Establishment of a human 3D pancreatic adenocarcinoma model based on a patient-derived extracellular matrix scaffold

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ARTICLE INFO

Keywords:
- pancreatic cancer
- microenvironment
- extracellular matrix
- disease modelling
- 3D culture model
- decellularization
- drug test
- response to treatment

ABSTRACT

Pancreatic cancer is likely to become one of the leading causes of cancer-related death in many countries within the next decade. Surgery is the potentially curative treatment for pancreatic ductal adenocarcinoma (PDAC), although only 10%–20% of patients have a resectable disease after diagnosis. Despite recent advances in curative surgery the current prognosis ranges from 6% to 10% globally. One of the main issues at the pre-clinical level is the lacking of model which simultaneously reflects the tumour microenvironment (TME) at both structural and cellular levels. Here we describe an innovative tissue engineering approach applied to PDAC starting from decellularized human biopsies in order to generate an organotypic 3D in vitro model. This in vitro 3D system recapitulates the ultrastructural environment of native tissue as demonstrated by histology, immunohistochemistry, immunofluorescence, mechanical analysis, and scanning electron microscopy. Mass spectrometry confirmed a different extracellular matrix (ECM) composition between decellularized healthy pancreas and PDAC by identifying a total of 110 non-redundant differently expressed proteins. Immunofluorescence analyses after 7 days of scaffold recellularization with PANC-1 and AsPC-1 pancreatic cancer cell lines, were performed to assess the biocompatibility of 3D matrices to sustain engraftment, localization and infiltration. Finally, both PANC-1 and AsPC-1 cells cultured in 3D matrices showed a reduced response to treatment with FOLFIRINOX if compared to conventional bi-dimensional culture. Our 3D culture system with patient-derived tissue-specific decellularized ECM better recapitulates the pancreatic cancer microenvironment compared to conventional 2D culture conditions and represents a relevant approach for the study of pancreatic cancer response to chemotherapy agents.

Introduction

Pancreatic cancer represents one of the most lethal malignancies worldwide, ranking as the 12th diagnosed cause of cancer and the 7th cancer-related death in the 2020.1 Pancreatic cancer is likely to become one of the leading causes of cancer-related death in many countries within the next decade.2 Pancreatic ductal adenocarcinoma (PDAC) constitutes approximately 85%–90% of all exocrine pancreatic tumors3,4 often diagnosed at advanced stages, when the tumor has progressed and treatment options are very limited.5 Surgery is the potentially curative treatment for PDAC, although only 10%–20% of patients have a resectable disease after diagnosis. Despite recent advances in the introduction of first-line multi-agent adjuvant chemotherapy with Folfirinox (fluorouracil, folinic acid, 5-fluorouracil).
Tissue decellularization

Surgically obtained HDE and PDAC specimens were kept in cold and sterile phosphate buffered saline (PBS) for no longer than 2 hours before processing. All the decellularization steps were performed with sterile solutions and under tissue culture hood. HDE and PDAC destined to be used as fresh samples (HDE and PDAC pre-DET) were rinsed with sterile PBS and consequently treated according to the methodology with which they were analyzed. Healthy mucosa and CRC destined to decellularization process (HDE and PDAC post-DET) were treated with 1 to 3 detergent-enzymatic treatment (DET) cycles. Each DET cycle was composed of deionized water at 4°C for 24 hours, 4% sodium deoxycholate (Sigma) at room temperature (RT) for 4 hours, and 2000 kU DNase-I (Sigma) in 1 M NaCl (Sigma) at RT for 3 hours, after washing in water. After decellularization, matrices were rinsed in PBS + 3% penicillin and/or streptomycin (pen/strep) for at least 5 days changing the solution two times a day and then stored at -80°C.

DNA isolation and quantification

To assess total DNA content within the fresh healthy pancreatic and PDAC compared with respective decellularized samples, specimens were treated using DNeasyBlood&Tissue kit (Qiagen) under manufacturer’s instruction. DNA samples were then quantified using Nanodrop 2000 at the 260/280 nm ratio (Thermo scientific).

Immunofluorescence (IF) and Immunohistochemistry (IHC) analyses on tissue sections

Decellularized and recellularized tissues were fixed in 4% paraformaldehyde (PFA) for 1 hour at 4°C. Samples were then dehydrated with a sucrose gradient: 10%, 15%, 30%. Finally, they were included in the cryostat embedding medium optimal cut temperature (OCT, Bio-Optica) and frozen in liquid nitrogen. The samples were stored in -80°C until the tissues were sectioned in 8 µm sheets, using a cryostat (Leica CM 1520). Immunofluorescence staining were performed as in 13 (time and dilution depend on the antibody, Supplementary table 1). For immunohistochemistry, native and decellularized tissues were fixed in 4% PFA and embedded in paraffin. After sections (5 µm) were stained with Haematoxylin and Eosin, Masson’s Trichrome, Alcian Blue and Periodic Acid Schiff assays according to routine protocols. Images were collected using a fluorescence inverted microscope (Leica B5000).

Table 1

Clinical and pathological characteristics of the 20 enrolled patients

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- patients with a known history of a hereditary cancer syndrome were excluded;
- patient that underwent neoadjuvant treatments were excluded;
- age > 18 years;
- written informed consent.
**Atomic Force Microscopy (AFM)**

**Sample preparation:** The starting samples, HDE and PDAC pre-post-DET, were uniformly prepared by cutting them with a double-blade scalpel in order to have a constant thickness (thickness average 35 mm). All samples were then embedded in 2% low melting agarose (Sigma) and sliced in cold PBS at 400 μm thickness using Leica VT1000S Vibratome with a speed of 0.1 mm/sec and frequency of 90 Hz and amplitude of 0.6 μm. Slices were then collected and dried for further analysis. At least 3 slices per sample were used for each study.

All measurements were performed using aXE Bio-AFM (Park Systems, South Korea) equipped with an inverted microscope (Nikon Eclipse Ti). The force-displacement curves were acquired using PPP-CONTSCR-10 pyramidal tips mounted on Si3N4 cantilevers with a nominal spring constant of 0.2 N/m (NanoSensors, Neuchatel, Switzerland), calibrated by the manufacturer prior to use. Before each test, the sensitivity of the AFM photodetector was calibrated by measuring the slope of the force-indentation curve acquired on a silicon standard. All experiments were performed at room temperature, in a fluid environment (PBS). Typically, indentation curves were acquired at 2 μm/s piezo displacement rate along the Z axis and using an indentation depth of 2 μm. At least four force curves were recorded on each sample, at different positions. Young’s modulus were calculated applying a fit of the Hertz model to each individual force-indentation curve, assuming a Poisson ratio of 0.5, as it is typical for soft biological material where incompressibility is assumed.

**Diffusion measurement**

Sample slices of 3 × 3 mm size were placed onto a glass slide and covered by a round coverslip. 5 μl of fluoresceinylthiocarbamoyl-dextran (FTC-dextran) (40KDa) solution (2mg/ml) were injected between the 2 glass surfaces. The fluorescence intensity of the sample was monitored by acquiring images at Zeiss confocal microscope (LSM800 Airyscan) after 1, 5, 10, and 20 minutes from the FTC-Dextran injection, and fluorescence intensity data were collected via Fiji software extracting the value of fluorescence at each point along a 500 μm line orthogonal to the FTC-dextran contact interface. Data from three individual orthogonal lines were collected per sample. For each collected data set, the spatial distribution of the fluorescence intensity was obtained at different times, normalizing the fluorescence intensity in the direction orthogonal to the contact interface, I, by its value at the tissue-solution interface, I0. The resulting curves, f(x,t) = I(x,t)/I0(t), were fitted using the one-dimensional version of Fick’s second law, assuming a diffusion coefficient dependent only upon the nature of the tracer. For each time, the apparent (ie, effective) diffusion coefficient, ADC, was determined by a GRG non-linear least-square regression analysis. The ADC of each sample is represented by the average of the fitting results at different times.

**Cells maintenance and expansion**

The human pancreatic adenocarcinoma cell lines PANC-1 and AsPC-1 were obtained from American Type Culture Collection (ATCC HTB-38TM) and maintained in DMEM and RPMI-1640 medium, respectively, supplemented with 10% fetal bovine serum, 1% L-Glutamine and 1% Pen/Strep antibiotic solution (growth medium [GM]) in humidified atmosphere at 37 °C in 5% CO2. Medium was changed every 3 days.

**Tissues Recellularization**

HDE and PDAC decellularized matrices were incubated overnight with growth medium containing primocin antibiotic (InviVoGen, Kemapenhout, Belgium) at 4°C. In order to normalize the intra-sample variability, scaffolds were cut into comparable dimensions before seeding. All matrices were then injected with 2.5 × 10^3 cells, resuspended in 10 μL of collagen I (diluted 2:3 with GM), using a 30G syringe needle. Samples were incubated for 4 hours in a humidified incubator at 37 °C and 5% CO2. The GM was carefully changed every 2 days.

**Scanning electron microscopy (SEM)**

Samples were fixed with 2% glutaraldehyde in 0.1 M phosphate; following washing they were cut into segments of approximately 1 cm length and cryoprotected in 25% sucrose, 10% glycerol in 0.05 MPBS (pH 7.4) for 2 hour, then fast frozen. Analysis were performed as reported in.

**ECM component quantification**

Collagen and sulphated glycosaminoglycan (sGAG) content in HDE and PDAC pre- and post-decellularization were quantified using respectively the SIRCOL collagen assay and Blyscan GAG Assay Kit (all from Biocolor) under manufacturer’s instruction.

**Drug Treatment and Cytotoxicity Assay**

For the 5-Fluorouracil (5-FU) and FOLFIRINOX treatments in 3D setting, HDE and PDAC DET scaffolds were repopulated with 2.5 × 10^5 pancreatic and AsPC-1 cells in 24-well plates. The FOLFIRINOX chemotherapy cocktail, made up from 5-FU, Irinotecan and Oxaliplatin, was used in an equal drugs ratio. Seven days post-seeding, the cells were treated with 1 μM, 10 μM, 20 μM, 50 μM, 70 μM, 100 μM for 72 hours. For the treatment of 2D cultures, PANC-1 and AsPC-1 were seeded at 5 × 10^3 cells per well in 96-well plates and treated with 0.1 μM, 0.5 μM, 1 μM, 5 μM, 10 μM, 50 μM and 100 μM of 5-FU and FOLFIRINOX for 72 hours. Following incubation, the medium was aspirated from the wells, and 20 μL of resazurin dye (Abcam) was added to the wells and incubated for 2 hours. Fluorescence was read at 530/590 nm using the Tecan Microplate Reader Spark microplate reader (Tecan LifeScience). Cytotoxicity was determined as the percentage of fluorescence in exposed cells compared to the untreated cells. The Inhibitory Concentration 50% (IC50) index was used to indicate the drug cytotoxicity calculated using GraphPad Prism software 6. (GraphPad Software, San Diego, CA, USA).

**The second harmonic generation (SHG)**

The second harmonic generation (SHG) were detected using a custom-built multimodal microscope, as described in. Coherence, (C) was calculated for collagen to verify the local dominant orientation in representative regions of interest using OrientationJ, an ImageJ plugin, as reported in.

**Mass Spectrometry analysis**

**Sample preparation:** HDE and PDAC decellularized matrices (range 2–5 mg) were digested following the protocol proposed by Naba et al. After digestion, samples were purified by SPE (Supel-Select HLB SPE tubes, Supelco). Eluted peptides were finally dried under vacuum and stored at -20°C until analysis. The Protein identification and Data processing were performed as reported in.

**Statistical analyses**

All graphs and statistical analyses were performed using the GraphPad Prism Software 6. Data were expressed as means ± Standard Deviation. For the comparison of paired experimental groups, the two-sided Student’s t-tests (for parametric dataset) and Mann-Whitney test (for non-parametric dataset) were used. One-way ANOVA with Bonferroni’s post-test (for parametric dataset) and Kruskal-Wallis test with Dunns post-test (for non-parametric dataset), were performed for multiple comparisons. A P-value < 0.05 was considered statistically significant (**: P-value < 0.05; **: P-value < 0.01; ***: P-value < 0.001; ****: P-value < 0.0001).
Results

Decellularization and Characterization of 3D Patient-Derived Scaffolds

Matched samples from both HDE and PDAC specimens were decellularized using the detergent-enzymatic treatment (DET). A gross appearance of fresh (pre-HDE and pre-PDAC) and decellularized (post-HDE and post-PDAC) biopsies underlined the typical color change from pink-red to white which is associated to an increase in translucence of decellularized samples (Fig 1, A). Both HDE and PDAC samples were decellularized after two DET cycles with a reduction in DNA amount of 95.1% and 96.3% respectively compared to native fresh tissue (P-value = 0.03 and P-value = 0.01, respectively) (Fig 1, A–C). At first, a general overview of tissue architecture maintenance was performed through laminin immunofluorescence. We observed that laminin, one of the major components of cell basement membranes, was maintained both in post-DET HDE and PDAC compared to native fresh tissues. Even if we counterstained decellularized sample with DAPI, there are no visible nuclei, confirming the occurred decellularization in comparison to fresh tissue in which we observed the presence of nuclei (Fig 1, B, upper panel). Scanning electron microscopy (SEM) analysis in fresh and decellularized samples. Scale bar: 20 μm (lower panel) (C) DNA quantification of pre- and post-DET HDE and PDAC tissue samples (* P-value < 0.05; ** P-value < 0.01) (D) Histological staining of HDE and PDAC samples pre- and post-DET. H&E, Hematoxylin and Eosin; AB, Alcian blue; PAS, Periodic Acid-Schiff. Scale bar: 100 μm. (E) Quantification of glycosaminoglycans (GAG) in pre- and post-DET in HDE and PDAC samples. (F) Young’s modulus quantifications in pre- and post-DET in HDE and PDAC samples. (means ± Standard Deviation) (**** P-value < 0.0001) (G) Confocal microscope images at the moment of FITC-Dextran injection (t = 0 min.) and after 20 minutes from injection (t = 20 min.) in pre- and post-DET in HDE and PDAC samples. Scale bar: 100 μm (left); Apparent Diffusion Coefficient (ADC) quantification 20 minutes from injection of FITC-Dextran in pre- and post-DET in HDE and PDAC samples (right) (means ± Standard Deviation) (* P-value < 0.05). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
glycoprotein, proteoglycans typically found in the connective tissues and mucus. Moreover, an increase of mucins secretion was revealed especially at duct level where the positivity resulted increased in columnar cell border, suggesting an increased mucin secretion function compared to HDE tissue. The same trend was preserved in post-DET samples in which a marked PAS signal was preserved at duct level (arrows A) and where the connective deposition was increased compared to post-DET HDE (arrows B) (Fig 1, D, lower panel). Absolute glycosaminoglycans (GAG) quantification confirmed the histology qualitative data in both HDE and PDAC scaffolds, where no significant loss of these ECM components was observed after DET process (Fig 1, E). Alcian blue (AB) histology demonstrated the presence of the mucopolysaccharides such as glycogen, glycoproteins, glycolipids and acid mucins both in pre- and post-DET conditions of both HDE and PDAC, with an increase of

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**Fig 2.** Tissue collagen analysis before and after decellularization. (A) Collagen I (Col I) and Collagen IV (Col IV) immunofluorescence analysis in healthy ductal epithelium (HDE) and Pancreatic ductal adenocarcinoma (PDAC) tissue samples, before (pre-detergent-enzymatic treatment (DET)) and after (post-DET). Nuclei are counterstained with DAPI (40,6-diamidin-2-fenilindolo (blue); Scale bar: 100 μm. (B) Masson’s Trichrome (MT) stains and (C) Second Harmonic Generation (SHG) analysis. Scale bar: 100 μm. (D) Fast Fourier Transform (FFT) analysis provided a measure of collagen fibers in relation with the values of coherency (C) (means ± Standard Deviation). (E) Quantifications of Soluble and Insoluble Collagens and SHG intensity (** P-value < 0.01). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
hyaluronic acid in proximity of tumor lesion (Fig 1, D, middle panel). Finally, SEM analysis confirmed the ultrastructure and topology maintenance after decellularization process with no signs of collapse or degradation of ECM components (Fig 1, B, lower panel).

Mechanical measurements on native and decellularized scaffolds

AFM was used to assess the changes in stiffness of both HDE and PDAC tissues. Mechanical measurements at micro-scale were carried out on native and decellularized scaffolds and an average value of Young’s modulus was derived from the indentation data of each tissue. PDAC exhibit a significantly higher Young’s modulus compared to HDE both pre- and post-DET and this data demonstrating that stiffness increases with fibrosis (HDE pre-DET vs PDAC pre-DET P-value < 0.0001 and HDE post-DET vs PDAC post-DET P-value < 0.0001). Young’s modulus resulted significantly reduced after the decellularization process within the PDAC samples (PDAC pre-DET vs PDAC post-DET P-value = 0.0002) (Fig 1, F). This trend is determined by the removal of the cells that undoubtedly conferred rigidity to the tissue by interacting with each other and with the ECM. Furthermore, in order to evaluate the permeability of the tissues we performed diffusion measurement. Each sample, native and decellularized, was characterized by the diffusion coefficient. In particular, an apparent (effective) diffusion coefficient (ADC) has been employed, because of the tissue cannot be considered as homogeneous at the length scale at which the measurement is performed due to variations in its microstructure. In Fig 1, G, it was observed a significantly decrease in diffusivity between post-DET PDAC compared to post-DET HDE and also underlined in images at confocal microscope 20 minutes after injection of FITC-Dextran. This quantification of the qualitative data confirmed that fibrosis and stiffening associated with cancer limited the diffusion across the tissue (HDE post-DET vs PDAC pre-DET P-value = 0.0430 and HDE post-DET vs PDAC post-DET P-value = 0.0127) (Fig 1, G).

Collagen analysis in pre- and post-DET HDE and PDAC tissue

Immunofluorescence analysis showed that the expression and distribution of key PDAC ECM collagens, such as interstitial collagen I and basement membrane collagen IV were maintained in post-DET HDE and PDAC tissues compared to native tissue (Fig 2, A). Masson’s Trichrome (MT) staining confirmed the preservation of collagen structures after decellularization and emphasized the completely absence of cell component, in post-DET compared to pre-DET samples (Fig 2, B). In particular, PDAC counterpart, highlighted by perineural invasion with dense desmoplastic stroma surrounding the adenocarcinomatous glands resulted maintained also after decellularization from the green branches of collagen presented in PDAC post-DET. This pattern was confirmed also in post-DET samples. Second-harmonic generation (SHG) analysis remarked as the tight organization of collagen I observed in pre-DET tissue was maintained also after decellularization (Fig 2, C). The qualitative collagens analysis was coupled to the analysis of their spatial orientation that is a crucial factor for the determination of mechanical properties of a tissue. Both pre- and post-DET HDE tissue resulted more anisotropic, coherent and uniformly oriented in the same direction in comparison to pre- and post-DET PDAC tissue (HDE pre-DET, C: 0.122 ± 0.05; HDE post-DET, C: 0.059 ± 0.03) (Fig 2, D). Both soluble and insoluble collagen quantifications in the HDE post-DET scaffold, demonstrated no

![Fig 3. Proteomic analyses of decellularized tissue secretome.](image-url)
significant changes when compared to HDE pre-DET scaffold. Differently, PDAC post-DET tissue showed an increased collagen content compared to pre-DET tissue (respectively, $P = 0.0065$ and $P = 0.0013$) (Fig 2, E). The same trend was also confirmed with the quantification of SHG intensity (Fig 2, E).

**Proteomic analysis of decellularized tissue secretome**

To better analyze tissue protein maintenance after decellularization, mass spectrometry analysis was performed. A total of 601 proteins have been obtained after LC-MS/MS data processing, of them 181 were assigned by the identification of at least 2 non-redundant peptides. After filtering by FDR < 1%, 110 non-redundant proteins were finally identified. Of them, 52 were classified as “core-matriome” or “matriome-associated” proteins using Matrisome Project. Collagens family was the most representative ECM component (Fig 3, A). Indeed, fibril-forming collagens (COL1, COL2, COL3, COL5, and COL11), basal lamina collagens (COL4), cell-matrix bridging collagen (COL6) and Fibulin Associated Collagens With Interrupted Triple helices collagen (FACIT) (COL12, COL14, and COL16) were present (Fig 3, C). Beside collagens, several proteoglycans, ECM regulators and glycoproteins have been identified (Fig 3, A and Figure 3C). Venn diagram reported in Fig 3, B compares differences in detected proteins to identify key components in post-DET PDAC with respect to the post-DET HDE counterpart. We found 67 proteins PDAC-specific and only 5 proteins HDE-specific, while 31 proteins were present in both post-DET HDE and PDAC. Shared proteins were mostly represented by collagens and other ECM insoluble components like ELN, DCN, FN1, N1, VIM, and fibrinogens. This is in agreement with literature and highlights that collagens are an abundant ECM component in PDAC, with COL1 responsible for the majority of the desmoplasmic reaction. Similarly, specific proteins found in PDAC samples were still related to stroma composition and included: collagens (COL10, COL12, COL14, and COL16), glycoproteins (FBLN1 and 2, EMILIN1, MAFAP4 and 5, POSTN, VTN, TGBF1, THBS1 and 2), and proteoglycans (BGN, VCAN, HRG, HSPG2). The five HDE-related proteins (GP2, PNLIP, KRT1, CLPS, CUZD1) were markers of pancreatic stroma. Indeed, glycprotein GP2 is the major component of pancreatic secretory granule membrane and CUZD1 is involved in trypsin activation in pancreatic acinar cells. PNLIP and CLPS are secreted pancreatic proteins involved in dietary lipid metabolism.

**Repopulation and Characterization of the 3D patient-derived scaffolds**

PANC-1 and AsPC-1 pancreatic cell lines were chosen to repopulate the three-dimensional patient-derived healthy and tumor scaffolds (3DHS and 3DTS), qualitative and quantitative characterization of 3DHS and 3DTS were compared with cells grown in conventional plastic plates, called 2D.

Firstly, at a general overview, we observed that AsPC-1 cells were able to easily penetrate both HDE and PDAC scaffolds compared to PANC-1 that tended to occupy predominantly their outermost portion (Fig 3, A and C). To test the ability of 3DHS and 3DTS to support cells engraftment and viability, we evaluated their proliferation index through the Ki67 marker (Fig 4, A–C). After 7 days of culture, as represented by IF, the number of Ki67-positive cells in 3DTS specimens (71% ± 8.6%) was higher compared with 3DHS (62% ± 4%) although not statistically significant. On the contrary, a significantly decreased proliferation rate in the 3DTS and 3DHS where evident when compared with the cells grown in conventional 2D condition (89% ± 3.34%, 3DTS vs 2D: $P$-value < 0.05; 3DHS vs 2D: $P$-value < 0.05) (Fig 4, A). A similar trend was observed also in AsPC-1 repopulated scaffolds in which we found a significant decrease of the proliferation rate in 3DHS (66% ± 9.5%, $P$-value < 0.05) and a similar trend in 3DTS (79% ± 3%, $P$-value = 0.07) compared with the cells grown in 2D setting (92% ± 0.76%) (Fig 4, C).

The effect of both HDE and PDAC scaffolds on cells movement and migration, were examined through F-actin and Vimentin staining. In the case of F-actin, no differences were observed in the scaffolds, both 3DHS (81% ± 4.6%) and 3DTS (76% ± 6.6%) repopulated by PANC-1 cells compared to cells grown in 2D (99% ± 0.5%) (Fig 4, A and C). Conversely, a statistically significant decreased expression of F-actin was observed in both 3DTS and 3DHS for both cell line (respectively, 76.3% ± 2.9%, $P$-value < 0.002 and 81.6% ± 2.6%, $P$-value < 0.007 for PANC-1 and 37.7% ± 4.9%, $P$-value < 0.0001 and 50.7% ± 9%, $P$-value = 0.0001 for AsPC-1, compared to the cells grown in 2D (99% ± 0.2%) (Fig 4, A and C).

![Fig 4. Evaluation of proliferation and polarization of PANC-1 and AsPC-1 in 3DHS, 3DTS and 2D models. (A-C) Immunofluorescence staining and relative quantification regarding PANC-1 and AsPC-1 cells proliferation in 3DTS, 3DHS and conventional 2-dimensional model (2D): Ki67, as a proliferation marker (red); F-actin, to evidence the microfilaments of cytoskeleton (red), Vimentin, as a mesenchymal marker (red); Laminin to highlight basement membrane structure (green); DAPI to counterstain nuclei. (scale bar = 75µm).](image-url)
In parallel, the expression of Vimentin, was evaluated in the same settings. We found a reversal of behavior for both cell lines when analyzed in the 2D and 3D context. High levels of Vimentin were found in 2D setting for both PANC-1 (97\%±0.2\%) and AsPc-1 (99\%±0.3\%) (Fig 4, A−C). Simultaneously, a statistically significant decreased expression of Vimentin was observed in 3DTS and 3DHS for both cell lines (respectively, 34\%±3.9\%, P-value <0.0001 for PANC-1 and 41\%±5.1\%, P-value <0.0001 for AsPc-1) (Fig 4, A−C). SEM analyses confirmed the homogeneous presence of cells within the 3DHS and 3DTS, we appreciated that both cell lines were able to adhere to collagen fibers especially near the injection site with several cell clusters (Fig 4, B for PANC-1 and 4D for AspC-1).

**Effect of chemotherapy drugs treatments on cells cultured in 2D and 3D Model**

Finally, we sought to determine if the 3DTS model were able to affect pancreatic cancer cell lines response to conventional single and multi-agent chemotherapy. Firstly, we calculated the percentage of viable PANC-1 (Fig 5, A) and AsPc-1 cells (Fig 5, E) treated with an increasing drug concentration in 2D setting. PANC-1 and AsPC-1 treated with 5-FU showed an IC50 of respectively 9.64 \(\mu\)M and 8.10 \(\mu\)M (Fig 5, B). Similarly, PANC-1 and AsPC-1 treated with FOLFIRINOX showed an IC50 of respectively 6.14 \(\mu\)M and 4.49 \(\mu\)M (Fig 5, F). Then we replicated the drug treatments with both 5-FU and FOLFIRINOX in 3DTS. In accordance with the 2D model, chemotherapy-response for both cell lines in the 3DTS maintained a dose-dependent trend (Fig 5,C−G). However, if compared to the 2D conventional culture, both PANC-1 and Aspc-1 cells grown in 3DTS displayed a reduced sensitivity to 5-FU and FOLFIRINOX with increased IC50 values. Especially, PANC-1 showed an IC50 of 40.87\(\mu\)M to 5-FU (Fig 5, B) and 25.48\(\mu\)M to FOLFIRINOX administration after the 3DTS repopulation (Fig 5, F). Similarly, in 3DTS repopulated with AsPc-1 IC50 resulted of 42.91\(\mu\)M and 19.24\(\mu\)M when we respectively treated with 5-FU and FOLFIRINOX drugs (Fig 5, B−F). Table in Fig 5, D summarizes the IC50 values obtained with PANC-1 and Aspc-1 cells following treatments with 5-FU and FOLFIRINOX chemotherapy treatments.
Discussion

The pancreatic cancer is predicted to be the second cause of cancer-related death by 2030. The most common pancreatic cancer is the PDACs, characterized by a pronounced resistance to radiation, cytotoxic, and molecularly targeted therapies. The chemo- and radio-therapeutic resistance of PDAC is thought to be mediated, in large part, by its prominent stroma, composed of a variety of non-neoplastic cell types and ECM. The deposition of abundant amounts of ECM is termed desmosplastion and exerts mechanical as well as biochemical effects on PDAC cells thereby impairing tumour perfusion and thus delivery of antitumor drugs. In this context, the possibility of mimicking the tumour microenvironment in vitro, with a particular focus on ECM compartment, will be a crucial tool to cover the gap between the in vitro and in vivo experimental models. Here we present a tissue decellularization approach using a detergent-enzymatic method, able to provide a patient-derived in vitro 3D scaffold from healthy pancreas and PDAC that maintains peculiar biochemical, bio-structural and mechanical properties that mimic the original in vivo physio-pathological conditions.

Firstly, we demonstrated that the detergent-enzymatic method used in our study successfully deplete the cellular compartment from the tissue, while preserving the ECM composition and distribution. Histological, immunohistochemical, and ultrastructural analysis of post-DET tissue confirmed the preservation of architecture and major ECM components compared to native tissue in both HDE and PDAC. In particular, PAS and AB histology qualitatively suggested a conserved total amount and distribution of glycosaminoglycan which was orthogonally confirmed by the absolute quantification of these ECM component. In PDAC stroma, the GAG family are mainly represented by hyaluronic acid (HA). HA has been shown to be abundantly accumulated in the surrounding stroma of malignant tumor. The HA-rich microenvironment may promote tumour progression by enhancing cell proliferation, migration, invasion, metastasis, angiogenesis, and resistance to chemotherapeutic agents. Several studies have shown increased expression of HA and its receptors in PDAC. Importantly, the abnormal accumulation of HA correlates with worsened prognosis in patients with PDAC. In addition, the conservation of GAGs during decellularization is important to maintain tissue mechanical properties and to preserve growth factors in the tissue. In accordance with literatures and with in vivo models quantitative findings demonstrated that pancreatic tumors are stiffer than normal pancreas.

Therefore, the PDAC stroma, with changes in its mechanics, contributes to the disease’s malignancy, progression, and therapeutic resistance.

As we have demonstrated, the ECM in PDAC significantly differs in composition and architecture from HDE. Considering its physical-mechano properties, the PDAC ECM is more abundant, denser, and stiffer. These features can modulate response to therapy, in fact, an excessive accumulation of stiff ECM can perform as a barrier, protective the cells from therapeutic treatments. This effect is directly linked to a reduced diffusion of oxygen, nutrients, metabolites and drug. Finally, we demonstrated that native tumor tissue in which was present the interaction between ECM and cells increased tissue stiffness respect to ECM alone and we believed that this phenomenon can directly contribute to chemo-resistance in cancer patients.

Collagens are by far the most abundant and well-characterized component of the ECM in PDAC stroma. In particular, collagen I and IV were responsible for the majority of the desmosplastic reaction. Multiple lines of evidence show that interstitial collagens, such as collagen I, foster pro-tumorigenic features, including invasion and EMT. Collagen type I increased the cell proliferation of the pancreatic cancer cell line AsPC-1 and attenuated apoptosis induced by 5-fluorouracil. Cancer cell-derived collagen type IV stimulates cell growth and migration, which was confirmed by RNA interference-mediated knockdown experiments. The qualitative evaluation of collagen I and IV distribution in post-DET scaffolds, showed a phenotypic appearance similar to pre-DET condition. In agreement with the literature, a general increase in collagen I and IV deposition was observed in PDAC tissue compared to HDE. The analysis of collagen fibre coherency suggested that, in PDAC tissue compared to HDE, the collagen fibres are not only quantitatively deregulated but also have differences in collagen fibre angle and spatial organization. In fact, PDAC stroma showed a reduction in collagen I signal coherency, which in accordance to literature data has been shown in other solid tumour such as lung, breast and thyroid. Among the non-collagen proteins characterizing PDAC samples, several glycoproteins involved in the cell adhesion have been identified: FBLN1, FBLN 2, EMI-1, MAFAP4 and 5, POSTN, VTN, TGFBI, THBS1 and THBS2. In particular the extracellular matrix protein TGF beta-induced protein (TGFBI) and the secreted cell adhesion protein peristin (POSTN) are known to play a role in a wide range of physiological and pathological conditions, including tumorigenesis. PDAC proteoglycans were mostly represented by BGN, VCAN, HRG, and HSPG2. Among these, the Basement membrane-specific heparan sulphate proteoglycan (HSPG2) and the versican core protein (VCAN) seem to be the most interesting ones, due to their involvement in the regulation of cell motility, growth and differentiation. Of note, both proteins contain an EGF-like domain, which is usually masked in intact membranes, but become exposed when the membrane is destroyed, as in the case of tumour invasion. Conversely, the five HDE-related proteins (GP2, PNLIP, KRT1, CLPS, CUZD1) are secreted proteins involved in dietary food metabolism and are markers of healthy pancreatic tissue.

According to recreate an in vitro 3D model of PDAC, we showed that AsPc-1 cells were more capable to penetrate the scaffolds than PANC-1 that are prone to occupy the outermost portion of the scaffolds. This evidence probably is due to AsPC-1 intrinsic ability to invade tissues considered their metastatic origin. Since we used a primary PDAC cancer cell line and a metastatic cancer cell line to repopulate the patient-derived HDE and PDAC scaffolds, we firstly highlighted any possible difference in terms of cell behaviour and cell-ECM interactions. On these basis, Ki-67 analysis in PANC-1 and AsPC-1, showed that in the face of an almost totality of positive cells in the 2D conventional culture plate those grown in 3DTS and 3DHS have a reduced percentage of positive cells. This result seems to bring the 3D culture model closer to the in vivo situation compared to conventional 2D cultures. In fact, different studies which evaluated the degree of Ki-67 expression in specimens obtained from PDAC tissue block in order to correlate it with disease free and overall survival, reported an in vivo Ki-67 cells rate between 12% and 39%.

A further characterization of the 3DHS and 3DTS repopulated scaffolds with SEM analyses showed that AsPC-1 cell line were able to better engrave and colonize the scaffolds with a marked invasive phenotype compared to PANC-1 that appear to remain close to the injection site. This result seems to be in line with what Miknyoczki SJ and colleagues observed in in vivo mouse model. In fact, after 21 days from the subcutaneous injection of the AsPC-1 and PANC-1, the two tumor lines showed a different tumorigenic potential: PANC-1 generated a tumor mass of 750 mm3 while the injection of AsPc-1 generated a mass of 1450 mm3. Subsequently, we decided to analyse the expression pattern of vimentin and F-actin which are considered markers of mesenchymal differentiation. In 2D conventional culture plate a percentage of positive cells of 92% compared to pancreatic adenocarcinomas with prominent expression in liver metastases. Naito and Iacobuzio-Danahue, found an increased percentage of vimentin positive cells in metastatic pancreatic cancers as compared to primary site. Moyer et al found a percentage of positive tumor cells to vimentin of 10% and in most cases of 50%. Interestingly, these data are in complete accordance on what we obtained in the 3DHS and 3DTS repopulated scaffolds with both cell lines.
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Regarding the F-actin expression, compared to cells grown in 2D conventional plastic plate, both cell lines in the 3D acellular scaffolds showed a down-regulation of F-actin expression with a peculiar pattern of staining which highlighted how only the cells in contact with the patient-derived ECM showed a positivity for this marker. This pattern became even more evident in the AsPC-1 metastatic cell line. This data is in line with the fact that the reorganization of the actin cytoskeleton and the formation of migratory membrane protrusions are the key mechanical drives for morphological changes and gained invasive properties of metastatic cancer cells. Our results suggested that both cancer cells line react to the bioactive microenvironment of 3DHS and 3DTS by modulating the expression of this cytokin proteins.63

Finally, we decided to evaluate the contribution of the patient-derived ECM in response to chemotherapy treatment. We evaluated cells response after treatment with 5-FU, which is one of the common backbone-drug used to treat different types of solid cancer, included PDAC. Later we evaluated their chemo-response in the multi-agent chemotherapy setup, FOLFIRINOX, that nowadays resulted the first-line treatment both for locally, advanced and metastatic PDAC.64 We observed a reduced sensitivity to 5FU and FOLFIRINOX in 3D patient-derived PDAC as compared to 2D which could be attributed to the decreased compound access or reduced drug sensitivity in response to hypoxic and more slowly cycling cells under 3D culture conditions.65

Moreover, as we demonstrated, the rigid, dense, fibrotic PDAC ECM forms a physical barrier for the drugs delivery to the cancer cells, and therefore this accounts for the therapeutic resistance observed in our 3D model. In addition, we demonstrated a decreased IC50 of FOLFIRINOX compared to 2D regimen both for Panc-1 and AsPC-1 in the 3DTS recellularization conditions confirming the increased cytotoxicity of multi-agent chemotherapy compared to single-agent.66 We highlight that ECM environment is crucial in producing a model system that adequately recaps the growth and mechanical behavior of the PDAC tissue for real therapeutic assessment. The tumor stiffness is related to the resistance of the tumor to drugs, yet it is only partially considered for in current models as therapeutic assessment of potential drugs is initially performed 2D flat model. In order to develop a realistic 3D patient-derived model for drug screening it is fundamental to incorporate the correct biophysical microenvironment in the tool. To conclude, in the present paper, we obtained a PDAC patient-derived ECM that could represent a useful scaffold for the generation of a 3D patient-derived in vitro model able to assess the effects of acellular tumor microenvironment in: cells maintenance, proliferation, migration and response to different chemotherapy protocols used today in clinics.

Acknowledgments

The authors would like to thank Dr. Roberta Salmaso for her invaluable technical support. Prof. Filippo Romanato, Dr. Giulia Borile and Dr. Deborah Sandrin for their technical support with confocal microscopy and second harmonic generation analysis. Centro di Analisi e Servizi for the Certification (CEASC; http://ceasc.unipd.it/) of the University of Padova for the technical support with scanning electron microscopy. Funding: LIFELAB Program, Veneto Region; Università degli Studi di Padova, Budget Integrato per la Ricerca dei Dipartimenti: BIRD199592; Fondazione istituto di Ricerca Pediatrica- Città della Speranza: Progetto IRP2021; Università degli Studi di Padova: Progetto di Sviluppo Dipartimentale DiSCOG (Progetto Biobanca - University of Padua); Takeda Pharmaceuticals: Takeda Open Innovation Award 2020.

All authors have read the journal’s policy on disclosure of potential conflicts of interest. There are no conflicts of interest to declare. All authors demonstrate that they participated sufficiently in the work to take public responsibility for the content, including participation in the conception, design, analysis, writing, or revision of the manuscript. All authors have read the journals authorship agreement, reviewed and approved the manuscript.

Supplementary materials

Supplementary tables related with this article can be found in the online version at doi:10.1016/j.trsl.2022.08.015.

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