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Establishment of Primary Cell Lines in Pancreatic Cancer

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1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is by far the most common type of tumor in the exocrine pancreas, accounting for 85% to 100% of all malignant pancreatic tumors (Kloppel et al., 2004). During the last 30 years, little improvement in the prognosis of patients with PDAC has been achieved (Jemal et al., 2009). A better understanding of the biological nature of this neoplasm might improve the prognosis of patients. For this purpose, permanent cultured cell lines are helpful, since their convenience of use facilitates a variety of experiments (Ku et al., 2002). However, the amount of viable tumor-derived material is limited. The majority of research in PDAC has been done in a few cell lines; only 19 pancreatic carcinoma cell lines are broadly available for research. In addition to their small number, permanent cell lines have another disadvantage: the long culture times leave the cells prone to genetic drift (Kato et al., 1999; McQueen et al., 1991).

To obtain a greater phenotypic heterogeneity of the disposable cell lines, and to circumvent the use of "old" cell lines, it is advantageous for research laboratories that focus on pancreatic cancer to establish their own primary carcinoma cell lines (Ruckert et al., 2011). Before using these cell lines in basic research, the origin of the cell line should be confirmed to exclude cross-contamination by existing cell lines. This is important, because some studies suggest that 15–20% of the cell lines used in experiments have been misidentified or contaminated with another cell line, which is also true for primary cell lines (Cabrera et al., 2006; Drexler et al., 1999). A characterization of the cell lines is further necessary to analyze and document the typical biochemical and pathophysiological features of the tumor cell lines (Henderson et al., 1996). In the present article we give information on how to isolate and characterize primary pancreatic cancer cell lines.

2. Techniques for the isolation of primary pancreatic cancer cell lines

The principle of tissue culture was established by Wilhelm Roux in the 19th century. However, it took nearly 60 years before the establishment of the first human cancer cell line (Gey et al., 1952). The first report on the culture of pancreatic cancer cell lines was in l963 (Dobrynin, 1963). Cells that are cultured directly from a subject are known as *primary cell lines* and different techniques have been used to establish such pancreatic cancer cell lines.

Some of these primary cells can be cultured successfully over a long time period and are then named *permanent* or *immortal cell lines*.

The most frequent used techniques are explant culture (where the culture grows from a solid tumor sample), xenografting, enzymatic digestion and culture of malignant bodily fluids. In the following, we describe these techniques in detail.

2.1 Explant cultures

The two most frequently used explant culture techniques are the "mechanical dissociation" and the "outgrowth method". To obtain sterile conditions, the tumor specimen should be rinsed three times in wash medium.

Most scientist use the mechanical dissociation to establish pancreatic cancer cell lines (**Table 1**). In this technique after washing, the specimen is transferred to a sterile petridish and cut up into smaller pieces about 1-2 mm^3 using sterile scalpel and forceps. The fragments and the spilled cells are then placed in a tissue culture flask and medium is added. The production of proteolytic factors (Diamantidis et al., 2008; Mahadevan et al., 2007) and augmented cell motility (Hotz et al., 2007; Rückert et al., 2010) enables pancreatic cancer cells to actively leave the tissue fragment. Complexes of tumor cells can be seen within the medium after 24 h (**Figure 1, C and D**). The presence of these "STC´s" (small trabecular complex) is generally a good predictive parameter for the successful isolation of primary tumor cell lines and under standard cell culture conditions cancer cell colonies will grow, as well as other contaminating cell types such as fibroblasts or lymphocytes (Rückert et al., 2011).

The other explant culture technique is the outgrowth method. As in the mechanical dissociation- technique, little pieces of the solid tumor are produced by dissection of the tumor sample with an sterile scalpel. Those little fragments are placed within a 6-well plate and left to dry until the pieces stick on to the surface. Medium is then added cautiously. Tumor cells as well as fibroblast will grow out of the tissue fragment (**Figure 1, A and B**; for a pictured tutorial see Rückert et al., 2011).

2.2 Xenografts

Xenografting of tumor samples into athymic mice is a way to establish pancreatic tumor lines with a good success rate and is frequently used by scientist for this purpose (Hotz et al., 2000). By repeated passages of the tumor lines in nude mice the cells are said to become more aggressive. Some of these tumor lines are used as source for the establishment of cell lines by either explant cultures or enzymatic digestion (Dexter et al., 1982; Yachida et al., 2011) (**Table 1**).

2.3 Enzymatic digestion

Enzymatic digestion can be used to free cancer cells from adherent connective tissue and produce a suspension of cells. For this purpose different enzymes are used, such as trypsin (Kaku et al., 1980) or collagenase type IV (Chifenti et al., 2009; Kalinina et al., 2010). However, this method is laborious and rarely used to establish pancreatic cancer cell lines (**Table 1**).

Author and year	Name	Method used	Medium	Characterisation
(Dobrynin et al., 1963)	CaPa	Explant culture	Medium 199, 5% human serum, 5% FCS	M, DD, chr
(Lieber et al., 1975)	Panc-1	Explant culture	DMEM, 10% FCS	M, DD, chr, xeno, FA
(Owens et al., 1976)	Hs 700T HS 766T	Enzymatic digestion	DMEM, 10% FCS	M, DD, chr, plating efficiency
(Yunis et al., 1977)	MIA PaCa-2	Explant culture	DMEM, 10% FCS, 2.5% horse serum	M, DD, chr, HC, FA, EM
(Akagi & Kimoto, 1977)	HCG-25	Ascites w/ medium	RPMI 1640, 10% FCS	M, DD, chr, xeno, HC, FA, EM, plating efficiency
(Fogh et al., 1977)	Capan-1	n.n.	RPMI 1640, 15% FCS	M, DD, HC, xeno, FA
(Grant et al., 1979)	Ger	Explant culture	(Ham's F12 + Eagle's) or DMEM, 10% FCS, amino acids, 1 mM glutamine, AA, 50 tzg/ml soya-bean trypsin inhibitor	M, DD, chr, xeno, FA,TM
(Kaku et al., 1980)	QCP-1	Enzymatic digestion	Medium 199, 10% FCS	M, DD, chr, HC, FA
(Morgan et al., 1980)	COLO 357	Explant culture	RPMI 1640, 20% FCS	M, DD, chr, HC, FA, plating efficiency
(Chen et al., 1982)	AsPC-1	Ascites w/ medium	RPMI 1640, 10% FCS	M, HC, chr, xeno, FA
(Metzgar et al., 1982)	HPAF	Ascites w/ medium	EMEM, 10% FCS	xeno
(Dexter, 1982)	RWP1 RWP2	Xenograft	RPMI 1640, 20% FCS, buffered	M, DD, chr, HC, xeno, FA, plating efficiency
(von Bulow et al., 1982)	PancTuI-I	n.n.	n.n.	
(Kyriazis et al., 1983)	SW-1990	Explant culture	L-15-CI	M, DD, chr, HC, xeno, FA, plating efficiency
(Yamaguchi et al., 1983)	HPC-Y1	n.n.	RPMI 1640, 10% FCS	M, xeno, TM
(Okabe et al., 1983)	T3M-4	Xenograft	F10, 15% FCS, AA	M, DD, chr, HC
(Kyriazis et al., 1986)	Capan-2	n.n.	RPMI 1640, 15% FCS, AA	M, DD, chr, HC, xeno, FA, plating efficiency

Author and year	Name	Method used	Medium	Characterisation
(Yamada et al., 1986)	PSN-1	Xenograft	RPMI 1640, 10% FCS	M
(Yamaguchi et al., 1986)	HPC-YT	n.n.	RPMI 1640, 10% FCS	M, DD, chr, xeno, FA
(Tan et al., 1986)	BxPC-3	Explant culture	RPMI 1640, 20% FCS, buffered	M, DD, chr, HC, xeno
(Kobari et al., 1986)	PK-1 PK-8 PK-9 PK-12 PK-14 PK-16	Explant culture	MEM, 20% FCS	M, DD, chr, HC, xeno, TM, doubling time
(Iwamura et al., 1987)	SUIT-2	Explant culture	DMEM, 5% FCS	M, DD, xeno, TM, EM
(Sujino et al., 1988)	JHP-1	Ascites w/ medium	n.n.	M, DD, chr, xeno, TM, plating efficiency
(Drucker et al., 1988)	SU.86	Explant culture	RPMI 1640, 20% FCS	M, DD, HC, chr, xeno, TM
(Nagata et al., 1989)	FA6	Xenograft	RPMI 1640, 10% FCS	FA
(Frazier et al., 1990)	MDA Panc-3	Explant culture	(L-15 + DMEM), 16 kg/ml glutathione, 5 kg/rnl insulin, 5 pg/ml transferring, 5 ng/ml selenium, 5 ng/ml EGF, 10% FCS, AA	M, DD, chr, HC, EM
(Fujii et al., 1990)	SOJ	Explant culture	RPMI 1640, 10% FCS	M, DD, chr, xeno, TM
(Schoumacher et al., 1990)	CFPAC-1	Explant culture	RPMI 1640, 10% FCS	IHC, xeno, FA
(Ikeda et al., 1990)	KP-1N KP-2 KP-3	Xenograft	Daigos T, 10%FCS	IHC, xeno, TM
(Yamaguchi et al., 1990)	HPC-Y0 HPC-Y1 HPC-Y5 HPC-Y9 HPC-Y11 HPC-Y15 HPC-YP HPY-YS HPC-Y25	Explant culture	RPMI 1640, 10% FCS	M, DD, IHC,chr, xeno, FA

Author and year	Name	Method used	Medium	Characterisation
(Chen et al., 1990)	PC-1 PC-2	Xenograft	Ham´s F12, 15% FCS	M, DD, chr, IHC, xeno, TM
(Elsasser et al., 1992)	PA-TU-8988S PA-TU-8988T	Explant culture	DMEM, 10% FBS, 10% Horse serum, 2 mM glutamine, AA	M, DD, chr, HC, xeno, EM
(Nishimura et al., 1993)	HuP-T3 HuP-T4	Ascites w/ medium	MEM, 10% FBS, 1% amino acids, 1% sodium pyruvate	M, DD, xeno, TM
(Elsasser et al., 1993)	PA-TU-8902	Explant culture	DMEM, 10% FCS, 2 mM glutamine	M, DD, chr, IHC, xeno, TM, EM
(Kalthoff et al., 1993)	PT45	n.n.	n.n.	n.n.
(Vila et al., 1995)	IMIM-PC-1 IMIM-PC-2 SK-PC-1 SK-PC-3	Explant culture	E4, 10% FCS	M, IHC, TM, FA
(Heike et al., 1995)	MZ-PC-1	Pleural effusion w/ medium	CMRL, 15% FCS, 2 mM glutamine, 1% amino acids	M, IHC, xeno, EM
(Lehnert et al., 1999)	A818	Explant culture	RPMI 1640, 10% FCS, 2 mM glutamine, 1 mM sodium pyruvate, AA	TM, FA
(Kato et al., 1999)	KMP-1 KMP-2 KMP-3 KMP-4 KMP-5 KMP-6	Explant culture	RPMI 1640 + Ham's F12, 20% FCS, AA	M, DD, chr, IHC, xeno, TM, seeding efficiency
(Hou et al., 1999)	PC-EN	Xenograft	n.n.	M, DD, chr, mut, IHC, xeno, TM
(Fralix et al., 2000)	UK Pan-1	Explant culture	DMEM, 10% FCS	M, DD, chr, mut, xeno, FISH
(Ku et al., 2002)	SNU-213 SNU-324 SNU-410 SNU-494	Explant culture	ACL-4, 5% FCS	M, DD, chr, mut, IHC
(Eisold et al., 2004)	FAMPAC	Explant culture	RPMI 1640, 10% FCS, AA	M, DD, chr, mut, xeno, TM
(Kawano et al., 2004)	SUIT-4	Ascites w/ medium	DMEM, Ham´s F12, AA	M, DD, chr, mut, IHC, xeno, FA

Author and year	Name	Method used	Medium	Characterisation
(Starr et al., 2005)	p34	Pleural effusion w/ medium	DMEM, 10% FCS, 2 mM glutamine, AA	M, DD, chr, IHC, xeno, FA
(Kong et al., 2007)	SPH	Ascites w/ medium	n.n.	M, DD, chr, xeno, TM
(Chifenti et al., 2009)	PP78 PP109 PP117 PP161	Enzymatic digestion	RPMI 1640, 1% glutamine, 10% FCS, AA	M, DD, STR, chr, mut, IHC, FA
(Kalinina et al., 2010)	PaCa 5061	Enzymatic digestion	RPMI 1640, 10% FCS, AA, 50 nmol/ml transferrin, 0.01 µg/ml insulin, 0.01 µg/ml EGF, 0.01 µg/ml basic FGF	M, DD, IHC, xeno, FA, TM, FISH
(Rückert et al., 2011)	PaCaDD-43 PaCaDD-60 PaCaDD-119 PaCaDD-135 PaCaDD-137	Explant culture	Dresden-Medium	M, DD, STR, mut, FA
(Yachida et al., 2011)	a99	Xenograft	DMEM, 20% FCS	M, DD, chr, mut, xeno, colony formation

(DD= doubling rate/ growth curve; M= morphology; STR= standard tandem repeat/DNA-fingerprint; chr= chromosomal analysis; mut= mutational analysis; xeno= xenograft; FA= functional analysis; HC= histochemistry; IHC= immunohistochemistry; EM= electron microscopy, TM= tumor marker; AA= antibiotics).

Table 1. Human pancreatic tumor cell lines (adapted from Iwamura & Hollingsworth, 1998). This list is not exhaustive. Some of the cell lines are available at ATCC (www.lgcstandards-atcc.org) or the German Collection of Microorganisms and Cell Cultures (www.dsmz.de)

2.4 Processing of malignant pleural effusion or ascites

This method is frequently used to obtain primary cancer cell lines. Although the success rate is not higher than in other methods many scientist favour this technique as material is easily obtainable. For establishing cancer cell lines with this method, bodily fluids are added to variable amounts of cell culture medium (Akagi et al., 1977; Chen et al., 1990; Nishimura et al., 1993) (**Table 1**).

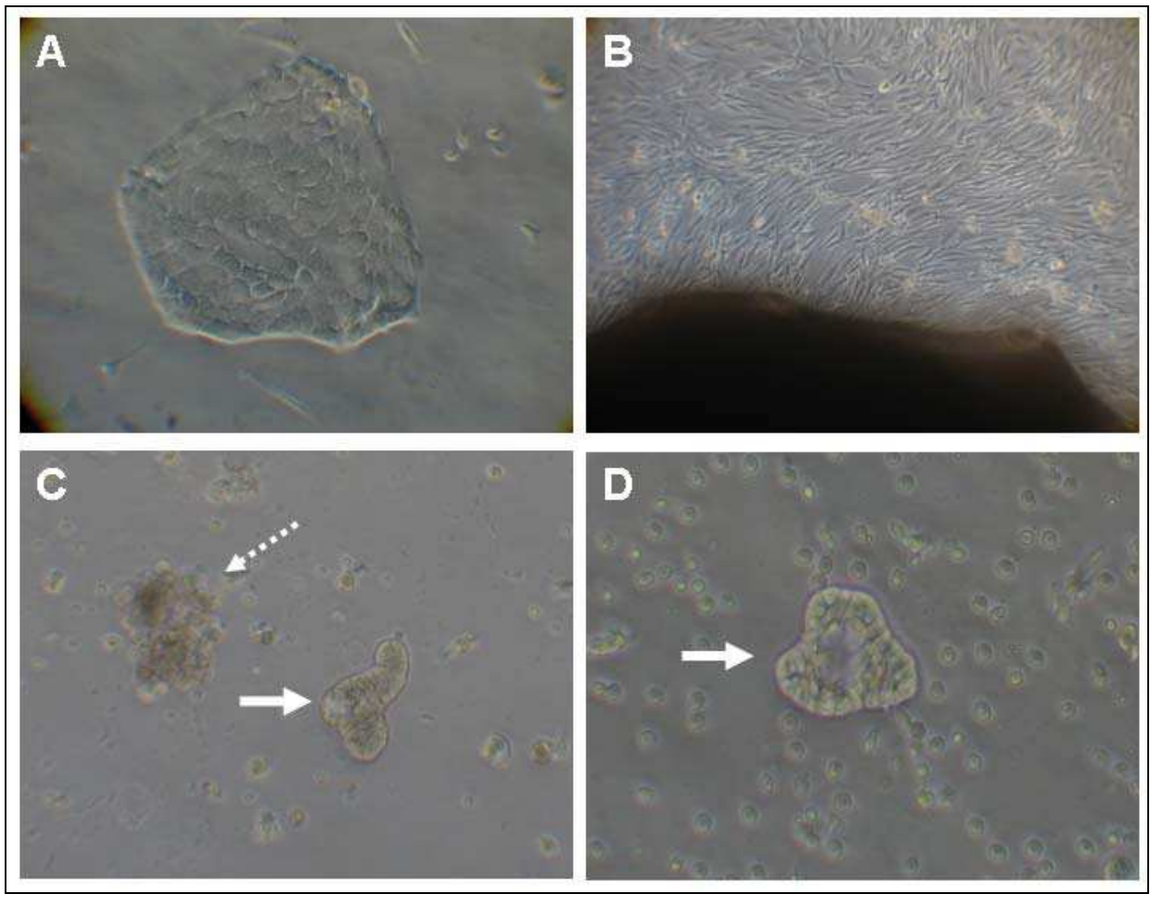

Fig. 1. Typical morphology of a primary pancreatic cancer colony (**A**). Fibroblasts growing out of a tissue fragment (**B**). Figures **C** and **D** depict complexes of cancer cells (full arrow) which the author calles "STC´s" (small trabecular complex). In (**C**) there is also a cluster of mesenchymal cells (interrupted arrow).

2.5 General remarks on the establishment of primary cell lines

The processing of samples should be as fast as possible. The success rate for the establishment of cell lines is considerably shortened if processing starts more than 2 hours after harvesting of the sample.

Parenchymal cells in culture are generally obvious due to their close cell-cell contacts and their tendency to grow in colonies (**Figure 1, A**). However, parenchymal cells are often overgrown by cells from the stromal compartment (**Figure 1, B**). Those fibroblasts can be removed by different techniques. One possibility is to mechanically remove fibroblasts by a sterile cannula under phase contrast microscope. Another method uses a solution of trypsin to enzymatic remove fibroblasts. This enzymatic removement is possible because tumor cells adhere much more firm to the surface than fibroblasts do.

The successful establishment of primary cell lines is often impeded by contamination by fungi and bacteria. For this purpose we advise to always use antibiotics within the cell culture media. A way to protect cell lines from fungal infection is reported in Rückert et al,

2011. Cell lines should be cryoconserved early and often during the early passages. As a general rule, cultures of pancreatic tumor cell lines should be split at high density (l : 2) (Iwamura et al., 1998; Rückert et al., 2011).

3. Cell culture conditions and medias

The majority of pancreatic cancer cell lines are grown *in vitro* in a basic culture medium such as DMEM or RPMI 1640 substituted with foetal calf serum (FCS) in varying percentages (**Table 1**). However, there are also successful isolations using other media. Generally, after a primary cell culture is established it should be assessed if growth might be optimized by a different media and/or a different ratio of FCS. Some pancreatic tumor cells can be adapted to growth in reduced serum (0.5% or less) or serum free conditions**.**

Cell cultures are generally maintained at 37°C in a humidified atmosphere of 5% CO_2 in air, and the medium should be replaced every 3 d in established cultures.

To guarantee the availability of primary cell lines for the further experiments, all cell lines should be cryoconserved. Storage of cryotubes should always be performed in liquid nitrogen.

4. Characterisation of primary tumor cell lines

Characterization of primary cell lines should be conducted to *proof the origin* from the parent tumor and to *proof the absence of cross-contamination*. Only by this the researcher can be sure that *in vitro* studies with a certain cell line can be related to a distinct tumor and its clinicophysiological properties. Most malignant tumors are heterogeneous in morphology and biological properties, e.g. degree of differentiation, malignant transformation, and metastatic properties. This fundamental facet of all solid tumors is called tumor heterogeneity (Heppner, 1984). Therefore it is essential to further *define the cytostructural and pathophysiological characteristics* of the cell line.

For these purposes different standards have been defined, which will be further explained below (Henderson & Kirkland, 1996; Iwamura & Hollingsworth, 1998).

4.1 Morphology and cytostructural characteristics

4.1.1 Growth pattern

Pancreatic tumors are generally categorized according to morphological criteria as grades of differentiation and invasiveness. Many cell lines exhibit morphological characteristics that are consistent with the grades of differentiation reported for the tumor from which the cell line was derived. In cell lines, morphology is normally assessed by phase contrast microscopy, but some authors also use electron microscopy or histochemical staining.

Features that should be described are:

- the growth pattern (e.g. epithelial, disorganized, mesenchymal)
- size of the cells (e.g. homogenous, inhomogenous, small, big)
- form of the cell (e.g. round, polygonal, elongated, swirly)
- size and form of the nucleus (e.g. small, big, ovoid, round)

Some examples of growth patterns of primary cells are given in **Figure 2**.

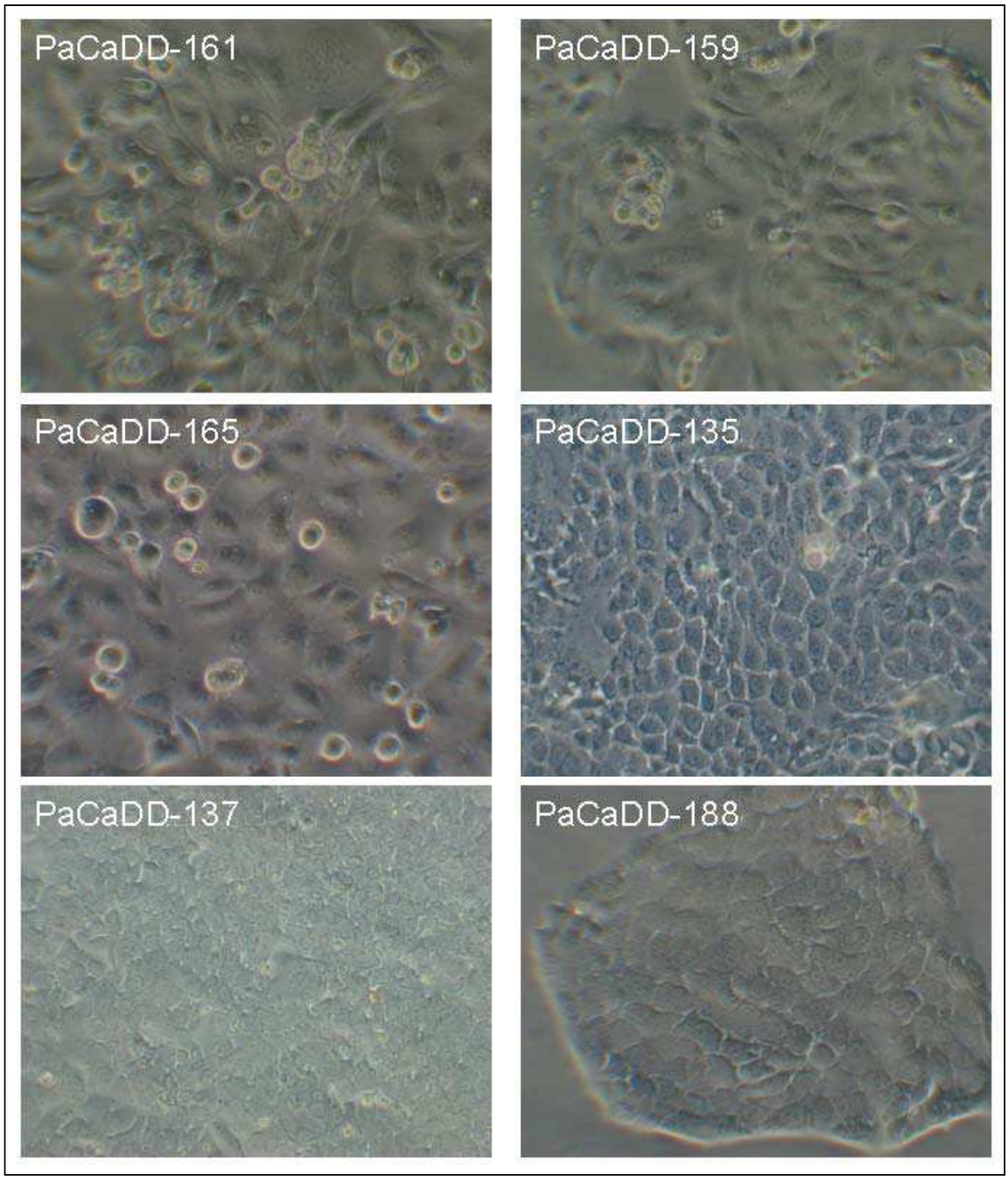

Fig. 2. Examples of the morphology and growth pattern of the primary carcinoma cell lines from our research lab as seen with phase contrast microscopy (Details from 40x, pictures by Felix Rückert).

4.1.2 Immunohistochemistry

Cell surface antigen characteristics can be used to discriminate pancreatic cancer cells from contaminating cell types like stromal and immune cells. The immunohistochemical phenotype can be compared to the native tumor tissue and thereby further proof its origin. Typical markers for pancreatic cancer are CK 8/18, E-cadherin, ezrin, p53, SMAD4, and Vimentin (Rückert et al., 2011).

4.2 Pathophysiological characteristics

4.2.1 Doubling time

The doubling time is an important feature of each cell line, because it can be correlated with an important pathophysiological parameter: aggressive growth. Furthermore, conclusion can be drawn on how often medium should be changed and in which ratio cell lines should be split.

The cell doubling time can be determined by plating a certain number of viable cells in to a well. Cells should then be counted at 24 h intervals for 7 d in triplicate. The doubling time of the cell population can then be calculated from the logarithmic growth curve by the following formula:

$$\vartheta = \lg N - \lg N0/\lg 2\ (t-t0),$$

with doubling time = $1/\vartheta$

Of course there are other possibilites to define the growth rate e.g. BrdU-staining or ki-67 staining.

4.2.2 Tumorigenicity

One notable feature of pancreatic cancer, which is amenable to study through the use of cell lines, is invasiveness and metastasis. Tumorigenicity can be measured by assessing the tumor formation of cell lines in athymic mice (Henderson & Kirkland, 1996). This can be done by orthotope (pancreas) or ectope (subcutaneous) injection of tumor cells. The number of cells required ranges from 100,000 to 2,000,000 cells. Pancreatic cancer cell lines should form a tumor within 3-4 weeks. If the cell line has tumorigenic potential, the progression of tumor volume/time as well as the ability to form metastasis should be documented.

4.3 Chromosomal and mutational analysis

Chromosomal and mutational analyses are used to proof the origin and the uniqueness of each cell line. They give further evidence on the role of mutations for the pathophysiology of the tumor. The origin of the cell lines, however, can also be proofed by other techniques as microsatellite analyses (Rückert et al., 2011).

4.3.1 Chromosomal analysis

Chromosomal analysis has long been the standard method to proof the uniqueness of an certain cell line. Classically, karyotype analysis was undertaken by chromosome banding.

By this method numerical and structural chromosomal aberrations could be identified. However, the chromosomal origins of markers, subtle translocations, or complex chromosomal rearrangements were often difficult to identify with certainty (Schrock et al., 1997). Because of this, new methods are recommended like the spectral karyotyping (SKY) or the comparative genomic hybridisation (array CGH).

During SKY, chromosomes are hybridized simultaneously with 24 chromosome-specific painting probes. The measurement of defined emission spectra allows for the definitive discernment of all human chromosomes in different colors (Schrock, 1997). The array CGH is a method to analyse copy number changes (gains/losses) in the DNA content of a given cell. By this, gains or losses of genes in tumor chromosomes compared to normal DNA can be detected (Wessendorf et al., 2002).

4.3.2 Mutational analysis

Pre-malignant and malignant tumors evolve by clonal expansion of mutant cells that have either a reproductive or survival advantage over other cells in the tissue. This competitive advantage is acquired by the overexpression of oncogenes and inactivation of tumor suppressor genes. Numerous studies have found a relatively unique molecular fingerprint in PDAC that is comprised of frequent alterations of four genes: kRAS, $p16^{INK4A}$, DPC4 and p53 (Bardeesy et al., 2002; Krautz et al., 2011). Because of the importance of these genes for the pathophysiology of PDAC the mutational analysis of these genes is recommended.

5. Conclusion

Basic research in cancer is absolutely dependent on cancer cell lines. The establishment of primary pancreatic cancer cell lines in laboratories with focus on pancreatic cancer has considerable advantages and can be done with reasonable expense. We hope that the present chapter encourages more scientists to start establishing own cell lines.

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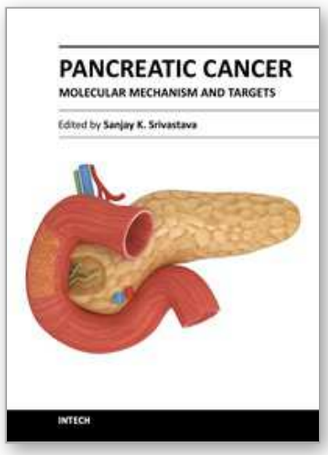

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This book provides the reader with an overall understanding of the biology of pancreatic cancer, hereditary, complex signaling pathways and alternative therapies. The book explains nutrigenomics and epigenetics mechanisms such as DNA methylation, which may explain the etiology or progression of pancreatic cancer. Book also summarizes the molecular control of oncogenic pathways such as K-Ras and KLF4. Since pancreatic cancer metastasizes to vital organs resulting in poor prognosis, special emphasis is given to the mechanism of tumor cell invasion and metastasis. Role of nitric oxide and Syk kinase in tumor metastasis is discussed in detail. Prevention strategies for pancreatic cancer are also described. The molecular mechanisms of the anti-cancer effects of curcumin, benzyl isothiocyante and vitamin D are discussed in detail. Furthermore, this book covers the basic mechanisms of resistance of pancreatic cancer to chemotherapy drugs such as gemcitabine and 5-flourouracil.

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