Advances in Bioengineering Pancreatic Tumor-Stroma Physiomimetic Biomodels

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Abstract

Pancreatic cancer exhibits a unique bioarchitecture and desmoplastic cancer-stoma interplay that governs disease progression, multi-resistance, and metastasis. Emulating the biological features and microenvironment heterogeneity of pancreatic cancer stroma in *vitro* is remarkably complex, yet highly desirable for advancing the discovery of innovative therapeutics. Diverse bioengineering approaches exploiting patient-derived organoids, cancer-on-a-chip platforms, and 3D bioprinted living constructs have been rapidly emerging in an endeavor to seamlessly recapitulate major cancer-stroma biodynamic interactions in a preclinical setting. Gathering on this, herein we showcase and discuss the most recent advances in bio-assembling pancreatic tumor-stroma models that mimic key disease hallmarks and the native desmoplastic biosignature. A reverse engineering perspective of pancreatic tumor-stroma key elementary units is also provided and complemented by a detailed description of biodesign guidelines that are to be considered for improving 3D models physiomimetic features. This overview provides valuable examples and starting guidelines for researchers envisioning to engineer and characterize stroma-rich biomimetic tumor models. All in all, leveraging advanced bioengineering tools for capturing stromal heterogeneity and dynamics, opens new avenues toward generating more predictive and patient-personalized organotypic 3D *in vitro* platforms for screening transformative therapeutics targeting tumor stroma.

Keywords: Pancreatic Tumor-Stroma, *In Vitro* Models, Organoids, 3D Bioprinting, Cancer-on-a-chip

1. Introduction

Pancreatic cancer is a highly lethal malignancy that is becoming increasingly prevalent worldwide.(Makohon-Moore and Iacobuzio-Donahue, 2016) Among all types of pancreatic cancer sub-types identified to date, pancreatic ductal adenocarcinoma (PDAC) is the most prevalent and the most challenging to tackle clinically, exhibiting a 5-year survival rate of approximately 8%.(Kuen et al., 2017; Orth et al., 2019) The poor prognosis of this neoplasia is intimately correlated with the unique bioarchitecture of its stromal components.(Gaviraghi et al., 2011; Pandol et al., 2009) Indeed, mounting evidence regarding PDAC tumor microenvironment (TME) indicate that its highly heterogeneous and desmoplastic distal/juxtatumoral stroma is a key effector in disease progression.(Schnittert et al., 2019; Weniger et al., 2018) In PDAC, the stromal compartment is particularly prevalent and bioactive having a major role in disease progression and drug resistance when compared to those of other solid tumors.(Kleeff et al., 2016) In this intricate setting, tumor-associated stromal elements actively communicate with their surrounding microenvironment and specifically with pancreatic cancer cells via numerous routes significantly modulating gene expression patterns, metabolic signatures, invasion/metastasis and resistance mechanisms activation.(Zhan et al., 2017) Improving our understanding and recapitulation of such multifactorial cancer-stromal interactions is crucial for discovering innovative biological targets.

Up-to-date, remarkable efforts and advances have been made toward generating increasingly physiomimetic 3D *in vitro* models that can more accurately recapitulate the biological and biophysical complexity of the TME. Such living 3D models greatly surpass the limitations of 2D monolayered cell cultures and the costly/low-throughput animal models which generally fall short in recapitulating the heterogeneous and highly fibrotic stroma components of PDAC.(Fang and Eglen, 2017; Laschke and Menger, 2017) The available toolbox of bioengineered 3D models for mimicking human disease in an *in vitro* setting includes: (i) cell-rich randomly assembled 3D spheroids, (ii) patient-derived organoids, (iii) cell-laden hydrogel platforms, (iv) dynamic microfluidics-based cancer-on-a-chip platforms, as well as 3D biofabricated constructs, and/or their combinations thereof.(Baker et al., 2016; Cao et al., 2019a; Ferreira et al., 2021; Huang et al., 2015; M. V. Monteiro et al., 2020a; Yu and Choudhury, 2019) In effect, evermore organotypic patient-derived organoids combined with microfluidic chips and 3D additive manufacturing living constructs are rapidly emerging as proficient platforms for recapitulating key aspects of the TME.(Monteiro et al., 2021b) Such capacity to mimic critical flow dynamics, as well as the biochemical, genetic, and biophysical cues that underly cancer progression are expected to contribute for unveiling critical aspects that ultimately influence therapeutics efficacy evaluation.(Cao et al., 2019b; Li et al., 2019; Zhang et al., 2017) In many ways these rapidly emerging platforms have also potential for transforming the foundations of the field of preclinical cancer modelling owing to their inherent modularity and bioengineering versatility, offering researchers the possibility for precisely introducing key TME elements and tumor tissue-stroma dynamics that are still challenging to be recapitulated *in vitro*. Achieving the successful inclusion of key stromal cellular effectors, and of the supporting pancreatic cancer ECM matrix, in a mode that recapitulates patient tumor cellular landscape, disease stage and desmoplastic environment, is anticipated to provide significant breakthroughs.

Gathering on the relevance of mimicking tumor-stroma interactions in *in vitro* models, herein we showcase and critically discuss the most recent and significant advances in exploring 3D platforms for modeling the unique PDAC tumor-stroma interplay. A comprehensive overview of key design blueprints for bioengineering stoma-rich p models is

also provided in light of the key unitary elements and analytical tools that can be used to intelligently generate physiomimetic living systems for better predicting candidate therapeutics performance before their translation to a clinical setting.

2. Pancreatic Tumor – Stromal Cells Interplay – An Undesirable Alliance

Pancreatic tumors stroma is unique in comparison to other malignancies in the sense that stromal constituents provide key signaling and bioprotective barriers that fuel disease progression and protect cancer cells from anti-cancer therapeutics.(Ho et al., 2020) In a bottom-up deconstructive perspective, PDAC stroma consists of key fundamental building blocks and core effectors including: (i) cancer-associated fibroblasts (CAFs), (ii) endothelial cells, (iii) immune system cells (*e.g.*, TAMs, Myeloid-derived suppressor cells (MDSCs), etc.), alongside with basement membrane and extracellular matrix (ECM) components.(Pothula et al., 2020; Stopa et al., 2020) Pancreatic tumors also actively influence surrounding lymphatic and autonomic nervous system elements, through direct and indirect means of communication using soluble/insoluble biomolecular mediators (*e.g.*, growth factors, cytokines, extracellular vesicles).(Tomás-Bort et al., 2020)

Up-to-date highly relevant clinical findings have highlighted that CAFs play an essential part in establishing the fibrotic stromal barrier that engulfs the tumor mass, and that ultimately impedes therapeutics access.(Grünwald et al., 2021) In PDAC, cancer-associated pancreatic stellate cells (CAFs) assemble in a core-shell like structure with distinct cellular and matrix composition surrounding the primary tumor site.(Grünwald et al., 2021; Sun et al., 2018) CAFs have been hypothesized to arise from pancreatic stellate cells (PaSCs).(Apte et al., 2013) In healthy tissues PaSCs exhibit a star-shaped morphology, recognized by the expression of both ectodermal and mesenchymal markers and by their capacity to store key retinoids such as vitamin A-rich in lipid droplets.(Pothula et al., 2020; Schnittert et al., 2019) While PaSCs have been speculated to play a minor role as regulators of pancreatic acinar secretions and of localized immune response, these cells are well recognized as crucial mediators of pancreatic ECM function.(Ferdek and Jakubowska, 2017; Suklabaidya et al., 2018) During PDAC development, PaSCs found in the periacinar region, can be activated in response to inflammatory cues and cancer cells-derived factors, acquiring a myofibroblast phenotype capable of deregulating ECM homeostasis, and also actively interfere with immune cell response (Wang et al., 2020). Generally, PaSCs transformation to CAFs is expedited by cancer cells-mediated secretion of growth factor β (TGF-β), tumor necrosis factor α (TNF-α), platelet-derived growth factor (PDGF), and several interleukins (*e.g.*, IL-1, -6, and -10).(Bynigeri et al., 2017)

Once transformed, CAFs establish complex a complex autocrine and paracrine signaling interplay with cancer cells, by secreting increased levels of cytokines (*e.g.*, IL-1, -6, -8 and -10) and growth factors (*e.g.*, transforming growth factor β (TGF-β), insulin-like growth factor 1 (IGF-1), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factor 2 (FGF-2), connective growth factor (CTGF), and C-X-C motif chemokine 12 (CXCL12).(Norton et al., 2020; Sun et al., 2018) All these soluble molecules contribute to the desmoplastic reaction and promote cancer cells proliferation, migration, invasion, and resistance.(Hosein et al., 2020) On the other hand, CAFs also exhibit an important role on cancer cells metabolic reprogramming by providing necessary biomolecular cues that support cancer survival under nutrient-deprived conditions.(Schnittert et al., 2019)

In this biodynamic microenvironment, CAFs are in turn stimulated by cancer cell-derived mediators such as hepatocyte growth factor (HGF) and fibroblast growth factor

(FGF)(Pereira et al., 2019), leading to increased matrix deposition and remodeling.(Luo et al., 2012) Such interplay further promotes tumor hypoxia, and surrounding blood vessels collapse, promoting epithelial-to-mesenchymal transition (EMT), increased cancer cells malignant behavior, and hampering anti-cancer therapeutics delivery and performance.(Kleeff et al., 2007; Sahai et al., 2020) More importantly, CAFs also exhibit extensive reciprocal signaling with TME infiltrating immune cells.(Watt and Kocher, 2013) This direct contact with cancer cells and crosstalk with immune cells is hypothesized to further increase pancreatic cancers ability to evade immune response.(Bynigeri et al., 2017; Norton et al., 2020)

Adding to this, pancreatic stroma is also populated by a complex immune cell niche. The immune compartment is rich in effector T cells, NK cells, and macrophages, which in all are counteracted by competing immunosuppressive tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), and regulatory T cells (Tregs), that promote an immunosuppressive microenvironment when bioinstructed by CAFs and cancer cells (Wang et al., 2020). In pancreatic cancer TAMs comprise a major component of immune cell populations. The synergistic crosstalk between cancer cells and CAFs (*e.g.*, via IL-10 and IL-13 secretion), in turn promotes macrophage polarization towards a TAM phenotype (*i.e.*, $CD136^{+}$ and $CD204^{+}$), that exerts tumor-promoting functions by secreting several growth factors, namely IL-10 which prevent dendritic cell-mediated antitumor immune responses.(Murakami et al., 2019; Wang et al., 2020) Moreover, a crucial subset of pancreatic CAFs expressing MHC class II and CD74, has shown to exhibit antigen presenting capacity, stimulating $CD4^{+}$ T cells and consequently modulating the immune response in pancreatic cancer.(Elyada et al., 2020) Recognizing and modulating the nature of this complex and evolving tumor-stroma crosstalk is crucial for bioengineering evermore physiomimetic *in vitro* PDAC models with improved in pre-clinical/clinical correlation of therapeutics performance.(Sahai et al., 2020)

3. Engineering Blueprints for Pancreatic Tumor - Stroma Models: Elements and Tools

Considering the multifarious nature of pancreatic cancer TME, the identification and biophysical characterization of its fundamental cellular and matrix elements, followed by their re-engineering from the bottom-up can unlock the generation of highly organotypic tumor-stroma *in vitro* platforms for screening candidate therapeutics targeted to malignant and/or stromal components.

Up to date, a plethora of techniques can be leveraged for a comprehensive deconstruction and characterization of native pancreatic tumor-stroma heterogeneity, hallmarks (*e.g.*, gene expression patterns, activation/de-activation of signaling pathways), as well as of major biomarkers (*e.g.*, specific growth factors, cytokines, etc.). Such techniques can in turn also be employed for subsequent physicochemical characterization of user-programmed 3D *in vitro* platforms, allowing researchers to evaluate models' similarity to native human tumors **(Fig.1)**. In this focus, methodologies based on high-content approaches such as: (i) single-cell RNA sequencing (W. Lin et al., 2020), (ii) imaging mass cytometry (Chang et al., 2017), (iii) multi-dimensional fluorescence imaging (Little et al., 2020), (iv) metabolomics/lipidomics (Gaspar et al., 2019), (v) multiplex ELISA (Hachey and Hughes, 2018) and (vi) cells and ECM proteomic profiling, conjugated with advanced bioinformatics analysis, have truly opened new opportunities to deepen our understanding of intricate tumor-stroma interactions and to pinpoint the numerous sub-populations/phenotypes present within pancreatic cancer TME.(Steele et al., 2020)

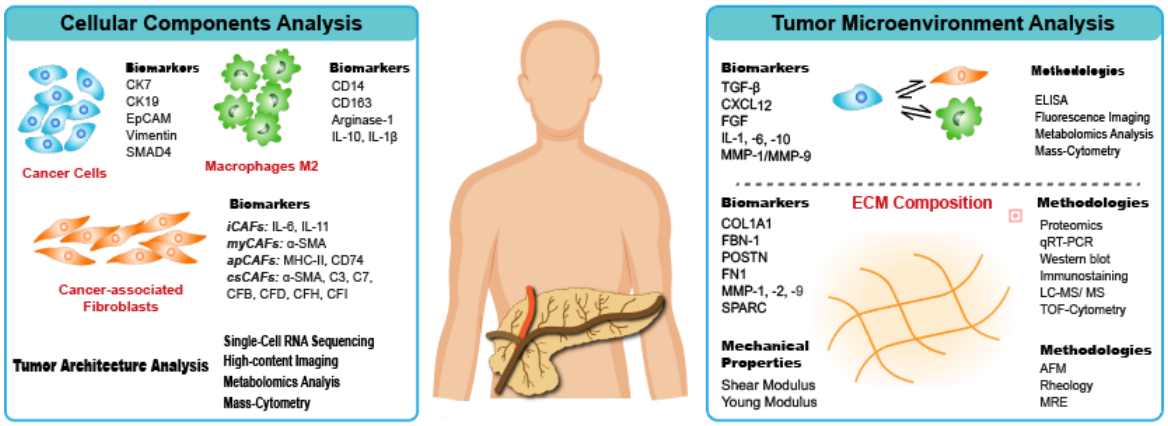

Fig.1. Schematic overview of major pancreatic cancer TME components, biomarkers, and advanced methodologies for their analysis. Characterization of key disease hallmarks during and after the bioengineering of 3D tumor-stroma platforms can open new avenues in improving their ability to emulate the *in vivo* scenario. Moreover, patients' tumor characterization a priori to the engineering of tumor-stroma *in vitro* models may unlock the potential for personalized and precision medicine approaches.

The highly relevant data libraries generated by such methodologies constitutes a unique opportunity for identifying and recapitulating the correct cellular elements and phenotypes in engineered preclinical models. This bioengineering roadmap based on the use of advanced characterization tools for supporting an informed generation of 3D *in vitro* tumors is widely transversal beyond pancreatic cancer. Indeed, this multi-dimensional strategy based on an initial TME profiling followed by rational 3D models biodesign and subsequent physiological characterization upon *in vitro* culture along time, can be viewed as a universal blueprint for engineering other malignancies in which tumor-stroma interactions are recognized to play a crucial role (*e.g.*, breast, lung, colorectal, etc.). This strategy could also be further extended toward personalized medicine approaches if one considers that patients' tumor-stroma could be profiled and then re-engineered in a laboratory setting for screening precision therapeutics.

Focusing on the early design stages specific for pancreatic tumor-stroma *in vitro* models, researchers must consider the inclusion of a wide array of elements and features including: (i) biological gradients establishment (*i.e.*, nutrients, metabolites, gas exchange), (ii) malignant and stromal phenotypes/heterogeneity, (iii) tumor-stroma cytoarchitecture, (iv) cell population ratios (*i.e.*, cancer-to-stroma ratios), as well as (v) tumor supporting ECM composition/mechanical properties. Recapitulating many of these aspects, as well as characterizing their influence in 3D pancreatic cancer model's physiology is key for bioengineering increasingly biomimetic testing platforms. All in all, introducing major stromal cells and ECM components is the key to recapitulate TME hallmarks as these are the main orchestrators of pancreatic cancer pathophysiology.

3.1. Living Stromal Elements in 3D Pancreatic Cancer Models – Bioengineering and Phenotyping

Considering that CAF stromal units are major living orchestrators of desmoplastic reaction in pancreatic cancer TME, their rational inclusion in a mode that accounts for malignant-stroma ratios is an important aspect that needs to be emulated for improving 3D tumor models correlation with the *in vivo* scenario.(Tsai et al., 2018) For introducing CAFs in microtumor models in a biomimetic way that recapitulates patient tumors occupation,

researchers have employed histopathological analysis of human tumor tissues.(Tanaka et al., 2020) Such informed design approach has evidenced that PDAC stroma may comprise 40 to 80 % of the entire tumor mass. On this focus, a seminal report has recently opened new avenues toward advancing histology-based tumor profiling through optimization and validation of an *in situ* 3D characterization/reconstruction of pancreatic cancer tumor anatomy with single cell resolution and in a patient-personalized mode.(Kiemen et al., 2020) The highly relevant data extrapolated from such volumetric analysis may provide major advances to researchers aiming toward recapitulate multiple tumor-stroma interactions including those taking place at the cellular level or more at a macroscale in the whole tumor volume. Localizing CAFs distribution in 3D is a highly desirable feature that will assist in bioengineering in vitro models with a more precise tumor-stroma bioarchitecture. Controlled cell deposition technologies such as 3D bioprinting, bottom-up cell-laden hydrogel assemblies among other will definitely play a major role in materializing these concepts in disease modeling.(Gaspar et al., 2019)

The growing evidences of CAFs abundance in pancreatic cancer TME has led researchers to better acknowledge the importance of mimicking their biological effects. As such, various researchers have endeavored to recapitulate human PDAC stroma occupancy by precisely tunning CAFs:cancer cells ratios (*i.e.,* CAF-to-cancer cell density introduced in cancer models), in an attempt to better mimic the *in vivo* scenario in an *in vitro* setting.(Tanaka et al., 2020) This parameter is crucial for enabling researchers to manipulate the desmoplastic fibrotic reaction and stroma-tumor signaling in 3D *in vitro* models (*i.e.,* via growth factors, cytokines, vesicles, etc.). Tunning this ratio according to different disease stages (*i.e.,* stage 0 - carcinoma *in situ*, up to stage 4 – confirmed spreading to other organs), has remained rather underexplored in 3D *in vitro* models and we anticipate that the combination of high-content cell characterization methodologies combined with advanced bioengineering tools will unlock the generation of stage-specific tumor surrogates for precision medicine approaches.

Adding to this, during 3D models design stages CAFs heterogeneity is another key aspect that must be considered, since a growing body of evidence indicates that these sub-cellular populations exhibit different phenotypes, ultimately influencing tumor progression and drug resistance through multiple cell-protecting mechanisms.(Pereira et al., 2019) Exploiting this heterogeneity may unlock new avenues and strategies regarding the discovery of novel biological targets for disrupting the pancreatic tumor-stroma interplay.

Generally, CAFs phenotype is characterized by an altered expression of alpha-smooth muscle actin (α-SMA), fibroblast activation protein (FAP), and S100A4.(Grünwald et al., 2021; Olive, 2015) CAFs can also display increased proliferative markers and motility, as well as cytoskeletal re-arrangement.(Erdogan and Webb, 2017; Han et al., 2020). Importantly, most activated CAFs secrete soluble growth factors and chemokines such as TGF-β, platelet derived growth factor (PDGF), chemokine (C-X-C motif) ligand 2 (CXCL2) and endothelin. (Schnittert et al., 2019; Zhan et al., 2017) CAF-associated secretome has been routinely characterized through conventional approaches (*i.e.*, ELISA or western blot). More recently, the establishment of advanced mass-spectrometry characterization approaches has led to the discovery of an additional array of characteristic biomarkers correlated with CAFs bioactivity having enabled the identification of multiple phenotypes.(X. Liu et al., 2017; Santi et al., 2017) Gathering on these approaches, up-to-date 4 main subtypes of pancreatic CAFs have been identified and classified according to their biomarkers/phenotypes, namely: (i) inflammatory CAFs (iCAFs), which lack α-SMA expression and exhibit high expression of inflammatory mediators (*e.g.*, IL-6, IL-11) being

located in the peripheral regions of the main tumor, (ii) juxtatumoral myofibroblasts (myCAFs), which express high levels of α-SMA and low levels of inflammatory mediators, (iii) antigen-presenting CAFs (apCAFs) that exhibit a combination of iCAF and myCAF biomarkers expressing low levels of α-SMA and IL-6, but expressing MHC-II and CD74, and (iv) complement-secreting CAFs (csCAFs) which express high levels of α-SMA and complement associated factors (*e.g.*, C3, C7, CFB, CFD, CFH, CFI), having only been recently identified.(Chen et al., 2021) Interestingly, the direct interplay of csCAFs with pancreatic cancer cells has only been observed in early tumor development, leading to important insights regarding their inclusion in 3D tumor models that aim to mimic different disease stages.(Chen et al., 2021; Sun et al., 2018)

Such heterogeneity not only highlights CAFs multifactorial influence in pancreatic cancer TME but also accounts for their possible role as important mediators of tumor immune response.(Schnittert et al., 2019) CAF-mediated desmoplasia is in turn correlated with increased pancreatic tissue stiffness that consequently induces blood vessel collapse.(Chronopoulos et al., 2017; Sugimoto et al., 2014) Currently available cell processing/engineering technologies such as cell sheets, hydrogel stacking, 3D bioprinting and/or organoid engineering could foreseeable help further develop models capable of integrating such population specific interactions in a mimetic platform.(Reid et al., 2019)

Metabolic profiling may also provide important insights in 3D pancreatic cancer models bioengineering since aberrant metabolism is a well-established hallmark of this neoplasia.(Knudsen et al., 2016) CAFs play an important role on PDAC metabolism shift as they undergo a metabolic transition from oxidative phosphorylation to glycolysis (*i.e.*, Warburg effect), over producing lactate, ketone bodies, glutamine, and fatty acids, which are then secreted and exploited by surrounding cancer cells to sustain their proliferation.(Broekgaarden et al., 2019; Sazeides and Le, 2018) Moreover, it has been evident that CAFs-derived alanine used in the TCA cycle also promote tumor growth in low-nutrient environments. Such, allows glucose to be used in nucleic acids synthesis, further accelerating cancer cells proliferation.(Sazeides and Le, 2018) As metabolic reprogramming plays a key role in carcinogenesis and therapy responsiveness, the recapitulation and study of such metabolic profiles *in vitro* can be useful to develop diagnostic techniques and to facilitate the identification of novel therapeutic targets.

Other living stromal elements such as immune system cells, particularly TAMs, are also key effectors in pancreatic cancer stroma, exhibiting a major influence in tumor progression and therapy resistance. These microenvironment reactive cells are generally recruited to the vicinity of cancer cells with increasing evidences demonstrating that their bioactivity and phenotype is closely related with M2-like polarized macrophages.(Lankadasari et al., 2019) Although full consensus regarding the secretome of such cells is still yet to be obtained, it is known that polarized TAMS contribute to tumor progression and drug resistance through the secretion of major tumor supporting growth factors (*e.g.*, EGF, MMPs, VEGF, PDGF, FGF, among others) and chemokines that stimulate tumor growth and trigger metastatic events.(Daniel et al., 2019) During tumorigenesis, cancer cells recruit monocytes and macrophages trough the secretion of specific factors (*e.g.*, CCL5, CXCL8, CXCL12, etc.) that ultimately bioinstruct monocytes polarization toward an M2-like phenotype. Interestingly, apart from cancer cell-derived signals, CAFs also secrete important factors (*i.e.*, TGF-β and IL-10) that promote macrophages polarization. Translating such environment to 3D *in vitro* platforms is critical, especially if the screening of immunomodulating therapeutics is envisioned.

From a bioengineering perspective, TAMS installation in *in vitro* models can be materialized through a number of different methodologies. In an simple, yet elegant approach, researchers have been co-culturing cancer cells with monocytes (*e.g.*, derived from cell lines or patient-derived cells), under specific conditions that promote the establishment of M2-like polarized macrophages following exposure to cancer cells secreted bioinstructive biomolecules.(Rebelo et al., 2018) However, such approach can be challenging from a logistic perspective since monocytes are generally cultured in suspension which can increase the complexity of their co-culture with cancer cells during regular culture media changes. On a different approach, monocytes can be firstly differentiated into M0 macrophages (adherent cells) via a stimulating culture media (i.e., generally supplemented with phorbol 12-myristate-13-acetate (PMA)), and co-cultured with cancer cells, enabling researchers to evaluate the potential of these engineered models to recapitulate the immunosuppressive pancreatic TME.(Kuen et al., 2017) Ultimately, monocytes may also be differentiated and polarized towards "M2"-like TAMs *in vitro* by stimulating monocyte-differentiated macrophages with IL-4/IL-13.(Yang et al., 2021) Successfully established immuno-active models via this methodology may be particularly useful for screening therapeutics that inhibit monocytes differentiation (*e.g.*, Pexidartinib, PF-04136309, among others), opening new opportunities to tackle pancreatic cancer.(Lankadasari et al., 2019; Mantovani et al., 2017; Xiang et al., 2021) However, prior to being used for advancing therapeutics screening researchers must evaluate the phenotype of differentiated macrophages through specific methodologies that enable the clear detection of TAMS-associated biomarkers (*e.g.*, IL-4, IL-10, IL-6, Arginase 1, CCL2, CD163, etc.) **(Fig.1)**.(Kuen et al., 2017)

3.2. Tumor and Stromal ECM in 3D Pancreatic Cancer Models – Bioengineering and Characterization

Throughout life, healthy tissue ECM provides cells with specific biological cues recognized to activate downstream signaling events exhibiting a key role in cellular signaling and cell fate. In contrast to normal pancreatic tissues, tumor ECM is highly fibrotic, operating both as a cell bioinstructive component that drives tumor progression, resistance and metastasis, as well as constituting a major physical barrier to therapeutics delivery.(Tomás-Bort et al., 2020)

During disease progression pancreatic ECM experiences several alterations in its nano/micro-topography, stiffness, viscoelasticity and biochemical composition.(Feig et al., 2012; Nia et al., 2020; Winkler et al., 2020) Collagen is the most abundant ECM component in cancer, with fibrillar collagens (*i.e.*, COL1A1, COL1A2, and COL3A1) representing the major elements of pancreatic cancer ECM. Interestingly, an approximate 2.6-fold increase in these components has been reported to occur during progression from healthy to malignant pancreatic tissues.(Liot et al., 2021; Nabavizadeh et al., 2020; Tian et al., 2019) Such uprise in fibrillar collagen is generally mediated by enzyme-mediated collagen crosslinking, by the action of lysyl oxidase (LOX) and transglutaminase 2.(Rice et al., 2017) Overall, pancreatic cancer-associated desmoplasia results in a stiffer microenvironment exhibiting dense collagen fiber assemblies, as well as increased laminin and fibronectin deposition.(Akhter et al., 2020) Additional ECM components that are significantly overrepresented in pancreatic cancer include Fibrillin-1 (FBN-1), fibrinogens (FGA, FGB, and FGG), and periostin, most of them being commonly associated with increased invasive capacity and disruption of surrounding tissue basement membrane.(Tian et al., 2019)

Hyaluronan (HA) is also a critical ECM component found *in vivo*, playing a key role in increasing malignant tissues stiffness due to its abundant accumulation in tumor surrounding stroma along time.(Sato et al., 2016) Considering that stromal cells are the major effectors in *de novo* matrix deposition, it is important to emphasize that HA is more prevalent in pancreatic stroma ECM rather than in the main tumor, an important aspect that is yet to be widely emulated in predictive 3D preclinical PDAC models.(Bulle and Lim, 2020; Jiang et al., 2020) The widely reported aberrant HA buildup and dynamic degradation in pancreatic TME is closely associated with its poor prognosis, as demonstrated by mounting clinical evidence.(Kim et al., 2020; Sato et al., 2016) Hyaluronan with different biopolymer backbone sizes have also been found to distinctly influence tumor development, with significant deposition/degradation of high molecular weight HA (> 500 kDa) promoting an anti-inflammatory and anti-angiogenic response, while lower molecular weight HA(20–200 kDa), having been recognized to bioinstruct angiogenic and pro-inflammatory pathways.(Chang and Lin, 2021; Sato et al., 2016)

To recapitulate such biomolecular features, researchers have been focusing on engineering ECM-mimetic hydrogel biomaterials, especially proteinaceous biomaterials (*e.g.*, gelatin, collagen, human-based platelet lysates) and/or tumor tissue decellularized extracellular matrix (dECM)/basement membrane extracts which exhibit cell adhesive and bioinstructive cues in an endeavor to stimulate cancer and stromal cells bioactivity similarly to that posed by native tumor-stroma ECM elements.(Blanco-Fernandez et al., 2021; Ferreira et al., 2020; C. F. Monteiro et al., 2020; Pinto et al., 2017) Up-to-date these biomaterials have been mainly exploited for 3D disease models in the form of fibers, sponges, microcarriers and/or bulk hydrogels.(Ajeti et al., 2017; Antunes et al., 2019; Blanco-Fernandez et al., 2021; Brancato et al., 2017; Ricci et al., 2014) Owing to their high-water content, biophysical properties and similarity to tissues ECM, hydrogel scaffolds have been

the most widely explored scaffolds to engineer organotypic 3D models.(Liaw et al., 2018; Lin and Korc, 2018) Various reports focusing on recapitulating pancreatic cancer-stroma ECM have taken advantage of a wide range of biologically tunable hydrogels and biocompatible crosslinking approaches (*e.g.*, photo-induced, enzyme, supramolecular, bioorthogonal click-chemistry, etc.) which can be leveraged to better control ECM mimetic cell laden platforms, bioactivity, porosity, topography and dynamic mechanical properties (*i.e.*, viscoelasticity, stiffness) (Lin and Korc, 2018; H. Y. Liu et al., 2017; Liu et al., 2018).

Tumor-stroma ECM biophysical properties are known to be of high interest for *in vitro* tumor modelling owing to constant de novo matrix deposition/remodeling through time. To bioengineer ECM biomimetic tumor-stroma platforms that emulate matrix mechanics either in a *'one-fits-all'* approach or in a more patient tumor-matched mode, researchers require highly sensitive characterization techniques and methodologies capable of providing tumor-stroma ECM mechanical characterization. Recent advances in mesoscale indentation force–displacement, harmonic motion/shear wave elastography (HME/SWE), atomic force microscopy (AFM) and magnetic resonance elastography have opened new opportunities for identifying native pancreatic cancer ECM stiffness.(Nguyen et al., 2016a; Zanotelli et al., 2020) Following a comprehensive analysis of literature reports employing these tools one can observe that pancreatic tumors mechanical features is highly heterogeneous, ranging from ~1 kPa to above 44 kPa **(Table 1)**.(Nabavizadeh et al., 2020) Such heterogeneity may be correlated with two main factors, (i) the lack of correlation and standardization regarding ECM analysis tools/methodologies, and (ii) the intra-tumoral heterogeneity generally observed in tumor tissue samples.(Guimarães et al., 2020) All in all, this ultimately impacts 3D tumor-stroma models engineering with researchers being uncertain which mechanical properties should be emulated.

Table 1. Pancreatic tissues mechanical stiffness characterization.

Sample Type	Characterization Technique	Population size (N)	Young's modulus (kPa)	Disease Class	Ref.
Murine Pancreatic Tumor	Harmonic motion elastography	30	11.3 ± 1.7	Stage I-II	(Nabavizadeh et al., 2020)
	Atomic force microscopy	52	4 ± 1.6	N.D.	(Rice et al., 2017)
	Harmonic motion elastography	N.D.	2.1 - 6.7	N.D.	(Nabavizadeh et al., 2018)
Healthy Human Pancreatic Tissue	Magnetic resonance elastography	22	1.13 - 2	N.A.	(Kolipaka et al., 2017)
	Mesoscale indentation	22	1.06 ± 0.25	N.A.	(Rubiano et

					al., 2018)
	Shear wave elastography	84	4.39 - 7.84	N.A.	(Yoshikawa et al., 2021)
Pancreatic Cancer Cell Lines	Atomic force microscopy	25-35	MIA PaCa-2: 1.7 ± 1.0 PANC-1: 2.4 ± 1.1 HPDE: 3.7 ± 1.2 Hs766T: 3.0 ± 2.0	N.D.	(Nguyen et al., 2016 b)
Human Pancreatic Tumors	Magnetic resonance elastography	26	3.22 - 5.11	N.D.	(Shi et al., 2018)
	Magnetic resonance elastography	8	6.06 ± 0.49	N.D.	(Itoh et al., 2016)
	Harmonic motion elastography	32	15 - 44.8	Stage II-III	(Nabavizadeh et al., 2020)
	Mesoscale indentation	-	6 - 18	N.D.	(Rubiano et al., 2018)
	Mesoscale indentation	59	1.4 - 5.1	Stage II-IV	(Sugimoto et al., 2014)
	Shear wave elastography	22	3.48 – 11.55	Stage II-IV	(Yoshikawa et al., 2021)

***N.D. – non defined; N.A. – not applicable**

From a critical perspective, not only further improvement and standardization of mechanical characterization methods is highly required but also our understanding of the dynamic mechanical alterations that occur from early stages to later stages of tumor progression must improve to accelerate the design of increasingly organotypic *in vitro* models. On this focus, the recent exploitation of advanced liquid chromatography–tandem mass spectrometry (LC-MS/MS) characterization techniques has provided extensive portrays of pancreatic cancer ECM evolution and patient heterogeneity.(Tian et al., 2019; Weniger et al., 2018) Such big data technologies revealed an up-regulated group of matrisome proteins present in both PDAC tumor and stroma compartments and allowed researchers to associate their deposition in a cell specific manner, highlighting the importance of the stromal compartment in PDAC desmoplasia.(Tian et al., 2019) Furthermore, by defining the ECM cellular origins in LC-MS/MS these studies revealed that although the pancreatic stroma is responsible for 90 % of *de novo* ECM deposition, elevated

levels of ECM proteins derived exclusively from cancer cells can be directly correlated with poor patient survival. Integrating these methodologies in 3D models early design stages, as well as during their *in vitro* maturation may provide important information regarding different models' correlation with the *in vivo* setting.

Holistically, the rational design of 3D pancreatic tumor-stroma models both at a cellular and matrix level benefits from exploring advanced tools to characterize human tumor tissues. Further down the screening pipeline, the same tools and methodologies can be leveraged to follow up and characterize preclinical tumor models biomarkers and bioactivity serving as a quasi-validation of 3D models' ability to recapitulate such major disease hallmarks.

4. Advances in *In vitro* Models for Capturing Pancreatic Tumor – Stroma Interplay

Gathering on the importance of living stromal elements and supporting ECM interplay with pancreatic cancer cells, researchers have been rapidly moving forward toward developing more organotypic *in vitro* platforms that account for these dynamic interactions. On this focus, long-term existing and rapidly emerging cell/matrix 3D culture technologies (*e.g.*, cell-rich and ECM mimetic biomaterial-based platforms), alongside with big data characterization tools are being actively explored as the bioengineering cornerstones for materializing human disease surrogates **(Fig. 2)**. Such unique synergy between fundamental tumor knowledge and engineering as already let to major advances on establishing 3D *in vitro* tumor-stroma pancreatic cancer models for preclinical validation of candidate anti-cancer therapies as it will be showcased in the following sections.

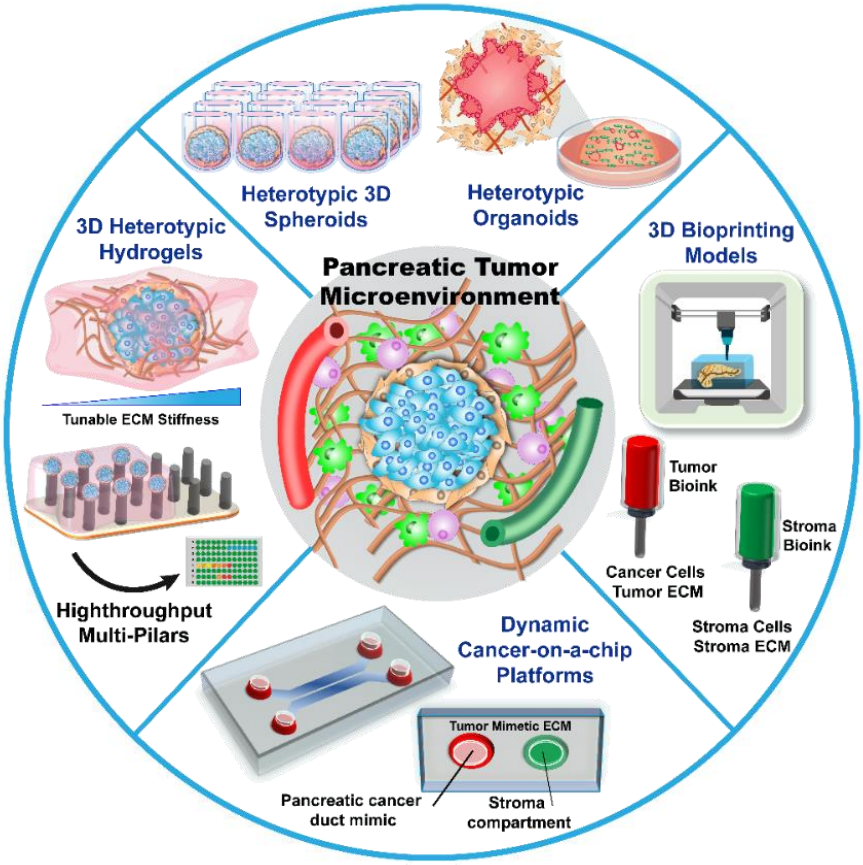

Fig. 2. Overview of advanced technologies for bioengineering pancreatic tumor-stroma physiomimetic *in vitro* models.

4.1. Cell-rich Tumor-Stroma 3D Models

Spheroid models comprising randomly agglomerated cells have been among the most widely explored platforms for *in vitro* tumor modelling. Spheroids highly modular nature enables the introduction of multiple cell types, this combined with their easy to establish unsupervised self-assembly renders them highly attractive for high-throughput screening assays.(Ferreira et al., 2018; M. V. Monteiro et al., 2020b) Most importantly, 3D spheroids allow researchers to reproduce key features found in *in vivo* solid tumors, including the 3D architecture, the establishment of close cell-cell interactions, pH/nutrient/oxygen gradients, gene and protein expression profiles, as well as activation of drug resistance mechanisms.(Costa et al., 2016) Currently, various technologies are available for 3D spheroids generation, including: (i) ultra-low attachment (ULA) surfaces, (iii) hanging drop technique, (iii) stirring bioreactors, and (iv) magnetic levitation, among others.(Ferreira et al., 2018; Tomás-Bort et al., 2020)

Aiming to recapitulate the stromal components of pancreatic cancer, a 3D *in vitro* cell-rich model was recently established through direct co-culture of different pancreatic cancer cell lines (PANC-1, AsPc-1, BxPC-3, Capan-1 and MIA PaCa-2) and PaSCs. Heterotypic 3D Spheroids incorporating PaSCs were more compact than their monotypic counterparts and exhibited a prominent desmoplastic reaction with increased collagen deposition, indicating the importance of including these stromal elements.(Ware et al., 2016)

Seeking to further investigate the role of fibrotic elements within pancreatic cancer TME, researchers recently devised an elegant heterotypic cell-rich living platform that enables a precise tunning of the ratio of fibrotic elements *in vitro*. The established 3D cell-culture technique was based on the use of a culture platform (*i.e.,* transwell type inserts) to enable high cell density 3D tissues generation devoid of cell supporting biomaterials facilitating the visualizations and analysis of 3D microtissues and respective ECM composition. To materialize the tumor models, normal human dermal fibroblasts (NHDF) and PSCs were embedded in fibronectin:gelatin and combined with PDAC cells with user-programmed cellular ratios. The cell suspensions were then cultured in transwell cell culture inserts coated with fibronectin enabling the formation of a stroma-rich compartment. PDAC cell-lines were then combined either with NHDF or human PDAC-derived PSCs at various seeding ratios to cover the clinically observed range of stroma proportion in PDAC tissues. Although such methodology was functional for several PDAC cells:NHDF/PSCs combinations, the assembly of living 3D microtissue models is highly dependent on tumor cell-associated expression of E-cadherin. Moreover, researchers were able to analyze the molecular mechanisms that lead to the acquisition of a myofibroblastic phenotype by normal fibroblasts when co-cultured with PDAC cells, also having demonstrated that the acquisition of such phenotype is dependent of the concerted activities of SMAD2/3 and YAP in fibroblasts **(Fig. 3A)**.(Tanaka et al., 2020)

Similarly, a tumor-stroma 3D PDAC spheroid model comprising heterotypic triple co-culture of pancreatic cancer cells (PANC-1), fibroblasts and vascular endothelial cells was bioengineering in non-adherent plates **(Fig. 3B)**.(Lazzari et al., 2018) The triple co-culture aimed to replicate the *in vivo* microenvironment, being observed that cancer cells exhibited reduced sensitivity to chemotherapeutics when compared to their monotypic 3D spheroid counterparts, thus more closely mimicking tumor resistance generally observed *in vivo* **(Fig. 3C)**. These evidences further supp–ort the relevance of recapitulating key cancer-stromal elements in preclinical models' bioengineering and validation stages.

Despite such 3D models recreate PDAC tumor-stroma interplay, they still fail to emulate the native compartmentalized tumor architecture which is widely recognized to significantly impact cancer cells response to therapeutics.(Koikawa et al., 2018; Kota et al., 2017; Pothula et al., 2020) Aiming at advancing cell-rich models in this direction, 3D organotypic spheroids comprising pancreatic cancer cells and CAFs at specific ratios were recently generated in an endeavor to simulate the native PDAC-stroma stratified bioarchitecture and desmoplastic features.(Monteiro et al., 2021a) Such models – so termed STAMS- were assembled in ultra-low adhesion (ULA) plates following an easy to implement two-step strategy. Firstly, pancreatic cancer cells we self-assembled into 3D spheroids and matured for 6 days to establish a template tumor core. Subsequently, CAFs were administered to pre-formed 3D spheroids and allowed to autonomously self-organize, ultimately establishing a cell-rich semi-enclosed layer around the original tumor core **(Fig. 3D)**. This *in vitro* model was shown to better recapitulate the native pancreatic tumor bioarchitecture in which cancer cells are enveloped by the highly fibrotic stroma. Interestingly, the *in vitro* assembled STAMS exhibited key PDAC biosignatures found in human tumors including abundant collagen deposition, secretion of key molecular markers (*e.g.*, TGF-β, FGF-2, IL-1β and MMP-9), as well as resistance to standard-of-care and precision therapeutics **(Fig. 3E)**.(Monteiro et al., 2021a) Such spatially organized tumor-stromal models may represent a valuable strategy with increased potential for drug discovery and preclinical screening of breakthrough therapies targeted to the tumor-stroma axis.

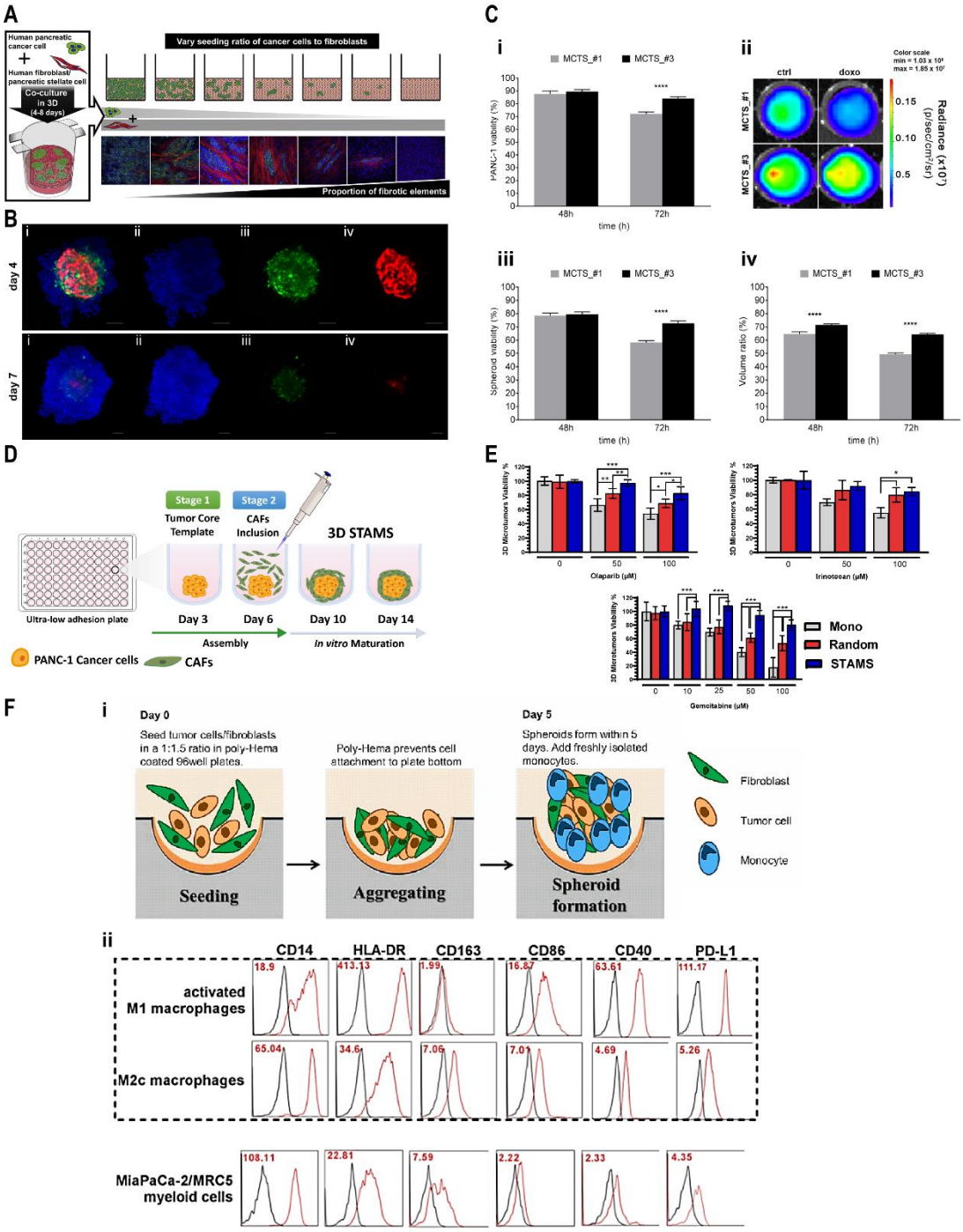

Fig.3. Pancreatic cancer tumor-stroma, cell-rich 3D *in vitro* models. (A) Schematic of 3D PDAC fibrotic tissue assembling by pancreatic cancer and PaSCs co-culture at different ratios. Adapted from (Tanaka et al., 2020) with permission of Elsevier. (B) Fluorescence micrographs of 3D MCTS comprising PANC-1: MRC-5: HUVECs, at day 4 and day 7. Blue: nuclei, green: GFP-expressing MRC-5 fibroblasts and red: RFP-expressing HUVECs. (C) Heterotypic tumor-stroma triple co-culture model. (i) 3D Pancreatic cancer cells response to doxorubicin treatment (0.5 μM). (ii) Representative bioluminescence images of control and treated monotypic (MCTS_#1) and heterotypic (MCTS_#3) spheroids. (iii) Spheroid viability after doxorubicin exposure (0.5 μM) for 48 h and 72 h. (d) Inhibition of spheroid growth following doxorubicin treatment. Adapted from (Lazzari et al., 2018) under the Creative Commons CC-BY-NC-ND license. (D) Schematics of 3D stratified PDAC models assembly by using ultra-low adhesion plates. (E) Cell viability analysis of:

PANC-1 monoculture 3D spheroids (Mono); PANC-1:CAF spheroids, were cells are randomly distributed (Random); and Stratified PANC-1:CAF spheroids (STAMS), following treatment with Olaparib, Irinotecan or Gemcitabine, at day 14 of culture. Adapted from (Monteiro et al., 2021a) with permission from Wiley-VCH. (F) (i) Schematics of 3D tri-culture PDAC model generation, and (ii) Polarization of monocytes-derived macrophages into M2 phenotype after co-culture with cancer cells:fibroblasts spheroids. 3D co-culture macrophages cell surface markers were compared to *in vitro* M2 differentiated macrophages by flow cytometry. 3D co-culture macrophages exhibited increased expression of CD14 and CD163, typical markers of M2 macrophages. Adapted from (Kuen et al., 2017) with permission from PLOs One under the terms of the Creative Commons Attribution License.

Adding to stromal fibroblasts, pancreatic cancer TME is also affected by immune cells infiltration and clinical evidences indicate that the presence of pro-tumoral immune cells such as regulatory T-cells, TAMs with M2-like polarization and myeloid-derived suppressive cells (MDSCs) in primary tumors might be correlated with tumor progression.(Karamitopoulou, 2019; Wörmann et al., 2014) Among these, TAMs have been extensive associated with poor prognosis in more than 80 % of all pancreatic cancers owing to immune-suppressive cytokines secretion (*e.g.,* IL-1, -6, -10 and TGF-β).(Pathria et al., 2019)

Recently, an immunocompetent 3D heterotypic triple model comprising pancreatic cancer cells, lung-derived fibroblasts and monocytes was established to emulate these tumor-stroma interactions (**Fig.2F,i**).(Kuen et al., 2017) In this TME surrogate setup the dynamic interplay between cancer cells and fibroblasts led to the release of immunosuppressive mediators and consequently to the differentiation of 3D cultured monocytes in TAMS exhibiting an M2-like phenotype, a major aspect considering that this event also occurs *in vivo* (**Fig.2F,ii**). Following the administration of T-cells to the tri-culture immunocompetent spheroid, macrophages inhibited CD4+ and CD8+ T-cell proliferation and activation. Such findings are particularly relevant and advantageous to model *in vitro* if the screening of candidate immunotherapeutics is envisioned. In fact, considering that pancreatic cancer is one of the most challenging neoplasia's to tackle via immunotherapy due to its renowned immunosuppressive environment, developing new testing platforms that recapitulate this major hallmark and multi-cellular population dynamics may pave the way to accelerate the discovery of bio-relevant immunotherapies.

Nevertheless, from a critical perspective, the groundbreaking advances in pre-clinical drug screening provided by cell-rich 3D spheroid platforms are not without some limitations, being one of the most important the lack of a pre-existing ECM in early culture time points.(Pradhan et al., 2017) Installing, ECM-associated biomolecular cues will activate mechanotransduction pathways, trigger different cellular phenotypes and introduce additional mass transport limitations, all of which are critical aspects that cannot be accurately replicated in standard 3D spheroids.(Pradhan et al., 2017) As such, the development of more physiomimetic PDAC models that accurately mimic pancreatic cancer *in vivo* TME cellular and ECM stromal components are being actively pursued.

4.2. Biomaterial-based Pancreatic Cancer-Stroma Models

Engineered biomaterial-based models comprising naturally-derived and/or synthetic biomaterials aiming to function as ECM-mimetic cell-supporting scaffolds have proven to be a valuable tool for recapitulating this key stromal component found in living tissues.(Wang et al., 2014) Gathering on this, natural-derived biomaterials arise as a particularly attractive alternative to recapitulate this component *in vitro* owing to their ECM-

like features (*i.e.,* viscoelasticity, high water content, tunable mechanical properties, display of bioinstructive/cell adhesive motifs). Up-to-date, a wide range of ECM mimicking biomaterials have been employed for modelling tumor ECM components *in vitro* including: (i) gelatin, (ii) collagen, (iii) hyaluronic acid and (iv) dECM.(Lin and Korc, 2018) Aiming to recapitulate *in vivo* tissues, collagen matrices have been exploited to model the migration behavior and invasion profile of pancreatic cancer cells. These hydrogels were installed in a custom-built high-throughput, high-content drug screening platform for providing the establishment of co-cultured 3D spheroid models comprising PDAC cells and CAFs surrounded by oligomeric type I collagen, (**Fig. 4A**).(Puls et al., 2018) Such high-throughput platform can accelerate the screening and preclinical validation of novel drugs in an effective manner when introduced in automated bioimaging systems, overcoming the laborious aspects and limitations of standard platforms.

On a similar focus, Matrigel has also been considered as a gold-standard for 3D hydrogel-based tumor modelling. Matrigel is a complex protein mixture derived from the basement membrane of Engelbreth-Holm-Swarm (EHS) mouse sarcoma a highly complex biomolecule mixture rich in collagen IV, laminin, heparin sulfate proteoglycans, as well as a variety of growth factors (*e.g.*, TGF-β, FGF, epidermal growth factor, PDGF) that constitute the original tumor TME from which this processed basement membrane is derived from.(Hughes et al., 2010; Lin and Korc, 2018) Due to its biological origin, Matrigel-based scaffolds have been widely exploited for engineering 3D *in vitro* PDAC models for investigating cells invasion potential and anti-cancer drugs efficacy, among other applications.(Lin and Korc, 2018) Despite being successful in supporting human cancer cells culture, Matrigel is an animal-derived biomaterial, is highly variable from batch-to-batch and its mechanical properties are challenging to be tailored to those of pancreatic cancer tissues. These undesirable features render Matrigel a sub-optimal option for accurately recapitulating pancreatic TME biophysical and biochemical properties.(Benton et al., 2014)

Hydrogel-based scaffolds generated from well-defined synthetic materials (*e.g.*, PEG, PLA, PCL, etc.) have also been exploited to model the pancreatic tumor-stroma interplay *in vitro*. Due to their poor bioactivity and low correlation with ECM components, biomimetic peptides (*e.g.*, MMP cleavable peptides, RGD peptides, etc.) alongside with chemical crosslinking moieties (*e.g.*, acrylate, tyrosine, etc.) are commonly conjugated with synthetic polymers to imprint organotypic features to these scaffolds.(H. Y. Liu et al., 2017) However, such approaches fail to fully recapitulate the intrinsic bioactivity of proteinaceous biomaterials.

Hyaluronic acid (HA) is yet another important stromal component that must be introduced during the engineering of pancreatic tumor-stroma models since it is widely recognized that this glycosaminoglycan (GAG) is over-expressed and accumulated in PDAC stroma. Importantly, the presence HA has been closely related with a poor patient outcome, owing to its contribution for cancer cells proliferation, activation of invasion mechanisms and multi-drug resistance.(Olive, 2015) Although unmodified HA does not support integrin-mediated cell adhesion, it can interact molecularly and activate CD44 and RHAMM (CD168) receptors in cells present in the pancreatic TME.(Sapudom et al., 2020) In addition to its biological relevance, from a chemical engineering point of view HA backbone is highly versatile being amenable for chemical modification through the conjugation with numerous moieties (*i.e*, norbornene, thiol, amine, boronate, etc).(Liu et al., 2018) Most commonly, HA has been chemically modified with methacrylate and thiol moieties to produce photocrosslinkable hydrogels with tunable mechanical properties and biodegradation.(Liu et al., 2018; Shih et al., 2016) This versatility aids on its processing into ECM mimetic

hydrogels and renders HA one of the most researched biopolymers for disease modelling. Particularly, due to its relevance in PDAC stroma, several studies have employed HA-based hydrogel scaffolds to assemble 3D *in vitro* pancreatic tumor models.(Liu et al., 2018; Wong et al., 2019)

Recently hyaluronan grafted chitosan (CS-HA) platforms were engineered as a strategy to recapitulate tumor pathophysiology, probe cancer cell-CAFs interactions and to screen for anti-cancer therapeutics effectiveness. 3D heterotypic spheroids were assembled on these systems by co-culturing pancreatic cancer cells and PSCs (1:9 cell-to-cell ratio).(Wong et al., 2019) In essence, such HA-rich scaffolds were employed to mimic the PDAC TME where the abundance of HA is an indicator for the lower prognosis.(Apte et al., 2013) The established co-culture spheroids exhibited a 3D core-shell type structure and up-regulated expression of stemness and migration markers, displaying potent *in vitro* tumorigenicity (**Fig. 4B,C**). The developed PDAC model enabled to recapitulate the HA-enriched TME, and exhibited *in vivo*-like chemoresistance, with cells also displaying a more physiomimetic invasive and metastatic phenotype. Despite providing an interesting advancement, the mechanics of cell-supporting ECM remained unaddressed, a particularly relevant feature if one envisions to mimic the natural ECM dynamics occurring *in vivo*.

Aiming to replicate ECM mechanical properties and the increased stiffness of pancreatic cancer TME, a mechanically tunable 3D *in vitro* system was designed by modulating collagen I hydrogel stiffness to achieve PDAC-tissue specific mechanics. In this approach, the viscoelastic properties of human malignant tumor, pancreatitis and healthy tissue were evaluated and compared with the developed 3D *in vitro* model.(Rubiano et al., 2018) As previous stated, PDAC lethality is largely correlated associated with its protective desmoplastic barrier, promoting their survival and delaying the chemotherapeutics agents delivery.(McCarroll et al., 2014) In addition to malignant tissue, pancreatitis is also accompanied by increased stiffness, therefore, mechanical characterization of healthy, pancreatitis and PDAC tissues could be an important insight to avoid misdiagnosis. In this particular study, both pancreatitis (2.15 ± 0.41 kPa) and tumors (5.46 ± 3.18 kPa) exhibit higher stiffness (in shear modulus) than normal tissue (1.06 ± 0.25 kPa).(Rubiano et al., 2018) To mimic the PDAC remodeling behavior, stromal cells were isolated from human PDAC tumors, laden in collagen hydrogels and cultured in cancer cell-conditioned medium, as a strategy to prime their response to paracrine signaling and modify their microenvironment. This has demonstrated the importance of tunning the mechanical properties of the cell supporting matrix employed for in vitro maturation of the models, however, a physiomimetic and dynamic stiffening of the matrix in a user programmed mode was still challenging to implement in this set-up. The construction of an *in vitro* system that recapitulates the *in vivo* stiffening of PDAC is an invaluable asset to probe the biomechanics that underly tumor growth and metastasis, ultimately assisting in the discovery of innovative therapeutics.(Rubiano et al., 2018)

On this focus, an elegant biomaterial-based model was developed for mimicking the unique ECM stiffening dynamics and fibrotic PDAC microenvironment that are at play during disease progression.(Liu et al., 2018) To recapitulate the stiffening events a double-network dynamic gelatin-hyaluronic acid hybrid hydrogel with modular thiol-norbornene photopolymerization (*i.e.*, U.V. light) and on-demand enzyme-triggered matrix stiffening was developed. Following thiol-norbornene gelation, the tyrosine residues present in gelatin macromers, were used as substrates to establish a secondary polymerization by exogenously added tyrosinase, which catalyzes di-tyrosine crosslinking and increases hydrogel crosslinking density and stiffness. This enables researchers to recapitulate the mechanical

changes suffered by TME during the desmoplastic reaction and to evaluate the influence of matrix composition and dynamic stiffening on PDAC cells phenotype and bioactivity **(Fig.4D)**. In this hydrogel platform encapsulated cancer cells exhibited human tumor-like phenotypes and increased invasiveness in stiffened gelatin-HA containing hydrogel (**Fig.4E**). Despite unlocking dynamic PDAC matrix stiffening, relevant stromal cell populations were yet to be included in these systems. One could hypothesize that the inclusion of stromal CAFs in conjugation with on-demand stiffening could provide a fine tuning of *in vitro* microenvironment mechanical properties. These approaches are envisioned to increase the similarity of these models with the native tumor tissue biophysical features according to tumor stage, an aspect that remains largely underexplored in disease modelling.

Besides the fibrotic and dense stroma, tumor-stroma hydrogel-based models have been developed to resemble the unique PDAC bioarchitecture. In such work, PDAC microtissue (μtissue) models were bioengineered in order to recreate PDAC bioarchitecture as found *in vivo*, where tumor niche is surrounded by a fibrotic stroma mainly composed by CAFs and ECM components in a juxtatumoral position (**Fig.4F**). The developed tumor-stroma PDAC models were assembled by seeding a pre-maturated PANC-1 spheroid in a collagen-based matrix populated by human PSCs, recapitulating the human PDAC stroma. By immunostaining authors confirmed the successfully envelopment of PANC-1 spheroid by the PSCs-laden collagen hydrogel as $CK19^{+}$ cells are only present in the core of the μtissue model, surrounded by the stroma-biomimetic compartment, recapitulating the native scenario (**Fig.4G**). Additionally, heterotypic μtissues exhibited significantly higher expression of key tumor markers including POSTN, FN1, COL1, IL-6 and VIM highlighting the biomimetic potential of the developed platform for understanding tumor-stroma interactions and high-throughput assays.

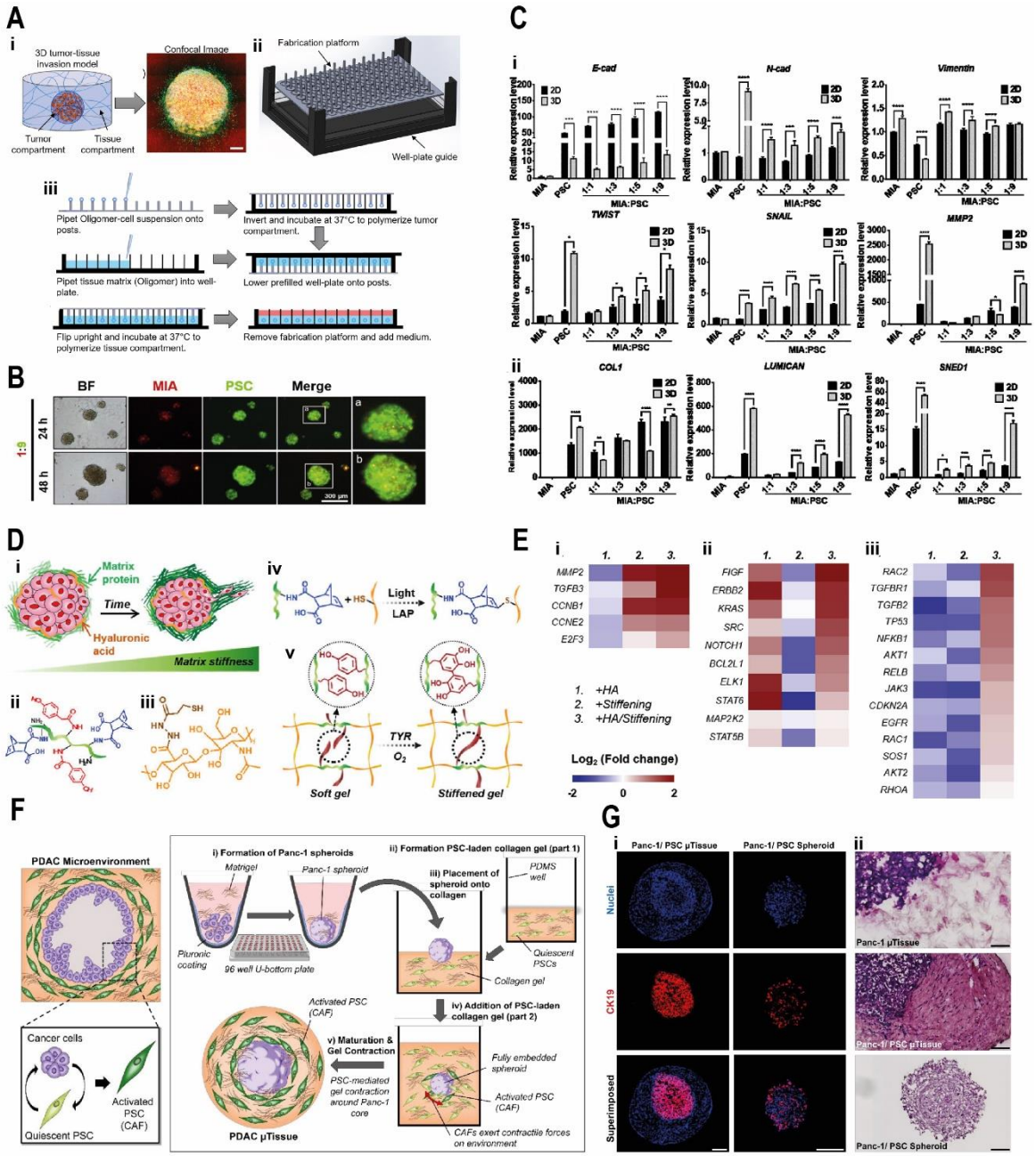

Fig. 4. Engineered biomaterial-based pancreatic cancer *in vitro* models. (A) 3D co-culture tumor like tissue-invasion model. (i) Schematic of 3D tumor-stroma invasion set-up, (ii) CAD construct of custom-designed platform for generating the tumor compartment, (iii) Step-wise methodology for establishing pancreatic tumor-stroma *in vitro* models. Adapted from (Puls et al., 2018) with permission of Springer Nature. (B) The morphology and viability of 3D spheroids comprising pancreatic cancer cells (MIA cells) and PaSCs seeded in a ratio 1:9 in Chitosan-Hyaluronan (CS-HA) platforms, for 24 or 48 h. (C) Gene expression profiling of 2D and 3D models cultured in CS-HA platforms. Adapted from (Wong et al., 2019) with permission from Elsevier. (D) Overview of dual crosslinked ECM-mimetic platforms. (i) Schematic of a fibrotic tumor microenvironment comprising matrix proteins and glycosaminoglycans, (ii) Norbornene (blue moiety) and hydroxyphenylacetic acid (HPA) (red moiety) functionalized gelatin, (iii) Thiol functionalized hyaluronic acid backbone. (iv and v) Schematics of thiol-norbornene U.V. light-mediated photocrosslinking

and on-demand tyrosinase-triggered di-HPA crosslinking. (E) Up-regulated genes in PDAC cells laden hydrogel. (ii) Gelatin-Norbornene-HPA hydrogel devoid of HA, (ii) Gelatin-Norbornene-HPA/HA hydrogel, and (iii) On-demand stiffened Gelatin-Norbornene-HPA/HA hydrogel. Adapted from (Liu et al., 2018) with permission from Elsevier. (F) Schematics of 3D PDAC μtissues generation and the inherent cellular organization. (G) Evaluation of PDAC μtissues cellular arrangement by (i) immunofluorescence, and (ii) hematoxylin-eosin staining. Adapted from [125] under the Creative Commons Attribution (CC BY) license.

4.2.1. Pancreatic Cancer-Stroma Organoid-in-Biomaterial Models

3D tumor organoids, are rapidly emerging as valuable preclinical screening platforms owing to their unique ability to reproduce key cellular features found in solid tumors *in vivo*.(Granat et al., 2019) Unlike their spheroid counterparts, tumor organoids self-organize into 3D architectures in a fully autonomous, cell-driven mode without requiring forced adhesion to generate 3D living architectures. Most importantly, tumor organoids often display tumor-specific cellular heterogeneity, gene and protein expression patterns, histomorphological features and a high degree of *in vitro/in vivo* correlation in preclinical drug screening set-ups.(Drost and Clevers, 2018) Particularly, patient-derived organoids (PDOs) provide an unprecedented level of predictiveness and constitute a truly corelative *in vitro* platform that can assist clinical decision making. PDOs are thus recognized as the next-generation of microtumor surrogates owing to their potential for modelling original tumors pathophysiological hallmarks (*e.g.*, driver mutations, resistance mechanisms activation, genetic drift, etc.), as well as their cellular heterogeneity and cytoarchitecture. These living microtissues can be readily established from surgically resected human tumors enabling the establishment of organoids from different disease stages and with different genetic traits, opening new avenues toward patient-personalized and precision medicine approaches.

Pancreatic tumor organoids are specifically characterized by nuclear irregularity and nucleolar prominence.(M. Lin et al., 2020) Moreover, when cultured *in vitro* pancreatic tumor organoids also retain gene/protein expression profiles and cellular 3D self-assembly features over several passages.(Fiorini et al., 2020; Nagle et al., 2018) Conventionally, tumor organoids are generated by encapsulation in ECM-mimetic biomaterials of animal-origin (*i.e.*, Matrigel or collagen I). Apart from these supporting hydrogels, the successful generation of pancreatic organoids requires the culture of their precursor cells under precisely controlled conditions and well-defined culture media supplemented with growth factors (*e.g.*, EGF, FGF), morphogens (*e.g.*, WNT modulators, Noggin), inhibitors (*e.g.*, the TGFβ inhibitor A8301), and supplements (*e.g.*, B27, Nicotinamide, N-Acetyl Cysteine).

Gathering on their remarkable potential but also recognizing their inherent limitations, researchers have been pursuing the establishment of patient-derived pancreatic organoids to shed further insights into tumor-stroma crosstalk, biology, progression and metastasis, as well as for screening candidate therapeutics targeted at these axis.(Fiorini et al., 2020) Recently, a biobank of patient derived tumor organoids, CAFs, and peripheral blood lymphocytes was established to function as the starting ground for engineering more organotypic models.(Tsai et al., 2018) Leveraging the isolated cells, heterotypic PDAC organoids co-cultured with stromal and immune cells were successfully established in ECM-mimetic hydrogels. For generating monotypic organoids, primary tumor tissues were subjected to enzymatic digestion, embedded inside Matrigel domes and matured *in vitro*. For heterotypic models, patient-matched CAFs alongside with organoid precursor cells were laden in Matrigel domes and incubated with $CD3^+$ T-lymphocytes. This highly

physiomimetic platform was able to recapitulate the biological barriers that impair T-cells migration to the juxta-tumoral stroma compartment, ultimately protecting cancer cells. The established PDAC organoids also expressed key pancreatic cancer biomarkers, such as CK7, CK19 and P53. The developed organoid-based model offers a unique platform to investigate innovative strategies aiming to improve lymphocyte infiltration into PDAC tissues. Despite providing considerable advances with CAFs installation into tumor organoids, the stromal cellular component is another major aspect that must be considered when aiming to engineering increasingly physiomimetic models. In fact, growing evidences indicate that different CAFs spatial organization in the TME originate multifarious sub-populations with specific phenotypes and roles in tumor progression/drug resistance.

Aiming to emulate such diverseness, PDAC organoids were recently combined with patient-derived CAFs isolated from different TME regions.(Grünwald et al., 2021) Initially, researchers extensively characterized two co-occurrent stroma states - reactive and deserted - with distinct spatial organization, as well as tumor promoting and chemoprotective roles through multi-omics analysis. While the reactive stroma is vascularized, exhibits immune infiltrates, promotes tumor progression and is more sensitive to chemotherapy. Conversely, the deserted TME supports tumor differentiation and is more chemoprotective. This seminal analysis generated important insights on PDAC organoids increased proliferation when exposed to reactive-type CAFs conditioned media, highlighting their tumor-supporting role. Having successfully established such living microenvironments, this approach was also leveraged to model the effect of TME stage in organoids chemoresistance. Interestingly, upon stimulating PDAC organoids with deserted-type CAF conditioned media, a higher resistance to the standard-of-care Gemcitabine was observed, in comparison to organoids cultured in reactive-type CAFs media. These important findings are suggestive that deserted-type stroma is closely associated with resistance to therapeutics. Overall, this study showed that PDAC spatially organized and heterogeneous stroma has distinct roles in promoting tumor growth and response to therapeutics. Focusing on the latter, patient-derived PDAC-derived organoids have been extensively employed for screening precision therapeutics (*e.g.*, gemcitabine, nab-paclitaxel, irinotecan, 5- fluorouracil, and oxaliplatin), and the generated data was then correlated to patients' responses. A high degree of correlation and predictive potential concerning PDAC organoids enabled an informed therapeutics selection.(Huang et al., 2015)

Owing to their organotypic features, tumor organoid-stroma models have also been exploited for screening invasion/metastasis processes *in vitro*. To investigate the molecular mechanisms of PDAC invasion process, human-derived PDAC organoid models were established in collagen gels.(Huang et al., 2020) During the invasion assay PDAC organoids exhibited two distinct patterns of invasion, one in which single cells with mesenchymal and amoeboid morphology invaded the surrounding collagen matrix and another one in which cells invaded collagen matrix as cohesive multicellular units. The authors found that invasive phenotype is correlated with clinical features, giving the human samples rise to organoids with predominantly mesenchymal invasion displaying significantly increased risk of death. Moreover, they demonstrated that SMAD4 *in situ* inactivation promoted collective invasion stimulated by TGF-β via non-canonical signaling, while organoids with wild-type SMAD4 mutation invade with mesenchymal phenotype. Overall, these organoid models can be promising for studying the mechanisms underlying the PDAC invasion and investigating possible strategies to inhibit PDAC invasion.

Tumor organoids have been commonly cultured in animal-derived matrices, most prominently, Matrigel or collagen matrices. Although such hydrogels have demonstrated be

suitable for support organoids growth, they suffer from batch-to-batch variations and fails to/ are devoid of emulate native tumor ECM mechanical properties, limiting the recapitulation of native cell-ECM interactions. To surpass such issue, tumor-stroma PDAC organoids have been established in synthetic hydrogels (e.g., polyethylene glycol (PEG)) owing to the easily mechanical tunability and modification with cell-adhesion cues enabling to resemble human tumor tissue stiffness and cell-ECM interactions. In this work, a custom-designed eight-arm PEG hydrogel chemically modified with adhesion-mimetic peptides (i.e., fibronectin-mimetic peptide PHSRN-K-RGD, collagen– mimetic GFOGER peptide, and a basement membrane-binding peptide) was synthesized to emulate cells secreted proteins. The results demonstrated that PDAC organoids established relevant cell–ECM interactions in PEG hydrogels allowing a similar growth kinetics and the establishment of a cellular architecture and polarity similar to Matrigel cultures.(Below et al., 2021) Interestingly by tunning PEG hydrogel physical properties to achieve a increasing stiffness similar to the native tumor, PDAC organoids engage signaling consistent with mechano-sensing showing increased nuclear translocation of YAP1 and increased Ctgf levels in stiffened hydrogels. Moreover, PDAC organoids co-cultured with fibroblasts and macrophages in PEG hydrogel exhibited a relevant tumor-stroma interactions, an invasive and migratory behavior, demonstrating that such synthetic platform successfully support tumor-stroma PDAC organoids culture.(Below et al., 2021) Overall, the established tumor-stroma platform in PEG hydrogel demonstrated to support heterotypic PDAC organoids growth and recapitulate key tumor hallmarks including the tumor morphology, native ECM mechanical traits, the dynamic crosstalk between cancer-stroma components and invasive behavior.

Ongoing human clinical trials exploring patient-derived organoids for drug screening and metastasis are currently underway (NCT03544255, NCT03500068). Regardless of their accuracy to model pancreatic malignancies and better predict clinical response, organoids are still limited in their ability to represent angiogenesis and metastasis to secondary organs. Therefore, combining tumor-stroma organoids with advanced bioengineering strategies that enable precise cell spatial positioning, inclusion in tumor-mimetic ECM and culture under physiological flow conditions are rapidly emerging as a fresh take to further improve our understanding of PDAC microenvironment and improve drug discovery/screening.

4.2.2. Modelling Pancreatic Tumor-Stroma Interplay – Emulating Form and Scale Through 3D-bioprinting

Recapitulating the complex morphology, spatial cellular arrangements and anatomic scale of human tumors microenvironment is highly desired in *in vitro* disease modelling. On this focus, rapidly emerging advances in additive manufacturing technologies such as 3D-bioprinting are enabling a precise and sequential build-up of tissue-like constructs with well-defined, user-programmed, geometries and chemical/biological gradients, among many other features that are unattainable with common manufacturing technologies (*i.e.*, micro-molding, surface patterning, solvent evaporation, etc.).(Datta et al., 2018) In this approach, computer aided design (CAD) encoded models are generally exploited for fabricating 3D tissue-specific living architectures comprising a cell-laden ECM-mimetic biomaterial (*i.e.*, so termed bioink), while allowing an accurate control over constructs physicochemical properties and cellular distribution, on the fly during printing.(Yin et al., 2018) Considering these advantages 3D-Bioprinting arises a valuable technology for rapidly generating biomimetic tumor constructs, with functional complexity, tailored biological components, reproducible geometry and programable/time-adaptable mechanical properties, resembling

those of *in vivo* malignant tissues.(Pereira and Bártolo, 2015) The capacity to distribute different cell types and ECM components in a biologically relevant 3D spatial arrangement at an anatomic scale, indeed opens a myriad of possibilities form improving tumor-stromal surrogates physiomimetic potential.

Up-to-date, *in vitro* tumor models of numerous types of malignancies including: glioblastoma, breast cancer, prostate cancer and pancreatic cancer have been materialized through 3D-Bioprinting technologies.(Duchamp et al., 2019; Hakobyan et al., 2020; Ma et al., 2018) Aiming to replicate pancreatic cancer early development stages, high-throughput spheroid arrays were recently generated by using 3D laser-assisted bioprinting (LAB).(Hakobyan et al., 2020) LAB enabled the generation of different 3D microdroplets comprising acinar and ductal cells and subsequent deposition in ECM-mimetic GelMA receiving substrates. The fabricated models were able to mimic the earlier events in PDAC, including EGFR translocation to the cell membrane and acinar-to-ductal transformation, representing an excellent platform for accessing key factors that contribute to disease progression.

In a different approach, 3D-bioprinting technology was also leveraged for investigating tumor-stroma cells interaction and recapitulate the native tumor architecture. For this purpose, a 3D-bioprinted heterotypic PDAC model comprising patient-derived cancer cells, HUVECs and PaSCs, was successfully established.(Langer et al., 2019) In the fabricated 3D constructs cancer cells were surrounded by stromal components leading to the establishment of autocrine and paracrine signaling. All in all, such heterotypic microtissues exhibited biomimetic tumor architectures and cellular distribution, as well as increased resistance to standard of care pancreatic cancer therapeutics as researchers observed a dose-dependent response of cancer cell death to treatment.(Langer et al., 2019)

Emulating bio-architecture, scale and physiology in 3D-bioprinted *in vitro* models of pancreatic cancer is an exciting advance that is envisioned to give rise to a new generation of models encoding anatomic-like scale in their design. Nevertheless, despite the fabricated model recapitulates more features of human tumor-stroma interplay when compared to other strategies, 3D-Bioprinting potential for recapitulating all the hallmarks of pancreatic cancer is still to be fully unraveled. Particularly, the formulation of tumor ECM mimetic bioinks that recapitulate major ECM components, the inclusion of key glycosaminoglycans such as hyaluronan (HA) and the installation of on-demand/programmable stiffening dynamics during long term maturation remain to be thoroughly explored in macro-scale tumor-stroma biomimicking platforms.

4.2.3. Modelling Pancreatic Tumor-Stroma Interplay On-a-chip

Organ-on-a-chip technologies have recently unlocked the opportunity for introducing nutrient feed dynamics coupled to physiological fluid flow, shear stress/mechanotransduction modulation events, and cellular/physical barriers in bioengineered *in vitro* models, enabling to recreate natural features occurring in *in vivo* tumors. Owing to their modular features and compatibility with optical/fluorescence microscopy, microfluidic systems can also be readily adaptable for high-throughput screening/high-content imaging/analytes sensing *in situ* (*i.e.*, sensing lab-on-a-chip systems), enabling real-time readouts and live-imaging follow-up of 3D tumor-stroma models maturation or response to therapeutics.(Carvalho et al., 2015; Shang et al., 2019) Such unique features render these platforms highly attractive for bioengineering pancreatic tumor-stroma heterotypic models and to probe their interplay under interchangeable/user-programmed dynamic conditions that are easily controllable and reproduced *in vitro*.

Pioneering achievements in this direction have been recently reported via the engineering of a tumor-stroma-on-a-chip for exploiting the role of tumor-stroma cell-ECM interactions in a spatially-controlled 3D architecture that closely mimics the *in vivo* TME.(Drifka et al., 2013) Such system was engineering by employing a microfluidic device with three inlet channels that converge to form a single culture channel (**Fig. 5A**). The cell-laden ECM-mimetic biomaterial was introduced through inlet ports to originate cell-rich ECM hydrogel tri-layer patterns (a central cancer cell-rich layer, two flanking stromal cell-rich layers) over the length of the central channel, allowing the compartmentalization of different cell types without requiring artificially pre-programmed physical barriers. Aiming to accurately recapitulate the heterogenous TME, a supporting ECM-mimetic dual-component hydrogel (*i.e.,* collagen type I collagen/HA) was combined with human pancreatic ductal adenocarcinoma PANC-1 cells and PaSCs, with the latter operating as a representative stromal component due to their important role in PDAC malignancy. Collagen organization was modulated via the manipulation of key polymerization parameters (*i.e.,* pH, temperature, salt concentration, flow rate) to study tumor-stroma interplay and cell-cell interaction migration under precise conditions. This compartmentalized design additionally enabled to probe the performance of candidate therapeutics in a relevant TME-like context, and most importantly to investigate the biophysical effects of innovative therapeutics in ECM compactness and collagen re-organization following treatment with different doses. Developing platforms for assessing biophysical effects in ECM stromal components is a highly desirable feature, especially considering the emerging evidences that targeting these biological barriers in pancreatic tumor could provide a therapeutic benefit. In a similar approach and aiming to better understand and resemble such tumor-stroma paradigm, a 3D vascularized PDAC model was established by co-culturing human PDAC organoids, fibroblasts, and endothelial cells in a perfusable platform suited in a 96 well plate.(Lai et al., 2020) The combination of patient-derived tumor organoids with organ-on-a-chip technology offers a remarkable advance on the 3D modelling field, allowing to include key tumor building blocks, namely tumor, stroma, and vasculature compartments, in a very integrative way recapitulating not only the native tumor architectural and cellular features offered by organoids but also the perfusable vasculature network, the fluid flow sensed by cells and drug delivery mechanisms of native environment. Furthermore, the inclusion of a perfusable vasculature enabled to recreate the drug diffusion mechanisms through the endothelium and tumor ECM until reach the tumor mass, suggesting the potential of such platform to study drug diffusion mechanism and evaluate anti-cancer drugs performance. The co-culture of PDAC organoids with activated myofibroblasts promoted tumor organoid growth and exhibited a high degree of ECM deposition followed by increased tissue stiffness suggesting the key role of cancer cells- myofibroblasts crosstalk in tumor growth/proliferation and ECM remodeling (Figure4BC). Particularly, heterotypic demonstrated higher deposition of ECM proteins such as collagen and pro-tumoral cytokines than monotypic counterparts, highlighting the biomimetic potential of the engineered model to recapitulate key pancreatic tumor hallmarks. To elucidate the contribution of stromal fibroblasts into tumor organoids resistance mechanisms, the heterotypic microtissues exhibited higher cellular viability after gemcitabine exposure than their monotypic counterparts. Such results should be correlated with the abundant collagen deposition and fibrotic matrix generated in such system. Overall, the designed platform enabled to recapitulate important tumor hallmarks and opened new avenues regarding to the co-culture of PDAC organoids with other stromal components and their integration in a high-throughput dynamic/perfusable system.

Adding to therapeutics screening, tumor-on-a-chip platforms also offer the possibility to investigate other complex biological hallmarks of pancreatic cancer, particularly its hypovascularity. Aiming to better emulate such pancreatic cancer-vasculature interactions, a rationally designed dual-channel microfluidic was recently developed.(Nguyen et al., 2019) The designed tumor-vasculature-on-a-chip platform was configured into two parallel hollow cylindrical channels embedded within a ECM-mimetic collagen-based 3D matrix. Microfluidic channels were laden with pancreatic cancer cells and HUVECs respectively, establishing the tumor and vascular compartments (**Fig. 5B**). During dynamic culture it was observed that PDAC cancer cells invaded into the matrix toward the endothelial lumen and removed endothelial cells originating b tumor-lined and tumor filled luminal structures, a phenomenon described as endothelial ablation. These remarkable observations were also validated in *in vivo* PDAC models, being verified that in both approaches PDAC invades blood vessels and ablates the endothelium. In addition, this platform enabled to validate that TGF-β receptor signaling reduces the ablation of endothelial cells by pancreatic cancer cells.(Nguyen et al., 2019). In fact, leveraging on this platform PDAC-driven endothelial ablation via activin-ALK7 pathway (TGF-β family receptors), was demonstrated to be a potential key mechanism underlying PDAC poor vascularization. These are major discoveries, considering that tumor re-vascularization strategies are evermore recognized as potential therapeutic targets.

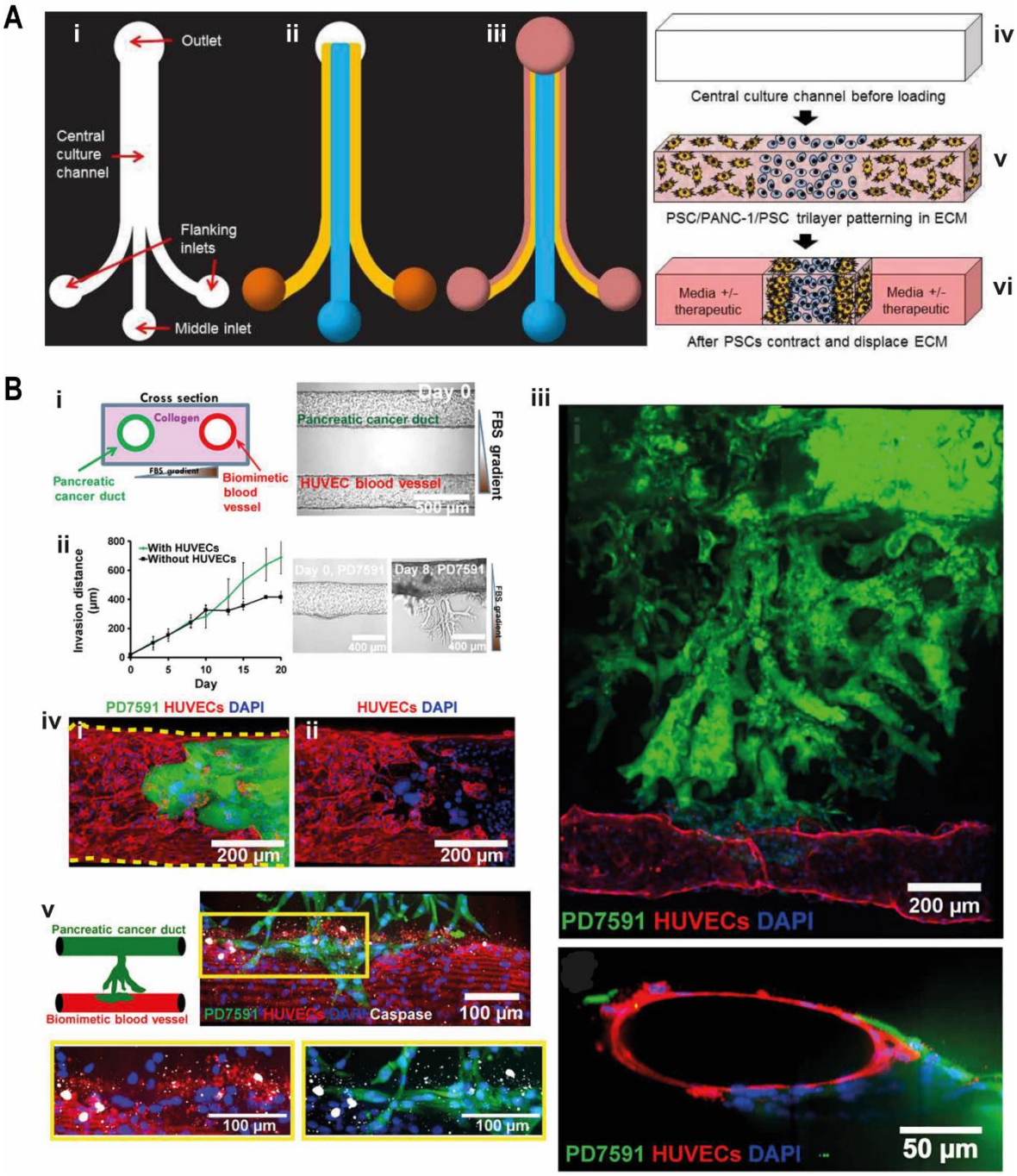

Fig. 5 Advanced organ-on-chip platforms to modulate pancreatic tumor-stroma interplay at a preclinical level. (A) Design and operation of the microfluidic device. (i - vi) Representation of the tri-layer patterning scheme and maturation following incubation with cultured media (pink color). Adapted from (Drifka et al., 2013) with permission from The Royal Society of Chemistry. **(**B) Tumor-on-a-chip organotypic model to study cancer cells vascular invasion. The microfluidic device comprises two hollow cylindrical channels that aims to recapitulate pancreatic duct and blood vessel structures embedded within a collagen matrix. Blood channel compartment was seeded with endothelial cells to form a biomimetic blood vessel. The pancreatic duct mimic was seeded with cancer cells. Adapted from (Nguyen et al., 2019) with permission from American Association for the Advancement of Science.

5. Concluding Remarks and Future Perspectives

The desmoplastic tumor microenvironment of pancreatic cancer and its unique stromal compartment renders it one of the most challenging to replicate in a preclinical setting. Gathering on the urgent necessity to develop increasingly physiomimetic models for both fundamental tumor biology studies and for innovative therapeutics screening, herein we showcased and discussed the most recent advances in bioengineering physiomimetic 3D *in vitro* platforms that recapitulate the unique tumor-stroma interplay naturally occurring in pancreatic cancer.

All in all, while traditional 3D spheroid models and bulk hydrogel-based platforms are valuable for emulating key tumor-stroma interactions, generally they still fail on recapitulating 3D tumor bioarchitecture and evolution under flow. Such hallmarks can be matched by the synergistic combination of 3D biofabrication technologies with rationally designed tumor-on-a-chip devices, an emerging trend that is expected to yield important advances in the future. Moreover, the combination with patient-specific tumor organoids that exhibit organ-like self-organization and patient-matched pathophysiology have potential for giving rise to highly personalized therapeutic regimes.

Although significant advances are envisioned with these platforms, and despite they already offer a great contribution for understanding tumor biology as well as to clinical decision making, the underlying complexity of the stromal compartment of this tumor still needs to be further addressed and more precisely emulated. Particularly, it will be valuable in the future to evaluate therapeutic responses in anatomic-sized heterotypic models comprising representative CAFs sub-populations and the various immune system stromal cells. Improvements in these aspects can shed light on several unanswered questions regarding cancer survival and metastasis mechanisms. Materializing this heterogeneity in cell-supporting matrices that better mimic the composition, architecture, biomolecular components, and mechanics of native ECM is also anticipated to boost the predictiveness of the new generation of pancreatic cancer preclinical models. Holistically, incorporating multiple engineering approaches to generate highly advanced tumor-stroma models and combining them with big data analytical tools (*e.g.,* omics-based analysis) prior and following *in vitro* design could be the key for recapitulating major biological hallmarks, biomarker signatures and resistance mechanisms. Importantly, these fundamental design blueprints and accumulating knowledge are not limited to pancreatic cancer being in fact widely applicable to a number of different neoplasias where a stroma-rich compartment is recognized to play a major role in disease progression (*i.e.,* breast and prostate cancers). The broader applicability of such bioengineering approaches further supports a more active development of tumor-stroma physiomimetic *in vitro* models in the foreseeable future.

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