Different Capabilities of Morphological Pattern Formation and Its Association with the Expression of Differentiation Markers in a Xenograft Model of Human Pancreatic Cancer Cell Lines

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Key Words

Pancreatic carcinoma cell lines · Xenografts · Transitions states · Transdifferentiation · Dedifferentiation

Abstract

Aims: New concepts of tumorigenesis favor an unregulated process recapitulating different stages of embryonic development with dysregulation of transition states. The aim of our study was to investigate the possibility of differentiation pathways of human pancreatic cancer cell lines in vivo. **Material and Methods:** Different human pancreatic cancer cell lines (YAPC, DAN-G, CAPAN-1, PANC-1 and MIA PaCa-2) were implanted subcutaneously (3×10^6 cells) for 28 days in nude mice. Xenotransplants were characterized with histochemistry (HE, PAS), immunohistochemistry (cytokeratin (CK)7, CK8, CK18, CK19, CK20, vimentin, chromogranin A (Chr-A), α_1 -antichymotrypsin (α_1 -chym), β -catenin, laminin-5, pancreatic and duodenal homeobox gene 1 (pdx-1), sonic hedgehog protein (shh), Patched (ptc)), Western

blotting and real-time PCR (CK7, CK8, CK20, Chr-A, pdx-1, shh, ptc). **Results:** Depending on three major morphologic phenotypes of tumor cell xenotransplants (ductal (YAPC), ductal/solid (DAN-G, CAPAN-1), solid (PANC-1, MIA PaCa-2)), a decrease of CK7/CK19 was found, accompanied by an increase of CK8/18 and vimentin. Predominantly the CK7-positive ductal phenotype (YAPC and DAN-G) was associated with pdx-1 expression, whereas the CK8-positive solid phenotype was associated with shh/ptc expression on protein and mRNA level. Additionally, CK-20 expression was mainly linked to the ductal phenotype, co-localized with nuclear β -catenin. The endocrine-exocrine transdifferentiation, as assessed by Chr-A and α_1 -chym, was on a constant low to moderate level in all xenotransplants. Finally, an intensive epithelial-mesenchymal interaction was observed by overexpression of laminin-5 at the invasion front. **Conclusion:** The observed patterns of morphology and molecular differentiation in human pancreatic cancer xenografts indicate that these cancer cell lines have different capabilities of pattern formation in vivo associated with molecular differentiation markers, especially of embryonic pancreatic development.

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Introduction

Pancreatic carcinoma is among the most aggressive solid malignancies, being the fifth leading cause of cancer death in the Western world. Despite its rising incidence, little progress has been made in overall survival [1].

The pancreas contains three different cell types: duct cells, exocrine cells and endocrine cells. Transition states of ductal and endocrine cells were observed during embryonic development [2]. Similar phenomena reflecting these processes have been described during tumorigenesis of the pancreas [3].

Newer concepts postulated that differentiation pathways, especially dedifferentiation, recapitulating early stages of tissue morphogenesis and cell differentiation during pancreas ontogeny [4–6], are key events in the initiation of tumorigenesis [7, 8]. This hypothesis is supported by the observation of re-expression of genes regulating embryonic pancreas development like sonic hedgehog pathway protein (shh) and its receptor Patched (ptc) [9, 10] as well as the pancreatic and duodenal homeobox gene 1 (pdx-1) [11, 12] in pancreatic adenocarcinoma and its precursor lesions of pancreatic intraepithelial neoplasia (PANINA). In part, dedifferentiation was detected by analyzing cytokeratin (CK) expression [13, 14].

Phenomena of transdifferentiation that are associated with phenotype switches (e.g. from exocrine to ductal [15, 16], exocrine to endocrine [17, 18] as well as from exocrine to mesenchymal [19, 20] and vice versa) are reported in pancreas tumorigenesis and reparation as well as in cell culture experiments. For the phenotype switches in transdifferentiation, it is postulated that cells must transiently dedifferentiate to a stem-cell-like phenotype linking de- and transdifferentiation pathways [21]. This is supported by the observation that transdifferentiation is linked with expression of the pancreatic duodenal homeobox-containing transcription factor pdx-1 [19, 22–24]. Furthermore, the process of trans- and dedifferentiation might be influenced by interactions with the environment, especially by laminins or β -catenin, which are important mediators of epithelial-mesenchymal transitions and cell-cell adhesion as well as survival [25–29].

Although these phenomena are specified and have in part been investigated in vitro [20, 30], systematical analyses using a xenograft model of human pancreatic cancer cell lines are still rare.

Therefore, the aim of our study was to investigate the possibility of differentiation pathways of human pancreatic cancer cell lines in vivo by a phenotype analysis using various specific markers of differentiation.

Material and Methods

Cell Culture

YAPC, DAN-G, CAPAN-1, PANC-1 and MIA PaCa-2 pancreatic adenocarcinoma cells were cultured in RPMI-1640 medium (Biochrom, Berlin, Germany) containing 10% fetal bovine serum (Biochrom), penicillin (10^7 U/l, Biochrom) and streptomycin (10 mg/l, Biochrom) at 37°C and 5% CO₂. YAPC, DAN-G and CAPAN-1 were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and PANC-1 and MIA PaCa-2 from the ATCC (Manassas, Va., USA), respectively.

In vitro Analysis

For in vitro assays, 150,000 cells were seeded per well of a 6-well culture plate (BD Biosciences, Heidelberg, Germany) or on chamber slides (Nunc, Wiesbaden, Germany) until confluence.

Xenograft Model

Pancreatic carcinoma cell lines were harvested and resuspended in sterile physiologic NaCl solution. 3×10^6 cells were injected subcutaneously into the flank of 4- to 6-week-old male NMRI mice (Harlan Winkelmann GmbH, Borcheln, Germany). For each cell line, 5 animals were used. Animals were kept in a light- and temperature-controlled environment and provided with food and water ad libitum. Tumor size was determined daily by measurement with a caliper square. After 28 days, animals were sacrificed by cervical dislocation and specimens of tumor were either fixed in 10% phosphate-buffered formalin or snap-frozen in liquid nitrogen. Ethical approval was achieved before the beginning of experiments.

Phenotypic Characterization of Pancreatic Cancer Cell Lines and Xenograft Specimens

Total protein was extracted from snap-frozen tumor samples, pooled from all animals of one group and subjected to SDS-PAGE as described previously [31]. Membranes were probed with the antibodies given in table 1. Reactive bands were detected with the ECL chemiluminescence reagent (Amersham Pharmacia Biotech, Freiburg, Germany), digitized on a LAS-1000 luminescence image analyzer (Fuji Photo Film, Düsseldorf, Germany) and analyzed using GelScan 5 software (BioSciTec, Frankfurt, Germany).

Fixed and paraffin-embedded tumor sections were subjected to routine hematoxylin and eosin (HE) staining and PAS reaction. Immunohistochemistry (IHC) was performed on pancreatic cancer cells xenografts as described [31]. Samples were analyzed with the antibodies given in table 1. HE staining was used to evaluate basic histomorphology of the specimens, especially ductal and solid morphology which was assigned if the tumor displayed ductal and solid formations in >75% of the whole specimen. The epithelial mucopolysaccharides were detected by the PAS reaction.

To evaluate the extension of the IHC, the slides with the immunohistochemical stainings were divided in quarters, digitized completely and analyzed quantitatively (% positive area) with the Ce2001 Cell Explorer software (BioSciTec). The intensity of the IHC staining was evaluated semiquantitatively (– = negative; () = focally positive; + = weakly positive; ++ = positive; +++ = strongly positive).

Table 1. Primary antibodies and antigen retrieval methods used for immunohistochemical analysis

Antigen specificity	Type	Dilution	Antigen retrieval	Vendor
CK7	Mouse monoclonal	1:1,000	P	BioGenex
CK8	Mouse monoclonal	1:200	MW	BioGenex
CK18	Mouse monoclonal	1:1,000	P	Sigma
CK19	Mouse monoclonal	1:500	P	DakoCytomation
CK20	Mouse monoclonal	1:50	MW	DakoCytomation
Vimentin	Mouse monoclonal	1:200	MW	DakoCytomation
Chr-A	Mouse monoclonal	1:1,000	MW	DakoCytomation
α_1 -chym	Rabbit polyclonal	1:10,000	P	Sigma
Laminin 5 (laminin γ_2)	Mouse monoclonal	1:30	Pt	Chemicon
β -Catenin	Rabbit polyclonal	1:100	MW, TRS	Sigma
Mib-1	Mouse monoclonal	1:100	MW	DakoCytomation
pdx-1	Rabbit polyclonal	1:250	MW	Chemicon
shh	Goat polyclonal	1:100	MW	SantaCruz
ptc	Goat polyclonal	1:50	MW, TRS	SantaCruz

P = Pronase (2 mg/ml, PBS, pH 7.4, 10 min at 37°C; Sigma, Germany); Pt = protease XXIV (0.05 mg/ml, PBS, pH 7.2, 10 min at 37°C; Sigma, Germany); MW = microwave (10 mmol/l citrate buffer, pH 6.0; 10 min at 800 W and 10 min at 560 W); TRS = target retrieval solution (pH 6.0; DakoCytomation, Denmark) used instead of citrate buffer.

RNA Isolation and Reverse Transcription

Total cellular RNA was extracted by use of the RNeasy Kit in combination with DNase treatment (Qiagen, Hilden, Germany), as described in the manual. The RNA was eluted in 30 μ l of RNase-free water. RNAs were pooled from all animals of one group. First-strand cDNA was synthesized using Superscript II RNase H-Reverse Transcriptase (Life Technologies, Karlsruhe, Germany). 4 μ l of RNA elute were added to 1 μ g of dT15 primers (TIB-Biomol, Berlin, Germany) and 1 μ M random hexamers (Promega, Heidelberg, Germany). After annealing for 10 min at 70°C and immediate cooling on ice, 1 $\times$ first strand buffer (Life Technologies), 10 mM DTT (Life Technologies), 0.5 μ l enzyme (100 units) and 0.5 mM deoxynucleotide triphosphate (Carl Roth, Karlsruhe, Germany) were added to get a total reaction volume of 10 μ l. The reaction was allowed to proceed for 50 min at 42°C, followed by 15 min at 70°C to inactivate the enzyme. After cDNA synthesis, distilled water was added to achieve a final volume of 100 μ l in each sample.

Quantitative Real-Time PCR

Primers were designed by use of the Primer Express software, Version 2.0.0 (Perkin-Elmer Applied Biosystems, Foster City, Calif., USA). The sequences of primers are listed in table 2. Each PCR assay was repeated three times for each cDNA sample. The 25 μ l total PCR volume consisted of 5 μ l cDNA, 12.5 μ l SYBRGreen PCR Master Mix (Applied Biosystems, Martinsried, Germany) and primers (MWG-Biotech, Ebersberg, Germany) in the following concentrations: 800 nM each for GAPDH and 400 nM each for the residual genes. After denaturation and activation of the hotstart enzyme for 10 min at 95°C, the PCR assays were carried out for 40 cycles, with denaturation at 95°C for 15 s, annealing and extension at 60°C for 1 min. To monitor amplification of pos-

sible contaminated DNA, distilled water served as a negative control.

Fluorometric PCR was performed with the ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems). After target amplification, SYBRGreen specifically binds to double-stranded DNA, which leads to an increase in detectable fluorescence signals. The amount of product detectable through fluorescence signals at any given cycle within the exponential phase of PCR is proportional to the initial number of template copies. The number of PCR cycles needed to detect the amplicon is therefore a direct measure of the template concentration. Threshold cycle was set when emission intensity of measurable fluorescence calibrated to starting level became equal to 10 standard deviations of the baseline.

Absolute quantification was achieved through generation of standard curves using serial dilutions of known concentrations of the different cDNAs. The RT-PCR results were expressed as the ratio of molecules of target gene (e.g. CK7) per 1,000 molecules of housekeeping gene (GAPDH) normalized to the mean expression level of all five cell lines. To monitor amplification of genomic DNA due to pseudogenes, RNA aliquots without reverse transcription were always amplified as negative controls.

Melting curve analysis was performed after each PCR run. After denaturation at 95°C for 20 s, a temperature ramp from 60 to 95°C in 20 min was run with simultaneous acquisition of fluorescence signals. At the melting point of double-stranded PCR products, an accelerated decline of fluorescence can be recognized. Using negative first-deviation plots, we checked each sample for primer dimers and unspecific products due to mispriming.

Table 2. Sequences of human primers used for real-time PCR experiments

CK7 (NCBI reference sequence No. X13320, amplicon 75 bp)
Forward primer (exon 4): 5'-TGAATGATGAGATCAACTTCTCAG-3'
Reverse primer (exon 5): 3'-TGTCGGAGATCTGGGACTGC-5'
CK8 (NCBI reference sequence No. BC000654, amplicon 101 bp)
Forward primer (exon 7): 5'-CTGGGATGCAGAACATGAGTATTC-3'
Reverse primer (exon 8): 3'-GTAGCTGAGGCCGGCTTGT-5'
CK18 (NCBI reference sequence No. BC000180, amplicon 86 bp)
Forward primer (exon 5): 5'-GAGACGTACAGTCCAGTCCCTTGG-3'
Reverse primer (exon 6): 3'-CCACCTCCCTCAGGCTGTT-5'
CK20 (NCBI reference sequence No. X73502, amplicon 105 bp)
Forward primer (exon 5): 3'-TCCCAGAGCCTTGAGATAGAACTC-5'
Reverse primer (exon 6): 5'-GTTGGCTAACTGGCTGCTGTAAC-3'
pdx-1 (NCBI reference sequence No. NM_008814, amplicon 64 bp)
Forward primer (exon 1): 3'-TTCCCATGGATGAAGTCTACCAA-5'
Reverse primer (exon 1+2): 5'-CGTAGGCGCCGCTG-3'
Chr-A (NCBI reference sequence No. NM_001275, amplicon 83 bp)
Forward primer (exon 2): 3'-GCGGTGGAAGAGCCATCAT-5'
Reverse primer (exon 2): 5'-TCTGTGGCTTCACCACTTTCTC-3'
shh (NCBI reference sequence No. NM_000193, amplicon 74 bp)
Forward primer (exon 3): 5'-GGAAGCAGCCTCCCGATT-3'
Reverse primer (exon 2): 3'-CGAGTCCAAGGCACATATCCA-5'
ptc (NCBI reference sequence No. NM_000264, amplicon 139 bp)
Forward primer (exon 3): 5'-CCAATCTTCTGGCGAGTATAATTTAAT-3'
Reverse primer (exon 2): 3'-TGGTTGTGGGCCTCCTCAT-5'
GAPDH (NCBI reference sequence No. NM_002046, amplicon 226 bp)
Forward primer (exon 4): 5'-GAAGGTGAAGGTCCGAGTC-3'
Reverse primer (exon 2): 3'-GAAGATGGTGATGGGATTTC-5'

Furthermore, agarose gel electrophoresis of selected samples out of each PCR run was performed to check for unspecific amplification products.

Results

Human Pancreatic Cancer Cell Line Xenografts in vivo

The tumor implants showed a continuous growth in all cases measured by planimetry. The mean tumor volume ($\pm$ SD) after 28 days as well as the Mib-1 proliferation rate (%) was (in increasing order): CAPAN-1 ($364 \pm 148 \text{ mm}^3$; 75%), PANC-1 ($990 \pm 530 \text{ mm}^3$; 80%), YAPC ($1,089 \pm 740 \text{ mm}^3$; 80%), MIA PaCa-2 ($1,143 \pm 1,290 \text{ mm}^3$; 85%) and DAN-G ($1,963 \pm 235 \text{ mm}^3$; 90%).

Morphology (see table 3, fig. 1)

Besides transitional areas, three different morphological growth patterns of the human pancreatic cancer cell lines were found in the xenografts: YAPC showed predominantly typical duct like structures with cuboid tu-

mor cells, DAN-G and CAPAN-1 formed a mix of ductal and solid structures, while PANC-1 and MIA PaCa-2 showed solid tumor formations with different tumor cell morphology (cuboid and focally sarcomatoid). The nuclear polymorphology increased with the loss of ductal differentiation which was associated with the loss of PAS-positive mucopolysaccharides.

Immunophenotype of Xenografts (see table 3, fig. 1)

CK7/CK19 and CK8/CK18 Profile. The expression of typical pancreatic CKs (CK7/CK19 and CK8/CK18) and of the typical mesenchymal marker vimentin correlated with histological pattern and tumor grading. Typical ductal structures showed a strong expression of CK7/CK19, which was decreased in solid tumor formations, whereas CK8/CK18 was increased, accompanied by an increased expression of vimentin. Overall a clear zonal distribution of these CKs could not be observed. CK7/CK19 and CK8/CK18 were mainly found in the periphery of DAN-G and PANC-1 xenografts. An association between tumor cell morphology and expression of CK7/CK19 as well as CK8/CK18 could not be established.

Table 3. Immunohistochemical quantification of typical trans- and dedifferentiation patterns of subcutaneous human pancreatic cancer cell line xenografts in nude mice

Cell line	Extensity ¹ , intensity ² , zonal distribution ³ of stainings inside xenografts				
	YAPC	DAN-G	CAPAN-1	PANC-1	MIA PaCa-2
Major morphology (HE staining)	Ductal	Ductal/solid	Ductal/solid	Solid	Solid
Mucin account (PAS reaction)	7% +/(++) c	3% (+)/– c	12% +/(++) d	<1% (+)/– d	<1% (+)/– d
<i>Cytokeratin profile</i>					
CK7/CK19	79%/30% ++/+++ d/d	48%/15% +/++ p/p	53%/18% +/(++) d/d	20%/11% +/(–) d/d	8%/9% +/- c/d
CK8/CK18	45%/30% +/++ d/d	40%/40% +/++ d/p	78%/30% ++/+++ d/d	63%/88% ++/+++ p/p	87%/60% ++/+++ d/d
<i>Transdifferentiation</i>					
Vimentin (mesenchymal)	8% +/- p	25% +/++ p	15% +/(++) p	73% ++/+++ d	66% ++/+++ d
CK20 (gastrointestinal)	7% +/(++) p	4% +/(++) p	6% +/(++) p	3% (+)/– p	<1% – p
Chr-A (endocrine)	1% +/- d	3% +/- d	4% +/- d	2% (+)/– d	<1% (+)/– d
α_1 -chym (acinar)	3% +/- p	<1% (+)/– p	4% +/- p	7% +/- d	9% +/- d
<i>Dedifferentiation</i>					
pdx-1	7% +/+++ d	17% ++/(+++) d	<1% (+)/– d	<1% (+)/– d	<1% (+)/– d
shh	12% +/(++) d	14% +/(++) d	25% +/+++ p	28% +/+++ p	31% +/+++ p
ptc	11% +/(++) d	10% +/(++) d	22% +/+++ p	33% ++/(+++) p	35% ++/(+++) p

¹ Quantitative evaluation of the stainings (% of all cells).

² Semiquantitative evaluation of the stainings: – = negative, + = weak, ++ = moderate; +++ = strong.

³ Distribution inside one xenograft: d = disseminated, c = central; p = peripheral; dependent on the localization of >50% of positive cells.

Expression of Transdifferentiation Markers. CK20 expression was mainly found in tumor xenografts with ductal differentiation (YAPC) or mixed ductal and solid structures (DAN-G and CAPAN-1). In these cases a predominant peripheral location of CK20 expression was found and was associated with a tubulus-like phenotype

of ductal tumor formation (CAPAN-1) which mimicked PANINA.

β -Catenin was diffusely localized at the cytoplasm membrane in the xenografts of YAPC, DAN-G and CAPAN-1. Focally, a nuclear expression of β -catenin, co-localized with the expression of CK20, was also observed

Fig. 2. Western blot analysis of CK7, CK20, Chr-A, pdx-1, shh and ptc in different human pancreatic cancer cells in vitro and in xenotransplants. Shown are representative examples of Western blotting from pooled total protein of 5 xenografts per cell line and the naïve cell lines (**A**). The bar diagrams show the mean densitometric analysis of two independent Western blots ($\pm$ SEM) in xenotransplants (**B**) and in vitro (**C**).

in CAPAN-1. An obviously enhanced cytoplasmic expression of β -catenin in combination with a loss of membranous staining was found in human pancreatic cancer cell lines PANC-1 and MIA PaCa-2 in the periphery of the xenografts.

Chromogranin A (Chr-A), being a marker for endocrine differentiation, was detected mainly in solid areas of tumor samples. Yet, expression was observed only in disseminated cells with a low overall expression level. The expression of α_1 -antichymotrypsin (α_1 -chym), which was used as a marker for acinar differentiation, increased

from low in YAPC, DAN-G and CAPAN-1 xenografts (1–4% of all cells) to moderate in PANC-1 and MIA PaCa-2 (7–9% of all cells). Finally, a laminin-5 overexpression was observed at the tumor invasion front in all cancer cell lines (data not shown).

Expression of Dedifferentiation Markers. Interestingly, pdx-1 was exclusively expressed in differentiated ductal tumor formations of YAPC and DAN-G cancer cell lines while shh and ptc were linked to CAPAN-1, PANC-1 and MIA PaCa-2. Similar to CK expression, no association between tumor cell morphology and expression of pdx-1, shh and ptc could be established.

Western Blot Analysis and mRNA Analysis of Xenografts (see fig. 2A, B, 3A)

Overall, the Western blot and mRNA analyses confirmed the immunohistochemical findings. Although all human cancer cell lines expressed CK7, the highest levels

Fig. 3. Quantitative real-time PCR of CK7, CK8, CK20, pdx-1, shh and ptc mRNA of different human pancreatic cancer cells in vivo (A) and in vitro (B). Shown are mean mRNA levels of two independent experiments ($\pm$ SEM), expressed relative to the level of GAPDH mRNA.

of protein and mRNA were found in tumors with a ductal or ductal/solid phenotype. Comparable to the IHC results, the loss of morphologic differentiation was associated with increased levels of CK8. The expression levels of CK7 and CK8 were associated with the mRNA levels of dedifferentiation parameters: In cases with high amount of pdx-1 (YAPC and DAN-G) higher levels of CK7 and lower levels of CK8 and shh/ptc than in all other cancer cell lines were detected. Protein and mRNA levels of CK20 were associated with pdx-1 and CK7-expression. Finally, low levels of Chr-A protein were detectable in DAN-G, PANC-1 and CAPAN-1 while MIA PaCa-2 showed high levels of Chr-A mRNA (data not shown) and protein.

Human Pancreatic Cancer Cell Lines in vitro (see fig. 2A, C, 3B)

Pancreatic carcinoma cell lines express the classical ductal human pancreatic cancer CK pattern profile (CK7/CK19 and/or CK8/CK18) in vitro as proven by immunocytochemistry (data not shown), Western blotting and mRNA analysis. In detail, expression of CK7 in combination with CK8 was found in YAPC, DAN-G and CAPAN-1, whereas CK8 was only detected in PANC-1 and MIA PaCa-2 pancreatic cancer cell lines. Expression of markers of trans- and dedifferentiation (Chr-A, pdx-1 and ptc) was only marginally detectable in PANC-1 and MIA PaCa-2 cell lines. Expression of CK20 (determined by Western blot and mRNA analysis) was only faintly seen in YAPC. Interestingly, these results were not paralleled for DAN-G and PANC-1 on mRNA levels regarding CK20 and shh.

Comparison of Protein and mRNA Expression in vitro and in vivo

The typical CK profile of human pancreas could be detected in vitro and in vivo. In contrast to in vitro experiments, PANC-1 and MIA PaCa-2 show a novel expression of CK7, a marker of ductal differentiation, in vivo.

Although focal expression of markers of trans- and dedifferentiation could already be observed in vitro. These phenomena were more enhanced on protein level in all human pancreatic cancer cell line xenografts, especially for CK20 (YAPC, DAN-G, CAPAN-1, MIA PaCa-2), Chr-A (all), pdx-1 (YAPC, DAN-G, PANC-1), shh (PANC-1 and MIA PaCa-2) and ptc (all except CAPAN-1). Although single genes were overexpressed in vivo (e.g. CK20 in YAPC, pdx-1 in YAPC and DAN-G, shh and ptc in MIA PaCa-2), the overall expression pattern of mRNA was heterogeneous between in vitro and in vivo experiments.

Discussion

In this model of human pancreatic carcinoma xenografts in nude mice, we demonstrated that different human ductal pancreatic carcinoma cell lines exhibit distinct morphological patterns and molecular markers of trans- and dedifferentiation. In detail, we detected two major specialized morphologic and molecular pathways

inside different human ductal pancreatic carcinoma cell lines: (i) a ductal tumor pattern (YAPC and DAN-G) with predominance of CK7 and pdx-1 and (ii) a more dedifferentiated solid tumor pattern (CAPAN-1, MIA PaCa-2 and PANC-1) with increased expression of CK8 and shh/ptc. These findings are of special interest as they indicate that xenografts of human pancreatic tumor cell lines have the potency to express markers known in embryonic pancreatic development, showing that these cell lines can reactivate otherwise inactive signaling pathways of development.

Embryologic development of the pancreas is under control of several homeodomain box proteins [for detailed reviews, see 4–6]: Among these, members of the sonic hedgehog protein family (shh) and their cognate receptors, Patched (ptc), are involved in pancreatic morphogenesis, regulating the formation of the dorsal and ventral endodermal buds with later fusion [32–34]. Additionally, ductal and endocrine differentiations arise from exocrine parts of the embryonic pancreas and are controlled by the pancreas duodenum homeodomain protein pdx-1, which is expressed in the whole pancreas in early embryonic development but is only found in β -cells of endocrine islets in the adult pancreas [35, 36]. Additionally, shh and pdx-1 are also upregulated in pancreatic tumorigenesis [9, 12], while the functional significance of this is so far unknown. There is some evidence that shh and pdx-1 act contrary in some circumstances (amniote embryo) [37], while we and others observed synergistic regulation of shh and pdx-1 on the protein level in mature pancreas (at least in mammals) [38] and in zebrafish embryo [39]. Ductal alterations preceding carcinoma formation, so-called PANINA, are spatiotemporally regulated by differential expression of shh [10] and pdx-1 [11]. In concordance with our observations, recent investigations on epithelial intermediate filament proteins demonstrated that dedifferentiation is associated with different expression of CKs [13, 14].

Interestingly, we found that these factors of embryologic development (pdx-1 and shh/ptc) as well as the CK profile are associated with morphological signs and protein expression patterns of transdifferentiation. A tubular transdifferentiation with enhanced expression of CK20 was observed in the ductal group [40, 41]. This was associated with an increased expression of β -catenin in well-differentiated human carcinoma cell lines. Additionally, we confirmed the findings of Joo et al. [28] and Qiao et al. [29] that grading of pancreas cancer is associated with reduced membranous and cytoplasmatic expression of β -catenin. Expression of vimentin, a classical

marker of mesenchymal differentiation, was mainly observed in dedifferentiated human carcinoma cell lines like MIA PaCa-2, which corroborates earlier findings [13, 20]. Furthermore, the vimentin-positive cell lines PANC-1 and MIA PaCa-2 showed a reduced expression of pdx-1 and elevated levels of ptc which is in line with the current literature [19]. These cell lines also displayed a higher expression of α_1 -chym indicating a true acinar transdifferentiation. Endocrine transdifferentiation was observed at low levels in all human pancreas carcinoma cell lines, indicating that this is not limited to the known pdx-1 ductal pathway [22, 23].

In particular, expression of molecular markers of trans- and dedifferentiation were more pronounced in a physiologic environment compared to cell culture conditions.

The observed overexpression of laminin-5 at the tumor border confirmed that epithelial-mesenchymal interactions are essentially involved in the tumor progression, also in pancreatic cancer [26].

Overall, growing evidence suggests that tumorigenesis repeats embryologic development pathways in many aspects: Neoplastic cells re- or dedifferentiate into a stem-cell-like phenotype with the potential of proliferation [25, 27]. One of the best examples in this field is the adenocarcinoma of the colon. Intensive morphologic analyses of colon tumors showed that tumor formation imitates typical morphological patterns of budding and branching during embryogenesis which was confirmed by molecular analyses [42]. Additional studies of gastric carcinomas showed that neoplastic changes in the gastric cancer sequence are associated with dedifferentiation imitating fetal morphogenesis [43], indicating that these mechanisms are commonly found in gastrointestinal tumor development and might also apply to pancreatic carcinogenesis.

According to our study design, we are aware that this is a relatively static glance at the differentiation potential of the various human pancreatic cancer cell lines in a nude mouse xenograft model, regardless of immediate changes after xenografting due to technical reasons (injection of tumor cell suspension).

In summary, the observed findings indicate that (i) human pancreatic cancer cell lines have the complete potential for differentiation *in vivo*, (ii) imitating different stages of embryonic pancreatic development, (iii) which could possibly be used further as a tool for designing specific tumor therapies.

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