Three-Dimensional Primary Cell Culture: A Novel Preclinical Model for Pancreatic Neuroendocrine Tumors

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Keywords

Pancreatic tumor · Neuroendocrine tumor · 3D culture · Preclinical model · Primary cells · Drug screening · Islet-like tumoroids · Spheroids · Organoids

Abstract

Molecular mechanisms underlying the development and progression of pancreatic neuroendocrine tumors (PanNETs) are still insufficiently understood. Efficacy of currently approved PanNET therapies is limited. While novel treatment options are being developed, patient stratification permitting more personalized treatment selection in PanNET is yet not feasible since no predictive markers are established. The lack of representative in vitro and in vivo models as well as the rarity and heterogeneity of PanNET are prevailing reasons for this. In this study, we describe an in vitro 3-di-

mensional (3-D) human primary PanNET culture system as a novel preclinical model for more personalized therapy selection. We present a screening platform allowing multicenter sample collection and drug screening in 3-D cultures of human primary PanNET cells. We demonstrate that primary cells isolated from PanNET patients and cultured in vitro form islet-like tumoroids. Islet-like tumoroids retain a neuroendocrine phenotype and are viable for at least 2 weeks in culture with a high success rate (86%). Viability can be monitored continuously allowing for a per-well normalization. In a proof-of-concept study, islet-like tumoroids were screened with three clinically approved therapies for PanNET: sunitinib, everolimus and temozolomide. Islet-like tumoroids

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display varying in vitro response profiles to *distinct therapeutic regimes*. Treatment response of islet-like tumoroids differs also *between patient* samples. We believe that the presented human PanNET screening platform is suitable for personalized drug testing in a larger patient cohort, and a broader application will help in identifying novel markers predicting treatment response and in refining PanNET therapy.

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Introduction

In contrast to many other malignancies, there are no molecular characteristics and biomarkers supporting treatment decisions in pancreatic neuroendocrine tumors (PanNETs). While molecular mechanisms underlying PanNET development and disease progression are continuously further deciphered [1, 2] and numbers of clinically approved therapies are steadily rising, the treatment options for PanNET are still primarily chosen based on clinician judgment.

The lack of appropriate models and the rarity of PanNET disease are two major factors that hinder further advances in PanNET translational research. Testing more effective therapies as well as performing predictive studies are lagging behind. Currently, only a limited number of human PanNET cell lines are available – with BON1, QGP1 and CM being the most commonly studied [3–6]. It is pertinent to note that while these cell lines were used to experimentally dissect molecular mechanisms of NETs, they do not represent well-differentiated slow-proliferating PanNETs. All of these cell lines are highly proliferative and were found to differ fundamentally in their mutational genetic background compared to PanNETs. In fact, studies have shown a strong resemblance to poorly differentiated pancreatic neuroendocrine carcinomas rather than PanNETs [7–11]. Moreover, other authors have even questioned the tissue of origin of the aforementioned cell lines, raising a significant debate as to the translational relevance of work performed using these cell lines [11, 12]. Recently, Benten et al. [13] described NT-3 as a novel cell line that better reflects well-differentiated slow-proliferating PanNETs, which present the bulk of PanNETs. Nevertheless, the full molecular profile of NT-3 cells remains to be determined so that its similarity to the primary well-differentiated slow-proliferating subtype can be established. Moreover, neither available cell lines nor genetically engineered mouse models recapitulate the spectrum of different molecular subtypes found in human primary Pan-

NETs [2, 14]. A stronger focus on developing more personalized in vitro models for studying these tumors is therefore demanded urgently. Cultivation and expansion of patient-derived neuroendocrine cells has been challenging owing to their intrinsically poor capacity for in vitro proliferation. However, due to advances in cell culture techniques, cell models of well-differentiated slow-proliferating PanNET derived from primary tissue have recently been used to study drug response and dissecting its underlying molecular mechanisms. For example, studies on isolated human primary PanNET cells cultured in vitro indicated that such a model might be utilized to determine patient response to treatment [15–18]. However, the major limitations of all these studies are the short cultivation window of the cells, the non-physiological 2-dimensional (2-D) format with limited cell-cell interactions, as well as their small scale in terms of investigated treatments and patient numbers.

In recent years, there have been tremendous advances in the development of 3-dimensional (3-D) tissue culture techniques, including scaffold-free setups in ultra-low attachment plates or scaffold-based encapsulation cultures to allow cell growth in 3 dimensions [19–23]. Culture of cells in 3 dimensions mimics a more physiological architecture of a tumor tissue, including cell-cell contact and allowing the development of spatial differences in the culture system with respect to proliferation, cell death and hypoxia within spheroids [21, 24–26]. Additionally, cells kept in a 3-D format can be cultured and treated longer than in 2-D monolayers [21]. Cells cultured in 3 dimensions frequently display increased therapy resistance compared to cells cultured in 2 dimensions [27–29], where 3-D culture most likely better reflects the in vivo situation [30, 31]. For this reason, lately high-throughput screenings of pharmacological compounds were preferentially performed in 3-D-cultured cells [32–34].

With the presented study, we aimed for developing a platform to collect PanNET samples from multiple surgical centers, to isolate primary cells and to cultivate these cells in 3 dimensions retaining NET characteristics and finally to measure short- and long-term in vitro treatment response.

Lead Contact and Material Availability

Further information and request for resources and reagents should be directed to and will be fulfilled by the Lead Contact Dr. I. Marinoni (Ilaria.marinoni@pathology.unibe.ch).

Experimental Model and Subject Details

Primary Cell Culture

Isolated primary PanNET cells were maintained in AdvDMEM + GF medium (DMEM-F12, 5% FBS, Hepes 10 mM, 1% L-glutamine (200 mM), 1% penicillin (100 U/mL), 1% streptomycin (0.1 mg/mL), 1% amphotericin B (0.25 mg/mL) (Merck, Switzerland), 20 ng/mL EGF, 10 ng/mL bFGF (Thermo Fisher Scientific, USA), 100 ng/mL PlGF, 769 ng/mL IGF-1 (Selleckchem, USA)) and in 24-well Corning® Costar® ultra-low attachment (ULA) plates (Corning, USA) (500 μ L/well, $3-5 \times 10^5$ cells/well) in a humidified cell incubator (21% O₂, 5% CO₂, 37°C). For drug screen cells were resuspended in fresh AdvDMEM + GF medium supplemented with 123 μ g/mL growth-factor-reduced Matrigel® (Corning, USA) and plated in 96-well ULA plates (50 μ L/well, $3-4 \times 10^3$ cells/well).

To set up the PanNET screening platform including drug screening, we made use of primary material from a total of 16 PanNET patients depicted in online supplementary Table 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000507669). Additionally, key resources used in this study are provided in Table 1.

Patient Studies

All patients agreed on the use of residual material and had signed an institutional informed consent. Patient characteristics are shown in online supplementary Table 1. The study was approved by the cantonal authorities (Kantonale Ethikkommission Bern, Ref.-Nr. KEK-BE 105/2015) and the Italian ethic commission (Comitato Etico, CE 252/2019).

Methods

Patient Samples and Cryopreservation

Fresh human PanNET tissue was obtained from patients diagnosed with PanNET undergoing surgery at the Inselspital Bern, Switzerland, or at the Pancreatic Surgery Unit, Pancreas Translational and Clinical Research Center, San Raffaele Scientific Institute, Milan, Italy. Tumor tissues of 16 PanNET patients were used for 19 isolations, 12 isolations for method establishment and 7 for a proof-of-concept drug screening. Patient characteristics are summarized in online supplementary Table 1.

Upon surgical resection a pathologist processed one mirror block of the tumor tissue to 8-mm³ blocks under sterile conditions avoiding necrotic regions. These blocks

were suspended in recovery cell culture freezing medium (Thermo Fisher Scientific, USA), cryopreserved using an isopropyl alcohol freezing container (Nalgene, USA) and stored in liquid nitrogen. The other mirror block was embedded in a microcassette, and fixation was performed with a PAXgene Tissue System according to the manufacturer's instructions. In short, tissue was incubated in a PAXgene Tissue FIX Container (Qiagen, Germany) at room temperature overnight. Fixated tissue was transferred into a PAXgene Tissue FIX Container (Qiagen, Germany) at 4°C until paraffin embedding (1–2 days) or kept at –20°C if embedding was not performed instantly.

Primary Cell Isolation and Culture

For primary cell isolation, tissue was thawed for 45–60 s in a 37°C water bath and cut into 1-mm³ pieces and washed with medium (advanced DMEM-F12, Hepes 10 mM, 1% L-glutamine, 1% penicillin-streptomycin-amphotericin B) and Dulbecco's phosphate-buffered saline (DPBS; Thermo Fisher Scientific, USA). After aspiration of the medium, the tissue was incubated in 5 mL digestion medium (10 mg/mL collagenase IV (Worthington, USA), 0.25% Trypsin-EDTA (Sigma-Aldrich, Switzerland), 10 mg/mL DNase (Roche, Switzerland) in advanced DMEM-F12, Hepes 10 mM, 1% L-glutamine, 1% penicillin-streptomycin-amphotericin B) in a gentleMACS™ dissociator (Miltenyi Biotec, Switzerland) for 1 h at 37°C (programme TDK_1). After digestion, trypsin was deactivated with AdvDMEM (advanced DMEM-F12, 5% FBS, Hepes 10 mM, 1% L-glutamine, 1% penicillin-streptomycin-amphotericin B), and cells were filtered through a 70- μ m smart strainer (Miltenyi Biotec, Switzerland) to remove debris of collagen. Red blood cells were lysed for 3 min with ACK lysis buffer (Thermo Fisher Scientific, USA) at room temperature. After 180 g centrifugation and aspiration of supernatant, the pellet was resuspended in AdvDMEM + GF medium (DMEM-F12, 5% FBS, Hepes 10 mM, 1% L-glutamine, 1% penicillin-streptomycin-amphotericin B, 20 ng/mL EGF, 10 ng/mL bFGF (Thermo Fisher Scientific, USA), 100 ng/mL PlGF, 769 ng/mL IGF-1 (Selleckchem, USA)). The cell suspension was plated in 24-well plates (cell + coated and tumor cells tested, Sarstedt, Germany) followed by a short spin for 30 s, at 200 g and incubation for 1 h (21% O₂, 5% CO₂, 37°C) to partially segregate fibroblasts by attachment. The supernatant was collected. For single cell dissociation the cell suspension was transferred into a 5-mL falcon tube and shortly spun down depending on cell/aggregate size. If large aggregates were present, cells were spun at 100–200 g; if smaller aggregates were present, cells were spun at 200–

Table 1. Key Resource Table

Reagent or resource	Source	Identifier
Antibodies		
Anti-human Ki-67 (1:200)	Dako	M7240
Anti-human synaptophysin (1:4,000)	Novocastra	27G12
Anti-human insulin (1:100)	Sigma	I-2018
Biological samples		
Human PanNET specimen	This study	n.a.
Primary human PanNET cell culture	This study	n.a.
Devices and consumables		
Gentle MACS™ dissociator	Miltenyi Biotec	n.a.
Cell + coated and tissue culture tested 24-well plates	Sarstedt	83.3922.300
24/96-well Corning® Costar® ultra-low attachment plates	Corning	CLS4515
PAXgene Tissue FIX Container	Qiagen	765312
PAXgene Tissue STABILIZER	Qiagen	765512
Infinite® 200 PRO plate reader	Tecan	n.a.
Automated immunostainer Bond RX	Leica Biosystems	n.a.
Automated Slide Scanner panoramic 250	3DHistech	n.a.
Chemicals and peptides		
Recovery freezing medium	Thermo Fisher Scientific	12648-010
Advanced DMEM-F12	Thermo Fisher Scientific	D6421
Collagenase IV	Worthington	LS004188 CLS-4
Trypsin-EDTA (0.25%)	Sigma Aldrich	T4049
DNase	Roche	10104159001
Ammonium-chloride-potassium lysis buffer	Thermo Fisher Scientific	A1049201
Epidermal growth factor	Thermo Fisher Scientific	PHG0311
Basal fibroblast growth factor	Thermo Fisher Scientific	PHG0026
Placenta growth factor	Selleckchem	264-PGB-010
Insulin-like growth factor 1	Selleckchem	100-11
Accutase	Thermo Fisher Scientific	A11105-01
Growth-factor-reduced matrigel	Corning	354230
Real-time Glo™ MT cell viability assay	Promega	G9712
Human plasma	Interregional Blood Transfusion SRC Epalinges	92040
Thrombin	Diagnostec	100-125
Sunitinib (S1042)	Selleckchem	S1042
Everolimus (S1120)	Selleckchem	S1120
Temozolomide (S1237)	Selleckchem	S1237
n.a., not available.		

300 g. The cell pellet was washed with DPBS and incubated in Accutase (Thermo Fisher Scientific, USA) supplemented with DNase (10 mg/mL) (Thermo Fisher Scientific, USA). Cells were carefully dissociated by repeated (10–15×) passage through a 1-mL syringe and 26 G 0.45 × 13 mm Microlance™ (BD, Switzerland) until aggregates were not visible anymore. The cell number was estimated using a hemocytometer, and cells were seeded in AdvDMEM + GF medium in 24-well ULA plates (5 × 10⁵ cells/mL/well). After 2 days of recovery phase, cellular aggregates were collected in 15-mL falcon tubes and differ-

entially centrifuged at 120 g for 5 min to separate cells and aggregates from debris/apoptotic cells. Supernatant was aspirated to remove semi- and nonviable cells. Cells were counted and resuspended in fresh AdvDMEM + GF medium supplemented with 123 µg/mL growth-factor-reduced Matrigel and plated in 96-well ULA plates (50 µL/well, 3–4 × 10³ cells/well). The setup consisted of 6–9 DMSO-positive control wells, 6 no-cell-negative control wells and technical triplicates for each drug concentration.

Viability Measurement

RealTime-Glo™ MT Cell Viability (RTG) assay (Promega, Switzerland) was used to repeatedly monitor cell viability in 3-D human primary PanNET culture. The RTG assay was performed according to the manufacturer's instructions, and luminescence was measured in an Infinite® 200 PRO plate reader (Tecan, Switzerland). In brief, after 3 days of sphere formation, conditioned medium of each well was supplemented with additional 50 µL of fresh AdvDMEM + GF medium containing Matrigel and RTG assay reagents (2×) to a final volume of 100 µL. Growth factors and FBS were replenished from a concentrate (0.77 µL GFs (130×) + 5 µL FBS) every 3–4 days in each well using a multichannel pipette. A 6-h RTG baseline before adding drug compounds was recorded for every well at day 0 of the drug screen. For calculating the in vitro growth curve, relative luminescence unit (RLU) values were normalized to corresponding baselines. For calculating the in vitro drug response, RLU values were normalized first to corresponding baselines followed by normalization to the DMSO control wells of a particular day as described in more details in the paragraph “curve fitting and drug sensitivity data.”

Micro-Cell-Block from Islet-Like Tumoroids

Islet-like tumoroids corresponding to $3\text{--}5 \times 10^4$ cells were collected in a 1.5-mL Eppendorf tube (either directly on the day of isolation [D0] or from 6–9 wells of a 96-well ULA plate at the end of a drug screen [D15]). Tumoroids were washed with DPBS and pelleted at 350–500 g. The supernatant was aspirated, and the cells were resuspended in human plasma derived from whole blood (Interregional Blood Transfusion SRC, Epalinges, Switzerland) and Thrombin (Diagnostec, Switzerland) (ratio 5:1) followed by 3-min incubation at room temperature. The clot was fixed with 4% PFA for 30–60 min protected from light. After a DPBS wash, the supernatant was aspirated and cells were incubated in a hematoxylin and DPBS solution (ratio 1:8) on a rocker shaker for 10–15 min at room temperature. The counterstained clot was transferred to a plastic microcassette for paraffin embedding. For immunohistochemistry the embedded material was cut into 2.5-µm-thick serial sections followed by deparaffinization, rehydration and antigen retrieval using an automated immunostainer (Bond RX, Leica Biosystems, Germany). Antigen retrieval was performed for Ki-67 (Dako M7240) with Tris for 30 min at 95°C, insulin (Sigma I-2018) and synaptophysin (Novocastra 27G12) with Tris for 30 min at 100°C. Antibodies were diluted as follows: Ki-67 1:200, insulin 1:4,000, syn-

aptophysin 1:100. Slides were counterstained with hematoxylin. Scans were acquired with an automated slide scanner Panoramic 250 (3DHitech, Hungary) at 20× magnification. Images were acquired using QuPath software [35].

Drug Preparation

Compounds (sunitinib (S1042), everolimus (S1120) and temozolomide (S1237)) were obtained from commercial vendors and stored as stock aliquots at –80°C. A 5-point, 625-fold concentration range was used for all compounds in order to have enough data points and a sufficient large drug concentration window to calculate reliable absolute IC₅₀ values [36]. The starting dosage for each compound was selected based on IC₅₀ screens in cancer cell lines publicly available online (see Cancerrxgene.org, PharmacDB, Cancer Drug Resistance DB), from literature search and/or from in vitro data from pilot human primary cell cultures and/or from PanNET cell lines (QGP1, NT3, BON1).

Curve Fitting and Drug Sensitivity Data

Drug-response curve data consisted of 6–9 DMSO-positive controls, 6 no-cell-negative controls, and 5 drug-response points for a 625-fold concentration range. For IC₅₀ calculation RLU values that were derived from an RTG assay from short-term treatment on day 3 and long-term treatment on day 7 of each well were weighted and normalized as the following: RLU values from each 6-h RTG baseline measurement (RLU_x d0) were scaled with the overall minimal value of day 0 for each plate (RLU_{min} d0) and transferred into a baseline weight (RLU_x weight) for each well to minimize well-to-well variability:

$$RLU_x \text{ weight} = \frac{RLU_x \text{ d0}}{RLU_{\min} \text{ d0}} \quad (1)$$

Each RLU value from day 3 was then accordingly weighted to its baseline weight:

$$RLU_x \text{ d3/7 weight} = \frac{RLU_x \text{ d3/7}}{RLU_x \text{ weight}} \quad (2)$$

The percentage response from weighted RLU was calculated by normalizing each value to no-cell-negative control (0%) and DMSO-positive control (100%) intervals. These data points were fitted in a 4-parametric linear-regression model [34, 37] with two constraints, top = 100% and bottom = 0%, to estimate corresponding IC₅₀. IC₅₀ value differences of >4-fold were clustered in strong-responder and weak-responder groups. In case of an IC₅₀

value >2.5-fold higher than the highest tested target-concentration samples were considered as non-responder (NR).

Quantification and Statistical Analysis

QuPath Image Analysis

Using QuPath software [35] digital-scanned hematoxylin-eosin and synaptophysin tissue sections were first preprocessed in the built-in visual stain editor using default settings for estimation of stain vectors to improve staining quality. Each tissue section was then superimposed with a 1,000- μ m grid box. In each tissue section one representative tile out of the grid box was selected by a cytopathologist (M.T.) as a training set. Using a watershed segmentation method, positive and negative cells were automatically detected within each representative tile. Two pathologists (M.T., A.P.) then manually reconfirmed positive cell detection based on histomorphological features including cellular and nuclear shape, tumor cell nest formation, tumor columns, nuclear “salt and pepper” structure, nuclear and cytoplasmic staining intensity. A minimum of $\geq 1,000$ cells were selected for each training set and a total of 67 parameters (including perimeter, circularity, staining optical density, etc.) were included for training of the random-trees machine learning classifier. The auto-update tool within QuPath allowed real-time reconfirmation of training efficiency/accuracy. These cell detection parameters were applied on the whole tissue slides by creating a script which performed automated cell classification/annotation. Detection results were extracted from QuPath and imported and analyzed within R.

Graph Pad Prism (Version 8.2.1) and R statistical environment were used for data analysis and visualization in the R version 3.6.2 (2019-12-12) platform: x86_64-w64-mingw32/x64 (64-bit). Attached base packages: Grid; stats; graphics; grDevices; utils; datasets; methods; base. Other attached packages: 81) scales_1.1.0; (2) MASS_7.3-51.4; (3) reshape2_1.4.3; (4) ConsensusClusterPlus_1.50.0; (5) circlize_0.4.8; (6) ComplexHeatmap_2.2.0; (7) RColorBrewer_1.1-2; (8) Rmisc_1.5; (9) plyr_1.8.5; (10) lattice_0.20-38; (11) plotrix_3.7-7; (12) cowplot_1.0.0; (13) forcats_0.4.0; (14) stringr_1.4.0; (15) dplyr_0.8.3; (16) purrr_0.3.3; (17) tidyr_1.0.0; (18) tibble_2.1.3; (19) ggplot2_3.2.1; (20) tidyverse_1.3.0; (21) broom_0.5.3; (22) readr_1.3.1.

Hierarchical Clustering Analysis of Drug Response Profiles

Using the ConsensusClusterPlus pipeline [38], the number and membership of clusters was determined for drug response profiles based on patient-specific IC_{50} values of all three drug treatments. Distances were calculated using Pearson's correlation sorted by an agglomerative hierarchical clustering algorithm. The WardD2 algorithm was used for subsampling, and the final consensus matrix was determined by group average.

Gene Expression Analysis

Mean expression values of growth factor receptors were analyzed in publicly available data of 26 PanNET patients. RNAseq data were downloaded from the ICGC Data Portal (PAEN-AU project). QC, mapping/alignment and raw count quantification is described in Scarpa et al. [2]. From RSEM data output for our downstream analysis we chose the FPKM (fragments per kilobase of exon per million fragments mapped) normalization method to account for sequencing depth and gene length for all raw read counts. A list of all available growth factor receptor was acquired from the UniProt Knowledgebase [39]. Expression values of all targets were transformed into a 0-to-1 scale for each patient to allow inter-patient comparability. Mean values for each target receptor were then calculated in all of the 26 PanNET patients:

$$\text{Expression value} = \text{mean}(\text{scaled}_{0,1}[\text{FPKM normalized raw counts}]) \quad (3)$$

χ^2 Test and Monte-Carlo Simulation

A χ^2 test of independence was conducted among all variables of interest. In order to meet requirements for χ^2 test statistic and to account for relatively small expected cell frequencies our data set was resampled using a Monte Carlo simulation (replication = 1×10^5) allowing to calculate p value estimates.

Data and Code Availability

The original RNAseq data set from human primary PanNET is publicly available at the ICGC Data Portal (PAEN-AU project). The complete expression data of growth factor receptors are available in the supplementary data sheet. The code supporting the current study has not been deposited in a public repository because the analysis code was generated from generic R packages, but the code is available from the corresponding author on request.

Fig. 1. Human primary PanNET cells form islet-like tumoroids and retain a neuroendocrine phenotype in vitro. **a** Schematic representation of human PanNET screening platform. **b** Venn diagram displaying usage of human PanNET patient material (outer circle) and individual patients (inner circle). The success rate for drug screening in PanNET patient material was 86% (6/7). Detailed log of cell isolation is provided in the online supplementary data file 1. **c** Representative hematoxylin-eosin (HE) and immunohistochemistry staining of islet-like tumoroids from B992 and

original primary tumor tissue B992 (top) and islet-like tumoroids from B563m and original metastatic tumor tissue from B563m (bottom). Cultured cells were formalin-fixed and embedded after 14 days in PanNET culture medium. Formalin-fixed primary PanNET tissue or cultured cells were stained for hematoxylin-eosin (HE), synaptophysin (SYN), insulin (INS) and Ki-67. Immunohistochemistry slides were counterstained with hematoxylin. Scale bar, 50 μ m.

Results

Cryopreservation Allows a Multicenter Approach

PanNET tumors are rare, therefore a collaborative network is crucial. Here we propose a novel platform for an effective multicenter approach which permits biobanking of cryopreserved PanNET tissues from multiple surgical centers by a single central institution which performs primary cell isolation and drug testing (Fig. 1a). After tumor resection, half of the specimen was formalin-free PAX-gene-fixed and paraffin embedded. These so-called mirror blocks served as controls for sample quality and were used to preassess patient-specific PanNET characteristics and tumor cell content in hematoxylin-eosin stainings and synaptophysin immunohistochemistry – a NET biomarker routinely used in clinics for diagnosis of PanNET. The other half was immediately cryopreserved in recovery freezing medium and later shipped and processed for primary cell isolation and in vitro drug screening.

Development of a 3-D Human Primary PanNET Cell Culture Model

Within this study, we performed 19 isolations from samples of 16 PanNET patients. Patient characteristics are summarized in online supplementary Table 1. In the first part of the study (12 patients), we set up the sampling, isolation and culture conditions. Two additional isolations were used for live-cell imaging to observe tumoroid formation. In the proof-of-concept part of the study (7 patients, including 3 patients who were also included in the method and development cohort), we tested the suitability of the setup for in vitro drug screening (Fig. 1b). During method development, we successfully isolated small aggregates and single cells from cryopreserved patient material in 73% (8/11) (online supplementary data file 1). Age, sex and other clinical parameters such as WHO grade, TNM staging and Ki-67 indices did not reveal a significant association with the isolation success and/or cellular yield (online suppl. Fig. 1A).

In 3 patient samples, isolation did not yield aggregates or single cells, which we attribute to the collection of largely acellular fibrotic or necrotic tissue as revealed from hematoxylin-eosin staining of corresponding mirror blocks (online suppl. Fig. 1B). Therefore, careful macroscopic selection of viable cellular tumor regions is crucial. Upon implementation of SOPs for sampling at the surgical centers, we observed strong quality improvements. In 2 patient samples (P005, P051) yielding successful cell isolation, we detected overgrowth of fibro-

blasts after 15 days (online suppl. Fig. 1C). This led us to implement a low FBS concentration in the culture medium and to include a partial fibroblast reduction step during cell isolation. Thus, the cell suspension was plated on coated plastic for 2 h followed by gently rinsing for the collection of low-adhesive nonstromal cells. Two tumor cell extracts (P032x, P033) were negative for synaptophysin immunohistochemistry (IHC) staining on conventional cytospin preparations and hence excluded for further culture. However, a post hoc analysis by a cytopathologist (M.T.) revealed that these cells were tumoral cells (online suppl. Fig. 1D). Consequently, to assess tumor cell content accurately, we implemented a formalin-fixating paraffin-embedding technique termed Micro-Cell-Block. Micro-Cell-Blocks retain cellular- and tumoroid morphology and require only a low cell number. Micro-Cell-Blocks on the day of isolation (D0) served as an internal quality control to assess successful fibroblast removal and to guide decision for continuation of the drug screening pipeline. Micro-Cell-Blocks at the end of the experiment (D15) allowed to quantify tumor cell content from synaptophysin immunohistochemistry and to reconfirm target cell identity on hematoxylin-eosin staining.

To account for more physiological cell culture conditions a PanNET-specific culture medium was developed combining literature and human transcriptomic data from 26 low-grade PanNETs [2]. We selected growth factors that were frequently reported in the PanNET literature [13, 15, 40] and for which – except of EGFR – all the target receptors (FGFR1, IGFR1/2, FLT1/VEGFR1, EGFR) were within the upper expression quintile (<28/151) of all currently available growth factor receptors and related proteins [40] in human PanNET patients (online suppl. Fig. 1A). Additionally, PanNET culture medium was supplemented with a low percentage of extracellular matrix. Several findings showed improved in vitro culture from Matrigel complementation due to scaffolding support [41–45]. Low concentration Matrigel supplementation stabilized PanNET culture, without leading to a transient artificially increased cellular growth as seen with higher supplementation (data not shown).

Human Primary PanNET Cells Form Islet-Like Tumoroids and Retain a Neuroendocrine Phenotype in vitro

After isolation and cell culture refinement we performed live-cell imaging in two human PanNET samples. We isolated single cells from cryopreserved primary PanNET tissue (B992) and PanNET liver metastasis (B563m).

Fig. 2. 3-D human primary PanNET in vitro model for a personalized drug-screening platform. **a** Detailed schematic representation of in vitro 3-D drug-screening pipeline in human primary PanNET cells. GF, growth factor replenishment; thin ticks + digit, RTG viability assessment; MCB, Micro-Cell-Block. **b** In vitro growth curve of all screened primary PanNET samples using the metabolic surrogate assay RealTime-Glo (RTG) in 3-D human pri-

mary PanNET culture. Cells were cultured in AdvDMEM + GF and a low percentage of matrigel in 0.16% DMSO for 10 days. Normalization was calculated based on per-well 6-h RTG baseline measurement. Data represent means $\pm$ SEM ($n = 7$, 3 technical replicates). RLU, relative luminescence unit.

Isolated cells from both patient specimens were viable. Live-cell imaging for 12 days revealed that isolated cells formed structures similar to extracted murine islets [46] which we hence termed islet-like tumoroids (online suppl. Fig. 2B). Islet-like tumoroids reached a more defined round structure after 72 h through aggregation and thereafter remained stable in volume (online suppl. Fig. 2C, suppl. video). Fourteen days after isolation, histomorphology of the islet-like tumoroids was examined and compared to corresponding mirror blocks. Islet-like tumoroids from primary and metastatic PanNET patient samples retained expression of synaptophysin, confirming that most of the cells consisted of tumor cells with preserved neuroendocrine phenotype (Fig. 1c). Furthermore, islet-like tumoroids from B992 expressed insulin as the original tumor tissue. The low percentage of Ki-67-positive tumor cells (2%) in vitro matched with the prolifera-

tion index described in the original tumor tissue (Ki-67 index <2%) (Fig. 1c, top, online suppl. Table 1). Similarly, the percentage of proliferating cells was retained in the metastatic PanNET sample (B563m), with a Ki-67 index of 12% in the original tumor tissues and 15% in cultured cells, respectively (Fig. 1c, bottom, online suppl. Table 1).

Setting Up an in vitro Drug Screening Pipeline for Islet-Like Tumoroids

After the successful pilot experiment, we sought to implement a pipeline for in vitro drug screening (Fig. 2a). Following a 2-day recovery phase after initial isolation, cells were replated from a 24-well format into a 96-well format. By this time, the majority of semi- and nonviable cells from isolation had segregated from viable cells. As seen in previous live-cell imaging analysis (online suppl. Fig. 2B, C), during 72-h incubation in the 96-well plate,

Fig. 3. Effect of standard of care pharmacological treatments on cell viability in 3-D human primary PanNET culture. **a** Representative in vitro viability curves using the metabolic surrogate assay RealTime-Glo (RTG) in 3-D human primary PanNET culture (P050) treated with 0.16% DMSO (control, Ctrl) or indicated treatment sunitinib (SUN), everolimus (EVE) and temozolomide (TEM) for 10 days. Normalization was calculated based on per-well 6-h RTG baseline measurement and corresponding DMSO control of the respective day. For all tested compounds a 5-point, 625-fold concentration range was used based on vast literature research and in-house in vitro preliminary studies. Data represent means $\pm$ SEM ($n = 1$ per patient, 3 technical replicates). RLU, rela-

tive luminescence unit. **b** In vitro dose response curves of screened PanNET patient displaying IC_{50} for SUN, EVE and TEM after short-term (72 h) treatment. Treatment responses (means $\pm$ SEM) were fitted into a 4-parameter logistic regression model in GraphPad software to calculate absolute IC_{50} . Data represent fitted curve means ($n = 7$). Dotted line, absolute IC_{50} . **c** Heat map comparing absolute IC_{50} s for SUN, EVE and TEM in 3-D human primary PanNET culture after short-term (72 h) treatment. The heat map was derived using the WardD2 clustering method with displaying Pearson's clustering distance using ComplexHeatmap R-package [38]. The color code represents the scaled IC_{50} (Z score) for each drug. Each row represents an individual patient.

cells formed islet-like tumoroids with only minor changes thereafter indicating a suitable time point for starting the drug treatment. Growth factors were replenished on days D2, D5, D8 and D12 after initial isolation. Due to low cell numbers available from PanNET specimens we selected RTG – a metabolic nonlytic assay – as a surrogate of cell viability. Pretreatment 6-h baseline measurements were recorded to normalize on an individual well basis and to correct for variability in cell number. Viability of

islet-like tumoroids in each well was repeatedly ($8\times$) monitored over a time course of 10 days before storing the cell material for further downstream analysis.

PanNET Screening Pipeline in Control Conditions

As a proof-of-concept experiment, we tested the PanNET drug-screening pipeline with 7 patient samples. Tumor cells were successfully isolated in all 7 PanNET specimens. Quantification of tumor cells in mirror blocks of

original tumor tissue showed variable tumor content within tissues and among patients ($70 \pm 18\%$, $n = 7$) (online suppl. Fig. 3A, B). Yet, assessment of hematoxylin-eosin stainings and synaptophysin immunohistochemistry on Micro-Cell-Blocks on the day of isolation (D0) by a cytopathologist (M.T.) reconfirmed successful selection of tumor cells after isolation and fibroblast depletion in all samples used for drug screenings ($93 \pm 15\%$, $n = 7$) (online suppl. Fig. 3A, B). Moreover, successful culture of tumor cells was also reconfirmed in Micro-Cell-Blocks at the end of each experiment (D15) ($95 \pm 11\%$) (online suppl. Fig. 3A, B). In all patient samples, islet-like tumoroids were formed and remained viable in 85% (6/7) for 15 days in culture.

Following the metabolic activity of untreated islet-like tumoroids over the time course of 10 days, we observed an association between in vitro proliferation and Ki-67 index in original tumor tissue in the majority of samples: the metastatic patient sample (B563m) with a Ki-67 index of 15% in the original tumor tissue displayed the highest signal increase (3.8-fold), while in 4 patient samples with lower Ki-67 indices (P049, P050, P051, B931) the signal only increased between 1.1- and 1.7-fold (Fig. 2b). In patient sample P044 this association was weak exhibiting a 1.6-fold increase despite a Ki-67 index of 18% in the original tumor tissue. P040 was the only sample with a decreasing signal in the untreated condition; hence, long-term time points (>72 h) from this particular patient sample were not included in further analysis.

In vitro Drug Response in Islet-Like Tumoroids Shows Distinctive Sensitivity Profiles

To assess whether 3-D human primary PanNET culture could be exploited to predict patient drug response in vitro, we evaluated the effect of three clinically approved PanNET treatments on cell viability [47]. 3-D human primary PanNET cultures from 7 different patients were screened for response to sunitinib, everolimus and temozolomide. A 5-point, 625-fold drug concentration range ensured a sufficient exploratory drug screening window for accurate IC_{50} estimation based on mathematical modeling [36]. As starting points IC_{50} s from publicly available databases were interrogated for each drug, followed by pilot assessments of their antiproliferative effect in PanNET cell lines and murine primary cells (data not shown) as well as further literature research. Cells were treated for 10 days, and viability was repeatedly monitored at 8 time points during drug screening. Drug response profiles differed clearly among the three standard of care treatments. Dose-dependent effects of sunitinib and everolimus were observed in all tested patient sam-

ples (Fig. 3a, online suppl. Fig. 4A). Interestingly, comparing interpatient drug responses we detected varying treatment sensitivities among patients (online suppl. Fig. 4A). IC_{50} values determined after short-term (72 h) treatment displayed two clearly distinct groups within sunitinib treatment and within everolimus treatment harboring >4 -fold differences in respective IC_{50} (Fig. 3b). Also consensus clustering matrix and hierarchical cluster analysis ($k = 4$) displayed robust response groups for short-term (72 h) treatments (Fig. 3c): a strong-responder group with samples sensitive to both treatments (P049), a group responding either primarily to everolimus (P049, P040, B563m) or to sunitinib (P050, B931, P051) – which was considered as mixed-responder group – and a weak-responder group including one sample insensitive to all treatments (P044). Importantly, in an integrative hierarchical cluster analysis, short-term treatment IC_{50} s – for the majority of patients – clustered closely together with long-term treatment IC_{50} s emphasizing robustness of the readouts (online suppl. Fig. 4B). Interestingly, in 1 case (B931) differences between short-term and long-term treatment were detected (online suppl. Fig. 4B).

Discussion/Conclusion

Current murine and human cell line models do neither accurately represent human well-differentiated slow-proliferating PanNETs, nor distinct human molecular subtypes, nor interpatient variability. In this study, we present a human PanNET screening platform allowing multicenter sample collection of cryopreserved patient specimens with an 86% success rate in primary cell isolation and cell culture. Isolated cells of well-differentiated slow-proliferating PanNET can be cultured in 3 dimensions and screened in vitro assessing response profiles to standard of care treatments for PanNETs. Since the cell number was the major limiting factor, we established protocols that are optimized for minimal amounts of resection specimens.

We present cryopreservation as a solution to make multicenter studies possible, thereby overcoming the issue of the rarity of PanNET samples. While difficult to implement in different centers, this generation of “living cell repositories” is promoted as innovative biobanking setting [48] and increasingly used in translational cancer research [49]. To account for more physiological conditions, growth factor supplementation for our PanNET culture medium was based on a combination of literature research [13, 15, 40], transcriptomic analysis of growth factor receptors as well as pilot experiments testing different growth factor concen-

trations and combinations. Final PanNET culture medium composition was selected according to best retention of viability during a 15-day period to minimize selection. This approach is clearly different from classical organoid approaches, where the culture medium selects for stem cell-like cells, and where these cells are kept individually in a biomatrix in order to produce clonal organoids [50, 51]. The aim of our presented “tumoroid” model is to in vitro treat a similar tumor cell composition as present in the patient. Following this approach, we can obtain a remarkable success rate of 86%, but we acknowledge that classical organoid models have many other advantages such as the potential to intervene mechanistically [52–54]. With a retention of $\pm 70\%$ of isolated cells in experiments before drug screening we believe that selection bias is minimal and that we are capable of treating the majority of cells representing the original tumor [55]. Compared to the limited number of studies using primary PanNET tissues in 2-D culture [15–18], we observe a reproducibly extended life span of the isolated cells up to 15 days. Longer experiments would also be possible at least in a subgroup of tumors; however, typically we did observe major responses already during the first 3 days of treatment.

We show that primary cells isolated from PanNET express original tumor characteristics and retain their neuroendocrine phenotype after 15 days. Interestingly, isolated cells form islet-like tumoroids in vitro. Similarly, nonneoplastic endocrine pancreatic cells are physiologically structured as islets. Kojima et al. reviewed the history of abundant findings which revealed that single cell suspension of endocrine pancreatic tissue from several species form islet-like structures and reconstitute their original architecture in vitro [56]. Currently, we do not know whether this reflects an endocrine specific phenotype or an even broader epithelial phenotype.

With the conditions presented, isolation and culture were successful in 6 out of 7 patients (86%). Only 1 sample (P040) showed a loss of viability after 7 days and was excluded from further long-term treatment analysis. During 3-D culture, all G1 PanNET patient samples expectedly displayed only marginal growth, whereas the metastatic patient sample B563m (Ki-67 index of 15%) showed the highest growth in vitro (3.8-fold in 10 days). One G2 sample (P044, Ki-67 index of 18%) exhibited a somewhat lower growth of 1.6-fold in 10 days. It seems not surprising to infrequently observe a rather weak association of Ki-67 index and in vitro proliferation. Indeed, a clear linear correlation of Ki-67 index to tumor growth rate has not been demonstrated clinically, to our knowledge. Biologically, proliferation represents only one aspect out of

many: we neither have knowledge about a different fraction of cell death within our PanNET specimen nor do we know exact durations of cell cycles for the isolated cells from individual patient specimens.

In vitro treatment with clinically approved chemotherapeutics for advanced PanNET disease revealed overall distinctive response profiles and drug sensitivities based on IC_{50} s. Comparing short-term (72 h) versus long-term treatment (7 days and 15 days) showed identical results in the majority of samples, indicating that these different time windows are potentially of minor importance. However, in 1 tumor (B931) we observed differences between short-term and long-term treatment. While it might not be important to use long-term treatment for detecting primary response in sunitinib and everolimus treatment, prolonged treatment could be of potential importance for other chemotherapeutics. In our series, we do not see a clear response to temozolomide in all of the 7 PanNETs examined. A possible explanation for this is the mechanism of action of temozolomide, which is strongly linked to cellular proliferation. Cytotoxicity of temozolomide is mediated by O_6 -methylguanine adducts, which can mispair with thymine during DNA replication. The resulting futile cycles of DNA processing induce cytotoxic double-strand DNA breaks that trigger apoptosis [57, 58]. Due to the low proliferation rate of our samples a time window of 10 days may be still too short for a detection of measurable effects. In line with that, the metastatic patient sample (B563m) that proliferated in vitro shows at least a faint response to temozolomide in our screen – even if the IC_{50} estimation is still far from our tested drug concentration window (online suppl. Fig. 4A) and even if the sample has been scored as weak responder. We can exclude nonpotency of the chemotherapeutic compound itself since our implemented 625-fold concentration window (0.46–288.00 μM) covers a sufficiently large drug window tested in PanNET and glioblastoma cell lines (data not shown) to eliminate this as a potential bias.

In other tumor entities it has been shown that ex vivo drug response correlates with patients’ response in primary cell culture approaches similar to our setup (e.g. esophageal adenocarcinoma, breast cancer, and head and neck squamous cell carcinoma) [59–61] and in patient-derived xenograft models [62, 63]. Further studies are needed to evaluate whether the observed in vitro sensitivity will correlate with clinical response in PanNET patients as well. To answer this question, first a prolonged clinical follow-up is crucial. Secondly, a larger patient cohort will be needed to perform correlation analysis and to have enough statistical power. Full clinical follow-up data of all enrolled patients

are therefore collected. Chemotherapy-specific in vitro treatment duration and concentration range with the highest predictive value will be defined by comparison of clinical data with the in vitro drug response. Due to the non-lytic approach, the islet-like tumoroids are collected after the experiment and are available for next-generation-sequencing end point analysis, as is the original tumor material from patients. The presented PanNET screening platform might therefore serve as a basis for developing personalized treatment of PanNET patients, performing molecular fingerprinting on the original tissue to be able to potentially detect predictive markers.

We are well aware that the present protocol still bears limitations. It depends on surgical resection specimens of PanNET metastases and high-stage tumors; however, most of these patients are diagnosed via biopsies of liver metastases. With further experience the protocol has the potential of being adapted to biopsy specimens as well – yet – some biological role must be proven first to ethically justify additional biopsies. The composition of growth factors could be further refined, and the culture system does not factor in stromal and inflammatory features potentially contributing also to tumor response.

In conclusion, we present a 3-D human primary PanNET screening platform as a new preclinical model, which reflects the characteristics of an individual tumor and has the capability to detect differential treatment response. Therefore, this model has the potential to pave the way towards more personalized medicine for PanNET patients in the future, including better patient stratification and identification of novel and experimental treatments.

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Statement of Ethics

Subjects (or their parents or guardians) have given their written informed consent, and the study protocol was approved by the cantonal authorities (Kantonale Ethikkommission Bern, Ref.-Nr. KEK-BE 105/2015) and the Italian ethic commission (Comitato Etico, CE 252/2019).

Disclosure Statement

All the authors declare no competing financial interest. There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author Contributions

A.P., I.M., T.W. and S.L.A.M. designed the study. T.W. and S.L.A.M. developed the tumoroid methodology. T.W., M.S. and S.L.A.M. performed experiments and data acquisition. S.L.A.M. analyzed and visualized data. M.T. analyzed, scored and quantified immunohistochemistry. M.F., C.D., M.S.L., F.M., S.P., C.K.F. and B.G. provided patient biopsies and patient clinical information. R.M., M.S.L., A.D., V.A., M.C.Z., C.K.F., B.G. and M.S. provided administrative, technical or material support. A.P., I.M., T.W. and S.L.A.M. wrote the paper.

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