Pancreatic ductal adenocarcinoma cell lines display a plastic ability to bi-directionally convert into cancer stem cells

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Abstract. Pancreatic ductal adenocarcinoma (PDAC) is often diagnosed when metastatic events have occurred. Cancer stem cells (CSCs) play an important role in tumor initiation, metastasis, chemoresistance and relapse. A growing number of studies have suggested that CSCs exist in a dynamic equilibrium with more differentiated cancer cells via a bi-directional regeneration that is dependent on the environmental stimuli. In this investigation, we obtain, by using a selective medium, PDAC CSCs from five out of nine PDAC cell lines, endowed with different tumorsphere-forming ability. PDAC CSCs were generally more resistant to the action of five anticancer drugs than parental cell lines and were characterized by an increased expression of EpCAM and CD44v6, typical stem cell surface markers, and a decreased expression of E-cadherin, the main marker of the epithelial state. PDAC CSCs were able to re-differentiate into parental cells once cultured in parental growth condition, as demonstrated by re-acquisition of the epithelial morphology, the decreased expression levels of EpCAM and CD44v6 and the increased sensitivity to anticancer drugs. Finally, PDAC CSCs injected into nude mice developed a larger subcutaneous tumor mass and showed a higher metastatic activity compared to parental cells. The present study demonstrates the ability to obtain CSCs from several PDAC cell lines and that these cells are differentially resistant to various anticancer agents. This variability renders them a model of great importance to deeply understand pancreatic adenocarcinoma biology, to discover new biomarkers and to screen new therapeutic compounds.

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a highly lethal disease characterized by early spread to local and distant organs, and most patients having an unresectable tumor at the time of diagnosis (1). Even for patients who initially present with localized disease and undergo curative surgery, the median survival is only 18 months (2). Although the improved understanding of pancreatic cancer biology and genetics, no significant advances in treatment have been realized in >10 years (2).

Recently, gene expression analyses of highly aggressive tumors have shown a compelling overlap of their gene expression profiles with those of normal stem cells (3). This observation strongly supports the relevance of cancer stem cell (CSC) isolation, first described in the early 1990s in hematologic malignancies (4). Since then, CSCs have been identified in a growing number of malignancies, including pancreatic adenocarcinoma (5,6). The unique functional properties of CSCs, such as self-renewal, anchorage-independent growth, long-term proliferative capacity, and chemotherapy resistance, suggest that they play an important role in tumor relapse. Their phenotype is generally associated with epithelial-to-mesenchymal transition (EMT) in which epithelial cells lose their characteristics, acquiring stem cell-like features. Recent data have demonstrated that, in a mouse model, pancreatic CSCs circulate in the blood before tumor growth and lead to metastasis formation (7). Furthermore, a growing number of studies have demonstrated an association between the presence of CSCs and worse clinical outcomes (8,9). Therefore, the identification of the specific molecular features of pancreatic CSCs is of crucial relevance for the development of more efficient therapies and for the discovery of specific markers.

The high level of heterogeneity of CSCs, which originates from genotypic and phenotypic plasticity, and their

low presence in cancer sample tissues, make their isolation and identification a strong limitation for the realization of biochemical studies. Thus, in order to obtain valid and reproducible results, the biochemical approach to study CSC pathophysiology can take advantage from the observation that CSCs can be isolated and enriched from several human cancer cell lines (5,10-12), in agreement with the recently proposed concept of CSC plasticity. Indeed, it has been shown that CSCs and non-CSCs exist in the tumor in a dynamic equilibrium and that both types of cells are capable to interconvert in response to environmental cues (3,8,13,14). Therefore, identification of factors responsible for bi-directional conversion of tumor cells will be of fundamental importance not only to target CSCs but also to prevent CSC generation from non-CSCs.

The present study was conducted to achieve the following three primary goals: i) to test the capacity of several PDAC cell lines to generate CSCs in selective medium; ii) to characterize biological features of the obtained CSCs, both *in vitro* and *in vivo*; and iii) to analyse CSCs for their sensitivity to different chemotherapeutic drugs in order to identify various CSC models for biochemical drug resistance studies. Interestingly, only five out of nine PDAC cell lines were able to form tumorspheres in selective medium and each of them showed a distinctive pattern of sensitivity to drugs. These findings suggest that the potentiality of PDAC cell lines to dedifferentiate depends on the origin of the cell line and that each cell line dedifferentiates into cells with a different phenotype and expression profile that determine the differential drug sensitivity.

Materials and methods

Drugs and chemicals. Gemcitabine (Jemta; Sandoz) and zoledronic acid (Zometa; Novartis) were solubilized in water, sorafenib (BAY43-9006; Bayer AG), tipifarnib (Zarnestra; Johnson & Johnson), and everolimus (RAD001; Novartis) were solubilized in DMSO. Gemcitabine was stored at -80˚C, zoledronic acid, everolimus and tipifarnib at -20˚C, sorafenib at room temperature until use.

Cell lines. The human cell lines PaCa44, HPAF-II, PT45P1, CFPAC1, PSN1, PC1J, PaCa3, Panc1, MiaPaCa2 (pancreatic adenocarcinoma cell lines), and VIT-1 (normal primary pancreatic mesenchymal cells) were grown in RPMI-1640 supplemented with 10% FBS, 2 mM glutamine, and 50 μg/ml gentamicin sulfate (Gibco/Life Technologies). Adherent cells were maintained in standard conditions for a few passages at 37˚C with 5% CO_2. To generate suspension cells and separate stem-like sphere-growing cells, adherent cells were washed twice in 1X PBS (Gibco/Life Technologies) and then cultured in CSC medium, i.e., DMEM/F-12 without glucose (US Biological Life Sciences) supplemented with 1 g/l glucose, B27, 1 μg/ml fungizone, 1% penicillin/streptomycin (all from Gibco/Life Technologies), 5 μg/ml heparin (Sigma), 20 ng/ml epidermal growth factor (EGF), and 20 ng/ml fibroblast growth factor (FGF) (both from PeproTech). Adherent cells were left in CSC medium for at least 1-3 weeks or until the appearance of floating cell aggregates, referred to as tumorspheres. Tumorspheres were cultured in CSC medium for at least three passages before initiating the characterization experiments.

Tumorsphere formation assay/vitality assay. PSN1, PC1J, PaCa3, Panc1, and MiaPaCa2 CSCs were plated in 96-well cell culture plates ($3x10^3$ cells/well) and incubated at 37˚C with 5% CO_2 in CSC medium. Tumorspheres were counted after 5 days.

PSN1, PC1J, PaCa3, Panc1, and MiaPaCa2 parental cell lines, CSCs, and ex-CSCs were plated in 96-well cell culture plates ($5x10^3$ cells/well) and incubated at 37˚C with 5% CO_2. After 24 h the cells were treated with increasing dose of five drugs (gemcitabine, zoledronic acid, sorafenib, tipifarnib, and everolimus). After 72 h, cell viability was measured by Cell Proliferation Reagent WST-1 (Roche Diagnostics).

For proliferation cell assay, Panc1 parental cell line and CSCs were plated in 96-well cell culture plates ($5x10^3$ cells/well) and incubated at 37˚C with 5% CO_2. Viable cells were counted by Trypan Blue dye exclusion after 1, 2, 3, 4, and 7 days. The doubling time was calculated using the formula $T = (T_2-T_1) \times \log 2/\log (Q_2/Q_1)$, where: T_1, day 3; T_2, day 7; Q_1, cell number at day 3; and Q_2, cell number at day 7. For CSCs, this experiment was performed using CSC medium containing 1 or 3 g/l glucose and similar results were obtained.

Flow cytometry analysis. Panc1 parental cell line and CSCs were harvested, washed, resuspended in 1X PBS and stained for 30 min on ice with FITC-conjugated monoclonal anti-CD326 (anti-EpCAM, no. 130-098-113), PE-conjugated monoclonal anti-CD133/2 (no. 130-090-853) (both from Miltenyi Biotech), PE-Cy7-conjugated polyclonal anti-CD66c (no. bs-6032R; Bioss, Inc.), APC-H7-conjugated monoclonal anti-CD44 (no. 560532; BD Biosciences), FITC-conjugated monoclonal anti-CD44v6 (no. ab30437; Abcam), and PE-Cy7-conjugated monoclonal anti-CD24 (no. 311119; BioLegend). Isotype-matched irrelevant antibodies were used as negative controls.

Approximately 20,000 gated events were acquired for each sample on a BD FACSCanto (BD Biosciences) and analyzed using FlowJo software (TreeStar, Inc.). Dead cells and debris were excluded based upon forward scatter and side scatter measurements.

RNA extraction and qPCR. Total RNA was extracted from 10^6 cells using TRIzol Reagent (Life Technologies) and 1 μg of RNA was reverse transcribed using first-strand cDNA synthesis. Real-time quantification was performed in triplicate samples by SYBR-Green detection chemistry with Power SYBR-Green PCR Master Mix (Applied Biosystems) on a 7000 Sequence Detection System. The primers used were: E-cadherin forward, 5'-GAC ACC AAC GAT AAT CCT CCG A-3' and reverse, 5'-GGC ACC TGA CCC TTG TAC GT-3'; ribosomal protein large P0 (RPLP0) forward, 5'-ACA TGT TGC TGG CCA ATA AGG T-3' and reverse, 5'-CCT AAA GCC TGG AAA AAG GAG G-3'.

The following cycling conditions were used: 95˚C for 10 min, 40 cycles at 95˚C for 15 sec, 60˚C for 1 min, 95˚C for 15 sec, and 60˚C for 15 sec. The average of cycle threshold of each triplicate was analyzed according to the $2^{-\Delta\Delta Ct}$ method.

Immunoblot analysis. Cells were collected, washed in 1X PBS, and resuspended in RIPA buffer, pH 8.0 (150 mM NaCl, pH 8.0; 50 mM Tris-HCl; 1% Igepal; 0.5% Na-Doc; and 0.1% SDS), 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF, 2.5 mM

EDTA, and 1X protease inhibitor cocktail (Calbiochem; Merck Millipore) for 30 min on ice. The lysate was centrifuged at 2,300 x g for 10 min at 4˚C and the supernatant was used for western blot analysis. Protein concentration was measured with the Bradford Protein Assay Reagent (Thermo Fisher Scientific) using bovine serum albumin as a standard. Thirty micrograms of protein extracts were electrophoresed through a 10% SDS-polyacrylamide gel and electroblotted onto PVDF membranes (Merck Millipore). Membranes were then incubated for 1 h at room temperature with blocking solution, i.e., 3% low-fat milk in TBST (100 mM Tris, pH 7.5, 0.9% NaCl, and 0.1% Tween-20), and probed overnight at 4˚C with the monoclonal rabbit E-cadherin primary antibody (1:20,000 in blocking solution, no. ab40772; Abcam). Horseradish peroxidase conjugated IgG polyclonal (1:8,000 in blocking solution, no. 12348; Merck Millipore) was used to detect specific proteins. Immunodetection was carried out using chemiluminescent HRP substrates (Merck Millipore) and recorded using an Amersham Hyperfilm ECL (GE Healthcare). To quantify E-cadherin expression, bands were scanned as digital peaks and the areas of the peaks were calculated in arbitrary units using the public domain NIH Image software (http://rsb.info.nih.gov/nihimage/), normalized with Ponceau S, and reported as fold induction relative to controls.

Subcutaneous in vivo model. Panc1 parental cell line and CSCs at three different dilutions ($1x10^4$, $1x10^5$ and $1x10^6$ cells/mice) were s.c. injected into the dorsal flank of five nude female mice for each condition (5 weeks of age; Charles River Laboratories, Inc.). For the control groups, mice received 100 μl injections of 1X PBS. Body mass was recorded weekly for each animal. Tumor size was monitored weekly in two perpendicular dimensions parallel to the surface of the mouse using a caliper. Tumor volume was calculated using the formula of $V = \pi/6$ x [(w x L)^(3/2)]. Animals were sacrificed at the volume of 2 cm^3. Immediately after death, neoplastic masses were collected for flow cytometry and histological assessment. To perform flow cytometry analysis, tumor masses were dissociated enzymatically and mechanically (MACS Dissociation kit; Miltenyi Biotech) and $5x10^5$ of the obtained cells were resuspended in 100 μl of RPMI without phenol red (Gibco/Life Techonologies) and then analyzed with 7-AAD (BD Biosciences) and with the antibodies described in flow cytometry analysis section. To perform histological analysis, tissue samples not used in the cytometry analysis were fixed in 10% (v/v) neutral-buffered formalin for 24-48 h and were processed routinely. Serial histological sections (4-6 μm thick) were obtained from each paraffin block and stained with hematoxylin and eosin (H&E) for histology assessment. Animal studies were approved by the Verona University Review Board.

Metastasis in vivo model and optical imaging (OI) acquisition. Mice were randomly allocated into the two groups (n=5 mice/group) and were anesthetized with 1.5% isofluorane-air mixture. As previously described (15), a small left abdominal flank incision was created, and the spleen was carefully exteriorized. MiaPaCa2-RFP ($1x10^6$) (expressing the red fluorescence protein) parental cells and CSCs were inoculated into the spleen with a 30-gauge needle. After 5 min, the spleen was removed using a high-temperature cautery (Aaron; Bovie Medical Corp.), in order to avoid the formation of a primary tumor. The abdominal wall was closed in one layer with wound clips. MiaPaCa2 RFP parental cells were a kind gift from Professor Turco (University of Fisciano). Optical images were performed using the IVIS Spectrum optical imager (Perkin-Elmer). The instrument is equipped with a charge-coupled device (CCD) camera cooled at -90˚C. Images were collected every 4 days, starting on the 14th day and up to the 36th day after the cancer cell injection. The image parameters were: exposure time = 1 sec, binning (B) = 8, diaphragm f/2 and field of view (FoV) = 19 cm. Four combinations of excitation/emission filters were used: 535 nm/580 nm, 535 nm/600 nm, 535 nm/620 nm and 570 nm/620 nm. The last combination showed the best signal to background ratio, thus the reported results refer to this modality. The measures were done on regions of interest (ROIs) traced on the optical images. During the acquisitions the animals were anaesthetized with 1.5% isofluorane-air mixture and placed on the heated stage of the optical device. Animal studies were approved by the Verona University Review Board.

Statistical analysis. ANOVA (post hoc Bonferroni) analysis was performed by GraphPad Prism 5 (GraphPad Software, Inc.). P-values <0.05, 0.01, or 0.001 show significant difference.

Results

The ability of PDAC cell lines to form tumorspheres and to reconvert to the adherent phenotype. In order to evaluate whether PDAC cell lines were able to dedifferentiate into cancer stem-like cells, we cultured nine PDAC cell lines (PaCa44, HPAF-II, PT45P1, CFPAC1, PSN1, PC1J, PaCa3, Panc1, MiaPaCa2), and one normal primary pancreatic mesenchymal cell line (VIT-1) in a selective medium containing EGF, FGF, and low glucose (1 g/l). Only five of them, all of tumoral origin (PSN1, PC1J, PaCa3, Panc1, MiaPaCa2), lost their characteristic epithelial morphology and were able to form tumorspheres after 1-3 weeks of culturing (Fig. 1A), preserving the undifferentiated state throughout numerous cycles of cell division. The CFPAC1 cell line was able to form tumorspheres, which however died after the first cell division. The remaining cell lines maintained their epithelial morphology or died, even when they were grown in diverse media, used to obtain CSCs from other tumor types. In order to test the *in vitro* capacity to form spheres, we counted the number of tumorspheres of PSN1, PC1J, PaCa3, Panc1 and MiaPaCa2 after 5 days of incubation (Fig. 1B). Interestingly, Panc1 cancer stem-like cells showed a 2-fold higher tumorsphere-forming ability relative to the least active PC1J cancer stem-like cells. As expected, parental cell lines grown in RPMI-1640 supplemented with FBS did not show any tumorsphere-forming ability (data not shown). PDAC cancer stem-like cells were able to re-differentiate into adherent cells (ex-CSCs) re-acquiring epithelial morphology after 2-7 days of culturing in RPMI medium supplemented with FBS (Fig. 1A).

To evaluate the effect of the phenotypic transformation from epithelial to sphere morphology on cell growth, we measured the proliferation time of Panc1 cancer stem-like cells compared

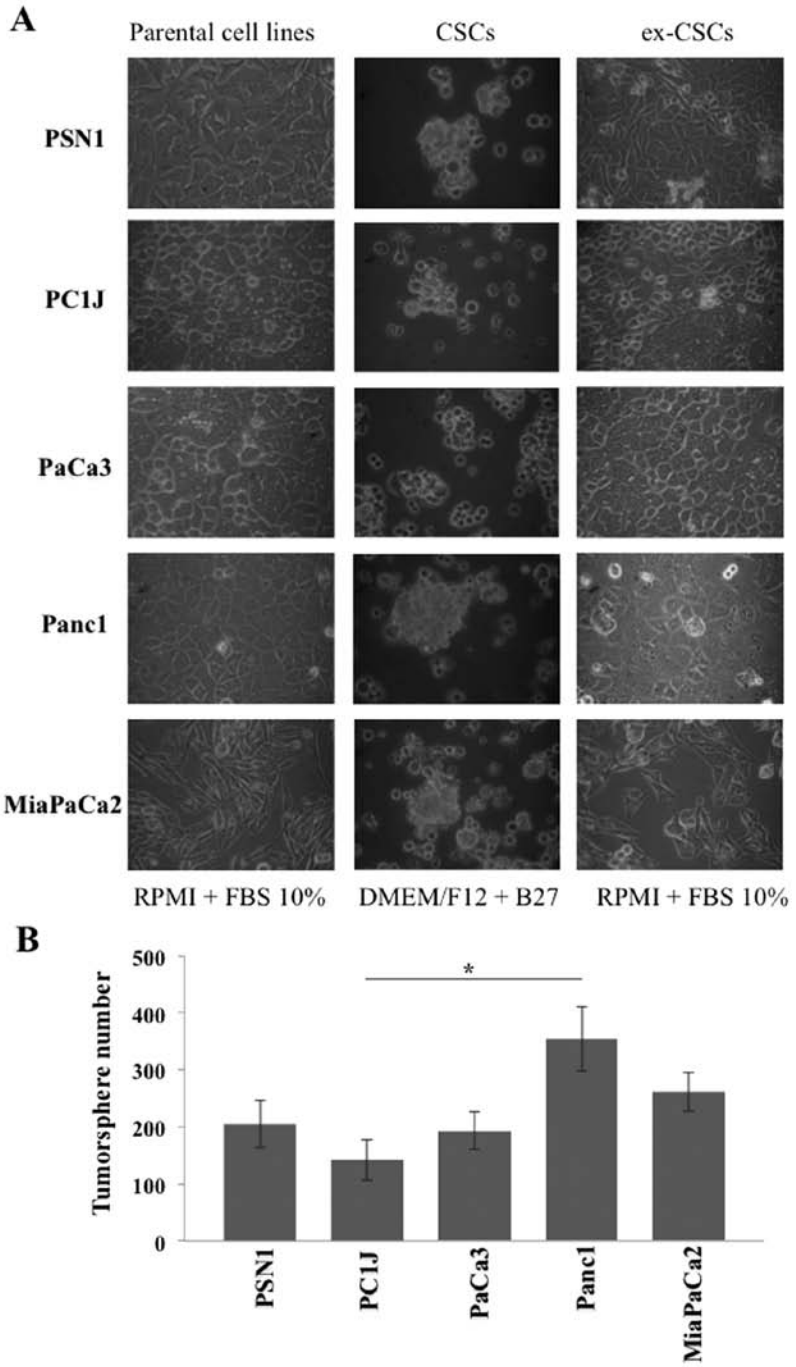


Figure 1. (A) Phase-contrast microscopy images of the indicated parental cell lines, tumorspheres [cancer stem cells (CSCs)], and reverted tumorspheres (ex-CSCs). (B) Tumorsphere formation assay of PSN1, PC1J, PaCa3, Panc1 and MiaPaCa2 CSCs. Tumorsphere number is the mean ± SEM of three independent experiments each performed 5-fold. *P<0.05.

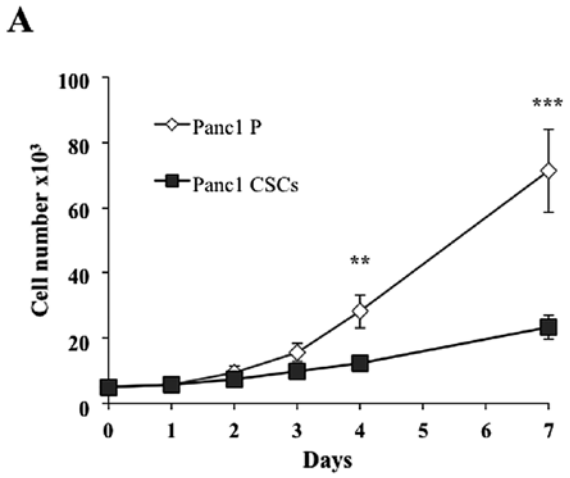
to the parental cell line and we observed that the doubling time was 3.26 and 1.83 days, respectively (Fig. 2A and B), suggesting a significant alteration of the mechanisms regulating the metabolic and cell division pathways.

Increased resistance of cancer stem-like cells to chemotherapeutic agents. Since it is known that CSCs are more resistant to chemotherapeutic treatments, to compare the chemosensitivity of the parental cell lines and the corresponding cancer stem-like cells, we evaluated the anti-proliferative activity of a panel of five anticancer compounds, gemcitabine (pyrimidine nucleoside analogue, gold standard treatment for PDAC), tipifarnib (farnesyl transferase inhibitor), sorafenib (multi-kinase inhibitor), everolimus (mTOR inhibitor), and zoledronic acid (farnesyl diphosphate synthase inhibitor). We treated PSN1, PC1J, PaCa3, MiaPaCa2, Panc1 parental cells, the corresponding cancer stem-like cells, and ex-CSCs for 72 h with the compounds and we determined the IC_{50} values, which are shown in Table I. These data indicated that cancer stem-like cells, in particular those obtained from PaCa3 and Panc1 cell lines, were more resistant to the action of the anticancer drugs than parental cell lines and that four out of

Table I. IC_{50} ± SEM values at 72 h in CSCs, parental cell lines and ex-CSCs.

IC_{50}	Gemcitabine (μM)	Tipifarnib (μM)	Sorafenib (μM)	Everolimus (μM)	Zoledronic acid (μM)
PSN1					
CSC	>2.5[a]	3.1±2.7[a]	20±3.9[a]	8.2±0.3	>500[a]
Parental cell line	1±0.13	17±2.5	2.4±0.05	7.9±1.3	395±57
ex-CSC	1.1±0.15	11.8±0.71	4.7±0.34	13.8±3.6	455±64
PC1J					
CSC	>5	16±0.6	18.9±0.55[a]	15.7±0.4	87±4.1[a]
Parental cell line	>5	20.5±3.3	10±1.2	15.7±0.3	>250
ex-CSC	>5	23±0.83	13.7±1.3	18±1.3	>250
PaCa3					
CSC	2.5±0.78	9.3±2.5[a]	>25[a]	17.9±0.9[a]	80±31
Parental cell line	3.5±0.24	1±1.5	12.6±3.1	9.6±0.8	45±26
ex-CSC	3.4±0.58	2.8±0.61	13.4±3.2	9.9±1.3	62±71
MiaPaCa2					
CSC	4.8±0.82	16.8±1.7	10.3±1.6	11.5±3.3	80±17[a]
Parental cell line	5±0.74	18.4±1.2	8.6±1.1	15.3±0.8	>500
ex-CSC	>5	16±1.3	8.2±0.95	15.8±0.26	>500
Panc1					
CSC	>5	>25[a]	24±0.8[a]	20±1.6[a]	237±97
Parental cell line	>5	17.5±0.89	8.5±0.7	11.6±1.6	250±87
ex-CSC	>5	19.7±2.7	15.7±3.0	18.8±1.7	>250

[a]P<0.05 CSCs vs. parental cell line. CSC, cancer stem cell.



B

Panc1	Doubling Time (day)
Parental cell line	1.83±0.32
CSCs	3.26±1.74

Figure 2. (A) Proliferation curve of Panc1 parental cells (P) and cancer stem cells (CSCs). Viable cells were counted by Trypan Blue dye exclusion. Values are the means ± SEM of four independent experiments. (B) Panc1 P and CSCs doubling time ± SEM was obtained from the data of (A). **P<0.01, ***P<0.001.

five cancer stem-like cells were more resistant to the action of sorafenib than parental cell lines. Furthermore, ex-CSCs showed IC_{50} values statistically comparable to those of parental cells for all the drugs.

Increased expression of EpCAM and CD44v6 on cancer stem-like cells. We characterized Panc1 cancer stem-like cells for the expression of various typical surface stem cell markers. The percentage of cells expressing EpCAM, CD44v6, CD44, CD133, CD66, and CD24 was determined in comparison to the parental cell line (Fig. 3A). As shown in Fig. 3B, EpCAM and CD44v6 expression increased 5- and 2-fold, respectively, in Panc1 cancer stem-like cells with respect to the corresponding parental cells. CD44 was expressed on parental cell line surface at high level and did not increase in cancer stem-like cells. CD133, CD66, and CD24 were expressed at low levels in both Panc1 parental and cancer stem-like cells.

In order to evaluate whether in the transition non-CSC to CSC the Panc1 cancer stem-like cells were subjected to EMT, we analyzed the expression of E-cadherin, the main marker of the epithelial state (14,16). Fig. 3C and D shows that E-cad decreased about 2-fold, both at mRNA and protein levels, in Panc1 cancer stem-like cells in comparison to the parental cell line. Taken together, all the above results strongly suggest that cells derived from PDAC cell lines by using the CSC selective medium possess CSC features, both phenotypically and genotypically.

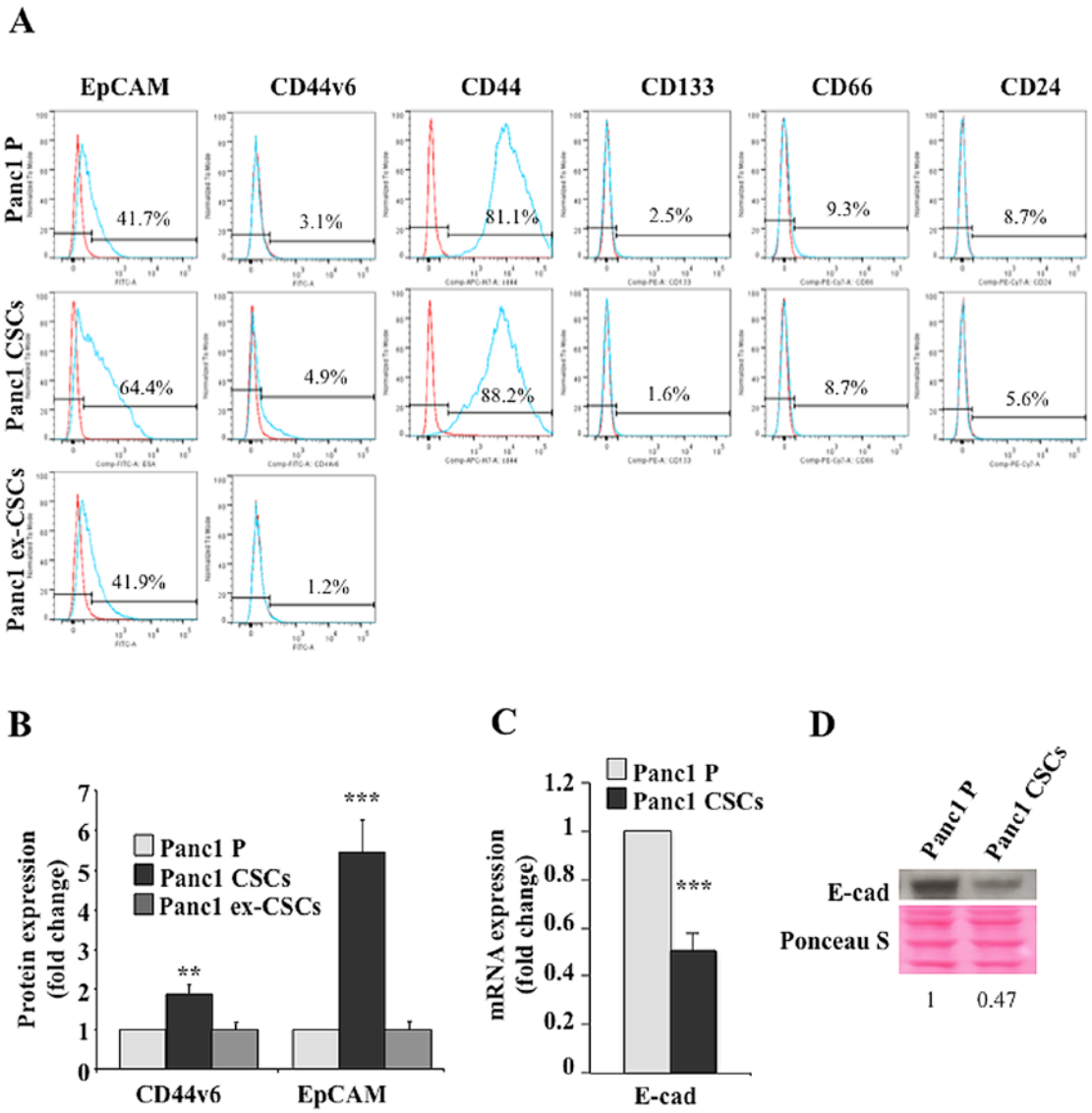


Figure 3. (A) Representative flow cytometry curves of Panc1 P, cancer stem cells (CSCs), and ex-CSCs stained with the indicated experimental antibodies (blue) or the negative-control IgG antibody (red). The percentage of positive cells is reported on the flow cytometry curves. (B) Histograms of CD44v6 and EpCAM protein expression in Panc1 P, CSCs, and ex-CSCs. Values are the means ± SEM of four independent experiments and are reported as the ratio between median fluorescence intensity (RMFI) obtained with the indicated experimental antibodies versus the negative-control IgG antibody (fold change relative to Panc1 P). (C) Histograms of E-cad mRNA expression in Panc1 P and CSCs. Real-time PCR values are the means ± SEM of three independent experiments each performed in triplicate and are reported as fold change relative to Panc1 P. (D) Western blot analysis of E-cad performed on Panc1 P and CSCs. The bands were scanned as digital peaks and the areas of the peaks were calculated in arbitrary units, as described in Materials and methods. The value of Ponceau S was used as a normalizing factor. Values are the means of three independent experiments. **P<0.01, ***P<0.001.

Re-differentiation of PDAC CSCs into adherent cells (ex-CSCs), morphologically observed in Fig. 1A, was associated with the re-establishment of the marker expression level measured in parental cells (Fig. 3A and B).

CSC tumorigenicity and stem cell marker expression in nude mice. To evaluate the tumor-initiating capabilities of pancreatic CSCs *in vivo*, Panc1 CSCs and the parental cell line ($1x10^4$, $1x10^5$ and $1x10^6$ cells/mouse) were subcutaneously injected into nude female mice and the tumor size was monitored weekly. Fig. 4A shows that Panc1 CSCs possessed an increased tumor-seeding ability compared to the parental cell line. Furthermore, $1x10^6$ Panc1 CSCs generated a larger tumor than the parental cell line (Fig. 4B) without influencing the body mass of mice (Fig. 4C). Histological assessment of tumor tissues revealed that CSC tumors were composed by a homogeneous population of cancer cells characterized by small nuclei, evident nucleoli and oncocytic cytoplasm, whereas parental cell tumors were constituted by a heterogeneous population of cells with clear/lipoblast-like features (Fig. 4D). No significant differences were observed concerning tumor necrosis or its distribution.

To evaluate whether injected CSCs maintained stem cell marker expression *in vivo*, we analyzed the expression levels of all the surface markers tested *in vitro* (Fig. 3A) on cells obtained by dissociating the tumor masses. Fig. 4E shows that EpCAM and CD44 were expressed in a higher percentage of cells dissociated from CSC tumors, in comparison to parental cell tumors. These results suggest that CSCs are more tumorigenic than parental cells and, even when subcutaneously

A

	Mice developing tumor	
Cell injected	Parental cell line	CSCs
10^4	0/5	1/5
10^5	1/5	2/5
10^6	3/5	5/5

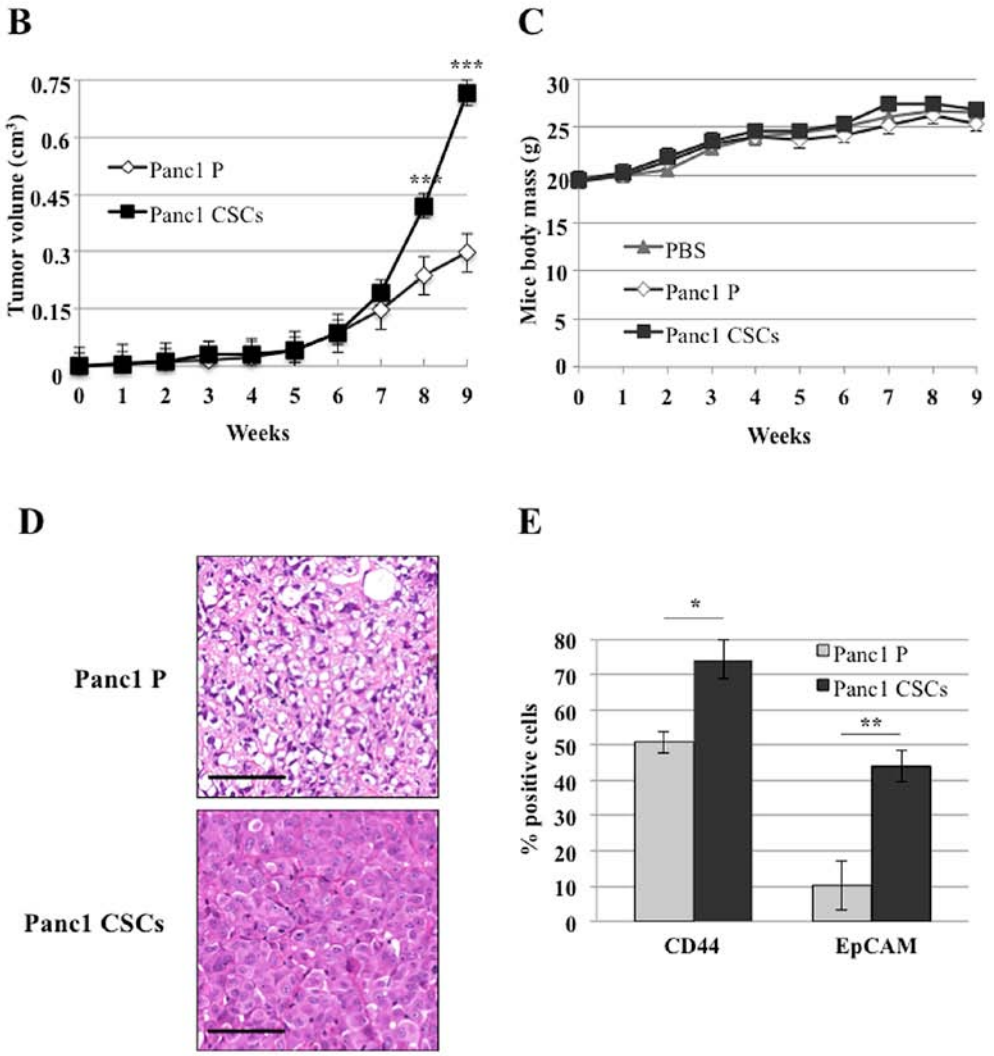


Figure 4. Parental and cancer stem cell (CSC) subcutaneous growth in nude mice. (A) The number of mice-developing tumors of total injected mice is indicated. Panc1 P or CSCs ($1x10^6$ cells/mouse) were subcutaneously injected into female nude mice. Values are the means ± SEM of (B) mouse tumor volume or (C) body mass measured every week. (D) Hematoxylin and eosin (H&E) staining of representative tumor tissues derived from Panc1 P and CSCs (scale bar, 100 μm). (E) Histograms of the percentage of positive cells for CD44 and EpCAM protein expression in dissociated masses derived from Panc1 P and CSCs. *P<0.05, **P<0.01 and ***P<0.001.

injected in mice, tend to maintain morphologic and molecular differences.

To evaluate the metastatic activity of pancreatic CSCs, we used an *in vivo* model of nude mice by injecting fluorescent cells (MiaPaCa2-RFP, as in Materials and methods) into the spleen. The growth of both parental and CSCs was evaluated and the optical images acquired at 14, 18 and 22 days after cell injection are shown in Fig. 5A. CSC metastases were well-detectable at the first time point, whereas parental metastases were detectable 4 days later. As shown in Fig. 5B, the total flux of the emitted visible light increased in both experimental groups. At all the three time points, CSC metastases showed a higher mean light emission with respect to parental metastases, but only at the 14th day the difference was statistically significant.

Discussion

Many studies have identified subpopulations of cells within tumors that drive tumor growth and recurrence, termed CSCs.

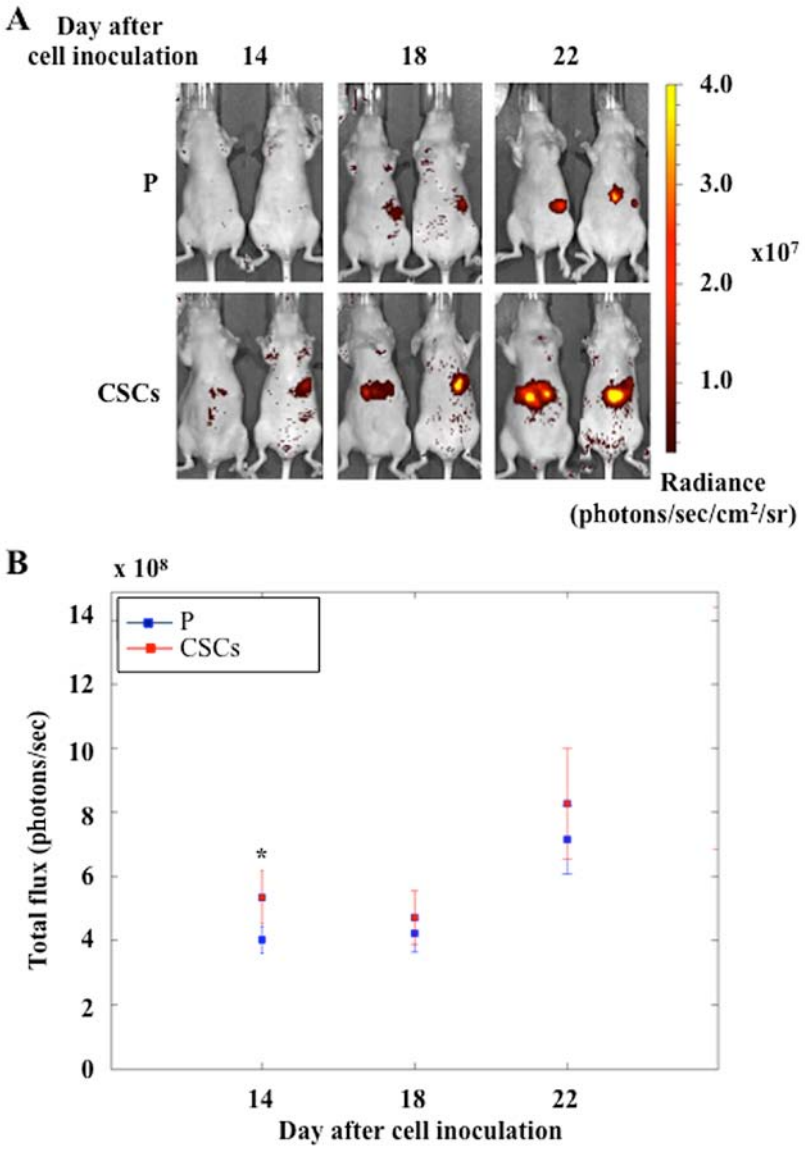


Figure 5. Parental and cancer stem cell (CSC) metastatic growth in nude mice. (A) Optical images of two representative mice injected with MiaPaCa2-RFP P and CSCs at 14, 18 and 22 days after cell inoculation into the spleen. Fluorescence images are superimposed on the photographic images in order to localize the cancer masses. False colors represent the radiance (photons/sec/cm^2/sr) of the light emitted as indicated in the color bar on the right. (B) Mean total flux ± SEM expressed in photons/sec measured in the two experimental groups. *P<0.05.

These cells are resistant to the majority of current cancer treatments, including radiation and chemotherapy, suggesting that most of the cancer therapies, while killing the bulk of tumor cells, may ultimately fail because they do not eliminate CSCs, which survive to regenerate new tumors. Consequently, the development of a reliable model of CSCs becomes crucial for basic and clinical cancer research.

Several techniques have been used to isolate CSCs from cancers (17,18). Initially, specific surface markers, previously identified in normal stem cells, were used to isolate CSCs (4,19). However, the observation that stem cell marker-negative cells were also able to grow as spheres and to give rise to very aggressively growing tumors *in vivo* did not allow reaching a consensus on the best marker to be used for the identification of CSCs in any particular cancer (20). Other methods used to isolate CSCs, such as sorting the side populations of cancer cells via intracellular Hoechst 33342 exclusion (17,21,22) or selecting the chemotherapeutic drug-resistant cells (23), yield only a small number of CSCs, which is inadequate for further biochemical experimentation. Recent studies have demonstrated that several cell lines can be enriched in spheres with stem-like features when cultured in serum-free medium supplemented with adequate growth factors (5,10-12,24).

In the current study, we first established the ability of several pancreatic cell lines to form spheres with the aim to correlate this ability to cell resistance to several chemotherapeutic agents. We found that only five out of nine PDAC cell lines had the capacity to form spheres after 1-3 weeks of culturing in a selective medium and the remaining cell lines were unable to form spheres even in other stem cell media. Panc1 cells, described as particularly resistant to gemcitabine (25,26), had the highest sphere-forming activity. Interestingly, the capacity to form spheres was independent of the nature of the tumor, whether primary or metastatic, from which each cell line originated (27). As expected, the

normal primary pancreatic mesenchymal cells, VIT-1, did not give rise to spheres. PDAC CSCs were able to regain the epithelial morphology and marker expression of parental cells after only 1 week of culturing in RPMI medium supplemented with FBS. Furthermore, Panc1 CSCs and the parental cell line had a doubling time of 3.26 and 1.83 days, respectively. These latter two findings strongly support a plastic CSC model, in which non-CSCs can re-acquire a CSC phenotype and that this bi-directional conversion is a common and essential component of tumorigenicity (3,8,13,20).

Evidence of enhanced therapeutic resistance to CSCs has been reported (17,18,28). In our study, we demonstrated that PDAC CSCs were generally more resistant than parental cells to several drugs and that ex-CSCs show sensitivity similar to parental cells. Differences among the cell types are likely ascribable to specific gene expression profiles, which will be analyzed in future studies. Notably, Panc1 CSCs further increased drug resistance compared to the parental cell line.

Several surface markers have been used to identify and isolate CSCs, including CD24 (29), CD66 (30), CD133 (31), CD44 (29), and EpCAM (29). However, none of them seems to univocally identify CSCs. Our flow cytometry data showed that EpCAM and CD44v6 expression increased in Panc1 CSCs in respect to the parental cell line, while CD133, CD66, and CD24 were lowly and CD44 was highly expressed on both cell types. This latter result correlates with the observations that CD44 plays a role in drug resistance (32,33) and that Panc1 are highly resistant to several chemotherapies (26).

When subcutaneously injected in nude mice, Panc1 CSCs developed larger tumor masses composed by a homogeneous population of cancer cells characterized by larger size compared to the heterogeneous population generated by parental cells. This result suggests a significant alteration of the mechanisms regulating the metabolic and cell division pathways. Interestingly, the tumor masses originated from CSCs and parental cells show a decreased expression of EpCAM and CD44 compared to the cells analyzed *in vitro*. This reduction was higher for the parental cells, suggesting that the *in vivo* subcutaneous environment has a higher differentiating activity on these cells. CSC metastasis obtained by injecting fluorescent cells into the spleen of nude mice showed higher mean values of light emission with respect to parental metastasis. These results further support the higher tumorigenic activity of Panc1 CSCs demonstrated in the subcutaneous mouse model experiments.

Taken together, our present study has demonstrated that the pancreatic CSCs isolated from PDAC cell lines have all the characteristics of the clinically relevant tumors. This model will be of great importance to deepen our understanding of the biology of pancreatic adenocarcinoma and will also be employed to early marker discovery and screening of compounds for therapeutic intervention.

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