PEA3 AND ER81:
ROLES IN TRANSFORMATION AND MAMMARY GLAND DEVELOPMENT

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By

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ABSTRACT

PEA3 is the founding member of a subfamily of closely related Ets transcriptional regulatory proteins that includes ERM and ER81. The PEA3 subfamily members share greater than 95% identity in their ETS DNA binding domain and 50% sequence similarity overall, suggesting these genes may serve redundant functions. The overexpression of each member is positively correlated with HER2 mediated breast tumorigenesis in humans and mice, suggesting a role for this subfamily in mammary development and oncogenesis. This study first addresses the role of PEA3 in cellular transformation mediated by oncogenic Ras and Neu. Wildtype and PEA3-null mouse embryo fibroblast cell lines were infected and tested for focus formation. PEA3-null fibroblasts are refractory to transformation as compared to their wildtype counterparts. Ras and Neu transformed foci show elevated PEA3 subfamily mRNA transcripts and PEA3 protein. ERM and ER81 are expressed in PEA3-null fibroblasts and do not appear to compensate for loss of function mutations in the PEA3 gene resulting in the transformation-defective phenotype. Expression of candidate PEA3 target genes (MMP-3 and MMP-9, which have known roles in transformation) is compromised in PEA3-null fibroblasts. Re-expression of PEA3 in these cells rescues the transformation-deficient phenotype and restores expression of MMP-3 and MMP-9. Hence, PEA3 appears to be a crucial effector in Ras and Neu mediated transformation, in addition to serving an important regulatory role of genes involved in cell motility and invasive tumor behaviour. This study also addresses the role of ER81 in normal mammary gland development. PEA3 is required for normal mammary gland development, as displayed by the reduced branching phenotype in PEA3-null female mice. Mice lacking functional ER81 were generated to determine if ER81 serves a similar role in mammary gland development. ER81 is expressed in the epithelial cells of mammary buds at E10.5, when these structures first appear during mouse embryogenesis. ER81 is then differentially expressed during postnatal mammary gland development, with highest expression occurring at times of extensive epithelial branching. During puberty, expression is observed in undifferentiated cap and body cells of terminal end buds, in differentiated luminal and myoepithelial cells of ducts. During pregnancy, expression in luminal epithelial cells is lost, but persists in the myoepithelial cells within the ducts and alveoli. Targeted disruption of both ER81 alleles result in severely runted mice that die by 4 weeks of age, thereby precluding study of mammary gland development in these mice beyond this developmental stage. However, loss of a single ER81 allele results in healthy looking mice, comparable in size and lifespan to wildtype littermates. Studies employing ER81 heterozygous mice reveal a 50% allelic dose is sufficient for normal mammary gland development. Loss of a single ER81 allele did not result in any overt phenotypes in ductal branching, lobulo-alveolar development, or morphology of the surrounding fat pad.

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CONTRIBUTIONS BY OTHERS

- Silvia Arber from Dr. Tom Jessell's Lab, Columbia University generated ER81 locus disruptions.

- Dinsdale Gooden of the Institute for Molecular Biology and Biotechnology, McMaster University synthesized the required synthetic oligonucleotides

- Laura Hastings immortalized the PEA3 and the ER81 mouse embryo fibroblasts, in addition to generating Ras and Neu transformed cell lines.

- Anatomical Pathology, McMaster Hospital embedded and sectioned mammary gland tissues.

- Dr. Martin McMahon, University of California supplied the retroviral vector pWZL BLAST 3 (pWB3).

- Dr. Howard Crawford, Vanderbilt University supplied mouse MMP3 and mouse MMP9 templates.

- Dr. Bill Muller, McMaster University, provided pMAM Neo template.

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LIST OF ABBREVIATIONS

DMEM	Dubelcos Modified Eagle's Medium
EGF	Epidermal Growth Factor
ER81	Ets Related 81
ERK	Extracellular Regulated Kinase
ERM	Ets Related Molecule PEA3 Like
FGF	Fibroblast Growth Factor
GAPDH	Glyceraldehyde Phosphate Dehydrogenase
IRES	Internal Ribosome Entry Site
MAPK	Mitogen Activated Protein Kinase
MEF	Mouse Embryo Fibroblasts
MoMuLV LTR	Moloney Murine Leukaemia Virus Long Terminal Repeat
MMP	Matrix Metalloprotease
MMTV	Mouse Mammary Tumour Virus
NLS	Nuclear Localised
PDGF	Platelet Derived Growth Factor
PEA3	Polyoma Enhancer Activator 3
TBP	TATA-binding Protein
TEB	Terminal End Bud

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INTRODUCTION

I. The *Ets* Gene Family

The *Ets* gene family contains over 30 members encoding transcription factors involved in normal and pathological processes. *Ets* genes are widely distributed in metazoan genomes (humans, rodents, flies, fish, chicken, worms, frogs and sea urchins) of multicellular organisms, but have not been identified in plants, fungi or protozoans (reviewed by Graves, 1998). *V-ets*, the founding family member was identified in the genome of an avian leukemia virus (E-twenty-six); E26 induces myeloblastosis and erythroblastosis in infected chickens (Leprince et al., 1983). Many cellular homologs of *v-ets, c-ets*-1 and *c-ets*-2 among the first, were subsequently isolated from different species (Watson et al., 1983).

Ets proteins share a highly conserved DNA binding region of approximately 85 amino acids known as the ETS domain (Karim, 1990). Structural analysis of this domain in various Ets proteins reveals a winged-helix-turn-helix structure, akin to that of the *E. coli* catabolite activator protein (Graves and Petersen, 1998; Donaldson et al., 1994; Donaldson et al., 1996; Liang et al., 1994; Kodandapani et al., 1996). This domain recognizes and binds a 10 base pair purine-rich sequence containing a centrally located 5'GGA,A/T3' sequence, whereby specificity is provided by the flanking bases (Nye et al., 1992; Klemsz et al., 1990; Urness and Thummel, 1990). Typically, Ets proteins bind DNA as monomers and either activate or repress transcription (Graves and Petersen, 1998). However, most Ets proteins have weak transactivation domains and tend to

enhance their transcriptional abilities by co-operating with other factors. This occurs primarily via a functional or physical interaction (Crepieux et al., 1994). For example, GABP alpha requires GABP beta as a partner to provide a transactivation domain and to stabilize binding to DNA (Gugneja et al., 1995).

Ets proteins are further organized into subfamilies based on sequence identity within the ETS domain, position of the ETS domain within a given cDNA, and the presence or absence of other domains that lie outside of the ETS domain which may modulate their activity (Laudet et al., 1999; Wasylk et al., 1993; and Janknecht and Nordheim, 1993).

II. Normal Role of Ets Proteins

Ets proteins are involved in a number of developmental processes throughout embryogenesis and adult physiology. These include: hematopoiesis, feather development, mammary gland development, lymphocyte differentiation and oocyte maturation (Graves and Petersen, 1998; Albagli et al., 1996; Scott et al., 1994; Yamamoto et al., 1998; Chen et al., 1990; and Wasylyk et al., 1993). More precisely, each *Ets* gene is thought to subserve a unique developmental process and physiological function. During development for example, particular *Ets* genes are expressed in different temporal and spatial patterns, as compared to adult physiology where distinct *Ets* genes are expressed in specific tissues and organs. Also, mutations of Ets genes result in various developmental irregularities. For example, mutations in *Drosophila Ets* genes suggest that *E74* is required for salivary gland development (Fletcher and Thummel,

1995), *pnt* for proper tracheal branching and for differentiation of the midline glial cells during development of the central nervous system (Klambt et al., 1993; Schultz et al., 1993; Krasnow, 1996; and Klaes et al., 1994), and *yan* for photoreceptor cell differentiation during eye development (O'Neil et al., 1994; and Lai and Rubin, 1992). Comparable studies in vertebrates also point to a unique role for each *Ets* gene. For example, disruption of either the PU1 or TEL gene results in embryonic lethality; PU1 is required for differentiation of myeloid and lymphoid cell lineages, and TEL in yolk sac angiogenesis (Scott et al., 1994; and Wang et al., 1997). Similarly, the Ets-2 null genotype also results in early embryonic lethality, attributed to defects within the extraembryonic tissues that are required to form the placenta (Hynes and Stern, 1994; Yamamoto et al., 1998). A partial disruption in the FLI1 gene on the other hand, does not affect embryo viability, but causes reduced production of T cells (Melet et al., 1996).

III. Regulation of Ets Activity by Signal Transduction Pathways

The activity of several Ets proteins (members of the Ets, Yan, Elk and PEA3 subgroups) is regulated by extracellular ligands acting through the Ras signalling pathway (Yang et al., 1996; Dickson et al., 1995; Treisman et al., 1996). Ras is a membrane localised GTPase that acts through mitogen-activated protein kinase (MAPK) cascades and affects the phosporylation of such Ets proteins. This modification may then affect multiple functions, including changes in transactivation, repression, or DNA binding properties. The Ras signalling pathway itself is frequently activated in a diversity of human cancers (Bishop, 1991) and the *RAS* gene found oncogenically

mutated in ~30% of all human cancers (Bos, 1998). Hence, it is likely that one or another Ets protein is correspondingly upregulated in these tumours.

Ets proteins are required in the initiation and maintenance of transformation mediated by activated Ras and Neu. Cell culture studies using 3T3 cells show dominant negative Ets proteins such as Ets1, Ets2, and PU.1 are able to block transformation by activated Neu and Ras and their introduction into previously transformed cells reverts the phenotype (Wasylyk et al., 1994; Galang et al., 1996).

IV. Ets Proteins and Cancer

Ets proteins are implicated in the genesis and progression of human cancers. Principally, this occurs via two mechanisms leading to the aberrant expression of *Ets* genes as a result of transcriptional upregulation or chromosomal translocations.

Chromosomal translocations involve fusions of EWS to *Ets* genes. Currently reported are fusions between EWS and FLI-1 (Delattre et al., 1992; May et al., 1993), ERG (Shimizu et al., 1993; Sorensen et al., 1994; Ichikawa et al., 1994; and Peter et al., 1997), ER81 (Jeon et al., 1995) and PEA3 (Kaneko et al., 1996; and Urano et al., 1996). These fusions result in the expression of hyperactive Ets fusion proteins bearing amino-terminal residues of EWS and carboxy-terminal sequences and ETS DNA binding domain of one or another Ets protein. Usually, these resulting chimeric proteins possess increased transcriptional activity by comparison to the Ets protein from which they were derived (Bailly et al., 1994), suggestive of their importance in human carcinogenesis. Indeed, translocations of this nature are known to occur in Ewing's sarcoma, peripheral

primitive neuroectodermal tumours, and in several types of leukemia. For example, EWS-FLI1/PU1 viral DNA integrations result in murine erythroid tumours and leukemias (Ben-David, 1991), whereas EWS-FLI1/ERG/ER1/PEA3 translocations result in Ewing's sarcomas (Delattre et al., 1992; Zucman et al., 1993; Jeon et al., 1995; and Urano et al., 1996). Furthermore, translocations involving human *Ets* genes with partners other than EWS also result in several leukemias. For instance, in chronic myelomonocytic leukemia, the ETS domain of ERG or TEL is fused by translocations to TLS/FUS (an RNA binding protein) or MN1 (a transcription factor), respectively (Golub et al., 1994). By contrast, in acute myeloid leukemia, the fusion partner is one of several tyrosine kinases including platelet derived growth factor (PDGF), Abl, or JAK2, whereby TEL contributes a protein-protein dimerization surface (Shimizu et al., 1993 and Panagopoulos et al., 1995).

Ets proteins are also implicated in human cancers due to their uncontrolled expression in a variety of tumour derived cell lines and individual tumours. Studies show Ets1 to be overexpressed in 64% (n=121) of gastric adenocarcinomas, with highest levels correlating to the more invasive type carcinomas having lymph node metastasis (Nakayama et al., 1996). ESX overexpression is reported in human ductal carcinoma in-situ (Chang et al., 1997); PEA3 is similarly overexpressed in 76% (n=74) of human breast cancers, with 93% of HER2-positive subclass of tumours, representing 20-30% of total breast cancer cases (Benz et al., 1996). Lastly, Ets2 overexpression occurs in human prostate cancer (Liu et al., 1997) and experimental liver cancer in rats (Liao et al., 1996).

V. Ets Target Genes

Only a small number of *bona fide* Ets target genes have been identified to date for distinct Ets proteins. This is due to the confounding relatedness of DNA binding specificities and the co-expression of multiple Ets proteins in the same cells of particular tissues and organs. Generally, the identification of Ets target genes are inferred from the occurrence of Ets binding sites in the promoters of a wide variety of viral and cellular genes including growth factors and cytokines, membrane integrins and receptors, tyrosine kinases, proteases and transcription factors (Macleod et al., 1992). In rare instances, target genes have been identified whereby Ets proteins bound and activated transcription of reporter genes coupled to these promoters in transient transfection assays. However, recent applications involving representational difference analysis (RDA) using subtractive cDNA cloning techniques show stromelysin 1 (MMP3), cytochrome P450F1, cytokeratin 15, EAT2, and manic fringe as EWS-FLI target genes in 3T3 cells (Braun et al., 1995; Hubank et al., 1995; Arvand et al., 1998; and May et al., 1997).

VI. PEA3 Subfamily

PEA3 (E1AF, ETV4) (Xin et al., 1992; Higashino et al., 1993) is the founding member of the PEA3 subfamily, which also includes ERM (ETV5) (Monte et al., 1994; Chotteau-Lelievre et al., 1997) and ER81 (ETV1) (Brown et al., 1992; Monte et al., 1995). Each *pea3* subfamily gene is positioned on a different chromosome (Jeon et al., 1998), but all three genes share a common architecture of fourteen equivalently sized exons that encode similar sequences and functional domains of the respective proteins

(Monte et al., 1996; de Launoit et al., 1997; and Jeon et al., 1998). Specifically, the proteins encoded by these genes are comprised of approximately 500 amino acids, share 95% identity in the ETS DNA binding domain, 85% identity within the 5' acidic region, and an overall amino acid identity of 50% (de Launoit et al., 1997; Monte et al., 1995). These findings suggest each gene may likely bind to the same sequence elements in target gene promoters and therefore serve redundant functions during embryogensis and tumorigenesis.

The PEA3 subfamily genes are expressed in distinct as well as overlapping temporal and spatial compartments. Each gene is expressed during mouse embryogenesis in cells derived from ectodermal (CNS, neural crest), mesodermal (kidney, limbud, skeletal muscle) and endodermal (endothelium, lining of digestive tract) layers, in regions undergoing cellular proliferation and migration (Chotteau-Lelievre et al., 1997; and Brown et al., 1998). Specifically, during early gastrulation, PEA3 and ERM are co-expressed in the same regions of the developing embryo, as compared to ER81, which is not expressed at this time. However, at the onset of organogenesis, all three genes are co-expressed in many of the same tissues that express them during adult physiology. As a result, it is thought that transcription of these subfamily genes is governed by common trans-acting factors (Xin et al., 1992; Higashino et al., 1993; Brown and McKnight, 1992; Monte et al., 1995; Monte et al., 1994; and Chotteau-Lelievre et al., 1997).

CHAPTER 1
ROLE OF PEA3 IN TRANSFORMATION OF MOUSE EMBRYO FIBROBLASTS

1.1 INTRODUCTION

1.1.1 The PEA3 Transcription Factor

Mouse PEA3 (Polyoma Enhancer Activator 3) was the first Ets binding site to be identified; initially described as a DNA binding activity that bound a sequence in the polyomavirus enhancer to activate transcription (Martin et al., 1988). PEA3 was subsequently isolated from FM3A cells, a mouse mammary epithelial cell line, and shown to be a member of the Ets family of transcription factors (Xin et al., 1992). The human PEA3 gene, named E1AF and thereafter ETV4 was also isolated and characterised (Higashino et al., 1993). Florescence in-situ hybridization studies have mapped both the mouse and human PEA3 locus to chromosome 11, band D and chromosome 17q21.3, respectively (Barrett and Hassell, unpublished; Isobe et al., 1995). Two additional proteins, ERM (Monte et al., 1994) and ER81 (Brown and McKnight, 1992), were subsequently identified by virtue of their similarity to PEA3, and currently comprise the PEA3 subfamily.

1.1.2 Role of PEA3 in Embryonic and Adult Physiology

During early mouse embryogenesis, PEA3 is expressed both temporally and spatially in ectodermal cells fated to differentiate into mesodermal and endodermal cells.

Later in embryogenesis, PEA3 RNA is expressed in the branchial mesoderm, somites, urogenital ridge, and in the developing limbs (Chotteau-Lelievre et al., 1997; Laing and Hassell, unpublished), suggesting an important role for PEA3 in differentiation of multiple tissues and organs including muscle, connective tissue, kidney, epididymus, and motor neurons. In adult mice, PEA3 has a more restricted pattern of expression, occurring only in a small subset of tissues that express ERM and ER81. The highest levels of PEA3 expression are found in the brain and epididymus, and at lower levels in the testes, ovaries, mammary gland, uterus, small intestine, skeletal muscle, colon, spinal cord, hair follicle, and kidney (Xin et al., 1992; Monte et al., 1994; and Chotteau-Lelievre et al., 1997).

Genetic analyses have begun to uncover the physiological function of PEA3 in the mouse. PEA3-null mice are viable however, analyses of males revealed an unexpected role for PEA3 in male sexual dysfunction. PEA3-deficient males are sterile due to a sexual ejaculatory dysfunction. Specifically, these mice fail to reproduce and set copulatory plugs, despite their normal mating behaviour and ability to evoke erections (Laing et al., 2000). Furthermore, PEA3 is required for normal postnatal mammary gland development. PEA3 is expressed as early as day 10.5 of gestation in the mammary bud and thereafter throughout postnatal mammary gland development. Highest levels of PEA3 expression occur at times of extensive epithelial outgrowth and branching. However, during this time, PEA3-null females display reduced secondary, tertiary, and quaternary ductal branching of their mammary glands as compared to their wildtype counterparts (MacNeil et al., unpublished). Lastly, PEA3 is expressed in progenitor

mammary epithelial cap cells of terminal end buds, in differentiated myoepithelial cells of ducts and alveoli, and in mammary epithelial tumour cells. Hence, there is a possibility that PEA3 may control proliferation or migration of mammary epithelial stem cells, or regulate the differentiation program of these cells.

1.1.3 Regulation of PEA3 Activity

Mouse PEA3 comprises 480 amino acids, bears an 85 amino acid ETS domain near its carboxyl terminus, and has a strong activation domain near its amino terminus. Early characterization of PEA3 shows it binds DNA with specificity and activates transcription of reporter plasmids bearing PEA3-responsive promoters (Xin et al., 1992). Furthermore, DNA binding and PEA3 transcriptional activity is subject to elaborate negative control, implying that mechanisms exist to regulate PEA3 activity (Bojovic and Hassell, 2001). Studies involving a series of unidirectional amino and C-terminal mutants of PEA3 and GAL4-PEA3 chimeras reveal two negative regulatory regions, which independently repress PEA3 activity; one flanking the activation domain, and the other flanking the ETS domain.

PEA3 is a nuclear phosphoprotein, phosphorylated exclusively at serine residues by different members of the mitogen activated protein kinase (MAPK) family. Commonly, MAP kinases phosphorylate serine or threonine residues that are immediately followed by a proline residue in target genes, generally resulting in an increase in their activity. PEA3 contains 8 proline-directed serine residues; mutations of each reveals the activity of MAP kinases acting through the Ras pathway, including

extracellular regulated kinase (ERK) 1 and Jun kinases can phosphorylate these sites both in-vitro and in-vivo (Tozer et al., unpublished). However, the mechanism by which phosphorylation of PEA3 increases its transactivation potential remains enigmatic. Interestingly, most PEA3 phosphorylation sites are located within negative regulatory domains, suggesting a post-translational mechanism may exist to relieve inhibition of its activity. Interaction with partner proteins is another mechanism for regulating PEA3 transcriptional activity. For example, studies involving zebrafish PEA3, reveal the transcription factor USF1 may regulate PEA3 activity in this species. Interestingly, zebrafish PEA3 is also subject to stringent regulation by autoinhibitory mechanism similar to those in murine PEA3 (Brown et al., 1998). In this species, USF1 can bind the PEA3 ETS domain and the autoinhibitory motifs flanking this region, to first relieve their inhibitory action, and second to stimulate the assembly of a ternary nucleoprotein complex (Greenall et al., 2001). Lastly, DNA binding activity of murine PEA3 is also regulated via physical interactions with other partners. These include binding to Non-O and PSF, proteins previously implicated in RNA splicing, TBP (TATA-binding protein) (Xin and Hassell, unpublished) and Pax3 (Dr. Phillipe Gros, collaboration studies) general transcription factors.

1.1.4 PEA3 and Tumorigenesis

PEA3 is implicated in initiating tumorigenesis, tumour invasion and metastasis. Several studies show PEA3 binding sites are present in promoters of cellular and viral genes, thereby supporting this potential role in proliferation, migration and metastasis

(Macleod et al., 1992; Wasylyk et al., 1989; Gutman and Wasylyk, 1990). Many PEA3 candidate target genes encode matrix metalloprotease (MMP) genes (Macleod et al., 1992; Borden and Heller, 1997; Matrisian et al., 1994), enzymes that degrade extracellular matrix, and have established roles in tumour invasion, angiogenesis and metastasis (Sato and Seiki, 1993; Bernhard et al., 1994; Hua and Muschel, 1996). Specifically, PEA3 is known to stimulate transcription of reporter genes directed by TIMP1 (Edwards et al., 1992), MMP1 (collagenase), MMP3 (stromelysin1), MMP7 (matrilysin), and MMP9 (gelatinaseB) promoters in transient transfection assays (Matrisian et al., 1994; Higashino et al., 1995; Crawford et al., 2001). In support of these findings, PEA3 (EIAF) is capable of conferring an invasive phenotype both *in-vitro* and *in-vivo* to MCF-7 cells, a human breast cancer cell line, due to MMP9 upregulation (Kaya et al., 1996). Furthermore, studies from another group (Hida et al., 1997), show transfection of antisense PEA3 (EIAF) into HSC3 cells, a highly invasive human oral squamous cell carcinoma cell line, results in reduced mRNA transcripts and protein levels for MMP1, MMP3, and MMP9. Lastly, increased levels of PEA3 (E1AF) in a non-metastatic mouse fibrosarcoma cell line QR32, results in induction of MT1-MMP, activation of MMP-2, and metastasis related activity of these cells (Habelhah et al., 1999). However, PEA3 function is not exclusively linked to the regulation of MMPs, as illustrated by its ability to transactivate other promoters. These include the Vimentin gene that encodes an intermediate filament protein involved in cell motility (Chen et al., 1996), the β-enolase gene that is expressed in proliferating myoblasts (Taylor et al., 1997), the inducible isoform of prostaglandin synthase, cyclooxygenase-2 (COX2) gene

(Howe et al., 2001), in addition to two different cell adhesion molecules, VE-cadherin, and ICAM1 (Gory et al., 1998; and de Launoit et al., 1998).

The PEA3 gene is translocated in Ewing's sarcoma (Urano et al., 1996; Kaneko et al., 1996; and Jeon et al., 1994) and in undifferentiated metastatic sarcoma (Bailly et al., 1994), consistent with a potential role for PEA3 in these cancers. Recent studies utilising intestinal tumours of Min (multiple intestinal neoplasia) mutant mice (Crawford et al., 2001) and several breast cancer studies further expand the potential role for PEA3 in tumorigenesis. Specifically, PEA3 is overexpressed in MMTV-Wnt1 transgenic mammary tumours (Howe et al., 2001), in addition to mouse (Trimble et al., 1993) and human HER2-positive breast tumors (Benz et al., 1996) by comparison to other *Ets* genes such as Ets1, Ets2, and GABPα, which are expressed in normal mammary glands but not overexpressed in tumors (Scott et al., 1994; Shepherd and Hassell, 2001). Furthermore, these increased RNA levels are thought to occur due to increased transcription of the PEA3 gene or the action of other transcription factors acting on the PEA3 promoter, rather than gene amplification. This model is consistent with *in-vitro* studies showing PEA3 transcriptional activity is increased by HER2 (O'Hagan et al., 1996), in addition to studies showing PEA3 can transactivate its own promoter and that of HER2 (Benz et al., 1997). Furthermore, dominant negative studies involving PEA3 bi-transgenic female mice bearing both the MMTV-NEU and MMTV-ΔNPEA3EN transgenes suggest PEA3 is an obligate intermediate in HER2 tumorigenesis. In these studies, MMTV-NEU and bitransgenic mammary tumours share similar expression levels of HER2, however, the bi-transgenic females exhibited delayed onset, reduced incidence and size of HER-2

tumors by comparison to HER2-positive mice. (Shepherd and Hassell, unpublished). Lastly, studies from our lab show dominant negative PEA3 abrogates the function of all three PEA3 subfamily members and reduces transformation of mouse 3T3 cells by oncogenic Neu.

1.1.5 Experimental Rationale

PEA3 may potentiate cell invasion and migration during oncogenesis. PEA3 is overexpressed in HER2 breast cancers that correlate with poor patient prognosis due to their highly invasive and metastatic nature (Benz et al., 1997). This phenotype may be due to PEA3 being a downstream nuclear effector of oncogenes such as HER2 and Ras, leading to the overexpression of specific MMPs, thereby contributing to metastatic potential. In support of this model, PEA3 is overexpressed in metastatic mammary adenocarcinomas of MMTV-neu transgenic mice (Trimble et al., 1993; Guy et al., 1992; Shepherd and Hassell, unpublished). Furthermore, dominant negative PEA3 abrogates the function of all three subfamily members, delays the appearance of mammary tumours, reduces the number and size of these tumours in bitransgenic mice bearing both the MMTV-HER2 and MMTV-ΔNPEA3EN transgenes, and reduces Ras and Neu mediated transformation of mouse 3T3 cells in culture. Together, these findings suggest PEA3 is an obligate intermediate in HER2 tumorigenesis (Shepherd and Hassell, unpublished). Lastly, PEA3 subfamily transcripts are increased following Raf activation in a series of mouse 3T3 fibroblasts that are inducibly transformed by Ras and its downstream effector proteins (McCarthy et al., 1995; Greenberg and Ziff, 1984; and Xin, unpublished),

inferring activation of Raf likely leads to the activation of the MAPK pathway downstream of Ras. These findings further suggest a role for PEA3 in transformation mediated by these oncogenes.

In order to test whether PEA3 *per se* is required for transformation, immortal fibroblasts derived from wildtype and PEA3-null embryos were tested for their capacity to be transformed by activated Ras and Neu. Since it was initially found that PEA3-null fibroblasts are refractory to transformation, PEA3 was re-expressed in one of the PEA3-null fibroblast cell lines. Various clones showing PEA3 re-expression were then tested for their capacity to become transformed, thereby addressing whether PEA3 is potentially a Neu/Ras effector, capable of complementing transformation. Lastly, the identification of transformation-specific PEA3 target genes will also be assessed. Target genes showing compromised expression in PEA3-null fibroblasts will be assessed together with PEA3-null fibroblasts engineered to re-express PEA3 to determine whether PEA3 *per se* is indeed a critical mediator of transformation.

1.2 OBJECTIVES

1. Determine the levels of expression of PEA3 protein and PEA3 subfamily transcripts in wildtype PEA3 fibroblast cell lines transformed by Ras61L or NeuNT.

2. Determine the expression pattern of PEA3 subfamily members in various PEA3 (+/+) and (-/-) mouse embryo fibroblasts to assess for variability between the different lines.

3. Compare the ability of PEA3-null and wildtype mouse embryo fibroblasts to become transformed by retroviral infections using activated Ras and Neu.

4. Determine if PEA3 can complement transformation by Ras61L and NeuNT in PEA3-null fibroblasts by co-infection assays.

5. Generate stable clones that express ectopic PEA3 derived from a PEA3-null cell line, and determine if these clones are sensitive to transformation by activated Ras or Neu.

6. Northern blot analysis of potential PEA3 target genes using PEA3-null, wildtype and retransformant mouse embryo fibroblast cell lines.

7. Establish ERM and ER81 expressing cell lines from PEA3 (-/-) mouse embryo fibroblasts to assess whether ERM or ER81 can functionally substitute for loss of function of PEA3.

1.3 MATERIALS AND METHODS

1.3.1 Maintenance of Cells in Culture

Cells were grown on 100mm dishes in a humidified Water Jacketed™ cell culture incubator (Forma-Scientific) under an atmosphere of 5% CO_2 at 37°C. PEA3 mouse embryo fibroblast cell lines were grown in Dubelcco's modified Eagle's medium (DMEM) supplemented with 10% calf serum (Gibco BRL), 1X penicillin/streptomycin (pen/strep) (Gibco BRL) and fungizone. COS-1 and Rat-1 cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS) (Gibco BRL), 1X pen/strep and fungizone. BOSC-23 cells were grown in GPT selective media [DMEM supplemented with 10% FBS, 1X pen/strep, 1X xanthine (Sigma), 1X glutamine (Gibco BRL), 1X HAT (Sigma), 1X mycophenolic acid (Gibco BRL), 1X aminopterin (Sigma), and 1X thymidine (Sigma)].

1.3.2 Preparation and Titration of Retroviral Supernatant

BOSC-23 cells, based on a human embryonic kidney cell line (293T) were used as the retroviral packaging cell line. Transfection of these cells with a retroviral vector either by calcium phosphate or lipid-based transfection is known to produce high titre recombinant virus within 24 hours (Pear et al., 1993; Warren et al., 1993). Retroviral supernatants were prepared for pBabe puro, pBabe puro Myc-PEA3, pBabe puro Ras61L, and pBabe puro NeuNT. BOSC-23 cells were maintained in GPT selective media and spit at low densities to avoid clumping. Cells were plated at 2.5 X 10^6 cells per 60mm

dish, 24 hours prior to transfection in DMEM supplemented with 10% FBS and 1X pen/strep, without selection. Retroviral supernatants were prepared via calcium phosphate transfections. 10μg of each plasmid DNA listed above were diluted in distilled water, and mixed with 62μL of 2M $CaCl_2$ in a total volume of 0.5mL. An equal volume of 2X HBS (50mM HEPES pH7.05, 10mM KCL, 12mM dextrose, 280mM NaCl, 1.5mM Na_2HPO_4) was added to the $CaCl_2$/DNA mix by pipetting up and down several times. This solution was immediately added to the cells, and allowed to incubate overnight at 37°C. After this time, the cells were replenished with 2.5mL of fresh media (DMEM containing 10% FBS with 1X pen/strep and no selection). Viral supernatants were collected 48 hours post transfection, when the cells were confluent, taking care not to disturb the confluent cell monolayer. The supernatants were filtered as 1mL aliquots through a 0.45μm filter into sterile 1.5mL tubes and stored at –80°C.

Rat-1 cells were used to calculate viral titres. Cells were seeded at 6.8 X 10^4 cells/well on a 6-well plate (Falcon) 24 hours prior to infection. Various serial dilutions (10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}) of each viral stock were prepared in DMEM supplemented with 10% FBS and 4ug/mL polybrene. Each well of cells was washed twice with 1XPBS and incubated with 0.45mL diluted virus. The cells were spun at 1800 rpm at 32°C in a tabletop centrifuge (Sorval RT6000B) for 45 minutes. After the spin, the plates were transferred to a humidified 32°C Water Jacketed™ cell culture incubator for 2 hours and 15 minutes. After viral incorporation, 1.5mLs of complete medium (10% FBS, 1X pen/strep, 1X fungizone) were added to each well and the plates incubated at 37°C overnight. The following day, the cells from each well were transferred onto 100mm

plates in media containing 2ug/mL puromycin selection. Media was then changed every other day for 3 weeks, during which time, cell death and colony formation was monitored. Cells were then fixed in 10% formalin (Fisher Scientific) for two hours and stained with giemsa stain (4% stain in 1XPBS) (Fisher Scientific) overnight. The following day, excess stain was rinsed with warm tap water, the plates allowed to dry, the numbers of puromycin resistant colonies scored, and the titers of each retrovirus calculated (appendix 1.7.1).

1.3.3 Focus Forming Assay

PEA3 mouse embryo fibroblast cells were seeded at 7.2 X 10^4 cells/well on 6-well Falcon dishes 24 hours prior to infection. For co-infections involving pBabe puro Myc-PEA3, a two-step infection was performed. For these infections, cells were washed twice in 1XPBS, incubated with 0.4mL of concentrated PEA3 viral supernatant, spun at 1800 rpm for 45 minutes at 32°C and subsequently transferred to a 32°C cell culture for an additional 2 hours and 15 minutes. After this time, the cells were supplemented with 1.5mL of complete medium (10% FBS, 1X pen/strep, 1X fungizone) and incubated at 37°C overnight. The following day, cells previously infected with PEA3 were subsequently infected with pBabe puro (1:1000), or pBabe puro Ras61L (1:100), or pBabe puro NeuNT (1:100) viral dilutions prepared in DMEM supplemented with 10% FBS and 4ug/mL polybrene, under the same conditions as described for PEA3. 24hrs following the second infection, the cells from each well were transferred onto 100mm dishes. Media was then changed every other day for 3 weeks, allowing stable

monolayers and foci to form. After 3 weeks, cells were fixed in 10% formalin (Fisher Scientific) for two hours and stained with giemsa stain (4% stain in 1XPBS) overnight. Excess stain was removed with warm tap water, the plates inverted to dry, and the numbers of foci scored.

1.3.4 Transformation and Purification of Plasmid DNA

Plasmid DNA was transformed according to Gibco BRL recommendations. Approximately 5ng of plasmid DNA was mixed with 50μL of MAX Efficiency® DH5α competent cells (Gibco BRL) and incubated on ice for 30 minutes. The cells were heat shocked for 45 seconds at 42°C and placed on ice for an additional 2 minutes. The cells were diluted with 0.9mL of SOC medium (2% bactotryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 20mM glucose) and grown with shaking at 225 rpm for 1 hour at 37°C. Cells were diluted 1:10 and plated on LB-agar plates containing 100μg/mL ampicillin and incubated at 37°C for 16 hours. Isolated colonies were picked and inoculated into a 2mL LB culture containing 100μg/mL ampicillin and grown for 6 hours at 37°C, prior to being inoculated and grown overnight at 37°C in a 200mL LB (10% bacto-tryptone, 5% bacto-yeast extract, 10% NaCl) culture with 100μg/mL ampicillin. Plasmid DNA was amplified and purified according to maxiprep protocol supplied by Qiagen. Large-scale DNA preps were then quantified by OD_{260} spectrophotometer analysis and stored at 4°C.

1.3.5 Cloning Methodology

Vector and insert DNA used for ligations were each digested in a total volume of 20μL for several hours at 37°C using one unit of restriction enzyme in the appropriate 1X digestion buffer. Vector DNA was dephosphorylated with 2μL of 10X CIAP buffer and 1 unit CIAP (Calf intestinal alkaline phosphatase) (Boehringer Mannheim) at 37°C for an additional hour. Enzymatic reactions were terminated with a 30-minute incubation at 65°C. Loading dye (0.5% orange G, 40% sucrose) was added to each sample to a final 1X concentration and the DNA electrophoresed on a 1% low melting point agarose gel containing 2μg/mL ethidium bromide in 1X TAE buffer (40mM tris-acetate, 2mM EDTA pH 8.5) at 70 volts. Vector and insert bands were excised from the gel and placed in 1.5mL microcentrifuge tubes. Gel fragments were melted at 65°C for 10 minutes with periodic vortexing and transferred to 37°C for 2 minutes. Ligations were set up using various ratios of vector-to-insert DNA. Typically, 1-5μL of vector DNA were aliquoted with 10-15μL of insert DNA to a final volume of 31μL with water. A negative control using vector DNA alone was also set up to account for proper dephosphorylation of the vector. Insert and vector DNA were quickly spun down and mixed with 9μL of a ligation master mix [4μL of 10X ligase buffer (NEB), 4μL of 10mM ATP, and 1μL T4 DNA ligase (NEB)]. Ligation reactions were incubated overnight at room temperature. The following day, 2 volumes of cold TCM buffer (10mM Tris pH7.5, 10mM $CaCl_2$, 10mM $MgCl_2$) were added to each low-melt ligation reaction followed by a 10-minute incubation at 65°C. MAX Efficiency® DH5α competent cells (Gibco BRL) were transformed with 10μL of each ligation reaction as outlined in section 1.3.4. At the end

of the transformation, all the bacterial cells were pelleted, resuspended in 100μL LB medium, spread onto LB agar plates containing 100μg/mL ampicillin and incubated at 37°C overnight.

Isolated colonies were screened by preparing miniprep DNA. A 3mL LB miniprep culture containing 100ug/mL ampicillin was inoculated and grown overnight at 37°C. Miniprep DNA was isolated and lysed using the boiling method (Sambrook et al., 1989). One mL of the miniprep culture was centrifuged for one minute at 14K rpm and the remainder of the culture stored at 4°C. Bacterial pellets were resuspended in 400uL STET buffer (8% sucrose, 0.5% Triton X-100, 50mM EDTA pH8, 10mM Tris pH8). Bacterial lysis was performed with 30uL of lysozyme (10ug/mL), vortexing briefly, and boiling the samples for one minute. After boiling, minipreps were centrifuged at 14K rpm for 15 minutes at room temperature. Chromosomal DNA was removed from each sample using sterile toothpicks and the miniprep DNA precipitated with 0.5mL isopropanol with centrifugation at 14K rpm for 15 minutes at room temperature. Pellets were washed in 0.1mL of 70% ethanol and centrifuged an additional 15 minutes. Pellets were dried at room temperature, resuspended in 50uL TE with 1uL RNase (2mg/mL), and stored at 4°C.

1.3.6 Generation of PEA3 Stable Cell Lines

Two drug-resistant lines of PEA3-expressing stable cell clones were derived in PEA3 (-/-) MEF-1 cells; one line being puryomycin resistant, the other blasticidin resistant. Puromycin clones were isolated by seeding PEA3 MEF1 cells at 1 X 10^5 per

well of 6-well plates (Falcon) 24 hours prior to infection. Infection of cells was performed as outlined above (section 1.3.3) with concentrated pBabe puro Myc-PEA3 retroviral supernatant. The following day, cells were plated onto 100mm plates containing a range of puromycin concentrations (0.5-1.5ug/mL). Media was changed every other day and the cells monitored for cell death and colony formation. Stable clones were isolated at a puromycin dosage of 1.5ug/mL which killed all the control cells infected with pBabe puro alone and allowed colony formation in Myc-PEA3 infected cells. Isolated clones were expanded onto 150mm plates and nuclear lysates isolated for Western blot analyses.

A second line of PEA3-expressing stable cell clones was isolated by first cloning the Myc-PEA3 cDNA into the retroviral vector (pWB3). This vector contains an internal ribosome entry site (IRES) and a blasticidin resistance gene driven by the moloney murine leukaemia retroviral long terminal repeats (LTR). Myc-PEA3 was cleaved from pBabe puro Myc-PEA3 by EcoRI digestion and cloned into the EcoRI site of pWB3 by setting up low melting point ligations as previously described (section 1.3.5). Miniprep DNA was prepared from isolated colonies using the boiling method and screened for the Myc-PEA3 insert by digesting with one unit of EcoRI restriction enzyme. Positive colonies were subsequently screened for insert orientation by digesting with one unit of BamHI restriction enzyme.

Expression of Myc-PEA3 from positive colonies was tested in COS-1 cells, by seeding the cells at a density of 3.5 X 10^5 cells per 60mm dish. The following day, 6ug of DNA and 15uL of lipofectamine (Gibco BRL) were each diluted in 0.3mL of DMEM.

The lipofectamine dilution was added to the diluted DNA and DNA-liposome complexes allowed to form for 30 minutes at room temperature. The reaction was then terminated using 2.4mL of DMEM. The COS-1 cells were washed once in 1XPBS and subsequently with DMEM, prior to being transfected with 3mL of the DNA-liposome complexes and incubated at 37°C for 5 hours. After this time, the cells were washed twice, supplemented with complete medium (DMEM supplemented with 10% CS, 1X pen/strep, and 1X fungizone) and incubated at 37°C. Nuclear lysates were prepared 48 hours post transfection, run on a 10% SDS-PAGE and immunoblotted with a mix of MP13 and MP16 PEA3 monoclonal antibodies.

Once expression of pWB3 Myc-PEA3 was confirmed in COS-1 cells, PEA3 (-/-) MEF-1 cells were seeded at 1 X 10^5 cells per 35mm well on a 6-well dish (Nunc), 24 hours prior to transfection. Lipofectamine mediated transfections were performed by diluting 2ug DNA (pWB3, or calf thymus, or pWB3-Myc PEA3), and 6uL lipofectamine each into 0.1mL DMEM. The lipofectamine and DNA dilutions were mixed and incubated at room temperature for 30 minutes. The reaction was then quenched with 0.8mL DMEM. PEA3 (-/-) MEF-1 cells were washed in 1XPBS, then with DMEM and transfected with 1mL of the DNA-lipid complexes for one hour at 37°C. After this time, the cells were washed twice and incubated with complete medium (DMEM supplemented with 10% CS, 1X pen/strep, and 1X fungizone). The following day, the cells were plated onto 100mm plates containing a range of blasticidin concentrations (0.5-4.0ug/mL). Media was changed every other day and cells monitored for cell death and colony formation. Stable cell clones that grew a dosage of 1.0ug/mL blasticidin were expanded

onto 150mm plates and nuclear lysates isolated for Western blot analyses. Cells transfected with calf thymus DNA all died at this blasticidin dosage. Nuclear lysates from puromycin and blasticidin resistant clones were resolved on a 10% SDS-PAGE gel, transferred onto nitrocellulose membrane and immunoblotted with a mix of MP13 and MP16 PEA3 monoclonal antibodies to screen for expression of PEA3 protein.

1.3.7 Protein Isolation and Quantification

Nuclear lysates were isolated and prepared (Schreiber et al., 1989) from cells grown on 150mm plates. Cells were washed twice in 20mL of cold 1XPBS, scraped in 0.8mL 1XPBS using a plastic spatula (Costar) and transferred to a 1.5mL tube. The cells were centrifuged at 7000rpm for 3 minutes at 4°C. The supernatant was aspirated from each sample and the pellet resuspended in 0.4mL of cold Buffer A (10mM HEPES pH7.6, 10mM KCL, 0.1mM EDTA pH8, 0.1mM EGTA pH8, 1mM DTT, 0.2mg/mL PMSF, 10ug/mL leupeptin, 10ug/mL pepstatin, 10ug/mL aprotinin). Cell membranes were lysed for 15 minutes on ice. Cellular proteins were precipitated by the addition of 25µL of 10% Igepal in Buffer A and vortexing for 15 seconds. The samples were centrifuged at 13K rpm for 30 seconds at 4°C, the supernatant aspirated and the pellet resuspended in 25µL of cold Buffer C (20mM HEPES pH7.6, 0.4M NaCl, 1mM EDTA pH8, 1mM EGTA pH8, 1mM DTT, 0.2mg/mL PMSF, 10ug/mL leupeptin, 10ug/mL pepstatin, 10ug/mL aprotinin). Nuclear membranes were lysed for 1.5 hours by vortexing at 4°C. The samples were then centrifuged at 13K rpm for 5 minutes at 4°C, the nuclear supernatants transferred to fresh tubes, and stored at –80°C. Total protein

concentration was determined by diluting 1-2μL of lysate with 1mL of 1X Bradford reagent (Biorad) in duplicate. The absorbance of each sample nuclear lysate sample, in addition to a series of BSA (bovine serum albumin) dilutions (0-15μg) were measured by spectrophotometer (Beckman DU 640) analysis at 595nm. A standard BSA curve was prepared using Microsoft Excel by plotting OD_{595} readings versus μg of BSA protein and finding the line of best fit. Protein concentrations of unknown nuclear lysates were determined from the standard curve by interpolating the concentration of the nuclear lysates from the known O.D. reading.

1.3.8 Western Blot Analysis

Protein samples were mixed with 4X loading buffer (200mM Tris-HCL pH6.8, 0.5% bromophenol blue, 40% glycerol, 20% BME (Beta-mercaptoethanol), 8% SDS), boiled for three minutes and resolved on a 10% SDS-PAGE gel with Tris-Glycine electrophoresis buffer (25mM Tris, 250mM glycine pH8.3, 0.1% SDS) at 160 volts for 5 hours at room temperature. Nitrocellulose Immobilon-Plus membrane (Millipore) was rinsed in 100% methanol, followed by water and soaked in Transfer Buffer (20mM Tris-HCL, 150mM glycine, 20% methanol) for 10 minutes. The protein was transferred onto the presoaked Immobilon-Plus membrane at 4°C overnight at 20 volts. Visual evidence of electrophoretic transfer was confirmed by staining the membrane in a 1:10 dilution of Ponceau S (2% Ponceau S, 30% tricholoroacetic acid, 30% sulfosalicylic acid) in water.

Western blots were blocked overnight at 4°C in a heat seal bag with blocking buffer (5% skim milk powder in TBS-T [10mM Tris-HCL pH7.3, 150mM NaCl, 0.05%

Tween 20]). The membrane was then placed in a new bag and incubated with primary antibody appropriately diluted in blocking buffer. Mouse α-PEA3 primary antibody consisted of a mixture of MP13 and MP16 monoclonal antibodies diluted 1:10 and 1:100, respectively. These antibodies recognize different PEA3 epitopes: MP13 recognizes amino acids 157-256 and MP16 recognizes amino acids 256 to 337. Mouse α-Tata-binding protein (BD Transduction Laboratories) antibody was diluted 1:500. Following incubation with the primary antibody, the blots were washed three times for 10 minutes in TBS-T, blocked for one hour in blocking buffer at room temperature and incubated with secondary antibody for an additional hour. Goat α-mouse (KPL) secondary antibody was diluted 1:5000 in blocking buffer and used for blots incubated with PEA3 or TBP primary antibodies. Western blots were washed three times for 10 minutes in TBS-T and incubated with Renaissance Western blot Chemiluminescence Reagent Plus (NEN Life Science Products). Protein was visualized by exposure to X-Omat Blue film (Kodak).

1.3.9 Western Blot Quantification by Kodak Image Station

Western blots previously incubated with chemiluminescence reagent were exposed to real-time imaging on the Kodak Image Station. Either one capture of 10 minutes or five captures of two minutes each were taken. Rectangular objects were drawn surrounding the bands of interest. The net intensity of each band was subtracted from the local background and the quantification data exported to Microsoft Excel, where each sample was then normalized to TBP, the internal control.

1.3.10 RNA Isolation and Quantification

Total RNA was isolated from mouse embryo fibroblast cells using 2mLs of TRIzol® Reagent (Gibco BRL) per 100mm plate as outlined in the manufacture's protocol. RNA was resuspended in Diethyl pyrocarbonate (DEPC) treated water and quantified by OD_{260} spectrophotometer analysis (Beckman DU 640). 20μg of total RNA was aliquoted into separate microcentrifuge tubes and stored at –80°C.

1.3.11 Generation of Radioactively Labelled Probes for Northern Analysis

DNA templates were excised from their plasmid vectors by the appropriate enzyme digestion and separated on low melting point agarose. Templates were gel purified using Qiaquick gel extraction kit (Qiagen) as per manufacturers protocol. DNA templates used for Northern analyses included a 480bp KpnI/HincII mouse MMP 3(matrix metalloproteinase) fragment, an 850bp EcorRI/KpnI MMP9 fragment, a 300bp KpnI PEA3 fragment from PGEM-PEA3 which corresponds to 559-864bp of murine PEA3, a 500bp EcoRI 5'ERM fragment from PCRII-5'ERM, a 1Kb HindIII/BamHI 5'ER81 fragment, and a 650bp XhoI GAPDH fragment corresponding to 181-830bp. Probes were synthesized as described in Strip-EZ DNA kit (Ambion) with the exceptions that 40ng of template DNA were used and probe synthesis incubation time was extended to one hour. Probes were purified using ProbeQuant™ G-50 micro columns (Amersham) as suggested by the manufacturer, and boiled for five minutes prior to overnight hybridization with the Northern blot.

1.3.12 Northern Blot Analysis

RNA samples were run on a 1% agarose /formaldehyde denaturing gel, prepared by boiling 1% (w/v) agarose in 1% 10X MOPS in a microwave. Once cooled, 9% (v/v) formaldehyde was added to the agarose mix prior to casting of the gel. RNA samples were mixed with 1% [3-(Npmorpholino)-propanesulfonic acid] 10XMOPS (200mM MOPS pH 7, 50mM sodium acetate, 10mM EDTA pH 8), 16.5% formaldehyde and 50% formamide in a final volume of 40μL. The samples were then heated at 65°C for 10 minutes and transferred onto ice for 5 minutes. 2μL of ethidium bromide (10mg/mL) and 2μL of formaldehybe loading buffer (1mM EDTA pH 8, 0.25% bromophenol blue, 0.25% xylene cyanol, 50% glycerol) were added to each sample prior to electrophoresis in 1% 10X MOPS and 9% formaldehyde running buffer. Samples were run at 70 volts until the xylene cyanol ran off the gel. Ribosomal bands were visualized under UV light and photographed. A ruler was used to measure the distance between the wells where the sample was loaded to each of the two ribosomal bands to serve as size markers. Excess formaldehyde was removed from the gel by rinsing in 10XSSC for an hour at room temperature and the RNA transferred overnight by capillary action onto a nylon membrane (GeneScreen). RNA was fixed to the membrane by a UV crosslinker (Stratagene), placed in a heat-sealed bag and either stored at –20°C or hybridized at 42°C overnight with a radiolabelled probe described above (section 1.3.11). After hybridization in UltraHybe solution (Ambion), blots were washed twice in 2XSSC,

0.1%SDS at 42°C for 5 minutes; once for 15 minutes in 0.1XSSC, 0.1%SDS at 42°C and another 15 minutes in 0.1XSSC, 0.1%SDS at 55-60°C. Northern blots were visualised by autoradiography using BioMax MS Film (Kodak) together with a BioMax MS Intensifier Screen (Kodak). Hybridized DNA probes were stripped from Northern blots as outlined in the Strip-EZ™ DNA kit protocol (Ambion).

1.3.13 Generation of RNase Protection Probes

In order to synthesize antisense RNA probes, 0.5μg of linearized plasmid DNA was mixed with 10mM ATP, GTP, CTP (Gibco BRL), 1μL RNAguard (Gibco BRL), 5μL enzyme buffer, 5μL [α-^{32}P] UTP (Amersham) and 20 units of RNA polymerase to a final volume of 25μL. The reaction was incubated at 37°C for 45 minutes. One μL of 10mM unlabelled UTP was then added to complete unfinished product and incubated for an additional 10 minutes prior to DNase treatment. Template DNA was digested with 2μL DNaseI (Gibco BRL), 1μL 0.5M $MgCl_2$ and incubating for an additional 15 minutes at 37°C. DNaseI was removed by phenol-chloroform extraction and riboprobes precipitated by the addition of 20μg yeast tRNA, two volumes of 2.5M NH_4OAc pH5.2 and 7.5 volumes ethanol. Pellets were dried, resuspended in 20μL of RNA loading buffer (80% formamide, 50mM EDTA, 1% bromophenol blue, 1% xylene cyanol), heated for 5 minutes at 85°C and run on a 6% acrylamide gel containing 7M Urea in TBE (Sambrook et al., 1997) for 1 hour. The gel was then wrapped in saran wrap and exposed to BioMax-AR Film (Kodak). The film was aligned with the gel and the radioactive probe bands excised and eluted at 37°C overnight in 400μL elution buffer (0.5M NH_4OAc

pH5.2, 1mM EDTA, 0.1% SDS). Supernatant was removed and the probes precipitated using 10μg yeast tRNA and 1mL absolute ethanol. Pellets were resuspended in 50μL hybridization buffer (1 part 10X PIPES buffer, 4 parts formamide). Probes were counted in a scintillation counter by the addition of 1μL of probe to 1mL scintillation fluid (Beckman).

The PEA3 riboprobe was synthsized using SP6 RNA polymerase to generate a 500nt (nucleotide) antisense message corresponding to a DraI/PvuII fragment at 3' end of the PEA3 cDNA. Antisense ERM was generated using T3 RNA polymerase (Boehringer Mannheim) to synthesize a 280nt product corresponding to an EcorRI/PstI fragment of the mouse cDNA. The ER81 riboprobe was synthesized using T7 RNA polymerase (Boeringher Mannheim) to generate a 250nt product corresponding to a HindIII/BamHI fragment of ER81. L32 was synthesized using T3 RNA polymerase to generate a protected fragment of 195nt corresponding to a XhoI/DraI region of L32.

1.3.14 RNase Protection Analysis

Antisense riboprobes were synthesized as described above (section 1.3.13). 30μg of total RNA was mixed with 1 X 10^5 counts per minute probe and denatured at 85°C for 5 minutes. RNA samples were then hybridized overnight at 50°C. Yeast tRNA was hybridized as a negative control. The following day, samples were chilled on ice, treated with 15μg RNaseA (Pharmacia), 0.45μL RNaseT1 (Boehringer Mannheim) and digested for 30 minutes at 37°C in digestion buffer (300mM NaCl, 10mM Tris-HCL, 5mM EDTA pH7.4). The digestion reactions were terminated by the addition of 20μL 10% SDS and

50μg Proteinase K (Boehringer Mannheim) per sample and incubating an additional 20 minutes at 37°C. Proteinase K was removed by phenol-chloroform extraction and RNA samples precipitated with 40μg yeast tRNA and two volumes of ethanol. Samples were precipitated at -80°C for 30 minutes and centrifuged at 13K rpm for 30 minutes. Pellets were resuspended in 5μL of RNA loading buffer (80% formamide, 50mM EDTA, 1% bromophenol blue, 1% xylene cyanol), heated for 5 minutes at 85°C and loaded onto a 6% acrylamide gel containing 7M Urea in TBE (Sambrook et al., 1997). Once the gel was finished running, the gel was transferred onto Whatman paper, dried for 2 hours at 80°C and exposed to Xomat-AR film (Kodak).

1.3.15 Transcript Quantification by PhosphorImager Analyses

Northern blots were exposed to a cleared Phosphor Screen (Molecular Dynamics) overnight and then scanned into the program ImageQuant. Equally sized rectangular objects were drawn where transcript bands were expected or observed. Rectangular objects were also drawn below each transcript area and defined as background. The volume of each rectangular area was then integrated after background subtraction. The quantification data was exported into Microsoft Excel for further analysis. The sum above background value for each sample was then normalized to GAPDH or L32, the internal loading controls used in Northern blotting or RNase Protection assays, respectively.

1.5.4 RESULTS

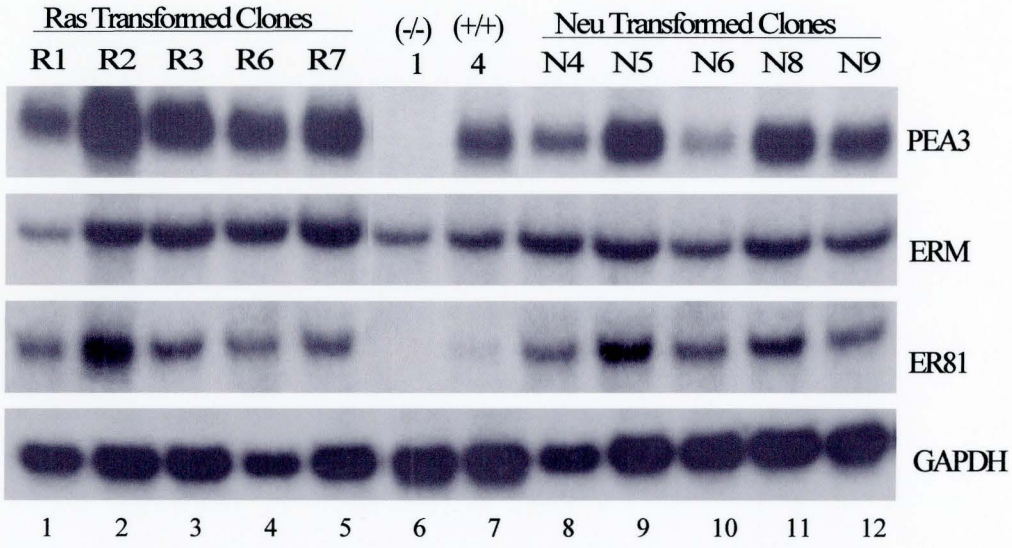
1.4.1 PEA3 Subfamily is Upregulated in Ras and Neu Transformed Clones

The observation that PEA3 subfamily transcripts are elevated in HER2 induced mammary tumours suggests transcription of these genes are increased in tumour cells, and hence might also be elevated in transformed fibroblast cell lines *in-vitro*. In order to address this possibility, the PEA3 MEF 4 (+/+) fibroblast cell line was infected with either pBabe puro Ras61L or pBabe puro NeuNT, transformed foci were isolated and total RNA prepared. Levels of PEA3 subfamily transcripts were analyzed by Northern and RNase protection assays. From a representative Northern blot (Figure 1.4.1, part A), it was observed that all PEA3 subfamily member transcripts were generally elevated in various Ras61L and NeuNT transformed clones examined, as compared to their untransformed (MEF 4) counterpart. However, ER81 showed the highest levels of upregulation, being highly expressed in every transformed clone (lanes 1-5 and 8-12) and barely detectable in the parental cell line (lane 7). The data from two independent experiments was quantified using the PhosphorImager analysis (Figure 1.4.1, part B), the levels of each transcript was normalized to GAPDH and compared to the endogenous levels present in the parental cell line. ERM transcript levels were upregulated only 2-3 fold. In contrast, PEA3 and ER81 transcripts were increased from 2 to 8 fold higher than the parental MEF 4 cell line.

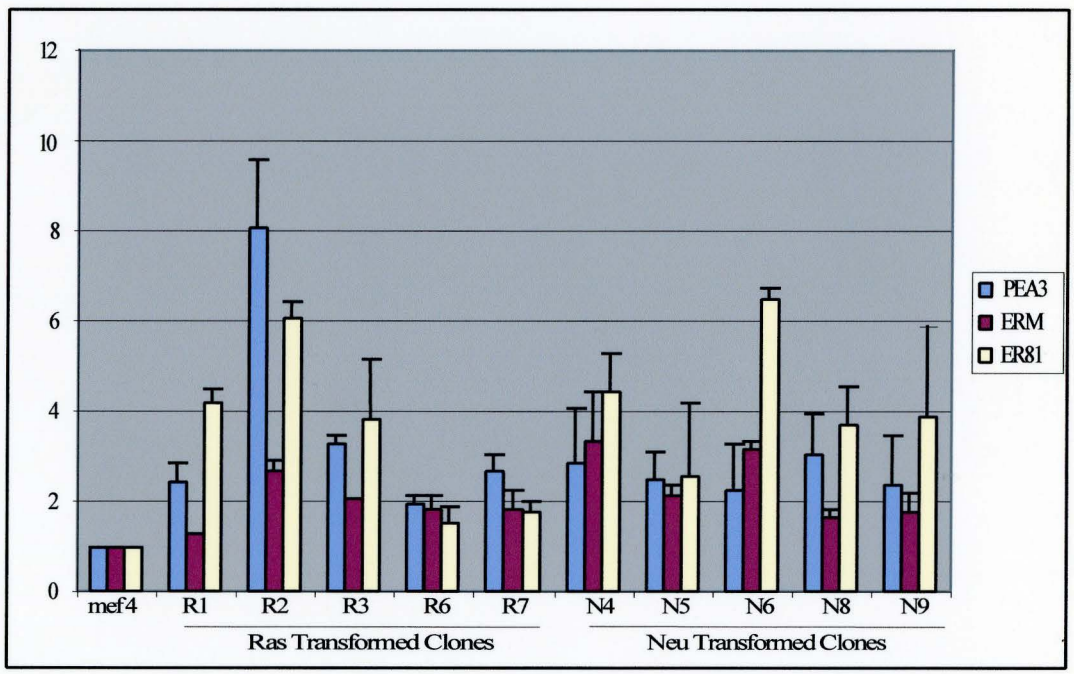
The observation that PEA3 transcript levels were elevated in Ras and Neu transformed cell lines as compared to the untransformed parental cell line, suggests that

Figure 1.4.1: PEA3 subfamily transcripts are upregulated in Ras61L and NeuNT transformed cell lines in comparison to the parental cell line (MEF 4). Wildtype PEA3 fibroblasts (MEF 4) were infected with pBabe puro Ras61L or pBabe puro NeuNT retroviral supernatants in a focus formation assay. (A) Total RNA was isolated from Ras (R1, R2, R3, R6, R7) and Neu (N4, N5, N6, N8, N9) foci and 30ug analyzed by Northern analysis. Hybridization was subsequently performed using riboprobes for the PEA3 subfamily (PEA3, ERM, and ER81). GAPDH serves as the internal loading control. MEF 1 serves as a negative control for PEA3 expression. (B) Quantification of PEA3 subfamily transcript levels in Ras and Neu transformed cells using PhosphorImager analyses. Levels of mRNA expression for each PEA3 subfamily member were normalized to GAPDH and compared to the endogenous levels found in the parental cell line (MEF 4). Quantification data represents two independent experiments. Error bars correspond to the standard deviation between the two experiments. Lanes 5&6 represent spliced lanes from the original audioradiogram.

A.


Ras Transformed Clones
(-/-)
(+/+)
Neu Transformed Clones
R1 R2 R3 R6 R7
1 4
N4 N5 N6 N8 N9
PEA3
ERM
ER81
GAPDH
1 2 3 4 5 6 7 8 9 10 11 12

B.


12
10
8
6
4
2
0
PEA3
ERM
ER81
mef4
R1
R2
R3
R6
R7
N4
N5
N6
N8
N9
Ras Transformed Clones
Neu Transformed Clones

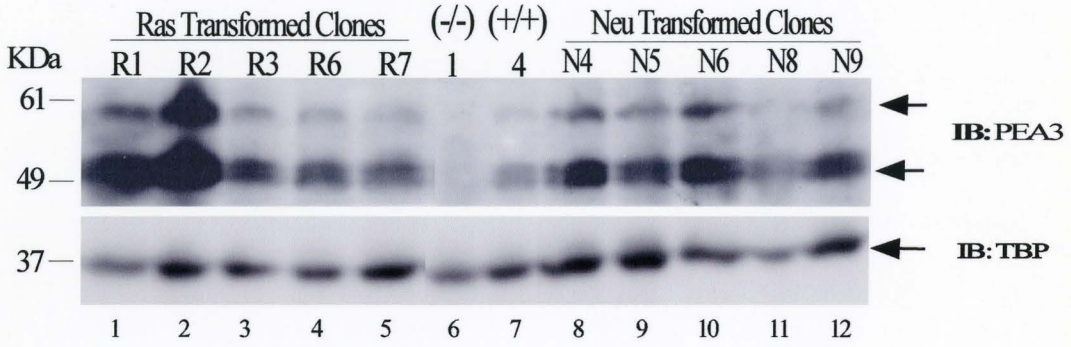
protein levels might also be elevated in these clones. Western analysis was performed and the levels of PEA3 protein detected by immunoblotting using a mixture of two anti-PEA3 monoclonal antibodies, MP16 and MP13 (Figure 1.4.2, part A). PEA3 protein levels were elevated in each of the transformed cell lines as compared to the MEF 4 (+/+) parental line; the highest levels were observed in two Ras transformed clones (R1 and R2; lanes 1&2) and three Neu transformed clones (N4-6; lanes 8-10). TBP was used as an internal loading control to ensure equal loading of nuclear lysate samples. Analysis of these cell lines was repeated and similar results observed; the data from the two independent experiments was quantified using the Kodak Image Station, and the protein levels of each clone normalized to TBP (Figure 1.4.2, part B). PEA3 protein levels were elevated 2 to 4 fold over the parental clone, in the majority of Ras and Neu transformed clones. However, one Ras transformed cell line (R2; lane 2), consistently expressed PEA3 protein levels exceeding 20 fold that of the parental cell line.

1.4.2 PEA3 is Required for Transformation Mediated by Activated Ras or Neu

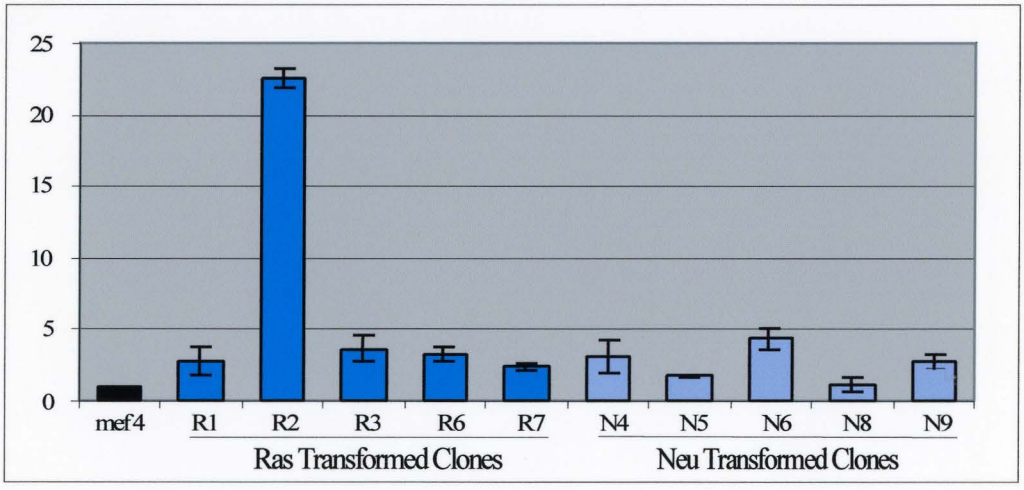
The observation that PEA3 is overexpressed in mouse and human HER2-positive mammary tumours and that PEA3 is a potential target of HER2 and Ras, suggests a role for PEA3 in oncogenesis. To learn whether PEA3 *per se* is required for transformation by oncogenically activated Ras and Neu, populations of wildtype and PEA3-null mouse embryo fibroblasts were infected with retroviruses expressing these oncoproteins. Retroviral supernatants to be used in these infections were produced for pBabe puro, pBabe puro myc PEA3, pBabe puro Ras61L and pBabe puro NeuNT in BOSC 23 packaging cells via calcium phosphate transfection assay. Retroviral titers were then

Figure 1.4.2: PEA3 protein levels are elevated in Ras and Neu transformed cell lines in comparison to the parental cell line (MEF 4). Ras and Neu transformed clonal cell lines were derived in a focus-forming assay, whereby MEF 4 (PEA3 +/+) cells were infected with pBabe puro Ras61L or pBabe puro NeuNT retroviral supernatants. (A) Nuclear lysates were isolated from various Ras (R1, R2, R3, R6, R7) and Neu (N4, N5, N6, N8, N9) foci and 300ug analyzed by Western Analysis using PEA3 antibodies. TBP serves as a loading control. MEF 1 serves as a negative control for PEA3 expression. (B) Quantification of PEA3 protein levels in Ras and Neu transformed cell lines using Kodak Image Station. Protein levels for each transformed cell line was normalized to TBP and compared to the endogenous levels in the parental cell line (MEF 4). The quantification data is representative of two independent experiments. Error bars represent the standard deviation between the two experiments. Lanes 5 & 6 depicts spliced lanes from the original audioradiogram.

A.


Ras Transformed Clones
(-/-)
(+/+)
Neu Transformed Clones
KDa
R1 R2 R3 R6 R7 1 4 N4 N5 N6 N8 N9
61
49
37
IB: PEA3
IB: TBP
1 2 3 4 5 6 7 8 9 10 11 12

B.


25
20
15
10
5
0
mef4 R1 R2 R3 R6 R7 N4 N5 N6 N8 N9
Ras Transformed Clones
Neu Transformed Clones

tested in Rat-1 cells, and found to be in the order of 2-6 x 10^5 focus forming units/mL (appendix 1.7.1).

PEA3-null (MEF 1) and wildtype (MEF 4) fibroblast cell lines were tested for their capacity to be transformed by oncogenically activated Ras and Neu. It was found that PEA3-null fibroblasts were refractory to transformation; no foci were detected in numerous independent experiments. By contrast, the wildtype PEA3 fibroblast cell line was readily transformed by the same oncogenes; at the multiplicity of infection used, between 20-30 foci appeared on the PEA3 (+/+) monolayers (Table 1.4.1). These observations suggest that PEA3 is required for Ras and Neu mediated transformation. To substantiate these findings, additional experiments were carried out with multiple wildtype (n=6) and PEA3-null (n=9) cell lines. The vast majority of PEA3-null fibroblast cell lines (n=7) were refractory to transformation, and all wildtype cell lines developed foci. (Hastings, unpublished). In addition, dominant-negative PEA3 blocks transformation by Ras or Neu in these mouse 3T3 cells. Furthermore, the inability to transform these knockout fibroblasts did not result from the ability of these oncogenes to selectively kill these cells, nor due to differences in DNA uptake or integration (Hastings and Shepherd, unpublished). During these experimental analyses however, two variant PEA3-null cell lines (MEF 110 and 114) were found to be sensitive to transformation by Ras and Neu (Hastings, unpublished). The sensitivity of these cell lines to transformation may have resulted from their capacity to express ER81 and / or ERM, which may complement for the loss of PEA3 (section 1.4.2).

Table 1.4.1: PEA3-null mouse embryo fibroblasts are refractory to transformation by constitutively activated Ras and Neu in focus formation assays. PEA3-null (MEF 1) and wildtype (MEF 4) fibroblast cell lines were seeded on 35mm dishes 24h prior to infection. Retroviral supernatants of pBabe puro (1:1000), pBabe puro Ras61L (1:100), and pBabe puro NeuNT (1:100) were diluted in DMEM supplemented with 10% calf serum and 4ug/ml polybrene. The cells were infected with 450uL of each viral supernatant, spun for 45mins at 1800rpm and incubated at 32°C for 2.5h to allow viral adsorption. Cells were then supplemented with complete media, placed at 37°C for 16h and passaged onto 100mm dishes. Media was changed every other day for 3 weeks, at which time foci were enumerated. Each independent experiment represents the average number of foci obtained from duplicate samples ± standard deviation.

CELL LINE	VIRUS	Expt. #1	Expt. #2	AVG
MEF 4 (+/+)	pBabe puro	0	0	0
	Ras 61L	25	30	27±3.54
	NeuNT	19	20	19±0.71
MEF 1 (-/-)	pBabe puro	0	0	0
	Ras 61L	0	0	0
	NeuNT	0	0	0

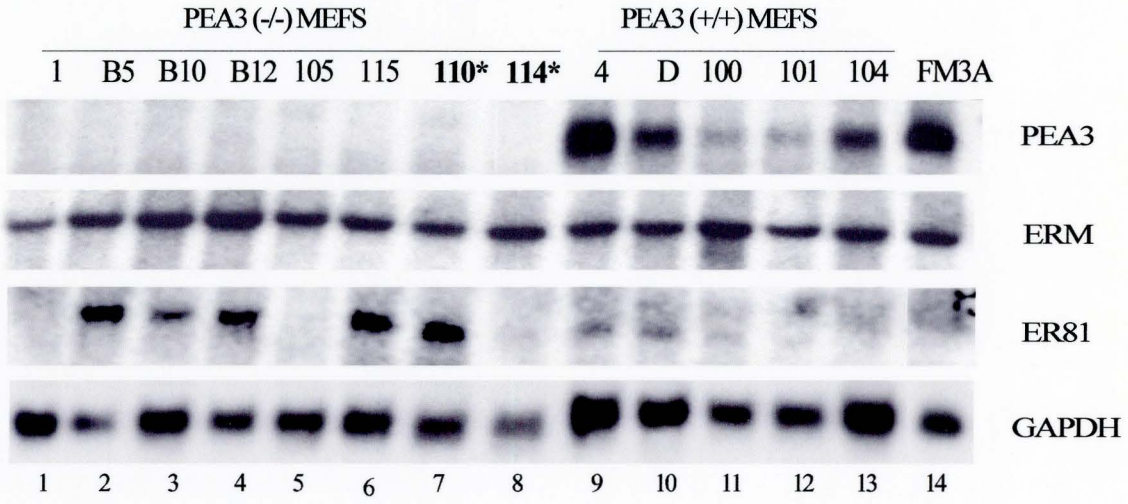
1.4.3 Expression Profile of PEA3 Subfamily in Wildtype and PEA3-null Mouse Embryo Fibroblasts

To determine whether the sensitivity of these two PEA3-null (MEF 110 and MEF 114) fibroblast cell lines to transformation is due to ER81 and / or ERM expression, the expression profile of the PEA3 subfamily members was compared among various wildtype (n=5) and PEA3-null (n=8) cell lines (Figure1.4.3, part A). Quantitation by PhosphorImager analysis was also carried out for each PEA3 subfamily transcript, and the data normalized to GAPDH RNA levels (Figure 1.4.3, part B). PEA3 gene transcripts were detected in each of the PEA3 (+/+) cell lines (lanes 9-14) but not in any of the PEA3 (-/-) cell lines (lanes 1-8). From the quantitation, MEF 4 expressed the highest levels of endogenous PEA3. ERM transcripts were detected in both PEA3-null and the PEA3 (+/+) cell lines. ER81 transcripts were variably expressed; ER81 RNA was present in five null cell lines (B5, B10, B12, 110, 115) and three wild-type cell lines (4, D, 100). When present, ER81 transcript levels were much higher in the PEA3-null cell lines relative to the wildtype cell lines. The FM3A mouse mammary tumour cell line from which PEA3 cDNAs were originally isolated (Xin et al., 1992), expressed both PEA3 and ERM RNA, but not ER81 RNA. GAPDH was used as an internal control to normalize the data and account for differences in sample loading.

Neither ERM nor ER81 was overexpressed in the variant transformation sensitive PEA3-null cell lines (MEF 110 and MEF 114; lanes 7&8) by comparison to the other

Figure 1.4.3: PEA3 subfamily mRNA expression in PEA3-null and PEA3 (+/+) mouse embryo fibroblast cell lines. Total RNA was isolated from wildtype (4, D, 100, 101, 104) and PEA3-null (1, B5, B10, B12, 105, 115, 110, 114) immortalized mouse embryo fibroblast cell lines growing in log phase. (A) Northern blot analysis was performed using 20ug RNA and hybridizing with cDNA probes for each PEA3 subfamily member (PEA3, ERM, and ER81). FM3A cells were used as a positive control for PEA3 expression. GAPDH serves as a control for RNA loading. (B) Quantification of Northern blot using PhosphorImager analysis. The data is represented for each independent cell line as a ratio of each transcript over GAPDH.

A.



B.

	PEA3/GAPDH	ERM/GAPDH	ER81/GAPDH
1	0.009677	0.054735	0.005521
B5	0.022607	0.279044	0.038072
B10	0.012655	0.171463	0.011593
B12	0.019994	0.353629	0.019361
105	0.012058	0.177205	0.002078
115	0.008568	0.144077	0.020362
110	0.016217	0.142833	0.024790
114	0.005944	0.277967	0.007302
4	0.115548	0.096717	0.006950
D	0.065375	0.103337	0.009773
100	0.042395	0.270420	0.009499
101	0.029742	0.134728	0.003581
104	0.041054	0.083086	0.001003
FM3A	0.209945	0.191558	0.002381

PEA3 (-/-) cell lines that were refractory to transformation (MEFs 1, B5, B10, B12, 105, 115; lanes 1-6). MEF 110 (lane 7) expressed ERM and ER81 RNA to levels comparable to MEFs B5, B10, B12 and 115 (lanes 2-4, 6); whereas MEF 114 (lane 8) was comparable to MEFs 1 and 105 (lanes 1&5). Hence, it would appear that the expression of the PEA3-related transcripts, ERM and ER81 do not account for the sensitivity to transformation of these two PEA3-null cell lines. In order to address this hypothesis, preliminary studies were performed to generate clones in the MEF1 fibroblast cell line (PEA3 -/-) that would overexpress ERM or ER81. In order to accomplish this, Myc-ER81 and Myc-ERM were cloned into the retroviral vector pWB3, shown to be an efficient vector in deriving clones expressing the gene of interest (section 1.4.4). Specifically, Myc-ER81 cDNA was removed from a pRSV vector by EcoR1 digestion, and cloned into the EcoR1 site in the MCS of the pWB3 vector. On the other hand, Myc-ERM was PCR amplified from a pCAN vector with primers containing a 5' Xho I linker and a 3' Sal I linker. The PCR product was subsequently cloned into the XhoI and SalI sites of pWB3 (appendix 1.7.2). The pWB3-Myc-ERM and pWB3-Myc-ER81 vectors were transfected into PEA3 (-/-) MEF1 cells. Blasticidin resistant colonies (0.5μg/mL) were isolated for both ERM (n=6) and ER81 (n=5) as described for PEA3 (section 1.4.4), to be used in future studies testing the hypothesis of ER81 and ERM redundancy in PEA3 null fibroblasts.

1.4.4 Ectopic Expression of PEA3 in PEA3-null Fibroblasts

A number of findings suggest that PEA3 is required for Ras and Neu mediated transformation of mouse embryo fibroblast cell lines. These include: (i) PEA3-null fibroblast cell lines are refractory to transformation, (ii) PEA3 RNA and protein levels are elevated in Ras and Neu transformed fibroblasts compared to their untransformed counterparts, and (iii) dominant-negative PEA3 reduces transformation by oncogenically activated Ras and Neu (Shepherd, personal communication). A possible hypothesis to account for these observations is that PEA3 is a Ras/Neu effector and thus required for transformation mediated by these oncoproteins. In order to test this hypothesis, the PEA3 cDNA was reintroduced into PEA3-null fibroblasts to determine whether re-expression of PEA3 in these cells can rescue transformation. Initially, MEF 1 (-/-) and MEF 4 (+/+) fibroblast cell lines were co-infected with concentrated pBabe puro PEA3 retroviral supernatant and subsequently infected with either activated Ras or Neu (Table 1.4.2). This method however, as tested by multiple trials, failed to show PEA3 complementation in Ras and Neu mediated transformation. There were no foci present in the null cell line, and 20-35 foci on the wildtype cell line. DNA-mediated transformation analyses yielded identical results.

In order to assess whether stable expression of PEA3 was required to rescue transformation, stable cell clones expressing ectopic PEA3 expression were isolated from the MEF1 (PEA3-null) cell line. pBabe puro myc-PEA3 retroviral supernatant was used to infect the MEF 1 cell line and puromycin resistant colonies (1.5 μg/mL) were isolated.

Table 1.4.2: Co-infection of PEA3 with activated Ras or Neu fails to complement transformation in PEA3-null mouse embryo fibroblast cell line. Wildtype (MEF 4) and PEA3-null (MEF 1) cells were seeded on 35mm dishes 24h prior to infection. The cells were then infected with either pBabe puro (1:1000) or concentrated pBabe puro Myc-PEA3 retroviral supernatants in DMEM supplemented with 10% calf serum and 4ug/ml polybrene, spun for 45mins at 1800rpm and incubated at 32°C for 2.5h. Cells were then supplemented with complete media and allowed to recover for 16h at 37°C. Cells previously infected with pBabe puro Myc-PEA3 was subsequently infected with pBabe puro Ras61L (1:100) or pBabe puro NeuNT (1:100), as described for PEA3. On the following day, the cells were passaged onto 100mm plates. Media was changed every other day for 3 weeks, at which time foci were enumerated. Numbers for each independent experiment represent the average number of foci obtained from duplicate samples ± standard deviation.

CELL LINE	VIRUS	EXPT #1	EXPT #2	AVG (expt 1+2)
MEF 4 (+/+)	pBabe puro	0	0	0
	Ras 61L + PEA3	66±16.97	34±2.12	50±22.63
	NeuNT + PEA3	20±4.24	28±4.34	24±5.65
MEF 1 (-/-)	pBabe puro	0	0	0
	Ras 61L + PEA3	2±2.82	0	1±1.41
	NeuNT + PEA3	0	0	0

These clones were screened for PEA3 expression by Western analysis (Figure 1.4.4); only one clone (C5-1; lane 5) was positive for ectopic PEA3 expression. Transfection of pBabe puro Myc PEA3 into COS1 cells was used as a positive control (lane 12) and MEF1 lysate served as a negative control (lane 1) for PEA3 expression.

Due to the inefficiency of producing PEA3 expressing cell lines, a second approach was initiated with a replication-defective retroviral vector, pWB3. This bicistronic vector was selected since it contains a blasticidin selection gene, retroviral LTR sequences and an IRES site immediately downstream of the cloned gene of interest. This IRES site is optimally aligned thereby allowing enhanced transcription and efficient cap-independent translation of the foreign gene, thus resulting in high titre virus and efficient infection of 3T3 cells. The Myc-PEA3 cDNA was removed from the pBabe puro vector (Figure 1.4.5, panel A) by EcoR1 digestion and cloned into the EcoR1 site in the MCS of the pWB3 vector (Figure 1.4.5, panel B). Expression of pWB3 Myc-PEA3 (lane 2) was confirmed via a transient transfection assay in COS1 cells and Western analysis (Figure 1.4.6). Transfections of pBabe puro Myc-PEA3 (lane 3) and the empty vector pWB3 (lane 1) served as positive and negative controls for PEA3 expression, respectively. Lipofectamine mediated transfection of pWB3-Myc-PEA3 DNA was performed in MEF1 cells; blasticidin resistant colonies were isolated and screened for PEA3 expression. Western analysis of the isolated cell lines (n=7) showed they were all positive for ectopic PEA3 expression (Figure 1.4.7; lanes 2-8). MEF 1 lysate (lane 1) and pBabe puro Myc-PEA3 transfected COS1 cell lysates (lane 9) were used as negative and positive controls for PEA3 expression, respectively.

Figure 1.4.4: Western blot screen for PEA3 re-expression in puromycin resistant clones infected with pBabe puro Myc-PEA3. PEA3-null mouse embryo fibroblast cell line (MEF 1) was infected with pBabe puro Myc-PEA3 retroviral supernatant in a focus formation assay. 24h after infection, cells were passaged onto 100mm plates and supplemented with 1.5ug/ml puromycin. Nuclear lysates were prepared from puromycin resistant colonies and 200ug analyzed by Western blotting using MP13 and MP16 monoclonal PEA3 antibodies. MEF 1 cells, the parental cell line from which the clones were derived, serves as a negative control for PEA3 expression. pBabe puro Myc-PEA3 was transfected into COS1 cells and serves as a positive control for PEA3 expression (represented as a spliced lane). TBP was used as an internal loading control.

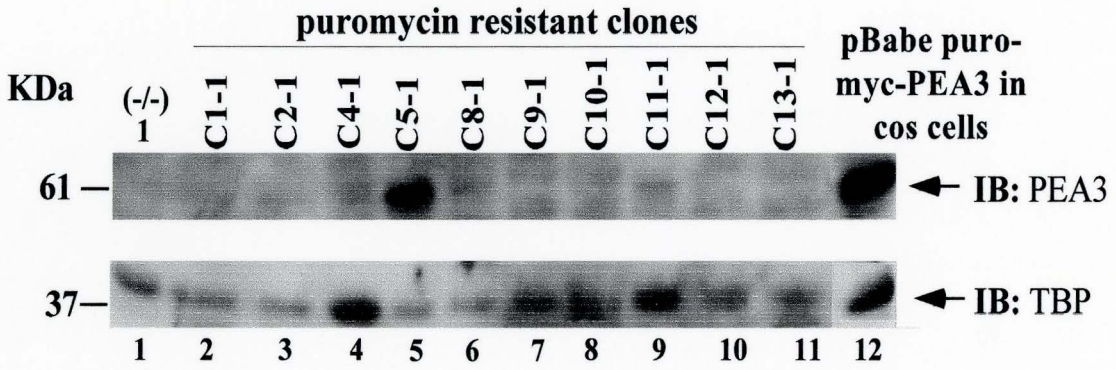
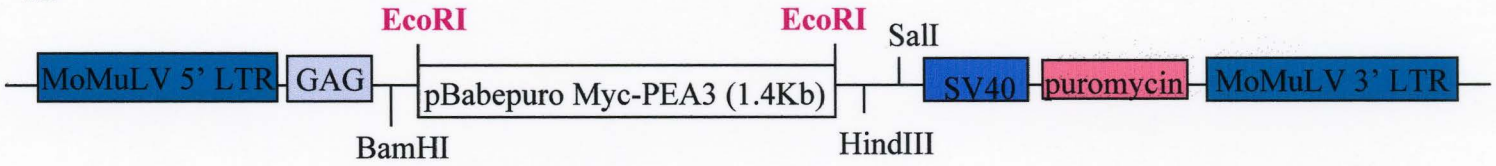

puromycin resistant clones
KDa
(-/-) 1
C1-1
C2-1
C4-1
C5-1
C8-1
C9-1
C10-1
C11-1
C12-1
C13-1
pBabe puro-myc-PEA3 in cos cells
61
IB: PEA3
37
IB: TBP
1 2 3 4 5 6 7 8 9 10 11 12

Figure 1.4.5: Structure of vectors used in cloning the cDNA insert of pBabe puro-Myc-PEA3 into pWB3. (A) Myc-PEA3 was digested and purified from pBabe puro by EcoRI restriction digestion and cloned into the EcoRI site found within the multiple cloning region of pWB3 (B). Orientation of the PEA3 cDNA inserted into pWB3 was verified by digesting the new construct with a BamHI restriction digest.

A.



B.

pWB3 vector

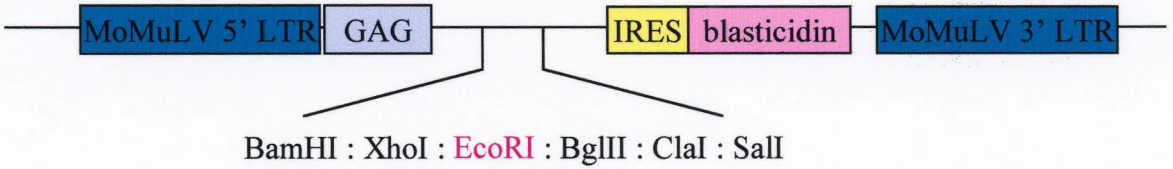


Figure 1.4.6: Myc-PEA3 protein expression in COS-1 cells from newly cloned retroviral vector, pWB3-Myc-PEA3. COS-1 cells were transfected with empty vector, pWB3 or pWB3 Myc-PEA3 24h after seeding the cells. Nuclear lysates were collected 48h post-transfection and PEA3 expression assayed via Western Analysis using MP13 and MP16 monoclonal PEA3 antibodies. pBabe puro Myc-PEA3 was also transfected into COS-1 cells and used as a positive control. 100ug of nuclear lysates were loaded for each sample. TBP was used as an internal loading control.

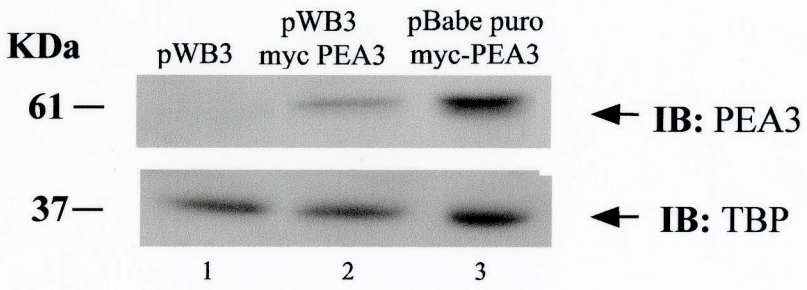
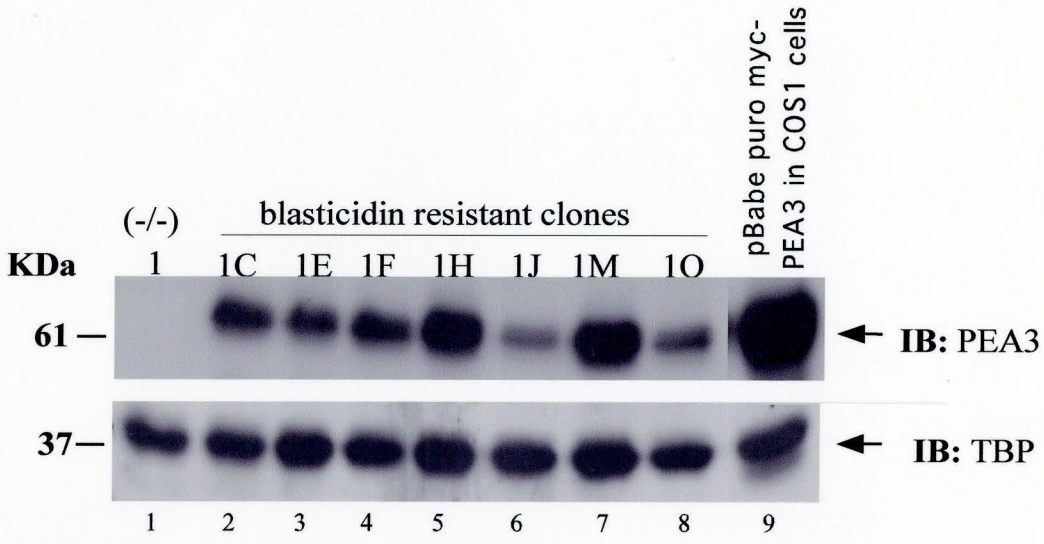

KDa
pWB3
pWB3
myc PEA3
pBabe puro
myc-PEA3
61
37
IB: PEA3
IB: TBP
1
2
3

Figure 1.4.7: Western blot screen for PEA3 re-expression in blasticidin resistant clonal cell lines transfected with pWB3-Myc-PEA3. PEA3-null (MEF 1) mouse embryo fibroblast cell line was seeded on 35mm dishes and transfected with pWB3 Myc-PEA3 after 24h. The following day, the cells were passaged onto 100mm plates and supplemented with 1ug/ml blasticidin. Nuclear lysates were prepared from blasticidin resistant colonies and 250ug analyzed by Western Analysis using PEA3 antibodies. pBabe puro Myc-PEA3 was transfected into COS-1 cells and served as a positive control (represented as a spliced lane from the original audiogram). TBP was used as an internal control.


(-/-)
blasticidin resistant clones
pBabe puro myc-PEA3 in COS1 cells
KDa
1
1C
1E
1F
1H
1J
1M
1O
61
IB: PEA3
37
IB: TBP
1
2
3
4
5
6
7
8
9

Three clonal cell lines derived from PEA3-null fibroblasts expressing PEA3 (C5-1, 1C, 1M) were tested for their capacity to be transformed by oncogenic Ras and Neu (Table 1.4.3). As observed previously, PEA3-null fibroblasts were still refractory to transformation, while wildtype fibroblasts displayed 20-30 foci per experiment. Furthermore, all three clones that re-express PEA3 rescued the PEA3-null transformation defective phenotype; two clones (C5-1, 1C) displayed comparable numbers of foci to those observed with the wildtype fibroblast cell lines. Hence, these observations show that re-expression of PEA3 in the PEA3-null cell line restores its capacity to be transformed by either oncogenic Ras or Neu.

1.4.5 Induction of PEA3 Target Genes by FGF8

To discover the molecular basis for the inability of PEA3-null fibroblast cell lines to be transformed by Ras or Neu, the expression of candidate PEA3 target genes was assessed in the various mouse embryo fibroblast cell lines. PEA3 binding sites occurs in the promoters of the serine proteinase urokinase (Nerlov et al., 1991; and Nerlov et al., 1992) and of various matrix metalloproteases (Crawford et al., 2001, Matrisian et al., 1994; and Higashino et al., 1995), suggesting that these are direct PEA3 target genes. Furthermore, expression of candidate PEA3 target genes is affected by loss-of-function mutations in PEA3; EGF stimulated MMP-3 and MMP-9 expression is ablated in PEA3-null cell lines as compared to their wildtype counterparts (Xin, unpublished). Based on these observations, northern analyses were performed to determine if cells engineered to re-express PEA3 have the restored ability to express candidate PEA3 target genes. FGF

Table 1.4.3: PEA3 re-expression in PEA3-null mouse embryo fibroblast cells can rescue Ras and Neu mediated transformation. MEF 1 (PEA3 -/-) cells were engineered to re-express PEA3 by infection or transfection assays with retroviral vectors encoding a Myc-PEA3 cDNA and a selectable marker. Puromycin or blasticidin resistant clonal cell lines were selected with puromycin or blasticidin and subsequently screened for ectopic PEA3 protein expression. Focus formation assays were performed on clones demonstrating positive PEA3 re-expression (MEF C5-1, MEFS 1C and 1M) by infecting the cells with pBabe puro (1:1000), pBabe puro Ras61L (1:100) or pBabe puro NeuNT (1:100) in DMEM supplemented with 10% calf serum and 4ug/ml polybrene. The cells were then spun for 45mins at 1800rpm, incubated at 32°C for 2.5h, supplemented with complete media and allowed to recover for 16h prior to being passaged onto 100mm dishes. Media was changed every other day for 3 weeks, at which time foci were scored. MEF 1 and MEF 4 serve as negative and positive controls for PEA3 expression, respectively. The number of foci shown for each independent experiment represent averages from duplicate samples ± standard deviation.

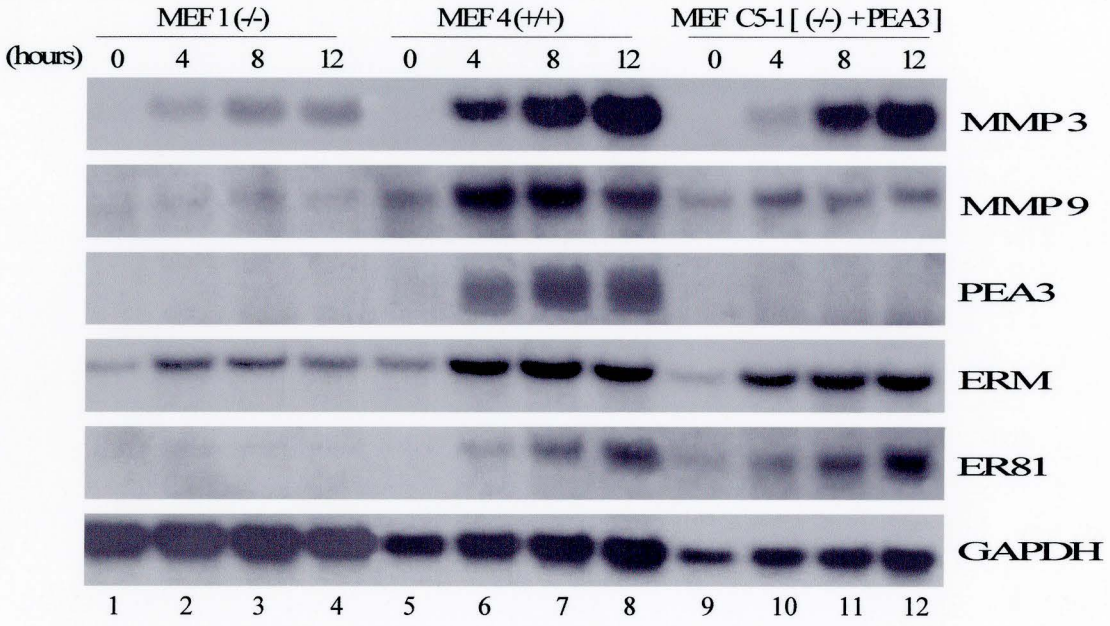
CELL LINE	VIRUS	EXPT #1	EXPT #2	AVG (expt 1+2)
MEF 4 (+/+)	pBabe puro	0	0	0
	Ras 61L	18±1.14	35±0.71	26±0.50
	NeuNT	18±5.66	36±2.12	27±2.50
MEF 1 (-/-)	pBabe puro	0	0	0
	Ras 61L	0	0	0
	NeuNT	0	0	0
C5-1 [(-/-) + PEA3]	pBabe puro	0	0	0
	Ras 61L	33±6.36	40±4.24	36±1.50
	NeuNT	13±2.12	33±2.82	23±0.50
1C [(-/-) + PEA3]	pBabe puro	0	0	0
	Ras 61L	28±2.12	13±4.24	20±1.50
	NeuNT	36±5.66	11±1.01	23±3.29
1M [(-/-) + PEA3]	pBabe puro	0	0	0
	Ras 61L	8±1.41	9±3.54	8±1.51
	NeuNT	8±1.41	9±0.71	8±0.50

however, was used as the stimulating ligand, as it has been shown to regulate the PEA3 subfamily gene expression in zebrafish and xenopus (Roehl and Nussen-Volhard, 2001; Raible and Brand, 2001; Munchberg et al., 1999). PEA3 (-/-), (+/+) and [(-/-) + PEA3] cells were serum starved for 36hrs and then stimulated with 7nM of murine recombinant FGF8 (isoform b) for 0, 4, 8 or 12hrs. Total RNA was collected, 20μg run on a denaturing formaldehyde gel, transferred to nitrocellulose and sequentially probed with mouse MMP-3, MMP-9 and each of the PEA3 subfamily members (Figure 1.4.8, part A). The data was quantified using a PhosphorImager and the levels of each transcript normalized to GAPDH. The zero hour timepoint in each cell line was set to one and the fold induction at 4, 8, and 12 hours calculated for each transcript (Figure 1.4.8, part B). Induction of MMP-3 and MMP-9 was observed in the PEA3 (+/+) cell line to high levels (lanes 6-8) compared to the PEA3-null cell line (lanes 2-4). Furthermore, ectopic re-expression of PEA3 in the null cell line rescued FGF8b induction of MMP-3 and MMP-9 transcripts (lanes 10-12). MMP-3 RNA was induced 13 fold in the wildtype cell line and 25 fold in the PEA3 retransformed cell line, as compared to only 2 fold in the PEA3-null cell line. Similarly, MMP-9 was induced nearly 6 fold in the wildtype cell line and 2 fold in the PEA3 retransformed cell line.

Induction of the PEA3 subfamily transcripts was also examined. PEA3 transcripts were absent in the PEA3-null cell line as expected (lanes 2-4). PEA3 RNA was induced in the wildtype fibroblast cell line (lanes 6-8) up to 22 fold. However, PEA3 levels were not induced to detectable levels in the PEA3 retransformed cell line (lanes 10-12). ERM RNA was induced in each of the three cell lines examined. Specifically,

Figure 1.4.8: Induction of candidate PEA3 target genes (MMP3 and MMP9) by FGF8b is affected by loss of function mutations in PEA3 mouse embryo fibroblast cell lines. (A) MEF 1 (PEA3-null), MEF 4 (PEA3 +/+), and MEF C5-1 [PEA3 (-/-) engineered to express PEA3] cells were grown to confluence, serum starved for 36h, and incubated with 7ng/mL FGF8b. Total cellular RNA was isolated at various timepoints (0, 4, 8, 12h) following FGF8 stimulation and 20ug analyzed by hybridizing with cDNA probes for candidate PEA3 target genes (MMP3 and MMP9) and the PEA3 subfamily (PEA3, ERM, and ER81). GAPDH serves as control for RNA loading. (B) Quantification of Northern blot data using PhosphorImager analysis. The mRNA transcripts for each gene were normalized to GAPDH and compared to the endogenous levels present at 0 hours for each independent cell line.

A.



B.

	mmp3	mmp9	pea3	erm	er81
MEF 1-0H	1.0	1.0	1.0	1.0	1.0
MEF 1-4H	1.1	1.1	0.9	3.2	0.6
MEF 1-8H	2.2	2.6	0.9	2.5	0.2
MEF 1-12H	2.2	1.7	0.8	2.3	0.3
MEF 4-0H	1.0	1.0	1.0	1.0	1.0
MEF 4-4H	9.0	5.7	19.4	4.2	3.2
MEF 4-8H	15.5	4.6	28.9	4.6	9.6
MEF 4-12H	25.4	2.7	22.3	3.2	13.5
MEF C5-0H	1.0	1.0	1.0	1.0	1.0
MEF C5-4H	2.3	1.9	1.1	4.8	3.8
MEF C5-8H	9.1	1.1	0.8	3.7	4.2
MEF C5-12H	13.0	1.1	1.6	4.1	4.8

ERM was induced up to 3 fold in the PEA3-null fibroblast cell line by 4hrs, as compared to 5 fold in both the wildtype and PEA3 retransformed cell line by 4 and 8 hours of FGF8 stimulation, respectively. Levels of ERM then decline slightly in each cell line. Lastly, ER81 transcripts were absent in the PEA3-null cell line (lanes 2-4), they were induced up to13 fold in the wildtype cell line (lanes 6-8) and approximately 5 fold in the PEA3 retransformed cell line (lanes 10-12). These observations suggest induction of MMP3 and MMP9 by FGF8b are dependent on PEA3 subfamily expression.

1.5.4 DISCUSSION

1.5.1 PEA3 is an Oncogenic Effector of HER2/Neu and Ras

Several *in-vivo* studies are consistent with the hypothesis that PEA3 is a nuclear target of oncogenes such as HER2 and Ras. First, PEA3 is overexpressed in mouse and human HER2 mammary tumours (Trimble et al., 1993; Benz et al., 1996). Also, expression of the dominant negative PEA3 transgene under the control of the MMTV-HER2 promoter abrogate the function of the PEA3 subfamily members, delay the appearance of mammary tumours, as well as reduce the numbers and size of these tumours (Shepherd et al., 2001). The emerging thought from these studies is that HER2 acts primarily through the Ras pathway to effect transformation and mammary oncogenesis, thereby resulting in increased PEA3 levels in these mammary tumours (Dankort and Muller, 2000). To further uncover the role of PEA3 in transformation and the mechanism whereby Ras and Neu increase PEA3 levels, *in-vitro* studies were undertaken using wildtype and PEA3-null mouse embryo fibroblasts. Initial studies using dominant negative PEA3 showed Ras and Neu mediated transformation of mouse 3T3 cells is reduced (Shepherd, unpublished), suggesting a potential role for PEA3 in transformation mediated by these oncoproteins. However, interpretation of such experiments is complex as this mutant may interfere with the function of PEA3 subfamily members, as well as other ets genes. Therefore, in order to test the hypothesis that PEA3 *per se* is required for cellular transformation, we employed immortalized PEA3-null fibroblast cell lines. These cell lines were infected and assayed for focus formation. Retroviral infections were employed instead of the traditional calcium phosphate or lipid

based methods of gene transfer due to its 100% efficacy in infecting cells with the desired DNA construct (Cepko et al., 1988). In addition, retroviruses can stably integrate into the genomes of infected cells, resulting in prolonged gene expression and genetic transmission to daughter cells (Boxhorn et al., 1998). The cDNAs contained in the retroviral vectors used in this study each contain point mutations making them constitutively active. pBabe puro Ras61L contains a point mutation in codon 61 changing it into a leucine residue, while pBabe puro NeuNT contains a valine to glutamic acid substitution in codon 659 of the transmembrane region (Bargmann et al., 1986; Bargmann et al., 1988). In contrast to the PEA3 wildtype fibroblast lines, cells harbouring a homozygous null mutation of the PEA3 gene are refractory to transformation by activated Ras and Neu (Table 1.4.4). Similar findings were previously published for SV40-immortalized c-Jun-null fibroblasts, which proved to be refractory to transformation by activated Ras and thought to be a nuclear target of the Ras signalling pathway (Johnson et al., 1996). The results obtained with PEA3 may also be compared to studies evaluating the role of c-fos in neoplastic transformation by Ras proteins. c-fos is not required in the Ras mediated transformation of fibroblasts (Hu et al., 1994). However, it plays a critical role in a mouse model for the malignant progression of multi-step skin cancer induced by activated Ras and topical applications of phorbol esters (Saez et al., 1995). Lastly, previous experiments showed that the inability of Ras and Neu to transform PEA3-null fibroblasts could not be attributed to differences in DNA integration and / or oncogene expression in these cells. Furthermore, this expression did not prove to be cytotoxic in these cell lines (Hastings, unpublished). Hence, these findings strongly

suggest that PEA3 is required for Ras and Neu mediated cellular transformation of these fibroblast cell lines.

PEA3 subfamily mRNA transcripts (Figure 1.4.1) and PEA3 protein (Figure 1.4.2) levels are elevated in Ras61L and NeuNT transformed mouse embryo fibroblasts as compared to their untransformed counterparts. Correspondingly, Ras also induces the overexpression of c-Jun protein in Ras transformed wildtype fibroblasts (Johnson et al., 1996; Pfarr et al., 1994). Moreover, it is also known that both the c-Jun and the PEA3 genes can positively upregulate their own protein products, thereby increasing the existing levels of these transcription factors even further. From these *in-vitro* studies, it appears that constitutive activation of Ras somehow upregulates the activity and overexpression of both PEA3 and c -Jun, as occurs in many tumours *in-vivo*. Her2 stimulation of the Ras pathway is known to activate downstream mitogen-activated protein kinase (MAPK) cascades, which to consequently effects the increased activity of PEA3 subfamily proteins in tumours (Janknecht, 1996; Janknecht et al., 1996). Additionally, previous studies showed that PEA3 could be specifically phosphorylated by the ERK1 and JNK MAP kinases both *in-vitro* and *in-vivo* (Benz et al., 1997; Perron and Tozer, unpublished). However, it is not known if these kinases are physiologically relevant in regulating PEA3 activity; nor is it clear whether phosphorylation is required for the activity changes that result from activation of the Ras pathway. One mechanism that may account for increased PEA3 activity and expression is that the activation of Ras and Neu could effect the activation of a series of transcription factors that then target the PEA3 gene for regulation by interacting with certain binding sites in the PEA3 promoter.

In this regard, there are a number of highly conserved AP-1 binding sites in the PEA3 promoter region from four different species (humans, mice, chickens, and fish), in addition to Ets binding sites in the mouse and human promoters (Kann, unpublished). Both ETS and AP-1/ATF families of transcription factors are known to be key mediators of Ras; inhibition of either AP-1 or Ets activation successfully blocks Ras mediated cellular transformation (Lloyd et al., 1991; Granger-Schnarr et al., 1992; Langer et al., 1992; Wasylyk et al., 1994; Gum et al., 1996). In addition, transfection studies showed that these two sites were responsible for conferring Ras responsiveness (Yang et al., 1996). Analyses previously done on the HB-EGF promoter for example, also identifies these sites as being regulated by Ras in addition to a downstream effector of this pathway, Raf (McCarthy et al., 1997). Lastly, the importance and requirement of increased AP-1 DNA binding activity was characterized in the transformation process, as regulating a switch between protease dependent invasive phenotype from a urokinase plasminogen activator (uPA) to a cathepsin L (CL) dependent invasive phenotype (Smeal et al., 1991; Johnson et al., 1996; Galang et al., 1996; Janulis et al., 1999; Silberman et al., 1999; Behrens et al., 2000). Interestingly, the AP-1 and Ets-1 binding sites are positioned relative to the start site of transcription the PEA3 promoter. Hence, Ras activation during transformation may lead to increased protein activity of c-Jun that can interact with Fos family proteins and bind AP-1 sites as homodimers or heterodimers ultimately activating transcription of PEA3. Consistent with the premise requiring stable levels of PEA3 for transformation is the finding that co-infection of PEA3 with Ras or Neu is not sufficient to complement transformation in PEA3-null fibroblasts (Table

1.4.2). Furthermore, when clonal cell lines showing stable expression of PEA3 were isolated from PEA3-null fibroblasts (Figure 1.4.4 and 1.4.7), they demonstrate a restored capacity to become transformed by constitutively activated Ras and Neu (Table 1.4.3). Hence, PEA3 is a Ras/Neu effector and thus is required for Ras and Neu mediated transformation of mouse embryo fibroblasts.

1.5.2 Functional Redundancy of the PEA3 Subfamily During Transformation

PEA3, ERM, and ER81 share greater than 95% homology within their ETS DNA binding domains and an overall amino acid identity of 50% (deLaunoit et al., 1997). This suggests these proteins may serve redundant functions and therefore be capable of regulating overlapping sets of target genes. Interestingly, all PEA3-null fibroblast cell lines that are refractory to transformation show differing levels of ERM expression, with some lines also showing ER81 expression (Figure 1.4.1). Given that at least one other subfamily member is expressed in these PEA3-null fibroblast cell lines, it appears that neither ERM nor ER81 can compensate for loss of PEA3 in the transformation-defective phenotype of these fibroblasts. This observation may therefore be an indication that each of the PEA3 subfamily proteins has distinct roles. Studies involving *c-jun* show similar findings; *c-jun* like PEA3 is a member of a gene family, yet other *Jun* family members, JunB and JunD, do not functionally substitute for *c-jun* in transformation (Vandel et al., 1996). Low expression levels of ERM and ER81 in PEA3-null mouse embryo fibroblast cell lines may explain why these two proteins cannot compensate for lack of PEA3 in Ras and Neu mediated transformation. However, activated Ras and Neu transform two

variant PEA3-null fibroblast cell lines, MEF 110 and MEF 114. It was hypothesized that ERM and ER81 might be overexpressed in these two cell lines, thus accounting for transformation. For this reason, the levels of ERM and ER81 expression, in the two variant PEA3-null cell lines were compared to the other PEA3-null fibroblasts (n=9) that are refractory to transformation. Interestingly, neither ERM nor ER81 was overexpressed in either of these variant cell lines. Furthermore, there was no unique expression profile in comparison to the other MEF cell lines that are refractory to transformation (Figure 1.4.1). This further suggests these subfamily members are not compensating for loss of function mutations in PEA3 within these cells. In order to address this hypothesis, stable cell clones that overexpress ERM were generated in a PEA3-null cell line (Figure 1.7.1). In future, it will be imperative to test their ability to become transformed by activated Ras and Neu in focus formation assays. Lastly, the ability of MEF 110 and MEF 114 to become transformed may be due to genetic alterations in these cells, which might have occurred during the immortalization process. A second possibility is other *Ets* genes of related DNA binding specificity may be expressed at high levels in these two cell lines and this in turn may be compensating for loss of function mutations in PEA3. This hypothesis could be easily addressed by: (i) expressing dominant negative PEA3 in these cell lines to determine if it could block Ras or Neu mediated transformation and (ii) examining transcript levels of all known Ets genes to test for their overexpression in these cells.

1.5.3 PEA3 Target Genes

PEA3 is thought to be a critical mediator of transformation downstream of the Ras signalling pathway, functioning to regulate the expression of various target genes required in the transformation process. Several studies show PEA3 can bind and regulate the expression of various MMPs genes including MMP-1, MMP-3, MMP-7, and MMP-9 (Matrisian et al. 1994; Higashino et al., 1995; Crawford et al., 2001). Typically, the activity of MMPs is regulated by gene transcription, pro-enzyme activation and inhibition by tissue inhibitors of MMPs (TIMPs). Gene expression of MMPs is regulated by growth factors such as EGF and FGF, both of which modulate the activity of various transcription regulatory proteins acting on MMP promoters (Matrisian; 1994). Various MMPs promoters contain PEA3 binding sites closely associated to AP-1 binding sites. Co-operation between these two complexes is often required for transcriptional activation and upregulation of these target genes by various mitogens (Nerlov et al., 1992; Matrisian, 1994; Borden and Heller, 1997; Su et al., 2000). Similar findings were previously observed for c-Jun and c-Fos proteins (Logan et a., 1996). Several other studies also suggest MMPs are candidate PEA3 target genes. For example, forced expression of E1AF (human PEA3) in the non-metastatic human breast tumour cell line MCF-7 induces the expression of several MMPs and confers an invasive phenotype *in-vitro,* whereas transfection studies with antisense E1AF results in reduced mRNA transcripts and protein production of these MMPs (Hida et a., 1997). In addition, dominant negative PEA3 can block reporter gene expression from the MMP1 promoter, as well as invasion of MCF-7 cells induced by activated Neu (Kaya et al., 1996). Hence,

these findings suggest that PEA3 may indirectly affect the invasive and metastatic phenotype of many tumors by regulating the expression of MMPs.

In order to identify transformation specific PEA3 target genes, wildtype and PEA3-null fibroblasts were stimulated with FGF. This family of growth factors is involved in various biological processes from normal development and wound healing, to angiogenesis, tumour development and tumour progression (reviewed by Powers et al., 2000; Mason et al., 1994). FGFs bind specific receptor tyrosine kinases in the context of heparan-like glycosaminoglycans. Once bound, this induces receptor phosphorylation, dimerization and activation, ultimately triggering the activation of various signal transduction cascades and altering gene expression (reviewed by Powers et al., 2000). Importantly, the role of FGF was validated in transformation of 3T3 cells, as well as regulation of PEA3 and ERM expression in zebrafish and ER81 expression in xenopus (MacArthur et al., 1995; Kouhara et al., 1994; Roehl and Nusslein-Volhard, 2001; Raible and Brand, 2001; Munchberg et al., 1999). Expression of candidate PEA3 target genes were shown previously to be differentially expressed between wildtype and PEA3-null mouse embryo fibroblasts upon EGF stimulation (Xin, unpublished). Consistent with this finding is FGF8 stimulated expression of MMP-3 and MMP-9, whereby expression of these target genes is compromised in PEA3-null fibroblasts in comparison to their wildtype counterparts (Figure 1.4.8). Furthermore, expression of these target genes is rescued by ectopic expression of PEA3 in the PEA3-null fibroblasts, to levels similar or greater than those observed in the wildtype PEA3 fibroblasts. Therefore, expression of MMP-3 and MMP-9 appears to be dependent on PEA3. Two other Ras effectors, c-Jun

and c-fos, are also known to regulate the expression of several MMPs in a manner similar to PEA3. More specifically, c-Jun null fibroblasts do not express stromelysin-1, in contrast to wildtype c-Jun fibroblasts. In addition, c-fos null fibroblasts do not express stromelysin-1, nor do they express collagenase-1 after stimulation with PDGF and EGF (Johnson et al., 1996; Hu et al., 1994).

Ectopic expression of PEA3 in the PEA3-null cell line restores expression of MMP-3 and MMP-9. Unexpectedly however, there was no co-ordinate increase in PEA3 mRNA transcript levels in this cell line upon FGF8 stimulation. Expression of PEA3 from this clonal cell line is driven by the Moloney Murine Leukaemia virus (MoMuLV) LTR promoter sequences. Therefore, a possible explanation for the lack of PEA3 induction may be a result of this promoter being sensitive to serum starvation. Consistent with this hypothesis are the low levels of PEA3 that are present in the absence of serum without FGF stimulation. A second possibility is FGF8 may be acting indirectly to regulate gene expression from this promoter. For example, nuclear targets of this FGF pathway may affect the expression of other transcription factors that in turn induce PEA3 expression. To validate this theory, there are a series of binding sites for genes such as AP1, C/EBP, NFKB, and c-ets1 (MatInspector analysis; Quandt et al., 1995) found on this promoter. Furthermore, it is also possible that some residual PEA3 protein is present in the retransformant cell line following serum starvation. In this scenario, stimulation with FGF8 may lead to further increases in PEA3 protein activity, thereby resulting in activation of MMP-3 and MMP-9. Nonetheless, the two other PEA3 subfamily members, ERM and ER81 may also be functionally substituting for PEA3 in this cell

line; each of these two genes are induced in the retransformant and wild-type cell lines, but not in the PEA3-null fibroblast cell line.

1.5.4 Conclusions and Future Directions

PEA3 is an oncogenic effector of Ras and Neu mediated transformation of mouse embryonic fibroblast cell lines. PEA3 also regulates the transcription of two potential target genes, MMP-3 and MMP-9, which are thought to play pivotal roles in the transformation process. The overall mechanism of how and which pathways, besides the Ras pathway, regulate PEA3 expression, remain unclear. For this reason, it is essential to identify the Ras-response sequence elements and cognate DNA binding factors within the PEA3 gene. In addition, the use of other oncogenes such as constitutively activated *src, mek, erk, rac1, raf, v-jun,* and *c-fos* that lie in the Ras pathway would help to elucidate the position of PEA3 in this pathway. Furthermore, other oncogenes that lie outside the Ras pathway such as E2F1, *v-myc,* E1A, E1B, and SV40 for example, should also be tested for their ability to transform PEA3-null fibroblast cell lines. Lastly, it is important to determine whether the PEA3 subfamily members ERM and ER81, or another ETS transcription factors can substitute for loss of function mutations in PEA3 during the transformation process.

1.5.4 APPENDIX

1.7.1 Calculation of Virus Titers

Titers were calculated for each viral supernatant based on the average number of colonies obtained from serial dilutions used to infect Rat-1 cells. The average number of colonies obtained for duplicate samples for each viral dilution is summarized in the table below:

	No virus	10^{-2} dilution	10^{-3} dilution	10^{-4} dilution	10^{-5} dilution	Virus Titer
pBabe puro backbone	0	monolayer	208	33.5	3	6.2×10^5
pBabe puro Myc-PEA3	0	monolayer	152.5	34.5	3.5	6.3×10^5
pBabe puro Ras61L	0	monolayer	64	11	2	2.8×10^5
pBabe puro NeuNT	0	monolayer	48.5	11.5	1.5	2.3×10^5

Table 1.7.1: Calculation of virus titers for pBabe puro, pBabe puro Myc-PEA3, pBabe puro Ras61L, and pBabe puro NeuNT retroviral supernatants.

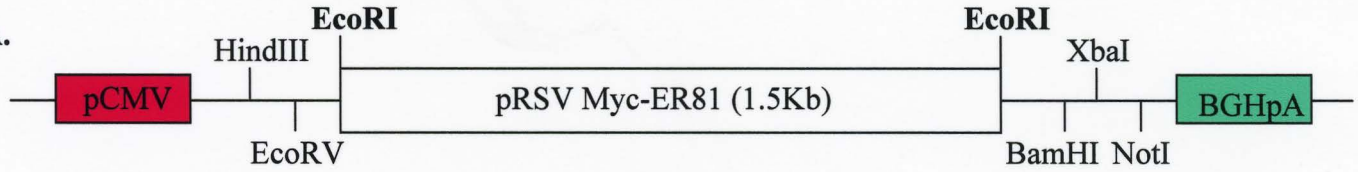
A sample calculation of how viral titer was calculated is shown below for pBabe puro: Each dilution (10^{-3} to 10^{-5}) produced 2.08×10^5, 3.45×10^5 and 3.5×10^5 focus forming units, respectively per 450µL of retroviral supernatant used for each infection.

- The average of the three dilutions is: 2.81×10^5 focus forming units
- Viral titer = (2.81×10^5 focus forming units) X (1000µL/1mL) X (1unit/450µL)

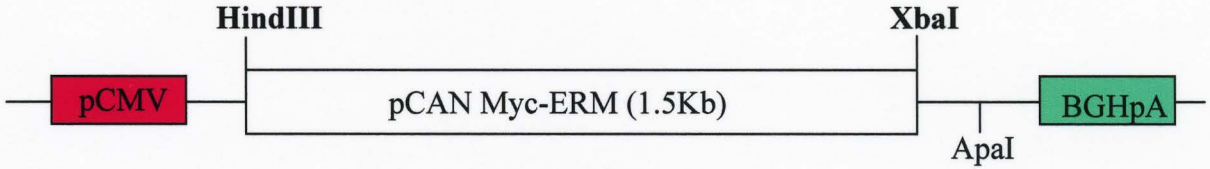
  = 6.2×10^5 focus forming units/mL.

Figure 1.7.1: Structure of vectors used in cloning pWB3-Myc-ERM and pWB3-Myc-ER81. (A) pRSV-Myc-ER81 was digested with EcoRI and cloned into the EcoRI site in the multiple cloning site of pWB3 (C). Orientation of the insert was verified by digesting with a BamHI restriction digest. (B) The Myc-ERM cDNA insert was PCR amplified from the plasmid pCAN-Myc-ERM with primers designed to have a 5'XhoI linker and a 3'SalI linker. The amplified Myc-ERM product was then purified and cloned into the XhoI/SalI sites of pWB3.

A.



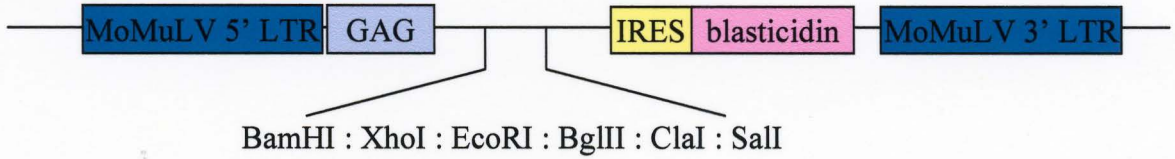
B.



FWD: 5' CCG CTC GAG ATG GAG CAG AAG CTG ATC TCC 3'
REV: 5' CCG GTC GAC TTA GTA AGC GAA GCC TTC G 3'

C.

pWB3 vector



CHAPTER 2
ROLE OF ER81 IN MAMMARY GLAND DEVELOPMENT

2.1 INTRODUCTION

2.1.1 The ER81 Transcription Factor

ER81 (Ets-Related 81) is a transcription factor that belongs to the PEA3 subfamily of Ets transcription factors. ER81 was initially identified by Brown and McKnight (1992) by screening a mouse embryo cDNA library using degenerate oligonucleotides designed to recognize conserved regions in the DNA binding domain of Ets related proteins. A human homolog of ER81, termed ETV1 (Ets translocation variant 1) was also identified by looking at Ewings sarcoma translocations (Jeon et al., 1995). A second homolog was subsequently identified while screening a human kidney cDNA library using a murine ER81 probe (Monte et al., 1995). ER81 shows 50% homology to PEA3 throughout the ORF, 95% in the ETS domain and 85% in the acidic domain.

ER81is expressed in a spatial and temporal pattern distinct from either PEA3 or ERM during mouse embryogenesis (Chotteau-Lelievre et al., 1997). Specifically, ER81 expression is not observed during early gastrulation events, unlike PEA3 and ERM. Later, at the onset of organogenesis however, ER81 is co-expressed with the other two PEA3 subfamily genes in many of the same tissues that express them into adulthood. Specifically, ER81 is ubiquitously expressed, primarily in mesenchymal compartments, as compared to PEA3 and ERM, which are preferentially expressed in epithelial cells (Chotteau-Lelievre et al., 1997; Laing and Hassell, unpublished). Expression is also found in cells having neural crest origin, such as jaw, tongue, and tracheal cartilage

(Chotteau-Lelievre et al., 1997). Lastly, ER81 mRNA expression is highest in brain, kidney, lung, low in spleen, intestine, thymus, and devoid in liver (Brown and McKnight, 1992). ER81-null mice are viable, however they are severely runted, suffer from limb paralysis due to flexor-extensor posturing of the limbs, and exhibit postnatal lethality at approximately 4 weeks of age probably due to neurological defects (S. Arber, personal communication; Bartel et al., 2000).

Mouse ER81 is a nuclear protein that comprises 477 amino acids, bears an 85 amino acid ETS domain near its carboxyl terminus and two strong activation domains; one at the amino terminus, the other at the carboxy terminus (Janknecht, 1996). ER81 binds DNA with specificity and functions as a transcriptional regulatory protein (Brown and McKnight, 1992). Furthermore, existing studies show both DNA binding and ER81 transcriptional activity is subject to elaborate negative control, implying that mechanisms exist to regulate ER81 activity. In these studies, a series of unidirectional amino and C-terminal mutants of ER81 and GAL4-ER81 chimeras reveal there exists two negative regulatory regions flanking the activation domain, which independently repress ER81 activity, in addition to two other regulatory modules that flank the ETS domain (Janknecht, 1996). In this respect, studies by Janknecht (2001) have shown that MAPKAP kinase 2 (MK2), an important intracellular mediator of stress, signals to regulate ER81 activity. In particular, MK2 can regulate ER81 activity in a manner dependent as well as independent of phosphorylating ER81 within its inhibitory domain, on serine residues 191 and 216. Lastly, ER81 is a target of the Ras/Raf/Mek/Erk signalling pathway (Janknecht, 1996; and Janknecht et al., 1996). Specifically, ER81 is

able to bind the ETS binding site of E74 and activate transcription upon stimulation of this pathway. Also, overexpression of oncogenic Ras, leading to *in-vivo* activation of ERK1, or constitutively activated MEK or Raf1, each result in an increase of ER81 mediated transcription. *In-vitro* studies support this theory as ER81 is phosphorylated by ERK1 at amino acids 63 to 182 within the amino terminus (Janknecht, 1996; Papoutsopoulou et al., 2000).

2.1.2 ER81 and Tumorigenesis

ER81 is thought to play a role in tumorigenesis. Initial studies of Ewing's sarcoma resulting from a t(7;22)(p22;q12) chromosomal translocation between EWS and ER81 synthesize a hyperactive fusion protein analogous to fusions produced by EWS-Fli1 and EWS-ERG translocations, which are thought to trigger cellular transformation. Furthermore, it is thought that abnormal regulation of ER81 target genes due to increased expression of these downstream targets is critical for cancer development (Jeon et al., 1995). Consistent with this hypothesis are studies showing ER81 is highly expressed in association with proliferation and migration events. Specifically, ER81 mRNA is found at high levels in certain tumour cell lines including tetrocarcinoma cell lines, prostate adenocarcinoma cell lines (Janknecht, 1996). In addition, ER81 is also overexpressed in HER 2 / Neu mouse mammary gland tumours (Shepherd et al., 2001) and several breast cancer cell lines (Chotteau-Lelievre et al., 1997; Janknecht et al., 1996; Baert et al., 1997) by comparison to other *Ets* genes, which are expressed in the normal mammary gland, but not overexpressed in tumours. Furthermore, it is thought that these high transcript

levels are due to increased transcription of the ER81 gene or increased stability of its mRNA, but not a result of gene amplification.

2.1.3 Mammary Gland Development

Development of the mouse mammary gland is initiated in the embryo, however the major part of development in this organ takes place during postnatal life. Mammary gland development begins between embryonic day 10 and 11 of gestation. At this time, the enlargement of a raised ridge of single ectodermal cells called the mammary streak forms, which runs along the mid-ventral body wall of the embryo, from the caudal base of the forelimb to the rostral base of the hindlimb (Sakakura, 1987). By embryonic day 12, these epidermal cells migrate to different positions and a rudimentary mammary anlage is formed, consisting of 5 pairs of glands. Each primordial gland forms a lens-shaped structure at 12 days and then develops into a "light-bulb" shaped structure, by day 14, composed of several layers of epidermal cells. Sexual differentiation of the gonads normally occurs by embryonic day 14 thereby determining the sexual phenotype of the mammary gland; in male embryos, testosterone induces the regression of the mammary buds, whereas in female embryos, the absence of the androgen allows the epithelial cells within the bud to enter a slow proliferative state. The mammary bud then undergoes a resting phase between embryonic days 11 to 16 where there is very little differentiation. After this stage, the buds enter a rapid phase of proliferation, leading to the formation of mammary sprouts, which in turn elongate into the underlying fatty stroma, giving rise to the primary ducts present by birth. This process is initiated and maintained by inductive

signals passing between the epithelial cells that form the ducts and the surrounding stroma that later form the mammary fat pad (reviewed by Sakakura, 1991).

Shortly after birth, each mammary gland averages 15-20 branched ducts, with no progressive development in morphology occurring until the stages of sexual maturation, between four and six weeks of age. At the onset of puberty, systemic hormones and locally acting growth factors accelerate ductal growth from structures called terminal end buds (TEBs). During puberty, TEBs initiate both ductal elongation and ramification, leading to the formation of primary and secondary branching structures with each estrus cycle (Daniel and Silberstein, 1987). At eight to twelve weeks of age, the mammary ducts reach the edge of the fat pad, the TEBs regress and the period of accelerated growth ceases. Functional differentiation of the mammary gland takes place during pregnancy. At this time, extensive lateral branching increases the size of the mammary ductal tree and lobulo-alveolar growth gives rise to specialized secretary epithelial cells. Production of milk proteins such as β-casein and WAP begin during mid-pregnancy in the alveoli where milk droplets are retained and released into the lumen during lactation and up to one to two days post-weaning (Daniel and Silberstein, 1987). Alveoli are composed of alveolar cells and basket like myoepithelial cells. These cells function to synthesize and squeeze the milk into the ducts, respectively. After weaning, accumulation of milk in alveoli and ducts signals the involution process. During involution, extensive remodelling of the mammary gland occurs due to decreased levels of prolactin (Traver et al., 1996), thereby reducing milk production levels and restoring the mammary gland to a virgin-like state (Lund et al., 1996; Walker et al., 1989). During this time, apoptosis of

the endothelial cells and milk synthesizing cells occur as a result of increased MMP activity and excess milk is secreted by myoepithelial cell contractions in response to oxytocin

2.1.4 Cell Populations During Mammary Gland Development

The mammary gland is made up of various cell populations; some cells being terminally differentiated, together with a subset of stem cells having the ability to differentiate into other cell types. Mammary ducts for example, comprise two differentiated cell layers: an inner lining of cuboidal luminal epithelial cells and a discontinuous outer lining of myoepithelial cells. TEBs, the structures responsible for elongation and increased number of mammary ducts, are large sac-like structures that consist of various cell types (Neville et al., 1987). These include an undifferentiated cap cell layer lining the distal tip of the TEB, a cuboidal epithelium of body cells residing below the cap cell layer, a luminal epithelial cell layer lining the lumen of the subtending duct and a myoepithelial cell layer on the periphery of the subtending duct at the proximal region of the TEB. Alveoli are found during pregnancy at the ends of ducts. These structures contain a network of differentiated myoepithelial cells on the outer surface, encasing a single layer of secretory luminal epithelial cells facing the lumen. The mammary epithelium is also thought to contain a subpopulation of pluripotent stem cells; inferred from transplant studies whereby a fully differentiated normal mammary tree containing branching structures and alveoli, which is capable of lactating, could be reconstituted from a single cell (DeOme et al., 1959; and Kordon and Smith, 1998).

Specifically, it is thought that there is a stem cell population enriched in advancing edge of TEBs, presumably within the cap cell layer (Williams and Daniel, 1983). However, other stem cells have been identified in the embryonic and adult mammary gland at various stages of mammary gland development (Daniel and Silberstein, 1987). Initially, stem cells were believed to reside within the myoepithelial cell layer, however recent studies have shown that a subset of luminal epithelial cells are capable of differentiating into myoepithelial cells, thereby suggesting these luminal cells may contain precursors of myoepithelial cells (Pechoux et al., 1999). Nonetheless, these stem cells are of specific interest to development as well as cancer biology, in part because they are likely the cells of origin of mammary carcinomas (Ruso et al., 1979).

2.1.5 Experimental Rationale

Members of the PEA3 subfamily share 95% amino acid identity within their ETS DNA binding domain and 50% amino acid identity overall (de Launoit et al., 1997), therefore implying these proteins may serve redundant functions. Breast cancer results from alterations that affect or disrupt the normal function of proteins. Like PEA3, ER81 is expressed in the embryonic mammary gland and co-ordinately expressed during periods of increased proliferation during postnatal mammary gland development. In addition, ER81 is similarly overexpressed in HER 2 / Neu mammary gland tumours in mice (Shepherd et al., 2001), and several breast cancer cell lines (Chotteau-Lelievre et al., 1997; Janknecht et al., 1996; Baert et al., 1997). Lastly, targeted disruption of PEA3 results in a reduced ductal branching phenotype during mammary gland development.

These observations therefore raise the possibility that ER81 may also fulfil similar roles in normal mammary gland development and neoplasia. In order to explore this possibility, ER81 was targeted for disruption in the mouse germ line in collaboration with Dr. Tom Jessell.

In order to understand the possible role of ER81 in embryonic and postnatal mammary gland development, the cell-type expression of ER81 was examined using mice carrying a β-galactosidase marker driven by the ER81 promoter. The consequences of loss-of-function mutations in the ER81 gene were also assessed using wholemount and histological analyses on postnatal mammary gland development. However, inactivation of both ER81 alleles results in death of these mice at approximately 4 weeks of age, hence precluding their analysis beyond this time. Heterozygotes on the other hand are viable and healthy; hence the analysis of their mammary glands presented no problems and was therefore pursued. In this regard, it is important to note that PEA3 heterozygous mice possess a branching phenotype that is intermediate in nature between wildtype and PEA3-null littermates. Therefore, it may be possible to discern a phenotype using heterozygous ER81 animals.

2.2 OBJECTIVES

1. Determine the cellular expression profile of ER81 at various stages (embryogenesis, puberty, pregnancy, and involution) of mammary gland development.

2. Assess the consequences of loss of function mutations in the ER81 subfamily gene on mammary gland development.

3. Introgress ER81-heterozygous mice into FVB/n strain background for future studies.

2.3 MATERIALS AND METHODS

2.3.1 Embryonic Stem Cell Derived Mice

Two lines of ER81 targeted knockout mice were generated in Dr. Jessell's Lab, Columbia University, New York, New York. The mice were generated in the C57/Sv129 mixed strain background. The first line of mice was engineered to carry a targeted interruption of exon 11. Specifically, these mice contained an IRES-Tau-LacZ gene in addition to a PGK-Neo cassette at this site, thereby disrupting part of the ETS domain. A second line of mice was generated because the LacZ gene in this original construct did not get expressed. The second line of mice contained an exon 2 knockin of a nuclear localized (NLS) LacZ construct, in addition to a PGK-Neo cassette at the ER81 ATG start site. The structures of the targeted alleles are shown in Figure 2.4.1.

2.3.2 Collection of Adult Mice and Embryos

Mice were weaned and genotyped at 3 weeks of age. Matings were set up once the mice reached 6 weeks of age. Virgin mice were collected at various timepoints after their date of birth. Mice used for pregnant time points were set up in the evening and pregnancy assessed the following morning by the presence of a vaginal plug. The presence of a vaginal plug was considered day 0-0.5 of pregnancy. Lactating glands were collected one to two hours after removal of the pups to allow the glands to accumulate milk. For involuting timepoints, mice were allowed to lactate for 14days and then were separated from their pups for 2, 5 or 7 days. All mice required for experimentation were euthanized by cervical dislocation.

For embryonic timepoints, timed pregnancies were set up overnight and embryos collected based on the presence of vaginal plugs. Embryos were dissected from the uterus and extraembryonic tissues. The yolk sac surrounding the embryo was collected for DNA preparation used in genotyping by the same methods described for tail clips (section 2.3.3).

2.3.3 Preparation of Genomic Tail DNA

Animals were ear tagged with a designated number and one centimetre piece of tail cut for genotyping. Tails were digested overnight at 56°C with 100ug/ml Proteinase K in 500µL tail lysis buffer (0.2% SDS, 100mM Tris, pH 8, 200mM NaCl, 5mM EDTA). An equal volume of phenol:chloroform:iso-amyl alcohol (25:24:1) was added to each tail followed by vortexing for one minute. The aqueous layer was separated from the organic phase by centrifugation at 13K rpm for 15 minutes. The aqueous phase was removed to a new tube and the DNA precipitated using 500µL of isopropanol. The DNA was then spooled using a heat sealed glass pipette, washed in 70% followed by 100% ethanol immersion, resuspended in 50µL TE and stored at 4°C.

2.3.4 Genotyping of Animals by PCR

Genotyping by PCR was performed on embryos for studies on embryonic mammary gland development and generating mouse embryo fibroblast cell lines. Different primers were used for each of the targeted alleles. For the original line containing the exon 11 mutation, the primers used were: 5'ATT TCA TTG CCT GGA

CTG GAC GAG3' and 3'TCA CTC ACA GAA TGT TGT CTC TCC5'. Primers for the exon 2 mutation were: 5'GGG CTG TCG AGG GTA ATT AGC TAT3' and 3'CTC CGT CTC CTC TTG CAC TCA TCG5'. Genomic tail DNA was diluted 1:10 with water. The diluted DNA was mixed with 14.8μL of ER81 primer PCR mix (1XPCR buffer, 3mM $MgCL_2$, 0.4mM dNTP, 0.4μM primers) and 0.2μL of Taq DNA polymerase (Gibco BRL). Samples were then denatured at 95°C for 30 seconds, annealed at 60°C for 40 seconds and extended at 72°C for 40 seconds in a total of 35 cycles. The PCR reaction was run on a 1% agarose gel. The absence of a 500bp product indicated a knockout animal. In order to identify heterozygous or wild-type animals, a second PCR was conducted using neo primers: 5'CAC GCA GGT TCT CCG GCC3' and 3'CGG CGA GGG CTA AGC GTC5'. DNA for PCR was diluted 1:10 and mixed with 14.8μL of neo primer PCR mix (1XPCR buffer, 2mM $MgCL_2$, 0.3mM dNTP, 1μM primers), and 0.2μL of Taq DNA polymerase (Gibco BRL). These samples were denatured at 92°C for 30 seconds, annealed at 58°C for 30 seconds and extended at 72°C for one minute, for a total of 30 cycles. The absence or presence of a 700bp product depicted a wild type or heterozygous animal, respectively.

2.3.5 Synthesis of Radiolabelled Probes

The template used to probe ER81 southern blots corresponds to a 1Kb SmaI/BglII fragment of the neomycin cDNA. The DNA template was excised from the plasmid vector pMAM and separated on a 0.09% low melting point agarose gel. The expected 1Kb fragment was gel purified using Qiaquick gel extraction kit (Qiagen) as described in

the Qiagen protocol. Probes were synthesized by the random priming protocol of Feinberg and Vogelstein (1983) using 100ng of purified neomycin template. Random hexamers were obtained from Boehringer-Mannheim, deoxyribonucleotides from Pharmacia and [α-^{32}P] dCTP from Amersham. Labelling reactions were performed using the Large Fragment of DNA Polymerase I (Gibco-BRL). Probes were purified using ProbeQuant™ G-50 micro columns (Amersham) as suggested by the manufacturer and boiled for 5 minutes prior to being used in overnight hybridization.

2.3.6 Southern Blot Analysis

Southern blots were utilised to distinguish between wildtype and heterozygous adult mice whereas knockout animals were distinguished by their severely runted appearance. 10µL of genomic tail DNA was digested overnight with 50U high concentration BamHI (Gibco BRL) restriction enzyme. Digests were run on a 1% agarose gel for a few hours at 100V. The gel was then denatured (1.5M NaCl, 0.5M NaOH) for 45 minutes and neutralized (1M Tris-Cl, 1M NaCl) for an additional 45 minutes, prior to being transferred overnight onto a nylon membrane (GeneScreen). The following day, the DNA was fixed to the membrane using a UV crosslinker (Stratagene) and the blot hybridized to a Neomycin radiolabelled probe described above (section 2.3.6) at 65°C overnight. Membranes were then rinsed briefly in 2XSSC at room temperature, washed three times each in 2XSSC, 0.5% SDS at 65°C for 10 minutes and once for 45 minutes in 0.2XSSC, 0.5% SDS at 65°C. A 4Kb band on the southern is

representative of a heterozygous mouse, while the lack of a band indicates a wildtype animal.

2.3.7 B-galactosidase Activity Assay

Mammary glands and embryos isolated for β-galactosidase activity assay were fixed (2% paraformaldehyde, 0.25% glutaraldehyde, 10% 0.1M phosphate buffer, pH 7.4) at room temperature for one hour. Tissues were then washed twice, each for 30 minutes in solution A [0.01% Na-desoxycholate, 0.2% NP40 (Sigma), 10% 0.1M phosphate buffer, and 2mM $MgCl_2$ in 0.05M Na_2HPO_4 buffer]. The tissues were washed an additional two times for 30 minutes in the same buffer containing decreased Na-desoxycholate (0.001%). Mammary gland tissues and embryos were then incubated either 5 days at room temperature or overnight at 37°C, respectively in X-gal staining solution [2mg/mL X-gal (Gibco-BRL), 30mM $K_4Fe(CN)_6$, 30mM $K_3Fe(CN)_6$-H_2O, 2mM $MgCl_2$, 0.001% Na-desoxycholate, 0.02% NP-40] to assay for β-galactosidase activity. Tissues were subsequently washed twice in phosphate buffered saline (PBS) for one hour. Wildtype glands and embryos were stained to control for endogenous β-galactosidase activity. Tissues were then prepared for wholemount or histological analysis.

Mammary glands used for wholemount analysis were fixed overnight at 4°C. The glands were then defatted for 4 hours in acetone and dehydrated in 70% ethanol followed by 100% ethanol, each for an hour. Lastly, the glands were cleared in xylene for 4 hours and mounted on glass slides with Permount (Fisher Scientific).

Tissues and embryos used for histological analyses were embedded in paraffin and sectioned at 8μm by anatomical pathology, McMaster Hospital. Sections were placed on glass slides, dehydrated overnight at 37°C and excess paraffin removed by incubating the sections twice in 100% xylene for 7 minutes. Tissue sections were then rehydrated in 100% ethanol, 70% ethanol, followed by distilled water, each for 5 minutes and counterstained with eosin Y (Sigma) for 15 seconds. Sections were then dehydrated in 70% ethanol followed by 100% ethanol and cleared in xylene, each for 5 minutes, prior to being mounted with Permount.

2.3.8 Mammary Gland Wholemount and Histological Analysis via Hematoxylin Staining

Inguinal (#4) mammary glands were isolated, spread onto glass slides and air dried overnight. The glands were then defatted with overnight incubation in acetone and stained overnight in Harris' modified hematoxylin (Fisher Scientific). Excess stain was removed with several washes with 1% HCL in 70% ethanol until the epithelial component within the gland was seen as a sharp contrast to the light background of the fat pad. The stain was then fixed in 0.02% ammonium hydroxide for one minute. The glands were first dehydrated in 70% ethanol and subsequently in 100% ethanol, each for two hours and the tissues cleared by overnight exposure to 100% xylene. Mammary glands were subsequently mounted with a cover slip in Permount (Fisher Scientific), allowed to dry and photographed.

Contralateral inguinal mammary glands were isolated for histological studies, fixed overnight at 4°C in 4% paraformaldehyde (BDH Laboratory supplies) and transferred to 70% ethanol. The mammary glands were then embedded into paraffin wax and sectioned at 4μm by anatomical pathology, McMaster Hospital. Tissue sections were placed on glass slides and dehydrated overnight at room temperature. Excess wax was removed by incubating slides in 100% xylene for 5 minutes. The samples were rehydrated in 100% ethanol, followed by 95% and 75% ethanol, each for 5 minutes. Sections were stained in Harris' modified hematoxylin for 5 minutes, followed by a quick 30 second destaining in 1% HCL diluted in 70% ethanol. Sections were then counterstained in Eosin Y solution (0.25% eosin in 80% alcohol, 0.5% glacial acetic acid) for one minute and immersed into running tap water for 5 minutes to remove excess stain. The samples were then dehydrated in increasing alcohols (70-100%) each for 30 seconds, cleared in xylene for 5 minutes, mounted with Permount and photographed.

2.3.9 Isolating and Immortalizing Mouse Embryo Fibroblasts

Timed matings were set up between heterozygous mating pairs in the evening. Pregnancy was assessed the following morning by the presence of a vaginal plug and mouse embryo fibroblasts collected from 12.5 day old embryos. Embryos were dissected and individually placed into sterile 1X PBS. The maternal uterine lining was removed from each embryo, in addition to the embryonic sac, which was used for preparation of genomic DNA (section 2.3.3) and genotyping (section 2.3.4). Embryos were individually trypsinized in 5mLs of 1X trypsin (Gibco BRL) and minced into small pieces with a

sterile scalpel on 60mm dishes. Once trypsinized, 10mLs of DMEM supplemented with 10% calf serum was added to each embryo. Single cells were generated by passaging the minced embryo pieces through a glass pipette several times. The cells were then centrifuged at 1000 rpm for 5 minutes at room temperature. Cell pellets for each embryo were resuspended in 10mLs of complete media (DMEM supplemented with 10% calf serum, 1X pen/strep, 1X fungizone) and plated on a 100mm dishes. The cells were immortalised according to the same 3T3 protocol used to generate the PEA3 MEF cell lines (Todaro and Green, 1963) and frozen down at various passage states (appendix 2.7.1).

2.3.10 Backcrossing into FVB/n Background

A heterozygous ER81-NLS (SV129/C57) mouse was mated to an FVB mouse. The litter produced from this mating (generation 1) was genotyped and analysed for animals heterozygous for the ER81 allele. These mice were subsequently mated to other FVB animals in order to produce the second generation. The ER81 allele was introgressed into the FVB background by repeating the above screening and mating process for up to 10 generations.

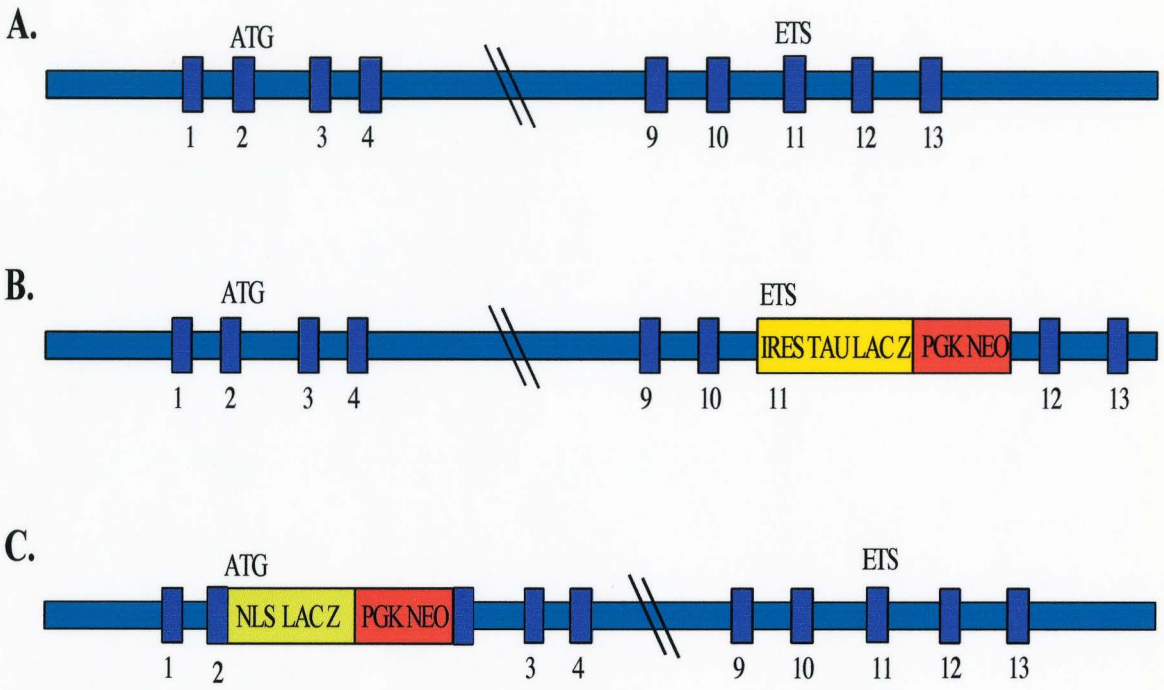
2.5. RESULTS

Multiple lines of ER81 knockout mice were isolated; two of these, were used in experiments outlined below. One line contained a disruption of the ER81 locus within sequences encoding the ETS domain, in which exon 11 contained an insertion of an IRES-Tau-LacZ construct and a PGK-Neo cassette (Figure 2.4.1, panel B). This line of mice expressed the Tau-β-galactosidase fusion protein at very low levels. However, since this line was readily available, it was employed to determine the consequences of loss-of-function mutations in the ER81 gene on mammary gland development using hematoxylin and eosin studies (section 2.4.3). A second disruption of the ER81 locus was made bearing an insertion of a nuclear localized LacZ and a PGK-Neo cassette in exon 2 at the ATG start site (Figure 2.4.1, panel C). Because NLS-β-galactosidase enzymatic activity was successfully directed from the ER81 promoter in this line of mice, they were employed in studies to determine the cellular expression pattern of ER81 during embryonic and postnatal mammary gland development (sections 2.4.1 and 2.4.2).

2.4.1 ER81 Expression During Embryonic Mammary Gland Development

Expression of ER81 during embryonic mammary gland development was assessed by setting up timed pregnancies between heterozygous ER81 mice, checking plugs the following morning, isolating embryos on daily intervals from E10.5-E15 and assaying the appropriate tissue for NLS-beta-galactosidase activity using X-Gal. ER81expression, as measured by beta-galactosidase activity, was first observed in the embryonic mammary gland at day 10.5 (Figure 2.4.2; panel A, arrowhead) when the

Figure 2.4.1: Structure of the ER81 locus disruptions. (A) The ER81 (C57/SV129) locus targeted for disruption. (B) The ETS domain was disrupted in the first line of mice generated. An IRES-Tau-LacZ construct and a PGK-Neo cassette were inserted into exon 11. This line of mice expressed the Tau-beta-galactosidase fusion protein at very low levels and was therefore used for wholemount analysis using hemotoxylin. (C) Second disruption of the ER81 locus, expressing a nuclear-beta-galactosidase fusion protein from the ER81 promoter. This line contained a nuclear localized (NLS) LacZ construct inserted into the ATG start site of exon 2, in addition to a PGK-Neo cassette. IRES = Internal ribosome entry site. PGK-Neo cassette mediates Neomycin resistance.

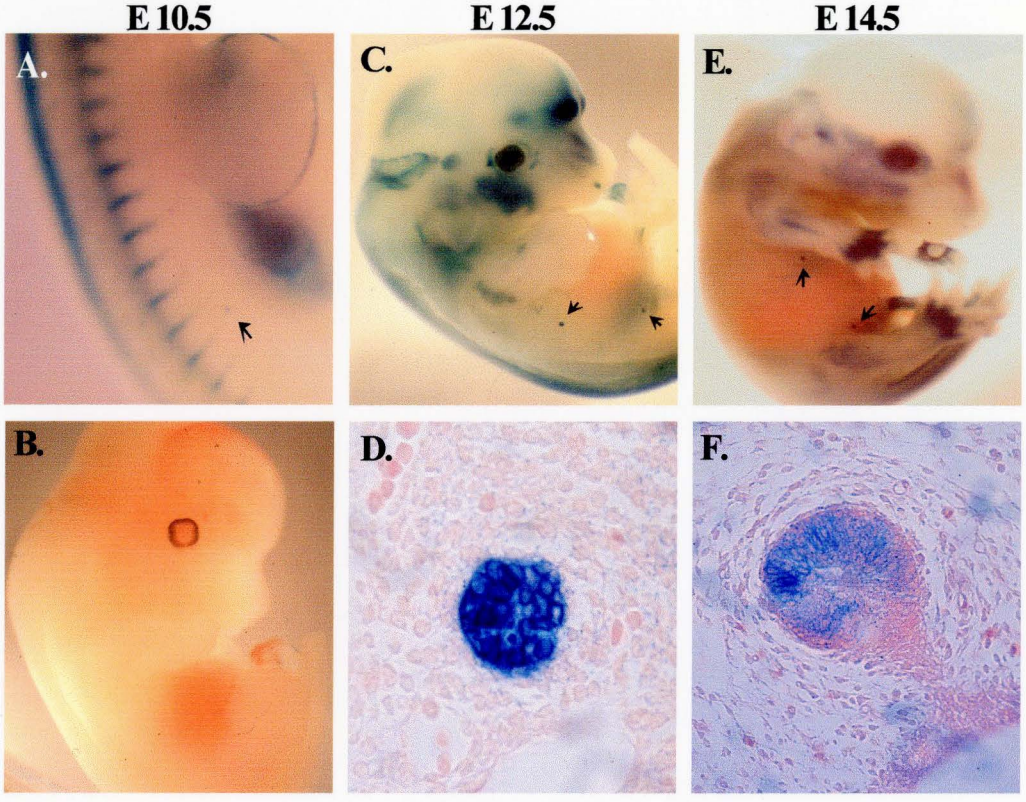

A.
ATG
ETS
1 2 3 4 9 10 11 12 13
B.
ATG
ETS
IRES TAU LAC Z
PGK NEO
1 2 3 4 9 10 11 12 13
C.
ATG
ETS
NLS LAC Z
PGK NEO
1 2 3 4 9 10 11 12 13

mammary gland first appears as a mammary bud (Henninghausen and Robinson, 2001). Expression continued in the mammary epithelium of all embryos until embryonic day 14.5 (Figure 2.4.2; panels C&E), at which time, expression was lost in approximately 50% of the littermates, corresponding to male embryos. Histological sections prepared along the longitudinal plane of each embryo revealed ER81 to be expressed in all the epithelial cells of the mammary bud (Figure 2.4.2; panels D&F). In addition, these histological sections facilitate the visualisation of morphological changes taking place during development of this gland; at E12.5, the gland has a round appearance, versus E14.5 where it is a bulb-shaped structure with a narrow neck, preparing for formation of the mammary sprout. No endogenous β-galactosidase activity was observed with any ER81 (+/+) embryos, as represented by the lack of beta-galactosidase activity in these embryos (Figure 2.4.2; panel B).

2.4.2 Expression Profile of ER81 During Postnatal Mammary Gland Development

Analysis of ER81 expression during postnatal mammary gland development was performed in ER81 heterozygous mice on both #4 mammary glands from each mouse; one gland was used for wholemount preparation, and the other for histological analysis. Expression of ER81 β-galactosidase activity was assayed by incubating the glands with X-gal. No endogenous β-galactosidase activity was observed in wildtype ER81 mammary glands, as represented by the lack of β-galactosidase activity in these glands

Figure 2.4.2: ER81 is expressed in epithelial cells during embryonic mammary gland development. Timed matings were set up and pregnancy assessed by the presence of vaginal plugs. Embryos were collected at various timepoints during embryonic development [E10.5 (A&B), E12.5 (C&D), and E15.5 (E&F)] and assayed for Beta-galactosidase activity. No endogenous Beta-galactosidase activity was observed in wild-type ER81 animals (B) as compared to heterozygous (A, E, F) and ER81-null (C&D) littermates. Embryos (C, E) were embedded in paraffin wax, sectioned longitudinally and counterstained with eosin (D, F). Original magnifications of embryos are: 10X (B, C, E), 40X (A), and 200X (D&F).


E 10.5
E 12.5
E 14.5
A.
C.
E.
B.
D.
F.

(Figure 2.4.3 panel A; and Figure 2.4.5 panel G).

Wholemount analyses show that ER81 is expressed throughout the entire ductal tree at various virgin timepoints (3, 5, 8, and 12wks) (Figure 2.4.3, panels B, D, F, H). During this time, there was little or no β-galactosidase activity in the ducts near the nipple area at 3weeks of age (Figure 2.4.3; panel B); expression was highest in the proliferating TEBs during puberty (Figures 2.4.3 panel B, arrows; and Figure 2.4.4 panels B&C), and begins to decline with sexual maturity. Histological analysis revealed that ER81 was expressed in body cells and highly proliferative cap cells of TEBs (Figure 2.4.4; panels D&E), in addition to cuboidal luminal epithelial cells of ducts (Figure 2.4.3; panels C, E, G (lc), I). At higher magnifications, it appeared that myoepithelial cells, located on the periphery of the ducts also showed some variable expression of ER81. This observation was likely a result of the estrous cycle (Figure 2.4.3; panel G, mc).

During pregnancy, ER81 was expressed throughout the entire ductal tree, as well as in alveolar structures (Figure 2.4.5; panels A, C, E). Histological analysis during this time revealed that ER81 was expressed in myoepithelial cells, but not luminal epithelial cells (Figure 2.4.5; panels B, D, F). This contrasts with the findings during puberty in non-pregnant females, which revealed that both cell types expressed ER81. Interestingly, expression in the ductal tree is lost during involution (Figure 2.4.4, panel H, arrows) and appears restricted to the regressing alveoli, as confirmed by histological analyses (Figure 2.4.5; panel I).

Figure 2.4.3: Beta-galactosidase activity during virgin mammary gland development of ER81-NLS-LacZ mice. Mammary glands were isolated from ER81-null (B&C) and heterozygous mice (D-I) at various stages of virgin mammary gland development (3, 5, 8, 12 weeks) and assayed for Beta-galactosidase activity. Contralateral glands were stained, sectioned and couterstained with eosin (C, E, G, I). At three weeks of age (B), staining is observed predominantly in the terminal end buds (arrows), with no staining present within the ductal tree in the area closest to the nipple (np). Between 5-12 weeks (B, D, F, H), ER81 expression is observed throughout the entire ductal tree. ER81 expression in ducts is located primarily within luminal epithelial cells (lc), found juxtaposed to the lumen (C, E, G, I), however, at higher magnifications, it appears that myoepithelial cells (mc) on the periphery of the ducts also express ER81 (G). No endogenous beta-galactosidase activity was detected in wildtype animals (A). Original magnifications are: 25X (A&B), 50X (D, F, H), 100X (C, E, I), and 400X (G).

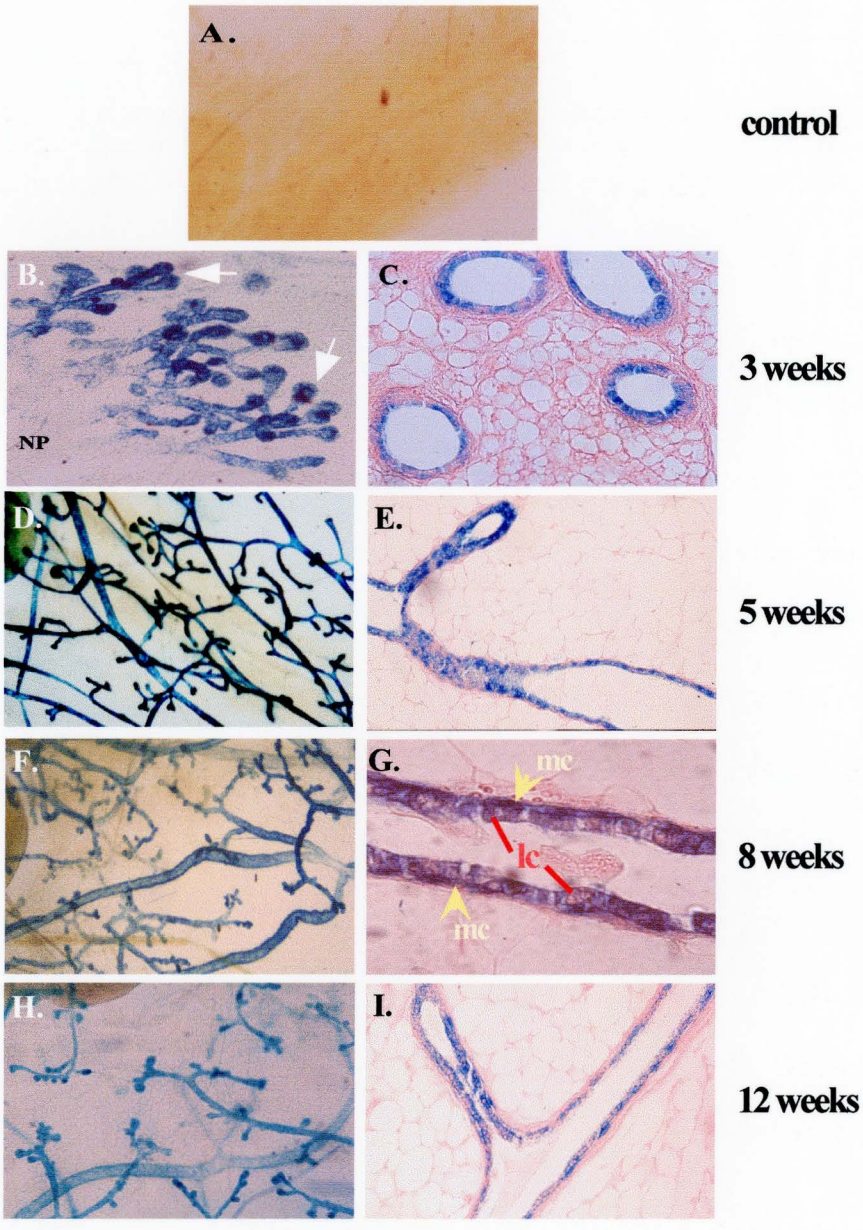

A.
control
B.
NP
C.
3 weeks
D.
E.
5 weeks
F.
G.
mc
lc
mc
8 weeks
H.
I.
12 weeks

Figure 2.4.4: Beta-galactosidase activity within terminal end buds of ER81-NLS-LacZ heterozygous females at 5 weeks of age. Inguinal and corresponding contralateral mammary glands were isolated and stained with X-gal to assay for beta-galactosidase activity. The inguinal mammary gland was prepared as a wholemount (B&C), and the contralateral gland was sectioned and counterstained with eosin (D&E). A diagrammatic representation of a terminal end bud structure taken from Daniel and Silberstein (1987) (A) depicts a single layer of cap cells (cp) on the periphery of the bud, large cuboidal body cells (bc) behind the cap cell layer, flattened myoepithelial cells (mc) in the subtending duct and a basal lamina (bl) which lines the entire structure. ER81 expression is observed in the terminal end buds in wholemount analysis (B&C), in cells corresponding to cap and body cells as depicted in histological sections (D&E). Original magnifications are: 50X (B&C), and 400X (D&E).

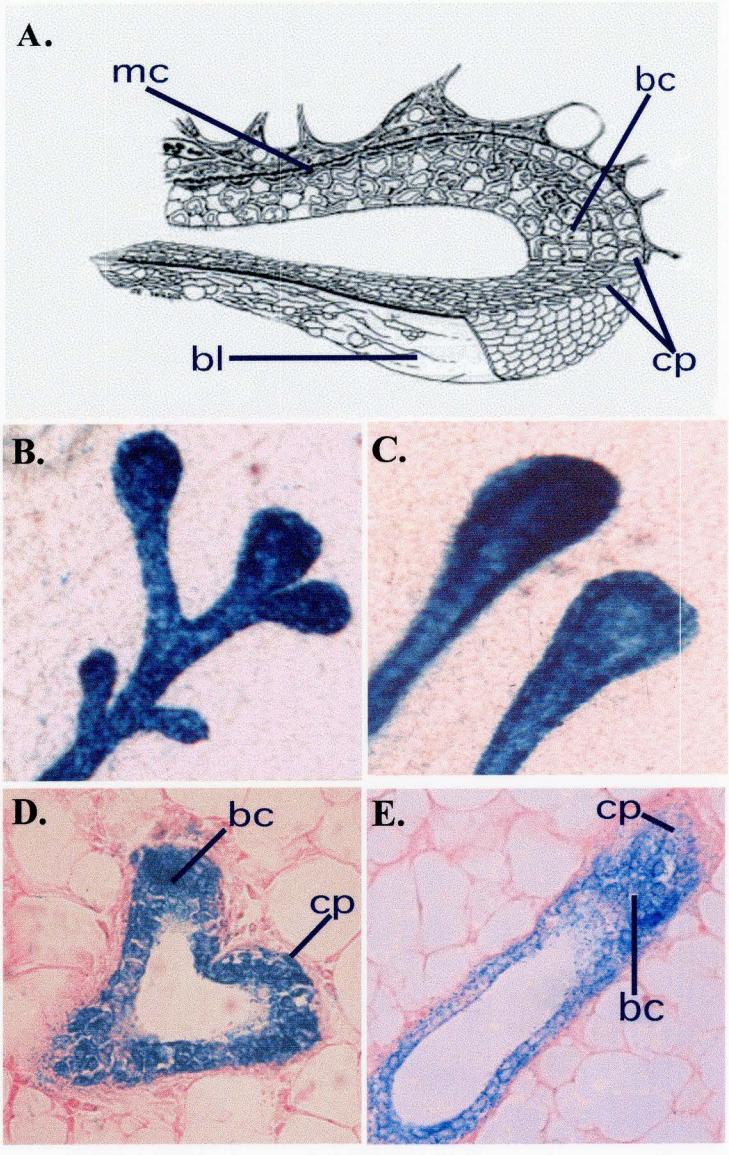
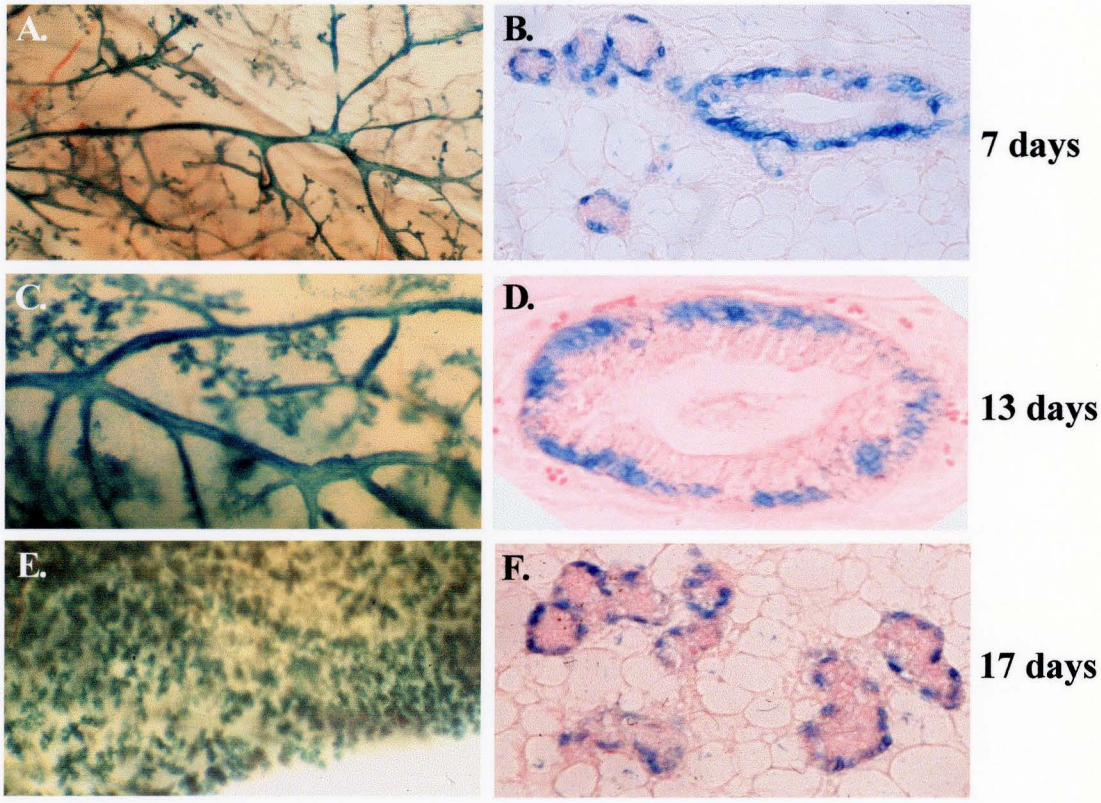
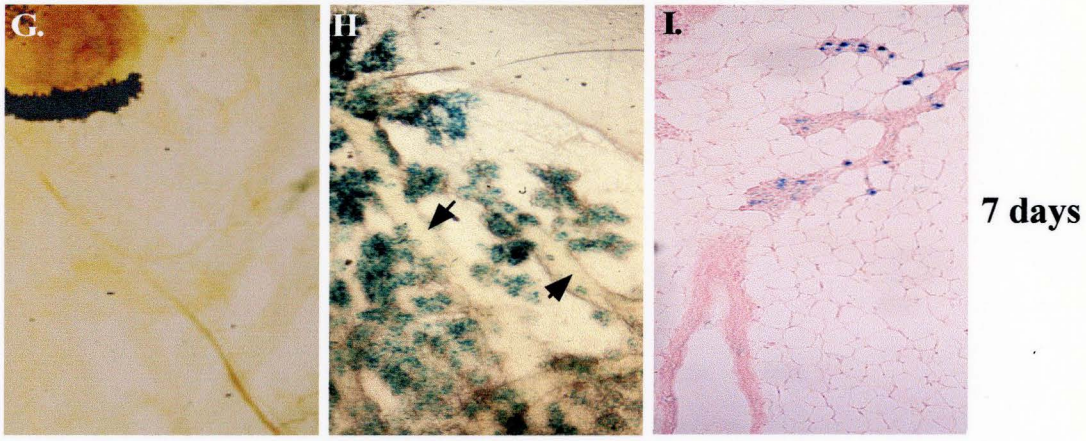

A.
mc
bc
bl
cp
B.
C.
D.
bc
cp
E.
cp
bc

Figure 2.4.5: Beta-galactosidase activity during various stages of pregnancy and involution in ER81-NLS-Lac Z heterozygous (A-F, H&I) females. Staining is observed throughout the mammary tree at 7 days (A&B), 13 days (C&D), and 17 days (E&F) of pregnancy. Ductal expression of ER81 is lost by 7 days of involution (H&I, arrows). At a cellular level, Beta-galactosidase activity is observed in myoepithelial cells of the ducts at 7 and 10 days (B&D) of pregnancy, surrounding the alveoli at 7 and 17 days of pregnancy (B&F), and in regressing alveoli during involution (I). No endogenous Beta-galactosidase activity was observed in wildtype animals (G). Original magnifications are: 25X (H), 64X (C&G), 100X (A, E, I), 200X (E), and 400X (B, D, F).

Pregnancy



Involution

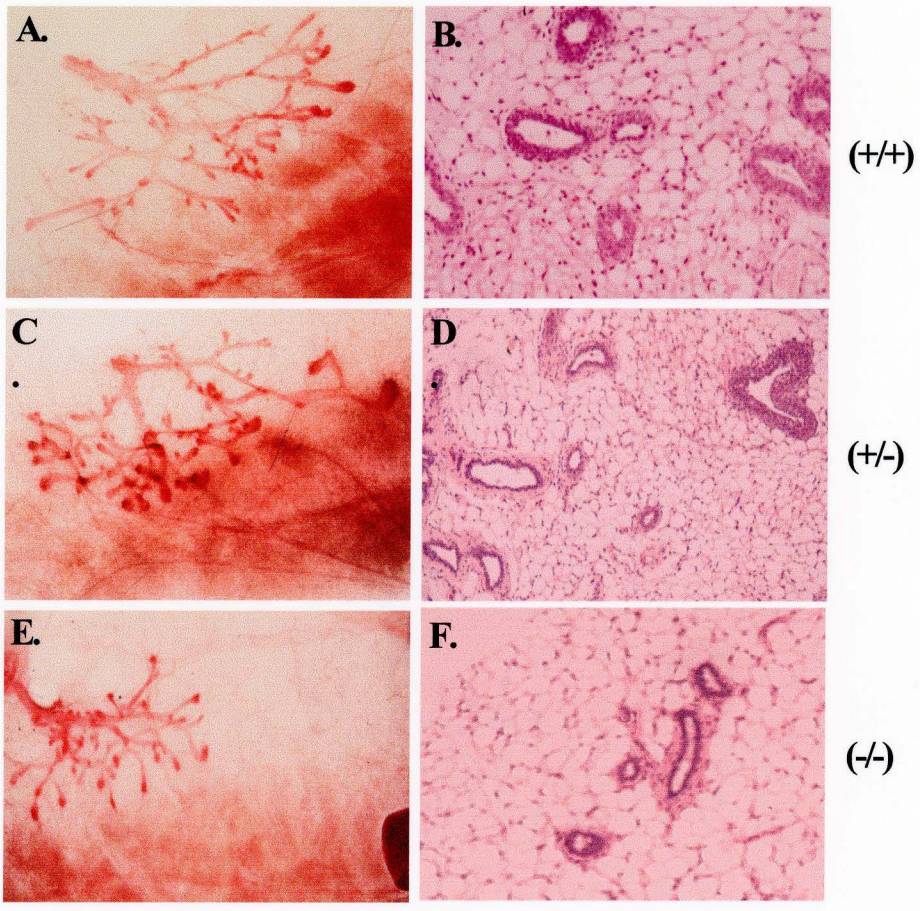


2.4.3 Role of ER81 in Mammary Gland Development

To address the consequences of loss-of-function mutations in the ER81 gene on mammary gland development, wholemounts and histological tissue sections were prepared by hematoxylin staining techniques. ER81-null mice die by 4 weeks of age, therefore precluding studies of mammary gland development past this stage. As a result, comparisons were carried out between wildtype and heterozygous animals in order to identify any aberrations resulting in mammary organogenesis. In this regard, it is important to note that discerning a phenotype due to loss of one ER81 allele may be possible because it was shown that PEA3 heterozygous mice have a reduced ductal branching phenotype that is less severe than that of PEA3-null mice but readily discernable from wildtype mice.

Comparisons between wildtype, heterozygous and ER81-null sibling mammary glands were carried out at 3 weeks of age (Figure 2.4.6). As a consequence of their severely runted nature, it was noted that mammary glands from ER81-null mice were much smaller in size as compared to their littermates. As illustrated in Figure 2.4.6, where each wholemount was photographed at the same magnification, only the ductal tree and nipple area was observed in wildtype (panel A) and heterozygous (panel B) mice. In contrast, the entire mammary gland of ER81-null mice was visible at the same magnification, with the lymphnode specifically positioned half way down the mammary gland (panel C) as a reference point. However, the shape of the mammary tree and the number of TEBs present in these mice does not appear to differ significantly from WT or heterozygous animals. Analysis of histological sections at this time did not reveal any

Figure 2.4.6: Hemotoxylin analysis of ER81 sibling mammary glands at 3 weeks of age. ER81 +/+ (A&B), +/- (C&D), and -/- (E&F) inguinal mammary glands were isolated, in addition to being stained with hemotoxylin for wholemount (A, C, E) and histological (B, D, F) analyses. Loss of function of one ER81 allele does not appear to affect the appearance of the mammary ductal tree (A&C, B&D). ER81-null animals have smaller mammary glands as a result of their smaller body size, but they do not display any noticeable phenotype in their ductal tree and ductal morphology (E&F) as compared to wildtype and heterozygous animals. Original magnifications are: 25X (A, C, E), 100X (B&D), and 200X (F).


A.
B.
(+/+)
C
.
D
.
(+/-)
E.
F.
(-/-)

noticeable differences in duct morphology or in the surrounding fat pad of these animals (Figure 2.4.6; panels B, D, F).

Only heterozygous and wildtype animals were compared beyond this time point in development due to premature death of the ER81 knockout animals. No major differences were noted in comparisons between wildtype and heterozygous animals during various virgin timepoints (5, 8, 12 weeks) (figure 2.4.7). Both wildtype and heterozygous animals developed TEB by 5 weeks of age with similar rates of growth near the lymphnode area (panels A&B). Throughout sexual maturation, both wildtype and heterozygous animals displayed normal primary (panels C&D) and lateral branching (panels E&F) thereby filling up the entire fat pad.

Mammary gland analyses were also carried out at various timepoints during pregnancy (7, 10, 17 days) (Figure 2.4.8); a time for added proliferation and differentiation of the mammary gland. No striking phenotypes resulted from the loss-of-function of a single ER81 allele. Both wildtype and heterozygous animals displayed normal lateral branching as displayed by the formation of tertiary and quaternary ductal branches, filling the fat pad in all dimensions (Figure 2.4.8; panels A-D). By late pregnancy, the formation of lobulo-alveolar structures were prevalent in both wildtype and heterozygous animals (Figure 2.4.8; panels E&F) with relatively the same abundance of these structures between both genotypes. Lastly, histological analyses of mammary glands during pregnancy revealed no abnormalities between wildtype and heterozygous mice. Specifically, no differences were noted in the surrounding fat pad, the

Figure 2.4.7: Wholemount analysis of ER81 mammary gland development during puberty. Inguinal mammary glands of wildtype (A, C, E) and heterozygous (B, D, F) ER81 mice were isolated at 5 (A&B), 8 (C&D) and 12 weeks (E&F) of age, and stained with hemotoxylin. Loss of function of a single ER81 allele does not appear to affect the development of the mammary ductal tree. Note the lateral branching (arrows) present in both wildtype and heterozygous animals at 12 weeks of age (E&F). Original magnifications are: 25X (C&D), and 50X (A&B, E&F).

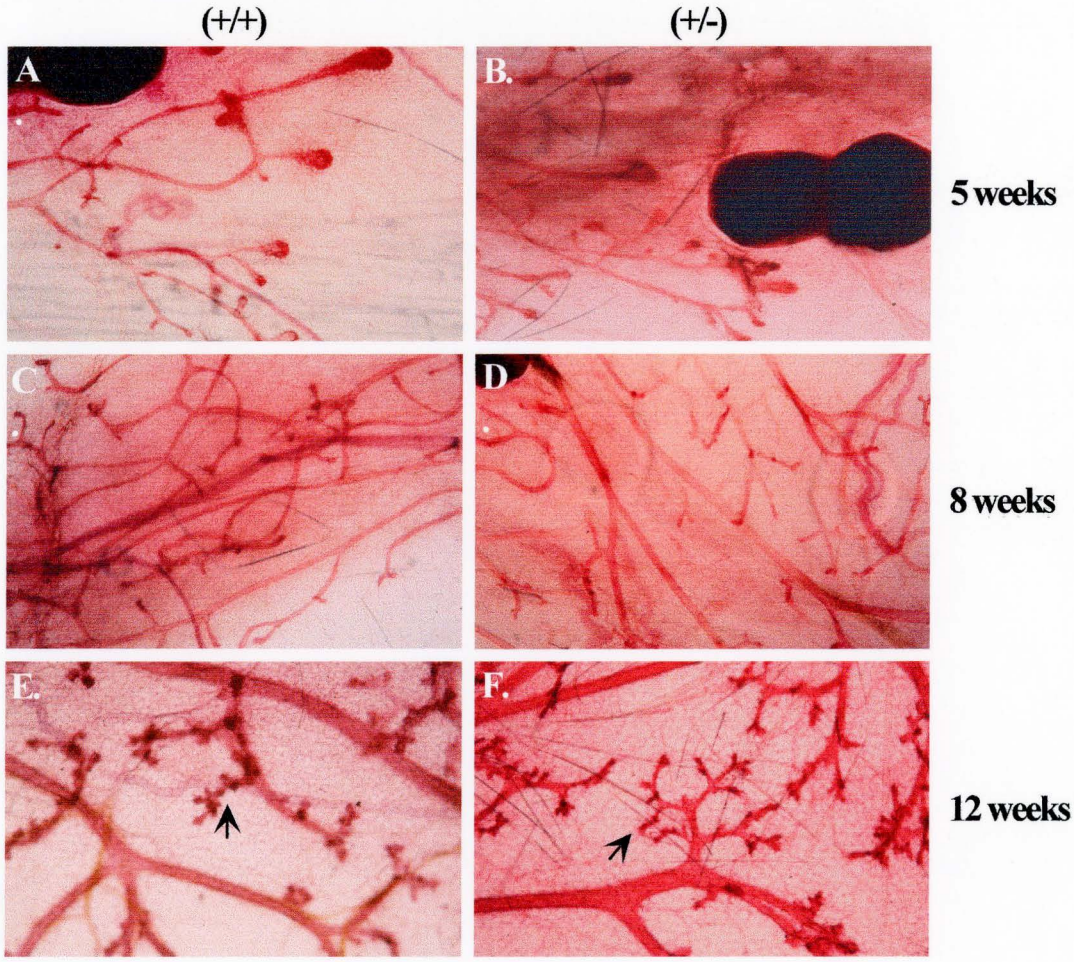
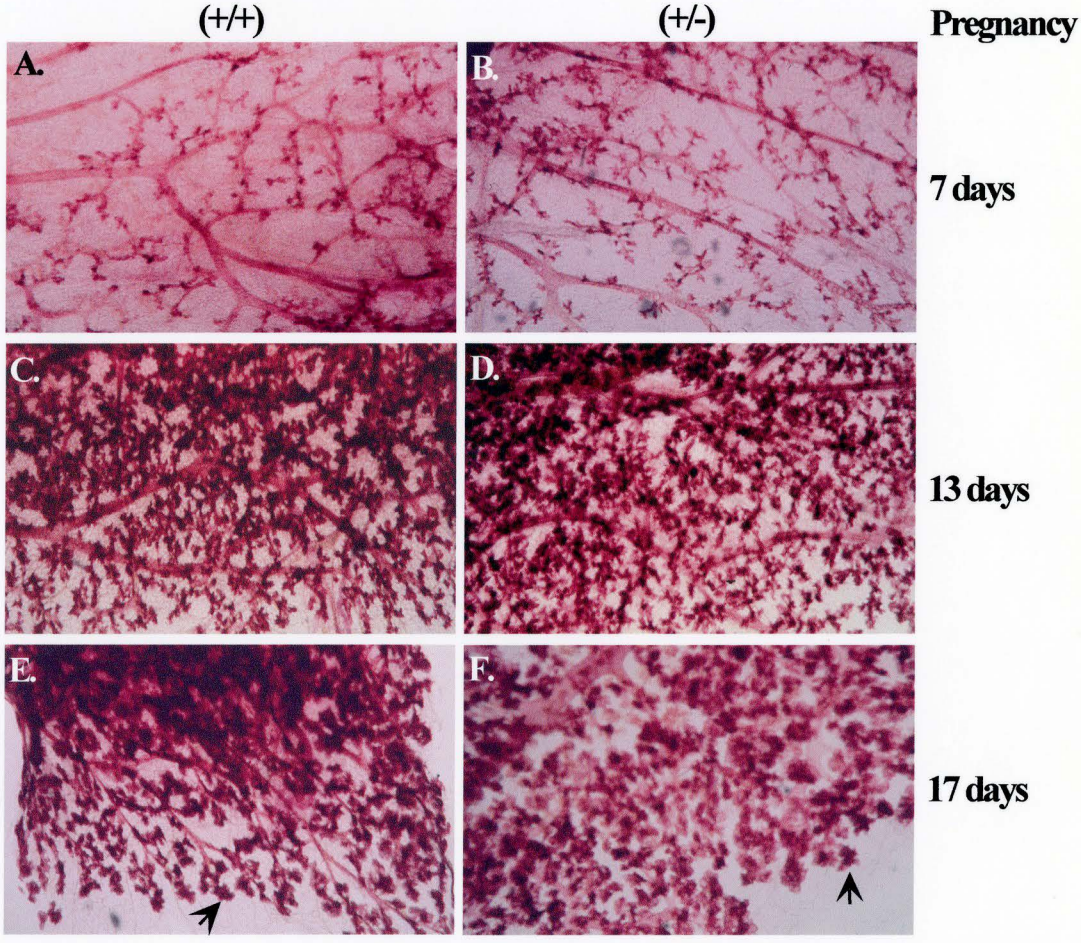
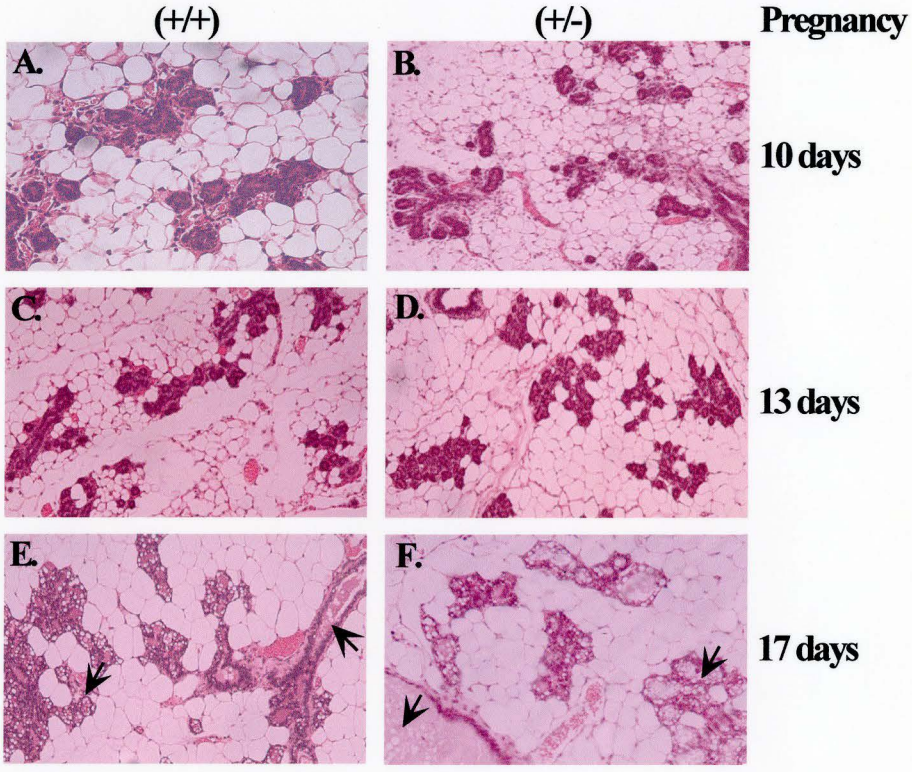

(+/+)
(+/-)
A
B.
5 weeks
C
D
8 weeks
E.
F.
12 weeks

Figure 2.4.8: Wholemount analysis of ER81 mammary gland development throughout various stages of pregnancy. Mammary glands of ER81 +/+ (A, C, E) and +/- (B, D, F) mice were isolated at 7 (A&B), 13 (C&D), and 17 days (E&F) of pregnancy, and stained with hemotoxylin. Loss of function of a single ER81 allele has no visible consequences on branching or development of alveolar structures (arrows). Original magnification of each: 25X.


(+/+)
(+/-)
Pregnancy
A.
B.
7 days
C.
D.
13 days
E.
F.
17 days

development of alveolar bundles (Figure 2.4.9; panels A-D), nor in the accumulation of milk droplets within the ducts and alveoli (Figure 2.4.9; panels E&F, arrows).

Figure 2.4.9: Hemotoxylin and eosin stained histological analysis of ER81 mammary glands during pregnancy. Mammary glands of wildtype (A, C, E, G) and heterozygous (B, D, F, H) ER81 mice were isolated at 10 (A&B), 13 (C&D), and 17 days (E&F) during pregnancy. Loss of a single ER81 allele appears to have no visible consequences on duct morphology and alveolar development as compared to wildtype animals. Milk droplets (arrows) are visible by 17 days of pregnancy within the ducts and the alveoli (E&F). Original magnifications are: 100X (B-H) and 200X (A).


(+/+)
(+/-)
Pregnancy
A.
B.
10 days
C.
D.
13 days
E.
F.
17 days

2.5. DISCUSSION

The PEA3 subfamily genes are co-expressed in the mammary anlage at E15.5, suggesting they play a role in the development of this organ (Chotteau-Lelievre et al., 1997). Since the PEA3 subfamily genes share greater than 95% amino acid identity in the DNA binding domain, these proteins likely bind the same DNA sequences and hence may exhibit a significant degree of functional redundancy. Targeted disruption of PEA3 significantly reduces mammary ductal branching during all stages of mouse mammary gland development. Given the high degree of similarity and the emerging phenotype in PEA3-null female mice, these factors raise the possibility that ER81 may also play a role in the development of this organ. However, another possibility is that each family member plays a distinct as well as redundant role in mammary gland development because neither ER81 nor ERM expression were able to compensate for the loss of PEA3 which lead to a ductal phenotype in the null mice.

2.5.1 Expression of ER81 in the Developing Mammary Gland

The embryonic mammary gland develops from the ectoderm and is first visible as a mammary bud at 10.5 days of embryogenesis. Beta-galactosidase activity assays confirm expression of the ER81 gene in all undifferentiated epithelial cells of the embryonic mammary bud as early as E10.5 (Figure 2.4.2), which coincides with the onset of mammary gland development. Whereas similar findings were observed for PEA3 (MacNeil, unpublished; Shepherd and Hassell, 2001), expression of ERM was not

detected until 12.5 days of embryogenesis (Kurpios, unpublished). Hence, ER81 and PEA3 may be two of the earliest known molecular markers of embryonic mammary gland development. ER81 expression continues in the developing mammary gland between E10.5 and E14.5. Interestingly, at the onset of sexual determination, expression of ER81 is extinguished in male embryos but continues in female embryos. Loss of ER81 expression in male embryos by E14.5 is likely due to sexual determination of the gland. At this time, the dense mesenchyme responds to fetal androgen produced by the testis, causing it to condense around the epithelial bud. The bud is then severed from the epidermis and undergoes complete or partial destruction, thereby inhibiting further development of male mammary gland (Robinson et al., 1999).

Expression of ER81 continues postnatally in the branching epithelium during puberty and pregnancy, both of which are periods of extensive cellular proliferation and migration. Hence, ER81 may play a role in these processes during mammary gland development. These observations are consistent with RNase protection analyses illustrating co-ordinate expression of the PEA3 subfamily genes during various stages of postnatal mammary gland development (MacNeil and Shepherd, unpublished). In further agreement with this hypothesis is the fact that ER81 is expressed in both cap and body cells of adult TEBs (Figure 2.4.4). More specifically, the undifferentiated cap cell layer in these TEB structures is thought to produce a supply of differentiated luminal and myoepithelial cells that are responsible for driving ductal growth and morphongenesis, thereby causing elongation of the subtending ducts (Knight and Peaker, 1982). Furthermore, these cap cells also represent a population of undifferentiated mammary

epithelial stem cells or progenitors both of the epithelial cell lineages. Although the TEBs are enriched in these mammary epithelial stem cells, these cells are also found in the embryonic mammary gland and various areas of the adult mammary tree (Daniel and Silberstein, 1987; Williams and Daniel, 1983). Moreover, these cells are of particular interest since they are likely the cells of breast tumour origin (Russo and Russo, 1987). The overexpression of ER81 in mammary tumours, as well as its expression in cap cells and in the embryonic mammary bud further supports the theory that ER81 plays a role in proliferation, migration, or differentiation of mammary epithelial cells.

In quiescent ducts, ER81 is expressed in luminal and myoepithelial cells during puberty (Figure 2.4.3). However, at the onset of pregnancy, expression is lost from the luminal epithelial cell layer and persists only in the myoepithelial cells of ducts and alveoli. In addition, ER81 is expressed in the myoepithelial cells of regressing alveoli during involution (Figure 2.4.5). Similar variations in patterns of expression were observed for the EGF related protein, CD44v6 and various PKC isoforms. The CD44v6 epitope is expressed in luminal epithelial cells during puberty, this expression gets turned off during lactation and then reappears during involution. Although the role of this epitope in mammary gland development remains enigmatic, it was also found overexpressed in ErbB/Her4 tumours and mammary carcinomas (Hebbard et al., 2000; Srinivasa et al., 2000). In addition, various PKC isoforms are also elevated in breast tumours and display differences in their cellular localization throughout normal mammary gland differentiation similar to ER81 (Gordge et al., 1996; O'Brian et al., 1989). PKC isoform *eta*, for example, is preferentially expressed in the luminal

epithelium and not the myoepithelium during puberty. This expression changes during pregnancy; it is lost from the ductal luminal epithelial cells, upregulated in the alveoli and secreted into milk (Masso-Welch et al., 1998). Although it is unclear why expression of the ER81 gene is being turned off in luminal epithelial cells during pregnancy, it may be hormone sensitive. The initial phases of mammary gland development and morphogenesis are primarily governed by epithelial-mesenchymal interactions, with a minimal contribution made from endocrine hormones. During sexual maturation, proliferation and ductal outgrowth are controlled by estrogen and prolactin. However, during pregnancy, the final phase of growth and functional differentiation is governed by additional systemic, local and intracellular signals and hormones. Some of these hormones include progesterone, insulin, thyroid hormone, growth hormone and hydrocortisone, in addition to the pre-existing pubertal hormones (Dickson et al., 1991; Hennighausen and Robinson, 1998). The process of involution is then triggered by a drop in prolactin levels (Traver et al., 1996), resulting in apoptosis of milk synthesizing cells and endothelial cells (Walker et al., 1989). The inability to detect ER81 expression in ducts during involution via beta-galactosidase activity assays may reflect loss of ER81 expression or decreased levels of ER81. Alternatively, the increased size of the gland may also present difficulties in substrate penetration. Loss of ER81 expression in luminal epithelial cells at the onset of pregnancy was not expected due to the fully differentiated nature of these cells. However, the presence of mammogenic hormones at this time may have caused ER81 expression to be shut down directly in the luminal epithelial cells. Alternatively, these hormones may have effected changes to these cells causing them to

lose some aspect of their basic functional identity resulting in loss of ER81 expression. In support of this theory, Sapino and colleagues (1990) showed terminally differentiated cells in ducts and alveoli can be induced to proliferate and change as a result of varied mammogenic stimuli. Furthermore, another study (Michalczyk et al., 2001) demonstrated that hormones present during pregnancy and lactation affected the expression pattern of intermediate filaments, keratins 8 and 18 in human luminal epithelial cells in either a filamentous or punctate form in a resting versus lactating mammary gland, respectively. In this regard, the traditional concept of stem cell differentiation relies on an irreversible switch into a terminally differentiated cell, whereas the evolving view focuses on cellular plasticity. Hence, a possibility exists that as a cell lineage matures, differentiated adult cells can change their fate or protein expression based on new extracellular signals (Blau et al., 2001).

ER81 is expressed in the embryonic mammary bud (Figure 2.4.2) and in putative stem cells within the TEBs (Figure 2.4.4). Furthermore, ER81 expression is also observed in luminal and myoepithelial cells of quiescent ducts during puberty (Figure 2.4.3) and myoepithelial cells of ducts and alveoli during pregnancy (Figure 2.4.5). These findings suggest ER81 expression is not restricted to undifferentiated cells or actively dividing cells, nor is it sufficient to retain cells in an undifferentiated state. However, the identity of these beta-galactosidase expressing cells was inferred from their position and morphology within the various mammary structures. It will therefore be essential to confirm the identity of these ER81 expressing cells using immunohistochemical techniques with antibodies to molecular markers that are known to

distinguish the various mammary epithelial cell types (Pechoux et al., 1999; Rudland et al., 1997; Dulbecco et al., 1983). ER81 expression in luminal epithelial cells may have important implications in tumour formation. Based on evidence from a number of studies, it is thought that deregulated proliferation of luminal epithelial cells results in loss of normal tissue architecture and allows malignant tumour cells to arise. Typically, malignant tumour cells are phenotypically equivalent to luminal epithelial cells; they proliferate without contacting myoepithelial cells or the basement membrane due to loss of expression and function of adhesion molecules (reviewed in Alford et al., 1998). Furthermore, monoclonal antibodies that are expressed in luminal epithelial cells such as keratin 6, keratin 19 are also expressed in tumours (Bartek et al., 1985; Smith et al., 1990). Lastly, Russo and colleagues (1983) identified an intermediate population of luminal epithelial cells that represent a small population in normal ducts and 90% of those in DMBA (7,12-dimethylbenzanthracene) induced tumours. The fact that ER81 expression is lost in luminal epithelial cells with the onset of pregnancy but retained in myoepithelial cells makes these implications in tumour formation less clear. Unlike luminal epithelial cells, myoepithelial cells are thought to play a tumour suppressive role during tumour formation; conditioned media from these cells have anti-proliferative effects on a variety of mammary epithelial cell lines (Shao et al., 1998). Typically, myoepithelial cells are important in maintaining normal mammary morphology because of their role in synthesizing and assembling the basement membrane. Furthermore, these cells are often lost in highly invasive mammary tumours (Guelstein et al., 1993; Sappino et al., 1988).

Each PEA3 subfamily member is co-ordinately expressed throughout normal mammary gland development and upregulated in tumours, thereby suggesting these proteins may be regulated by the same signalling mechanism. During puberty, ER81 is expressed in both luminal and myoepithelial cells. Furthermore, ER81 expression in the luminal epithelial compartment at this time is unique and distinct in comparison to the other subfamily members, PEA3 and ERM. This may confer a distinct role for ER81 in mammary gland development. One possibility is the ER81 protein is more highly expressed in luminal epithelial cells and is thus more active in these cells. Hence, the lumin specific expression of ER81 may therefore provide as to why ER81 is unable to rescue the PEA3 branching defect in PEA3-null mice. Since each PEA3 subfamily member is additionally expressed in myoepithelial cells, this further suggests these proteins are not functionally redundant and may play distinct roles during mammary gland development.

2.5.2 Role of ER81 During Mammary Gland Development

Targeted disruption of the ER81 gene (Figure 2.4.1) results in lethality of these mice by approximately 4 weeks of age, thereby precluding further study of ER81 inactivation on mammary gland development. At 3 weeks of age, targeted disruption of both ER81 alleles does not appear to affect the initial mammary tree present at birth. However, ER81-null mice are severely runted and consequently have mammary glands that are much smaller in contrast to wildtype and heterozygous littermates. This is evident by comparing the position of the mammary tree relative to the lymphnode (Figure 2.4.6). In

wildtype mice at 3-4 weeks of age, terminal end buds begin to appear at the ductal tips and growth increases slightly both in rate and degree of branching (Daniel and Silberstein, 1983). In contrast to PEA3-null mice, which had a discernable phenotype at this age (MacNeil, unpublished), ER81-null mammary glands appear normal with no reduction in the numbers of ducts or terminal end buds.

In order to characterize the role of targeted disruption of ER81 in both mammary gland development and tumorigenesis, the ER81 null allele was introgressed into the FVB/n background strain. In future, mammary gland development may be studied by transplanting mammary epithelial cells from ER81-null mice into the cleared fat pad of wildtype mice bearing the same strain genetic background (DeOme et al., 1959). However, a future problem with this approach is that reciprocal transplants will not be possible due to the early lethality of the mice. Hence, the transplanted ER81-null epithelium will not provide the necessary proof that ER81 plays a role in mammary gland development. Alternatively, in order to circumvent this problem, a cre / lox approach could be employed in order to create a conditional mammary specific knockout.

Previous studies demonstrate PEA3 heterozygous females possess a reduced branching phenotype intermediate in nature between wildtype and PEA3-null littermates (MacNeil, unpublished). Since ER81 heterozygous mice are viable and healthy, they were analysed for the possibility of a similar phenotype throughout postnatal development. In general, mammary glands from ER81 hemizygotes displayed a normal pattern of arborization comparable to wildtype littermate animals. Specifically, TEBs formed normally and drove ductal morphogenesis to produce primary ducts with side

branching that filled the fat pads of both heterozygous and wildtype females (Figure 2.4.7). Hence, the mammary epithelial cells in ER81 heterozygous mice are able to respond to proliferation signals from the estrogen receptor transcription complex that drives ductal elongation throughout the mammary fat pad (Hennighausen and Robinson, 1998). This suggests loss of a single ER81 allele may not be sufficient to adversely affect cell proliferation, migration, and differentiation. Furthermore, during pregnancy, ER81 also does not have a negative impact on lateral branching, alveolar development and differentiation, nor in lactation or milk secretions (Figure 2.4.8 and Figure 2.4.9). However, in this regard, droplets of milk fat were observed in late pregnancy as well as involution within the ducts and alveoli; a phenomena that typically occurs due to leakiness of the cell junctions (Richert et al., 2000).

Whereas loss of a single PEA3 allele results in a discernable branching phenotype intermediate in nature between wildtype and null mice, there does not appear to be an importance in the required levels of ER81 for its role in mammary gland development. Loss of a single ER81 allele did not result in any apparent phenotype or have a discernable consequence on mammary gland development. This observation suggested that a 50% dosage of ER81 is sufficient for effecting the development of this organ. Alternatively, PEA3 and / or ERM may also be partially compensating for the loss of ER81 during mammary gland development in these mice. Although loss of a single allele did not manifest a phenotype, this does not preclude the possibility that loss of both alleles may still reveal an essential function in mammary gland development. However, the possibility exists that loss of both ER81 alleles may result in normal mammary gland

development. In this respect, many unrelated knockout mice were generated with no overt phenotypes in any stage of mammary gland development. These include the targeted disruptions of the heart fatty acid binding protein (H-FABP), and those of the alpha 3 and alpha 6 integrin subunits (Clark et al., 2000; Klinowska et al., 2001). These results proved interesting since it was expected that mammary phenotypes would result as a consequence of knocking out these molecules.

2.5. Conclusions and Future Directions

ER81 was expressed in every epithelial cell of the mammary bud at the onset of mammary organogenesis and may therefore serve as one of the earliest known markers of embryonic mammary gland development. Expression continued postnatally and was associated with proliferation and migration events. ER81 is expressed in undifferentiated cap and body cells within the TEBs, in differentiated luminal and myoepithelial cells of ducts during puberty, in myoepithelial cells of ducts and alveoli during pregnancy and in regressing myoepithelial cells of alveoli during involution. Future studies should focus further on confirming the identity of these cells via immunohistochemical analyses. Lastly, the effects of targeted disruption of both ER81 alleles and its role in mammary gland development was not fully addressed due to the early lethality of ER81-null mice. Hence transplant experiments should be performed to adequately address the functional consequences of ER81 mutations in development of this organ. Studies using heterozygous mice revealed a single ER81 allele was sufficient for mammary gland

development. Loss of a single allele did not result in any overt phenotypes during ductal branching or alveolar development.

2.5. APPENDIX

ER81 mouse embryo fibroblasts were isolated from 12.5day embryos as described (section 2.3.9). Various wildtype and ER81-null cell lines were frozen down at early passage stages (P2-P6). One wildtype and one knockout cell lines were immortalized by passaging the cells according to a specific 3T3 protocol (Todaro and Green, 1963) and frozen down at passage 24-28 once they came out of crisis. Each of the cell lines listed below is stored in liquid nitrogen.

ER81 MEF	GENOTYPE	PROCESSING STATUS
B1	+/+	Early Passage; frozen
G1	-/-	**Immortalized; frozen**
D2	+/+	**Immortalized; frozen**
A3	-/-	Early Passage; frozen
B3	-/-	Early Passage; frozen
C3	-/-	Early Passage; frozen
D3	-/-	Early Passage; frozen
E3	-/-	Early Passage; frozen
A4	+/+	Early Passage; frozen
B4	+/+	Early Passage; frozen
F4	+/+	Early Passage; frozen
D6	+/+	Early Passage; frozen
A7	-/-	Early Passage; frozen
B7	-/-	Early Passage; frozen
E7	+/+	Early Passage; frozen
F7	+/+	Early Passage; frozen

Table 2.7.1: Genotype and status of ER81 mouse embryo fibroblast cell lines.

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