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In vitro models of pancreatic cancer for translational oncology research

Georg Feldmann, **Sherri Rauenzahn**, and **Anirban Maitra**
Department of Pathology, The Sol Goldman Pancreatic Cancer Research Center, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Abstract

Background—Pancreatic cancer is a disease of near uniform fatality and the overwhelming majority of patients succumb to their advanced malignancy within a few months of diagnosis. Despite considerable advances in our understanding of molecular mechanisms underlying pancreatic carcinogenesis, this knowledge has not yet been fully translated into clinically available treatment strategies that yield significant improvements in disease free or overall survival.

Objective—Cell line-based *in vitro* model systems provide powerful tools to identify potential molecular targets for therapeutic intervention as well as for initial pre-clinical evaluation of novel drug candidates. Here we provide a brief overview of recent literature on cell line-based model systems of pancreatic cancer and their application in the search for novel therapeutics against this vicious disease.

Conclusion—While *in vitro* models of pancreatic cancer are of tremendous value for genetic studies and initial functional screenings in drug discovery, they carry several imanent drawbacks and are often poor in predicting therapeutic response in humans. Therefore, in most instances they are successfully exploited to generate hypothesis and identify molecular targets for novel therapeutics, which are subsequently subject to further in-depth characterization using more advanced *in vivo* model systems and clinical trials.

Keywords

pancreatic cancer; cell line models; mouse models of pancreatic cancer; translational research; drug discovery

1. Introduction

Ductal adenocarcinoma of the pancreas (a.k.a. pancreatic cancer; the terms pancreatic cancer and pancreatic ductal adenocarcinoma will be used synonymously in the text) is an almost uniformly lethal malignancy, which accounts for an estimated 213,000 annual deaths worldwide and is the fourth most common cause of cancer-related mortality in the United States, with an overall median survival of less than 6 months [1, 2]. The five year survival rate is below 5% when combined for all stages, and <2% for the majority of cases that present at with advanced (metastatic) disease, which are amongst the most dismal seen in any human malignancy [1].

Correspondence: Georg Feldmann, MD, Department of Pathology, The Sol Goldman Pancreatic Cancer Research Center, Cancer Research Building 2, Room 316, 1550 Orleans Street, Baltimore, MD 21231, USA, gfeldma4@jhmi.edu, Phone: +1 410-955-3511, Fax: +1 410-614-0671.

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Ongoing research efforts over the past decade have led to significant advances in our understanding of the underlying etiological and molecular mechanisms facilitating pancreatic carcinogenesis. Nevertheless, this increase in knowledge has not yet been translated into clinically tangible advances in terms of novel therapeutic strategies with marked improvement of overall patient survival [3].

Cell-line based *in vitro* model systems of pancreatic cancer provide powerful tools for discovery of molecular targets for novel therapeutics as well as for preclinical evaluation of drug candidates. A brief review of available *in vitro* models and its use and limitations in drug research is given in the following text.

2. *In vitro* culture of non-neoplastic pancreatic cells

The human pancreas is a complex organ consisting of several tissue compartments and as of to date we are still far from fully understanding all of the physiologic interactions underlying regulation of organ development and homeostasis, as well as those governing the development of malignant neoplasia. The question of the 'cell of origin' of pancreatic cancer has long been a controversial issue. While the traditional model, which is mostly based on morphologic similarities observed by light microscopy on histological specimens, suggests that pancreatic cancer arises from the ductal cell compartment, there is an opposing hypothesis suggesting that pancreatic cancer arises from transdifferentiated acinar cells [4–6]. A variation of the latter theory is the concept of pancreatic cancer possibly arising from a yet to be defined population of pancreatic stem/progenitor cells, which some schools of thought believe might reside within the acinar cell compartment, or in centro-acinar cells [7–9]. Establishment and *in vitro* culture of non-neoplastic pancreatic cells is of interest with regard to pancreatic cancer research in at least two aspects: firstly, it allows distinct examination of conditions regulating growth and differentiation of the respective distinct cell compartments in an isolated *in vitro* setting, as well as determination of the genetic alterations required for malignant transformation of these cells. Secondly, such non-neoplastic cells provide valuable controls in functional studies using novel experimental therapeutic approaches, specifically identifying therapeutic targets that cancer cells depend on in order to maintain a fully malignant phenotype, while exerting little, or ideally, no effects on these non-malignant cells.

2.1 Pancreatic ductal cell culture

Despite the immense relevance for pancreatic cancer research, surprisingly few cases of long-term propagated *in vitro* cultures of pancreatic ductal cells have been reported. This may be due to several factors, such as the relative scarcity of ductal cells in the human pancreas (<5% of the total pancreatic volume), a general lack of knowledge regarding physiologic properties regulating their growth and differentiation, thus hampering establishment of appropriate culture conditions, and the frequent occurrence of senescence in *in vitro* cultures of ductal cells, which often prevent successful long-term culture [10–12]. Therefore, initially it has proven to be a challenge to propagate human pancreatic ductal cells in culture for more than 1–2 months [13–15].

Generation of two distinct models of epithelial cell lines that could readily be maintained in long term culture has been described, and these were generated either through immortalization by introduction of the human papillomavirus 16 gene E6E7 proteins, or by stable transfection with human telomerase reverse transcriptase (hTERT) and growth in a special culture medium containing epidermal growth factor (EGF) [16–18]. Unfortunately, these immortalized lines do not represent ideal *in vitro* models of genuinely 'normal' human pancreatic ductal cells, since introduction of viral proteins abrogates the function of key tumor suppressor pathways like p53 or Rb, while the presence of EGF activates several oncogenic pathways, e.g. downstream of EGFR. Nevertheless, both of these cell lines lack key functional features of fully malignant

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pancreatic cancer cells, e.g. anchorage independent growth in soft agar or the ability to engraft as tumors in athymic mice, and have thus been of tremendous value as controls in functional *in vitro* assays. Moreover, they have been successfully used in various *in vitro*-transformation models [17, 19, 20] (see example in Figure 1).

2.2 Culture of acinar cells

While isolation and *in vitro* propagation of rat pancreatic acinar cells under special conditions has been described 21, 22, long-term culture of human or mouse pancreatic acinar cells has proven to be challenging [23]. Short term (primary) explants of mouse acinar cells have been established and have been successfully utilized for a variety of ex vivo functional assays over their limited period (<7 days) of viability [24].

2.3 Culture of islet cells

Isolation of human islet cells has so far proved to be difficult and long-term survival under *in vitro* culture conditions has been limited [25–31]. As observed with ductal cells, cultured islet cells undergo senescence in *in vitro* culture. However, as opposed to duct cells, immortalization by stable transfection techniques has so far been mostly unsuccessful in the case of islet cells [23].

3. Generation of pancreatic cancer cell lines

There are two general approaches for the generation of cell lines from human pancreatic cancers that are frequently used: Cell lines can either be directly established from primary patient-derived tumor tissue samples or from murine xenografts of human pancreatic cancers in athymic nude or SCID mice [32–35]. The latter method carries the advantage that *ex vivo* passaging in mice is a relatively innocuous way to enrich the neoplastic cell compartment, while at the same time eliminating stromal components of human origin, thus enhancing the probability for successful establishment of a cancer cell line 36, 37. Addition of matrigel can further increase the take rate of xenografted tumor tissue samples [38]. Disadvantages of this method include the requirement of significantly more time (propagation of a subcutaneous xenograft from primary human pancreatic cancer tissue samples typically requires some weeks up to several months), the chance of contamination with murine fibroblasts and the risk of acquiring additional genetic alterations during the period of *in vivo* growth as a murine xenograft.

Success rates for direct establishment of pancreatic cancer cell lines from primary tissues are generally relatively low, and appear to be only slightly higher when metastatic tumor tissues are used as starting material as opposed to primary tumor tissue samples [35, 39–47]. Jaffee et al. systematically optimized a protocol for establishment of cell lines from primary pancreatic cancer tissues [48]. In this study, the authors describe four key factors as being critical for successful *in vitro* establishment of pancreatic cancer cell lines: firstly, freshly harvested tissues have to be cut into fragments of one to five mm in diameter and digested by collagenase (300 units/mL) and hyaluronidase (200 units/mL) overnight on a shaker at 37 °C. Secondly, before initial plating stromal cells have to be removed from the digested samples as far as possible by two 20 min centrifugation steps at gravity. Plating of resuspended cells is then done at a density of 1–2 million viable cells per milliliter of fresh Panc media. Thirdly, for optimum growth of primary carcinoma cells RPMI-1640 base medium containing 15% of fetal bovine serum (FBS), 200 μM of L-glutamine, 1× non-essential amino acids solution and 1% sodium pyruvate has to be supplemented with human insulin (0.2 units/mL) and insulin-like growth factors (IGF) I and II (0.01 μg/mL each). Lastly, during the first weeks of *in vitro* culture stromal overgrowth has to be avoided and contaminating fibroblasts have to be eliminated by repeated differential

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trypsinization. Using this optimized protocol, the authors report a success rate of ~30% for the establishment of pancreatic cancer cell lines from primary tissue samples.

In our experience complete purification of a primary cancer cell line from contaminating human (in the case of primary human tumor samples as starting material) or murine fibroblasts (in the case of xenograft tissues) can often be challenging despite repetitive differential trypsinization. It is therefore tempting to speculate, whether novel, improved techniques might become available in the near future that allow for reliable and more rapid purification of primary cell lines.

4. *In vitro* methods for translational research in pancreatic cancer

Pancreatic cancer is a complex genetic disease and the underlying genetic and functional alterations required during the multistep pancreatic cancer progression cascade are still not fully understood [49]. Isolation of patient derived pancreatic cancer cell lines provides the only means available to date to accurately mimic the entirety of genomic alterations involved in the human disease in an *in vitro* model system that can be maintained in culture for more than a few days 50 (see [51] for a review on molecular genetics of pancreatic cancer).

The use of cell line-based *in vitro* approaches for drug discovery in pancreatic cancer carries some obvious immanent shortcomings which often limit the applicability of such techniques. For example, while direct drug effects on the neoplastic cells themselves can generally be studied on cell lines in a relatively straightforward manner, the vast majority of known or supposed tumor-host interactions are difficult to impossible to accurately mimic in a purely cell culture-based system. These include - to various degrees – provision of a specific growth environment formed by the extracellular matrix of tumor stromal cells, tumor-associated neo-angiogenesis, secretion of cytokines and chemokines and possibly other growth-modulating factors by the tumor microenvironment as well as anti-neoplastic defense mechanisms conferred by the host immune system [51, 52].

A good example for possible shortcomings of cell culture based *in vitro* models as compared to animal models was given in a recent report by Curran and co-workers. While spontaneously developing medulloblastomas in $Ptch^{+/-}$; $p53^{-/-}$ mice were dependent on Hedgehog-signaling and thus showed dramatic response to pharmacological Hedgehog blockade *in vivo*, no signs of Hedgehog pathway-activity or therapeutic response to Hedgehog-inhibition was observed by the same group in medulloblastoma cells after establishment as cell lines *in vitro* [53, 54]. This was almost certainly as a result of the absence of tumor-stroma interactions in tissue culture models. Some of the key advantages and disadvantages of different *in vitro* and *in vivo* model systems of pancreatic cancer with respect to their application in drug discovery are summarized in Table 1.

4.1 *In vitro* models of tumor-stroma interactions

In humans, pancreatic cancer is characterized by a pronounced deposition of extracellular matrix components and proliferation of stromal cells, namely surrounding fibroblasts, commonly referred to as “desmoplasmic reaction” 55. An increasing body of evidence suggests that this process is centrally involved in regulating neoplastic cell growth, invasiveness and metastatic spread and is thus of pivotal interest both for a deeper understanding of pathophysiological mechanisms governing pancreatic cancer progression as well as in order to identify processes that might be exploited as molecular targets for novel therapeutic approaches (see 56–58 for more comprehensive recent reviews on the topic of tumor-stromal cell interactions in pancreatic cancer). A striking example for the importance of the tumor microenvironment in mediating cancer progression was recently given by studies reporting somatic mutations and copy number alterations within cancer-associated fibroblasts which

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might be involved in promoting enhanced cancer cell growth 59–66. An ever growing number of secreted growth factors, cytokines and chemokines that are produced by stromal cells have been suggested to be pathophysiologically involved in pancreatic cancer progression, among them transforming growth factor-beta (TGF-beta), connective tissue growth factor (CTGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF), nerve growth factor (NGF), leukemia inhibitory factor (LIF), oncostatin M, interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), chemokine (C-C motif) ligand 2 (CCL2), C-X-C chemokines CXCL1, CXCL2, CXCL8, CXCL12 and winglesstype MMTV integration site family members 1 and 3 (WNT1 and WNT3) [56, 67–69]. Moreover, a growing body of evidence suggests that extracellular matrix components expressed by stromal cells can stimulate membrane-bound receptors on pancreatic cancer cells and mediate cell survival and motility 56.

A more recent finding is the observation that pancreatic stellate cells (PSC) are activated in pancreatic cancers and are potentially involved in modulating the malignant phenotype and inducing desmoplasmic reaction [70–74]. Cell culture models aimed at studying potential synergistic effects on *in vitro* growth and motility of pancreatic cancer cells – by either using co-culture techniques or conditioned media - have been tested, but their value and applicability for functional studies in translational research is only beginning to be well understood at present [68, 75–77].

4.2 Functional *in vitro* assays

Finally, and especially in the case of pancreatic cancer, metastatic dissemination is a major predictor of therapeutic success and overall survival in solid tumors. The complex cascade of metastatic spread involves several steps which a neoplastic cell has to overcome in order to form a metastatic tumor, including proliferation of neoplastic cells at the primary tumor site, active migration and invasion into surrounding tissue, intravasation into lymph or blood vessels, anchorage independent survival in the blood stream, accumulation and extravasation and autonomous growth at a distant organ site, and induction of neo-angiogenesis at the metastatic tumor site (Figure 2). To date, most of these steps are far from being sufficiently well understood on a pathophysiological level and are therefore difficult to simulate in an *in vitro* setting in a way that would allow to accurately mirror each and every of the biological aspects involved in regulating these events in situ [78, 79].

Of note, an ever growing number of *in vitro* assays has been developed, attempting to mimic some of the aspects of neoplastic tumor growth and dissemination named above. For example, net tumor cell growth can readily be assessed *in vitro* by direct cell counting or by colorimetric 3-(4,5-dimethylthiazolyl- 2)-2,5-diphenyltetrazolium bromide (MTT) assays, proliferation can clearly be quantified by assays such as BrdU or tritium incorporation, immunolabeling for Ki67 and other surrogate markers of proliferation, CFSE assays etc., while a significant number of assays exists to accurately quantify different stages of apoptosis *in vitro* (e.g. DNA fragmentation, TUNEL, annexin binding, caspase activation assays, etc.) [80–83]. Also, a number of *in vitro* assays exist which simulate and quantify some of the more complex steps pointed out above. Cell motility can readily be assessed *in vitro* by time-lapse observations under the microscope, wound healing or Boyden chamber assays; introduction of a layer of matrigel or other coating material can be used to mimic tumor cell invasion and breach through the epithelial basement membrane [84]. Anchorage independent growth can be simulated *in vitro* by means of soft agar, methylcellulose or other three-dimensional culturing techniques [85], and re-plating of highly diluted cell suspensions and subsequent ability to form colonies has been used as a means to determine clonogenecity *in vitro*. Finally, various *in vitro* models of tube formation and angiogenesis are commonly used [86].

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All of the above-mentioned assays have been utilized for assessing *in vitro*-effects of novel candidate drugs on these distinct central cell functions, and in many cases, a reasonable correlation between these *in vitro* assays and the *in vivo* scenario has been documented. Moreover, these *in vitro* assays enable scientists to specifically examine functional effects of manipulating single proteins by means of genetic techniques. Gene function can be specifically abrogated through gene knockout, RNAi-mediated knockdown of gene expression or introduction of dominant negative protein. Enhanced gene function on the other hand is readily achieved by forced overexpression or introduction of constitutively active mutant gene products. This approach can be applied to link specific genes and signaling pathways to distinct cellular functions involved in pancreatic carcinogenesis and metastatic spread, with the final goal being the identification of potential targets for the development of novel therapeutics [87]. One promising *in vitro* screening method to emerge in recent times has been pharmacological synthetic lethal screening, wherein isogenic cell lines differing in a single gene product are screened against large scale chemical libraries to identify small molecules that are lethal only to that line in the isogenic pair which has a defective gene [88]. The use of synthetic lethal screen was first employed in yeast to identify genetic pathways that are lethal to survival when mutated in concert, and its extrapolation to cancer cells as a tool for drug discovery was first proposed by Hartwell and colleagues in 1997 [89]. Recently, using such synthetic lethal screening of isogenic pancreatic cancer cell lines differing only in Dpc4 function, von Hoff and colleagues were able to isolate compounds that are specifically toxic to Dpc4- null cells, a finding with immediate translational significance given that 55% of pancreatic cancer lack Dpc4 function [90].

4.3 *In vitro* models for genetic studies

While these techniques are undoubtedly of immense value as screening tests to identify potential new drug targets, there are some obvious limitations of these cell culture-based *in vitro* model systems and the *in vivo* relevance of obtained *in vitro* results is often unclear [91]. Firstly, as mentioned above, it is almost impossible to accurately mimic the complex network of tumor-host interactions in its entirety. This crosstalk includes direct effects conferred by the physical interaction of neoplastic cells with the extracellular matrix of surrounding stromal tissue, humoral factors, e.g. secretion of cytokines and growth factors by host cells, and effects mediated by the host immune system. Secondly, and possibly more importantly, pancreatic cancer cells might progressively alter genetically over time with increasing duration of *in vitro* culture.

There are various reports demonstrating that pancreatic cancer cells cultured *ex vivo* as xenografts or as cell lines *in vitro* accurately mirror the genetic setup and dependence on known oncogenic signaling pathways of their parent tumors and remain fairly stable over time [36, 92]. However, there are also several lines of evidence suggesting that additional genetic alterations might in fact occur during long-term *in vitro* propagation of pancreatic cancer cells.

Firstly, from a theoretical point of view it seems likely that pancreatic cancer cell lines might not have the ability to maintain their genetic fidelity over longer periods of time. Genomic instability is a key feature observed in almost all cases of pancreatic cancer and is among the earliest aberrations found during pancreatic carcinogenesis [93–96]. Therefore, under cell culture conditions optimized for maximum survival of neoplastic cells and in the absence of other selection mechanisms usually found in the *in vivo* situation (i.e. lack of immune-surveillance, absence of anatomical barriers etc.) there might likely be a greater tendency towards acquiring additional, heterogeneous genetic alterations.

Secondly, there are also various lines of empirical evidence suggesting that accumulation of additional genetic changes might in fact occur in pancreatic cancer cell lines with increasing passage numbers. In a recent global sequence analysis study on 24 cases of pancreatic cancer

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covering the majority of genes represented in the RefSeq database, Jones et. al found an average of 48 somatic mutations per pancreatic cancer sample (ranging from less than 30 to over 140 mutations in individual samples) [49]. While this number is significantly lower than the average number of mutations found in breast (on average 101 per tumor) and colorectal cancers (77 per tumor) using a similar approach 97, it is still by far higher than what might have been expected based on *in vitro* and *in vivo* transformation experiments (Table 2).

For example, in a recently described genetically engineered mouse model of pancreatic cancer it was found, that introduction of one single genetic alteration, i.e. pancreas-specific overexpression of an oncogenic $Kras^{G12D}$ allele, was sufficient to induce formation of murine pancreatic intraepithelial neoplasia (mPanIN) lesions. In two of 29 mice followed longitudinally (i.e. less than 10%) these precursor lesions progressed into frank malignancy after 6 and 8 months, respectively [98]. In this particular model, the long latency and low frequency of actual cancers suggest, that additional mutations must occur in order to result in a fully malignant phenotype, whereas the majority of $Kras^{G12D}$ expressing cells might undergo ras-induced senescence and be eliminated, thereby failing to accumulate additional genetic alterations required for complete malignant transformation [99]. These observations are in line with data from our own group showing that expression of $Kras^{G12D}$ is sufficient to generate mPanIN lesions in adult mouse pancreata when targeted under the control of tamoxifen-inducible Ela-Cre^{ERT2} or Mist1-Cre^{ERT2} constructs [7]. However, progression to fully malignant pancreatic cancer was not observed in this model during 12 months of follow-up.

A later report by Hingorani et al. showed that additional expression of a dominant negative form of the tumor suppressor gene p53 ($Trp53^{R172H}$) under the control of Pdx1-Cre led to development of moderately to well-differentiated metastatic pancreatic cancers with a median survival of 5 months and 100% lethality after 12 months of follow-up [100]. As observed in pancreata overexpressing $Kras^{G12D}$ alone, development of pancreatic cancer in this latter model was preceded by the appearance occurrence of mPanIN lesions. Of note, LOH of the p53 gene locus was reproducibly observed in cell lines derived from murine pancreatic cancers in this model system, but expression analysis and direct sequencing did not show any evidence for alterations in the Cdkn2/Ink4a, Cdk4 or Smad4 signaling pathways [100].

These data are in line with another report of specific expression of $Kras^{G12D}$ and concomitant abrogation of p53 expression by Cre-mediated excision under the control of Pdx1-Cre [101]. In this model, pancreatic adenocarcinomas occurred with an average latency of 6.2 weeks. In yet another example, DePinho and collegues reported that pancreas-specific targeting of oncogenic $Kras^{G12D}$ expression in combination with abrogated Ink4a/Arf (murine p16 and p19) function by crossing on a Pdx1-Cre genetic background led to the rapid formation of poorly differentiated pancreatic cancers [102]. These mice reproducibly die of metastatic disease between 7 to 11 weeks of age 103. Similarly, pancreas-specific $Kras^{G12D}$ expression in combination with targeted silencing of transforming growth factor beta-signaling by specific Tgfbr2- knockout led to the development of well-differentiated ductal adenocarcinomas in the murine pancreata with 100% penetrance and a short median survival of only 59 days [104].

While all of the above mentioned transformation models were done in mice, i.e. in a different species and conclusions drawn from these models can therefore be applied to the human disease only with some caution [105, 106], there are nevertheless also some *in vitro* transformation models of human cells suggesting that the number of genetic alterations required for malignant transformation might actually be considerably smaller than the average of 48 alterations observed by global direct sequencing [49].

In a recent study, Campbell and collegues found that only 4 genetic alterations are necessary for *in vitro* transformation of immortalized human pancreatic epithelial cells (HPNE) 20,

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107. hTERT-HPNE cells are immortalized through ectopic expression of the catalytic subunit of telomerase (hTERT) 108–110. Upon subsequent stable transfection of the human papillomavirus 16-derived genes E6 and E7 (E6/E7), constitutively active $Kras^{G12D}$ and SV40 small t (st) antigen, HPNE cells readily showed colony formation and anchorage independent growth, as well as spontaneous migration and invasion into a matrigel layer in modified Boyden chamber assays, in line with acquisition of a malignant phenotype.

Using a similar experimental approach, Tsao and collegues found that isolated non-neoplastic human pancreatic ductal epithelial cells, which had previously been immortalized by transfection with the human papillomavirus 16 genes E6 and E7 (E6/E7) 16, 17, showed xenograft tumor formation upon subcutaneous injection of one million cells subcutaneously into SCID mice in 4/7 cases (57%) and in 2/5 cases (40%) upon orthotopic (intrapancreatic) injection of two million cells 19. Curiously, these cells did not shown signs of *in vitro* transformation, specifically no colony formation was observed in soft agar assays.

Taken together, these observations suggest that the actual number of genetic alterations required for pancreatic carcinogenesis in humans might be considerably smaller than the average of 48 alterations found in the global sequencing approach by Jones et. al [49]. Not only is this of immense interest for our understanding of underlying pathophysiological mechanisms leading to the development of pancreatic cancer, but this idea also holds extremely promising implications for the identification of molecular targets for therapeutic intervention. Despite the lack of clinically tangible progress in the development of novel therapeutic strategies for pancreatic cancer over the last decades, it seems likely that in the end pancreatic cancer is caused by only a limited number of defined genetic alterations, many or most of which are already known to date and hence readily accessible for the development of novel therapeutics.

5. Expert opinion

From the discussion above, it is fair to conclude that panels of freshly generated low-passage pancreatic cancer cell lines mirror genetic features and response to therapeutic interventions with better fidelity than cell lines which have been cultured *in vitro* for longer periods of time [36, 47, 48].

In the case of pancreatic cancer, a significant limitation for the use of *in vitro* models for translational research and drug discovery is the lack of cell line models for pancreatic cancer precursor lesions. In recent years it has increasingly become commonly accepted that virtually all cases of pancreatic cancer arise via the development of tangible and genetically defined precursor lesions, including pancreatic intraepithelial neoplasia (PanIN), mucinous cystic neoplasms (MCN) and intraductal papillary mucinous neoplasms (IPMN) 111, 112. Better understanding of the genetics of these precursor lesions has direct implications for early detection as well as for secondary prophylaxis. Moreover, studying the genetics of precursor lesions allows us to separate “early” (disease initiating) alterations from “late” (disease promoting) changes. While nearly all or majority of genetic changes in precursors are likely to be “driver” lesions, many of the genetic alterations in frank malignancies are likely to be “passenger” mutations, acquired as a result of progressive genetic instability.

While genetically engineered mouse models mimicking mPanINs [98], MCNs [113, 114] and IPMNs [115] could be successfully developed in recent years, long term *in vitro* cultures of the human counterparts of these precursor lesions are currently not available. The lack of such cell line models seems to be most likely due to technical difficulties. For example, PanIN lesions are, by definition, microscopic in nature and nearly impossible to localize macroscopically, such that enrichment for these cells through *ex vivo* passage in mice is not an option with currently available techniques. Moreover, due to their likely limited growth potential, the ability of PanINs to engraft and be serially propagated in immunocompromised mice is also

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questionable. It remains an interesting point of speculation, whether 17 or not improvements of *ex vivo* and *in vitro* culturing techniques will enable generation of such cell line models of pancreatic cancer precursor lesions in the near future.

One can conclude from the mentioned points that although cell line models are undoubtedly of immense value for drug discovery in translational research related to pancreatic cancer, the use of animal models is nevertheless indispensible for a more concise preclinical evaluation of novel drug candidates. This is even more so, since acquisition of toxicity data and estimation of adverse effect profiles can only be done using test animals in a preclinical setting. Estimation of toxicity profiles based on cell line data alone is almost uniformly less useful.

Therefore, the main scope for the use of cell line based models remains in the early phase of drug discovery, wherein rapid, repetitive screening of large numbers of candidate substances, often in an automated or semi-automated manner, is required. As opposed to the use of experimental animal models, cell lines enable to cheaply and rapidly obtain virtually unlimited amounts of cancer cells and are therefore often ideal for such screening setups.

Also, at an early phase of drug discovery, where a direct molecular target or oncogenic signaling pathway has already been identified, genetically manipulated cell line models can often be used successfully for high-throughput efficacy screening *in vitro*. Candidate small molecules with satisfactory efficacy can then undergo further biological testing, including assessment of toxicity profiles, *in vivo* pharmacokinetics and pharmacodynamics in animal models.

A recent example for this approach is the search for small-molecule Hedgehog inhibitors as novel cancer drug candidates. Aberrantly re-activated Hedgehog signaling has been described in basal cell carcinomas [116], medulloblastomas [117, 118] as well as in various cancers of the gastrointestinal [119] and respiratory tract [120, 121], including pancreatic cancer [119, 122]. Pharmacological blockade of Hedgehog signaling by means of the plant alkaloid cyclopamine [123, 124] has been identified as valid experimental therapeutic approach in preclinical *in vivo* and *in vitro* model systems [117, 121]. However, cyclopamine itself is not an ideal drug candidate for clinical application due to its poor water solubility and bioavailability as well as a relatively low affinity to Smo. Therefore, cell based high-throughput screens have been used to identify other small molecule Hedgehog inhibitors with more favorable pharmacokinetics and – dynamics that are more suitable as drug candidates [125]. The *in vitro* model system for this screen consisted of C3H.10T1/2 cells stably transfected with a Hh-responsive (Gli_{8x}-luciferase) reporter construct, and a lipid-modified form of Hedgehog-ligand with enhanced potency which had an octyl moiety attached to its N-terminus (Shh^{OCT}) [126] was used for basal induction of Hedgehog pathway activity. This reporter cell line was assayed against a library of ~100,000 small synthetic organic molecules. One small molecule Hedgehog-inhibitor thus identified, designated Hh-Antag691 [127], inhibited pathway activity at ~10fold lower concentrations as compared to cyclopamine and was subsequently shown to inhibit medulloblastoma growth in a genetically engineered $Ptch1^{+/-}$ $p53^{+/-}$ mouse model [54]. Similar reporter systems used in other studies included stably transfected NIH-3T3 cells and a palmitoyl- and cholesteryl-modified ShhN polypeptide (ShhN) [128,] 129. Of note, these examples also unmasked some of the inherent drawbacks of this approach for drug discovery, namely that pharmacokinetics, *in vivo* efficacy and toxicity profiles in humans are often difficult to estimate based on the initial preclinical data alone. Moreover, potential off-target-effects of a given substance in the *in vivo* situation can greatly affect its suitability as a drug candidate – either by conferring undesirable adverse effects or by contributing additional but unexpected favorable effects on tumor cell survival. Therefore, in scenarios like the one mentioned here, it is quite common that drug candidates which performed well in the initial discovery screens and early preclinical characterization phases fail at later stages during preclinical and clinical development, so that they need to be modified in chemical composition

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and reevaluated or completely abandoned from further development towards clinical application. Only a minority of candidate substances identified in discovery screens can be developed all the way towards successful application as novel drugs in the clinical arena. In the case of Hedgehog-Antagonists described here follow-up drug candidate small molecule Hedgehog-inhibitors are currently evaluated in clinical phase I and II trials in various malignant solid tumors including pancreatic cancer (www.clinicaltrials.gov) and others are likely to follow in the near future.

Another example demonstrating the amount of tedious work and time-consuming steps involved in translating an identified molecular target into clinical application as novel therapeutic strategy is given by attempts to target tumor-neoangiogenesis in pancreatic cancer as well as other solid tumors over recent years. While various studies reported striking successes of such strategies in mouse models of various cancers beginning over a decade ago [130–135], drugs resulting from these efforts have only recently been introduced into the clinical arena and have so far shown overall disappointing results in terms of improving overall survival of pancreatic cancer patients, although additional evaluations are still ongoing [136] (www.clinicaltrials.gov).

Additional examples of signaling pathways recently identified to be pathogenetically involved in genesis and maintenance of pancreatic cancer and thus representing potential targets for the development of novel therapeutics, for which similar screening and preclinical evaluation strategies are being followed include the Notch [137–140], Wnt [141, 142], TGF-beta [143], EGF [144–146], Raf/Mek/Erk [147, 148], PI3K/Akt [149] and mTOR [150–153] signaling pathways.

Acknowledgments

Declaration of interests

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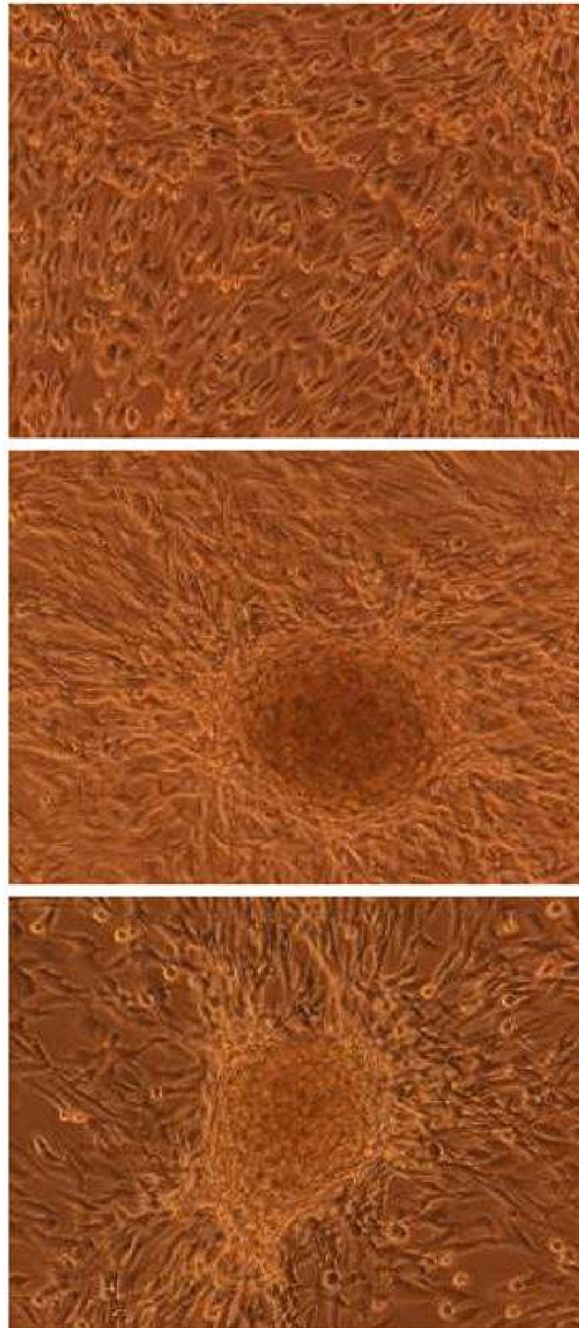

Figure 1.

(top) Non-transformed hTERT-immortalized HPNE cells grow as a monolayer in tissue culture. (middle and bottom) Examples of transformed HPNE cells with multi-layering ("focus" formation). In this example, the gene(s) responsible for this neoplastic phenotype are not known, as transformation was induced by transient activation of a Sleeping Beauty transposon, leading to disruption and/or activation of cancer-associated genes in the HPNE genome. In vitro models of pancreatic epithelial cells can be used for such "forward" genetics experiments, as well as for more traditional reverse genetics using known combination of oncogenes or tumor suppressor genes, as discussed in the text.

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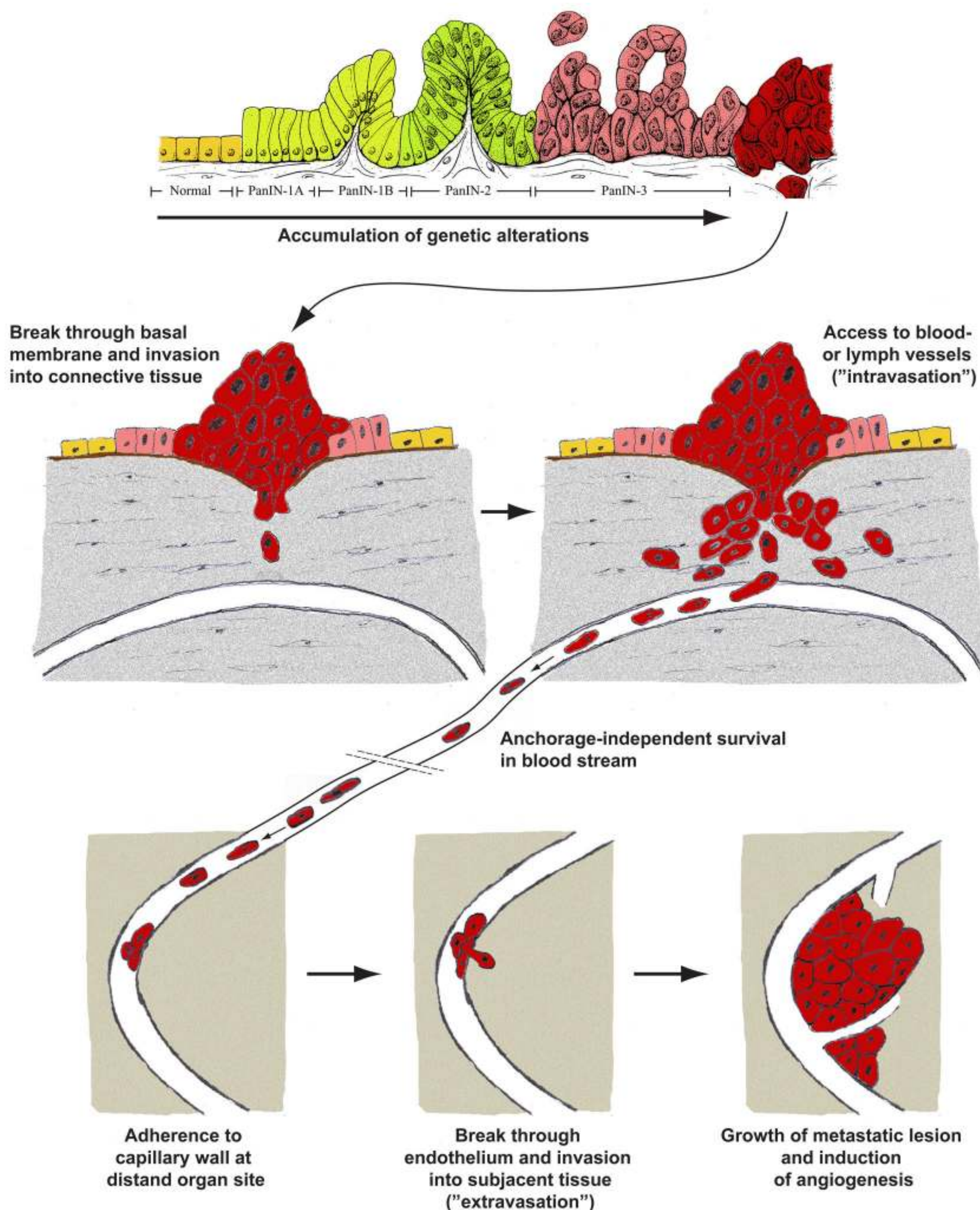

Figure 2.

Pancreatic cancer does not occur 'de novo' but via development of clearly defined tangible precursor lesions that correlate with step-wise accumulation of genetic alterations. In order to develop systemic metastases, an invasive clone must overcome several additional selection barriers, including invasion into surrounding tissue, anchorage-independent survival in the blood stream, active migration and invasion at a distant organ site and establishment of a metastatic tumor lesion. While a variety of in vitro methods exist trying to mimic single aspects of this complex process, it is nevertheless impossible to faithfully mimic in its entirety by means of cell line-based in vitro models alone as of to date.

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Table 1

Key advantages and disadvantages of different in vitro and in vivo model systems of pancreatic cancer in drug discovery

Model system	Advantages	Disadvantages
Patient-derived cell lines	Accurately mirror entire spectrum of genetic alterations of human pancreatic cancers Cell-line based studies are comparably cheap and less time- and labor-consuming than studies on animal models Easily accessible Virtually unlimited amounts of cancer cells can be generated with relatively little required technical equipment	Functional studies generally highly prone to artificial results Purely cell-line based treatment studies aimed at determining efficacy of novel drug candidates are commonly extremely poor in predicting actual therapeutic response or adverse effects in humans Do not allow to study important in vivo factors such as pharmacokinetics and toxicity profiles Do not reflect effects conferred by tumor-stroma interactions May genetically change over time
Co-cultures	Can to some degree mimic effects of secreted growth-modulating stimuli and direct physical contact with the ECM conferred by stromal cells	Difficult to co-culture more than two different cell compartments in one experiment Difficult to accurately control for the physiological ratio of neoplastic to stromal cells Most of the other disadvantages listed above for “patient-derived cell lines” apply here as well
Patient-derived xenografts	Accurately mirror entire spectrum of genetic alterations of human pancreatic cancers Can be used to enrich for neoplastic cell compartment and eliminate contaminating human stromal cells Testing of candidate drugs or other novel experimental therapeutic strategies by using xenografts usually faster and cheaper as compared to transgenic models Orthotopic xenograft models allow to study effects of locally invasive growth and systemic metastasis	Xenogenic chimera between human and murine cells Do not accurately mirror effects conferred by host immune system Not suitable to accurately mimic tumor-stroma interactions in humans Different anatomies in mice versus humans May genetically change over time Require experimental exploitation of and potentially inflicting harm on mammals
Genetically engineered mouse models	Overall reliable, usually highly reproducible results Allow for distinct studies of virtually any desired combination of genetic alterations in an in vivo setting More recently described models enable to mirror most of the key features of the human disease surprisingly realistically, including local intrapancreatic growth, invasion into surrounding structures, systemic metastasis, development via mPanIN precursor lesions, histological microarchitecture resembling aspects of human pancreatic cancers Readily available for pre-clinical evaluation of novel experimental therapeutic approaches	Do not reflect the entire spectrum of genetic alterations found in human cancer tissues Xenogenic model systems Different anatomies in mice versus humans Require experimental exploitation of and potentially inflicting harm on mammals Usually highly cost-, labor- and time-intensive studies Though usually providing valuable data on efficacy, pharmacokinetics and toxicity profiles of novel experimental therapeutic interventions, results from these studies nevertheless often fail to accurately predict response in humans, i.e. can not substitute subsequent clinical evaluation

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Table 2

Studies estimating numbers of genetic alterations required in pancreatic carcinogenesis

Reference	Summary	Species	Genetic model used	Number of genetic alterations	Comments
49	direct sequencing of 24 human pancreatic cancer samples; DNA isolated from cell lines or murine xenografts	human	Genuine human pancreatic ductal adenocarcinoma cells	average of 48 per tumor sample	first and so far only global sequencing study of human pancreatic cancer samples
91	pancreas-specific expression of an oncogenic $Kras^{G12D}$, which is achieved by combination to a lox-stop-lox (LsL) cassette and expression of Cre-recombinase under control of Pdx1 or P48 promoters, led to development of mPanIN precursor lesions, which eventually progressed to fully invasive cancers at low frequency	mouse	LsL-$Kras^{G12D}$; Pdx1-Cre LSL-$Kras^{G12D}$; $P48^{+/Cre}$	1	
7	targeting of oncogenic $Kras^{G12D}$ expression to the adult pancreas by means of an inducible Ela-Cre^{ERT2} construct leads to formation of mPanIN lesions but not fully malignant pancreatic cancer	mouse	LsL-$Kras^{G12D}$; Ela-Cre^{ERT2}	1	
93	pancreas-specific targeting of combined $Kras^{G12D}$ and $Trp53^{R172H}$ expression led to development of metastatic pancreatic carcinomas with median survival of 5 months	mouse	LsL-$Kras^{G12D}$; LsL-$Trp53^{R172H}$; Pdxl-Cre	2	no acquired mutations found in Cdkn2/Ink4a, Cdk4 or Smad4
94	development of pancreatic adenocarcinomas with average latency of 6.2 weeks in mice expressing $Kras^{G12D}$ in the absence of p53 und control of Pdx1-Cre construct	mouse	LsL-$Kras^{G12D}$; $p53^{lox/lox}$; Pdx1-Cre	2	
95	rapid development of poorly differentiated metastasizing cancers in murine pancreata expressing oncogenic $Kras^{G12D}$ in combination with abrogation of Ink4a/Arf expression	mouse	LsL-$Kras^{G12D}$; Ink4a/$Arf^{lox/lox}$; Pdx1-Cre	3	
97	development of well-differentiated pancreatic ductal adenocarcinomas with high penetrance and short median survival of only 59 days upon concomitant oncogenic Kras-signaling and Tgfbr2-knockout	mouse	LsL-$Kras^{G12D}$;$Tgfbr2^{flox/flox}$; $Ptf1a^{cre/+}$	2	
100	Immortalization and in vitro transformation of cultured non-neoplastic human pancreatic ductal cells (hTERT-HPNE)	human	hTERT-HPNE; E6/E7; $Kras^{G12D}$;SV40st	5	
19	Expression of oncogenic Kras in cultured immortalized non-neoplastic human pancreatic ductal cells (HPDE) leads to tumorigenecity in SCID mice in 50% of cases	human	HPDE; E6/E7; $Kras4B^{G12V}$	3	absence of in vitro clonogenecity