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# Role of Ets Transcription Factors in Mammary Gland Development and Oncogenesis

**Trevor Shepherd[1] and John A. Hassell[1,2]**

*PEA3* is the founding member of a subfamily of closely related *ets* genes that includes *ER81* and *ERM*. *PEA3* is expressed in the epithelial cells of mammary buds at the time that these first appear during mouse embryogenesis, and it is differentially expressed during postnatal mammary gland development. *PEA3* expression is highest at the onset of puberty and during early pregnancy, times of extensive epithelial outgrowth and branching. *PEA3* is expressed in undifferentiated epithelial cap cells of terminal end buds, and in differentiated myoepithelial cells of ducts and alveoli. Loss-of-function mutations in the *PEA3* gene compromise mammary ductal branching at the onset of puberty and early during pregnancy. *PEA3* is overexpressed in the vast majority of human breast tumors and in nearly all of the HER2-positive subclass of such tumors. *PEA3* is similarly overexpressed in transgenic mouse models of this malignancy. Expression of dominant-negative *PEA3* in the mouse mammary gland of MMTV-HER2 transgenic mice dramatically delays the onset and reduces the incidence of mammary tumors. Hence *PEA3* and/or its close relatives play key regulatory roles in both mammary gland development and oncogenesis.

**KEY WORDS:** Ets; PEA3; HER2; stem cells; development; oncogenesis.

## INTRODUCTION

The Ets family transcription factors play key regulatory roles in development and oncogenesis (1). These proteins share an evolutionarily conserved, ~85 amino acid ETS DNA binding domain that defines them as members of this family. Structural analyses of the ETS domain of several Ets proteins reveal a winged-helix-turn-helix structure akin to that of the *E. coli* catabolite activator protein (2–5). Ets proteins bind to an ~10 base pair DNA sequence in the promoters of their target genes and generally activate transcription; rare members of the family repress this process (1). An invariant feature of all Ets binding sites is the occurrence of a central 5′-GGA, A/T-3′ motif; sequences flanking this central element govern the specificity of binding by particular Ets proteins (1).

*Ets* genes are found exclusively in multi-cellular organisms and multiple members of the *ets* gene family are present in all species studied to date. The human *ETS* gene family currently comprises 30 paralogs; orthologs of most of these genes have also been identified in the mouse. Alignment of the ETS domains of all known Ets proteins from different species allows definition of 13 subfamilies (Fig. 1) (6). Sequence comparison of subfamily members reveals that they possess nearly identical ETS domains and share additional regions of sequence similarity outside the borders of this region.

## *ETS* GENES AND CANCER

Ets proteins are thought to play causative roles in the genesis and progression of human tumors

[1] Institute for Molecular Biology and Biotechnology, McMaster University, 1280 Main Street West, Hamilton, Ontario, Canada L8S 4K1.

[2] To whom correspondence should be addressed. e-mail: hassell@mcmaster.ca



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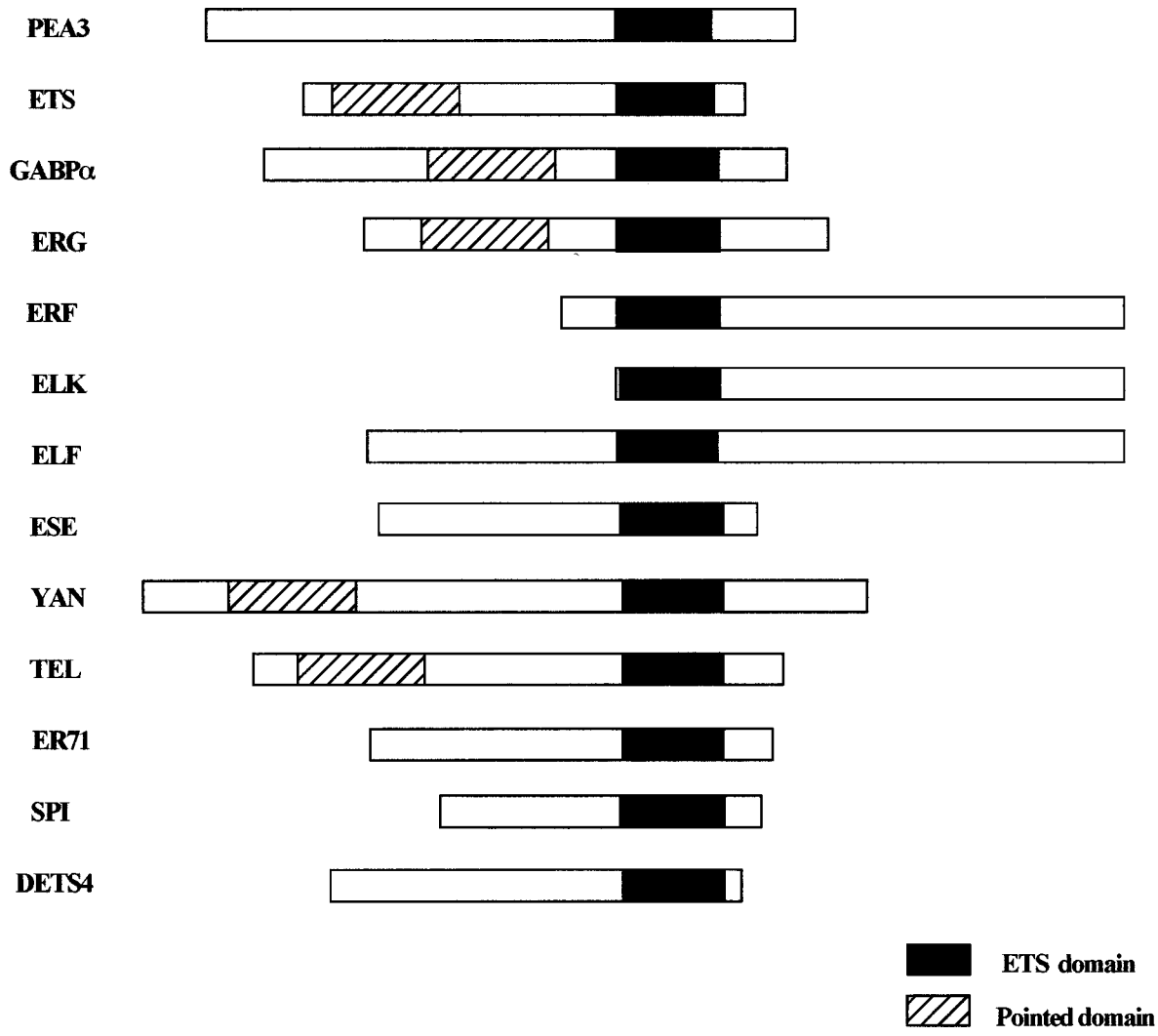


**Fig. 1.** The *ets* gene family of transcription factors can be divided into 13 subfamilies based on the sequence similarity of their ETS domains. Illustrated are schematic diagrams of a representative member from each Ets subfamily protein. Most Ets proteins possess a carboxyl-terminal ETS domain, and only a subset of Ets proteins contains the helix-loop-helix Pointed domain (1,6,7).

including breast cancer (7). Two principal mechanisms appear to account for the "activation" of *ETS* genes in different human malignancies: chromosomal translocations leading to the expression of hyperactive Ets fusion proteins and transcriptional upregulation resulting in increased levels of an otherwise normal Ets protein. Chromosomal translocations occur in Ewing's sarcomas and in several types of leukemia; increased *ETS* gene transcripts are observed in breast and gastric tumors.

Ewing's sarcoma and the related primitive neuroectodermal tumors are commonly associated with translocations that juxtapose a segment of the *EWS* gene to one of five different *ETS* genes including *FLI-1* (8); *ERG* (9,10); *ER81* (11); and *PEA3* (12,13) in individual tumors. Of these, the *FLI-1* and *ERG* genes are most commonly translocated in Ewing's sarcomas. The *EWS* gene is ubiquitously expressed and is thought to encode an RNA binding protein. Transcription of translocated *ETS* genes is governed by *EWS* promoter elements; the encoded fusion proteins bear amino-terminal residues of EWS and carboxyl-terminal sequences that include the ETS DNA binding domain of one or another of the aforementioned Ets proteins. The EWS portion of these chimeric proteins harbors a strong activation domain and hence

EWS-Ets fusion proteins generally possess increased transcriptional activity by comparison to the parental Ets protein from which they were derived (14). This observation suggests that it is the increased activity of the Ets proteins and the commensurate increased expression of their downstream target genes that is critical for oncogenesis.

Translocations involving human *ETS* genes and partners other than *EWS* also occur in chronic myelomonocytic leukemia (CML)[3] (15) and acute myeloid leukemia (AML) (16,17). In CML the ETS domain of ERG2 or TEL is fused by translocations to TLS/FUS or MN1 respectively. *TLS/FUS* encodes an RNA binding protein related in structure to EWS, whereas *MN1* may encode a transcription factor (18). Given the structure of these fusion proteins it is likely that they too function as constitutively-active Ets transcription factors.

By contrast, AML translocations involving TEL do not result in fusion proteins bearing the ETS DNA binding domain, and the fusion partner is not an RNA binding protein (7). Instead TEL contributes a protein:protein interaction surface and the fusion partner is one of several tyrosine kinases, including the platelet-derived growth factor receptor, ABL and Janus kinase 2. The TEL sequences effect the dimerization of these kinases thereby stimulating their activity.

Uncontrolled expression of *ETS* genes is also implicated in human cancer. A survey of a large number ($n = 121$) of gastric tumors revealed that *ETS1* was overexpressed in 64% of gastric adenocarcinomas (19). *ETS1* is expressed to particularly high levels in the more invasive carcinomas and its overexpression correlates positively with lymph node metastasis. Similarly we have reported that *PEA3* is overexpressed at the RNA level in 76% of human breast tumors ($n = 74$) (20). Interestingly, *PEA3* is overexpressed in nearly all of the HER2-positive subclass of these tumors. *HER2* (also named *ERBB2* or *NEU*) encodes a membrane-associated receptor tyrosine kinase, which is upregulated in 20–30% of all breast cancer cases; its increased expression is associated with poor clinical outcome largely because of the propensity of these tumors to metastasize (21).

[3] Abbreviations: mouse mammary tumor virus (MMTV); chronic myelomonocytic leukemia (CML); acute myeloid leukemia (AML); Ets translocation variant (ETV); extracellular regulated kinase (ERK); nuclear localized $\beta$-galactosidase (NLS-*lacZ*); polyomavirus middle T antigen (PyMT); ductal carcinoma *in situ* (DCIS); dominant-negative PEA3 (ΔNPEA3En).

## *PEA3* SUBFAMILY OF *ETS* GENES

Mouse *PEA3* (22) (the human gene is named Ets translocation variant 4 [*ETV4*] and has also been termed *E1A-F*) (23) is the founding member of a subfamily of *ets* genes, which also includes *ER81* (*ETV1*) (24,25) and *ERM* (*ETV5*) (26,27). Each *PEA3* subfamily gene is located on a different chromosome (28), but all three genes share a common architecture comprising fourteen equivalently sized exons that encode similar sequences of the respective proteins (27–30) (Fig. 2).

The proteins encoded by these three genes comprise about 500 amino acids and share an overall sequence identity of 50%; their ETS domains are 95% identical (29). The high sequence identity of the ETS domains of PEA3, ER81 and ERM predict that they very likely bind to the same sequence elements in target gene promoters.

Functional analyses of PEA3 subfamily Ets proteins reveal that they commonly activate transcription (1,29). The activation domain of each protein is located near the amino terminus, and comprises an acidic amino acid segment, whose sequence is conserved among all three proteins [(29); Bojovic and Hassell, in press]. The PEA3 activation domain is flanked by two negative regulatory regions, which independently repress its activity [Bojovic and Hassell, in press]. It is not known whether ER81 and ERM possess equivalent negative regulatory regions.

The ETS domain of each protein is required and sufficient for sequence-specific DNA binding. Recent analyses of the zebrafish and mouse PEA3 proteins reveal that DNA binding is also negatively controlled by two regulatory modules that flank the ETS domain [(29); Bojovic and Hassell, in press]. Hence both the transcription activation and DNA binding functions of PEA3 are negatively regulated implying that mechanisms exist to overcome repression of the activity of this transcription activator. It is noteworthy that PEA3 is a phosphoprotein (31) and that different members of the mitogen activated protein kinase family (extracellular regulated kinase [ERK] 1 and ERK2, as well as Jun kinases) phosphorylate PEA3 *in vitro* at the same sites that are phosphorylated *in vivo* [Tozer *et al*., unpublished]. Indeed all of the phosphopeptides derived from trypsin cleavage of PEA3 labeled *in vivo* with radiolabeled orthophosphate are obtained by trypsin cleavage of PEA3 phosphorylated *in vitro* with radiolabeled ATP and a combination of ERK2 and Jun kinase 1. Post-translational

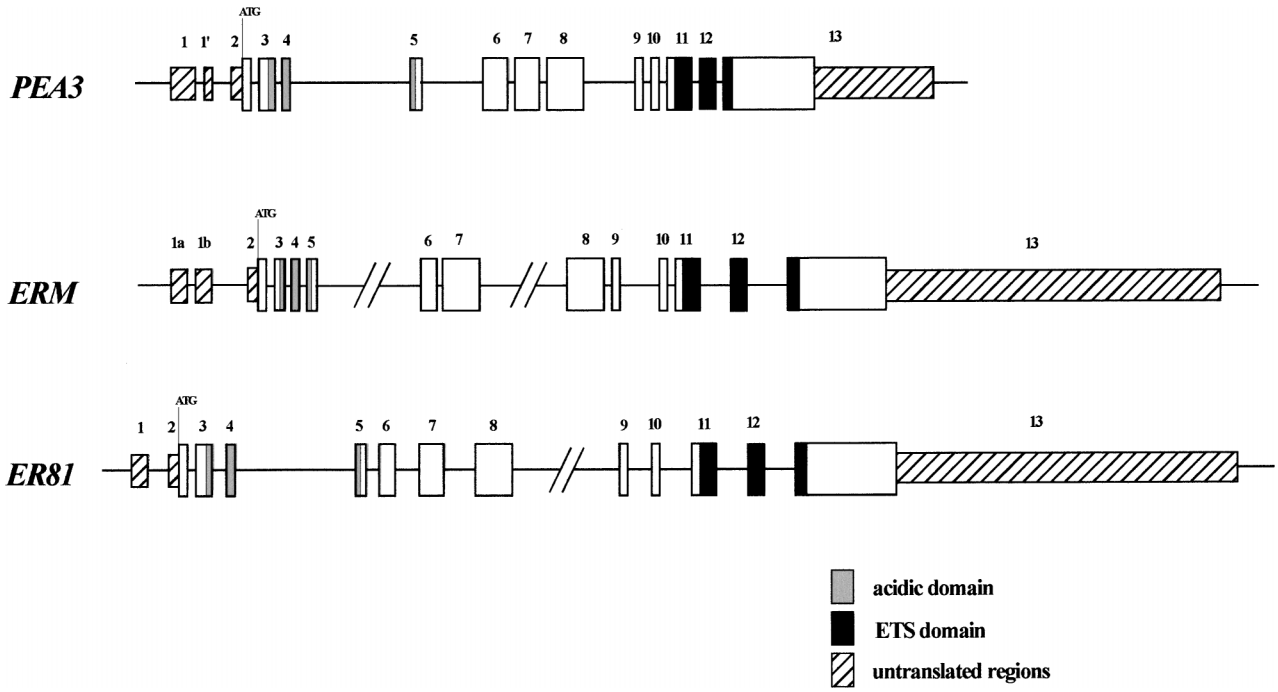


**Fig. 2.** All three *PEA3* subfamily genes have equivalently-sized exons encoding similar regions of their respective proteins. Introns are represented by a horizontal line, and exons are illustrated by rectangles. The cross-hatched areas represent non-coding, untranslated sequences embodied in the mRNAs of each gene. The sequence (ATG) encoding the translation initiation codon is present in exon 2 of each gene. The ETS domain is encoded by exons 11, 12, and 13. The acidic transactivation domain is encoded by exons 3, 4, and 5. The divergence in structure among these three genes occurs primarily in the untranslated regions as well as in the size of several introns. This diagram is not drawn perfectly to scale because the size of several introns in *ERM* and *ER81* is not known (27,30).

phosphorylation may provide one means of regulating PEA3 activity, but other mechanisms are equally plausible.

*PEA3*, *ER81*, and *ERM* are co-expressed in several tissues and organs of embryonic and adult mice suggesting that their transcription is governed in part by a common pathway(s) impinging on the same *trans*-acting factor(s) (22,24–27). *ERM* is nearly ubiquitously expressed in the adult mouse; only the liver appears devoid of its transcript (26). The expression profile of *ER81* is more limited, but all tissues that express *ER81* also express *ERM* (24–26). The expression of *PEA3* is most restricted occurring principally in the brain and epididymis; lower levels of *PEA3* mRNA are also found in the spinal cord, kidney, intestine, skeletal muscle, testis, hair follicles and mammary gland (22). *ERM* and *ER81* are co-expressed in these same tissues (26), but it is not known whether the same or distinct cells in each organ express the *PEA3* subfamily genes.

The *PEA3* subfamily genes are expressed in unique as well as overlapping temporal and spatial compartments during mouse embryogenesis in cells derived from each of the three germ layers and in regions of the embryo undergoing cellular proliferation and migration [(32); Laing *et al.*, submitted]. Indeed these three *ets* genes appear to be preferentially expressed in tissues and organs at sites of epithelial-mesenchymal transitions. Early during gastrulation *PEA3* and *ERM* are co-expressed in the same regions of the developing embryo, whereas *ER81* is not expressed at this time. Later at the onset of organogenesis all three *PEA3* subfamily genes are co-expressed in many of the same tissues that express them into adulthood. The embryonic expression profiles of these genes suggests that they may fulfill both distinct and overlapping roles. Indeed the very similar expression pattern of *PEA3* and *ERM* suggests the potential for a high degree of functional redundancy between these two proteins. This potential may be clarified by defining the cellular expression profile of these three *ets* genes in different tissues and organs, and by analyses of the consequence of their targeted disruption in the mouse germ line.

## *PEA3* AND MAMMARY GLAND DEVELOPMENT

*PEA3* subfamily genes are co-expressed in the mammary anlage at day 15.5 of embryogenesis (E15.5) suggesting that they play a role in the development of this organ (32). To examine *PEA3* gene expression during embryonic and postnatal mammary gland development in greater detail, and to learn whether *PEA3* is required for mammary gland development, we derived mice bearing a targeted *PEA3* allele. Exons encoding the ETS domain were deleted and replaced with sequences bearing an internal ribosome entry site and encoding nuclear localized $\beta$-galactosidase (NLS-*lacZ*). In mice heterozygous for the targeted allele ($PEA3^{nlslacZ}$), the profile of $\beta$-galactosidase activity mimics the pattern of *PEA3* expression during embryogenesis strongly suggesting that *NLS-lacZ* transcription is governed by the *PEA3* promoter [MacNeil *et al*., unpublished].

The embryonic mammary gland develops from the ectoderm and is first visible as a ridge or bud at this stage of embryogenesis (33). Analyses of $\beta$-galactosidase activity as a surrogate for *PEA3* in the heterozygous $PEA3^{nlslacZ}$ mutant, revealed that *PEA3* is expressed as early as E10.5 in all the epithelial cells of the mammary buds [MacNeil *et al*., unpublished]. Hence *PEA3* is expressed in epithelial cells commensurate with the onset of mammary organogenesis and is one of the earliest known molecular markers of embryonic mammary gland development. *PEA3* expression continues in the developing mammary epithelium between E10.5 and E15.5. At the onset of sexual determination (E15.5), *PEA3* expression is extinguished in male embryos but continues in female embryos.

Analyses of *PEA3* subfamily gene expression by quantitative multiplex RNase protection assays reveal that these genes are coordinately expressed during the various stages of postnatal mammary gland development [MacNeil *et al*., unpublished]. *PEA3* subfamily genes are expressed at the highest levels at the onset of puberty and early during pregnancy, times of extensive epithelial outgrowth and branching, but their expression declines at mid pregnancy, and remains relatively low during lactation and at the onset of involution. The expression of the targeted *PEA3* allele, $PEA3^{nlslacZ}$, as assessed by measurement of $\beta$-galactosidase activity at the cellular level, is regulated similarly during postnatal mammary gland development further confirming that the targeted allele is subject to the same controls as is the wild type gene.

Analyses of the cellular expression profile of $\beta$-galactosidase activity in $PEA3^{nlslacZ}$ heterozygote females during postnatal mammary gland development revealed that PEA3 is expressed in the cap cells of terminal endbuds, and in the myoepithelial cells of ducts and alveoli [MacNeil *et al*., unpublished].

We have also carried out preliminary analyses of $\beta$-galactosidase activity at the cellular level as a surrogate for ERM and ER81 expression during postnatal mammary gland development using heterozygous mice bearing similarly targeted alleles [Kurpios *et al*., unpubished]. These analyses suggest that ERM is expressed in the same repertoire of mammary epithelial cells as those that express PEA3. ER81 like PEA3 and ERM is also expressed in cap cells of terminal endbuds; however, unlike its relatives, ER81 appears to be expressed in differentiated luminal epithelial cells of ducts. Whereas additional and more rigorous analyses need to be carried out to confirm these findings, these observations imply a role for PEA3, ERM and ER81 in mammary epithelial cell differentiation. The PEA3 subfamily proteins may control proliferation or migration of mammary epithelial stem cells or they may regulate the differentiation program of these cells.

A regulatory role for *PEA3* in mammary ductal outgrowth and branching is inferred from the observation that these processes are compromised in female mice lacking functional *PEA3* alleles. *PEA3*-null mice are viable and healthy, but male mice are sterile [Laing *et al*.]. Whereas females reproduce normally and rear their pups, the postnatal branching of the mammary ductal tree is significantly reduced prior to puberty and during pregnancy revealing a role for *PEA3* in mammary gland development [MacNeil *et al*., unpublished]. The roles, if any, of *ER81* and *ERM* in mammary gland development are not yet known, but these are currently being assessed by analysis of mice bearing targeted mutations in these genes.

## ROLE OF *ets* GENES IN MAMMARY ONCOGENESIS

### Role of *PEA3* Subfamily *ets* Genes in Mammary Oncogenesis

The potential that *PEA3* subfamily *ets* genes play a role in mammary oncogenesis stems from our finding that *PEA3* transcripts are elevated in

the mammary tumors of transgenic mice expressing one of two different oncoproteins under the control of the mouse mammary tumor virus (MMTV) promoter (34). Analyses of mammary tumors and lung metastases arising in multiple different strains of MMTV-HER2 and MMTV-polyomavirus middle T antigen (PyMT) transgenic mice revealed that *PEA3* transcripts are expressed at much higher levels in mammary tumors and lung metastasis than in morphologically normal tissue adjacent to the tumors. Whereas all mammary tumors analyzed overexpressed *PEA3* mRNA, the extent thereof varied from one tumor to another likely reflecting differences in the fraction of tumor cells comprising individual tumors. Commonly the magnitude of *PEA3* overexpression in mammary tumors of HER2 transgenic mice varied from three- to ten fold.

Upregulation of *PEA3* expression appears to be an early event in mammary oncogenesis as suggested by the observation that hyperplastic mammary lesions arising in these mice contain elevated levels of *PEA3* transcripts [Shepherd and Hassell, unpublished]. Recent analyses of mammary tumors in MMTV-HER2 transgenic mice bearing a targeted $PEA3^{nlslacZ}$ allele clearly demonstrate that *PEA3* ($\beta$-galactosidase activity) is overexpressed in the epithelial tumor cells [Shepherd *et al.*, unpublished]. *PEA3* is not expressed at these levels in the normal mammary epithelial cells or in mesenchymal cells (fibroblasts and adipocytes) in the mammary gland.

We have reported similar findings for human breast tumors (20). An analysis by *in situ* hybridization of 74 breast tumor samples of known HER2 status and of 5 normal breast samples revealed that *PEA3* is overexpressed in the epithelial compartment of 76% of these breast tumors. *PEA3* is overexpressed in 73% of ductal carcinoma *in situ* (DCIS) tumors ($n = 41$), and in a similar percentage (79%) of invasive breast tumors ($n = 33$). 93% of all HER2-positive breast tumors overexpressed *PEA3*; 92% of DCIS and 95% of invasive tumors overexpress both *HER2* and *PEA3*. *PEA3* overexpression in these tumors does not correlate with nuclear grade, estrogen receptor status or S-phase fraction, and does not result from amplification of the *PEA3* gene. Hence in mammary tumors of both mice and humans *PEA3* gene transcripts are elevated in epithelial tumor cells, appearing early during oncogenesis. Moreover, *PEA3* appears to be invariably overexpressed in the HER2-positive subclass of mammary tumors in both species.

### All Three *PEA3* Subfamily Genes are Coordinately Upregulated in HER2-Positive Mouse Mammary Tumors

The similar expression profile of *PEA3* subfamily genes during mammary gland development, and the expression of *PEA3* in presumptive mammary epithelial stem cells, the likely cells of origin of breast tumors, prompted us to determine whether other *PEA3* subfamily *ets* genes were also overexpressed in HER2-positive mammary tumors. Remarkably all three *PEA3* subfamily genes were coordinately overexpressed in HER2-induced mouse mammary tumors by comparison to age-matched normal mammary tissue or normal mammary tissue adjacent to the tumors [Shepherd *et al.*, unpublished]. As previously stated, these genes are also co-regulated during postnatal mammary gland development. Hence the signaling pathways regulating *PEA3* subfamily gene expression during mammary gland development may be the same as those downstream of the HER2 receptor tyrosine kinase.

By contrast to the *PEA3* subfamily genes, other *ets* genes expressed in the mammary gland (*GABP*$\alpha$, *ets1*, and *ets2*) are expressed at reduced levels in mammary tumors by comparison to normal mammary tissue. The apparent reduced expression of these *ets* genes in mammary tumors may result from *bona fide* downregulation of their expression in epithelial tumor cells. Alternatively, these genes may normally be expressed in the stromal compartment; the fraction of stromal cells in normal mammary tissue likely supersedes that in mammary tumors possibly accounting for the apparent decreased expression of their transcripts in the tumors.

### *PEA3* Subfamily Genes are Required for HER2-Induced Mouse Mammary Tumorigenesis

To determine whether *PEA3* subfamily genes play a causative role in mammary oncogenesis, transgenic mice were isolated expressing dominant-negative PEA3 ($\Delta$NPEA3En) under the control of the MMTV promoter, which directs transgene expression primarily to the mammary epithelium of mice (35). Subsequently, MMTV-$\Delta$NPEA3En mice were mated to MMTV-HER2 transgenic mice. The onset of mammary tumors was significantly delayed and their incidence dramatically reduced in bi-transgenic virgin female offspring by comparison to matched MMTV-HER2 transgenic mice [Shepherd *et al.*, unpublished].

This effect apparently did not result from reduced expression of the HER2 transgene because the variation in the expression of HER2 was the same in MMTV-HER2 and bi-transgenic mammary tumors. This observation suggests that PEA3 is an obligate intermediate for the tumorigenic role of HER2.

Interestingly, dominant-negative PEA3 transcripts were not expressed in 6 of 7 mammary tumors from the bi-transgenic females. The mechanism whereby dominant-negative PEA3 expression is compromised in these tumors is not known. *MMTV-ΔNPEA3En* transgene expression was assessed by RNase protection assays and deletions in the transgene may not have been readily detected by this method. Hence either reduced transcription of the transgene or deletions in the transgene could account for our observations. Although many more tumors need to be analyzed to confirm these preliminary findings, they suggest that, in this model, mammary tumors arise primarily from epithelial cells that fail to express dominant-negative PEA3 or that express mutant forms of the protein. Whatever the explanation, these findings strongly suggest a requirement for PEA3 subfamily proteins or other Ets proteins with related DNA binding specificity for HER2-mediated mammary oncogenesis.

As discussed later, dominant-negative Ets2 does not affect mammary tumorigenesis in the PyMT model although PyMT-induced tumors arising in the mammary glands of MMTV-PyMT transgenic mice also overexpress *PEA3*. Hence dominant-negative PEA3 apparently acts with specificity to compromise the function of one or more Ets proteins in mammary epithelial cells required for oncogenesis.

Unexpectedly, the expression of dominant-negative PEA3 in mammary epithelial cells under MMTV promoter control did not compromise normal mammary gland development [Shepherd and Hassell, unpublished], contrasting with the observation that ductal branching in the mammary gland during puberty and early pregnancy was impaired in *PEA3*-null mice. That this phenotype is manifest in mice heterozygous for the targeted *PEA3* allele suggests that only a twofold difference in PEA3 protein levels affect ductal branching. These observations can be reconciled if the temporal and/or spatial expression profiles of PEA3 and dominant-negative PEA3 differ. For example, the *MMTV-ΔNPEA3En* transgene may not be expressed or it may be expressed poorly in the mammary epithelial cells that normally express *PEA3*. Alternatively the *ΔNPEA3En* transgene and the endogenous *PEA3* gene may be expressed in the same mammary epithelial cells but at different times during mammary gland development. The expression of ΔNPEA3En in mammary epithelial cells after ductal outgrowth and branching has occurred is unlikely to affect these processes despite their dependence on PEA3.

## Regulation of *PEA3* Subfamily Gene Expression in Mammary Tumors

The mechanisms leading to elevated *PEA3*, *ER81*, and *ERM* transcripts in HER2 and PyMT-induced mammary tumors are not known. HER2 and PyMT are known to signal through common signaling pathways (i.e., the Ras pathway) to effect transformation in cell culture and mammary oncogenesis in mice. The Ras pathway may be dysregulated in the majority of human breast tumors whether they possess elevated levels of HER2 or not and this finding could account for increased expression of the *PEA3* subfamily genes in the epithelial cells of such tumors.

We suspect that the downstream effectors of these signaling pathways include transcription activators that target the *PEA3* subfamily genes for regulation. In this regard it is noteworthy that the *PEA3* promoters of four different species, including humans and mice, have been shown to possess a highly conserved AP-1 binding site in the same position relative to the start site for transcription (Fig. 3) [Kann *et al.*, unpublished]. Ras signaling results in the increased activity of c-Jun, which is capable of binding to AP-1 sites as a homodimer or heterodimer with Fos family proteins thereby activating transcription. The *PEA3* promoters of both humans and mice also possess binding sites for Ets transcription factors; the position of these sites too is generally conserved relative to the start site of transcription. The activity of several Ets proteins is also increased by Ras signaling. The *ER81* and *ERM* upstream regulatory elements are not well characterized, but the transcription of these genes may be regulated similarly.

The fact that the expression of the *PEA3* subfamily genes is coordinately regulated during normal mammary gland development and tumorigenesis suggests that common signaling pathways and transcription factors regulate their expression. However, it bears mention that the signaling pathways affecting *PEA3* subfamily gene regulation have not been identified, and it is currently not known whether elevated

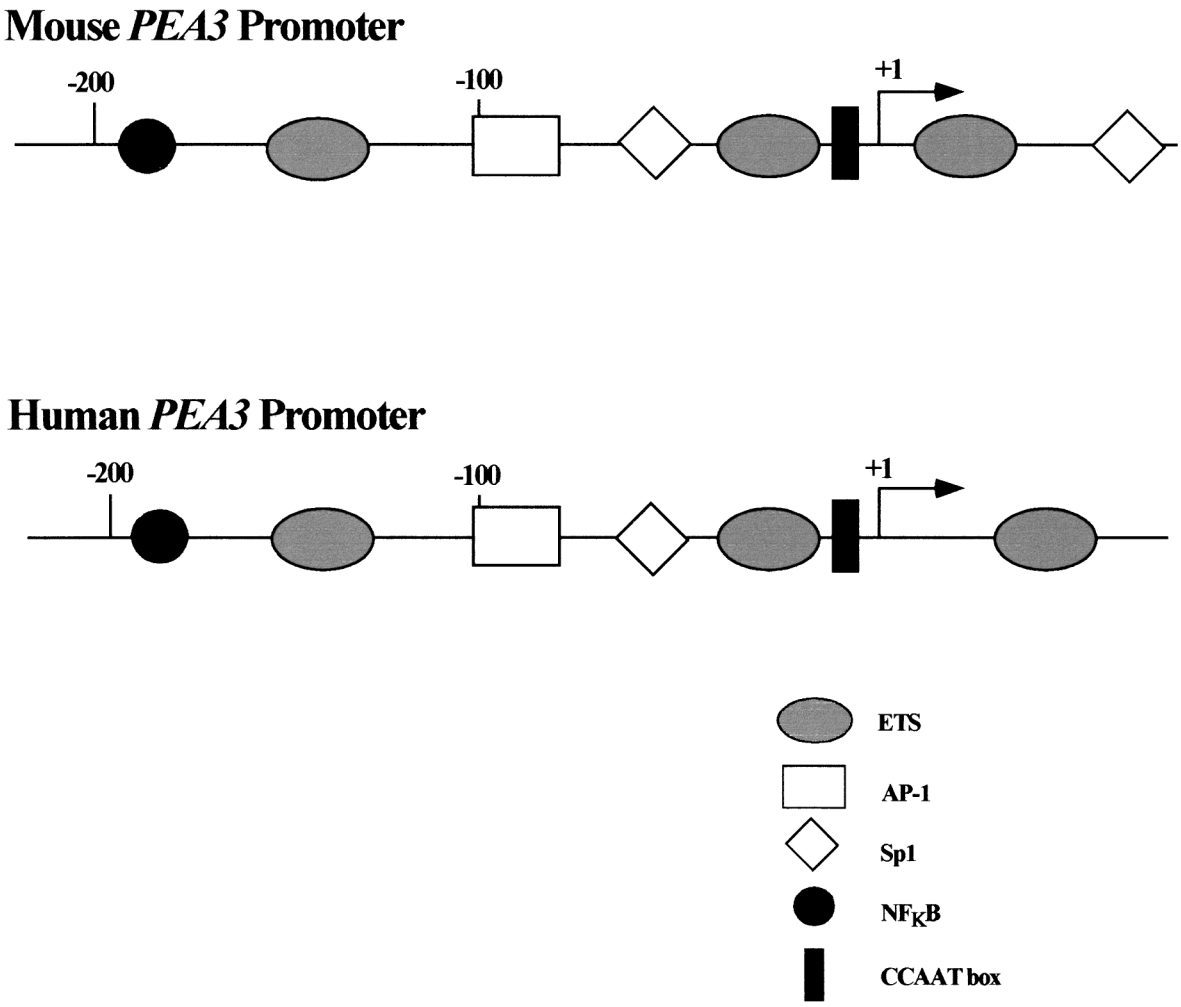


**Fig. 3.** The candidate promoters of the mouse and the human *PEA3* genes contain conserved candidate transcription factor binding sites. The relative position of the indicated transcription factor binding sites conserved in sequence and position relative to the start site of transcription between the mouse and human PEA3 promoters is illustrated. The location of the 5′ end of the mouse transcript has been mapped and is presumed to represent the transcriptional start site (+1) of the mouse and human genes. Neither the mouse nor human promoters bear a canonical TATA box.

*PEA3* subfamily transcripts in breast tumors arise from increased transcription of the *PEA3* subfamily genes or increased stability of their mRNAs.

### Role of Other *Ets* Genes in Mammary Oncogenesis

*Ets2* has also been implicated in mammary oncogenesis in mice. PyMT-induced mammary tumors occurring in transgenic mice heterozygous for a loss-of-function *ets2* allele are reduced in size (36). *Ets2*-null embryos fail to implant due to defects in extraembryonic trophoblasts; this phenotype can be rescued by aggregation with tetraploid embryos, which contribute wild type extraembryonic tissue (37). The mammary glands of *ets2*-null mice or mice heterozygous for the mutant *ets2* allele develop normally prior to puberty and during pregnancy, and otherwise appear indistinguishable from those of their age-matched wild type counterparts (36). Hence the effect of loss-of-function mutations in *ets2* is not an indirect consequence of retarded mammary gland development.

Recently, N. Kanno and R. Oshima (personal communication) derived transgenic mice that express dominant-negative Ets2 under MMTV control. Female offspring obtained by mating these mice to MMTV-PyMT transgenic mice develop mammary

tumors of the same size, and at the same rate and incidence as those that arise in MMTV-PyMT transgenic strains. Hence if dominant-negative Ets2 is functioning as expected in mammary epithelial cells expressing PyMT, then it clearly does not compromise mammary tumorigenesis effected by this oncoprotein.

This finding apparently contradicts the observation of a reduction in the size of PyMT-induced tumors in mice bearing a single functional *ets2* allele. One explanation, which could reconcile these data, is that reduced levels of active Ets2 in the mammary stroma, but not the mammary epithelium, compromise tumor growth. The cellular localization of *ets2* in the mammary gland has not been reported, but this gene appears to be ubiquitously expressed in mice (37,38).

A sampling of a number ($n = 10$) of human HER2-positive DCIS tumors revealed overexpression of *ESX* (also known as *ESE1*, *ERT*, *JEN*, and *ELF3*) transcripts by *in situ* hybridization compared to normal breast ductal epithelium ($n = 3$) (39). However, analysis of additional clinical samples is required to firmly link *ESX* overexpression to breast tumorigenesis, and to learn whether *ESX* expression is correlated with HER2 status or other molecular and clinical correlates of breast tumors. It is noteworthy that *ESX* is expressed in the normal mouse mammary gland and that its expression in this organ fluctuates during postnatal mammary gland development (40).

## *ETS* Gene Expression in Breast Tumor Cell Lines

*ETS* gene expression has also been described in human breast tumor cell lines growing in culture although the relevance of these findings to human breast tumorigenesis remains unclear. For example, an analysis of *PEA3* subfamily transcripts in 20 human breast tumor cell lines by comparison to two normal human mammary epithelial cell strains revealed that only two tumor cell lines failed to express any of the *PEA3* subfamily transcripts (41). The two normal cell strains expressed all three *PEA3* subfamily genes as did about half (9/20) of the cell lines. Nearly all (16/20) of the cell lines expressed both *PEA3* and *ERM*. *ER81* was expressed in slightly less than half (9/20) of the tumor cell lines and all of these cell lines also expressed *PEA3* and *ERM*. One cell line expressed only *PEA3*, and another only expressed *ERM*. Hence *PEA3* and *ERM*, and to a lesser extent *ER81* are generally coexpressed in breast tumor derived cell lines.

These findings in human tumor cell lines approximate our observation that the three *PEA3* subfamily genes are coordinately overexpressed in HER2-positive mouse mammary tumors. It is noteworthy that the expression of *PEA3* subfamily genes in primary human breast tumors has not been assessed. Hence it is not clear whether the expression profile of these genes in human breast tumor cell lines correlates with their expression profile in primary breast tumors or not.

By contrast to HER2-positive mammary tumors in mice, the extent of expression of the *PEA3* subfamily genes in human breast tumor-derived cell lines generally did not exceed that of the two normal mammary epithelial cell strains. One plausible explanation is that the conditions used to propagate cells *in vitro* modify *ETS* gene expression thereby obscuring differences between the normal mammary epithelial tumor cells and their transformed counterparts. Indeed neuregulin, which activates the tyrosine kinase activity of HER2 heterodimers, stimulates expression of *PEA3* [C. Benz, personal communication] and *ESX* in normal human mammary epithelial cell strains growing in culture (40). The expression of *ETS* genes needs to be studied under conditions *in vitro* that more closely mimic those existing *in vivo* to make meaningful comparison of their expression levels in normal and malignant cells.

## PEA3 Target Genes and HER2-Mediated Mammary Oncogenesis

The *HER2* gene is frequently overexpressed not only as a consequence of gene amplification but also as a result of transcriptional upregulation in many human breast tumor cell lines (42). The *HER2* upstream regulatory region contains a conserved Ets binding site and mutation of this sequence reduces transcription of linked reporter genes in several different mammalian cell lines, including human breast tumor cell lines (43). These findings are consistent with the hypothesis that one or more Ets proteins regulate transcription of the *HER2* gene and may account for its increased expression in breast tumor cells.

The fact that *PEA3* is overexpressed in breast tumors of both mice and humans suggested to us that it might fulfill this function (43). In light of our more recent findings it is also possible that *ERM* or *ER81* may also serve this role. Indeed, *E. coli* expressed PEA3 binds to the *HER2* promoter *in vitro* (43), and

transient transfection of a PEA3 expression vector with a *HER2* promoter-reporter in COS monkey kidney cells increased expression of the reporter in direct proportion to the amount of PEA3 expressed in the cells. Luciferase expression from the *HER2* promoter-reporter was also dependent on the presence of the conserved Ets binding site in the *HER2* promoter (20). Hence PEA3 seemingly has the potential to positively regulate *HER2* transcription in these cells. Other Ets proteins, including ESX, also bind to the *HER2* promoter and activate transcription of linked reporter genes (39).

However, a recent report suggests that PEA3 represses *HER2* promoter-reporter expression, apparently in a dose-dependent fashion, in a human ovarian (SKOV-3) and a breast (MDA-MB-453) carcinoma cell line contingent on the occurrence of the conserved PEA3 binding site in the *HER2* promoter (44). Mutation of the PEA3 binding site in the promoter severely compromised reporter gene expression in both cell lines suggesting that one or more Ets proteins endogenous to these cells regulate *HER2* transcription.

These findings are difficult to reconcile with the observation that *PEA3* is overexpressed in HER2-positive human breast tumors. Clearly if PEA3 acts as a repressor of *HER2* transcription in HER2-positive breast tumor cells, then *HER2* expression could only occur in these cells if another strong Ets transactivator functioned to regulate *HER2* transcription, or if a PEA3-specific repressor antagonized the activity of PEA3 in these cells. Alternatively the *HER2* promoter may be modified in HER2-positive breast tumor cells thereby precluding the specific binding of PEA3 to this promoter.

Whatever the explanation it bears mention that PEA3 and its related subfamily members commonly function as transcriptional activators in all cell types tested, including human breast tumor-derived cell lines (20,22,25,45,46). However, like other transcription activators, PEA3 squelches transcription from PEA3 responsive reporter plasmids when expressed at very high levels. Perhaps transcriptional squelching accounts for the reduced expression of the reporter gene from the *HER2* promoter in these recent studies (44). It is also not clear to what extent these two cell lines are representative of HER2-positive primary human tumor cells. Further experiments are required to resolve these issues.

Few *bona fide* PEA3 subfamily target genes are known, but PEA3 binding sites have been identified in the regulatory regions of many genes associated with tumorigenesis (47). A significant fraction of these encode proteases required for degradation of the extracellular matrix; their deregulated expression has been associated with the propensity of tumor cells to metastasize (48). For example, PEA3 binding sites occur in the promoters of the serine protease, urokinase plasminogen activator (49,50), and in those of several matrix metalloproteinases (45,51). PEA3 activates transcription of reporter genes directed by the MMP-1, MMP-9, and MMP-11 promoters (45). The metastatic potential of the MCF7 human breast cell line was increased by forced expression of PEA3 (52). Moreover, expression of anti-sense PEA3 in human tumor cells reduced their invasiveness (53). It is noteworthy that *PEA3* expression is also associated with migratory stem cells during embryogenesis (32,54). Hence its dysregulated expression in breast tumor cells may recapitulate its regulation during development. Whether any of these candidate PEA3 target genes plays a role in mammary gland development and tumorigenesis remains unknown. However, it is apparent that further progress in elucidating the role of *PEA3* and other *ets* genes in these processes will require the identification of the target genes of these transcription factors.

## CONCLUSIONS

*PEA3* subfamily *ets* genes are expressed in the epithelial cells of the embryonic mammary gland and their expression is coordinately regulated during postnatal mammary gland development. All three *PEA3* subfamily genes are also coordinately overexpressed in HER2-positive tumors. *PEA3* is expressed in cap cells and in myoepithelial cells of ducts and alveoli. In view of these observations it is tempting to speculate that *PEA3* subfamily *ets* genes are expressed in multipotential mammary stem cells and that they play a role in the differentiation program of these cells. Mammary epithelial stem cells may be the cells of origin of breast tumors. The overexpression of *PEA3* subfamily genes in these cells could accelerate their proliferation and/or prevent their differentiation. The study of the *PEA3* subfamily *ets* genes in mammary gland development and oncogenesis should shed light on both of these processes and may lead to the discovery of new therapeutic agents to treat breast cancer.

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