was isolated as described above.

For preparation of radiolabelled DNA probes for use in Southern analysis (Feinberg and Vogelstein, 1983), approximately 50 ng of DNA template in low-melting point agarose was mixed with 20 U of Random Hexamers (Roche Biochemicals) and boiled for 5 minutes. The remaining reaction components of 1x RP buffer (90 mM HEPES pH 6.6, 10 mM $MgCl_2$), 1 mM dATP, 1 mM dGTP, 1 mM dTTP, 2 mM DTT, 50 µCi α^{32}P-dCTP, and 3 U Klenow polymerase (GibcoBRL/Life Technologies) were added and the samples were incubated at 37°C for 2 hours. Note that at no time during the setup of this reaction were the samples placed on ice, or were ice-cold solutions added to the samples, in order to minimize the possibility of solidification of the agarose. After DNA synthesis, the radiolabelled DNA was separated from the unincorporated α^{32}P-dCTP using a ProbeQuant™ G-50 Microcolumn (Amersham Pharmacia) and the probe was quantified using a scintillation counter.

For Southern analysis (Southern, 1975) of transgenic mouse DNA, approximately 5 μg of genomic DNA was digested overnight using 40 U of *Bam*HI in a reaction volume of 30 μL at 37°C. The following day, the digested DNA was electrophoresed on a 1% agarose/1xTAE gel for several hours until the bromophenol blue dye was 1 cm from the end of the gel. Alternatively, for Southern analysis of *Pea3*-null mouse DNA, the genomic DNA was digested overnight using 40 U of *Eco*RI. The following day, the digested DNA was electrophoresed on a 0.8% agarose/1xTPE gel overnight at 25 V until adequate resolution of the 6 kb and 7 kb bands of the 1 kb DNA ladder (wild-type *Pea3* allele is 6.3 kb and *Pea3*-null allele is 6.7 kb). A photo was taken of the ethidium bromide stained gel, the gel was incubated at room temperature with agitation for 30 minutes in denaturing solution (0.5 M NaOH, 1.5 M NaCl), then incubated for a similar length of time in neutralizing solution (1 M Tris-Cl pH 7.5, 1 M NaCl). The DNA was transferred overnight to a GeneScreen Plus hybridization transfer membrane (NEN Life Science Products) using the wick-method of Southern blotting. The following day the DNA was crosslinked to the blot using a UV Stratalinker 2400 (Stratagene) set at $1.2x10^5$ μJ. The blot was incubated at 65°C with agitation in prehybridization solution containing 5x Denhardt's (0.1% BSA Fraction V, 0.1% polyvinylpyrrolidone, 0.1% Ficoll Type 400), 5x SSC, 1.5% SDS, and 100 μg/mL sheared salmon sperm DNA in a sealed bag. After the radiolabelled DNA probe was boiled for 5 minutes, approximately $1x10^6$ cpm/mL of probe was added to the prehybrization solution directly into the bag, the bag was sealed twice, the probe was mixed throughly into the solution, and placed back at 65°C with agitation overnight. The following day the blot was removed from the bag and

washed three times for 10 minutes at 65°C in wash buffer (1x SSC, 0.5% SDS). The blot was wrapped in plastic wrap and exposed to either Kodak X-ray film at –80°C for several days, or to a PhosphorImager screen overnight with subsequent analysis using ImageQuant 3.3 software.

2.17 Whole-mount preparation of mouse mammary glands

Preparation of haematoxylin-stained mammary gland whole-mounts were performed as previously described (Vonderhaar and Greco, 1979). The #4 inguinal mammary gland of a female mouse was spread on a glass slide then left to air-dry overnight. The glands were fixed and defatted in acetone overnight, crushed using another slide, and placed in acetone for another two hours. The glands were then placed in Harris' modified haematoxylin (Fisher) overnight or until the gland was thoroughly stained. The glands were destained by several washes in 70% ethanol/1% HCl, fixed for one minute in 0.02% ammonium hydroxide, then dehydrated through a graded series of ethanol washes. The glands were incubated overnight in xylenes and mounted with Permount™ (Fisher Scientific) under a coverslip.

2.18 Histological preparation of mouse organs and tissues

Dissected mouse tissues were placed directly into 10% phosphate-buffered formalin (Fisher Scientific) and left to fix at 4°C overnight. The glands were sent to Anatomical Pathology (McMaster University Medical Centre) to be embedded in paraffin, sectioned at 3 μm, and prepared for standard haematoxylin-eosin staining.

2.19 β-galactosidase activity assays in mouse tissues

X-gal staining of mouse tissues to visualize β-galactosidase activity was performed essentially as described previously (Robinson et al., 1995). Mammary glands, tumour tissue, or lungs were isolated from female mice and placed into fixative (2% paraformaldehyde, 0.2% glutaraldehyde, 0.1 M phosphate buffer, 2 mM $MgCl_2$) to fix for one hour at room temperature. The glands were washed twice for 30 minutes in Rinse Buffer A (0.1 M phosphate buffer, 2 mM $MgCl_2$, 0.1% IGEPAL CA-630 (Sigma), 0.1% sodium desoxycholate) then twice for 30 minutes in Rinse Buffer B (0.1 M phosphate buffer, 2 mM $MgCl_2$, 0.1% IGEPAL CA-630, 0.01% sodium desoxycholate). The glands were then incubated for 2 to 4 days shaking in the dark at room temperature in Rinse Buffer B containing 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 1 mg/mL X-gal substrate (GibcoBRL/Life Technologies). After sufficient colour reaction had occurred, the glands were washed twice for one hour in 1x PBS then post-fixed at 4°C overnight in the same 2% paraformaldehyde/0.2% glutaraldehyde fixative used previously. The glands were washed twice in 1x PBS for one hour then stored in 70% ethanol until further processing. For wholemount preparation, the glands were defatted in acetone for approximately 4 hours, incubated in xylenes for 1 to 2 hours, then mounted on a glass slide under a coverslip using Permount™. For histological preparation, the glands were sent to Anatomical Pathology to be embedded in paraffin and sectioned at 8 μm. The serial sections were processed for either standard haematoxylin-eosin staining or mounted as is to visualize X-gal staining.

2.20 Tumour studies

All mice used in this study were housed in a centralized animal facility (McMaster University Medical Centre) under normal day-night cycles and fed chow and water *ad libitum*. Mice involved in tumor studies were palpated for mammary gland tumor nodules every week beginning at 5 months of age. Once a tumor was identified, the mouse was monitored on a weekly basis until the 2-month endpoint was reached at which time the tumor-bearing mouse was sacrificed for dissection and subsequent analyses.

2.21 Immunohistochemistry

Formalin-fixed, paraffin-embedded tissues were sectioned at 2 μm and mounted onto silanized slides. The tissues were deparaffinized with xylenes, rehydrated in a graded series of ethanol washes, with a final soak for 30 minutes in 1xPBS. The slides were incubated for 30 minutes at approximately 95°C in DAKO® Target Retrieval Solution to facilitate antigen retrieval, followed by several washes in 1xPBS after the samples had been allowed to return to room temperature. Excess wash solution was wiped off of the slides, the tissue sections were blocked with 5% skim milk in 1xPBS for 1 hour, followed by another incubation with 10% goat serum, each at room temperature in a humidified chamber. The primary antibodies, anti-9E10 c-*myc* monoclonal antibody (1:20), anti-neu rabbit polyclonal antibody (1:40; Oncogene Research Products; Ab-1), or anti-β-

galactosidase monoclonal antibody (1:100; Sigma; Clone GAL-40), were diluted in 1% BSA and placed directly on the tissue sections. Incubation with the primary antibody was carried out at 4°C overnight in a humidified chamber. The following morning, the tissue sections were rinsed briefly in 1xPBS prior to incubation with the secondary antibodies. Each of the secondary antibodies were diluted 1:40 in 1% BSA. The tissue sections were incubated for one hour with sheep anti-mouse Texas Red-conjugated secondary antibody (Jackson ImmunoResearch Laboratories; 515-076-062) at room temperature in the dark in a humidified chamber. The slides were rinsed thoroughly with 1xPBS followed by a second incubation with goat anti-rabbit FITC-conjugated secondary antibody (Calbiochem; 401314) under identical conditions. The slides were rinsed twice with 1xPBS, once with 1xPBS containing 0.01% Triton-X-100, then mounted using DAKO® Mounting Media under a coverslip. Immunofluorescence was visualized within 24 hours using a Zeiss Axioskop and images were captured using an Axiocam and the Axiovision 4.0 software program. Merged images were generated using both Adobe Photoshop 6.0 and Axiovision 4.0 software programs.

CHAPTER THREE

Pea3 SUBFAMILY EXPRESSION IN THE MAMMARY GLAND AND IN MAMMARY TUMOURS

Results

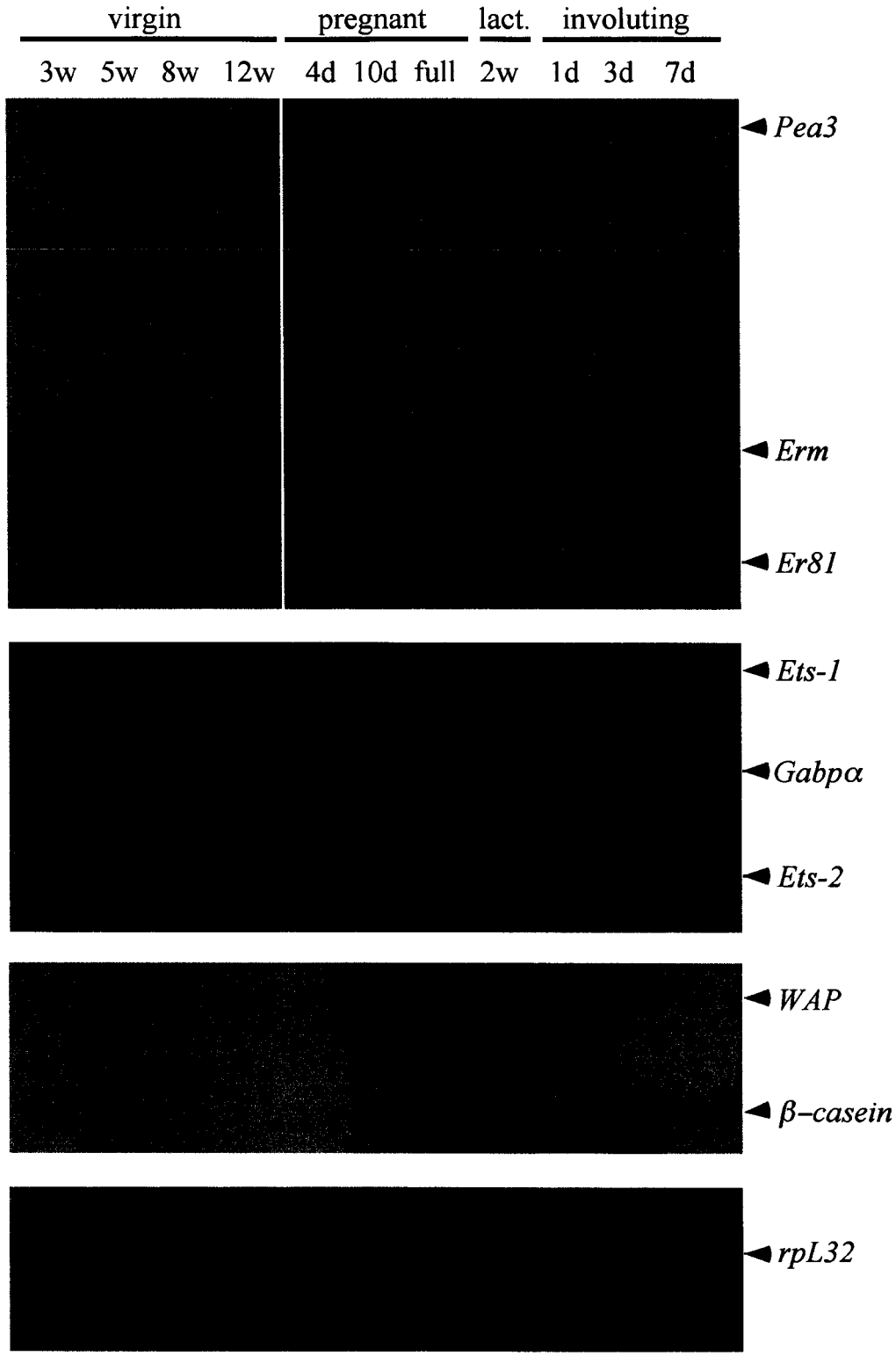
3.1.1 Analysis of *ets* gene expression in the mouse mammary gland

Postnatal mouse mammary gland development is a dynamic process involving cyclic events of cell proliferation, differentiation and apoptosis. This involves the expression of a variety of different genes, some of which may be specific *ets* genes. To address this issue, total RNA isolated from the mammary glands of several FVB/N mice at various developmental timepoints were pooled then analyzed by RNase protection assay using probes specific for the *Pea3* subfamily genes, as well as those for *Ets-1*, *Ets-2*, and *Gabpα*. Probes directed against whey acidic protein (*Wap*) and *β-casein*, two highly expressed and tightly regulated mammary gland differentiation-specific genes, were used as developmental markers of the mammary gland.

Each of the *Pea3* subfamily genes was expressed in the mouse mammary gland (Figure 3.1). A slight increase was observed during puberty (5 weeks) and at the onset of pregnancy (4 days). However, all three genes were dramatically reduced in abundance during late pregnancy and lactation, with a gradual increase then observed during the process of involution. Expression of *Erm* was relatively higher than that of *Pea3* and *Er81* in the mammary gland at each timepoint analyzed.

Figure 3.1 Several *ets* genes are expressed during postnatal mouse mammary gland development.

RNase protection assays were performed on total RNA isolated from the mammary glands of FVB/N mice at the indicated stages of postnatal mouse mammary gland development using radiolabelled riboprobes specific for each *ets* gene shown. Each developmental timepoint represents pooled RNA from 3 to 5 mice. Full pregnancy is at the 19-day timepoint and within approximately 24 hours prior to birth of the pups. Each of the *Pea3* subfamily genes is expressed in the virgin mammary gland with a slight elevation during puberty (ie. 5w sample). These three genes are expressed during early pregnancy as well but are dramatically downregulated at mid-pregnancy and do not begin to be expressed again until late involution. Three other *ets* genes that are highly expressed in the mouse mammary gland, *Ets-1*, *Ets-2*, and *Gabpα*, display a similar expression profile during development. The expression of *WAP* and *β-casein*, two major milk protein genes, is induced during pregnancy and lactation, and thus they serve as markers for functional differentiation of the mammary gland. The *rpL32* riboprobe was used as an internal control for RNA loading.


virgin
pregnant
lact.
involuting
3w 5w 8w 12w 4d 10d full 2w 1d 3d 7d
Pea3
Erm
Er81
Ets-1
Gabpα
Ets-2
WAP
β–casein
rpL32

Similar analyses of the *ets* genes *Ets-1*, *Ets-2* and *Gabpα* revealed that they too were expressed in the mouse mammary gland with a very similar dynamic expression profile to that of the *Pea3* subfamily genes (Figure 3.1). The *Ets-1* and *Ets-2* genes were expressed at much higher levels relative to *Gabpα*.

Expression of *WAP* and *β-casein* mRNAs correlated with the functional differentiation of the mammary gland (Figure 3.1). Detection of *β-casein* gene expression was slightly earlier than that of the *WAP* gene, with both genes being maximally expressed at late pregnancy and lactation. Rapid downregulation of the *WAP* and *β-casein* genes was observed with the onset of involution. It should be noted that 30 times less total RNA was used to detect the *WAP* and *β-casein* mRNA (1 μg) versus that of the various *ets* genes (30 μg) which illustrates the tremendous switch in gene expression towards milk protein production during pregnancy and lactation in the mammary gland.

3.1.2 The *Pea3* subfamily is coordinately overexpressed in MMTV-*neu*-induced mammary tumours

Pea3 mRNA is overexpressed in the mammary tumours of MMTV-*neu* transgenic female mice (Trimble et al., 1993). In addition, several different *ets* genes are expressed in the normal mouse mammary gland. Therefore, the profile of gene expression in MMTV-*neu*-induced mammary tumours was determined for the same set of *ets* genes as those described above. Again RNase protection assays were performed using total RNA,

but in this instance the RNA was isolated from several independent tumours isolated from MMTV-*neu* transgenic mice or from normal virgin FVB/N female mouse mammary glands.

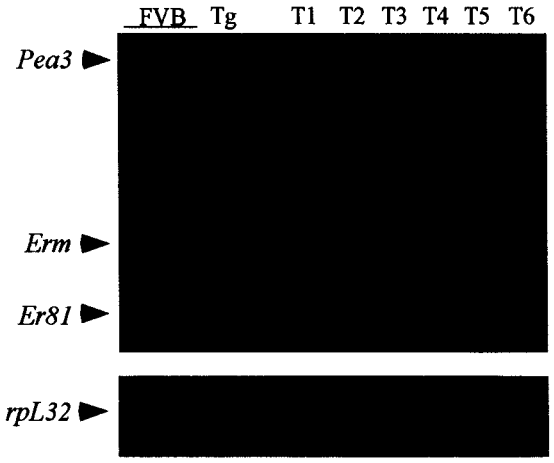
As illustrated in the previous experiments, each of the *Pea3* subfamily genes was expressed at relatively low levels in the normal virgin female mouse mammary gland (Figure 3.2). However, the *Pea3* subfamily genes were coordinately upregulated in every MMTV-*neu*-induced mammary tumour RNA sample. Indeed, quantification of expression revealed an average increase in expression of 5- to 10-fold across most samples, with the highest upregulation being for *Er81* of approximately 60-fold in one sample. Expression of *Pea3* subfamily genes in the non-tumour bearing mammary glands isolated from a virgin MMTV-*neu* mouse was identical to the levels observed in FVB/N mammary gland samples.

Analysis of *Ets-1*, *Ets-2* and *Gabpα* gene expression in a similar set of mammary gland and MMTV-*neu* tumour samples revealed that although these *ets* genes were expressed at relatively high levels in normal mammary tissue, each of these three mRNAs were decreased in the tumour samples (Figure 3.3). The level of *Ets-1* mRNA was reduced by nearly 20-fold in the tumours, and both *Ets-2* and *Gabpα* were decreased by 5- to 10-fold in almost every tumour sample studied.

Figure 3.2 The *Pea3* subfamily mRNA are elevated in the mammary tumours of MMTV-*neu* transgenic mice.

(***a***) RNase protection assays were performed on 10 μg of total RNA from normal mammary glands (FVB and Tg) and from several mammary tumours of MMTV-*neu* transgenic virgin female mice (T1-T6). Radiolabelled riboprobes specific for the *Pea3* subfamily mRNA were used, and *rpL32* served as an internal RNA loading control. Each of the *Pea3* subfamily members is expressed at low levels in the normal mammary gland of virgin female mice (FVB) and of non-tumour bearing MMTV-*neu* transgenics (Tg), but are dramatically increased in all MMTV-*neu* tumour RNA samples studied (T1-T6). (***b***) Quantification of the above RNase protection assay by PhosphorImager analysis using ImageQuant 3.3 software. The *Pea3* subfamily genes are elevated by approximately 5- to 20-fold in most tumour samples, after normalization to *rpL32*, when compared to their expression in the normal FVB/N mammary gland that is set to one.

a


FVB
Tg
T1
T2
T3
T4
T5
T6
Pea3
Erm
Er81
rpL32

b

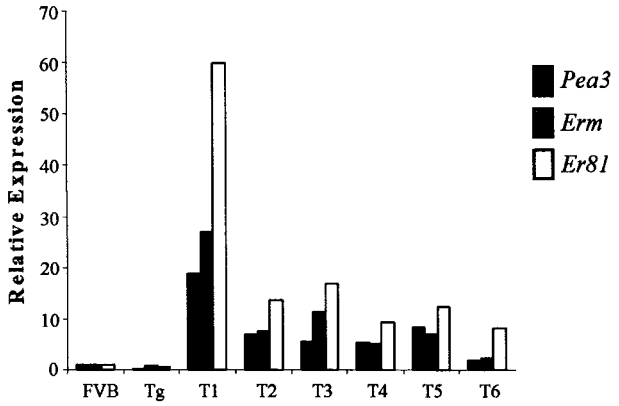
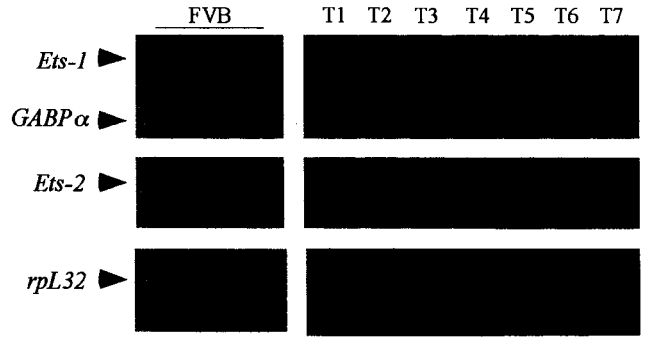

Relative Expression
70
60
50
40
30
20
10
0
FVB
Tg
T1
T2
T3
T4
T5
T6
Pea3
Erm
Er81

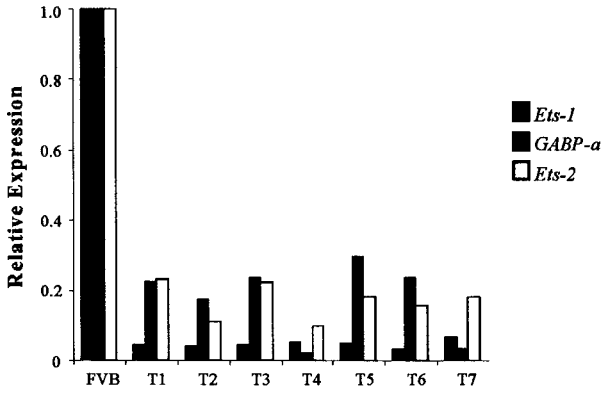
Figure 3.3 *Ets-1*, *Ets-2*, and *Gabpα* mRNA are reduced in the mammary tumours of MMTV-*neu* transgenic mice.

(***a***) RNase protection assays were performed on 10 μg of total RNA from normal mammary glands (FVB) and from several mammary tumours of MMTV-*neu* transgenic virgin female mice (T1-T7). Radiolabelled riboprobes specific for *Ets-1*, *Ets-2*, and *Gabpα* mRNA were used, and *rpL32* served as an internal RNA loading control. Each of these three *ets* genes are expressed at moderate to high levels in the normal mammary gland of virgin female mice (FVB), but are consistently decreased in all MMTV-*neu* tumour RNA samples studied (T1-T7). (***b***) Quantification of the above RNase protection assay by PhosphorImager analysis using ImageQuant 3.3 software. *Ets-1* mRNA is reduced by about 20-fold, and *Ets-2* and *Gabpα* mRNA by 5- to 10-fold, after normalization to *rpL32*, when compared to their expression in the normal FVB/N mammary gland that is set to one.

a


FVB
T1 T2 T3 T4 T5 T6 T7
Ets-1
GABPα
Ets-2
rpL32

b


Relative Expression
1.0
0.8
0.6
0.4
0.2
0
FVB T1 T2 T3 T4 T5 T6 T7
Ets-1
GABP-a
Ets-2

3.1.3 *Pea3* is expressed in the Neu-positive epithelial cells of MMTV-*neu*-induced mammary tumours

Previous results have illustrated that *Pea3* mRNA is overexpressed in the mammary tumours and lung metastases of MMTV-*neu* and MMTV-PyVMT transgenic female mice (Trimble et al., 1993). However, the precise localization of *Pea3* expression in mammary tumours was not analyzed. To perform this experiment, mice bearing a nuclear-localized β-galactosidase gene (*nls-lacZ*) knocked into the *Pea3* locus were crossed with MMTV-NDL2-5 transgenic mice (MacNeil, 1999; Siegel et al., 1999). This strain of transgenic mice bears a mutated *neu* cDNA sequence originally identified in an MMTV-*neu*-induced tumour (Siegel et al., 1994) which leads to increased Neu receptor tyrosine kinase activity (Siegel and Muller, 1996) and increased tumour kinetics (Siegel et al., 1999). Efficient translation of β-galactosidase in the $Pea3^{nls\text{-}lacZ}$ mice is facilitated by the placement of an internal ribosomal entry site upstream of the *nls-lacZ* gene.

Tumours arising in virgin female MMTV-NDL2-5 mice were isolated along with contralateral mammary glands and lungs between 4 to 6 weeks after initial palpation of the tumour. Tissues derived from both wild-type and $Pea3^{nls\text{-}lacZ/+}$ mice were processed for β-galactosidase activity. MMTV-NDL2-5 female mice typically develop multifocal hyperplastic lesions throughout the mammary gland, some of which develop into solid adenocarcinomas (Siegel et al., 1999). MMTV-NDL2-5 female tumour mice bearing the $Pea3^{nls\text{-}lacZ}$ allele exhibited numerous blue-staining, β-galactosidase-positive lesions and

tumours throughout the mammary gland (Figure 3.4). Lesser staining is observed along the ducts in mammary tissue adjacent to neoplastic lesions. *Pea3* is normally expressed in the ducts of mature virgin female mouse mammary glands (MacNeil, 1999). Histological tissue sections of these same samples revealed that β-galactosidase activity is localized to the tumour with very little staining being observed in surrounding tissue. No endogenous β-galactosidase activity was observed in tissues derived from MMTV-NDL2-5 mice with wild-type *Pea3*.

Several mice from these studies developed lung metastases as previously reported for the MMTV-NDL2-5 mice (Guy et al., 1992; Siegel et al., 1999). Similar to the primary mammary tumours generated in $Pea3^{nls\text{-}lacZ}$ female mice, the lung metastases stain positive for β-galactosidase activity. These results indicate that *Pea3* is specifically overexpressed in mammary tumours and lung metastases induced by activated Neu.

To directly assess whether *Pea3* is expressed in the same cells that express the MMTV-*neu* transgene, co-immunohistochemical analysis was performed using tissue sections derived from tumours isolated from the same mice described above (Figure 3.5). Although expression of the Neu protein appeared to be variegated throughout the tumour, it was readily detectable with the anti-*neu* primary antibodies in each section. Co-incubation with antibodies directed against β-galactosidase revealed strong co-localization of *Pea3* expression with that of Neu.

Figure 3.4 *Pea3* expression is localized to mammary tumours and lung metastases of MMTV-NDL2-5 transgenic mice.

(***a***) X-gal-stained wholemount of the #4 inguinal mammary gland isolated from a tumour-bearing MMTV-NDL2-5/*Pea3*$^{nls\text{-}lacZ/+}$ virgin female mouse. Several neoplastic lesions stain positive for β-galactosidase activity (ie. *Pea3* expression). (b) Wholemount preparation of X-gal-stained lungs isolated from a tumour-bearing MMTV-NDL2-5/*Pea3*$^{nls\text{-}lacZ/+}$ transgenic mouse. Numerous β-galactosidase-positive lung metastases are present, illustrating that *Pea3* is also expressed in the mammary tumour-derived lung metastases that develop in these mice. (***c-f***) Histological tissue sections of X-gal-stained mammary tumours isolated from either MMTV-NDL2-5/*Pea3*$^{+/+}$ (***c*** and ***e***) or MMTV-NDL2-5/*Pea3*$^{nls\text{-}lacZ/+}$ (***d*** and ***f***) transgenic mice were stained with haematoxylin and eosin (***c*** and ***d***) or mounted immediately after staining with X-gal (***e*** and ***f***). *Pea3* expression (β-galactosidase activity) is localized specifically to mammary tumour cells (***f***), whereas no endogenous β-galactosidase activity is evident in the cells comprising MMTV-NDL2-5/*Pea3*$^{+/+}$ tumours (***e***). Original magnification = 6x (***a*** and ***b***), 200x (***c-f***)

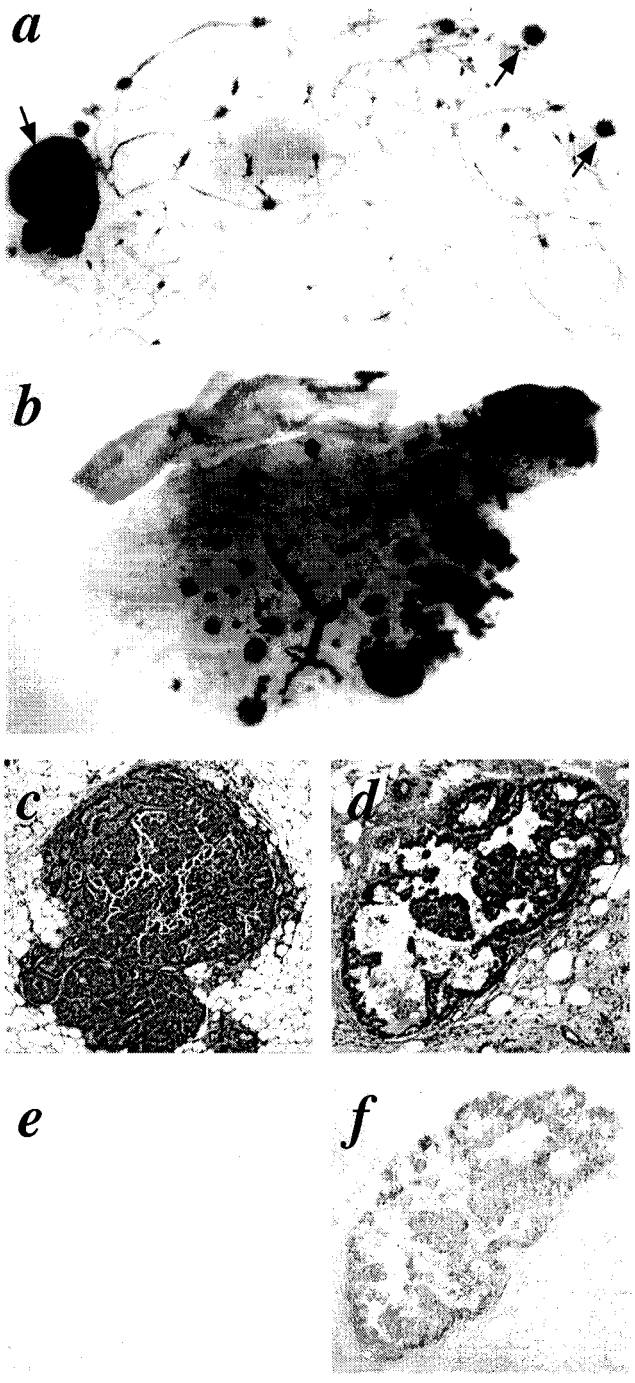
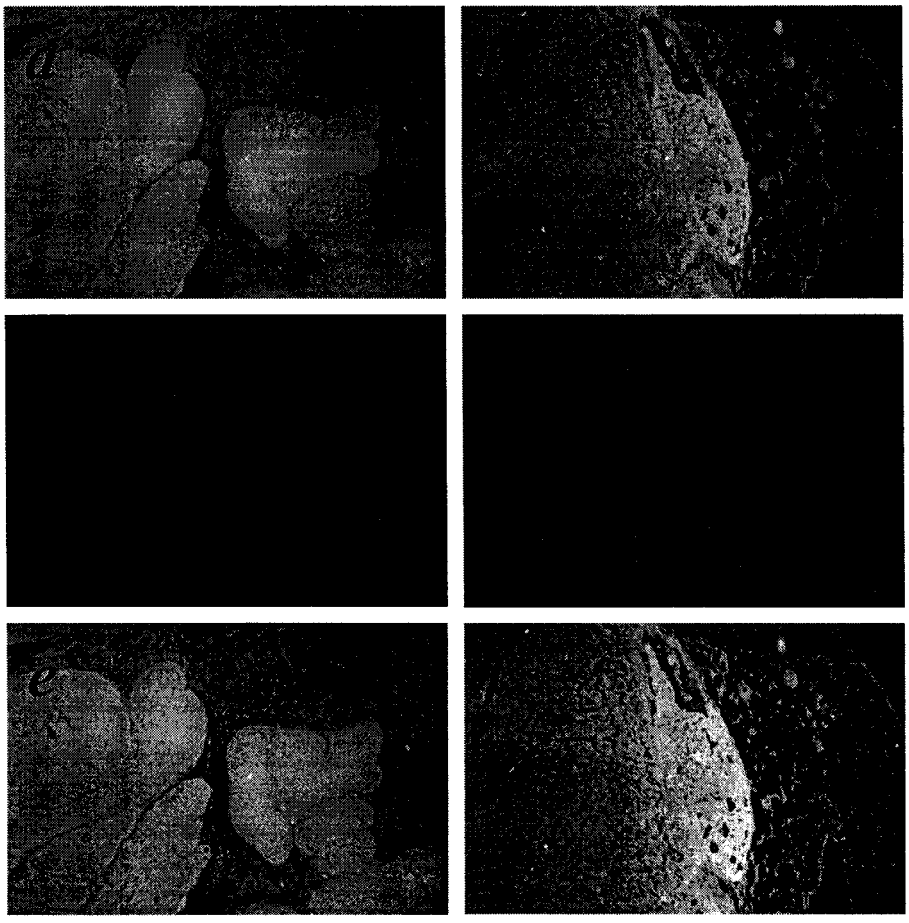

a
b
c
d
e
f

Figure 3.5 *Pea3* expression co-localizes with Neu in mammary tumours of MMTV-NDL2-5 transgenic female mice.

Immunohistochemical analysis was performed by co-incubation of formalin-fixed, paraffin-embedded tissue sections with the Ab-1 anti-*neu* rabbit polyclonal antibody to detect Neu protein (***a*** and ***b***) and the anti-β-gal monoclonal antibody to detect β–galactosidase protein (***c*** and ***d***) that is expressed by the endogenous *Pea3* promoter. The goat anti-rabbit FITC-conjugated and sheep anti-mouse Texas Red-conjugated secondary antibodies were used to detect expression by immunofluorescence microscopy to allow merging of images to detect co-localization (***e*** and ***f***). Tissue sections of mammary tumours isolated from MMTV-NDL2-5/*Pea3*$^{+/+}$ (***a***, ***c*** and ***e***) and MMTV-NDL2-5/*Pea3*$^{+/nlslacZ}$ (***b***, ***d*** and ***f***) female mice were analyzed. Images were captured at each wavelength with a fixed shutterspeed of 2 000 ms using an Zeiss Axiocam and Axiovision 4.0 software, and the resultant images were then merged using both Adobe Photoshop 6.0 and Axiovision 4.0 software. The Neu protein is detectable in both tumour samples (***a*** and ***b***). The tumour sample derived from the MMTV-NDL2-5/*Pea3*$^{+/nlslacZ}$ mouse is highly immuno-reactive for β-galactosidase protein (***d***) that co-localizes strongly with the Neu protein (***f***). Background immuno-reactivity is observed for the anti-β-gal antibody in the MMTV-NDL2-5/*Pea3*$^{+/+}$ tumour sample (***c***), but it does not result in a positive co-localization signal when the images are merged (***e***). Original magnification = 100x



3.2 Discussion

3.2.1 *Pea3* subfamily expression during postnatal mammary gland development

The majority of mammary gland development from the primitive mammary anlagen occurs postnatally (Daniel and Silberstein, 1987). Times of extensive epithelial proliferation are during puberty with the progression of terminal end buds through the stromal mesenchyme of the fat pad and with the onset of pregnancy when numerous secondary and tertiary ducts appear. Functional and terminal differentiation occurs when lobulo-alveolar structures form and begin to produce and secrete milk from the secretory epithelium. Epithelium of the mammary gland also exhibits massive cell death and remodelling during the process of involution after the weaning of pups from the mother.

Several *ets* genes are expressed in the mammary glands of mice. The relative expression level among the six genes analyzed varies, with *Ets-1* and *Ets-2* expressed at the highest, *Gabpα* and *Erm* expressed at moderate levels, and *Pea3* and *Er81* expressed at low but detectable levels. Their expression varies during the different stages of mammary gland development. Expression is observed in mammary glands isolated from nulliparous female animals from the ages of 3 weeks to several months. Their expression is either slightly elevated or is at least maintained with the onset of pregnancy, but dramatically reduced by late pregnancy and lactation. Expression slowly returns to near adult virgin levels by approximately 7 days involution. The dynamic profile of *ets* gene expression may at least be partially due to the massive upregulation of milk protein gene expression at mid- to late pregnancy and during lactation. It has been observed that β-

casein and *WAP* mRNA levels increase by 100- to 1000-fold at these stages of mammary gland development (Rosen, 1987) and therefore total RNA produced in the mammary gland increases. Thus, less expressed genes are diluted in the total RNA population isolated from the mammary gland at these developmental stages, specifically in this case of the various *ets* genes analyzed.

However, putative function of these *ets* genes in the mammary gland can still be hypothesized based on their expression pattern. There appears to be a slight elevation in *Pea3* subfamily expression during puberty (ie. 5-week sample) in the nulliparous mouse mammary gland. Their expression implies possible function during this highly proliferative stage. Other experimental evidence has illustrated localization of *Pea3* subfamily gene expression in the highly proliferative pluripotent cap cells of the terminal end buds during puberty (MacNeil, 1999)(N. Kurpios, G. Fidalgo and J. A. Hassell, unpublished). β-galactosidase activity was used as an indirect marker for the expression of each *Pea3* subfamily member in these studies, and thus precludes accurate quantification. However, at a strictly qualitative level, it appeared that the level of expression was elevated in these cells versus the more differentiated subtending ductal epithelial cells.

As already mentioned, several of the *ets* genes expressed in the mammary gland appear to increase slightly at the onset of pregnancy. Pregnancy can be divided into basically two different yet equally important phases: the proliferative stage in early pregnancy during which tertiary ducts form and lobulo-alveoli begin to develop, and the functional differentiation stage of late pregnancy during which the fat pad fills with

lobulo-alveoli capable of producing and secreting milk. Thus, the correlative expression of *ets* genes during the first half of pregnancy suggests function in the proliferative rather than the functional differentiation of the mammary gland. Indeed, forced overexpression of PEA3 during pregnancy and lactation via the MMTV promoter results in a reduced capacity to form lobulo-alveoli and reduced ability to nurse young (see Section 5). This implies that downregulation of the *Pea3* gene, and possibly that of *Erm* and *Er81* as well, in the epithelium during late pregnancy and lactation may be important in facilitating the onset of the functional differentiation pathway. It is noteworthy that expression of the *Pea3* subfamily members during embryonic development is highest in developing organs and tissues involving epithelial-stromal interactions, like those of the mammary gland (Chotteau-Lelievre et al., 1997). However, their expression is usually dramatically decreased upon differentiation of these tissues (Chotteau-Lelievre et al., 1997).

Localization of *Ets-1*, *Ets-2* and *Gabpα* in the mouse mammary gland has not been performed. However, *Ets-1* expression has been observed in cells of different haematopoietic lineages, as well as in endothelial cells (Watson et al., 1988), and *Ets-2* and *Gabpα* expression has been reported to be widespread in the adult mouse (Brown and McKnight, 1992; Watson et al., 1988). Mammary glands contain not only epithelial cells (luminal and myoepithelial), but fibroblasts juxtaposed to the basal lamina of the mammary ducts, adipocytes of the fat pad, endothelial cells forming the blood vessels present throughout the mammary gland, and cells of the lymph nodes. Therefore, it is likely that numerous cell types within the mammary gland express any or all three of

these *ets* genes, including the mammary epithelium. However, it must be noted that the *Pea3* subfamily *ets* genes are specifically expressed only in the epithelial component of the mammary gland. This unique characteristic is consistent for all three *Pea3* subfamily genes with their overlapping expression in the primordial mammary epithelium of the developing mouse embryo (MacNeil, 1999)(N. Kurpios, G. Fidalgo and J. A. Hassell, unpublished).

Although not analyzed in this thesis, the expression of other *ets* genes has been identified in the mammary gland. The three related *ets* genes of the *ESE* (epithelial-specific ets) subfamily, namely *ESE-1* (also known as *ESX*) (Choi et al., 1998), *ESE-2* (Oettgen et al., 1999) and *ESE-3* (Kas et al., 2000) are expressed during mouse mammary gland development. These genes have been identified as being epithelium specific in many tissues, but their localization in mammary epithelial cells has yet to be determined. Thus, whether these *ets* genes are expressed in a similar subpopulation of cells of the mammary gland as *Pea3* subfamily genes is not clear.

3.2.2 Coordinate overexpression of the *Pea3* subfamily genes in MMTV-*neu* induced mammary tumours

Previous findings have illustrated the overexpression of *Pea3* mRNA in mammary adenocarcinomas and lung metastases of MMTV-*neu* and MMTV-PyVMT transgenic female mice (Trimble et al., 1993). So too, *PEA3* is overexpressed in 76% of human breast tumour specimens studied (Benz et al., 1997). There was a high correlation

of *PEA3* overexpression with that of *HER2*-positivity (93%) whereas no correlation was observed with other markers such as nuclear grade, estrogen receptor positivity, or S-phase fraction.

Analysis of total RNA isolated from mammary glands of normal FVB/N virgin female mice versus that isolated from several mammary tumours of MMTV-*neu* transgenic females revealed that *Pea3* subfamily genes are coordinately upregulated in tumours relative to normal mammary gland controls. The *Pea3* subfamily genes are expressed in mammary epithelium of the normal mammary gland. Since, the tumours of these MMTV-*neu* transgenic mice arise in, and are composed primarily of, mammary epithelial cells (Cardiff et al., 1991; Guy et al., 1992), it is formally possible that *Pea3* subfamily mRNA are increased due to a higher proportion of epithelial cells present in the tumour. Thus, quantification of *Pea3* subfamily gene expression on a per cell basis in tumour cells versus that of adjacent normal mammary epithelial cells would be necessary to test for upregulation.

However, the *Pea3* subfamily genes may be upregulated at the transcriptional level in MMTV-*neu* tumours. NIH 3T3 cells engineered to induce activated Raf signalling in response to an androgen analogue R1881, coordinately increase expression of each *Pea3* subfamily mRNA within two to four hours post-induction (J-H Xin and J. A. Hassell, unpublished). Similar stimulation by activated Raf kinase in the MCF10A immortalized human mammary epithelial cell line leads to upregulation of both *PEA3* and *ERM* transcripts at 8- and 72-hour timepoints as determined by Affymetrix DNA-chip arrays (Schulze et al., 2001). These results imply that *PEA3* subfamily genes share a

similar response to oncogenic signalling downstream of Neu and Raf kinases. The *Pea3* subfamily genes have a common genomic architecture comprising 13 exons that encode similar regions of the translated protein product (de Launoit et al., 2000). Due to their high protein sequence similarity and common structure of their genetic loci, the *Pea3* subfamily genes more than likely represent a recent set of gene duplication events prior to their separation onto different chromosomes (Jeon and Shapiro, 1998). Thus, they may contain a subset of common DNA-binding sites within their promoters and/or enhancers that respond similarly to Neu-Ras-Raf-MAPK signalling by increasing gene transcription. A candidate AP1-ETS composite DNA binding site within the proximal promoter of the *Pea3* gene conserved among several species has been mapped by deletion analysis as a putative Raf-responsive element in NIH 3T3 cells (C. Messier and J. A. Hassell, unpublished). The composite DNA element consisting of juxtaposed ETS and AP1 sites within the promoters of various genes has already been defined as the Ras-responsive element (RRE) (Wasylyk et al., 1998). It remains to be determined whether or not this RRE present in the *Pea3* promoter exists within the *Erm* and *Er81* gene promoters.

3.2.3 Reduction in *Ets-1*, *Ets-2* and *Gabpα* gene expression in MMTV-*neu* mammary tumours

As discussed above, several *ets* genes are expressed in the developing mouse mammary gland. The *ets* genes studied, other than the *Pea3* subfamily, were *Ets-1*, *Ets-2*, and *Gabpα*. In the adult nulliparous female mouse mammary gland, each of these

genes was expressed at high to moderate levels relative to the *Pea3* subfamily. However, analysis of several MMTV-*neu* tumour RNA samples revealed a marked reduction in the level of mRNA for each of these *ets* genes. Since it was previously argued that their expression might be localized to cell types other than the mammary epithelium, it is quite possible that the apparent decrease in expression of these genes is due to the lower contribution of these specific cell types to the tumour proper.

The decrease of *Ets-1* mRNA in the MMTV-*neu* tumours is unusual since *Ets-1* has been shown to be involved in the process of angiogenesis during tumour growth (Iwasaka et al., 1996; Sato, 1998). In addition, *Ets-1* expression is correlated with urokinase-type plasminogen activator (*uPA*) gene expression during invasion (Grevin et al., 1993; Iwasaka et al., 1996). It has been well documented that the *uPA* gene enhancer contains a canonical RRE that can be regulated by Ets proteins like Ets-1 (Nerlov et al., 1992; Nerlov et al., 1991; Stacey et al., 1995). Dominant-inhibitory mutant Ets-1 is able to revert Ki-Ras transformation (Wasylyk et al., 1994) and reduce NeuNT transformation of mouse fibroblasts (Galang et al., 1996) implicating Ets-1 function in oncogenesis downstream of the Neu and Ras signalling pathways in some cell types. Blocking Ets-1 function with either a dominant-negative mutant or with antisense oligonucleotides is able to block angiogenesis (Chen et al., 1997; Iwasaka et al., 1996; Nakano et al., 2000). Although these data suggest a role for Ets-1 in carcinogenesis, the decreased *Ets-1* expression in MMTV-*neu* mammary tumours suggests that this is not the case in Neu-induced breast cancer.

The expression of *Ets-2* is ubiquitous in the adult mouse (Watson et al., 1988). Specific function of this gene is required early in development, since *Ets-2*-null animals die due to implantation defects caused by defective trophoblast function (Yamamoto et al., 1998). Rescue of such animals by tetraploid aggregation to bypass embryonic lethality illustrated a role for *Ets-2* in the hair follicle (Yamamoto et al., 1998). To assess the potential role for *Ets-2* in mammary oncogenesis, *Ets-2* heterozygous female animals bearing the MMTV-PyVMT transgene were studied (Neznanov et al., 1999). These mice displayed delayed tumour kinetics due to the loss of a single allele of the *Ets-2* gene, thus implicating a critical role for *Ets-2* in the development of these tumours. However, it is believed that *Ets-2* is expressed in both the mammary epithelium and the stroma, and that complete Ets-2 function may actually be required in the surrounding stromal compartment rather than the tumour itself (R. Oshima, personal communication). It should be noted that loss of *Ets-2* leads to reduced expression of *Mmp-9*, *Mmp-3*, and *Mmp-13* in *Ets-2*-null mouse embryonic fibroblasts after stimulation with FGF (Yamamoto et al., 1998). Many MMPs are expressed in mammary stroma (Rudolph-Owen and Matrisian, 1998); thus Ets-2 may be required for their expression in the mesenchyme surrounding MMTV-PyVMT-induced mammary tumours. If Ets-2 expression is more prevalent in the stroma, rather than the epithelial-derived tumour, this may explain the observed decrease in *Ets-2* mRNA abundance in MMTV-*neu* mammary tumours.

3.2.4 *Pea3* expression is localized to the epithelial cells of MMTV-*neu* mammary tumours

Utilizing genetically engineered mice that bear the nuclear localized β-galactosidase reporter transgene embedded within the *Pea3* gene, surrogate *Pea3* expression was observed in epithelial cells of MMTV-*neu* mammary tumours and resultant lung metastases. Thus, *Pea3* expression occurs in the same cells that express oncogenic Neu.

The *Pea3* gene is normally expressed in mammary epithelium of adult nulliparous female mice, principally in what appears to be the myoepithelial cells (MacNeil, 1999). Thus, whether *Pea3* is truly overexpressed in the mammary tumours at the level of a single cell is not known. Analysis of *Pea3* gene expression in the mammary gland and in MMTV-*neu* tumours by β-galactosidase activity assays may not accurately quantify the levels of expression in these two tissue types. It is formally possible that single epithelial cells that normally express *Pea3*, which comprise a small subset of the total number of cells in the entire mammary gland, are the cells which eventually give rise to the tumours of MMTV-*neu* transgenic mice. Upregulation of *Pea3* in mammary tumours as compared with the normal epithelial cells that express it would need to be established at the single cell level. However, it should be noted that X-gal-staining of mammary tumours results in a rapid colour reaction (complete staining within twenty-four hours) relative to what is observed with staining of normal mammary glands (several days are required to observe positive staining).

CHAPTER FOUR

CONSTRUCTION AND CHARACTERIZATION OF DOMINANT-NEGATIVE PEA3 (ΔNPEA3En)

4.1 Results

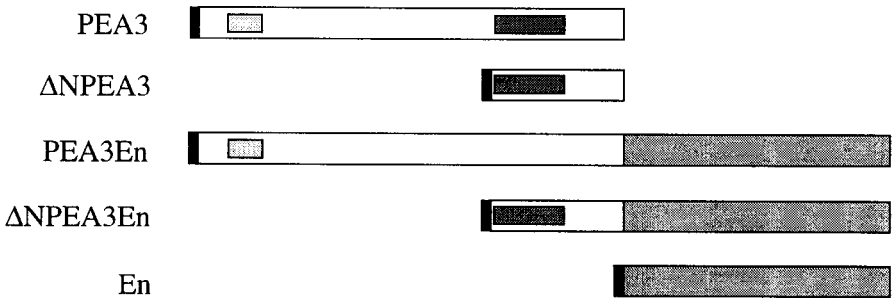
4.1.1 Generation of dominant-negative PEA3 mutants

To block the function of PEA3, several different mutants of mouse PEA3 protein were generated (Figure 4.1). These constructs included: (1) ΔNPEA3 comprising the carboxyl-terminus of PEA3 (amino acids 334-480 of mouse PEA3), (2) PEA3En comprising the full-length PEA3 sequence (amino acids 1-480) fused at its carboxyl terminus to the *Drosophila* Engrailed repression domain (En; amino acids 2-298 of En protein) (Han and Manley, 1993), (3) ΔNPEA3En consisting of the carboxyl terminus of PEA3 fused to the En repression domain, and (4) the En repression domain alone to serve as negative control for non-specific transcriptional repression. The region of mouse PEA3 used in ΔNPEA3 and ΔNPEA3En contains the ETS DNA-binding domain of PEA3 but lacks the strong acidic transactivation domain present at the amino terminus of the protein (Bojovic and Hassell, 2001; Xin et al., 1992). The sequences of En protein being used have been characterized as a potent transcriptional repression domain when fused to heterologous DNA-binding domains (Han and Manley, 1993). In addition, similar constructs have been used to generate dominant-negative Myb proteins

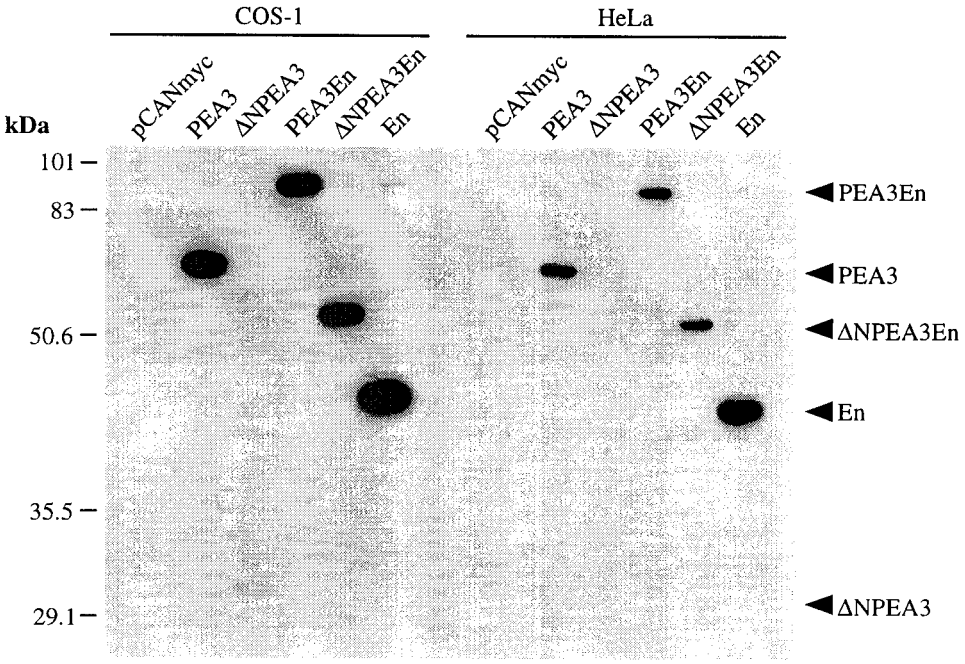
Figure 4.1 Generation of putative dominant-negative PEA3 mutants.

(***a***) Schematic illustration of the various dominant-negative PEA3 protein constructs as compared to the full-length wildtype PEA3 protein. PEA3 protein coding sequences are illustrated in white with those containing the ETS DNA-binding domain (red) and the acidic transactivation domain (yellow). The *Drosophila* Engrailed repression domain (En; gray) is fused to the carboxyl-terminus of two constructs, PEA3En and ΔNPEA3En, and one construct serving as a negative control is the En repression domain alone. Each protein construct has the 11-amino acid 9E10 c-*myc* epitope tag (black) fused to the amino-terminus, to facilitate detection of protein expression. (***b***) Expression of each protein construct in transiently transfected COS-1 and HeLa cells as detected by Western immuno-blotting using the anti-9E10 monoclonal antibody. Each protein migrated at its approximate predicted molecular weight, although the ΔNPEA3 mutant was expressed at much lower levels than the other proteins in COS-1 cell lysates, and was undetectable in HeLa cell lysates.

a


PEA3
ΔNPEA3
PEA3En
ΔNPEA3En
En

b


COS-1
HeLa
pCANmyc
PEA3
ΔNPEA3
PEA3En
ΔNPEA3En
En
kDa
101
83
50.6
35.5
29.1
PEA3En
PEA3
ΔNPEA3En
En
ΔNPEA3

that have been used successfully both in cell culture and in transgenic mice to inhibit Myb function (Badiani et al., 1994). Each construct was cloned into the pCANmyc expression vector whereby expression was controlled by the human cytomegalovirus promoter and each protein was tagged at its amino terminus with the 11-amino acid 9E10 human c-*myc* epitope to facilitate detection in Western immuno-blotting assays. Each DNA sequence that was generated via PCR was verified by complete DNA sequencing. Efficient protein expression was tested by transient transfection of 1 μg of each effector plasmid into both COS-1 and HeLa cells using LipofectAMINE Reagent™, followed by Western immuno-blotting of total cell extract using the anti-*myc* 9E10 monoclonal antibody (Figure 4.1). Each protein, except for ΔNPEA3, was readily detectable in both transfected cell extracts. A relatively low level of ΔNPEA3 was only visible in transfected COS-1 cell protein extracts.

4.1.2 Characterization of putative dominant-negative PEA3 mutants

To test the function of each putative dominant-negative PEA3 construct, the 3xPEA3-*luc* reporter plasmid was cotransfected into FM3A cells with either the pCANmyc empty expression vector or each of the different constructs listed above. The PEA3-responsive elements in the luciferase reporter were derived from the 4xPEA3-CAT reporter plasmid containing four reiterated copies of the ETS site found in the polyoma virus enhancer (Xin et al., 1992). These sites have been shown previously to be

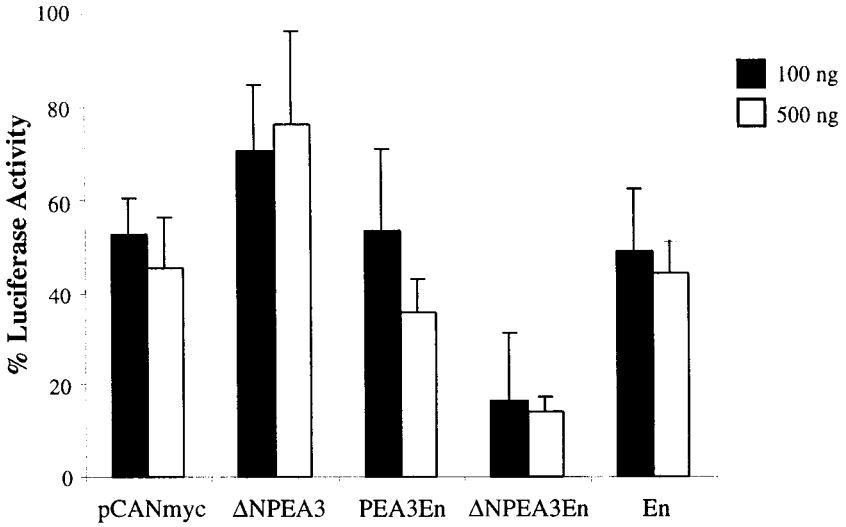
responsive to transfected mouse PEA3 in HeLa cells. FM3A cells were chosen to do the analysis with the dominant-negative PEA3 clones since this mouse mammary adenocarcinoma cell line contains elevated levels of PEA3 protein (Xin et al., 1992). Only ΔNPEA3En exhibited a consistent reduction in reporter gene expression, at both doses used (Figure 4.2). Slight repression was observed at 500 μg of transfected PEA3En expression vector, however this was not consistent among experiments, and reduction in luciferase expression was observed at the same dosage using the empty vector and the construct containing the En repression domain alone. Transfection of ΔNPEA3 appeared to slightly increase reporter expression. This is consistent with the mapping of a putative weak transactivation domain in PEA3 carboxyl terminal to its ETS domain (Bojovic and Hassell, 2001). Total cell extracts were isolated from these transfected FM3A cells to perform Western immuno-blotting analysis to accompany the luciferase expression results. Each of the four constructs was expressed in FM3A cells. In fact, ΔNPEA3 and the En repression domain appeared to be more efficiently expressed in these cells than in COS-1 or HeLa cells.

Overexpression of PEA3 in the mammary tumours of both humans and various transgenic mice strains implicated PEA3 in the oncogenic transformation process (Benz et al., 1997; Trimble et al., 1993). In addition, similar dominant-negative Ets proteins have been shown to inhibit both activated Ki-Ras and NeuNT-mediated transformation of fibroblasts in cell culture (Galang et al., 1996; Wasylyk et al., 1994). To further characterize the inhibitory function of these mutants on PEA3 activity, focus assays were

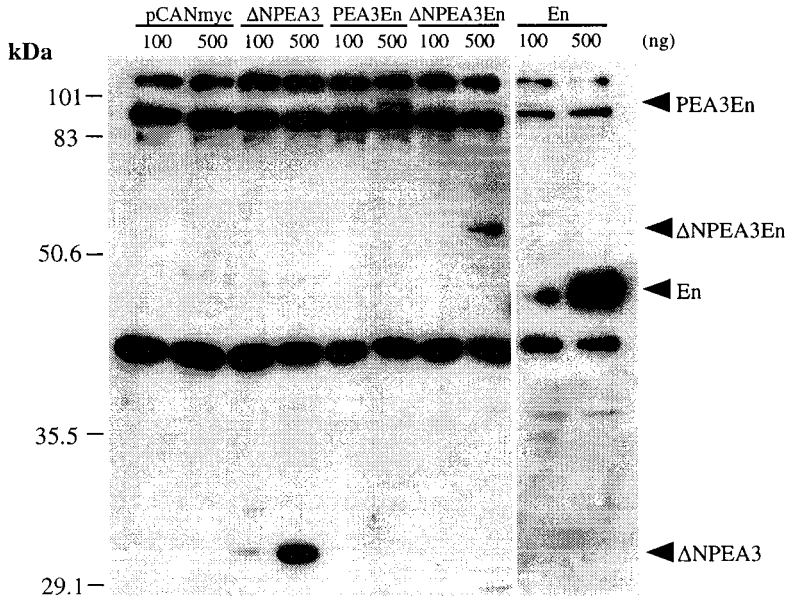
Figure 4.2 Analysis of the ability of various dominant-negative PEA3 mutants to repress expression of a 3xPEA3-*luc* reporter in FM3A cells.

(***a***) FM3A cells were transiently transfected with 100 ng of 3xPEA3-*luc* reporter plasmid and two different amounts (100 and 500 ng) of each expression vector as indicated. ΔNPEA3En showed the only decrease in reporter gene expression (4- to 5-fold). Both the PEA3En and En repression domain control had no effect on luciferase expression, whereas the ΔNPEA3 mutant had a slightly higher level of reporter expression. (***b***) Expression of each protein from the above experiments was verified by Western immuno-blotting using 75 μg of total protein lysate and the anti-*myc* 9E10 monoclonal antibody. The En repression domain was expressed at much higher levels relative to the other protein constructs, thus a shorter autoradiographic exposure is shown for those lanes. In contrast to the expression analysis in COS-1 and HeLa cells, the ΔNPEA3 mutant protein is expressed in FM3A cells at relatively similar levels to the PEA3En and ΔNPEA3En proteins.

a


100
80
60
40
20
0
% Luciferase Activity
100 ng
500 ng
pCANmyc
ΔNPEA3
PEA3En
ΔNPEA3En
En

b


pCANmyc
ΔNPEA3
PEA3En
ΔNPEA3En
En
100 500 100 500 100 500 100 500 100 500
(ng)
kDa
101
83
50.6
35.5
29.1
PEA3En
ΔNPEA3En
En
ΔNPEA3

performed in NIH 3T3 fibroblasts using point-activated NeuNT as a transforming agent and co-transfecting each putative dominant-negative PEA3 construct (Figure 4.3). Consistent with the results obtained in transient transfection reporter assays in FM3A cells, ΔNPEA3En showed the most significant effect in reducing focus formation induced by NeuNT. PEA3En was able to reduce the number of transformed foci as well. The pCANmyc empty expression vector and En repression domain had a slightly reductive effect at the highest doses used (500 μg of plasmid) and ΔNPEA3 had no effect on oncogenic NeuNT-mediated focus formation.

These analyses of the various putative dominant-negative PEA3 mutant constructs revealed that ΔNPEA3En had the most consistent and significant effect on inhibiting both PEA3-responsive reporter gene expression, as well as on reducing NeuNT-mediated transformation of NIH 3T3 cells. Thus, ΔNPEA3En was chosen to perform additional experiments, addressing its dominant-inhibitory function in cell culture.

4.1.3 ΔNPEA3En represses reporter transactivation by PEA3 subfamily proteins

To further characterize the specific dominant-inhibitory function of ΔNPEA3En a number of transient transfection assays were performed. First, the 3xPEA3-*luc* reporter was used and both ΔNPEA3En and the pCANmyc empty vector were titrated over a greater range of concentrations than previously used. Co-transfections into FM3A cells revealed that ΔNPEA3En repressed reporter gene expression in a dose-dependent fashion as compared with the empty vector control (Figure 4.4). Second, the *uPA-luc* reporter

Figure 4.3 Analysis of the ability of various dominant-negative PEA3 mutants to affect NeuNT-mediated focus formation in NIH 3T3 cells.

NIH 3T3 cells were transfected with 50 ng of pJ4ΩNeuNT expression vector and two different amounts (100 and 500 ng) of each of expression vector as indicated. PEA3En and ΔNPEA3En were able to reduce focus formation mediated by oncogenic NeuNT by about 5-fold. At the highest amounts of expression vector used (500 ng) the pCANmyc empty expression vector reduced focus formation by approximately 2-fold. The ΔNPEA3 mutant and En repression domain control had little or no effect on the number of foci formed versus the empty vector.

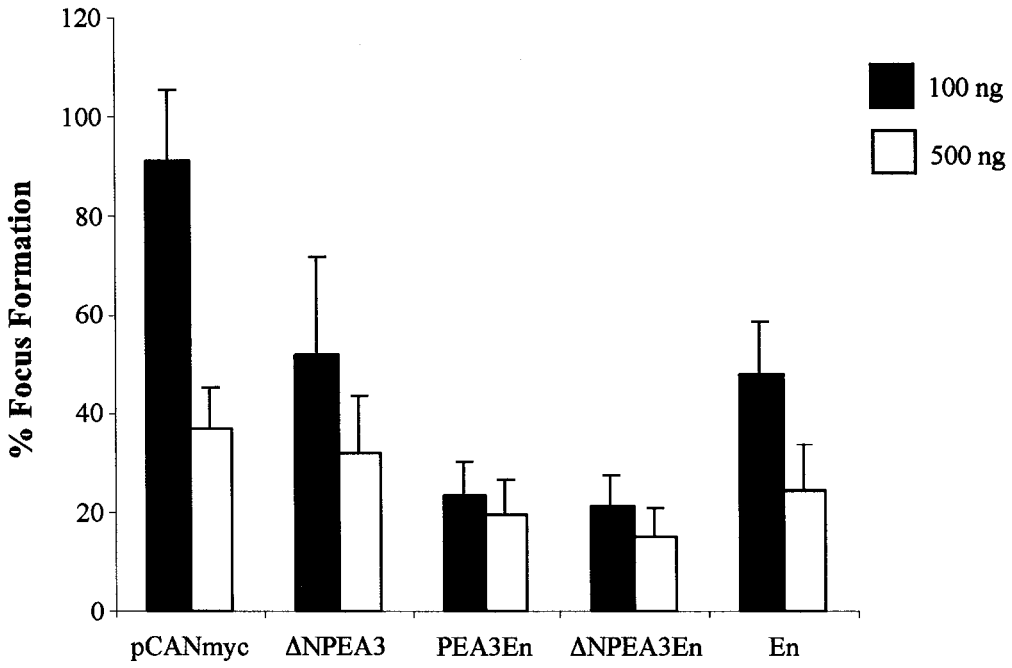
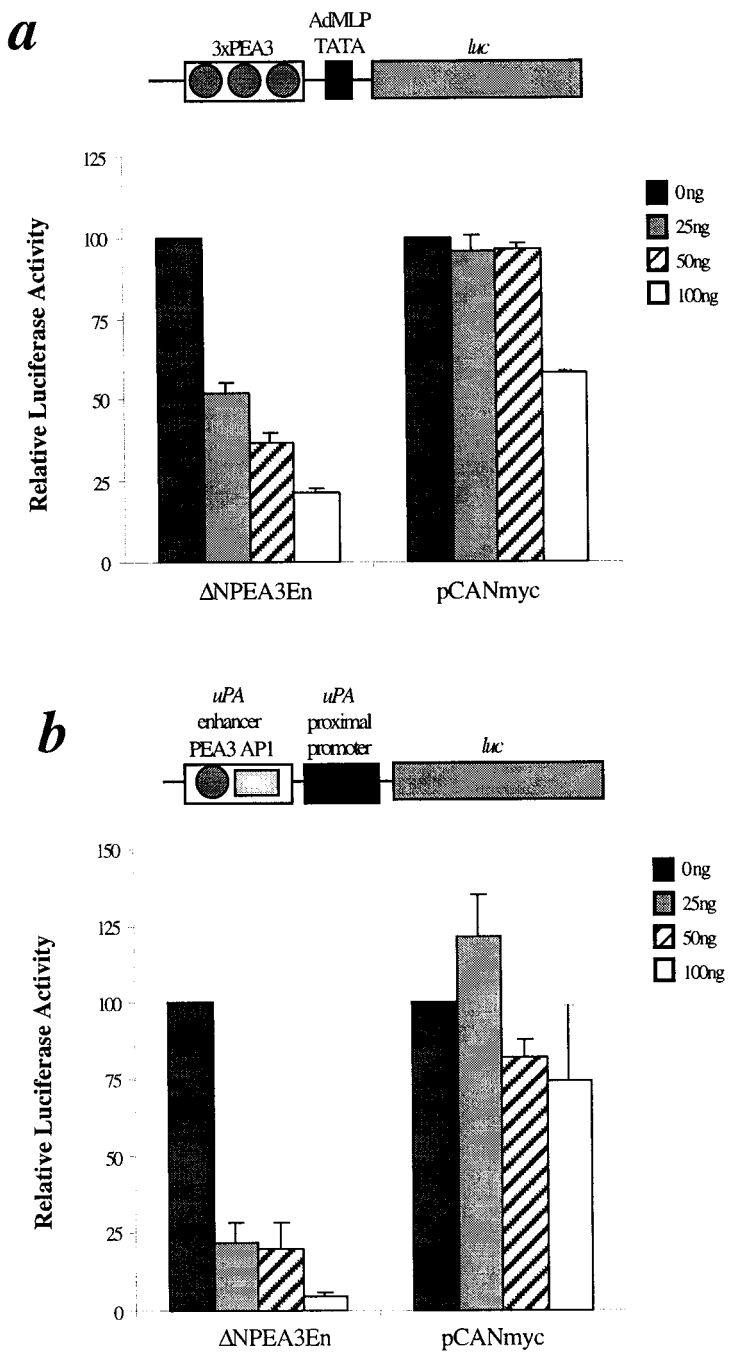

120
100
80
60
40
20
0
% Focus Formation
100 ng
500 ng
pCANmyc
ΔNPEA3
PEA3En
ΔNPEA3En
En

Figure 4.4 ΔNPEA3En is able to repress expression of the 3xPEA3-*luc* and *uPA-luc* reporters in FM3A cells.

(***a***) FM3A cells were transiently transfected with 100 ng of the 3xPEA3-*luc* reporter plasmid and increasing amounts of either the pCANmyc empty expression vector or the ΔNPEA3En expression construct. As illustrated, the 3xPEA3-*luc* reporter contains three PEA3 elements of the polyomavirus enhancer element linked to the adenovirus major late promoter TATA box placed upstream of the luciferase gene. ΔNPEA3En reduces luciferase expression of this reporter in a dose-dependent fashion versus that of the control vector. (***b***) FM3A cells were transiently transfected similar to those described above but instead using 100 ng of the *uPA-luc* reporter vector. The *uPA-luc* reporter contains the urokinase plasminogen activator gene enhancer, which includes PEA3 and AP1 elements, linked to the proximal promoter of this same gene placed upstream of the luciferase protein coding sequence. ΔNPEA3En represses expression of the *uPA-luc* reporter in FM3A cells by up to 10-fold as compared to the pCANmyc empty vector control.


a
AdMLP
TATA
3xPEA3
luc
Relative Luciferase Activity
125
100
75
50
25
0
0ng
25ng
50ng
100ng
ΔNPEA3En
pCANmyc
b
uPA
enhancer
PEA3 AP1
uPA
proximal
promoter
luc
Relative Luciferase Activity
150
125
100
75
50
25
0
0ng
25ng
50ng
100ng
ΔNPEA3En
pCANmyc

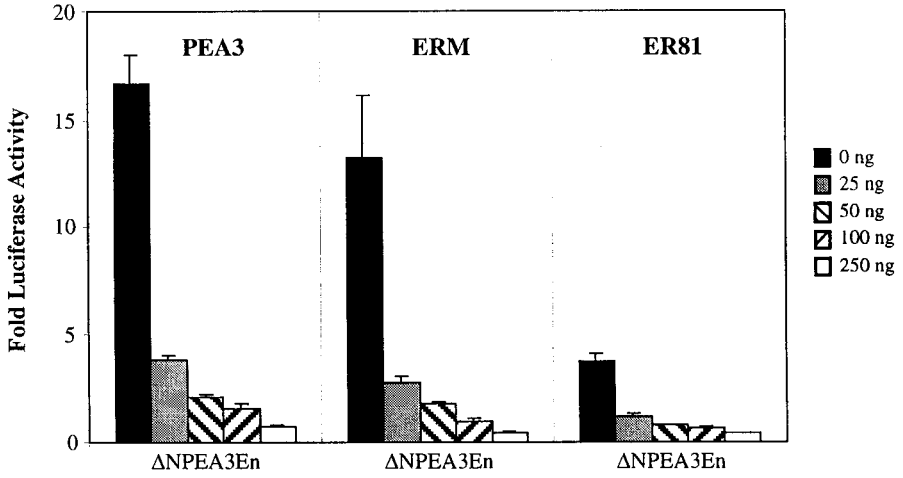
plasmid was used in similar experiments in FM3A cells. This reporter contains the *uPA* gene enhancer containing a single PEA3-AP1 Ras-responsive element fused to the proximal promoter of the *uPA* gene (Stacey et al., 1995). When ΔNPEA3En was co-transfected with the *uPA-luc* reporter, luciferase expression was efficiently reduced versus co-transfection of pCANmyc and *uPA-luc*. In fact, the overall ability of ΔNPEA3En to repress expression of *uPA-luc* appeared to be greater than that observed for the 3xPEA3-*luc* reporter.

The PEA3 subfamily comprises three members which have high amino acid sequence similarity within their ETS domain, and each member is coordinately overexpressed in MMTV-*neu*-induced mammary tumours. Thus, the ability of ΔNPEA3En to block the function of each PEA3 subfamily protein was assessed in transient transfections performed in COS-1 cells using the 5xPEA3-*luc* reporter. This PEA3-responsive reporter plasmid differs from the 3xPEA3-*luc* plasmid in that it contains five reiterated copies of an optimal PEA3 binding site (5'-CCGGAA-3', termed 4_4; S. Bowman and J. A. Hassell, unpublished) and has been shown to be highly responsive to PEA3-dependent transcriptional activation in COS-1 cells (Bojovic and Hassell, 2001). The amount of each transfected PEA3 subfamily expression vector yielded approximately equal amounts of expressed protein (determined empirically prior to these analyses). PEA3, ERM, and ER81 were able to transactivate the 5xPEA3-*luc* reporter by approximately 16-, 13-, and 4-fold, respectively (Figure 4.5). Increasing amounts of co-transfected ΔNPEA3En dramatically reduced the transcriptional activation

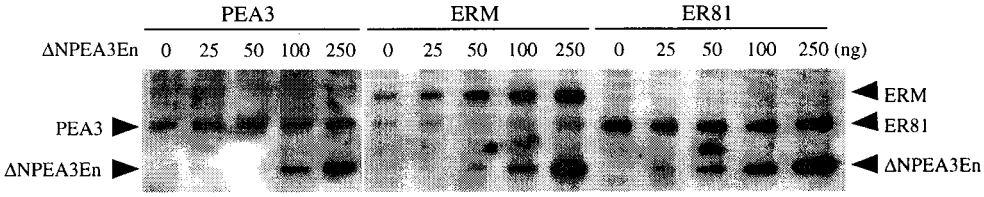
Figure 4.5 Dominant-negative PEA3 is able to repress transactivation by all three PEA3 subfamily proteins.

(***a***) COS-1 cells were transfected with 100 ng of the 5xPEA3-*luc* reporter plasmid and either 25 ng of pCANmyc/PEA3, 250 ng of pCANmyc/ERM, or 1 μg of pCANmyc/ER81 expression vectors. An increasing amount (25, 50, 100, and 250 ng) of the pCANmyc/ΔNPEA3En expression vector was co-transfected with the samples as indicated and the pCANmyc empty vector was used to bring up the total amount to 1.25 μg in all samples. PEA3, ERM and ER81 were able to transactivate the 5xPEA3-*luc* reporter by 16-, 13-, and 4-fold, respectively. ΔNPEA3En was able to reduce luciferase expression induced by each PEA3 subfamily protein to near basal levels (~1). (***b***) Expression of the PEA3 subfamily proteins and of the ΔNPEA3En mutant was verified by Western immuno-blotting of 10 μg of total protein lysates using the anti-*myc* 9E10 monoclonal antibody. The amounts of expression vector used for PEA3, ERM, and ER81 were determined empirically in previous assays to yield approximately equal amounts of each expressed protein. Increasing amounts of the ΔNPEA3En protein is observed in each set of co-transfected samples. It should be noted that at 100 ng of ΔNPEA3En transfected expression vector, an equivalent amount of ΔNPEA3En protein and each of the PEA3 subfamily proteins are expressed, but luciferase expression has been reduced to basal levels (***a***).

a


20
15
10
5
0
Fold Luciferase Activity
PEA3
ERM
ER81
0 ng
25 ng
50 ng
100 ng
250 ng
ΔNPEA3En
ΔNPEA3En
ΔNPEA3En

b


PEA3
ERM
ER81
ΔNPEA3En 0 25 50 100 250 0 25 50 100 250 0 25 50 100 250 (ng)
PEA3
ΔNPEA3En
ERM
ER81
ΔNPEA3En

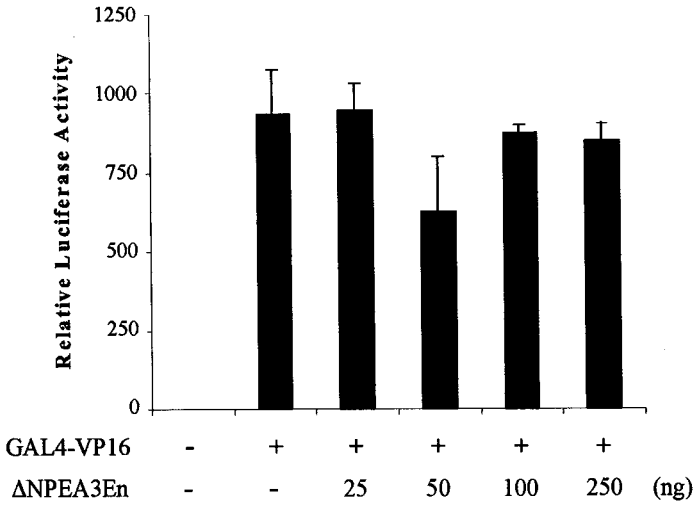
mediated by each PEA3 subfamily member in a dose-dependent manner. Since this result could be due to reduced expression of the PEA3 subfamily proteins by ΔNPEA3En, whole cell protein extracts were isolated and subjected to Western immuno-blot analysis using the anti-*myc* 9E10 antibody, since each ectopically-expressed protein used in these experiments contained the human c-*myc* epitope tag at its amino-terminus. The expression level of PEA3, ERM, and ER81 does not change with increasing amounts of ΔNPEA3En protein. In fact, when 100 ng of ΔNPEA3En expression vector was used, approximately equal amounts of ΔNPEA3En protein and each PEA3 subfamily member was expressed in transfected cell lysates. Since luciferase expression was reduced to near basal levels at this dose of ΔNPEA3En, then ΔNPEA3En appears to be acting as a truly definitive dominant-negative mutant.

To test if ΔNPEA3En repression of PEA3 reporter gene expression was dependent on the presence of ETS sites in the promoter, experiments were performed in COS-1 cells using the 5xGAL4-*luc* reporter plasmid and the GAL4-VP16 effector as a transcriptional activator. As previously observed (Bojovic and Hassell, 2001), the 5xGAL4-*luc* plasmid exhibited very high levels of luciferase expression when co-transfected with GAL4-VP16 (Figure 4.6). Increasing amounts of ΔNPEA3En, the same amounts used in the previous experiments, failed to decrease the activity of 5xGAL4-*luc*. Western immuno-blotting revealed that similar levels of ΔNPEA3En protein was expressed in these experiments as was observed in the assays in which ΔNPEA3En was able to efficiently repress PEA3 subfamily protein function.

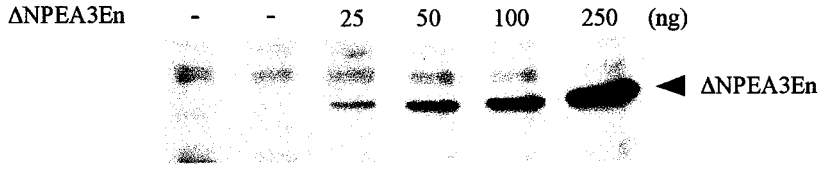
Figure 4.6 ΔNPEA3En does not repress transactivation of the 5xGAL4-*luc* reporter by GAL4-VP16.

(***a***) COS-1 cells were transfected with 100 ng of the 5xGAL4-*luc* reporter plasmid and 250 ng of pSG424/GAL4-VP16 expression vector. An increasing amount (25, 50, 100, and 250 ng) of pCANmyc/ΔNPEA3En expression vector was co-transfected into the samples as indicated with the pCANmyc empty vector being used to bring up the total amount to 250 ng in all samples. GAL4-VP16 was able to potently transactivate the 5xGAL4-*luc* reporter, but ΔNPEA3En had no effect on luciferase expression. (***b***) Expression of ΔNPEA3En was verified by Western immuno-blotting of 10 μg of total protein lysate using the anti-*myc* 9E10 monoclonal antibody. The amount of ΔNPEA3En protein expressed in these samples is similar to that observed in experiments in which ΔNPEA3En was able to efficiently inhibit PEA3 subfamily protein function (see Figure 4.5).

a


Relative Luciferase Activity
1250
1000
750
500
250
0
GAL4-VP16 - + + + + +
ΔNPEA3En - - 25 50 100 250 (ng)

b


ΔNPEA3En - - 25 50 100 250 (ng)
ΔNPEA3En

4.1.4 ΔNPEA3En reduces NeuNT-induced focus formation on NIH 3T3 fibroblasts

To further characterize the ability of ΔNPEA3En to reduce NeuNT-mediated focus formation on fibroblasts, experiments were performed similarly to those done previously, except lower amounts of transfected pCANmyc and ΔNPEA3En expression vectors were used. Again ΔNPEA3En reduced the number of foci induced by oncogenic NeuNT in NIH 3T3 cells by about 4- to 5-fold as compared to the empty vector control (Figure 4.7).

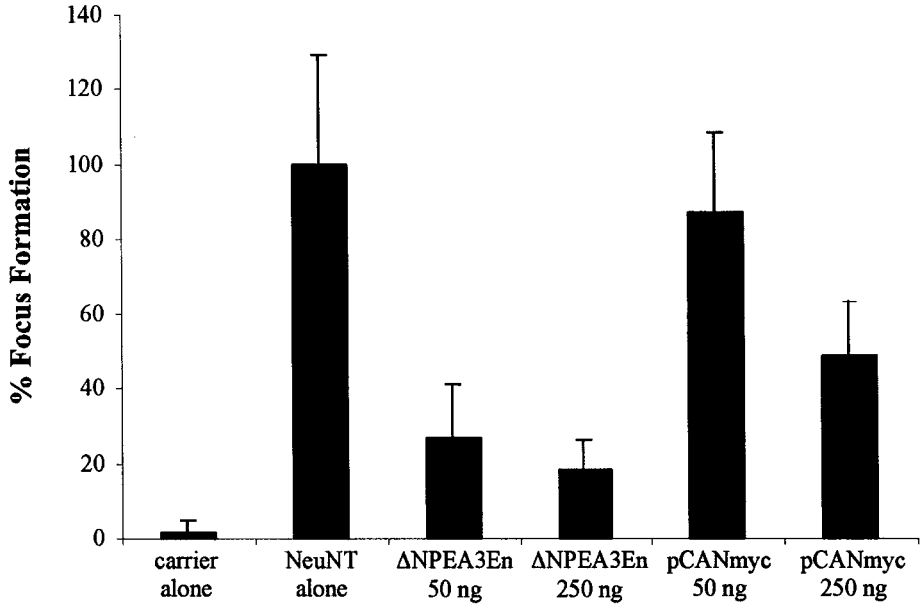
Since the ability of ΔNPEA3En to reduce focus formation in these assays could be a result of either its general toxicity and/or its effects on cell growth, colony formation assays were performed in NIH 3T3 cells. Cells transfected with the same amounts of vector used in the focus formation assays were selected using neomycin-resistance as a selectable marker since the pCANmyc expression vector contains the neomycin resistance gene driven by the SV40 promoter/enhancer. There was no observable difference in the number colonies formed by cells transfected with ΔNPEA3En versus the pCANmyc empty expression vector (Figure 4.8).

4.1.5 Dominant-negative PU.1 Ets mutants act similar to ΔNPEA3En

Previous experiments using dominant-inhibitory Ets mutants of PU.1 revealed similar results in their ability to revert Ki-Ras transformation of 3T3 fibroblasts (Wasylyk et al., 1994). To test whether similar dominant-negative PU.1 mutants would behave similar to ΔNPEA3En, two different expression constructs were generated. The first comprising the

Figure 4.7 ΔNPEA3En reduces NeuNT-induced focus formation on NIH 3T3 cells. (***a***) NIH 3T3 cells were transfected with 50 ng of pJ4ΩNeuNT expression plasmid and two different amounts (50 and 250 ng) of either the pCANmyc empty expression vector or the ΔNPEA3En expression construct. ΔNPEA3En reduces the number of foci induced by oncogenic NeuNT by 4- to 5-fold as compared to the control vector. 250 ng of pCANmyc expression vector reduced the number of foci slightly by about 2-fold. (***b***) Representative sample of formalin-fixed and Giemsa-stained NIH 3T3 cells from a single focus formation assay as described above.

a


% Focus Formation
140
120
100
80
60
40
20
0
carrier alone
NeuNT alone
ΔNPEA3En 50 ng
ΔNPEA3En 250 ng
pCANmyc 50 ng
pCANmyc 250 ng

b

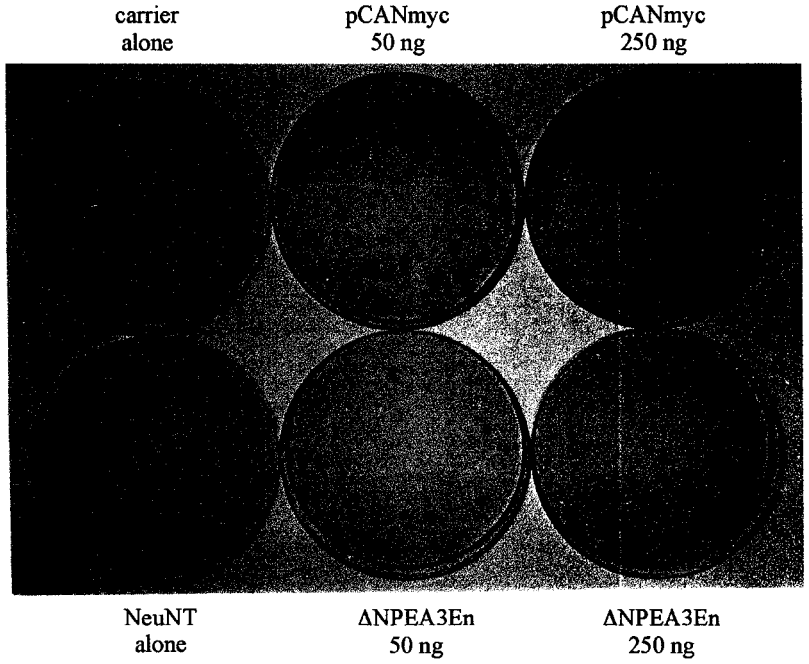
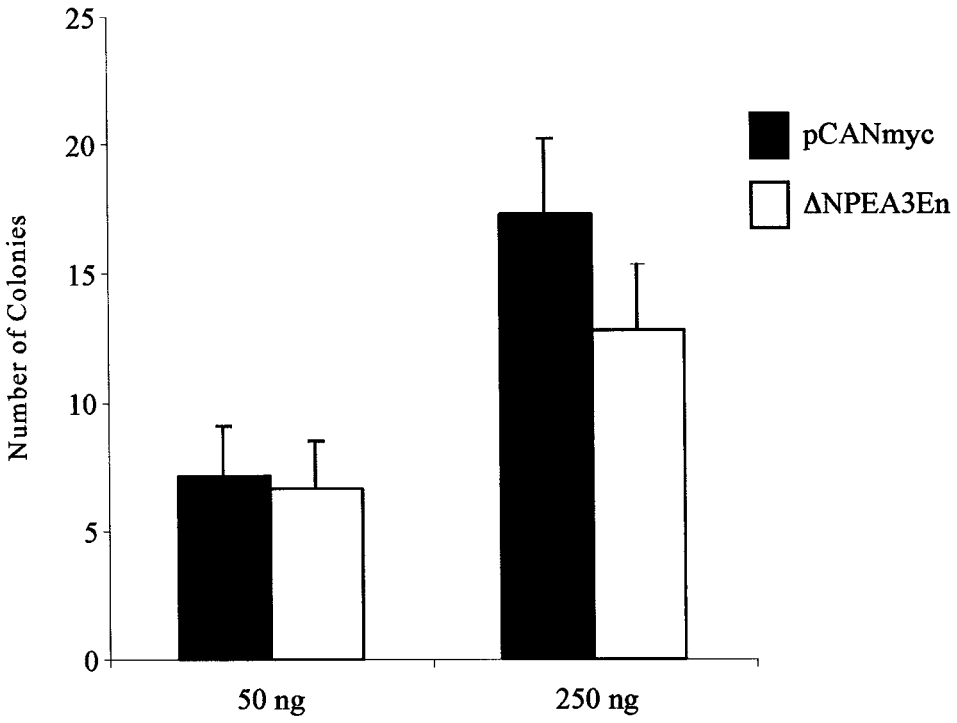

carrier alone
pCANmyc 50 ng
pCANmyc 250 ng
NeuNT alone
ΔNPEA3En 50 ng
ΔNPEA3En 250 ng

Figure 4.8 ΔNPEA3En is not toxic and does not generally inhibit growth of NIH 3T3 cells.

NIH 3T3 cells were transfected with two different amounts (50 and 250 ng) of pCANmyc/ΔNPEA3En or pCANmyc empty expression vectors. These expression vectors also bear the SV40 promoter/enhancer directing expression of the neomycin-resistance selectable marker. G418-resistant colonies were scored two weeks post-transfection. Data presented are the mean number of colonies per 35 mm well for each sample from two independent experiments, with the error bars representing the standard deviation. These analyses reveal no significant difference in the number of colonies at each amount of plasmid DNA transfected between ΔNPEA3En and the control.


25
20
15
10
5
0
Number of Colonies
50 ng
250 ng
pCANmyc
ΔNPEA3En

carboxyl-terminus of PU.1 (ΔNPU.1) that contains the ETS DNA-binding domain, and the second which fuses the En repression domain to the carboxyl-terminus of ΔNPU.1 (ΔNPU.1En) (Figure 4.9). Each of these PU.1 mutants were cloned into the pCANmyc vector analogous to the dominant-negative PEA3 constructs, and protein expression was verified by transient transfection into COS-1 cells followed by Western immuno-blotting using the anti-*myc* 9E10 antibody.

NIH 3T3 cell focus formation assays were performed using pJ4ΩNeuNT expression plasmid and co-transfection of pCANmyc or pCANmyc containing ΔNPEA3En, ΔNPU.1, or ΔNPU.1En. Each of the dominant-inhibitory Ets mutants was able to reduce NeuNT-induced transformation of NIH 3T3 cells to a similar extent (Figure 4.9). It should be noted that dominant-negative Ets-1 and Ets-2 mutants have also been shown to revert Ki-Ras transformation of fibroblasts and NeuNT-induced focus formation in NIH 3T3 cells (Galang et al., 1996; Wasylyk et al., 1994). Thus, it appears that expression of dominant-inhibitory mutants of various Ets proteins is sufficient to reduce the capacity of Neu and Ras to transform fibroblasts in culture.

4.2 Discussion

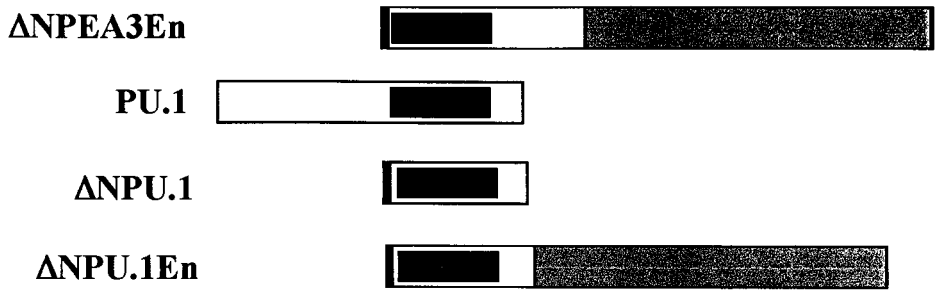
4.2.1 Dominant-negative PEA3 inhibits PEA3 subfamily protein function

The PEA3 subfamily proteins act as DNA-binding proteins to activate target gene expression. The three proteins share nearly identical DNA-binding domains, as well as high amino acid sequence homology within the acidic transactivation domain situated at

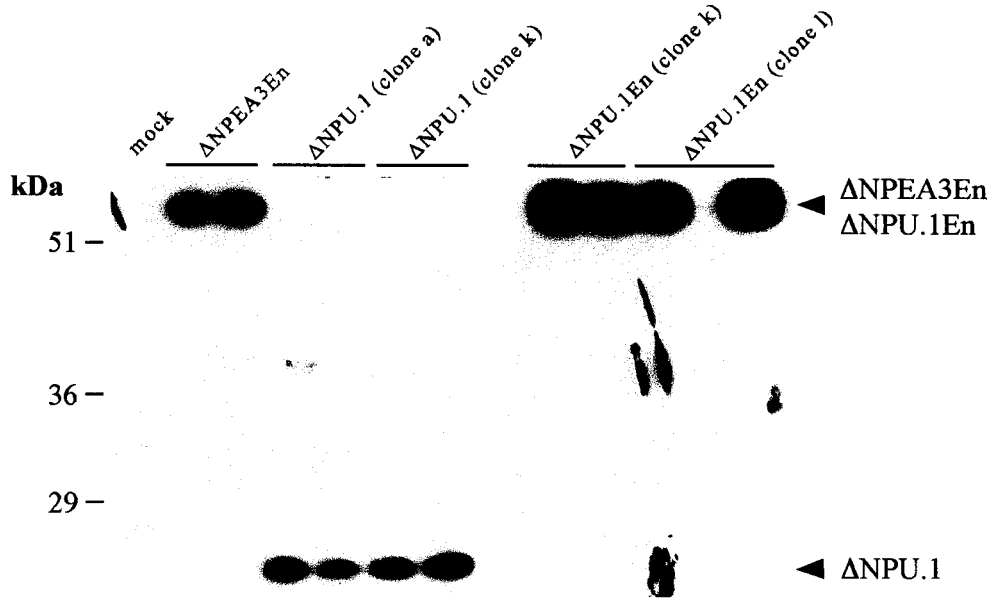
Figure 4.9 Dominant-negative PU.1 mutant proteins can function similar to ΔNPEA3En in reducing NeuNT-mediated focus formation.

(***a***) Schematic illustration of the full-length wild-type PU.1 protein and two dominant-negative mutants, ΔNPU.1 and ΔNPU.1En. The ΔNPEA3En protein is also presented for comparison. The ETS DNA-binding domains of PU.1 and PEA3 are shown in red and the En repression domain fused to the carboxyl-terminus of the ΔNPU.1En and ΔNPEA3En proteins is in grey. All DNA constructs were cloned into the pCANmyc expression vector that provides an 11-amino acid 9E10 c-*myc* epitope tag (black) at the amino-terminus of each expressed protein. (***b***) COS-1 cells were transiently transfected with pCANmyc expression vectors containing ΔNPU.1, ΔNPU.1En, and ΔNPEA3En coding sequences. Western immuno-blotting was performed on 10 μg of total protein lysates using the anti-*myc* 9E10 monoclonal antibody to verify expression of each construct. Two different clones of both ΔNPU.1 (a and k) and ΔNPU.1En (k and l) were chosen to perform these analyses, whereas only one of each was used in subsequent studies. (***c***) Dominant-inhibitory mutants of PU.1 and PEA3 are capable of reducing NeuNT-induced focus formation in mouse fibroblasts. NIH 3T3 cells were transfected with 50 ng of the pJ4ΩNeuNT expression plasmid and 50 ng of either pCANmyc/ΔNPEA3En, pCANmyc/ΔNPU.1, or pCANmyc/ΔNPU.1En expression vectors. Transfection of the pCANmyc plasmid served as a negative control. The number of foci that formed due to NeuNT was reduced to similar levels by all three Ets transdominant mutant proteins.

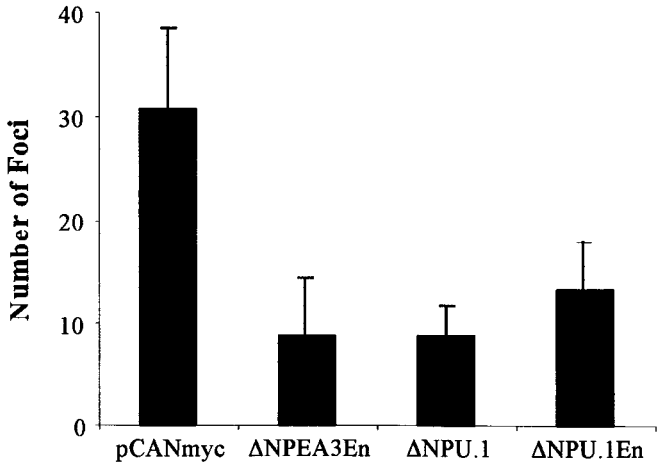
a


ΔNPEA3En
PU.1
ΔNPU.1
ΔNPU.1En

b


mock
ΔNPEA3En
ΔNPU.1 (clone a)
ΔNPU.1 (clone k)
ΔNPU.1En (clone k)
ΔNPU.1En (clone l)
kDa
51 –
36 –
29 –
ΔNPEA3En
ΔNPU.1En
ΔNPU.1

c


Number of Foci
40
30
20
10
0
pCANmyc
ΔNPEA3En
ΔNPU.1
ΔNPU.1En

the amino-terminus (de Launoit et al., 2000). Thus, it is likely that the PEA3 subfamily proteins can function in an analogous fashion when expressed in cells. This argues that expression of a dominant-negative mutant of PEA3 could potentially inhibit all three PEA3 subfamily proteins. Indeed, the capacity of PEA3, ERM, and ER81 to transactivate a PEA3-responsive reporter gene is significantly repressed by increasing amounts of co-transfected ΔNPEA3En expression plasmid. The ability of ΔNPEA3En to act as a true dominant-negative mutant is evidenced by the fact that in samples where approximately equal amounts of PEA3 subfamily protein and ΔNPEA3En are expressed reporter activity is reduced to near basal levels. This likely represents the ability of the Engrailed repression domain present in ΔNPEA3En to actively repress transcription by interfering with TFIID function (Ohkuma et al., 1990) when bound to nearby ETS sites. Passive repression by competition for ETS sites between ΔNPEA3En and the PEA3 subfamily proteins is less likely to completely account for the observed inhibition, since the region of PEA3 protein present in ΔNPEA3En does not exhibit enhanced ability to bind to PEA3 binding sites versus the wild-type PEA3 protein (Bojovic and Hassell, 2001).

Since ΔNPEA3En is able to inhibit the ability of PEA3 subfamily proteins to stimulate gene expression in this artificial reporter gene assay, it may be able to repress the expression of PEA3 target genes. ΔNPEA3En is able to dramatically reduce the *uPA* gene enhancer and promoter driven transcription in FM3A cells, which express both PEA3 and ERM. The *uPA* gene is considered an Ets target gene since activation of the

uPA enhancer by oncogenes, growth factors, and phorbol esters has been shown to require an ETS site present in the ETS-AP1 composite Ras-responsive element (D'Orazio et al., 1997). The gene for a secreted extracellular matrix component, osteopontin (*Opn*), has been identified as a potential PEA3 subfamily target gene, and the *Opn* promoter is repressed by dominant-negative PEA3 (J. Wong and J. A. Hassell, unpublished). The genes for several different adhesion molecules, such as *ICAM*-1 and *ALCAM*, have been identified as target genes for ERM and PEA3, respectively (de Launoit et al., 1998; Ellison, 2001). The ALCAM promoter is transactivated by PEA3, and likewise is repressed by expression of ΔNPEA3En (Ellison, 2001). Several other target genes involved in the initiation and progression of human cancers, including those encoding matrix metalloproteinases, *CCND1*, and *HER2*, may be repressed similarly by ΔNPEA3En. Thus, in cells that aberrantly overexpress the PEA3 subfamily proteins, especially in breast tumour cells, ΔNPEA3En would be predicted to efficiently inhibit their function and possibly alter the transformed phenotype.

4.2.2 Dominant-negative PEA3 reduces oncogenic Neu-mediated focus formation

Several *ets* genes have been shown to function in transformation of mouse fibroblasts via the use of transdominant mutants of the respective proteins. Expression of the DNA-binding domain of Ets-1, Ets-2, and PU.1/Spi-1 revert the transformed phenotype of Ki-Ras-transformed fibroblasts (Wasylyk et al., 1994). Similarly, co-expression of activated Neu receptor tyrosine kinase and dominant-negative mutants of

Ets-2 and Ets-1 yielded reduced number of transformed foci, possibly due to ablation of Ets protein function downstream of the Neu signalling pathway (Galang et al., 1996). The results presented herein illustrate the similar ability of a dominant-negative mutant version of PEA3 to reduce transformation of NIH 3T3 cells by point-activated NeuNT. Similar mutant PU.1 constructs were generated that also had the same quantitative effect on transformation in this system. The results from these studies taken with the previous published data illustrates the functional role for one or more Ets proteins in mediating oncogenesis induced by the Neu-Ras signal transduction cascade. Although Ets proteins can be divided into subgroups based on differing affinities for distinct ETS binding sites *in vitro*, overexpression of any one Ets protein may lead to the promiscuous binding to both low and high affinity sites. Therefore, the similar ability of various different dominant-negative Ets proteins in reducing tranformation may be due to this effect.

The question must now be posed as to which *ets* genes are expressed in transformed cells, and what function they serve in Neu and/or Ras oncogenic signaling? The majority of *Pea3*-null mouse embryonic fibroblasts are refractory to transformation by activated Ras and Neu (G. Fidalgo, L. Hastings and J. A. Hassell, unpublished). Introduction of PEA3 back into these cells complements loss-of-function of PEA3, and restores the capacity of these cells to be transformed (G. Fidalgo and J. A. Hassell, unpublished). Immortalized mouse embryonic fibroblasts when transformed by Ras and Neu generally exhibit coordinate overexpression of the *Pea3* subfamily *ets* genes (G. Fidalgo and J. A. Hassell, unpublished) reminiscent of what is observed in the majority of MMTV-*neu* induced mammary adenocarcinomas of female transgenic mice (see Figure

3.2). Also, the *Pea3* subfamily genes are upregulated in response to induced Raf serine/threonine kinase signalling in NIH 3T3 cells (J-H Xin and J. A. Hassell, unpublished). *PEA3* and *ERM* mRNA are increased by 11- and 5-fold respectively in human MCF10A breast epithelial cells following Raf stimulation (Schulze et al., 2001). Thus it appears that *PEA3* subfamily *ets* genes represents a common target of this signalling pathway in various cell types. It is noteworthy that the *Pea3* subfamily is overexpressed in mammary tumours of MMTV-*Wnt-1* transgenic mice (Howe et al., 2001), as well as in the intestinal tumours of *min* mice that lack a functional adenomatous polyposis coli (*Apc*) gene (Crawford et al., 2001). Wnt-1 signalling via the frizzled receptor acts through β-catenin that is negatively regulated by both APC and GSK3-β (Bienz and Clevers, 2000). This represents another independent signalling pathway involved in different epithelial cancers that may also require the function of PEA3 subfamily proteins.

CHAPTER FIVE

GENERATION AND CHARACTERIZATION OF MMTV-PEA3, MMTV-ΔNPEA3En, AND MMTV-EWSΔN268PEA3 TRANSGENIC MICE

5.1 Results

5.1.1 Generation of transgenic mice

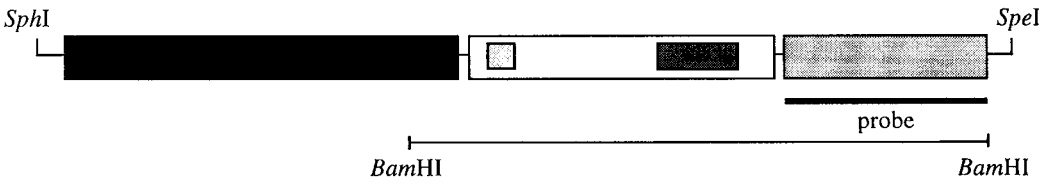
The results obtained in cell culture illustrated that ΔNPEA3En acts as a dominant-inhibitory mutant of PEA3, and it has the ability to block the function of ERM and ER81. Thus, ΔNPEA3En was expressed in the mammary glands of female mice to elucidate the role of PEA3 in mammary tumorigenesis induced by MMTV-*neu*. In addition, transgenic mice were created that overexpressed wildtype PEA3, and a naturally occurring potentially oncogenic mutant, EWSΔN268PEA3 (Crnac, 1997) (Figure 5.1). The latter had been isolated as a rare translocation involving the EWS and PEA3 genes in human Ewing's sarcoma (Kaneko et al., 1996; Urano et al., 1996), whose chimaeric protein product has increased transcriptional activity versus that of the wildtype PEA3 protein (Crnac, 1997).

Each of the DNA sequences corresponding to the three different constructs were placed downstream of the mouse mammary tumour virus-long terminal repeat (MMTV-LTR) and the resultant constructs were linearized, purified, and injected into the male pronucleus of fertilized one-cell embryos. The resultant mice were screened by Southern analysis using a probe corresponding to the simian virus 40 polyadenylation cassette

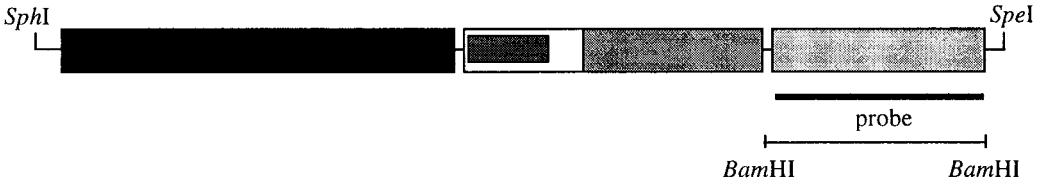
Figure 5.1 Schematic diagrams of MMTV-PEA3, MMTV-ΔNPEA3En, and MMTV-EWSΔN268PEA3 transgenes.

Schematic representation of each MMTV transgene construct used to generate transgenic lines of mice. Sequences coding for PEA3 are shown in white, with those containing the ETS DNA-binding domain (red) and acidic transactivation domain (yellow) also illustrated. The En repression domain of ΔNPEA3En is indicated in green and the EWS portion of EWSΔN268PEA3 is shown in blue. Each expression construct is cloned downstream of the MMTV-LTR (black) and contains DNA sequences at the 3' end comprising an intron and polyadenylation signal from SV40 (grey). The SV40 intron/polyadenylation cassette (SPA) was used as a probe for both Southern analyses of *Bam*HI-digested genomic tail DNA, and RNase protection assays of RNA isolated from various tissues of the transgenic mice. The unique restriction endonuclease sites used to digest the transgene DNA constructs prior to embryo microinjection are shown at the termini of each.

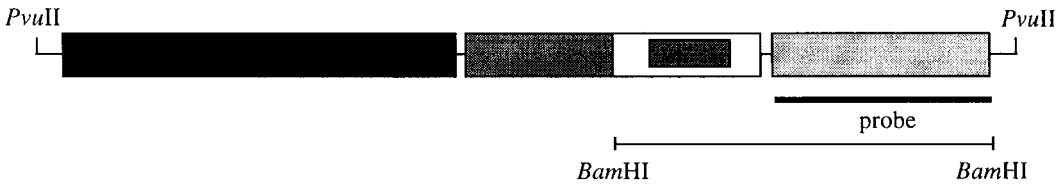
MMTV-PEA3-SV40pA



MMTV-ΔNPEA3En-SV40pA



MMTV-EWSΔN268PEA3-SV40pA



(SPA) located in the 3' untranslated region of each transgene. The number of mice from each potential transgenic strain which carried the respective transgene were five for MMTV-PEA3, four for MMTV-ΔNPEA3En, and only two for MMTV-EWSΔN268PEA3 (Table 5.1). It should be noted that two independent rounds of microinjections were performed for the MMTV-EWSΔN268PEA3 transgene construct, with one potential founder mouse being identified from each. In establishing the various lines, it became evident that one potential MMTV-PEA3 founder did not transmit the transgene to its offspring. The remaining lines of transgenic mice were screened for transgene expression by RNase protection assay using total RNA isolated from female mouse mammary glands, both virgin and lactating, and from male epididymis and testis. These organs are known to elicit potentially high levels of MMTV-LTR directed transgene expression (Webster and Muller, 1994) and thus were chosen for the initial characterization. Of the mice screened by this method, several lines of MMTV-PEA3 transgenics expressed the transgene either very highly (988 line) or at moderate levels (969 and 955 lines) when compared with the internal control, rpL32. There appeared to be only one transgenic line (921 line) that expressed the MMTV-ΔNPEA3En transgene at moderate levels with two others expressing ΔNPEA3En at much lower levels (979 and 985 lines). Both MMTV-EWSΔN268PEA3 transgenic lines did not express the transgene in the female mammary glands or in the male reproductive organs as detected by this method.

Further analyses of transgene expression in specific lines of MMTV-PEA3 and MMTV-ΔNPEA3En transgenic mice were performed using total RNA isolated from various other

Table 5.1 Generation of MMTV-PEA3, MMTV-ΔNPEA3En, and MMTV-EWSΔN268PEA3 transgenic mice.

Several lines of transgenic mice were generated that successfully expressed MMTV-PEA3 and MMTV-ΔNPEA3En. Expression was observed to varying extents in the mammary gland of virgin (v. mgld.) and lactating (l. mgld.) female mice, and in the epididymis (epid.) and testis of male mice. No lines were isolated that expressed the MMTV-EWSΔN268PEA3 transgene.

a- Two independent rounds of embryo microinjections were performed for the MMTV-EWSΔN268PEA3 transgene construct.

b- The 978 founder died before successfully transmitting the transgene to its progeny.

c- Relative expression was determined by RNase protection assay using the SPA riboprobe and 20 μg of total RNA from the indicated tissues. The rpL32 riboprobe was used as an internal control for RNA loading.

++++ - very high expression (≥2-fold relative to rpL32)

+++ - high expression (1- to 2-fold relative to rpL32)

++ - moderate expression (0.5- to 1-fold relative to rpL32)

+ - low expression (<0.5-fold relative to rpL32, but detectable above background)

- - not detectable above background

n.d.- not determined

n.a. - not applicable

Transgene	Embryos Implanted	Live Births	Founders	Ear Tag	Germline	Expression[c]			
						v. mgld	l. mgld.	epid	testis
MMTV-PEA3	235	29	5	955	+	+	+	++	-
				969	+	++	n.d.	n.d.	+
				976	+	-	-	-	-
				978	-[b]	n.a.	n.a.	n.a.	n.a
				988	+	+++	++++	++++	+
MMTV-ΔNPEA3En	208	47	4	906	+	-	n.d.	n.d.	n.d.
				921	+	++	n.d.	++	-
				979	+	+	+	n.d.	n.d.
				985	+	-	n.d.	n.d.	-
MMTV-EWSΔN268PEA3	266[a]	94	2	494	+	-	-	-	-
				638	+	-	-	-	-

mouse organs to determine the specificity of transgene expression. These tissues included the heart, lung, liver, kidney, spleen, salivary gland, as well as the mammary gland from virgin and lactating female mice and the testis, epididymis, and seminal vesicle of male mice. The 988 and 955 lines of MMTV-PEA3 transgenics exhibited the highest level of expression in the lactating mammary gland, with relatively high expression being observed in the mammary gland of virgin mice, the salivary gland, and the epididymis (Figure 5.2). Similar analysis of the 921 line of MMTV-ΔNPEA3En transgenic mice revealed the highest level of transgene expression in the mammary glands of virgin female mice and seminal vesicle of male mice, with slightly less expression in the lactating mammary gland of females and epididymis in males (Figure 5.3). All subsequent experimental analyses that are presented herein were performed using the 988 line of MMTV-PEA3 transgenics and the 921 line of MMTV-ΔNPEA3En transgenics.

To approximate the transgene copy number in MMTV-PEA3 and MMTV-ΔNPEA3En transgenic mice, Southern blotting analysis was performed on genomic tail DNA using a probe that would recognize both the endogenous *Pea3* gene and each transgene (Figure 5.4). The bands corresponding to the expected DNA fragments were quantified by PhosphorImager analysis, and made relative to the endogenous *Pea3* gene (ie. two copies) in each sample. MMTV-PEA3 transgenic mice possess approximately 12 copies of the transgene, whereas the MMTV-ΔNPEA3En transgenics harbour about 4 copies.

The *Pea3* gene is expressed at low levels in the normal mouse mammary gland

Figure 5.2 Analysis of MMTV-PEA3 expression in tissues of the 988 and 955 transgenic mice.

(***a, b***) RNase protection assays were performed using the radiolabelled SPA riboprobe and 20 μg of total RNA isolated from the indicated organs. (***a***) In the 988 line, the MMTV-PEA3 transgene is expressed highest in the mammary gland of virgin and lactating female mice, as well as in the salivary gland and the male epididymis with weaker expression being detected in a few other organs. (***b***) In the 955 line, the MMTV-PEA3 transgene is expressed in the same target organs, but to a lesser extent than in the 988 line. (***c, d***) Quantification of the above RNase protection assays by PhosphorImager analysis and using ImageQuant 3.3 software. MMTV-PEA3 transgene expression in the 988 line (***c***) and 995 line (***d***) were normalized to rpL32 that served as an internal loading control.

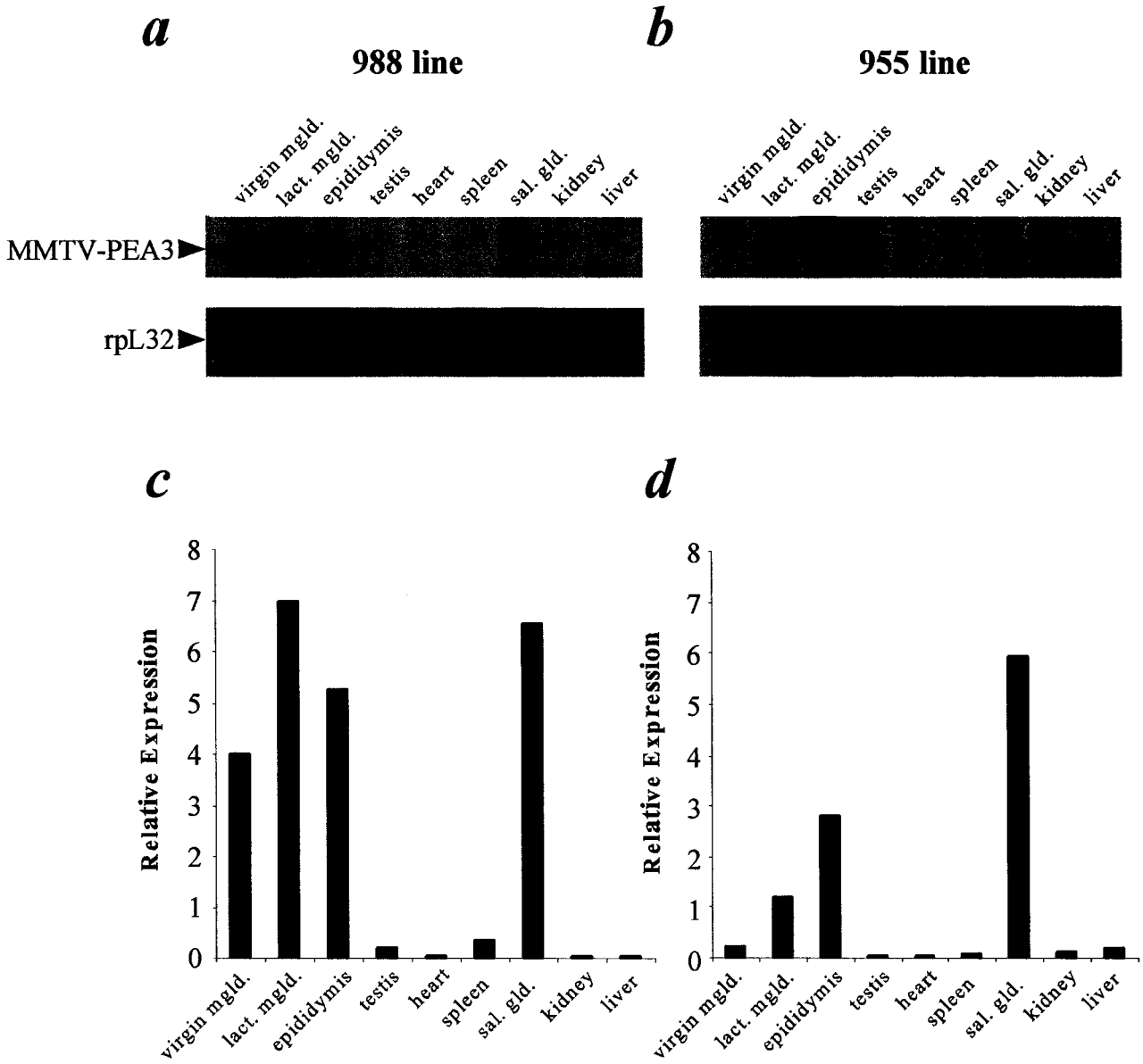
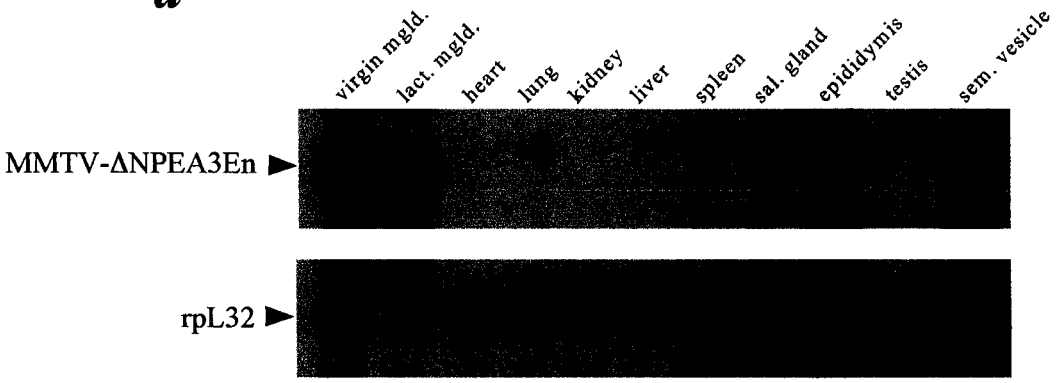

a
988 line
virgin mgld.
lact. mgld.
epididymis
testis
heart
spleen
sal. gld.
kidney
liver
MMTV-PEA3
rpL32
b
955 line
virgin mgld.
lact. mgld.
epididymis
testis
heart
spleen
sal. gld.
kidney
liver
c
Relative Expression
0
1
2
3
4
5
6
7
8
virgin mgld.
lact. mgld.
epididymis
testis
heart
spleen
sal. gld.
kidney
liver
d
Relative Expression
0
1
2
3
4
5
6
7
8
virgin mgld.
lact. mgld.
epididymis
testis
heart
spleen
sal. gld.
kidney
liver

Figure 5.3 Analysis of MMTV-ΔNPEA3En expression in tissues of the 921 transgenic mice.

(***a***) RNase protection assays were performed using the radiolabelled SPA riboprobe and 20 μg of total RNA isolated from the indicated organs. The MMTV-ΔNPEA3En transgene is expressed highest in the mammary gland of virgin and lactating female mice, as well as in the seminal vesicle and the epididymis of male mice, with weaker expression being detected in a few other organs. (***b***) Quantification of the above RNase protection assays by PhosphorImager analysis and using ImageQuant 3.3 software. MMTV-ΔNPEA3En transgene expression was normalized to rpL32 that served as an internal loading control.

a


virgin mgld.
lact. mgld.
heart
lung
kidney
liver
spleen
sal. gland
epididymis
testis
sem. vesicle
MMTV-ΔNPEA3En
rpL32

b

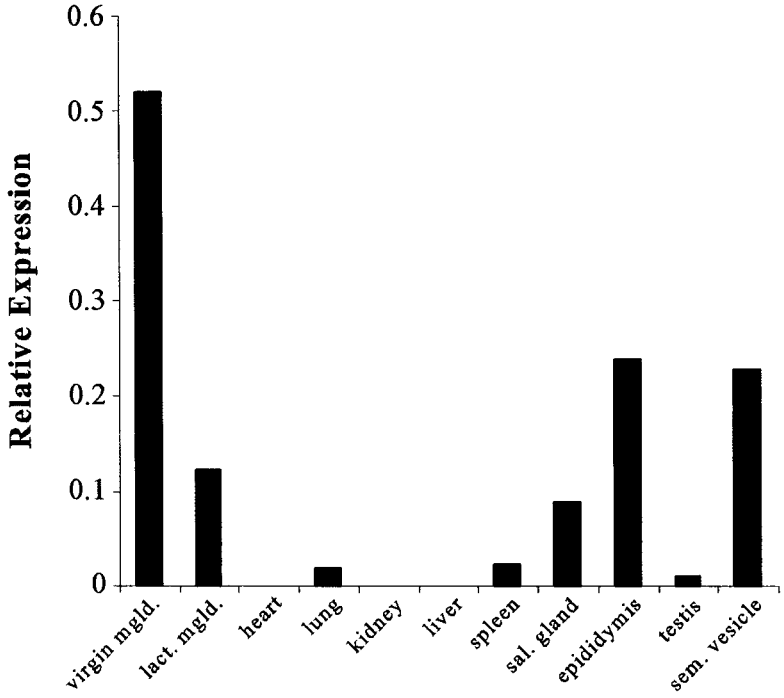
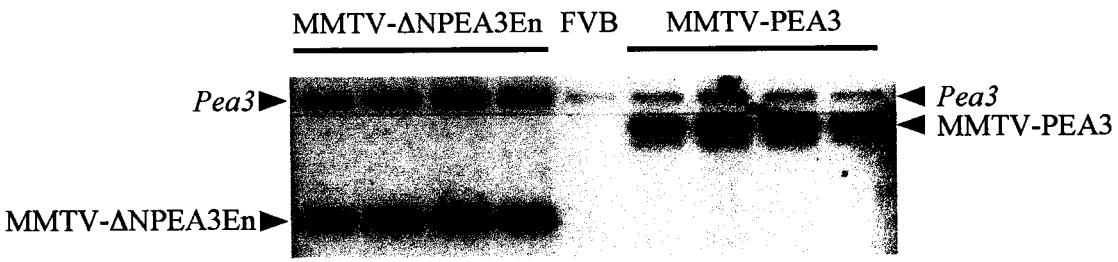

Relative Expression
0.6
0.5
0.4
0.3
0.2
0.1
0
virgin mgld.
lact. mgld.
heart
lung
kidney
liver
spleen
sal. gland
epididymis
testis
sem. vesicle

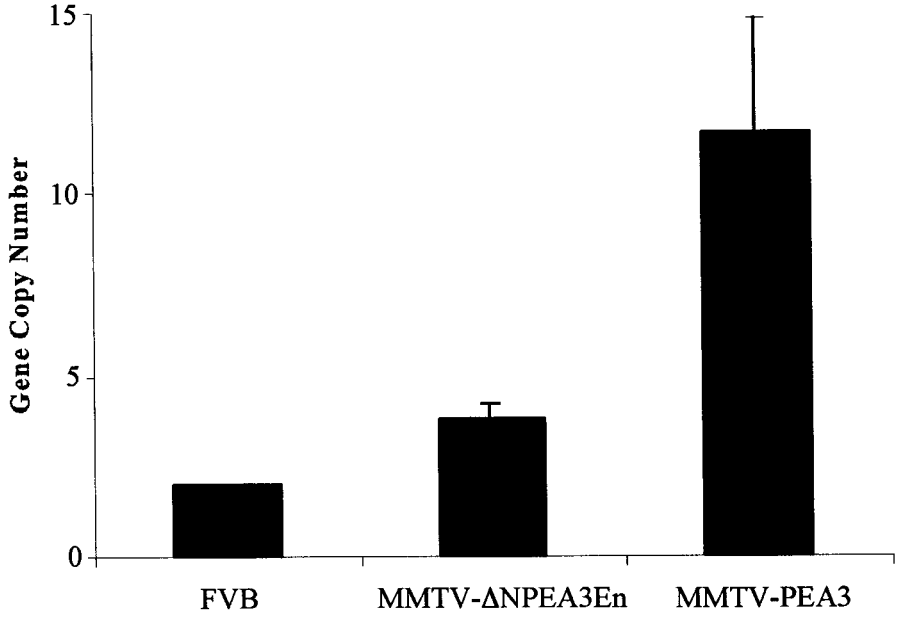
Figure 5.4 Estimation of transgene copy number in MMTV-PEA3 and MMTV-ΔNPEA3En transgenic mice.

(***a***) Southern analysis of *Bam*HI-digested genomic tail DNA isolated from several MMTV-PEA3 and MMTV-ΔNPEA3En transgenic mice and using the radiolabelled *Dra*I-*Pvu*II fragment of the mouse *Pea3* cDNA as a probe. The bands corresponding to endogenous *Pea3*, MMTV-PEA3, and MMTV-ΔNPEA3En are indicated. Endogenous *Pea3* served as a control for equal loading of DNA among samples. (***b***) Quantification of each DNA band was performed by PhosphorImager analysis using ImageQuant 3.3 software and normalizing each transgene band to the corresponding endogenous *Pea3* signal representing 2 copies of the gene in that sample. The mean of the samples from each line of transgenic mice is illustrated, with the error bars indicating the standard deviation among the samples. This analysis reveals that the MMTV-PEA3 transgene copy number for the MMTV-PEA3 transgenic mice is approximately 12, and for the MMTV-ΔNPEA3En transgenic mice it is approximately 4.

a


MMTV-ΔNPEA3En
FVB
MMTV-PEA3
Pea3
MMTV-ΔNPEA3En
Pea3
MMTV-PEA3

b


Gene Copy Number
15
10
5
0
FVB
MMTV-ΔNPEA3En
MMTV-PEA3

and is readily detectable in male epididymis (Xin et al., 1992) (see Figure 3.1). To directly quantify the level of PEA3 overexpression and ectopic dominant-negative PEA3 expression, versus that of the endogenous *Pea3* gene in the mammary gland of female mice and epididymis of male mice, RNase protection assays were performed (Figure 5.5). A single riboprobe that would recognize and differentiate between endogenous *Pea3* mRNA, as well as each of the two transgene messages, was used with total RNA isolated from the mammary gland and epididymis of FVB/N and each strain of transgenic mice. In the virgin female mouse mammary gland, the MMTV-ΔNPEA3En transgene was expressed at approximately 250-fold higher than the endogenous *Pea3* gene, whereas the MMTV-PEA3 transgene was overexpressed about 100-fold. The apparent lower level of MMTV-PEA3 transgene expression observed was due to the increase in endogenous *Pea3* mRNA of approximately 4-fold in the mammary gland sample of the 988 line of MMTV-PEA3 transgenic female mice. In the male epididymis, MMTV-PEA3 is overexpressed by 400-fold over *Pea3*, whereas the MMTV-ΔNPEA3En transgene is expressed 150-fold above the endogenous *Pea3* gene.

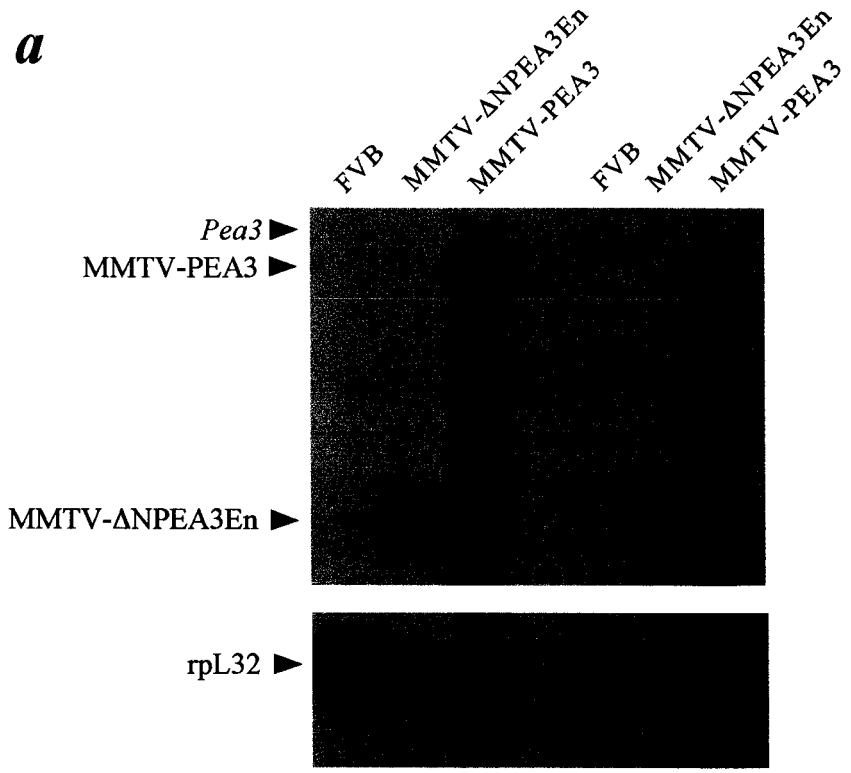
5.1.2 Analysis of mammary gland development

Since both wildtype and dominant-negative forms of PEA3 were overexpressed in the mammary glands of their respective transgenic lines, wholemounts and histological analyses were performed on the mammary glands at specific stages of postnatal development.

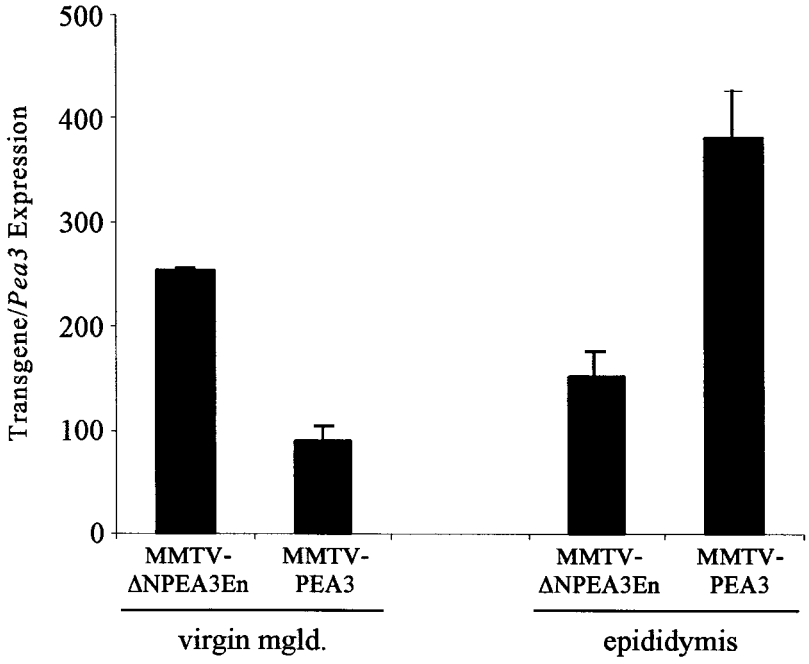
Figure 5.5 The MMTV-PEA3 and MMTV-ΔNPEA3En transgenes are overexpressed as compared to the endogenous *Pea3* gene.

(***a***) RNase protection assay performed on 20 μg of total RNA isolated from the mammary glands of 8-week-old virgin female mice and from the epididymides of male mice from either the 921 line of MMTV-ΔNPEA3En transgenics, the 988 line of MMTV-PEA3 transgenics, or the wildtype FVB/N strain. The PEA3DP riboprobe was used since it recognizes both endogenous *Pea3* mRNA and each transgene mRNA species in the same sample but yields differentially protected RNA species for each. The *Pea3* gene is expressed, albeit at low levels, in the mammary gland and epididymis of mice, whereas both the MMTV-PEA3 and the MMTV-ΔNPEA3En transgenes are expressed at significantly higher levels. The rpL32 riboprobe was used as an internal control for RNA loading. (***b***) Quantification of transgene expression in the mammary gland and epididymis of MMTV-PEA3 and MMTV-ΔNPEA3En transgenic mice versus the endogenous *Pea3* gene as determined by PhosphorImager analysis using ImageQuant 3.3 software. MMTV-PEA3 is expressed between 100-fold higher in the mammary gland and 400-fold higher in the epididymis than is *Pea3*, and MMTV-ΔNPEA3En is expressed about 250-fold higher in the mammary gland and 150-fold higher in the epididymis.

a


FVB
MMTV-ΔNPEA3En
MMTV-PEA3
FVB
MMTV-ΔNPEA3En
MMTV-PEA3
Pea3
MMTV-PEA3
MMTV-ΔNPEA3En
rpL32

b


Transgene/Pea3 Expression
500
400
300
200
100
0
MMTV-ΔNPEA3En
MMTV-PEA3
MMTV-ΔNPEA3En
MMTV-PEA3
virgin mgld.
epididymis

The site of most extensive epithelial cell proliferation and migration occurs in the TEB of the mouse mammary gland during puberty (Daniel and Silberstein, 1987). Wholemount analysis revealed no difference in the number, or progression, of the TEBs among FVB/N, MMTV-PEA3 transgenics, and MMTV-ΔNPEA3En transgenic female mice (Figure 5.6). This observation was upheld upon histological observation of the TEB structures, where the general cellular make-up of the TEB was normal in the MMTV-PEA3 and MMTV-ΔNPEA3En samples as compared to FVB/N controls (Figure 5.6).

The mammary glands of virgin female MMTV-PEA3 transgenic mice exhibited a slightly increased number of ductal branches versus that of age-matched FVB/N controls (Figure 5.7). In addition, near the periphery of the mammary gland the terminal ducts appeared to have numerous side buds along their length in a much higher density as compared with the analogous regions of the FVB/N gland (Figure 5.7).

In contrast, the MMTV-PEA3 transgenic female mice had a marked decrease in the extent of lobulo-alveolar development of the mammary gland during pregnancy, as compared with age-matched FVB/N control mice (Figure 5.8). Since this observation implies a potential defect in the ability of MMTV-PEA3 transgenic female mice to produce milk, histological analysis was performed on mammary glands isolated from pregnant female FVB/N and MMTV-PEA3 mice. Although mammary glands of pregnant MMTV-PEA3 female mice had an overall reduced number of lobulo-alveoli, these structures were still able to differentiate, as determined by the presence of milk droplets within secretory epithelial cells (Figure 5.9).

Similar analyses were performed on the mammary glands of virgin female

Figure 5.6 The mammary gland terminal end buds of MMTV-PEA3 and MMTV-ΔNPEA3En transgenic female mice are normal.

The #4 inguinal mammary glands were isolated from 5-week-old virgin female wild-type FVB/N (***a*** and ***b***), MMTV-ΔNPEA3En transgenic (***c*** and ***d***), and MMTV-PEA3 transgenic (***e*** and ***f***) mice. There is no apparent difference among the three strains of mice in the number or progression of TEBs as observed in haematoxylin-stained wholemounts (***a***, ***c*** and ***e***). In addition, histological make-up of the TEB is similar in the haematoxylin- and eosin-stained histological tissue sections (***b***, ***d*** and ***f***). Original magnification = 10x (***a***, ***c*** and ***e***), 200x (***b***, ***d*** and ***f***)

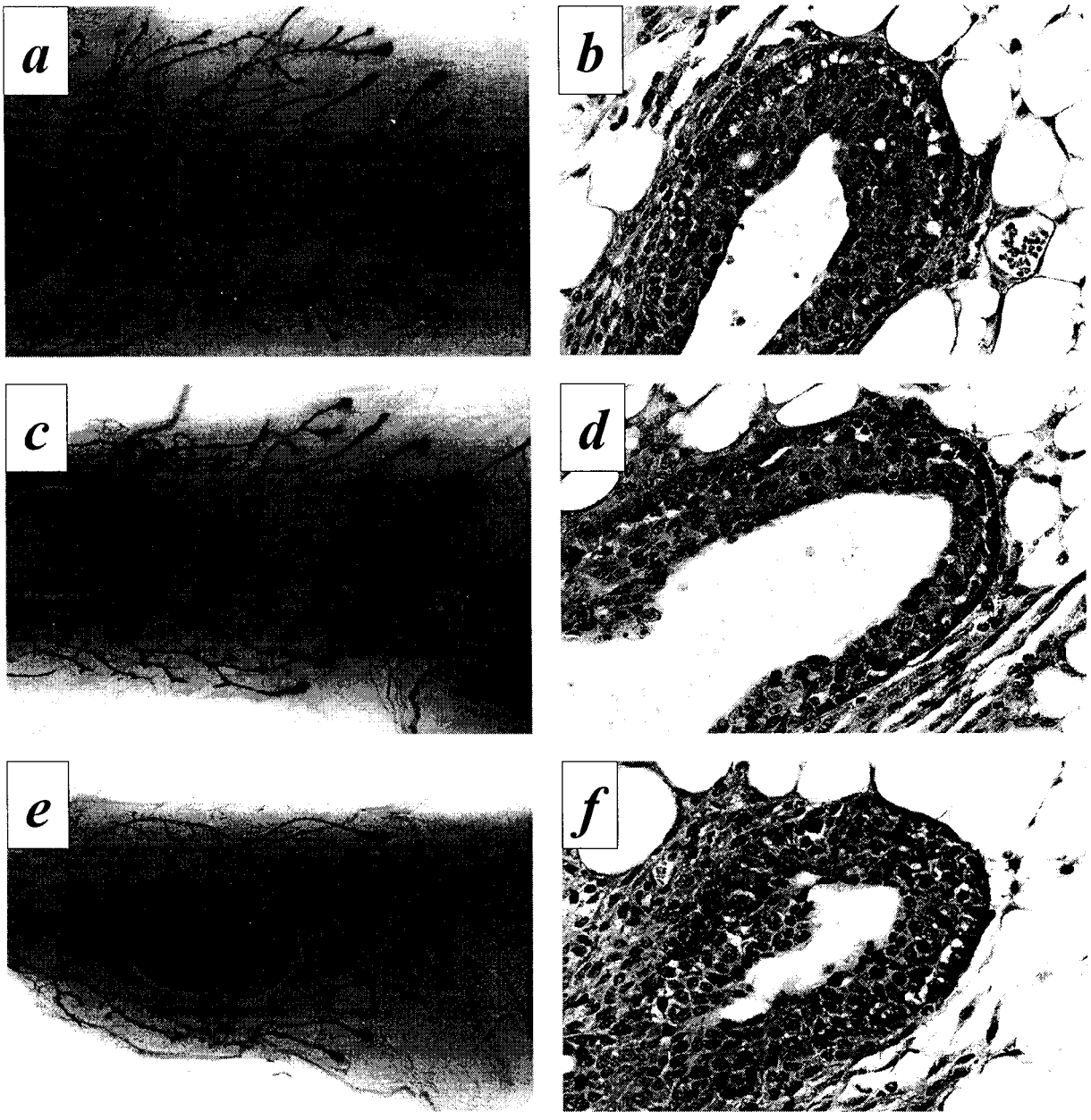

a
b
c
d
e
f

Figure 5.7 The mammary glands of virgin female MMTV-PEA3 transgenic mice have increased ductal branching and side bud formation.

The #4 inguinal mammary glands were prepared as haematoxylin-stained wholemounts (***a-d***) or as histological tissue sections stained with haematoxylin and eosin (***e*** and ***f***). Mammary glands were isolated at 8 weeks of age from female FVB/N (***a***, ***c***, and ***e***) and MMTV-PEA3 transgenic (***b***, ***d***, and ***f***) mice. The mammary glands of MMTV-PEA3 transgenic virgin female mice have an increased number of ductal branches (***b***) versus their wildtype littermates (***a***). Numerous side buds along various ducts are readily visible in the mammary glands of MMTV-PEA3 transgenic mice (***d***, arrows) as compared with those of age-matched FVB/N mice (***c***). Histological preparations reveal the presence of numerous acini along the ducts of MMTV-PEA3 transgenics (***f***) that are not seen in the FVB female mammary gland (***e***). These acini most likely represent the side buds seen in mammary gland wholemounts prepared from these mice (***d***). It must be noted, however, that these mice were not matched to stage in estrus cycle. Original magnification = 6x (***a*** and ***b***), 50x (***c*** and ***d***), 200x (***e*** and ***f***)

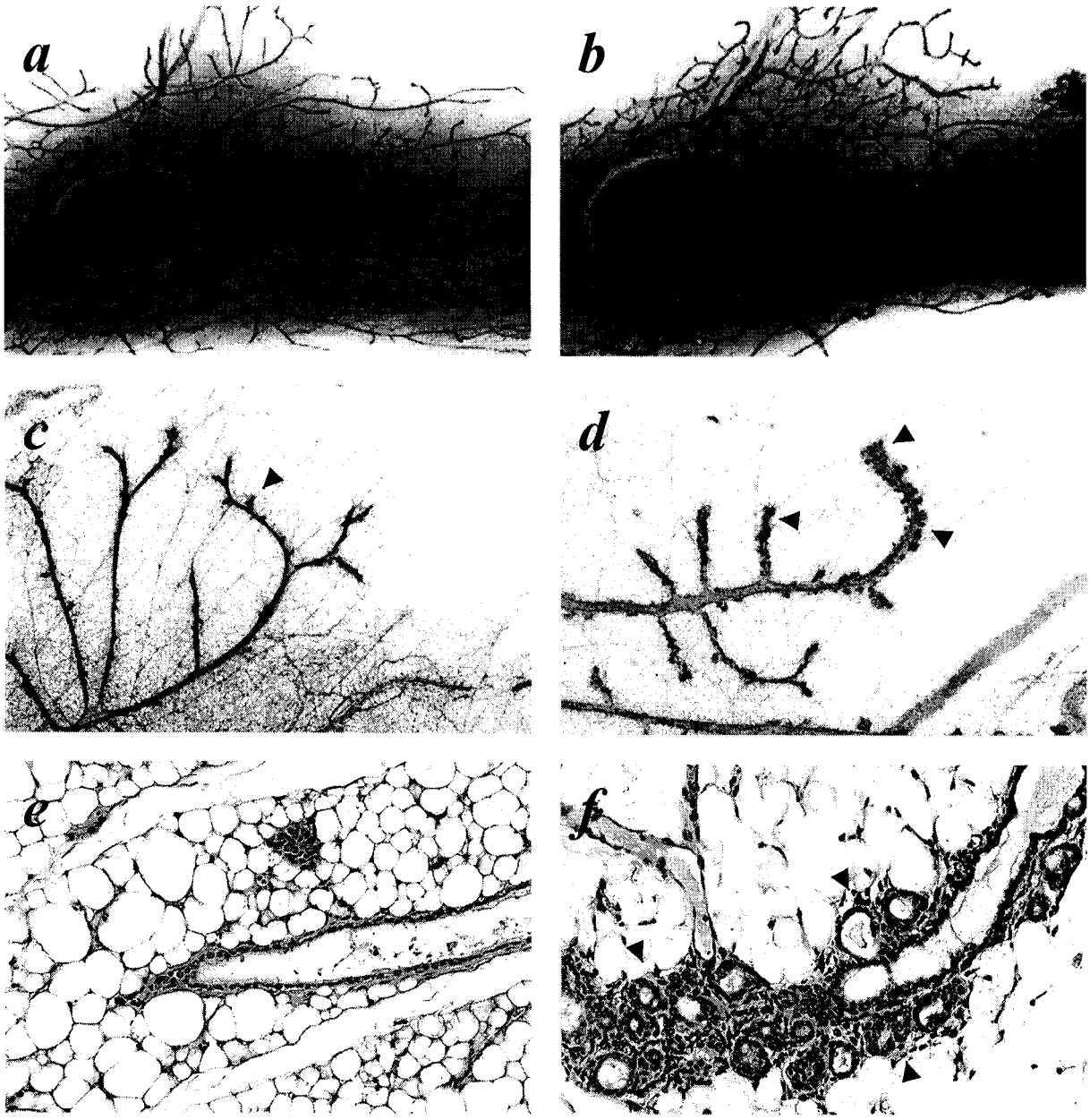

a
b
c
d
e
f

Figure 5.8 Female MMTV-PEA3 transgenic mice have reduced lobulo-alveolar development of their mammary glands during pregnancy.

The #4 inguinal mammary glands from female mice were isolated at 4 (***a*** and ***b***), 10 (***c*** and ***d***), and 19 days of pregnancy (***e*** and ***f***). Pregnancy was determined by the observance of a copulatory plug, followed by inspection of the uterus upon dissection. Haematoxylin-stained mammary gland wholemounts of FVB/N mice (***a***, ***c***, and ***e***) and age-matched MMTV-PEA3 mice (***b***, ***d***, and ***f***) were prepared. At all stages of pregnancy the mammary glands of MMTV-PEA3 transgenic female mice have a reduced number of lobulo-alveoli versus FVB/N mice. This MMTV-PEA3 mammary gland phenotype is still manifest at the end of pregnancy (***f***) whereas the FVB/N mammary gland has completely filled the fat pad with lobulo-alveoli (***e***). Original magnification = 6x

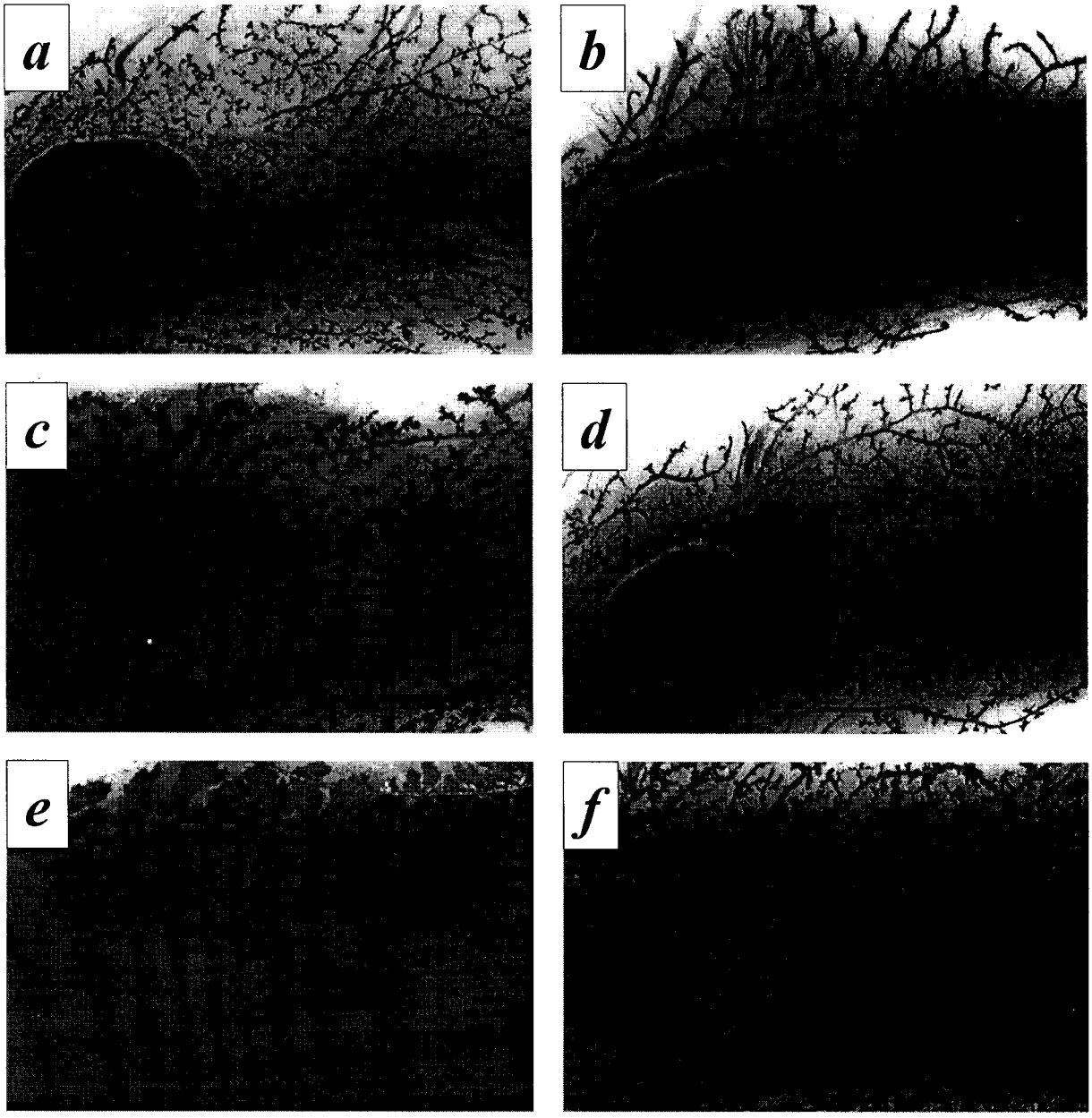
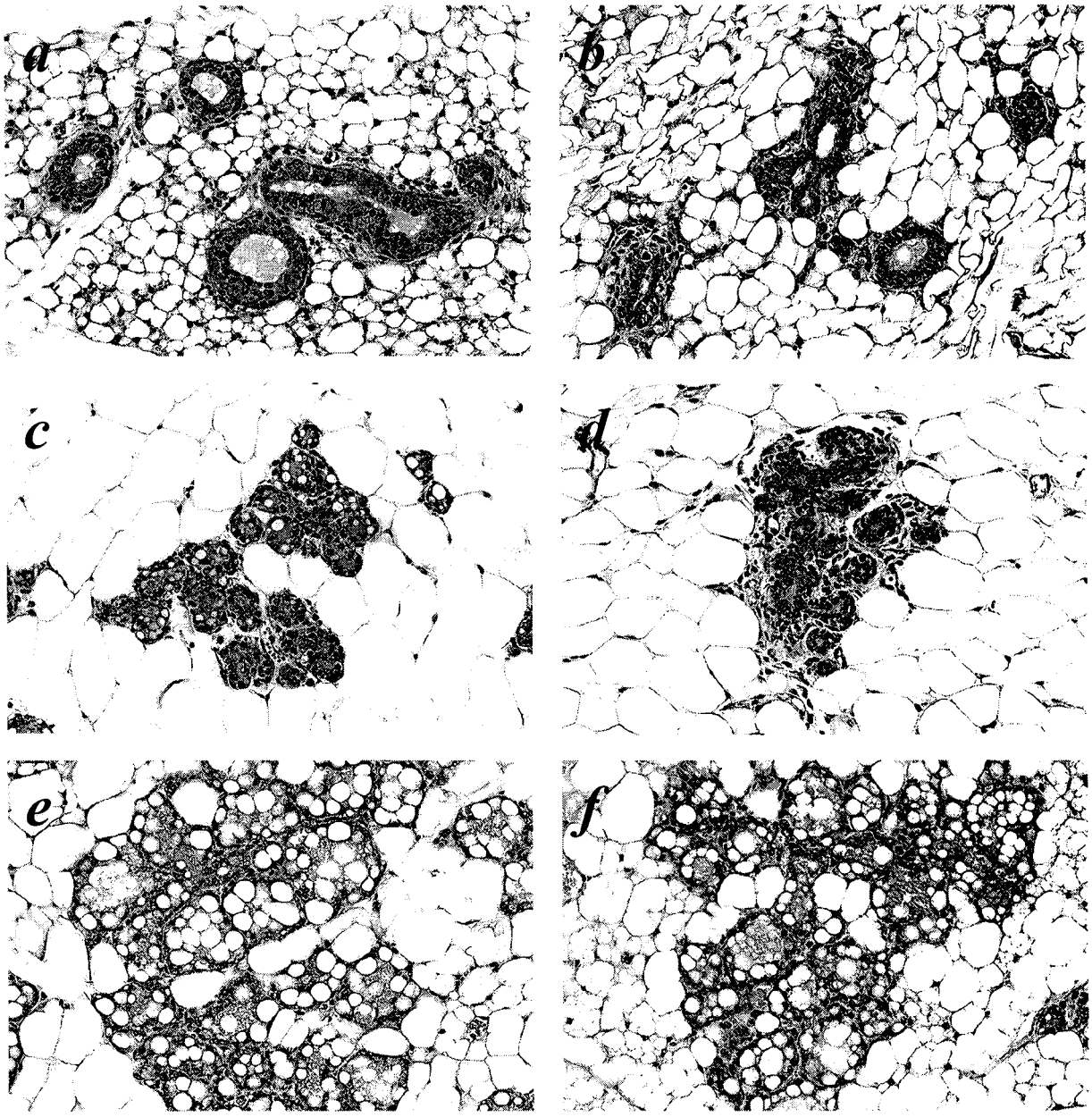

a
b
c
d
e
f

Figure 5.9 The mammary glands of MMTV-PEA3 transgenic female mice are able to form functional lobulo-alveoli during pregnancy.

The #4 inguinal mammary glands from female mice were isolated at 4 (***a*** and ***b***), 10 (***c*** and ***d***), and 19 days of pregnancy (***e*** and ***f***). Haematoxylin- and eosin-stained histological tissue sections of the mammary glands of FVB/N mice (***a***, ***c***, and ***e***) and age-matched MMTV-PEA3 mice (***b***, ***d***, and ***f***) were prepared. Although MMTV-PEA3 female mice have reduced number of lobulo-alveoli in their mammary glands, they are still able to produce milk as evidenced by the formation of milk droplets in the secretory epithelial cells of the lobulo-alveoli near the end of pregnancy (compare ***e*** and ***f***). Original magnification = 200x


a
b
c
d
e
f

MMTV-ΔNPEA3En transgenic mice. Wholemount analysis of the mammary glands from these mice revealed what appeared to be a buildup of material within the lumen of the primary and secondary ducts that was not evident in the control FVB/N glands (Figure 5.10). This phenotype was more evident with increasing age of the mouse. The material within ducts of MMTV-ΔNPEA3En transgenic mammary glands appeared to be cellular in origin as determined by histological preparations of these tissues. However, with the onset of pregnancy, this phenotype was no longer apparent, and it also did not affect the function of the mammary gland since females were eventually able to produce milk and nurse their young comparable to wildtype FVB/N females.

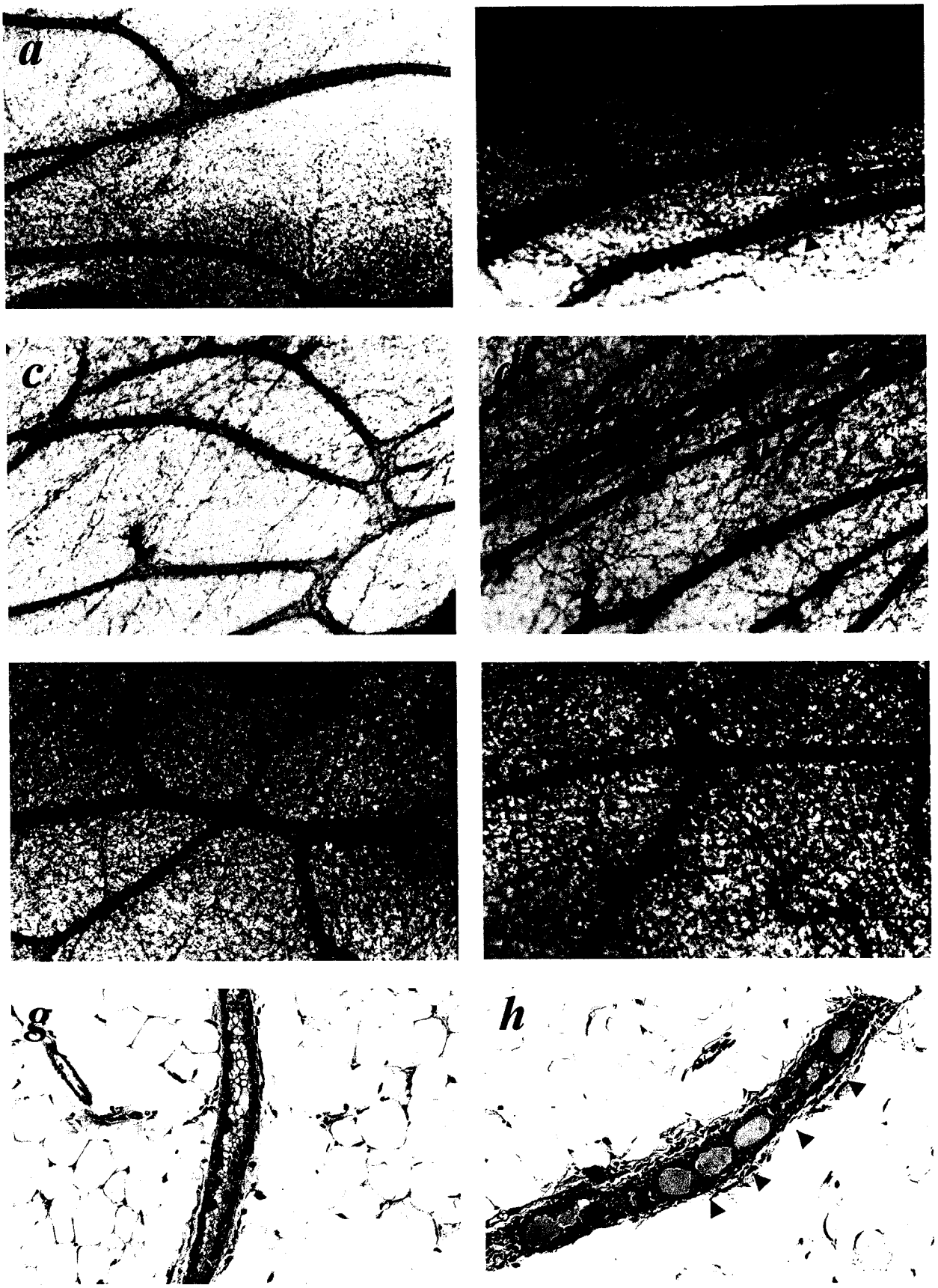
Thus, it appeared that female MMTV-PEA3 transgenics had aberrant mammary gland development both in the nulliparous mouse with increased branching and side bud formation, and in the pregnant mouse with decreased number of lobulo-alveoli. The phenotype of cellular debris within the lumen of the mammary gland ducts of virgin female MMTV-ΔNPEA3En transgenic mice, however, had no observable detrimental effect on their function.

5.1.3 Analysis of male reproductive organs

Analysis of transgene expression in the MMTV-PEA3 and MMTV-ΔNPEA3En transgenic mice revealed that they are highly expressed in several male reproductive organs. Relatively high levels of expression were observed in the epididymis in both strains of transgenic mice, with lower but detectable expression in the testis. Since *Pea3*

Figure 5.10 Virgin female MMTV-ΔNPEA3En transgenic mice have altered mammary ducts with luminal debris.

The #4 inguinal mammary glands from virgin female mice were isolated at 3 (***a*** and ***b***), 5 (***c*** and ***d***), and 6 months of age (***e-h***). Haematoxylin-stained mammary gland wholemounts of FVB/N mice (***a***, ***c***, and ***e***) and age-matched MMTV-ΔNPEA3En mice (***b***, ***d***, and ***f***) and histological tissue sections stained with haematoxylin and eosin (***g*** and ***h***, respectively) were prepared. The ductal lumen of the mammary gland of MMTV-ΔNPEA3En female mice contains material (arrows) that may be cellular in origin (***h***) and is not present in the mammary glands of age-matched FVB/N control mice. This phenotype is visible at as early as 3 months of age and is accentuated with increasing age of the animal. However, this phenotype of the MMTV-ΔNPEA3En virgin female mammary gland is not detectable during pregnancy, and it does not interfere with the ability of the mothers to produce milk and nurse their young. Original magnification = 25x (***a-f***), 200x (***g*** and ***h***)


a
c
g
h

is normally expressed in these tissues, overexpression of wildtype PEA3 or ectopic expression of dominant-negative PEA3 may affect the normal development and/or function of these organs. In addition it should be noted that *Pea3*-null male mice are unable to mate successfully possibly due to a defect in the function of male reproductive organs (Laing et al., 2000).

To directly assess whether there is a phenotype resulting from MMTV-PEA3 or MMTV-ΔNPEA3En transgene expression in the male reproductive organs, the testis and epididymis from mature male transgenic and wildtype FVB/N mice were isolated and prepared for histological analysis. There was no observable phenotypic difference among the samples obtained from MMTV-PEA3 transgenic, MMTV-ΔNPEA3En transgenic, and FVB/N male mice (Figure 5.11). This agrees with the observation that MMTV-PEA3 and MMTV-ΔNPEA3En male transgenics are both able to reproduce successfully.

5.1.4 MMTV-PEA3 rescues the ductal branching defect of the *Pea3*-null mammary gland

The mammary glands of nulliparous *Pea3*-null female mice have reduced ductal branching in comparison with age-matched wildtype control mice (MacNeil, 1999). Since the MMTV-PEA3 transgene is expressed in the organs that are affected by loss-of-function of PEA3, MMTV-PEA3 transgenic mice were bred with mice harbouring the *Pea3*-null allele introgressed onto the FVB/N background. Mammary gland wholemounts from resultant female offspring were performed using mice of each genotype to address whether or not MMTV-PEA3 could complement the *Pea3*-null

Figure 5.11 The testis and epididymis of MMTV-PEA3 and MMTV-ΔNPEA3En transgenic male mice are histologically normal.

Haematoxylin and eosin-stained histological tissue sections of the testis (***a-c***), and the initial segment (***d-f***) and caput regions (***g-i***) of the epididymis, were prepared from age-matched adult male FVB/N mice (***a***, ***d*** and ***g***), MMTV-ΔNPEA3En transgenic mice (***b***, ***e*** and ***h***), and MMTV-PEA3 transgenic mice (***c***, ***f*** and ***i***). No overt histological differences are observed among these samples, and mature sperm is produced in the testis and present in the epididymis. Original magnification = 200x

b
c

phenotype.

Mammary glands isolated from *Pea3*-null virgin female mice at 12 weeks of age had a reduced number of branches versus that of their wildtype littermates, thus reproducing the original phenotype (Figure 5.12). It should be noted, however, that the severity and penetrance of the phenotype in the FVB/N background strain of mice is less than previously observed in the BalbC/Sv129 mixed strain (MacNeil, 1999). Female mice that lacked the endogenous *Pea3* gene but possessed the MMTV-PEA3 transgene had a ductal branching pattern that was comparable to the wildtype gland. Similar to previous results, MMTV-PEA3 transgenic female mice with wildtype *Pea3* had increased ductal branching in their mammary glands as compared with those of FVB/N female mice.

5.1.5 MMTV-PEA3 cannot complement the reproductive defect of *Pea3*-null male mice

Previous analyses illustrated that *Pea3* mRNA is expressed in the epididymis and to a lower extent in the testis of male mice (Xin et al., 1992). To expand these analyses, RNase protection assays were performed on total RNA isolated from several different male reproductive organs of wildtype FVB/N mice (Figure 5.13). *Pea3* is moderately expressed in the epididymis, vas deferens, and prostate glands, but to lower levels in other organs including the testis. As a positive control for comparison, *Pea3* expression is readily detectable in RNA isolated from adult mouse brain and from the FM3A mammary adenocarcinoma cell line.

Figure 5.12 The MMTV-PEA3 transgene is able to rescue the mammary gland branching defect of *Pea3*-null virgin female mice.

The *Pea3*-null allele was first introgressed onto the FVB/N background, then these mice were mated with MMTV-PEA3 transgenics to eventually yield female mice of the various genotypes. Haematoxylin-stained mammary gland wholemounts were prepared from 12-week-old virgin female wildtype *Pea3* mice (***a***), *Pea3*-null mice (***b***), MMTV-PEA3 transgenic mice (***c***), and *Pea3*-null mice that bear the MMTV-PEA3 transgene (***d***). The reduced ductal branching phenotype of *Pea3*-null virgin female mice that was originally observed in a BalbC/Sv129 mixed strain background is not as pronounced in the FVB/N inbred strain (***b***), but is still observable when compared with age-matched wildtype controls (***a***). MMTV-PEA3 transgenic mice have increased ductal branching (***c***) and when crossed with *Pea3*-null mice the MMTV-PEA3 transgene can rescue the branching deficit due to lack of the endogenous *Pea3* gene (***d***). Original magnification = 6x

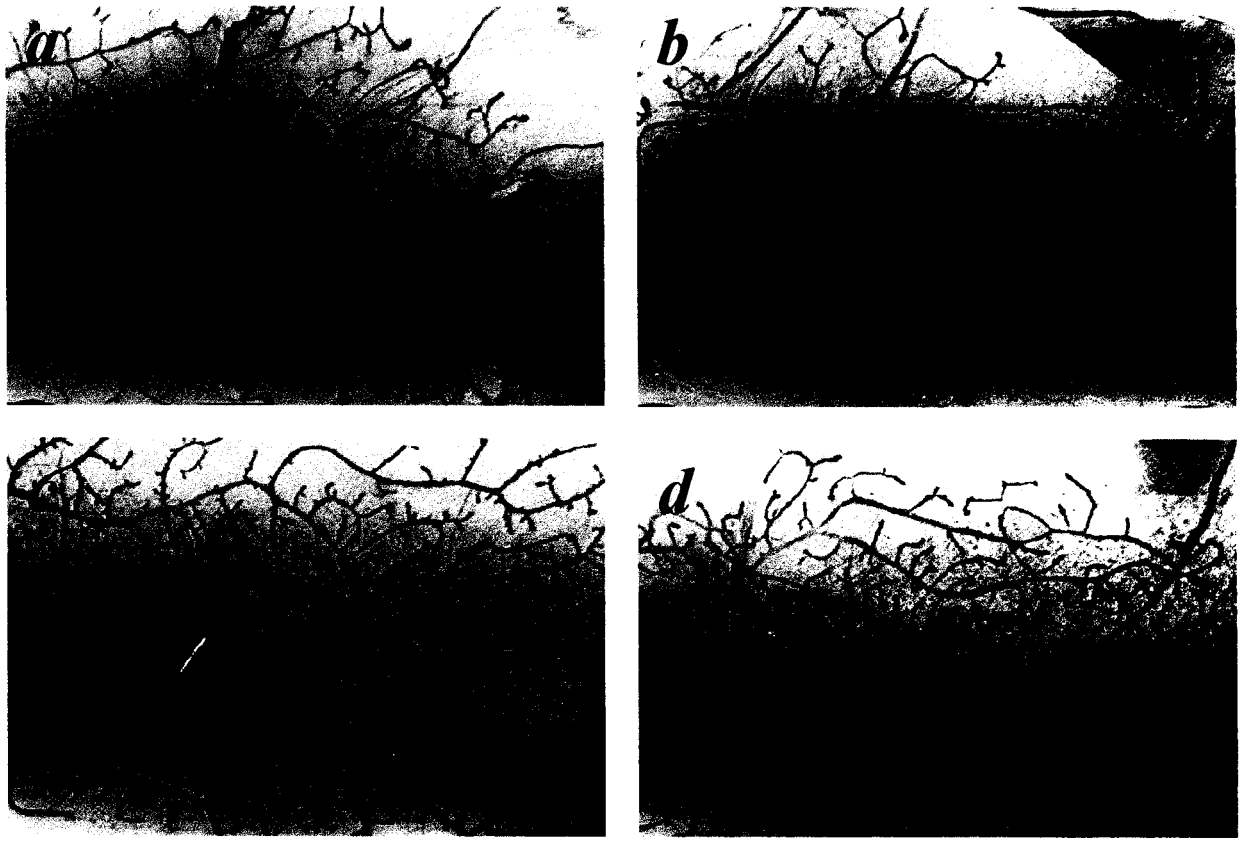
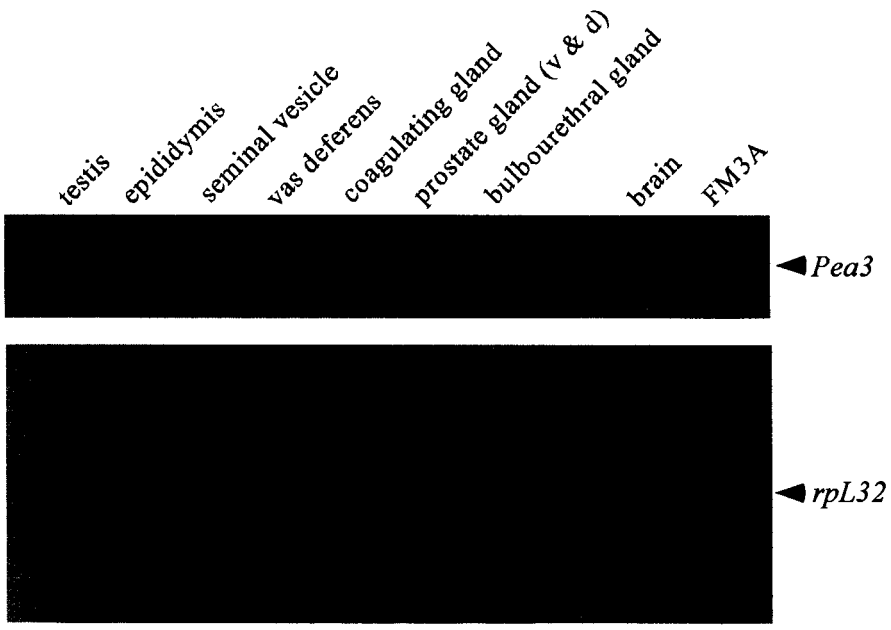

a
b
d

Figure 5.13 *Pea3* mRNA is expressed in several reproductive organs of the male mouse.

RNase protection assays were performed on 30 μg of total RNA isolated from the indicated tissues using a radiolabelled riboprobe specific for mouse *Pea3*. The *Pea3* gene is expressed in the epididymis, and to a lesser extent in the prostate glands (v, ventral; d, dorsal), and the vas deferens. RNA isolated from the mouse brain and the FM3A mouse mammary adenocarcinoma cell line were used as positive controls for *Pea3* mRNA expression.


testis
epididymis
seminal vesicle
vas deferens
coagulating gland
prostate gland (v & d)
bulbourethral gland
brain
FM3A
Pea3
rpL32

Male mice resulting from the crosses between *Pea3*-null and MMTV-PEA3 transgenic mice were housed with age-matched female FVB/N mice beginning at the approximate age of sexual maturity (8 to 10 weeks). The females of these mating pairs were observed for either the presence of a copulatory plug within the first few days of initiating the breeding pairs, or within one to two weeks for noticeable pregnancy. Both wildtype and heterozygous *Pea3* mutant male mice were able to mate successfully (Table 5.2). However, homozygous *Pea3*-null male mice were unable to mate, even after an extensive period of time (~2 to 3 months) with the female FVB/N mice. This mimics the phenotype observed in the original *Pea3* knockout strain (Laing et al., 2000). Male mice harbouring the MMTV-PEA3 transgene but lacking both functional alleles of the *Pea3* gene were also unable to sire litters. Thus, the re-expression of PEA3 under the control of the MMTV-LTR in several reproductive organs was unable to rescue the male reproductive defect of *Pea3*-null male mice.

5.2 Discussion

5.2.1 MMTV-PEA3 virgin female transgenic mice have increased ductal branching and number of side buds

The *Pea3* subfamily *ets* genes are expressed at low to moderate levels in the mammary gland of nulliparous female mice (see Figure 3.1). Their expression has been localized to epithelial cells of the developing mammary gland, especially the pluripotent cap cells of

Table 5.2 The MMTV-PEA3 transgene cannot functionally rescue the mating defect of *Pea3*-null male mice.

The *Pea3*-null allele was first introgressed onto the FVB/N background, then these mice were mated with MMTV-PEA3 transgenics to eventually yield male mice of the various genotypes indicated. At approximately 8 weeks of age the male mice of each genotype were set up to mate with age-matched FVB/N female mice. Successful matings were scored by either visualization of copulatory plugs or pregnancy of the female mice by two months after the mating experiment was initiated. These experiments revealed that the *Pea3*-null phenotype of male mice is still present in the FVB/N inbred background (previous observations were performed using the BalbC/Sv129 mixed strain), and that crossing these mice with those that express the MMTV-PEA3 transgene in several male reproductive organs does not rescue this defect.

Genotype	Proportion of Males Mated Successfully
Pea3$^{+/+}$	4/4
Pea3$^{+/-}$	16/16
Pea3$^{-/-}$	0/7
Pea3$^{+/+}$/MMTV-PEA3	6/6
Pea3$^{+/-}$/MMTV-PEA3	10/10
Pea3$^{-/-}$/MMTV-PEA3	0/6

the progressing TEBs during puberty (MacNeil, 1999) (N. Kurpios, G. Fidalgo and J. A. Hassell, unpublished). Analysis of mammary glands from virgin female *Pea3*-null mice revealed reduced ductal branching as compared with wild-type littermate controls (MacNeil, 1999). Ectopic overexpression of wildtype PEA3 via the MMTV-LTR in the mammary epithelium of virgin female mice leads to increased ductal branching. So too is the presence of an increased number of side buds along numerous ducts in MMTV-PEA3 transgenic mice versus those of their wildtype littermates. The increased number of ducts and side buds in MMTV-PEA3 female mice implicates PEA3, or its related subfamily members, ERM and ER81, in the proliferation of epithelial cells in the mammary gland.

TEBs, or more specifically the cap cells at the leading edge of the TEB, represent highly proliferative subpopulation of epithelial cells in the mammary gland (Daniel and Silberstein, 1987). All three *Pea3* subfamily *ets* genes are expressed in the mitotically active cap cells of the terminal end bud. Expression of PEA3 under the control of the MMTV-LTR results in a greater than 100-fold increase in *Pea3* mRNA levels in the mammary gland over that of the endogenous transcript. The *Pea3* subfamily genes are expressed at lower levels in the mammary glands of adult virgin female mice as compared with those during puberty, whereas MMTV-PEA3 expression is fairly constitutive at all stages of mammary gland development. Thus, MMTV-PEA3 expression is most likely present in a greater proportion of mammary epithelial cells and sustained for a much longer period of time than that of the endogenous *Pea3* gene. This

may lead to aberrant PEA3 activity either in cells that do not normally express PEA3, or at times when PEA3 is normally reduced to much lower levels. However, this is speculative since localization of MMTV-PEA3 expression versus that of the endogenous *Pea3* gene is not known.

The Met receptor tyrosine kinase and its cognate ligand, hepatocyte growth factor/scatter factor (HGF/SF), are involved in ductal branching of the mammary gland (Niemann et al., 1998; Soriano et al., 1995; Soriano et al., 1998; Yang et al., 1995). ETS sites are present in the promoter of the *met* gene, and Ets-1 has been shown to transcriptionally activate the *met* promoter (Gambarotta et al., 1996). Perhaps overexpression of PEA3 in mammary epithelial cells via the MMTV-LTR in transgenic mice leads to greater Met receptor production. This may enhance the HGF/SF-Met receptor signalling pathway which in turn increases ductal branching in MMTV-PEA3 virgin female mice.

Alternatively, the TGFβ signalling pathway is involved in negatively regulating ductal branching and inhibiting the formation of side buds (Pierce et al., 1993; Robinson et al., 1991; Silberstein et al., 1992). Placement of pellets containing TGFβ into the mammary fat pad (Daniel et al., 1989; Silberstein and Daniel, 1987), or targeted overexpression in the mammary epithelium via the MMTV-LTR (Pierce et al., 1993), results in retarded ductal outgrowth. At least one function of TGFβ signalling is to restrict cell proliferation. Thus, if forced overexpression of PEA3 results in increased mammary epithelial cell growth, this may bypass the growth inhibitory signal of TGFβ in

these cells. The possible mechanism by which PEA3 may be able to do this, whether it is direct or indirect, has not been addressed. However, it should be noted that the translocation variant EWS-FLI-1, an oncogenic mutant of the FLI-1 Ets protein, is able to repress the promoter of the TGFβ type II receptor (Hahm et al., 1999; Im et al., 2000). Therefore, Ets proteins may be involved directly in the regulation of TGFβ receptor signaling.

5.2.2 MMTV-PEA3 pregnant female mice have reduced lobulo-alveolar development of the mammary gland

The *Pea3* subfamily genes are expressed in the mammary gland during the first half of pregnancy, then markedly decrease in expression during the latter half of pregnancy and lactation (see Figure 3.1). Again, as in the mammary gland of virgin mice, *Pea3* subfamily expression parallels the stages that involve mammary epithelial cell proliferation. Their expression declines during the stages of functional differentiation of the mammary gland. Ectopic overexpression of PEA3 in the mammary epithelium of MMTV-PEA3 transgenic mice throughout pregnancy results in reduced lobulo-alveolar development of the mammary gland as compared with wildtype FVB/N control female mice. Thus, downregulation of *Pea3* subfamily gene expression may be important in the functional differentiation of the mammary gland during the second half of pregnancy.

Pregnancy involves two fundamentally different processes: cell proliferation and cell differentiation. Mammary epithelial cell proliferation occurs during the first half of

pregnancy to increase the number of secondary and tertiary ducts, whereas differentiation occurs primarily during the second half of pregnancy to allow the development of lobulo-alveoli that will eventually produce and secrete milk. If PEA3 functions in the proliferation of mammary epithelial cells, perhaps overexpression of PEA3 can inhibit the normal differentiation program of these cells during pregnancy. PEA3 may actually function in maintaining mammary epithelial cells in a less-differentiated, or multipotent, state. The cap cells of the TEB, where *Pea3* and its related *ets* genes, *Erm* and *Er81*, are highly expressed during puberty, are considered to be a putative stem cell population in the mammary gland due to their less-differentiated, pluripotent characteristics (Daniel and Silberstein, 1987). Thus, continued expression of PEA3 in mammary epithelial cells during pregnancy via the MMTV-PEA3 transgene may maintain many of these cells in a less-differentiated state, thereby reducing lobulo-alveolar development of the mammary gland. However, the pregnancy phenotype of MMTV-PEA3 transgenic mice may also be due to its effect on the production of ovarian and/or pituitary hormones which are known to be critically involved in the differentiation of the mammary gland. Indeed, MMTV-PEA3 female mice produce fewer litters and reduced average litter size when compared to FVB/N female mice. This may be the result of transgene expression in the ovary and/or pituitary gland; however, these analyses have not been performed. Progesterone and prolactin act on the mammary gland during pregnancy to facilitate lobulo-alveolar development and milk production (Hennighausen and Robinson, 1998). Loss-of-function of the progesterone receptor, prolactin, or the prolactin receptor lead to defects in the functional differentiation of the mammary gland, albeit with much more extreme

phenotypic consequences than that observed in the MMTV-PEA3 transgenic female mice (Horseman et al., 1997; Humphreys et al., 1997; Lydon et al., 1995).

5.2.3 MMTV-PEA3 rescues the mammary gland ductal branching phenotype but not the male mating defect of *Pea3*-null mice

Mice that lack the *Pea3* gene are viable, however male mice are unable to mate successfully and female mice have reduced mammary gland ductal branching (Laing et al., 2000; MacNeil, 1999). *Pea3* mRNA is normally expressed in several male reproductive organs and in the mammary gland of female mice (Xin et al., 1992) (see Figure 3.1 and Figure 5.14). Since MMTV-PEA3 transgenic mice overexpress PEA3 in these same affected tissues, it was thought that mating MMTV-PEA3 transgenics with *Pea3*-null animals may functionally complement loss-of-function of PEA3. Female mice that lacked *Pea3* but harboured the MMTV-PEA3 transgene had increased branching equivalent to that observed in wildtype female mice. However, MMTV-PEA3 male mice in a *Pea3*-null background failed to mate with female mice identical to the original observed phenotype. Thus, ectopic expression of PEA3 in *Pea3*-null mice rescued the ductal branching defect in the female mammary gland, but was unable to complement the mating impairment in male mice.

In the mammary gland of female mice, *Pea3* gene expression is localized to the myoepithelial cells of mature ducts (MacNeil, 1999). Although not assessed directly in MMTV-PEA3 transgenic female mice, it can be reasoned that the MMTV-LTR driven transgene is expressed in epithelial cells as observed by other groups (Deckard-Janatpour

et al., 1997). Since the MMTV-PEA3 transgene appears to rescue the reduced branching phenotype of *Pea3*-null female mice, it is likely that the ectopic expression of PEA3 in these mice occurs in the same cells that normally express the endogenous *Pea3* gene. This result also implies that PEA3 function is required in the epithelial component of the mammary gland for normal development of this organ, and not the in the stroma or systemically.

The *Pea3* gene is normally expressed in several male reproductive organs in mice. The MMTV-PEA3 transgene is expressed in the epididymis and to lower levels in the seminal vesicle and testis. However re-introduction of PEA3 expression into the reproductive organs of *Pea3*-null male mice failed to rescue the mating defect observed in these mice. In fact, *Pea3*-null male mice are able to produce sperm that are capable of fertilization *in vitro* implying no obligatory PEA3 function in spermatogenesis and maturation (Laing et al., 2000). More evidence indicates a role for PEA3 in the innervation of smooth muscles of the male urethra necessary for successful penile erection and copulation. MMTV-PEA3 is more than likely expressed in cells of the male reproductive tract other than those that require PEA3 function, thus explaining why the MMTV-PEA3 transgene is unable to complement the *Pea3*-null male mating deficiency.

5.2.4 MMTV-PEA3 and MMTV-ΔNPEA3En transgenic male mice have normal reproductive development and function

Pea3 is expressed in several male reproductive organs of mice, and more importantly male mice that lack the *Pea3* gene are unable to mate successfully (Laing et

al., 2000; Xin et al., 1992) (Figure 5.14 and Table 5.2). In addition, transgenes regulated via the MMTV-LTR are typically expressed in several male reproductive organs (Webster and Muller, 1994). However, overexpression of either PEA3 or ΔNPEA3En in male reproductive organs (ie. epididymis, testis and seminal vesicles) of MMTV-PEA3 and MMTV-ΔNPEA3En transgenic mice, respectively, did not result in an altered phenotype. Throughout the testis and the epididymis there were no observable differences in cell morphology or stage of germ cell differentiation between transgenic male mice and wildtype littermates. In addition, male mice of both transgenic strains are able to successfully sire litters.

Although *Pea3*-null male mice are unable to mate, most of the evidence to date does not point to a defect in the male reproductive organs involved in sperm production due to the loss-of-function of PEA3. In fact, PEA3 may play a role, either directly or indirectly, in the innervation and/or stimulation of smooth muscle cells within the male urethra required for penile erection and proper function during copulation (Laing et al., 2000). Thus, the MMTV-PEA3 and MMTV-ΔNPEA3En transgenes are more than likely not expressed in the cells in which PEA3 function is required. This would explain why the MMTV-PEA3 transgene cannot functionally rescue the male mating defect of *Pea3*-null mice, and that the MMTV-ΔNPEA3En male mice are fully capable of mating successfully even though they produce a dominant-inhibitory mutant that is able to block PEA3 function. Thus, the evidence obtained from transgenic mice further illustrates that PEA3 does not function in the epithelial component of male reproductive organs, such as that of the epididymis, and is consistent with previous data obtained from the analyses of

Pea3 knockout mice. However, these results do not exclude the possibility that the expression of *Pea3* may be regulated temporally during the development of the male reproductive organs. Perhaps expression of PEA3 or ΔNPEA3En via the MMTV promoter does not overlap temporally and/or spatially with the endogenous *Pea3* gene thereby precluding the observance of any reproductive phenotype in MMTV-PEA3 and MMTV-ΔNPEA3En transgenic male mice.

MMTV-PEA3 and MMTV-ΔNPEA3En transgenic male mice have normal reproductive organs and are able to mate. This is not surprising since there are only a few examples of MMTV transgenic mice that harbour defects in this organ system. MMTV-*MAT* transgenic mice that express the matrix metalloproteinase matrilysin develop what appears to be abnormal proteolytic activity within the testis in older adult males leading to decreased fecundity (Rudolph-Owen et al., 1998). Several other transgenic mice that bear oncogenes under the control of the MMTV promoter develop hyperplasia within male reproductive tissues. MMTV-*neuNT* (Guy et al., 1996; Muller et al., 1988), MMTV-*Ha-ras* (Sinn et al., 1987), MMTV-*int3* (Jhappan et al., 1992), and MMTV-*int2* (Muller et al., 1990) transgenic male mice develop epididymal hyperplasia, with the latter also exhibiting prostatic hyperplasia. Overexpression of these transgenes results in malignant transformation of the mammary gland, and in some instances hyperplasia in various other organ systems, illustrating the dominant acting nature of their gene products on cell growth and function. In general a phenotype within the male reproductive organs of MMTV transgenic mice is rare, and when observed it is less severe than the phenotype of the mammary gland of female mice. Therefore, the absence of a mutant phenotype in

the male reproductive organs of MMTV-PEA3 and MMTV-ΔNPEA3En mice is not unexpected.

CHAPTER SIX

ANALYSIS OF MAMMARY TUMORIGENESIS IN MMTV-*neu*/ΔNPEA3En AND MMTV-*neu*/PEA3 BI-TRANSGENIC FEMALE MICE

6.1 Results

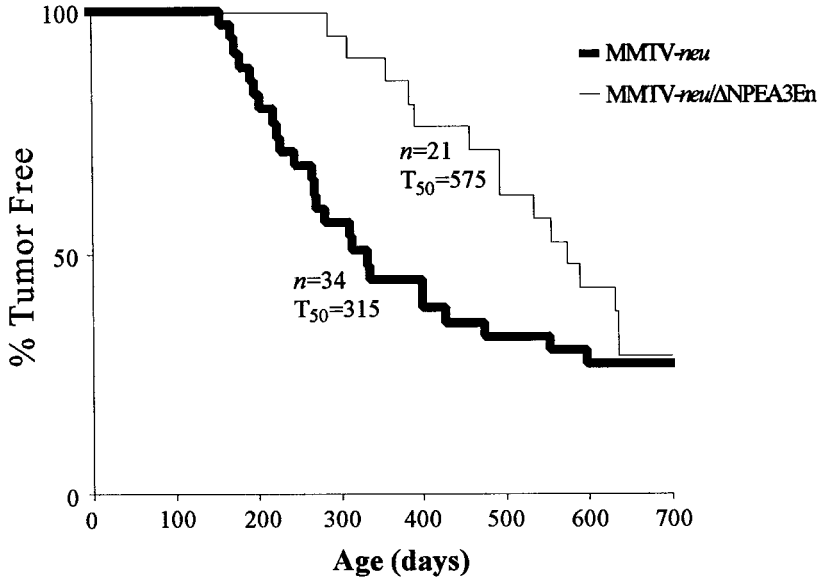
6.1.1 ΔNPEA3En negatively affects mammary tumorigenesis in MMTV-*neu*/ΔNPEA3En bi-transgenic mice

To address the potential role of PEA3, as well as that of ERM and ER81, in MMTV-*neu*-mediated mammary oncogenesis, MMTV-ΔNPEA3En transgenic mice were crossed with the N#202 line of MMTV-*neu* mice. The resultant virgin female offspring from these crosses were palpated weekly beginning at 5 months of age and scored for the appearance of mammary tumours. MMTV-*neu* transgenics developed palpable mammary tumours with a median age of onset of approximately 315 days (Figure 6.1). This is slightly longer than what was initially observed for this line (N#202) of MMTV-*neu* transgenic mice (Guy et al., 1992). However, their bi-transgenic littermates harbouring both MMTV-*neu* and MMTV-ΔNPEA3En transgenes exhibited a significant delay in the onset of tumour formation with a median age of 575 days, nearly double that observed in MMTV-*neu* mice. Statistical analysis using the log-rank test revealed that this difference was significant ($p<0.003$).

During the 2-month period between palpation of the initial mammary tumour and experimental endpoint at which time the animal is sacrificed, MMTV-*neu* mice typically

Figure 6.1 ΔNPEA3En delays MMTV-*neu*-induced mammary tumour onset in MMTV-*neu*/ΔNPEA3En bi-transgenic virgin female mice.

MMTV-ΔNPEA3En transgenic mice were crossed with MMTV-*neu* transgenic mice that heritably develop stochastic focal mammary tumours after a long latency. The mammary glands of nulliparous female mice were palpated weekly starting at 5 months of age for the occurrence of tumours. MMTV-*neu* transgenic female mice developed mammary tumours with a median age of onset of 315 days. However, MMTV-*neu*/ΔNPEA3En bi-transgenic female mice had a marked delay in the formation of mammary tumours (median age of onset of 575 days) almost double that of MMTV-*neu* mice. This difference is statistically significant ($p<0.003$) as determined by the log-rank test. Eventually the same proportion of mice (approximately 75%) from both cohorts developed at least one mammary tumour by the end of the study (700 days).


100
50
0
% Tumor Free
0
100
200
300
400
500
600
700
Age (days)
MMTV-neu
MMTV-neu/ΔNPEA3En
n=21
T50=575
n=34
T50=315

develop more than one mammary tumour (Siegel et al., 1994). Indeed, 71% of MMTV-*neu* female mice (n=13) that developed mammary tumours had two or more tumours at the end of 2 months (Table 6.1). In contrast, MMTV-*neu*/ΔNPEA3En bi-transgenic virgin female mice less often developed more than a single mammary tumour (21%, n=14).

The mass of primary tumour from several MMTV-*neu* and MMTV-*neu*/ΔNPEA3En female mice was also determined. Although the difference was slight, the primary tumours that arose in MMTV-*neu*/ΔNPEA3En bi-transgenics were smaller (mean of 1.8 g, n=14) than those that developed in the MMTV-*neu* transgenic mice (mean of 3.0 g, n=13) (Table 6.1). However, the tumours were histologically identical between the two sets of transgenic mice (see Figure 6.2).

Upon necropsy the lungs were isolated and scored for the presence of metastases. Although in some instances lung metastases were readily visible macroscopically, haematoxylin-eosin stained tissue sections of the lungs obtained from these mice were visualized microscopically for the presence or absence of smaller neoplastic lesions. There was no difference in the propensity for mammary tumours to metastasize to the lungs between MMTV-*neu* and MMTV-*neu*/ΔNPEA3En transgenic mice (Table 6.1).

6.1.2 MMTV-ΔNPEA3En Transgene Expression is Reduced or Absent in MMTV-*neu*/ΔNPEA3En Bi-transgenic Mammary Tumours

MMTV-*neu*/ΔNPEA3En bi-transgenic mice exhibited a dramatic delay in the onset of mammary tumour formation. Yet eventually the same proportion of mice developed

Table 6.1 Summary of data from tumour studies involving MMTV-*neu* transgenic and MMTV-*neu*/ΔNPEA3En bi-transgenic mice.

a- At two months after palpation of the first mammary tumour, female mice were sacrificed and the number of distinct tumours present in the mammary glands were scored. Mice were grouped into those having a single tumour (1) or those with 2 or more (≥2). The difference between MMTV-*neu* and MMTV-*neu*/ΔNPEA3En female mice was statistically significant (p=0.0039) as determined by the one-sided z test.

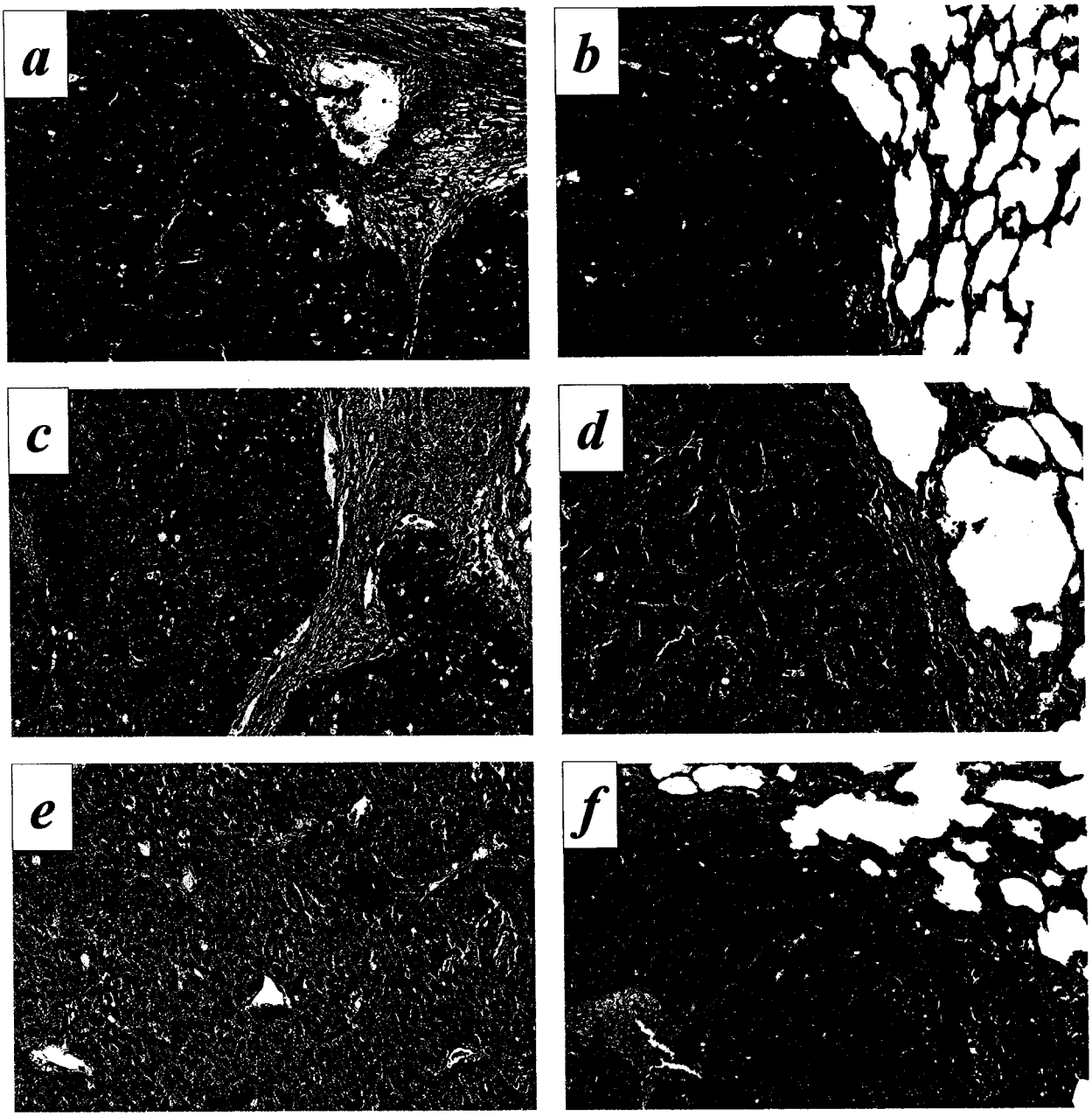
b- At the two-month endpoint, the mass of the primary tumour was determined. This difference is statistically significant ($p \leq 0.025$) as determined by the Student's t-test.

c- The presence of lung metastases was scored by analysis of several haematoxylin- and eosin-stained tissue sections isolated from the lungs of tumour-bearing mice at the 2-month endpoint. The proportion of mice developing lung metastases was similar between both strains of mice (p=0.268) as determined by the one-sided z test.

Strain	Tumour onset (days)	Number of tumours per mouse (%)[a]		Primary tumour mass (g) ± SD[b]	% lung metastases[c]
		1	≥2		
MMTV-*neu*	315	4/14 (28.6)	10/14 (71.4)	2.96±1.54 (*n*=13)	33.3 (*n*=15)
MMTV-*neu*/ΔNPEA3En	575	11/14 (78.6)	3/14 (21.4)	1.77±1.07 (*n*=14)	45.4 (*n*=11)

Figure 6.2 Mammary tumours and lung metastases of MMTV-*neu*/ΔNPEA3En bi-transgenic mice and MMTV-*neu*/PEA3 bi-transgenic mice are histologically identical to those of MMTV-*neu* transgenic female mice.

Mammary tumours and lungs were isolated from MMTV-*neu* (***a*** and ***b***), MMTV-*neu*/ΔNPEA3En (***c*** and ***d***), and MMTV-*neu*/PEA3 (***e*** and ***f***) female mice at the 2-month endpoint after initial palpation of the mammary tumour and prepared as haematoxylin and eosin-stained tissue sections. Representative tissue sections of mammary tumours (***a***, ***c***, and ***e***) and of lung metastases (***b***, ***d***, and ***f***) are presented from each strain of mice. Similar cellular structure, as visualized by nuclear and cytoplasmic staining, and gross morphology of the tumours themselves is observed among the MMTV-*neu* and bi-transgenic tumours. The metastases present in the lungs of these same mice are histologically similar to the primary tumours that arose in the mammary gland. Original magnification = 200x


a
b
c
d
e
f

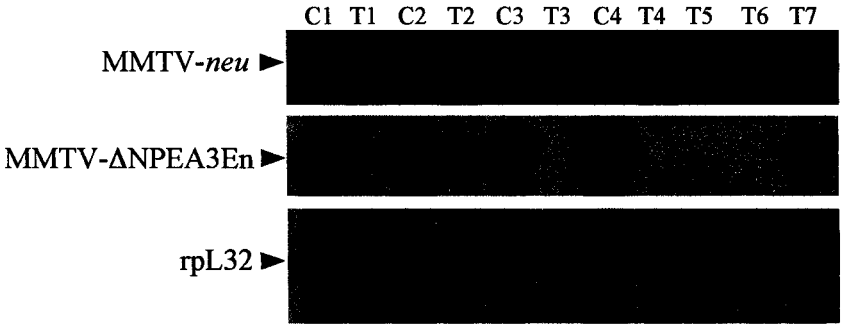
tumours that were histologically identical to MMTV-*neu* tumours, and these tumours retained the capacity to metastasize to the lungs. To address whether the tumours continued to express the two transgenes, RNase protection assays were performed using RNA from normal contra-lateral mammary glands and from several bi-transgenic derived tumours. MMTV-*neu* was expressed in all RNA samples analyzed with its expression being elevated in most tumour samples versus the contra-lateral mammary glands (Figure 6.3). This result is in keeping with previous observations of MMTV-*neu* tumours (Guy et al., 1992; Siegel et al., 1994). Expression of the MMTV-ΔNPEA3En transgene was readily detected in all four contra-lateral mammary glands; however, its expression was reduced or absent in all seven mammary tumours samples analyzed. Only two tumour samples, represented by T2 and T7, had detectable but markedly reduced MMTV-ΔNPEA3En mRNA expression. Quantification of the difference in ΔNPEA3En transgene expression in contra-lateral versus tumour tissue was performed by PhosphorImager analysis of the data. Indeed, the MMTV-ΔNPEA3En transgene is reduced in the tumour samples as compared with the contra-lateral mammary gland RNA samples.

To address whether ΔNPEA3En protein is expressed in the two tumour samples that have detectable MMTV-ΔNPEA3En transgene RNA, immunohistochemical analyses were performed on formalin-fixed tissue sections isolated from these tumours. Tissue samples were also obtained from an MMTV-*neu* tumour and a mammary gland isolated from a multiparous MMTV-ΔNPEA3En transgenic female mouse to serve as controls. The Neu protein was detectable in all three of the tumour samples that were analyzed, but

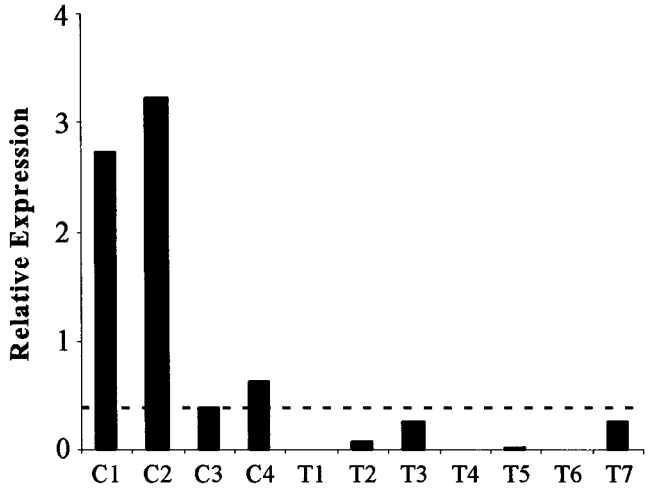
Figure 6.3 Expression of the MMTV-ΔNPEA3En transgene is reduced or absent in tumours of MMTV-*neu*/ΔNPEA3En bi-transgenic mice.

(***a***) RNase protection assays were performed on 5 μg of total RNA isolated from the mammary tumours and the normal contra-lateral mammary glands of several MMTV-*neu*/ΔNPEA3En bi-transgenic female mice using riboprobes specific for the MMTV-*neu* and MMTV-ΔNPEA3En transgenes. The rpL32 riboprobe was used as an internal control for RNA loading in each sample. The MMTV-ΔNPEA3En transgene is highly expressed in all four contra-lateral RNA samples (C1-C4), however, only two of seven tumours (T2 and T7) expressed the transgene. The MMTV-*neu* transgene mRNA is present in all samples, but is elevated in most of the tumour samples relative to the normal contra-lateral mammary gland. (***b***) Quantification of MMTV-ΔNPEA3En transgene expression by PhosphorImager analysis using ImageQuant 3.3 software. Expression of MMTV-ΔNPEA3En in the tumour samples T2 and T7 is reduced when compared with the four contra-lateral mammary gland samples (C1-C4). The dashed horizontal line represents the putative lower threshold level of MMTV-ΔNPEA3En transgene expression below which the MMTV-*neu* transgene is able to induce mammary tumours. Expression in the other tumour samples was negligible above background.

a


C1 T1 C2 T2 C3 T3 C4 T4 T5 T6 T7
MMTV-*neu*
MMTV-ΔNPEA3En
rpL32

b


Relative Expression
4
3
2
1
0
C1 C2 C3 C4 T1 T2 T3 T4 T5 T6 T7

it was not detected above background in the multiparous mammary gland tissue section (Figure 6.4). Expression of ΔNPEA3En was readily detectable in the mammary gland sample obtained from the multiparous MMTV-ΔNPEA3En female mouse. However, the T2 bi-transgenic tumour sample failed to co-localize ΔNPEA3En protein with Neu, and co-localization was patchy in the T7 bi-transgenic tumour tissue. Thus, these results imply that most tumours that develop in MMTV-*neu*/ΔNPEA3En bi-transgenic mice fail to express ΔNPEA3En at both the mRNA and protein level, with a small proportion exhibiting variegated transgene expression.

6.1.3 PEA3 overexpression attenuates mammary tumorigenesis in MMTV-*neu*/PEA3 bi-transgenic Mice

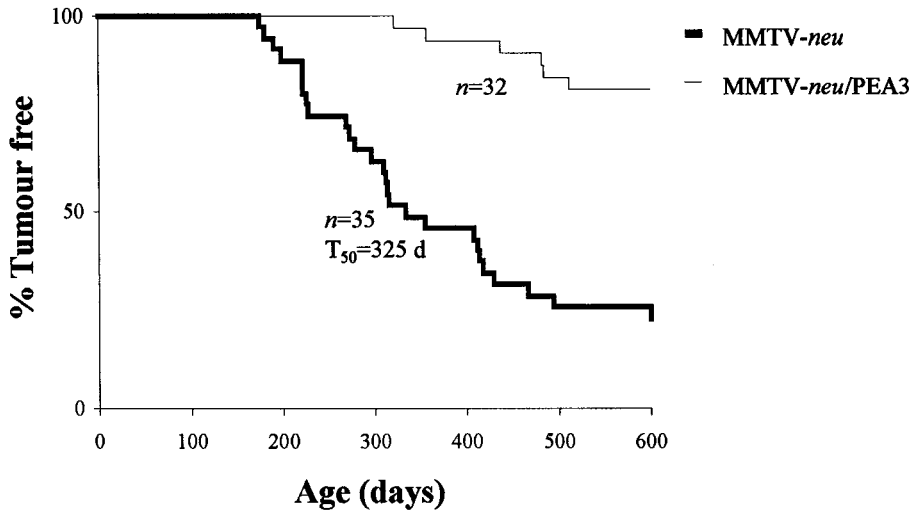
Pea3 mRNA is upregulated in the mammary tumours of MMTV-*neu* transgenic female mice versus the normal mammary gland of wildtype mice (Trimble et al., 1993)(see Figure 3.2). Since MMTV-PEA3 transgenic female mice already overexpress PEA3 prior to mammary tumour formation mediated by oncogenic Neu, these mice were mated with MMTV-*neu* transgenic mice to determine the functional consequence of PEA3 overexpression on the initiation and progression of tumour formation. The mammary glands of virgin female offspring from such crosses, bearing the MMTV-*neu* or both MMTV-*neu* and MMTV-PEA3 transgenes were palpated every one to two weeks after 5 months of age to detect tumour formation. The incidence and latency of tumour onset in the MMTV-*neu* female mice were similar to data presented earlier (Figure 6.5;

Figure 6.4 ΔNPEA3En protein rarely co-localizes with Neu in mammary tumours of MMTV-*neu*/ΔNPEA3En bi-transgenic mice.

Immunohistochemical analysis was performed by co-incubation of formalin-fixed, paraffin-embedded tissue sections with the Ab-1 anti-*neu* rabbit polyclonal antibody to detect Neu protein (***a-d***) and the anti-*myc* 9E10 monoclonal antibody to detect ΔNPEA3En protein (***e-h***). The goat anti-rabbit FITC-conjugated and the sheep anti-mouse Texas Red-conjugated secondary antibodies were used to detect expression by immunofluorescence microscopy and to allow the merging of images to detect co-localization (***i-l***). Tissue sections isolated from the mammary tumours of two bi-transgenic mice that apparently express MMTV-ΔNPEA3En mRNA (T7, ***b***, ***f***, and ***j***; T2, ***c***, ***g***, and ***k***; see also Figure 6.2) were examined. In addition, sections of a mammary tumour from an MMTV-*neu* transgenic mouse (***a***, ***e***, and ***i***) and a mammary gland from a multiparous MMTV-ΔNPEA3En female mouse (***d***, ***h***, and ***l***) served as positive controls for the anti-*neu* and anti-*myc* primary antibodies, respectively. Images were captured using a Zeiss Axiocam and Axiovision 4.0 software, and the resultant images were merged using both Adobe Photoshop 6.0 and Axiovision 4.0 software. Minimal co-localization of ΔNPEA3En and Neu proteins is observed in a portion of the T7 tumour sample (***j***), and no co-localization occurs in the T2 tumour tissue section (***k***). Neu protein is expressed in the MMTV-*neu* tumour sample (***a***) with no cross-reactivity of the anti-*myc* antibody (***e***); likewise, ΔNPEA3En is readily detectable in regressed alveoli of the MMTV-ΔNPEA3En multiparous female mammary gland (***h***), whereas endogenous Neu/ERBB2 protein is absent (***d***). Original magnification = 200x

Figure 6.5 MMTV-PEA3 reduces MMTV-*neu*-induced mammary tumour onset in MMTV-*neu*/PEA3 bi-transgenic virgin female mice.

MMTV-PEA3 transgenic mice were crossed with MMTV-*neu* mice and the mammary glands of nulliparous female offspring were palpated every one to two weeks starting at 5 months of age for the occurrence of tumours. Approximately 75% of MMTV-*neu* transgenic female mice developed mammary tumours with a median age of onset of 325 days. However, MMTV-*neu*/PEA3 bi-transgenic female mice had a marked increase in latency and reduction in overall mammary tumour formation with only 20% of mice developing a palpable tumour before 600 days of age. This difference is statistically significant ($p<0.000002$) as determined by the log-rank test.


100
50
0
% Tumour free
0
100
200
300
400
500
600
Age (days)
n=32
n=35
T50=325 d
MMTV-neu
MMTV-neu/PEA3

compare with Figure 6.1). The median age of tumour onset was approximately 325 days for the MMTV-*neu* mice with close to 75% (n=35) of the mice eventually developing mammary tumours before the end of the study (600 days) (Figure 6.5). Surprisingly, less than 20% (n=32) of MMTV-*neu*/PEA3 bi-transgenic female mice developed mammary tumours by the completion of the study, which is significantly different from the tumour curve of MMTV-*neu* mice (p<0.000002) as determined by the log-rank test.

Most tumour-bearing MMTV-*neu* female mice develop more than one mammary tumour within the two months after initial palpation and the experimental endpoint at which time the animal is sacrificed. In this study, 66.7% (n=15) of MMTV-*neu* mice that harboured mammary tumours had two or more by the two-month endpoint (Table 6.2). However, of the six MMTV-*neu*/PEA3 bi-transgenic female mice that developed mammary tumours, only 33.3% had more than a single primary tumour. Although this may represent a trend similar to what was observed in MMTV-*neu*/ΔNPEA3En bi-transgenics, the small sample size of tumour-bearing MMTV-*neu*/PEA3 bi-transgenics did not yield a statistically significant difference from the MMTV-*neu* transgenic mice (p=0.0823) as determined by the one-sided z test.

Although these data appear to reflect a negative affect of PEA3 overexpression on the initiation of mammary tumorigenesis mediated by MMTV-*neu*, MMTV-PEA3 expression in the mammary tumours of bi-transgenic mice may influence tumour progression and eventual metastasis to the lungs. The mass of each initial primary tumour at the two-month endpoint was determined for several mice of each genotype. The

Table 6.2 Summary of data from tumour studies involving MMTV-*neu* transgenic and MMTV-*neu*/PEA3 bi-transgenic mice.

a- At two months after palpation of the first mammary tumour, female mice were sacrificed and the number of distinct tumours present in the mammary glands were scored. Mice were grouped into those having a single tumour (1) or those with 2 or more (≥2). The difference between MMTV-*neu* and MMTV-*neu*/PEA3 female mice was not statistically significant (p=0.0823) as determined by the one-sided z test.

b- At the two-month endpoint, the mass of the primary tumour was determined. This difference was not statistically significant ($p \leq 0.10$) as determined by the Student's t-test.

c- The presence of lung metastases was scored by analysis of several haematoxylin- and eosin-stained tissue sections isolated from the lungs of tumour-bearing mice at the 2-month endpoint. The proportion of mice developing lung metastases was similar between both strains of mice (p=0.288) as determined by the one-sided z test.

Strain	Tumour onset (days)	Number of tumours per mouse (%)[a]		Primary tumour mass (g) ±SD[b]	% lung metastases[c]
		1	≥2		
MMTV-*neu*	325	5/15 (33.3%)	10/15 (66.7%)	2.70±1.26 (*n*=14)	21.4 (*n*=14)
MMTV-*neu*/PEA3	>600	4/6 (66.7%)	2/6 (33.3%)	3.95±1.67 (*n*=6)	33.3 (*n*=6)

average mass of MMTV-*neu* derived tumours was 2.70 grams (n=14) and of MMTV-*neu*/PEA3 tumours it was 3.95 grams (n=6) (Table 6.2). Although the trend may seem to indicate that mammary tumours of MMTV-*neu*/PEA3 bi-transgenics are larger than those that develop in MMTV-*neu* female mice, the small sample size of tumour-bearing bi-transgenics rendered the data outside statistical significance ($p \leq 0.10$). Likewise, there was no statistically significant difference in the proportion of tumour-bearing mice that developed lung metastases (p=0.288) (Table 6.2).

6.1.4 PEA3 and ΔNPEA3En do not repress MMTV-LTR-mediated transcription

Dominant-negative PEA3 acts to inhibit PEA3 function essentially by binding to consensus ETS sites in target genes and repressing gene expression (see Chapter 4). Overexpression of PEA3 protein may cause repression by squelching of transcription by titrating away necessary co-factors required for MMTV-LTR promoter activity. Alternatively, PEA3 may act as a transcriptional repressor in certain cellular contexts (Xing et al., 2000). Since there are candidate ETS sites present in the MMTV-LTR that may be functionally important (Aurrekoetxea-Hernandez and Buetti, 2000; Welte et al., 1994), then ΔNPEA3En and PEA3 may reduce the onset and tumour incidence in MMTV-*neu*/ΔNPEA3En and MMTV-*neu*/PEA3 bi-transgenics by repressing expression of the MMTV-*neu* transgene in these mice. To address whether the MMTV-LTR is responsive to PEA3 and can be transcriptionally repressed by ΔNPEA3En, the MMTV-*neu* mammary tumour derived NDL cell line was transiently transfected with an MMTV-

luc reporter plasmid and either the pCANmyc/PEA3 or pCANmyc/ΔNPEA3En expression plasmids. NDL cells were chosen to perform these analyses because they represent a cell culture system that is analogous to the mammary epithelial cells of transgenic mice *in vivo*. Increasing amounts of transfected PEA3 and ΔNPEA3En expression plasmids were unable to affect MMTV-*luc* expression in NDL cells (Figure 6.6). Since this could be due to the lack of PEA3 and ΔNPEA3En expression in the transfected cells, Western immuno-blotting was performed to address this point. Both PEA3 and ΔNPEA3En proteins were detected in their respective transiently transfected NDL protein lysates. Therefore, PEA3 and ΔNPEA3En are unable to affect expression regulated by the MMTV-LTR in mammary tumour cells, thus arguing against repression of MMTV-*neu* gene expression as a mechanism for reduced tumorigenesis in MMTV-*neu*/ΔNPEA3En and MMTV-*neu*/PEA3 bi-transgenic female mice.

6.2 Discussion

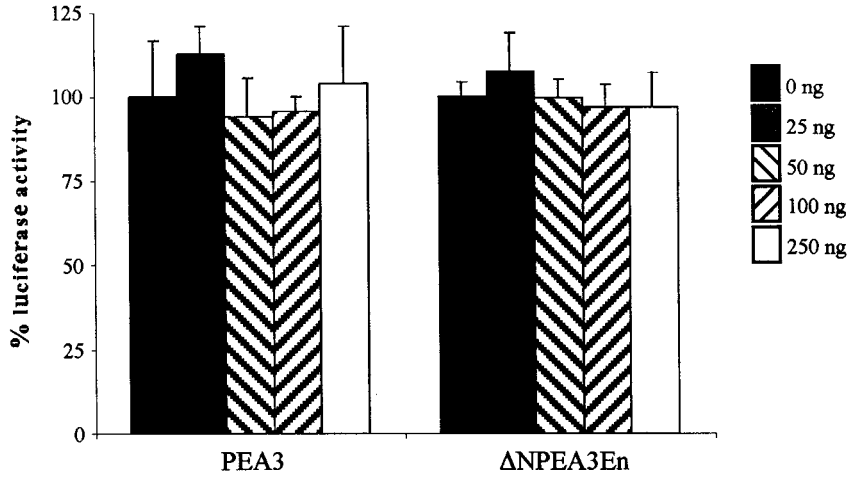
6.2.1 Dominant-negative PEA3 inhibits MMTV-*neu*-induced mammary tumorigenesis

The *Pea3* subfamily *ets* genes are overexpressed in mammary tumours of MMTV-*neu* transgenic female mice (see Figure 3.2). Their function in the initiation and/or progression of these tumours was evidenced by the increased tumour latency, and reduced number and size of tumours, in MMTV-*neu*/ΔNPEA3En bi-transgenic mice.

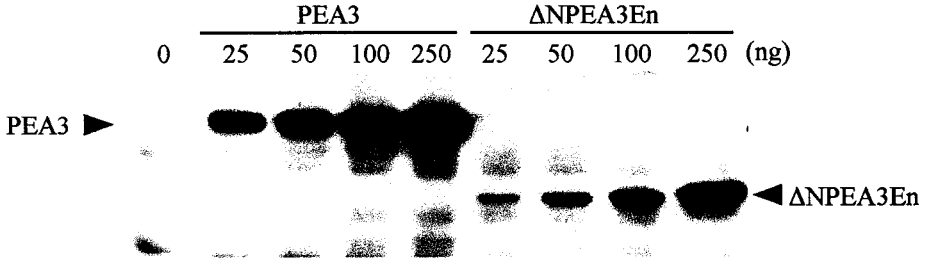
Figure 6.6 Expression of the MMTV-*luc* reporter is not affected by PEA3 or ΔNPEA3En in NDL mouse mammary tumour cells.

(***a***) NDL cells were transiently transfected with 500 ng of the MMTV-*luc* reporter and increasing amounts (25, 50, 100, and 250 ng) of the pCANmyc/PEA3 or the pCANmyc/ΔNPEA3En expression vectors as indicated. The total amount of pCANmyc plasmid in each sample was maintained at 250 ng. Luciferase expression driven by the MMTV-LTR was unaltered with increasing amounts of either PEA3 or ΔNPEA3En effector plasmids. (***b***) Expression of PEA3 and ΔNPEA3En in these experiments was verified by Western immuno-blotting using 100 μg of total protein lysates and the anti-*myc* 9E10 monoclonal antibody to detect the myc-tagged recombinant proteins as indicated.

a


125
100
75
50
25
0
% luciferase activity
PEA3
ΔNPEA3En
0 ng
25 ng
50 ng
100 ng
250 ng

b


PEA3
ΔNPEA3En
0
25
50
100
250
25
50
100
250
(ng)
PEA3
ΔNPEA3En

Indeed, expression of ΔNPEA3En was reduced or absent in all mammary tumours that arose in the bi-transgenic mice when compared with contra-lateral mammary glands.

There appears to be a requirement for secondary activating mutations in MMTV-*neu* induced tumours (Siegel et al., 1994), and also for reduction of MMTV-ΔNPEA3En expression below a critical threshold for tumours to initiate and/or progress fully in MMTV-*neu*/-ΔNPEA3En bi-transgenic female mice. The MMTV-*neu* transgene was expressed in each tumour, and its level increased in almost all tumours as compared with morphologically normal contra-lateral mammary glands in keeping with previous analyses (Guy et al., 1992; Siegel et al., 1994). Since mammary tumours arise less frequently in MMTV-*neu*/ΔNPEA3En bi-transgenic mice versus MMTV-*neu* mice, and those that do develop fail to express appreciable amounts of ΔNPEA3En, then these results suggest an obligatory role for the PEA3 subfamily proteins in Neu-mediated mouse mammary tumorigenesis. ΔNPEA3En is able to inhibit the function of all three PEA3 subfamily proteins (Figure 4.5), although its overexpression would most likely block the function of other Ets proteins with similar DNA-binding activity.

Female MMTV-*neu* transgenic mice generally develop more than one mammary tumour between the time of palpation of the initial tumour and the time at sacrifice. Each of these tumours represents independent lesions since each possesses different MMTV-*neu* transgene deletion patterns (Siegel et al., 1994). In contrast, in the majority of

MMTV-*neu*/ΔNPEA3En bi-trangenic mice that eventually developed mammary tumours, only a single tumour arose. This supports the result of overall increase in latency of tumour onset in bi-transgenic mice since each mammary tumour represents an independent lesion. Therefore, although a single tumour has arisen from a clonal cell due to the loss or reduction in ΔNPEA3En expression, the remaining contra-lateral mammary glands lacking tumours more than likely continue to co-express both MMTV-*neu* and MMTV-ΔNPEA3En transgenes thereby inhibiting further tumour formation. However, verification of co-localization in the normal mammary gland of MMTV-*neu*/ΔNPEA3En bi-transgenic mice has not been performed.

The apparent smaller size of mammary tumours derived from bi-transgenic mice may be due to the stochastic re-expression of the ΔNPEA3En transgene in the progressing Neu-induced tumour. Variegation of transgene expression is common in both cell culture and in transgenic mice (Cardiff, 1996; Dobie et al., 1996). Immunohistochemical analyses of tumours isolated from bi-transgenic mice revealed patchy expression of both Neu and ΔNPEA3En proteins (Figure 6.4). Thus, heterogeneous ΔNPEA3En expression in these tumours may influence the growth and progression of these tumours. However, unstable expression of ΔNPEA3En in the subset of tumour cells that have become invasive, have extravasated into the bloodstream, and have metastasized to the lungs obviously fails to inhibit the formation of these secondary lesions.

ETS sites are present in the enhancer of the MMTV-LTR and at least one was shown to be involved in mediating non-hormonal transcriptional regulation (Welte et al.,

1994). Multimeric complexes containing the Ets protein GABPα are able to bind to and cooperate with activated glucocorticoid receptor to stimulate the MMTV-LTR (Aurrekoetxea-Hernandez and Buetti, 2000). Therefore, it is plausible that ΔNPEA3En acts to reduce tumorigenesis in MMTV-*neu*/ΔNPEA3En bi-transgenics by inhibiting expression of *neu* from the MMTV promoter. However, the ability of ΔNPEA3En to affect expression of the MMTV-*neu* transgene specifically without affecting its own expression is unlikely. In addition, all normal mammary gland samples isolated from MMTV-*neu*/ΔNPEA3En bi-transgenic female mice exhibited high levels of expression for both transgenes (Figure 6.3). Even in isolated regions of mammary tumours derived from bi-transgenic mice, co-localization of Neu and ΔNPEA3En was observed. Specific analyses performed in cell culture illustrated that neither PEA3 nor ΔNPEA3En were able to affect expression mediated by the MMTV-LTR. Therefore, when taken together, these data argue against PEA3 and ΔNPEA3En inhibiting MMTV-*neu*-induced mammary tumorigenesis by simply blocking MMTV-*neu* transgene expression.

6.2.2 Overexpression of PEA3 significantly reduces mammary tumorigenesis in MMTV-*neu*/PEA3 bi-transgenic female mice

Pea3 is overexpressed in the mammary tumours and the lung metastases of MMTV-*neu* and MMTV-PyVMT transgenic female mice (Trimble et al., 1993). The results presented herein (see Chapter 3) have repeated these previous findings, and quantification revealed an approximate 5- to 10-fold increase in *Pea3* mRNA expression level in the mammary tumours of MMTV-*neu* mice versus normal mammary gland

control tissue. *PEA3* mRNA is elevated in the majority of human breast tumour samples and its overexpression is highly correlated with *HER2*-positivity in these tumours (Benz et al., 1997). However, in bi-transgenic mice that bear both the MMTV-*neu* and MMTV-PEA3 transgenes, the onset of mammary tumour formation is dramatically reduced (Figure 6.5). Only 20% of the MMTV-*neu*/PEA3 transgenic female mice developed mammary tumours versus approximately 75% of MMTV-*neu* mice by the completion of the study (600 days). Thus, although the *PEA3* gene is upregulated in the majority of mammary tumours in both mice and humans, overexpression of PEA3 to the levels facilitated by the MMTV-LTR in the mammary glands of mice retards the initiation of tumour formation.

PEA3 protein typically functions to activate target gene expression at the level of transcription (Bojovic and Hassell, 2001; Crawford et al., 2001; Xin et al., 1992) (Figure 4.5). However, overexpression of a transcription factor may lead to repression of target gene expression by squelching mechanisms. If co-activators present at rate-limiting amounts are required for PEA3 to function normally in modulating gene transcription, excess amounts of free PEA3 protein may titrate these components away from where they are required. Increasing amounts of transfected PEA3 expression vector leads to a dose-dependent increase in vimentin reporter gene expression, but at the highest doses a decrease in reporter expression is observed (Chen et al., 1996). Evidence that PEA3 acts to repress the *HER2* promoter (Xing et al., 2000) may also represent a similar transcriptional repression via squelching, since others have shown that the *HER2* promoter is positively responsive to several Ets proteins including PEA3 (Benz et al.,

1997). The MMTV-PEA3 mRNA is expressed in the mammary glands of female transgenic mice approximately 100-fold higher than the endogenous *Pea3* gene (Figure 5.5). Although experiments to detect and quantify PEA3 protein expression in these mice were not performed, one can infer that the amount of the resultant protein expressed from the MMTV-PEA3 mRNA would be much greater than the endogenous PEA3 protein level in mammary epithelial cells.

Ets-2 is an Ets protein that functions as a strong transcriptional activator. Various analyses have been performed using deleted versions of the Ets-2 protein which render them as a dominant-negative mutants capable of inhibiting wild-type Ets-2 function (Galang et al., 1996; Wasylyk et al., 1994). Overexpression of *trans*-dominant mutant Ets-2 represses Ets-responsive reporter gene expression (Galang et al., 1996; Wasylyk et al., 1994), blocks oncogenic NeuNT-induced focus formation (Galang et al., 1996), and reverts the Ki-Ras transformed phenotype of mouse fibroblasts (Wasylyk et al., 1994). However, overexpression of Ets-2 protein in cultured cells is capable on its own to elicit phenotypes similar to those observed with its dominant-negative mutants (Foos et al., 1998). This observation hearkens to the similarity in tumorigenesis phenotypes between MMTV-*neu*/PEA3 and MMTV-*neu*/ΔNPEA3En bi-transgenic female mice. In fact, overexpression of the amino-terminus of the Ets-2 protein that contains the transcriptional activation domain but no DNA-binding activity, is able to act as a dominant-inhibitory mutant of Ets-2 similar to those which comprise just the carboxyl-terminal DNA-binding domain (Galang et al., 1996). This transactivation domain mutant of Ets-2 may block normal Ets-2 function by sequestering necessary co-factors. This

supports the argument that overexpression of an Ets protein, such as PEA3, can result in inhibition of its normal function as a transcriptional activator by putative squelching effects. It should be noted that the amino-terminus of the PEA3 protein contains a strong transcriptional activation domain bounded by negative-regulatory regions (Bojovic and Hassell, 2001). Studies are currently being performed to determine what protein-protein interactions occur with other transcription factors and/or co-factors via this region.

CHAPTER SEVEN

CONCLUSIONS

7.1 Summary

The *Pea3* subfamily *ets* genes are expressed throughout mouse embryonic development. Their expression is primarily localized to structures involved in established epithelial-mesenchymal interactions as well as in organ systems undergoing branching morphogenesis (Chotteau-Lelievre et al., 1997). In the postnatal female mouse, the mammary gland undergoes the most dynamic changes of any of the organ systems (Daniel and Silberstein, 1987). Intercellular communication between the mammary epithelium and the stromal fat pad are essential for normal branching morphogenesis and functional differentiation of the mammary gland (Cunha and Hom, 1996; Sakakura, 1991). Each of the *Pea3* subfamily genes is expressed in the developing mammary gland of the female mouse. Their expression is most readily detected during times of cell proliferation and branching morphogenesis. Their cellular expression is localized to the epithelial component of the female mouse mammary gland and expression of all three are detected as early as embryonic day E10.5 within the primordial mammary epithelial bud (MacNeil, 1999)(Kurpios, Fidalgo, and Hassell, unpublished results). Highest expression is localized to the terminal end buds of the mammary gland in female mice during puberty; these structures represent a population of highly proliferative, pluripotent mammary epithelial cells. A potential function of the *Pea3* subfamily in the mammary

gland was evidenced by reduced ductal branching of the mammary gland in *Pea3*-null female mice (MacNeil, 1999). *Pea3* is expressed specifically in epithelial cells of the mammary gland, and the requirement for PEA3 in the epithelial component is supported by the fact that MMTV-PEA3 expression in the mammary epithelium of *Pea3*-null female mice restores wildtype branching morphogenesis. Overexpression of PEA3 in the mammary gland of MMTV-PEA3 results in increased ductal branching and side bud formation in virgin animals and reduced number of lobulo-alveoli in the differentiated mammary gland of pregnant mice. The results obtained from studies of both *Pea3*-null and MMTV-PEA3 transgenic female mice support the hypothesis that PEA3 functions in the proliferation and branching morphogenesis of the female mouse mammary gland. Down-regulation of PEA3, and possibly that of ERM and ER81 is potentially required for normal functional differentiation of the mammary gland.

The spatial and temporal expression pattern of *Pea3* subfamily genes in the female mouse mammary gland implies a common signaling pathway may be involved in their coordinated regulation. The hepatocyte growth factor/scatter factor (HGF/SF) and the Met receptor are important in mediating branching morphogenesis of the mammary gland (Niemann et al., 1998; Soriano et al., 1995). HGF/SF is produced in the mesenchyme of the mammary gland, and binds to and stimulates the Met receptor present on the surface of epithelial cells (Soriano et al., 1998; Yang et al., 1995). Each of the *Pea3* subfamily genes is expressed in the epithelial component of the mammary gland. Perhaps this signal transduction pathway impinges on promoters of *Pea3*, *Erm*, and *Er81* in mammary epithelial cells. The fact that PEA3 is required for normal branching

morphogenesis implicates that at least PEA3 may be an important mediator of HGF/SF-Met receptor function during this developmental process.

Normal signaling by the HER2/Neu receptor has been implicated in formation of lobulo-alveolar structures and differentiation of the mammary epithelium (Niemann et al., 1998). However, oncogenic signaling by Neu in mammary epithelium of nulliparous MMTV-*neu* transgenic female mice leads to tumour formation and metastasis (Siegel et al., 2000). The *Pea3* subfamily genes are expressed at low levels during the differentiation of the mouse mammary gland during the latter half of pregnancy, implying that they are not targets of normal HER2/Neu signaling in the mammary epithelium. In fact, downregulation of PEA3 expression levels may be required for normal mammary gland differentiation, since MMTV-PEA3 females have reduced lobulo-alveoli in their mammary glands. However, the *Pea3* subfamily is coordinately overexpressed in the tumours of MMTV-*neu* transgenic female mice. Since the *Pea3* subfamily is upregulated in response to oncogenic HER2/Neu signaling, but possibly unaffected by normal HER2/Neu function, perhaps PEA3, ERM and ER81 are important mediators specifically in the oncogenic transformation of the mammary epithelium induced by mutant HER2/Neu. However, this characteristic of coordinate *Pea3* subfamily overexpression in oncogenesis is not restricted to just HER2/Neu-induced mammary tumours. MMTV-*Wnt1* mammary tumours also display increased expression of the *Pea3* subfamily (Howe et al., 2001). Wnt1 functions through a different signaling cascade than that of HER2/Neu (Bienz and Clevers, 2000), yet its overexpression results in transformation of the mammary epithelium and coordinate upregulation of the *Pea3* subfamily as is observed

in HER2/Neu-induced mammary tumours. Therefore, the *Pea3* subfamily *ets* genes likely represent a common set of target genes involved in mammary tumorigenesis induced by a variety of oncogenic signaling pathways. This implies that *Pea3* subfamily genes may represent useful and consistent diagnostic markers for detection of human breast cancer.

In addition, overexpression of PEA3 or a dominant-inhibitory mutant of PEA3 significantly reduced the onset of mammary tumours in MMTV-*neu* transgenic female mice. Theoretically, this may be reproduced in any mammary tumour that overexpresses one or more of the *Pea3* subfamily *ets* genes, for instance, in the mammary tumours of MMTV-*Wnt1* transgenic female mice. Thus, therapeutic agents that target the function of PEA3 subfamily proteins in breast tumour cells, the majority of which overexpress one or more *PEA3* subfamily genes, may prove efficacious in treating breast cancer in humans.

7.2 Future directions

In vivo, the *Pea3* subfamily genes are expressed in mammary epithelium. Culturing of mammary epithelium and stimulation of ductal morphogenesis and differentiation *in vitro* is well-established (Ip and Darcy, 1996). Analysis of *Pea3* subfamily gene expression can be monitored using these techniques to elucidate which signaling pathways are involved in their regulation. Isolation of mammary epithelial cells from mice harbouring the nuclear-localized β-galactosidase gene inserted into each respective *Pea3* subfamily gene locus would be useful for carrying out such experiments. Cells derived from homozygous knockout animals of *Pea3* and *Er81* would allow for the direct assessment of the requirement of these two genes in mammary epithelium.

Alternatively, mammary epithelial cells from MMTV-ΔNPEA3En transgenic female mice, or transduction of normal mammary epithelial cells with viral vectors capable of expressing dominant-negative PEA3, could be used to address the functional requirement of all three PEA3 subfamily members downstream of these signaling pathways. If these studies implicated one or more of the *Pea3* subfamily genes downstream of particular pathways in the normal growth and differentiation of the mammary gland, then further analyses could be undertaken: (1) to dissect which sites are required within their promoters and/or enhancers; (2) to determine which transcription factors mediate their expression; and (3) to differentiate transcriptional regulation in normal mammary epithelium from that which is observed in mammary tumours.

Overexpression of dominant-negative PEA3 in the mammary epithelium of transgenic female mice resulted in a delayed onset of mammary tumorigenesis mediated by MMTV-*neu*. However, eventually the same proportion of mice developed tumours due to the selective loss or reduced expression of the dominant-negative PEA3 transgene in the tumours. Thus, the functional requirement of the PEA3 subfamily proteins in MMTV-*neu*-mediated mammary tumorigenesis can only be implied from the fact that the majority of tumours that arose in bi-transgenic mice required the loss of dominant-negative PEA3 expression. In addition, since dominant-negative PEA3 expression is absent in mature tumours, the function of PEA3 subfamily proteins in metastatic progression of tumorigenesis could not be addressed. Regulated induction of dominant-negative PEA3 transgene expression at various stages of tumorigenesis could be used to assess the role of PEA3 subfamily proteins throughout progression of the disease.

Transgenic mice bearing the reverse tetracycline repressor fused to the VP16 transcriptional activation domain (rtTA) expressed from the MMTV-LTR could be crossed with mice that contain dominant-negative PEA3 sequences downstream from multiple tetO elements (D'Cruz et al., 2001). This system allows for a tightly controlled, on-off mechanism of transgene expression using the tetracycline analogue doxycycline as has been observed in mice generated using *Ha-ras*, *myc*, *neuNT*, and *Wnt-1* oncogenes (D'Cruz et al., 2001)(L. Chodosh, personal communication). Female offspring from crosses of MMTV-rtTA/tetO-ΔNPEA3En transgenics with MMTV-*neu* mice would allow for analyses of PEA3 subfamily function to be performed in much greater detail at several different stages of mammary tumorigenesis.

This transgenic system has the capacity to control the level of transgene expression depending on the dose of doxycycline administered. Overexpression of wildtype PEA3 in MMTV-PEA3 female mouse mammary glands by more than two orders of magnitude above the endogenous *Pea3* gene transcript level leads to a potent inhibitory effect on MMTV-*neu*-induced mammary tumorigenesis, most likely due to transcriptional squelching mechanisms. Thus, by utilizing this coupled transgenic system, MMTV-rtTA/tetO-PEA3 female mice could potentially address the role of PEA3 overexpression *in vivo*. Perhaps at lower levels of expression, PEA3 acts to potentiate mammary tumorigenesis, whereas at much higher levels it acts to inhibit this process.

Both MMTV-rtTA/tetO-ΔNPEA3En and MMTV-rtTA/tetO-PEA3 transgenic female mice could be used in crosses not only with MMTV-*neu* transgenics, but also in other transgenic mouse models of mammary tumorigenesis in which the *Pea3* subfamily

genes have been implicated, such as MMTV-*Wnt-1* mice (Howe et al., 2001). These models systems would further dissect the function of PEA3 subfamily proteins at all stages of mammary tumor progression and metastasis. For instance, profiling gene expression patterns at specific timepoints after transgene induction within the tumours could lead to identification of mammary tumour-specific PEA3 subfamily target genes that are relevant to specific stages of disease progression.

Research on the PEA3 subfamily has been focused on their involvement in mammary gland development and mammary tumorigenesis in mice. *PEA3* mRNA is overexpressed in the majority of human breast tumour samples (Benz et al., 1997), and almost every human breast tumour cell line expresses the *PEA3* subfamily gene transcripts at high levels (Baert et al., 1997). Thus, it is imperative to address the function of the PEA3 subfamily proteins in human breast cancer more directly. Overexpression of PEA3 in a non-invasive mammary tumour cell line MCF7 confers increased invasiveness and ability to form tumours in mice coordinate with the upregulation of several matrix metalloproteinases (Higashino et al., 1995; Kaya et al., 1996). It would be important to extend these analyses by deriving both normal human breast epithelial cells, and breast tumour cell lines of varying tumorigenic potential, which inducibly overexpress each of the PEA3 subfamily proteins. The reciprocal system could be set up to address the functional requirement of PEA3 subfamily proteins by overexpressing either dominant-negative PEA3 in these same cells, or antisense RNA molecules specific for each *PEA3* subfamily member mRNA. Analysis of cell growth, morphology, invasiveness, motility, tumour formation, metastasis, angiogenesis and

apoptosis could all be addressed with the focus on PEA3 subfamily function in each of these processes. The PEA3 subfamily proteins act as transcription factors and potentially regulate the expression of key target genes involved in mammary tumorigenesis. Thus, development of a cell culture system could lead to the identification of known and/or novel target genes, and directly relate their expression to a specific phenotypic process involved in breast cancer.

In conjunction with the stable cell culture system outlined above, it would be advantageous to develop an approach to ablate PEA3 subfamily protein function *in vivo*. This may be performed by the construction of viral vectors to express dominant-negative PEA3 in transduced tumour cells. Alternatively, one could design a small molecule inhibitor that mimics the sites of DNA to which the ETS domain of PEA3 subfamily proteins bind with high affinity (Chiang et al., 2000). Both of these strategies could be thoroughly tested in both cell culture and animal models, and they potentially offer a relevant and efficacious therapy for breast cancer in cooperation with other molecular targeting strategies.

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