Single-cell-resolved differentiation of human induced pluripotent stem cells into pancreatic duct-like organoids on a microwell chip

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Creating in vitro models of diseases of the pancreatic ductal compartment requires a comprehensive understanding of the developmental trajectories of pancreas-specific cell types. Here we report the single-cell characterization of the differentiation of pancreatic duct-like organoids (PDLOs) from human induced pluripotent stem cells (hiPSCs) on a microwell chip that facilitates the uniform aggregation and chemical induction of hiPSC-derived pancreatic progenitors. Using time-resolved single-cell transcriptional profiling and immunofluorescence imaging of the forming PDLOs, we identified differentiation routes from pancreatic progenitors through ductal intermediates to two types of mature duct-like cells and a few non-ductal cell types. PDLO subpopulations expressed either mucins or the cystic fibrosis transmembrane conductance regulator, and resembled human adult duct cells. We also used the chip to uncover ductal markers relevant to pancreatic carcinogenesis, and to establish PDLO co-cultures with stellate cells, which allowed for the study of epithelial–mesenchymal signalling. The PDLO microsystem could be used to establish patient-specific pancreatic duct models.

Pancreatic ductal cells are organized in tubular ductal networks. Ductal cells secrete a bicarbonate-rich alkaline aqueous solution to transport zymogens produced by acinar cells. Dysfunction of ductal cells can affect fluid composition in cystic fibrosis, leading to a subsequent deterioration of the entire organ. Dysplastic events within the ductal epithelium can lead to pancreatic ductal adenocarcinoma (PDAC), one of the most lethal cancer types. Shortage of healthy and early disease-affected primary ductal material hampers the discovery of biomarkers for diagnostics and drug development. To overcome the limitation imposed by this shortage of donor material, lumen-forming pancreatic organoid cultures have been derived from resected healthy and diseased pancreata. Pancreatic organoids preserve the cellular heterogeneity of the human pancreas and can be used to establish functional tests or discover biomarkers for PDAC in vitro. Pancreatic cancer-derived organoids: (1) exhibit an undefined genetic background, (2) are generated from a tumour origin, (3) mimic the end stage of cancer, and (4) are unsuitable for biomarker discovery in the earliest stages of pancreatic dysplasia. Adult human pancreatic organoids are challenging to establish and to culture in an untransformed state and, moreover, do not provide access to developmental intermediates. Lineage-committed pancreatic ductal cells generated from human pluripotent stem cells (hPSCs) could be an alternative source of pancreatic organoids to overcome these obstacles.

The engineering and translation of in vitro ductal disease models require a full mechanistic understanding of the in vitro ductal differentiation landscape, cell-type composition, and functionality at the ductal stage. Unfortunately, there is a dearth of knowledge on human embryonic duct development. Previous studies in rodents revealed that all pancreatic cell lineages evolve from pancreatic-progenitor cells, a common pancreatic cell type derived from endoderm. Pancreatic progenitors organized in the pancreatic bud undergo tip–trunk patterning. The acinar cells evolve from the tip domain, and ductal cells together with subsequently delaminating endocrine cell types evolve from presumably bipotential trunk cells. Time-resolved immunostaining of developing human embryos suggest a similar mechanism for human pancreatic cell-type development. Recently, we and others developed a differentiation protocol to guide hPSCs to form pancreatic duct-like organoids (PDLOs). Single-cell RNA sequencing (scRNA-seq) has become the method of choice for studying cell differentiation in organoids or tissue development to reveal cell heterogeneity, quantify cell types, and resolve molecular mechanisms leading to cell lineage bifurcations and their subsequent transitions. scRNA-seq of primary human pancreatic tissue is well-established, but to our knowledge, there is no time-resolved single-cell transcriptional roadmap of developmental trajectories from hPSCs towards ducts.

Organoid formation and stem cell differentiation are non-linear deterministic systems; thus, slight variations in initial conditions...
can influence the outcome of the process. Therefore, controlling the initial organoid size and shape in microwells is thought to improve the reproducibility of differentiation\textsuperscript{29,30}. Microwells are the most simplistic chip technologies for assisting the formation of organoids and their culture. Further, microenvironmental signals of confined organoids and the organoids themselves are directly accessible for downstream analysis from the open microwells. Thus, a microwell chip design offers simple cell sample collection for scRNA-seq, and proteome and/or secretome analyses during ductal differentiation of hPSCs.

In this study, we designed a microwell chip to generate defined 3D aggregates of human induced pluripotent stem cell (hiPSC)-derived pancreatic progenitors and, subsequently, to induce their differentiation towards PDLOs. Time-resolved scRNA-seq combined with cleared immunofluorescence imaging provided a deep understanding of in vitro ductal cell-type differentiation. By defining the emergent cell types at each stage of differentiation on the basis of their gene-expression profiles and organoid structures, we provide a precise cell-by-cell description of the in vitro differentiation trajectory. Transcriptional data of PDLOs were complemented by their proteome and secretome, enabling the identification and validation of prognostic cancer markers. Thus, we show the applicability of hiPSC-derived PDLOs on a microwell chip for future ductal disease modelling.

**Results**

**A microwell chip to form 3D aggregates from hiPSC-derived pancreatic progenitors.** To enable engineering and long-term culture of duct-like organoids from hiPSC-derived pancreatic progenitors, we designed a microwell chip technology (Fig. 1a–d). The microwell chip was produced by soft lithography with polydimethylsiloxane (PDMS), where the casting moulds were prepared using 3D stereolithography printing (Supplementary Fig. 1a). Each microwell chip contained four hexagonal arrays with cone-shaped wells, and 12 round pillars surrounded each array. The function of the pillars was to retain an aqueous drop (20–40 µl) above the array by surface tension (Fig. 1a,b). A 180-µm-thin PDMS bottom layer allowed high-resolution imaging of confined organoids (Fig. 1c). Together with roundings between wells, the pillars enabled an efficient and homogeneous cell seeding process without blind spots. Details of the production method and workflow for cell seeding and culturing are given in Supplementary Fig. 1b. First, we validated the stem cell viability within differently sized microwells (150, 300 and 600 µm). Next, we systematically altered the initial cell number and well size to optimize the formation of hiPSC-derived aggregates of pancreatic progenitors. Pancreatic-progenitor aggregates formed on the microwell chip within 4 h with uniform size according to variable well diameters and cell numbers (Fig. 1d,e). A small number of cells (less than 50 cells per well) compromised the aggregation step in the microwells, and the generation of cell aggregates with diameters of more than about 250 µm is known to impair nutrient supply\textsuperscript{31}. For the subsequent ductal differentiation, we selected an average initial pancreatic-progenitor-aggregate size of 96 µm (600 cells in 300-µm-diameter wells).

**Ductal differentiation on the microwell chip.** We differentiated 3D pancreatic-progenitor aggregates towards PDLOs using the microwell chip. Pancreatic progenitors generated in monolayer culture formed aggregates on the chip, followed by a two-step differentiation process with specific growth factors (Fig. 2a). Subsequently, pancreatic-progenitor aggregates underwent large morphological changes within the microwells, as indicated in the representative bright-field images (Fig. 2b and Supplementary Fig. 3a). In the first ductal induction phase, the uniform round-shaped pancreatic-progenitor aggregate structure was broken up by newly formed multi-layered epithelial protrusions (Fig. 2b). In the second ductal differentiation phase, multi-layered epithelium organoids reduced their number of layers and cystic organoids were segregated from the outer layer of the PDLOs. Notably, a few cystic PDLO structures remained connected to the multi-layered epithelial PDLOs at day 31 of differentiation. The morphological transformation of the PDLOs is visualized in Supplementary Video 1. High-resolution immunofluorescence images of cleared organoids confirmed cellular re-organization towards the end of phase 1 of ductal differentiation (Fig. 2c,d). Epithelial nature and pancreatic ductal identity of the cells at the final stage were confirmed by...
the upregulation of E-cadherin (CDH1), cytokeratin 19 (KRT19), aquaporin 5 (AQP5), mucin 1 (MUC1), carbonic anhydrase II (CA2), cytokeratin 7 (KRT7), claudin 1 (CLDN1) and cystic fibrosis transmembrane conductance regulator (CFTR) (Fig. 2c,d and Supplementary Fig. 3b). Expression of ductal markers, which were already detected at the pancreatic-progenitor stage, such as cytokeratin 8 (KRT8), SRY-box transcription factor 9 (SOX9), hepatocyte nuclear factor 1 homeobox B (HNF1B), and pancreatic and duodenal homeobox 1 (PDX1) were maintained in PDLOs (Fig. 2c,d and Supplementary Fig. 3b,c). By contrast, the progenitor marker homeobox protein NKX6-1, which becomes restricted to endocrine cells during pancreatic development, was downregulated at the protein level (Supplementary Fig. 3c). Stemness marker SRY-box 2 (SOX2) and octamer-binding transcription factor 4 (OCT4) were absent (Supplementary Fig. 3d), as were non-ductal pancreas markers, except in a few endocrine cells located at the periphery of PDLOs (Supplementary Fig. 3e). In agreement with the marker panel, a forskolin-induced swelling assay confirmed pancreatic ductal functionality (Supplementary Fig. 4). To demonstrate that the microwell chip-derived PDLOs are lineage-committed, we transplanted PDLOs differentiated until day 27 orthotopically into the pancreas of immunocompromised mice (Fig. 3a). PDLO
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engraftments formed tubular duct-like tissue after eight weeks, homogeneously expressed epithelial ductal markers such as SOX9, KRT19, AQP5 and CDH1, and were negative for endocrine cell types (Fig. 3b,c).

PDLOs exhibited a predominant apical-out polarity, although apical markers tight junction protein-1 (ZO1), cilia marker acetylated tubulin (acTUB), and ADP-ribosylation factor-like protein 13B (ARL13B) were also partly visible on the opposing membrane side, indicating very small lumen within the organoid (Fig. 3d and Supplementary Fig. 5). One explanation for the observed PDLO apical-out polarity could be the lack of extracellular matrix (ECM) deposition in suspension culture format32–34. We hypothesize that providing a basement membrane, as mimicked by Matrigel, for the microwell chip-derived PDLOs could facilitate the formation of an epithelium with a strictly apical-inside organization. Indeed, an apical-out to apical-in polarity switch was observed upon transfer of microwell chip-derived PDLOs into a 3D Matrigel culture or after transplantation (Fig. 3d).

Fig. 3 | Apical-out polarity of the microwell chip-derived PDLOs switched upon orthotopic transplantation or embedding into Matrigel. a, Schematic of the Matrigel and orthotopic transplantation experiment. PDLOs were transplanted on day 27 and mice were euthanized after 8 weeks. PP, pancreatic progenitor. b, Haematoxylin and eosin staining and magnification of the engraftment site depicted by the dashed square (n = 2 mice). Scale bars, 500 µm (left and middle) and 50 µm (right). c, PDLOs formed human epithelial duct-like tissue in vivo. CHGA, chromogranin A; H-NUCL, human-specific nucleoli; VIM, vimentin. Scale bar, 50 µm. d, Immunofluorescence images of the apical markers ZO1 and acTUB and basal markers COL4A1 and ARL13B on 3D pancreatic-progenitor aggregates, PDLOs, Matrigel PDLOs and transplanted PDLOs. Complementary images are shown in Supplementary Fig. 5. Nuclei were counterstained with DAPI. Scale bars, 50 µm.

Single-cell characterization of duct-like organoids. To reconstruct ductal cell-type development in our microwell chip model and to define time-resolved cell composition according to the transcriptional identity, we performed scRNA-seq analysis of 14,811 cells (Fig. 4a and Supplementary Fig. 6a). Louvain clustering and scRNA-seq data analysis with the two-dimensional data reduction algorithm uniform manifold approximation and projection (UMAP) identified nine cell clusters (Fig. 4b,c). With the progression of the growth factor-induced differentiation process, the
recorded single-cell transcriptomes changed substantially, indicated by the time-dependent emergence of the distinct cell clusters (Fig. 4b). All cell clusters could be assigned to cell types by matching known pancreatic developmental genes to the differentially expressed genes (DEGs) in the respective cluster, including three duct-like clusters (Fig. 4c–e and Supplementary Data 1). To test the
robustness of the differentiation approach on the single-cell level, we sequenced the end stages of two independent experiments. In both cases, approximately 90% of pancreatic progenitors developed into cells with a transcriptomic profile of one of the duct-like clusters (Fig. 4d). Violin plots highlight a selection of DEGs specific for each cluster (Fig. 4f). The major cell populations identified included four presumptive progenitor cell types (clusters I–IV), three types of duct-like cells (clusters V–VII), a small endocrine-like cell population (cluster VIII) and a subset of non-pancreatic cells, namely endothelial-like cells (cluster IX). Intriguingly, our starting pancreatic-progenitor-cell population, which was generated in a 2D cell culture, contained cells with different transcriptional profiles suggestive of multipotent and unipotent ductal progenitor cells. In both cell clusters, common progenitor markers were expressed, including PDX1, HNF1B and SOX9 (Fig. 4e,f and Supplementary Fig. 6c). Cluster III, hereafter designated unipotent ductal progenitors (UDPs), showed high expression of KRT8 and low expression of NKKx6-1, suggesting that these cells committed to a ductal-primed fate at the end of the 2D cell differentiation58,59 at day 13. Upon aggregation of the 2D cultured progenitors on microwell chips, both corresponding 3D progenitor clusters were transcriptionally re-identified; however, a subset of pancreatic-progenitor markers showed specific alteration patterns (Fig. 4b1): 3D UDPs (cluster IV) upregulated the markers HES family bHLH transcription factor 1 (HES1) and S100 calcium-binding protein A10 (S100A10), whereas cluster II showed an increased expression of glycoprotein 2 (GP2) and pancreas-associated transcription factor 1a (PTF1A), which were specific to this subpopulation (Fig. 4f and Supplementary Fig. 6c). PTF1A and NKKx6-1 were co-expressed only in cluster II, suggestive of multipotent pancreatic progenitors (MPPs), which can give rise to acinar, endocrine and ductal cells in mice42,59. With the progression of differentiation, three duct-like cell clusters emerged, characterized by high KRT19 and SOX9 and intermediate CA2 expression; the latter being a hallmark enzyme of mature pancreatic ducts60 (Fig. 4f and Supplementary Fig. 6c). Differential gene expression of the combined three duct-like clusters revealed significant upregulation of ductal epithelial markers such as CLDN1 and S100A10. While duct-like 1 cells specifically expressed HCO3−secretion-related proteins such as CFTR, duct-like 2 cells were enriched for mucin-related genes; for example, MUC13 and trefoil factor 1 (TFFI). The duct-like 3 cluster contained only 134 cells and showed similarities to duct-like 2 cluster with significantly higher expression of CLDN4 and MMP1. We also resolved a small fraction of endothelial cells and pancreatic endocrine cells. Doubling the initial cell number of the pancreatic-progenitor aggregates did not influence the outcome of the ductal differentiation (Supplementary Fig. 6b).

Ductal cell subpopulations in PDLOs. To validate duct-like cell types identified in single-cell transcriptomics on a protein level, we performed immunofluorescent staining for specific cluster markers on microwell chip-derived PDLOs. We stained PDLOs differentiated on our microwell chip to day 23, 27 and 31 for CFTR and MUC13. CFTR was expressed only in cells of multi-layered epithelial PDLOs, and MUC13 was expressed in a different subset of cells at the outer side of multi-layered epithelial and in all cystic PDLOs (Fig. 5a,b). A differential expression pattern of a mucin-rich (MUC1+) and a CFTR+ duct subtype within the pancreas has been reported previously42. While MUC1 transcripts were not detected in the PDLO scRNA-seq data, MUC1 protein expression was readily found by immunostaining and label-free mass spectrometry on bulk PDLOs (Fig. 5b, Supplementary Fig. 7a and Supplementary Data 4). Next, we performed combinatorial staining of PDLOs for further proteins encoded by duct-like subcluster DEGs. Indeed, we found distinct expression patterns and fluorescence intensities across multi-layered epithelial PDLOs for MUC1, CFTR, BICC1, MMP1 and TFF1 (Fig. 5b and Supplementary Fig. 7a). For instance, BICC1 was broadly detected but often appeared weaker in MUC1-positive cells. In larger PDLOs, MMP1 was localized to the peripheral layers, while CFTR also appeared in luminal structures inside the organoid (Fig. 5b and Supplementary Fig. 7a). Overall, protein expression of the scRNA-seq based duct-like subtype markers were not mutually exclusive, in agreement with the single-cell transcriptomic data (Fig. 4e,f).

To translate these spatial expression patterns from microwell chip-derived PDLOs to primary human pancreatic tissue, we stained healthy pancreata and a chronic pancreatitis specimen for the cluster-specific markers (Fig. 5c,d and Supplementary Fig. 7b,c). MUC1 was restricted to acinar structures, centro-acinar cells and connected intercalated ducts; the connected intercalated ducts also expressed CFTR (Fig. 5c, first and second row and Supplementary Fig. 7b). We also stained KRT19, confirming ductal identity. The marker pattern changed according to the size of the branching ducts, indicating a transition between different co-expression patterns. For example, CFTR expression decreased in intralobular ducts and was hardly expressed in larger ducts, where BICC1 became more prominent (Fig. 5c, third row). In addition, BICC1 was rarely detected in cells in direct proximity to the MUC1-positive cells. Conversely, MMP1 was essentially absent in intercalated ducts but showed a variable staining intensity within larger ductal structures (Fig. 5c, fourth row and Supplementary Fig. 7b). MUC13, TFF1 and SCRT could not be detected in healthy ducts (Fig. 5c, fifth row and Supplementary Fig. 7b). Immunostaining of chronic pancreatitis tissue further confirmed duct specificity, exhibiting differential expression patterns of the described markers and revealing SCRT expression in metaplastic ductal epithelium (Fig. 5d and Supplementary Fig. 7c). Together, these data demonstrate spatially changing expression patterns of the ductal cell-type markers MUC1, CFTR, BICC1, MMP1, TFF1 and SCRT at protein level in PDLOs and in human primary tissue. Thus, expression states are probably more dynamic and complex than the initial transcriptomic subgrouping had suggested.

Trajectory of the in vitro ductal development. To resolve time-dependent relationships during PDLO differentiation, we performed dynamic RNA velocity analysis61,62. First, we calculated a latent time on the basis of the balance of spliced and unspliced RNA transcripts within the single-cell transcriptomes (Fig. 6a). Indeed, the theoretical latent time matched true chronological differentiation times (compare with Fig. 4a). The corresponding RNA velocity streamlines indicate two differentiation routes from pancreatic progenitors towards duct-like cells: (1) duct-like 1 cells evolved from MPPs, and (2) duct-like 2 cells mainly evolved from UDPs, which were already present at the pancreatic-progenitor stage (Fig. 6b). Velocity streamlines also directed from duct-like 1 to duct-like 2 cells, indicating a relevant degree of plasticity as reported previously in the pancreas42. Few endocrine cells emerged from the MPP cell cluster. Consistent with the velocity analysis, partition-based graph abstraction analysis showed a connectivity (edges) between clusters (dots) along the second ductal differentiation route (Fig. 6c).

Evaluation of cell cycle states showed that maturation of duct-like cells was accompanied by a gradually decreasing fraction of cells in S phase (Fig. 6d). Subsequently, we plotted the changes in the expression of common stage-specific markers along the latent time to trace ductal differentiation. In line with mouse development data, expression of pancreatic-progenitor markers including GP2, tweety family member 1 (TTYH1), PDX1 and PTF1A decreased over time. Conversely, ductal markers such as S100A14, CFTR, TFF1 and CA2 were upregulated (Fig. 6e). Concordance between transcriptional dynamics of MPP markers and genes that are essential during mitosis (topoisomerase 2 (TOP2) and cyclin B2 (CCNB2)) was observed, all of which decreased in level during the differentiation process.
The top 300 dynamic genes are listed in Supplementary Data 2. Upon ductal cell fate commitment, genes characteristic for pancreatic secretion (CFTR and SCTR) were transiently induced, accompanied by gradual increases in genes involved in mineral absorption (metallothionein 1E (MT1E)). Further, MUC13 was upregulated, showing a similar temporal expression profile as genes associated with lipid transport or metabolism (apoliprotein B (APOB)) and genes less studied in the pancreatic duct such as TFF3 or macrophage stimulating 1 (MST1), the latter being critical for maintaining exocrine differentiation status and tissue integrity. Since we also detected dynamical expression of several ECM-related genes, we analysed the scRNA-seq data in more detail regarding changes in expression of ECM components during PDLO maturation. Duct-like cells increased the expression of laminin-α3 and -α5 subunits (Supplementary Fig. 8a). At the same time, corresponding laminin-binding integrins were expressed, supporting...
ECM formation along the differentiation trajectory. Concomitantly, duct-like cells downregulated the expression of basal matrix collagens accompanied by upregulation of collagenases MMP1 and MMP10 (Supplementary Fig. 8b). Pancreatic tubulogenesis requires the basement membrane laminin-1 and an α6-containing integrin receptor for proper initiation, provided by the pancreatic mesenchyme during physiological development. Accordingly, progenitor and duct-like cluster 1 cells expressed laminin-α1, duct-like cells 2 and 3 expressed laminin-α3 and laminin-α5 (Supplementary Fig. 8a). Of note, laminin-α4 and integrin-α5, which are central for β-cell formation, were not expressed in PDLOs or in the progenitor state. By contrast, αVβ5 integrins were upregulated in duct-like cells (Supplementary Fig. 8b), in agreement with previous findings. Thus, PDLOs are likely to secrete soluble ECM and corresponding binding proteins as seen under in vivo conditions. In the open microwell culture format, however, the proteins are most probably resolved in the medium, which would explain the polarity switch of the microwell chip-derived PDLOs upon either in vivo transplantation or 3D Matrigel culture (Fig. 3d).

In addition to the ECM genes, we integrated a set of signalling pathways related to the applied growth factor stimuli and found that EGF- and FGF10-mediated MAPK–ERK and ErbB signalling peaked in the duct-like clusters 1 and 2. Compatibly, charting gene signatures representative for processes occurring in mature ducts such as pancreatic secretion or mucin type O-glycan biosynthesis further supports that duct-like cells were generated on the microwell chip (Supplementary Fig. 9a–d).

\( CFTR^+ \) and mucin\(^+ \) subpopulations in primary duct tissue. One demand for the microwell chip–derived PDLOs is that the duct-like cell types closely resemble human tissue; we therefore integrated three scRNA-seq datasets from primary human pancreas tissue into our PDLO differentiation kinetics (Fig. 7a). Within the re-clustered combined dataset, duct-like clusters from PDLOs mapped with primary duct cells. Ductal progenitors from the in vitro differentiation trajectory clustered separately, as did endocrine, acinar and endothelial cells. A comparison of the initial cell-type assignments with the cluster location in the combined dataset further substantiated the integration approach (Fig. 7b). Previous reference data from primary ducts described two ductal cell types—one expressing \( MUC1 \) or \( TFF1 \) and a second with \( CFTR \) as the cluster marker gene. Highlighting \( CFTR \) and \( TFF1 \) within the combined dataset confirmed the presence of ductal cell subtypes in the PDLOs (Fig. 7a and Supplementary Data 3). In fact, PDLO cells positive for \( CFTR \) clustered with the primary \( CFTR \) duct cell type, and PDLO cells positive for \( MUC13 \) or \( TFF1 \) clustered with primary \( MUC1 \) or \( TFF1 \) duct cells. Data integration can lead to an overinterpretation of cell-type similarities. To confirm our ductal cell subpopulation analysis without data integration, we re-clustered the largest primary human ductal single-cell dataset (Supplementary Fig. 10a,b) and searched for our cluster markers (Supplementary Fig. 10c–e). Indeed, the duct-like 1 cluster markers \( CFTR, BICC1 \) and \( SCTR \) were expressed in cell clusters separated from the duct-like 2 and 3 cluster markers \( MMP1 \) and \( TFF1 \) (Supplementary Fig. 10d,e). In addition, we calculated a gene-expression enrichment score of the top 100 DEGs of the primary \( CFTR^+ \) and \( MUC1^+ \) duct subpopulations from Baron et al. across the PDLO differentiation kinetics. \( CFTR^+ \) and \( MUC13^+ \) PDLO cells again correlated with the corresponding primary ductal subpopulations (Fig. 7c).

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Applications of the microwell chip. The microwell chip could be used for various applications. One example was for the investigation of cell–cell communication between pancreatic ducts...
Four fluidic separable hexagonal arrays on the microwell chip were exploited to establish a cross-contamination-free co-culture of PDLOs and human pancreatic stellate cells (HPaSteCs) (Supplementary Fig. 11a). The latter resemble quiescent stromal cells in the pancreas able to convert during inflammation, injury or cancer development to a

Fig. 7 | Duct-like cells of the PDLOs clustered with primary ductal cells and resembled \( CFTR^+/-\) /mucin\(^+-\) subpopulations. a, Integration of three primary pancreas scRNA-seq datasets\(^{3,24,26}\) (bottom left) into 10 Louvain clusters (middle plot, duct-like PDLO cells are highlighted by areas in their cluster colour). UMAP expression plots display cell-type-specific marker gene expression (surrounding graphs). Bottom right illustrates the location of our PDLO differentiation kinetics. Analysis of the \( CFTR^+/-\) /mucin\(^+-\) subpopulations in the isolated scRNA-seq dataset of Qadir et al.\(^{26}\) is shown in Supplementary Fig. 10. b, Annotated cell types with their percentile distribution of the original clusters. c, Enriched expression of the marker genes for the \( CFTR^+\) /mucin\(^-\) and mucin\(^+\) /\( CFTR^-\) subpopulations defined in Baron et al.\(^{24}\) during PDLO differentiation.
metabolically active state via autocrine and paracrine signals, then serving as a central player in the pathogenesis of pancreatic disease. Quantitative proteomic analysis of PDLOs and HPaStEcS separated co-cultured cells from their individually cultured counterparts (Supplementary Fig. 11b). None of the high-abundance proteins of the individual cultured HPaStEcS and only 2% of those of the PDLOs were found in the upregulated protein set of the co-cultured PDLOs and HPaStEcS, which suggests that there was negligible cross-contamination on the microwell chip (Supplementary Fig. 11c). Enrichment for similar Gene Ontology (GO) terms indicated reciprocal signalling between the two cell types. Pathways involved in energy metabolism and cellular signalling were enriched in co-cultures compared with single cultures (Supplementary Fig. 11c). An in silico constructed protein network in co-cultured PDLOs and HPaStEcS resembled a mitogenic pattern in support of metabolic activation (Supplementary Fig. 11d).

Another application of the microwell chip is the discovery of prognosticators and/or early-stage pancreatic cancer biomarkers. Secretomes derived from either wild-type or genetically modified pancreatic ducts could be a promising resource and the open accessibility of microwells enabled their characterization from the hydrogel-free environment of the PDLOs by collecting the supernatant. To obtain sufficient protein, we made use of label-free mass spectrometry and upscalled the microwell chip to 1,196 PDLOs (Fig. 8a). Mass spectrometry identified 2,528 secreted proteins with high confidence level (Supplementary Data 4), retrieved over a time interval of 8 h, out of which 167 contained a signal peptide for active secretion into the extracellular space. GO-term analysis of the PDLO secretome showed an enrichment of enzymes with hydrolytic and lipid-binding function (Fig. 8b). Scoring the filtered secretome against all human tissue types revealed significant enrichment for the terms ‘pancreas, glandular cells’ (Fig. 8b). Finally, we used the secretome and scRNA-seq data to identify potential prognostic markers of PDAC by using our microwell technology. Within the top 200 DEGs of the duct-like clusters and the 2,528 proteins of the filtered PDLO secretome, 30 and 186 unfavourable prognostic markers for pancreatic cancer were found, respectively (Fig. 8c). Eleven of the transcriptomic proteins and 38 of the markers identified in the secretome were predominantly expressed in pancreatic duct cells.

Filamin b expression in human pancreatic cancer cohorts. One unfavourable PDAC marker in the overlapping protein set was filamin B (FLNB), which has been identified in a secretome screen from pancreatic cancer cell lines without validation in human patients (Fig. 8d). FLNB exerts tissue- and context-dependent functions in distinct cancers, whereas both gain and loss of function have been shown to foster cancerous properties. To investigate FLNB expression during pancreatic carcinogenesis, we assessed FLNB protein expression in an independent cohort of resected PDACs by immunohistochemistry (Fig. 8e). Normal pancreatic ductal epithelium and some acinar glands were faintly FLNB positive at their luminal surface. As expected, microwell chip-derived PDLOs were also FLNB positive (Fig. 8e and Supplementary Fig. 12a,b). By contrast, PDAC strongly expressed FLNB in the cytoplasm and on the entire cell surface in concordance with the loss of polarity in the carcinoma cells (Fig. 8e and Supplementary Fig. 12b). Immunohistochemical observation was specified using the semi-quantitative H-score method followed by correlation with clinical data. Comparing normal ductal epithelium with corresponding cancer tissue revealed significantly higher H-scores in PDAC (Fig. 8f), whereas H-scores did not correlate with any clinical parameter including survival (Fig. 8g). Pancreatic intraepithelial neoplasias (PanIN) represent the most relevant PDAC precursor lesions and can be frequently found adjacent to PDAC and their presence is of prognostic relevance. Interestingly, H-scores were also elevated in PanINs compared with normal ducts (Fig. 8f). FLNB expression in PanIN lesions significantly correlated with increased survival of patients (modified overall survival; \( P = 0.0019 \)) (Fig. 8g and Supplementary Fig. 12c). A gradual increase from normal to preneoplastic lesions has been described, for example, for the epigenetic silencer enhancer of zeste homologue 2 (EZH2)—in established cancers, higher expression levels were associated with better prognosis. As alternative splicing can lead to shorter FLNB isoforms being strongly associated with gene signatures of epithelial–mesenchymal transition in basal-like breast cancer patient samples, distinct forms might also be present in pancreatic cancer precursor lesions, leading to distinct biological outcomes. To probe the feasibility of FLNB as a biomarker for liquid biopsy, we consulted an independent cohort of human patients with metastatic PDAC and measured FLNB levels in peripheral blood. When comparing FLNB levels in peripheral blood, no obvious differences were observed between patients with metastatic PDAC and healthy volunteers; however, metastatic samples clustered into two FLNB groups. To dissect this clustering in more detail, we correlated clinical and histological characteristics of the PDAC patients with individual FLNB levels in peripheral blood. Intriguingly, differentiated tumours (grade 2 or less) had significantly higher FLNB peripheral blood levels when compared to less differentiated tumours (grade 3 or more) or healthy donors, the latter two sample sets having more or less similar levels (Fig. 8h). We conclude that FLNB might be a suitable blood biomarker for differentiated PDACs and could therefore complement biomarker panels detecting early PDAC formation or discriminating differentiated and dedifferentiated PDAC.

Discussion
We have developed a microwell chip to engineer PDLOs from hiPSCs, and charted their cellular heterogeneity during the differentiation trajectory. The advances of the microwell chip are (1) the low consumption of cells and materials, (2) defined and homogeneous size of generated 3D aggregates, (3) the possibility for long-term 3D cell culture, (4) sample retrieval for downstream analysis with
minimal perturbation, and (5) the possibility to establish co-cultures. We challenged the potential of this microwell chip for application and comprehensively analysed the secretome and proteome to identify potential prognostic and diagnostic PDAC biomarkers.

Microwell culture is a low-cost cell culture technology offering uniformity for the aggregation process and flexibility in design for multiple downstream applications. The microwell chip implements 3D pancreatic-progenitor aggregates to generate two morphologically

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**GO-term analysis of PDLO whole secretome**

| Molecular function terms                  | $P_{adj}$ | $-\log_{10}(P_{adj})$ |
|------------------------------------------|-----------|-----------------------|
| Hydrolase activity                       | $1.1 \times 10^{-16}$ | 16.0                  |
| Oxireductase activity                    | $2.1 \times 10^{-14}$ | 14.0                  |
| Exopeptidase activity                    | $1.6 \times 10^{-13}$ | 13.0                  |
| Lyase activity                           | $2.1 \times 10^{-9}$  | 9.0                   |
| Peptidase inhibitor activity             | $1.3 \times 10^{-7}$  | 7.0                   |
| LDL particle binding                     | $2.2 \times 10^{-5}$  | 5.0                   |
| Lipid binding                            | $4.4 \times 10^{-5}$  | 5.0                   |
| Phospholipid binding                     | $1.7 \times 10^{-4}$  | 4.0                   |
| Hydrolase activity                       | $1.9 \times 10^{-7}$  | 7.0                   |
| Tissue match to human protein atlas      | $P_{adj}$ | $-\log_{10}(P_{adj})$ |
| Pancreas, glandular cells                | 5.2 x $10^{-27}$     | 27.0                  |
| Bile duct cell                           | 1.9 x $10^{-13}$     | 13.0                  |

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**Prognostic marker for pancreatic cancer**

| (1) Ductal DEG marker (scRNA-seq) | (2) Secretome protein (LC–MS/MS) |
|----------------------------------|---------------------------------|
| Identified unfavourable marker mainly duct expressed | 30 (log 200) | 11 |
| Identified favourable marker mainly duct expressed | 2 | 87 |

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**FLNB overall survival: PDAC**

- H-score < 150
- H-score ≥ 150
- $P = 0.93$

**FLNB overall survival: PanIN**

- H-score < 150
- H-score ≥ 150
- $P = 0.0019$

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**FLNB concentration (ng ml⁻¹)**

- Healthy control
- PDAC grading ≤ 2
- PDAC grading ≥ 3

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**Established PDAC biomarker**

- LMO7
- SDCBP2
- TAX1BP1

**Weak literature link to PDAC**

- FLNB
- PSL1

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**Examples:**

- ANXA2
- ANXA3
- ESR
- LYPLA1
- KRT19
- AREG
- CAPN2
- LDLR
- LMO7
- SDCBP2
- TAX1BP1

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**Molecular function terms**

- Hydrolase activity
- Oxireductase activity
- Exopeptidase activity
- Lyase activity
- Peptidase inhibitor activity
- LDL particle binding
- Lipid binding
- Phospholipid binding
- Hydrolase activity

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**H-score**

- Healthy
- PanIN
- PDAC

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**NS**

- **:** $P = 0.006$
- ***:** $P = 0.024$
- **:** $P = 0.006$
distinct PDLO types: a multi-layered epithelial type and a cystic PDLO type. Pancreatic organoids generated from healthy or cancerous human pancreata grown in hydrogels show cystic morphologies\(^\text{2,12,13}\) with an apical-in polarity. By contrast, microwell-chip-derived PDLOs exhibited predominantly, but not entirely, apical-out polarity. After engraftment in mice, they formed ductal structures exhibiting their apical side to the lumen. The same switch to apical-in polarity was found when PDLOs were transferred from the microwell chip to an embedding laminin-rich hydrogel culture as mimicked by Matrigel. This indicates that ECM components are also involved in the induction of ductal epithelial polarity. The inversion of apical-in to apical-out cell polarity upon transferring organoids from hydrogel to suspension cultures has been previously described for enteroids\(^\text{41}\). The dominant apical-out configuration of PDLOs enhances constant exposure to external stimuli, allows easy access to the secretome of cells, and makes the apical membrane accessible for various studies, such as the study of membrane barrier functions or pathogen infections. Nevertheless, swelling in cystic PDLOs upon stimulation with forskolin suggests a relevant degree of luminal CFTR activity at the inner membrane of PDLOs.

PDLO development in microwells also facilitates the stepwise study of synchronous human duct development. Time-resolved single-cell transcriptomics characterized the entire cell populations evolving from the pancreatic progenitor to duct-like cell stage. Interestingly, the duct-like cells differentiated on microwells clustered together with three reference primary duct cell types in published scRNA-seq datasets and could be stained in primary human pancreas tissue. Furthermore, counterparts of the previously identified ductal cell subtypes in the adult pancreas with mucosal restitution (MUC1) and HCO\(_3\)\(^-\) secretion (CFTR) transcriptomic profile\(^\text{14}\) were identified within the in vitro-generated PDLOs. Besides ductal heterogeneity at terminal differentiation stages, emerging intermediates revealed important details about ductal cell-type commitment. Hitherto, it has been suggested that endocrine precursors delaminate from a common trunk domain arising at embryonic day 12.5 in mice, which further undergoes tubular morphogenesis to subsequently form the ductal network\(^\text{46}\). Although the timing of particular marker expression is slightly different, detection of similar markers suggests the presence of a trunk and tip domain during human pancreas development\(^\text{15,16}\). An unexpected finding of our analysis was the lack of a trunk domain subcluster, at least based on the in vitro marker gene analysis. Velocity analysis suggests that the microwell-derived duct cell types generated from distinct temporally co-existing progenitor populations harboured distinct potency. The few endocrine cells present in PDLOs evolved from the 3D MPPs, which were positive for classical tripotent pancreatic-progenitor labels, including GP2 and PTF1A, contradicting a trunk-like state\(^\text{46}\). We did not detect a PTF1A/\(\text{\~{N}}\)KX6.1+ cell cluster in close proximity to endocrine and ductal cells that would represent an intermediate population giving rise to these two lineages. Possible explanations for the absence of such a trunk domain—as described in mouse development\(^\text{19}\)—could be an early ductal priming of PDLOs or insufficient scRNA-seq sampling time intervals. By contrast, MUC13-positive ductal cells evolved from UDPs, being present at day 13, and to a smaller extent, from the CFTR-positive duct cell type. We cannot exclude that the applied chemical induction protocol does not entirely resemble the in vivo development of the pancreas, and that artificial aberrations of the trunk model are induced. Differences between in vitro and in vivo progenitors have been observed during endocrine development, which also did not report a distinct trunk-like stage\(^\text{6,15}\). Additional developmental routes of pancreatic progenitors have been postulated from in vivo mouse scRNA-seq data\(^\text{46}\), and the development of a ductal subpopulation from Pdx1+/Ptf1a+ pancreatic-progenitor cells has been directly demonstrated in vivo by lineage tracing experiments\(^\text{46,47}\). Thus, it appears that the heterogeneity of pancreatic progenitors is larger than expected. Only single-cell data of primary human ductal embryonic development, which we currently lack, could further refine the in vitro PDLO engineering approach. Our finding that CFTR+/mucin+-duct-like cell types in the PDLOs resemble primary adult human duct cells indicates that PDLOs can be applicable for modelling diseases of the pancreatic ductal compartment, especially pancreatic cancer.

There is currently no efficient screening approach for early tumour detection. Personalized treatment of pancreatic cancer is still in its infancy, and predictive biomarkers are largely missing. Thus, secreted biomarkers from early neoplastic ducts could overcome this lingering unsolved obstacle. In this regard, the microwell chip can be a central interface tool for analytical methodologies such as mass spectrometry. PDLOs expressing oncogetic driver genes and probed on our microwell chip could serve as a hub to dissect oncogene-specific secretomes. Our data provide a proof of concept by determining the secretome of the genetically unaltered PDLOs and implementation of a systematic downstream biomarker classification, which we use to investigate potential diagnostic and prognostic pancreatic cancer markers against a comprehensive PDAC biobank.

**Methods**

**Microwell chips.** All microwell chips used within this study consisted of four hexagonal microwell arrays surrounded by 12 pillars. Each pillar had a diameter of 0.6 mm and a height of 4 mm. The three different microwell chips had well diameters of 150 \(\mu\)m, 300 \(\mu\)m and 600 \(\mu\)m with 251, 61 and 19 wells per array, respectively. The well diameter:well depth ratio on each microwell chip was 1.15. For visualization, the 3D print model of the 600 \(\mu\)m microwell chip is displayed in Fig. 1a.

Moulds of the PDMS microwell chips were printed using the Freeprint mould (Detax) resin in a SLA 3D printer (Asiga PICO2 HD 27) with a slice thickness of 10 \(\mu\)m. After washing the prints twice with isopropanol for 10 min, moulds were incubated at 80 °C for 1 h. Post-curing of the parts was achieved with an Otoflash G171 (NK-Optik) unit by exposing each side of the mould to 2,000 flashes under nitrogen environment. To ensure complete evaporation of isopropanol, moulds were incubated at 80 °C overnight. PDMS microwell chips were produced by standard soft lithography. In short, 3D printed moulds were precoated with 0.1% hydroxypropylmethylcellulose (Fluka Analytical) dissolved in 0.2 M sodium phosphate (Sigma) \(\text{pH} 3\), adjusted with 0.1 M citric acid (Sigma)) for 10 min, based on the method of Gitlin et al.\(^\text{71}\). The 3D-printed moulds were washed with deionized water and dried with nitrogen. 1:10 PDMS (SYLGARD 184 Silicone Elastomer Kit, Dow Chemical Company) was cast and degassed in a vacuum chamber. A glass substrate was placed onto the microwell chip spacer structures and the PDMS was cured for 1.5 h at 80 °C.

**Cell seeding onto the microwell chip technology.** Before cell seeding, microwell chips were coated with 10% Pluronic F-127 (Sigma) overnight and sterilized for 30 min using 254 nm UV light (NK-Optik). On the next day, microwell chips were washed twice with Dulbecco’s Phosphate Buffered Saline (PBS) (Gibco) and once with DMEM:F12 (Gibco). For ductal differentiation on microwell chips, pancreatic progenitors were washed with PBS, then incubated for 3–8 min with TrypLE Select (Gibco) at 37 °C for detachment. Pancreatic progenitors were centrifuged at 200g for 5 min and then seeded in 35 \(\mu\)l PDLO medium per array. The surface tension on top of the arrays allowed an equal distribution of the cell-suspension. After cell settling at 37 °C for 45 min, additional 660 \(\mu\)l PDLO medium was carefully added to the side of the microwell chip. For preliminary determination of ideal cell numbers for high-density formation, hiPSCs were seeded on the microwell chip in mTeSR1 medium (Stemcell Technologies), supplemented with 10 \(\mu\)M ROCK inhibitor Y-27632 (Abcam) during the first 24 h.

**Culture of hiPSCs.** Generation and culture of the hiPSC line has been described previously\(^\text{46}\). In brief, hiPSCs were cultured on hESC Matrigel precoated plates according to the manufacturer’s recommendations (Corning) in mTeSR1 medium at 5% CO\(_2\), 5% O\(_2\), and 37°C in a humidified nitrogen environment. The general scientific use of the cells was approved by the local ethics committee at Ulm University (reference no. 68/11-UBB/bal.). The exact isolation method, culture and pancreatic differentiation of hiPSCs, and the study of such hiPSC derivatives was approved by the local ethics committee at Ulm University (reference no. 159/19) under informed consent of donors.

**hiPSC differentiation to PDLOs.** hiPSCs were differentiated into pancreatic progenitors in a monolayer culture based on a fusion protocol from two previously published studies\(^\text{44,45}\). In brief, 2.5×10\(^6\) hiPSCs were seeded in mTeSR1 with...
caerulein (Sigma). Ten microliters of a 50 µM ROCK inhibitor per well of a 24-well plate, precoated with growth factor reduced (GFR) Matrigel (1:8 in DMEM:F12, Corning). For the first 4 d, cells were seeded in microwells. BE3 medium was supplemented with 1% Glutamax (Gibco), 0.75 g l⁻¹ l-ascorbic acid (all Sigma) were added. From day 9 until day 13, BE3 medium was supplemented with 20 µM FGF10, 0.044 g l⁻¹ nicotinamide (Sigma), 330 nM Indolactam V (StemCell Technologies) and 500 ng ml⁻¹ SANT-1, 2 µM rosiglitazone (Aldrich 43328), 1:800 anti-goat Alexa488 (Invitrogen A11055) and 1:800 anti-rat antibodies were used: 1:500 anti-rabbit Alexa488 (Invitrogen A21200), 1:200 anti-rabbit atto565 (Sigma Aldrich 43328), 1:800 anti-goat Alexa488 (Invitrogen A10155) and 1:800 anti-rat Alexa488 (Invitrogen A12208).

Orthotopic transplantation of PDLOs. Microwell chip-derived PDLOs at day 27 were collected and singularized. After pipetting PBS directly on top of the microwells, cells were washed 2 more times with PBS and singularized with Accutase (Sigma) at 37°C for 30 min. The enzymatic reaction was neutralized with 1% BSA (Prolab) 1% penicillin/streptomycin (Thermo) in DMEM:F12 (Gibco), cells were washed in BE3 medium and resuspended in PDLO medium phase II supplemented with 20 µM Y 27632 and GFR-Matrigel in a 1:1 ratio. Aliquots with cell–Matrigel mixture were kept on ice until transplantation. Mice were anaesthetized starting 3 d before transplantation by injection of 1 mg ml⁻¹ Tramadol (Grünenthal) to the drinking water. To improve the take-up rate when transplanting low numbers of PDLO cells, an acute pancreatitis was induced using cærulin (Sigma). Ten microlitres of a 5 g ml⁻¹ cælulin, 0.9% NaCl solution was injected intraperitoneally every hour 6 times before transplantation. After anaesthesia with isoflurane, a small cutaneous midline incision was followed by a small incision into the peritoneum. A volume of 30 µl with 100,000 cells was injected per mouse directly into the pancreatic tail. Carefully, pancreas and spleen were repositioned in the abdomen before the abdomen was closed by medical sewing using 5-0 polyglactin coated vicryl suture (Ethicon). Surgical staples were used for closing the skin and removed one week after transplantation, when Tramadol treatment was also stopped. Mice were killed after 8 weeks and pancreas were collected, fixed with 4% PFA at 4°C overnight, and processed for histological analysis. All animal experiments were performed in compliance with the institutional guidelines, under ethical and animal protection regulations of Ulm University.

Fast acrylamide free tissue clearing and immunofluorescence staining. For imaging of whole organoids on microwell chips, the organoids were cleared using a protocol based on fast acrylamide free tissue clearing (FACT)17. Organoids were washed once with PBS and then fixed with PFA for 3 d at 4°C. Washing with PBS was followed by incubation at 37°C for 1–3 h with 8% ultra-pure SDS (Invitrogen) in PBS for actual clearing. After washing the organoids twice with PBS and PBS-T (0.01% Tween-20 (Roti)), the organoids were blocked and post-fixed with 1% BSA (Roche), 22.5 mg ml⁻¹ glycine (Roti) and 0.1% Tween-20 for 1 h. The primary antibody was diluted in 1% BSA and 0.1% Tween-20 and samples were stained for 3 d at 4°C. After washing twice with PBS-T (Duolink), organoids were incubated with the secondary antibody diluted in 1% BSA overnight at 4°C. The organoids were washed twice with PBS (Duolink) and PBS-T (Duolink) before incubation with the primary antibodies 1:200 PDX1 (R&D AF2419), 1:200 CDH1 (Cell Signaling 3159), 1:200 SANT1, 2 µM ZnSO4, 50 ng ml⁻¹ SANT-1, 1:800 anti-goat Alexa488 (Invitrogen A11055) and 1:800 anti-rat Alexa488 (Invitrogen A12208).
The images were graded according to PDAC and PanIN cells. The intensity of FLNB was denoted from 0 to 3 (0: negative; 3: strongly positive). The percentage of cells with positive FLNB cells within cancerous structures was graded from 1 to 5 (1 denotes 20% and 5 denotes 100% of FLNB-positive cells). All healthy ductal cells were slightly positive and rated 5 for number of positive cells and 1 for intensity.

The H-score was calculated by multiplication of intensity scores (intensity of staining) with percentage scores (number of positive cells) in PDAC, PanIN and healthy cells. For the Kaplan–Meier plots, the maximal H-Score per lesion was used for each individual. Individuals who died from non-tumour-related causes were treated as alive. For the bar plot in Fig. 8a a Mann–Whitney U-test was used to calculate statistical significance.

**Forskolin swelling assay.** For the swelling assay, organoids were transferred to a bright-field imaging microwell chip, which was composed of microwells with a diameter of 800µm and a well bottom that allowed bright-field imaging. Cells were stimulated at day 28 with either 20µM forskolin (Abcam) and 100µM 3-Iobutyl-1-methylxanthine (Sigma) (forskolin treatment) or 1:1,000 DMSO (control) in DMEM:F12 medium. Live-cell imaging was performed on a Zeiss Axio Observer Z1 microscope for 18h after treatment. Images of 16 different positions were taken for each sample every 20 min.

**Pancreatic stellate cells.** Human pancreatic stellate cells (HPaSteCs) isolated from a chronic pancreatitis resection and immortalized by SV40 large T antigen and the catalytic subunit of hTERT, were provided by M. Löhr (Karolinska Institute). Cells were cultured in DMEM supplemented with 10% FCS and 1% penicillin-streptomycin and split using 0.05% Trypsin-EDTA (Sigma) twice a week in a 1:6 ratio.

**PDLO–stellate co-culture.** PDLOs and HPaSteCs were seeded on a fluidic hexagonal microwell chip, facilitating paracrine signalling between PDLOs and HPaSteCs in the same microwell chip without direct cellular contact. For this, 150 cells were seeded per microwell following the protocol described (shown in Supplementary Fig. 10a). The PDLOs were derived on the upscaled microwell chip (Fig. 8a), collected at day 31, and transferred onto 2 arrays of the microwell chip (Fig. 1). The microwell chip was filled with phase II ductal media and co-cultured for 3d without medium change. The 3D HPaSteC aggregates and PDLOs were collected at day 34. Therefore, the media was removed and 40µl drops of PBS were placed on top of each array. Using surface tension, the HPaSteC aggregates and PDLOs were collected without cross-contamination. They were washed 3 times with PBS with an incubation time of 10 min between washes. The 3D HPaSteC aggregates and PDLOs were centrifuged at 200g for 5min within the washing steps. The dry pellet was frozen and kept at −80°C upon sample preparation for proteomic measurements.

**PDLO secretome sample preparation for mass spectrometry.** To characterize the secretome of PDLOs, an upscaled microwell chip was manufactured. The microwell chip contained 1,196 microwells with a well diameter of 400µm, a well depth of 600µm and 600 cells were seeded per well (Fig. 8a). At day 28 of differentiation, PDLOs were washed three times with blank DMEM:F12 medium. Ten minutes of incubation time between the washing steps ensured settling of eventually washed out PDLOs. Then, 700µl blank DMEM:F12 was added and the supernatant was taken after 8h for subsequent analysis by mass spectrometry. For the parallel PDLO proteome determination, PDLOs were collected at the end of the experiment from the microwell chips and washed twice with ice-cold PBS and then lysed for 15 min on ice in 200µl RIPA buffer (Thermo Fisher Scientific). For disruption of the DNA, the samples were additionally sonicated and then incubated for another 15 min on ice. Protein lysates were centrifuged at 13,000g for 5min at 4°C and the supernatant was collected. Each 10µg lysate and 20µg supernatant were subjected to tryptic digest, applying a modified filter aided sample preparation (FASP) procedure as described. Peptides were collected by centrifugation (10min at 14,000g) and acidified with 0.5% trifluoroacetic acid and stored at −20°C.

**Mass spectrometry measurements.** Liquid chromatography–mass spectrometry (LC–MS/MS) analysis was performed in data-dependent acquisition mode. Mass spectrometry data were acquired on a Q-Exactive HF-X mass spectrometer (Thermo Scientific), coupled to a nano-RSLC (Ultimate 3000 RSLC; Dionex). Tryptic peptides were automatically loaded on a C18 trap column (300µm inner diameter × 5mm, Acclaim PepMap100 C18, 5µm, 100 Å, LC Packings) at 30µl/min flow rate. For chromatography, a 18 reversed phase analytical column (nanoEase M2, HSS T3 Column, 100 Å, 1.8 µm, 75µm × 250mm; Waters) at 250 nl/min flow rate in a 95min non-linear 3–40% acetonitrile gradient in 0.1% formic acid was used. The high-resolution (60,000 full width at half-maximum) mass spectrum was acquired with a mass range from 300 to 1,500 m/z with automatic gain control target set to 3 × 10^6 and a maximum of 30 ms injection time. From the mass spectrometry pre-scan, the 15 most abundant peptide ions were selected for fragmentation (MS/MS) if at least doubly charged, with a dynamic exclusion of 30s. MSMS spectra were recorded at 15,000 resolution with automatic gain control target set to 5 × 10^5 and a maximum of 50 ms injection time. The normalized collision energy was 28, and the spectra were recorded in profile mode.

**Protein identification.** Proteome Discoverer 2.4 software (Thermo Fisher Scientific; version 2.4.1.15) was used for peptide and protein identification via a database search (Sequest HT search engine) against the Swiss-Prot human database (release 2020_02, 20,349 sequences), considering full tryptic lysine and peptide acceptance only the top-scoring hit for each spectrum, and satisfying the cut-off values for false-discovery rate <1% and posterior error probability <0.01. The final list of proteins complied with the strict parsimony principle.

**Label-free quantification.** Proteins were quantified on the basis of abundance values for unique peptides. Abundance values were first normalized to the total amount of peptides in each sample to account for sample loading errors. The protein abundances were calculated summing up the abundance values for admissible peptides. In the following only proteins with more than one unique peptide hit were used for downstream analysis.

**Secretome data were filtered as follows:** First, proteins that were not detected in the parallel recorded proteome or scRNA transcriptome datasets of the duct-like clusters were filtered. Second, proteins that had not been detected previously in the pancreas were filtered out using the ‘not detected proteins in the pancreas’ protein list of the Human Protein Atlas project. To determine the fraction of proteins that were actively secreted, we matched the PDLO secretome against the refined human secretome. Only proteins with the labels blood secretion and extracellular space were used.

Protein abundance changes within the co-culture experiment were calculated by normalizing the proteomes to the mean abundance levels. The principal components were calculated on the normalized abundances. In the following, the loadings of the principal components were used for the visualization of the protein expression in the PDLO–stellate co-culture. Proteins that were upregulated in co-culture experiments compared with the respective separately cultured PDLOs or HPaSteCs were further subjected to overrepresentation analyses. Enrichment of upregulated proteins against gene sets in common databases (GO, KEGG and Reactome) was tested using gProfiler (version: e100_eg7_p14_773832) browser tool, and Enrichr allowed expansion to an EnrichR collective database comprising transcription factor protein–protein interaction networks. The co-culture signalling networks were generated with the X2Kweb interface.

**Patient material.** Archival samples of formalin-fixed paraffin-embedded material and clinical data from clinical reports originate from the previously published ULM cohort, which included 122 patients with resected PDAC. Data collection was done retrospectively and included cases from 1997 to 2008. The study was approved by the local ethics committee at Ulm University (reference no. 105/98, 211/2002 and 268/2008).

**Blood plasma of patients with metastasizing PDAC was provided by the biobank of Ulm University Hospital. A group of healthy subjects was used as controls. The study was approved by the local ethics committee at Ulm University (reference no. 159/19). Written informed consent of all patients was given for material extraction and scientific use.

**Enzyme-linked immunosorbent assay.** Levels of human FLNB in PDAC patient serum were analysed using the FLNB ELISA kit (MyBioSource, MSB731914) according to the manufacturer’s guidelines. Before the assay, plasma was centrifuged at 1,000g for 15 min at 4°C and the undiluted supernatant was added in duplicates to the ELISA plate. Absorbance at 450 nm was measured with Tecan Infinite M200 Pro plate reader. Concentration of the samples was interpolated from the standard curve that was determined within the same experiment run. The results and patient characteristics are shown in Supplementary Data 5. Statistical significance between the control patients and PDAC patients with grade 2 and 3 tumours was calculated by t-test (Fig. 8b). One patient with a tumour grading of 2–3 was excluded for the calculation of statistical significance.

**Image analysis.** Immunofluorescence, immunohistochemistry and bright-field images were cropped, rotated, aligned, and enlarged (with black background to unify the picture size), and brightness and contrast was edited with ImageJ. The measurements of the organoid diameters for the comparison of different cell numbers and well diameters were performed in ImageJ. In Fig. 1c, at least 58 3D pancreatic-progenitor aggregates from three different microwell chip arrays were measured for each condition. In Supplementary Fig. 2b, 3D hiPSC aggregates from four technical replicates were measured over 3 d. Size changes were analysed by one-sided Student’s t-test in R. Normal distribution was confirmed by the Shapiro–Wilk test. For the image analysis and editing, ImageJ version 1.52p was used.

**Sample preparation for scRNA-seq.** While pancreatic progenitors at day 13 were collected with TrypLE Select as described above, organoids on the microwell chip were collected on days 14, 16, 20, 23, 27 (samples from experiment 2: 600 cells, 300µm microwell diameter) and 31 (samples from experiment 1: 300 cells, 300µm
microwell diameter and 600 cells, 600 µm microwell diameter) by washing the microwell chip three times with PBS. For the dissociation of PDLs into single cells, organoids were incubated in Accutase for 30–45 min at 37°C. In experiment 2, single cells were cryopreserved in DMEM with 10% heat-inactivated FBS (Thermo Fisher Scientific) and 10% DMSO based on a previously described scRNA-seq sample preparation protocol84. For sequencing, cryo-preserved cells were thawed in DMEM:F12 and then live–dead filtered as described in the 10x Genomics protocol CGG000093. Cells from Experiment 1 (Fig. 2a) were not cryo-preserved or filtered, but directly processed for actual scRNA-seq measurements. An RNA library was generated using Chromium Single Cell 3’ library and Gel Read Kit v3.1 (10X Genomics). The amplified cDNA library was sequenced on a NovaSeq 6000 S2 flow cell from Illumina. The sequenced cell numbers are presented in Supplementary Fig. 6b.

scRNA-seq data pre-processing. Raw sequencing data files were demultiplexed, aligned (reference genome hg38_ensr96), filtered, barcodes and UMIs were counted, and subjected to a quality filter with CellRanger (10x Genomics). The pre-processing and downstream analysis were performed with the package Scantpy API in Python with default parameters, unless stated otherwise. First, death stressed cells, identified by a percentage of mitochondrial genes higher than 15%, were filtered out. Next, cells with less than 1,200 or more than 104 expressed genes and genes expressed in less than 3 cells were excluded. Then, the datasets from different days and experiments were concatenated, normalized to 104 gene counts per cell and log-transformed. Batch effects were corrected using Combat. Later, the top 4,000 highly variable genes were used for the downstream analysis. As described by Luecken and Theis8, we corrected for the total gene counts, percentage of mitochondrial genes, and the cell cycle distribution of S, G2 and M phase to investigate differentiation-dependent changes on the transcriptome level.

Dimensionality reduction, clustering, and cell-type annotation. The single-cell neighbourhood graph was computed with the first 50 principal components and 10 nearest neighbours and the cells were clustered with the Louvain algorithm87 at a resolution of 0.4. For visualization, the dimensionality of the data was reduced using UMAP9. For cell-type annotation, 30 DEGs for each of the nine Louvain clusters were calculated by ranking the clusters against all remaining cells with the t-test method (Supplementary Data 1). The clusters were then annotated on the basis of known marker genes.

RNA velocity through dynamical modelling. To investigate developmental trajectories, we analysed the RNA velocity by recovering directed dynamic gene information through splicing kinetics. Information such as clustering and UMAP coordinates were retrieved from the Scany Phylo tool. The pre-processing and downstream analysis were performed with scvelo using default parameters. Splice variants and cells were filtered, normalized, and logarithmized with the function scv.pp.filter_and_normalize (parameters: min_cells=3, min_counts=200, min_shared_count=20, n_top_genes=500). In a subsequent step, the moments, based on the connectivities, were calculated with 30 principal component analyses and 30 neighbours. After recovering the dynamics, the latent time was calculated with a root cell from day 13. On the basis of this latent time, the velocity was calculated as a dynamical model.

For the poly fit from Fig. 5c, we excluded the endocrine and the endothelial cell clusters, as well as cells from the progenitor clusters with a latent time higher than 0.5. This mainly excluded the progenitor cells present at late time points. Afterwards, the cells were sorted by their latent time and the gene expression was fitted to a third-degree polynomial following the code published by Bastidas-Ponce et al.10.

Enriched gene expression of gene sets. The gene-enrichment UMAP plots from Fig. 8c and Supplementary Fig. 9 were generated using the scany command sctl.score_genes. The score function subtracts the average expression of a set of genes with a reference gene-set expression, randomly sampled from the whole gene pool. The gene lists for the pathway analysis were downloaded with the R package KEGGREST11.

Integration of primary pancreas datasets. For the integration of the primary pancreas, three human datasets were used. While GSE4841331 (human samples GSM2230757, GSM2230759 and GSM2230760) and GSE8154723 focused mainly on endocrine cells, GSE13188626 described more ductal cell types. Before the datasets were concatenated, they were preprocessed, clustered and aligned as described for the PDLO scRNA-seq data. To calculate the neighbourhood graph, 32 principal component analyses (based on an elbow plot) and 20 nearest neighbours were considered. For integration and correction of the datasets, we applied bbknn to the datasets (neighbors_within_batch=5, n_pcs=32, trim=0, copy=True) and then re-clustered the cells with the Louvain algorithm12 at a resolution of 1.3.

Re-clustering of the GSE1318866 dataset. To further investigate our identified duct-like cell-cluster markers, the GSE1318866 dataset was re-clustered in a similar way to our main analysis. Cells with fewer than 800 different genes and more than 15% mitochondrial gene counts were filtered out. For the re-clustering a Louvain algorithm with a resolution of 0.06 was applied, and 2,000 variable genes and 27 principal components were taken into account.

Software specifications. The scRNA-seq alignment was run in CellRanger version 3.0.1 and the analyses were run in Python 3.7.4 with Scrapy API version 1.4.4 or 1.5.1, anndata version 0.6.22 or 0.7.4, umap version 0.3.10, numpy version 1.17.4, sciPy version 1.5.1, pandas version 0.25.3 or 1.0.5, scikit-learn version 0.22, statsmodels version 0.10.1, Python-igraph version 0.7.1, Louvain version 0.6.1, scvelo version 1.2.6, and matplotlib version 3.2.1, seaborn version 0.9.0, looumpy version 3.0.6, XlsxWriter version 1.2.6, biknn version 1.3.6 and scrublet version 0.2.1.

The plots in Figs. 1 and 8 and Supplementary Fig. 2 were generated in RStudio with R version 3.6.0 with the R packages readxl version 1.3.1, ggplot2 version 3.3.0, dplyr version 1.0.4, survminer version 0.4.8, ggpubr version 0.4.0, reshap2 version 1.4.4, survival version 3.1-12 and ggplot version 0.6.0. Dot plots in Fig. 8c and bar graphs in Supplementary Fig. 12 were generated using GraphPad Prism version 8.4.3. Principal component plots (Supplementary Fig. 11b) were calculated with stats version 3.6.0 and plotted with factoextra version 1.0.7.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The main data supporting the results in this study are available within the paper and its Supplementary Information. Raw data, read counts and the analysed datasets served from scRNA-seq can be accessed from the Gene Expression Omnibus repository using the accession code GSE162547. Mass spectrometry data have been deposited on the PRIDE database and can be accessed with the identifier PXD024461.

Code availability. The code for scRNA-seq analysis is available on Zenodo at https://doi.org/10.5281/zenodo.6736625.

Received: 27 August 2020; Accepted: 1 June 2021; Published online: 8 July 2021

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Acknowledgements

This work is supported by the Helmholtz Pioneer Campus, the German Ministry of Education and Research (INDIMED-PancChip), the Baden-Württemberg Stiftung (project ExPoChip), ERC (Consolidator Grant Number 772646), Deutsche Forschungsgemeinschaft (DFG) Sachbeihilfe KL 2544/7-1, KL 2544/1-2, KL 2544/5-1, GRK 2254/1 and 2, Heisenberg-Programm KL 2544/6-1, German Cancer Aid (Grant 111879), Else Kröner-Fresenius-Stiftung (supporting A.K. with an Excellence grant and M.H. with a First Application grant) and Bausteinprogramm of Ulm University (granted to M.H.). We thank NK-Optik for instrumental support. We thank M. Lohr (Karolinska Institute) for providing the human pancreatic stellate cells and T. Walzthöni for bioinformatics support provided at the Bioinformatics Core Facility, Institute of Computational Biology, Helmholtz Zentrum München.

Author contributions

S.W., M.B., A.K. and M. Meier designed the study. S.W. designed and produced the microwell chips, based on work from M. Moussus. S.W., M.B., J.M. and M.H. executed the biological experiments. S.W., M.B., M.H. and T.G. did the imaging and image analysis. S.W., M.S. and H.L. performed the scRNA-seq processing and S.W. did the analysis. C.v.T. and S.M.H. did the mass spectrometric measurements and data processing. S.E.W. and P.M. stained and evaluated the FLNB patient cohort. I.S. and T.S. took the serum samples of the PDAC patient cohort and M.H. performed the ELISA. S.W., M.B. and M. Meier analysed the MS/MS and FLNB screening results. M.H., A.K. and M. Meier received the funding and supervised the study. The manuscript was written by S.W., M.B., M.H., A.K. and M. Meier. All authors corrected and approved the paper.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41551-021-00757-2.

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Peer review information *Nature Biomedical Engineering* thanks the anonymous reviewers for their contribution to the peer review of this work. Peer reviewer reports are available.

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- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on [statistics for biologists](https://www.nature.com/) contains articles on many of the points above.

Software and code

**Policy information about availability of computer code**

**Data collection**

All microscope images were obtained with the Zeiss Axio Observer LSM 880 or Z1 with either Zen 2.3 SP1 FP1 (black) or Zen 2 (blue).

IHC images were captured with a Leica Typ DMS500, digital camera DFC 420C with the LAS software Leica application Suite Version 3.1.0.

Chip pictures were taken with a Nikon D7200.

Proteome Discoverer 2.4 software (Thermo Fisher Scientific; version 2.4.1.15) was used for peptide and protein identification via a database search (Sequest HT search engine) against the Swissprot human database (Release 2020_02, 20432 sequences).

**Data analysis**

The scRNA-seq alignment was run in CellRanger version 3.0.1 and the analyses were run in python 3.7.4 with Scanpy API version 1.4.4 or 1.5.1, anndata version 0.6.22 or 0.7.4, umap version 0.3.10, numpy version 1.17.4, scipy version 1.5.1, pandas version 0.25.3 or 1.0.5, scikit-learn version 0.22, statsmodels version 0.10.1, python-igraph version 0.7.1, louvain version 0.6.1, scvelo version 0.1.26 development, matplotlib version 3.2.1, seaborn version 0.9.0, loopy version 3.0.6, XlsxWriter version 1.2.6, bbknn version 1.3.6 and scrublet version 0.2.1.

The plots from Fig. 1 and Supplementary Fig. 2 were generated in RStudio with R version 3.6.0 with the R packages readxl version 1.3.1, ggplot2 version 3.3.0 and ggplot2 version 3.3.0.

The codes used for the scRNA-seq analysis are available on Zenodo at https://doi.org/10.5281/zenodo.4738625

ELISA results were analysed using GraphPad Prism 8 (GraphPad Software, San Diego, California USA, www.graphpad.com).

For the image analysis and editing, ImageJ version 1.52p and ZEN Blue imaging software (ZEISS) was used.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.
Data

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All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
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The main data supporting the results in this study are available within the paper and its Supplementary Information. Raw data, read counts and the analysed datasets from scRNA-seq can be accessed from the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE162547) repository via the accession code GSE162547. Mass-spectrometry data have been deposited on the PRIDE database (https://www.ebi.ac.uk/pride/archive/projects/PXD024461) and can be accessed via the identifier PXD024461.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

**Sample size**
No statistical methods were used to calculate sample sizes for our biological experiments. In the scRNA-seq experiments, the number of analysed cells was selected according to standard protocols, on the basis of relevant previous studies, and to comply with technical requirements. Difficulties in the retrieval of cells, sample preparation, or in the quality filter applied during data processing resulted in a variable number of sequenced cells, ranging from 500 to 6,700 cells (Supplementary Fig. 6a). The number of PDAC patients analysed in Fig. 8e–g and in Supplementary Fig. 12c was specified by the previously published ULM cohort. Sample size for the analysis of FLNB serum levels (Fig. 8h) was limited by availability of (material from) donors/PDAC patients. Precise numbers and details on the experimental replicates are provided in the paper.

**Data exclusions**
Some cells from scRNA-seq were filtered out as part of a standard quality-check procedure. For the analysis of the PDAC patient cohort, twelve patients were excluded because of the absence of tumour in FFPE tumour specimens (Fig. 8f,g). In Fig. 8h, one patient with a tumor grading of 2–3 could not be assigned to one of the predetermined groups (grading ≤2 or ≥3) and was therefore excluded for the calculation of statistical significance.

**Replication**
In general, the results of biological experiments were determined from 2 or more technical or biological replicates, with numbers stated in Methods and in the figure captions. The findings of scRNA-seq analysis could be reproduced in a second independent ductal differentiation experiment (Fig. 4d). Regarding the reproduction of the expensive and comprehensive analyses over the time series of differentiation, only the end stages were assessed. To identify duct-like cell subtypes in healthy human primary pancreas tissue and in tissue from pancreatitis patients (Fig. 5c,d, and Suppl. Fig. 7b,c), samples of at least two different donors were analysed for each condition.

**Randomization**
Randomization was not relevant to the study.

**Blinding**
The data analyses were not blinded because the analyses were based on quantitative measurements.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

**Materials & experimental systems**

| n/a | Involved in the study |
|-----|------------------------|
| ☐   | Antibodies             |
| ☒   | Eukaryotic cell lines  |
| ☒   | Palaeontology and archaeology |
| ☒   | Animals and other organisms |
| ☒   | Human research participants |
| ☒   | Clinical data           |
| ☒   | Dual use research of concern |

**Methods**

| n/a | Involved in the study |
|-----|------------------------|
| ☒   | ChIP-seq               |
| ☒   | Flow cytometry         |
| ☒   | MRI-based neuroimaging |

Antibodies

**Antibodies used**

| Primary antibodies:          | Secondary antibodies:                                      |
|-----------------------------|------------------------------------------------------------|
| acTUB (host: rabbit, dilution: 1:1000, Abcam, ab179484, clone: EPR16772, lot: GR3240369-6), AMY2A (host: rabbit, dilution: 1:1000, Sigma, A2837-3VL, polyclonal, lot: 06884796), AQP5 (host: rabbit, dilution: 1:200, Abcam, ab92320, clone: EPR3747, lot: GR3273694-1), ARL13B (host: mouse, dilution: 1:1000, Abcam, ab136648, clone: N295B/66, lot: GR3272548-1), BICC1 (host: rabbit, dilution: 1:75, Sigma, NB194171, polyclonal), C-KIT (host: mouse, dilution: 1:100, Invitrogen, CD11705, clone: 104D2, lot: 2086609), CA2 (host: rabbit, dilution: 1:500, Abcam, ab124687, clone: EPR5195, lot: GR155503-7), CDH1 (host: mouse, dilution: 1:1000, BD Bioscience, D10182, clone: 36, lot: 9315423), CDH1 (host: rabbit, dilution: 1:200, Cell Signaling, 3195, clone: 2E10, lot: 13), CFTR (host: mouse, dilution: 1:200, R&D, MAB1660, clone: 13-1, lot: BLO820050A), CFTR (host: rabbit, dilution: 1:200-800, Invitrogen, A11055, Lot: 2180272), anti-mouse alexa488 (host: donkey, dilution: 1:200, Invitrogen, A21208, Lot: 21476188), anti-mouse alexa568 (host: donkey, dilution: 1:500, Invitrogen, A21206, Lot: 2289872), anti-rabbit atto550 (host: goat, dilution: 1:1000, Sigma Aldrich, 43328, Lot: BC01523), anti-rat alexa488 (host: rabbit, dilution: 1:800, Invitrogen, A21208, Lot: 2180272), anti-goat alexa488 (host: donkey, dilution: 1:500-800, Invitrogen, A11055, Lot: 2211210) |
| Human pancreatic stellate cells, isolated from a chronic pancreatitis resection and immortalized by SV40 large T antigen and the catalytic subunit of hTERT (Jeschowski et al., 2005), were kindly provided by Prof. Matthias Lösér (Karolinska Institute). |

**Validation**

Validated on primary human tissue and/or used as recommended by the supplier.

**Eukaryotic cell lines**

Policy information about cell lines

**Cell line source(s)**

hiPSCs were established in-house from healthy donor keratinocytes. Written consent of the patient was given for material extraction and scientific use; reprogramming was permitted by the “Ethikkommission” from Ulm University (reference no. 68/11-UBB/bal.)

Human pancreatic stellate cells, isolated from a chronic pancreatitis resection and immortalized by SV40 large T antigen and the catalytic subunit of hTERT (Jeschowski et al., 2005), were kindly provided by Prof. Matthias Lösér (Karolinska Institute).

**Authentication**

Isolation, culture and pancreatic differentiation of iPSCs, and the study of iPSC derivatives, were approved by the local ethics committee at Ulm University (reference no. 159/19) under the informed consent of donors.

**Mycoplasma contamination**

Mycoplasma tests were regularly performed on the cell line, with negative results.

**Commonly misidentified lines**

No commonly misidentified cell lines were used.

**Animals and other organisms**

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

**Laboratory animals**

All animals were NSG mice (strain: NOD.1-Cg-Pkdcsdcsdh1tm1Wjl/SzJ; GVO) from Charles River, RRID:BCB-4142, female, 11-weeks old, with a weight of 25 g.

**Wild animals**

The study did not involve wild animals.

**Field-collected samples**

The study did not involve samples collected from the field.
Ethics oversight

All animal work was done under ethical and animal protection regulations of the German animal protection law, and were previously approved by the respective governmental review board of the state of Baden-Württemberg (TVA-1406).

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Human research participants

Policy information about studies involving human research participants

Population characteristics

The previously published ULM cohort included 122 patients with resected pancreatic ductal adenocarcinoma (Schmid et al, Pancreas 2013; Feld et al., Oncotarget 2015). PDACs were classified with the International Union Against Cancer (UICC) according the 7th Edition of the TNM Classification of Malignant Tumors. In few cases, metastatic PDAC has also been resected (Hackert et al., Eur J Surg Oncol 2017). All 110 tumor samples analysed in this study were treatment-naïve. 40 patients received adjuvant chemotherapy after primary resection. In detail: 24 patients received gemcitabine monotherapy and 14 received a combination of gemcitabine with either capecitabine (2 patients), erlotinib (8 patients) or cetuximab (4 patients). 5-Fluorouracil was administered to one patient. One patient received a not-further-specified study drug. FLNB protein expression was determined by immunohistochemistry in 110 samples. Twelve patients were excluded because of the absence of tumour in FFPE tumour specimens. FLNB protein-expression level within the tumour compartment was evaluated by a board-certified pathologist at Ulm University. FLNB expression level was scored from zero (negative) to five (strong). FLNB expression levels were correlated to the clinical characteristics of patients. The analysis of FLNB serum levels was restricted to patients with metastasizing PDAC and healthy control subjects. Detailed patient characteristics are available as Supplementary Information.

Recruitment Data collection of the ULM cohort (archival samples of FFPE material and clinical data from clinical reports) was done retrospectively, and included cases from 1997 to 2008. Blood plasma from patients was provided by the biobank of Ulm University Hospital.

Ethics oversight

The studies were approved by the local ethics committee at Ulm University (reference no. 159/19).

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

Confirm that:
- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Differentiation efficiencies were controlled on day 3, 9 and 13. The percentage of definitive endoderm cells was assessed by staining the surface marker CXCR4 and c-KIT on day 3. Pancreatic endoderm and pancreas progenitor cells were formaldehyde fixed (4% PFA) (Thermo Fisher Scientific) on day 9 and 13 for intracellular staining of PDX1, and PDX1 + NKX6-1, respectively. Details of the staining procedure have been previously described in Hohwieler et al. (Gut 2017).

Instrument

LSRII flow cytometer (BD Biosciences)

Software

FlowJo (v10; BD Bioscience)

Cell population abundance

Not applicable.

Gating strategy

First, cells were gated on FSC-A/SSC-A; and second, single cells were gated on FSC-H and FSC-A. Positive signal was gated based on isotype controls and undifferentiated hiPSCs as the negative control.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.