C-Terminal-PEDF Reduces IC50 Doses and Chemoresistant Population of CD133 and BCRP1-Positve Cancer Stem Like Cells

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Abstract: The cancer stem cell hypothesis suggests that cancer contains cancer cells with stemness characteristics, and also in immortalised cancer cell lines. This work analyzes the characteristics of the "cancer stem cell" population in C6 tumour cell line. We found a small population defined by the expression of two biomarkers, previously reported in both stem and cancer stem cells, with an aggressive phenotype injected in nude mice. We found 0.6% BCRP1+ cells, the principal protein responsible for the Side Population (SP). On the other hand, we found a small $CD133^+$ positive population, a self-renewal related protein. Moreover, the entire $CD133^+$ population matched with part of $BCRP1^+$ cells suggesting that $CD133^+$ cells could be a subpopulation of $BCRP1^+$ cells, and therefore, a more specific cancer stem cell population than previously described for glioma C6 cell line. These $CD133^+/BCRP1^+$ cells display an aggressive phenotype when injected into NOD/SCID mice. We also eventually found this cancer stem cell like population ($BCRP1^+/CD133^+$) in other types of cancer, even in a brain tumour patient sample with aggressive disease, but not in patient sample with good prognosis. Besides, the important finding in this study is that inhibition of cancer stem cell self renewal could reflex a decrease in resistance to chemotherapy. IC50 and percentage of resistant cells is reported after treatment with a stem cell self renewal inhibitor.

Keywords: BCRP1, ABCG2, ABC transporter family, EpCAM, DFFDA, long retaining labelling cells, Cancer stem cell, self renewal inhibition, asymmetric division, Pigmented epithelium derived factor (PEDF).

INTRODUCTION

Cancer Stem Cell (CSC) hypothesis postulates the presence of cells with stem cell characteristics inside the tumours [1]. This is a novel hypothesis with less than 11 years and the concept of the cancer stem cell has been treated in innumerable scientific texts [2-5]. Nevertheless, there are many controversies on the texts that support this hypothesis [6]. Much has been suggested about the existence of a single tumour stem cell like population [7-9], and for the moment, different markers have been related with the tumour stem cells of different types of tumours Table **1**. Even in immortalised tumour cell lines it have described the side population, a putative cancer stem cell population, for instance in glioma C6 cell line or B104 cell line of neuroblastoma [10, 11].

CSC properties, such as quiescence, multidrug resistance and enhanced DNA repair ability, may be responsible for their resistance to conventional therapies. Surface markers of this CSC population could be a useful tool to predict tumour relapse, and the key to separate and study the self-renewal of these cells to improve new anti-neoplasic therapies.

Recent studies have revealed different possible markers for cancer stem cells, including cell surface and transporter proteins, enzymatic, functional markers, niche signalling or activated pathways [12-15]. We present herein a list of markers of different cancer stem cells to attempt to understand their role in CSC specific properties Table **1**. However, the specificity of these markers to locate CSC is controversial [6, 16]. Particularly in brain tumours, surface protein CD133 has been related to this type of cells [15, 17-19]. The main difference between tumour stem cell like populations and the rest of heterogeneous cells in the tumour is their capacity of quiescence and self-renewal [20], as well as their

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Table 1: List of Markers that have been Related with Different Cancer Stem Cells of Different Types of Tumours or Tumour Cell Lines with References

Tumour/ cancer stem cell	Specific Biomarkers/ Stem cell-like properties
HEMATOLOGICAL MALIGNANCY [20, 47]	
Acute myeloid leukaemia (AML)	$CD34^+/CD38^-$, $Thy\text{-}1^-$, KIT^-, Leukemic grafts in vivo
Multiple myeloma and RPMI 8226 and NCI-H929 myeloma cells	$CD38^-$, Leukemic grafts in vivo
BRAIN TUMOR [17, 48]	
Primary meduloblastoma, ganglioma, astrocytoma, multiform glioblastoma and ependymoma	$CD133^+/nestin^+$, Neurospheres, Tumorogenic in vivo
MELANOMA [49]	
Metastatic melanoma, primary WM115 and metastatic WM239A melanoma cell lines	$CD20^+$/ melanoma spheroids
BREAST CANCER [50]	
Primary and metastatic breast cancers	$CD44^+/CD24^{-/low}$, Oct3/4, mammospheres, Tumorogenic in vivo
MCF7 breast cancer cell line	$CD44^+/CD24^{-/low}$, mammospheres, Tumorogenic in vivo
OVARIAN CANCER	
A2 clone from primary ovarian cancer	$CD44^+/CD24^{-/low}$, Nanog, EGFR, Vimentin, E-cadherin, Tumorogenic in vivo
A4-T spontaneously transformed clone	$CD44^+/CD24^{-/low}$, Nanog, EGFR, Vimentin, Snail, Tumorogenic in vivo A4-T > A2 clone.
PROSTATIC CANCER [51]	
Primary and metastatic prostatic adenocarcinomas	$CD133^+/CD44^+/\alpha_2\beta_1\text{-integrin}^{high}$, protatespheres, Tumorogenic in vivo
LAPC-4, LAPC-9 and DU 145 prostatic cancer cell line or xenografts	$CD44^{high}$, SMO^+/β-catenin, Tumorogenic in vivo
PC3 prostatic cancer cell line	$CD44^{high}/CD133^+$
PANCREATIC CANCER [52]	
Primary pancreatic adenocarcinoma	$CD44^+/CD24^+/ESA^+$, Tumorogenic in vivo
PancTul and A81 pancreatic cancer cell line	$CD44^+/ABCG2^+$
COLORECTAL CANCER [53]	
Colorectal adenocarcinoma	$CD133^+$, colon-spheres,Tumorogenic in vivo
HEAD AND NECK CANCER [54]	
Head and neck squamous cell carcinoma	$CD44^+$, Tumorogenic in vivo

metastatic capacity [21] and the production of tumours in null mice [22], properties that are not present in 99% of tumour cells. Quiescence (slow or infrequent cellular cycles) probably confers greater effectiveness to these cells with regard to DNA repair mechanisms [23], helps toxic expulsion and could promote other molecular routes still not described to confer the observed chemotherapy resistance. In addition, due to their self renewal capacity, these cells could be the responsible for tumour relapse and metastasis.

Our main goal in this work is to study the relationship between markers of cancer stem cells, such as BCRP1 and CD133, in cells with slow cellular cycles in glioma C6 cell line, and the chemotherapy response after inhibition of self renewal capacity.

BCRP1 protein, which is involved in the expulsion of toxic agents from tumour stem cell-like cells, is expressed in this cell population, even in brain tumour patients. Our first results, although preliminary, show the need to study the cancer stem self renewal capacity to avoid tumour relapse and to verify that the appearance of these positive cells is an indication of a future recurrence of the neoplasic disease.

A better knowledge of the mechanisms that govern the self renewal of CSCs could, in the future, help to develop new targeted therapies, eliminating or controlling those cells responsible for the origin and recurrence of tumours.

On the other hand, cancer cell lines are a debatable model for the study of CSCs. The establishment of the culture and successive passages *in vitro* could involve a decrease in the cancer stem cell population. Nevertheless, it has been demonstrated that even the stable tumour lines used in the laboratories for years

have left a small population, or side population, with stem cell characteristics: self-renewal, quiescence and multipotence [10]. The culture of cancer cells found in effluvial liquid from patients with cancer metastasis has also this type of cells. The culture and analysis of these samples show the presence of cell with self-renewal capacity in the culture. These cells from ascitic liquid proliferate at a high division rate, adhering to the *Petri disc* and growing in the presence of serum [24]. The cells of these patients samples have been spontaneously immortalized in the laboratory and used in this study.

Self renewal of these stem-like cells from cancer could be controlled by similar process as normal stem cells self renewal. PEDF is a neurotrophic and neuroprotective factor with anti-angiogenic activity [25] and is the first soluble factor known to specifically activate B-cells. PEDF's activity towards NSCs indicates that it may contribute to the maintenance of a pool of undifferentiated stem cells in neurogenic niches, which is necessary to achieve a normal rate of neurogenesis and also protects from neurodegeneration [26]. The signalling pathway controlling how PEDF is affecting self-renewal is largely unknown; however, two different membrane proteins have been postulated as putative PEDF receptors. The first, PNPLA2 (PEDF-Receptor), has phospholipase activity [27], the second, Laminin receptor PEDF (LR-PEDF), is a lipase-linked cell-membrane protein [28].

MATERIAL AND METHODS

Cell Lines and Cultures

C6 Cell Line

The C6 glial cell strain was cloned from a rat glial tumour induced by N-nitrosomethylurea by Benda *et al*. [29] After a series of alternate culture and animal passages, S-100 production increased a ten-fold as cells grew from low density to confluence. PA00: is a primary culture from an original advanced metastatic breast cancer patient, with brain and digestive metastasis, described previously [21]. Cells were cultured from ascitic fluid obtained in the last period of the patient life. Sample patient cells grown in culture during four weeks. Then we have observed a period of quiescence and low percentage of life flat cells about three weeks and after that and then a fast grown of small cells with a rapid amplification rate and high survival capacity. Cells from the C6 cell line and PA00 primary culture were cultured in DMEN-F12 medium (Lonza Walldersville, Inc) supplemented with 10% (v/v) fetal bovine serum (FBS) (Lonza Walldersville, Inc), 5% (v/v) penicillin and 5% (v/v) glutamine (Biowhittaker). All cells cultures were grown in a humidified cell incubator at 37 ºC under a 5% CO_2 atmosphere.

Tumour Samples

Brain tumour samples were taken from patients by the neurosurgical team at the A Coruña Universitary Hospital (CHUAC) and the University Hospital Complex of Albacete (CHUA), with patients informed document signed and accepted. The samples were disaggregated and cells were plated in complete free serum media [30] supplemented with EGF(20ng/mL) and β-FGF (10ng/mL).

Animals

Severe Combined Immunodeficiency (SCID) mice, 2 months old males, were maintained in quarantine in positively pressurised isolator cages with barrier filtering at the Castilla-La Mancha University Animal House (UCLM).

PEDF and the Correspondent C-Terminal Construction

The C-terminal PEDF domain cDNA was sub-cloned in the pcDNA™3.1 (-)/myc-His from the vector pCMV6-XL5-Serpinf1 (NM_002615) human cDNA clone (OriGene Technologies, Inc.). The C-terminal domain was amplified in two different steps. The signal peptide was first amplified with PEDF1 and PEDF3 primers Table **2**, and then the carboxyl fragment comprising between 195 and 418 amino acids was also amplified using PEDF2 and PEDF4 primers Table **2**. PCR products were then fused and amplified with PEDF1 and PEDF2 in order to obtain a single C-terminal fragment containing the signal sequence and the restriction sites for enzymes *EcoRI* and *BamHI.* The PCR product was then cloned in the *EcoRI* and *BamHI* sites of pcDNA™3.1(-)/myc-His A vector. The construct was checked by sequencing (PCR Big Dye Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems Inc) in order to confirm that it had been inserted correctly and there were no undesirable mutations. Sequencing reactions were analysed with the automatic sequencer 3130 (Applied Biosystem).

PEDF C-Terminal Conditioned Media

HEK293T cells were calcium-phosphate-method transfected with pcDNA™3.1(-)-C-terminal-myc-His A (scheme in Figure **S2A**). In order to verify the process,

Table 2: Primers for the PEDF and C-Terminal Subcloning. The Nucleotides in Italic Characters Indicate the Palindromic Sequences of *EcoRI* y *BamHI* Enzymes. In Blue Letters, the Fusion Area Introduced by PEDF3 and PEDF4 Primers

PRIMER	SEQUENCE (5'→3')
PEDF1	CG *GAATTC* ATG CAG GCC CTG GTG CTA CTC C
PEDF2	GCG *GGATCC* GG GCC CCT GGG GTC CAG AA
PEDF3	ACCCACTGCCCCTTGAAGTGAGCTGCTGTGCCCGCAGGAGG
PEDF4	CCTCCTCGGGCACAGCAGCTCACTTCAAGGGGCAGTGGGT
T7	TAATACGACTCACTATAGGG
BGH	TAGAAGGCACAGTCGAGG

transfected cells were monitored by means of a plasmid which contained the green fluorescent protein (GFP) cDNA. Cells were cultured from 3 to 5 days. Cells transfected with the pcDNA™3.1(-)-C-terminal-myc-His plasmid were cultured at 30ºC in order to facilitate the secretion of the C-terminal domain. The presence and quantification of the recombinant protein present in the medium was checked (Figure **S2B**) by SDS-PAGE and *western blot* using the primary antibody anti-c-Myc (mouse monoclonal IgG1, Santa Cruz) and the primary phosphoserine antibody (4A4 clone, Millipore). The secondary antibody was a goat anti-mouse IgG-HKP (Santa Cruz).

Flow Cytometry and Cell Sorter Analyses

Flow cytometry analysis was carried out using CD133 (PE conjugated AC133 clone, Miltenyi Biotec), EpCAM (PE conjugated HEA-125 clone, Miltenyi Biotec) and BCRP1 (FITC conjugated 5D3 clone, Chemicon), according to manufacturer's instructions. Cells were analyzed and sorted using a FACS (Fluorescent Activated Cell Sorting) In-flux ™ system, from Cytopeia.

Immunomagnetic Cell Sorter

For magnetic cell sorting, cells were labelled with CD133 (clone AC133) MACS micro beads and sorted using the Miltenyi Biotec CD133 Cell Isolation Kit, according to the manufacturer's protocols (Miltenyi Biotec Inc., Auburn, CA).

Long Retaining Labelling Cell Monitoring Assay

The cell division monitoring assays are based on the use of fluorescent probes which are secreted as long as cells segregate. The trail used in this study was Oregon Green® 488 carboxylic acid diacetate (carboxy-DFFDA) - 6-isomer (Invitrogene Corporation). The cells were stained with DFFDA dye in a 1:10 dilution with BSA-EDTA supplemented PBS and incubated for 10 min at 37 ºC. Later, cells were washed with PBS and centrifuged for 5 min at 180g. The pellet was resuspended in PBS-BSA-EDTA, and the homogeneity of the staining was analysed by a LSR flow cytometer.

Xenograft Tumorigenicity Assay

The suspension of sorted cells was mixed in a 1:1 volume with BD Matrigel™ Basement Membrane Matrix (Benton Dickinson). 200µl were injected subcut-aneously into both sides of the mice with a 25 gauge needle. Tumour growth was monitored by measuring width and length with a gauge calibre. Tumour growth was monitored by measuring a calliper after dissection at seven weeks. Tumour volume was calculated according to the formula: V=2*(wide)*(length)*0.052 [31].

MTT Assay

For MTT assays we use p24 plates, seeding 5000 cells/well. Cells were incubated with 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Aldrich) 0.5 mg/mL for 20-40 minutes at 37 ºC. Then, dimethyl sulfoxide (DMSO; Sigma Aldrich) was added. The resulting solutions were measured at 540 nm by a spectrophotometer.

Histology

Sections were prepared in a cryostat 20 micron size. Initially, sections were dyed with haematoxylin and dehydrated. Finally, the samples were prepared with Ultramount Aqueous Permanent Mounting Medium (DAKO®). All preparations were observed under a microscope (Leica-DMRXA).

Immunofluorescence

Immunofluorescence was carried out using two different kinds of samples: tumour tissue and cell

culture. Fresh tumours were fixed with 4% formaldehyde and frozen. Then the tumours were embedded in Tissue-Tek® (O.C.T™ Compound). Tissues were cut into 12µm thick sections. Cells were fixed with 4% paraformaldehyde and incubated with a blocking solution. Fluorescent immunostaining was done using CD133 (PE conjugated AC133 clone, Miltenyi Biotec) and BCRP1 (5D3 clone FITC conjugated, Chemicon) antibodies. The concentration used for immunofluorescence in CD133 and BCRP1 assays was 1:10 for both primary antibodies. Cell integrity was determinated by staining cells with DAPI (Sigma) at 5ng/mL. Finally, the samples were prepared with Fluorescent Mounting Medium (DAKO®). All preparations were observed under a confocal microscope (Carl Zeiss LSM700).

Statistical Analysis

All data were statistically analysed using SPSS software (SPSS Ibérica, IBM Company). U de Mann Winey test was used to analyse de signification of the differences.

RESULTS

CSCs seem to be resistant to chemotherapy due to specific drug transporters present in the cell membrane of this population. Self-renewal properties have also been associated with this chemoresistance, since these chemoresistant cells do not disappear and could give rise to new tumour growth once chemotherapy stops. For this reason, our results are focused in these two proteins and also in its potential inhibition for tumorogenesis reduction and relapse control in future anti-neoplasic therapies:

BCRP1 and CD133 Proteins are Expressed in Tumour Cell Lines

Initially, we tried to discern whether BCRP1 and CD133 are characteristic proteins of cells with a slow rate division in their cell cycles. We have used the tumour cell line Glioma C6, in which *side population* (SP) has been described [10] with a 0.4% presence. In these cell lines, BCRP1 and CD133 positive cells were further detected Figure **1a-b**. by immunocytology and immunocytometry assays. The percentage of these positive cells in glioblastoma C6 cell line is close to 0.5% for both proteins, as it is show in cytometry pictures Figure **1a-b**. These two markers, BCRP1 and CD133 have been also observed by cytometry assays in a patient sample with bad prognostic but non in a sample of free disease patient Figure **S1a-b.**

Long Term Cells Express CD133 Self-Renewal Related Protein

In order to address quiescent cells in our experimental model, we performed long retaining labelling with DFFDA staining and culture assays. Figure **2a** shows the label-retaining capacity in the glioma C6 cell line. First panel shows the whole population before labelling with DFFDA. Second panel shows homogeneous labelling at time cero of our culture assay. Third panel shows the proportion of cells able to retain the colorant in the cytoplasm after 5 days

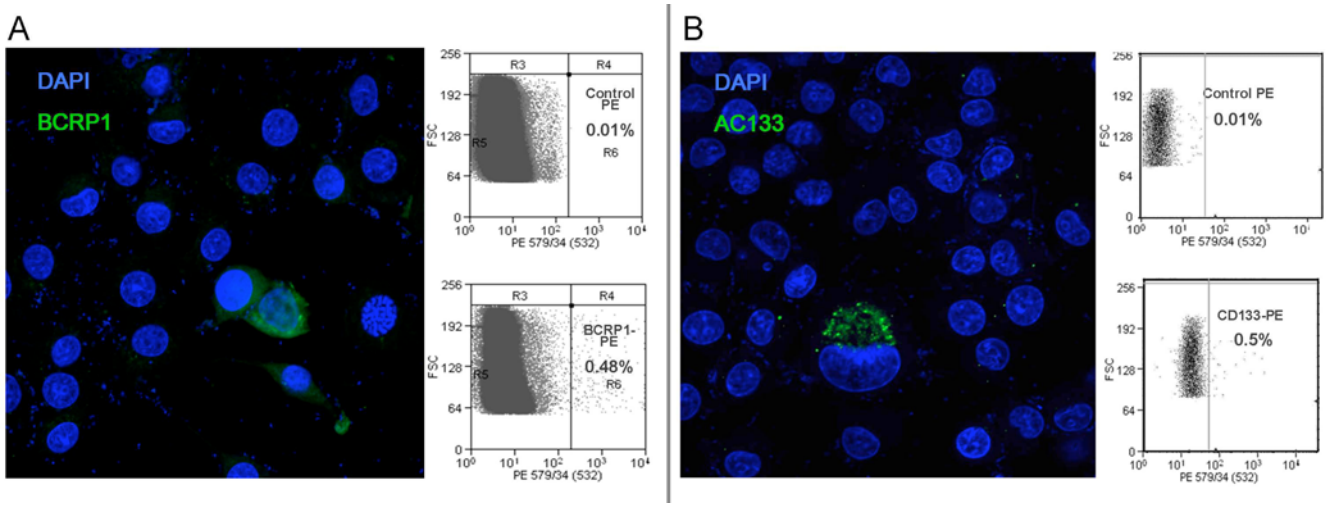

Figure 1: Protein Expression levels of AC133$^+$ and BCRP1$^+$ cells present in tumour cell line C6. a-b. Immunofluorescent images of C6 glioma cells (lens 63x magnification) show BCRP1 **a.** and AC133 **b**. expression as a *green signal* at the cell surface and DAPI nuclear expression as a blue signal. Next, the expression was assessed by flow cytometry. A percentage close to 0.5% was observed in the histograms of this glioma C6 cell line (down histograms). Upper histograms are isotype controls. (AC133 antibody recognises the glicosilated form of CD133 protein).

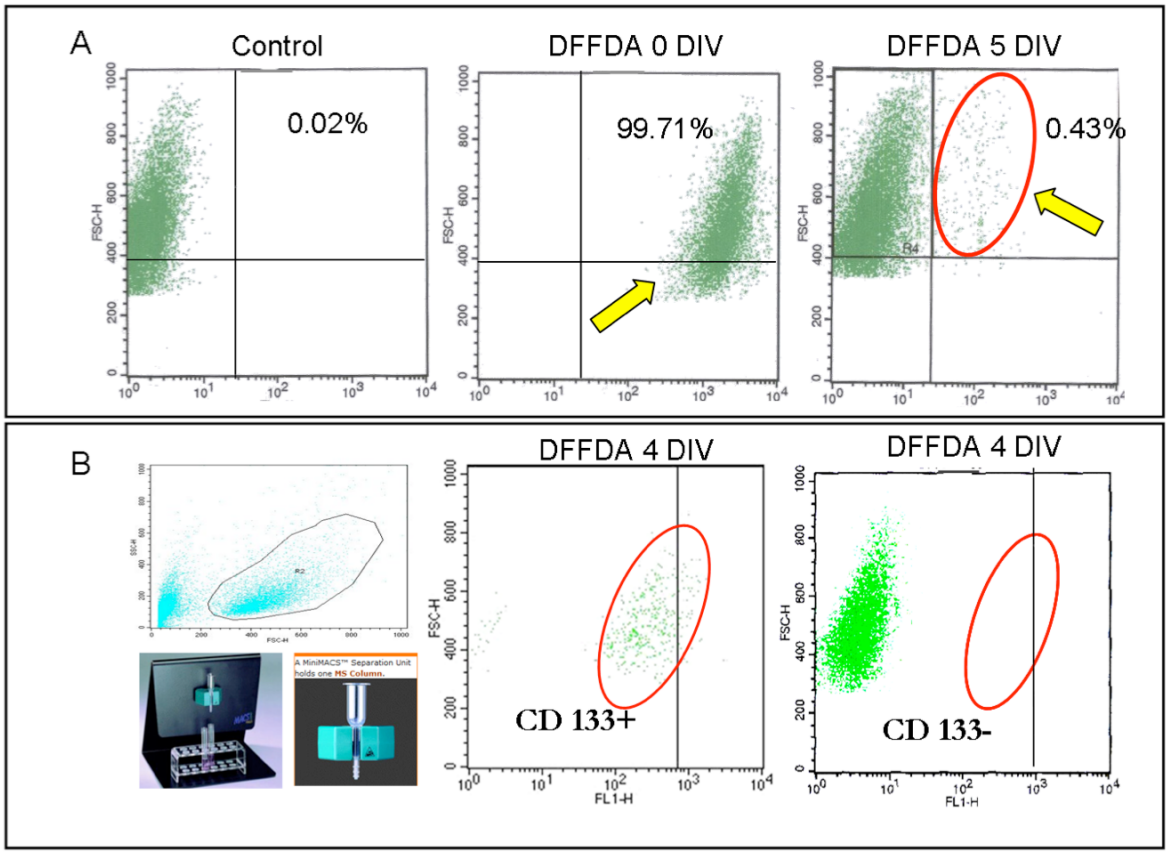

Figure 2: Long cell cycle cells express CD133 self-renewal related protein. a. C6 glioma cells were stained with the fluorescent dye DFFDA as described in the methods section. 99.71% of the populations (*green)* are represented at 0 DIV, and the red circle differentiates slow kinetics 4.35% after 5 DIV. **b.** C6 cells were stained with DFFDA and CD133 immunomagnetic antibodies and then separated using MACS technology based on their CD133 expression. Cells were seed and analyzed by flow cytometry for a period of 4 DIV.

cultured *in vitro* (5DIV). A 0,4% of C6 glioma cells retain the green labelling after this time, due to their slowly cell cycle. To correlate this quiescence property with self renewal, a feature typical of stem cells, we performed double labelling with DFFDA and CD133 protein which has been related with self renewal, Figure **2b**. CD133 antibody was covalently fused to a magnetic particle to facilitate magnetic separation and subsequent cell culturing. Figure **2b** shows the general population after double labelling with DFFDA staining and CD133 antibody, and the schematic drawing of the magnetic separation equipment (bottom panel). Figure **2b** (central panel) also shows the results obtained after four days in culture of the DFFDA and CD133 labelled separated cells. CD133 positive cultures over 4 DIV show a higher DFFDA positive population than CD133 negative cells Figure **2b**, right panel).

Double BCRP1 and CD133 Protein Expression in Relation to Tumour Size

Double labelling of BCRP1 and CD133 cells was also performed Figure **3a**. Selected cells were injected into nude mice in order to study the tumorogenic capacity of those cells. Two cytometry plots, in Figure **3a**, show the isotopic control (left) and double labelling sorting assay (right). Double positive cell populations was selected (square) representing the 0,4% of the total population. Double negative cells were also sorted and injected into control nude mice. 4.000 cells were injected in all cases. Also Figure **3a** shows examples of the obtained tumours in nude mice after one month, with the different cell injections. The graphic in Figure **3a** represents the medium size of the positive and negative tumour obtained. This assay shows the results of two independent injections of each type of populations, and show the tendency of a bigger size in positive cells tumour than in tumour obtained from double negative cells with a p value of 0.058.

Seven weeks after cell inoculation, tumours were dissected and fixed for immunocytochemistry assay. Figure **3b** show abundance of BCRP1 positive cells. Figure **3c** shows mitosis rate and structure of the tumour and CD133 positive cells. Left panels

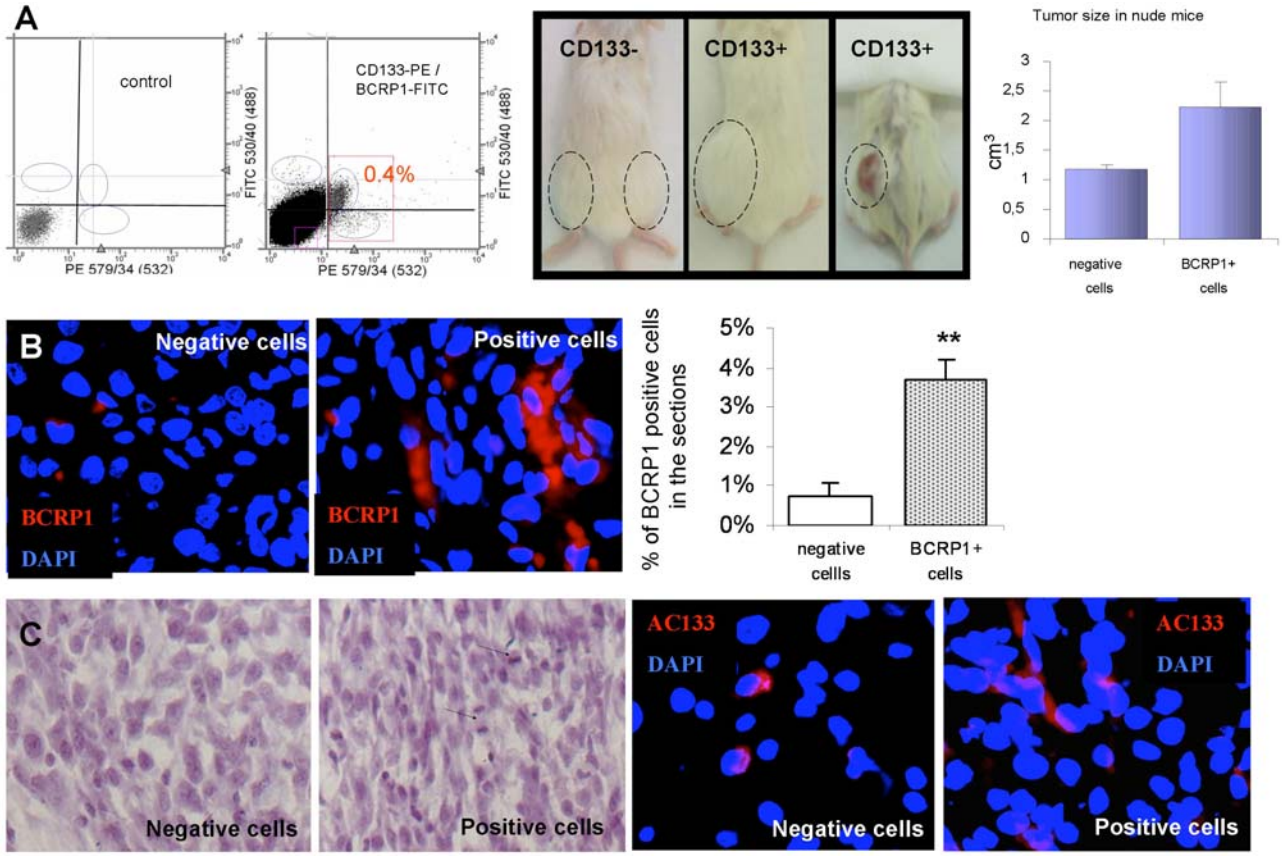

Figure 3: AC133 and BCRP1 positive cells xenograft in nude mouse model. (**A**) Sorted cells in the C6 glioma cell line. Left panel is the control of isotype. AC133$^+$ 0.4 % is the selected population to be injected (square). Positive and negative cells were injected intra-peritoneal into nude mice (4000 cells per shot). Four weeks after the injection, mice were photographed **a** and sacrificed. Tumors sizes were measured and tumor growth volume was obtained (histogram A on the right). The bars indicate differential tendency between AC133$^+$ and AC133$^-$ tumors size in **b**: Formed tumors were fixed and immunostained for BCRP1 protein detection and photographed by confocal microscopy. The graphic shows a significant difference ($p<0.01$) in number of BCRP1$^+$ cells between positive and the negatives tumors. **c:** Histology and AC133 tumors immunostaining. Histology was verified by hematoxiline staining. Tumors from positive cells have more mitotic figures and more AC133 positive cells than tumors from negative cells.

correspond to tumours obtained in the mice in which CD133 negative cells had been injected, and right panels correspond to injections of CD133 positive sorted cells. Tumours from injection of positive sorted cells show higher numbers of BCRP1 protein staining than tumours from negative cells injected, $p<0.01$, as is show in the Figure **3b**. In the same way as BCRP1 analysis, the tumours formed from double positive cells, show higher expression of CD133 protein by immunocytochemistry, and also higher numbers mitotic cells and small nucleus cells than tumours from injections of negative cells Figure **3c**.

BCRP1 Positive Cells and Chemoresistance

This BCRP1 and CD133 positive cells seem to be important in the tumorogenic process, and the study of its cellular-division control has been a central objective in this paper. As we have pointed, PEDF and the carboxyl-terminal part of PEDF (c-ter), have been related with stem cells self renewal [32]. To inhibit self renewal capacity of those BCRP1 and CD133 positive cells, c-ter molecule was added in culture to C6 glioma cell line for four to seven passes in culture. To study chemoresistance, a dose-response assay with growing chemotherapy concentrations was carried out. The results were analysed by MTT viability assay. Figure **4** shows cell survival rate and IC50 in a dose/response assay (solid line). In our hands, the IC50 of C6 glioma cells have a chemotoxicity around 2nM docetaxel and they are very resistant to irinotecan and temozolamide, touching the IC50 with 20 nM concentration of both drugs. By adding c-ter, the IC50 is halved in these assays (dots line). In Figure **4A** it is shown how IC50 of C6 cell line with temozolamide and c-ter treatment is 7.5nM and IC50 for irinotecan treatment adding c-ter is 5nM, Figure **4B**. In the case of docetaxel treatment combined with c-ter, IC50 decrees three times to 0.6nM

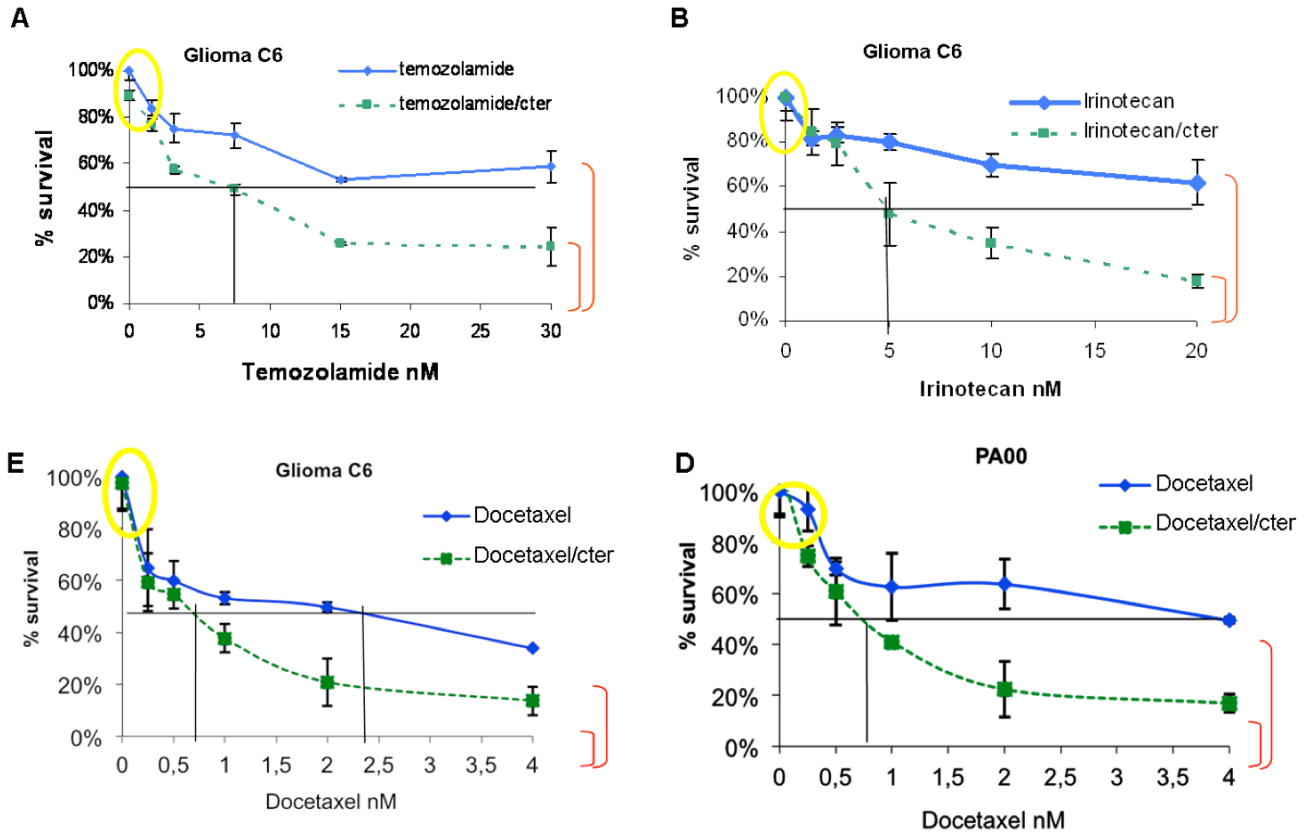

Figure 4: Chemotherapy dose-responses curves. C6 glioma cell line are shown in **A-C** and PA00 patient derived cell line in **D**, incubated for 72 hours in DMEM-F12 with 10% FBS containing the indicated amounts of chemotoxic (temozolamide, irinotecan or docetaxel as marked in the figure). **A:** Survival assay with MTT for Glioma C6 cells treated with temozolamide (solid line) or temozolamide plus c-ter (dots line). The IC50 with c-ter treatment decrees more than four times versus control assay. Resistant population at the maximum physiologic drug concentration is also reduced with c-ter treatment (brackets in the right part of the graphic). **B:** Idem **A** with irinotecan treatment. The IC50 of C6 glioma cells exposed to irinotecan decrease with c-ter more than four times. Resistant population at the maximum physiologic drug concentration is also reduced with c-ter treatment (brackets in the right part of the graphic). **C:** Idem **A** with docetaxel treatment. The IC50 of C6 glioma cells exposed to docetaxel decrease with c-ter more than three times. Resistant population at the maximum physiologic drug concentration is also reduced with c-ter treatment (brackets in the right part of the graphic). **D**: Idem **A** with docetaxel treatment in PA00 metastatic patient cells. The IC50 of PA00 cells exposed to docetaxel decrease with c-ter more than five times. Resistant population at the maximum physiologic drug concentration is also reduced with c-ter treatment (brackets in the right part of the graphic).

in C6 glioma cells, Figure **4C**, and more than five times in the case of the resistant cells from a metastatic patients as PA00 (from 4nM to 0.75nM adding c-ter) Figure **4C**. It is important to note that the remaining population, resistant to chemotherapy, is also reduced (brackets in the right part of the graphic). The addition of the c-ter molecule is not toxic for the culturing of these cells because in absence of chemotherapy the viability percentage is the same as in the control experiment (circle in y-axis).

As CD133 positive cells were separated from negative cells in Figure **2**, sorting assays were also performed for the BCRP1 protein, but this time a fluorescent antibody was used on a cell-sorting cytometer. BCRP1 positive and negative cells were cultured under the same conditions and cell density (5.000 cells in a 96 well with 100ul of DMEM plus 10% FBS). After sorting and culturing, the cells were used in a dose-response assay at an escalating chemotherapy concentration. The results were analysed by MTT viability assay as previously described. Figure **5A** shows a sorting experiment after chemotherapy treatment in separated C6 glioma cells. The bulk of BCRP1 negative cells (squares Figure **5A**) exhibit the same behaviour as that of the entire cell line (diamonds Figure **5A**), with a similar docetaxel IC50 between 2,5nM to 3nM. With the exception that negative BCRP1 cells have survival problems even without chemotherapy show by the 80% of culture survival without chemotherapy. On the other hand, BCRP1 positive cells demonstrate their resistance to chemotherapy treatment (triangles Figure **5A**).

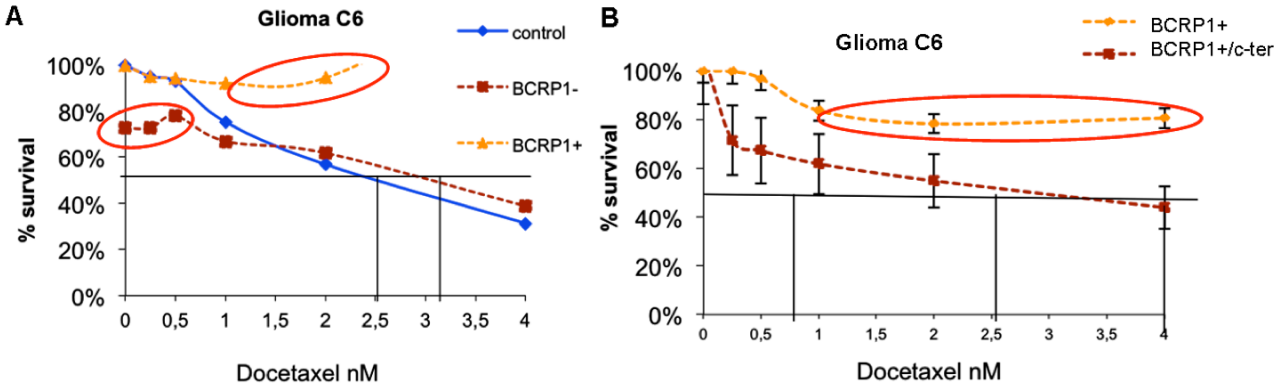

Figure 5: Dose-response assay with MTT for C6 glioma cells treated with docetaxel, total population and BCRP1$^-$ and BCRP1$^+$ cells after sorting. Note the high resistance rate show by the BCRP1+ cells (triangles **A**). Control cells and BCRP1 negative cells have a similar behavior with close IC50, but note that BCRP1 population have less viability even in control condition without chemotherapy (circle in point 0 x-axis). **B:** Chemotherapy dose-response curves of control (solid line) and c-ter treatment (dots line) on BCRP1 positive C6 glioma cells resistant to chemotherapy. C-ter treatment is able to decrease the IC50 of these resistant cells close to IC50 of BCRP1 negative cells or control population (both in **A**). And also the final resistant population BCRP1 positive at the maximum physiologic drug concentration used, is 50% less abundant (**B**). For statistical analysis U Mann Winey was used. At least three independent experiment have been performed every assay. Data are shown by means±SE of survival determinations.

In Figure **5B** we show the results of BCRP1 positive cells after dose-response assays in the presence and absence of the c-ter self-renewal regulator fragment. C6 glioma cells expressing BCRP1 trans-membrane protein were selected by cell sorting assay and culture in the same conditions described. c-ter treatment in combination with docetaxel chemotherapy was applied to BCRP1 positive population (black squares Figure **5B**). The c-ter protein fragment is able to induce a decrease in the resistance of this population, getting a IC50 close to that in de negative population (Figure **5A**), around 3nM, and a reduction in the remaining percentage of chemoresistant cells after chemotherapy (gray diamonds Figure **5B**) treatment about 50%, as is shown in Figure **5B**.

In sort, Figure **6** is a scheme shows the postulate hypothesis with the PEDF and c-ter effect over CSCs self-renewal and chemoresitance. PEDF protein increase self renewal and chemoresistance properties in CSCs, as in normal NSCs. C-ter function could be related to exit from quiescence and decreasing self renewal capacity; this signalling allow cells to accelerate the kinetic division becoming more sensible to chemotherapy. However, a short remaining population is still at the cultures after c-ter treatment, as is show in the four histograms in Figures **4** and **5**, at maxim physiologic doses for the different drug tested. So c-ter is not able to eliminate the full CSC population.

DISCUSION

Brain tumours account for 189,000 new cases and 142,000 deaths annually (1.7% of new cancers, 2.1% of cancer deaths). Glioma is the most common primary neoplasm of the central nervous system (CNS), accounting for over 40% of all such tumours, and 78% of CNS malignant tumours in adults. Almost without exception, virtually all patients with malignant brain tumours eventually develop local recurrence, despite aggressive and multimodal treatment (surgery, radiotherapy and chemotherapy) [33]. CSCs are thought to constitute a small subset of cells within a tumour that sets off both the primary disease and its recurrence [34]. This fate is thought to be due to their capacity for self-renewal and inherent chemo- and radio-resistance. CSCs have been identified in a growing number of hematopoietic and solid tissue malignancies, and are typically recognized by the expression of cell surface markers and their slow cell cycle which confers them the capacity to retain labelling in culture. Since persistence and tumour relapse after treatment is the real problem in cancer treatment, we sought to study the self-renewal and chemoresistance properties of CSCs [1].

The origin of tumours can be diverse, with several controversial hypotheses being currently debated [35]. Nevertheless, it has been proposed that those cells are possibly responsible for maintaining the cultures *in vitro* and the cells that cause disease relapse in patients. Different mechanisms are involved in this especial behaviour. The presence of drug transporter proteins, such as BCRP1 in this type of cells, could help toxic expulsion and confer chemotherapy resistance [36]. It is also postulated that while the bulk of tumour cells, after detecting flaws in the DNA structure, is not able to

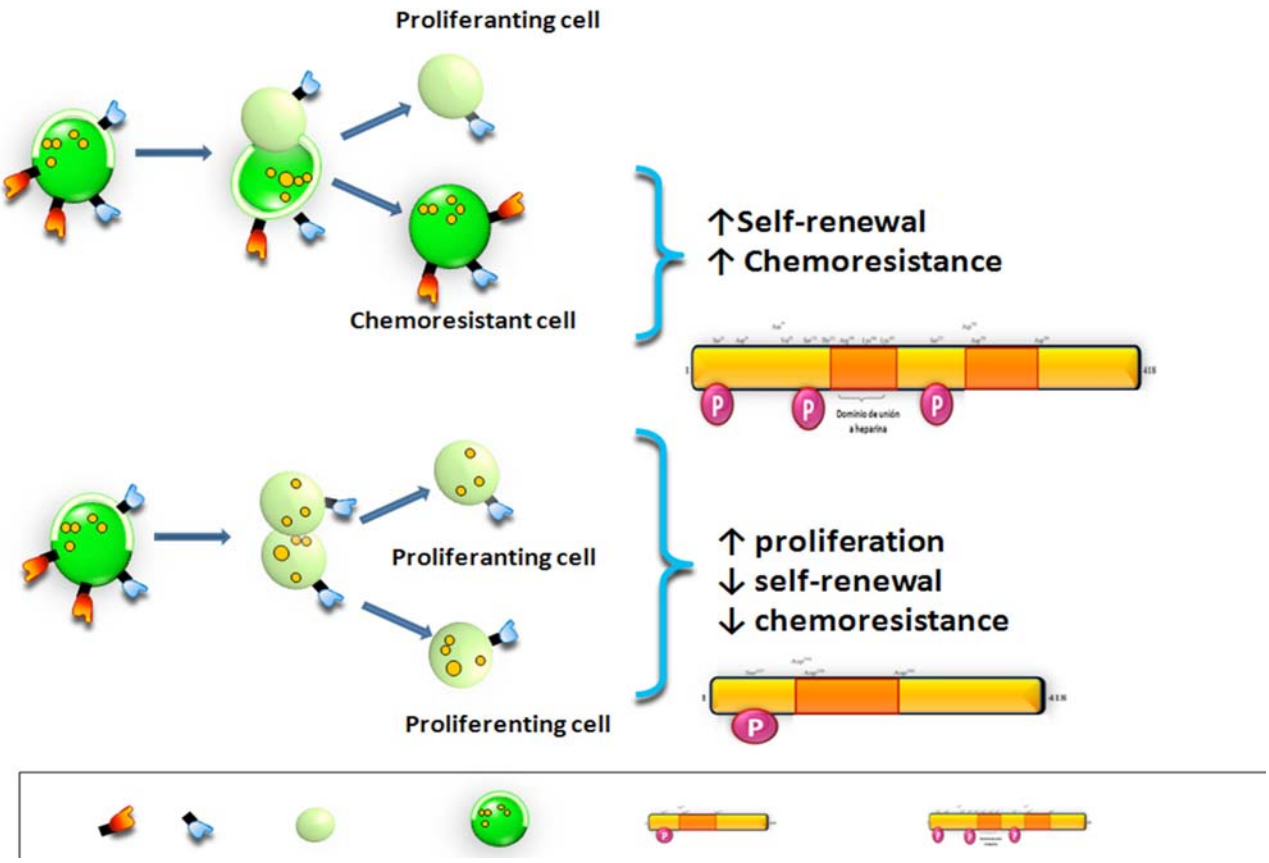

Figure 6: Scheme of the hypothesis obtained. PEDF C-terminal decreases chemoresistance by inducing CSC symmetrical cell division, and thus depleting the CSC population. CSCs divide in an asymmetrical manner, thus regenerating the CSCs pool. PEDF, which has been shown to increase neural stem cell self-renewal, could induce asymmetrical division in CSCs and therefore increase tumor resistance of the cell line. On the other hand, PEDF C-terminal domain could decrease chemoresistance by depleting the CSC population because of abolition of the CSC self-renewal PEDF pathway. However, other receptors of PEDF or pathways must be involved, because a resistant population of cells remains in the cultures even with chronic treatments.

pass the cell cycle checkpoint and undergo apoptosis, quiescent CSCs could in fact activate DNA reparation systems and withstand the chemotherapy effect [37, 38]. Finally, asymmetric divisions enable the self-renewal capacity, because the asymmetric division gives rise to two cells with different phenotypic determinants. One of the cells is exactly as the original cell and the second cell is one step more committed to their final phenotype, which rise to all the tumour lineages [34]. These three characteristics: self-renewal, drug exclusion and chemoresistance, would largely justify tumour relapse after treatment.

In our study, we have tried to address all these characteristics in tumour cell lines such as glioma C6 and patient cell samples. In addition, cells that express BCRP1 protein in their surface display the advantage of being able to expel certain toxic substances effectively, which confer chemotherapeutic resistance characteristics to the cells. We have found this membrane protein in both glioma C6 cells Figure **1**, and also in patient's samples (supplementary figure Fig 1s). Cells with sporadic cell cycles, which stay most of the time in quiescence state, retain DFFDA staining on *in vitro* assays (Figure **2**) and correlate with the expression of CD133 protein. These cells could have a greater likelihood to repair DNA damage and cell systems, and escape the apoptosis process. In our study, BCRP1 protein expression also correlates with CD133 protein expression. This surface marker, which is related to self-renewal, is co-expressed in the BCRP1 positive population Figure **3a**. The confirmation of which of these two types of markers appear in coincident populations is controversial [18]. Moreover, it is possible that the state of the culture, the degree of inhibition by contact and the cell density of the plate could be influencing factors, including those that arise from the own acidification of culture media. Without being able to control all these factors *in vitro*, it is not possible to definitely conclude whether the population of cells that express BCRP1 retain DFFDA staining and express self-renewal proteins. In fact, our results show

that double labelling BCRP1/CD133 tumour cells produce tumours of bigger volume than double negative cells Figure **3**. These tumours also have higher numbers of BCRP1, CD133 and condensed nucleus cells, which are specific characteristics to more aggressive, resistant and invasive tumours. Nevertheless, the production of tumours is not specific of this BCRP1/CD133 positive cell population. In SCID mice model, positive and negative BCRP1/CD133 cells produce tumours, but with different time progression and aggressive phenotype. Further studies will be needed to confirm the different aggressive phenotypes of these two types of tumours.

CD133 marker, whose function still remains unknown, has been extensively associated with the self-renewal of stem cells, both in neoplasic systems [9] as well as in the nervous [39] and the haematopoietic systems [40]. In addition, its function seems to be related with a process of protein glycosilation, although this has not yet been fully studied [41]. An antibody that recognizes a glycosilation-dependent CD133 epitope (AC133) has been described [42]. In this study, the authors have used this antibody to discern its function and level of expression in the population of tumour stem cells and other cell types of the tumour. CD133 positive cells have a subpopulation of AC133 positive cells which have been selected in this study. This effect could be also a factor meriting further research; more even so if the $CD133^{+}/AC133^{-}$ cell population is responsible for tumour formation in the control mice experiment.

The cancer stem cell hypothesis, which is the subject of this work, proposes the existence of cells with self-renewal capacity and resistance to drugs, resembling those of somatic stem cells and responsible of the neoplasic relapse. The regulation of self-renewal in the cancer stem cell could be the way to avoid patient relapse. The carboxyl-fragment of the PEDF protein (c-ter) has been related to the inhibition process of self-renewal in neural stem cells (NSCs) [32, 43]. This capacity, which has been equally demonstrated *in vivo* as well as *in vitro* assays, decreases the cell population that has the ability to form neurospheres in culture [32]. The cell cultures, we are working on, contain cells with CSCs properties that are modified after treatment with the c-ter truncated protein regulating self-renewal and chemoresistance of CSCs. Our results show that this self-renewal effect of c-ter could also be present in CSCs as it is in NSCs. The assays have been performed not only in tumour cell lines, but also in tumour cells derived from patient samples (Figure **4D**). Decreased resistance to chemotherapy treatment is observed after c-ter addition Figures **4** and **5B**. This decreased resistance, represented by the IC50 decrease, could be due to the loss of the cancer stem cell population as a result of the effect of the c-ter down-regulation of CSCs self renewal. c-ter domain of PEDF would induce symmetrical divisions Figure **6**, giving rise to two tumour cells and losing its self-renewal capacity. This loss of self-renewal capacity would produce tumour cells with a faster CSCs division cycle. The resulting loss of CSCs quiescence and the acquisition of fast cell cycles could allow improved performance of chemotherapeutic drugs, making cells go into apoptosis after treatment. The c-ter protein has also been involved in the decrease observed in the remaining chemoresistant cell population, even in cells with a slow cell division cycle and expression of resistance markers such as BCRP1, as is shown in Figure **5B**. BCRP1 positive cells are chemoresistant, but after c-ter protein treatment a decrease in the IC50 of BCRP1 positive cells is also observed in Figure **5B**. This phenomenon could be due to the decrease of the CSCs population by the inhibition effect caused by c-ter on self-renewal [32]. Those results are in agreement with the hypothesis that the c-ter protein is a good candidate molecule for the application of new therapies. PEDF carboxyl terminal domain is a secreted protein that does not need to be mobilized towards the outside of the cell. This protein can be purified from standard and accessible assays, which would facilitate its use as a therapeutic drug. However, a resistant cell population remains after c-ter and chemotherapy treatments, and this population is able to produce tumours. Even if we have got to decrease the IC50 of several chemotherapies treatments, the CSCs are not completely eliminates with this c-ter molecule. With the data of the PEDF full protein functionality and the different potential receptors [27, 28] for PEDF could be possible that other fragments of the PEDF protein will be also able to produce the desired effect on CSCs inhibition. Another issue that remains unsolved is whether the phosphorylable serine of the C-terminal PEDF domain plays a role on protein function [44, 45].

On the other hand, all these results on CSCs self-renewal inhibition are in agreement with the results obtained with the truncated and completed PEDF molecule in normal neural stem cells. This protein, reported to be involved in the self-renewal process of neural stem cells and which has recently been associated to Notch-induced self renewal [46], is able

to rescue the NSCs population. So, the study of the differences between stem cells and cancer stem cell self renewal in a big deal steal not solved.

At present it is not known whether expression of CD133 and BCRP proteins is related with PEDF self-renewal regulator and its c-terminal fragment; whether they are cause-effect, or whether a direct relationship exists between their signalling pathways. The effort to characterise new markers of CSC population is necessary to delimit this population responsible of the cancer relapse.

Also unknown are the signalling pathways through which the PEDF and c-ter molecules exert their effect. But it seems clear that a relationship between cancer stem cell, tumours, self-renewal and chemotherapy efficiency definitely exists in terms of tumour relapse, and probably in terms of self-renewal regulation factors. c-ter molecule could be a new target for future anti-neoplasic therapies aimed at cancer stem cell elimination.

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

SUPPLEMENTARY DATA

The supplementary Figures can be downloaded from the journal website with the article.

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