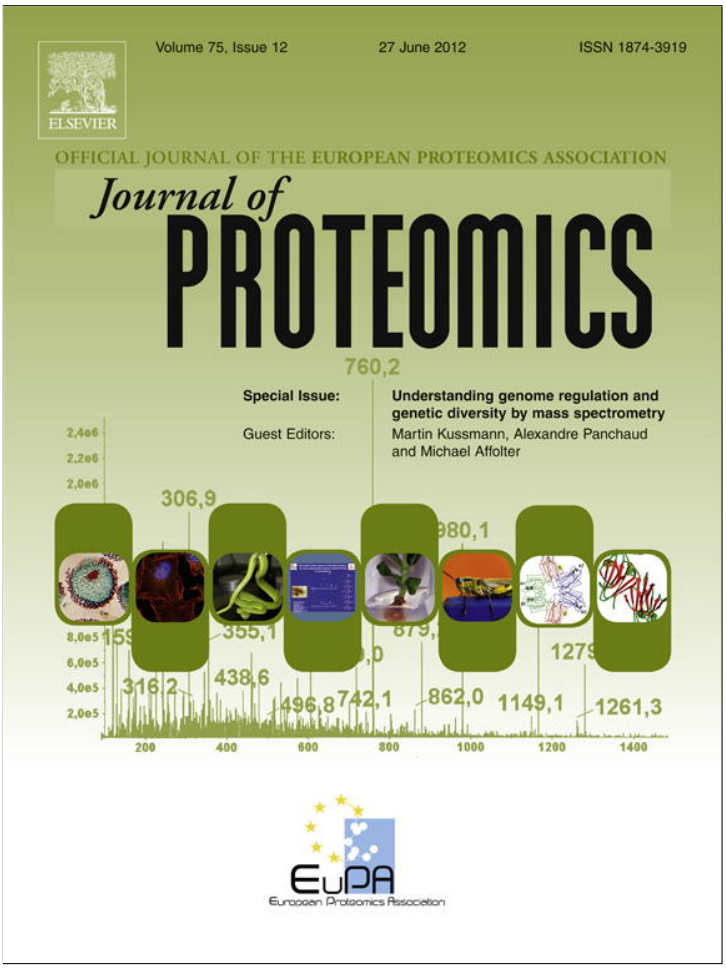

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Immunoassay-based proteome profiling of 24 pancreatic cancer cell lines

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ABSTRACT

Pancreatic ductal adenocarcinoma is one of the most deadly forms of cancers, with a mortality that is almost identical to incidence. The inability to predict, detect or diagnose the disease early and its resistance to all current treatment modalities but surgery are the prime challenges to changing the devastating prognosis. Also, relatively little is known about pancreatic carcinogenesis. In order to better understand relevant aspects of pathophysiology, differentiation, and transformation, we analysed the cellular proteomes of 24 pancreatic cancer cell lines and two controls using an antibody microarray that targets 741 cancer-related proteins. In this analysis, 72 distinct disease marker proteins were identified that had not been described before. Additionally, categorizing cancer cells in accordance to their original location (primary tumour, liver metastases, or ascites) was made possible. A comparison of the cells' degree of differentiation (well, moderately, or poorly differentiated) resulted in unique marker sets of high relevance. Last, 187 proteins were differentially expressed in primary versus metastatic cancer cells, of which the majority is functionally related to cellular movement.

1. Introduction

Pancreatic cancer is one of the most lethal malignancies. Most patients die within a year of diagnosis and only about 3% survive five years or longer [1]. The poor prognosis can be attributed to late presentation, aggressive local invasion, early formation of metastases, and poor response to chemotherapy [2,3]. Additionally, diagnosis by conventional means is very difficult due to the anatomic location of the pancreas [4]. Patients with pancreatic tumours often present themselves with vague complaints of gastrointestinal complications at late stages of the disease. Surgery on such late-stage patients is frequently impossible or of only limited effect [5]. The establishment of procedures for an earlier diagnosis of pancreatic cancer poses a challenge [6] since the appropriate molecular information is lacking. It is nearly impossible to obtain clinical samples from patients with premalignant or early stage malignant disease that may provide this data.

Although ductal cells make up only about 10% of all pancreatic cells and 4% of the pancreatic volume, more than

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90% of human pancreatic cancers are morphologically and biologically consistent with a classification as cells that belong to the ductal cell lineage of the exocrine pancreas [6]. The processes that lead to malignant alterations in the pancreas and the reasons why particularly ductal cells are affected are poorly understood. Current knowledge of the biological properties of pancreatic ductal adenocarcinoma (PDAC) is in part derived from in vitro studies of pancreatic tumour cell lines. Although they are an artificial system, cultured cells provide an important model for studying physiologic, pathophysiologic, and differentiation processes in a controlled manner. An in vivo investigation of individual cellular components can pose a problem in complex tissues. Pancreatic cancer cell lines have been suggested as a good source for the discovery of proteins that may act as biomarkers and drug targets [7]. A substantial number of PDAC cell lines of different characteristics have been established and provide a good material source for investigating some molecular aspects of the devastating disease (comprehensively reviewed in [8]).

Despite the remarkable progress of our understanding of the complex biological processes involved in disease pathogenesis at the level of nucleic acids, our insight into the molecular background of tumorigenesis remains partial. Many regulatory processes actually take place at the protein level. Proteins are involved in basically all vital biological processes and execute many cellular functions. Consequently, about 98% of all current therapeutic agents target proteins. Because of the obvious importance of variations that occur at the protein level, proteomic analyses have been performed on samples from pancreatic cancer patients for the identification of relevant biomarkers or possible therapeutic targets. Gel-based processes and mass spectrometry (reviewed in [9–11]) as well as antibody microarrays were applied. Antibody microarrays have become a robust proteomic tool for high-throughput analyses toward a better understanding of disease biology, its early detection, discrimination of tumours and the monitoring of disease progression [12,13]. The platform also serves as a complementary system to other comprehensive proteomic approaches [14]. The majority of the array-based analyses, however, investigated sera from pancreatic cancer patients [15–20]. While potentially important for early diagnosis, sera analyses have only limited relevance with respect to understanding the particular cellular features of pancreatic cancer.

In this study, the focus was on the identification of cellular variations that could be utilised as molecular evidence for disease characteristics. To this end, we took advantage of an antibody microarray made of 810 antibodies that permits the analysis of the expression levels of 741 distinct proteins [15]. They had been selected on the basis of a thorough analysis of transcriptional profiling data and other available information to be highly associated with the occurrence of pancreatic adenocarcinoma, colon and breast cancer. With this resource, the cellular proteomes of 24 pancreatic cancer cell lines and two control cells were studied. The molecular differences found in the analysis allow conclusions on the degree of cell differentiation, serve as indicator for defining the actual source of the tumour cells, and permit an assessment of their metastatic potential.

2. Experimental procedures

2.1. Chemicals and antibodies

All chemicals used in this study were purchased from Sigma-Aldrich, unless stated otherwise, and were of highest purity or protein grade. In the analysis, a set of 810 antibodies was used as reported earlier [15]. Their respective target proteins and origin are listed in Supplementary Table S1. The majority had been produced on the basis of transcriptional studies on different cancer entities e.g. [21–23], from which targets exhibiting differential expression were selected. For 668 of these targets, affinity-purified, peptide-specific, and polyclonal antibodies from rabbit were produced by Eurogentec (Seraing, Belgium). They were characterized by means such as immunohistochemical analyses. An additional 142 antibodies were purchased from different commercial providers or obtained from collaborating partners. Antibodies supplied as ascites fluid, antisera or with stabilizer proteins were purified using the Nab Protein G Spin Kit (Thermo Scientific, Rockford, USA). The antibodies were concentrated by filtration with Microcon 100 kDa (Millipore, Schwalbach, Germany) and the protein concentration for each antibody was adjusted to 2 mg/ml using Bicinchoninic Acid Protein Assay Reagent kit (Thermo Scientific). The binders were aliquoted and stored at −80 °C.

2.2. Cell culture

In total, 26 cell lines were used in this study (Table 1). All were tested with the Venor GeM Mycoplasma Detection kit (Minerva Biolabs GmbH, Berlin, Germany) and found to be mycoplasma negative. Usually, the cells were grown in IMDM medium (Invitrogen, Darmstadt, Germany) supplemented with 10% foetal calf serum, 50 U/ml penicillin and 50 μg/ml streptomycin at 37 °C and 5% CO_2. Human umbilical endothelial cells (HUVEC) were cultured in complete endothelial culture medium (PromoCell, Heidelberg, Germany). The immortalized pancreatic cell line HPDE [24] was grown in keratinocyte serum free medium supplemented with bovine pituitary extract and epidermal growth factor (Invitrogen).

2.3. Protein extraction

The cellular proteins were extracted using the optimized extraction process described in detail before [25]. In brief, at 90% confluence, cells were washed three times with ice cold phosphate buffered saline (PBS) and layered with the extraction buffer (Hepes-Mix: 20 mM Hepes buffer, pH 7.9, 1 mM $MgCl_2$, 5 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 1% NP-40 substitute, 0.5% sodium cholate, 0.25% n-dodecyl-β-D-maltoside (GenaXXon Bioscience, Ulm, Germany), 0.25% amidosulfobetaine-14, 1 U/μl of Benzonase (Merck Biosciences, Schwalbach, Germany) and Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, Bonn, Germany)). After an incubation on ice for 30 min with occasional mixing, the cells were scraped off and pipetted several times through a fine needle syringe, followed by centrifugation at 20,000 *g* at 4 °C for 20 min. The supernatant was aspirated with a fine needle in order not to disturb the upper layer or the pellet. Protein concentration in the

Table 1 – Cell lines used in the analysis.

#	Cell line	Source of cells	Cell type	Age of donor (years)	Gender	Disease	Class	Differentiation
1	HPDE	–	Ductal	–	–	Normal, immortalized	Exocrine	–
2	HUVEC	Umbilical vein	Endothelial	–	–	Human endothelial normal cell	Endothelial	–
3	BxPC-3	Primary tumour	Ductal	61	Female	Adenocarcinoma	Exocrine	Moderate
4	FAMPAC	Primary tumour	Ductal	43	Female	Adenocarcinoma; cystic fibrosis	Exocrine	Poor
5	IMIM-PC1	Primary tumour	Ductal	–	–	Adenocarcinoma	Exocrine	Moderate
6	IMIM-PC2	Primary tumour	Ductal	–	–	Adenocarcinoma	Exocrine	Well
7	MDA- Panc28	Primary tumour	Ductal and Acinar	69	Female	Adenocarcinoma	Exocrine	Poor
8	MIA PaCa-2	Primary tumour	Ductal	65	Male	Carcinoma	Exocrine	Poor–moderate
9	PANC-1	Primary tumour	Ductal	56	Male	Epithelioid carcinoma	Exocrine	Poor
10	SK-PC-1	Primary tumour	Ductal	–	–	Adenocarcinoma	Exocrine	Well
11	SU.86.86	Primary tumour	Ductal	57	Female	Ductal carcinoma	Exocrine	Moderate
12	Capan-1	Liver metastasis	Ductal	40	Male	Adenocarcinoma	Exocrine	Well
13	Capan-2	Liver metastasis	Ductal	56	Male	Adenocarcinoma	Exocrine	Well
14	CFPAC-1	Liver metastasis	Ductal	26	Male	Adenocarcinoma; cystic fibrosis	Exocrine	Well
15	Suit-2	Liver metastasis	Ductal	73	Male	Adenocarcinoma	Exocrine	Well
16	Suit-007	Liver metastasis	Ductal	73	Male	Adenocarcinoma	Exocrine	Moderate
17	Suit-020	Liver metastasis	Ductal	73	Male	Adenocarcinoma	Exocrine	Moderate
18	Suit-028	Liver metastasis	Ductal	73	Male	Adenocarcinoma	Exocrine	Moderate
19	Colo357	Lymph node metastasis	Ductal	–	–	Adenocarcinoma	Exocrine	Well
20	T3M4	Lymph node metastasis	Ductal	56	Male	–	Exocrine	Moderate
21	A818-1	Ascites	Ductal	75	Female	Adenocarcinoma	Exocrine	Moderate
22	A818-4	Ascites	Ductal	76	Female	Adenocarcinoma	Exocrine	Moderate
23	A818-7	Ascites	Ductal	77	Female	Adenocarcinoma	Exocrine	Moderate
24	AsPC-1	Ascites	Ductal	62	Female	Adenocarcinoma	Exocrine	Moderate–well
25	HPAF-II	Ascites	Ductal	44	Male	Adenocarcinoma	Exocrine	Moderate–well
26	BON-1	–	–	28	Male	Carcinoid tumor	Endocrine	–

supernatant was determined with the Bicinchoninic Acid Protein Assay Reagent kit.

2.4. *Protein labelling*

Extracted protein was labelled as described in detail earlier [15,26] using the fluorescence dyes DY-549 or DY-649 (Dyomics, Jena, Germany) at a molar dye-to-protein ratio of 7.5, with the assumption that 60 kDa is the average molecular weight of all proteins. The protein concentration was adjusted to 2 mg/ml. Labelling occurred in the dark in 0.1 M carbonate buffer, pH 8.5, at 4 °C for 2 h. Unreacted dye was quenched with 10% glycine for 30 min at 4 °C in the dark. Labelled samples were stored at −20 °C until analysis.

2.5. *Antibody microarray printing*

A protocol was used, which has been described in very detail earlier [26]. In short, the antibodies were spotted on epoxysilane-coated slides (Nexterion-E; Schott, Jena, Germany) using the contact printer MicroGrid-2 (BioRobotics, Cambridge, UK) and SMP6B pins (Telechem, Sunnyvale, USA) at a humidity of 40 to 45%. The printing buffer was composed of 0.1 M carbonate buffer, pH 8.5, containing 0.01% Tween-20, 0.05% sodium azide, 0.5% dextran sulphate, 5 mM magnesium chloride, 137 mM sodium chloride, and 1 mg/ml of the respective antibody. On each slide, the antibodies were printed in quadruplicates. After printing, the slides equilibrated at a humidity of 40 to 45% overnight and were stored in dry and dark conditions at 4 °C until use.

2.6. *Sample incubation*

Incubation of the microarrays with labelled samples was performed as reported [26]. Briefly, printed slides were washed once for 5 min followed by another wash for 15 min with PBS containing Tween-20 at a final concentration of 0.05% (PBST20). The slides were blocked with 5 ml of 10% non-fat dry milk (Biorad, Munich, Germany) in PBST20 for 3 h at room temperature using Quadriperm chambers (Greiner Bio-One, Frickenhausen, Germany) on an orbital shaker. Blocked slides were incubated in Quadriperm chambers with 50 μg of DY-649 labelled sample and 50 μg of a DY-549 labelled pool of all samples in 5 ml incubation buffer containing 10% milk in PBST20 in the dark at 4 °C overnight. The slides were then washed four times for 5 min in large volumes of PBST20, rinsed with deionised water, and dried in a ventilated oven at 22 °C. Scanning of slides was performed with a ScanArray-4000XL (Perkin Elmer, Waltham, USA) at constant laser power and PMT. The images were analysed with the software GenePix Pro 6.0 (Molecular Devices, Sunnyvale, USA).

2.7. *Data analysis*

GPR files of the scanned images were analysed with the Chipster software (v1.4.6, CSC, Finland). Data were normalized using *normexp* with background correction offset [0, 50] as reported [27]. The entire data set is accessible as supplemental information (Supplemental Table S2). Similarities and dissimilarities among cell lines were assessed globally using hierarchical clustering,

Non-Metric Multidimensional Scaling [28] and Detrended Correspondence Analysis [29]. Two-group comparisons, such as HUVEC versus all ductal cancer cells, primary versus metastatic cells and liver versus lymph node metastases, were performed using the Empirical Bayes test with Bonferroni–Hochberg multiple testing correction and a cut-off at a p-value of 0.05 [30]. Multiple-group comparisons, such as for cell origin and degree of differentiation, were performed using LIMMA with a p-value adjustment according to Bonferroni–Hochberg multiple testing correction [30]. Cluster analysis was conducted using Pearson correlations and dendrograms were constructed using the average linkage method (http://chipster.csc.fi). Biomarker and functional investigations were performed with the Ingenuity Systems Pathway Analysis tools (Ingenuity Systems, Redwood City, CA). The p-values were calculated using right-tailed Fisher's exact test (www.ingenuity.com). Protein annotation was conducted with the open-source STRAP software [31].

2.8. *Immunofluorescence imaging*

The expression of cell surface markers collagen-XV-α1 and tetraspanin-6 was studied in cell lines MDA-Panc-28, AsPC-1, BxPC-3, Capan-1 and Panc-1. Cells were cultured on Nunc Lab-TecII glass slide chambers (Fisher Scientific, Schwerte, Germany). At 75–85% confluency, the medium was removed and cells were washed with PBS. Slides were fixed with ice-cold methanol: acetone (1:1) for 20 min and blocked for 3 h with 3% milk in PBST20. After blocking, slides were incubated for 1 h at room temperature with goat anti-collagen-XV-α1 or rabbit anti-tetraspanin-6 antibodies diluted at 1:500 in PBST20 containing 3% milk. Subsequently, the slides were washed 4 times for 5 min with PBST20 and incubated in the dark for 1 h at room temperature with 3% milk in PBST20 containing donkey anti-goat or goat anti-rabbit secondary antibody labelled with Texas Red or Cy3, respectively. Slides were again washed 4 times for 5 min with PBST20, incubated for 1 min with 0.5 μg/ml Hoechst dye, and then rinsed briefly with PBS. Imaging was done with a DeltaVision Core microscope (Applied Precision Inc., Issaquah, USA) using a 40× objective. Images were deconvolved using the built-in SoftWoRx software utilising the "enhanced ratio" algorithm, "medium noise" filtering and 10 iterations per channel.

3. Results

3.1. *Cell line selection criteria*

For the study, we collected several ductal adenocarcinoma cell lines so as to represent different cellular characteristics repeatedly (Table 1). Also, by using cells that exhibit cellular characteristics in variable combinations and have their origin from different sources, the chances were higher to identify common aspects rather than findings that are only specific to the particular tumour of the individual patient, from whom the respective cell line was isolated. As controls, two cell lines were utilized (Table 1). The Human Umbilical Endothelial Cell (HUVEC) has frequently been used as a control in comparative studies of pancreatic cancer [32,33]. More recently, the Human Pancreatic Duct Epithelial (HPDE) cell line was introduced, which exhibits a near normal phenotype and genotype except for the loss of the p53 functional pathway [34]. HPDE was immortalized after transfection with LXSN16-E6E7 retroviral expression vector for the E6 and E7 genes of human papilloma virus 16 [24]. Of the cancer cell lines, nine had their origin from primary tumour, seven from liver metastasis, two from lymph node metastasis and five from ascites (Table 1). Since they all originated from exocrine tumours, we also studied BON-1 cells for comparison, which had been established from a pancreatic endocrine carcinoid tumour [35].

3.2. *Cell line comparison*

First, the protein levels of the various cell lines were compared to each other at a global level, taking into account the complete data set produced on the antibody arrays. Analysis was done by hierarchical clustering, Non-Metric Multidimensional Scaling [28] and Detrended Correspondence Analysis [29]. From all three analysis types, it became apparent that overall the HPDE cells had at the protein level much more in common with the cancer cell lines than HUVEC, presumably due to the absence of cellular senescence. In the resulting plots, the HPDE cells fall in between the cancer cell lines while HUVEC is more segregated (e.g., Fig. 1). As a consequence of this resemblance of HPDE to PDAC cells, all comparisons were done with the HUVEC proteome acting as the basic control to which all expression measurements were related. The hierarchical clustering (Fig. 1) reflects to some extent the origin of the cell lines from primary tumour, metastases or ascites, respectively, although substantial differences were observed within groups. Cell lines that are a sub-population of a parental cell line, like the Suit panel and the A818 cells, cluster intimately. Others, less directly related cells show similar results, such as the poorly differentiated primary cells Mia Paca-2 and PANC-1. In contrast, the two lymph node cell lines T3M4 and Colo-357 are comparatively different. The cells IMIM-PC2 and SU.86.86, which both have their origin in primary tumours, exhibit a relatively high degree of similarity but are rather distant from the other cells from primary tumours. The FAMPAC cell line was established from a patient with a family history of pancreatic cancer [36]. However, on the basis of the 741 proteins studied, no particular expression differences to the other pancreatic ductal cells were recorded. AsPC-1 seems to be exceptionally different from the other ascites cells and exhibited closer resemblance with cell lines from liver metastases or primary tumours. Finally, BON-1 is the cell line that is furthest apart from all others, which is not surprising, since it is an endocrine carcinoid tumour. The comparison with the exocrine tumour cells revealed a surprisingly large number of 490 proteins that were expressed at significantly different levels (Supplemental Table S3). In a cluster analysis based on a comparison of all cells to HPDE (not shown), HUVEC was as distant from the PDAC cell lines as BON-1.

3.3. *Variations in the protein expression of PDAC cells*

The protein levels in the PDAC cell lines (all cell lines but BON-1 and HPDE) were compared to the proteome of HUVEC. There were 132 significantly regulated proteins (49 up- and 83 down-

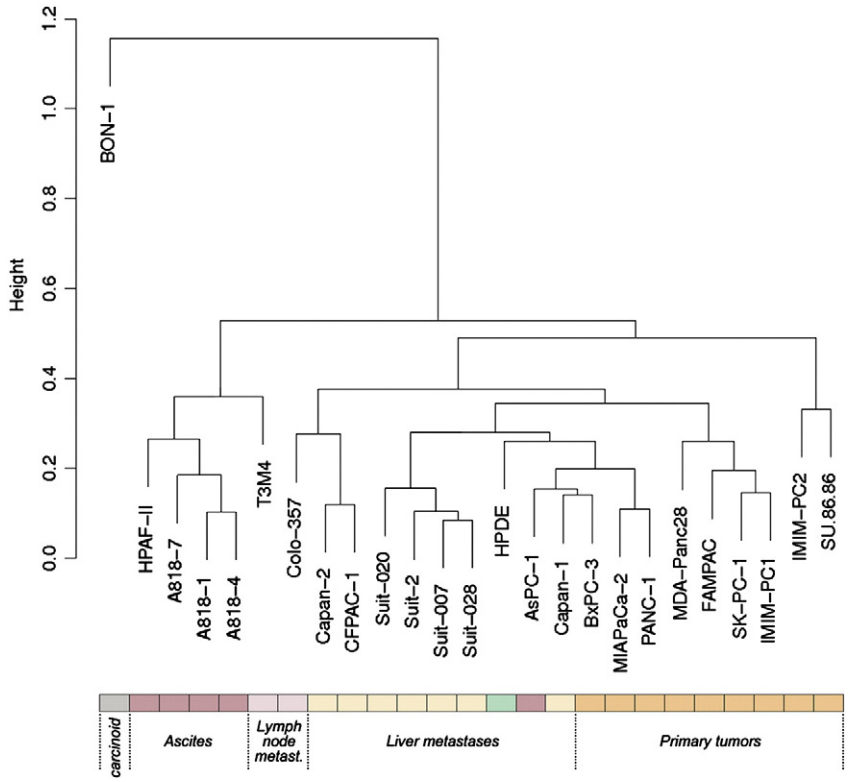

Fig. 1 – Hierarchical clustering of the cell lines. Based on the variations in protein expression compared to HUVEC, a cluster analysis was performed. At the bottom, the origin of each cell line is indicated. The colour-code used is identical to that of Table 1.

regulated). They all could also be observed as significant variations in individual comparisons to HUVEC of at least 20 of the 23 cell lines studied. A comparison to the Pancreas Expression Database (www.pancreasexpression.org), which holds the results of 54 mostly transcriptional studies, revealed that 60 (45%) of them had already been reported as biomarkers at the transcript or protein level before (Supplemental Table S4). The relatively high percentage is not surprising, since many antibodies used in the analysis had been selected on the basis of transcriptional variations observed in various tumour forms including pancreatic cancer. Therefore, some are bound to identify known biomarkers. It is actually more surprising that more than half of the differentially regulated molecules – altogether 72 proteins (Table 2) – had not been defined before as markers for pancreatic cancer. An analysis of the functional aspects of these PDAC-specific proteins revealed strong connections to cell death, cellular development, movement, growth and differentiation (Supplemental Table S5).

3.4. *Comparison of cell lines from primary tumours and metastases*

Cell lines originating from primary tumours (BxPC-3, Capan-2, FAMPAC, IMIM-PC1, IMIM-PC2, MDA-Panc28, MIA-PaCa-2, PANC-1, SK-PC-1 and SU.86.86) and metastatic tumour cells (Capan-1, CFPAC-1, Colo357, Suit-007, Suit-020, Suit-028, Suit-2 and T3M4) were compared in order to find metastasis-specific changes. In total, 187 proteins were found to be differentially expressed (106 down, 81 up) in cells from metastases as compared to those from primary tumours (Supplemental Table S6). An analysis of the functional annotations associated with the proteins revealed that 39% of them are involved in regulating cellular movement, migration, invasion and chemotaxis. Also their GO terms imply that the majority (84%) are incorporated in the regulation of such cellular processes or the function as binding molecules, interacting selectively but not covalently (Supplemental Fig. S1).

3.5. *Assignment of the cell source*

The PDAC cells in our analysis fall mainly into three groups that reflect the source from which they had been isolated: primary tumours (BxPC-3, Capan-2, IMIM-PC1, IMIM-PC2, MDA-Panc28, MIA-PaCa-2 and PANC-1), ascites fluid (A818-1, A818-4, A818-7, AsPC-1 and HPAF-II), and liver metastasis (Capan-1, CFPAC-1, Suit-007, Suit-020, Suit-028 and Suit-2). For each group, distinctively regulated proteins were found: 10 proteins were specific for primary tumours, 50 for ascites and 102 for liver metastases. (Fig. 2; Supplemental Table S7). There was an overlap of these proteins with the molecules that were found to be metastasis-specific. After their removal, 8, 47 and 63 unique proteins remain that act as indicators of the cellular source rather than the metastatic status.

Table 2 – Newly reported proteins that were up- (positive fold change (Log-FC)) or down-regulated (negative fold change) in PDAC as compared to HUVEC cell lines.

ID	Symbol	Entrez gene name	Location	Family	p-Value	Log-FC
O43865	AHCYL1	Adenosylhomocysteinase-like 1	Cytoplasm	Enzyme	7.69E-03	0.497
Q9H8T0	AKTIP	AKT interacting protein	Cytoplasm	Other	2.22E-02	−0.204
P02743	APCS	Amyloid P component, serum	Extracellular space	Other	2.48E-02	0.257
O75947	ATP5H	ATP synthase, H+transporting, mitochondrial Fo complex, subunit d	Cytoplasm	Enzyme	1.79E-02	−0.197
Q16864	ATP6V1F	ATPase, H+transporting, lysosomal 14 kDa, V1 subunit F	Cytoplasm	Transporter	7.65E-03	0.366
Q16611	BAK1	BCL2-antagonist/killer 1	Cytoplasm	Other	4.80E-02	−0.250
P38398	BRCA1	Breast cancer 1, early onset	Nucleus	Transcription regulator	4.32E-02	−0.305
Q9H5K9	C4orf41	Chromosome 4 open reading frame 41	Unknown	Other	1.00E-02	0.362
O43497	CACNA1G	Calcium channel, voltage-dependent, T type, alpha 1 G subunit	Plasma membrane	Ion channel	2.20E-02	−0.311
P51671	CCL11	Chemokine (C–C motif) ligand 11	Extracellular space	Cytokine	4.64E-04	−0.522
P13501	CCL5	Chemokine (C–C motif) ligand 5	Extracellular space	Cytokine	4.30E-05	−0.470
P20248	CCNA2	Cyclin A2	Nucleus	Other	1.58E-03	−0.402
P51959	CCNG1	Cyclin G1	Nucleus	Other	1.90E-02	−0.433
P50991	CCT4	Chaperonin containing TCP1, subunit 4 (delta)	Cytoplasm	Other	2.99E-02	0.247
Q99832	CCT7	Chaperonin containing TCP1, subunit 7 (eta)	Cytoplasm	Other	7.65E-03	0.564
O15519	CFLAR	CASP8 and FADD-like apoptosis regulator	Cytoplasm	Other	1.33E-04	−0.408
P02778	CXCL10	Chemokine (C-X-C motif) ligand 10	Extracellular space	Cytokine	1.17E-04	−0.622
O95865	DDAH2	Dimethylarginine dimethylaminohydrolase 2	Unknown	Enzyme	4.73E-02	0.417
Q8IX18	DHX40	DEAH (Asp-Glu-Ala-His) box polypeptide 40	Unknown	Enzyme	4.54E-02	−0.231
P24534	EEF1B2	Eukaryotic translation elongation factor 1 beta 2	Cytoplasm	Translation regulator	3.85E-02	0.402
P21860	ERBB3	v-erb-b2 erythroblastic leukaemia viral oncogene homolog 3 (avian)	Plasma membrane	Kinase	2.70E-05	−0.373
P21781	FGF7	Fibroblast growth factor 7	Extracellular space	Growth factor	1.98E-02	0.388
P15328	FOLR1	Folate receptor 1 (adult)	Plasma membrane	Transporter	9.90E-03	−0.979
P62993	GRB2	Growth factor receptor-bound protein 2	Cytoplasm	Other	1.11E-02	−0.259
P48637	GSS	Glutathione synthetase	Cytoplasm	Enzyme	7.20E-05	−0.447
P05204	HMGN2	High mobility group nucleosomal binding domain 2	Nucleus	Other	4.10E-02	−0.203
Q92506	HSD17B8	Hydroxysteroid (17-beta) dehydrogenase 8	Cytoplasm	Enzyme	1.02E-02	−0.328
NP_008831	IFNA1/ IFNA13	Interferon, alpha 1	Extracellular space	Cytokine	2.22E-02	−0.125
P29459	IL12A	Interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35)	Extracellular space	Cytokine	3.10E-03	−0.195
P01583	IL1A	Interleukin 1, alpha	Extracellular space	Cytokine	4.93E-03	−0.418
P60568	IL2	Interleukin 2	Extracellular space	Cytokine	1.16E-02	−0.290
P05112	IL4	Interleukin 4	Extracellular space	Cytokine	2.70E-05	−0.678
P05113	IL5	Interleukin 5 (colony-stimulating factor, eosinophil)	Extracellular space	Cytokine	1.07E-02	−0.353
P05231	IL6	Interleukin 6 (interferon, beta 2)	Extracellular space	Cytokine	3.70E-04	−0.311
Q96CN7	ISOC1	Isochorismatase domain containing 1	Cytoplasm	Enzyme	5.89E-04	−0.434
Q8TB96	ITFG1	Integrin alpha FG-GAP repeat containing 1	Plasma membrane	Other	4.86E-02	−0.189
Q02241	KIF23	Kinesin family member 23	Cytoplasm	Other	2.47E-02	−0.224
O43474	KLF4	Kruppel-like factor 4 (gut)	Nucleus	Transcription regulator	3.41E-02	0.201
Q53G59	KLHL12	Kelch-like 12 (Drosophila)	Unknown	Other	1.98E-02	0.249
P20700	LMNB1	Lamin B1	Nucleus	Other	4.43E-02	−0.249
P33992	MCM5	Minichromosome maintenance complex component 5	Nucleus	Enzyme	1.55E-02	0.520
P25325	MPST	Mercaptopyruvate sulfurtransferase	Cytoplasm	Enzyme	1.03E-02	0.524
P21757	MSR1	Macrophage scavenger receptor 1			2.81E-02	−0.240

Table 2 (*continued*)

ID	Symbol	Entrez gene name	Location	Family	p-Value	Log-FC
			Plasma membrane	Transmembrane receptor		
Q02505	MUC3A	Mucin 3A, cell surface associated	Extracellular space	Other	4.80E-02	0.331
Q07826	NA	X-linked retinopathy protein	–	–	2.22E-02	−0.125
Q14934	NFATC4	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4	Nucleus	Transcription regulator	1.10E-03	−0.660
Q02962	PAX2	Paired box 2	Nucleus	Transcription regulator	2.81E-02	0.369
Q16342	PDCD2	Programmed cell death 2	Nucleus	Other	2.82E-02	−0.196
P49763	PGF	Placental growth factor	Extracellular space	Growth factor	3.29E-02	−0.263
P19387	POLR2C	Polymerase (RNA) II (DNA directed) polypeptide C, 33 kDa	nucleus	Enzyme	2.40E-02	0.348
P09086	POU2F2	POU class 2 homeobox 2	Nucleus	Transcription regulator	2.20E-02	−0.162
P62906	RPL10A	Ribosomal protein L10a	unknown	Other	2.47E-02	0.228
Q02878	RPL6	Ribosomal protein L6	Cytoplasm	Other	8.02E-03	0.541
P18124	RPL7	Ribosomal protein L7	Cytoplasm	Transcription regulator	3.26E-02	0.207
P62841	RPS15	Ribosomal protein S15	Cytoplasm	Other	9.90E-03	0.468
P39019	RPS19	Ribosomal protein S19	Cytoplasm	Other	2.97E-02	0.322
P21453	S1PR1	Sphingosine-1-phosphate receptor 1	Plasma membrane	G-protein coupled receptor	3.97E-02	−0.305
Q15393	SF3B3	Splicing factor 3b, subunit 3, 130 kDa	Nucleus	Other	1.08E-02	0.301
Q8N474	SFRP1	Secreted frizzled-related protein 1	Plasma membrane	Transmembrane receptor	1.10E-03	−0.428
Q16586	SGCA	Sarcoglycan, alpha (50 kDa dystrophin-associated glycoprotein)	Plasma membrane	Other	4.30E-05	−0.529
Q13485	SMAD4	SMAD family member 4	Nucleus	Transcription regulator	3.85E-02	0.234
Q13318	SPI1	Spleen focus forming virus (SFFV) proviral integration oncogene spi1	nucleus	Transcription regulator	1.10E-03	−0.215
Q9Y5Y6	ST14	Suppression of tumorigenicity 14 (colon carcinoma)	Plasma membrane	Peptidase	4.93E-02	−0.224
Q96FV9	THOC1	THO complex 1	Nucleus	Transcription regulator	1.13E-03	−0.374
P35625	TIMP3	TIMP metallopeptidase inhibitor 3	Extracellular space	Other	4.30E-05	−0.587
Q9UDY2	TJP2	Tight junction protein 2 (zona occludens 2)	Plasma membrane	Kinase	4.18E-03	−0.338
Q99805	TM9SF2	Transmembrane 9 superfamily member 2	Plasma membrane	Transporter	2.74E-02	−0.348
O43557	TNFSF14	Tumour necrosis factor (ligand) superfamily, member 14	Extracellular space	Cytokine	3.80E-04	−0.453
P19474	TRIM21	Tripartite motif containing 21	Nucleus	Enzyme	7.42E-03	−0.282
P19320	VCAM1	Vascular cell adhesion molecule 1	Plasma membrane	Other	3.19E-03	−0.424
P15692	VEGFA	Vascular endothelial growth factor A	Extracellular space	Growth factor	2.48E-02	−0.222
Q9Y6I7	WSB1	WD repeat and SOCS box containing 1	Unknown	Other	1.40E-03	0.306

3.6. *Comparison of PDAC cells based on their degree of differentiation*

In this analysis, only cell lines were included, for which a well-defined and particularly consistent degree of differentiation has been reported in the literature. For some of the studied cell lines, controversial results exist and they were not considered for this reason. The status of other cell lines has been described in the literature just once. Thus, no independent confirmation about their degree of differentiation exists. We tried to corroborate the published information for these cell lines but were not successful in all cases. FAMPAC and MDA-Panc28 produced results that are in contrast to literature data. They had been described as poorly differentiated cells, based on the secretion of ductal proteins like MUC1 and cytokeratin-7 [36,37]. In our cultures, however, we could not confirm this (data not shown). Because of this disagreement, they were not included in the analysis.

Comparing the protein expression of PDAC cells with different degrees of differentiation (well differentiated: Capan-1, Capan-2, CFPAC-1 and SK-PC-1; moderately differentiated: A818-1, A818-4, A818-7, AsPC-1, T3M4 and BxPC-3; and poorly differentiated: MIA PaCa-2 and PANC-1), unique sets of proteins were identified that are associated with the differentiation degree (Fig. 3). Inversely to the decrease in the degree of cellular differentiation from well via moderate to poor, an increase in the number of regulated proteins from 5 via 34 to 107 proteins was observed (Supplemental Table S8). Of all the 741 protein targets represented on the chip

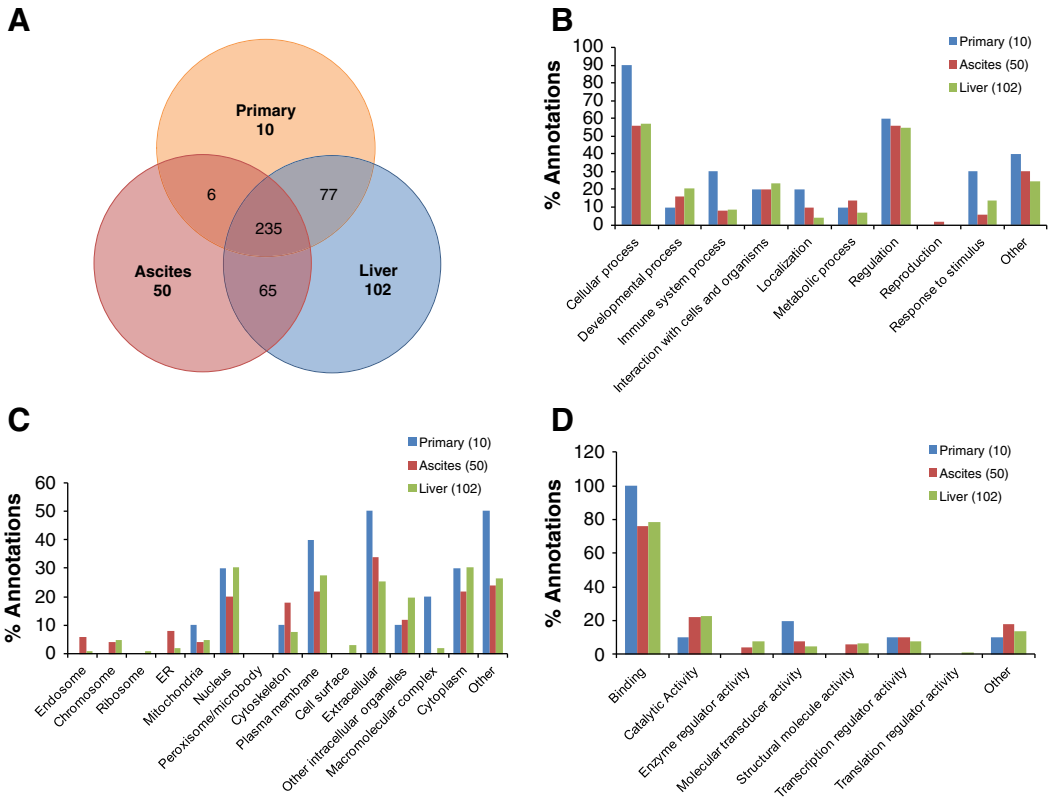

Fig. 2 – Number of tissue-type specifically expressed proteins. A Venn-diagram (A) shows the number of proteins that were regulated in cell lines originating from primary tumour, ascites or liver. In panels B, C and D, GO annotations of the regulated proteins are shown for biological processes, cellular components and molecular functions, respectively.

in terms of antibodies, 156 are involved in the regulation of cellular degree of differentiation. We found 61 proteins of the 156 to be significantly regulated among the three groups.

3.7. *Acinar versus ductal cell type*

All exocrine cell lines exhibit a ductal phenotype except of MDA-Panc-28, which displays both ductal and acinar features [37]. A comparison revealed 87 proteins that differentiated MDA-Panc-28 from the purely ductal tumour cells (Supplemental Table S9). In particular the levels of the cell surface proteins collagen-XV-α1, mucin-2, tetraspanin-6 and C-C chemokine receptor type 7 were differentially expressed, suggesting that these molecules could be used for an immunohistochemical detection of cells from an acinar lineage. Using immunofluorescence staining with antibodies against collagen-XV-α1 and tetraspanin-6, for example, this was confirmed experimentally (Fig. 4).

4. Discussion

Cell lines provide a model to investigate parts of the molecular basis of cancer and could consequently permit an identification of both biomarkers and new therapeutic avenues. For example, markers were detected that are associated with pancreatic cancer metastasis using the SW1990 cell line [38]. In another study, the association between cell-surface markers and cancer tumorigenicity had been assessed [39]. Here, we evaluated the cellular proteome of 24 pancreatic cancer cell lines using an antibody microarray with binders against 741 target proteins [15].

A problem posed by studies with cell lines is the definition of an appropriate control. We applied two cells for this purpose, the Human Umbilical Endothelial Cell (HUVEC), which was frequently used before as a control in comparative studies of pancreatic cancer [32,33] and the Human Pancreatic Duct Epithelial (HPDE) cell line [34], which was introduced more recently. Although the latter does not form tumours in immune-deficient animals and has only relatively few reported genomic mutations, the overall comparison at the protein level indicated that it is more similar to the cancer cell lines than HUVEC. Consequently, HUVEC was used as control. It is to be expected that a thorough analysis of the genomes of the many established cell lines may contribute additional information about which cell line could act best as a control in future comparative studies.

Nearly half of the proteins that were identified as differentially expressed in a comparison of cancer and normal cell lines had already been described for pancreatic cancer, for example acting as markers for the treatment efficacies with drugs like erlotinib (ERBB3 and VEGFA), bevacizumab (VEGFA), celecoxib (PTGS2), pioglitazone hydrochloride (INS), tegafur, gimeracil, oxonic acid and gemcitabine (IL8, TP53 and VEGFA) or for predicting the success of radiotherapy (AREG and VEGFA)

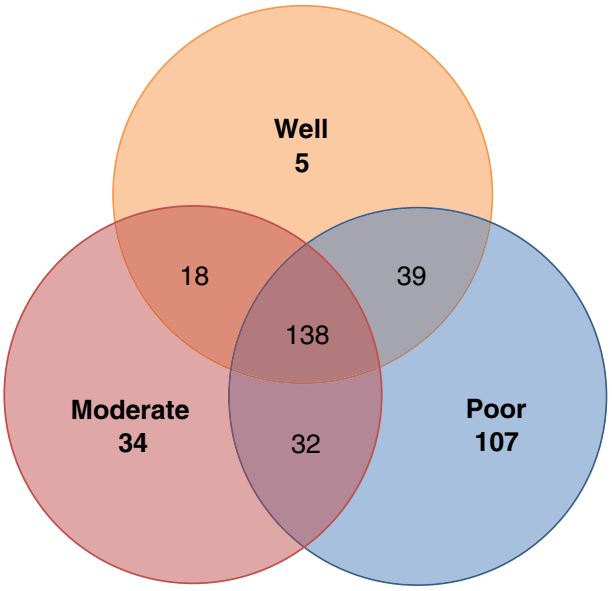

Fig. 3 – Comparison of cells based on their degree of differentiation. In the Venn-diagram, the number of regulated proteins are shown that exhibited variation in well, moderately or poorly differentiated cell lines.

(ClinicalTrials.gov by Ingenuity). Also, IL10, TNF and VEGFA had been used as prognostic markers. Except for TP53 and AREG, the above mentioned markers were down-regulated in all tested cell lines, which is in agreement with previous reports which showed lower expression in the pancreatic cell lines PANC-1 [40,41], MIA PaCa-2 [42] and MDA Panc-28 [41] as well as in the pancreatic duct of human adults [43,44]. Interestingly, all extracellular proteins with a regulatory effect on PDAC had a lower expression level in the cancer cell lines. This could be explained by their concomitantly strongly increased abundance in the secretome of PDAC cell lines that we found in a separate, still preliminary study. Especially IL6 was strongly secreted by 16 of the pancreatic cancer cell lines (unpublished data). Fittingly, in patients, a higher diagnostic and prognostic value of serum IL-6 was documented than that of CRP, CEA and CA 19–9 [45].

Proteins IL6, IL10, TNF, TP53 and VEGFA, along with other, up-regulated proteins like BRCA1, RPS19, RRM2 and SMAD4, have been reported to affect the development of PDAC. It was shown, for example, that RRM2 contributes significantly to the invasiveness of PDAC [46], potentially through the up-regulation of MMP9. Silencing of the *RRM2* gene was associated with a decrease in cancer cells viability [47]. Targeting RRM2 with gemcitabine is among the common therapeutic approaches for treating pancreatic cancer [48]. Higher expression of this enzyme was documented in the pancreatic cell lines MIA PaCa-2 [46], Suit-2 [47] and PANC-1 [49]. Fittingly, RRM2 was one of the most up-regulated proteins in the present study, exhibiting an increase in all cancer cell lines. Similarly, SMAD4 was shown to be expressed at an elevated level in several cancer cell lines [50–53], which is in agreement with our findings. BRCA1, which plays a central role in DNA repair through promoting the cellular response to DNA damage [54] and functions as a tumour suppressor protein [55], was found significantly less abundant in cancer as compared to normal cells. Mutations of the *BRCA1* gene have been reported for breast and ovarian [56] as well as pancreatic cancer [57].

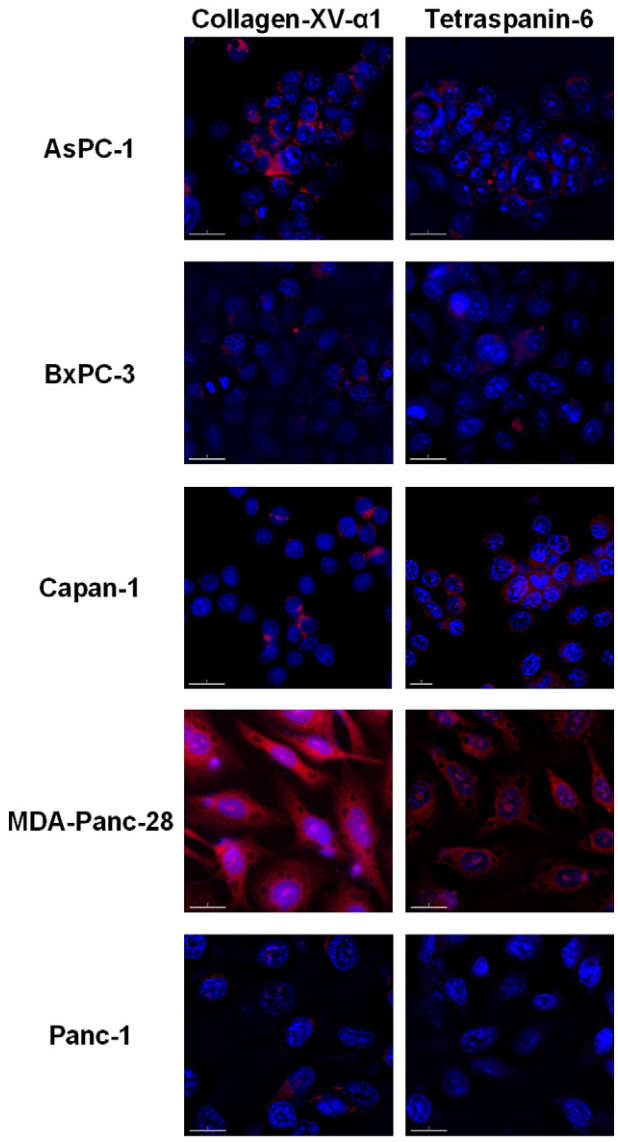

Fig. 4 – Immunohistochemical analysis of five cell lines. Using fluorescently stained antibodies against collagen-XV-α1 and tetraspanin-6, only cell line MDA-Panc-28 exhibited strong signal intensities, which is consistent with the microarray data and indicates the specific expression of the two proteins in cells of acinar origin. The other four cells are of ductal lineage.

Distant metastases and multi-organ involvement are very common in advanced pancreatic cancer and among the main factors responsible for its dismal prognosis. Over 85% of patients with pancreatic cancer are presented with disease metastasis at the time of diagnosis. Surgery is contraindicative at this level and radio- and chemotherapy are the only available options yet disconcerting owing to tumour resistance. Better insight to the prime molecules involved in the interaction between the cancer cells and the affected organ as well as the surrounding environment could allow for more effectively targeting pancreatic cancer metastases. Comparing cell lines that originated from pancreatic cancer metastases with cells from primary tumour, we found alteration in the expression of

several cell surface proteins that promote invasion and metastasis, including cell adhesion molecules such as ICAM1, VCAM1, Junctional Adhesion Molecule B (JAM2), Occludin and Selectin, molecules mediating homotypic interaction during invasion and metastasis such as E-Cadherin, Afadin (MLLT4), molecules mediating the interaction with the extracellular matrix such as MMP1, MMP2 and TIMP1, antigenic glycoproteins like CEACAM5, MADCAM1 and LAMP2, receptors like FAS, IL1A, IL1RN, IL2R and IL2RG and recognition proteins for immune cells like CD44, GCA.

A closer look at the molecular feature of metastatic pancreatic cancer may provide valuable answers why PDAC cells harbour and thrive in certain organs more than others. At autopsy, the liver is the most frequent site of distant metastases, followed by the peritoneum, pleura and lung, bones, and adrenal glands [58]. Unlike the case with other cancers, such as colorectal cancer [59], surgical intervention of advanced pancreatic cancer is not an option for most cases [60], and chemotherapy may be the only treatment at this level, although again of little overall benefit. Additionally, pancreatic cancer commonly develops resistance to currently available therapeutic agents and drugs. The analysis of primary tumours, ascites and liver metastases yielded proteins that may be potential targets for therapy. Among the proteins that were highly regulated only in cells from liver metastasis is serine/threonine-protein kinase B-raf, a cytoplasmic enzyme that is targeted with the compounds sorafenib and PLX4032 [61,62]. Sorafenibin in combination with gemcitabine has shown cytotoxicity against MIA PaCa-2, Capan-1 and PANC-1 cell lines [63]. Unfortunately, however, similar findings could not be reproduced in phase II clinical trials [64,65]. Drugs like XR9576, OC 144–093, and valspodar have been used as inhibitors of the protein ATP-binding cassette (ABCB1), which involves in drug-resistance of certain tumours [66–68]. ABCB1 is a P-glycoprotein that is not expressed by many cell types [66]. P-glycoproteins have been suggested as potential targets in cancer therapy [69]. Overexpression of ABCB1 was observed in our study only in cells that originated from liver metastasis. Targeting ABCB1 in pancreatic cancer patients with liver metastasis may serve as good route to treat liver metastases specifically. Transforming growth factor beta 2 (TGF-β2) was also among the proteins overexpressed in cells from liver metastases. This growth factor is known to affect negatively the immune response to cancer cells and promote cancer progression through proliferation, metastasis, and angiogenesis [70]. Modulation of TGF-β2 level with AP-12009 has shown promising results in clinical trial for treatment of malignant gliomas [71].

Matrix metaloproteinases (MMPs) are known proteolytic enzymes used by tumour cells for detachment and invasion. In pancreatic cancer, an imbalance between MMPs and their tissue inhibitors has been reported [72]. Our results showed an increased expression of MMP-7 and MMP-12 in cells that originated from ascites, MMP1 in cells from liver metastasis, MMP-11 and MMP-14 in both the primary tumour and liver groups, and MMP-2 in all groups. Inhibition of MMP-2 by RO28-2653 or MMI-166 showed an effective reduction of liver metastasis in an animal model of pancreatic cancer [73].

The degree of cancer cell differentiation corresponds strongly with the disease progression and its aggressiveness and therefore indicates processes that may help in accurate prognosis and therapeutic success. The majority of PDAC tumours are well to moderately differentiated, while poorly differentiated tumours are less common [74]. In PDAC, the hallmarks of cell differentiation are tubular structures, papillae, cysts and secreted mucins [75]. The last are a group of glycoproteins that are mainly produced by ductal and granular epithelial cells. Some of them, such as MUC4, were suggested as potential tumour markers for a diagnosis of pancreatic cancer [76]. Among all secreted mucins, MUC2 is considered to be the molecule most correlated to inflammation and cancer [77]. In our analysis, a significant decrease in MUC2 expression was observed across all differentiation stages, with the lowest level being found in poorly differentiated cell lines, which is in agreement with previous records [76,78]. MUC1 was significantly higher only in the poorly differentiated cell lines. Since our control cell line, HUVEC, has the capacity to express MUC1 [79], it could be that the level of secreted MUC1 is comparable to that of the well and moderately differentiated cancer cells and thus the reason for the significant difference to poorly differentiated cells. Apart from the mucins, also 61 other proteins involved in the regulation of differentiation were similarly regulated in cancer cells irrespective of the actual differentiation stage. The majority are extracellular and nuclear proteins and, to a lesser extent, proteins bound to the plasma membrane. Most function as transcription regulators, cytokines, growth factors and trans-membrane receptors. Some are well-established key players in the events leading to PDAC, such as p53 [80], NFκB [81], ERBB2 [82], p38 MAPK [83], CDK4 [84] and SMAD4 [85], which all were found to be overexpressed in the cancer cells. However, besides the proteins common to all cancer cell lines, also proteins were found that were regulated only at a particular stage of differentiation. It is intriguing that the number of specifically regulated proteins increased with the decrease of cell differentiation, being highest in the poorly differentiated cell lines.

In conclusion, the comparative analysis of a substantial number of pancreatic cancer cell lines provided information about the degree of heterogeneity of cell lines and their suitability to act as model systems. Also, many proteins were identified, which at the level of cell lines exhibited significant expression variations between tumour and normal cells, primary tumours and metastases, different stages of differentiation and cells with different origin. Besides the potential of these proteins as biomarkers, interesting candidates for potential new therapeutic approaches were found, such as for a targeted treatment of metastatic cells. Based on the results with the cell lines, more directed studies with patient samples are made possible and are the next step in any therapeutic development. Gathering more molecular information on the disease and the definition of appropriate model systems are prerequisite to the establishment of novel therapies, which are very urgently needed for pancreatic cancer, for which basically no treatment modality exists at the moment but for surgery in few cases.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2012.04.042.

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