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Research article

Keywords: Breast cancer, Tumour initiating cells, PEDF, relapse, tumoral biomarkers, self-renewal

Posted Date: October 23rd, 2020

DOI: https://doi.org/10.21203/rs.3.rs-93992/v1

Moonlighting role of PEDF in breast cancer

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ABSTRACT

Background: Breast cancer is the leading cause of death among females in developed countries. Although the implementation of screening tests and the development of new therapies has increased the probability of remission, relapse rates still remain high. Numerous studies have indicated the connection between cancer initiating cells and slow cellular cycle cells, identified by their capacity to retain long labelling (LT+).

Methods: We have designed a transgenic protein consisting in the C-terminal part of this protein, which acts by blocking endogenous PEDF in culture cell assays. Present work is based in doses-response in vitro assays as well as flow cytometry analysis of surface markers and cell cycle kinetic study of the tumour initiating cells.

Results: In this study we show that this type of cells is present not only in cancer cell lines but also in cancer cells from patients with metastatic and advanced stage tumours. We also present new assays showing how stem cell self-renewal modulating proteins, such as PEDF, can modify the properties, expression of markers, and carcinogenicity of cancer stem cells.

This protein has been involved in self-renewal in adult stem cells and has been described as anti-tumoral because of its anti-angiogenic effect. However, we show that PEDF enhances resistance in breast cancer patient cells in vitro culture by favoring a slow cellular cycle population (LT+). The PEDF signalling pathway could be a useful tool for controlling cancer stem cells self-renewal, and therefore control patient relapse.

Conclusions: We demonstrate that it is possible to interfere with the self-renewal capacity of cancer stem cells, induce anoikis *in vivo*, and reduce resistance against Docetaxel treatment in cancer patient cells *in vitro* culture. We have also demonstrated that this PEDF modified protein produces a significant decrease in cancer stem cell markers. All these properties make this protein a potential application in clinical cancer therapies via co-administration with chemotherapy for relapse cancer treatment.

KEYWORDS

Breast cancer, Tumour initiating cells, PEDF, relapse, tumoral biomarkers, self-renewal

BACKGROUND

The incidence of breast cancer has increased in recent years, due to an aging population[1]. In fact, breast cancer is the leading cause of death among females in developed countries, although the implementation of screening tests and the development of anti-neoplasic therapies such as Trastuzumab has increased the probability of a cure in those patients[2]. However, the relapse rate remains very high in different breast cancer types[3] and this is why further study of new pharmacological drugs and diagnostic methods is still needed.

Relapse is mainly due to the tumour cell population's resistance, characterised by their capacity for self-renewal, resistance to drugs, and a slow cellular cycle[4,5]. This last characteristic allows for detection of this population, since this produces long retained labelling[6–8]. The auto-renewal capacity of these cells is essential for stem cells to be maintained throughout the life of the organism. Pigment Epithelium-Derived Factor (PEDF)

protein has been related to this self-renewal mechanism[9,10]. This protein may induce cellular differentiation and promote apoptosis in a variety of tumour cells[7,8], and it is also able to inhibit tumour proliferation, vascularization, cell migration, and metastasis[11–13] affecting the division of fast tumour cells. In addition, PEDF is a niche-derived regulator of adult neural stem cells [9,14,15] that activates slowly dividing cells without inducing proliferation nor differentiation [9,10]. Together with the fact that tumour initiating cells (TICs) within a tumor are capable of self-renewal, this is why this protein-signalling pathway could be important not only as an anti-neoplasic agent but also as a regulator of self-renewal in TICs and patient relapse. As has been widely described, PEDF is a pleiotropic molecule, presenting two domains with clearly differentiated functions, an anti-angiogenic part and a second domain with neurotrophic properties, each activating different signalling pathways. By using this fragmentation of the molecule into these two domains we are able to take advantage of their different effects on signalling pathways, including the carboxy-terminal fragment´s inhibition of the crucial TICs population´s self-renewal ability, which thus hinders tumour recurrence. This is why we suggest a new therapeutic mechanism that consists in the co-administration of carboxy-terminal PEDF protein fragments and chemotherapy.

In order to detect TICs, four different epitopes were analysed; these epitopes are implicated in the cells' different processes and have been previously related with cancer stem cells in literature. BCRP1 is a drug transporter from the ABC transporter family, but while it is not as ubiquitous as other family members, such as MDR1, it is commonly expressed in the population[16,17]. EpCAM is a transmembrane glycoprotein that is involved in cell signalling, migration, proliferation, and differentiation[18,19]. This whole process is closely related to the epithelial-mesenchymal transition, essential in the metastatic mechanism in which TICs could play an important role. CD133 is a pentaspan membrane glycoprotein that has been used as a stem cell biomarker since its discovery in 1999, although its function is still unknown. AC133 is a glycosylated-isoform of CD133, recognised by a specific antibody

which has been described as a biomarker for human hematopoietic stem cells and different cancer stem cell models[20–23].

All these proteins have been involved in different metastatic processes, and could be a target for new therapies but, more interestingly, they can be indicators of the process' progress and could suggest what the mechanism and the signalling pathway of these cells involved in the resistance to chemotherapeutic treatments is. One of the cellular mechanisms that may be involved in these resistance processes is anoikis[24–28]. Anoikis is a form of apoptosis that occurs in anchorage-dependent cells and in a niche context, when the cells detach from the surrounding extracellular matrix (ECM) and lose the connection to the nurse surrounding cells[28,29]; this is one of the processes we observed after the combination of chemotherapy and treatment with the carboxy-terminal part of PEDF protein.

In this scenario, we have addressed the problem of locating breast tumour-initiating cells, and the study of the self-renewal mechanism by inhibiting its functionality and potential tumour recurrence. We also postulate that the PEDF signalling pathway could be a potential therapeutic target for control of cancer initiating cells' self-renewal and tumour relapse, as has been postulated in other neoplastic models[30].

MATERIALS AND METHODS

CELL CULTURE

MCF7 (ATCC® HTB-22™), MDA-MB-231 (ATCC® HTB-26™) and 293 HEK-293 (ATCC® CRL-1573™) cell lines were acquire from ATCC company (Manassas, Virginia, United States). Pa00 was derived in the laboratory from metastatic patient pleural effusion[31]. Pa00, MCF7 and MDA-MB-231 cells were maintained in DMEM (Lonza. Basel, Switzerland) 10% FBS (Lonza), 1% Glutamine (Lonza) (0,2M) and 1% Penicillin/streptomycin (Lonza) (100 units+100 ug/10ul) in a 5% CO_2 humidified incubator at 37ºC.

STAIN WITH DDAO AND SORTING

Cells for cell cycle dynamic assays were plated (100 000 cells). Next, these cells were washed with PBS, disaggregated and then incubated at 37ºC with Cell Trace ® Far-Red-DDAO-SE (DDAO-SE, Molecular probe ref C34564. Eugene, Oregon, United States), at the concentration recommended in the product data sheet. After 10 min, an aliquot of freshly labeled cells (approx. 200 000 cells) was fixed with 0,5% of paraformaldehyde to use as a positive control for the experiment. Then, cells were centrifuged at 180g for five minutes and suspended in the culture medium to grow under standard conditions. Eight days *in vitro* later, cells were disaggregated and sorted by In-fluxTM (Becton Dickinson. Franklin Lakes, New Jersey, United States) sorting equipment depending on their fluorescent retaining labeling level. Positive cells were considered slowly dividing cells, and thus potential tumour initiating cells.

CYTOMETRY ASSAY

Cytometry assays were performed with a MACSQuant Analyzer 10 cytometer (Miltenyi Biotec ref 130-096-343. Bergisch Gladbach, Germany). Samples were first washed in PBS and incubated with Miltenyi Biotec FcR blocking Reagent (human), in 100 microliters of sample. The immunostaining was performed per the standard protocol recommended by the commercial houses of the different antibodies. The antibodies and the working dilutions used were as follows: AntiBCRP-FITC 5D3 Chemicon (Temecula, California, United States) and AntiBCRP-PE 5D3 Chemicon incubated for 20 minutes at a 1:10 dilution; while AntiEpCAM- PE Clon HEA-125, AntiAC133-PE Clon AC133, AntiCD133-PE 293C3, Anti CD44-FITC and CD24-PE, all from Miltenyi Biotec, were incubated 10 minutes at a 1:11 dilution. Sorting experiments were performed with a BD Influx™ cell sorter. FITC or PE are used as abbreviation of Fluorescein isothiocyanate and Phycoerythrin respectively.

XENOGRAFTS

FOXn1nu females, at 1 month of age were obtained from Charles River International Laboratories (Wilmington, Massachusetts, United States) to use in these experiments. Animals were housed and bred under 20-25 ºC, humidity of 50-60% and a 12 hours' light-dark cycle. All experiments were performed in accordance with relevant guidelines and regulations and the animals were treated in accordance with the approval of the local ethics committee (University of Castilla-La Mancha PI081746). The experiments were performed as previously laboratory assays[30]. In sort, untreated cells (control) and treated cells (8nM CTE-PEDF) suspended in PBS were injected subcutaneously on both flanks of those immunocompromised mice. 5 000 cells were injected in a final volume of 200 uL 1:1 dilution Matrigel™ Basement Membrane Matrix (Becton Dickinson) with a 25 gauge-needle. Tumour growth was monitored weekly with a caliper. The final tumour volume was calculated as $V= 2 \times L_1 \times L_2 \times \pi/6$. Tumours were mechanically and chemically dissociated with collagenase and trypsin at 37ºC. They were then washed with PBS and seeded in DMEM medium, 10% FBS, 1% glutamine (200 mM), 1% Penicillin/streptomycin, 0.5% EGF and 0.04% FGF in a 5% CO_2 humidified incubator at 37ºC 12h before start the rest of the experiments.

PEDF and CTE-PEDF PRODUCTION

PEDF and Cter-PEDF were cloned into pcDNA™3.1/myc-His A, B, & C Mammalian Expression Vectors (Invitrogen™. Carlsbad, California, United States) following the protocol previously described by [32]. After cloning, vectors were checked by sequencing (3130 Applied Biosystems. Foster City, California, United States). Serine 227 was mutated into a glutamate (E) residue using *ser227glu-dw* (5'-CCA AGT AGA AAT CCT CGA GCT CAG TCT TTC TGG AGT-3') and *ser227glu-up* (5-GTT TGA CTC CAG AAA GAC TGA GCT CGA GGA TTT CTA-3) primers. Proteins were produced by HEK-293-T cells after transfecting with phosphate calcium. Conditioned mediums were collected after 3 DIV, quantified by western-blot using anti-c-myc mouse monoclonal IgG1 (Santa Cruz Biotechnology. Dallas, Texas, United States), and anti-phosphoserine clone 4A4, (Millipore. Burlington,

Massachusetts, United States) and stored at -20ºC or purified using GE Healthcare Life Sciences™ HisTrap™ FF Crude columns (Thermo Fisher Scientific. Waltham, Massachusetts, United States).

TREATMENT WITH PEDF AND CTE-PEDF

Cells were treated with PEDF or CTE-PEDF at a final concentration of 8 nM in the medium. The acute treatment consists of a single treatment: two hours of peptide exposure before chemotherapy treatment. Chronic treatment consists of six peptide treatments (culture medium with CTE once per week), totalling six weeks of peptide exposure before chemotherapy treatment.

DOSE-RESPONSE CURVE

Cells were plated in a 24-well plate at a final concentration of 15 000 cells/well in a total volume of 200 microliters or 200 cells/well in a 96-well plate for sorted cells. Half of the plate was plated with CTE-PEDF medium. The next day, the drug was added in increasing concentrations 4 nM, 2 nM, 1 nM, 0.5 nM, 0.25 nM. These were cultured for 3 days under drug exposure in *in vitro* conditions, and then developed with an MTT assay or methyl purple assay.

MTT ASSAY

Supernatant was removed, after which 100 microliters/well of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide () was added. After 30 minutes of incubation, the supernatant was again removed, and the precipitated crystals were dissolved in 100 microliters of DMSO. The plate was read in a spectrophotometer at 540 nm.

METHYL PURPLE ASSAY

This method[30] is used to quantify surviving cells. 5 000 cells/well were seeded in 24-well plates, in a final volume of 250 µL. The next day, cells were treated with increasing doses

of chemotherapeutic agents and stored in a humidified incubator at 37ºC, 5% CO_2 for 4 days. Then, cells were fixed with 0.5% glutaraldehyde (Sigma. St. Louis, Missouri, United States) for 10 minutes. Next, cells were stained with 0.1% crystal violet for 20 minutes. After several washes with PBS, 10% acetic acid was used to solubilize the sample. Finally, a spectrophotometric reading was performed at a wavelength of 590 nm. IC50 value was determined as the dose of drug necessary to eliminate 50% of the cell population, obtained through logarithmic regressions made with the DE.0 plus v 1.0 program.

HISTOLOGY

Cryostat sectioning slides were performed using a cryostat (Microm HM 550, Thermo Scientific). Tumour samples were fixed with formaldehyde at 4%, placed in sucrose 30% overnight, included into Tissue-Tek® OCT™ (Sakura ® Finetek USA. Torrance, California, United States) and then frozen with liquid nitrogen. Slides (12µm) were stained with hematoxylin and mounted with Dako Ultramount Aqueous Permanent Mounting Medium (Agilent Thechnologies. Santa Clara, California, United States) and analyzed with a Leica-DMRXA-photomicroscope (Leica. Wetzlar, Germany).

ANALYSIS OF CELL MORPHOLOGY

500 000 treated and control cells were seeded in p100 plates. After 24 hours, cells were incubated for 30 minutes with Hoechst (5µg/mL). Ten random microscopy images were taken (Motic AE31.Barcelona, Spain) using an ultraviolet light. Analysis of the cytoplasmic area and the separation between cells was analyzed using the Image J application (https://imagej.nih.gov/ij/).

STATISTICAL ANALYSIS

Data was analyzed with R software 3.5.1 version (https://www.r-project.org/). The statistical analysis was carried out using Mann-Whitney U tests (one tailed, significance level=0.05). The data is expressed as the mean plus the standard error (SE). At least n=3 independent

192 experiments were performed for every assay. The results obtained are considered
193 statistically significant when $p<0.05$(*), $p<0.01$(**) and $p<0.005$(***). Blood cell data was first
194 transformed into a quadratic variable to improve data homogeneity. Then, normality was
195 tested using the Sapiro-Wilk test, Q-Q plots, and Levene's test for homogeneity of variance.
196 Next, groups were compared using non-parametrical Kruskall-Wallis test by ranks and
197 Wilcoxon's not paired test to analyse data in pairs. Finally, logistic regression was used to
198 model the relationship between the number of cells and progression. The odds ratio was
199 used to strengthen the association between variables (Confidence Interval 95%).

RESULTS

1. Long retaining labelling cells exhibit characteristics of cancer stem cells, in cancer cell lines and in cancer patient cells.

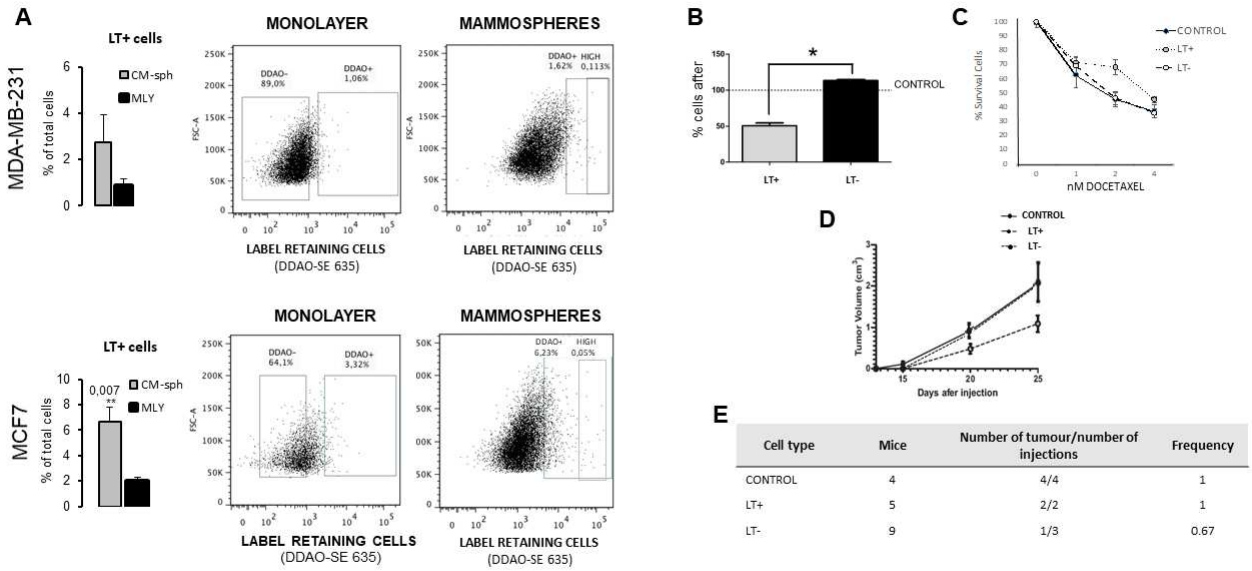
MDA-MB-231 and MCF7 breast adenocarcinoma cell lines exhibit a cell population characterized by long retained labelling growing either in adherent or mammosphere conditions (Figure 1A). A population of 0.9 ± 0.3 % and 3 ± 1% of long retaining labelling cells is present when the MDA-MB-231 cell line is grown in an adherent monolayer and in mammosphere conditions, respectively. In mammosphere conditions, it is possible to see 0.1% of total cells with high labelling. This result is also seen for the MCF7 cell line where the percentage of long labelling cells is 3.32% and 6.28% (0.5% high labelled) in monolayer culture and mammosphere conditions, respectively.

Ascitic fluid cells from a patient with a metastatic adenocarcinoma (Pa00 cells) also present long retaining labelling cells after 8 days in culture. A methyl purple assay proves that LT- cells show the same growth rate as our control cells but have a higher growth rate than LT+ cells (Figure 1B). Those cells underwent a dose-response assay with Docetaxel chemotherapy. As we can see in Figure 1C, even though LT+ cells grow less than LT- or control cells, they are more resistant to chemotherapy than control or LT- cells (IC50 value is double in the case of LT+ cells compared to control or LT- cells). Docetaxel treatment eradicates 53% of LT- cells at 2 nM, while only 27% of LT+ were affected by the same concentration.

Finally, Pa00 LT+ and LT- sorted cells were injected in nude mice to study the carcinogenicity of those populations in vivo. This experiment shows that the tumour volume is similar when injecting LT- cells and control non-separated cells but smaller when injecting LT+ cells (Figure 1D). In addition, Pa00 LT- cells failed to form tumours after cell injection.

That´s why the frequency of tumour formation is lower when injecting LT- cells than LT+ cells or control non-separated cells (Figure 1E).

In short, LT+ cells show a lower growth rate and are more resistant to chemotherapy than LT- or control non-separated cells. In addition, *in vivo* assays reveal a decrease in the frequency of tumour formation in LT- cells when compared to LT+ or control cells.



Cell type	Mice	Number of tumour/number of injections	Frequency
CONTROL	4	4/4	1
LT+	5	2/2	1
LT-	9	1/3	0.67

Figure 1. LT+ population display cancer stem cells characteristics. (A) Cells were stained with DDAO and cultivated 8DIV in monolayer or as mammospheres. Both cell lines showed LT+ population and it was higher in mammosphere assays (cytometry assay). Quantification of LT+ cells (n=3) is shown. (B) Growing patterns of LT+ and LT- cells. Pa00 cells were stained, grown 8DIV (400cells/well) and then sorted according to their DDAO content. It was checked the number of living cells after 3DIV by methyl purple assay. LT- cells grew similar to control and faster than LT+ cells. (C) Docetaxel dose-response curves for LT+, LT- and control cells. Cells were stained with DDAO and grown for 8DIV, sorted by their content of DDAO and grown with increasing concentration of docetaxel. LT+ cells showed more resistance against docetaxel than LT-. (D) 5000 cells were injected in nude mice in each case. The

volume of the tumour is similar when injecting LT- cells and control non-separated cells but smaller when injecting LT+ cells. All tumours were palpable at the same time. LT+ tumours grew slowly compared to control or LT- group. (E) Pa00 LT- cells failed to form tumours when injected in a small amount of cells**.** All set of tumour cells were able to form tumours when 5000 cells were injected. However, LT- cells displayed a less frequency in tumour forming when 1200 or 350.000 cells were injected.

2. PEDF modulate CSC properties, producing an increase of drug resistance and increasing LT+ cells proportion.

PEDF is a verified modulator of stem cell self-renewal[14,33]. PEDF chronic treatment produces morphological changes in size and cytoplasm shape of Pa00 cells in culture (Figure 2A). An increment of 170 μm^2 of the cytoplasmic area is shown in PEDF treated cells (730±80 μm^2 in PEDF treated and 560±30 μm^2 in control medium). However, there are no significant differences between the nucleus size with and without treatment (Figure 2B).

We have also studied the growing pattern of chronically PEDF-treated patient cells. Close to a 40% decrease in cell growth can be seen in treated cells compared to controls (Figure 2C). This slower growth rate of treated cells translates into a lower tumour volume and less necrotic area when comparing with control untreated cells. The histological analysis of the xenograft, showed that PEDF treated tumours present smaller necrotic areas than control xenografts. PEDF treatment also produces a compact growth of the tumoral cells with dense cytoplasm and compact external matrix compared to control tumours (Figure 2D-E).

These cells were also checked in a dose-response assay, and despite their low growth rate, PEDF treated cells exhibit higher IC50 values and resistant population, as 47% of PEDF treated cells survived, which means there were 16% more resistant cells than in

the control. This result implies a higher drug resistance to Docetaxel than control cells (Figure 2F).

We carried out the next assay to prove that the slow growth observed *in vitro* and *in vivo* is due to the appearance of a higher number of slow-cycle cells after PEDF treatment. The quantification of LT+ cells after 3DIV revealed a significant increase after PEDF treatment compared to the control (Figure 2G). A dose-response assay was performed, and the results demonstrate that the resistance to docetaxel is 38% higher in PEDF treated cells at 4 nM than in the control group (Figure 2H).

To sum up, PEDF treated cells show changes in the cytoplasm size, a decrease of cell cycle kinetics, an increase in drug resistance, and the capacity to produce tumours with not only a lower growth rate but also lower tumour volume and fewer necrotic areas.

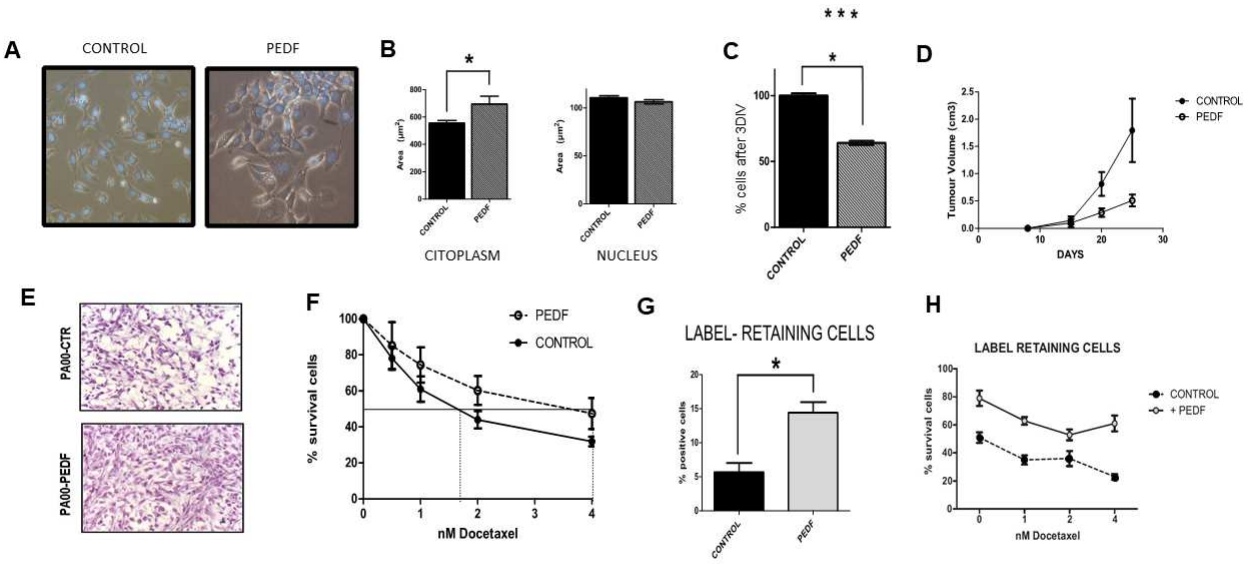


Fig 2. Pigmental Epithelium Derived Factor (PEDF) increases the number of LT+ cells and the Docetaxel resistance of breast cancer cells. (A) Cells treated with chronic PEDF showed a different morphology than control. (B) Quantification of morphology differences induced by PEDF treatment. (C) Growing pattern n=3 after 3DIV of PEDF treated cells and control. PEDF chronic treated cells grew slower than control. (D)

Docetaxel dose-response curve of PEDF treated cells and control, n=3. PEDF chronic treated cells were more resistant against Docetaxel. (E) PEDF chronic treatment increased the number of LT+ cells in vitro (n=3). (F) Docetaxel dose-response curve of LT+ PEDF treated cells and LT+ cells, n=3. LT+ PEDF chronic treated cells were more resistant against Docetaxel than LT+. (G) PEDF treated cells and control cells were injected into nude mice. PEDF chronic treated cells grew slower than control. (H) Histology of PEDF treated tumours and control tumours. Necrotic areas are bigger in control compared to PEDF treatment.

3. Cter-PEDF counteracts the effects of native PEDF, decreasing the resistance in tumours and inducing anoikis and depleting CSC.

As the truncated protein CTE-PEDF does not exhibit the same effects in stem cells as the full-size PEDF protein, we decided to check if this peptide would counteract the effects of the native protein. This protein presents phosphorylation sites that are important for the protein's function. In order to maintain the negative charge, a glutamate was introduced in position 227 of the protein's sequence, where a serine was previously located. This modification leads to the CTE-PEDF protein (Figure 3A). In vitro treatment with CTE-PEDF (200ng/ml) was performed in patient cultured cells. This experiment shows an anoikis effect (Figure 3B) and morphological changes that can be measured by cytoplasmic and nuclear areas and cell distances (Figure 3C). Nuclear and cytoplasmic areas are reduced 29% (Nuclear area: control 110±2 μm^2, CTE 81±2.34; Cytoplasmic area control: 556±18 μm^2, CTE: 394±17 μm^2) while cell distances double their size (control 12±1 μm^2, CTE: 23.0±0.2 μm^2).

The next approach was a dose-response assay with Docetaxel. The result demonstrates that CTE-PEDF treated cells are less resistant to the drug than control cells. Just the CTE-PEDF treatment produces a 20% reduction of the initial population

(Figure 3D). The IC50 value is double in the control group when compared to CTE-PEDF treated cells (Figure 3E).

The results demonstrate that CTE-PEDF produces anoikis in cancer patient cells and reduces the drug resistance to Docetaxel.

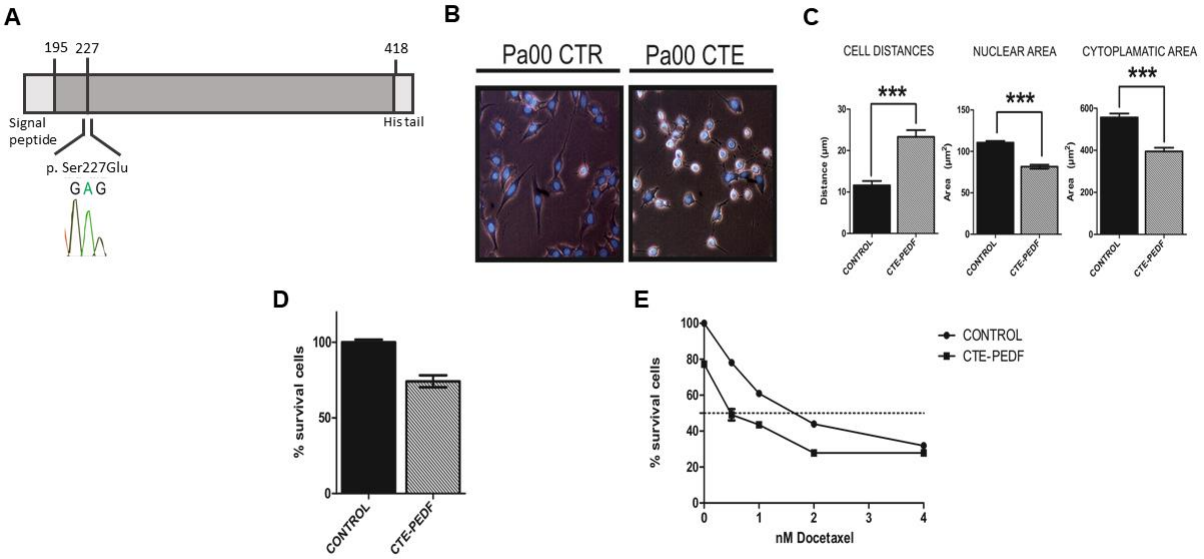


Fig 3. CTE-PEDF induces anoikis in vivo and reduces resistance against Docetaxel without affecting LT+ population. (A) CTE-PEDF construction (B) Pa00 cells were treated chronically with CTE (200ng/uL). After a week they showed an increase in anoikis. (C) Quantification of CTE induced morphology. (D) Growing pattern n=3 after 3DIV of CTE treated cells and control. CTE chronic treated cells grew slower than control. (E) Docetaxel dose-response curve of CTE treated cells and control, n=3. CTE chronic treated cells were less resistant against Docetaxel.

4. **CTE-PEDF depletes CSC expression markers.**

We already know the effect produced in cells decreasing their resistance and inducing anoikis as is show in previos results, when cultures are treated with CTE-PEDF but, what we are now considering is whether this effect is produced because of a reduction in CSC number and thus, in CSC expression markers. To solve this question,

Pa00 cells were injected with CTE-PEDF or PBS in nude mice. Tumours were dissected and dissociated to study stemness marker expression by flow cytometry. Four different epitopes, that have been previously related with cancer stem cells, were analysed: BCRP1, EpCam, CD133 and AC133. CTE-PEDF treatment produces a significant reduction in all studied markers in patient tumour cells Pa00. The same experiment was performed with cells chronically treated in culture with CTE-PEDF, and the same reduction in marker expression was observed (Figure 4).

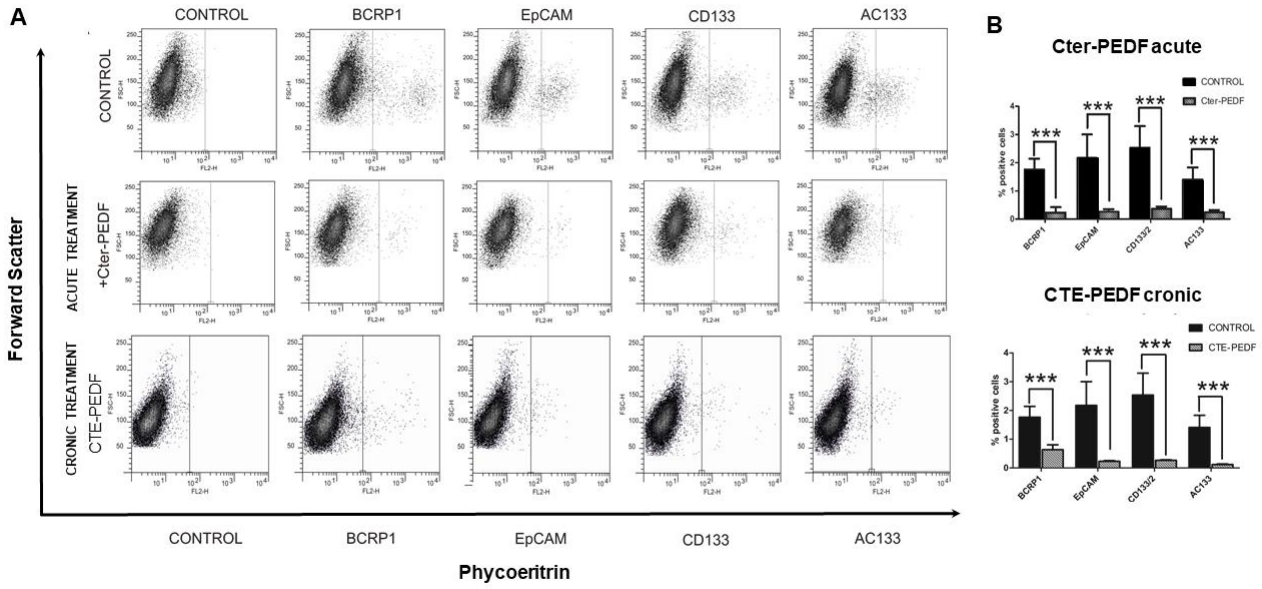


Fig 4. Cter-PEDF and CTE-PEDF treatments decrease CSC in vivo in Pa00 ascitis cell line. (A) 5000 Pa00 cells were injected in matrigel with 200ng/uL of Cter-PEDF, CTE-PEDF or PBS (control). A cytometry assay showed that the number of CSC is lower in both treatments compared to control. (B) Quantification of positive cells in each treatment compared to control (n=3). Significant differences are shown.

In conclusion, CTE-PEDF decreases putative stemness markers involved in self-renewal and patient release. They could be interesting proteins to control drug-resistant cells populations in patient samples.

DISCUSSION

Breast cancer is the most common cancer in women worldwide. We study how to prevent relapse, taking breast cancer as an example because of its impact on the population. It's important to distinguish tumour initiating cells, responsible for tumour formation, and also probably for tumour relapse, and this is why we have focused on marker expression to detect this kind of cells (TICs). A combination of different epitopes found in the literature was applied to detect these cells[34–36]. Later, our objective was to discover a possible new treatment for patients in which TICs were detected. We demonstrate that PEDF (and derivatives) have a moonlighting role in breast cancer development and relapse, and it has a potential application in clinical cancer therapies by the co-administration with chemotherapy for relapse cancer treatment. This is the case of CTE-PEDF, that decreases drug resistance[37,38]. The co-administration with chemotherapy would lead to a less resistant population because TICs would have changed and lost their stemness characteristics, as we discuss in the following sections.

About the stemness characteristics of long retaining labelling cells, in cancer cell lines and in cancer patient cells.

We have shown that MDA-MB-231 and MCF7 breast adenocarcinoma cell lines exhibit a cell population characterized by long retained labelling growing either in adherent or mammosphere conditions, as has been previously described in other tumour cell lines[39–41]. Other researchers have detected a similar percentage of stemness and invasiveness for breast cancer cells in these cell lines, which was modified by oestrogen through Gli1 activation[42]. Even in mammosphere cultures, we have observed that a high DDAO positive population remains after 8 days in culture. These cells, with slow kinetic cell division, have probably only divided once in this period. This subpopulation could be related to the resistant cells maintained by IL-6 in some breast cancer treatments, such as the inhibitor of the human epidermal growth factor receptor 2 (HER2) Lapatinib [43].

Ascitic fluid cells from a patient with a metastatic adenocarcinoma (Pa00 cells) have been used in this paper parallel to the use breast cancer cell lines. Ascitic fluid should be an acellular liquid resulting from inflammation events. After metastasis progression, ascitic fluid could contain tumour cells that grow in culture conditions[44,45]. These cells also present long retained labelling after 8 days in culture, so we sorted these cells according to their DDAO content. This experiment showed that LT+ cells grow more slowly than LT- cells and respond less to chemotherapy than control or LT- cells. We postulated that this is the reason for the resistance observed in some treatments. Similar differences in resistance have also been observed in other colon and breast cancer cell lines[6] and even in other more aggressive type of tumours as a glioblastoma C6 cell line[37]. The final percentage of resistant cells after maximum physiological doses of Docetaxel treatment is also higher in LT+ positive cells than in the rest. This LT+ population that supports higher Docetaxel IC50 than LT- or control cells could be the origin of patient relapse and related to a higher BCRP1 and CD133 marker expression. To investigate this hypothesis, Pa00 LT+ and LT- sorted cells were injected in nude mice to study the tumorigenicity of those populations. We have considered not only the final volume of the xenograft tumours, but also the timing of the tumours' appearance. LT- cells formed tumours at the same time as control non-separated samples but LT+ Pa00 cells, which have a decrease in the kinetic division of their cell cycle, took longer to form tumours. Accordingly, the percentage of final tumour growth after LT+ cell injection is 1/3 higher than in LT- xenografts. This result, together with the previously shown data, leads us to consider that the difference between these two cell types is the initial tumour capacity. This data correlates with previous data from research groups that correlate chemorresistance, tumorigenicity potential, and slow-cycling in some tumour cells[39–41].

All this data supports the hypothesis that LT+ cells present slow cell cycle division, higher chemotherapy resistance, and higher frequency of tumour formation than LT- or control cells.

These characteristics confirm the idea that LT+ cells could be involved in relapse and metastasis progression in breast cancer.

PEDF modulates tumour initiating cells and causes an increase of drug resistance and LT+ population.

PEDF chronic treatment produces a decrease in the growth rate *in vitro* and *in vivo* due to an increase in the number of LT+ cells (slow-cycle cells). Despite their low growth rate, PEDF treated cells show higher IC50 value and resistant populations than control, indicating higher resistance to chemotherapy. One possible explanation for this effect could be that the low growth rate leads to more time for these cells to repair chemotherapy damage, even though another hypothesis could contribute to this stage, such as higher expression of ABC drug transporters in these cells.

CTE-PEDF counteracts the effect of native PEDF and depletes TICs markers expression

As we have shown here, CTE-PEDF treated patient cells showed anoikis and morphological changes in culture. What is known as the anoikis effect is the apoptosis induced by the lost, insufficient, or inappropriate interactions between the cell and the extracellular matrix[46]. This effect could be the base for the interaction of TICs with the surrounding niche, and could be involved in the loss of stemness properties and chemotherapy resistance. Docetaxel resistance decreases when cells are treated with CTE-PEDF. It counteracts the effect of PEDF, which is a niche protein[9] involved in self-renewal and trophic maintenance of pluripotent cells. PEDF is secreted by endothelial cells and is one of the proteins involved in the self-renewal stem cell capacity. Endothelial cells also play a significant role in tumour progression and metastasis[47], and the niche signals could be key to understanding tumour progression, epithelial-mesenchymal transition, and distant metastasis at patient relapse. A significant difference is shown

between treated cells and controls, with PEDF or derived peptides, but the protocol followed in this experiment should be standardized to obtain a better result. The resistance of these cells is because they come from a patient in advanced metastasis ascites.

Expression of markers that had been previously related to cancer stem cells decreases when cells are treated with CTE-PEDF. This effect confirms the idea that CTE-PEDF produces a decrease in resistance to drugs such as Docetaxel, because it reduces the number of tumour initiating cells. That indicates a possible potent application as a future treatment for this protein when co-administered with chemotherapy. Although further studies need to be carried out, these experiments point to a possible new strategy for relapse cancer treatment.

CONCLUSIONS

The PEDF stem cell self-renewal modulator protein modify the carcinogenicity of cancer stem cells and could be a useful tool to control its self-renewal and therefore control patient relapse. We have designed a transgenic peptide derived from PEDF to interfere with the self-renewal capacity of cancer stem cells, inducing anoikis in vivo and reducing resistance in cells from cancer patients. We have also shown that this PEDF-derived protein produces a significant decrease in cancer stem cell markers, making this protein a potential tool for delaying patient relapse.

LIST OF ABBREVIATIONS

LT+: retain long labelling

PEDF: Pigment Epithelium-Derived Factor

TICs: tumour initiating cells

ECM: extracellular matrix

HER2: epidermal growth factor receptor 2

DECLARATIONS

- Ethics approval and consent to participate: The Ethics Committee of the “Complejo Hospitalario de Albacete” gave its approval for the present study to be carried out with the corresponding informed consents.
- Consent for publication: All authors give our consent for the publication of the data reflected in this article.
- Availability of data and materials: All data and materials are described in the corresponding sections of this article. There is no supplementary material.

CONFLICTS OF INTEREST: The authors declare no competing financial and non-financial interests in relation to the work described.

FUNDING: Research Project from the “Asociacion Española Contra el Cancer”, Albacete Regional Division. Programa Propio Universidad Politécnica de Madrid.

AUTHOR CONTRIBUTION STATEMENT: 1. Conception or design of the work: Carmen Ramírez-Castillejo; Francisco Sánchez-Sánchez 2. Data collection. Carmen Gil Gas; Paloma Honrubia Gómez; Carmen Belén Alvarez-Simón; Jose Luis Sánchez; Sebastiá Sabater. 3. Data analysis and interpretation. Carmen Ramírez-Castillejo; Carmen Gil Gas; Francisco Sánchez-Sánchez; Marta Sánchez-Díez. 4. Drafting the article. Carmen Gil Gas; Marta Sánchez-Diez; Francisco Sánchez-Sánchez 5. Critical revision of the article. Carmen Ramírez-Castillejo; Carmen Gil Gas; Francisco Sánchez-Sánchez; Marta Sánchez-Díez, Paloma Honrubia-Gómez, Jose Luis Sánchez, Sebastiá Sabater. 6. Final approval of the version to be published. Carmen Ramírez-

Castillejo; Francisco Sánchez-Sánchez; Marta Sánchez-Díez, Paloma Honrubia-Gómez, Jose Luis Sánchez, Sebastiá Sabater.

AC K N OW L E D G M E N T S:

We thank C.E. Gavira O Neill for reading the manuscript and English correction. This work was supported by grant from The Asociación Española Contra el Cáncer (División provincial de Albacete). C.R-C. was investigator of the Programa Ramon y Cajal from the MEC. ADAO association for her unconditional help and support.

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FIGURE LEGENDS

Figure 1. LT+ population display cancer stem cells characteristics. (A) Cells were stained with DDAO and cultivated 8DIV in monolayer or as mammospheres. Both cell lines showed LT+ population and it was higher in mammosphere assays (cytometry assay). Quantification of LT+ cells (n=3) is shown. (B) Growing patterns of LT+ and LT- cells. Pa00 cells were stained, grown 8DIV (400cells/well) and then sorted according to their DDAO content. It was checked the number of living cells after 3DIV by methyl purple assay. LT- cells grew similar to control and faster than LT+ cells. (C) Docetaxel dose-response curves for LT+, LT- and control cells. Cells were stained with DDAO and grown for 8DIV, sorted by their content of DDAO and grown with increasing concentration of docetaxel. LT+ cells showed more resistance against docetaxel than LT-. (D) 5000 cells were injected in nude mice in each case. The volume of the tumour is similar when injecting LT- cells and control non-separated cells but smaller when injecting LT+ cells. All tumours were palpable at the same time. LT+ tumours grew slowly compared to control or LT- group. (E) Pa00 LT- cells failed to form tumours when injected in a small amount of cells**.** All set of tumour cells were able to form tumours when 5 000 cells were injected. However, LT- cells displayed a less frequency in tumour forming when 1 200 or 350 000 cells were injected.

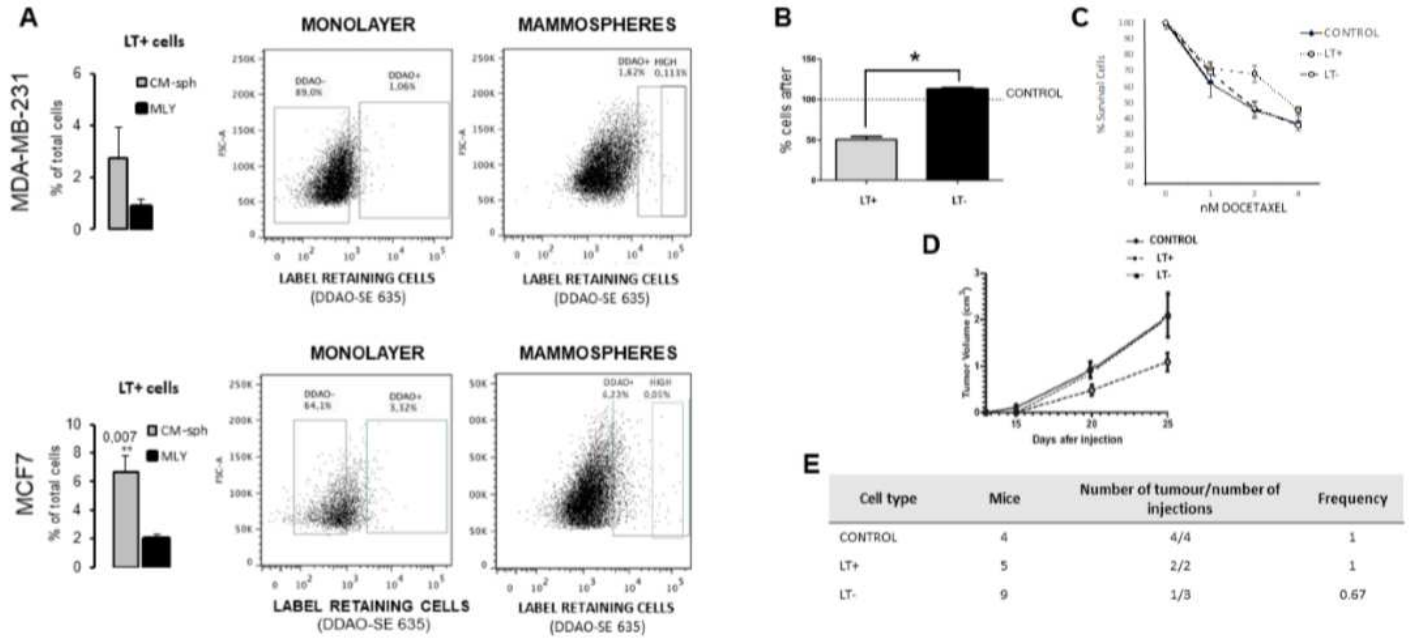
Fig 2. Pigmental Epithelium Derived Factor (PEDF) increases the number of LT+ cells and the Docetaxel resistance of breast cancer cells. (A) Cells treated with chronic PEDF showed a different morphology than control. (B) Quantification of morphology differences induced by PEDF treatment. (C) Growing pattern n=3 after 3DIV of PEDF treated cells and control. PEDF chronic treated cells grew slower than control. (D) Docetaxel dose-response curve of PEDF treated cells and control, n=3. PEDF chronic treated cells were more resistant against Docetaxel. (E) PEDF chronic treatment increased the number of LT+ cells in vitro (n=3). (F) Docetaxel dose-response curve of LT+ PEDF treated cells and LT+ cells, n=3. LT+ PEDF chronic treated cells were

more resistant against Docetaxel than LT+. (G) PEDF treated cells and control cells were injected into nude mice. PEDF chronic treated cells grew slower than control. (H) Histology of PEDF treated tumours and control tumours. Necrotic areas are bigger in control compared to PEDF treatment.

Fig 3. CTE-PEDF induces anoikis in vivo and reduces resistance against Docetaxel without affecting LT+ population. (A) CTE-PEDF construction (B) Pa00 cells were treated chronically with CTE (200ng/uL). After a week they showed an increase in anoikis. (C) Quantification of CTE induced morphology. (D) Growing pattern n=3 after 3DIV of CTE treated cells and control. CTE chronic treated cells grew slower than control. (E) Docetaxel dose-response curve of CTE treated cells and control, n=3. CTE chronic treated cells were less resistant against Docetaxel.

Fig 4. Cter-PEDF and CTE-PEDF treatments decrease CSC in vivo in Pa00 ascitis cell line. (A) 5000 Pa00 cells were injected in matrigel with 200ng/uL of Cter-PEDF, CTE-PEDF or PBS (control). A cytometry assay showed that the number of CSC is lower in both treatments compared to control. (B) Quantification of positive cells in each treatment compared to control (n=3). Significant differences are shown.

Figures



Cell type	Mice	Number of tumour/number of injections	Frequency
CONTROL	4	4/4	1
LT+	5	2/2	1
LT-	9	1/3	0.67

Figure 1

LT+ population display cancer stem cells characteristics. (A) Cells were stained with DDAO and cultivated 8DIV in monolayer or as mammospheres. Both cell lines showed LT+ population and it was higher in mammosphere assays (cytometry assay). Quantification of LT+ cells (n=3) is shown. (B) Growing patterns of LT+ and LT- cells. Pa00 cells were stained, grown 8DIV (400cells/well) and then sorted according to their DDAO content. It was checked the number of living cells after 3DIV by methyl purple assay. LT- cells grew similar to control and faster than LT+ cells. (C) Docetaxel dose-response curves for LT+, LT- and control cells. Cells were stained with DDAO and grown for 8DIV, sorted by their content of DDAO and grown with increasing concentration of docetaxel. LT+ cells showed more resistance against docetaxel than LT-. (D) 5000 cells were injected in nude mice in each case. The volume of the tumour is similar when injecting LT- cells and control non-separated cells but smaller when injecting LT+ cells. All tumours were palpable at the same time. LT+ tumours grew slowly compared to control or LT- group. (E) Pa00 LT- cells failed to form tumours when injected in a small amount of cells. All set of tumour cells were able to form tumours when 5 000 cells were injected. However, LT- cells displayed a less frequency in tumour forming when 1 200 or 350 000 cells were injected.

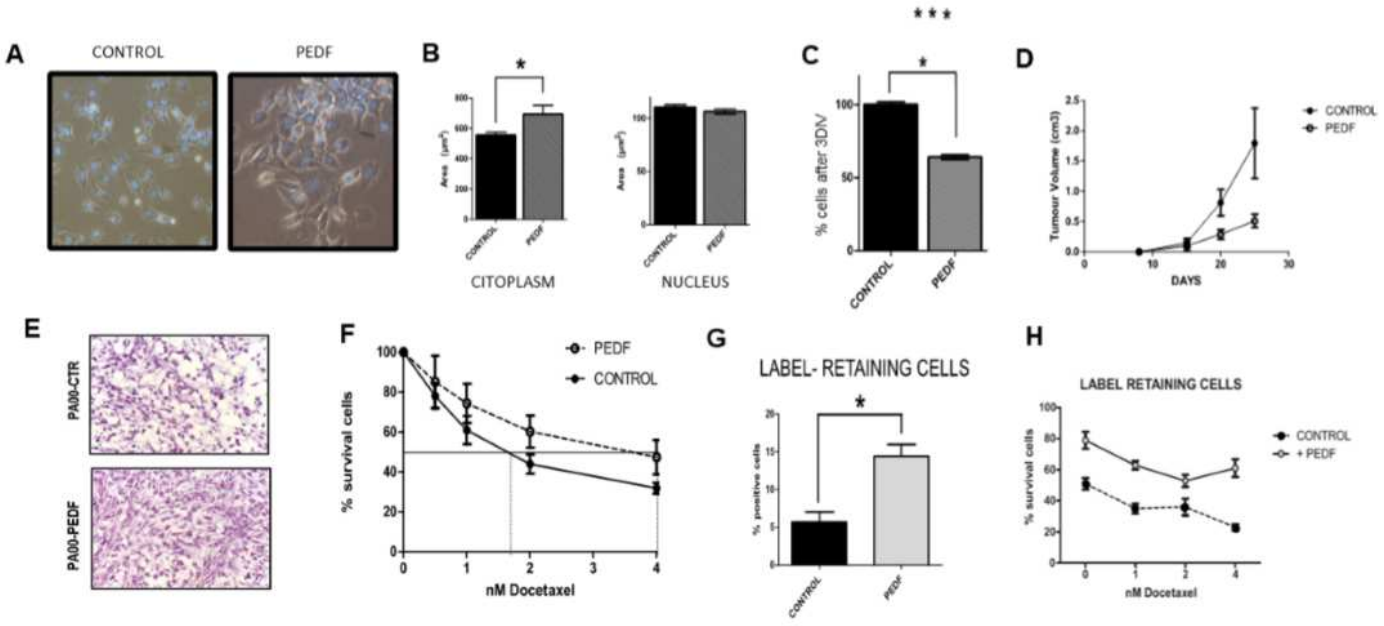


Figure 2

Pigmental Epithelium Derived Factor (PEDF) increases the number of LT+ cells and the Docetaxel resistance of breast cancer cells. (A) Cells treated with chronic PEDF showed a different morphology than control. (B) Quantification of morphology differences induced by PEDF treatment. (C) Growing pattern n=3 after 3DIV of PEDF treated cells and control. PEDF chronic treated cells grew slower than control. (D) Docetaxel dose-response curve of PEDF treated cells and control, n=3. PEDF chronic treated cells were more resistant against Docetaxel. (E) PEDF chronic treatment increased the number of LT+ cells in vitro (n=3). (F) Docetaxel dose-response curve 683 of LT+ PEDF treated cells and LT+ cells, n=3. LT+ PEDF chronic treated cells were more resistant against Docetaxel than LT+. (G) PEDF treated cells and control cells were injected into nude mice. PEDF chronic treated cells grew slower than control. (H) Histology of PEDF treated tumours and control tumours. Necrotic areas are bigger in control compared to PEDF treatment.

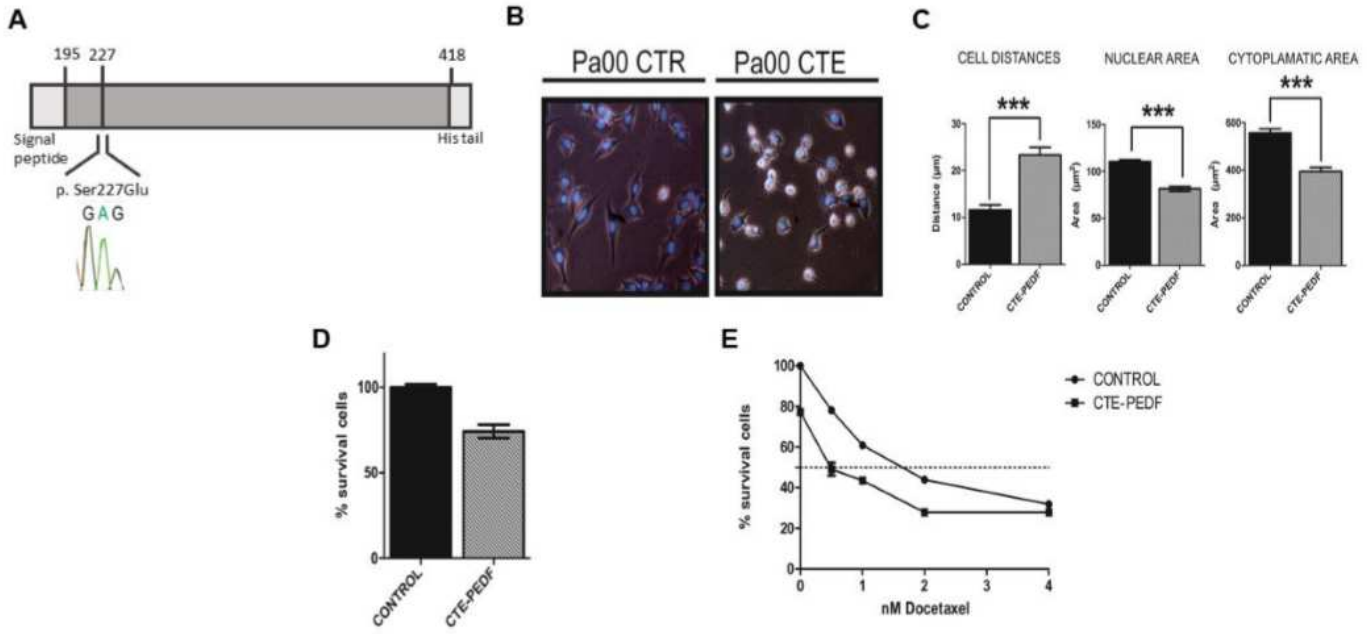


Figure 3

CTE-PEDF induces anoikis in vivo and reduces resistance against Docetaxel without affecting LT+ population. (A) CTE-PEDF construction (B) Pa00 cells were treated chronically with CTE (200ng/uL). After a week they showed an increase in anoikis. (C) Quantification of CTE induced morphology. (D) Growing pattern n=3 after 3DIV of CTE treated cells and control. CTE chronic treated cells grew slower than control. (E) Docetaxel dose-response curve of CTE treated cells and control, n=3. CTE chronic treated cells were less resistant against Docetaxel.

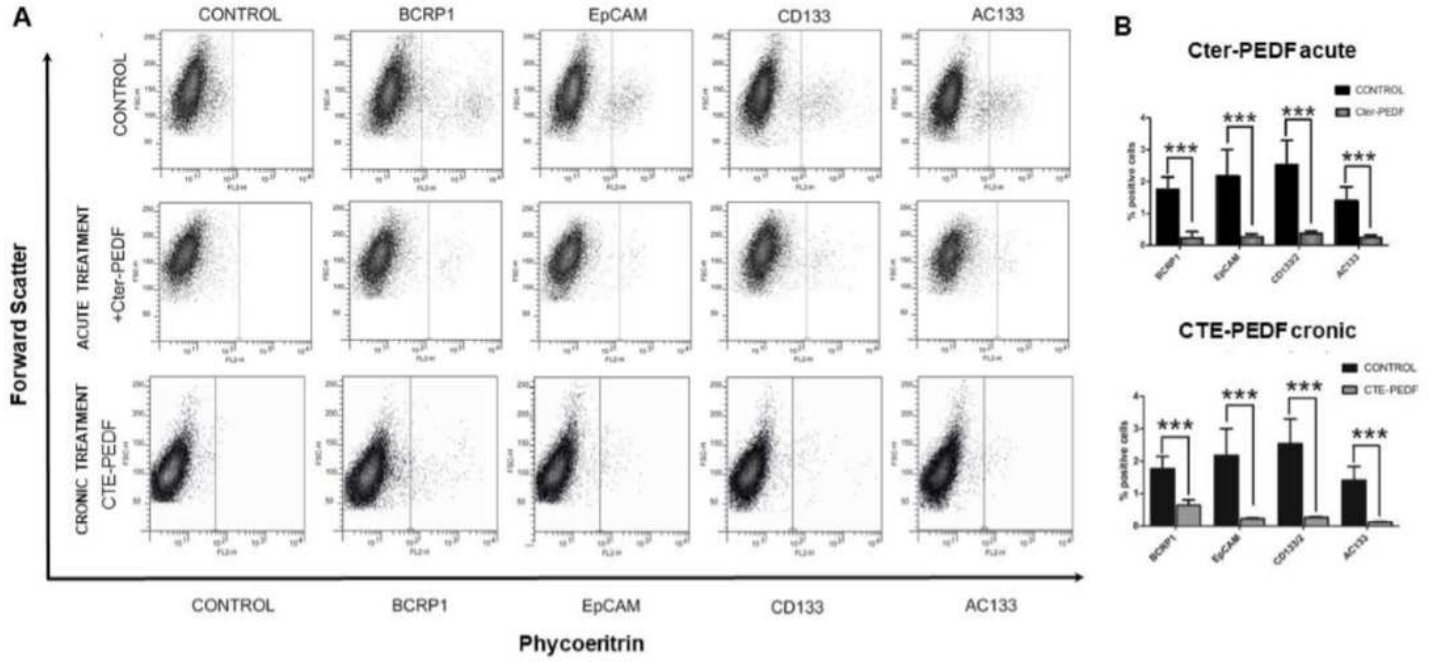


Figure 4

Cter-PEDF and CTE-PEDF treatments decrease CSC in vivo in Pa00 ascitis cell line. (A) 5000 Pa00 cells were injected in matrigel with 200ng/uL of Cter-PEDF, CTE- PEDF or PBS (control). A cytometry assay showed that the number of CSC is lower in both treatments compared to control. (B) Quantification of positive cells in each treatment compared to control (n=3). Significant differences are shown.