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Pre-clinical Models of Pancreatic Ductal Adenocarcinoma

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Abstract

Pancreatic ductal adenocarcinoma (PDA) is one of the most difficult human malignancies to treat. Five-year survival rate of PDA patients is 7%, and PDA is predicted to become the second leading cancer-related cause of death in the United States of America. Despite intensive efforts, the translation of findings in preclinical studies has been ineffective, due partially to the lack of preclinical models that faithfully recapitulate features of human PDA. Here we review current preclinical models for human PDA (e.g. human PDA cell lines, cell line-based xenografts and patient-derived tumor xenografts). In addition, we discuss potential applications of the recently developed pancreatic ductal organoids, three-dimensional culture systems and organoid-based xenografts as new preclinical models for PDA.

Keywords

Pancreatic cancer; organoid; preclinical model; patient-derived tumor xenograft

Introduction

Pancreatic ductal adenocarcinoma (PDA) is currently the fourth leading cause of cancer-related mortality in the United States [1], and due to an increasing incidence and lack of efficacious treatments it is projected to become second behind lung cancer by 2030 [2]. Molecular pathology studies and extensive genomic analyses have established a model of the progression of pancreatic adenocarcinoma. Activating mutations of *KRAS* are present in tumours of most PDA patients (>90%), indicating that oncogenic *KRAS* contributes to its inception [3]. It is also well-established that other genetic alterations affecting *CDKN2A*,

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Conflict of Interest: Hans Clevers and Sylvia Boj are named as inventors on patents pending and granted on the organoid technology.

Author contributions

CIH, SFB, HC and DAT wrote the manuscript. CIH and SFB contributed equally to this review.

TP53, and *SMAD4* cooperate with oncogenic *KRAS* to accelerate progression of PDA [4]. The signature mutations of PDA have been identified in microscopic premalignant lesions associated with the pancreatic ducts; these are referred to as pancreatic intraepithelial neoplasia (PanIN) and they are characterized by alterations in cellular architecture such as mucinous cytoplasm, nuclear crowding and nuclear atypia [5]. Various genetically engineered mouse models (GEMMs) support this model of genetic progression. While activation of oncogenic *Kras* in pancreatic epithelial cells is sufficient to initiate PDA in murine models, combinations with *Trp53*, *Cdkn2a* or *Smad4* mutations accelerates PDA progression, and recapitulates many features of the cognate human disease [6–8].

Recent genome-wide approaches have comprehensively characterized the human PDA genome and have identified additional genes and pathways operant in disease pathogenesis [9, 10]. Furthermore, the application of transposon-mediated insertional mutagenesis approaches in GEMMs has implicated an additional cadre of genes that cooperate with oncogenic *Kras*, including chromatin remodellers, ubiquitin proteases and transcription factors [11–13]. Beyond these mutational events, the PDA genome is also characterized by diverse, large-scale chromosomal changes with frequent amplifications, deletions and rearrangements [9].

Although we now have a better understanding of PDA biology and disease progression, the translation of this knowledge for patient benefit has been slow. In this regard, a potential contributing factor may be the inappropriate use of certain preclinical models of human PDA. Traditional PDA preclinical models consist of GEMMs where PDA develops *in situ*, and transplantation models where human PDA cells and tumour fragments are implanted into immune deficient mice. Recently, we described organoid models of PDA, which appear to closely mimic the development of human pancreatic cancer in mice. In this review, we will describe the pertinent features of these distinct models of PDA, and discuss how they may be applied to translational studies for human benefit.

Human PDA cell lines

Although the first human cancer cell line was established in 1951 from a cervical carcinoma [14], over ten years passed before the first human pancreatic cancer cell line was reported [15]. To date, many human PDA cell lines have been well characterized in terms of their cellular phenotypic characteristics and molecular aberrations [16]. Additionally, PDA cell lines have played important roles in drug development.

There are several practical advantages of working with cell lines: they are homogenous, easy to propagate and grow indefinitely in defined media conditions. Thereby, it is feasible to perform high throughput screens with chemical compounds or genetic tools [17–19]. For example, William Hahn and his colleagues investigated the importance of 11,194 genes in 102 human cancer cell lines, including 13 PDA cell lines, by using RNA interference (RNAi) [20]. Using this synthetic lethal approach, they identified 23 essential genes in the PDA cell lines, which represent potential therapeutic targets for this disease. Since PDA cell lines harbour a distinct set of mutations with corresponding transcription profiles, it has been possible to develop prognostic classifiers for patients and predictive biomarkers for drug

response. For example, such approaches have been useful for stratifying patient groups based on expression profiling and mutational status [21]. Additionally, novel anti-cancer drug targets have been proposed by integrating drug response with genomic sequence, transcriptional profiling and DNA copy number detection datasets [22].

Even though cancer cell lines bear considerable promise as preclinical models, there are several caveats. First, most published studies have been performed in a restricted number of cell lines, with only 15 PDA cell lines being commonly used by the research community [23]. Therefore, currently available cell lines do not represent the full spectrum of mutations found in pancreatic cancers. Indeed, since many cell lines have been generated from metastatic and rapidly growing tumours, primary PDA and more slowly growing variants are likely under-represented. Second, while epithelial cells proliferate as polarized and well-organized structures *in vivo*, PDA cell lines propagated as monolayer cultures lack this structural organization and functional differentiation [24, 25]. Third, another weakness of studying PDA cell lines in vitro is the absence of the tumour microenvironment, which is a prominent feature in pancreatic cancer. It is well understood that the tumour microenvironment is not a bystander but rather an active participant in tumour progression [24, 26]. It has been reported that cell lines have a distinct expression profile when compared to patient tissues or xenografts, suggesting the importance of the tumour microenvironment [27]. Fourth, an important consideration is the possibility that selection or genetic drift may occur in PDA cell lines when established from tumours. Not only do newly established PDA cell lines lose the tumour heterogeneity present in the original cancer specimens, but additional molecular changes might also evolve, effectively obscuring the genetic alterations present in the primary tumour [16]. Often it has been described that cell lines have different genetic status compared with primary tissues, suggesting that the procedure for cell line establishment selects or generates a sub-population of aggressive clones [28]. Therefore, therapeutic approaches with these PDA cell lines may identify drugs that only target the most advanced clones, while sparing the bulk of the cells that comprise tumours in vivo [25].

Thus, although PDA cell lines offer an important starting point for investigations in this disease, additional approaches are needed to delve into the complexity of pancreatic cancer biology and therapy.

Cell-line based xenografts

Xenograft tumour models have been widely used as an *in vivo* counterpart to cell lines by cancer researchers [29]. Human cancer cell lines are injected either orthotopically or ectopically (usually subcutaneously) into immune-compromised mice, and these engrafted neoplastic cells form a three-dimensional architecture and recruit a host stromal microenvironment that may better recapitulate human tumours. Such xenograft tumours were projected to provide a better in vivo representation of cancer pathobiology and drug responsiveness and thereby guide prioritization of clinical development [30]. However, extensive screening for over 10 years by the National Cancer Institute (NCI) suggests a moderate predictive value for their xenograft models, and even less concordance between *in vitro* testing data and clinical utility [31]. In fact, it has been also reported that cell line-

based xenografts inconsistently predict therapeutic response in many cancers, including PDA [32–35].

For example, Bruns and colleagues used a pancreatic orthotopic xenograft model to evaluate the anti-EGF-R (epidermal growth factor–receptor) antibody C225, and found a dramatic tumour reduction (85%) when C225 was combined with gemcitabine [36]. However, in a phase III clinical trial where patients with advanced pancreatic cancer were treated with either gemcitabine plus placebo or gemcitabine plus the anti-EGFR antibody Cetuximab, no benefit was observed [37]. Also, Trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor, in combination with gemcitabine was effective in ten different cell lines and the corresponding xenograft models [38, 39]. However, adding the HDAC inhibitor CI-994 (Tacedinaline) to gemcitabine for the treatment of advanced pancreatic carcinoma patients did not improve overall survival, response rate or time to progression compared to single-agent gemcitabine [40]. Many other examples of discordance between preclinical studies based on cell line-based xenografts and clinical trials exist for other types of cancers [41–43].

The lack of predictive drug responsiveness using xenograft models is likely due to multiple factors. First, it should be noted that the measurement of preclinical response in preclinical studies might differ from that deployed for clinical trials. For example, a 70% tumour reduction in xenograft models means that 30% of tumour cells still remain and did not respond to the drug, which could correlate with therapeutic failure in clinical trials [44]. Second, there are many neoplastic cell features inherent to the establishment of the cell line, as previously discussed. Third, although xenografts do recruit a host stroma, cell line-based xenografts grow primarily as homogenous masses of tumour cells with limited stromal infiltration. This may be particularly relevant for PDA, since PDA tumours contain a minority of neoplastic cells and are predominantly comprised of a stromal compartment consisting of an acellular extracellular matrix and a variety of non-neoplastic cell types including cancer associated fibroblasts, immune cells, and vascular cells [45]. Fourth, the interactions with the acquired immune system (lymphocytes, NK cells), and certain paracrine interactions (e.g. HGF/c-MET) are aberrant or absent in the interactions between human neoplastic cells and murine stromal cells [46]. To address some of these issues, additional mouse models of human tissues have been developed.

Patient-derived tumour xenografts

An improvement to traditional xenograft models has been the development of patient-derived tumour xenograft models (PDTX). PDTX models are obtained by directly implanting freshly resected tumour pieces ectopically (usually subcutaneously) or orthotopically into immune-compromised mice, and passaging tumour fragments in subsequent generations [47, 48]. PDTX models have been reported to better predict therapeutic responses than traditional xenografts. Indeed, retrospective reviews published by the National Cancer Institute (U.S.) in 2001 and the National Cancer Institute of Canada in 2003 concluded that cell-line based xenografts rarely accurately predict drug responses [32, 33, 49]. However, in several retrospective studies, it has been noted that PDTX models for certain cancers closely mirror drug responses in human patients over other cell-line based

xenograft models [49]. This may reflect the fact that PDTXs are not comprised of neoplastic cell populations isolated from human tumours that have initially survived and adapted to the artificial environment of monolayer tissue culture. It has been postulated that since PDTX models are generated from whole tumour pieces, the tumour neoplastic cell architecture is maintained while the murine stroma replaces human stroma and therefore this growing chimeric tumour better reflects the properties of the original human tumour [50–52]. Also, importantly, serially passaged PDTXs generally show relatively consistent biological properties and stable phenotypes across multiple passages at the histological and molecular levels [44].

PDTX models of human cancer have been proposed as a means to develop therapeutic approaches for individual patients. Following the establishment of the sentinel tumour-bearing mouse, PDTX tumours are serially passaged and expanded for testing with different drugs in order to identify the best drug or drug combination that can be proposed for the care of that patient. For example, Hidalgo and colleagues performed a pilot study in which several advanced pancreatic cancer patients were successfully treated on the basis of the drug response of PDTXs derived from their previously resected primary cancers [53].

However, PDTX models still have several limitations as preclinical models. First, PDTX generation is only modest successful, with aggressive tumours engrafting best. In some instances, it has been shown that the ability of engraftment itself can serve as a negative predictor of the patients' disease free survival in resected PDA patients [54]. Second, PDTX models require a large amount of tumour tissue for transplantation, and this can only usually be obtained from surgically resected tumour material. Given the reality that only 15% of PDA patients have surgically resectable tumours at the time of diagnosis [55], PDTX models may not benefit the majority of patients who present with advanced disease. Third, establishing PDTX models for drug testing is a lengthy and therefore costly process since it can take up to 6 months to establish the first generation tumour bearing mouse, and this mouse is then serially passaged to generate enough secondary and tertiary carriers for the actual therapeutic trials [56]. Waiting 6–12 months to identify active therapies for a particular pancreatic cancer patient can easily be untenable for the management of that patient, since the disease often progresses swiftly [55].

A potential additional confounding situation is that PDTXs, although much more similar to the native human tumour than traditional xenografts, may still not accurately reflect the neoplastic and stromal features present in the original human tumour. Few studies have probed whether PDTX models accurately recapitulate the clonal heterogeneity of the primary PDA tumour [57]. Indeed, although PDTX models do not undergo prior adaptation to tissue culture conditions, the generation of these models still requires the outgrowth of malignant cells and therefore a selection process of the most proliferative clones that survive engraftment occurs in vivo [44, 54]. It is also worrisome that clonal evolution has been observed in the passaging of PDTX models, resulting in the recommendation that PDTX models should be used within three generations for conducting therapeutic experiments [47]. A closer analysis of the clonal features of PDTX tumours as compared to the original human tumour is an unmet need for the field, and could lead to improvements for this model.

Regarding the tumour microenvironment, although the architecture of PDTX tumours mimics that of the original human tumour much better than traditional xenografts, PDTX models share the same shortcomings with xenografts of harbouring an impaired immune infiltrate and possessing certain aberrant cross-species ligand-receptor interactions. Interestingly, the choice of an ectopic as compared to orthotopic engraftment site appears to influence the expression of PDA specific genes. Most PDTXs are ectopic, subcutaneous models to facilitate the logistics of tumour measurements. However, Rubio-Viqueira, et al. compared the expression of 15 selected genes between primary tumours and PDTXs from subcutaneous injections and found that only three genes showed statistically significant correlations [56]. In contrast, a recent study using orthotopic injections of 45 PDA patient tumours show a close correlation in genome-wide gene expression profile between primary tumours and passaged PDTX tumours [58]. Nonetheless, for serial passaging and expansion for drug screening, orthotopic PDTX models would be technically challenging on a large scale.

In spite of these limitations, PDTX models may still be useful for a select group of pancreatic cancer patients [53]. Indeed, Dr. Hidalgo and colleagues recently reported that the positive predictive value of PDTX models in PDA patients is more than 80% [44]. Thus, PDTXs may be useful for certain PDA patients who have a predicted survival of greater than 6 months and who can provide sufficient tissue for model generation, although it might not be applicable to most PDA patients. Therefore, it is necessary to develop suitable models that avoid the aforementioned limitations of PDTXs and are applicable to all PDA patients. Recently established pancreatic cancer organoids cultures established from primary pancreatic tumours or fine-needle biopsy samples may address this need [59, 60].

Three-dimensional tumour organoids

The three-dimensional (3D) culturing of normal cells and their malignant counterparts was introduced as early as the 1970s [61]. In semisolid matrices, epithelial cells can develop polarized structures due to the assembly of cell-cell contacts and cell-matrix interactions that simulate the basement membrane. Abundant extracellular matrix components including collagen, laminin and fibronectin are required to support these cultures. Unsurprisingly, studies that have compared gene expression profiles between 2D and 3D cultures have shown that cells are greatly influenced by cell-matrix interactions [62–64]. A classic example is the 3D culturing of normal breast epithelial cells, which form acinar structures with hollow lumens [65]. In contrast, breast cancer cells form highly disorganized structures in the same conditions [66]. Unlike breast epithelial cells, normal pancreatic ductal cells do not passage in similar 3D conditions, and although various PDA cell lines can grow as spheroids in 3D cultures [24], it is unclear how well they reflect the properties of the original human tumour.

In past five years an alternative *in vitro* 3D model for human tissues, termed organoids, has been developed. Adult stem cells are prepared from human or mouse adult tissues and embedded in a three-dimensional matrix where they self-organize into epithelial structures that resemble the respective organ of origin [67–71]. The protocol for growing human organoids was developed by mimicking the homoeostatic environment of the normal tissue

stem cells. Indeed, normal and healthy (disease-free) human cells in organoid cultures are able to self-renew and expand long-term while remaining genetically stable [71, 72]. More recently, organoid cultures of pancreatic epithelium have enabled the propagation of normal and neoplastic pancreatic epithelial cells for both murine and human [59, 73].

To generate pancreatic organoid cultures, cells are embedded in Matrigel™, which contains the critical components of the basement membrane, and supplemented with the minimal requirements for sustainable growth of pancreatic epithelial cells without mesenchyme. In short, EGF (mitogen), R-spondin-1 (enhances Wnt signalling), Noggin (inhibits BMP signalling) and Wnt3a are indispensable maintenance factors. Human pancreatic organoids additionally require FGF10 (mitogen), nicotinamide, A83-01 (Alk inhibitor) and prostaglandin E_2 (PGE_2, mitogen) for long-term expansion. One of the challenges in conventional tissue culture models has been the lack of normal or pre-neoplastic counterparts to compare with neoplastic/tumour cells. Indeed, while neoplastic cells can proliferate well in monolayer cultures, normal or preneoplastic cells have a limited life span and slow proliferation rate, making it extremely difficult to study the early stages of disease progression. In contrast, organoid cultures generate normal pancreatic ductal cells with a comparable proliferation rate to neoplastic cells. In addition, the success rate of establishing organoid cultures from normal and PDA patient specimens is higher than 2D cell lines and other xenografts [59]. Organoids can be readily established from surgically resected tumours, and when the specimen contains normal pancreas it is possible to grow both neoplastic and matching normal organoids from that patient. Since the organoid conditions can propagate both normal and neoplastic cells, this suggests that selective pressures that favour the growth of certain neoplastic clones may not be operant in organoids, and by extension that tumour heterogeneity may be better preserved compared to other models. This hypothesis is now undergoing direct assessment. Additionally, because it is possible to simultaneously support the proliferation of normal and neoplastic cells, if one wishes to establish a pure neoplastic cell organoid sample the sample must be isolated carefully or different culturing conditions can be used in order to avoid cross-contamination by a normal ductal cell population. In principle, selective culture conditions can be applied for the majority of PDA samples given the high penetrance of activating *KRAS* mutations [3], by withdrawing EGF or adding EGFR inhibitors.

For the PDA organoid approach to be relevant to most patients, they would need to be readily prepared from small biopsies over a short time period. Importantly, organoids can be generated in several weeks from small fine needle aspiration (FNA) biopsies obtained endoscopically from patients with advanced PDA, raising the possibility that these models can be available within a time frame to enable therapeutic testing for most patients. Furthermore, since FNA-based organoids are relatively simple to generate, this enables the possibility of evaluating the tumour response longitudinally and following tumour evolution during the course of treatment or disease progression.

Organoid cultures of PDA enable various molecular and cellular studies that have not been previously possible. For example, as organoids are pure epithelial populations, they circumvent the stromal suppression of molecular measurements that primary tumours harbour, and the comparisons to normal ductal pancreatic cells are possible. Indeed, RNA,

DNA, and protein-based assays are readily accomplished with organoids [59]. Furthermore, organoid cultures are genetically amenable for transfection and viral infection, enabling genetic screening or targeted evaluations of particular genes.

Various pertinent topics regarding pancreatic organoids are currently being pursued, including the possibility that co-cultures with PDA stromal cells may recapitulate the heterogeneous cellular interactions *in vivo*. Additionally, therapeutic testing and diagnostic studies using organoids are currently underway, to determine their predictive utility. Other types of cancer organoids such as colon and prostate cancers have been developed and shown to represent a robust platform to identify mutational aberrations and drug responses [74, 75].

Organoid-based xenografts

Similar to cell line based xenografts, it is also possible to generate organoid-based xenografts. Indeed, transplantation of pancreatic tumour organoid cultures into immune-compromised mouse pancreata recapitulates the full spectrum of disease progression, forming early and advanced PanIN-like stages and progressing towards invasive and metastatic pancreatic ductal adenocarcinoma [59]. Unlike other xenograft models from human specimens, organoid-based xenografts give a unique opportunity to investigate human PDA progression. Upon orthotopic transplantation, human organoids derived from PDA patients form PanIN-like structures, architecturally and cytologically resembling all the features of the different PanIN-stages. The only exception is that PanIN-like structures (intraepithelial) are not intra-ductal epithelial structures within the host murine pancreas. In contrast, conventional 2D cell line-based xenografts simply repopulate again in the host environment, without generating any PanIN-like structure. Moreover, tumours derived from organoids faithfully recapitulate the massive collagen deposition observed in human PDA tissues and tumours from GEMMs, another characteristic lacking in cell-based xenografts [59, 76]. To our knowledge, the organoid-based xenograft model is the first xenograft model that mimics human PDA progression (Figure 1).

How engrafted PDA organoids form PanIN-like structures still remains to be determined. Since organoid cultures may better preserve tumour neoplastic cell heterogeneity, it is possible that the recovery of a variety of stem cells that reflect the different stages of disease progression manifest their potential upon transplantation, including early pre-cancer lesions such as PanINs. Alternatively, it is also plausible that organoid transplantation of PDA samples may reflect cellular plasticity and epigenetic changes due to the organoid culture conditions. For example, certain malignant features of melanoma can be reverted by ECM signalling pathways [66]. By extension, the interaction of pancreatic cancer cells in organoid cultures with basement membrane components may be reverted into PanIN-like biological state. The concept of clonal selection or cellular plasticity we are presenting in the organoid orthotopic transplantations is distinct from a recent study by Zaret and colleagues in which one patient derived PDA cell line was reprogrammed to generate induced pluripotent stem (iPS) cells. Upon subcutaneous implantation, these iPS cell line developed early PanIN-like structures within teratomas that later progressed to invasive carcinoma [77].

With organoid-based xenografts, it is possible to study human PanIN-like structures, allowing the investigation of early biomarkers and PDA progression in vivo. Ongoing work will assess the utility of the organoid transplantations for therapeutic evaluation and diagnostic development, and compare them to more traditional models of PDA.

Discussion

A variety of preclinical models for human PDA are available for basic and translational studies, and they have helped establish the overall genetic features of this disease. However, individualized models of PDA are not possible for most patients when traditional cell lines or xenografts are required, and organoid models may provide an improved alternative. At a translational level, included amongst the critical unanswered questions are: whether PDA organoid models truly reflect the genomic complexity of the original tumour; whether the genomic complexity of organoids is indefinitely stable; if the therapeutic predictive utility of organoid models *in vitro* and *in vivo* is superior to other models; and whether organoids can be used to identify novel diagnostic strategies for PDA patients. Organoids can also be applied for fundamental scientific inquiry, such as whether a minimal assortment of genetic alterations can fully transform a normal human pancreatic ductal cell into PDA as has been performed for intestinal organoids [78, 79]. Despite these unknowns concerning organoids, the next several years promises to be very active as the field determines the most optimal preclinical systems to evaluate pancreatic cancer for human benefit.

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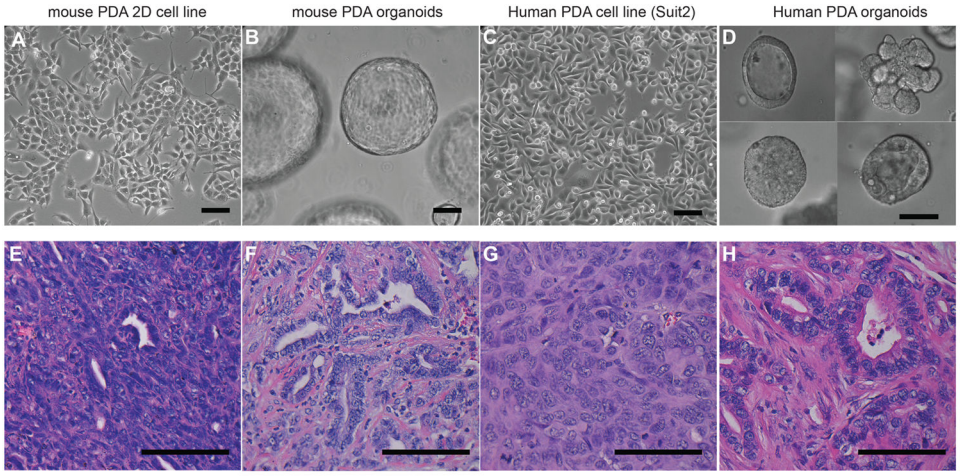

Figure 1.

Models of murine and human pancreatic adenocarcinoma (PDA). (A) Mouse PDA 2D cell line derived from KPC mice, (B) Mouse PDA organoid culture derived from KPC mice, (C) Suit2 human PDA cell line, (D) human PDA organoid culture, (E) Haematoxylin & Eosin (H&E) stain of tumour derived from orthotopic transplantation of KPC cells, (F) H&E stain of tumour derived from orthotopic transplantation of mouse PDA organoids, (G) H&E stain of tumour derived from orthotopic xenograft tumour of Suit2 human cell line in *Nu/Nu* mouse, and (H) H&E stain of tumour derived from orthotopic xenograft tumour of human PDA organoids in *Nu/Nu* mouse. Scale bars, 100 μm.