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Establishment of PANDA - a new human pancreatic ductal adenocarcinoma cell line with 3D cell culture technology

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Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive type of malignancy with one of the worst prognoses amongst any type of cancer. Surgery is applicable only to the limited number of patients with locally resectable tumors and currently represents the only curative treatment option. Treatment with chemotherapy and radiotherapy can only extend patient survival. Despite advances in conventional therapies, the five-year survival of PDAC remained largely unchanged. New *in vitro* and *in vivo* models are therefore urgently needed to investigate this type of cancer. Here, we present the establishment and characterization of a novel pancreatic cancer cell line, isolated from a patient with PDAC. Cell line abbreviated as PANDA (PANncreatic Ductal Adenocarcinoma) was established with an optimized 3D culture protocol published previously by our group. The new cancer cell line "PANDA" represents a novel *in vitro* approach for PDAC cancer research and new therapy testing.

Key words: pancreatic ductal adenocarcinoma, cancer cell line, in vitro models, 3D culture

Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive type of cancer that is predicted to be the second most common cause of death within the next 10 years [1]. Despite advances in conventional therapies and diagnostic procedures, the prognosis is poor and the five-year survival of PDAC remained largely unchanged to date [2, 3]. Surgical resection of the primary tumor that is applicable only to the limited number of patients with locally resectable tumors currently represents the only curative treatment option [4]. However, the tumors in the pancreas have a high tendency to metastasize to the liver, as the liver milieu seems to be supportive for the engraftment of disseminated PDAC cancer cells [5]. The lack of adequate and fully characterized *in vitro* cell models to study human pancreatic tumors represents a major impediment towards the development of new therapies. The use of already established (2D) cell lines is associated with several problems. In particular, the overpassaged cells typically gain spontaneous mutations and frequently change their original phenotype [6,7]. For pancreatic ductal adenocarcinoma, several (2-dimensional) cancer cell lines are available at the global biorepository centers (e.g., ATCC), or cell banks [7], some of them are already used for decades. Cells grown on 2D monolayers currently represent a standard approach for *in vitro* modelling and drug testing. However, cancer cells cultured in 3D environment resemble more closely *in vivo* tissue architecture and environment. Cancer cells grown in form of 3D more accurately recapitulate the *in vivo* structure of tumors and therefore are more suitable for experimental drug testing [8–10]. The development of new 3D cancer models also fulfills the goals of worldwide efforts in replacement and reducing the number of animals used in research experiments. New 3D cancer

cell lines are therefore urgently needed in the field. Here, we present a new human pancreatic cancer cell line established with an improved 3D culture protocol, published previously by our team [11]. The cell line can be grown in 3D as well as in 2D conditions. Characterization of cell line abbreviated as „PANDA" (PANcreatic Ductal Adenocarcinoma) using several experimental approaches (ICC, IHC, flow cytometry, western blot analysis, and *in vivo* and *in vitro* assays) is shown in this manuscript.

Material and methods

Clinical case. Primary tumor tissue was taken from a 64-year-old male patient who underwent a total duodenopancreatectomy for pancreatic adenocarcinoma. Histopathological examination of the surgical specimen confirmed "high-grade" G3 ductal adenocarcinoma of the pancreas, which was staged pT2pN2 (19/4), L1,V1, Pn1, R0 ≤1mm. The patient did not undergo oncological treatment due to the generally poor condition. Six months later PET/CT examination revealed suspect MTS sites in the liver, abdominal wall, and around truncus coeliacus. The patient died 7 months after primary surgery.

Establishment of PANDA cell line. An experimental study was approved by the Ethics Committee of Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Slovak Republic. An informed consent letter was obtained from the patient before tumor removal and sample processing. The patient agreed to donate part of the tumor (residual tissue from histopathological examination of cancer) for research purposes. The viable tumor tissue (residual tissue from histological examination) was placed into a 50 ml Falcon tube containing DMEM/F12 Glutamax medium (Gibco, USA) supplemented with antibiotic-antimycotic supplement (Gibco, USA) and stored in the refrigerator. Tissue was then transferred to the laminar hood and processed as described previously [12]. Briefly, cell suspension isolated with collagenase IV was mixed with an ice-cold suspension of CSC medium and Matrigel (3:1 ratio) and plated on ultra-low attachment plates (Corning, USA). The next day, an extra small amount (1–2 ml) of CSC medium (for complete recipe for this medium see reference [12]) was added to the wells with solidified Matrigel:CSC medium mixture. Seven to twelve days after plating, the formation of spheroids was detected (Figures 1A–C). After 4 weeks of culture, formed 3D spheroids were isolated with dispase and accutase enzyme treatment and re-plated in fresh Matrigel:CSC medium mixture or transferred directly into normal culture plates for the establishment of a 2D culture of cancer cells. To increase the efficacy of attachment, some plates were coated with hESC-qualified Matrigel (Corning, USA). Epithelial cells surrounding spheroids attached to the plates started to appear approx. 7 days after attachment of spheroids (Figure 1D). Cells were expanded for several days and epithelial cell colonies were manually scratched and transferred by 1 ml pipette tip into fresh plates to get rid of the stromal cells. The manual selection was found to be faster and less stressful compared to FACS sorting. Cancer cells (containing no stromal cells with typical mesenchymal phenotype) were then further expanded (Figure 1E).

Karyotype analysis. For karyotype analysis, cells at approx. 70% confluency were treated with colchicine (Gibco, USA) for 2 hours in an incubator at 37 °C, then trypsinized, and stored in form of single-cell suspension on ice. The sample was then submitted to the Department of Clinical Genetics for karyotype analysis of G-banded chromosomes.

Cell viability assays and growth curve analysis. To test the sensitivity of PANDA cells to gemcitabine, a drug commonly used in the therapy of PDAC, cells at confluency around 70% were incubated with several different concentrations of the drug. Briefly, cells were plated on culture dishes at a concentration of 250,000 cells/well, and the 2D cell culture medium (DMEM/F12 Glutamax, 1% P/S, and 10% FBS) containing drug was added after 24 hours to the culture. Three days after drug application, cells were trypsinized and counted on an automatic Bio-Rad cell counter. Experiments were done in triplicates and representative results from three independent experiments are shown.

Sequencing experiments (NGS). The test was performed using the Illumina TruSight™ Oncology 500 (TSO500) (Illumina, USA) targeted hybrid-capture based next-generation sequencing assay on a NextSeq 550DX (Illumina, USA) instrument in RUO mode. Unique molecular identifiers (UMI) were used to enable the detection of variants at low VAFs (variant allele frequency) with a high degree of sensitivity and specificity. The data were analyzed using the Illumina Software TSO500 v2.0.0 Local App and a customized analysis pipeline within the Clinical Genomics Workspace software platform from PierianDx (Supplementary Figure S1).

Immunocytochemistry. Newly established PANDA cancer cells were grown on plastic microscopy chamber slides (Ibidi, USA) in DMEM/F12 Glutamax medium containing 10% FBS and 1% Pen/Strep. When confluency of cells reached approx. 60–70%, the culture medium was removed, cells were gently washed with PBS (pH 7.2 Gibco, USA), and fixed for 10 minutes with 4% buffered paraformaldehyde. After fixation, cells were washed twice with PBS and blocked for 1 hour in blocking buffer, containing PBS, 5% goat serum, and 0.3% Triton X-100. Incubation with primary antibodies was done overnight at 4 °C in the refrigerator; primary antibodies were diluted in blocking buffer. The next day, the cells were rinsed three times with washing buffer (containing PBS, 5% goat serum, and 0.3% Triton X-100) with a 5-minute soaking time between washes. Secondary antibody (goat anti-mouse or goat anti-rabbit antibodies), labeled with a fluorescent dye and prepared in blocking buffer was added to the cells and incubated for 1 hour at laboratory temperature. Both primary and secondary antibodies were diluted in blocking buffer as

recommended by the manufacturer. The list of antibodies used in the experiment (at the concentrations according to the manufacturer's recommendations) is part of Supplementary Table S1. Images were taken with an Olympus IX71 fluorescent microscope and processed with ImageJ software.

Flow cytometry analysis. For flow cytometry analysis, cultured cells were rinsed with sterile PBS, then trypsinized with 0.25% trypsin (Gibco, USA), filtered through 40 μm cell mesh (to get rid of cell clumps), and counted on BioRad automatic cell counter. The total number and viability of cells were recorded. Approximately 1×10^6 cells in 100 μl of FACS buffer (containing PBS, 1% mouse serum, and 0.001 mM EDTA) was used for staining with antibodies, conjugated to a fluorescent dye (FITC, PE, or APC). Briefly, cells were first blocked in FACS buffer for 30 minutes on ice and then stained with primary antibodies (1 hour, on ice, in dark) in 5 ml flow cytometry tubes. After incubation, cells were washed with 5 ml of PBS, centrifuged, and re-suspended in 400 μl of FACS buffer. Single-channel analysis of surface marker expression was performed with FACS ARIA II flow cytometer and sorter (BD Biosciences, USA). Dead cells and debris were excluded from the analysis by gating. Results and expression profiles were prepared using FACS Diva analysis software. The list of antibodies used in the experiment (at the concentrations according to the manufacturer's recommendations) is part of Supplementary Table S1. Staining for SSEA-4 was performed in freshly fixed cells according to the manufacturer's protocol with the H/M Pluripotent Stem Cell Multi-Color Flow Cytometry kit from R&D Systems.

Western blot analysis of protein expression. The expression of selected proteins was confirmed with western blot analysis. Briefly, cells in the pellet were washed twice with sterile PBS and stored at –80 °C until analysis. Protein lysates were prepared from pellets with RIPA buffer (Cell Signaling, USA), supplemented with Complete Protein Inhibitor Cocktail (Roche, Germany) according to the protocol, provided by the manufacturer. The concentrations of proteins in protein lysates were measured with the BCA Protein Assay (Pierce, USA). Fifty micrograms (50 μg) of total protein were loaded per well in 3–15% gradient gels (BioRad, USA) for electrophoretic separation. The list of antibodies used in the experiment (at the concentrations according to the manufacturer's recommendations) is also part of Supplementary Table S1. Signals were recorded with the chemiluminescence detection mode with a CCD camera (BioRad, USA).

DNA fingerprinting. To confirm the originality of the new cancer cell line and to exclude possible cross-contamination with other cell line(s), the DNA fingerprint profile was analyzed with a commercially available STR profiling service (ATCC, USA). The sample was processed using the ABI Prism® 3500xl Genetic Analyzer and data analyzed using GeneMapper® ID-X v1.2 software (Applied Biosystems). The test results for PANDA cells showing eight-core STR loci plus Amelogenin ATCC reference database profile are shown in Supplementary Figure S2.

Antibody array. For detection of expression of selected transcription factors, the Stem Cell Antibody Array Membrane System (R&D Systems, UK) was used. Briefly, protein lysates were prepared and incubated with membranes according to the manufacturer's protocol. For the chemiluminescence signal detection, Kodak Blue X-ray films were used and scanned subsequently with a high-resolution scanner. Images were processed with ImageJ software.

Mycoplasma testing. The cell line was tested for the presence of several Mycoplasma species using a commercially available PCR kit (ATCC, USA). The cell lysate was prepared and subsequent analysis with standard PCR method was performed in accordance with the manufacturer's protocol.

Xenograft experiments. Briefly, to test the *in vivo* tumorigenic potential of the newly developed PANDA cell line, cells (tested negative for 16 Mycoplasma species with commercial PCR kit), were transplanted (xenografted) into Crl:NU(NCr)-Foxn1nu 6-week-old male athymic nude mice (The Jackson Laboratory, USA). The cell suspension (100 μl of total volume) containing 5×10^6 cells in a mixture of ice-cold, sterile PBS (pH 7.4) and 50% Matrigel (Corning, USA) was prepared and kept on ice and applied subcutaneously with precooled syringe into right or left flank of nude mice. Tumor formation was detected 2–3 weeks after transplantation. Tumors with approx. size ~1 cm were extracted from animals euthanized by cervical dislocation and submitted for pathological evaluation to confirm the histological similarity with an original tumor. All experiments were performed in accordance with the institutional guidelines for animal care and approved by the Ethical Review Committee at Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Slovak Republic.

Histology. Extracted tumors were measured, rinsed with PBS buffer (Gibco, USA), and immediately placed into 10% buffered paraformaldehyde (Cell Signaling, USA) for fixation. Tissue samples were fixed for 3 days, then dehydrated in a series of graded ethanol, and embedded in paraffin. Paraffin blocks were sectioned into 5 μm tissue sections, mounted onto glass slides, and stained with Hematoxylin/Eosin (H/E).

Targeted metabolomic analysis of cell lysates. Targeted quantitative metabolomic analysis of intracellular metabolites was performed using cell lysates of 2D and 3D cell cultures, respectively. The cell lysates were prepared following the standard operating procedure (SOP) for cell lysis provided by the manufacturer of the metabolomic kit (see details below). Briefly, after the removal of the media, 2D and 3D cultures were washed twice with ice-cold PBS and lysed in ice-cold ethanol/0.01 M phosphate buffer (85:15, v/v) using 25 μl of lysis buffer per 10^6 cells. Subsequently, three sequences of sonication in an ice bath (3 min) followed by a freeze-thaw cycle (liquid nitrogen for 30 s) were done. Finally, the sonicated samples were centrifuged (5 min, 4 °C, 31,500×g) to collect the cell lysates, which were stored at –80 °C until metabolomic analysis (10 μl of the cell lysates were used for analysis). The metabolomic characterization of prepared cell lysates

was achieved by the AbsoluteIDQ p180 kit (BIOCRATES Life Science AG, Austria). The p180 kit enables simultaneous determination and quantification of 185 metabolites from six classes (21 amino acids, 21 biogenic amines, 40 acylcarnitines, 88 glycerophospholipids – consisting of lysophosphatidylcholines and phosphatidylcholines, 14 sphingolipids and a sum of hexoses – including glucose). The kit was analyzed by the liquid chromatograph ACQUITY UPLC™ I-Class (Waters, Czech Republic), composed of flow-through-needle sample manager (FTN-SM), binary solvent manager (BSM) pump, and column manager (CM) coupled with XEVO TQ-S triple quadrupole mass spectrometer (Waters, Czech Republic). Analytes from amino acid and biogenic amine classes were analyzed by the liquid chromatography-tandem mass spectrometry (LC-MS/MS) after derivatization with phenyl isothiocyanate (PITC) and separated on the ACQUITY UPLC™ BEH C18 (2.1 mm × 75 mm, 1.7 μm) column fitted with a VanGuard pre-column (Waters, Czech Republic). The rest of the analytes were determined by flow injection analysis-tandem mass spectrometry (FIA-MS/MS) – without derivatization and separation. The MS/MS signals were integrated using MassLynx software version 4.1 (Waters, Czech Republic), and metabolites' final concentrations were automatically calculated using MetIDQ™ software – version Oxygen (BIOCRATES Life Science AG, Austria). Detailed information and a list of analytes were described in our previous study [13]. During the metabolomic analysis, the QC2 level sample was measured four times to evaluate the coefficient of variation (CV) for each metabolite, which was considered as a marker of the reliability of measurement during analysis (metabolites with CV≥20% were excluded). Limit of quantification – LOQ (for amino acids and biogenic amines) and limit of detection – LOD (for acylcarnitines, glycerophospholipids, sphingolipids, and hexoses) were other criteria for exclusion. Metabolites with values under LOQ and LOD in both samples, respectively, were excluded from the analysis.

Results

Establishment of PANDA cell line. We have successfully generated a new pancreatic carcinoma cell line (PANDA) from resected tumor tissue from a 64-year-old patient diagnosed with pancreatic ductal adenocarcinoma (Figures 1A–B). The cell line was generated with the previously published protocol designed by our group. The protocol combines the advantages of the 3D culture of cancer cells with a standard, user-friendly 2D culture protocol. Initially, the cell suspension isolated with collagenase IV treatment of tumor sample is embedded in Matrigel-media matrix and cultured in 3D, in form of spheroids. During passaging, some spheroids were collected and embedded in the matrix, while some were left to grow in form of free-floating spheroids (Figure 1C). The 3D culture conditions preferably support the growth of cancer cells while the proliferation of contaminating stromal cells (fibroblasts) is suppressed. This approach is also widely used in stem cell research for removing the feeder-layer cells (mouse embryonic fibroblasts) from iPSC colonies. After a few passages in 3D, cancer spheroids were re-plated on 2D plates. Cells with epithelial phenotype started to appear around attached spheroids. Some remaining contaminating fibroblasts or stromal cells (Figure 1D) were still detected in culture; therefore, manual dissection of cancer cell colonies was performed. This approach led to the isolation of stromal cell-free culture of cancer cells with the polygonal epithelial phenotype (Figure 1E). Flow cytometry analysis indicated that the CD90+ cell population (stromal cells) disappeared after manual dissection of cancer cell colonies followed by passaging (Figures 3-B4).

Immunocytochemistry, immunohistochemistry, and flow cytometry analysis. We have confirmed the epithelial phenotype of the PANDA cancer cell line with bright field microscopy, immunohistochemistry, and flow cytometry. *In vitro* cultured cancer cells stained positive for Cytokeratin-17 and 19 (Figures 1F–1J), while surrounding stromal cells that were present in early passages were negative. PANDA cells were found to express PDX-1 (Figures 1K–1N and Figures 1S, 1T) and PAX-6 (Figure 1O), but were negative for SOX-2 (Figures 1P, 1R).

The histology of the original patient's tumor (Figures 2-II-B and 2-II-C) showed positivity for Cytokeratin-17 (Figure 2-II-D) and Cytokeratin-19 (Figure 2-II-E). PANDA cells that were isolated from the original tumor, when injected into athymic nude mice (Figure 2-II-F) developed tumor (Figures 2-II-G, 2-II-H, and Figures 2-II-N, 2-II-O) that showed expression of Cytokeratin-17 (Figure 2-II-L), Cytokeratin-19 (Figures 2-II-I, 2-II-J), and Cytokeratin-7 (Figure 2-II-M). Focal expression of Mucin-4 was also detected (Figure 2-II-K) in xenografts.

Flow cytometry analysis (Figure 3) of PANDA cells showed high expression (69.7%) of the epithelial cell-adhesion molecule (EpCAM, or CD326), CD24 (65.7%), and CD9 (95.0%). The similar expression pattern was found for CD166 (41.0%) and CD151 (71.0%). Mild expression was detected for CD44 (10.9%) and CD133 (7.1%) markers. Negligible expression was found for CD56, CD90, and CD109 surface markers (0.43%, 0.8%, and 0.3%, respectively). FACS analysis also showed the expression of SSEA-4 (marker associated with cancer stem cell phenotype) (Figure 3).

Triple staining for the CD24+CD44+CD133+ population (Figure 3F, considered by some studies to be a population of cancer stem cells) was also performed, and around 7.1% of cells stained positive for all three markers.

Western blot. Western blot analysis (Figure 1W) of lysed cells proved the expression of Nanog, Keratin 17, Keratin 17/19, and PanCadherin in cell lysates prepared from PANDA cells. Antibody targeting mutated KRAS (G12D mutation) did not detect any specific band suggesting that PANDA cells do not contain this mutation (this was in accordance with sequencing experiments that showed p.G12V mutation).

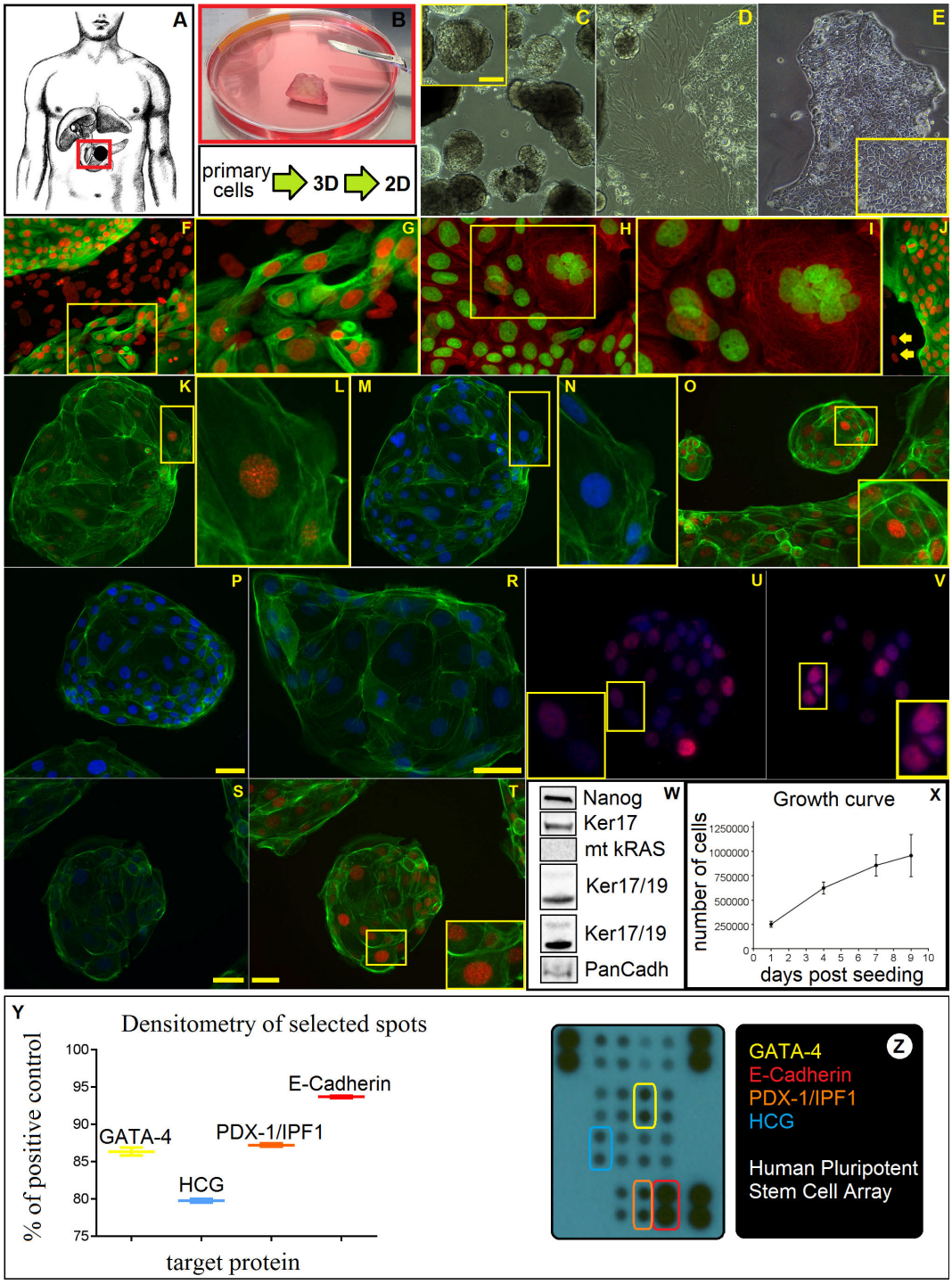

Figure 1. Derivation and *in vitro* characterization of the cancer cell line. The tumor was obtained from a 64-year-old male patient who underwent total duodenopancreatectomy for pancreatic adenocarcinoma (A) and processed (B) *in vitro* as described previously [12]. Primary cells that were initially cultured in 3D culture formed spherical tumorspheres (C); after replating on a standard 2D culture plate they attached and spread in form of cancer cells (D). Note some remaining stromal cells surrounding the colonies of epithelial cancer cells (D). Cancer cells purified by manual colony-picking and replated without contaminating stromal cells (E). Immunocytochemistry images showed colonies of PANDA cells stained positive for Keratin 17/19 (F–J). Yellow arrows indicate negative stromal cells (J). PANDA cells were found to express PDX-1 (K–N and S, T) and PAX-6 (O), but were negative for SOX-2 (P, R). Western blot analysis (W) of lysed cells proved expression of Nanog, Keratin 17, Keratin 17/19, and PanCadherin. Antibody targeting mutated KRAS (G12D mutation) did not detect any specific band (this was in accordance with sequencing experiments showing p.G12V mutation). Curve (X) showing the growth characteristics of PANDA cells. Antibody array protein chip shows relatively high expression of GATA-4, EpCAM, PDX-1, and HCG proteins in PANDA cell lysates (Y, Z).

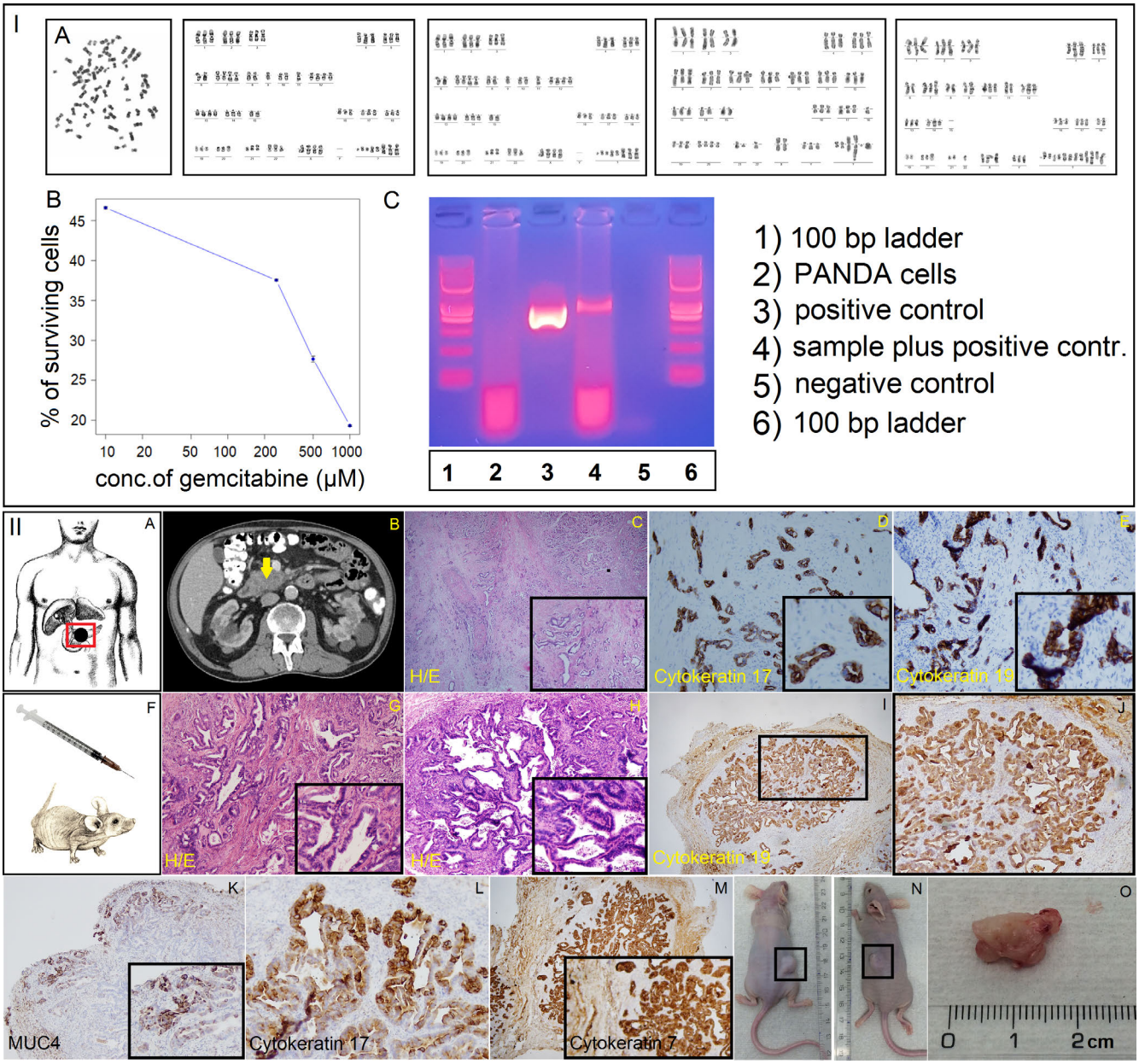

Figure 2. Karyotype analysis of PANDA cells. The analysis of G-banded chromosomes derived from cultured PANDA cells showed the complex hypertriploid male karyotype (I-A). Many chromosomes were affected by numerical and structural changes. Whole chromosome losses, including loss of chromosome Y, were more frequent than gains (I-A). Sensitivity of PANDA cells to gemcitabine (Figure 2I-B) shown on the graph. PCR testing for multiple Mycoplasma species shows PANDA cells to be negative for Mycoplasma infection (I-C). A computed tomography scan image shows localization of pancreatic tumor from a patient with pancreatic ductal adenocarcinoma (II-B). Histology of the original patient's tumor (II-B and II-C) showed positivity for Cytokeratin-17 (II-D) and Cytokeratin-19 (II-E). PANDA cells that were isolated from the original tumor, when injected into athymic nude mice (II-F) developed tumors (II-G, II-H and II-N, II-O) that showed expression of Cytokeratin-17 (II-L), Cytokeratin-19 (II-I, II-J), and Cytokeratin-7 (II-M). Focal expression of Mucin-4 was also detected (II-K) in xenografts.

Growth curve analysis. The growth curve was prepared from cells growing in triplets up to 9 days from seeding. The doubling time of PANDA cells was estimated to be around 36 hours as verified by the growth curve analysis (Figure 1X). To date, the PANDA cells have undergone more than 24 serial transfers (passages).

Antibody array. The stem cell antibody array (RND Systems, UK) used for detection of selected proteins and transcription factors indicated GATA-4, E-Cadherin, PDX-1, and HCG to be expressed significantly in PANDA cells (Figures 1Y–Z). All these factors were found to be connected with the pathology of pancreatic cancer.

Mutation analysis. Using a cut-off of 3% for variant allele frequency, KRAS mutation (p.G12V) was detected in the cell line sample. When also considering low-frequency variants, TP53 mutations were also found (Supplementary Figure S1).

Karyotyping experiments. The analysis of G-banded chromosomes derived from the cultured PANDA cells showed the complex hypertriploid male karyotype. Karyotype was as follows: 73<3n+>,XX,+X,+X,-Y,-6,-7,-7,+der(7)t(7;?)(?;?)x2,-8,-9,-9,-10,-11,-11,+12,+13,-15,-21,-21,+der(21)t(21;?)(p11.2;?)x2,-22,22,+der(22)t(6;22)(p21;p11.2)x2,+8mar. Many chromosomes were affected by numerical and structural changes. Whole chromosome losses, including loss of chromosome Y, were more frequent than gains (Figure 2-I-A). Structurally modified chromosomes included derivated chromosomes resulting from translocations, dicentric, and marker chromosomes. A thorough definition of these complex structural aberrations, designation of marker chromosomes, and breakpoints remained beyond the limits of standard cytogenetic G-banding procedure at the obtained resolution level (300-bands).

STR analysis. The new pancreatic ductal adenocarcinoma cell line (PANDA), authenticated using Short Tandem Repeat (STR) analysis service, was found to be unique as it shows a human profile with no match for any profile in the ATCC STR database. The list of loci (8 core STR loci and Amelogenin) is shown in Supplementary Figure S2.

Targeted metabolomic analysis of cell lysates. As a result, 134 out of 185 metabolites met the criteria mentioned above in Material and methods section. Subsequently, fold change (ratio of the measured value in 3D culture to 2D culture) for each metabolite was calculated to detect quantitative differences in cell metabolome (50% change was set as a threshold). A total of 64 metabolites met the fold change criterion (Supplementary Table S2). We identified 41 metabolites with a decreased concentration in 3D cell culture (mainly acylcarnitines, glycerophospholipids, and sphingolipids). The most reduced metabolites were propenoylcarnitine (C3) – decreased 6.25-fold, butyrylcarnitine (C4) – decreased 5.55-fold, and hexadecanoylcarnitine (C16) – decreased 5-fold. On the other hand, 23 metabolites were increased in the 3D cell culture (mostly from amino acids and biogenic amines classes). Among them, glutamine (Gln) was increased 8.02-fold, followed by symmetric dimethylarginine (SDMA) – increased 8-fold, and ornithine (Orn) – increased 7.75-fold. Besides, the role of selected metabolites in known metabolic

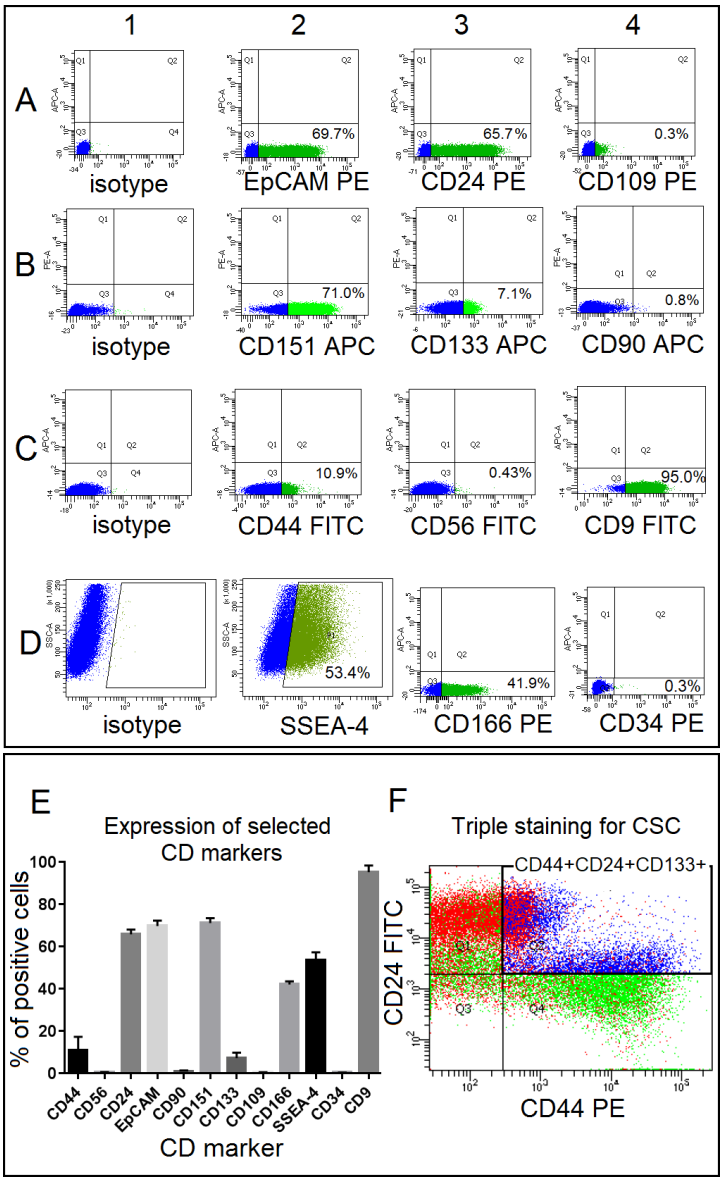

Figure 3. Flow cytometry analysis of cancer cells. A–D) Flow cytometry analyses of selected CD surface markers in PANDA cells. Isotype antibody served as a negative control. Dead cells were excluded from analysis by gating. E) Graph shows a summary of test results from three independent experiments. Triple positive staining for CD24+CD44+CD133+ CSC population (F).

pathways was analyzed using the Pathway Analysis module of MetaboAnalyst 4.0 [14]. Metabolites from glycerophospholipids and sphingolipids classes were not included for pathway analysis due to the lack of functionally relevant information on the detailed fatty acid side-chain compositions (for more information see Ref. [15]). The pathway analysis was performed using the following algorithms: over-representation analysis (ORA) was done with the hypergeometric test and pathway topology analysis was done with relative-betweenness centrality. Kyoto Encyclopedia of Genes and Genome (KEGG) database (version Oct-2019) with Homo sapiens pathway library was selected. Finally, nine altered pathways were

observed with FDR p-value <0.05 (Supplementary Table S3 and Supplementary Figure S3). Out of them, four pathways with the highest impact (the pathway impact value calculated from pathway topology analysis) were visualized with highlighted metabolites annotated with KEGG IDs (Supplementary Figure 4). Our results point to alteration in arginine biosynthesis; alanine, aspartate, and glutamate metabolism; arginine and proline metabolism; and D-glutamine and D-glutamate metabolism.

Discussion

The incidence of pancreatic tumors has increased significantly over the past two decades. Diagnosis of pancreatic ductal adenocarcinoma (PDAC), the most common and most aggressive cancer of the pancreas gives patients little chance for cure, and success of treatment depends on complete surgical removal of the tumor. However, about 80–90% of PDAC patients already show surgically incurable cancer at the time of clinical manifestation. So far, the survival of patients with pancreatic cancer has not improved significantly, which is partially caused by the lack of adequate research models for pancreatic cancer representing a major impediment for the development of new therapies. Therefore, new *in vitro* and also *in vivo* models are required to gain insights into the pathology of this type of cancer. *In vitro* cancer models (cell lines) represent important research tools to investigate disease mechanisms and to screen for new therapies. Ideally, *in vitro* cancer models should replicate the complexity and dynamic nature of the real tumor microenvironment. Conventionally employed 2-dimensional or monolayer cell culture models do not fully reflect these interactions. Although still being used mainly for practical reasons, a selection of drugs that turn out to be ineffective *in vivo* remains a serious problem. For PDAC cancer research, several cell lines utilizing mainly traditional, 2-dimensional model systems have been generated and are provided by ATCC to pancreatic cancer research groups. A major technological breakthrough and an important tool in research and clinical applications are 3D organoid cultures. 3D tumor cell models simulate the *in vivo* tumor microenvironment and potentially eliminate ineffective drugs at the pre-clinical stage. They can bridge the gap between 2D cell culture and *in vivo* experiments and therefore, an increasing number of researchers begin to emphasize 3D tumor cell culture in their research. To the best of our knowledge, a quite limited number of 3D human pancreatic cell lines is available. According to several studies, cancer cells cultured in the form of 3D spheroids gained cancer-stem cell properties including stem cell transcription factors and surface markers expression and showed a higher level of aggressiveness *in vivo* [17]. The development of new 3D cancer models is therefore an important and crucial step towards identifying new and more efficient therapies and reducing the attrition rate of pharmaceuticals [18].

Establishing new cancer cell models is important as already established cell lines are over passaged and gained new mutations that were not present in original cancer tissue. There is therefore a continuous need for the establishment of novel cell lines and their biobanking in cell bank centers. Submission of new cell lines to cell banks enables researchers from other research teams to get access to such models. Cancer cell lines that allow researchers to perform their experiments in 3D culture conditions are especially of interest when considering the advantages of 3D spheroid culture in new therapy testing. Newly generated cancer cell line PANDA is presented by our group to the research community dealing with this deadly type of cancer. The cell line is readily accessible via scientific collaboration and later will be submitted to ATCC for general use by the research community.

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