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

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Running title: Stemness and invasiveness markers define pancreatic cancer spheres

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Abstract

Pancreatic cancer stem-like cells are described to be characterized by the membrane expression of CD24, CD44 and ESA, and their capacity to grow as spheres in serum-free medium containing well-defined growth factors. The capacity of a panel of 4 pancreatic cancer cell lines (PANC-1, CFPAC-1, PancTu-1, PSN-1) to form spheres was tested. All cell lines with the exception of PancTu-1 developed spheres. Phenotypically, the sphere-growing cells demonstrated an increased *in vitro* invasion capability. Both gene and protein expression of markers of metastases, (CXCR4, Osteopontin, CD44v6) and components of active Hedgehog pathway signaling were assessed. Spheres clearly demonstrated increased expression of the above mentioned markers when compared to their adherent counterpart. With the aim to identify a minimum set of markers able to separate cells that have the capacity to form spheres from those incapable, a principal component analysis (PCA) 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, metastasis) of the spheres over the adherent cells. In conclusion, our study suggests that the sphere-growing cell population is not only composed of cells displaying classical stem membrane markers but 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.

Abbreviations List

BSA= Bovine Serum Albumine; CSM= Cancer Stem Cell Medium; CK-19= Serum cytokeratin 19; CXCR4= Chemokine (CXC motif) receptor 4; DM= Differentiation Medium; D-PBS= Dulbecco's Phosphate Buffered Saline; EGF= Epidermal Growth Factor; FACS= Fluorescence Activated Cell Sorter; FBS= Fetal Bovine serum; FGF= Fibroblast Growth Factor; GEM= Gemcitabine; Gli-1= Glioma-associated oncogene homolog 1; Gli-2= Glioma-associated oncogene homolog 2; HGF= Hepatocyte Growth Factor; H&E= Hematoxylin & Eosin; HBSS= Hank's Buffered Salt Solution; Isl-1= Insulin gene enhancer protein Isl-1; OPN= Osteopontin; PCA= Principal Component Analysis; PDX-1= Pancreatic and duodenal homeobox 1; Ptch= Patched homolog; human RPLP0= Ribosomal Protein, large, P0; SDF1= Stromal Derived Factor 1 alpha; Shh= Sonic Hedgehog; SM= Standard Medium

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 US Food and Drug Administration approved 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 Gemcitabine versus 5-fluorouracil observed in a relatively small randomized phase III study including 126 patients [2]. Despite the introduction of Gemcitabine in the current therapeutical treatment as a single agent, the efficacy 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 due to the fact that 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 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,..), first identified in a subset of AML (acute myelogenous leukemia) cells, were shown to be similar to normal haematopoietic stem cells and 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⁺ cells possess characteristics and properties of stem cells, as they showed a 100-fold increased tumorigenic potential compared to 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 is the receptor for the chemokine CXCL12 (also known as SDF1)) that localizes to the invasive edge of primary pancreatic carcinomas. This cell population exhibited a significantly stronger migratory activity *in vitro* and revealed to be more metastatic *in vivo* than CD133⁺/CXCR4⁻ cells. Kolb *et al.* [9] correlated the increased 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 Gemcitabine treatment resulted in an enrichment of pancreatic cancer stem cells (CD24⁺, CD44⁺) which prompted the repopulation of the proliferating cells after Gemcitabine release. Only the combined treatment of Gemcitabine 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 study, in which we have demonstrated that stemness markers do not suffice to completely describe sphere growing cells, confirm the hypothesis that sphere-growing cells not only display stem cell characteristics but can 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 that are specific to the pancreatic cancer stem cell niche.

Materials & Methods

Cells lines and culture conditions

Human pancreatic cancer cell lines PANC-1, PSN-1, CFPAC-1 and PancTu-1 cells (ATCC) were grown in RPMI (Cambrex Bioscience, Milan, Italy) supplemented with 10% fetal bovine serum (FBS, Cambrex Bioscience, Milan, Italy), 100 U ml⁻¹ penicillin and 0.1mg ml⁻¹ streptomycin. Adherent cells were maintained in standard conditions for a few passages at 37°C with 5% of CO₂ and detached using trypsin-EDTA solution (trypsin 0.25%, 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 D-PBS 1X (Cambrex Bioscience, Milan, Italy) and then cultured in DMEM+F-12 (1:1) supplemented with 1X B27, 1 Pg/ml Fungizone, 1% penicillin/ streptomycin (Gibco, Invitrogen), 5 µg/ml Heparin (Sigma, Inc) and 20 ng/ml EGF/ FGF (Peprotech, Inc). The medium is referred to as 'Cancer Stem Cell medium' (CSM). 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 sub-cultured in CSM for at least four passages before initiating the characterization experiments.

Drugs and chemicals

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

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 analyzed using Agilent Technology and 1 µg of RNA retro-transcribed into cDNA using the First Strand cDNA Synthesis Kit from Roche (Roche, Mannheim, Germany). SYBR-Green Technology (Applied Biosystems) was used for all quantitative real-time PCR-(QRT-PCR) experiments. Twenty µl of 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 used primers are: 5'-ACA TGT TGC TGG CCA ATA AGG T-3' 5'-CCT AAA GCC TGG AAA AAG GAG G-3' for RPLP0, 5'-GCA TTG GCA TCT TCT ATG GTT-3' 5'-CGC CTT GTC CTT GGT AGT GT-3' for CD133, 5'-TCC AAC TAA TGC CAC CAC CAA-3' 5'-GAC CAC GAA GAG ACT GGC TGT-3' for CD24, 5'- CCG CTG CGA GGA CGT AGA-3' 5'-TGT TGG CTG CGT CTC ATC AAA ACC-3' for ESA, 5'-AGA AGG TGT GGG CAG AAG AA-3' 5'-AAA TGC ACC ATT TCC TGA GA-3' for CD44, 5'-AGG AAC AGT GGT TTG GCA AC-3' 5'-CGA ATG GGA GTC TTC TCT GG-3' for CD44v6, 5'-AGG GAG GAA AGC AGA CTG AC-3' 5'-CCA GTC ATT TCC ACA CCA CT-3' for Gli-1, 5'-CGC CTA TGC CTG TCT AAC CAT GC-3' 5'-AAA TGG CAA AAC CTG AGT TG-3' for Ptch, 5'-CAA AAA GGA AAC GCA AAG ACG-3' 5'-AGC AAA TCA CTG CAA TTC TCA-3' for OPN, 5'-GGA CAG GCT GAT GAC TCA GA-3' 5'-CAA AAG GAA ACG CAA AGA CG-3' for Shh. Thermal cycle protocols were performed for 40 cycles of 10 sec at 95°C, 30 sec at 60°C. All PCR reactions were carried out using the ABI Prism 7000 Instrument (Applied Biosystems) and analyzed by the 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 most reliable housekeeping gene.

Quantitative Real-time PCR average value 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 analysis was performed with Matlab Statistics Toolbox (The MatWorksTM) and visualization of the PCA analysis shows the principal components.

Fluorescence-Activated Cell Sorting (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 in Hanks' balanced salt solution (Gibco, Invitrogen) containing 2% (v/v) FBS (FACS buffer) and resuspended in FACS buffer at a density of 100,000 cells/ 100 μ l. Direct -labeled 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 μ l. Direct-labeled antibodies anti-CD44v6 allophycocyanin and Mouse IgG1 Isotype Control-APC (R&D Systems, Inc) 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 analyzed 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 onto permanox chamber slides (Lab-Tek, Nunc) at a density of 100 cells/well. PANC-1 adherent cells were cultured in RPMI containing 10% FBS and 1% Penicillin/Streptomycin for 10 days. To favour cell attachment and differentiation of PANC-1 spheres, cells were incubated in CSM without EGF/FGF but with the addition of 1% FBS (referred to as differentiation medium (DM)). After the differentiation induction, the expression of progenitor and differentiation markers (PDX-1, Isl-1, CK-19) in PANC-1 spheres was compared to that in adherent PANC-1 cells using immunocytochemistry. Around ten million PANC-1 spheres were fixed for 10 min in 4% paraformaldehyde, resuspended in a warm solution of 1% agar dissolved in PBS and transferred in a bottom cut Eppendorf tube until complete agar solidification. Cells embedded in solidified agar were then embedded in paraffin, cut and further processed and analyzed for immunocytochemistry. (The protocol is available on: 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, Cambridge, Inc), and anti-Cytokeratin 19 (clone BA17, Dako) at a dilution of 1:5500, 1 μ g/ml, 1:100, respectively. For CK-19 antigen retrieval was performed for 5 minutes in citrate buffer (pH 6) at 99°C. All immunocytochemical reactions were developed using NovoLinK Polymer Detection System kit (Visionbiosystems, Novocastra, Uk). All images were acquired using a DMD108 microscope (Leica microsystems, Srl). 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 Inc., Corning, NY, USA). Cells were starved overnight in serum-free medium with and without SDF₄ (100 ng/ml). 100 μ l of cells diluted at 75 x10⁴ /ml in migration buffer (DMEM with 5% BSA) were placed in the upper wells whereas the chemo-attractants (HGF, SDF-1, FBS and CSM) diluted in 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% CO₂ 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, Infiniti, TECAN, USA). 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 weeks-old, Charles River). Typically, cells were resuspended in a total volume of 100 μl DPBS and injected subcutaneously. Tumour development was monitored for 30 days. At the end of the study, animals were sacrificed and tumour samples were formalin-fixed, embedded in paraffin, sectioned, deparaffinized using xylene, and hydrated by series of decreasing ethanol washes. Hematoxylin/Eosin and Ki67 immunohistochemical staining was performed. Ki67 (clone, MM1 Novocastra) was diluted 1:50 and antigen retrieval was performed at 95° for 15 min in pH6 buffer citrate.

Generation of orthotopic human pancreatic cancer xenografts in immunodeficient mice.

All animal experiments have been 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 spheres growing PANC-1 cells in a total volume of 30 μl of sterile PBS were injected into the pancreas of 10 female athymic CD1 mice (4 weeks old, Charles River): five received spheres cells, five adherent cells. Animals were anesthetized using ketamine/ xylazine cocktail at a ratio of 100 mg kg⁻¹:20 mg kg⁻¹ placing the mice under a heat lamp.

Magnetic Resonance Imaging

Mice were anesthetized by inhalation of a mixture of air and O₂ containing 0.5–1% isoflurane and placed into a 3.5 cm i.d. 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, United Kingdom). 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 hours from 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 four 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 to form 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

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were obtained after three-four 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. The majority of the cells maintained their capacity to grow in adherence even in 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, Smo, and of the 'aggressiveness' markers CXCR4, Osteopontin and CD44v6 was quantified in all cell lines by qRT-PCR. The obtained values were analyzed 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 which do not, a Principal Component Analysis 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 analysis was carried out including CD44v6, a protein marker of metastasis in pancreatic cancer. As shown in **Figure 1B** the PCA analysis 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 to 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 four 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 to 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+10% FBS) and CSM condition. The quantitative gene expression of the complete panel of stem cell markers (CD24, CD44, ESA, 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 higher 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 Osteopontin, 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 is most striking in spheres versus adherent cells (**Fig 2**). Indeed, FACS analysis revealed the presence of a restricted CD24⁺ positive subpopulation (8.5%) in adherent cells, whereas an enrichment of positive cells was observed (32%) in spheres (**Fig. 2A, B**). Likewise, a significant

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increase of triple positive cells (CD24⁺, CD44⁺, ESA⁺) was observed in PANC-1 spheres (21%) compared to adherent cells (7.9%) (**Fig. 2C, D**). 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 higher than in adherent cells in which no CD133 could be detected (data 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 cells and plated in a 96-well plate as single cells per well. After seven to ten 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 which 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 ten 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 **Fig 3** PANC-1 spheres are CK-19 (differentiation marker) negative (**Fig. 3 B**) and 70% of the cells are PDX-1 positive (**Fig. 3 E**). 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 - **Fig. 3D**). Adherent PANC-1 cells show high CK19 positivity. **Figure 3A** shows the quantitative analysis of the percentage of PDX-1, Isl-1 and CK-19 positivity in spheres, differentiated spheres (cells in differentiating medium 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 beta 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 Osteopontin [9]. Since Osteopontin was described to be a direct downstream target gene of the human Hedgehog signaling pathway [15], the gene expression analysis of PANC-1 spheres versus adherently-growing cells was extended to indicator genes of active Hedgehog signaling pathway (e.g., Shh, Gli-1, 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 Osteopontin in spheres in comparison to adherent PANC-1 was confirmed. Spheres showed the earlier described 350 (± 30 , $p < 0.01$) and 9.24-fold (± 0.52 , $p < 0.01$) higher mRNA expression of Osteopontin and CXCR4, respectively.

Moreover, the increased Osteopontin 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 , $p < 0.01$) and 3.27 (± 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 signaling pathway.

Increased CXCR4 expression in PANC-1 spheres regulates migration

Before investigating the potential functional role of increased CXCR4 expression in spheres as compared to 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 to their adherent counterparts (6.4%) (Fig 4A - B).

Potential association of increased membrane CXCR4 to 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 chemo-attractant (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 (Figs. 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 mice model. Typically, 2×10^6 cells of each cell type were injected subcutaneously in athymic female mice. After 35 days, mice were sacrificed and tumours were dissected from skin, formalin-fixed and embedded in paraffin. H&E staining (Figs. 5A, 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 significantly higher proliferation rate (70-80% positivity) as compared to adherent cell-derived tumours (40% positivity) (Figs. 5C, 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 spheres growing cells and five with adherent cells. Tumour growth was followed using magnetic resonance imaging for 3 months, setting the first control at 20 days after surgery. Mice injected with PANC-1 spheres growing cells developed faster tumour than mice injected with PANC-1 adherent cells. In animals receiving sphere-growing PANC-1 cells, small masses were detected at magnetic resonance imaging 6 week after surgery, whereas no tumors were seen at the same time-point for the adherent-cell group. The latter only developed detectable tumours after two additional weeks.

Putative metastasis 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 (Fig. 6A and B).

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 which 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 also stem-cell marker- negative cells were 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 Liu *et al.* [23], it became clear that the marker-positive cells were not considerably more chemoresistant as compared to the marker-negative cells. In fact, CD133 positive cells were only 15-20% more chemoresistant than CD133 negative cells upon standard of care treatment (temozolomide, carboplatin, taxol and VP16). The aim of the current 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 which have clearly demonstrated that cancer stem-like cells have the ability to grow as spheres and that these spheres do mirror the geno- and phenotype of patient-derived tumours [24, 25]. 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 which have previously been demonstrated to promote sphere-growing of cells (i.e. serum-free medium supplemented with growth factors present in the pancreatic microenvironment [26]). 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, who described 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 which 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 to the aggressive/ highly metastatic behaviour of pancreatic cancer [27, 28, 29, 30, 9] (such as CXCR4, Osteopontin 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 multi differentiation - spheres potentially display something more than stem cell characteristics alone. Therefore, in a subsequent phase of our 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 they 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 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 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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Figure Legends

Figure 1: PCA of CD44, ESA, CD24, 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, PSN-1). The first principal component does not distinguish cells able to form spheres from those which 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. *Nota bene:* In this graphical representation PANC-1 and PSN-1 are superimposed due to their vicinity.

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⁺.

Figure 3: (A) Analysis of PANC-1 spheres compared to differentiated 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 analysis, followed by Tukey-Kramer test for multiple comparisons was performed. CK-19 and Isl1 expression levels are significantly different between all three cell types ($p < 0.05$). Expression of PDX-1 is only significantly different between spheres and adherent cells.

(B) PANC-1 paraffin-embedded spheres were stained for CK-19; bar=1 μm . (C) CK-19 staining of PANC-1 adherent cells in chamber slides; bar= 5 μm . (D) CK-19 differentiation experiments with PANC-1 spheres growing in differentiation medium for 11 day; bar= 1 μm . (E) Representative image of PDX-1 immunostaining of PANC-1 spheres; bar=10 μm .

Figure 4: CXCR4 cell surface expression in PANC-1 adherently-growing cells (5A; 6,4%) and spheres (5B; 19.4%) using FACS analysis. All results are expressed as mean of three independent experiments. (5C) Transwell migration assay of PANC-1 spheres compared to adherently-growing cells. Average % of cells (normalized to DMEM serum-free medium as control) that migrated after 48h from the upper to the lower chamber is shown. The medium in the lower chamber was contained the following chemo-attractants: DMEM serum-free medium (as control ctrl); complete CSM (CSC); Hepatocyte Growth Factor at 200ng/ml (HGF); DMEM serum free medium supplemented with Stromal Derived factor 1 alpha (200ng/ml)(SDF) and DMEM supplemented with 10% FBS (10% FBS). Statistical significant (t -test *, $P < 0.05$) differences were observed between ctrl and SDF.

Figure 5: Hematoxylin & Eosin and Ki67 immunohistochemical stainings: comparison in tumours generated from PANC-1 adherently growing cells shown in A (bar= 10 μm) and C (bar= 20 μm) and from PANC-1 spheres shown in B (bar = 10 μm) and D (bar= 20 μm). Hematoxylin & Eosin show that sphere cells (A) generate a tumour with a higher cell pleomorphism as compared to that generated from adherently-growing cells (A). Tumours from sphere cells also proliferate at a higher rate as shown by Ki67 (a cellular marker strictly associated with cell proliferation) immunopositive nuclei are visualized in adherent cells (C, 40% positive nuclei) and in spheres (D, 70-80% positive nuclei).

Figure 6: Putative metastasis (black arrows), derived from spheres growing cells, were found in small intestine (A) and liver (B).

Fig. 1

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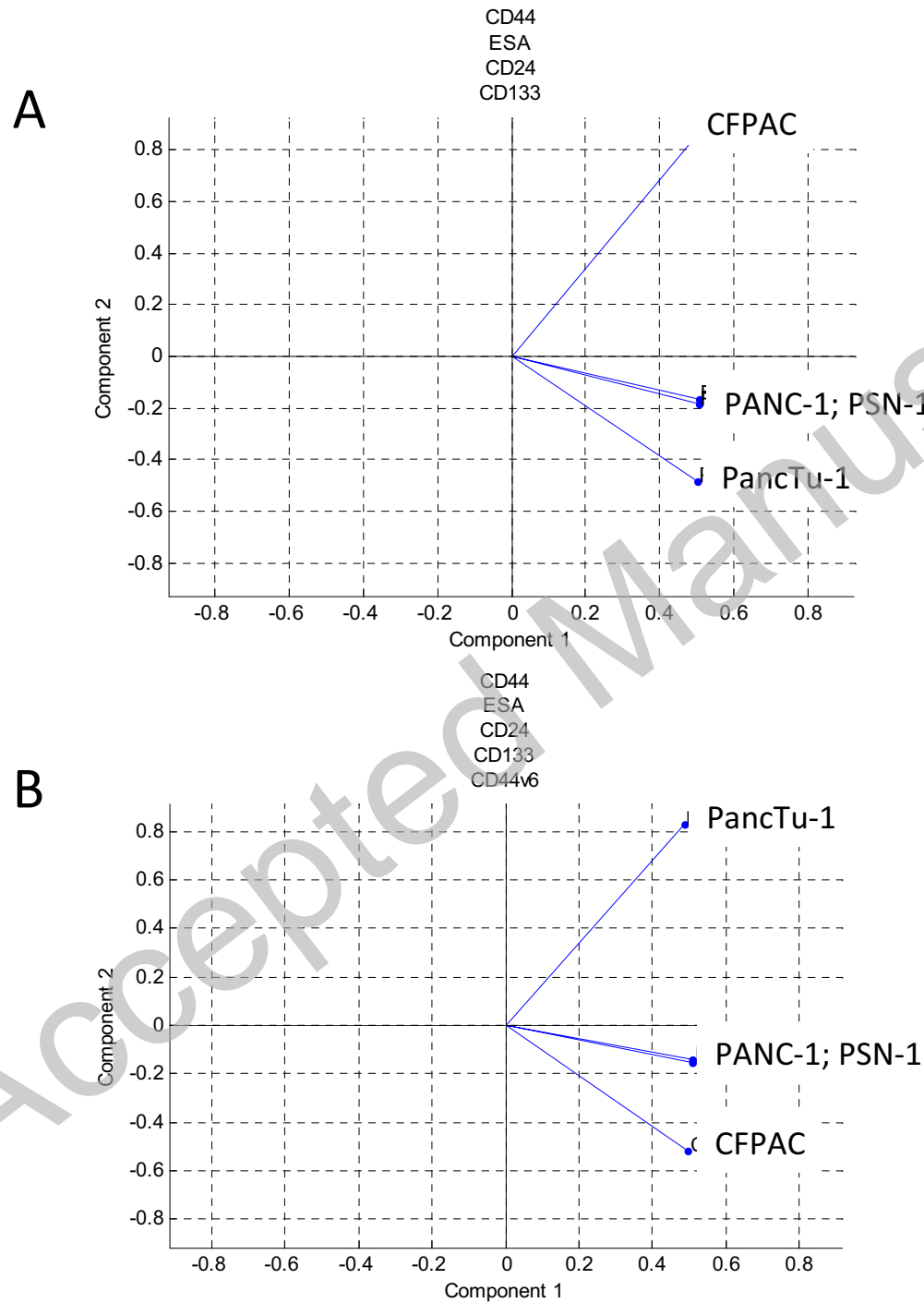
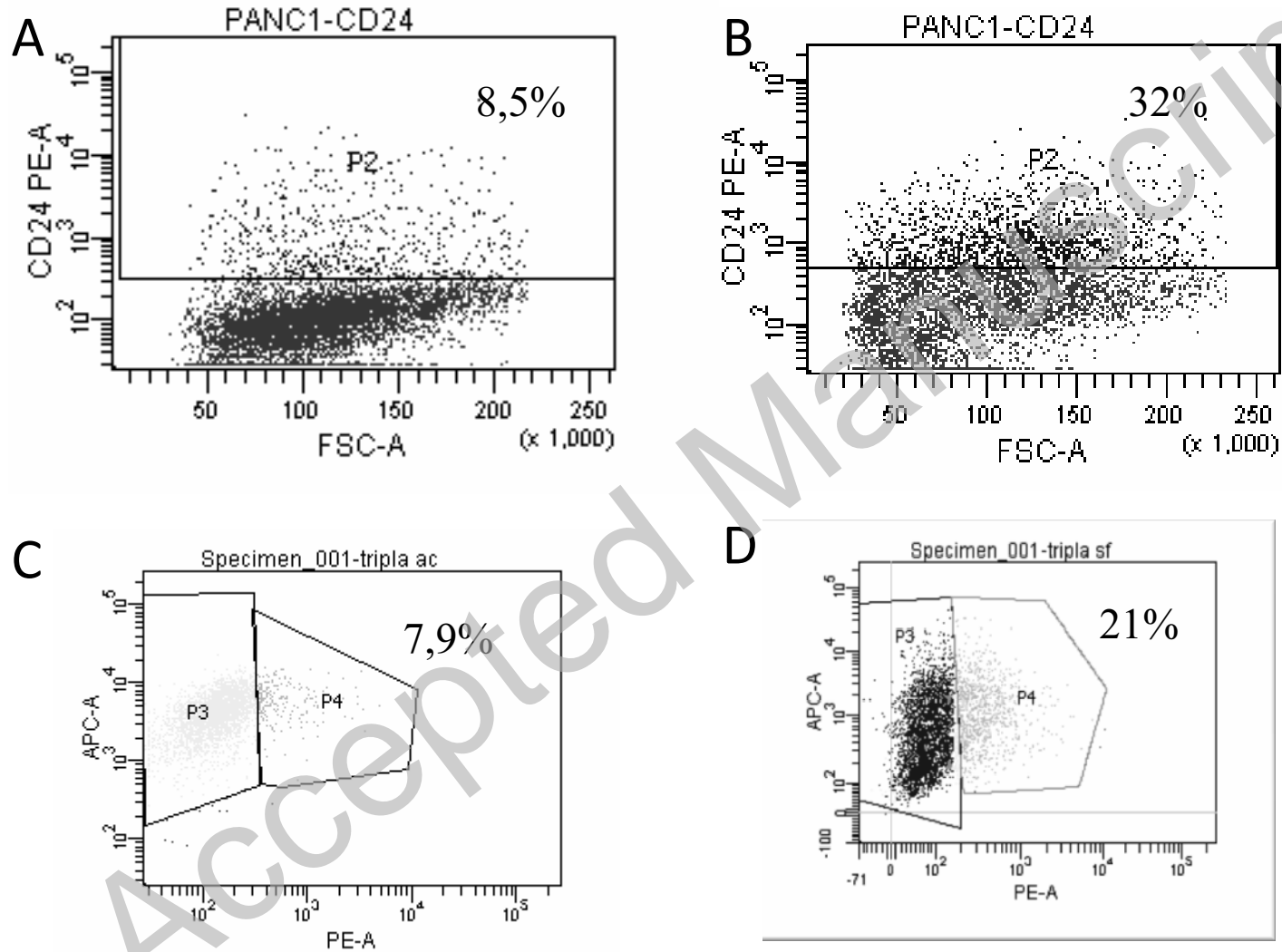
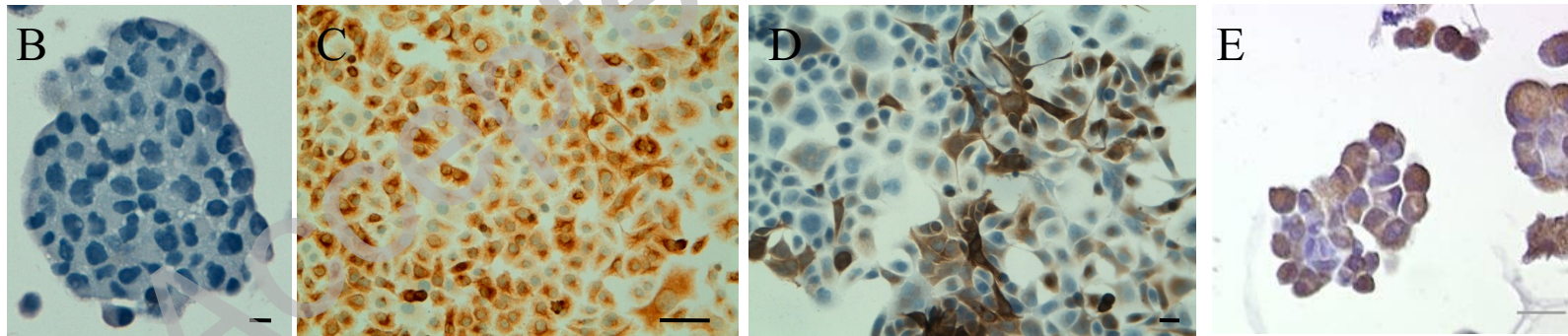
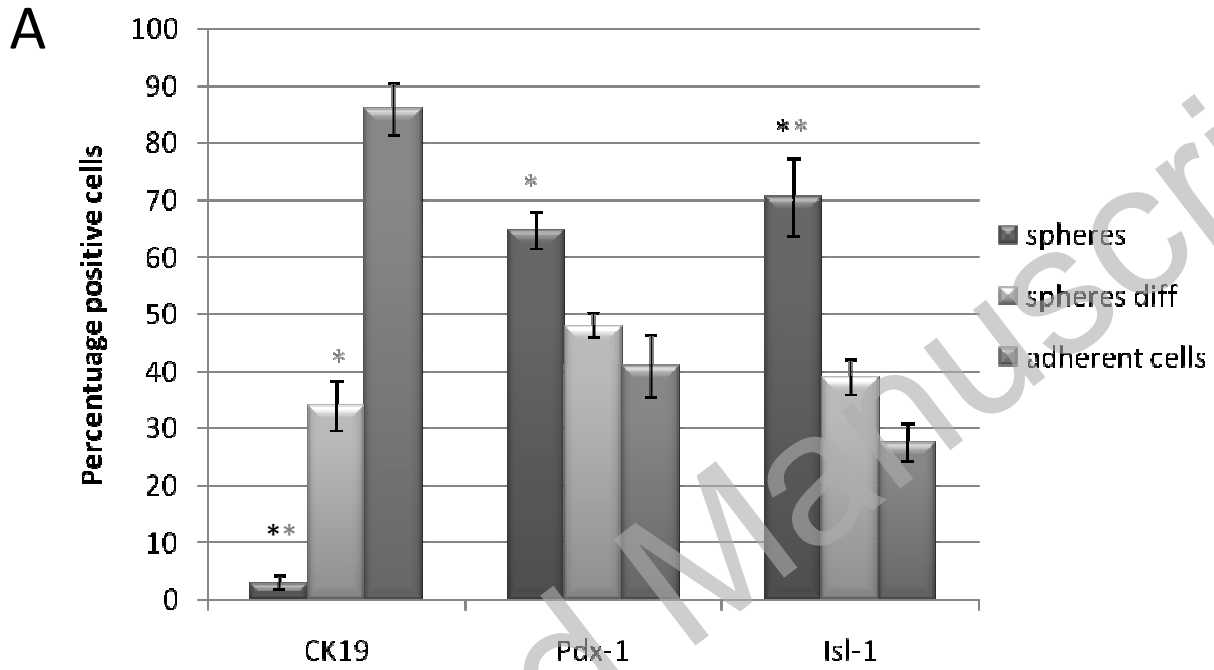


Fig. 2



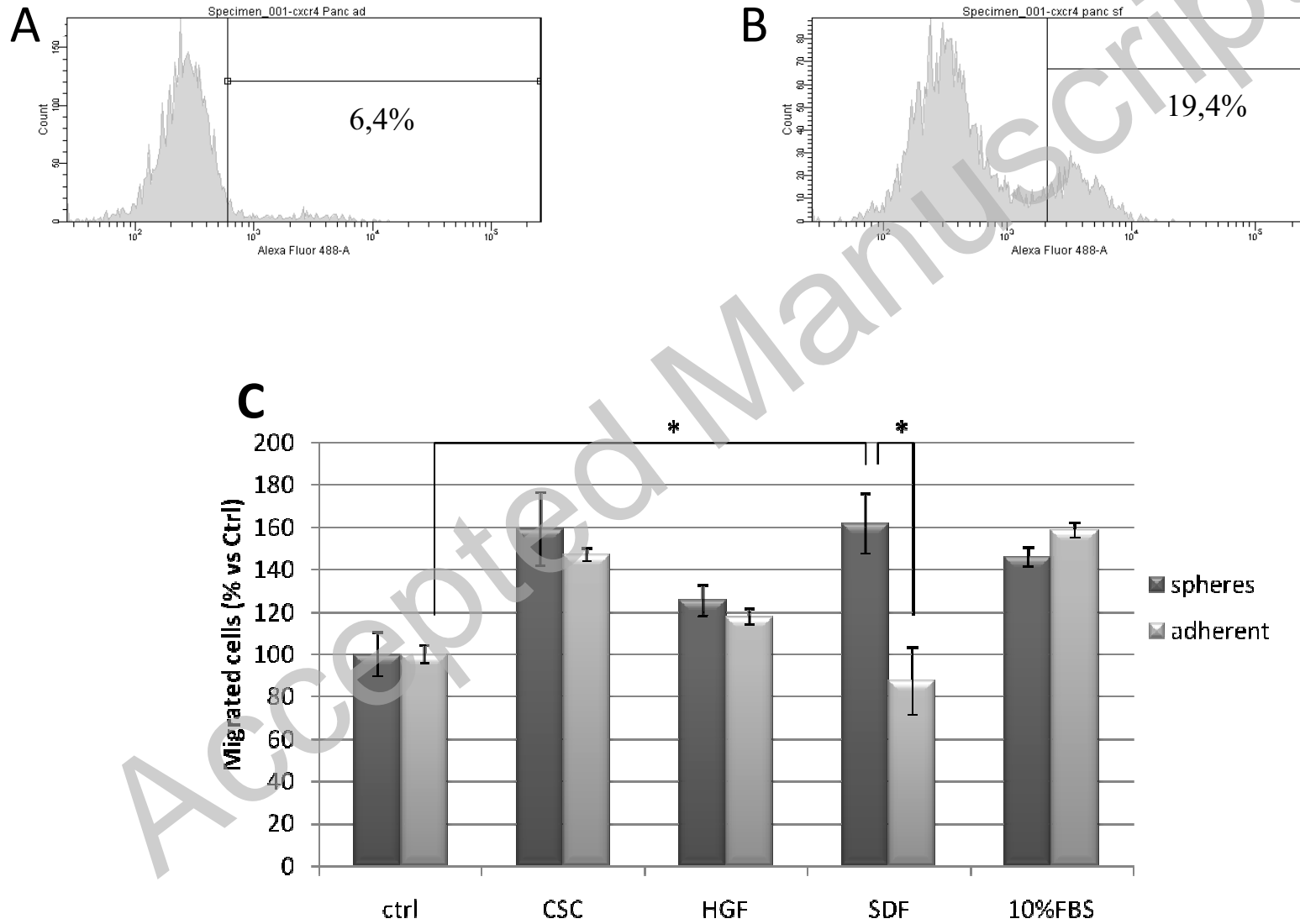
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Fig. 3



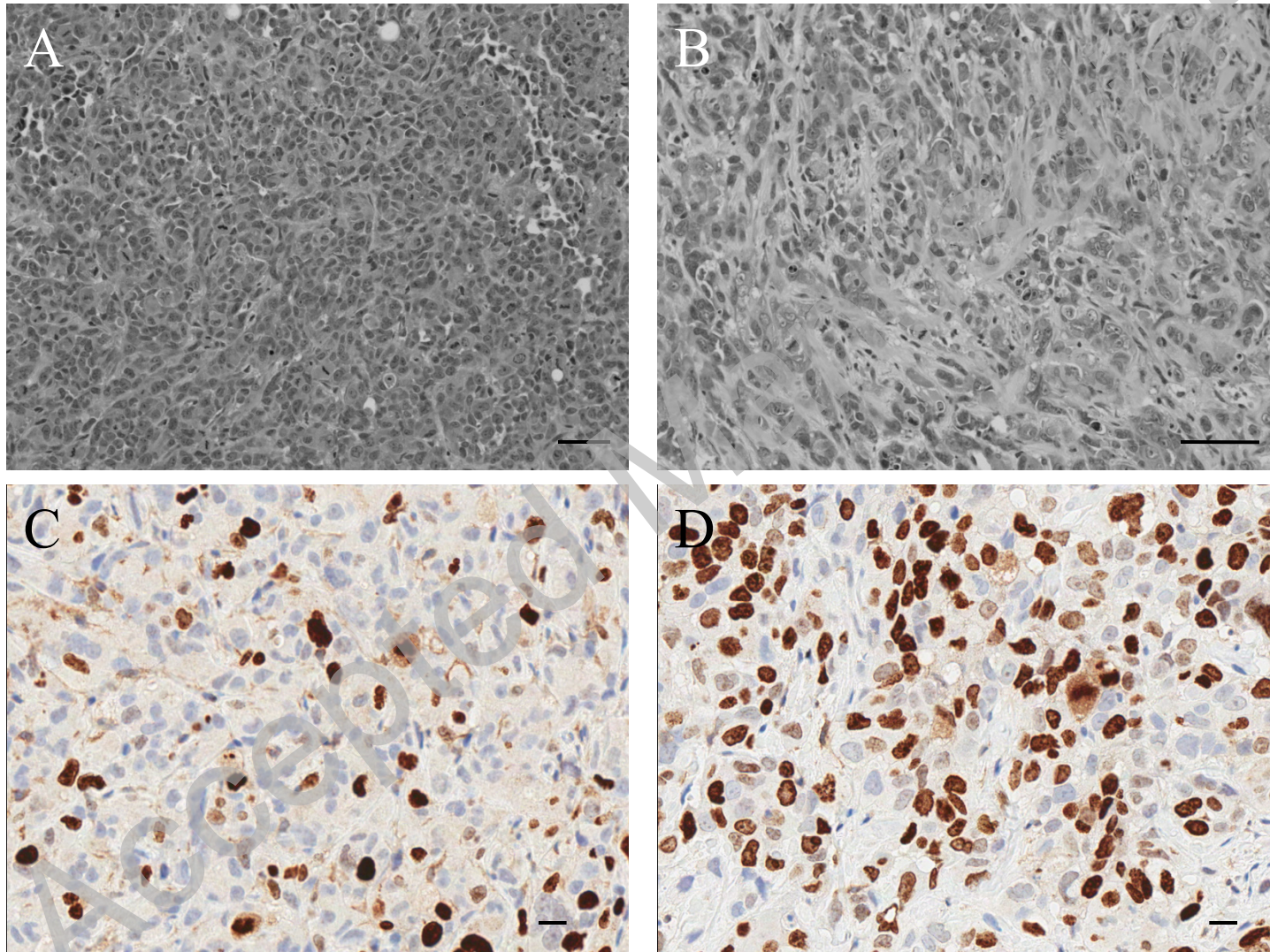
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Fig. 4



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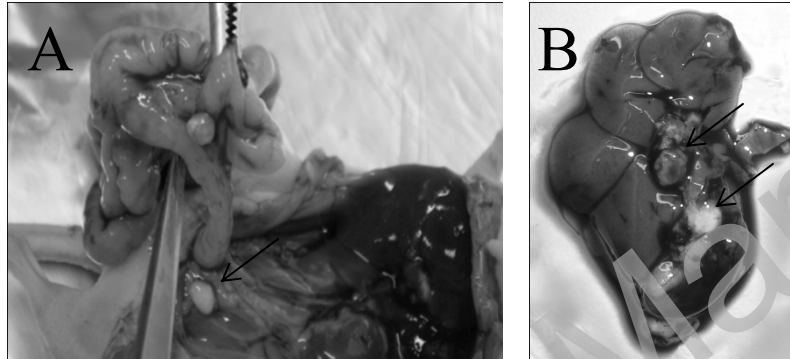
Fig. 5



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Fig. 6



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