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TOPIC HIGHLIGHT

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Pancreatic cancer organotypics: High throughput, preclinical models for pharmacological agent evaluation

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Abstract

Pancreatic cancer carries a terrible prognosis, as the fourth most common cause of cancer death in the Western world. There is clearly a need for new therapies to treat this disease. One of the reasons no effective treatment has been developed in the past decade may in part, be explained by the diverse influences exerted by the tumour microenvironment. The tumour stroma cross-talk in pancreatic cancer can influence chemotherapy delivery and response rate. Thus, appropriate preclinical *in vitro* models which can bridge simple 2D *in vitro* cell based assays and complex *in vivo* models are required to understand the biology of pancreatic cancer. Here we discuss the evolution of 3D organotypic models, which recapitulare the morphological and functional features of pancreatic ductal adenocarcinoma (PDAC). Organotypic cultures are a valid high throughput preclinical *in vitro* model that maybe a useful tool to help establish new therapies for PDAC. A huge advantage of the organotypic model system is that any component of the model can be easily modulated in a short timeframe. This allows new therapies that can target the cancer, the stromal compartment or both to be tested in a model that mirrors the *in vivo* situation. A major challenge for the future is to expand the cellular composition of the organotypic model to further develop a system that mimics the PDAC environment more precisely. We discuss how this challenge is being met to increase our understanding of this terrible disease and develop novel therapies that can improve the prognosis for patients.

Key words: 3D organotypic model; Pancreatic cancer; Pancreatic stellate cell; Stroma; Preclinical models

Core tip: Pancreatic cancer carries a terrible prognosis, as the fourth most common cause of cancer death in the Western world. One of the reasons no effective treatment has been developed in the past decade may in part, be explained by the influences exerted by the tumour microenvironment. The tumour stroma cross-talk in pancreatic cancer can influence chemotherapy delivery and response rate. Organotypic models of pancreatic cancer allow new therapies that can target the cancer, the stromal compartment or both to be tested in a model that mirrors the *in vivo* situation and can help improve patient prognosis.

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PANCREATIC CANCER

Pancreatic cancer has one of the highest mortality rates among malignancies, and is the fourth most common cause of cancer death in the Western world[1,2]. With an overall 5-year survival rate of 6% and median survival of less than six months, pancreatic ductal adenocarcinoma (PDAC) carries one of the bleakest prognoses in all of medicine. Surgery offers the only hope of a possible cure for patients; however even of those 10% of patients eligible for curative resection, only 21% will survive to five years[3]. This is due to the fact that, at diagnosis, distant metastases are common[4]. Clearly there is an urgent need for therapies for PDAC. One of the possible reasons that targeted therapies fail to improve the prognosis of patients with PDAC may, in part, be explained by the diverse influences exerted by the tumour microenvironment. Delineating the signalling networks within the tumour microenvironment, may help to explain the huge discrepancy between relative success and effectiveness of therapies in preclinical assay (predominately 2D cell based assays and xenograft mouse models) and their abject failure in human PDAC.

Many epithelial malignancies, including breast, prostate, skin and pancreatic cancers, often exhibit a significant stromal reaction around the tumour cells[5-9]. Once thought to be a bystander, it is becoming increasingly evident that the stroma not only functions as a mechanical barrier but also constitutes a dynamic compartment that is critically involved in the process of tumour formation, progression, invasion, and metastasis[10,11]. In particular, PDAC shows the most prominent stromal reaction or "desmoplasia" (defined as proliferation of fibrotic tissue with an altered ECM which contributes to tumour growth and metastasis) (Figure 1)[12]. This surrounding tumour environment is an highly heterogeneous and complex mixture of cells from different lineages; fibroblasts, pancreatic stellate cells, smooth muscle cells, immune, inflammatory, neural, adipose and endothelial cells[13-16].

The high proportion of stromal cells in pancreatic cancer (up to 80% of the tumour volume[17]) is associated with overexpression of a number of paracrine and autocrine signalling factors, such as vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factor β (TGFβ), insulin-like growth factor I (IGF- I), fibroblast growth factor (FGF) and their respective receptors as well as secretion of Matrix metalloproteinases (MMPS) and proteases which serve to fuel pancreatic cancer proliferation, metastasis and invasion[18-22]. In turn, pancreatic cancer cells secrete growth factors such as FGFs, TGFβ, IGF and platelet derived growth factor (PDGF)[23]. This interaction between cancer cells and stroma leads to altered transcription in stromal components, such as fibroblasts and inflammatory cells, promoting cancer cell motility and resistance to hypoxia. The net result is an unique tumour micro-environment, where tumour cells become inaccessible to chemotherapy and metastasise readily, leading to poor chemotherapy response rate[17].

These studies have highlighted the importance of stroma-cancer cross-talk. Thus, just studying pancreatic cancer cells without any stromal representation does not reflect accurately the *in vivo* situation. Cells grown on 2D tissue culture plates or in Transwell™ inserts differ in their morphology, differentiation and cell-cell and cell-matrix interactions compared to cells *in vivo*[24,25]. There is a need for physiologically relevant *in vitro* model systems that allow us to investigate and interrogate cancer and stromal cell behaviour and their interactions. Thus, 3D organotypic models are an invaluable research tool[26].

MODELLING PDAC

In vitro (2D) studies of tumour stroma interactions in PDAC

Improved understanding of the mechanisms that mediate epithelial-stromal interactions in PDAC is now possible due to the isolation, and *in vitro* culture, of pancreatic stellate cells (PSC), the key cells driving the desmoplastic reaction[21]. In the healthy pancreas, PSCs make up 4%-7% of all pancreatic cell types and exist in a quiescent state[27]. Quiescent PSCs are characterised by lipid droplets rich in vitamin A, resembling hepatic stellate cells (HSCs) first described by in the 19th century[28]. They express desmin and glial fibrillary acid protein (GFAP) marker which serve to distinguish them from pancreatic fibroblasts[29]. In acute and chronic inflammatory conditions, PSCs are activated. This is characterised a loss of fat droplets, expression of α-smooth muscle actin (αSMA), and an increased synthesis and secretion of several ECM proteins such as fibronectin, laminin and collagen type I and Ⅲ[27,30,31].

The isolation and immortalisation of PSCs from human and rat pancreas has provided an additional tool for studying PSC activation and can overcome the limitations of culturing primary stellate cells. While immortalised stellate cells have provided a valuable tool in the study of PSC function, it is important to validate findings using primary PSCs[14]. PSCs have been immortalised using either SV40 large T antigen or human telomerase in human PSCs as we have previously successfully done in our laboratory[24,32-38]. Immortalised PSCs display an activated phenotype in 2D culture. Importantly PSC cell line is comparable to activated PSCs, which include expression of αSMA and ECM proteins. Importantly, expression profiling of primary and PSC cell lines have shown only a few differences, with differential differences expression of ECM proteins, cytokines and integrins[37]. In addition, both immortalised and primary PSCs respond to TGF-β or PDGF in a similar manner[33]. Thus primary and immortalised PSCs have facilitated for the dissection of

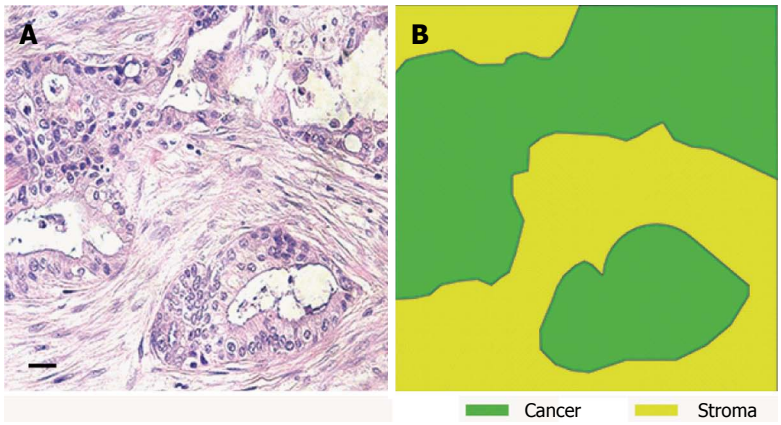

Figure 1 Human pancreatic ductal adenocarcinoma has a dense desmoplastic stromal component. A: HE of human pancreatic cancer shows an area of invasive tumour; B: Stromal and epithelial components of the tumour are highlighted from figure A (scale bar 100 μm).

important cross-talk between PSCs and pancreatic cancer cells and are an important source to explore the tumour promoting aspects of tumour myofibroblasts in PDAC[16].

The bidirectional interaction between PSCs and pancreatic cancer cells has been studied using co-culture or well-established 2D *in vitro* assays such as wound assays or Transwell™ inserts to study migration[39]. Co-culturing of PSCs and pancreatic cancer cells showed that PSCs can increase the proliferation and migration of pancreatic cancer cells, while inhibiting apoptosis by the release of several cytyokines and growth factors. Similarly, culturing PSCs in the conditioned medium of pancreatic cancer cells increases the proliferation, matrix synthesis and motility of PSCs, most likely *via* FGF-2, PDGF and TGF-β[22,40].

The desmoplasia in PDAC is believed to have a detrimental effect on the successful response to chemotherapy and radiotherapy[40,41]. *In vitro* experiments have shown that PSCs can increase the stem cell characteristic of pancreatic cancer cells, a possible mechanism of resistance to therapy[42]. Furthermore, in areas of the tumour that are hypoxic as a result of hypovascularity and profuse stroma provides a micro-environment in which pancreatic cancer cells thrive[43]. *In vitro* studies have shown that, co-culturing PSCs and pancreatic cancer cells under hypoxic conditions, PSCs are able to influence PCC invasion more strongly than in normoxic conditions[44]. Thus, pharmacological targeting of PSCs is an attractive option in treating PDAC.

Role of the stroma in PDAC-in vivo studies

Animal models, such as xenografts, orthotopic grafts or genetically engineered mice (GEM), have validated many *in vitro* findings. Early subcutaneous mouse models, in which PSCs and pancreatic cancer cells were injected into the flanks of immunocompromised mice, demonstratied that, in the presence of PSCs, pancreatic cancer cell proliferation increased and tumours formed more rapidly than when pancreatic cancer cells were injected alone[22]. Apte and colleagues showed that injection of pancreatic cancer cells (MiaPaCa-2 and AsPC-1 cell lines), together with primary human PSC into the mouse pancreas was able to stimulate fibrosis, tumour growth and metastasis[40]. More recently, sex mismatch studies (injection of male PSCs and female pancreatic cancer cells into the pancreas of female mice), have shown that Y chromosome positive PSCs are able to migrate through blood vessels, together with cancer cells, localising to distant sites, such as the liver and diaphragm, where they are able to facilitate seeding, survival and growth of pancreatic cancer cells[45].

The development of genetically engineered mouse (GEM) models of PDAC has provided the most physiologically relevant model that closely mimics the situation in human cancer. Most of the GEM models of PDAC are based on the conditional, pancreas-specific, expression of the Kras oncogene ($KRAS^{G12D}$), present in 90% of human PDAC cases[46], this is facilitated by expressing Cre recombinase under the control of the embryonic pancreas lineage determining transcription factor Pdx-1 or Ptf1/p48 ("KC" mice). KC mice develop pancreatic tumours ranging from precursor pancreatic intra-ductal neoplasms (PanINs) to fully invasive and metastatic disease[47,48], albeit with a long latency period of up to a year. These KC mice have been crossed with mice harbouring several additional mutations, to investigate their contribution to the rapid progression to PDAC. GEM models of PDAC have been developed with activating mutations in TGFβ receptor and/or inactivation of tumoral suppressors such as p53 ("KPC" mice), INK4A/ARF and Smad4, which are the most common PDAC drivers[49]. There are several excellent reviews on the various GEM models that have been developed for studying the development of PDAC[50-52]. The generation of complex allele combinations together with the latency period involved in the development of tumour makes these models inherently expensive. Further criticism against GEM models of PDAC has focused on the multi-focality of their PDAC, involvement of whole pancreas with tumours, histological variants commonly observed, presence of tumours in other organs as well as genetic homogeneity; features missing in the human PDAC[53]. Thus, 3D organotypic models may be an attractive option as a preclinical tool, bridging the gap between traditional 2D cell culture assays and the complex GEM models.

Organotypic models used in other cancers

The idea of recapitulating the physiomimetic 3D envi-

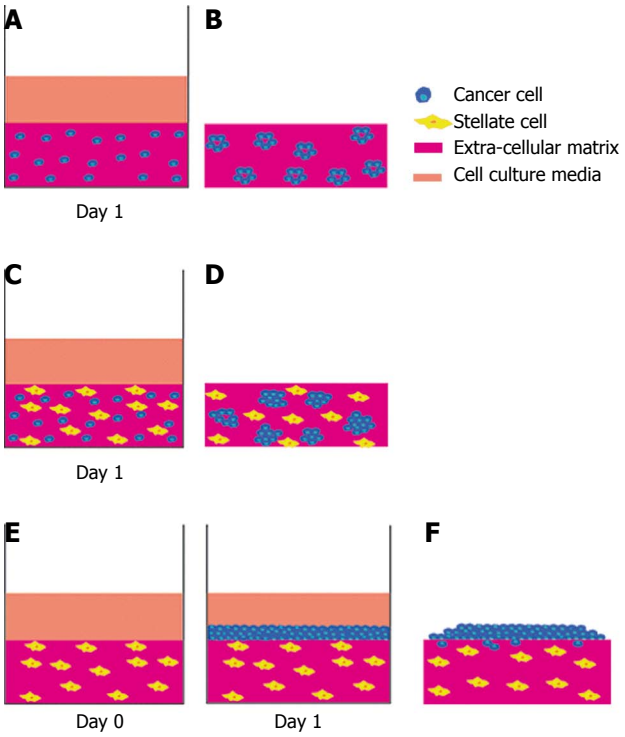

Figure 2 Submerged organotypic culture models used to investigate pancreatic ductal adenocarcinoma. A: Cancer cells were embedded in the extracellular matrix mixture before it was allowed to polymerise. These cells were then fed with culture media placed on top of the gel; B: Representative configuration of cells within the gel after 7 d of culture. The cancer cells forming duct-like structures within the gel. This model mimics the behaviour of invading cancer cells; C, D: Show the same model with both pancreatic stellate cells and cancer cells embedded within the extracellular matrix gel. Using this model the interaction between stellate cells and invading cancer cells can be examined; E: Stellate cells embedded in the gel prior to polymerisation, with cancer cells seeded on top of the gel 24 h later. In this model cancer cell invasion can be analysed in the presence of pancreatic stellate cells (F). Representatice HE of these organotypic models are reviewed in Froeling *et al*[26].

ronment started in the 1960's growing 3D tissue explants in tissue culture media (organ cultures). Organ cultures of neural tissue explants are perhaps the best established model[54]. These models are still being used to study the basis of neurological diseases and injuries[55]. Organ cultures are also used in the study of cardiovascular function[56], angiogenesis[57], thymus[58], skin[59], bone[60], and urogenital tissues[61].

In cancer research, there has been an abundance of evidence suggesting that 3D models are superior to the conventional 2D culture in plastic flasks. However, current preclinical research still relies heavily on the latter[62]. From the simplest form: the "monotypic" cell model, comprising just one epithelial cell type, 3D co cultures have progressively evolved to contain multiple cell types, thus enabling study of their respective contributions[63]. An early example was the the "skin equivalent", achieved by culturing keratinocytes either on de-epidermalised dermis or on collagen gels embedded with dermal fibroblasts[64,65].

The success of pioneering studies with breast epithelial cells cultured in, or on, a reconstituted basement membrane (*e.g.*, Matrigel) undergoing glandular differentiation forming with apico-basal polarity and a central hollow lumen[66], have led to similar experiments for the liver, salivary gland, bone, lung, skin, intestine, kidney and thyroid glands[64,67-71]. The choice of cell source and ECM is critical in developing a representative model. For example, human luminal epithelial cells, grown in laminin rich basement membrane analogue (Matrigel) form acini[72]; however when grown in collagen I, these same cells show an altered integrin profile and abnormal polarity[73].

These 3D models have increased our understanding of how cells perceive biochemical and physical cues from the surrounding microenvironment[74]. For example, β1 integrin is expressed in normal breast epithelial cells but is lost when cells transform into a malignant phenotype. Re-expression of β1 integrin in 3D matrices induces the reversion of the tumor phenotype by allowing the malignant cells to differentiate into glands[75].

The incorporation of tissue specific stromal cells is critical for approximation to the *in vivo* condition. Thus, the isolation and availability of human PSCs hase been critical to the development of PDAC organotypic cultures[26].

Pancreatic cancer organotypics

Pancreatic cancer cell lines and normal pancreatic ductal epithelial cells (HPDE) previously have been cultured on type I glycosaminoglycan scaffolds and in collagen type I or Matrigel. Given only epithelial cells were in these models, the effect of the stroma on tumour cell behaviour was absent[76-78]. However, these studies were able to show that pancreatic cancer cells embedded into Matrigel formed spheroids with a distinct morphology and loss of apico-basal polarity as compared to culturing in 2D[76].

The introduction of stromal cells in PDAC 3D organotypic cultures was first demonstrated by our laboratory[24]. Depending on the hypothesis being explored, the flexible 3D models of PDAC can be set up distinctly. Pancreatic cancer cells can be embedded into the ECM gel consisting of collagen and Matrigel to simulate cells that have already invaded into the stroma However, in order to understand the influence of PSCs on the behaviour of invaded pancreatic cancer cells these cells can be embedded in an ECM gel together with cancer cells (Figure 2).

Submerged ECM gels (when pancreatic cancer cells are grown on top of the gel and PSCs are embedded) are designed to model the early events in tumour progression. When pancreatic cancer cells are cultured on top of this model, they form luminal structures that resemble ducts (Figure 3). Using this model, we have shown that PSCs induce Ezrin translocation from the apical to the basal compartment of the cells is an early event in pancreatic cancer cell invasion[24,79]. This phenomenon has been validated across a range of human gastro-intestinal tumours[80,81]. Finally, in order to study the invasion of pancreatic cancer cells in the 3D model, the submerged

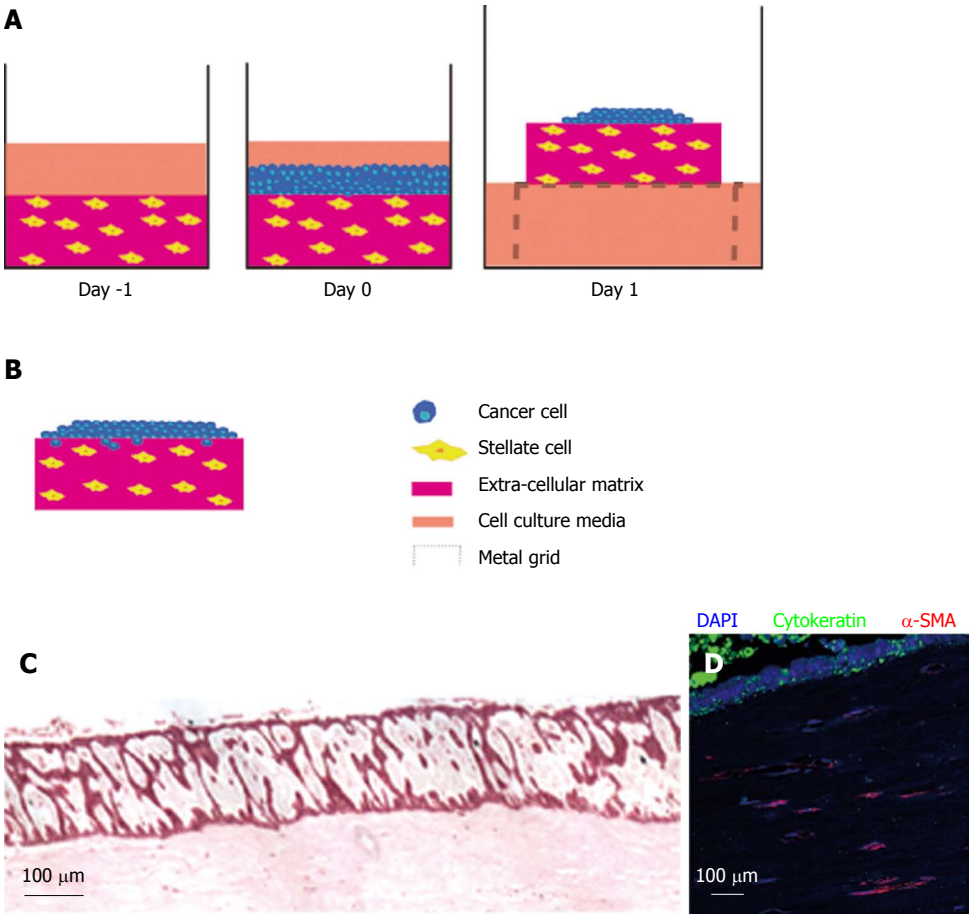

Figure 3 Raised organotypic model of pancreatic ductal adenocarcinoma with embedded pancreatic stellate cells. A: Extracellular matrix gels containing stellate cells were polymerised in 24-well plates before cancer cells were seeded on top and allowed to attach. These gels were then raised onto metal grids and fed from below creating a chemotactic gradient. Cells were cultured for up to 14 d; B: Illustration of cancer cells in a raised model containing stellate cells showing proliferation and invasion into the gel; C: HE section of a raised gel containing stellate cells with cancer cells seeded on top; D: Immunofluorscence in the same gel showing strong cytokeratin expression in the cancer cells and α-smooth muscle actin (α-SMA) expression in the embedded stellate cells.

culture system can be raised upon a grid ('air-liquid' model) and fed from underneath, creating a gradient that stimulates pancreatic cancer cells to invade while at the same time recapitulates cancer-stellate cell interaction *in vivo* (Figure 4).

Using the air liquid 3D model we have shown that the presence of PSCs leads to a significant increase, and altered sub-cellular distribution, of β-catenin in pancreatic cancer cells. Treating these 3D co cultures with All Trans Retinoic Acid (ATRA, which renders PSC quiescent) dampens Wnt-β catenin signalling resulting in reduced pancreatic cancer invasion[16]. Importantly, these results were confirmed *in vivo*, whereby treating KPC mice with ATRA led to disruption lead to disruption of the activated stroma and increase in apoptosis of tumour cells. These sets of observations validate the use of the organotypic model as a tool to assess new therapies in PDAC.

3D organotypic models provide a perfect intermediate between 2D cultures and GEM. Use of distinct cell types in these co-culture allows assessment of changes in signalling cascades and molecular targets resulting from cancer-stroma cross-talk in the absence of noise from other stromal elements present *in vivo*. Thus the relative contribution of each cell type in the complex microinvrionment can be assessed. Using this approach Kadaba *et al*[14] isolated cancer cells from organotypic models of various organ including pancreas, skin and oesophagus after the cancer cells were exposed in 3D to their respective stromal cells (Figure 5). They demonstrated that cancer cell stromal interactions significantly alter proliferation, cell cycle, cell movement, cell signalling and inflammatory response in addition to changing stiffness in the ECM gel. Importantly, changes in stiffness of ECM gels was particularly prominent as the proportion of PSC in the ECM gel increased, a finding highly pertinent to drug delivery and perfusion in PDAC[41]. This study also highlighted the possible need for multidrug targeting or use of pleiotropic agents in PDAC therapy.

Despite the importance of multiple pathways in PDAC, the proto-oncogene Src has been heralded as a potential single molecular therapeutic target[82]. The conundrum of promise of Src inhibitors in combination with chemotherapy *in vitro* and the *in vivo* reduction of metastasis in KPC mice by 50%, was explored in organotypic cultures[82]. Using fluorescence lifetime imaging microscopy (FILM) to measure fluorescence resonance

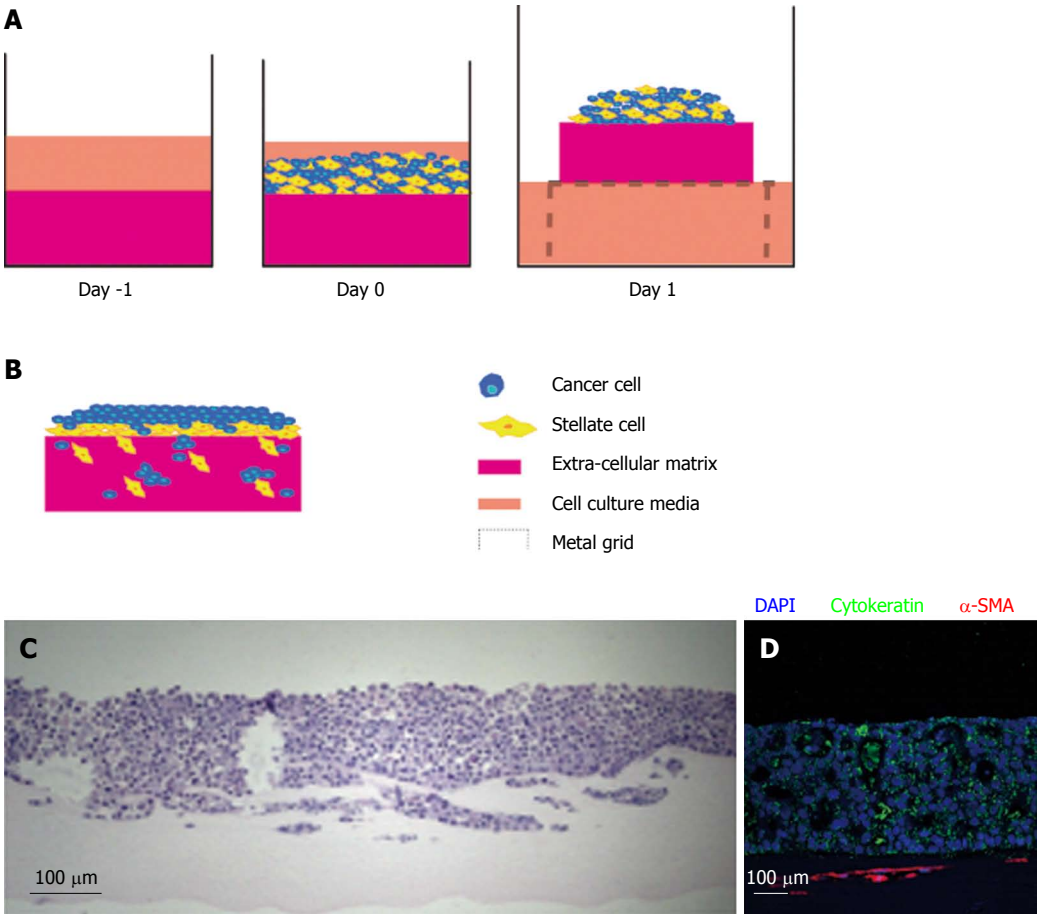

Figure 4 Raised organotypic model of pancreatic ductal adenocarcinoma with cancer cells and pancreatic stellate cell interaction. A: Extracellular matrix gels containing were polymerised in 24-well plates before cancer stellate cells were seeded on top and allowed to interact and attach. These gels are then raised onto metal grids and fed from below creating a chemotactic gradient. Organotypic models were cultured for up to 14 d; B: Illustration of cancer cells and stellate cells in a raised model showing increased proliferation and invasion of both stellate and cancer cells into the gel; C: HE section of a raised gel with cancer cells and pancreatic stellate cells seeded on top; D: Immunofluorescence in the same gel showing strong cytokeratin expression in the cancer cells and α-smooth muscle actin (α-SMA) expression in the stellate cells which form a layer below the cancer cells.

energy transfer (FRET) an ECFP-YFP Src reporter, in PDAC cells in organotypic cultures Anderson and colleagues investigated the influence of tumour microenvironment on Dasatinib delivery in PDAC[83]. In organotypic PDAC models with cancer cells expressing the Src biosensor cultured on top of an ECM gel with embedded primary human fibroblasts, they were able to show quantitatively that the microenvironment contribution to poor drug delivery to tumour cells is dependent on distance of cells from the invasive edge. This was validated in subcutaneous *in vivo* models due to the limitations of microscopy techniques precluding orthotopic or GEM models. This study demonstrated the adaptability of the organotypic model as powerful tool to address hypotheses at the molecular level in a complex microenvironment.

Future applications and challenges

3D organotypic models that mimic the morphological and functional features of their *in vivo* parental tissues have potential for bridging the gap between cell-based discovery research and animal models[84,85]. A huge advantage of the organotypic system is that any component of the model can readily be modulated in a short time-frame. For example, the matrix composition can be altered to reflect the *in vivo* situation. The increase in ECM stiffness exerts elevated force on transformed cells increasing cellular response and resulting in increased tumour growth, survival and motility[14,86].

The relative paucity of primary stellate cells to conduct all the experiments in sufficient replicates lead us to generate a mini organotypic culture system (Figure 6) which give comparable results to the conventional "air liquid" co culture model[14]. Additional cell types can be titrated in such as stellate cells[14] or endothelial cells (Di Maggio, unpublished observations). For example, to assess the role of stroma on angiogenesis, in oesophageal cancer endothelial cells on a 2D monolayer have been cultured with fibroblast and cancer cells embedded in a collagen gel layered on top[87]. Elsewhere investigation of the role of macrophages in malignant growth of human squamous cell carcinoma has been investigated in organotypic cultures[88]. Immune response and inflammation play an important role in the desmoplastic reaction and inflammation is thought to activate pancreatic stellate cells[13,89].

Therapeutic agents such as chemotherapy (Gemen-

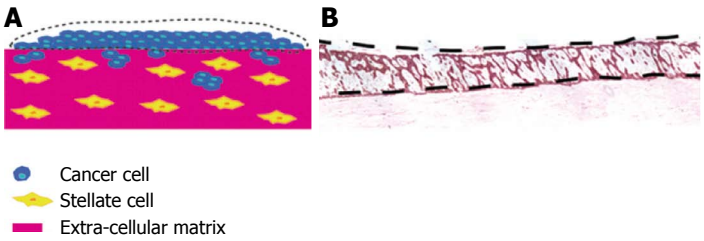

Figure 5 Use of organotypic model to isolate cell types grown together by laser microdisection. By using the raised organotypic model with pancreatic stellate cells embedded within the gel (Figure 4B) the sellate and cancer cells are kept separate. A, B: Laser microdissection of the cancer cells can then be performed to allow analysis of cancer cells grown in the presence of pancreatic stellate cells. Scale bar 100 μm.

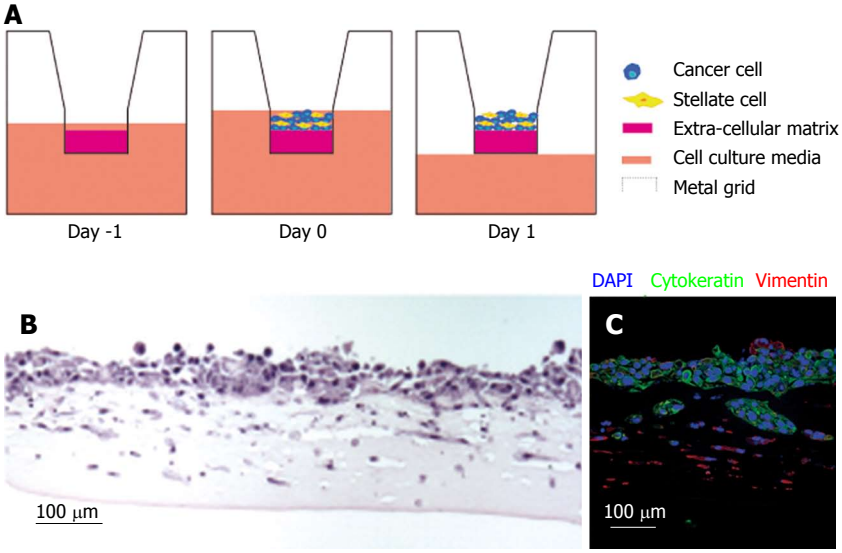

Figure 6 Mini-organotypic model of pancreatic ductal adenocarcinoma. Extra cellular matrix gel is polymerised within the insert of a standard migration assay plate. A: Cancer and pancreatic stellate cells are seeded on top of the gel and allowed to attach. The media is then removed and then replaced only in the bottom of the well to again create a chemotactic gradient cells are cultured for 7-10 d; B: HE image demonstrating a similar pattern of cell proliferation and invasion is seen, as in the raised model; C: Immunofluorscence in the same gel showing strong cytokeratin expression in the cancer cells and vimentin expression in the stellate cells which form a layer below the cancer cells and invade into the gel ahead of cancer cells.

zitdis and Carapuca and Ghallab, unpublished observations), small molecules[90] or RNAi (Arumugam and Watt, unpublished observations) can be tested in these organotypic cultures. The best dosage and regimen can then be taken in small animals thus reducing animal usage[16,83]. Examples from other related fields include testing Met-kinase inhibitor or COX-2 inhibitor in skin cancer models[91], tyrosine kinase inhibitors for breast cancers[92] and Eps8 and HAX1 or β6 integrin[93] RNAi in cancer cells prior to their incorporation into organotypic cultures to assess the effects on cell invasion.

Finally, many PDAC patients present very late with their disease when metastasis have already occurred. Thus, treating PDAC cells immediately after seeding in a 3D environment does not reflect the true clinical setting as tumours are well established at the time of patient treatment. We currently are investigating the effect of treating organotypic models once they are established and invasion of PDAC and/or stromal cells has begun. It is likely this would give a better understanding of the treatment regimen that is required when novel therapies emerge into a preclinical setting.

CONCLUSION

Organotypic culture models are valuable tools for studying the mechanisms of pancreatic cancer, providing an easily manipulated system in which specific questions can be addressed, thus facilitating the translation of basic science to the clinic. Allowing manipulation of cell types, matrix composition, and exogenous therapies, these physiologically relevant model systems are reproducible, experimentally flexible and offer targeted high-throughput platforms. Although the organotypic model provides a physiologically relevant means to study the tumour stroma interactions and the use of new therapies to target the cross talk, it remains a simplified representation of the complex *in vivo* situation and it still remains critical to test new therapies in orthotopic or transgenic models of the disease. However, the use of the organotypic model as a preclinical tool is becoming increasingly important and our group, as well as others, are modulating the 3D cultures to recapture other important aspects of the tumour microenvironment that can influence cancer cell behaviour. Thus, 3D organotypic models have potential for bridging the gap between cell based discovery and complex animal models. By providing an environment in which cell behaviour and novel treatment options can be investigated in an easily reproducible and controlled manner, these models more precisely mimic pancreatic cancer, thus providing a major contribution to preclinical drug and therapeutic discovery.

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