REVIEW

Pancreas 3D Organoids: Current and Future Aspects as a Research Platform for Personalized Medicine in Pancreatic Cancer

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SUMMARY

An organoid is as a group of epithelial cells growing in a 3-dimensional structure, with self-renewal and self-organization capacities, which recapitulates the tissue of origin. Use of organoids as a powerful tool for investigation and as a step toward personalized medicine is reviewed.

Pancreatic ductal adenocarcinoma is one of the most aggressive forms of cancer, and the third leading cause of cancer-related mortality in the United States. Although important advances have been made in the last decade, the mortality rate of pancreatic ductal adenocarcinoma has not changed appreciably. This review summarizes a rapidly emerging model of pancreatic cancer research, focusing on 3-dimensional organoids as a powerful tool for several applications, but above all, representing a step toward personalized medicine. (Cell Mol Gastroenterol Hepatol 2018;5:289–298; https://doi.org/10.1016/j.jcmgh.2017.12.004)

Pancreatic ductal adenocarcinoma (PDAC) is a rapidly progressing and usually fatal disease, and is the eighth leading cause of global cancer-related deaths in men and the ninth in women.[1] In the United States, PDAC is the third leading cause of cancer mortality and it is projected to be the second leading cause of cancer mortality by 2020.[2] According to the American Cancer Society, in 2016, a total of 53,670 people were diagnosed with PDAC in the United States and 43,090 died from this cancer. Pancreatic cancer accounts for about 3% of all cancers in the United States and for approximately 7% of cancer deaths. Although treatments have improved, especially in the fields of immunotherapy and adjuvant chemotherapy, PDAC has an average 5-year survival rate of only 7%–8%.[3]

The reasons for this morbid outcome are multifactorial, including diagnosis at late stages, rapid progression with metastasis to other organs, and resistance to conventional therapeutic modalities.[4] Most patients are diagnosed with metastases and succumb to disease within 6–12 months of diagnosis.[5] Therefore, understanding the mechanisms underlying disease initiation and progression are critical for early detection, risk stratification, and targeted therapeutic strategies.

The most frequent precursors of PDAC are microscopic pancreatic intraepithelial neoplasia (PanIN), followed by intrapapillary mucinous neoplasm and mucinous cystic neoplasm. PanINs are microscopic (<5 mm) mucinous-papillary lesions, which lead to invasive carcinoma.[6] Ductal adenocarcinomas display an intense stromal reaction that has been postulated to serve as a physical barrier to delivery of chemotherapy.[7] PDAC is associated with several gene alterations, such as activation of oncogenes (mutant *KRAS* is found in $>90\%$ of PDAC) and inactivation of tumor suppressor genes (*TP53*, *p16/CDKN2A*, *SMAD4*, and *BRCA2*).[8–10] Additionally, several genome-wide studies have been done. This has resulted in the identification of novel somatic mutations, although in low frequency, copy number variations, structural variations, and epigenetic alterations.[11–14] To that end, Bailey et al[11] performed a very elegant study where they combined the analyses of whole genomes, exomes, and RNA sequencing from 456 PDACs. Using this approach, they were able to define 4 different subtypes of PDAC (squamous, pancreatic progenitor, immunogenic, and aberrantly differentiated endocrine exocrine) and associated each subtype to specific molecular pathways, as well as histology and survival.

Despite these advances in the understanding of the mechanisms underlying PDAC pathogenesis, the impact on patient benefit is lagging. As a result, new model systems are being developed and used to fill this gap with the hope

of translation into improved diagnostics and therapeutics. This review covers the past and current model systems briefly, and then focuses on 3-dimensional (3D) organoids as a newer relevant model system.

Pancreatic Cancer Cell Lines

The first human pancreatic cancer cell line was generated and published in 1963,[15] and since then, many PDAC cell lines have been developed from murine and human tumors. The use of cell lines has many advantages: ease of use, minimal cost, and the ability for genetic and pharmacologic manipulation. Although cell lines have been very advantageous in pancreatic cancer research, this approach has several key limitations. First, many cell lines have been generated from metastatic tumors, and thus, primary PDAC and PanIN lesions are commonly underrepresented. Second, the lack of other cell types (fibroblasts, nerves, immune cells, adipocytes, endothelial cells) makes it difficult to reflect the physiological dynamics of the disease with accuracy. Furthermore, different expression profiles in the cell lines, as compared with the patient tumors or xenografts, have been reported,[16,17] with the potential selection of more aggressive clones during the generation of the cell lines.[18] Additionally, normal pancreatic ductal cells are challenging and difficult to maintain in culture, so the comparison between normal cells and tumor cells is often not possible. Finally, repeated passaging of cell lines inevitably results in genetic drift.

Xenografts

Xenograft models consist of implantation of pancreatic cancer cells either orthotopically or ectopically (usually subcutaneously) into immunodeficient mice, generating a tumor that might recapitulate a human tumor. Although this approach does offer a better representation of human tumors as compared with *in vitro* cell lines, problems with fidelity and therapeutic response has been reported.[19–21] One potential way of surmounting these limitations involves developing xenografts from patient tumors, which are known as patient-derived tumor xenografts (PDX). PDX helps recapitulate the genetic and phenotypic alterations of human tumors more effectively, which makes them a useful tool for determining drug response,[22] with a positive predictive value of more than 80%.[23] There are limitations with PDX, including the requirement for a large amount of tissue, expense, and time.[24]

Genetically Engineered Mouse Models

This model involves the use of genetic engineering techniques that can either activate or silence gene expression in mice to model disease characteristics. Genetically engineered mouse models (GEMMs) are an excellent approach to study disease progression from early stages (even PanIN) to primary/metastatic PDAC.[25] Some of the advantages in autochthonous models are that tumors develop spontaneously, interactions between the stroma and the tumor are maintained, tumor microenvironment dynamics are captured, and metastatic biology can be investigated. The use of GEMMs facilitates investigation of specific mutations under spatiotemporal control using the *Cre-LoxP* system, among other systems. The most frequently used mouse models take advantage of pancreas-specific cre-lines (eg, *Pdx1-Cre* or *Ptf1a-Cre*) in combination with a mutant allele of $Kras^{G12D}$ (which activates the gene driven by its endogenous promoter) and *TP53* loss.[26,27] This model phenocopies the human disease with high fidelity. More recently, the Saur group introduced the next-generation of GEMMs by combining 2 independent recombinase systems (*Cre-LoxP* and *Flp-FRT*), thereby allowing manipulation of distinct compartments (tumor and stroma) at specific time points within the organism. This allows investigators to ask specific questions regarding tumor-stroma interactions or to explore factors required for tumor maintenance.[28]

Although GEMMs are extremely valuable for understanding tumor biology, the breeding and maintenance of GEMMs are expensive and time consuming. Additionally, gene mutations are being introduced typically into the germline of the mouse, when they occur somatically in human tumors. Nonetheless, the use of next-generation mouse models and alternative approaches, such as unbiased genetic forward screens,[29] may allow for additional new insights.

Pancreatic Organotypic Model Systems

The ideal disease model should recapitulate the structure and genetic profile of the tissue under study, represent the heterogeneity and different stages of the disease, and respond to stimuli in a physiological manner. Moreover, from a practical point of view, this model needs to be feasible, reproducible, easy to maintain, and inexpensive. Therefore, even after recognizing the many strengths and applications of GEMMs, it is not a perfect model. Nonetheless, in recent years, there has been parallel attention to 3D cell culture and 3D organoid model systems.

Three-Dimensional Cell Culture

The 3D cell culture technique prevents cells from attaching to the bottom of the plate by maintaining the cells in suspension or embedding them in a matrix.[30] Initial attempts to establish 3D cultures from normal and tumor cells were unsuccessful, because of lack of cell viability and limited longevity.[31–33] However, in recent years, multiple laboratories, including ours, have developed 3D cultures of murine and human tissues, using slightly different conditions with specialized matrices to maintain cell-cell and cell-matrix interactions and polarized structures.[34–39]

3D cultures of epithelial cells give rise to spheres. Pancreatic spheres can be generated from pancreatic ductal and acinar cells.[40] Pancreatic spheroids may mimic some of the *in vivo* characteristics of PDAC, such as microenvironmental factors and drug responses.[37,41–43] 3D spheres from embryonic pancreatic cells resemble some aspects of pancreatic development, and display expression of PDX1 and SOX9.[44] Spheres generated from ductal cells have been used to study pancreatic carcinogenesis, especially the role of mutant *KRAS*, and with application to drug testing.[35,45–47]

In 2013, our laboratory published the first comprehensive protocol[39] for isolating mouse pancreatic ductal epithelial cells and duct-like cells directly, without requiring additional culture steps, for further use in 2-dimensional or 3D culture. This isolation protocol uses dolichos biflorus agglutinin lectin in combination with magnetic bead separation. Ductal cells can be maintained on top or within a collagen matrix (for 2-dimensional or 3D, respectively). Remarkably, this technique can be used in several physiological conditions (ie, embryonic development) and pathologic states (ie, inflammation and cancer).

Three-Dimensional Organoids

Based on the successful findings with 3D culture, a new *ex vivo* model was developed termed "organoids." This term refers to a group of cells growing in a 3D structure that is generated directly from primary tissues, embryonic stem cells, or pluripotent stem cells, with self-renewal and self-organization capacity, maintaining similar appearance and functionality as the original tissue. Organoids can be maintained through indefinite passage and preserve genetic stability.[48,49] 3D organoids share many characteristics with 3D spheres, so there is a tendency to consider them as a single model; however, strictly speaking, 3D cultures originate from cell lines in monolayer, whereas 3D organoids come directly from tissues as do the protocol we have described for 3D cultures.[30,39]

Briefly, 3D organoids are generated by the digestion (enzymatic or mechanical) of the original tissue into small pieces that are then embedded in a matrix. Collagen and Matrigel (Corning, Corning, NY) are the commonly used matrices for the generation of 3D organoids. Also, specific growth factors and differentiation modulators are needed to supply mesenchymal-based signals, such as epidermal growth factor, fibroblast growth factor 10 (mitogens), Rspo1 (enhances Wnt signaling), Noggin (inhibits BMP signaling), Wnt3a, nicotinamide, *N*-acetylcysteine, gastrin and A83-01 (Alk inhibitor). In addition, supplementation with prostaglandin E_2 is required in normal (untransformed) human 3D organoids. Flow cytometry or magnetic beads (coupled to dolichos biflorus agglutinin lectin) are recommended to isolate ductal cells, although there is evidence that nonductal cells are not viable and get eliminated after 1 passage (Figure 1).[50]

The Clevers' laboratory has been a pioneer in this field, developing a 3D system where epithelial organoids arise from adult tissue, maintaining the identity of the original tissue. The first 3D organoids were derived from the mouse small intestine[51] and gradually expanded to other mouse and human gastrointestinal epithelial tissues.[52–56] In 2013, Huch et al[57] described this system in pancreatic tissue. Later, in 2015, Boj et al[58] reported the first 3D organoid model from murine and human pancreatic adenocarcinoma by embedding cells in Matrigel (Table 1). They use serum-free Matrigel with the addition of several growth factors to propagate murine adult pancreatic duct cells. The media is characterized by Wnt activation (RSPO1), thereby allowing the expansion of ductal structures in a serum-free condition, with further

upregulation of *Lgr5* (stem cell marker and RSPO1 receptor) and subsequent self-renewal. Other important components of the media are Glutamax, Hepes, Noggin, Gastrin I, nicotinamide, epidermal growth factor, fibroblast growth factor 10, *N*-acetylcysteine, B_{27} supplement, and in the case of human samples, also addition of Wnt3a and Primocin. Their model recapitulates normal and tumor tissues from mice and humans, and even more interestingly, the 3D organoids are physiologically similar to the original tissues. The organoids exhibit ductal epithelial cell markers, and lack genes representative of acinar and endocrine lineages. Furthermore, they reported that orthotopic transplantation of tumor organoids into immunodeficient mice initially generated preinvasive lesions similar to PanIN that could progress to invasive adenocarcinoma and metastasis, representing an attractive model for cancer progression. The authors also performed gene expression and proteomic analysis (RNAseq and mass spectrometry, respectively) in murine pancreatic 3D organoids, observing that both gene and proteomic profiles correlated with pancreatic tumor progression.

A group led by Muthuswamy[59] developed pancreatic exocrine progenitor organoids from human pluripotent stem cells with global gene expression similar to the human pancreas, and expression of specific pancreatic markers (*NKX6.1*, *PTF1A*), higher levels of progenitor markers (*PDX1*, *NKX6.1*), lower levels of islet (*NKX2.2*), and acinar (*GATA4*) markers in comparison with the adult pancreas. The authors also developed another efficient model to generate 3D organoids from human pancreatic adenocarcinoma (Table 1). The Muthuswamy laboratory cultured the cells as an overlay in media on top of a Matrigel bed. This culture media is supplemented mainly with B27, ascorbic acid, insulin, hydrocortisone, fibroblast growth factor 2, all-*trans* retinoic acid, and Y267632. Interestingly, this media does not require stimulation of Wnt signaling. They also demonstrated similar drug sensitivity (using epigenetic regulators, specifically EZH2 inhibitors) between 3D organoids and the matched primary tumor. A similar approach, namely inducing pluripotency in pancreatic cancer patient–derived cells and differentiation into the pancreatic ductal lineage so as to recapitulate carcinogenesis, has been reported.[60] Remarkably, this study led to the identification of thrombospondin-2 in combination with the established serum marker CA19-9 to detect early stage pancreatic cancer, thus underscoring a translational potential.[61]

The Skala group published another methodology for pancreatic tumor organoids that allows propagation of fibroblasts and tumor cells within a mixture 1:2 of Matrigel and supplemented media.[62] The culture media contains 10% fetal bovine serum, 1% penicillin-streptomycin, and 10 ng/mL epidermal growth factor receptor. These conditions allow the formation of tumor cells with fibroblasts from human and murine PDAC (Table 1).

Finally, the Kuo group published a methodology based on an air-liquid interface that consists of inner collagen gel-containing Transwell (Corning) with direct air exposure and therefore having cells that are in contact with high levels of oxygen. This approach uses a matrix with type I collagen instead of Matrigel. This permits 3D organoid growth from
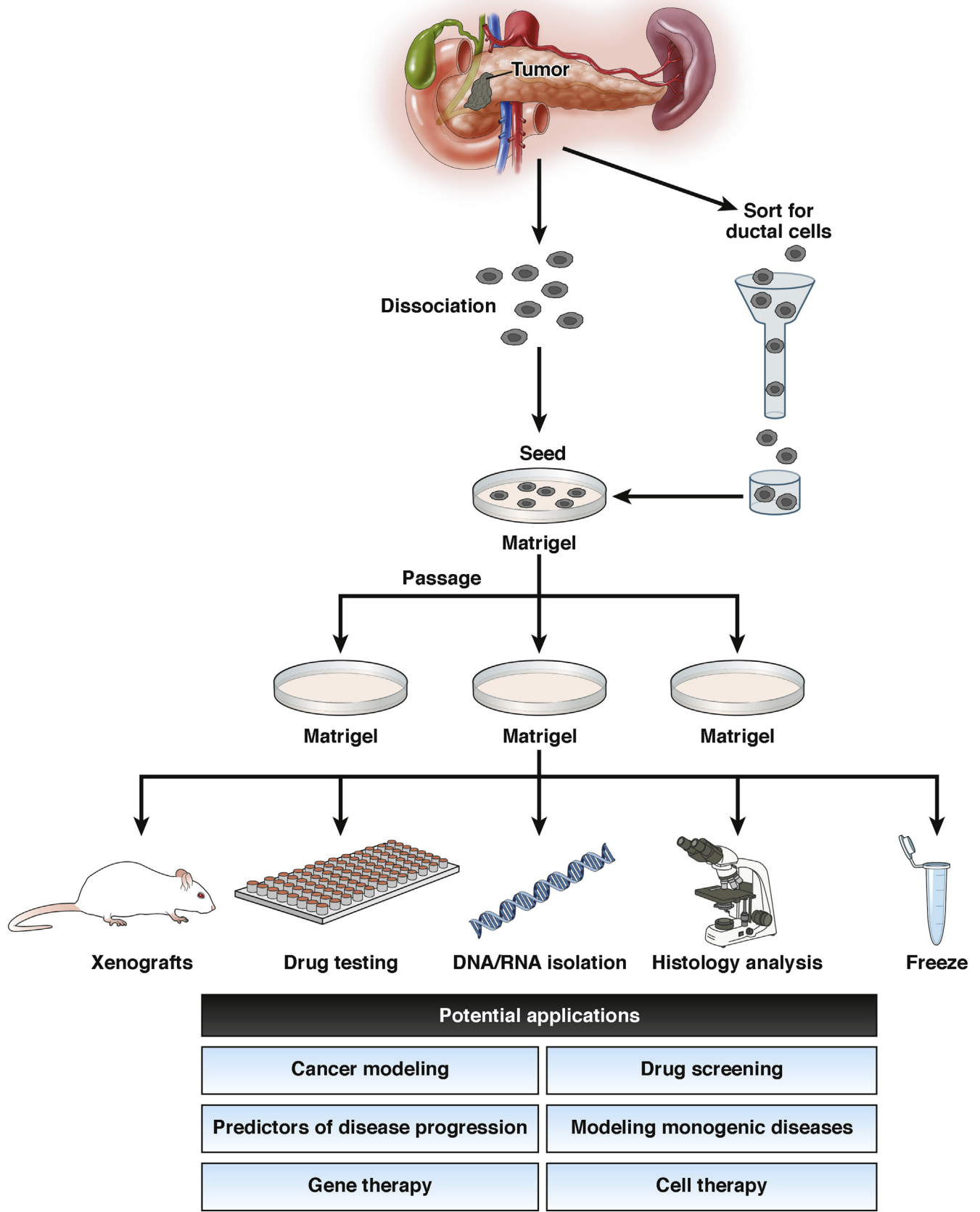
Figure 1. Three-dimensional organoids: generation and applications.

Table 1. Pancreatic Organoid Systems

	Clevers and Tuveson Laboratories[55]	Muthuswamy Laboratory[56]	Skala Laboratory[59]	Kuo Laboratory[60]
Tissue	Murine and human Normal pancreas, PanIN, and PDAC	Human pluripotent stem cells Human PDAC	Murine and human PDAC	Murine Neonatal or adult tissue Murine PDAC
Matrix	Matrigel	Matrigel	Matrigel	Collagen
Method	Cell embedded in Matrigel	Cells, media, and 5% Matrigel over a Matrigel bed	Cell embedded in Matrigel and media	Air-liquid interface Cells over a collagen bed
Passage/freeze	Yes	In human PDAC	Not reported	In context of *KRAS/p53* mutations
Transplantation	Resemble PanINs that progress to PDAC	Recapitulate primary tumor	—	Successful generation of PDAC
Drug testing	Reported	Reported	Reported	Not reported
Limitations	No mesenchymal component	Normal tissue model not reported	Transplantation model not reported	No human model

PanIN, pancreatic intraepithelial neoplasia; PDAC, pancreatic ductal adenocarcinoma.

neonatal or adult murine tissues without the need of exogenous growth factor supplementation (Table 1).[63] This approach is reminiscent of the 3D organotypic cultures we have published for the mouse and human esophagus.[64,65]

Applications of Three-Dimensional Organoids

Organoids represent a powerful tool for research with the capacity to be applied to many key aspects of pancreatic tissue pathology (Figure 1). Pancreatic 3D organoids can be generated from small amounts of tissue and done so in a short period of time, thereby allowing for drug testing and evaluation of potential diagnostic biomarkers. 3D organoids can be generated not only from surgical samples but also from endoscopic fine-needle aspiration or biopsy samples,[58] and therefore, a wide variety of disease-stages and clinical conditions can be recapitulated with this technique. It is critical to understand that almost all large-scale genomic and transcriptomic studies in human pancreatic cancers have focused on tumor samples from surgical specimens. However, only 20% of patients with pancreatic cancer are eligible for surgical resection. The remaining 80% of patients have advanced stage disease and poor prognosis. These shortcomings can now be resolved potentially using 3D organoid technology.

Organoids represent a novel platform for the analysis of gene expression in epithelial cells without contamination by hematopoietic, mesenchymal or immune cells. This model represents a novel approach to validate the genetic alterations that are required for cancer progression, and identify and elucidate genes associated with early stage to late stages of cancer progression, treatment response, and outcomes. For example, Chio et al[66] used an organoid model system to evaluate the role of *NRF2* in PDAC progression, by knocking down this transcriptional factor in human and murine organoids. They reported a low proliferation rate in human cancer organoids that did not express *NRF2*.

Moreover, the authors were able to relate *NRF2* with mRNA translation through redox regulation.

Organoids have an indefinite passaging capability and genetic stability and are attractive candidates to be manipulated genetically to study particular genes. These modifications can be done by retroviral transduction or plasmid transfection, and subsequent maintenance with drug selection using such agents as G418 or puromycin.[67] This technology is promising and can be exploited in regenerative medicine and gene therapy.

Recently, the CRISPR-Cas9 system is being used to mimic genetic alterations, especially in monogenetic hereditary syndromes, such as cystic fibrosis that involves mutations in the cystic fibrosis transmembrane conductor receptor.[68] Mutations in *CFTR* cause cystic fibrosis, a hereditary disease that affects multiple organs. Recently, the Kleger group derived human pluripotent stem cells organoids and pancreatic organoids from cystic fibrosis patients,[69] allowing drug testing with *CFTR* modulators (as described in cystic fibrosis rectal organoids[70]), and mRNA-mediated gene therapy. This is another example of the translational applications in 3D organoids.

Based on the ability of normal tissue to yield 3D organoids and the differentiation potential of organoids, it is possible that organoids could be a potential source of donor tissue for future autotransplantation. For example, organoids could be generated from the normal pancreas and differentiated to beta cells for cell therapy in diabetes mellitus.[71] One of the most relevant applications of organoids is their use in xenografts. Interestingly, transplantation of pancreatic tumor organoids into immunodeficient mice generates preneoplastic lesions (PanINs) that can progress to invasive adenocarcinoma and metastasis.[58] This model represents an attractive and convenient approach to study cancer progression and identify potential biomarkers for early stages of PDAC.[58] More importantly, the observed lesions closely resemble human PDAC.

Organoids can provide a platform for drug testing of individual tumors in a short period of time before or in parallel to implementation of clinical therapy for a PDAC patient. Although some groups have reported a 1-week time period (from biopsy to drug selection),[72] large-scale drug screens could be conducted within 3–4 weeks after receiving the biopsy of a given patient. This approach has the potential to identify individual therapeutic vulnerabilities (based on the genetic mutation profile and drug response in organoids), or to define second or third lines of treatment if standard therapy does not work. The possibility of drug screening can be evaluated and combined with a dynamic technique of vital imaging as functional optical metabolic imaging. This multiphoton microscopy technique detects changes in cellular metabolism through the measurement of autofluorescence intensity and the lifetime of reduced nicotinamide adenine dinucleotide and flavin adenine dinucleotide. It is also capable of detecting heterogeneity, identifying the nonresponding subclones, and differentiating between premalignant and invasive lesions.[62,73] Walsh et al[74] reported that optical metabolic imaging has high sensitivity for detecting metabolic changes 24–72 hours after exposure to effective drugs, and this response is correlated with the expected responses (ie, based on HER2 expression in breast cancer). Furthermore, it has been shown that optical metabolic imaging analysis can differentiate between cell types and drug response. For example, a recent study reported that fibroblasts from PDAC organoids also respond to drug therapy, although there is no significant increase in cell death. Therefore, this technique can be used to test the response to stroma-targeting treatments in organoids.[62]

To analyze potential biomarkers and stratify patients based on genetic profiles and drug response, the creation of biobanks of 3D organoids is a growing trend. Such biobanks from surgery or endoscopic ultrasound allows the cataloging of a wide spectrum of tumors from different stages. Interestingly, Walsh et al[75] demonstrated that it is possible to generate 3D organoids from frozen mammary tumor tissues, with comparable results from 3D organoids derived from fresh tissues in terms of morphology, viability, and metabolism properties.

It is important to keep in mind that although the 3D organoids systems and their potential applications are attractive, they are not inexpensive and are time-consuming. Additionally, one important limitation is that 3D organoids lack some important components present *in vivo*, such as fibroblasts, endothelial cells, immune cells, and neural cells. To surmount this limitation, efforts are ongoing to coculture organoids with other cell types, in order to generate a more "physiological" microenvironment, and to study potential cell-cell interactions. Cancer-associated fibroblasts are involved in the secretion of extracellular matrix components, and have been associated with tumor growth and chemoresistance.[76,77] Recently, Ohlund et al[78] have developed a coculture model of pancreatic cancer 3D organoids and pancreatic stellate cells. They observed an increase in the proliferative rate of organoids and fibroblasts in this method. Also, they found heterogeneity between cancer-associated fibroblasts, with different levels of smooth muscle actin and interleukin-6 as a function of proximity to the organoids. These findings reflect the complexity of the stroma and its influence in epithelial tumorigenesis.

Future Directions

This is only the starting point of the use of 3D organoids as a potential tool in pancreatic cancer research. There are several key points that require emphasis, such as the stability of features in the organoids that are shared with the original tumor, the predictive value, and the ease of drug testing. Clinical trials are ongoing to evaluate the potential use of 3D organoids as platforms pretreatment and posttreatment. From a practical point of view, standardization of protocols for 3D organoid generation is needed to achieve reproducibility. Ideally, optimization would include expansion of 3D organoid technology to other types of pancreatic lesions, such as cystic pancreatic cystic lesions (intrapapillary mucinous neoplasm, mucinous cystic neoplasm) and neuroendocrine tumors. In addition, cost-effective modifications of culture conditions have to be evaluated.

Various preclinical PDAC models have been developed, and each approach has contributed to important aspects in the investigation of PDAC pathogenesis. To that end, the development of 3D organoids is a promising model and powerful tool for several translational applications.

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