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Pancreatic cancer spheres are more than just aggregates of stem marker-positive cells

Margherita GAVIRAGHI*†[1], Patrizia TUNICI†, Silvia VALENSIN†, Marco ROSSI†, Cinzia GIORDANO†, Letizia MAGNONI‡, Mario DANDREA*, Licia MONTAGNA*, Rossana RITELLI§, Aldo SCARPA*§ and Annette BAKKER†

*Department of Pathology, Section of Anatomical Pathology, Policlinico GB Rossi, University of Verona, 37134 Verona, Italy, †Department of Oncology, Siena Biotech Spa, Strada del Petriccio e Belriguardo 35, 53100 Siena, Italy, ‡Department of Molecular Informatics, Siena Biotech Spa, Strada del Petriccio e Belriguardo 35, 53100 Siena, Italy, and §ARC-NET Center for Applied Research on Cancer, 37134 Verona, Italy

Synopsis

Pancreatic cancer stem-like cells are described by membrane expression of CD24, CD44 and ESA (epithelial-specific antigen) and their capacity to grow as spheres in a serum-free medium containing well-defined growth factors. The capacity of a panel of four pancreatic cancer cell lines (PANC-1, CFPAC-1, PancTu-1 and PSN-1) to form spheres was tested. All cell lines with the exception of PancTu-1 developed spheres. Phenotypically, the sphere-growing cells showed an increased *in vitro* invasion capability. Both gene and protein expressions of markers of metastases [CXCR4 (CXC chemokine receptor 4), OPN (osteopontin) and CD44v6] and components of active hedgehog pathway signalling were assessed. Spheres clearly demonstrated increased expression of the above-mentioned markers when compared with their adherent counterpart. With the aim of identifying a minimum set of markers able to separate cells that have the capacity to form spheres from those incapable of forming spheres, a PCA (principal component analysis) of the multidimensional dataset was performed. Although PCA of the 'accepted' stemness genes was unable to separate sphere-forming from sphere-incapable cell lines, the addition of the 'aggressiveness' marker CD44v6 allowed a clear differentiation. Moreover, inoculation of the spheres and the adherent cells *in vivo* confirmed the superior aggressiveness (proliferation and metastasis) of the spheres over the adherent cells. In conclusion, the present study suggests that the sphere-growing cell population is not only composed of cells displaying classical stem membrane markers but also needs CD44v6-positive cells to successfully form spheres. Our results also emphasize the potential therapeutic importance of pathways such as CXCR4 and hedgehog for pancreatic cancer treatment.

Key words: adherently growing cell, cancer marker, pancreatic cancer, principal component analysis (PCA), sphere-growing cell

INTRODUCTION

Pancreatic cancer is one of the most lethal human cancers with an overall 5-year survival rate of 3–5% and a median survival of less than 6 months. The very short survival rates may be due to the fact that there are no appropriate diagnostic tools for early detection. Metastatic seeding is one of the major features of pancreatic cancer (occurring in more than 80% of cases), and metastases to loco-regional lymph nodes or liver are often already present at the time of diagnosis. Despite the efforts made in the past 50 years, conventional treatment approaches have little impact on the course of this aggressive neoplasm [1].

Since 1997, the U.S. FDA (Food and Drug Administration) approved GEM (gemcitabine) for first-line treatment of patients with locally advanced or metastatic adenocarcinoma of the pancreas. This approval was based on a slight clinical benefit response and survival for GEM versus 5-fluorouracil observed in a relatively small randomized phase III study including 126 patients [2]. Despite the introduction of GEM in the current therapeutic treatment as a single agent, the effectiveness of chemotherapy still remains limited and highly inefficient [3]. A potential explanation for the failure of the classical therapeutic approaches for pancreatic cancer might be because the agents only target the proliferating cells [4]. In the last few years, it has been extensively demonstrated that tumours are composed of different cell

Abbreviations used: AML, acute myelogenous leukaemia; CSM, cancer stem cell medium; CK-19, serum cytokeratin 19; CXCR4, CXC chemokine receptor 4; DM, differentiation medium; DMEM, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's PBS; EGF, epidermal growth factor; ESA, epithelial-specific antigen; FBS, fetal bovine serum; FGF, fibroblast growth factor; GEM, gemcitabine; Gli, glioma-associated oncogene homologue; HGF, hepatocyte growth factor; H/E, haematoxylin/eosin; Isl-1, insulin gene enhancer protein 1; MRI, magnetic resonance imaging; OPN, osteopontin; PCA, principal component analysis; PDX-1, pancreatic and duodenal homeobox-1; Ptch, patched homologue; QRT-PCR, quantitative real-time PCR; RPLP0, ribosomal protein, large, P0; SDF-1, stromal-derived factor-1α; Shh, sonic hedgehog

[1]To whom correspondence should be addressed (email margherita.gaviraghi@tiscali.it).

populations, of which a small subset displays stem cell properties (e.g. high plasticity, multipotentiality, capacity to grow at clonal density and higher capacity to migrate) [5].

Cancer stem cells (also called cancer progenitor cells, cancer stem-like cells etc.), first identified in a subset of AML (acute myelogenous leukaemia) cells, were shown to be similar to normal haemopoietic stem cells and to be able to induce AML in immunodeficient mice [6]. Subsequently, the presence of a highly aggressive and potentially chemoresistant cancer stem cell population was demonstrated in a large panel of solid tumours.

As for other solid tumours, pancreatic cancers were demonstrated to contain cancer stem cells. Li et al. [7] reported that $CD44^+CD24^+ESA^+$ (where ESA is epithelial-specific antigen) cells possess characteristics and properties of stem cells, as they showed a 100-fold increased tumorigenic potential compared with the triple negative population when xenografted in immunodeficient mice.

Also, Hermann et al. [8] demonstrated that pancreatic cancers contain different subpopulations of cells. They defined a distinct subset of $CD133^+/CXCR4^+$ cells {CXCR4 (CXC chemokine receptor 4) is the receptor for the chemokine CXCl12 [also known as SDF-1 (stromal-derived factor-1α)]} that localizes to the invasive edge of primary pancreatic carcinomas. This cell population exhibited a significantly stronger migratory activity *in vitro* and was revealed to be more metastatic *in vivo* than $CD133^+/CXCR4^-$ cells. Kolb et al. [9] correlated the increased OPN (osteopontin) expression in pancreatic cell lines and tumour specimens with increased metastatic seeding.

Recently, Jimeno et al. [10] discovered in a pancreatic cancer xenograft that GEM treatment resulted in an enrichment of pancreatic cancer stem cells ($CD24^+$ and $CD44^+$), which prompted the repopulation of the proliferating cells after GEM release. Only the combined treatment of GEM with the hedgehog pathway inhibitor cyclopamine resulted in significant tumour shrinkage. Moreover, the hedgehog inhibitor was able to reduce both static and dynamic markers of cancer stem cells.

The results of our present study, in which we have demonstrated that stemness markers are not sufficient to completely describe sphere-growing cells, confirm the hypothesis that sphere-growing cells not only display stem cell characteristics but can also be considered as the aggressive subpopulation of pancreatic tumours. Based on our findings, we were able to generate a test system that can readily be applied to the identification of potential drug targets which are specific to the pancreatic cancer stem cell niche.

MATERIALS AND METHODS

Cell lines and culture conditions

Human pancreatic cancer cell lines PANC-1, PSN-1, CFPAC-1 and PancTu-1 cells (A.T.C.C.) were grown in RPMI 1640 (Cambrex Bioscience, Milan, Italy) supplemented with 10% (v/v) FBS (fetal bovine serum; Cambrex Bioscience, Milan, Italy), 100 units/ml penicillin and 0.1 mg/ml streptomycin. Adherent cells were maintained in standard conditions for a few passages at 37°C with 5% CO_2 and detached using trypsin/EDTA solution (trypsin 0.25% and EDTA 0.02%). Cells were routinely checked for mycoplasma contamination using a PCR-based method [11].

To generate suspension cells and separate stem-like sphere-growing cells, adherent cells were washed twice in Dulbecco s PBS 1× (Cambrex Bioscience, Milan, Italy) and then cultured in DMEM (Dulbecco's modified Eagle's medium)+F-12 (1:1) supplemented with 1× B27 (Gibco, Invitrogen), 1 pg/ml Fungizone, 1% penicillin/streptomycin (Gibco, Invitrogen), 5 μg/ml heparin (Sigma) and 20 ng/ml EGF (epidermal growth factor)/FGF (fibroblast growth factor; Peprotech). The medium is referred to as CSM (cancer stem cell medium). Adherent cells were left in CSM for at least 3–4 weeks or until the appearance of floating cell aggregates, referred to as pancreatic cancer spheres. Spheres were collected without disturbing the adherent cells and subcultured in CSM for at least four passages before initiating the characterization experiments.

Drugs and chemicals

GEM (Gemzar, Lilly) stocks (40 mg/ml solution) were stored in aliquots at –20°C and single aliquots were utilized for the drug treatment.

QRT-PCR (quantitative real-time PCR)

Total RNA was isolated from PANC-1 spheres and PANC-1, PSN-1, CFPAC-1 and PancTu-1 adherent cell lines using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the standard protocol provided by the manufacturer, with on-column DNA digestion. RNA integrity and concentration was analysed using Agilent Technology and 1 μg of RNA was retrotranscribed into cDNA using the First Strand cDNA Synthesis kit from Roche (Mannheim, Germany). SYBR-Green Technology (Applied Biosystems) was used for all QRT-PCR experiments. A 20 μl portion of the PCR reaction mixture containing 6 μl of template cDNA (diluted 1:25), 10 μl of SYBR-Green reagent and 0.5 μmol/l of specific primers was used for each QRT-PCR reaction. The primers used were: 5′-ACATGTTGCTGGCCAATAAGGT-3′ and 5′-CCTAAAGCCTGGAAAAAGGAGG-3′ for RPLP0 (ribosomal protein, large, P0), 5′-GCATTGGCATCTTCTATGGTT-3′ and 5′-CGCCTTGTCCTTGGTAGTGT-3′ for CD133, 5′-TCCAACTAATGCCACCACCAA-3′ and 5′-GACCACGAAGAGACTGGCTGT-3′ for CD24, 5′-CCGCTGCGAGGACGTAGA-3′ and 5′-TGTTGGCTGCGTCTCATCAAAACC-3′ for ESA, 5′-AGAAGGTGTGGGCAGAAGAA-3′ and 5′-AAATGCACCATTTCCTGAGA-3′ for CD44, 5′-AGGAACAGTGGTTTGGCAAC-3′ and 5′-CGAATGGGAGTCTTCTCTGG-3′ for CD44v6, 5′-AGGGAGGAAAGCAGACTGAC-3′ and 5′-CCAGTCATTTCCACACCACT-3′ for Gli-1 (glioma-associated oncogene homologue 1), 5′-CGCCTATGCCTGTCTAACCATGC-3′ and 5′-AAATGGCAAAACCTGAGTTG-3′ for Ptch (patched homologue), 5′-CAAAAAGGAAACGCAAAGACG-3′ and 5′-AGCAAATCACTGCAATTCTCA-3′ for OPN, 5′-GGACAGGCTGATGACTCAGA-3′ and

5′-CAAAAGGAAACGCAAAGACG-3′ for Shh (sonic hedgehog). Thermal cycle protocols were performed for 40 cycles of 10 s at 95°C, 30 s at 60°C. All PCR reactions were carried out using the ABI Prism 7000 instrument (Applied Biosystems) and analysed by ABI Prism 7000 SDS software. To confirm amplification specificity, PCR products were subjected to a melting curve analysis. In order to avoid potential interference of the cell culture conditions with the expression of typically used cytoskeletal markers, the ribosomal protein RPLP0 was selected and validated as the most reliable housekeeping gene.

QRT-PCR average values of PANC-1, PSN-1, CFPAC-1 and PancTu-1 adherent cells calculated for the ten selected genes were normalized by dividing each cell line dataset by its standard deviation. PCA (principal component analysis) was performed with Matlab Statistics Toolbox (The MatWorks™) and visualization of the PCA shows the principal components.

FACS

PANC-1 adherent cells were grown up to 70% confluency. The adherent cells were trypsinized for dissociation, while the spheres were mechanically dissociated avoiding all potentially sphere compromising manipulations. Subsequently, both cell suspensions were washed twice with Hanks balanced salt solution (Gibco, Invitrogen) containing 2% FBS (FACS buffer) and resuspended in FACS buffer at a density of 100 000 cells/100 μl. Direct-labelled antibodies, anti-CD44-allophycocyanin, anti-CD24-phycoerythrin (BD Bioscience, Pharmingen), ESA–FITC (BD Immunocytometry Systems) and PECXCR4 (BD Bioscience, Pharmingen) used at a dilution of 1:40, were incubated for 20 min on ice. For the analysis of CD44v6, cells were resuspended in FACS buffer at a density of 200 000 cells/50 μl. Direct-labelled antibodies anti-CD44v6 allophycocyanin and Mouse IgG1 Isotype Control-APC (R&D Systems) were used at a dilution of 1:2.5, and incubated for 45 min on ice. Subsequently, each sample was washed twice with FACS buffer and samples were analysed employing a FACSAria instrument (BD Immunocytometry Systems).

Differentiation profile of pancreatic cancer stem cells

To evaluate the differentiation pattern of spheres, the spheres were dissociated into single cells and plated on to Permanox chamber slides (Lab-Tek, Nunc) at a density of 100 cells per well. PANC-1 adherent cells were cultured in RPMI 1640 containing 10% FBS and 1% penicillin/streptomycin for 10 days. To favour cell attachment and differentiation of PANC-1 spheres, cells were incubated for 10 days at 37°C in CSM without EGF/FGF but with the addition of 1% FBS [referred to as DM (differentiation medium)]. After the differentiation induction, the expression of progenitor and differentiation markers [PDX-1 (pancreatic and duodenal homeobox-1), Isl-1 (insulin gene enhancer protein 1) and CK-19 (serum cytokeratin 19)] in PANC-1 spheres was compared with that in adherent PANC-1 cells using immunocytochemistry. Approx. 10 million PANC-1 spheres were fixed for 10 min in 4% (w/v) paraformaldehyde, resuspended in a warm solution of 1% agar dissolved in PBS and transferred to a bottom cut Eppendorf tube until complete agar solidification. Cells embedded in solidified agar were then embedded in paraffin, cut into sections and further processed and analysed for immunocytochemistry. (The protocol is available at http://icg.cpmc.columbia.edu/cattoretti/Protocol/immunohistochemistry/Cell_block.html.)

The adherent cells were fixed for 10 min with 4% paraformaldehyde, briefly washed with PBS and incubated for 15 min in PBS containing 1% BSA for specific antibody blocking. Cells were subsequently incubated overnight with anti-PDX-1, anti-Isl-1 (both from Abcam) and anti-CK-19 (clone BA17, Dako) at dilutions of 1:5500, 1 μg/ml and 1:100 respectively. For CK-19, antigen retrieval was performed for 5 min in citrate buffer (10 mM citric acid and 0.05% Tween 20, pH 6) at 99°C. All immunocytochemical reactions were developed using a NovoLinK Polymer Detection System kit (Visionbiosystems, Novocastra). All images were acquired using a DMD108 microscope (Leica Microsystems). The positivity of the antibody staining was quantified by counting the number of positive cells versus the total number of cells (minimum 500 cells were counted per condition) in both growth conditions.

Transwell migration assay

In vitro chemotaxis was assayed using the HTS Transwell-96 system from Corning (Corning, NY, U.S.A.). Cells were starved overnight in a serum-free medium with and without SDF-1 (100 ng/ml). A 100 μl portion of cells diluted at 75×10^4/ml in migration buffer (DMEM with 5% BSA) was placed in the upper wells, whereas the chemoattractants [HGF (hepatocyte growth factor), SDF-1, FBS and CSM] diluted in a serum-free medium were added to the lower wells. Polyester membranes with a pore size of 1.3 μm were used and incubation was performed at 37°C in a 5% CO_2 atmosphere for 48 h. At the end of the reaction, migrated cells were detached by placing transwell chambers for 15 min on ice, stained with CyQuant dye and counted using a fluorescence plate reader (M1000 Infinite®; Tecan). All experiments were performed in triplicate.

Subcutaneous xenografts generated from adherent and sphere-growing pancreatic cancer cells

All animal experiments were carried out according to the ethical standards of the Verona University Review Board, and the animals were maintained in accordance with the institutional guidelines. A total of 2×10^6 adherent and sphere-growing pancreatic cancer cells in a final volume of 100 μl were injected into the right flank of female athymic CD1 mice (4-week-old, Charles River). Typically, cells were resuspended in a total volume of 100 μl of DPBS and injected subcutaneously. Tumour development was monitored for 30 days. At the end of the study, animals were killed and tumour samples were formalin-fixed, embedded in paraffin, sectioned, deparaffinized using xylene and hydrated by a series of decreasing ethanol washes. H/E (haematoxylin/eosin) and Ki67 immunohistochemical staining

was performed. Ki67 (clone MM1; Novocastra) was diluted 1:50 and antigen retrieval was performed at 95°C for 15 min in pH 6 citrate buffer.

Generation of orthotopic human pancreatic cancer xenografts in immunodeficient mice

All animal experiments were carried out according to the ethical standards of the Verona University Review Board, and the animals were maintained in accordance with the institutional guidelines. A total of 5×10^5 adherent or sphere-growing PANC-1 cells in a total volume of 30 μl of sterile PBS were injected into the pancreas of ten female athymic CD1 mice (4 weeks old, Charles River): five received spheres cells and five adherent cells. Animals were anaesthetized using ketamine/xylazine cocktail at a ratio of 100 mg/kg:20 mg/kg and the mice were placed under a heat lamp.

MRI (magnetic resonance imaging)

Mice were anaesthetized by inhalation of a mixture of air and O_2 containing 0.5–1% isofluorane and placed into a 3.5 cm internal diameter transmitter–receiver birdcage coil. Images were acquired using a Biospec Tomograph (Bruker, Karlsruhe, Germany) equipped with a 4.7 T, 33-cm bore horizontal magnet (Oxford Ltd, Oxford, U.K.). The respiration rate was monitored. The imaging planes were both coronal and transverse.

RESULTS

Not all pancreatic cancer cell lines can form spheres

In order to test whether pancreatic adherent cells lines were able to form spheres, a panel of four established cells lines was selected: PANC-1, PSN-1 and PancTu-1 (derived from primary tumours) and CFPAC-1 (derived from liver metastasis) [12]. Cell lines were first cultured as adherent cells for several passages, washed with DPBS and subsequently cultured in CSM. Cells started to lose their characteristic epithelial morphology within 24–36 h after the incubation with CSM. They typically lost a rhomboidal epithelial shape and became floating cells or cell clusters. After 1 week, the floating spheroids were collected without disturbing the remaining adherent cells and grown until they reached a sufficient density to be transferred to a 75 cm flask. After 4 weeks a clear distinction between the cell lines that had the capability to form spheres and the ones that had not could be made. Three cell lines, PANC-1, PSN-1 and CFPAC-1, were capable of forming spheres. They already demonstrated a significant production of floating cells 5 days after the addition of CSM and a sufficient number of spheres for subsequent experiments were obtained after 3–4 weeks. Although there was a clear difference in morphology between the spheres from PANC-1, CFPAC-1 and PSN-1, they all demonstrated the capacity to grow in suspension and to proliferate under CSM culture conditions for a long time.

PancTu-1 cells did not have the capacity to produce spheres when cultured in CSM. Most of the cells maintained their capacity to adhere even in the presence of CSM, and only very few single floating cells were formed. Also, separating the floating cells from their adherent counterparts and replating them in CSM conditions did not result in the formation of spheres.

CD24, CD44 and ESA expression cannot successfully separate sphere- from non-sphere-forming pancreatic cancer cell lines

In order to further characterize the four pancreatic cell lines, the expression of the reported cancer stem-like markers CD24, CD44, ESA and CD133, as well as the expression of the hedgehog markers Gli-1, Gli-2 and Smo and of the 'aggressiveness' markers CXCR4, OPN and CD44v6, was quantified in all cell lines by QRT-PCR. The obtained values were analysed using multivariate statistical analysis, a quantitative method allowing interpretation of high-dimensional datasets. In order to be able to generate a minimum gene set necessary to distinguish those cell lines that have the capacity to form spheres from the ones that do not, a PCA was performed on the normalized data [13].

Using the pancreatic cancer stem-like markers CD24, CD44, ESA and CD133, it was not possible to distinguish the 'sphere-capable' from the 'sphere-incapable' cell lines. In fact, PancTu-1 resides in the same quadrant as the 'sphere-capable' cell lines PANC-1 and PSN-1 (Figure 1A). In a subsequent experiment, the same PCA was carried out including CD44v6, a protein marker of metastasis in pancreatic cancer. As shown in Figure 1(B), the PCA now successfully separated the 'sphere-capable' from the 'sphere-incapable' cell lines. CFPAC-1 is located in the first quadrant together with PSN-1 and PANC-1, and the sphere-incapable PancTu-1 is clearly separated from the others. Moreover, the fact that PANC-1 and PSN-1 reside closer to the axes origin may reflect the difference in the capability of the cell lines to form spheres. CFPAC-1 spheres have a different morphology as compared with PANC-1 and PSN-1 spheres: CFPAC-1 spheres form rounded, regular and melted spheres, which made them difficult to dissociate into single cells; PANC-1 and PSN-1 form very similar spheres. PancTu-1 cells were unable to form spheres even when they were cultured in CSM for more than 4 weeks. These cells are well separated in the fourth quadrant from the three others.

This analysis suggests that the earlier described stem cell markers alone are not sufficient to predict sphere formation and that CD44v6 is essential to successfully predict the sphere-forming capability of pancreatic cancer cell lines. Moreover, FACS analysis demonstrated an increased membrane expression of CD44v6 protein in PANC-1 spheres compared with their adherent counterpart. In a next step, a full characterization of sphere-forming cells was performed.

Pancreatic cancer spheres demonstrate increased expression of stem cell markers

PANC-1 cells were grown in two different medium conditions: the adherent standard cell condition (RPMI 1640+10% FBS) and

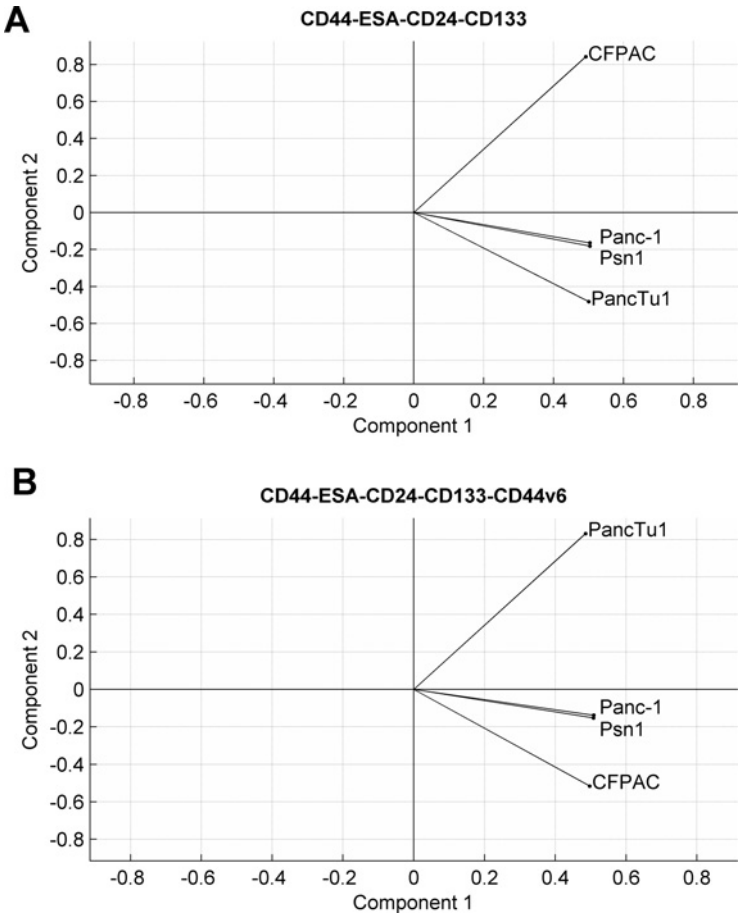

Figure 1 **PCA of CD44, ESA, CD24 and CD133 carried out in the absence and in the presence of CD44v6**
(**A**) Cell line projection using stemness markers on the two first principal components; the second principal component clearly separates CFPAC from the others (PANC-1, PancTu-1 and PSN-1). The first principal component does not distinguish cells able to form spheres from those that do not. (**B**) Cell line projection of the stemness markers including CD44v6 on the two first principal components clearly shows three clusters, in agreement with the biological ability to form spheres. Note: In this graphical representation, PANC-1 and PSN-1 are superimposed due to their vicinity.

CSM condition. The quantitative gene expression of the complete panel of stem cell markers (CD24, CD44, ESA and CD133) and of the metastatic marker CD44v6 was assessed in PANC-1 spheres and adherent cells. CD24 gene expression in PANC-1 spheres is 25-fold greater than in adherent cells (± 2). Although both CD44s (CD44 and CD44v6) and ESA did not change significantly their expression between spheres and adherent cells, the CD44v6 ligand OPN demonstrated a 350 (± 30, $P > 0.01$) fold increased mRNA in spheres versus adherent cells. Also at the protein level, increased positivity of the membrane marker CD24 was most striking in spheres versus adherent cells (Figure 2). Indeed, FACS analysis revealed the presence of a restricted $CD24^+$ subpopulation (8.5%) in adherent cells, whereas an enrichment of positive cells was observed (32%) in spheres (Figures 2A and 2B). Likewise, a significant increase in triple positive cells ($CD24^+$, $CD44^+$ and ESA^+) was observed in PANC-1 spheres (21%) compared with adherent cells (7.9%; Figures 2C and 2D). However, neither CD44 nor ESA expression levels varied significantly between adherent cells and spheres. Although there was variability in CD133 expression in sphere-growing cells, their expression was always significantly greater than in adherent cells in which no CD133 could be detected (results not shown). These data are in agreement with an earlier study in which Olempska et al. [14] demonstrated that PANC-1 adherent cells did not express CD133.

Pancreatic cancer spheres demonstrate typical stem cell functionalities

In order to investigate whether PANC-1 sphere-growing cells also display typical stem cell properties such as self-renewal and multipotentiality, these features were evaluated.

Self-renewal, defined as the ability of cells to go through numerous cycles of cell division while maintaining the undifferentiated state, was assessed by the spheres' capacity to produce second generation spheres. Cells were dissociated into single

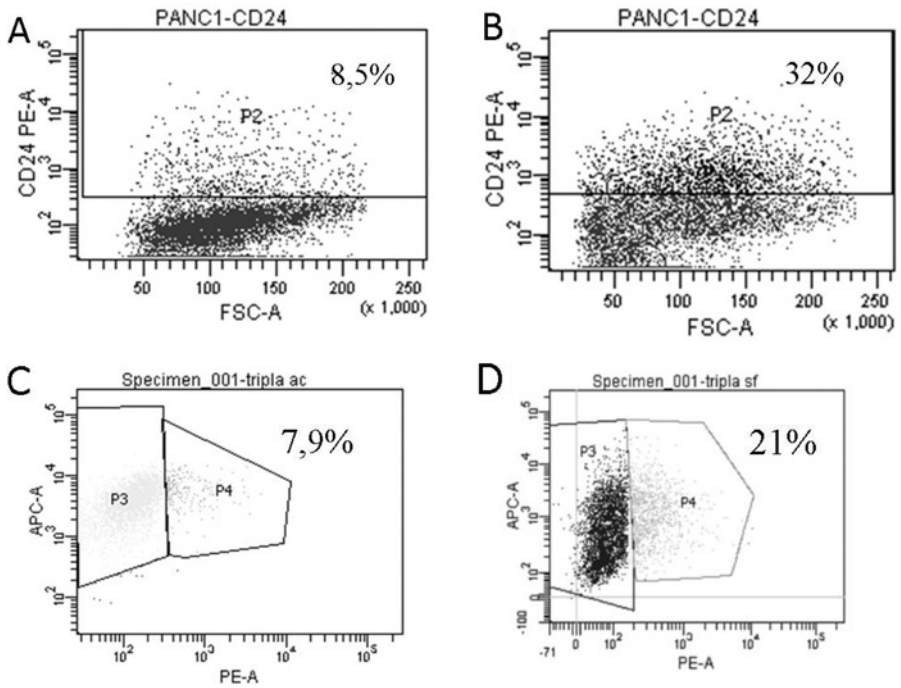

Figure 2 **Cell surface expression analysis of stemness markers in adherent and sphere-growing PANC-1 cells**
(**A**) PANC-1 adherent $CD24^+$, (**B**) PANC-1 spheres $CD24^+$, (**C**) PANC-1 adherent $CD24^+CD44^+ESA^+$ and (**D**) PANC-1 spheres $CD24^+CD44^+ESA^+$.

cells and plated on a 96-well plate as single cells per well. After 7–10 days, only 16% of the single cells were able to produce second generation spheres and the newly formed spheres were morphologically more regular than the initial spheres. These observations confirm that PANC-1 spheres represent a heterogeneous cell population that contains a stem-enriched subpopulation of cells, potentially responsible for more aggressive tumour behaviour.

Multipotentiality, defined as the capacity of progenitor cells to differentiate into cells from a limited number of lineages, was investigated by subjecting PANC-1 spheres to differentiating conditions. Typically, dissociated spheres were grown in differentiating conditions. After 10 days in DM, the cells completely attached to the plastic slides and acquired mixed (partly sphere, partly adherent) morphological shapes. Specific markers of different phases of pancreatic development (PDX-1, Isl-1 and CK-19) were selected in order to follow the differentiation profile of PANC-1 spheres in differentiating conditions.

As shown in Figure 3, PANC-1 spheres are CK-19 (differentiation marker) negative (Figure 3B) and 70% of the cells are PDX-1 positive (Figure 3E). Exposing spheres for 11 days to differentiating conditions (DM) results in attachment of the spheres to the slide increased CK-19 positivity (35% positive cells; Figure 3D). Adherent PANC-1 cells show high CK19 positivity. Figure 3(A) shows the quantitative analysis of the percentage of PDX-1, Isl-1 and CK-19 positivity in spheres, differentiated spheres (cells in DM for 11 days) and adherent cells. As depicted in the graph, the increased expression of the differentiation marker CK-19 coincides with a decreased expression of the early progenitor marker PDX-1 and the β-cell precursor marker Isl-1 in spheres exposed to DM.

PANC-1 spheres demonstrate increased aggressiveness

Aggressiveness marker expression

It has been demonstrated that there is a clear correlation between an increased metastatic potential of pancreas cancer and an increased expression of CXCR4 [8] and OPN [9]. Since OPN was described to be a direct downstream target gene of the human hedgehog signalling pathway [15], the gene expression analysis of PANC-1 spheres versus adherently growing cells was extended to indicator genes of the active hedgehog signalling pathway (e.g. Shh, Gli-1 and Ptch). Hedgehog pathway activation was assessed both at the receptor level (Shh) and at the intracellular 'target gene' level (Gli-1 and Ptch).

The overexpression of the reported 'aggressiveness' markers CXCR4 and OPN in spheres in comparison with adherent PANC-1 was confirmed. Spheres showed the earlier described 350-fold (± 30, $P < 0.01$) and 9.24-fold (± 0.52, $P < 0.01$) higher mRNA expression of OPN and CXCR4 respectively.

Moreover, the increased OPN levels were clearly in line with an increased hedgehog pathway activity as indicated by its markers: spheres demonstrated a 2 (± 0.2, $P < 0.05$), 5 (± 0.42,

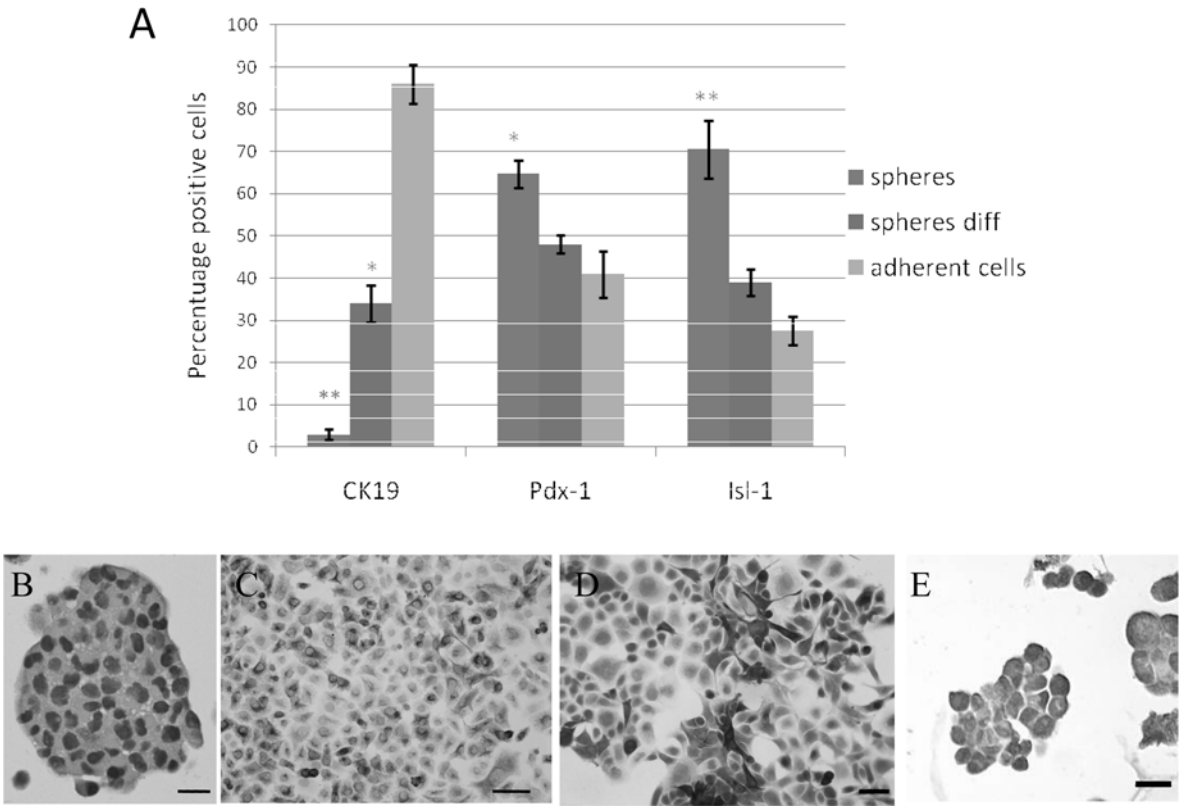

Figure 3 **Differentiation marker analysis of differentiated PANC-1 spheres and adherent cells**
(**A**) Analysis of PANC-1 spheres compared with differentiated (diff) spheres and adherent cells. The percentage of CK-19-, PDX-1- and Isl-1-positive PANC-1 cells is represented as the fraction of the total number of cells. One-way ANOVA followed by the Tukey–Kramer test for multiple comparisons was performed. CK-19 and Isl1 expression levels were significantly different between all three cell types ($P < 0.05$). Expression of PDX-1 was significantly different only between spheres and adherent cells. (**B**) PANC-1 paraffin-embedded spheres were stained for CK-19; scale bar, 1 μm. (**C**) CK-19 staining of PANC-1 adherent cells in chamber slides; scale bar, 5 μm. (**D**) CK-19 differentiation experiments with PANC-1 sphere-growing cells in DM for 11 days; scale bar, 1 μm. (**E**) Representative image of PDX-1 immunostaining of PANC-1 spheres; scale bar, 10 μm.

$P < 0.01$) and 3.27 ($\pm$0.47, $P < 0.01$) fold increase in Shh, Gli-1 and Ptch expression respectively. All differences were statistically significant, suggesting that sphere-growing cells show increases in aggressiveness markers and possess a more active hedgehog signalling pathway.

Increased CXCR4 expression in PANC-1 spheres regulates migration

Before investigating the potential functional role of increased CXCR4 expression in spheres as compared with adherent cells, the increased expression of CXCR4 at the plasma membrane was confirmed. FACS analysis demonstrated that PANC-1 spheres (19.4%) displayed an increased membrane protein expression of CXCR4 when compared with their adherent counterparts (6.4%; Figures 4A and 4B).

Potential association of increased membrane CXCR4 with increased migration was appraised in a transwell migration assay. Although FBS, CSM and HGF in the lower chamber did not result in a different migration pattern of spheres and adherent cells, the addition of the natural ligand of CXCR4 as chemoattractant (SDF-1) had a significant effect on the migration capacity of spheres (160% of control) and not on the adherent cells. Thus these results not only validate the functional activity of CXCR4 in spheres but also confirm the increased migratory capacity of spheres versus adherent cells (Figure 4C).

Pancreatic cancer spheres show a different morphology after in vivo *passage with respect to their adherent counterpart*

The morphology of tumours derived from PANC-1 spheres and adherent cells was investigated in a xenograft mouse model. Typically, 2×10^6 cells of each cell type were injected subcutaneously in athymic female mice. After 35 days, mice were

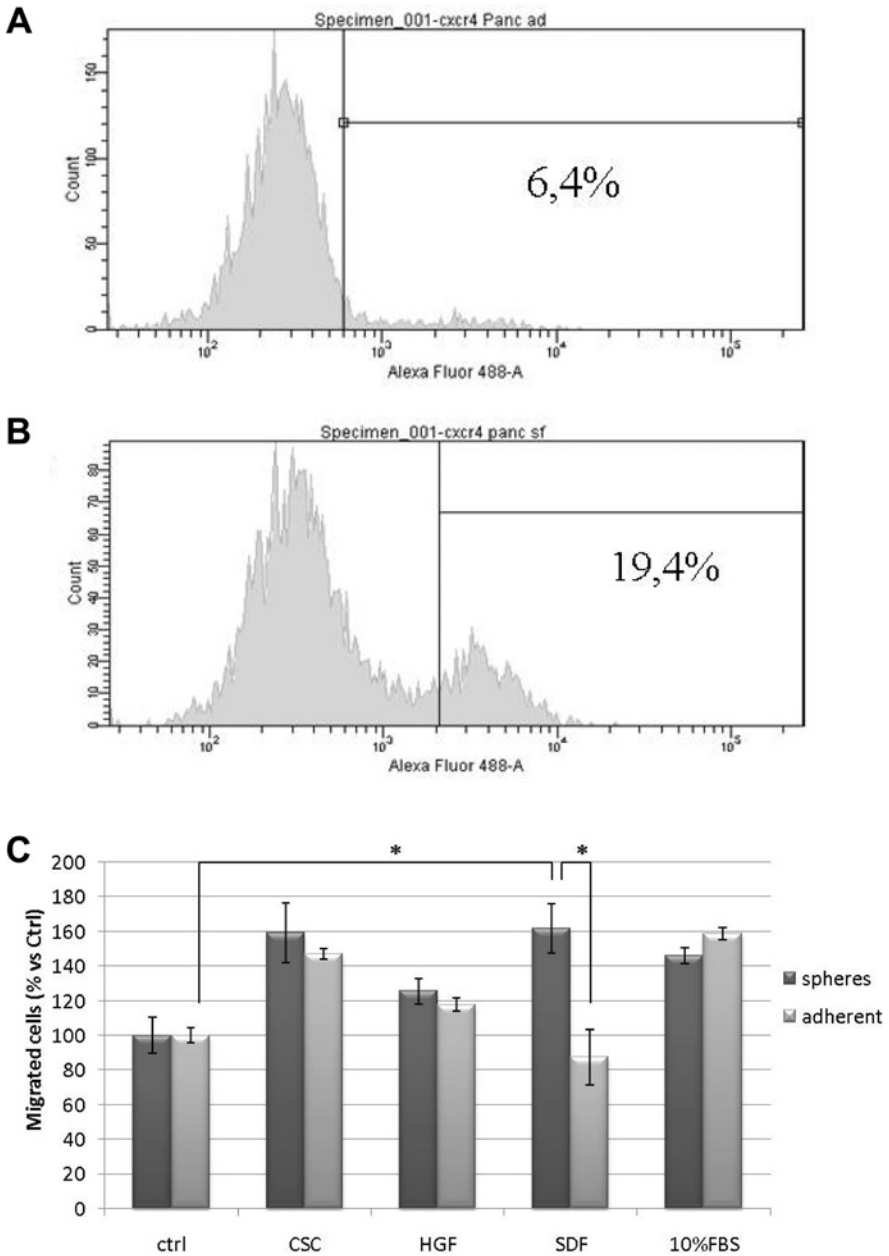

Figure 4 CXCR4 surface expression in PANC-1 adherent cells and spheres

CXCR4 cell surface expression in PANC-1 adherently growing cells (**A**; 6.4%) and spheres (**B**; 19.4%) using FACS analysis. All results are expressed as means of three independent experiments. (**C**) Transwell migration assay of PANC-1 spheres compared with adherently growing cells. Average percentage of cells (normalized to DMEM serum-free medium as the control) that migrated after 48 h from the upper to the lower chamber is shown. The medium in the lower chamber contained the following chemoattractants: DMEM serum-free medium (as the control, ctrl); complete CSM (CSC); HGF at 200 ng/ml; DMEM serum-free medium supplemented with SDF-1 (200 ng/ml; SDF) and DMEM supplemented with 10% FBS (10% FBS). Statistically significant (Student's *t* test; $*P < 0.05$) differences were observed between ctrl and SDF.

killed and tumours were dissected from skin, formalin-fixed and embedded in paraffin. H/E staining (Figures 5A and 5B) demonstrated that sphere-growing tumours established in nude mice showed more irregular cells undergoing mitosis than adherent cell-derived tumours. Moreover, adherent cells demonstrated a more organized architecture, whereas the spheres generated a more dysplastic tumour structure. Spheres were less organized, contained more apoptotic bodies and more cells undergoing mitosis as assessed by Ki67 immunostaining on paraffin-embedded tumour sections. In fact, Ki67 is a marker used to determine the growth fraction of a given cell population [16] and showed that sphere-derived tumours had a

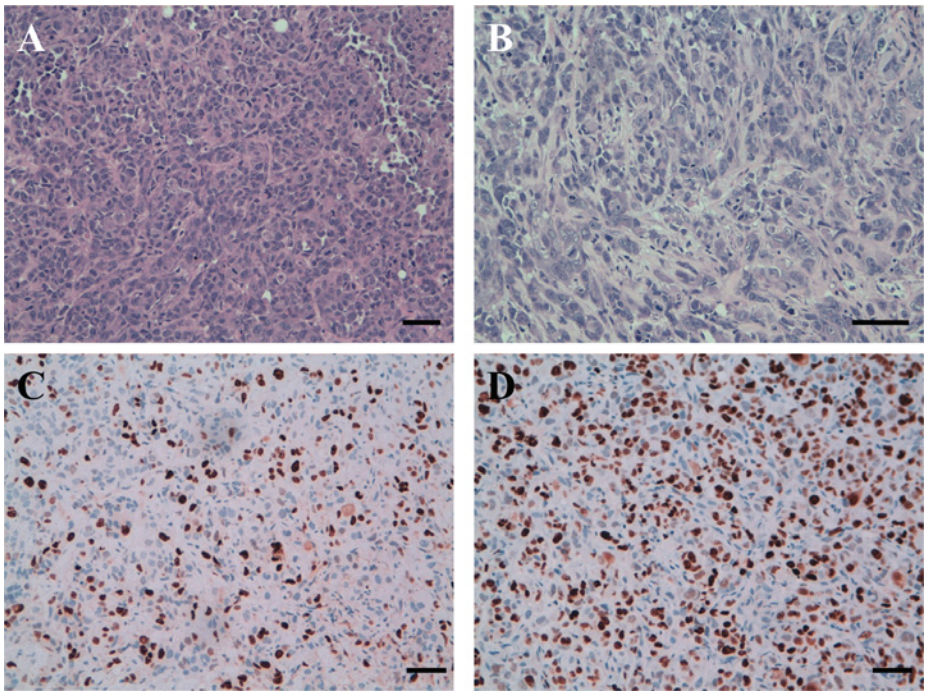

Figure 5 Histological and histochemical features of PANC-1 xenograft tumours
H/E and Ki67 immunohistohemical stainings: comparison in tumours generated from PANC-1 adherently growing cells shown in (**A**) (scale bar, 10 μM) and (**C**) (scale bar, 20 μM) and from PANC-1 spheres shown in (**B**) (scale bar, 10 μM) and (**D**) (scale bar, 20 μM). H/E in (**A**, **B**) clearly shows that sphere cells generate a tumour profile that is more aggressive when compared with the profile generated from adherently growing cells (more irregular mitotic signature, arrows). In (**C**, **D**), Ki67 (a cellular marker strictly associated with cell proliferation)-positive nuclei are visualized in adherent cells (40% positive nuclei) and in spheres (70–80% positive nuclei).

significantly higher proliferation rate (70–80% positivity) when compared with adherent cell-derived tumours (40% positivity) (Figures 5C and 5D).

Tumour growth profile from adherent cells and spheres in the orthotopic mouse model

Ten nude mice were used to obtain orthotopic tumours from 5×10^5 PANC-1 cells: five were injected with sphere-growing cells and five with adherent cells. Tumour growth was followed using MRI for 3 months, setting the first control at 20 days after surgery.

Mice injected with PANC-1 sphere-growing cells developed tumours faster than mice injected with PANC-1 adherent cells. In animals receiving sphere-growing PANC-1 cells, small masses were detected by MRI 6 weeks after surgery, whereas no tumours were seen at the same time point for the adherent-cell group. The latter only developed detectable tumours after two additional weeks.

Putative metastases were found in 5/5 animals injected with sphere-growing cells, as small masses were found on the surface of the small intestine and in the liver (Figures 6A and 6B).

Taken together, our results suggest that PANC-1 spheres (due to their increased aggressiveness) might be a more reliable model system for studies of pancreatic cancer.

DISCUSSION

Based on a large number of studies, it has become increasingly clear that many tumours contain a very small subpopulation of cancer stem-like cells that are characterized by a series of highly specific features. These cells, usually recognized by virtue of their membrane protein expression profile, exhibit typical stem cell characteristics and were hypothesized to be the more aggressively growing and chemoresistant cell population of a tumour [17–22]. Recently, some confusion has emerged in the 'cancer stem cell field' based on conflicting results obtained by different researchers. For instance, it was demonstrated that stem-cell marker-negative cells were also able to grow as spheres and to give rise to very aggressively growing tumours *in vivo*. In addition, from a chemosensitivity study conducted on glioma cells by

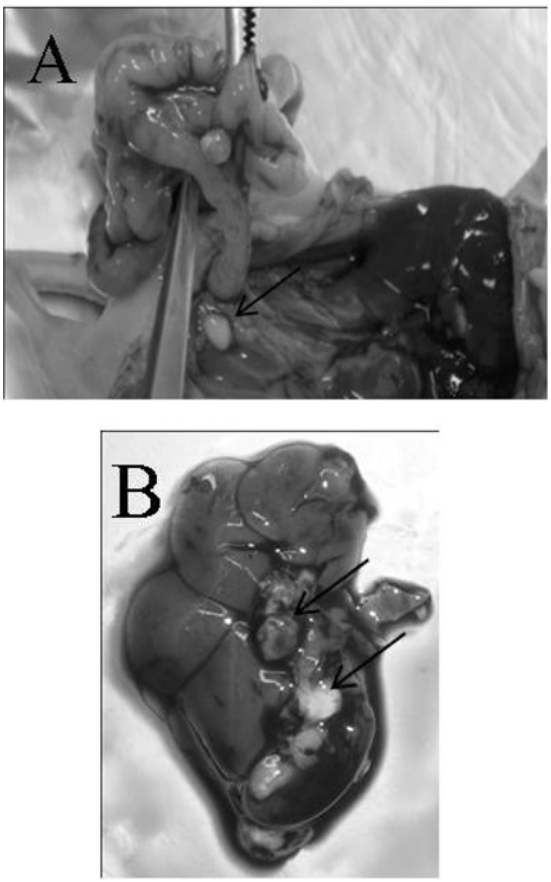

Figure 6 Putative metastasis (black arrows), derived from sphere-growing cells, were found in the small intestine (A) and liver (B)

Liu et al. [23], it became clear that the marker-positive cells were not substantially more chemoresistant when compared with the marker-negative cells. In fact, CD133-positive cells were only 15–20% more chemoresistant than CD133-negative cells upon standard care treatment (temozolomide, carboplatin, taxol and VP16). The aim of the present study was to enrich and characterize the subpopulation of tumour cells that can mimic the high aggressiveness of pancreas tumours observed in patients in terms of proliferation and metastatic capacity. We capitalized on previous observations in other solid tumours that have clearly demonstrated that cancer stem-like cells have the ability to grow as spheres and do mirror the genotype and phenotype of patient-derived tumours [24]. The opposite, showing that all sphere-growing cells need to be and are only stem-like, has not yet been investigated. Thus, instead of separating stemness marker-positive from negative cells, we studied the behaviour and marker expression pattern of pancreatic tumour spheres. The main question we wanted to answer was whether sphere-growing cells are homogeneous 'aggregates' of stem-like cells only. A panel of well-established pancreatic cancer cell lines was thus subjected to culture conditions that have previously been demonstrated to promote sphere forming cells (i.e. serum-free medium supplemented with growth factors present in the pancreatic microenvironment [25]). Interestingly, we found that only some pancreatic cancer cell lines had the capacity to form spheres under these conditions. In fact, PancTu-1 did not form spheres even after a long time of culture in the stem-cell-promoting medium. At first, this seemed contradictory to results published by Olempska et al. [14], who reported that PancTu-1 showed high expression levels of the typical stemness marker CD133. Besides the fact that in our hands the CD133 levels in PancTu-1 cells were relatively low, a multiparametric analysis (PCA) using expression values of all pancreatic cancer stem-associated markers [7] was unable to differentiate the lines that show a sphere-growing capacity from those that do not. Only when mRNA expression values from markers that in previous studies were reported to be associated with the aggressive/highly metastatic behaviour of pancreatic cancer [9,26–29] (such as CXCR4, OPN and CD44v6) were included, the two cell populations could be successfully separated by PCA. To our knowledge, these results point, for the first time, in the direction that (although spheres demonstrate the typical characteristics of stem-like cells, such as self-renewal and multidifferentiation) spheres potentially display something more than stem cell characteristics alone. Therefore, in a subsequent phase of our present study, we investigated functional features of pancreatic cancer sphere-growing cells. Our results demonstrated that the pancreatic cancer spheres were not only more invasive *in vitro* but also demonstrated a more aggressive proliferation, invasion and metastasis capacity *in vivo*. Thus these functional studies confirmed our initial observation based on marker analysis employing PCA. Taken together, our present study has demonstrated that the pancreatic cancer spheres are more than just cancer stem-like cells. In addition, as these sphere-growing cells can be maintained relatively easily we have also generated a cell line-derived pancreatic cancer model that demonstrates the characteristics of the clinically relevant tumour. This cell system might be very useful to further the understanding of certain aspects of pancreatic cancer and could also be employed to screen compounds for therapeutic intervention.

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