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Development of PEA-15 using a potent non-viral vector for therapeutic application in breast cancer

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Abstract

Advanced breast cancer requires systemic treatment, therefore developing an efficient and safe strategy is urgently needed. To ensure the success of target therapy, we have developed a breast cancer-specific construct (T-VISA) composed of the human telomerase reverse transcriptase (hTERT; T) promoter and a versatile transgene amplification vector VISA (VP16-GAL4-WPRE integrated systemic amplifier) to target PEA-15 (Phosphoprotein enriched in astrocytes) in advanced breast tumors. PEA-15 contains a death effector domain that sequesters extracellular signal-regulated kinase (ERK) in cytoplasm, thereby inhibiting cell proliferation and inducing apoptosis. T-VISA-PEA-15 was found to be highly specific, selectively express PEA-15 in breast cancer cells, and induce cancer-cell killing in vitro and in vivo without affecting normal cells. Moreover, intravenously treatment with T-VISA-PEA-15 coupled with liposome nanoparticles attenuated tumor growth and prolonged survival in mice bearing advanced breast tumors. Importantly, there was virtually no severe toxicity when PEA-15 is expressed by our T-VISA system compared with cytomegalovirus (CMV) promoter. Thus, our findings demonstrate an

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5. Conflicts of interest

The authors declare no conflict of interest.

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effective cancer-targeted therapy that is worthy of development in clinical trials eradicating advanced breast cancer.

Keywords

Breast cancer; T-VISA system; PEA-15; Target therapy

1. Introduction

Breast cancer is the first most common cancer among women and one of the leading causes of death due to cancer all over the world[1]. Many advanced breast cancer patients develop local recurrence or distant metastasis during the course of the disease[2; 3]. Therefore, additional treatment strategies are needed. Targeted gene therapy is an attractive strategy due to the benefit of cancer-specific expression of therapeutic genes[4; 5]. We previously showed that T-VISA vector containing hTERT promoter and VISA (VP16-GAL4-WPRE integrated systemic amplifier) system could target gene expression specifically in human breast tumor, without severe toxicity in normal tissues[6].

Recently, our studies showed that PEA-15 (phosphoprotein enriched in astrocytes) is a multifunctional protein, which can regulate cell proliferation, autophagy, and apoptosis[7; 8; 9]. PEA-15 controls cell proliferation by interfering with the extracellular signal-regulate kinase (ERK) pathway[10]. Other authors also demonstrated that high expression level of PEA-15 in cancer cells correlated with longer survival of cancer patients, such as astrocytic tumors and colorectal cancer[11; 12]. However, the role of PEA-15 in breast cancer has not been completely illuminated in the use for cancer targeted therapy.

It has previously been shown that PEA-15 can suppress the growth of triple-negative breast cancer cells[7]. Therefore, to investigate thoroughly the role of PEA-15 for therapeutic efficacy, we selectively expressed PEA-15 by integrating it into the T-VISA vector to generate T-VISA-PEA-15, and discussed its cancer-killing efficacy and safety. Fortunately, our study demonstrates that T-VISA-PEA-15 gene therapy not only has significant anti-cancer effects, but also assured the tumor-specific targeting effects without systemic toxicity in normal cells. Thus, the current study provides a promising treatment strategy worthy of further development in clinical trials for treating advanced breast cancer by a cancer-targeted gene therapy.

2. Materials and Methods

2.1 Cell lines and culture

Both breast cancer cell lines (T47D, MCF-7, MDA-MB-231, MDA-MB-468, MDA-MB-361, MDA-MB-453, BT474, 4T1, SKBR-3) and immortalized normal mammary epithelial (184A1, and MCF-10A) were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained according to the manufacturer's instructions. The above cell lines were passaged in our lab for less than six months after resuscitation of frozen aliquots. All cell lines were authenticated by short tandem repeat DNA profiling before use and found to be free of Mycoplasma infection. Primary human

breast cancer cell lines (P1, P2 and P3) were obtained from patients with breast cancer who underwent modified radical mastectomy. This study was approved by the Institutional Review Board of Sun-Yat Sen University Cancer Center of China. Tissues were collected under patients' agreement. Stable cell lines MDA-MB-231-Luc expressing firefly luciferase were constructed and maintained as described before[6].

2.2 Western blot analysis and immunohistochemistry

Western blotting was performed as described previously[7; 13] using anti-PEA-15(Cell Signaling Technology, Cambridge, MA), anti-ERK, anti-phospho-ERK (Ser217/Ser221), Elk-1, AP-1, caspase-3 and caspase-7(Cell Signaling Technology, Danvers, MA). The membranes were stripped and re-blotted with a Rabbit monoclonal anti-GAPDH antibody (Sigma, St Louis, MO) as a loading control.

The formalin-fixed, paraffin-embedded tissues were sectioned at Five-micrometer and immunostaining for PEA-15 was performed as our previous protocol by using the rabbit anti-PEA-15 antibody (Abcam), horseradish peroxidase-conjugated avidin biotin complex (Vector Laboratories, Burlingame, CA, USA) and 0.03% diaminobenzidine (DAB) chromagen.

2.3 Preparation of plasmids and liposome

To engineer the treatment plasmids, we replaced the luciferase gene with PEA-15, which was digested with BglII/NheI, followed by ligation to the vector backbone. In order to avoid ampicillin resistance in the clinic, the pUK21-T-VISA-PEA-15 containing kanamycin resistance gene was created by inserting the functional part of pGL3-T-VISA-PEA-15 vector into NotI and SalI sites of the pUK21 vector. Therapeutic plasmids purified by Qiagen Endo-Free Mega Prep kit (Qiagen, Valencia, CA, USA) according to the manufacturer' protocol. The endotoxin level was determined to be <10 endotoxin units/mg of DNA (QCL-1000 kit, BioWhittaker, Walkersville, MD, USA). Plasmid was incorporated into extruded DOTAP/Cholesterol liposome that was produced in our laboratory according to the previously published protocol[5; 14].

2.4 Luciferase reporter assay

Cells were transiently co-transfected with 2 µg indicated plasmid and 0.1 µg CMV-Luc (internal control) in 12-well plates using Lipofectamine (Invitrogen) for cell killing experiment. Forty-eight hours later, cells were subjected to lysis and assayed for luciferase activity using the dual-luciferase reporter assay system (Promega) according to the manufacturer's protocol.

2.5 FACS analysis

To evaluate apoptosis, cells were incubated with Annexin V and PI solution (BD Biosciences) according to the manufacturer's protocol. Flow cytometric analysis was performed immediately using a FACScalibur TM (Becton Dickinson, Heidelberg, Germany).

2.6 Breast tumor animal model and in vivo gene targeted therapy

Female BALB/c nu/nu mice at 4–6 weeks of age were used to establish human xenograft tumors model. All mice were maintained in a specific pathogen-free environment at the Animal Experiment Center of Sun Yat-Sen University, and all procedures were approved by the Animal Care and Use Committee of Sun Yat-Sen University and conformed to the legal mandates and national guidelines for the care and maintenance of laboratory animals. To test the antitumor effect of CMV-PEA-15 and T-VISA-PEA-15 in vivo, luciferase-expressing MDA-MB-231 cells (5×10^6) were injected into the left fourth inguinal mammary gland of mice. After inoculation, mice were noninvasively imaged by In vivo Imaging System (Xenogen) to assess tumor growth and then randomly assigned to one of three groups for every tumor model. Each group of mice received 100 μ l of DNA:liposome complexes that contained 20 μ g of pUK21-T-VISA (control), pUK21-CMV-PEA-15, or pUK21-T-VISA-PEA-15 administered through tail-vein injection, twice per week for 3 consecutive weeks. The growth of tumors was imaged as previously described. Before each imaging, mice were anesthetized and intraperitoneally injected with 100 μ l of D-luciferin (Xenogen; 15 mg/ml in phosphate-buffered saline) and then images of mice were taken by the IVIS imaging system, and Living Imaging software (both from Xenogen) was used to quantify the signals.

2.7 TUNEL staining analysis

To determine tissue undergoing apoptosis after PEA-15 expression, an in situ Apoptosis Detection Kit, peroxidase (POD) was conducted according to the manufacturer's instructions. The percentage of apoptotic cells were calculated by random selecting 3 fields.

2.8 Acute toxicity analysis

The analysis of acute toxicity was performed as previously described. Briefly, high doses (50 or 100 μ g) of DNA-liposome complex were injected into the intact Balb/c mice. Blood samples from mice were collected at indicated time and measured by automatic analyzer (Roche Cobas Mira Plus, Roche, Mannheim, Germany).

2.9 Statistical analysis

Data are given as mean \pm SD. Student's t-test was done to evaluate the differences between different groups. Survival curves were calculated by the Kaplan–Meier method. The difference in mouse survival time between two groups was assessed with the log-rank test. The significance level was set at a value of $P < 0.05$.

3. Results

3.1 Expression of PEA-15 in paired breast tumor tissues and adjacent normal breast tissues

In our study, we examined the expression of PEA-15 protein by IHC analysis in 8 pairs of fresh invasive breast tumor tissues (T) compared with adjacent normal breast tissues (N), respectively (Fig. 1). The results showed that protein expression had significantly higher levels in adjacent normal tissues than that in breast tumor tissues ($P < 0.01$).

3.2 T-VISA-PEA-15 selectively inhibits breast cancer cell proliferation

Up to now, PEA-15 has been reported to be correlated with low proliferation and our previous study have demonstrated that overexpression of PEA-15 can suppress triple-negative breast cancer cell growth[7]. In addition, our constructed T-VISA vector has been assured to be a robust cancer-specific promoter, and it hold the ability to drive target gene expression highly and specifically in breast cancer cells[6]. Therefore, to enhance the gene expression activity and specificity, we incorporated PEA-15 into our T-VISA system and then evaluated the potential anti-tumor effects of T-VISA-PEA-15 in numerous breast cancer cells (Fig. 2A). In order to balance the difference of gene transfection efficiency, we transiently co-transfected 9 human breast cancer cell lines with CMV-PEA-15, T-VISA-PEA-15 plus the indicator plasmid pGL3-CMV-Luc. The cell killing effects of T-VISA-PEA-15 were assessed by assaying luciferase activity 48 hours after transfection (Fig. 2B and 2C). The results showed that T-VISA-PEA15 significantly suppressed these cancer cells dose-dependently (Fig. 2B), but the killing effects has no obvious differences compared with CMV-PEA-15 treatment group (Fig. 2B and 2C). We further demonstrated that T-VISA-PEA-15 efficiently killed primary breast cancer cells obtained from three individual patients (P1, P2 and P3) compared with the control vector (Fig. 2D). Thus, T-VISA-PEA-15 has the ability to preferentially kill breast cancer cell lines and primary breast tumor cells. However, T-VISA-PEA-15 did not kill normal breast cell lines MCF10A and 184A1 (Fig. 2E). In contrast, CMV-PEA-15 induced death of not only breast cancer cells (Fig. 2B, 2C and 2D) but also primary breast tumor cells and normal cells tested (Figure 2E). In brief, T-VISA-driven PEA-15 selectively kills breast cancer cells in vitro.

3.3 T-VISA-PEA-15 sequesters extracellular signal-regulated kinase (ERK) in cytoplasm, and then induces cancer cell apoptosis

To investigate the mechanisms of cell killing effects of our T-VISA-PEA-15:liposome complex, we conducted western blotting to test the expression of Erk1/2 in cytoplasm. The results showed that both T-VISA-PEA-15 and CMV-PEA-15 sequesters pERK in cytoplasm, and the downstream nuclear transcription factor Elk-1 and AP-1 were all down-regulated (Fig.3A). Moreover, we found an increase in cleaved caspase 3 and cleaved caspase 7 protein expression (Fig. 3B), indicating that PEA-15 driven by CMV or T-VISA promoter does indeed increase apoptosis. Furthermore, we used Annexin V-FITC/PI staining and FACS to assess whether CMV-PEA-15 and T-VISA-PEA-15 induced cell apoptosis in MDA-MB-231 and MCF-7 cells. Consistent with our expectation, T-VISA-PEA-15-transfected cells had an obviously higher ratio of apoptotic cells compared with CMV-PEA-15 or Ctrl (T-VISA) (Fig.3C).

3.4 T-VISA-PEA-15 nanoparticles suppress tumor growth and prolong mouse survival time more effectively than CMV-PEA-15 in breast tumor mouse model

To investigate the cell killing effects of T-VISA-PEA-15 *in vivo*, we established an orthotopic animal model with human breast cancer cell line, MDA-MB-231-Luc, and delivered the indicated plasmid DNA in liposome complexes when tumors were palpable. We monitored the tumor growth by noninvasive IVIS imaging device in real time and the survival information of mice were also recorded. In MDA-MB-231-Luc model, expression

of PEA-15 driven by T-VISA or CMV treatment group demonstrated a strong reduction in tumor growth (both $P < 0.05$), but tumor inhibition by T-VISA-PEA-15 treatment was more substantial than CMV-PEA-15 treatment (Fig. 4A). What's more, T-VISA-PEA-15 significantly prolonged the survival time of mice more effectively than CMV-PEA-15 ($P < 0.05$), although the survival time of mice in treatment groups had all been extended compared to control group ($P < 0.05$ in both treatment groups) (Fig. 4B). By terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) assay, the results showed that T-VISA-PEA-15 induced apoptosis in tumor cells more remarkable than CMV-PEA-15 and Ctrl (Fig. 4C).

3.5 T-VISA-PEA-15 nanoparticles has virtually no acute toxicity in normal mice compared with CMV-PEA-15

In order to compare the treatment safety profile between T-VISA-PEA-15 and CMV-PEA-15, a single high dose of 50 or 100 μg plasmid DNA was injected by tail vein of BALB/c mice (Fig. 5A and B). Blood samples were collected and serum levels of liver alanine aminotransferase (ALT), aspartate aminotransferase (AST), and blood creatinine (CRE) were measured for mice that received 50 μg plasmid DNA. In the CMV-PEA-15 treatment group, the serum level of ALT, AST and CRE were significantly higher than either T-VISA-PEA-15 or control group but decreased by Day 4 (Fig. 5C, 5D and 5E). Moreover, T-VISA-PEA-15-liposome complexes and control groups showed 100% event-free survival, while 85% or 70% event-free survival for CMV-PEA-15 treated mice at a dose of 50 or 100 μg DNA, respectively (Fig. 5A and B). These results indicate that T-VISA-PEA-15-liposome-mediated systemic gene therapy is safer than the commonly used CMV promoter-driven treatment strategy.

4. Discussion

The delivery of therapeutic genes into tumor cells remains a key obstacle for cancer therapy [15; 16]. In this study, we examined the effectiveness of PEA-15 targeted therapy utilizing a T-VISA breast cancer specific promoter system. We first, examined the expression levels of protein levels of PEA-15 in the tissues of patients with breast cancer and the adjacent normal breast tissues of these patients. We observed that PEA-15 levels were significantly reduced in breast tumor tissues compared to their normal counterparts. The function of PEA-15 as tumor suppressor or as inhibitor of tumor cell growth and invasion in various cancers has been investigated [8; 9; 17; 18]. Our group and others reported that phosphorylation status of PEA-15 is associated with the anti-tumor functions of PEA-15. PEA-15 contains two major serine residues (Ser104 and Ser116) at C-terminus. When PEA-15 is unphosphorylated state, PEA-15 bind to ERK and reduces the transcriptional activity of ERK by inhibiting the transcription factor Elk-1 [19; 20; 21; 22; 23]. However, phosphorylation at Ser104 blocks PEA-15 binding to ERK, or double phosphorylation at Ser104 and Ser116 switches the binding specificity of PEA-15 from ERK to FADD, thereby stabilizing its anti-apoptotic function [24; 25]. Based on these observations, we proposed that unphosphorylated-PEA-15 at both Ser104 and Ser116 could result in more potent suppression of tumorigenicity than wild-type PEA-15 in breast cancer. We have shown that the double-unphosphorylated form (PEA-15-AA), strongly inhibited

tumor growth and Ki-67 expression in an ovarian cancer xenograft model[8; 9]. Several other studies also have shown that unphosphorylated form of PEA-15 acts as a tumor suppressor in cervical cancer and lung cancer cells [26; 27]. Moreover, recent studies indicate that serous ovarian cancer has a genomic pattern very similar to that of triple-negative breast cancer [28] which may indicate that potent form of PEA-15, such as PEA-15-AA, can be selectively targeted for TNBC using breast cancer-specific promoter.

In general, gene delivery systems are comprised of viral as well as non-viral systems[29; 30; 31], Viral systems possess higher gene transfer efficiency, however, which have many unsolved problems, such as immunogenicity, cytopathic and recombinogenic effects[32], while non-viral systems are confronted with low packaging and gene transfection efficiency, and so on[14; 33]. Therefore, to enhance the transgene expression of non-viral vector, we previously constructed T-VISA promoter, which have been demonstrated to hold high activity and specificity compared to CMV promoter in breast cancer cell lines. Moreover, our T-VISA can targetedly drive the expression of miR-34a and pro-apoptosis gene BikDD in breast tumor in vitro and in vivo[13]. Looking for clinical application PEA-15, we incorporated the PEA-15 cDNA into our T-VISA promoter system and engineered T-VISA-PEA-15 plasmid. To our delighted, forced expression of PEA-15 by T-VISA vector in breast cancer cell lines lead to inhibition of proliferation compared to CMV promoter in vitro, while the cell killing effects on normal cell lines were significantly weaker than CMV. To intensive study the biological function of PEA-15 in breast cancer, we found that cytoplasmic pERK were down-regulated by CMV-PEA-15 and T-VISA-PEA-15, whereas the level of ERK was not changed. In short, PEA-15 sequestered extracellular signal-regulated kinase (ERK) in cytoplasm[7; 34], leading to apoptosis of breast cancer cells and suggesting that T-VISA-PEA-15 may exert its tumor suppressive effect by enhancing multiple pro-apoptosis gene expression.

Based on the above investigation, we then studied the effectiveness of non-viral delivery system in breast cancer mouse models. The T-VISA-PEA-15: liposome-nanoparticles treatment had better therapeutic effect than Ctrl and CMV-PEA-15 complexed with in the MDA-MB-231-Luc animal model. What's more, the mouse survival in T-VISA-PEA-15 treatment group was prolonged more effectively than Ctrl and CMV-PEA-15 group. It could be explained by the differences of the duration of transgene expression and tumor cell apoptosis induced by CMV or T-VISA vector. In addition, the acute toxicity of T-VISA-PEA-15 treatment group in vivo was assessed and the results showed PEA-15 induced by T-VISA is more safer than CMV. The main mechanism of action of T-VISA-PEA-15 in breast cancer is summarized in Fig.6.

Now that we can surmount the disadvantage of efficient personalized gene expression through modified the non-viral vector, T-VISA-PEA-15 nanoparticles would undoubtedly be an excellent candidate for future development into clinical trial and the approach described here could also be easily applied to other cancer cell types via modifying the suppressor gene and cancer-specific promoter.

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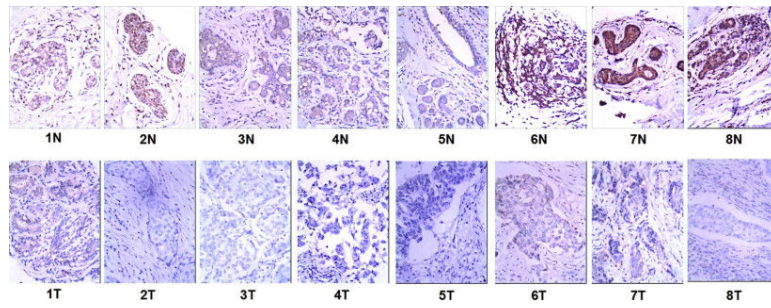


Fig. 1. Expression of PEA-15 is downregulated in breast tumor tissues compared with adjacent morphologically normal breast epithelial tissues from the same patient. IHC confirmed that PEA-15 protein was downregulated in the breast tumor tissue (T) compared with the paired adjacent normal breast epithelium (N) from the same patient.

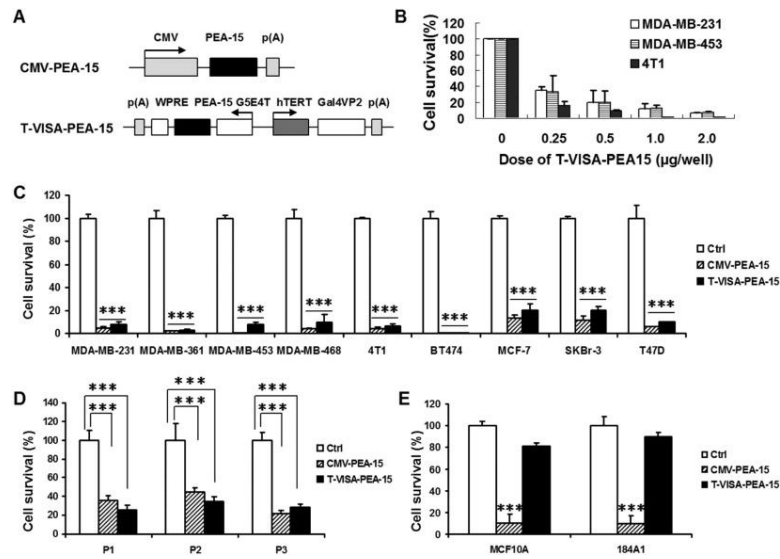
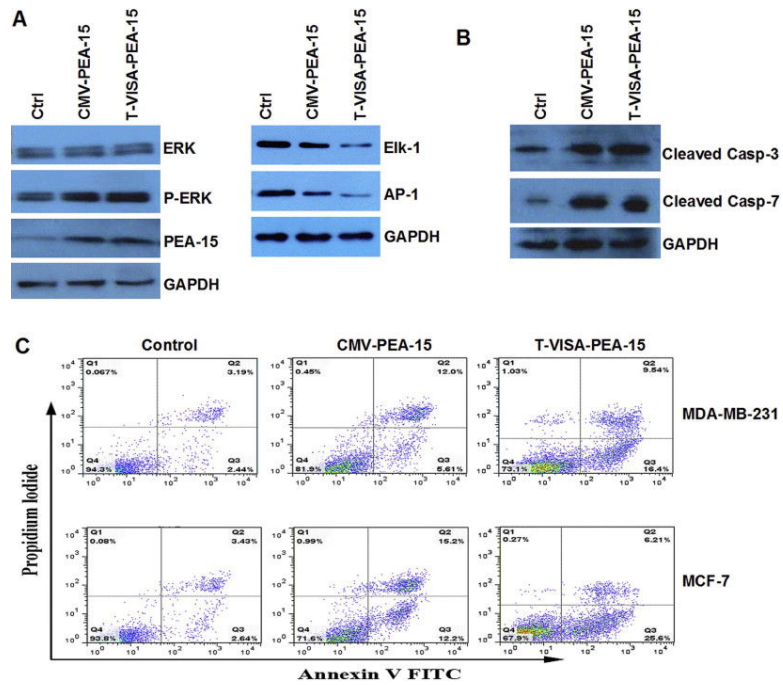


Fig. 2. T-VISA-PEA-15 selectively exerts an inhibitory effect in multiple breast cancer cell lines. (A) Schematic diagram of CMV-PEA-15 and T-VISA-PEA-15 constructs in the pUK21 backbone. (B) Different advanced breast cancer cells were respectively cotransfected increasing amount (0, 0.25, 0.5, 1.0, or 2 µg/well) of T-VISA-PEA-15 and 100 ng of CMV-Luc. (C) T-VISA-PEA-15 effectively inhibits breast tumor cells proliferation. The results are expressed as a percentage of T-VISA control transfected cells (100%) and represent the mean \pm SD of three independent experiments. ***indicates $P < 0.001$. (D) PEA-15 expression driven by T-VISA also kills primary human breast tumor cells effectively. ***indicates $P < 0.001$. (E) The killing effects of T-VISA-PEA-15 on normal breast cells are slower than CMV-PEA-15. ***indicates $P < 0.001$.

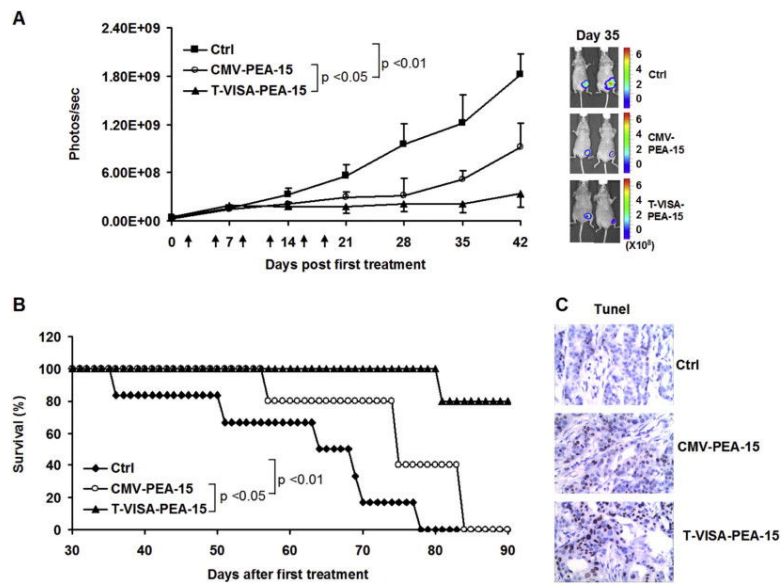
**Fig. 3.**

T-VISA-PEA-15 erk1/2 in cyto and induce the apoptosis.

(A) Western blot analysis of ERK, pERK and the downstream protein expression in MDA-MB-231 cell. GAPDH was used as an internal control.

(B) Western blotting verified cleaved caspase 3 and cleaved caspase 7 protein expression.

(C) Annexin V-FITC/propidium iodide staining and FACS quantification of the number of apoptotic cells in MDA-MB-231 and MCF-7 cells transfected with DNA(Ctrl, CMV-PEA-15 or T-VISA-PEA-15)-liposome complexes. FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate.

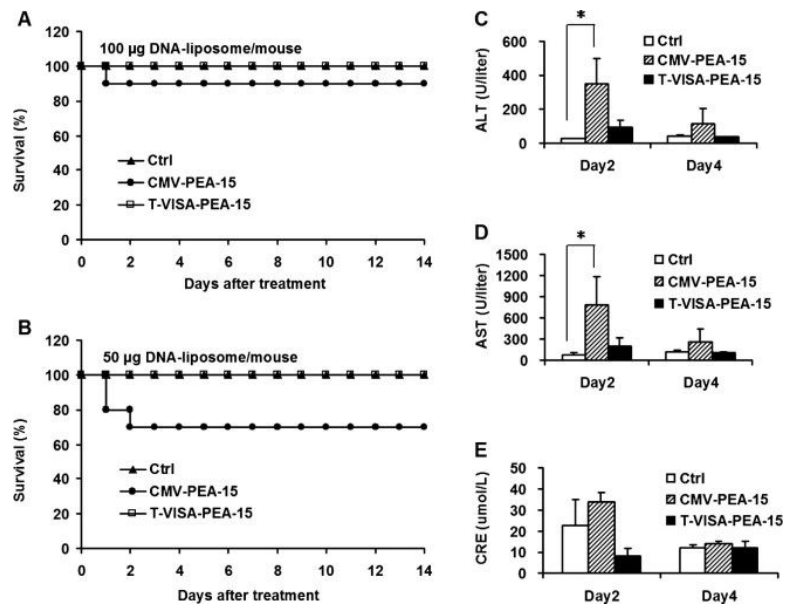
**Fig. 4.**

T-VISA-PEA-15:liposome complexes dramatically suppresses breast tumor growth and Significantly Improves Survival in the breast tumor model. BALB/c nude mice bearing MDA-MB-231-Luc tumors were treated with 20 μ g of liposomal plasmid DNA (CMV-PEA-15, T-VISA-PEA-15 or Ctrl), twice weekly for three consecutive weeks.

(A) The tumor cell growth photon signals were quantified (left panel) with Xenogen's software and representative images are presented (right panel). Error bars indicate SEM. Arrows indicate the treatment time points.

(B) Kaplan-Meier survival analysis from (A) was determined. Expression of PEA-15 driven by T-VISA vector prolonged mouse survival more effectively than PEA-15 driven by CMV promoter.

(C) Representative images of in vivo apoptosis of tumor by TUNEL assay.

**Fig. 5.**

T-VISA-PEA-15 nanoparticles have no systemically acute toxicity compared with CMV-PEA-15 in immunocompetent mice.

(A and B) Kaplan-Meier survival analysis of female BALB/c mice who were treated with single doses of 100 µg or 50 µg plasmid in a liposomal complex via the tail vein. (C, D, and E) Kinetics serum level of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and blood creatinine (CRE) in mice after a single dose of 50 µg plasmid injection. Asterisk, $P < 0.05$.

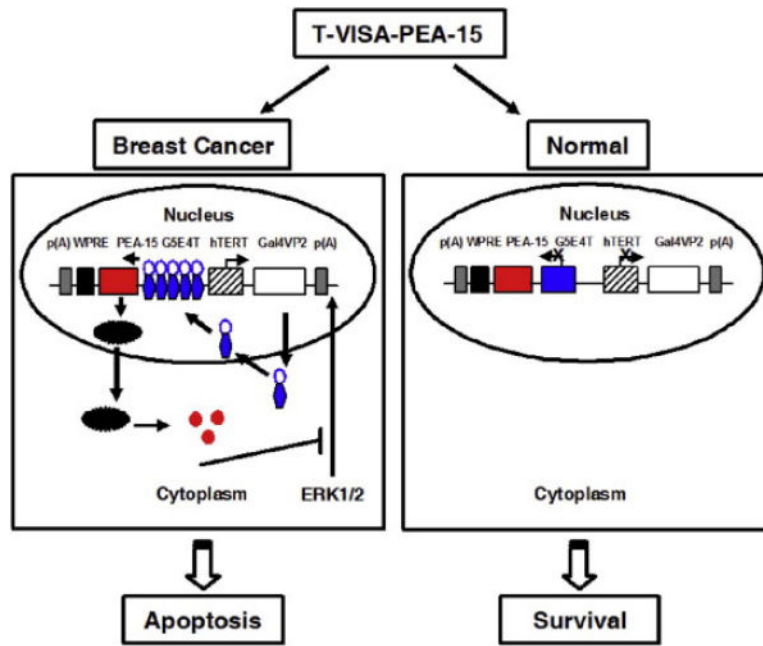


Fig.6. Diagrammatic sketch of the T-VISA-PEA-15 system targeting to breast cancer. The T-VISA-PEA-15 system involves a series of steps in human breast cancer: Firstly, the hTERT promoter drives the GAL4-VP2 fusion protein specifically expression in breast tumor and then open target gene PEA-15 mRNA transcripts. Afterwards, the PEA-15 mRNA transcripts enhance and prolong expression duration of PEA-15 protein, which can sequesters extracellular signal-regulated kinase (ERK) in cytoplasm, thereby leading to apoptosis of breast cancer cells. However, there's little non-specificity gene expression in normal cells for the activity of hTERT promoter is very lower, thereafter, normal cells survival.