The *pea3* subfamily *ets* genes are required for HER2/Neu-mediated mammary oncogenesis

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Background: The PEA3 Ets transcription factor is overexpressed in the vast majority of human breast tumors and in nearly all of those of the HER2/Neu-positive subclass. PEA3 is also overexpressed in various transgenic mouse models of this disease. Whether PEA3 plays an essential role in HER2/Neu-mediated oncogenesis has heretofore not been addressed.

Results: Here, we report that each of the three highly related *ets* genes of the *pea3* subfamily (*pea3*, *er81*, and *erm*) were coordinately overexpressed in mammary tumors of MMTV-*neu* transgenic mice. Other *ets* genes normally expressed in the mammary gland were not upregulated in these tumors. Expression of a dominant-negative *pea3* transgene under the control of the MMTV promoter in mammary epithelial cells of MMTV-*neu* transgenic mice dramatically delayed the onset of mammary tumors and reduced the number and size of such tumors in individual mice. Those tumors that arose in bitransgenic mice expressed the MMTV-*neu* transgene, but not the MMTV-dominant-negative *pea3* transgene.

Conclusions: These findings imply that one or more of the PEA3 subfamily Ets proteins or other Ets proteins with related DNA binding specificity play an essential role in Neu-mediated mammary oncogenesis. Hence, agents that inhibit the expression or activity of the PEA3 subfamily proteins may prove efficacious in the treatment of breast cancer.

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Background

The *HER2* gene (also known as *c-erbB2* and *neu*) is amplified and/or overexpressed in several human malignancies, including breast cancer [1], and is linked with an increased propensity of such tumors to metastasize, resulting in a poor prognosis for the patient [2]. Increased expression of HER2 in breast tumors is also correlated with a poor response to hormonal and chemotherapeutic agents [3–5]. The *HER2* gene encodes a receptor tyrosine kinase structurally related to the epidermal growth factor receptor family proteins, which act through downstream signaling modules, including the Ras and phosphoinositide 3′-kinase pathways, to alter gene expression [1].

Mouse *pea3* (the human gene is named *E1A-F* and *ETV4*) is the founding member of the *pea3* subfamily of *ets* genes, which also include *er81* (*ETV1*) and *erm* (*ETV5*) [6–11]. Ets proteins share conserved winged helix-turn-helix ETS DNA binding domains comprising approximately 85 amino acids [12]. These proteins bind to sequence elements bearing a centrally located conserved 5′-GGA, A/T-3′ motif; the DNA binding specificity of individual Ets proteins is dictated by sequences flanking this core. Commonly, Ets proteins activate transcription, but some members of the family repress this process. Ets proteins play key roles in embryonic development; their mutation and/or overexpression is associated with multiple malignancies [13].

We showed previously that PEA3 is invariably overexpressed in mammary tumors of transgenic mice that express the wild-type rat *neu* cDNA under the transcriptional control of the MMTV promoter (MMTV-*neu*) [14]. PEA3 is similarly overexpressed in 76% of all human breast tumors; 93% of the HER2-positive subclass of such tumors, which represent 20%–30% of all breast tumors [2], overexpress PEA3 [15]. Here, we show that all three *pea3* subfamily *ets* genes are coordinately upregulated in mammary epithelial tumor cells of MMTV-*neu* transgenic mice, but other *ets* genes, including those commonly expressed in the mouse mammary gland, are not. Interference with PEA3 subfamily protein function in mammary epithelial cells of MMTV-*neu* transgenic mice delayed the onset and progression of mammary tumors, suggesting a required role for these Ets proteins in oncogenesis.

Results

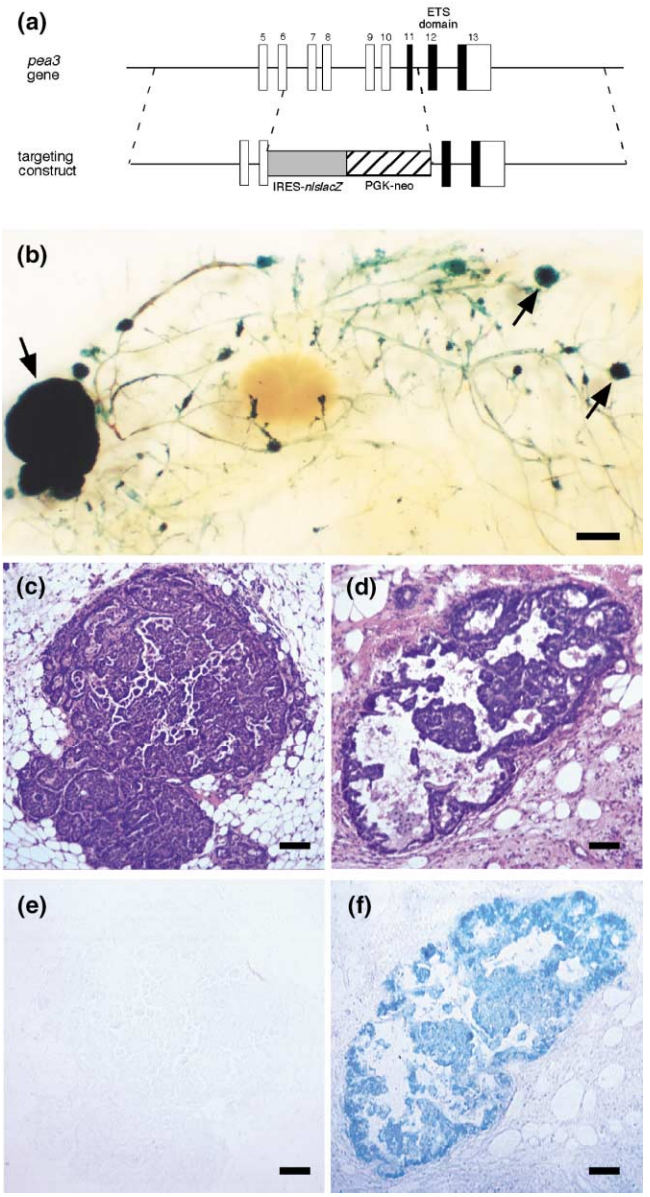
PEA3 is overexpressed in mouse mammary epithelial tumor cells

Whereas PEA3 is overexpressed in mouse mammary tumors appearing in mice bearing the MMTV-*neu* transgene, the cellular expression profile of PEA3 in these

tumors has not been assessed. To this end, we made use of mice bearing a mutation in the *pea3* gene; such mice harbor a deletion in the *pea3* gene, which is replaced by sequences representing an internal ribosome entry site and a nuclear localization signal linked to *Escherichia coli* β-galactosidase (*nlslacZ*) (Figure 1a). This mutant *pea3* allele ($pea3^{nlslacZ}$) is nearly identical in structure to that described previously; it only differs from the latter by the presence of sequences encoding *lacZ* at the site of the deletion in the *pea3* gene [16]. Mice homozygous for either *pea3* loss-of-function allele are viable; males are sterile, but the reproductive capacity of females is normal [16]. The profile of *lacZ* expression (β-galactosidase activity) in $pea3^{nlslacZ/+}$ mutant embryos and adults mimics that of the wild-type *pea3* gene.

To assess the expression of the $pea3^{nlslacZ}$ allele in mouse mammary tumors, we derived offspring from crosses between $pea3^{nlslacZ/+}$ mutant mice and MMTV-*neu* transgenic mice. The N#202 MMTV-*neu* transgenic strain, used throughout this study, bears the wild-type Neu cDNA. Mammary tumors arise in this transgenic strain due to deletions in sequences encoding the extracellular region of Neu [17], resulting in constitutive dimerization and consequent activation of its tyrosine kinase activity [18]. We prepared mammary gland whole mounts from the transgenic $pea3^{nlslacZ/+}$ female mice bearing mammary tumors and incubated these with X-gal to detect β-galactosidase activity. Multiple distinct tumor masses expressing β-galactosidase were readily apparent (Figure 1b). To examine the cellular expression profile of the mutant *pea3* allele, we incubated mammary tumors from MMTV-*neu* transgenic mice bearing either wild-type or a mutant *pea3* allele with X-gal and prepared serial sections. These sections were either stained with hematoxylin-eosin (Figure 1c,d) or processed without subsequent staining (Figure 1e,f). β-galactosidase activity was not detected in mammary tumors from mice bearing wild-type *pea3* alleles (Figure 1c,e), but β-galactosidase activity was present at high levels in the mammary tumor cells from MMTV-*neu* transgenic mice harboring a mutant *pea3* allele (Figure 1d,f). The stromal cells (principally fibroblasts and adipocytes) surrounding the mammary tumors of $pea3^{nlslacZ/+}$ mice did not express β-galactosidase activity, but the normal epithelial cells adjacent to the tumors expressed this activity at low levels (Figure 1f). Hence, PEA3 was selectively overexpressed in mammary epithelial tumor cells, the same cells that express the MMTV-*neu* transgene [19].

Figure 1

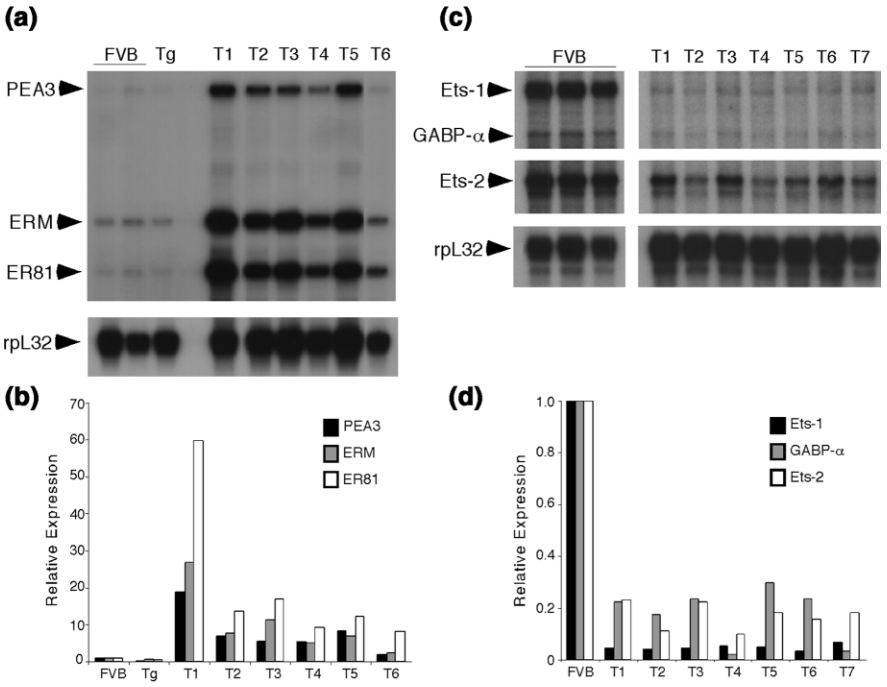


PEA3 is expressed in mouse mammary tumor cells of MMTV-*neu* transgenic mice. **(a)** Targeted knockin of a nuclear-localized β-galactosidase reporter gene into the *pea3* locus. **(b)** An X-gal-stained whole mount of the number 4 inguinal mammary gland isolated from a tumor-bearing MMTV-*neu*/$pea3^{nlslacZ/+}$ virgin female mouse. The arrows identify select mammary tumors. **(c–f)** Tissue sections of X-gal-stained mammary tumors isolated from either (c,e) MMTV-*neu*/$pea3^{+/+}$ or (d,f) MMTV-*neu*/$pea3^{nlslacZ/+}$ transgenic mice were (c,d) stained with hematoxylin and eosin or (e,f) mounted immediately after incubation with X-gal. The scale bars represent 1 mm in (b) and 100 μm in (c)–(f).

The *pea3* subfamily genes are coordinately overexpressed in Neu-induced tumors

To determine whether *ets* genes other than *pea3* are expressed in the mammary gland and overexpressed in mammary tumors, we compared the spectrum of Ets transcripts in RNA samples from two normal mammary glands of virgin mice and from two mammary tumors of MMTV-*neu* transgenic mice. We prepared cDNA from the RNA samples, amplified these with pairs of degenerate oligonucleotide primers complementary to ETS domain sequences, and cloned and sequenced 400 independent

Figure 2



PEA3 subfamily transcripts are increased in MMTV-*neu*-induced mammary tumors. **(a)** PEA3, ERM, and ER81 transcripts are expressed at low levels in the normal, virgin mouse mammary gland from either the FVB/N strain (FVB) or from asymptomatic MMTV-*neu* transgenic mice (Tg) but are increased substantially in mammary tumors (T1–T6) that arose in the MMTV-*neu* transgenic mice. A multiplex RNase protection assay was performed using riboprobes specific to mouse PEA3, ERM, and ER81 mRNA; mouse rpL32 was used as an internal control for RNA loading. **(b)** The PEA3 subfamily transcripts are increased in the MMTV-*neu* tumor samples compared to the normal mammary gland controls from either the nontransgenic FVB/N strain (FVB) or from asymptomatic MMTV-*neu* transgenic mice (Tg). Relative PEA3 subfamily transcript abundance was quantified by PhosphorImager analysis and was normalized to that of rpL32. The normalized abundance of each PEA3 subfamily transcript in the FVB/N samples was set to 1. **(c)** The abundance of Ets-1, Ets-2, and GABP-α mRNA levels is reduced in mammary tumors arising in the MMTV-*neu* transgenic mice. A multiplex RNase protection assay was performed using riboprobes specific to mouse Ets-1, Ets-2, and GABP-α mRNA; rpL32 was used as an internal control for RNA loading. All the lanes in this panel were derived from the same exposure of the original autoradiogram. **(d)** Quantification of the abundance of Ets-1, Ets-2, and GAPB-α transcripts in normal and mammary tumor tissue relative to that of the internal control transcript, rpL32. Transcript abundance was quantified as described above.

cDNAs (92 or more cDNAs from each RNA source). PEA3, ER81, ERM, GABP-α, Ets-1, Ets-2, Elf, and Tel cDNAs were readily isolated from all the samples. ER81 and ERM cDNAs were recovered at much higher frequencies from the tumor samples than from the normal samples, suggesting that *er81* and *erm* were overexpressed in mammary tumors compared to normal mammary tissue (data not shown).

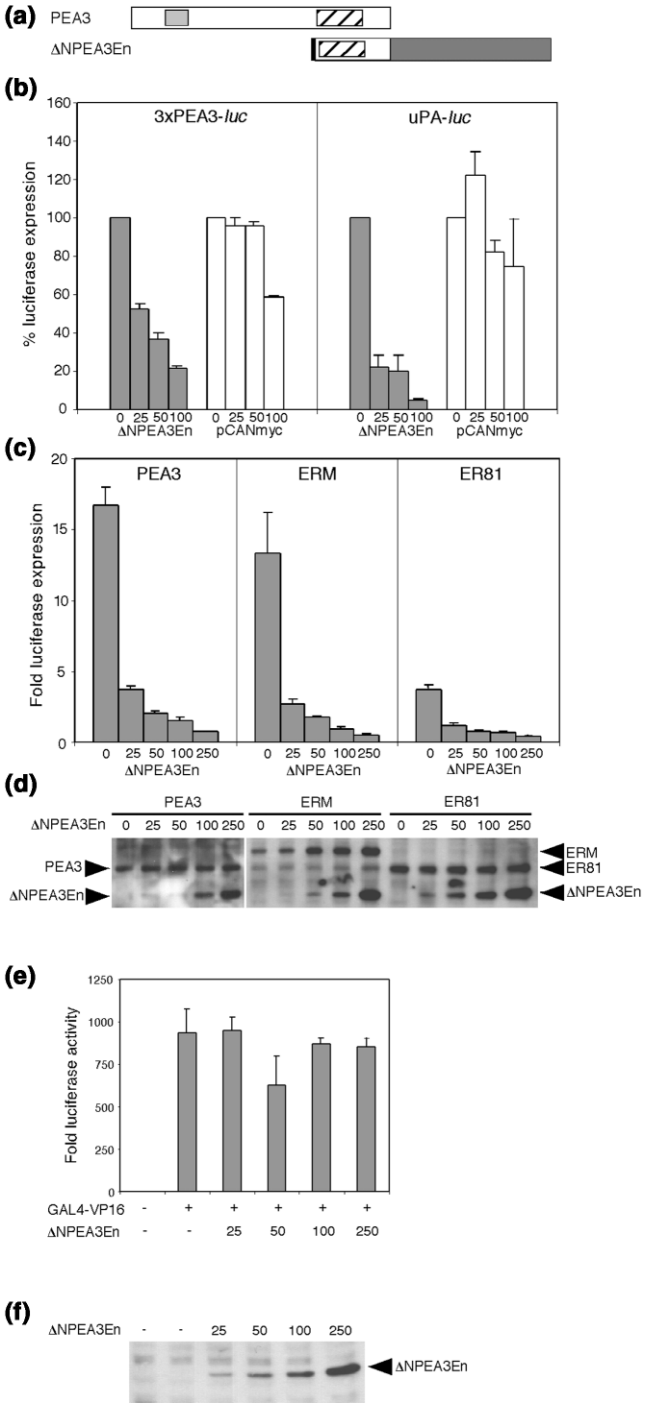
To investigate this potential, we compared PEA3, ER81, and ERM transcript levels in normal mammary gland samples with those in several mammary tumor samples from MMTV-*neu* transgenic mice by multiplex ribonuclease (RNase) protection analysis (Figure 2a,b). All three PEA3 subfamily transcripts were coordinately overexpressed in independent mammary tumors. The variation in the extent of overexpression of these transcripts among the tumors may reflect the cellular heterogeneity of the tumors. Mammary tumors comprise principally epithelial tumor cells, but multiple normal nonepithelial and epithelial cell types are intimately associated with the tumor mass or contaminate the margins of dissected tumor samples. The contribution of these cells to the tumor mass, particularly of small tumors, can affect quantification of transcript levels intrinsic to the tumor cells.

We also examined the transcript levels of three other *ets* genes (*ets-1*, *ets-2*, and *gabp*-α) in another set of similar samples (Figure 2c,d). These three *ets* genes were expressed at the same or greater levels than those of *pea3*, *er81*, and *erm* in the normal mammary gland samples. The abundance of the Ets-1, Ets-2, and GABP-α transcripts were reduced in the tumor samples compared to the normal mammary glands. The reduced expression of these *ets* genes in the tumor samples may reflect their downregulation in epithelial tumor cells. Alternatively, these genes may normally be expressed in nonepithelial cells; their apparent reduced expression in tumor tissue may reflect the reduced contribution of these stromal cells to the tumor mass compared to the normal mammary gland. Whatever the explanation, these data suggest that, among the *ets* gene transcripts examined, those of the *pea3* subfamily were selectively upregulated in Neu-induced mouse mammary tumors.

Dominant-negative PEA3 inhibits Neu-induced tumorigenesis

To learn whether the *pea3* subfamily *ets* genes play an essential role in Neu-mediated mammary oncogenesis in mice, we tested the capacity of a dominant-negative allele of *pea3* to affect this process. To this end, we deleted sequences encoding the activation domain of PEA3 [20]

Figure 3



Dominant-negative PEA3 reduces PEA3 subfamily protein function in cell culture in a dose-dependent fashion. **(a)** Structure of PEA3 and of ΔNPEA3En. PEA3 encompasses a strong activation domain within its amino terminus (light gray box) and an ETS DNA binding domain (hatched box) near its carboxyl terminus. ΔNPEA3En possesses a Myc epitope at its amino terminus (black box) coupled to a PEA3 carboxyl-terminal fragment bearing the ETS domain (hatched box) and the *Drosophila* Engrailed repression domain (residues 2–298; dark gray box). **(b)** Expression of luciferase from the 3xPEA3-*luc* and *uPA-luc* PEA3-responsive reporters is repressed in a dose-dependent fashion by ΔNPEA3En in mouse FM3A mammary adenocarcinoma cells. Different DNA doses (25–100 ng) of the pCANmyc vector encoding ΔNPEA3En or the empty expression vector (pCANmyc) were transfected as described in the Materials and methods. **(c)** Dominant-negative PEA3 reduces the capacity of PEA3, ERM, and ER81 to activate luciferase expression from the 5xPEA3-*luc* reporter plasmid in COS-1 cells in a dose-dependent fashion. The pCANmyc expression vector was used to effect the expression of ΔNPEA3En, PEA3, ERM, and ER81; the empty expression vector (pCANmyc) was also independently transfected in these experiments to serve as a control. Increasing doses (25–250 ng) of the appropriate expression vector were transfected as described in the Materials and methods. The basal luciferase activity resulting from transfection of the reporter plasmid with the empty expression vectors was set at 1. **(d)** A representative immunoblot illustrating the abundance of PEA3, ERM, ER81, and ΔNPEA3En in a single experiment. Each of these proteins bears an amino-terminal c-Myc epitope; their abundance was simultaneously determined on the same immunoblot with a monoclonal antibody directed against this epitope. **(e)** Dominant-negative PEA3 does not affect GAL4-VP16-mediated activation of a GAL4-responsive luciferase reporter plasmid in COS-1 cells. COS-1 cells were transfected with a fixed amount of the expression vector encoding GAL4-VP16 and with increasing doses (25–250 ng) of the pCANmyc/ΔNPEA3En expression vector as described in the Materials and methods. The basal luciferase activity resulting from transfection of the reporter plasmid with the empty expression vectors was set at 1. **(f)** A representative immunoblot illustrating the abundance of ΔNPEA3En in a single experiment. The abundance of the Myc-tagged ΔNPEA3En protein was assessed with a monoclonal antibody directed against the c-Myc epitope.

and appended sequences coding for the repression domain from the *Drosophila melanogaster* Engrailed (En) protein to derive ΔNPEA3En (Figure 3a). Previous analyses of a similar Myb-En fusion protein demonstrated that such proteins interfere with Myb function in specific tissues of transgenic mice [21].

To ensure that ΔNPEA3En functioned as expected, we assessed its capacity to affect reporter gene expression from two different PEA3-responsive reporters in transient transfection experiments. One reporter bears an artificial promoter comprising repeats of an optimal PEA3 binding site juxtaposed to a TATA box, whereas the other contains the urokinase plasminogen activator (*uPA*) enhancer/promoter. We assessed the capacity of ΔNPEA3En to affect expression of luciferase from these reporters in mouse FM3A cells, which express high endogenous levels of PEA3 [6]. ΔNPEA3En reduced expression of both reporters in a dose-dependent fashion in these cells (Figure 3b). We also assayed the capacity of ΔNPEA3En to affect PEA3 subfamily-activated reporter gene expression in COS-1 cells, which do not express PEA3 at detectable levels [6]. ΔNPEA3En suppressed PEA3-, ERM-, and ER81-activated luciferase expression in a dose-dependent fashion, suggesting that it can compromise the function of all three PEA3 subfamily proteins (Figure 3c). Western immunoblotting experiments revealed that the doses of ΔNPEA3En used in these experiments did not affect expression of PEA3, ERM, or ER81 from the pCANmyc vectors encoding these proteins (Figure 3d). ΔNPEA3En

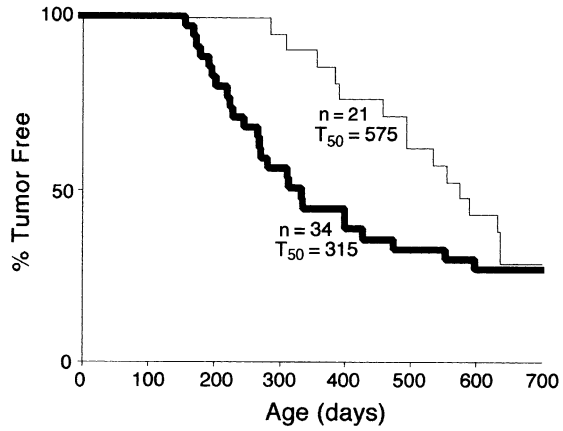
was specific in its ability to inhibit the function of the PEA3 subfamily proteins because it was unable to repress GAL4-VP16-mediated expression of luciferase from a GAL4-responsive luciferase reporter, which does not contain PEA3 binding sites (Figure 3e). Western immunoblotting experiments showed that ΔNPEA3En was expressed in a dose-dependent manner in these experiments (Figure 3f) to levels equivalent to those required to nearly completely repress PEA3-, ERM-, or ER81-activated luciferase expression from the PEA3-responsive reporter in the same COS cells (Figure 3d,f). These observations suggest that ΔNPEA3En inhibited the function of the PEA3 subfamily proteins likely by competing for their binding sites in the PEA3-responsive promoter of the reporter.

To determine whether ΔNPEA3En could similarly affect PEA3 subfamily protein function in mammary epithelial cells of transgenic mice, we placed the coding sequences for this protein under the transcriptional control of the MMTV promoter and used the resulting DNA to derive independent transgenic founder strains. One transgenic strain, which expressed ΔNPEA3En transcripts in organs characteristic of the tissue specificity of the MMTV promoter, was used in subsequent experiments. The mammary glands of both virgin and lactating female mice of this strain expressed ΔNPEA3 transcripts at high levels relative to other organs. Whole-mount analyses of the mammary glands of the MMTV-ΔNPEA3En mice revealed no obvious developmental abnormalities during postnatal mammary gland development. These mice nursed their pups, and the pups grew at normal rates, suggesting that the expression of ΔNPEA3En in the mammary gland did not affect the function of this organ.

To learn whether the expression of ΔNPEA3En affected mammary oncogenesis, we monitored the time of the appearance of mammary tumors in nulliparous female offspring resulting from crosses between MMTV-ΔNPEA3En and MMTV-*neu* transgenic mice. We compared the time of onset of mammary tumors in the MMTV-*neu*/ΔNPEA3En bitransgenic mice with that in MMTV-*neu* transgenic mice (Figure 4). Tumors occurred in 50% of the MMTV-*neu* transgenic mice by 315 days of age, but 575 days were required for tumors to appear in 50% of the bitransgenic mice. This difference is statistically significant, as determined by the log-rank test ($p < 0.003$). By the end of the experiment (700 days), approximately 75% of the mice in both cohorts developed mammary tumors.

Multiple tumors arise in different mammary glands of the MMTV-*neu* transgenic mice [17]. Generally, additional independent tumors appear and progress during the 2-month period after the primary tumor is first detected by palpation. Two or more tumors were found in 71% (n = 14) of the bitransgenic mice 2 months after the primary tumor was detected (Table 1). By contrast, the bitransgenic strain developed multiple tumors far less frequently: only 21% (n = 14) of the mice developed two or more tumors during this time frame. We noted upon autopsy that the primary tumors in the bitransgenic mice were generally smaller 2 months after they were initially detected than those in the MMTV-*neu* transgenic strain. To quantify this difference, we weighed the mammary tumors arising in each transgenic strain 2 months after they were first detected. The mean weight of tumors arising in the MMTV-*neu* strain was 3.0 g (n = 13), whereas that of the tumors in the bitransgenic strain was 1.8 g (n = 14) (Table 1). Primary mammary tumors arising in the MMTV-*neu* strain metastasize to the lungs at high frequency [22]. However, we did not detect a statistically significant difference in the percentage of mice bearing lung metastases 2 months after the primary tumors arose between the two transgenic strains (Table 1). Collectively, these observations suggest that the expression of ΔNPEA3En in the mammary glands of MMTV-*neu* transgenic mice delayed the appearance of mammary tumors and reduced their number and growth in individual mice.

Figure 4



ΔNPEA3En delays mammary tumor onset in MMTV-*neu*/ΔNPEA3En bitransgenic mice. Virgin female mice bearing either the MMTV-*neu* transgene (dark line) or the MMTV-*neu* and MMTV-ΔNPEA3En transgenes (thin line) were palpated for mammary tumors weekly.

To determine whether Neu and ΔNPEA3En transcripts were expressed in mammary tumors in the bitransgenic mice, we carried out RNase protection analysis (Figure 5a). Neu transcripts were generally overexpressed in mammary tumors compared to unaffected contralateral mammary glands from the same mice, in keeping with previous findings [17, 22]. The incidence of transgene deletions leading to constitutive activation of Neu was the same in the MMTV-*neu* and the bitransgenic mice,

Table 1

Summary of tumor data from studies of MMTV-*neu* and MMTV-*neu*/ΔNPEA3En transgenic mice.

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

[a] The median age of tumor onset as scored by identification of a palpable tumor.
[b] The number of tumors present at the end of the 2 months subsequent to initial identification of a tumor. Mice were grouped into those having a single mammary tumor (1) versus those having two or more (≥2) tumors. The difference is statistically significant ($p = 0.0039$) as determined by the one-sided z test.
[c] The weight of the earliest arising mammary tumor was measured. The difference between MMTV-*neu* and MMTV-*neu*/ΔNPEA3En tumors is statistically significant ($p < 0.02$) as determined by the Student's t test.
[d] The percentage of mammary tumor-bearing mice, which also possessed lung metastases, was determined by analyzing multiple haematoxylin- and eosin-stained tissue sections of the lungs 2 months after initial palpation of the mammary tumor. The number of metastatic lesions per lung was not assessed because these were too numerous to be accurately enumerated at this time.

suggesting that expression of ΔNPEA3 in the bitransgenic mice did not affect this process (data not shown).

Surprisingly, ΔNPEA3En transcripts were not expressed or were expressed at much lower levels in the mammary tumors compared to the normal contralateral mammary gland of the same mouse. Indeed, ΔNPEA3En was only expressed in the mammary tumors from two of seven mice examined (T2 and T7) and then only at levels substantially lower than those observed in the histologically normal mammary glands contralateral to the tumors. The fact that ΔNPEA3En was not generally expressed in the mammary tumors strongly suggests that this protein interfered with Neu-induced mammary oncogenesis. Like mammary tumors of MMTV-*neu* transgenic mice, those arising in the bitransgenic mice also overexpressed PEA3 compared to the normal control mammary tissue (data not shown).

The core sequence (5′-GGA, A/T-3′) characteristic of Ets protein binding sites (EBS) is present in the MMTV promoter [23], and the Ets-protein, GABP-α, can activate transcription from the MMTV promoter dependent on these sites [24]. Hence, it is formally possible that ΔNPEA3En can bind to these sites when expressed at high levels and thereby repress *neu* transgene expression, thus affecting mammary oncogenesis. To explore this potential, we used a mammary epithelial cell line (NDL) derived from a mammary tumor of an MMTV-*neu* transgenic mouse. The NDL cell line supported high-level expression of an MMTV-luciferase reporter plasmid (Figure 5b). Independent cotransfection of an expression vector encoding either PEA3 or ΔNPEA3En with the MMTV-luciferase reporter did not affect expression of luciferase under conditions in which both proteins were expressed to high levels in these cells (Figure 5b,c). Hence, it seems unlikely that either PEA3 or ΔNPEA3En binds to the EBS in the MMTV promoter even when they are overexpressed. These experiments also illustrate that ΔNPEA3En did not function in a nonselective manner to repress transcription from a complex viral promoter bearing EBS in Neu-transformed mammary epithelial tumor cells.

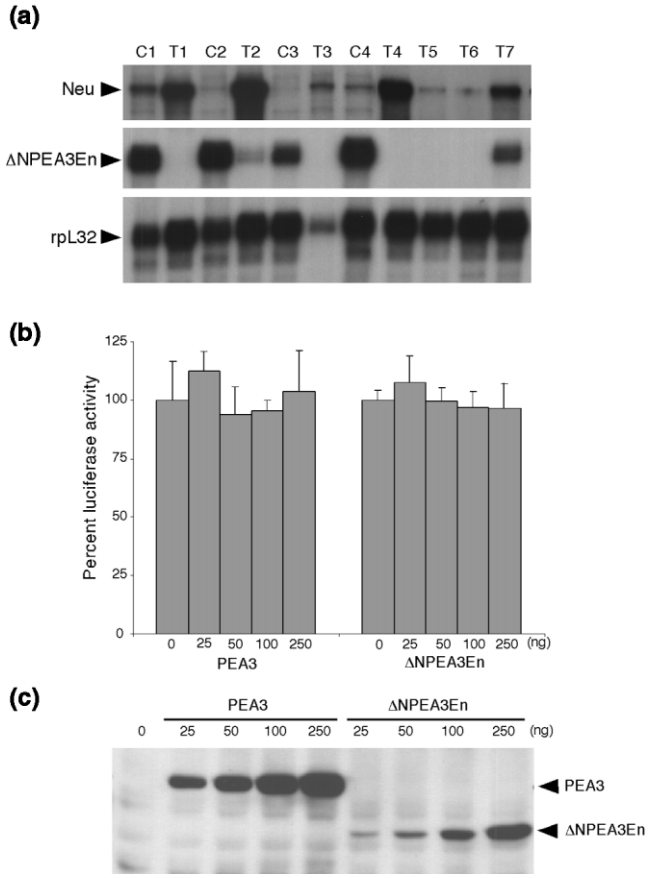
Discussion

The *pea3* subfamily genes were coordinately overexpressed in Neu-induced mouse mammary tumors. Three other *ets* genes, *ets-1*, *ets-2*, and *gabp*-α, which are normally expressed in the mammary gland, were not overexpressed in mammary tumors, suggesting that upregulation of the *pea3* subfamily genes was specific. The genes comprising the *pea3* subfamily have an identical architecture, strongly suggesting that they arose from a common ancestral gene [25]. Hence, these genes may also share common promoter elements; Neu may regulate the activity of transcription factors that bind to these sites, thus accounting for their coordinate overexpression in mammary tumors. This hypothesis is supported by the observation that induction of the oncogenically activated Raf kinase, which is known to act downstream of Neu, leads to the upregulation of *pea3* transcripts in human mammary epithelial cells [26].

The expression of dominant-negative PEA3 under the aegis of the MMTV promoter in mammary epithelial cells delayed the onset of mammary tumors, reduced the number of such tumors in individual mice, and retarded the growth of these tumors in the bitransgenic strain. Tumors arising in the bitransgenic mice did not express the MMTV-ΔNPEA3En transgene or expressed it at low levels. These observations are consistent with a requirement for one or another PEA3 subfamily Ets protein for Neu-mediated mammary oncogenesis.

The requirement to extinguish ΔNPEA3En function in Neu-positive mammary epithelial cells to ensure their progression to malignancy likely accounts for the delayed onset and the reduced size and incidence of multiple tumors in the bitransgenic mice. We imagine that both the MMTV-*neu* and MMTV-ΔNPEA3En transgenes are

Figure 5



(a) Expression of the MMTV-ΔNPEA3En transgene is reduced or undetectable in mammary tumors arising in MMTV-*neu*/ΔNPEA3En bitransgenic mice. **(b)** Increasing amounts of either PEA3 or ΔNPEA3En do not affect luciferase expression from the MMTV promoter in NDL mouse mammary tumor cells. The basal luciferase activity resulting from transfection of the reporter plasmid with the empty expression vector was set at 100%. **(c)** A representative immunoblot illustrating the abundance of PEA3 and ΔNPEA3En in a single experiment. The abundance of these Myc-tagged proteins was assessed with a monoclonal antibody directed against the c-Myc epitope.

initially expressed in the epithelial cells of the origin of mammary tumors. Oncogenesis is likely initiated by mutations in the *neu* transgene in rare epithelial cells, but the progression of these cells to full malignancy may be retarded by ΔNPEA3En. This barrier to tumor progression may be breached in rare epithelial cells expressing both transgenes by a genetic or an epigenetic mechanism that results in reduced ΔNPEA3En expression and function. The need to block ΔNPEA3En expression in initiated tumor cells, a condition that is not required for tumors to arise in the MMTV-*neu* transgenic strain, likely accounts for the long delay in time to progression and incidence of mammary tumors in the bitransgenic mice. The reduced size of mammary tumors in the bitransgenic strain may be due to the episodic reexpression of ΔNPEA3En in mammary tumors between the time these tumors first arise and are palpable and the time of sacrifice of the mice 2 months later. It is noteworthy that the expression of transgenes is unstable [19, 27].

It is formally possible that the effect of ΔNPEA3En on Neu-mediated tumor progression in the bitransgenic mice resulted from the inhibition of transcription of the MMTV-*neu* transgene. ΔNPEA3En may bind directly to EBS in the MMTV promoter and thereby inhibit *neu* transgene transcription or it may act indirectly to negatively affect expression of the *neu* transgene in bitransgenic mice. Such a model would require that the two MMTV transgenes respond differently to ΔNPEA3En despite the fact that the same promoter regulates expression of each transgene in the same cell. Both the MMTV-ΔNPEA3En and MMTV-*neu* transgenes were expressed in the normal contralateral glands of tumor-bearing bitransgenic mice and in the normal mammary glands of asymptomatic bitransgenic mice, suggesting that they can be coexpressed in mammary epithelial cells. Furthermore, we could not repress MMTV reporter gene expression by overexpression of ΔNPEA3En, nor could we activate this reporter by expression of PEA3 in Neu-transformed mouse mammary tumor cells despite the occurrence of EBS in the MMTV promoter. Hence, we think it is unlikely that ΔNPEA3En represses expression of the MMTV-*neu* transgene in mammary tumor cells and that this accounts for its effect on mammary oncogenesis.

Recently, Xing et al. [28] reported that PEA3 suppresses *HER2* promoter activity in transient transfection experiments in two different human tumor-derived cell lines dependent on the occurrence of a PEA3 binding site in this promoter and inhibits the capacity of these cell lines to form tumors in nude mice. These investigators suggested that PEA3 functions to repress transcription of the endogenous *HER2* gene, thereby reverting the transformed phenotype of these tumor cell lines, and offered the prospect that PEA3 might prove efficacious either alone or in combination with other agents to combat HER2-positive human tumors. By contrast, in addition to others, we have observed that PEA3, ERM, and ER81 are each capable of activating the expression of reporter genes coupled to PEA3-responsive promoters, including the human *HER2* promoter in various cell types including mammary epithelial tumor cells ([6, 20, 29–31]; this report). We have noted, however, that the expression of these same reporter genes is inhibited when each of the PEA3 subfamily proteins is expressed at high levels. We suspect that transcriptional squelching accounts for these observations. These findings coupled with our previous observations demonstrating increased PEA3 transcript levels in both mouse [14] and human [15] HER2/Neu-positive mammary tumors and those reported herein

strongly suggest that use of PEA3 as a therapeutic agent is contraindicated.

Conclusions

We hypothesize that PEA3 subfamily function is required for Neu-mediated mammary oncogenesis. This hypothesis is consistent with the finding that PEA3 subfamily transcripts were overexpressed in nearly all HER2/Neu-positive mammary tumors and with the observation that abrogating PEA3 subfamily function in the mammary gland suppressed Neu-mediated mammary oncogenesis. Whereas the inhibitory dominant-negative PEA3 protein may compromise the function of Ets proteins with DNA binding specificity related to that of the PEA3 subfamily members, there is no evidence to suggest that Ets transcription factors other than those of the PEA3 subfamily are overexpressed in mammary tumors. Hence, it seems likely that PEA3 Ets subfamily function is required for mammary oncogenesis. PEA3 is also overexpressed in mammary tumors induced by Wnt-1 [32] in MMTV-*Wnt-1* transgenic mice and in intestinal tumors of multiple intestinal neoplasia (Min) mice, which harbor inactivating mutations in the adenomatous polyposis coli (*Apc*) tumor suppressor gene [31]. Wnt-1 signals through the Wnt/β-catenin pathway, which includes Apc as one of its signaling elements, to effect oncogenesis in these two mouse strains. Hence, PEA3 and other members of the PEA3 Ets subfamily may play key roles in oncogenesis initiated by multiple signaling pathways, including the Neu/Ras and Wnt/β-catenin pathways, which are dysregulated in diverse malignancies. Consequently, we propose that agents that inhibit the expression or function of PEA3 and its close relatives ER81 and ERM, or of their target genes, may prove efficacious in the treatment of Neu/HER2-positive tumors and perhaps other tumors in humans characterized by PEA3 subfamily overexpression.

Materials and methods

DNA constructs

The pCANmyc expression vector (a gift from Dr. Paul Polakis) bears the human cytomegalovirus promoter upstream of sequences encoding an epitope endogenous to the c-Myc protein [33]. The molecular cloning and expression of cDNAs downstream of the c-Myc epitope leads to the synthesis of fusion proteins bearing this epitope at their amino termini. PCANmyc/ΔNPEA3En was constructed by PCR amplification of sequences encoding amino acids 334–480 of mouse PEA3 cDNA using the RSV-PEA3 expression plasmid [20] and the pSCDMSMenT plasmid (a gift from Dr. Ali Fattaey) bearing the *Drosphila* repression domain as templates. pCANmyc/PEA3 was isolated by cloning full-length mouse PEA3 cDNA from pGEM7PEA3 into pCANmyc1. Full-length mouse ERM cDNA was isolated by RT-PCR using mouse mammary tumor RNA and was cloned into pCANmyc1. Full-length mouse ER81 cDNA was isolated by PCR of ER81 sequences from pBS/ER81 (a gift from Dr. Tom Jessell) and was cloned into the pCANmyc1 expression vector to generate pCANmyc/ER81. The DNA sequence of all recombinant plasmids was determined to ensure that mutations were not introduced during PCR. The 3xPEA3-*luc* reporter was constructed by isolating three of the four PEA3 binding sites from 4xPEA3-CAT [6] and cloning them into pGL3-Basic (Promega) to generate 3xPEA3-*luc*. The *uPA-luc* reporter plasmid was a gift from Dr. Craig Hauser [34]. The 5xPEA3-*luc* reporter plasmid, the GAL4-VP16 expression vector, and the 5xGAL4-*luc* reporter plasmid have been described previously [20]. The MMTV-*luc* reporter was constructed by isolating the luciferase gene from pGL3-Basic and cloning it into the MMTV-SV40 plasmid [22]. To generate MMTV-ΔNPEA3En/SV40pA, the DNA encoding ΔNPEA3En was isolated from pCANmyc/ΔNPEA3En and was cloned into the MMTV-SV40 plasmid. The sequence of the DNA primers and details concerning the construction of the expression and reporter vectors are available from the authors upon request. The PEA3 riboprobe template encompasses nucleotides 1445–1941 of mouse PEA3 cDNA cloned into pBluescriptIIKS. The plasmid was linearized with BamHI to generate an antisense PEA3 RNA probe using T3 RNA polymerase and was used to detect PEA3 and ΔNPEA3En transcripts. The ERM riboprobe template encompasses nucleotides 162–438 of mouse ERM cDNA cloned into pSL301 (Stratagene); it was linearized with HindIII to generate an antisense ERM RNA probe using T3 RNA polymerase. The ER81 riboprobe template encompasses nucleotides 945–1197 of mouse ER81 cDNA cloned into pBluescriptIIKS; it was linearized with HindIII to generate an antisense ER81 probe using T7 RNA polymerase. The Ets-1 riboprobe template encompasses nucleotides 135–787 cloned into pBluescriptIIKS; it was linearized with XhoI to generate an antisense Ets-1 RNA probe using T7 RNA polymerase. The Ets-2 riboprobe template (a gift from Dr. Robert Oshima) has been described previously [35]; this DNA was linearized with EcoRI to generate an antisense Ets-2 RNA probe using SP6 RNA polymerase. The GABP-α riboprobe encompasses nucleotides 690–975 of mouse GABP-α cloned into pBluescriptIIKS. The plasmid was linearized with BamHI to generate an antisense GABP-α RNA probe using T3 RNA polymerase. The rpL32 riboprobe template has been described previously [14]; it was linearized with XbaI to generate an antisense rpL32 RNA probe using T3 RNA polymerase. The MMTV-*neu* riboprobe template has been described previously [17]; it was linearized with SmaI to generate an antisense Neu RNA probe using T7 RNA polymerase.

Cell culture and transient transfections

Cell lines were cultured as described previously [6, 20]. Transient transfections were carried out using LipofectAMINE Reagent (Life Technologies). FM3A cells were seeded at 5×10^5 cells per 35-mm well the day prior to transfection. A total of 0.1 μg reporter plasmid (3xPEA3-*luc* or *uPA-luc*) was cotransfected with varying amounts of either pCANmyc or the pCANmyc/ΔNPEA3En expression plasmid. The cells were lysed 24 hr posttransfection using Reporter Lysis Buffer, and luciferase assays were performed using Luciferase Assay Reagent (Promega) and a Lumat luminometer (Berthold, Perkin Elmer). Luciferase activity was normalized to the total protein concentration of the lysates, which were assessed using the Bradford assay kit (Bio-Rad). COS-1 cell transfections and analyses of luciferase activity were performed similarly, except for the following modifications. A total of 1.25×10^5 cells were seeded the day prior to transfection, and 0.25 μg 5xPEA3-*luc* reporter was cotransfected with either 0.025 μg pCANmyc/PEA3, 0.25 μg pCANmyc/ERM, or 1 μg pCANmyc/ER81. Similarly, 0.1 μg 5xGAL4-*luc* reporter and 0.25 μg GAL4-VP16 effector plasmid were transfected into COS-1 cells. Transient transfections of the mouse mammary epithelial NDL tumor cell line was carried out as described above after seeding 2.5×10^5 cells per plate a day earlier; 0.5 μg MMTV-*luc* reporter was used with varying amounts of the effector DNAs encoding PEA3 or ΔNPEA3En.

Western immunoblotting

Protein isolation from transfected cells and immunoblotting were performed essentially as described previously [20]. Total protein (40 μg) from COS-1 cells and from NDL cells (100 μg) was separated on a 10% SDS-polyacrylamide gel prior to transfer. Proteins was detected using the anti-9E10 c-*myc* monoclonal antibody and goat anti-mouse horseradish peroxidase-conjugated secondary antibody (KPL; 474–1806), followed by incubation with Western Blot Chemiluminescence Reagent Plus (NEN Life Science Products) and exposure to X-ray film (Kodak Scientific Imaging).

Knockout and transgenic mice

Mice bearing a *pea3*^{nlslacZ} allele were derived using a targeting vector of essentially the same structure as that used previously to functionally inactivate the *pea3* gene [16]. The targeting construct bears 1.8 kb of *pea3* genomic DNA on the 5′ flank and 10.8 kb on the 3′ flank, separated by an IRES linked to *nlslacZ* coding sequences coupled to a pA signal and a PGKneoPA expression cassette. Homologous recombination between the targeting construct and the endogenous *pea3* gene in ES cells results in deletion of sequences comprising a part of exon 6 and exons 7–11. Exons 11, 12, and part of exon 13 encode the PEA3 ETS DNA binding domain. The isolation and characterization of the N#202 MMTV-*neu* line of transgenic mice used in this study has been described previously [22, 36]. The generation of the transgenic mice expressing ΔNPEA3En under the control of the MMTV promoter was performed as described previously [22]. Both transgenic lines were established in and maintained on the FVB/N strain background. Mice were palpated for mammary gland tumor nodules every week beginning at 5 months of age, monitored weekly for 2 months subsequent to the appearance of tumors, and sacrificed thereafter.

β-galactosidase activity assays in mouse tissues

β-galactosidase activity was performed essentially as previously described using X-gal as a substrate [37]. The tumor tissues used in histological analyses were paraffin-embedded and sectioned at 8 μm, and serial sections were processed for either hematoxylin-eosin staining or mounted directly to visualize β-galactosidase activity. Histological slides were photographed using a Zeiss Axioskop, and adjustments of brightness and contrast were made using Adobe PhotoShop 3.0.1 software.

RNA isolation and RNase protection assay

Mouse tissue RNA was prepared and analyzed by RNase protection analysis as described previously [16]. The relative abundance of the various RNA species was assessed by using a PhosphorImager equipped with ImageQuant 3.3 software (Molecular Dynamics).

Supplementary material

A supplementary figure illustrating the tissue-specific expression of the MMTV-ΔNPEA3En transgene is available at http://images.cellpress.com/supmat/supmatin.htm.

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