

Award Number DAMD17-96-1-6223

TITLE: Development of PEA3 as a Potential Gene Therapy Agent for Breast Cancer

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REPORT DATE: October 1999

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE October 1999	3. REPORT TYPE AND DATES COVERED Final (1 Sep 96 - 31 Aug 99)	
4. TITLE AND SUBTITLE Development of PEA3 as a Potential Gene Therapy Agent for Breast Cancer			5. FUNDING NUMBERS DAMD17-96-1-6223	
6. AUTHOR(S) Mr. Zhenming Yu				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The University of Texas M. D. Anderson Cancer Center Houston, Texas 77030			8. PERFORMING ORGANIZATION REPORT NUMBER	
E-mail: zyu@gsbs3.gs.uth.tmc.edu				
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Deterick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words) <p style="text-align: center;">HER-2/neu overexpression plays an important role for cancer development. To suppress cell transformation mediated by HER-2/neu overexpression, we identified a DNA-binding protein PEA3 of the Ets gene family which specifically targeted a DNA sequence on the HER2/neu promoter and downregulated the promoter activity. Expression of PEA3 resulted in preferential inhibition of cell growth and tumor development of HER-2/neu-overexpressing cancer cells. A competition model to elucidate the molecular mechanism for the transcription inhibition of Her-2/neu by PEA3 was proposed. PEA3 directly binds to the HER-2/neu promoter and competition of PEA3 with other Ets transactivating factor(s) for a positive regulatory motif on the Her-2/neu promoter may account for the downregulation of Her-2/neu transcription by PEA3.</p>				
14. SUBJECT TERMS Breast Cancer, PEA3, Her-2/neu, Transcriptional Repression, Tumor Suppression.			15. NUMBER OF PAGES 73	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

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INTRODUCTION

Breast cancer is one of the major causes of death for women in the United States (1,2). Amplification/overexpression of the Her-2/neu gene was found in approximately 30% of human breast cancer (3-8). Overexpression of the Her-2/neu oncogene correlates with poor survival for breast cancer patients and enhances metastatic potential and chemoresistance of human cancer cells (3, 8, 9, 10, 11). Downregulation of Her-2/neu suppresses the malignant phenotypes of cancer cells with Her-2/neu overexpression, indicating that Her-2/neu oncogene is an ideal target for development of the novel therapeutic agent (12-17). In this study, we found that PEA3, a member of the Ets transcriptional factor family, could directly bind to the consensus motif on the Her-2/neu promoter and prevent Her-2/neu gene overexpression by suppressing the promoter activity. Downregulation of Her-2/neu expression by PEA3 resulted in reversing the transforming phenotype mediated by Her-2/neu overexpression, inhibiting cell growth *in vitro*, and, in a preclinical gene therapy setting, blocking tumor formation from Her-2/neu-overexpressing cancer cells and prolonging survival rate of treated animals.

BODY

1. The tasks outlined in the approved Statement of Work:

Task 1. Examination of tumor suppression activity of PEA3 in breast cancer cell lines. Months 3-8.

Task 2. Examination of the anti-tumor effect of PEA3 by using liposome-mediated gene transfer. Months 13-24.

Task 3. Construction of adenovirus vector carrying PEA3, and control without PEA3. Infection of human breast cancer cell lines and examination of *in vitro* and *in vivo* transformation phenotype of these infected cells, including cell growth rate, colony formation ability in soft agar and tumorigenicity of m.f.p. injection. Months 13-36.

Task 4. Determining of the therapeutic effect of Ad.PEA3 in HER-2/neu-overexpressing breast cancers. Months 12-24.

Task 5. Testing the synergistic effect of PEA3-mediated gene therapy and chemotherapy *in vitro* and *in vivo*. Months 24-48.

2. The research accomplishment to date with respect to the Statement of Work:

A. We have completed Task 1 and Task 2. The results are as the following:

(1). PEA3 suppress the HER-2/neu transforming phenotype *in vitro*

To test whether expression of PEA3 can repress HER-2/neu expression and therefore reverse the transforming phenotype, a genomic activated rat neu oncogene (cNul04) that was known to transform mouse fibroblast cells and result in enhanced focus-forming activity (18-20) was

subject to a focus forming assay by cotransfecting with the PEA3 cDNA or the control vector into the mouse fibroblast NIH3T3 cells. The results indicated that PEA3 dramatically suppressed the focus formation caused by cNu104 (Fig. 1A and B).

To test if ectopic expression of PEA3 can suppress cell growth of cancer cells, breast and ovarian cancer cells with high or basal level HER-2/neu expression were transfected with a plasmid carrying a neomycin resistance gene and the PEA3 cDNA controlled by a CMV promoter. Subsequent neomycin selection resulted in numerous resistant colonies from the HER-2/neu low expressing cell lines while only few colonies of cell lines with HER-2/neu overexpression were retained through the selection (Fig. 1C and Table 1). The surviving colonies were isolated and tested for ectopic expression of PEA3 by RT-PCR. Virtually all of the clones derived from the HER-2/neu low-expression cell line (MDA-MB-435), while only two clones derived from the HER-2/neu 6 overexpressing cell line (MDA-NO-453), contained PEA3 mRNA expressed from the plasmid (Fig. 1D). One of the two clones from MDA-MB-453 (clone No. 2) did not express detectable PEA3 protein. Another clone (clone No. 8) expressing ectopic PEA3 protein grew extremely slow and eventually lost during subsequent cell culturing (data not shown). The facts that transfection of PEA3 gene into HER-2/neu-overexpressing cells reduces the number of neomycin-resistant colonies and that the survived clones either do not express PEA3 protein or are unable to grow in cell culture strongly suggest that PEA3 inhibits growth of HER-2/neu-overexpressing cancer cells.

(2). PEA3 inhibits tumor growth *in vivo*

To further investigate the tumor suppression potential of PEA3, we used an established animal model to test whether PEA3 could suppress tumor development in animals and prolong animal survival (17, 21). Ovarian cancer orthotopic tumor xenografts derived from a HER-2/neu-overexpressing cell line (SKOV3-ipl) or a cell line with only basal level of HER-2/neu expression (2774c-10) were developed intraperitoneally in nude mice. Tumor-bearing mice were treated with or without PEA3-expressing plasmid DNA once per week delivered by a cationic liposome (DC-Chol) by intraperitoneal injection. Fifty percent of mice with SKOV3-ipl tumors survived without detectable tumors for longer than one year after treated by PEA3 plus liposome, while all mice in the control groups died within half year (Fig. 2A). On the other hand, no response to PEA3-liposome treatment was observed for mice bearing 2774c-10-derived tumors

(Fig. 2B). The therapeutic effect of PEA3 combined with the cationic liposome was associated with down-regulation of the HER-2/neu p185 protein product in the treated tumors of SKOV3-ipl xenograft (Fig. 2A insert).

B. To achieve the goals in Task 3 and Task 4, we had tried to construct the adenovirus vector carrying PEA3. However, we could not get the infectious virus carrying PEA3 so far.

We suspect that this is due to the growth inhibition effect of PEA3 on the recipient cell. Indeed, to our experience, we had not been able to obtain the infectious virus carrying pro-apoptotic gene Bik/Nbk either.

We would like to focus on using PEA3-liposome complex instead of adenovirus construct for therapy purpose later on.

C. Experiments proposed in Task 5 has just been initiated and we do not have any conclusive data to date.

We will search for other funding to continue our study on this important issue.

3. Besides the tasks outlined in the Statement of Work, we further investigated the molecular mechanism for PEA3 to repress the Her-2/neu promoter activity. We also found that DNA binding domain of PEA3 along is sufficient to repress Her-2/neu promoter. The results are presented as the following:

A. PEA3 binds to the HER-2/neu promoter

A DNA motif with the sequence of 5'AGGAAG3' has been identified on the HER-2/neu promoter (22). To test whether PEA3 can recognize and bind to this putative PEA3 binding site, purified GST-PEA3 fusion protein was prepared and incubated with ³²p-labeled oligonucleotide probes containing either the wild-type PEA3 binding site sequence or the same sequence but with the core PEA3 binding motif mutated (5'AGCTCG3'). DNA-protein association was

investigated with an electrophoretic mobility shift assay (EMSA). Specific binding between the fusion protein and the wild-type probe was identified (Fig. 3). The binding was diminished in the presence of unlabeled wild-type oligonucleotide. There was no detectable association between the fusion protein and the mutant oligonucleotide. GST alone does not bind to the probe (data not shown).

B. PEA3 represses the HER-2/neu promoter activity through a positive regulatory motif of the promoter

To investigate the effect of PEA3 on the promoter activity of HER-2/neu, luciferase reporter gene driven by the HER-2/neu promoter (pNulit) was cotransfected with different amount of PEA3 cDNA into the SKOV-3 ovarian cancer cell line (Fig. 4A). The PEA3 cDNA repressed the promoter activity of HER-2/neu in a dose-dependent manner. Similar results were observed in experiments using a breast cancer cell line MDA-MB-453. In these experiments both the luciferase (Fig. 4A, 5A) and the chloramphenicol acetyl transferase (HER2-CAT; Fig. 4B) reporter genes consistently demonstrated that PEA3 co-expression can repress the HER-2/neu promoter activity. These results indicate that PEA3 is a potent transrepressor for HER-2/neu gene expression. The intact PEA3 binding site on the HER-2/neu promoter is required for PEA3-mediated transcriptional repression because the HER-2/neu promoter with a mutated PEA3 binding sequence (5'AGGAAG3' to 5'AGCTCG3') was not subject to the negative regulation by PEA3 (Fig. 5A). The results suggest that PEA3-mediated HER2/neu down-regulation is through the PEA3 binding site on the HER-2/neu promoter. When the promoter activities were compared between the wild-type and mutant promoters in the absence of PEA3, the activity of the mutant promoter was significantly lower than that of the wild-type promoter (Fig. 5B), indicating that the PEA3 binding site on the HER-2/neu promoter actually acts as a positive regulatory element for elevated expression of HER-2/neu.

C. PEA3 competes with other transactivating factor(s)

The results shown above demonstrate that PEA3 down-regulates HER-2/neu expression and suppresses the transforming phenotype mediated by HER-2/neu overexpression. The fact that the PEA3 binding motif on the HER-2/neu promoter functions as a positive regulatory element for HER-2/neu gene transcription (Fig. 5B) implicates the presence of a yet unidentified

transactivating factor(s) which can recognize the same positive regulatory element and that PEA3 may compete with the transactivating factor(s) for the same DNA motif and result in repression of HER-2/neu transcription. To test this hypothesis, nuclear extract from the HER-2/neuoverexpressing cell line MDA-MB-453 was prepared and tested for binding activity to a oligonucleotide probe containing DNA sequence of the PEA3 binding site in the presence of increasing amount of GST-PEA3 fusion protein. The EMSA results revealed a specific nuclear binding activity to the PEA3 binding site (Fig. 6A). The binding of the unknown factor(s) is unique and can be distinguished from the PEA3-DNA complex. Furthermore, increasing doses of PEA3 fusion protein diminished the unique binding activity in MDA-MB-453 cells (Fig. 6B), indicating a competition relationship between PEA3 and the unidentified transcriptional factor(s). This competition model suggests that overexpression of the DNA-binding activity of PEA3 would be sufficient to suppress HER-2/neu gene expression by blocking the binding of other transactivators to the same DNA motif. To test this hypothesis, a PEA3 construct containing only the DNA binding domain was cotransfected with pNulit into MDA-MB-453 cells. Expression of the DNA binding domain of PEA3 (PEA3DBD) resulted in decrease of the luciferase reporter activity in a dose-dependent manner (Fig. 6Q). Thus, consistent with the hypothesis, either wild-type PEA3 or the DNA-binding domain of PEA3 can function as potent repressors of the HER-2/neu gene expression -

4. Discussion

(1). PEA3 as an Ets family protein

The PEA3 protein contains a domain of about 85 amino acids with extensive sequence similarity with the ETS domain, a conserved region shared by all members of the Ets family that characteristically bind to the cognate DNA binding site as a monomer through their ETS DNA-binding domain. The consensus DNA binding site of PEA3 seems to occur on the promoters of a cohort of genes and play a positive role for their expression. Examples include the stromelysine gene (23) and the tumor suppressor gene maspin (24, 25). For most of the cases, there are other Ets proteins rather than the PEA3 protein itself shown to target the consensus binding site and regulate the expression of the genes. The biological effects of the PEA3 protein to these genes remain to be determined. This study, to our knowledge, for the first

time shows the tumor suppression function of an Ets protein through suppressing the oncogenic activity of the HER-2/neu oncogene.

(2). The PEA3 protein down-regulates HER-2/neu promoter

We have identified a DNA motif on the HER-2/neu promoter that directly binds to the Ets-related transcription factor PEA3 and results in down-regulation of the HER-2/neu gene and consequently inhibition of cell transformation *in vitro* and tumor development *in vivo*. Our results indicated that PEA3 might act as a transcriptional repressor and likely compete with other transactivators through the same DNA motif. Since some other Ets family members which share the same binding site specificity as PEA3 may also bind to the same PEA3 binding site on the HER-2/neu promoter and transactivate the gene, it is conceivable that PEA3 may repress the HER-2/neu promoter by blocking the binding of other transactivating Ets proteins. There are precedents of other Ets family members, like ERF and Net, acting as repressors of gene expression (26, 27). On the other hand, promoters negatively regulated by Ets binding sites have also been reported (28, 29).

The effect of PEA3 on HER-2/neu promoter in this study is observed mainly in human breast and ovarian cancer cell lines, which are biologically relevant to the diseases. Other groups have previously reported transactivation of HER-2/neu promoter by PEA3 in COS-1 cells (30). Under our experimental condition, in COS-1 cells we did notice a very weak PEA3-mediated induction of HER-2/neu promoter. The potential species- or cell type-specific transactivating effect of PEA3 is an interesting phenomenon. It might be due to the disparity of regulatory mechanisms between different cell types and/or species. In this regard, it is worth noting that COS-1 cells were derived from monkey kidney cells transformed by SV40 T antigen (31).

(3). The PEA3 gene as a potential gene therapy agent

Recently targeting the protein product of HER-2/neu p185 with a humanized anti-p185 monoclonal antibody (Herceptin) has shown encouraging therapeutic effects for patients with HER-2/neu-overexpressing breast cancer though with significant side effect of cardiotoxicity (32, 33). The results, however, indicate that HER-2/neu overexpression is an excellent target for development of therapeutic strategies of cancer. In addition to the immunotherapy strategy, a

gene-therapy setting using either the adenovirus type 5 E1A or the SV40 large T antigen gene to down-regulate HER-2/neu overexpression has been reported (12,16,17,21,30, 34). In the case of E1A, a phase I clinical trial has just been completed with observation of HER-2/neu down-regulation associated with E1A expression (35). However, both E1A and SV40 large T antigen are viral proteins and very likely suppress HER-2/neu in an indirect manner as none of these proteins have been shown to bind the HER-2/neu promoter directly. The fact that PEA3 directly binds to the HER-2/neu promoter likely provides stronger efficacy and higher specificity to inhibit HER-2/neu promoter activity. We have presented the data to demonstrate that the DNA binding domain of PEA3 is sufficient to down-regulate HER-2/neu overexpression. These features make PEA3 an attractive candidate for further molecular manipulation to develop next generation therapeutic molecules with higher binding affinity and enhanced specificity. In addition, the strategy described in this study provides a general approach to identify potential transformation suppressors through searching for DNA-binding protein recognizing specific DNA sequence on the promoter of the targeted oncogenes or other disease-causing pathogenic genes.

5. Future work to better address this topic

(1). Test whether p300/CBP plays a similar role in PEA3-mediated inhibition of Her-2/neu transcription as in E1A-mediated inhibition of Her-2/neu transcription.

It was reported that p300/CBP interacts with Ets-1 and Ets-2 in the transcription activation of the human stromelysin promoter and the conserved Ets DNA binding domain along is sufficient to mediate the interaction between Ets-2 and p300/CBP *in vitro* (36, 37). Since we previously showed that the binding of E1A to p300/CBP and the binding motif of p300/CBP on Her-2/neu promoter are crucial for the ability of E1A to inhibit Her-2/neu promoter activity (38), it is worth investigating whether p300/CBP plays a similar role in PEA3-mediated inhibition of Her-2/neu transcription. If this is the case, it may provide another complementary mechanism for the transcription repression of Her-2/neu by PEA3.

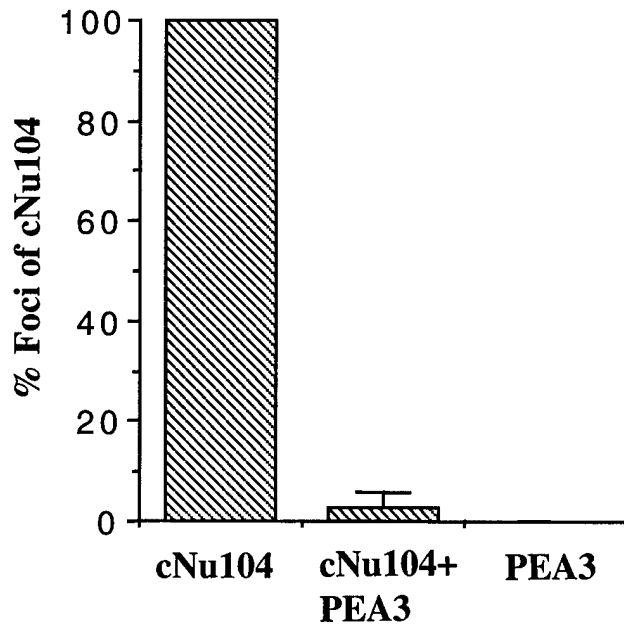
(2). Identify the Ets family member(s) that binds to the Ets consensus motif and activates transcription of Her-2/neu.

Although several candidate Ets members have been suggested to possibly involve in regulation of Her-2/neu transcription (39), it still remains unclear which Ets family member(s) is responsible for the positive regulation of Her-2/neu transcription. To make our competition model complete, we hope to identify the Ets family member(s) regulating Her-2/neu transcription and further confirm our hypothesis.

(3). Develop DNA binding domain of PEA3 (PEA3 DBD) as a potential gene therapy agent for breast cancer and ovarian cancer with Her-2/neu overexpression.

Many PEA3 target genes have been reported and a significant fraction of these genes encode proteases required for degradation of the extracellular matrix (40, 41, 42, 43). The unregulated expression of these enzymes is associated with metastatic propensity of tumor cells (44). Therefore, one withdraw to use PEA3 as a gene therapy agent is that the metastatic potential of cancer cells may possibly be enhanced. In this study we also showed that DNA binding domain of PEA3 (PEA3 DBD) alone is sufficient to repress Her-2/neu transcription. Presumably, PEA3 DBD does not have this problem in contrast to full length PEA3 as a gene therapy agent for breast cancer and ovarian cancer overexpressing Her-2/neu. Besides, PEA3 DBD may also be able to repress the transcription of PEA3 target genes encoding protease via a dominant-negative route and thus suppress the metastatic potential of cancer cells. It would be intriguing and worthwhile to test the feasibility of developing PEA3 DBD as a potential gene therapy agent for breast cancer and ovarian cancer overexpressing Her-2/neu.

A.



B.

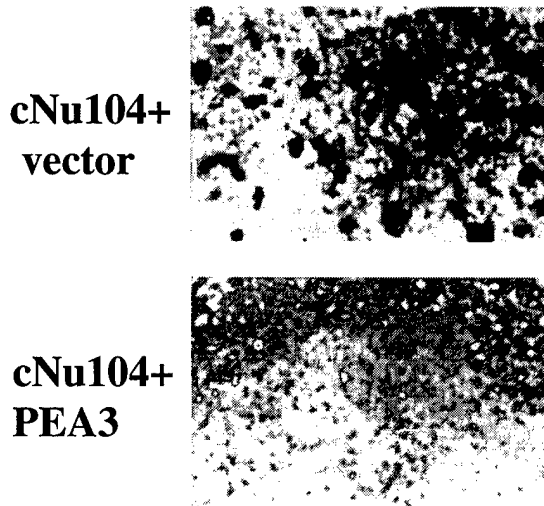


Fig.1 A&B

C.

HER-2/neu overexpressors

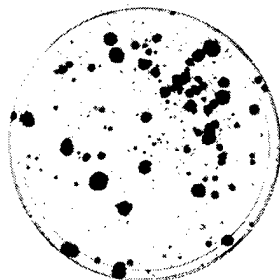
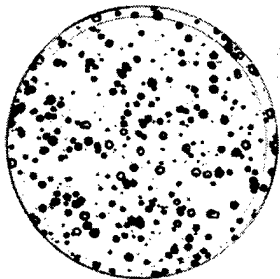
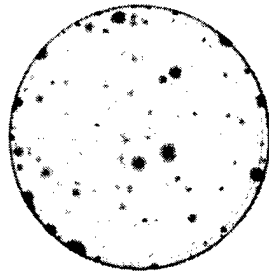
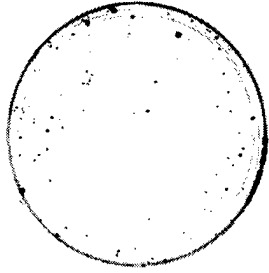
Basal HER-2/neu expressors

MDA-MB-453 SK-OV-3-ip1

MDA-MB-435

2774 c-10

PEA3



Vector
control

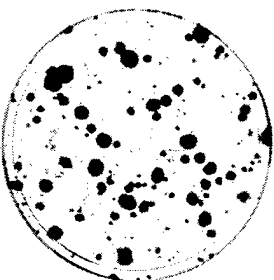
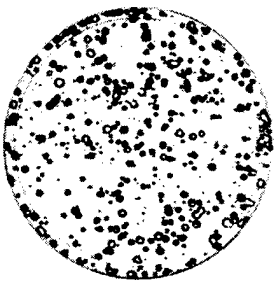
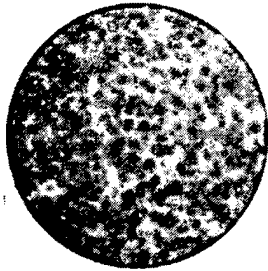
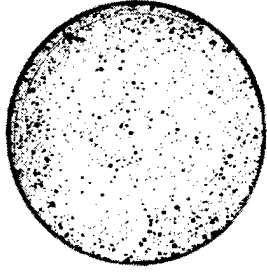


Fig.1 C

D.

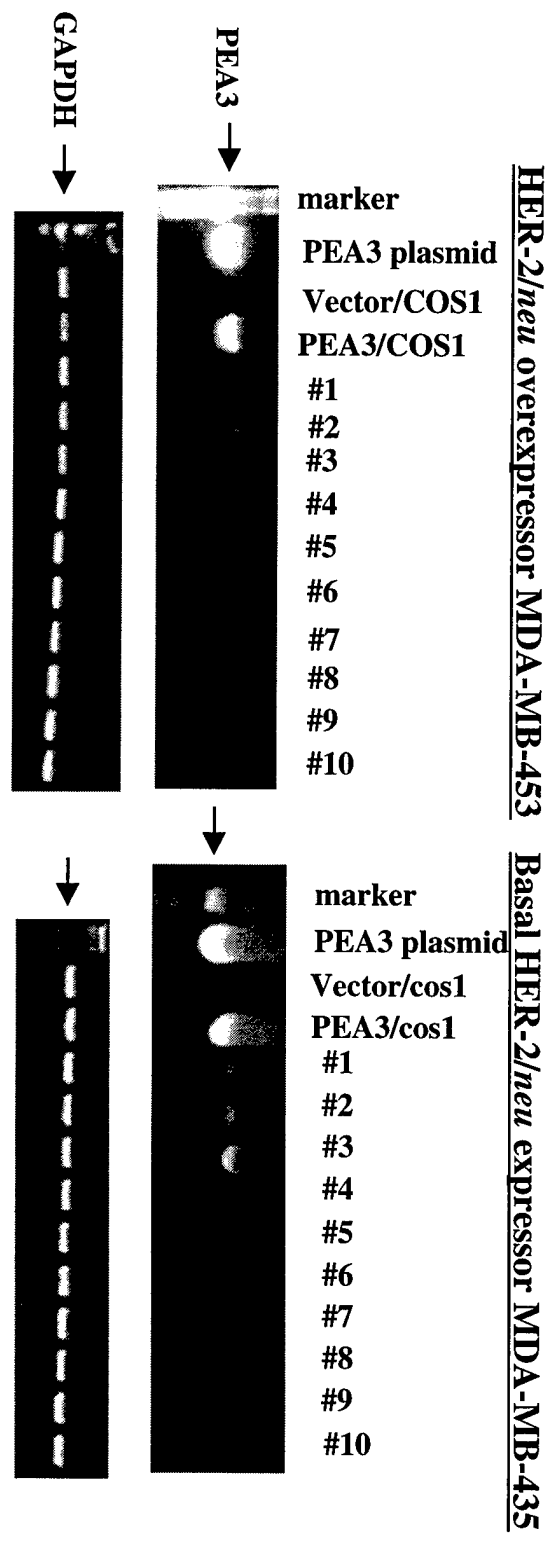


Fig.1 D

Figure 1. PEA3 represses neu-mediated transformation and inhibits cell growth of HER-2/neu-overexpressing cancer cells.

A. PEA3 represses the transforming activity of activated genomic rat neu. NIH3T3 cells (2×10^5) were plated to a 100-mm culture dish 24 hours before transfection. Cells were transfected with 0.5 μg of pSV2neo, 1 μg of cNu104 plus 5 μg of either pSR α PEA3 or the control vector pSV2E by calcium phosphate precipitation. Two days after transfection, cells were split and two plates were subject to selection in 500 $\mu\text{g}/\text{ml}$ of neomycin (G418; Life Technologies, Inc), and the other two were maintained in normal medium until foci appeared. Foci and neomycin-resistant colonies were stained and counted. The number of foci was normalized by the corresponding number of neomycin-resistant colonies. Results are expressed as ratio of the number of foci to the number of colonies from each transfection. The ratio of cNu104 transfection alone was set as 100 %.

B. An example of the PEA3-mediated suppression of the focus forming activity by cNu104.

C. Cell growth of HER-2/neu overexpressing breast and ovarian cancer cells was inhibited by PEA3 transfection as indicated by the decrease of colony numbers compared with vector transfection.

D. Ectopic PEA3 RNA expression was preferentially lost in the survival neomycin-resistant clones derived from the HER-2/neu-overexpressing breast cancer cell line MDA-MB453. Total RNA was extracted from cell clones resulted from stable transfection and RT-PCR was performed by following the manufacturer's instruction (SuperScript preamplification system; Life Technologies, Inc). The primers derived from the PEA3 coding sequence (5'-TGAATTATGACAAGCTGAGCCG-3') and from the expression vector pcDNA3 (5'-TCAGCGAGCTCTAGCATTTAGG-3') were use to amplified the ectopically expressed PEA3 transcript. Primers for GAPDH internal control were: 5'-AGGTGAAGGTCGGAGTCAAC-3' and 5'-TCCATTGATGACAAGCTTCCC-3'. COS-1 cells transiently transfected by the PEA3 cDNA were used as a positive control. Amplification was performed on a Perkin Elmer DNA Cyclor 480 for 35 cycles with denaturing at 94°C for 30 seconds, annealing at 58°C for 1.5 minutes and extension at 72°C for 1.5 minutes.

Table I. PEA3 suppresses growth of cancer cells overexpressing HER-2/neu.¹

HER-2/neu overexpressers

<u>Cell lines</u>	<u>pcDNA3</u>	<u>PEA3</u>
SK-BR-3	100	0.7 (±0.9)
MDA-MB-453	100	19.7 (±3.0)
SKOV3-ip1	100	14.3 (±4.1)

HER-2/neu basal-level expressers

<u>Cell lines</u>	<u>pcDNA3</u>	<u>PEA3</u>
MDA-MB-435	100	73.4 (±10.9)
2774 c-10	100	83.3 (±3.8)

¹ Experiment for each cell line was independently repeated 2-4 times using different batches of plasmid DNA and cell culture. Inhibition of colony formation by PEA3 transfection is shown in percentage with the number of neomycin-resistant colony resulted from pcDNA3 transfection arbitrarily set as 100 %.

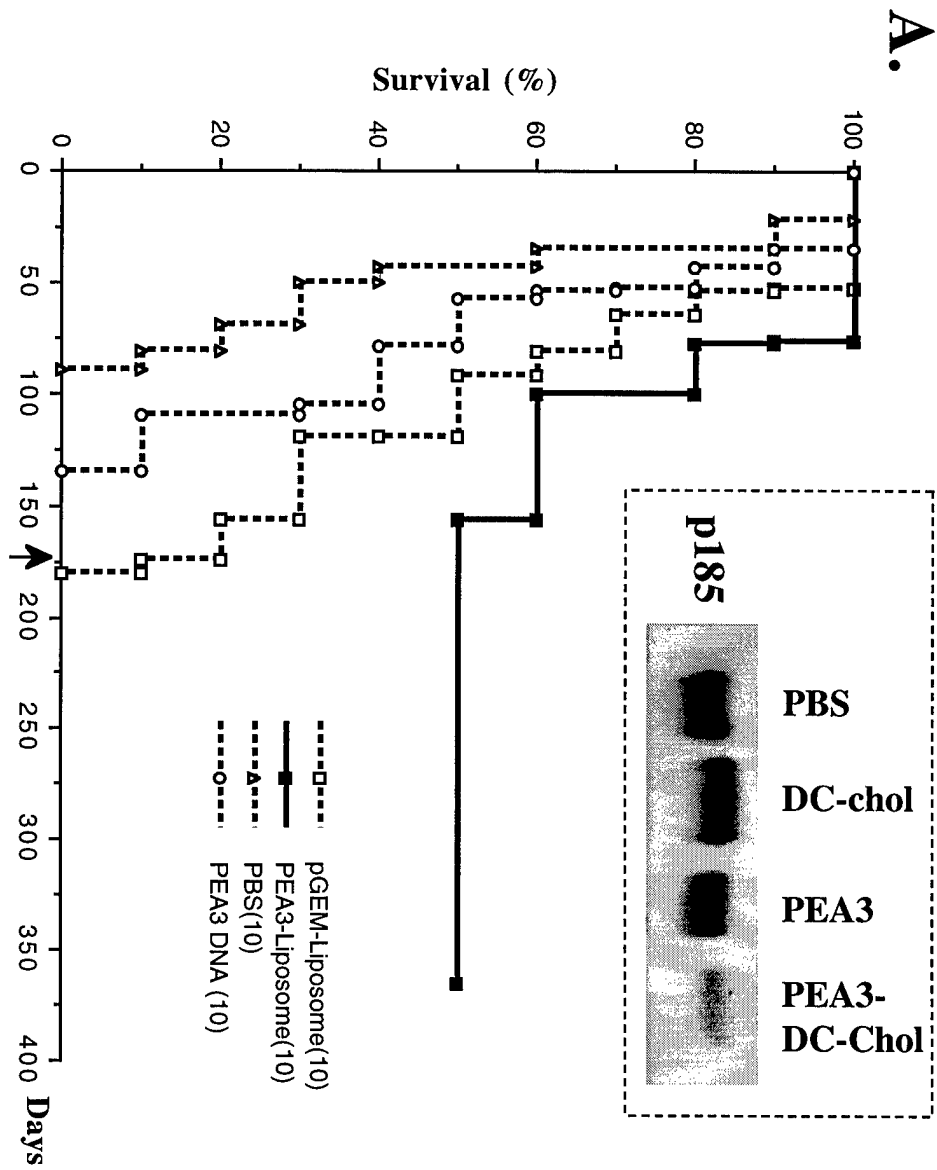


Fig. 2A

B.

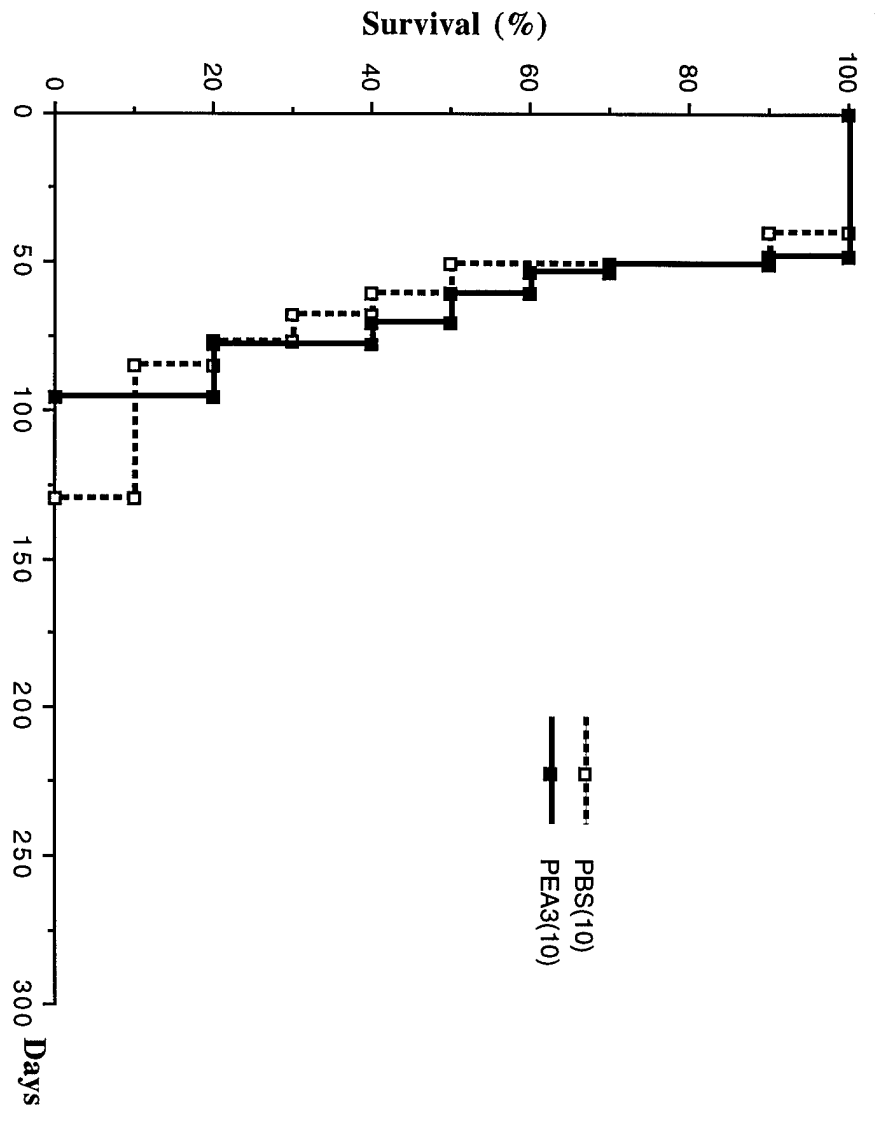


Fig. 2B

Figure 2. PEA3 inhibits growth of tumor xenografts derived from HER-2/neu overexpressing cancer cells.

A. Nude mice carrying intraperitoneal tumors derived from SKOV3-ip1 received weekly i.p. injections of a reagent containing PEA3 plasmid DNA complexed with liposome (PEA3-liposome), control DNA (pGEM) complexed with liposome (pGEM-Liposome), naked PEA3 plasmid DNA, or PBS. Number of mice in each group is shown in the parentheses. The arrow marks the last injection. Tumor samples were retrieved from mice treated with PBS, the lipid vehicle DC-Chol, PEA3 naked DNA, or PEA3-DC-Chol complex, and the levels of p 185 were measured by immunoblotting as shown in the insert. Loading was controlled by the endogenous α -actin expression (data not shown).

B. Nude mice carrying tumors derived from 2774 c-10 were treated with PEA3-liposome complex (the solid line) or PBS alone (the dashed line) by the same procedure as SKOV3-ip1 cells.

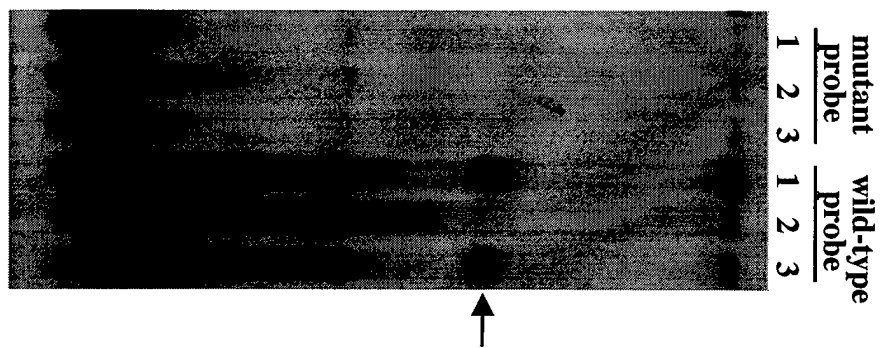
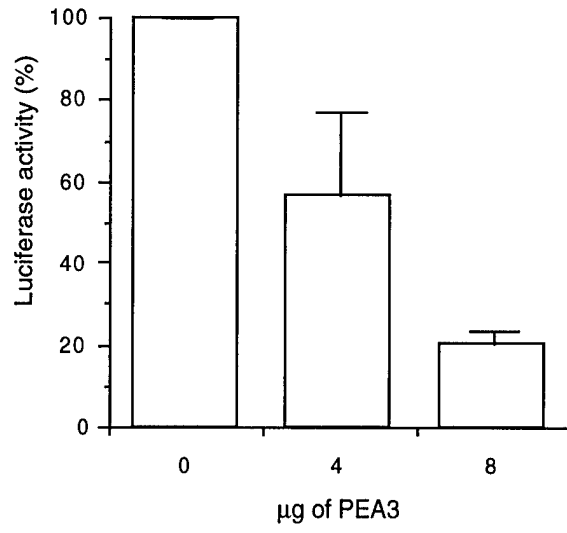


Fig. 3

Figure 3. PEA3 binds to the HER-2/neu promoter. GST-PEA3 fusion protein was incubated with ³²p-γ ATP end-labeled wild-type or mutated oligonucleotide, containing the consensus PEA3 binding site sequence on the HER-2/neu promoter (lanes 1). The binding specificity was tested by competition with unlabeled wild-type (lanes 2) or nonspecific oligonucleotide (lanes 3).

A.



B.

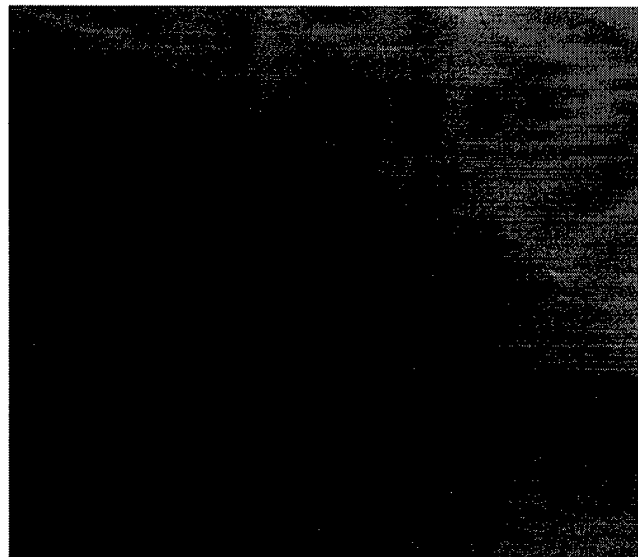
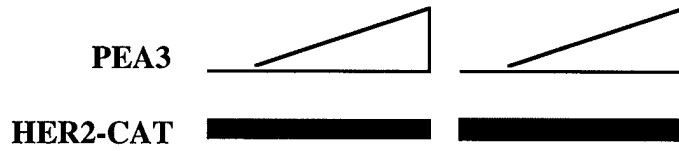
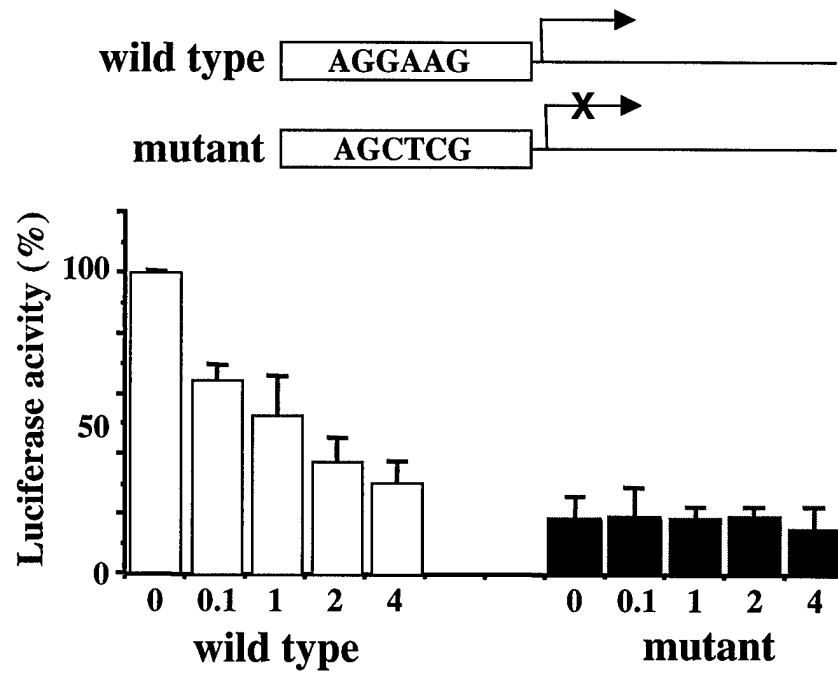


Fig. 4

Figure 4. PEA3 inhibits the HER-2/neu promoter activity in HER-2/neu overexpressing cancer cell lines. Human ovarian cancer cells SKOV-3 were cotransfected with 5 μ g of pNeulit or HER2-CAT with different amounts of PEA3 plasmid DNA (pcDNA3-PEA3) as indicated. The luciferase (**A**) or the chloramphenicol acetyltransferase (CAT) (**B**) reporter activity was measured. For the CAT transient assay, the doses of cotransfected pcDNA3-PEA3 were 0, 5, 10, and 15 μ g. The results of two independent experiments are shown.

A.



B.

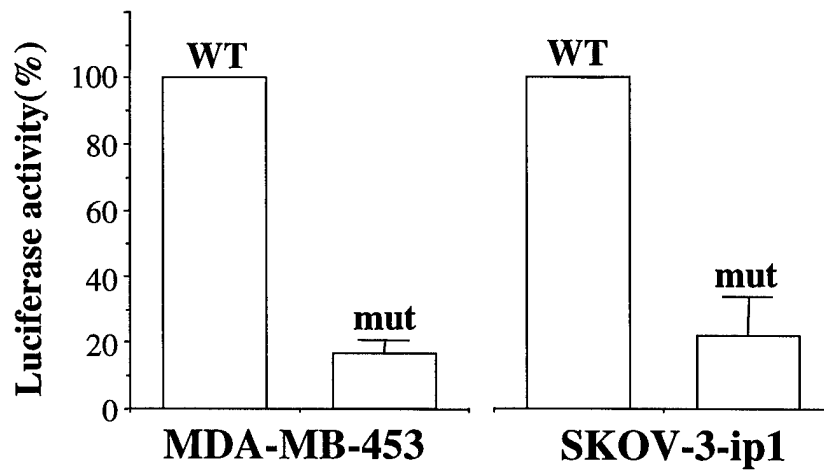


Fig. 5

Figure 5. PEA3 represses HER-2/neu promoter activity through a positive regulatory DNA motif.

A. Luciferase reporter gene driven by either wild-type (open bars) or the PEA3 site-mutated (close bars) HER-2/neu promoter was measured after co-transfection with PEA3 cDNA into MDA-NM-453 cells.

B. The luciferase activities derived from wild-type and the PEA3 site-mutated HER-2/neu promoter were compared by transfection into a breast cancer cell line (MDA-MB-453) and a ovarian cancer cell line (SKOV-3-ip1).

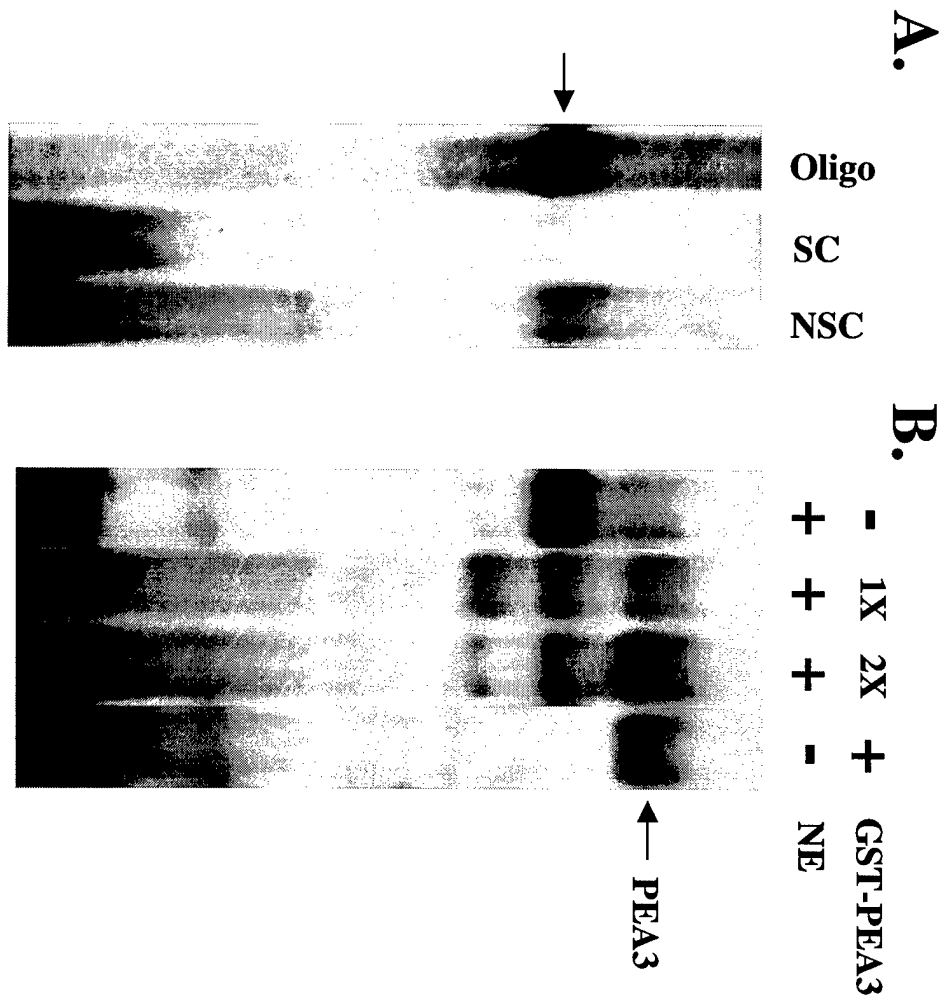


Fig. 6A&B

C.

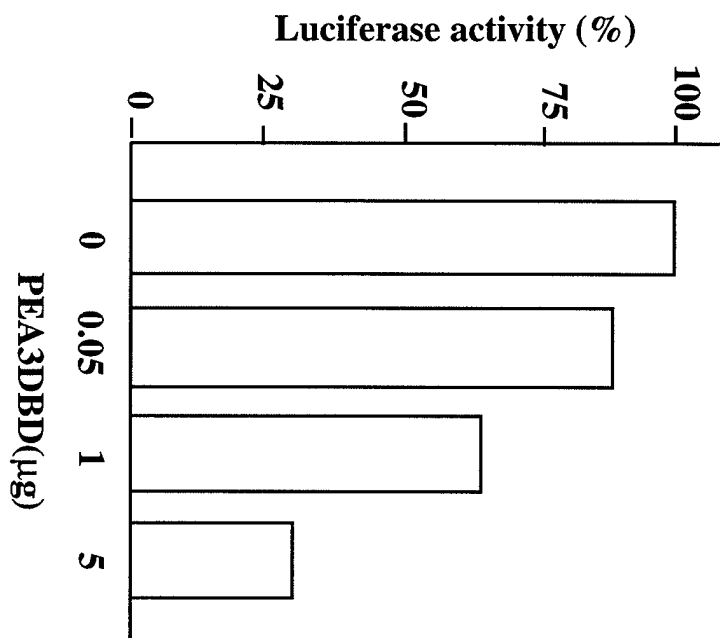


Fig. 6C

Figure 6. PEA3 competes with a distinct family member of the Ets family detected in HER-2/neu-overexpressing cancer cells.

A. Nuclear extract of MDA-MB-453 cells was incubated with the DNA oligonucleotide probe in the absence (Oligo) or presence of unlabeled competitor of the same specific oligonucleotide (SQ or non-specific oligonucleotide of shuffled sequences (NSC)). The signal of specific DNA-protein complex is indicated by an arrow.

B. Increasing amount of GST-PEA3 fusion protein was added into the binding reaction to test the competition between the unknown nuclear factor and PEA3 protein. The DNA-PEA3 complex is indicated by an arrow. NE, nuclear extract.

C. The DNA-binding domain of PEA3 is sufficient to down-regulate the HER-2/neu promoter as assayed by cotransfection of different doses of PEA3DBD and pNulite. The result from a representative experiment is shown.

KEY RESEARCH ACCOMPLISHMENTS

A. Explored and confirmed the feasibility of development of PEA3 as a potent gene therapy agent for breast cancer and ovarian cancer overexpressing Her-2/neu.

- (1). PEA3 suppress the HER-2/neu transforming phenotype in vitro.
- (2). PEA3 inhibits tumor growth in vivo.

B. Provided a competition model to elucidate the molecular mechanism for the transcription inhibition of Her-2/neu by PEA3.

- (1). PEA3 binds to the HER-2/neu promoter.
- (2). PEA3 represses the HER-2/neu promoter activity through a positive regulatory motif of the promoter; competition of PEA3 with other Ets transactivating factor(s) may account for the down-regulation of Her-2/neu by PEA3.

REPORTABLE OUTCOMES

(1). Results achieved in this study have been submitted to the journal of *Nature Medicine* (manuscript appended).

Xiangming Xing, Shao Chun Wang, Susan Miller, Leaf Huang, Wei Ya Xia, and Mien-Chie Hung. The Ets Protein PEA3 Suppresses HER-2/Neu Overexpression And Inhibits Tumorigenesis

(2). Supported by this award, Ms. Xiangming Xing obtained her Ph.D. degree from the Graduate School of Biomedical Sciences The University of Texas-Houston.

(3). Part of the graduate training of Mr. Zhenming Yu is supported by this award.

CONCLUSION

In this study, we explored and confirmed the feasibility of development of PEA3 as a potent gene therapy agent for breast cancer and ovarian cancer overexpressing Her-2/neu. PEA3 suppresses the HER-2/neu transforming phenotype in vitro and inhibits tumor growth in vivo. A competition model to elucidate the molecular mechanism for the transcription inhibition of Her-2/neu by PEA3 was proposed. PEA3 directly binds to the HER-2/neu promoter and competition of PEA3 with other Ets transactivating factor(s) for a positive regulatory motif on the Her-2/neu promoter may account for the down-regulation of Her-2/neu transcription by PEA3.

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APPENDICES

1. **A manuscript of the paper describing the result of this study is appended. This paper was submitted to the journal of *Nature Medicine*.**

Xiangming Xing, Shao Chun Wang, Susan Miller, Leaf Huang, Wei Ya Xia, and **Mien-Chie Hung**. The Ets Protein PEA3 Suppresses HER-2/Neu Overexpression And Inhibits Tumorigenesis

2. **Personnel receiving pay from this research effort:**

Xiangming Xing
Zhenming Yu

The Ets Protein PEA3 Suppresses HER-2/Neu Overexpression And Inhibits Tumorigenesis

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Key Words: PEA3, HER-2/neu overexpression, tumorigenecity, transformation

HER-2/neu overexpression plays an important role for cancer development. To suppress cell transformation mediated by HER-2/neu overexpression, we identified a DNA-binding protein PEA3 of the *ets* gene family which specifically targeted a DNA sequence on the HER-2/neu promoter and downregulated the promoter activity. Expression of PEA3 resulted in preferential inhibition of cell growth and tumor development of HER-2/neu-overexpressing cancer cells. This study demonstrates a novel approach targeting HER-2/neu overexpression and also provides a general and promising approach to design repressors for diseases caused by overexpression of pathogenic genes.

INTRODUCTION

Overexpression of the HER-2/neu gene (also known as c-erbB2) is frequently associated with many types of cancers including breast and ovarian cancers¹⁻⁷. The overall survival rate of breast and ovarian cancer patients whose tumors have HER-2/neu overexpression is significantly lower than that of patients whose tumors do not have HER-2/neu overexpression⁸⁻¹¹. A large body of evidence indicates that HER-2/neu overexpression is associated with elevated tumorigenicity¹², enhanced metastatic potential¹³⁻¹⁷, and, in certain circumstances, increased resistance to chemotherapy¹⁸⁻²².

On the other hand, downregulation of the HER-2/neu oncogene confers suppression to the transforming phenotype induced by the oncogene^{12, 23-27}. We have shown previously that the adenovirus type 5 E1A gene can indirectly downregulate HER-2/neu promoter activity through the coactivator p300²⁸. Downregulation of HER-2/neu overexpression by E1A significantly mitigated tumorigenic activity of human breast and ovarian cancer cells in nude mice^{12, 26, 29, 30}. These results strongly implicate the potential of inhibiting HER-2/neu-mediated cell transformation by transcriptional repressors which target the promoter of the oncogene.

One strategy to efficiently suppress oncogene-mediated transformation is to inhibit the activity of oncogene promoter by DNA-binding protein that can directly bind to specific DNA sequence on the promoter and result in inhibition of transcription activity. A DNA motif (5'AGGAAG3') containing the consensus binding site of PEA3³¹, a member of the *ets* transcription factor family, is present 26 nucleotides upstream from the major transcriptional start site on the HER-2/neu promoter³². The sequence and position of the motif is conserved among human, rat, and mouse HER-2/neu promoters³³. In this study, we show evidence that PEA3 directly binds to the consensus motif and prevents HER-2/neu gene overexpression by suppressing the promoter activity. Downregulation of HER-2/neu expression resulted in reversing the transforming phenotype

induced by HER-2/neu activation, inhibiting cell growth *in vitro*, and, in a preclinical gene therapy setting, blocking tumor formation from HER-2/neu-overexpressing cancer cells and prolonging survival rate of treated animals. Our study demonstrates a promising approach to design repressors targeting diseases caused by overexpression of pathogenic genes.

RESULTS

PEA3 binds to the HER-2/neu promoter

A DNA motif with the sequence of 5'AGGAAG3' has been identified on the HER-2/neu promoter³³. To test whether PEA3 can recognize and bind to this putative PEA3 binding site, purified GST-PEA3 fusion protein was prepared and incubated with ³²P-labeled oligonucleotide probes containing either the wild-type PEA3 binding site sequence or the same sequence but with the core PEA3 binding motif mutated (5'AGCTCG3'). DNA-protein association was investigated with an electrophoretic mobility shift assay (EMSA). Specific binding between the fusion protein and the wild-type probe was identified (Fig. 1). The binding was diminished in the presence of unlabeled wild-type oligonucleotide. There was no detectable association between the fusion protein and the mutant oligonucleotide. GST alone does not bind to the probe (data not shown).

PEA3 represses the HER-2/neu promoter activity through a positive regulatory motif of the promoter

To investigate the effect of PEA3 to the promoter activity of HER-2/neu, a luciferase reporter gene driven by the HER-2/neu promoter (pNulit) was cotransfected with different amount of PEA3 cDNA into the SKOV-3 ovarian cancer cell line (Fig. 2A). The PEA3 cDNA repressed the promoter activity of HER-2/neu in a dose-dependent manner. Similar results were observed in experiments using a breast cancer cell line MDA-MB-453. In these experiments both the luciferase (Fig. 2A, 3A) and the chloramphenicol acetyl transferase (HER2-CAT; Fig. 2B) reporter genes consistently demonstrated that PEA3 coexpression can repress the HER-2/neu promoter activity. These results indicate that PEA3 is a potent transrepressor of HER-2/neu gene expression. The intact PEA3 binding site on the HER-2/neu promoter is required for the

PEA3-mediated transcriptional repression because the HER-2/neu promoter with a mutated PEA3 binding sequence (5'AGGAAG3' to 5'AGCTCG3') was not subject to the negative regulation by PEA3 (Fig. 3A). The results suggest that PEA3-mediated HER-2/neu downregulation is through the PEA3 binding site on the HER-2/neu promoter. When the promoter activities were compared between the wild-type and mutant promoters in the absence of PEA3, the activity of the mutant promoter was significantly lower than that of the wild-type promoter (Fig. 3B), indicating that the PEA3 binding site on the HER-2/neu promoter actually acts as a positive regulatory element for elevated expression of HER-2/neu.

PEA3 suppress the HER-2/neu transforming phenotype *in vitro*

To test whether expression of PEA3 can repress HER-2/neu expression and therefore reverse the transforming phenotype, a genomic activated rat neu oncogene (cNu104) that was known to transform mouse fibroblast cells and result in enhanced focus-forming activity³⁴⁻³⁶ was subject to a focus forming assay by cotransfecting with the PEA3 cDNA or the control vector into the mouse fibroblast NIH3T3 cells. The results indicated that PEA3 dramatically suppressed the focus formation caused by cNu104 (Fig. 4, A and B).

To test if ectopic expression of PEA3 can suppress cell growth of cancer cells, breast and ovarian cancer cells with high or basal level HER-2/neu expression were transfected with a plasmid carrying a neomycin resistance gene and the PEA3 cDNA controlled by a CMV promoter. Subsequent neomycin selection resulted in numerous resistant colonies from the HER-2/neu low expressing cell lines while only few colonies of cell lines with HER-2/neu overexpression were retained through the selection (Fig. 4C and Table I). The surviving colonies were isolated and tested for ectopic expression of PEA3 by RT-PCR. Virtually all of the clones derived from the HER-2/neu low expressor cell line (MDA-MB-435), while only two clones derived from the HER-2/neu

overexpressing cell line (MDA-MB-453), contained PEA3 RNA expressed from the plasmid (Fig. 4D). One of the two clones from MDA-MB-453 (clone No. 2) did not express detectable PEA3 protein. Another clone (clone No. 8) expressing ectopic PEA3 protein grew extremely slow and eventually lost during subsequent cell culturing (data not shown). The facts that transfection of PEA3 gene into HER-2/neu-overexpressing cells reduces the number of neomycin-resistant colonies and that the survived clones either do not express PEA3 protein or is unable to grow in cell culture strongly suggest that PEA3 inhibits growth of HER-2/neu-overexpressing cancer cells.

PEA3 inhibits tumor growth *in vivo*

To further investigate the tumor suppression potential of PEA3, we used an established animal model to test whether PEA3 could suppress tumor development in animals and prolong animal survival^{27,29}. Ovarian cancer orthotopic tumor xenografts derived from a HER-2/neu-overexpressing cell line (SKOV3-ip1) or a cell line with only basal level of HER-2/neu expression (2774 c-10) were developed intraperitoneally in nude mice. Tumor-bearing mice were treated with or without PEA3-expressing plasmid DNA once per week delivered by a cationic liposome (DC-Chol) by intraperitoneal injection. Fifty percent of mice with SKOV3-ip1 tumors survived without detectable tumors for longer than one year after treated by PEA3 plus liposome, while all mice in the control groups died within half year (Fig. 5A). On the other hand, no response to PEA3-liposome treatment was observed for mice bearing 2774 10-c-derived tumors (Fig. 5B). The therapeutic effect of PEA3 combined with the cationic liposome was associated with downregulation of the HER-2/neu p185 protein product in the treated tumors of SKOV3-ip1 xenograft (Fig. 5A insert).

PEA3 competes with other transactivating factor(s)

The results shown above demonstrate that PEA3 down-regulates HER-2/neu

expression and suppresses the transforming phenotype mediated by HER-2/neu overexpression. The fact that the PEA3 binding motif on the HER-2/neu promoter functions as a positive regulatory element for HER-2/neu gene transcription (Fig. 3B) implicates the presence of a yet unidentified transactivating factor(s) which can recognize the same positive regulatory element and that PEA3 may compete with the transactivating factor(s) for the same DNA motif and result in repression of HER-2/neu transcription. To test this hypothesis, nuclear extract from the HER-2/neu-overexpressing cell line MDA-MB-453 was prepared and tested for binding activity to a oligonucleotide probe containing DNA sequence of the PEA3 binding site in the presence of increasing amount of GST-PEA3 fusion protein. The EMSA results revealed a specific nuclear binding activity to the PEA3 binding site (Fig. 6A). The binding of the unknown factor(s) is unique and can be distinguished from the PEA3-DNA complex. Furthermore, increasing doses of PEA3 fusion protein diminished the unique binding activity in MDA-MB-453 cells (Fig. 6B), indicating a competition relationship between PEA3 and the unidentified transcriptional factor(s). This competition model suggests that overexpression of the DNA-binding activity of PEA3 would be sufficient to suppress HER-2/neu gene expression by blocking the binding of other transactivators to the same DNA motif. To test this hypothesis, a PEA3 construct containing only the DNA binding domain was cotransfected with pNulit into MDA-MB-453 cells. Expression of the DNA-binding domain of PEA3 (PEA3DBD) resulted in decrease of the luciferase reporter activity in a dose-dependent manner (Fig. 6C). Thus, consistent with the hypothesis, either wild-type PEA3 or the DNA-binding domain of PEA3 can function as potent repressors of the HER-2/neu gene expression .

DISCUSSION

PEA3 as an *ets* family protein

The PEA3 protein contains a domain of about 85 amino acids with extensive sequence similarity with the ETS domain, a conserved region shared by all members of the *ets* family that characteristically bind to the cognate DNA binding site as a monomer through their ETS DNA-binding domain. The consensus DNA binding site of PEA3 seems to occur on the promoters of a cohort of genes and play a positive role for their expression. Examples include the stromelysin gene³⁷ and the tumor suppressor gene *maspin*^{38,39}. For most of the cases, there are other *ets* proteins rather than the PEA3 protein itself shown to target the consensus binding site and regulate the expression of the genes. The biological effects of the PEA3 protein to these genes remain to be determined. This study, to our knowledge, for the first time shows the tumor suppression function of an *ets* protein through suppressing the oncogenic activity of the HER-2/neu oncogene.

The PEA3 protein downregulates HER-2/neu promoter

We have identified a DNA motif on the HER-2/neu promoter which directly binds to the *ets*-related transcription factor PEA3 resulting in downregulation of the HER-2/neu gene and consequently inhibition of cell transformation *in vitro* and tumor development *in vivo*. Our results indicated that PEA3 can act as a transcriptional repressor and likely compete with other transactivators through the same DNA motif. Since some other *ets* family members which share the same binding site specificity of PEA3 may also bind to the same PEA3 binding site on the HER-2/neu promoter and transactivate the gene, it is conceivable that PEA3 may repress the HER-2/neu promoter by blocking the binding of other transactivating *ets* proteins. There are precedents of other *ets* family members, like ERF and Net, acting as repressors of gene expression^{40,41}. On the other hand, promoters

negatively regulated by Ets binding sites have also been reported ^{42, 43}.

The effect of PEA3 on HER-2/neu promoter in this study is observed mainly in human breast and ovarian cancer cell lines, which are biologically relevant to the diseases. Other groups have previously reported transactivation of HER-2/neu promoter by PEA3 in COS-1 cells ⁴⁴. Under our experimental condition, in COS-1 cells we did notice a very weak PEA3-mediated induction of HER-2/neu promoter. The potential species- or cell type-specific transactivating effect of PEA3 is an interesting phenomenon. It might be due to the disparity of regulatory mechanisms between different cell types and/or species. In this regard, it is worth noting that COS cells were derived from monkey kidney cells transformed by SV40 T antigen ⁴⁵.

The PEA3 gene as a potential gene therapy agent

Recently targeting the protein product of HER-2/neu p185 with a humanized anti-p185 monoclonal antibody (Herceptin) has shown encouraging therapeutic effects for patients with HER-2/neu-overexpressing breast cancer though with significant side effect of cardiotoxicity ^{46, 47}. The results, however, indicate that HER-2/neu overexpression is an excellent target to develop therapeutic strategies of cancer. In addition to the immunotherapy strategy, a gene therapy setting using either the adenovirus type 5 E1A or the SV40 large T antigen gene to demonstrate HER-2/neu overexpression has been reported ^{12, 26, 27, 29, 30, 48}. In the case of E1A, a phase I clinical trial has just been completed with observation of HER-2/neu downregulation associated with E1A expression ⁴⁹. However, both E1A and SV40 large T antigen are viral proteins and very likely suppress HER-2/neu in an indirect manner as none of these proteins have been shown to bind the HER-2/neu promoter directly. The fact that PEA3 directly binds to the HER-2/neu promoter likely provides stronger efficacy and higher specificity to inhibit HER-2/neu promoter activity. We have presented the data to demonstrate that the DNA-binding domain of PEA3 is sufficient to downregulate HER-2/neu overexpression. These

features make PEA3 an attractive candidate for further molecular manipulation to develop next generation therapeutic molecules with higher binding affinity and enhanced specificity. In addition, the strategy described in this study provides a general approach to identify potential transformation suppressors through searching for DNA-binding protein recognizing specific DNA sequence on the promoter of the targeted oncogenes or other disease-causing pathogenic genes.

METHODS

Plasmids To construct the pNeulit plasmid, the promoter region of human HER-2/neu was amplified with a pair of primers (primer A 5' - GATAGGATCCGGGGTCTGGAAGCC-3' and primer B 5' - GGGCAGATCTGGTTTTCCGGTCCCAATGGA-3'). The amplified DNA fragment was digested with BamHI and BglII, and ligated into the BamHI site of pGL2-Basic (Promega, Madison, WI). The sequence of this insert has been confirmed. For site-directed mutagenesis of the PEA3 binding site, two more primers with sequences spanning the PEA3 binding site on the HER-2/neu promoter were synthesized in which the PEA3 motif AGGAAG was changed to AGCTCG (primer C 5' - GGAGGAGGAGGGCTGCTTGAGCTCGTATAAGAATG-3' and primer D 5' - CATTCTTATACGAGCTCAAGCTCCTCC-3'). The PCR product amplified by primers A and D and the product amplified by primers B and C were annealed followed by Klenow extension. The double-stranded DNA fragment was then cloned into the pGL2-Basic vector as the wild-type promoter. The full-length PEA3 cDNA was cloned into pcDNA3 between the HindIII and BamHI sites (pcDNA3-PEA3). The pSR α PEA3 plasmid used in the focus forming assay contains the same PEA3 cDNA cloned into the pcDL-SR α 296 vector and is under the control of a promoter containing the simian virus 40 early promoter and the R-U5 region of the long terminal repeat of human T-cell leukemia virus type 1⁵⁰. The plasmid carrying the DNA-binding domain of PEA3 (PEA3DBD) was PCR-amplified and cloned into pcDNA3.1/His vector.

Cells Mouse fibroblast NIH3T3 and human cancer cells used in this study were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % bovine calf serum and 10 % fetal bovine serum, respectively.

Gel mobility shift assay The annealed double-strand oligonucleotide derived from the HER-2/neu promoter containing either the wild-type (5' - GGAGCTCGAGGGCTGCTTGAGGAAGTATAAGAATG-3' and the complementary strand) or a mutated PEA3 binding site (5' - GGAGCTCGAGGGCTGCTTGAGCTCGTATAAGAATG-3' and the complementary strand) was end labeled by ³²P- γ ATP. Binding of the protein factors to DNA sequences was achieved in a mixture containing 1X binding buffer (20 mM HEPES, pH 7.9, 5 mM

MgCl₂, 5 % glycerol, 0.1 M KCl, 0.2 mM EDTA, 2 mM DTT), 0.3 µg GST-PEA3 (or GST alone or 1 µg nuclear extract of MDA-MB-453), 2 to 8 µg poly d(I-C), 1 µg of bovine serum albumin, and 20,000 cpm of ³²P-labeled oligonucleotide. 100× the amount of competitor oligonucleotide was added when necessary. In the protein competition assay, 0.3 or 0.6 µg of GST-PEA3 protein was included in the reaction mixture. The binding reaction was performed at room temperature for 20 minutes. The samples were separated in a 5 % polyacrylamide. Gels were dried under vacuum and autoradiographed.

Transient transfection and luciferase assay Human cancer cells were seeded with 60-80 % of confluency and transfected by incubating with DNA : DC-chol liposome complex (13 nmol lipid : 1 µg DNA) in serum free medium for 2-5 hours, or by incubating with the DNA : polyethylenimine (PEI, 4.5 µg/µl, average MW 25,000; Aldrich, Milwaukee, WI) complex (0.5 µl PEI : 1 µg DNA) for 1-3 hours. Cell lysates were prepared 48 hours later for luciferase activity assay by following the manufacturer's instruction (Promega) using the monolight 2010 Luminometer (Analytical Luminescence Laboratory). The measured luciferase activities were normalized by measuring the co-transfected β-galactosidase activity. The β-galactosidase activity was measured by mixing equal amounts of cell extract and 2 X Buffer (120 mM Na₂HPO₄, 80 mM NaH₂PO₄, 2 mM MgCl₂, 100 mM β-mercaptoethanol, 1.33 mg/ml orthonitrophenyl galactose). After incubating at 37°C, enzyme activity was determined by absorbance at 420 nm.

Stable transfection and colony formation assay Human cancer cells under 80 % confluency were transfected with 1 µg of PEA3 expression plasmid or the pcDNA3 vector using DC-chol liposome as a carrier, and subject to neomycin selection. Neomycin-resistant colonies were stained by 1 % crystal violet and counted. Transfections of each cell line were repeated 2-3 times using cells of different passages and plasmid DNA from independent preparations.

Orthotopic ovarian cancer model and liposome-mediated *in vivo* gene transfer Four- to six-week-old athymic female *nu/nu* mice were purchased from Harlan Sprague Dawley, Inc.(Indianapolis, IN) and treated in accordance with

institutional guidelines. To establish tumors, 2×10^6 SKOV-3-ip1 cells were injected into the intraperitoneal (ip) cavity as described ^{27, 29}. Five days after ip injection of cancer cells, the mice that had tumors were separated in five groups and received weekly ip injections of 200 μ l of different reagents for a period of time. The responses and survival rates were observed for 1 year.

Immunoblotting Immunoblot analysis for PEA3 and HER-2/neu p185 expression in cells and tumor samples was performed as described previously ^{26, 48}.

ACKNOWLEDGMENTS

The authors gratefully thank Dr. J H Chen for providing PEA3 cDNA. This work was partially supported by NIH grants RO1-CA58880, RO1-CA77855 (to MCH), RO1-CA64654 (to LH), and supported by M. D. Anderson Breast Cancer Research Program as well as a NIH cancer center core grant (CA 16672). XX is a recipient of predoctoral fellowship from the Breast Cancer Research Program of the Department of Defense (DAMD17-96-1-6223).

FIGURE LEGENDS

Figure 1 PEA3 binds to the HER-2/neu promoter. GST-PEA3 fusion protein was incubated with 32 P- γ ATP end-labeled wild-type or mutated oligonucleotide containing the consensus PEA3 binding site sequence on the HER-2/neu promoter (lanes 1). The binding specificity was tested by competition with unlabeled wild-type (lanes 2) or non-specific oligonucleotide (lanes 3).

Figure 2 PEA3 inhibits the HER-2/neu promoter activity in HER-2/neu overexpressing cancer cell lines. Human ovarian cancer cells SKOV-3 were cotransfected with 5 μ g of pNeulit or HER2-CAT with different amounts of PEA3 plasmid DNA (pcDNA3-PEA3) as indicated. The luciferase (A) or the chloramphenicol acetyltransferase (CAT) (B) reporter activity was measured. For the CAT transient assay, the doses of cotransfected pcDNA3-PEA3 were 0, 5, 10, and 15 μ g. The results of two independent experiments are shown.

Figure 3 PEA3 represses HER-2/neu promoter activity through a positive regulatory DNA motif. A, Luciferase reporter gene driven by either wild-type (open bars) or the PEA3 site-mutated (close bars) HER-2/neu promoter was measured after cotransfection with PEA3 cDNA into MDA-MB-453 cells. B, The luciferase activities derived from wild-type and the PEA3 site-mutated HER-2/neu promoter were compared by transfection into a breast cancer cell line (MDA-MB-453) and an ovarian cancer cell line (SKOV-3-ip1).

Figure 4 PEA3 represses neu-mediated transformation and inhibits cell growth of

HER-2/neu-overexpressing cancer cells. A, PEA3 represses the transforming activity of activated genomic rat neu. NIH3T3 cells (2×10^5) were plated to a 100-mm culture dish 24 hours before transfection. Cells were transfected with 0.5 μg of pSV2neo, 1 μg of cNu104 plus 5 μg of either pSR α PEA3 or the control vector pSV2E by calcium phosphate precipitation. Two days after transfection, cells were split and two plates were subject to selection in 500 $\mu\text{g}/\text{ml}$ of neomycin (G418; Life Technologies, Inc), and the other two were maintained in normal medium until foci appeared. Foci and neomycin-resistant colonies were stained and counted. The number of foci was normalized by the corresponding number of neomycin-resistant colonies. Results are expressed as ratio of the number of foci to the number of colonies from each transfection. The ratio of cNu104 transfection alone was set as 100 %. B, An example of the PEA3-mediated suppression of the focus forming activity by cNu104. C, Cell growth of HER-2/neu-overexpressing breast and ovarian cancer cells was inhibited by PEA3 transfection as indicated by the decrease of colony numbers compared with vector transfection. D, Ectopic PEA3 RNA expression was preferentially lost in the survival neomycin-resistant clones derived from the HER-2/neu-overexpressing breast cancer cell line MDA-MB-453. Total RNA were extracted from cell clones resulted from stable transfection and RT-PCR was performed by following the manufacturer's instruction (SuperScript preamplification system; Life Technologies, Inc). The primers derived from the PEA3 coding sequence (5'-TGAATTATGACAAGCTGAGCCG-3') and from the expression vector pcDNA3 (5'-TCAGCGAGCTCTAGCATTAGG-3') were use to amplified the ectopically expressed PEA3 transcript. Primers for GAPDH internal control were: 5'-AGGTGAAGGTCGGAGTCAAC-3' and 5'-TCCATTGATGACAAGCTTCCC-3'. COS-1 cells transiently transfected by the PEA3 cDNA were used as a positive control. Amplification was performed on a Perkin Elmer DNA Cycler 480 for 35 cycles with

denaturing at 94°C for 30 seconds, annealing at 58°C for 1.5 minutes and extension at 72°C for 1.5 minutes.

Figure 5 PEA3 inhibits growth of tumor xenografts derived from HER-2/neu-overexpressing cancer cells. A, Nude mice carrying intraperitoneal tumors derived from SKOV3-ip1 received weekly ip injections of a reagent containing PEA3 plasmid DNA complexed with liposome (PEA3-liposome), control DNA (pGEM) complexed with liposome (pGEM-Liposome), naked PEA3 plasmid DNA, or PBS. Number of mice in each group is shown in the parentheses. The arrow marks the last injection. Tumor samples were retrieved from mice treated with PBS, the lipid vehicle DC-Chol, PEA3 naked DNA, or PEA3-DC-Chol complex, and the levels of p185 were measured by immunoblotting as shown in the insert. Loading was controlled by the endogenous α -actin expression (data not shown). B, Nude mice carrying tumors derived from 2774 10-c were treated with PEA3-liposome complex (the solid line) or PBS alone (the dashed line) by the same procedure as SKOV-3-ip1 cells.

Figure 6 PEA3 competes with a distinct family member of the *ets* family detected in HER-2/neu-overexpressing cancer cells. A, Nuclear extract of MDA-MB-453 cells was incubated with the DNA oligonucleotide probe in the absence (Oligo) or presence of unlabeled competitor of the same specific oligonucleotide (SC) or non-specific oligonucleotide of shuffled sequences (NSC). The signal of specific DNA-protein complex is indicated by an arrow. B, Increasing amount of GST-PEA3 fusion protein was added into the binding reaction to test the competition between the unknown nuclear factor and PEA3 protein. The DNA-PEA3 complex is indicated by an arrow. NE, nuclear extract. C, The DNA-binding domain of PEA3 is sufficient to down-regulate the HER-2/neu promoter as assayed by cotransfection of different doses of PEA3DBD and

pNulite. The result from a representative experiment is shown.

Table I. PEA3 suppresses growth of cancer cells overexpressing HER-2/neu.¹

HER-2/neu overexpressers

<u>Cell lines</u>	<u>pcDNA3</u>	<u>PEA3</u>
SK-BR-3	100	0.7 (\pm 0.9)
MDA-MB-453	100	19.7 (\pm 3.0)
SKOV3-ip1	100	14.3 (\pm 4.1)

HER-2/neu basal-level expressers

<u>Cell lines</u>	<u>pcDNA3</u>	<u>PEA3</u>
MDA-MB-435	100	73.4 (\pm 10.9)
2774 c-10	100	83.3 (\pm 3.8)

¹ Experiment for each cell line was independently repeated 2-4 times using different batches of plasmid DNA and cell culture. Inhibition of colony formation by PEA3 transfection is shown in percentage with the number of neomycin-resistant colony resulted from pcDNA3 transfection arbitrarily set as 100 %.

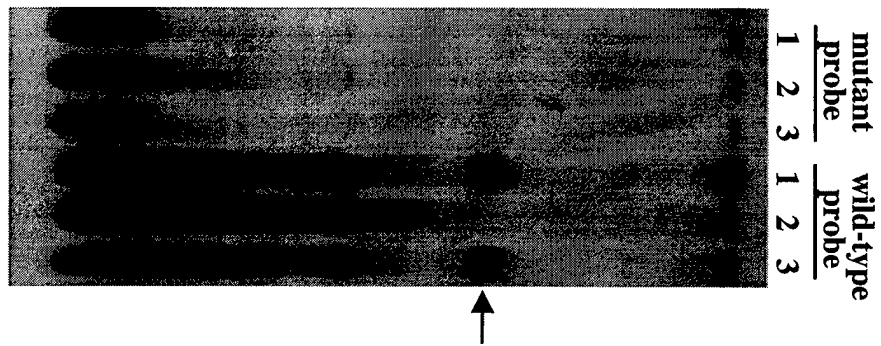
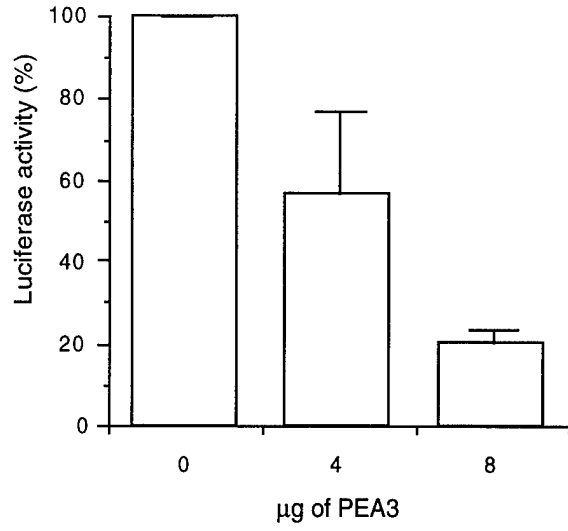


Fig. 1

A.



B.

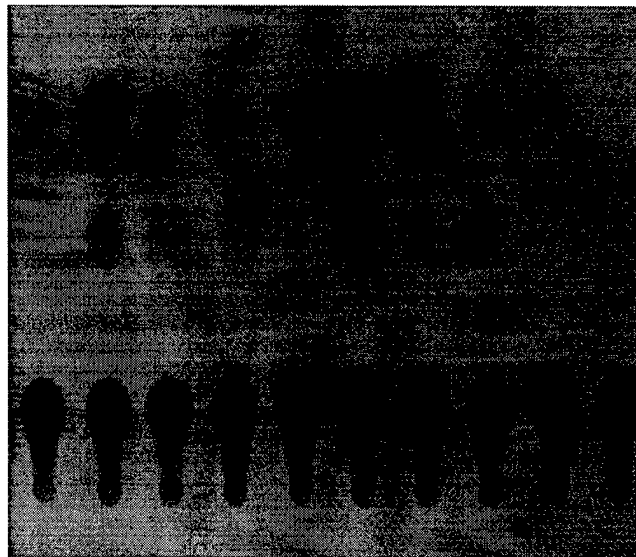
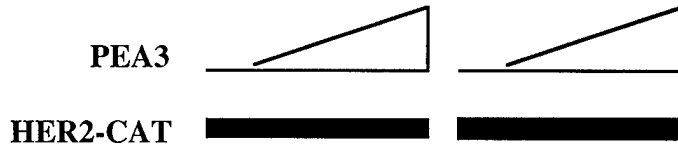
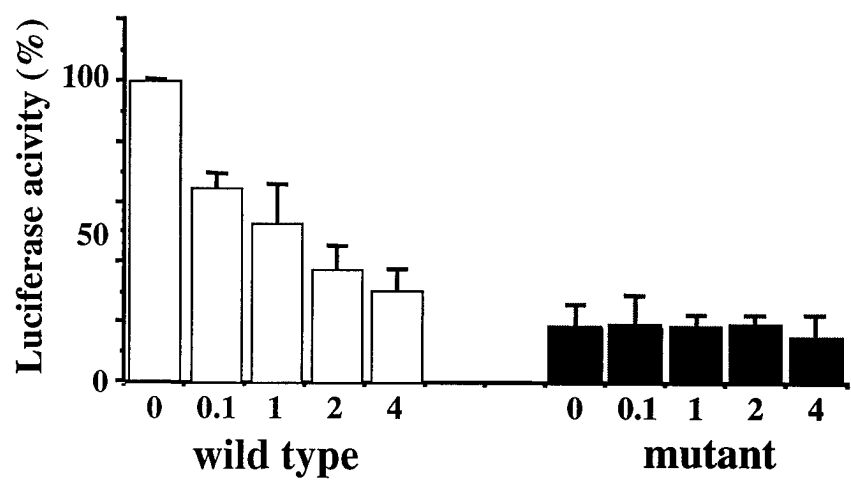
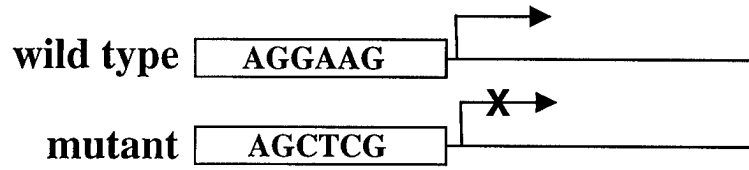


Fig. 2

A.



B.

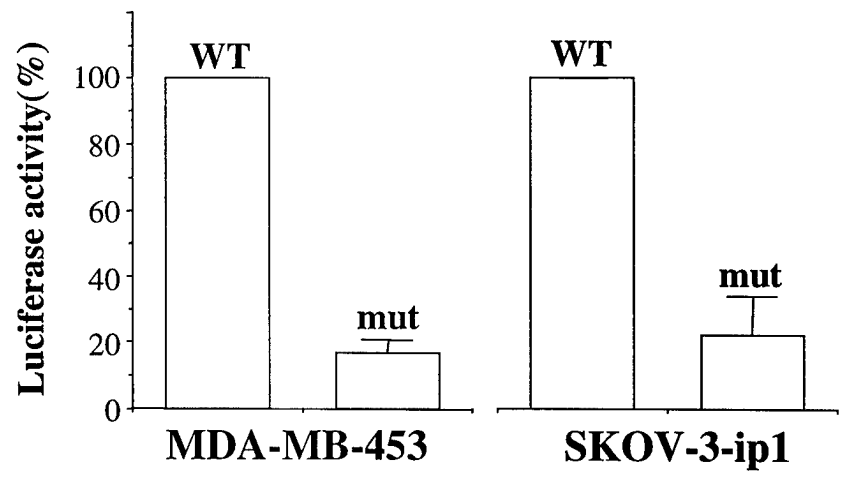
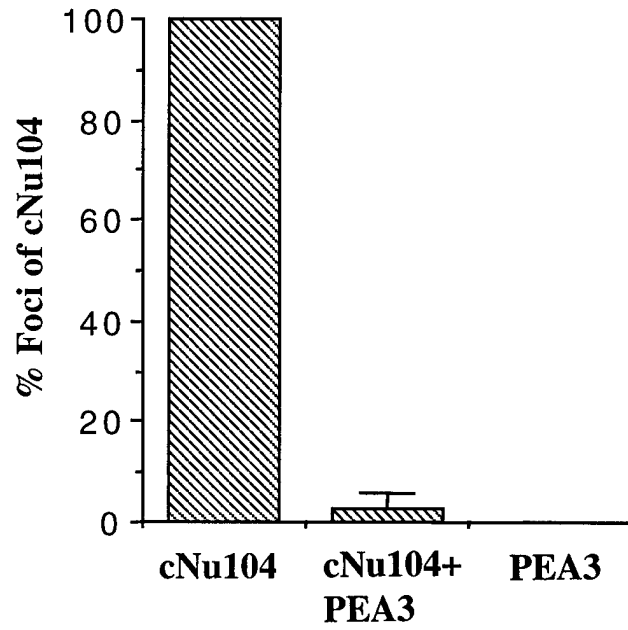


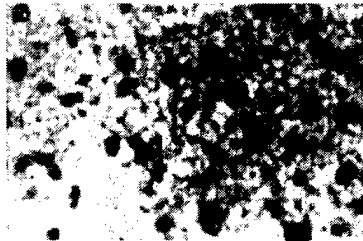
Fig. 3

A.



B.

cNu104+
vector



cNu104+
PEA3

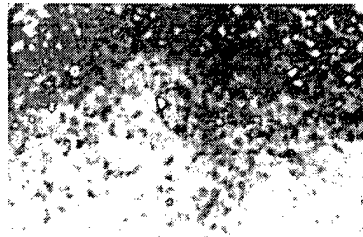


Fig. 4

C.

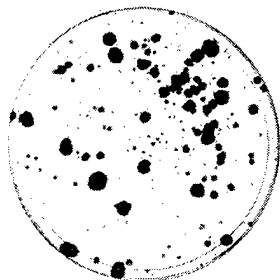
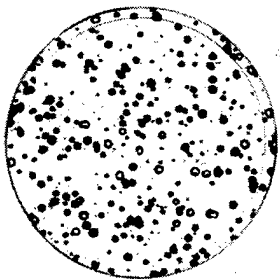
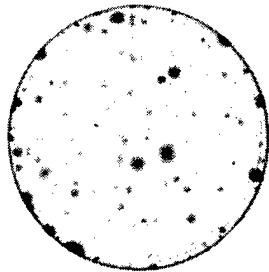
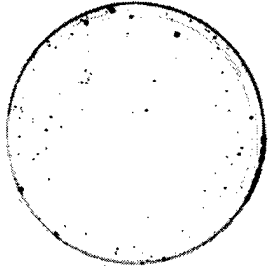
HER-2/neu overexpressors

Basal HER-2/neu expressors

MDA-MB-453 SK-OV-3-ip1

MDA-MB-435 2774 c-10

PEA3



Vector
control

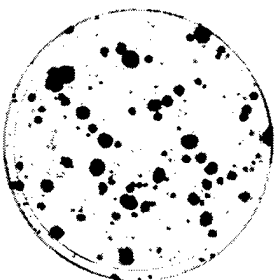
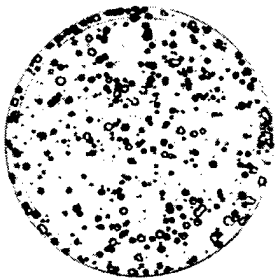
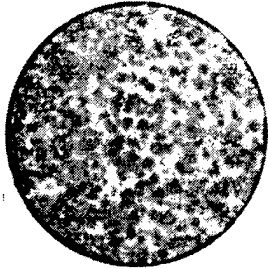
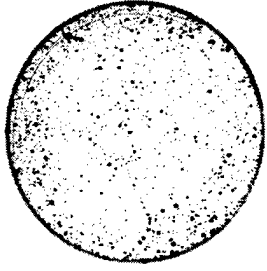


Fig. 4

D.

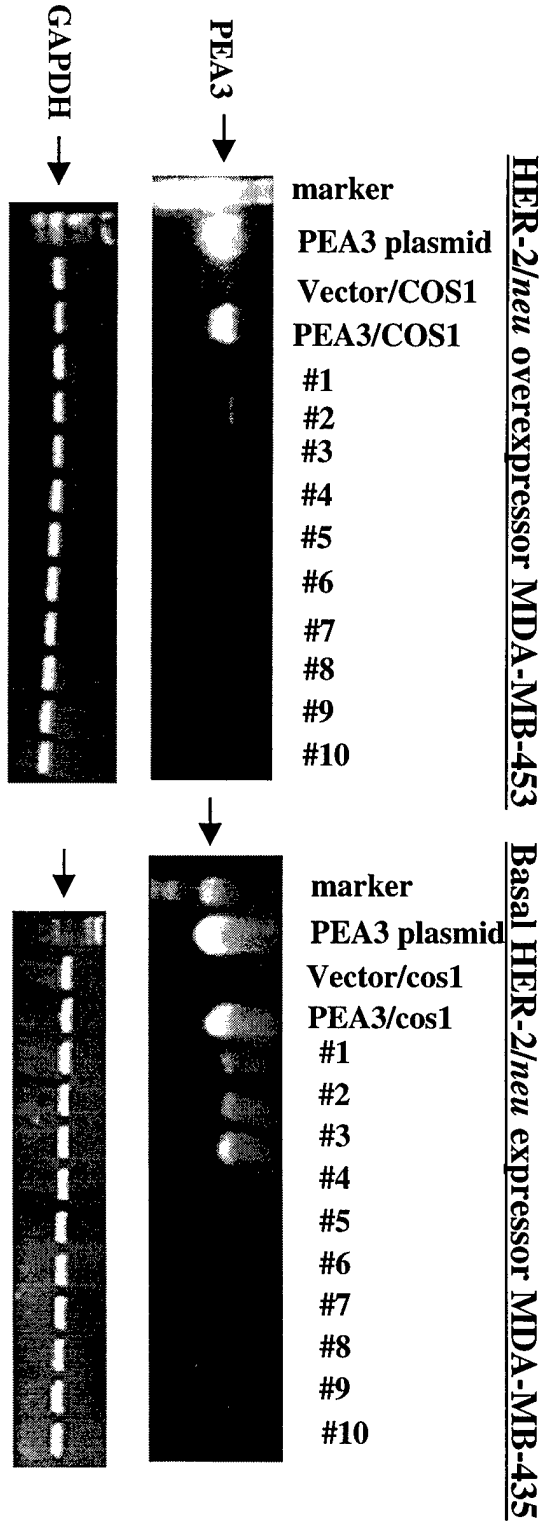


Fig. 4

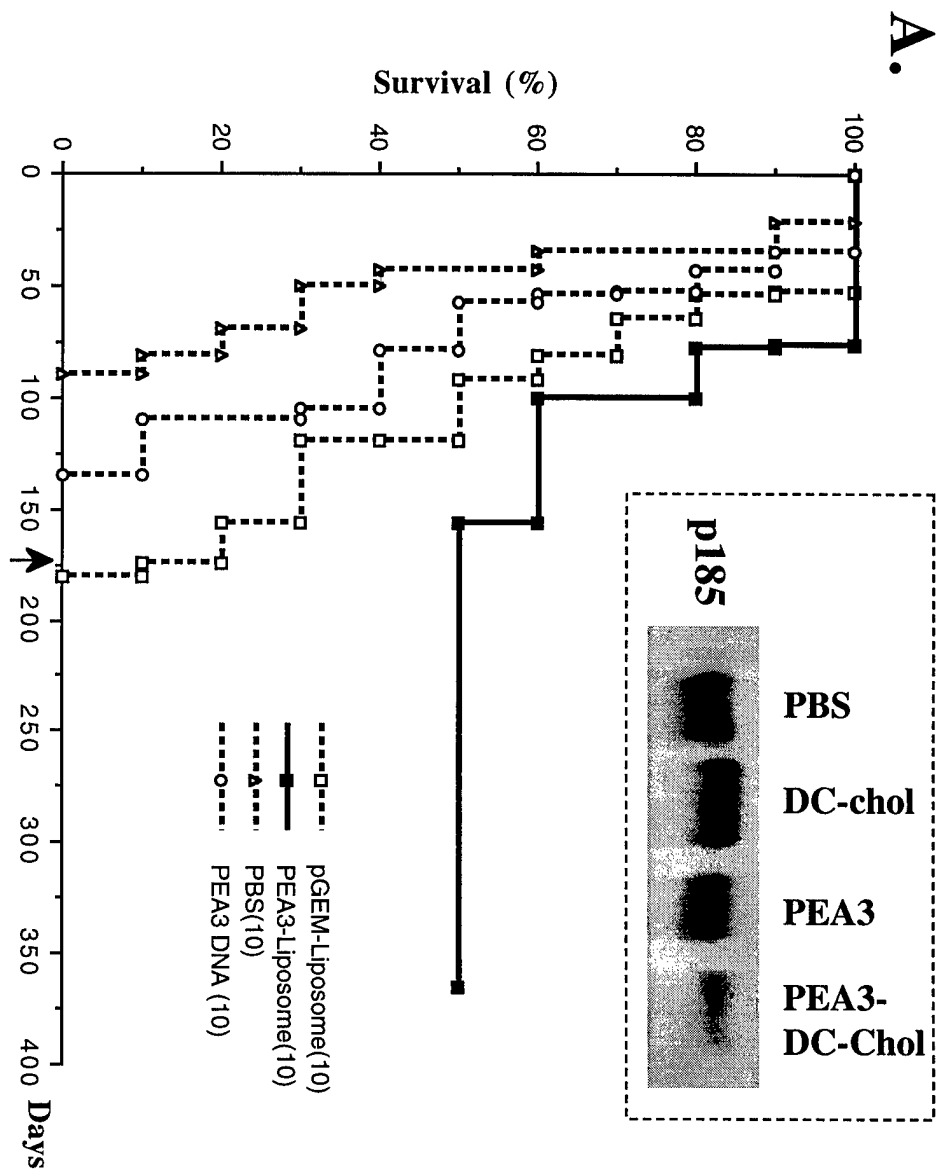


Fig. 5

B.

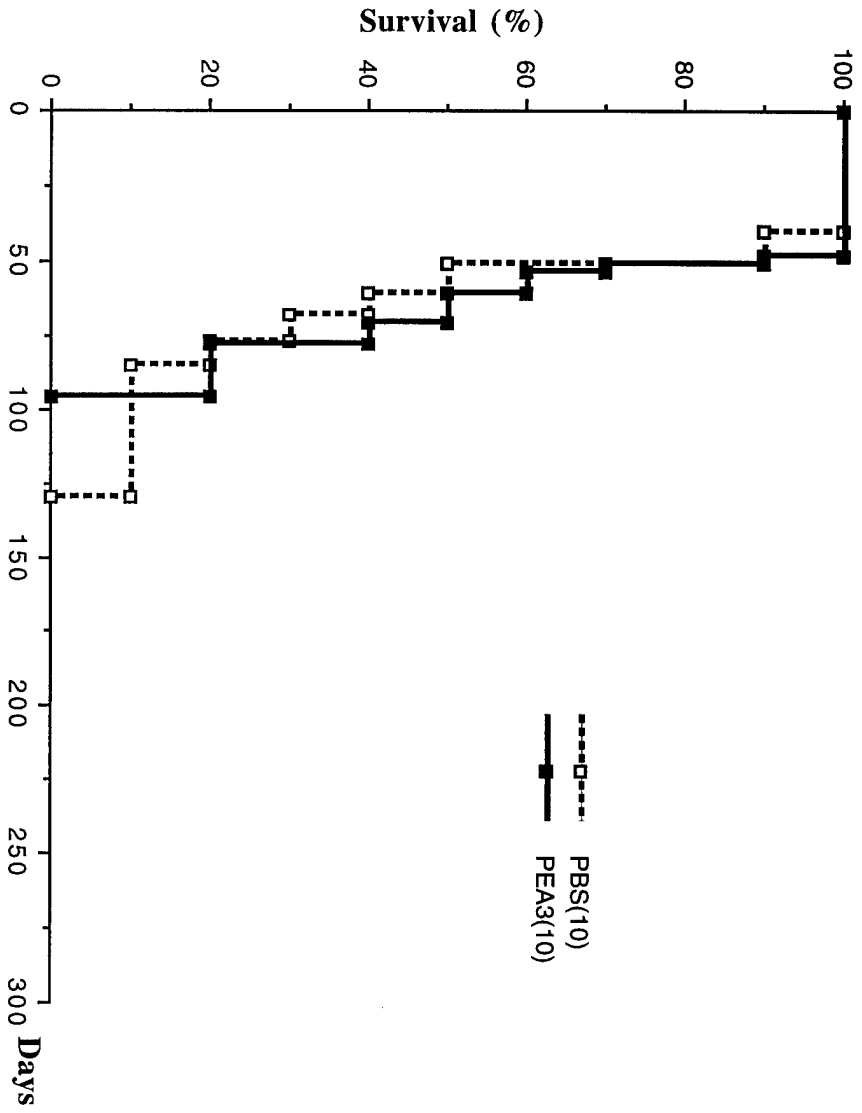


Fig.5

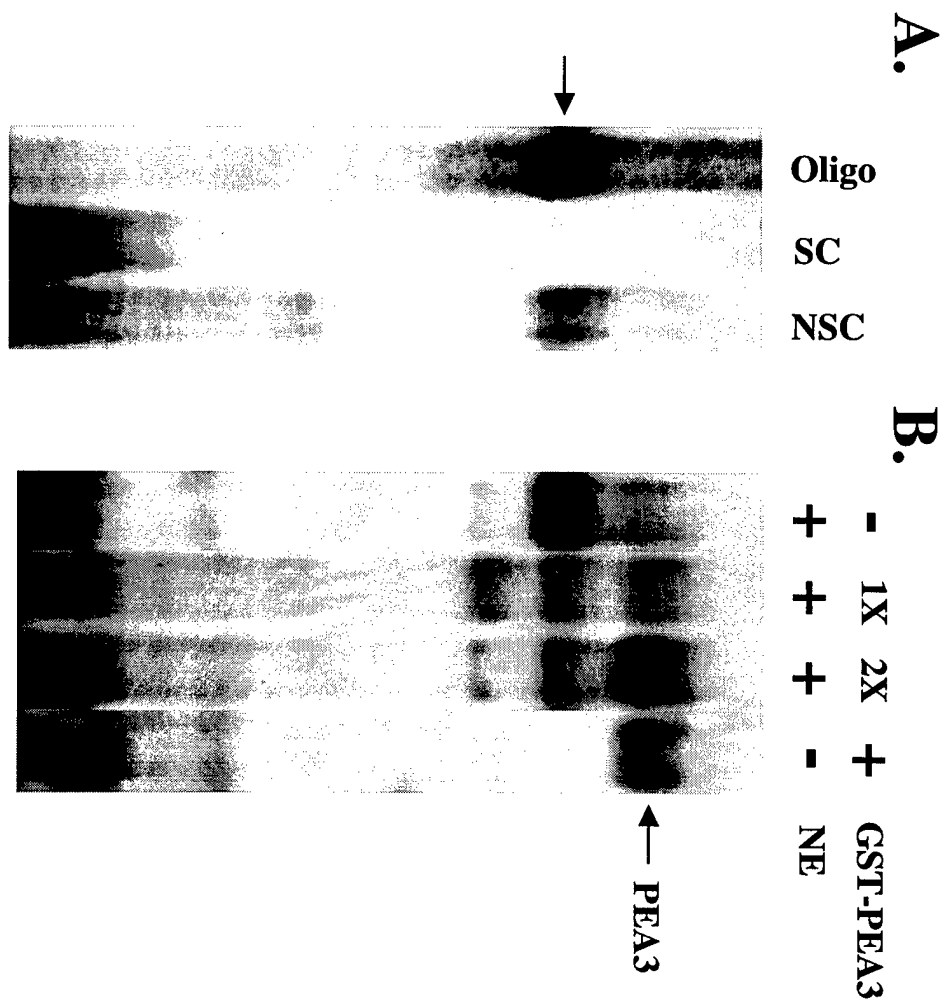


Fig. 6

C.

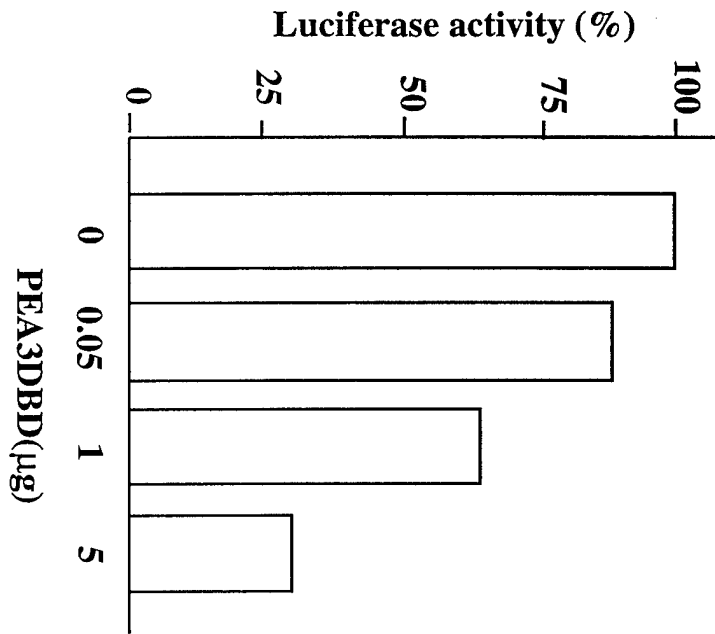


Fig. 6

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