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ARTICLE

Specific driving of the suicide *E* gene by the CEA promoter enhances the effects of paclitaxel in lung cancer

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Abstract

Classical chemotherapy for lung cancer needs new strategies to enhance its antitumor effect. The cytotoxicity, nonspecificity, and low bioavailability of paclitaxel (PTX) limits their use in this type of cancer. Suicide gene therapy using tumor-specific promoters may increase treatment effectiveness. We used carcinoembryonic antigen (CEA) as a tumor-specific promoter to drive the bacteriophage *E* gene (pCEA-*E*) towards lung cancer cells (A-549 human and LL2 mice cell lines) but not normal lung cells (L132 human embryonic lung cell line), in association with PTX as a combined treatment. The study was carried out using cell cultures, tumor spheroid models (MTS), subcutaneous induced tumors and lung cancer stem cells (CSCs). pCEA-*E* induced significant inhibition of A-549 and LL2 cell proliferation in comparison to L132 cells, which have lower CEA expression levels. Moreover, pCEA-*E* induced an important decrease in volume growth of A-549 and LL2 MTS producing intense apoptosis, in comparison to L132 MTS. In addition, pCEA-*E* enhanced the antitumor effects of PTX when combined, showing a synergistic effect. This effect was also observed in A-549 CSCs, which have been related to the recurrence of cancer. The in vivo study corroborated the effectiveness of the pCEA-*E*-PTX combined therapy, inducing a greater decrease in tumor volume compared to PTX and pCEA-*E* alone. Our results suggest that the CEA promoter is an excellent candidate for directing *E* gene expression specifically towards lung cancer cells, and may be used to enhance the effectiveness of PTX against this type of tumor.

Introduction

Twelve million new cases of lung cancer, the second most common cancer in both men and women, were diagnosed in 2012 [1, 2] and these showed a low survival rate related to the failure and/or limitation of chemotherapy. Paclitaxel, a drug of choice for lung and breast cancer treatment, induces neuropathy, hemotoxicity, and muscle and joint pain that limit its use [3, 4]. In addition, standard paclitaxel (PTX) contains Cremophor EL as a solvent, which can induce allergic reactions that require premedication with high doses of antihistamines and corticosteroids [5]. For this reason, it is necessary to develop new therapeutic strategies that improve the effectiveness of PTX in lung cancer.

Gene therapy is a treatment strategy that is able to modify or destroy tumor cells without damaging healthy ones, and it can be used to improve classical cytotoxic therapy. Recently, gene therapy using the retinoblastoma protein-interacting zinc finger tumor suppressor gene [6] improved PTX activity in cervical cancer cells, demonstrating stronger cell inhibition than PTX alone [7]. In this context, the *E* gene of bacteriophage φX174, a gene that codes for a 91-amino acid membrane protein with lytic function [8–10], was able to significantly decrease the proliferation of lung cancer cells through the apoptotic mitochondrial pathway [8, 9]. The advantage of this gene over classical suicide gene therapy systems, such as the thymidine kinase *(TK)* gene [11–13], is the fact it does not require a prodrug to cause cell toxicity. However, *E* gene

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shows low cellular specificity, affecting both tumor and healthy cells. Tumor-specific promoters whose proteins are overexpressed in tumor tissues and which have low values or are not expressed in healthy tissues, are tools for driving selective and localized gene expression. In fact, alpha-fetoprotein (AFP) [11], epithelial cell adhesion molecule (EpCAM) [12], survivin [13], vascular endothelial growth factor [14], and human telomerase reverse transcriptase promoters [15], have all been assayed for delivering suicide genes in cancer cells. We recently demonstrated that carcinoembryonic antigen (CEA) is an excellent tumor-specific promoter for directing *E* gene expression towards cancerous colon cells, but not normal colon cells, inducing high levels of cell growth inhibition [16]. The carcinoembryonic antigen, also known as CEACAM5 or CD66e, was discovered in malignant tumors of the endodermally derived epithelium of the gastrointestinal tract and pancreas, and has been used as an oncofetal tumor marker as it is overexpressed in several human carcinomas from the gastrointestinal, respiratory, and genitourinary systems, as well as breast cancers [17–20]. Patients with non-small-cell lung cancer show high serum CEA levels, which have been related to poor survival rates [19, 20]. This differential overexpression in lung cancer tissue compared to normal tissue therefore makes CEA a powerful tool for driving genes to lung cancer cells. Finally, tumors are characterized by a high heterogeneity, where the cancer stem cells (CSCs) are responsible for tumor progression and recurrence, as well as chemoresistance. In lung cancer, CSCs show resistance to paclitaxel [21], although the mechanism for avoiding the drug's cytotoxicity is not yet clear [22, 23]. This phenomenon may be relevant to preventing post-treatment enrichment of CSCs, something that has been related to the disconnection between sophisticated treatments and patient survival rate [24].

The aim of the present work was to analyze the improvement of PTX activity against lung cancer cells in a system where the CEA promoter specifically drove *E* gene expression towards this type of tumor cells. We conducted this study using culture cells (A-549 human and LL2 mice lung cancer cells, and the L132 human embryonic lung cell line) and their respective 3D tumor spheroids (MTS), a model that mimics the biological environment of the tumor and allows the penetration and distribution limits of therapeutic agents to be determined [25, 26]. In addition, we carried out an in vivo study using immunocompetent mice (C57BL/6) with lung tumors induced by subcutaneous injection. Finally, based on the relevance of CSCs, we extended the study to A-549 CSCs. Our results showed a greater expression of the *E* gene in the tumor cells lines and MTS than in normal cells, when regulated by the CEA promoter. The expression of *E* gene enhances the effect of paclitaxel in lung cancer cells. These results suggest that *E* gene may be a useful new tool for treating this type of cancer.

Materials and methods

Cell cultures

The A-549 human lung adenocarcinoma epithelial cell line, LL2 mice Lewis lung carcinoma cell line and L132 human embryonic lung cell line were provided by the Instrumentation Service Center of the University of Granada, Granada, Spain. All cell types were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin-penicillin (Sigma), under air containing 5% CO_2 and in an incubator at 37 °C.

Luciferase assay

Cell lines (7×10^3 cells/well) were seeded into 96-well culture plates. After 24 h, cells were cotransfected with 0.2 µg/well of *luciferase* reporter vectors (pGL2/CEA or pGL2) and 0.01 µg/well of CMV/*Renilla* vector as internal controls for normalization of the transcriptional activity of the reporter vectors (Dr. Miguel Burgos from Biotechnology Institute, Granada, Spain). Transfection was carried out using DNA:JetPRIME in vitro (Polyplus Transfection) (ratio 1:3) according to the manufacturer's instructions. Experiments were performed in three groups: cells transfected with pGL2/CEA and CMV/*Renilla*, cells transfected with pGL2 and CMV/*Renilla*, and untransfected cells (control). After transfection (48 h), luciferase activity was determined using the Dual-Glo Luciferase Assay System (Promega) according to the manufacturer's instructions. The luminescence was measured in a luminometer (96 GloMax® microplate). The luciferase activity in each well was normalized to CMV/*Renilla* using the formula Ln = L/R (Ln: Normalized luciferase activity; L: Luciferase activity reading; R: Renilla activity reading). The Ln was further standardized according to the transcriptional activity of pGL2 using the formula of RLU = L_n/pGL3-basic (RLU: relative luciferase unit). All transfections were performed in triplicate.

E gene transfection

Generation of pcDNA3.1/CEA/*E* (pCEA-*E*) vectors and transfection of cell lines (A-549, LL2 and L132) were carried out following our own experience [16]. Polymerase chain reaction (PCR) amplification of the *E* gene was performed to determine correct gene expression [16]. The integrity of the RNA was demonstrated using β-actin

primers. Amplified PCR products were separated by 1.5% agarose gel electrophoresis and visualized with RedSafe Nucleic Acid Staining Solution (iNtRON Biotechnology). Images were scanned using a Bio-Rad documentation system (Quantity One Analysis Software). E protein expression was tested using the anti-V5-FITC-antibody (Invitrogen, Madrid, Spain) as previously described [16].

In vitro cell proliferation assay

Cell lines were seeded in 24-well plates at the densities of 1.5×10^3 for A-549 and L132, and 1.2×10^3 for LL2, in 400 µl of their respective culture medium and incubated overnight. After that, cells were transfected at days 0 and 2 with 0.5 µg/well of the pCEA-*E* or pCEA (without gene) using DNA:JetPRIME in vitro (Polyplus Transfection) (ratio 1:3) and treated with PTX at the IC_{50} for either cell line (10 nM for A549 and LL2 and 3 nM for L132) at days 1 and 3. Experiments were performed in the following groups: cells transfected with pCEA, cells transfected with pCEA-*E*, cells transfected with CEA and treated with PTX, cells transfected with pCEA-*E* and treated with PTX, cells treated with PTX and control cells (cells neither transfected nor treated). MTT (3- (4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (5 mg/ml) was added to each well (20 µl) and incubated for 4 h at 37 °C. Two hundred microliters of dimethylsulfoxide were then added to each well after the medium had been removed. Optical density was determined using a Titertek multiscan colorimeter (Flow Laboratories) at 570 and 690 nm. The proliferation effect of the combined gene and cytotoxic therapy was determined every day for 4 days.

Multicellular tumor spheroids generation and treatment

A-549, LL2 and L132 cells (5×10^2 cells/well) were grown in a 96-well microplate (BD Biosciences) to generate MTS according to Rama et al. [25]. Once the cells were seeded, the plates were centrifuged at 500 ×*g* for 10 min to promote aggregation. Multicellular tumor spheroids were formed after 3 days. They were grown in DMEM (Sigma, St. Louis, MO, USA), supplemented with 10% FBS and 1% streptomycin-penicillin (Sigma), under air containing 5% CO_2 and in an incubator at 37 °C. Multicellular tumor spheroids were treated in the same groups and in a similar form that those described for cell cultures (see previously). Growth of the spheroids was monitored and measured to obtain a median relative volume (volume at day *x*/volume at day 0). Their volumes (V, mm^3) were estimated by measuring the largest diameter "*a*" and the second largest diameter "*b*" perpendicular to "*a*", then calculating the volume from, $V = a \times b^2 \times \pi/6$.

Apoptosis analysis

Multicellular tumor spheroids treated with pCEA-*E* (see above) were collected and fixed with 4% paraformaldehyde, for 3 h at room temperature. Then, a TUNEL assay (TUNEL kit, Roche, Mannheim, Germany) was performed following the manufacturer's instructions. Cell nuclei were counterstained with Hoechst. Fluorescence images were captured using confocal microscopy (Nikon A1, Nikon Corporation, Tokyo, Japan). The quantification of the tunel-positive nuclei was carried out with ImageJ software by obtaining the relative light intensity of each image for each cell line.

Lung cancer stem cells

Lung cancer stem cells were obtained from A-549 cells and characterized following Hu et al. [27] modified by Leiva et al. [28]. Agarose (1%) was added to 6-well plates to generate a non-adherent surface and 2×10^5 cells/well were seeded in induction medium (DMEM/Nutrient Mixture F-12 Ham) (2 ml) supplemented with EGF (20 ng/ml), with heparin (4 µg/ml), bFGF (20 ng/ml), 1% of penicillin-streptomycin (Sigma Aldrich) and hormone mixture B27 (1 ×) (Gibco, Spain). After treatment including transfection, PTX and combined therapy (see above), CSCs were collected on day 13, centrifuged at 1600 rpm for 10 min, and disaggregated with Tripsin/EDTA. Then, they were seeded in a 96-well plate (1×10^3 cells/well) and incubated in complete DMEM (100 µl) for 24 h at 37 °C in a 5% CO_2 atmosphere. Cell proliferation was determined with Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan). Briefly, the reagent of CCK-8 was added to the wells at a final concentration of 10% and the plates were incubated for 4 h at 37 °C. After the incubation time, the optical density of the wells was measured at 450 nm in a Titertek multiscan plate reader (Flow, Irvine, California).

In vivo study

Female C57BL/6 mice (Charles River, Barcelona, Spain) were used for the in vivo studies. All mice (body weight: 25–30 g) were kept in a laminar airflow cabinet located in an ambient-controlled room (37.0 ± 0.5 °C and a relative humidity of 40–70%) and subjected to a 12-h day/night cycle under specific pathogen-free conditions. Tumors were induced by subcutaneous injection of 7×10^5 LL2 cells in 100 µl of PBS into the left flanks of mice. Tumors were allowed to grow to 11 days (75 mm^3, a minimal size for ideal intratumor injection) before starting treatment. Mice were randomly assigned to the following groups ($n = 9$): treated with pCEA-*E*, treated with PTX + pCEA-*E*, treated with PTX and untreated (control). JetPEI in vitro (Polyplus

Transfection) was used as a transfection enhancer reagent according to the manufacturer's instructions. JetPEI/DNA complexes with a ratio of 1:6 were formed for 15 µg DNA/50 µl 10% glucose plus 1.8 µl JetPEI/50 µl 10% glucose. Paclitaxel (10 mg/kg) was injected intraperitoneally. Each mouse received JetPEI/DNA complexes intratumoral injections and/or PTX intraperitoneal injection every 2 days, up to a maximum total of five administrations. Furthermore, every 2 days the tumor dimensions were measured with a digital caliper. The tumor volume was calculated with the formula V (mm^3) = $(a \times b^2 \times \pi)/6$, ("*a*" being the largest diameter of the tumor, and "*b*" the largest diameter perpendicular to "*a*"). The final point of the experiment, when animals were sacrificed, was day 28. All studies were approved by the Ethics Committee of the Medical School at the University of Granada and complied with international standards (European Communities Council Directive 86/609).

Detection of *E* gene expression by RT-PCR in vivo

RNA was extracted from LL2 murine tumor cells with the Rneasy Mini kit (Qiagen). cDNA was generated by means of the Promega Reverse Transcription System (Promega, Madrid, Spain) using total cellular RNA (1 µg). Polymerase chain reaction (PCR) amplification of the *E* gene was performed to determine correct gene expression as described above.

Statistical analysis

All the results have been expressed as the mean ± the standard deviation [29]. Statistical analysis of the studies was carried out with the Student's *t* test and mice survival *P* was calculated with the Kaplan−Meier method and then analyzed with the log-rank test. All the tests were performed with the Statistical Package for the Social Sciences (SPSS) *v*. 15.0 with a significance level of 0.05 ($\alpha = 0.05$).

Results

CEA activity and pCEA-*E* transfection

Previous pCEA-*E* transfection, a determination of CEA activity in cancer and normal cell lines, was carried out using a luciferase assay. As shown in Fig. 1a, high levels of CEA activity were detected in A-549 and LL2 lung cancer cell lines (32.56% and 28.86%, respectively). By contrast, a low level of CEA activity was detected in L132 normal intestinal epithelial cells (2.7%). pCEA-*E* transfection was carried out to induce *E* gene expression under CEA promoter control which was confirmed using RT-PCR (Fig. 1b). Expression of E protein in cells transfected with pCEA-*E* was detected using the anti-V5-FITC-antibody (Fig. 1c), indicating the effectiveness of the construction to the subsequent in vitro experiment.

Inhibition of cell growth by PTX + pCEA-*E* combined therapy

Firstly, a proliferation assay demonstrated the effect of *E* gene expression during 96 h in A-549, LL2 and L132 cell lines. As shown in Fig. 2, A-549 and LL2 cell lines (with high levels of CEA expression) showed a significant and time-dependent decrease in growth, reaching the maximum percentage of inhibition at 96 h (64.1% and 68.5%, respectively) ($p < 0.01$). By contrast, a minimal growth inhibition was observed in L132 cells (with low levels of CEA activity) (16.3% at 96 h), demonstrating the selective target of CEA promoter to lung cancer cells and the cell growth inhibition by *E* gene. Cell lines transfected with pCEA-*LacZ* (control) showed similar proliferation to those of each parental cell.

Secondly, we determined the PTX IC_{50} for each cell line (10 nM ± 2.06, 10 nM ± 1.9, and 3 nM ± 0.2 for A549, LL2, L132, respectively) and use them in association with pCEA-*E*. This combined therapy displayed a significant reduction in A-549 and LL2 cell viability, which was greater than those found with the use of PTX (49.1% and 51.8%, in A-549 and LL2 at 96 h, respectively) or *E* gene alone. This reduction was time-dependent, reaching the maximum percentage of inhibition at 96 h (92.4% and 90.2% in A549 and LL2 cells, respectively) ($p < 0.01$). Interestingly, the combined therapy showed a synergistic antiproliferative effect in lung cancer cells that was more evident in LL2 (A-549: 4.2% and 4.3% at 24 and 48 h, respectively; LL2: 16.4% and 11.8% at 24 and 48 h, respectively) (Fig. 2). In contrast, minimum difference in inhibition of cell growth compared to that obtained with the use of PTX alone was observed in L132, being also maximal at 96 h (55 %) (Fig. 2). No increase of cell growth was observed in any cell line when PTX + pCEA-*LacZ* (control) was used.

PTX + pCEA-*E* combined therapy effect in 3D tumor spheroid models

Firstly, and as in monolayer study, we assayed the effect of *E* gene expression in 3D MTS. A-549 and LL2 MTS transfected with pCEA-*E* showed a significant and time-dependent decrease in volume growth (Fig. 3) which was maximum at 96 h (57.2% and 58.4% in A549 and LL2 MTSs, respectively) ($p < 0.01$). In contrast, minimal growth inhibition was observed in L132 (15.4% at 96 h) indicating again the selectivity of CEA against these cells. To determine apoptosis induction by pCEA-*E* in MTS, a TUNEL assay was carried out. As shown in Fig. 4, a larger apoptotic

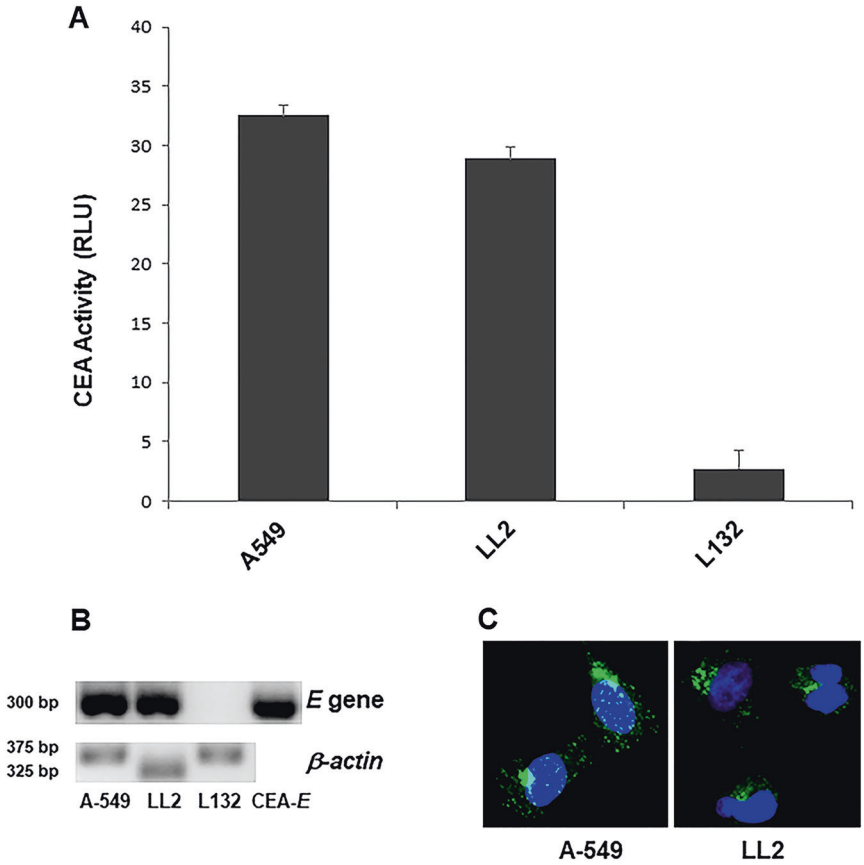

Fig. 1 CEA promoter activity and and pCEA-*E* transfection. **a** A-549, LL2 lung cancer and L132 normal cells were cotransfected with luciferase expression vectors pPGL2/CEA or pGL2 and the Renilla expression vector CMV/Renilla. Luciferase activity of each transfection was normalized by the Renilla reading. Luciferase activity was represented by the ratio of the specific promoter over the activity of pGL2. Data represent the mean value of three replicates ± the standard error of the mean (SEM). High levels of CEA activity were detected in A-549 and LL2 lung cancer cell lines, but not in L132. **b** A-549, LL2 lung cancer and L132 normal cells were transfected with pCEA-*E*. Total RNA was transcribed to cDNA using RT-PCR amplification of β-actin (mice for LL-2 and human for A-549 and L132) and *E* gene. pCEA-*E* vector was used as positive control. A correct expression of the *E* gene is observed in all cells transfected with pCEA-*E*. **c** Representative image of the recombinant E protein (E-V5) detected using anti-V5-FITC antibody (green) in both A-549 and LL2 cells with high CEA expression. Cell nuclei were stained by DAPI (blue). Magnification: ×40

area in both A549 and LL2 MTS was detected. In contrast, undetectable apoptosis was observed in MTS derived the L132 cells corroborating the selective antitumor activity of the therapeutic gene.

Secondly, PTX + pCEA-*E* combined therapy displayed a significant reduction in A-549 and LL2 MTS volume which was greater than those induced by PTX (42.3% and 41.7% in A-549 and LL2 MTS at 96 h, respectively) or *E* gene alone. The MTS volume decrease using PTX + pCEA-*E* was time-dependent, reaching the maximum percentage of inhibition at 96 h (87% in A549 MTS and 81.7% in LL2 MTS) (< 0.01). As in cell cultures, a synergistic effect was showed in A-549 MTS (8.8% and 11.7% at 24 and 48 h, respectively) and LL2 MTS (21.3% and 18.6% at 24 and 48 h, respectively). In contrast, few volume growth differences were observed between L132 MTS treated with PTX + pCEA-*E* (52.6% at 96 h) or PTX alone (41% at 96 h) (Fig. 3). Finally, the volume growth of MTS transfected with pCEA (control) was similar to that MTS obtained from parental cells. Findings obtained with PTX + pCEA combined therapy were similar to those obtained in non-transfected cells treated with PTX alone (Fig. 2).

Lung CSCs assay

A-549 CSCs were isolated to determine the effectiveness of the PTX + pCEA-*E* combined therapy in these radio- and chemoresistant cells. Figure 5 shows a significant and time-dependent decrease in A-549 CSCs proliferation which was greater at 96 h for pCEA-*E* and PTX alone (57% and 40.5%, respectively) ($p < 0.01$). However, as previously, the PTX + pCEA-*E* combined treatment induced the highest decrease in A-549 CSCs proliferation (76.7%). A synergistic effect was detected at 48 and 72 h (13.9% and 9.1%, respectively) although was lower than those described in A-549 culture cells or MTS.

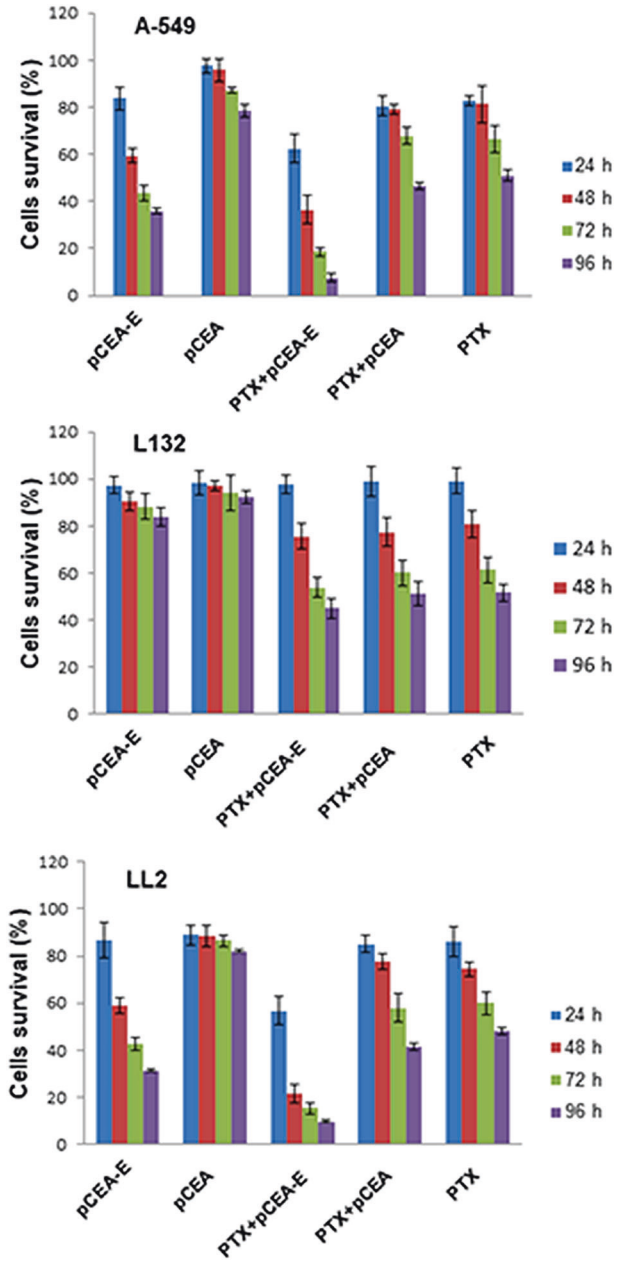

Fig. 2 PTX + pCEA-*E* combined therapy in lung cancer and normal cells. Cytotoxic drug PTX was used for the treatment of pCEA-*E* transfected and nontransfected A-549 and LL2 lung cancer cells (10 nM) and nontransfected and transfected L132 lung cells (3 nM). pCEA (empty) transfected cells were used as a control. Values at 24, 48, 72 and 96 h represent means ± SD of quadruplicate cultures. A-549 and LL2 cell lines showed a significant and time-dependent decrease in growth, but a minimal growth inhibition was observed in L132 cells

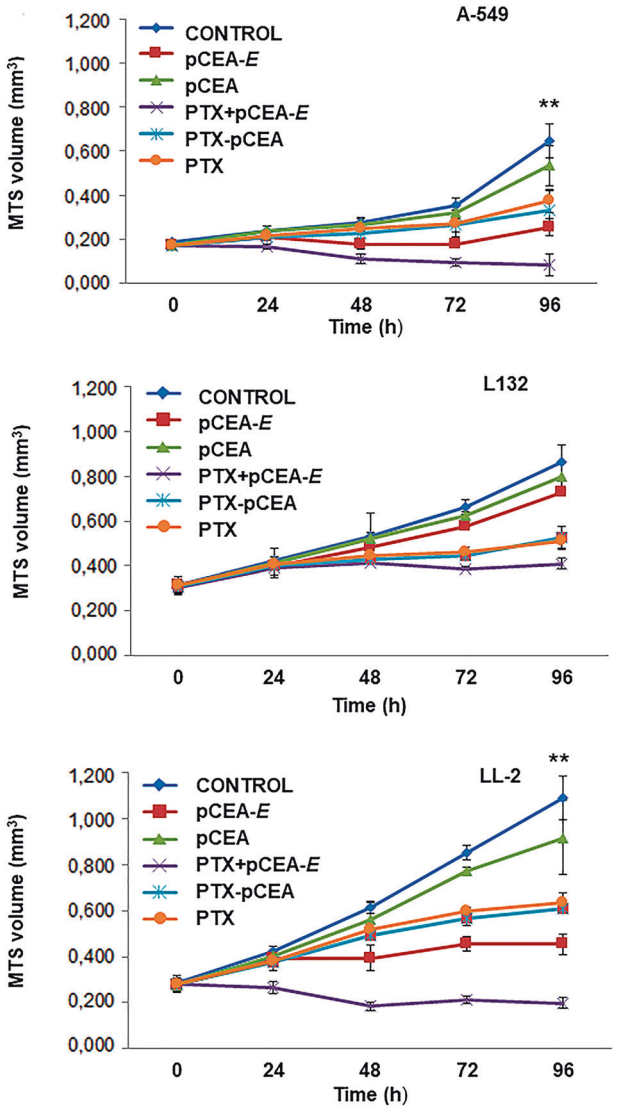

Fig. 3 PTX + pCEA-*E* combined therapy in multicellular spheroids (MTS). MTS generated from A-549, LL2 and L132 pCEA-*E* transfected and nontransfected cells were treated with PTX. Transfected A-549 and LL2 MTS pCEA-E showed a significant and time-dependent decrease in volume growth, but minimal growth inhibition was observed in L132. Values at 24, 48, 72 and 96 h represent means ± SD of quadruplicate experiences. $^{**}p < 0.05$

PTX + pCEA-*E* combined therapy effect in lung cancer xenografts

Tumor mass volume of mice was monitored throughout the treatment in order to determine the in vivo efficacy of the pCEA-*E* and combined therapy with PTX + pCEA-*E* (Fig. 6). Previously, *E* gene expression in tumors was assessed by RT-PCR. As Fig. 6b shows, an amplification 227 bp was found in LL2 tumors of mice treated with PTX + pCEA-*E* or pCEA-*E*, but not either in those without treatment (control) or treated with PTX alone, indicating the effectiveness of the construction. pCEA-*E* intratumoral treatment demonstrated significant reduction in tumor volume which was time-dependent. As Fig. 6c shows, treatment with PTX and pCEA-*E* alone showed similar tumor volume reduction (52.3 and 46%, respectively) ($p < 0.01$) in comparison to the control. However, a greater inhibition of tumor volume growth was obtained with the PTX + pCEA-*E* combined therapy (83.1%). In addition, a

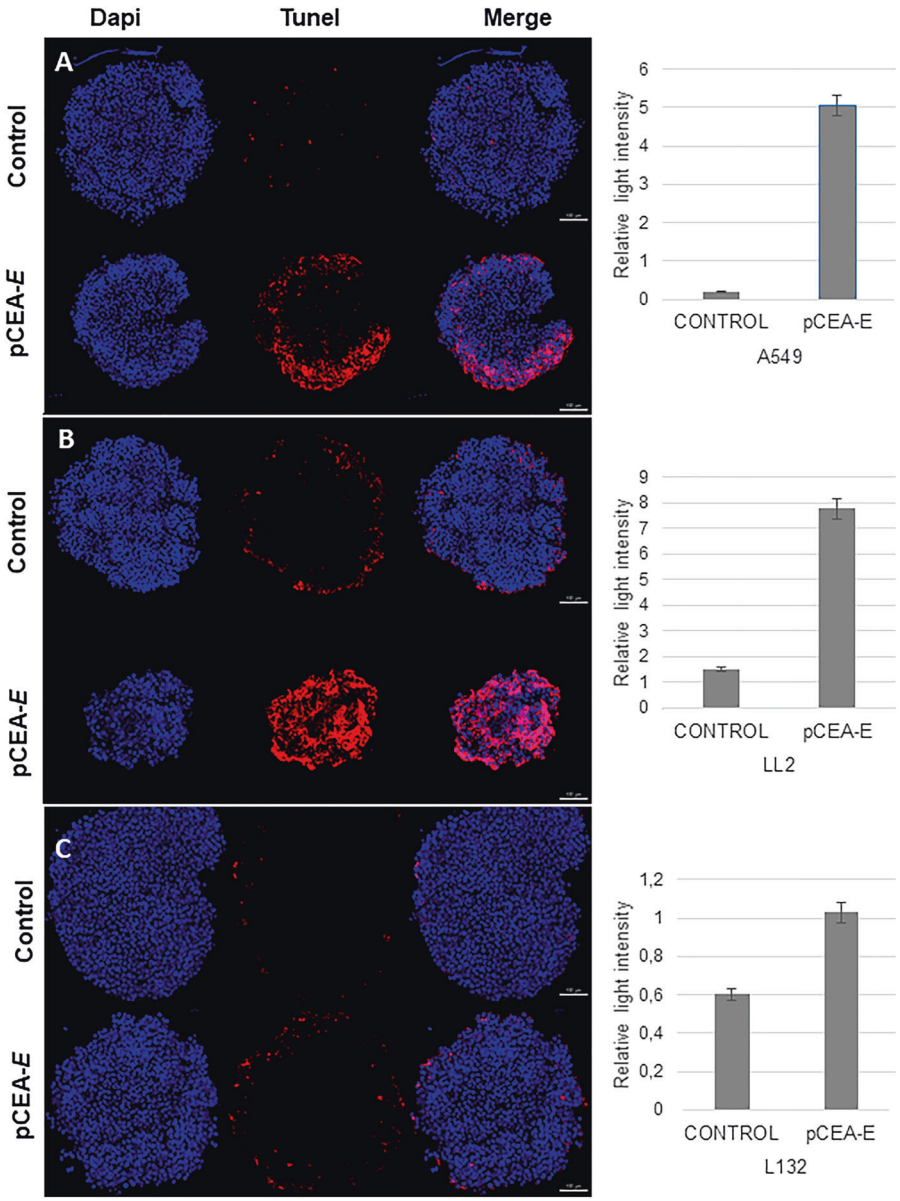

Fig. 4 Representative images of the apoptosis induced by *E* gene therapy on MTS. **a** MTS from A-459 (**a**), LL2 (**b**) and L132 (**c**) treated with pCEA/*E* (see Methods). Apoptosis (in red) was detected using a TUNEL assay. Nontreated MTS were used as controls. Nuclei were stained with Hoechst (blue). Original magnification: bar = 100 µm. **b** Quantification of the tunel-positive nuclei by ImageJ software. pCEA-E was able to significantly reduce the volume of MTS generated from A-549 and LL2 cells, inducing intense apoptosis which was not observed in MTS from L132 cells

significantly increased survival rate was detected in mice bearing lung tumors treated with PTX, pCEA-*E* and PTX + pCEA-*E* compared with the control group ($p < 0.05$). These results were reflected in the Kaplan−Meier curves where the fraction surviving in mice bearing lung tumors was almost the same for PTX and pCEA-*E*-treated mice and moderately higher in those treated with PTX + pCEA-*E*, although no statistically significant differences were found (Fig. 7).

Discussion

During oncogenesis certain proteins are deregulated. The difference in their expression between tumor and healthy tissues opens a new door in the search for specific therapies. Tumor-specific promoters induce protein overexpression in tumor tissues whereas in healthy tissues either only low values are detected or they are not expressed at all. These promoters are therefore tools that can be used to selectively drive suicide gene expression in various types of tumors. For example, the human osteonectin promoter hON-522E has been used to express the *TK* gene in human prostate cancer PC3M [30], FOS (FBJ murine osteosarcoma viral oncogene homolog) promoter to express *TK* in glioma [31], and MAR (nuclear matrix attachment region) promoter to express the *cytosine deaminase* (*CD*)/*TK* double suicide genes in human gastric cancer SGC-7901 cells [13]. Recently, the a*lpha-fetoprotein* (*AFP*) gene has been used as a tumor-specific promoter to express the *TK* gene in

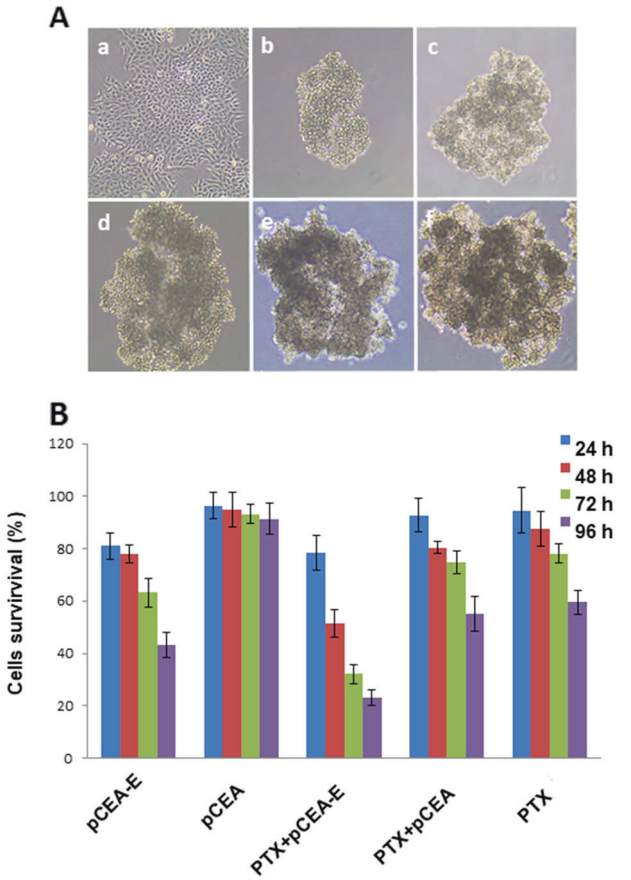

Fig. 5 Effect of PTX + pCEA-*E* combined therapy in A-549 CSCs. **a** Representative optical microscope images of the formation of CSCs spheres from the A-549 cell line exposed to an induction medium at 0, 2, 4, 6, 8, 12 days ("a" to "f", respectively) under conditions of low adherence (magnification: ×10). **b** Proliferation of CSCs exposed to PTX, pCEA-*E* and PTX + pCEA-*E* was analyzed by CCK-8 assay (see Methods) at different times (24−96 h). A significant and time-dependent decrease in A-549 CSCs proliferation was observed. The PTX + pCEA-E combined treatment induced the highest decrease, and a synergistic effect was detected at 48 and 72 h. Values represent means ± SD of quadruplicate cultures

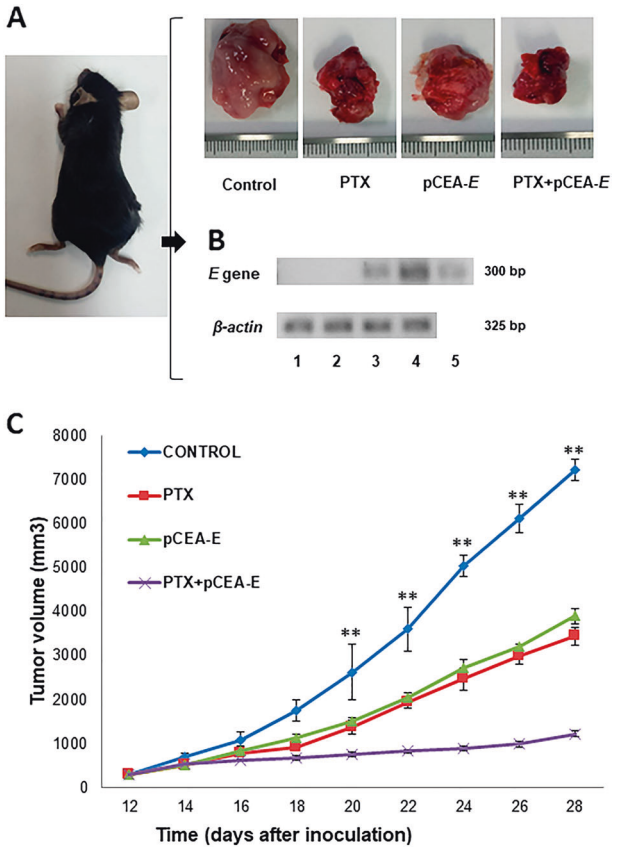

Fig. 6 Intratumoral PTX + pCEA-*E* combined therapy of subcutaneous tumor induced by LL2 murine tumor cells. **a** Representative image of the tumors excised from mice at the end of the experience (28 days) without treatment (control) and after PTX, pCEA-*E* and PTX + pCEA-*E* (combined therapy) treatments. A decrease in tumor volume can be observed, being greater in the combined therapy. **b** Determination of *E* gene expression in tumor mice. Total RNA isolated from LL2 tumors was transcribed to cDNA using RT-PCR amplification as described in "Materials and Methods". The integrity of the RNA was demonstrated using β-actin primers. Amplified PCR products of β-actin mRNA and *E* gene mRNA were separated by 1.5% agarose gel electrophoresis and visualized with Red Safe Nucleic Acid Staining Solution. Lane 1, no treated mice (control); Lane 2, mice treated with PTX; Lane 3, mice treated with PTX + pCEA-*E*; Lane 4, mice treated with pCEA-*E*; Lane 5, positive control (pCEA-*E*). A correct expression of the *E* gene is observed in mice treated with pCEA-*E* and PTX + pCEA-*E*. **c** Modulation of the tumor volume after treatments. pCEA-*E* induced a significant reduction in tumor volume at the end of the study period in comparison with the volume growth of untreated tumors (control) ($p < 0.01$). The greater inhibition of volume growth was observed with PTX + pCEA-*E* treatment. Data represent the mean value ± SEM. $^{**}p < 0.05$

hepatocellular carcinoma cells [11]. In lung cancer, the TTS (*TTF1* gene under the control of the hTERT promoter and hSPA1 promoter) system has shown selective activity [32]. Alekseenko et al. [33] combined tandem promoters of the human *telomerase* and *survivin* genes to achieve more efficient gene transcription in lung cancer cells without affecting normal lung fibroblasts. Recently, a surfactant protein B promoter-driven *TK* suicide gene (pSPB-tk) was assayed for possible gene therapy of human lung adenocarcinoma [34]. In this context, CEA is an oncofetal tumor marker positively expressed in several cancer tissues, including in colon [35, 36], gastric [37] and lung cancer [38], but not in normal tissues [16, 39, 40]. In fact, the CEA promoter has been used to express *TK* and *CD* genes in CEA-positive lung cancer cells [41]. Zhang et al. [39] used the CEA promoter to drive dual *tumor necrosis factor-related apoptosis-inducing ligand* (*TRAIL*) and *manganese superoxide dismutase* (*MnSOD*) genes in human lung carcinoma cell lines. A-549 cells displayed 60% cell death 96 h after infection with CEA-*TRAIL-MnSOD* whereas the human lung bronchial epithelial cell line BEAS-2B was not affected. We previously studied the effect of the *E* gene in

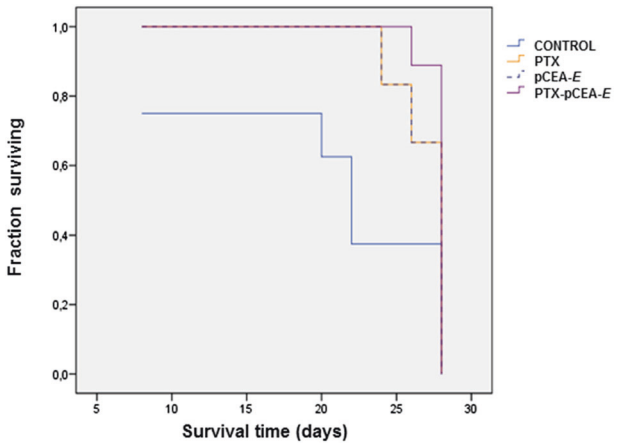

Fig. 7 Kaplan−Meier curves of mice survival rates after exposure to the treatments. Significantly increased survival rate was detected in mice bearing lung tumors treated with PTX, pCEA-E and PTX + pCEA-E compared with the control group ($p < 0.05$). Data are represented as the mean value ± SD. Comparison between treatment groups was performed with the log-rank test

lung, colon and breast cancer cells, although there was no discrimination between cancer and normal cells [8]. In this study, we used the CEA promoter to specifically express *E* gene in A-549 and LL2 lung cancer cells. Transfection with pCEA-*E* induced a significant and time-dependent decrease in proliferation in both A-549 and LL2 cells along with minimal growth inhibition in the normal L132 cells with no CEA promoter activity. In addition, pCEA-*E* was able to significantly reduce the volume of MTS generated from A-549 and LL2 cells, inducing intense apoptosis which was not observed in MTS from L132 cells. These results support the fact that pCEA-*E* specificity decreases the proliferation of lung cancer cells.

On the other hand, classical chemotherapy, one of the most effective approaches to cancer treatment, is limited by secondary effects, cytotoxicity and low bioavailability [42]. Combination with synergistic therapeutic strategies, such as gene therapy, is a promising tool for improving chemotherapy treatment in cancer [25, 43–45]. Considering this issue, we combined pCEA-*E* therapy with PTX. Paclitaxel is a strong antitumor agent currently used for treating a wide range of carcinomas, including non-small-cell lung cancer [46]. Previously, Cheng et al. [7] demonstrated that PTX may increase its antitumor activity using the *RIZ* gene. Transfection of the pcDNA3.1 (+)-EGFP-RIZ1 vector into four cervical cancer cell lines treated with PTX validated the synergistic effect of *RIZ1* gene therapy with this drug. The use of an adenoviral vector as a vehicle for the vascular endothelial growth factor (VEGFR2) combined with PTX and carboplatin significantly reduced tumor weights in an ovarian cancer xenograft model compared with the drug alone [47]. Gene therapy based on miR-24, which is downregulated in endometrial carcinoma (EC), was also able to increase PTX activity. In fact, upregulation of miR-24 by miR-24 agonist (agomiR-24) inhibited EC HEC-1A cell proliferation and significantly increased cell chemosensitivity to PTX [48]. Similar results were observed by Liu et al. [49] when they raised miRNA-203 levels in colon cancer. This miRNA, which is downregulated in colon cancer, correlated with PTX-resistant cells, so that its overexpression led to the sensitization of these cells. In order to determine the activity of PTX against lung cancer cells in association with *E* gene, we implemented a combined therapy. The PTX + pCEA-*E* combined therapy led to a significant reduction in both A-549 and LL2 cell viability, greater than that seen with either PTX or pCEA-*E* gene treatment alone, achieving greater therapeutic efficacy than standard chemotherapy. In fact, the combined therapy exhibited a synergistic antiproliferative effect at 24 and 48 h in both the A-549 and LL2 cell lines, although this effect was more apparent in LL2 cells (16.4% and 11.8% at 24 and 48 h, respectively). In contrast, PTX + pCEA-*E* caused inhibition of L132 cell growth similar to that obtained with PTX alone, suggesting that CEA could be used as a tumor-specific promoter to specifically driv*E* genes to lung cancer cells without damaging normal lung cells.

On the basis of these findings, we conducted a 3D MTS study. Previously, MTS has been used to determine the antitumor effect of the association between gene therapy and cytotoxics. In fact, Prados et al. [50] demonstrated that the bacterial *gef* gene associated with PTX decreased the volume of MTS derived from lung cancer cells (87.4%) compared with MTS controls. This inhibition was greater than that obtained using the *gef* gene or PTX alone. In our study, both A-549 and LL2 MTS showed a significant decrease in volume growth (57.2% and 58.4% at 96 h, respectively) after pCEA-*E* gene treatment. In contrast, minimal growth inhibition was observed in L132 MTS (control). These results corroborated the antiproliferative effect of *E* gene and the CEA promoter selectivity against lung cancer cells, as previously observed in cell cultures. In addition, the combined therapy (PTX + pCEA-*E)* demonstrated a greater MTS volume decrease (87% in A549 MTS; 81.7% in LL2 MTS) than that observed with PTX alone. As in the cell cultures, there was a synergetic antiproliferative effect at 24 and 48 h which was higher in LL2 MTS. The presence of a unique population of cells, referred to as CSCs, has been suggested to be responsible for lung cancer metastasis, disease relapse, and refractory responses to current chemotherapies [24]. In primary lung tumors and metastasis, differential sensitivity of CSCs to PTX has been described [21, 51]. In order to determine the activity of the PTX + pCEA-*E* combined therapy in lung CSCs, we carried out a study using isolated A-549 CSCs. Our results showed a significant and time-dependent decrease in the proliferation of CSCs using the combined

therapy (77.6% at 96 h), which was higher than that observed with *E* gene or PTX alone. This effect was lower than in cells, which may be due to the chemoresistance attributed to CSCs [24]. However, the synergistic effect demonstrated with the combined therapy in CSCs (13.9% at 48 h; 9.1% at 72 h) was somewhat greater than that observed in cell cultures.

Finally, to determine the effectiveness of our combined therapy in vivo, we used mice bearing lung tumors from LL2 CEA-positive cells. The ability of the CEA promoter to selectively drive genes to lung tumors in vivo has been documented. In fact, the CEA-*TK* and CEA-*TRAIL-MnSOD* system showed a greater suppression of tumor growth than single genes when assayed in lung cancer xenograft models in nude mice [39, 52]. Previously, *E* gene has been assayed successfully in mice bearing colon cancer tumors from MC-38 CEA-positive cells [16]. In our study, mice treated with pCEA-*E* showed a significant decrease (46%) in lung tumor volume compared with the control group. These results were very similar to those obtained in mice treated with PTX (52.6%). However, the greater inhibition of tumor volume was observed after PTX + pCEA-*E* combined therapy (83.1%). The expression of *E* gene in tumors induced by LL2 murine tumor cells was analyzed and seen to be correct in cells treated with pCEA-*E* and PTX + pCEA-*E*, demonstrating the efficacy of CEA as a specific tissue-promoter for driving *E* gene to target lung tumors. Finally, although PTX + pCEA-*E* combined therapy was accompanied by an increase in the survival rate, no clear difference was observed in comparison to simple treatments, probably due to the limited time of the experience. Therefore, more extensive assays will be necessary to corroborate this in vivo effect.

We have demonstrated that CEA is an excellent tumor-specific promoter for driving *E* gene expression in lung cancer cells, inducing apoptosis. In contrast, normal lung cells are not damaged. A combined therapy using PTX + pCEA-*E* enhanced the antitumor effect against lung cancer cells, inducing cell growth inhibition, and a decrease in both MTS volume and tumor volume. This effect was also observed in lung CSCs, responsible for the recurrence of lung cancer. These results suggest that *E* gene expression under CEA promoter control may be an excellent candidate for enhancing the activity of PTX in this type of tumor.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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