nature biomedical engineering | PEER-REVIEW INFORMATION

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

Corresponding author: Matthias Meier

Editorial note

This document includes relevant written communications between the manuscript's corresponding author and the editor and reviewers of the manuscript during peer review. It includes decision letters relaying any editorial points and peer-review reports, and the authors' replies to these (under 'Rebuttal' headings). The editorial decisions are signed by the manuscript's handling editor, yet the editorial team and ultimately the journal's Chief Editor share responsibility for all decisions.

Any relevant documents attached to the decision letters are referred to as **Appendix #**, and can be found appended to this document. Any information deemed confidential has been redacted or removed. Earlier versions of the manuscript are not published, yet the originally submitted version may be available as a preprint. Because of editorial edits and changes during peer review, the published title of the paper and the title mentioned in below correspondence may differ.

Correspondence

Wed 18 November 2020
Decision on Article nBME-20-2235

Dear Prof Meier,

Thank you again for submitting to *Nature Biomedical Engineering* your manuscript, "Charting ductal differentiation in a pancreas-on-chip model with single-cell resolution". The manuscript has been seen by 3 experts, whose reports you will find at the end of this message. You will see that although the reviewers have some good words for the work, they articulate concerns about the degree of support for the claims, and in this regard provide useful suggestions for improvement. We hope that with significant further work you can address the criticisms and convince the reviewers of the merits of the study. In particular, we would expect that a revised version of the manuscript provides:

* Clarification of the role of stroma cells in the model, as suggested by Reviewer #1.

* Clarification of the spatial and temporal development of matrix composition, as suggested by Reviewer #1.

* Discussion of the technological innovation of the work, as suggested by Reviewer #1.

* Demonstration of the presence of new cell subpopulations in vivo, as suggested by Reviewers #2 and #3.

* Evidence of whether biomarkers correlate with decreased survival, as suggested by Reviewer #2.

* Clarification of the interpretation of the results, in particular in the context of whether the markers are ductal and whether mouse data can be extrapolated to human, as suggested by Reviewer #3.

* Provision of clear images to support the claims, as suggested by Reviewer #3.

When you are ready to resubmit your manuscript, please upload the revised files, a point-by-point rebuttal to the comments from all reviewers, the (revised, if needed) reporting summary, and a cover letter that explains the main improvements included in the revision and responds to any points highlighted in this decision.

Please follow the following recommendations:

* Clearly highlight any amendments to the text and figures to help the reviewers and editors find and understand the changes (yet keep in mind that excessive marking can hinder readability).

* If you and your co-authors disagree with a criticism, provide the arguments to the reviewer (optionally, indicate the relevant points in the cover letter).

* If a criticism or suggestion is not addressed, please indicate so in the rebuttal to the reviewer comments and explain the reason(s).

* Consider including responses to any criticisms raised by more than one reviewer at the beginning of the rebuttal, in a section addressed to all reviewers.

* The rebuttal should include the reviewer comments in point-by-point format (please note that we provide all reviewers will the reports as they appear at the end of this message).

* Provide the rebuttal to the reviewer comments and the cover letter as separate files.

We hope that you will be able to resubmit the manuscript within 25 weeks from the receipt of this message. If this is the case, you will be protected against potential scooping. Otherwise, we will be happy to consider a revised manuscript as long as the significance of the work is not compromised by work published elsewhere or accepted for publication at *Nature Biomedical Engineering*. Because of the COVID-19 pandemic, should you be unable to carry out experimental work in the near future we advise that you reply to this message with a revision plan in the form of a preliminary point-by-point rebuttal to the comments from all reviewers that also includes a response to any points highlighted in this decision. We should then be able to provide you with additional feedback.

We hope that you will find the referee reports helpful when revising the work, which we look forward to receive. Please do not hesitate to contact me should you have any questions.

Best wishes,

Michelle

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Dr Michelle Korda
Senior Editor, *Nature Biomedical Engineering*

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Reviewer #1 (Report for the authors (Required)):

This manuscript reports the development of a pancreatic duck-like organoid model from iPSC. This manuscript is a timely report to address one of the main bottlenecks of in vitro tumor model development - overcome the limited supply and culture of primary pancreatic acinar and epithelial cells. The developed model mimic anatomical characteristics of pancreatic acinus and duct. The model was further analyzed via single-cell RNA-seq and identified a subpopulation of ductal cells during developments. The data were further analyzed to discover the markers for pancreatic cancers.

The manuscript is well written and logically structured. Its impact is potentially significant. However, several weaknesses are noted.
1. One of the major weaknesses is the lack of stroma cells, which play important roles in pancreas development and PDAC initiation/progression.

2. Matrix composition in both spatial and temporal contexts is overlooked. Analysis of this will significantly strengthen the manuscript.

3. The developed model is a smaller version of the spheroid culture well-plate. Technological innovation is moderate. The authors may want to articulate what is the major engineering innovation.

4. Another major limitation is prolonged culture durations - order of month cultures. The authors want to discuss how to address this technical challenge.

Reviewer #2 (Report for the authors (Required)):

Summary
The manuscript entitled 'Charting ductal differentiation in a pancreas-on-chip model with single-cell resolution' by Wiedenmann and Breunig et al. provides a novel pancreatic duct-on-chip culture platform to aid in the culture and study of pancreatic progenitor populations. Notably, the work used this new technique to chemically induce into two distinct pancreatic ductal progenitors: (i) intermediates and (ii) mature duct-like cells. This is an important step forward in charting the evolution of the ductal lineage in the pancreas. Finally, the authors utilize single cell transcriptomic analysis to uncover new ductal markers that may be important for pancreatic tumorigenesis.

Major Comments:
1. The identification of new duct-like clusters identified in Figure 3 requires significant elaboration. These populations should be thoroughly investigated in tissue to see these cells truly exist in vivo. For example, are "duct-like 1" clusters found to be Krt19+, Cftr+, Sctr+, Blcc1+ while the "duct-like 2" and "duct-like 3" are Krt19+, Cftr-, Sctr-, Blcc1-? Are these three sub-populations readily identifiable within the same tissue, or does one duct-like population predominate in individual animals? This is essential to establishing the physiological relevance of the new system.

2. Figure 4, as it currently stands, is lacking in depth. It appears to be a verification at the protein level of individual markers identified from the scRNA-seq experiment in Figure 3, which is just a confirmation that the transcriptional profiling is valid. It is also unclear why only 2 of the many new markers that were identified were chosen to be validated. Did the others not correlate at the protein level? If so, is there any way to combine Figure 3 and Figure 4 to only identify the transcriptional hits from Figure 3 that were validated at the protein level in Figure 4? It seems that that there are ~3 unique hits for each new sub-type of duct-like cells (i.e. "false hits" identified by the sequencing experiment that may have changes at RNA levels that don't correlate at the protein level). Nonetheless, the validation of a limited number of markers is another concern.

3. To truly demonstrate the benefit of this pancreas-on-chip as a biomarker development tool, the authors should take a few of the top hits identified (such as SDCBP2 or FLNB) and show that staining in human and mouse tissues confirms the expression of these proteins and that high staining correlates with decreased survival.

Minor Comments:
1. The authors show gain of ductal and epithelial genes as the organoids progress down the chemical induction (in Figure 2C-D). The authors should show loss of "stemness" genes, potentially indicating the irreversibility of this conversion.
2. ECAD appears to be lost in the Type II day 31 organoids. Do the authors think that epithelial-to-mesenchymal transition is occurring in this subtype?
3. It is unclear what parts of Figure 3F reached statistical significance.
4. Figure 5F contains many genes that might be worthy of more discussion regarding potential hierarchy of ductal development over time. This should be discussed further.

Reviewer #3 (Report for the authors (Required)):

The article by Wiedenmann and collaborators reports on the development of a 3D model of human pancreatic ductal development. It is a valuable study, which will be useful to help our understanding of human development and for disease modelling (Cystic fibrosis, PDAC, pancreatitis…), for which they provide some preliminary data. The study itself is deep and one of its strength is the use of single cell sequencing to characterize heterogeneity in the system and its time-progression.

Several points will need careful attention prior to publication:
- Most importantly, many markers presented as ductal markers are also present in pancreas progenitors. The authors should revisit the literature and revise the text and their conclusions (see some details below). Caution in extrapolating mouse data to human should also be applied.

- The authors draw many lineage conclusions which should be worded with more caution. The single-cell methods show the molecular proximity between cells, not lineage.

- Some documentation of markers by immunocytochemistry is of low quality and more compelling data need to be provided (see below)

- The benchmarking to published transcriptome datasets of in vivo samples should not be over-interpreted. Batch corrections are applied to merge the data, which “forces” the in vitro clusters to map in the vicinity of some populations in the dataset. It can’t be used to claim that the cells in vitro are the same as in vivo.

Detailed comments
(1) Line 41: reducing bicarbonate secretion to transport facilitation is somewhat reductionist.

(2) Line 51: Typo to correct “dysplasian”

(3) Lines 52-53: “Thus, lineage-committed pancreatic ductal cells generated from human inducible pluripotent stem cells (hiPSCs) could be an alternative source of pancreatic organoids to overcome these obstacles12,13”. This is true but it would be even more obvious from adult ductal-derived organoids. Alternative systems should not be dismissed.

(4) Lines 56-58: While previous studies have clearly shown that all pancreatic lineages derive from pancreatic progenitors and ref 15 is certainly appropriate as the first one, if the authors cite more than one, 14 would need to be complemented by many others (doi: 10.1016/j.devcel.2009.11.003; doi: 10.1242/dev.053843.; doi: 10.1242/dev.056499. or at single-cell level doi: 10.1038/s41467-017-00258-4.)(this is likely too many and citing only the first one is a possibility. Reference 16 does not show that all pancreatic lineages derive from pancreatic progenitors and to my knowledge this demonstration has not been made. It would be appropriate to say that by analogy to mouse, because the cells express similar markers, we assume that in human it is the same.

(5) Line 62: Please include a reference to your previous paper

(6) Lines 95-96: “Small diameters clearly compromised the aggregation step, while a large diameter is known to impair nutrient supply”. The sentence does not represent the results faithfully. In Fig1e, it seems that the number of cells has more impact on the diameter of the aggregate than the well diameter. For example, with 75 cells, the diameter seems a little larger but almost the same in 150 and 300 micrometer diameter wells. It would also be better not to mix in the same sentence a reference to the authors’ observations in the figure and reference to the literature. The nutrient supply problem is not shown in the present article.

(7) Lines 97-98: Please refer to the differentiation method used to obtain PP at this point.

(8) Lines 113-115: It would be good to know where the two-step protocol comes from. Why these components? Why the selected times?

(9) Lines 119-122: while is it likely that the type II structures are formed from the type 1 PDLOs it would be more convincing to show live imaging. Is any cell death involved in type II PDLOs? They are very small and the culture does not look very healthy by day 31.

(10) Lines 125-126: Are they really formed of pancreatic ductal cells? All the markers cited are also expressed in progenitors (see for example doi: 10.1677/joe.0.1810011. for CK19 in the pancreas of human foetuses as early as 41 days post conception and CFTR is widespread in 13-week foetuses https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2230211/?page=3- at these stages the ducts are expected to be composed of progenitors).

(11) Figure 2: The absence of CK19 in the center of organoids is puzzling. From ref doi: 10.1677/joe.0.1810011 Figure 5, CK19 is expressed throughout the epithelium in progenitors/ducts. The only negative cells during early development are endocrine cells and likely acinar cells. Are the central cells acinar or endocrine? It does not seem so from the transcriptome. What are they? Are CK19-negative cells found in the transcriptome?

(12) Supplementary Figure 3a, b: The images suggest that ZO1 is polarized with the apical side in or out PLDOs I. What is the proportion of PDLO Is of each type? Figure 4 suggests that there are PDLO Is with a mixed apical in- and out- polarity. This is important in the system for future functional studies as the ducts in vivo have their apical side in. The absence of polarization of PDLO IIs is another reason to doubt their health. However, they may express other apical markers. The paper investigates Muc13 at a later point. Is it polarized?

(13) Supplementary Figure 3c: Many of the images are not of publication quality and are not convincing, notably for the markers on rows 2 and 3 except for Ki67 and CFTR (both clear). Immunofluorescence would likely be more convincing as the DAPI can show the nucleus as a landmark and moreover several markers can be combined. When signals are in rare cells, more cells need to be documented, when they are weak, negative controls need to be provided and a mix of high and low magnification panels. This would be important to get a good understanding of what these cells are and their spatial arrangement.

(14) Lines 154-156: “The major cell populations identified included four different 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 type of non-pancreatic cells, namely endothelial-like cells (cluster IX).” And lines 156-158: “Intriguingly, our starting PP cell population, which was generated in a 2D cell culture, already contained multipotent and unipotent ductal progenitor cells.” The authors should use more careful wording as it is unclear what these cells really are. A suggestion follows “Intriguingly, our starting PP cell population, which was generated in a 2D cell culture, contained cells with different transcriptional profiles suggestive of ….” And see points below about the arguments relative to what these cells are.

(15) Lines 159: “PTF1A and NKX6-1 were expressed only in the multipotent progenitors (MPP)”. The authors don’t prove these cells are multipotent progenitors. This needs to be reworded into something in the following spirit “PTF1A and NKX6-1 were co-expressed in cluster, suggestive of multipotent progenitors (MPP) by analogy to mice (ref)”…. And so on for the next clusters. To 29-31, which are reviews, it would be nice to add original lineage tracing in mouse: for Ptf1a (DOI: 10.1242/dev.090159 and at single cell level doi: 10.1038/s41467-017-00258-4.) For Nkx6.1 the original tracing for Sox9 combined with images of absent Nkx6.1 can be found in doi: 10.1242/dev.056499.)

(16) Note that CD133/Prominin is not a marker of unipotent ductal progenitors. It is abundant in pancreas progenitors and endocrine and exocrine cells emerging from them (see doi: 10.1073/pnas.0609490104. for mouse and subsequent work of the Kim lab in human). It is not clear to this reviewer if CK7 is a ductal marker or is also expressed in progenitors. The best argument that these cells are on a ductal trajectory is the reduction of NKX6.1, based on the mouse references highlighted in the comment above..

(17) Figure 3 is very interesting. It is biased to some markers but the Tables are useful and show an unbiased analysis to potentially new markers. There are problems with p-values=0 in several of the columns in Tables 1 and 2

(18) The ducts like cluster 3 remains somewhat enigmatic, all the more that it seems detectable only in one of the two experiments. Is this a third ductal type or some cells enriched in CLDN4 and MMP1. Are CLDN4-high cells detected by immune?

(19) Line 192: Is CFTR expressed similarly in the Type I PDLOs with apical side in and apical-out? Were all the PDLO II MUC13+?

(20) Line 206: “In line with the trilineage differentiation potency of MPPs, these cells also produce endocrine cells without a clear evidence of an intermediate bipotent trunk domain” These experiments are not lineage tracing. They suggest a continuity between the endocrine cells and some populations with more progenitor hallmarks. The wording should be more careful. Whether there are bipotent progenitors in the culture should have been discussed earlier in the result section. From what we know in mouse (in the absence of human data) these cells should be NKX6.1+ but PTF1A-. PTF1A seems very low in the data. I am not sure it enables to make a strong statement here. I am sure some PTF1A- cells can be found but is it technical noise or do they cluster in UMAP?

(21) Line 200 and following: the authors should be careful not to over-interpret the velocity trajectories. The ductal populations seem to be linked to the other populations via multiple wide bridges made of few cells. The PAGA shows very well the uncertainty of trajectories.

(22) Line 215 and thereafter: again one needs to be careful about these markers being true ductal markers and whether they are expressed in progenitors (see the comments above, notably for CFTR).

(23) Line 239 and following: This is a very interesting analysis. The authors should however make it clear that a batch correction was done (here bbknn). This method will “force” the cells somewhere on the clusters detected in vivo. It by no means shows how close the cells grown in vivo are to the ductal cells but will find the most related. This should be made very clear in the results to the reader. The authors currently make claims that cannot be made with the data.

(24) In the discussion, it would be worth discussing the differences in media with the author's previous system which seemed to produce acinar cells.

(25) Lines 335-337: "Although the timing of particular marker expressions is slightly different, trunk/tip patterning is confirmed in human development15." It is not confirmed and was never shown. Marker expression suggests the presence of tip and trunk cells similar to those characterized functionally in mice.

(26) Line 426: It would be useful to indicate the differentiation efficiency used as a cut-off to proceed further to PDLOs.

(27) Line 593: The data should be made available in a public repository

Tue 04 May 2021
Decision on Article nBME-20-2235A

Dear Prof Meier,

Thank you for your patience in waiting for our feedback on your revised manuscript, "Charting ductal differentiation in a pancreas-on-chip model with single-cell resolution". Having consulted with Reviewers #2 and #3 (whose comments you will find at the end of this message; as noted in previous e-mail correspondence, despite our chasing efforts Reviewer #1 failed to provide a report), I am pleased to write that we shall be happy to publish the manuscript in *Nature Biomedical Engineering*, provided that the points specified in the attached instructions file are addressed.

When you are ready to submit the final version of your manuscript, please upload the files specified in the instructions file.

Also, please address the minor points of Reviewer #3, and provide a point-by-point reply.

For primary research originally submitted after December 1, 2019, we encourage authors to take up transparent peer review. If you are eligible and opt in to transparent peer review, we will publish, as a single supplementary file, all the reviewer comments for all the versions of the manuscript, your rebuttal letters, and the editorial decision letters. **If you opt in to transparent peer review, in the attached file please tick the box 'I wish to participate in transparent peer review'; if you prefer not to, please tick 'I do NOT wish to participate in transparent peer review'.** In the interest of confidentiality, we allow redactions to the rebuttal letters and to the reviewer comments. If you are concerned about the release of confidential data, please indicate what specific information you would like to have removed; we cannot incorporate redactions for any other reasons. If any reviewers have signed their comments to authors, or if any reviewers explicitly
agree to release their name, we will include the names in the peer-review supplementary file. More information on transparent peer review is available.

Please do not hesitate to contact me should you have any questions.

Best wishes,

Pep

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Pep Pàmies
Chief Editor, *Nature Biomedical Engineering*

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Reviewer #2 (Report for the authors (Required)):

The authors have re-submitted a rigorously revised manuscript that contains a substantial amount of new data with appropriate conclusions.

Reviewer #3 (Report for the authors (Required)):

The authors have gone a long way to address the reviewers' comments. They notably added:
- data showing co-culture with mesenchymal cells (in adjacent wells that communicate) and the analysis of their effect by transcription profiling. That's a nice addition. This is an important achievement as finding co-

culture conditions enabling multiple populations to co-exist has been a challenge in the organoid field. It remains superficially analysed as we don't know how well the cells proliferate and the effects of ratios. What the induced genes and the GO terms tell us about the effect of the mesenchyme on the ductal cells and of the ductal cells on the mesenchyme remains unclear. However, since the authors did so much already, this is beyond the scope of the paper.
- the ductal markers are much better documented and discussed.
- the discussion of progenitors is much improved and fits previous literature better. Transplantation experiments illustrate the ductal commitment.
- one of the prospective PDAC markers identified is further investigated, including expression by immunocytochemistry, its association with PANINs, tumors and its presence in the blood as a predictive marker.
- in a less important addition but addressing a reviewer comment, they show specific data on matrix proteins produced and deposited in the system (transcriptome data figure and immunofluorescence.
- they did some data reanalysis to satisfy comments on benchmarking

Remaining major technical criticisms or questions.
None

Remaining minor technical criticisms or questions.
1-Though transplantation experiments illustrate the ductal commitment only 2 animals were analysed. It is also unclear how rare cells would be identified in these experiments which do not appear to have a high number of engrafted tissue.

2-In figure 3d, the Zo1 immuno does not suggest that apical is outside at day 28 and that there is no polarity in 3D PP aggregates. Most of the staining seems to be inside. Of note, these panels are very small. The small insets would be better used to show a magnified area.

Missing or unclear details about statistics, protocols or materials (please check the reporting summary provided, and note that the form is a dynamic PDF file that when not flattened can only be properly viewed via Acrobat Reader
None noticed

Missing citations to relevant literature.
None

Optional suggestions for improvement.
None, experimentally

Stylistic issues or recommendations.
3-In the title, the authors state: "we uncover molecular ductal differentiation mechanisms". I would argue that the authors identify conditions conducive of molecular ductal differentiation but that they do not really uncover mechanisms (how do ductal cells differentiate? What is the medium component driving them? Which molecules do they rely on to trigger ductal differentiation?)
4-Incorrect sentence line 143: "Stemness marker SRY-box 2 (SOX2) and octamer-binding transcription factor 4 (OCT4) were absent (Supplementary Fig. 3c) similar to non-ductal pancreas markers except for few endocrine cells located at the periphery of PDLOs (Supplementary Fig. 3d)." Suggestion: "Stemness markers SRY-box 2 (SOX2) and octamer-binding transcription factor 4 (OCT4) were absent (Supplementary Fig. 3c), as were non-ductal pancreas markers, except for few endocrine cells located at the periphery of PDLOs (Supplementary Fig. 3d)."

5-Line 333: Typo- "To reconfirmed" should read "To confirm"

6-Line 362: "Gene ontology (GO) term analysis for protein signatures and transcription factors revealed activation of both cell types as indicated by enrichment of pathways governing stellate cell activation45, energy metabolism, and cellular signaling (Supplementary Fig. 11c). Alongside, a constructed protein network in co-cultured PDLOs or
HPaSteCs resembled a mitogenic pattern in further support of this notion (Supplementary Fig. 11d)." This sentence has a structural problem that prevents understanding. It is also not clear to this reviewer what these terms reveal about the association with mesenchyme.

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Rebuttal 1

Reviewer report

Reviewer #1:

1. *One of the major weaknesses is the lack of stroma cells, which play important roles in pancreas development and PDAC initiation/progression.*

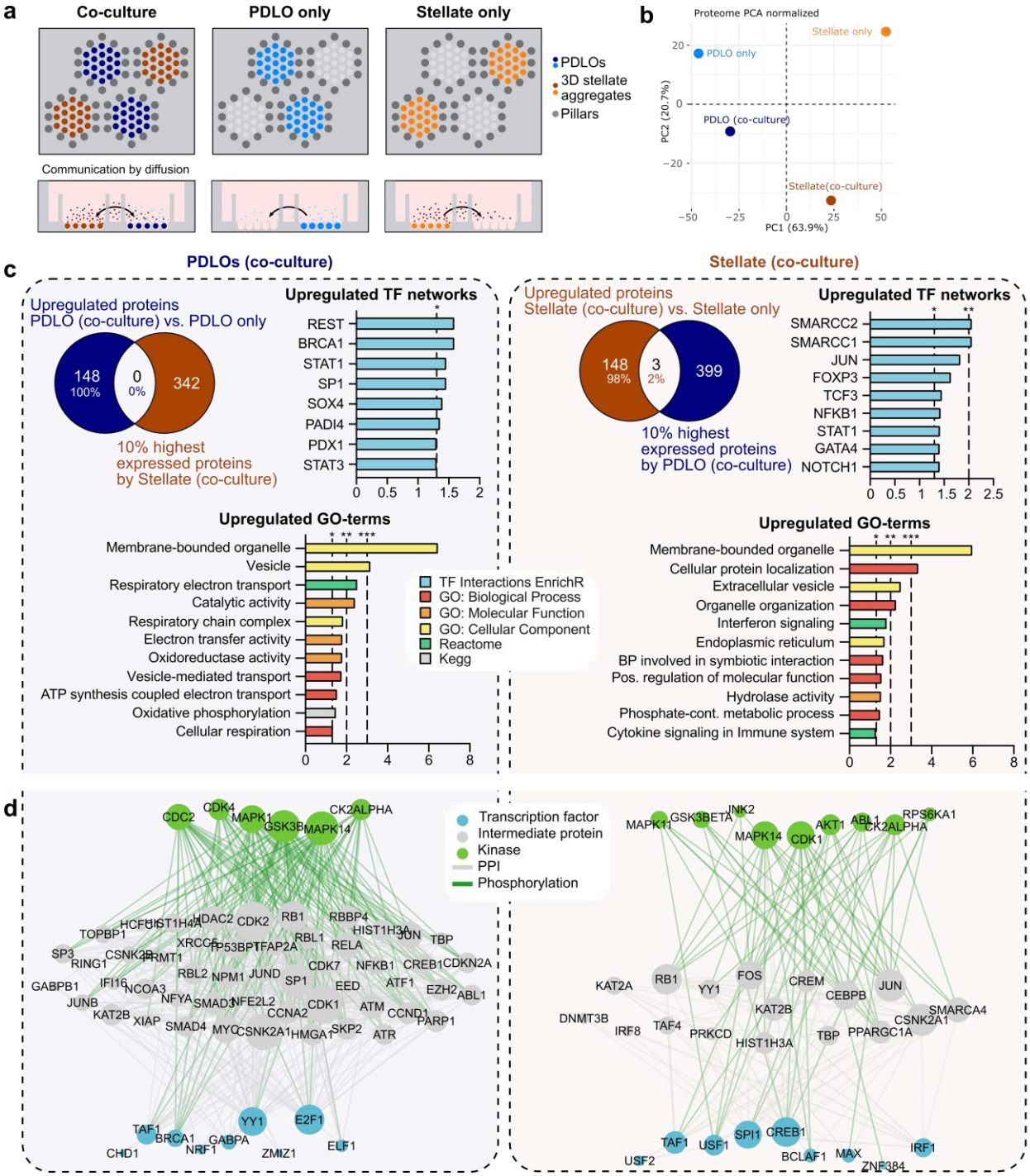
Answer: We fully agree that a complete PDAC model would include a tumor-specific microenvironment consisting of stromal cells and immune cells. We would like to stress, however, that our manuscript is focused on both (i) the development of a new bioengineering platform to generate ductal cells on a microchip device and (ii) to shed light on the differentiation trajectories via a comprehensive scRNA-seq analysis. Our advances are comparable to research attempts made in the development of pancreatic β-cells, for which also the understanding of differentiation trajectories, culture formats, and analytical platforms comes first and stands for its own (Veres et al., 2019). Accordingly, our study reports for the first-time developmental routes taken by pancreatic ductal cells during lineage commitment form pancreatic progenitors.

Nevertheless, we took the suggestion of the referee serious and demonstrate that our duct-on-chip platform is versatile and allows to mimic a stromal microenvironment by integrating co-cultures on-chip platform between PDLOs and quiescent pancreatic stellate cells. The latter cell type resembles inactive stromal cells in the human pancreas (Erkan et al., 2012). Quantitative proteomics allowed us in a proof-of-concept study to dissect the cross-talk between PDLOs and stromal cells inducing an activating response in both cell types. As our duct-on-chip platform could potentially host also inflammatory cells in the four separable fluidic hexagonal arrays, a virtually complete tumor-microenvironment could be generated. The co-culture experiment illustrated in **new Supplementary Fig. 11** has been designed as proof-of-principle to highlight the application possibilities of the chip platform. In future, more experiments will be needed to characterize the signaling networks between the two cell types in particular in context of PDAC and its activating paracrine signaling. However, we believe that this is beyond the scope of this manuscript.

The results, that we have compiled for the revision, are shown in the **new Supplementary Fig. 11** and we added the following sentences to our manuscript:

"The duct-on-chip platform can be applied for various applications. One example is to investigate cell-cell communication between pancreatic ducts and various kinds of stromal cells. For this, four fluidic separable hexagonal arrays on the micro-well chip were exploited to establish a cross contamination-free co-culture between PDLOs and human pancreatic stellate cells (HPaSteC) (**Supplementary Fig. 11a**). The latter resemble quiescent stromal cells in the pancreas capable to convert during inflammation, injury, and cancer development via auto- and paracrine signals to a metabolically active state, then serving as a central player in the pathogenesis of pancreatic disease (Erkan et al., 2012). Quantitative proteome analysis of both on-chip cultured 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 the PDLOs were found in the upregulated protein set of the co-cultured PDLOs and HPaSteCs, which suggest neglectable cross-contamination on-chip (**Supplementary Fig. 11c**). Gene ontology (GO) term analysis for protein signatures and transcription factors revealed activation

of both cell types as indicated by enrichment of pathways governing stellate cell activation (Erkan et al., 2012), energy metabolism, and cellular signaling (**Supplementary Fig. 11c**). Alongside, a constructed protein network in co-cultured PDLOs or HPaSteCs resembled a mitogenic pattern in further support of this notion (**Supplementary Fig. 11d**).”



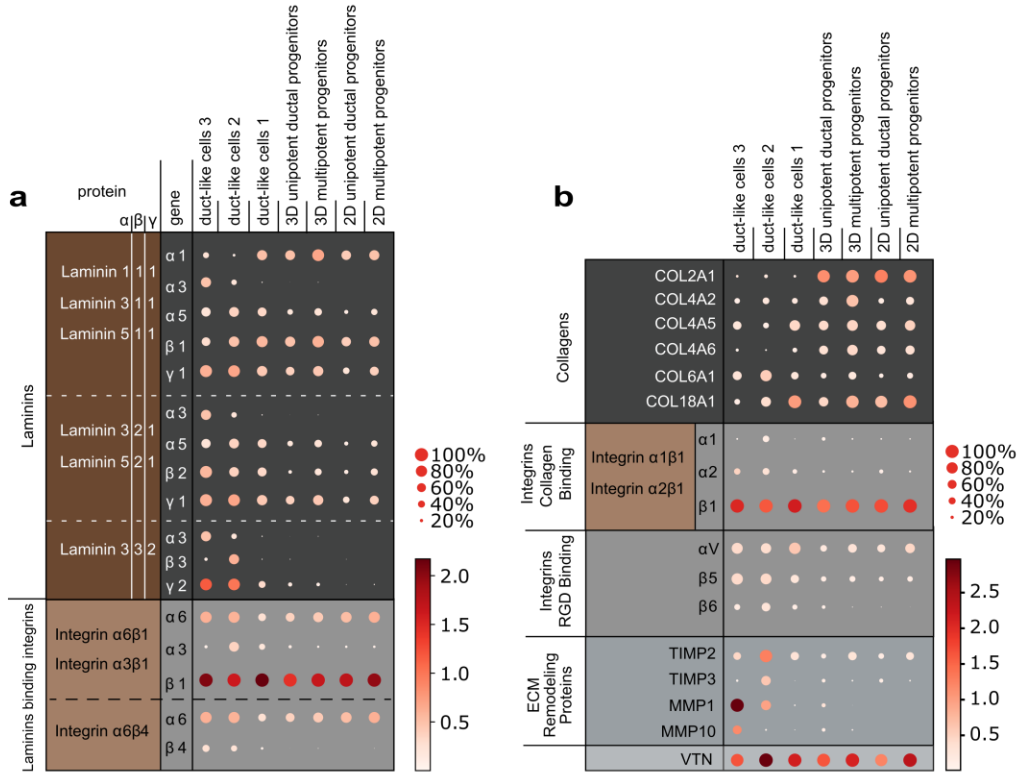
Supplementary Fig. 11: Co-culturing of PDLOs and human pancreatic stellate cell (HPaSteC) aggregates on-chip. **a**, Schematic of the micro-well chip for co-culturing PDLOs and 3D HPaSteC aggregates. The four fluidically separable hexagonal arrays on the micro-well chip enabled co-cultures of PDLOs and HPaSteCs without mixing

cell types. After 3 days of co-culture, both cell types were retrieved, and their proteomes were determined by label-free mass spectrometry. **b**, Principal component analysis (PCA) separating PDLOs and HPaSteCs after co-culture from their cultures on individual chips. **c**, Cross contamination test and GO term analysis of the upregulated protein sets in PDLOs and HPaSteCs. Enriched protein interactions of several transcription factors such as STAT1, STAT3 or JUN, STAT1, NFKB suggested an activation of PDLOs and HPaSteCs, respectively, in co-cultures. GO term analysis enquiring key databases further revealed an upregulation of vesicle trafficking indicative for increased autocrine and paracrine signaling (membrane-bounded organelle, extracellular vesicle, vesicle-mediated transport) and energy consumption (respiratory electron transport, respiratory chain complex, ATP synthesis, or oxidative phosphorylation). **d**, Protein network analysis(Clarke et al., 2018) of the upregulated protein sets in co-cultured PDLOs and HPaSteCs revealed paracrine signaling. In both cases, we find mitogen stimulation network signaling, which shows the diffusion-driven communication and activation of the co-cultured cells.

2. *Matrix composition in both spatial and temporal contexts is overlooked. Analysis of this will significantly strengthen the manuscript.*

Answer: We agree with the reviewer and included a transcriptomic analysis of differentially expressed ECM and matrix remolding genes (see **new Supplementary Fig. 8**). We added the following data and sentence to the manuscript:

"Since we also detected dynamical expression of several ECM-related genes, we analyzed the scRNA-seq data in more detail regarding changes 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/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 (Crisera et al., 2000; Jiang et al., 1999; Miner and Yurchenco, 2004). Accordingly, progenitor and duct-like cluster 1 cells expressed α1 laminin, duct-like cells 2 and 3 expressed α3, α5 laminin (**Supplementary Fig. 8a**). Of note, α4 laminin (Qu et al., 2014) and α5 integrin, which are central for β-cell formation (Mamidi et al., 2018), were neither expressed in PDLOs nor in the progenitor state. In contrast, αVβ5 integrins were upregulated in duct-like cells (**Supplementary Fig. 8b**) in agreement with previous findings (Cirulli et al., 2000). It can be concluded that on-chip-derived PDLOs are likely to secrete soluble ECM and corresponding binding proteins as seen under *in vivo* conditions. Within the open micro-well culture format, however, the proteins are most likely resolved in the media which would explain the polarity switch of the on chip-derived PDLOs upon either *in vivo* transplantation or 3D Matrigel culture (**Fig. 3d**)."



Supplementary Fig. 8: Single-cell transcript analysis of genes associated with extracellular matrix (ECM) showing dynamical expression during the differentiation trajectory of PDLOs. Dynamical genes were determined by the velocity analysis in main Fig. 5. **a,** Dot plot shows the change of laminin and laminin-binding integrin gene expression within the cells of the different clusters. **b**, Dot plot shows the change of collagen, collagen-binding integrins, RGD binding integrins, and ECM remodeling gene expression within the cells of the different cluster during differentiation.

3. *The developed model is a smaller version of the spheroid culture well-plate. Technological innovation is moderate. The authors may want to articulate what is the major engineering innovation.*

Answer: We agree that microarray culture approaches are not entirely new. However, commercial low attachment well plates are available for the formation of 3D cultures. The new impact of the presented approach is given on one hand by (i) the low cell and material consumption (**Fig. 1e, Supplementary Fig. 2**), (ii) defined and homogeneous size distribution of generated 3D cell cultures (**Fig. 1e, Supplementary Fig. 2**), (iii) the possibility for long-term culture of 3D aggregates (see **Fig. 2**), (iv) sample retrieval for downstream analysis including secretome analytics with minimal perturbation (e.g. **Supplementary Fig. 2, 3, 4, 5, 7**), and (v) the possibility to establish co-cultures (**Supplementary Fig. 11**). These advantages, however, are of central importance to the stem cell field. On standard low attachment well plates, formation of 3D cultures has size distribution of 1 order of magnitude, requires 3 order of magnitude more cells, and does not promote long-term culturing due to merging aggregates upon media exchange. For example, in low attachment well plates a sample loss of above 50% is observed when transferring a 2D into a 3D cell culture, whereas on our platform almost no cell loss occurred since all cells from the 20-40 µL seeding volume are collected in the wells. Equally important, the platform allows simple retrieval of the cell sample in low volume for downstream analysis. This also includes mass spectrometry for secretome analysis as illustrated in **new main Figure 8**. Again, on low attachment well plates removal of the supernatants will

lead to cell sample loss and thus a potential bias in measurements. With the simple microsystems design, we could overcome this problem. A further novelty is the very flexible design in view of specific applications such as enabling co-cultures as shown in **new Supplementary Fig. 11**. In summary, the versatility and concomitant novelty of the presented platform is clearly given from our perspective. We added the following sentence to the discussion:

"The advances of the duct-on-chip platform are (i) the low cell and material consumption, (ii) defined and homogeneous size of generated 3D aggregates, (iii) the possibility for long-term 3D cell culture, (iv) sample retrieval for downstream analysis with minimal perturbation, and (v) the possibility to establish co-cultures."

4. *Another major limitation is prolonged culture durations - order of month cultures. The authors want to discuss how to address this technical challenge.*

Answer: The general concept of generating specific human cell types *in vitro* from human pluripotent stem cells is to recapitulate human embryonic development. Therefore, pancreatic cell types will have to go through comparable developmental phases *in vitro* from definite endoderm to pancreas progenitor cells before specializing into the mature endo- or exocrine cell types (Pagliuca et al., 2014). This implies that we require new and standardized technologies for maintaining long-term cell cultures. In fact, our manuscript successfully fosters such an approach. Shortening of the cell culture system could be achieved upon establishing a stable pancreas progenitor cell line, however, reproducible conditions remain to be reported. Although this is an active research field so far expanding and freezing of intermediate cell types has not been achieved without some degree of clonal selection. We have to stress that the generation of genetically defined ductal cell types in a standardized and accessible analytical culture platform is a major forthcoming for establishing screening studies for the early onset of PDAC.

Reviewer #2

Major Comments:

1. *The identification of new duct-like clusters identified in Figure 3 requires significant elaboration. These populations should be thoroughly investigated in tissue to see these cells truly exist in vivo. For example, are "duct-like 1" clusters found to be Krt19+, Cftr+, Sctr+, Blcc1+ while the "duct-like 2" and "duct-like 3" are Krt19+, Cftr-, Sctr-, Blcc1-? Are these three sub-populations readily identifiable within the same tissue, or does one duct-like population predominate in individual animals? This is essential to establishing the physiological relevance of the new system.*

Answer: We thank this reviewer but would like to underpin that our work elaborates human not murine cells and thus we focused our analysis on establishing the physiological relevance in humans. The transcriptional state of cells is highly dynamic and thereby we identified ductal-like cell types on scRNA datasets that can describe the plasticity or continuum of cell expression states. The ductal-like cluster 3, however, contained only 134 cells and a Pearson correlation showed a similarity to cells of ductal-like cluster 2. In line we find that MMP1, a cluster marker was expressed in nearly all PDLO cells, although with variable signal intensity (**see new Fig. 5**). Expression of the scRNA-seq based duct-like subtype markers were not mutually exclusive, which was expected from the single-cell transcriptomic data (**Fig. 4e,f**). We therefore concentrated our efforts on demonstrating the existence of the two main ductal clusters *in vivo*.

In order to show the physiological relevance of the identified ductal subtypes we extended our marker panel and show differentially expressed genes (now MUC1, CFTR, BICC1, SCTR, MUC13, and MMP1) in the same healthy human pancreas tissue from two individuals as well as pancreatic tissue from two patients with chronic pancreatitis (**new main Fig. 5**). The latter is *per disease pathology* particular rich in ductal structures and in addition includes a certain degree of dysplastic ducts. Immune-co-stainings in both tissue types could demonstrate spatially changing expression patterns of the ductal cell type markers MUC1, CFTR, BICC1, MMP1, TFF1, and SCTR at the protein level in PDLOs and in human primary tissue. This suggests that there are transcriptional subtypes of ductal cells. The physiological function can not be given but it becomes clear that or current picture of ductal cell types is more complex than had suggested so far.

Furthermore, we challenged our scRNA-seq data sets with a recent data set derived from adult human pancreata and reproduced our *KRT19*$^{+}$, *CFTR*$^{+}$, *SCTR*$^{+}$, *BlCC1*$^{+}$, MUC1^{low}, TFF1^{-}, MMP1^{-} cluster and a *KRT19*$^{+}$, MUC1^{+}, TFF1^{+}, MMP1^{+}, *CFTR*$^{-}$, *SCTR*^{low}, *BlCC1*^{low} cluster (**new Supplementary Fig. 10**).

We added the findings to **new Fig. 5** and **new Supplementary Fig. 7** and rewrote this section of our manuscript:

"To validate duct-like cell types identified in single-cell transcriptomics on protein level, IF stainings for respective cluster markers were performed on-chip-derived PDLOs. For this, CFTR and MUC13 were stained in PDLOs differentiated on-chip to day 23, 27, and 31. While CFTR was only expressed in cells of multilayered epithelial PDLOs, MUC13 was expressed in a different subset of cells at the outer side of

multilayered epithelial and in all cystic PDLOs (**Fig. 5a,b**). A differential expression pattern of a mucin-rich (MUC1+) and a CFTR+ ductal subtype within the pancreas has been previously reported (Baron et al., 2016). 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; Supplementary Data 5)**. Next, we performed combinatorial staining of PDLOs for further duct-like subcluster DEGs. Indeed, we found distinct expression patterns and fluorescence intensities across multilayered epithelial PDLOs for MUC1, CFTR, BICC1, MMP1, and TFF1 (**Fig. 5b; 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; Supplementary Fig. 7a**). Overall, expression of the scRNA-seq based duct-like subtype markers were not mutually exclusive, which was expected from the single-cell transcriptomic data (**Fig. 4e,f**).

To translate these spatial expression patterns from on-chip-derived PDLOs to primary human pancreatic tissue, the cluster-specific markers illustrated above were stained in both healthy pancreata and chronic pancreatitis specimen. MUC1 was restricted to acinar structures, centroacinar cells, and connected intercalated ducts, the latter also expressing CFTR. KRT19 (**Fig. 5c**; first and second row) was additionally stained confirming ductal identity. In addition, BICC1 was expressed in such smaller ducts but was rarely detected in cells in direct proximity to the MUC1 positive cells. Dependent on the size of the branching ducts, the marker pattern changed pointing to a transition of different co-expressions. For example, CFTR expression decreased in intralobular ducts and was hardly found in larger ducts, while BICC1 became more prominent (**Fig. 5c**; third row). On the other hand, MMP1 was basically absent in intercalated ducts but showed a variable staining intensity within larger ductal structures (**Fig. 5c**; fourth row; **Supplementary Fig. 7b**). MUC13, TFF1, and SCTR could not be detected in healthy ducts (**Fig. 5c**; fifth row; **Supplementary Fig. 7b**). Immunostainings of chronic pancreatitis tissue further confirmed duct specificity with differential expression patterns of the described markers and revealed SCTR expression in metaplastic ductal epithelium (**Fig. 5d; Supplementary Fig. 7c**). Taken together, we could demonstrate spatially changing expression patterns of the ductal cell type markers MUC1, CFTR, BICC1, MMP1, TFF1, and SCTR on protein level in PDLOs and in human primary tissue. Herein, expression states are likely more dynamic and complex than initial transcriptomic subgrouping had suggested.”

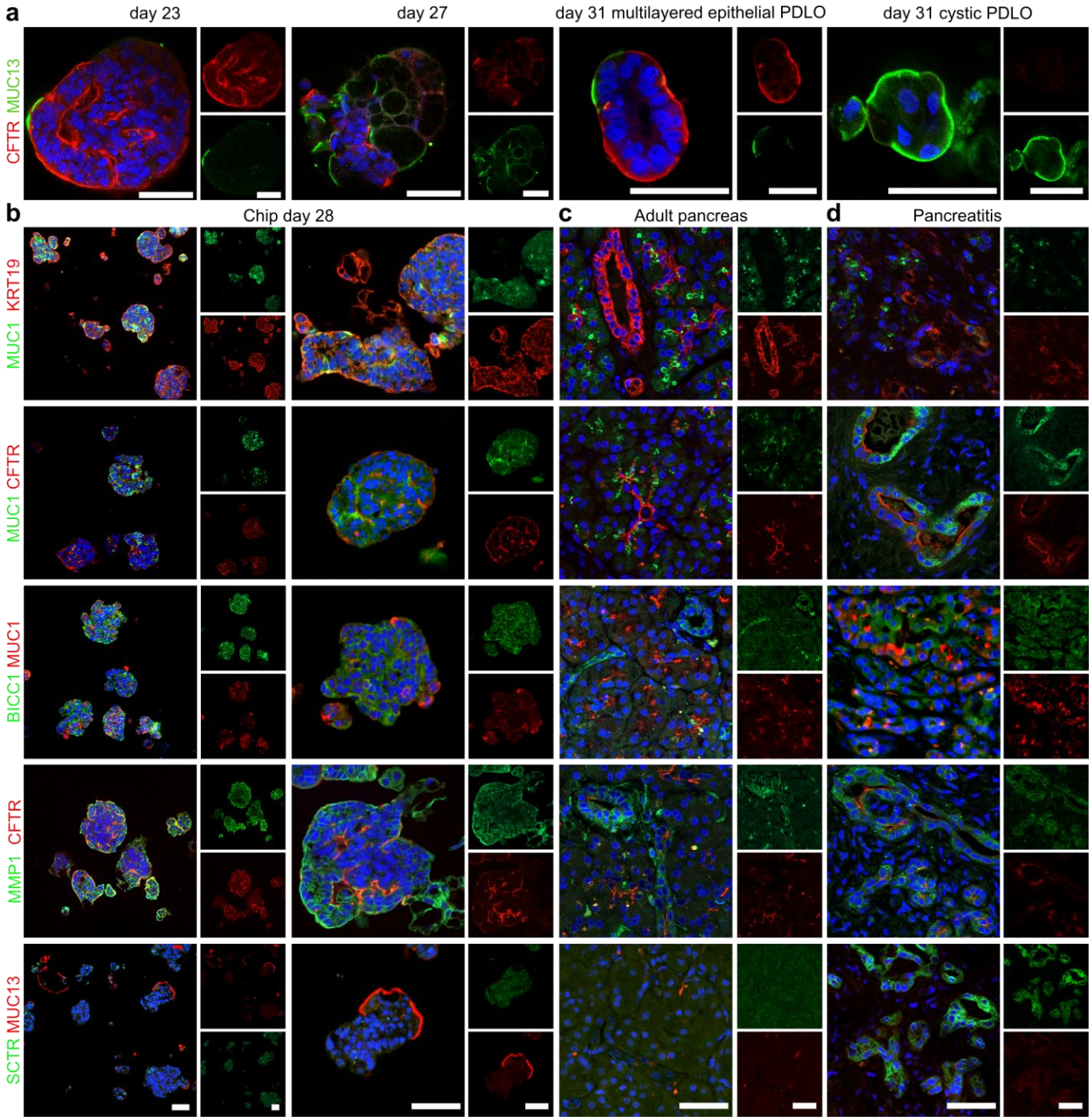
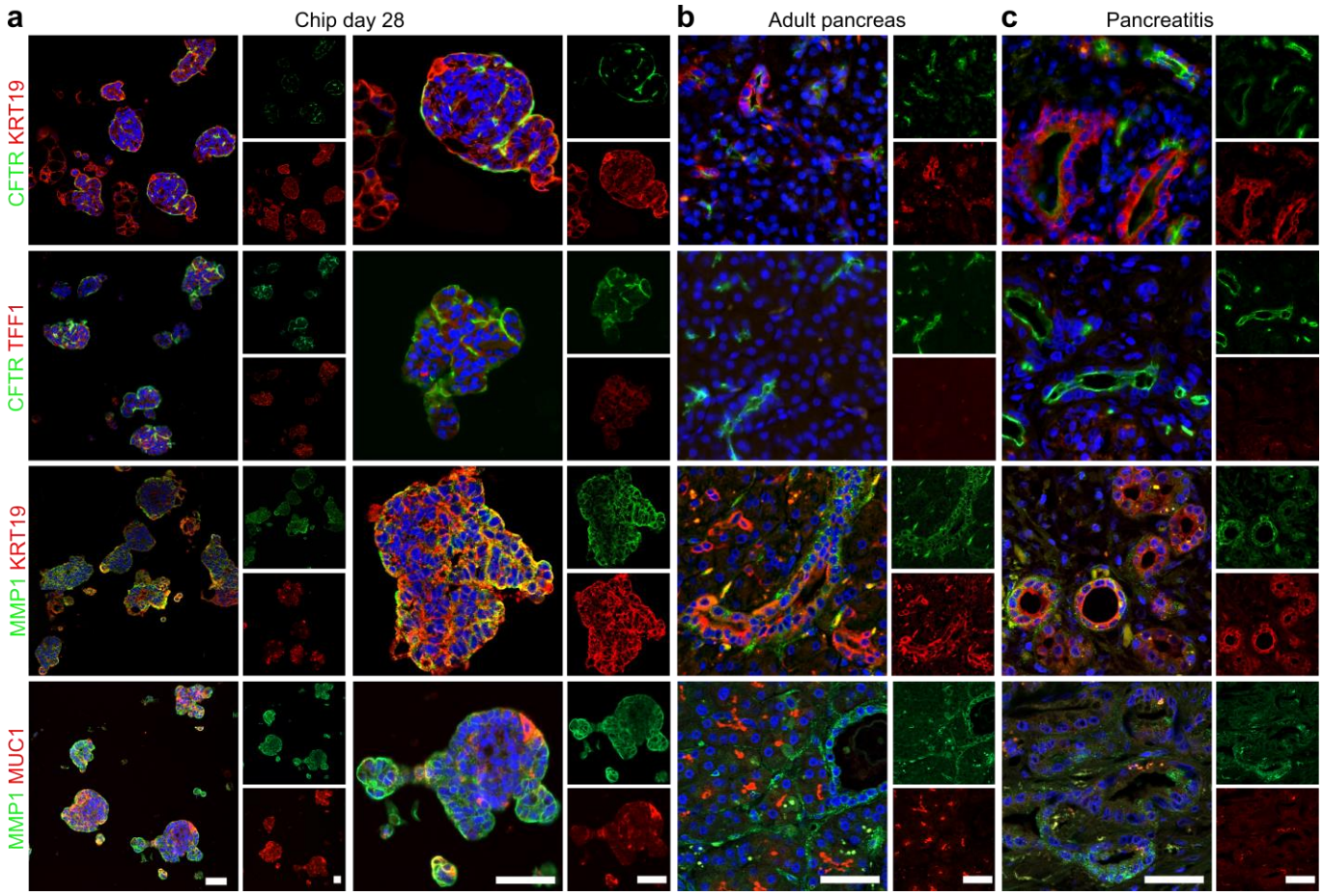


Fig. 5: Ductal subcluster-specific genes located within PLDOs and primary pancreas tissue on the protein level. **a**, PDLOs after 23, 27, and 31 DOD were stained for duct-like cluster 1 marker CFTR, and duct-like cluster 2 marker MUC13, respectively. **b**, PDLOs differentiated until day 28 stained for cell subtype markers identified by the scRNA sequencing analysis. **c,d**, The same markers are used to locate the *in-vitro*-generated duct-like cell subtypes in primary human pancreas tissue, either (**c**) healthy or (**d**) pancreatitis tissue. Scale bar denotes 50 µm.



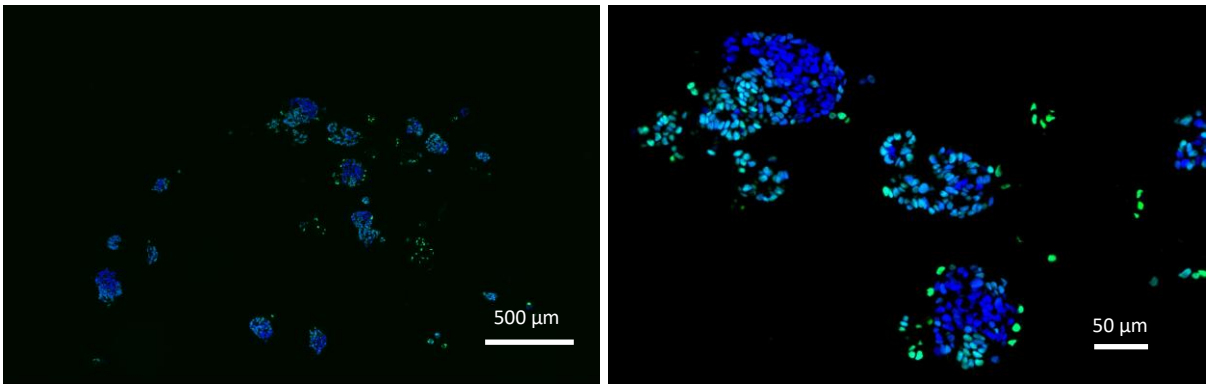
Supplementary Fig. 7: Ductal subcluster-specific genes located within PLDOs and primary pancreas tissue on the protein level. **a**, PDLOs differentiated until day 28 stained for cell subtype markers and pan-ductal marker KRT19. **b**,**c**, The same markers are used to locate the *in vitro* generated ductal-like cell subtypes in healthy human primary pancreas tissue and in tissue from pancreatitis patient, respectively. Scale bar denotes 50 µm.

2. *Figure 4, as it currently stands, is lacking in depth. It appears to be a verification at the protein level of individual markers identified from the scRNA-seq experiment in Figure 3, which is just a confirmation that the transcriptional profiling is valid. It is also unclear why only 2 of the many new markers that were identified were chosen to be validated. Did the others not correlate at the protein level? If so, is there any way to combine Figure 3 and Figure 4 to only identify the transcriptional hits from Figure 3 that were validated at the protein level in Figure 4? It seems that that there are ~3 unique hits for each new sub-type of duct-like cells (i.e. "false hits" identified by the sequencing experiment that may have changes at RNA levels that don't correlate at the protein level). Nonetheless, the validation of a limited number of markers is another concern.*

Answer: We fully agree with the reviewer and extended **new main Fig. 5** for additional cluster markers including now MUC1, CFTR, BICC1, SCTR, MUC13, MMP1, and TFF1 staining. The answer to question 2 goes along with our answer to question 1. Besides SCTR, we clearly detected all mentioned cluster markers on protein level. We included the results for SCTR since staining on chronic pancreatitis specimen showed specific staining results, indicating that PDLOs and healthy primary tissue did not meet an expression level to allow detection with the used antibody while in metaplastic or dysplastic ducts SCTR

seems to be upregulated (**new main Fig. 5d**). Albeit we clearly observed spatial organization of different markers. The significantly higher expression on the transcriptomic level does not mandatorily mean that the other two clusters do not express these genes. In particular, this was a concern for the markers of the third cluster, where cluster-specific markers such as *MMP1* and *CDX2* were not exclusively found in one cell type on protein level (**main Fig. 4d** and **review figure**). The similarity of duct-like cells 3 to duct-like cluster 2 stays high. However, as parameters for the clustering during the scRNA analysis were carefully chosen we did not omit this cell cluster. Nevertheless, we carefully revised the manuscript pointing to the similarity of cluster II and III.

CDX2 **PDLO**



Review Figure: CDX2 (green) DAPI (blue) co-staining confirming subset marker expression. Expression in a substantial part of PDLO cells indicates expression in ductal-like cells other than ductal-like type III.

3. *To truly demonstrate the benefit of this pancreas-on-chip as a biomarker development tool, the authors should take a few of the top hits identified (such as SDCBP2 or FLNB) and show that staining in human and mouse tissues confirms the expression of these proteins and that high staining correlates with decreased survival.*

Answer: To answer the reviewers question we validated one identified marker, i.e. FLNB against a clinical patient database. The result is added to the **new main Fig. 8, new Supplementary Fig. 12** and **Supplementary Data 5**. Further, we added the following text to our manuscript:

"One unfavorable 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 patients (Zubair et al., 2020) (**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 (Bandaru et al., 2014; Iguchi et al., 2015). To investigate FLNB expression during pancreatic carcinogenesis (Surcel et al., 2019), we assessed FLNB protein expression in an independent cohort of resected PDACs (Arnold et al., 2021; Feld et al., 2015; Schmid et al., 2013) by IHC (**Fig. 8e**). Normal pancreatic ductal epithelium and some acinar glands were faintly FLNB positive at their luminal surface. As expected on-chip-derived PDLOs were also FLNB positive (**Fig. 8e; 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; Supplementary Fig. 12b**). IHC observation was specified using the

semiquantitative H-score method followed by correlation with clinical data (Hirsch et al., 2003). Comparing normal ductal epithelium with corresponding cancer tissue revealed significantly higher H-scores in PDAC (**Fig. 8f**), while H-scores did not correlate with any clinical parameter including survival (**Fig. 8g**). Pancreatic intraepithelial neoplasia (PanIN) represent the most relevant PDAC precursor lesions and can be frequently found adjacent to PDAC and their presence is of prognostic relevance (Hassid et al., 2014; Kleger et al., 2014; Reichert et al., 2016). Interestingly, H-scores were also elevated in PanINs in comparison to normal ducts (**Fig. 8f**). FLNB expression in PanIN lesions significantly correlated with higher survival of patients (mOS; p=0.0019) (**Fig. 8g; Supplementary Fig. 12c**). A gradual increase from normal to preneoplastic lesions has been described e.g. for the epigenetic silencer enhancer of zeste homolog 2 (EZH2), albeit in established cancers higher expression levels ascribed better prognosis (Bremer et al., 2019). As alternative splicing can lead to shorter FLNB isoforms being strongly associated with EMT gene signatures in basal-like breast cancer patient samples, distinct forms might also be present in pancreatic cancer precursor lesions ascribing distinct biological outcome (Li et al., 2018). To finally probe FLNB feasibility as a liquid biopsy-based biomarker, we consulted an independent cohort of human metastatic PDAC patients and measured FLNB levels in peripheral blood (PB). When comparing FLNB levels in PB, there was no obvious difference in metastatic PDAC patients 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 PB. Intriguingly, differentiated tumors (≤G2) had significantly higher FLNB PB levels when compared to less differentiated tumors (≥G3) or healthy donors, the latter two having more or less similar levels (**Fig. 8h**). We conclude that FLNB might be a suitable blood biomarker for differentiated PDACs and therefore could complement biomarker panels detecting early PDAC formation or discriminating differentiated and dedifferentiated PDAC.”

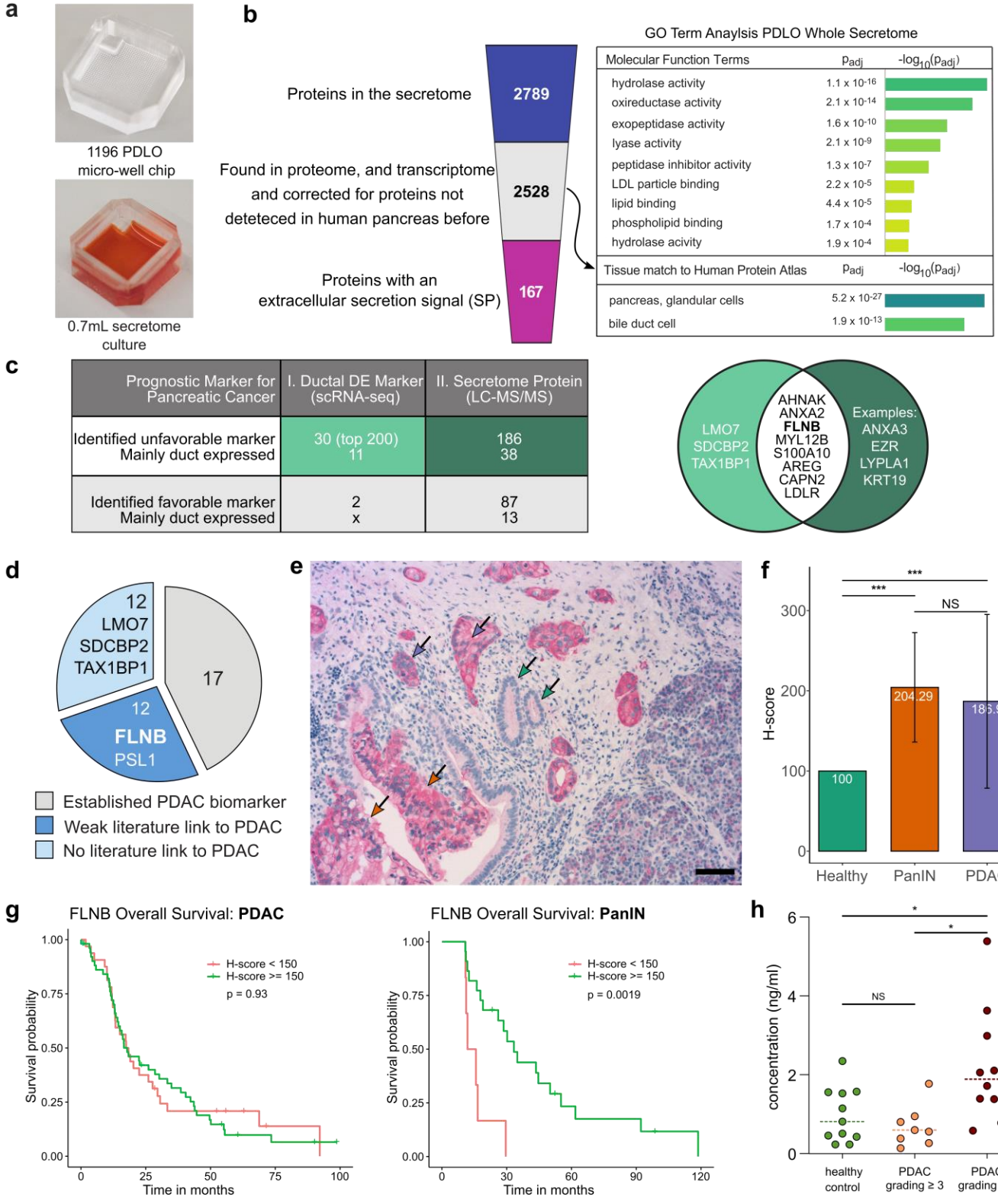


Fig. 8 Potential PDAC biomarkers within the secretome and transcriptome of PDLOs. **a**, Micro-well chip used for the determination of the PDLO secretome by LC-MS/MS. **b**, Left: Filter process applied to the secretome data. Right: GO terms enriched within the filtered PDLO secretome with relevance for duct function. **c**, Prognostic pancreatic

cancer markers within the top 200 DEGs of the combined duct-like clusters from the scRNA-seq analysis and within the PDLO secretome. The Venn diagram highlights the overlap between the revealed unfavorable diagnostic markers for pancreatic cancer from the scRNA-seq and secretome analysis. Pancreatic duct expression was assessed by examination of Human Protein Atlas staining (Uhlén et al., 2015). **d**, Literature survey for the set of unfavorable diagnostic markers from table c. **e**, IHC staining of FLNB within PDAC tissue. Purple arrows indicate PDAC, orange arrows PanINs, and green arrows healthy ducts. Scale bar denotes 50 µm. **f**, FLNB H-score in heathy, (n=86), PanIN (n=28) and PDAC (n=84) tissue. The Man-Whitney-U-test was applied to calculate statistical significance. Significance levels are indicated as follows: *: p-value<0.05; **: p-value< 0.01; ***: p-value<0.001. **g**, Overall survival curve for patients with high and low FLNB H-score. High FLNB H-scores in PanINs correlated with a favorable prognosis for patients. **h**, FLNB blood concentration in a healthy control group (n=11) and patients with PDAC grade ≤2 (n=10) and ≥3 (n=7). The t-test method was used to calculate statistical significance. Significance levels are the same as in f.

Minor Comments:

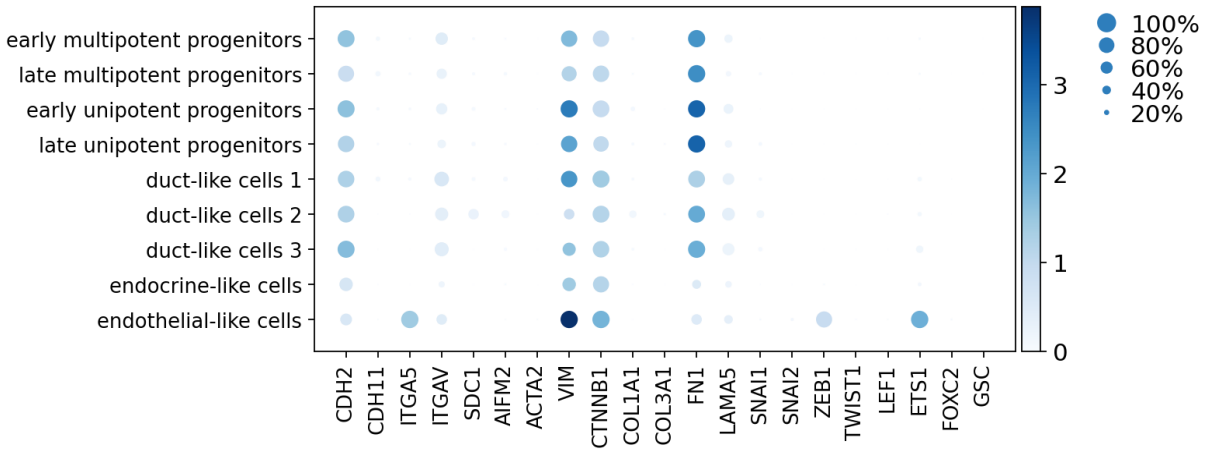
1. *The authors show gain of ductal and epithelial genes as the organoids progress down the chemical induction (in Figure 2C-D). The authors should show loss of "stemness" genes, potentially indicating the irreversibility of this conversion.*

Answer: We stained PDLOs, pancreas progenitor and hiPSC aggregates for the stemness proteins and added the images to **new Supplementary Fig. 3**. Key pancreatic progenitor markers such as NKX6-1 were reduced in PDLOs, and characteristic stem cell markers SOX2 and OCT4 were only detected in the hiPSC aggregates and not in the PDLOs. Further, we added the following sentence to the manuscript:

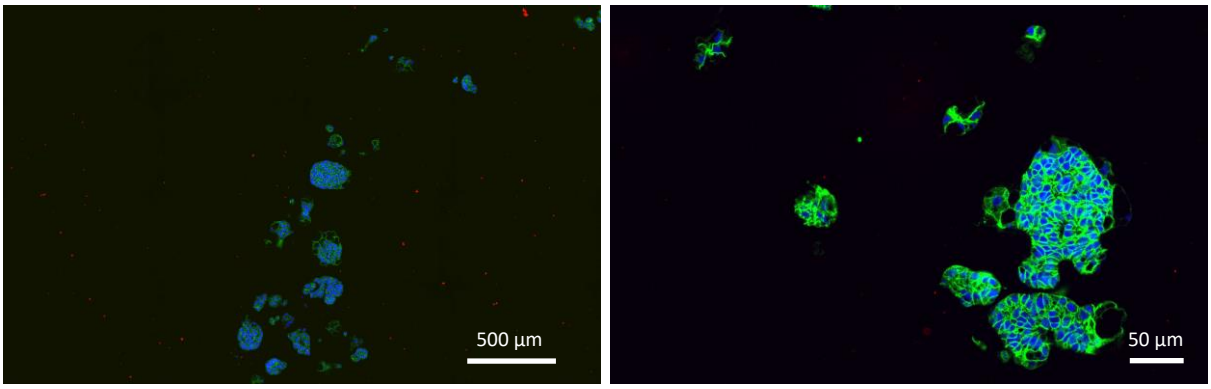
"Epithelial nature and pancreatic ductal identity of the cells at the final stage were confirmed by the upregulation of E-cadherin (CDH1), cytokeratin 19 (KRT19), aquaporin 5 (AQP5), mucin 1 (MUC1) carbonic anhydrase II (CA2), cytokeratin 7 (KRT7), claudin 1 (CLDN1), and cystic fibrosis transmembrane conductance regulator (CFTR) (**Fig. 2c,d; Supplementary Fig. 3a**). Expression of ductal markers, which were already detected at the PP 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; Supplementary Fig. 3a,b**). Differently, the progenitor marker homeobox protein NKX-6.1 (NKX6-1), which becomes restricted to endocrine cells during pancreatic development, was downregulated on protein level (**Supplementary Fig. 3b**). Stemness marker SRY-box 2 (SOX2) and octamer-binding transcription factor 4 (OCT4) were absent (**Supplementary Fig. 3c**) similar to non-ductal pancreas markers except for few endocrine cells located at the periphery of PDLOs (**Supplementary Fig. 3d**)."

2. *ECAD appears to be lost in the Type II day 31 organoids. Do the authors think that epithelial-to-mesenchymal transition is occurring in this subtype?*

Answer: The single-cell transcriptomic data did not indicate EMT within the ductal cells at day 31 of differentiation. The EMT marker plot is added below. This is further substantiated by negative staining of ZEB1 in PDLOs on day 28 of differentiation (see **new Supplementary Fig 3d** and **figure for reviewers only**).



CDH1 ZEB1 **PDLO**



Review Figure: (Top) EMT marker on scRNA analysis during ductal differentiation. (Bottom) Overview staining related to Supplementary Fig 3d, where magnifications of a representative PDLO is shown. ZEB1 staining is shown in red, CDH1 in green confirming the absence of mesenchyme in type I and type II PDLOs.

3. It is unclear what parts of Figure 3F reached statistical significance.

Answer: All differentially expressed genes are significant. Their corresponding p-values are given in **Supplementary Data 1**. The added common markers *KRT19*, *HNF1B,* and *CA2* were not significantly upregulated in one specific ductal subcluster but were added to the graph since they are generally accepted and frequently used pancreatic duct markers.

4. Figure 5F contains many genes that might be worthy of more discussion regarding potential hierarchy of ductal development over time. This should be discussed further.

Answer: We agree that the deciphering of the signaling network over the time trajectory is a central point of interest. Additional pathway analysis is given **in Supplementary Fig. 9**. Further, we evaluated in the **new Supplementary Fig. 8** some of the dynamical genes found in **Fig. 6f** and extended them with corresponding genes involved in the formation of the organoid ECM. All dynamical genes are given in **new Supplementary Data 2** as a resource for further explorations.

Reviewer #3

Several points will need careful attention prior to publication:

1. *Most importantly, many markers presented as ductal markers are also present in pancreas progenitors. The authors should revisit the literature and revise the text and their conclusions (see some details below). Caution in extrapolating mouse data to human should also be applied.*

Answer: We thank the reviewer for carefully reading our manuscript and thoughtful correction and comments. We revised our manuscript accordingly and addressed all requested comments. Please see also our answers to your questions (10, 11, 14, 15, 16, 22 and 25) below and **new Fig. 3, new Fig. 5, new Supplementary Fig. 3** and **Supplementary Fig. 7**. In particular, we addressed the reviewers concern in extrapolation mouse to human data and rephrased our sentences throughout the manuscript, e.g.

"Hitherto, it has been suggested that endocrine precursors delaminate from a common trunk domain arising at E12.5 in mice, which further undergoes tubular morphogenesis to subsequently form the ductal network (Nair and Hebrok, 2015). 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 (Jennings et al., 2013; Nair and Hebrok, 2015)."

and

"PTF1A and *NKX6-1* were only co-expressed in such cluster II, suggestive of multipotent progenitors (MPP), which can give rise to acinar, endocrine, and ductal cells in mice (Nair and Hebrok, 2015; Pan and Wright, 2011)."

2. *The authors draw many lineage conclusions which should be worded with more caution. The single-cell methods show the molecular proximity between cells, not lineage.*

Answer: We agree and changed the wording in our manuscript to "cell types" not lineages and generally toned down and interpreted our data with more caution.

3. *Some documentation of markers by immunocytochemistry is of low quality and more compelling data need to be provided (see below)*

Answer: We replaced immunocytochemistry images with immunofluorescence stainings and extended and improved other images in the **new Fig. 3, and 5** and in the **new Supplementary Fig. 3 and 7** in order to strengthen our findings. Please visit also our answer to question 2 and 3 of reviewer 2.

4. *The benchmarking to published transcriptome datasets of in vivo samples should not be over-interpreted. Batch corrections are applied to merge the data, which "forces" the in vitro clusters to map in the vicinity of some populations in the dataset. It can't be used to claim that the cells in vitro are the same as in vivo.*

Answer: We agree that data integration can lead to overinterpretation and have carefully revised the text to avoid any claim that PDLO cells are identical to adult to the investigated human ductal cells. Instead, we assume that our cells might resemble a fetal stage, but are a powerful tool to provide an outlook for ductal subtypes. As valid datasets resolving embryonic stage and pancreatic cell types are missing, unravelling the exact "age" of ductal cells in more detail is not possible. In order to demonstrate that our approach is valid to show a cellular similarity between our PDLO cells and primary human ductal cells, we used the raw scRNA-seq data of Qadir et al. (2020) and highlighted our marker combinations for the duct-like cell type 1 and 2 in the re-clustered UMAP plot (**new Supplementary Fig. 10**). Here, no data integration or batch correction has been performed. Strikingly, *CFTR/BICC1/SCTR* and *MUC1/TFF1/MMP1* positive cells clustered together, respectively. This suggests together with the IF stainings that the two described ductal transcriptional states in different duct cells exists *in vivo***.** Thereby, we also demonstrate the validity and relevance of our PDLO scRNA-seq data comparison to primary duct tissue data sets, in the revised version against two independent adult human scRNA seq data sets. In support of a clear difference between our PDLO and pancreatic progenitors, we clustered also pancreatic progenitor cells from our differentiation with the primary data set, however, no overlap was observed (see **Fig. 6**), whereas ductal and endocrine cells *in vitro* and *in vivo* subtypes clearly overlapped. Over regression of the data would inevitably lead to merging of not only ductal cells but also progenitor cells with the primary data set.

Detailed comments:

1. *Line 41: reducing bicarbonate secretion to transport facilitation is somewhat reductionist.*

Answer: We revised the sentence:

"Ductal cells secrete a bicarbonate-rich alkaline aqueous solution to transport zymogens produced by acinar cells (Lee et al., 2012)."

2. *Line 51: Typo to correct "dysplasian"*

Answer: We corrected the misspelling.

3. *Lines 52-53: "Thus, lineage-committed pancreatic ductal cells generated from human inducible pluripotent stem cells (hiPSCs) could be an alternative source of pancreatic organoids to overcome these obstacles12,13". This is true but it would be even more obvious from adult ductal-derived organoids. Alternative systems should not be dismissed.*

Answer: It was not our intension to exclude ductal organoids derived from primary tissue, however, the sentence states that hiPSC derived can be alternative and not exclusive. To acknowledge primarily derived duct organoids we changed the sentences as follows:

"Adult human pancreatic organoids (Boj et al., 2016; Georgakopoulos et al., 2020) 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 (hiPSCs) could be an alternative source of pancreatic organoids to overcome these obstacles (Hohwieler et al., 2017; Huang et al., 2015)."

4. Lines 56-58: While previous studies have clearly shown that all pancreatic lineages derive from pancreatic progenitors and ref 15 is certainly appropriate as the first one, if the authors cite more than one, 14 would need to be complemented by many others (doi: 10.1016/j.devcel.2009.11.003; doi: 10.1242/dev.053843.; doi: 10.1242/dev.056499. or at single-cell level doi: 10.1038/s41467-017-00258-4.)(this is likely too many and citing only the first one is a possibility. Reference 16 does not show that all pancreatic lineages derive from pancreatic progenitors and to my knowledge this demonstration has not been made. It would be appropriate to say that by analogy to mouse, because the cells express similar markers, we assume that in human it is the same.

Answer: We added three suggested references and changed the sentence to:

"Previous studies in rodents revealed that all pancreatic cell lineages evolve from a common pancreatic endoderm-derived cell type, named pancreatic progenitor (PP) cell(Zhou et al., 2007). PPs organized in the pancreatic bud undergo tip/trunk patterning. While the acinar cells evolve from the tip domain, ductal cells together with subsequently delaminating endocrine cell types evolve from presumably bipotent trunk cells(Schaffer et al., 2010; Villani et al., 2019). Time-resolved immunostaining of developing human embryos suggest a similar mechanism of human pancreatic cell type development(Jennings et al., 2013)."

5. Line 62: Please include a reference to your previous paper

Answer: The protocol development process was a study on its own and is currently under revision. We will add the reference upon acceptance of the work.

6. Lines 95-96: "Small diameters clearly compromised the aggregation step, while a large diameter is known to impair nutrient supply". The sentence does not represent the results faithfully. In Fig1e, it seems that the number of cells has more impact on the diameter of the aggregate than the well diameter. For example, with 75 cells, the diameter seems a little larger but almost the same in 150 and 300 micrometer diameter wells. It would also be better not to mix in the same sentence a reference to the authors' observations in the figure and reference to the literature. The nutrient supply problem is not shown in the present article.

Answer: We changed the sentence accordingly:

"A low number of cells (<50 cells/well) compromised the aggregation step in the micro-wells. Generation of aggregates with diameters over ~250 µm are known to impair nutrient supply(Freyer, 1988)."

7. Lines 97-98: Please refer to the differentiation method used to obtain PP at this point.

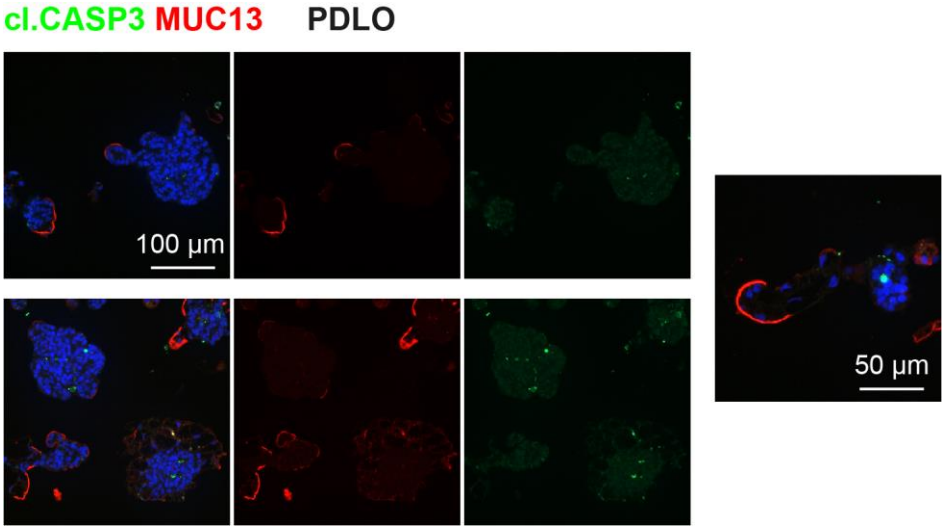
Answer: PPs were differentiated based on fusion protocol of our own work (Hohwieler et al., 2017)and the Nostro lab (Cogger et al., 2017). Both references are included in the material and method section.

8. Lines 113-115: It would be good to know where the two-step protocol comes from. Why these components? Why the selected times?

Answer: The protocol development process was a study on its own and is currently under revision. We will add the reference upon acceptance of the work.

9. Lines 119-122: "while is it likely that the type II structures are formed from the type 1 PDLOs it would be more convincing to show live imaging. Is any cell death involved in type II PDLOs? They are very small and the culture does not look very healthy by day 31."

Answer: We agree and added a movie file showing the development of the morphological organoid phenotypes. The movie file is added as **Supplementary Movie 1**. Further, we stained PDLOs for cleaved Caspase 3 and do not see indications for increased apoptosis in any cells including MUC13 positive cells. Further, we successfully transferred PDLOs after differentiation on-chip to Matrigel culture for long-term culturing or successfully transplanted chip-derived PDLOs (see **new Fig.3**).



Review Figure: MUC13 and cleaved Caspase 3 staining showing no signs of increased apoptosis in type II PDLOs.

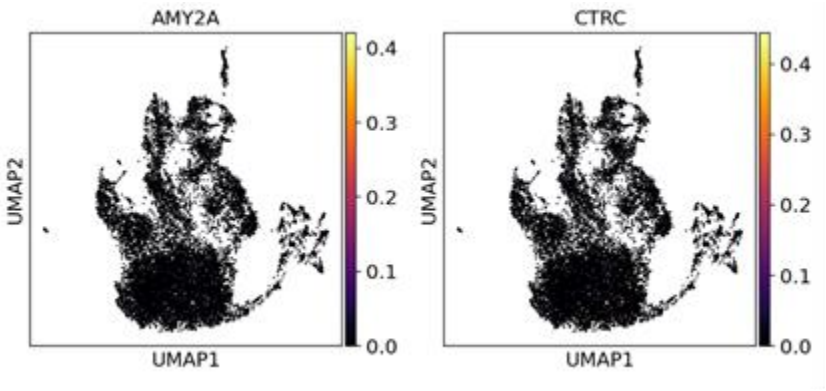
10. Lines 125-126: Are they really formed of pancreatic ductal cells? All the markers cited are also expressed in progenitors (see for example doi: 10.1677/joe.0.1810011. for CK19 in the pancreas of human foetuses as early as 41 days post conception and CFTR is widespread in 13-week foetuses https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2230211/?page=3- at these stages the ducts are expected to be composed of progenitors).

Answer: Please see our **new Supplementary Fig. 3**. Here, we provide a direct comparison of gene expression in PDLOs and pancreatic progenitors. While indeed many pancreatic markers such as KRT19, PTF1A, NKX6-1, PDX1, SOX9, and HNF1B are described to be widely expressed in PPs, lineage restriction to specific marker combinations defines the respective cell types. Note, KRT19 is clearly upregulated in PDLOs after performing identical staining, imaging, and processing. Upregulation of duct-restricted markers and downregulation of PP markers, which are not related to ducts, such as NKX6-1

thereby fosters cellular identity of PDLOs. Importantly, ductal markers associated with a ductal function such as CFTR and MUC1 were also upregulated in PDLOs without any detection in PPs (**Supplementary Fig. 3**). Please refer also to **new main Fig. 7** and **new Supplementary Fig. 10**, in which we show the transcriptional similarity of the PDLOs to primary duct cells. However, we do not want to argue that ductal differentiation protocols rather resemble fetal stages of development than adult tissues, but due to a lack of a time-resolved human single-cell fetal reference data set we were not able to define the exact "age" of our PDLOs. Of note, **Supplementary Fig. 3** also indicates that albeit CK19 is disorganizedly expressed in PPs, its expression becomes more organized and increases during ductal maturation. Thus, CK19 is a valuable marker for ductal maturation similarly indicated by an increase at the scRNA-seq level in PDLO clusters.

11. Figure 2: The absent of CK19 in the center of organoids is puzzling. From ref doi: 10.1677/joe.0.1810011 Figure 5, CK19 is expressed throughout the epithelium in progenitors/ducts. The only negative cells during early development are endocrine cells and likely acinar cells. Are the central cells acinar or endocrine? It does not seem so from the transcriptome. What are they? Are CK19-negative cells found in the transcriptome?

Answer: Thanks for the careful investigation and rising this valid concern. Importantly, we did not identify a relevant *KRT19* negative subpopulation in our transcriptome data (see **new Supplementary Fig. 6c**). Likewise, improved staining in **new Supplementary Fig. 3**. demonstrates that also the cells in the center of PDLOs are KRT19 positive, however, we observed a polarization and stronger KRT19 expression at the edges of PDLOs. The biological relevance of such KRT19 polarization remained unclear to us but it is possible that keratin polarization might be involved in tubulogenesis as shown for different expression levels of another epithelial marker E-Cadherin (Kesavan et al., 2009). Of note, no acinar markers were expressed in PDLOs (**review Fig.** and **new Supplementary Fig. 3d**). Further, only a few cells expressed CHGA (**new Supplementary Fig. 3d**) corresponding to a minor endocrine population in the transcriptome (**new main Fig. 4e,f**), which indeed loose *KRT19* expression at later time points of differentiation (please see **also new main Fig. 3b**). Importantly, such endocrine cells are very few and scattered and do not reconsitute the center of PDLOs.

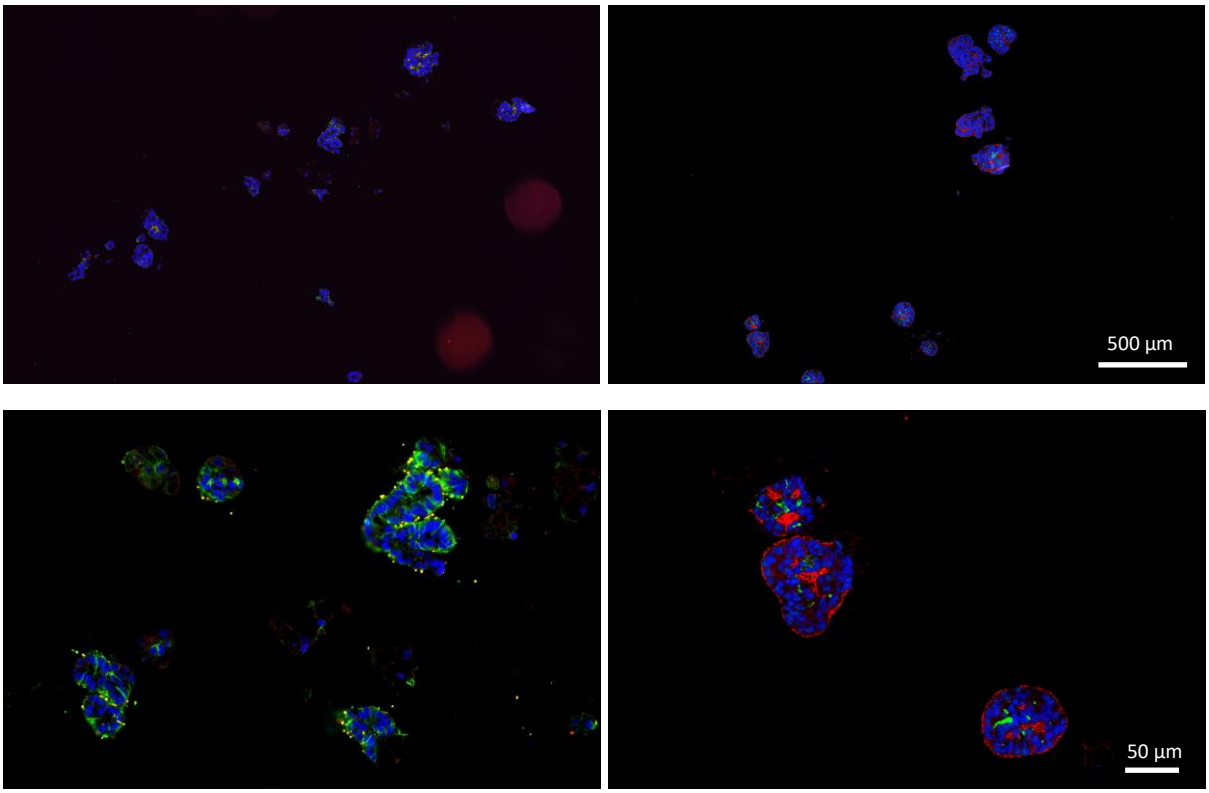


Review Figure: UMAP plot of *AMY2A* and *CTRC* during ductal differentiation.

12. Supplementary Figure 3a, b: The images suggest that ZO1 is polarized with the apical side in or out PLDOs I. What is the proportion of PDLO Is of each type? Figure 4 suggests that there are PDLO Is with a mixed apical in- and out- polarity. This is important in the system for future functional studies as the ducts in vivo have their apical side in. The absence of polarization of PDLO IIs is another reason to doubt their health. However, they may express other apical markers. The paper investigates Muc13 at a later point. Is it polarized?

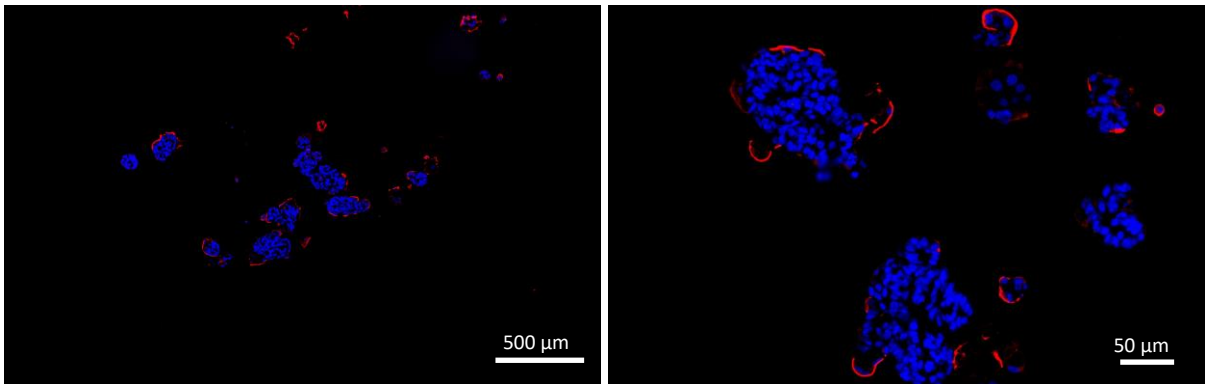
Answer: The multilayer epithelial PDLO type constitutes around 70 % of PDLOs and such PDLOs showed a dominating apical out configuration (>80 %) (see review figure). MUC13 staining shows the same apical out polarity and appears polarized in multilayered epithelial PDLOs (**new Fig. 3** and **review figure**).

acTUB ARL13B **PDLO** COL4A1 ZO1 **PDLO**



Review figure: (Left) apical marker acTUB (green) and ARL13B (red) staining and basal marker COL4A1 (green) and apical marker ZO1 (red) overview images related to **new Fig. 3**.

MUC13 **PDLO**



Review figure: Overview of MUC13 (red) DAPI (blue) staining related to **Fig. 5b** indicating polarization of apical marker MUC13 in multilayered and cystic PDLOs.

We added a further experiment to manipulate the polarization of our PDLOs. PDLOs were either engrafted into mice or transferred to Matrigel culture after day 28 of differentiation in order to investigate if *in vivo* or matrix components can induce an apical-in polarity of our PDLOs as it is reported for primary ductal organoids. **New main Fig. 3** shows the IF images of the polarity markers in PPs, PDLOs on-chip and transferred to Matrigel, as well as engrafted PDLOs. Indeed, we observed that in both latter cases our PDLOs give rise to organoids or duct-like tissue with an apical-in polarity. This highlights that first our PDLOs are healthy at the time of differentiation, and secondly that *in vivo* environment and ECM components in the Matrigel culture can induce a reorganization of the polarity of the PDLOs. Please note that the possibility of manipulating and directing organoid polarity might be beneficial for several applications, as an apical-outside orientation might exhibit an advantage for example in secretome analysis of PDLOs.

We added the following sentences to our manuscript:

"One explanation of the observed PDLO apical-out polarity could be the lack of extracellular matrix (ECM) deposition in suspension culture format (Crisera et al., 2000; Jiang et al., 1999; Miner and Yurchenco, 2004). We hypothesize that providing a basement membrane, as mimicked by Matrigel, to the on-chip-derived PDLOs could facilitate the formation of an epithelium with a strictly apical-inside organization. Indeed, upon transfer of on chip derived PDLOs into a 3D Matrigel culture or after transplantation an apical-out to apical-in polarity switch was observed (**Fig. 3d).**"

and

"It can be concluded that on-chip-derived PDLOs are likely to secrete soluble ECM and corresponding binding proteins as seen under *in vivo* conditions. Within the open micro-well culture format, however, the proteins are most likely resolved in the media which would explain the polarity switch of the on chip-derived PDLOs upon either *in vivo* transplantation or 3D Matrigel culture (**Fig. 3d**)."

13. Supplementary Figure 3c: Many of the images are not of publication quality and are not convincing, notably for the markers on rows 2 and 3 except for Ki67 and CFTR (both clear). Immunofluorescence would likely be more convincing as the DAPI can show the nucleus as a landmark and moreover several markers can be combined. When signals are in rare cells, more cells need to be documented, when they

are weak, negative controls need to be provided and a mix of high and low magnification panels. This would be important to get a good understanding of what these cells are and their spatial arrangement.

Answer: We agree and added higher quality images and provided marker combinations in the **new Supplementary Fig. 3**. Where suitable, we also included undifferentiated hiPSC aggregates or PP cells as controls (**new Supplementary Fig. 3a-c**). Overview images are added to the **new main Fig. 5**.

14. Lines 154-156: "The major cell populations identified included four different 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 type of non-pancreatic cells, namely endothelial-like cells (cluster IX)." And lines 156-158: "Intriguingly, our starting PP cell population, which was generated in a 2D cell culture, already contained multipotent and unipotent ductal progenitor cells." The authors should use more careful wording as it is unclear what these cells really are. A suggestion follows "Intriguingly, our starting PP cell population, which was generated in a 2D cell culture, contained cells with different transcriptional profiles suggestive of …." And see points below about the arguments relative to what these cells are.

Answer: We changed the sentences as suggested.

15. Lines 159: "PTF1A and NKX6-1 were expressed only in the multipotent progenitors (MPP)". The authors don't prove these cells are multipotent progenitors. This needs to be reworded into something in the following spirit "PTF1A and NKX6-1 were co-expressed in cluster, suggestive of multipotent progenitors (MPP) by analogy to mice (ref)"…. And so on for the next clusters. To 29-31, which are reviews, it would be nice to add original lineage tracing in mouse: for Ptf1a (DOI: 10.1242/dev.090159 and at single cell level doi: 10.1038/s41467-017-00258-4.) For Nkx6.1 the original tracing for Sox9 combined with images of absent Nkx6.1 can be found in doi: 10.1242/dev.056499.)

Answer: We changed the sentence structures accordingly and added the original literature (Kopp et al., 2011; Larsen et al., 2017; Pan et al., 2013).

16. Note that CD133/Prominin is not a marker of unipotent ductal progenitors. It is abundant in pancreas progenitors and endocrine and exocrine cells emerging from them (see doi: 10.1073/pnas.0609490104. for mouse and subsequent work of the Kim lab in human). It is not clear to this reviewer if CK7 is a ductal marker or is also expressed in progenitors. The best argument that these cells are on a ductal trajectory is the reduction of NKX6.1, based on the mouse references highlighted in the comment above.

Answer: We agree with the reviewer and changed our sentence as follows:

"Cluster III, hereafter named unipotent ductal progenitors (UDP), showed high expression of *KRT8* and low expression of *NKX6-1,..."*

As a proof that CK7 is not expressed in PPs but labels mature ductal cells, we show here for the reviewer only unpublished data from a CK7 reporter line.

Redacted unpublished data

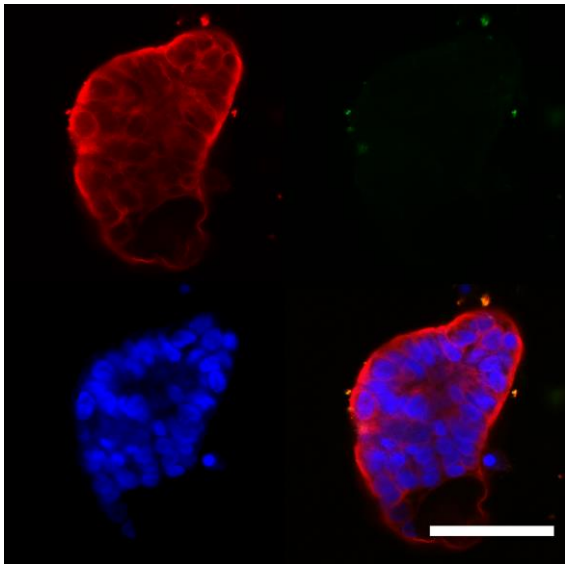
Review Figure: Flow cytometry analysis of a KRT7-Venus reporter cell line with corresponding gating strategy.

17. Figure 3 is very interesting. It is biased to some markers but the Tables are useful and show an unbiased analysis to potentially new markers. There are problems with p-values=0 in several of the columns in Tables 1 and 2

Answer: We revised the table and set the p-values.

18. The ducts like cluster 3 remains somewhat enigmatic, all the more that it seems detectable only in one of the two experiments. Is this a third ductal type or some cells enriched in CLDN4 and MMP1. Are CLDN4-high cells detected by immune?

Answer: The transcriptional state of cells is highly dynamical and identified ductal-like cell types on the scRNA datasets can describe the plasticity or continuum of cell expression states. Ductal-like cluster 3 contained only 134 cells. A Pearson correlation showed a similarity to cells of ductal-like cluster 2. Cluster 3 cells mainly showed elevated *CLDN4* and *MMP1* levels, which both were also found in duct-like cluster 2 (please see **Fig. 4e** and **f**). We stained for CLDN4/MMP1. MMP1 protein was found in PDLOs at variable level (**new Figure 5b** and **Supplementary Fig. 7**), whereas CLDN4 could not be detected (see Figure below).



Review Fig: PDLO differentiated to day 31 and stained for MMP1 (red), CLDN4 (green) and with DAPI (blue). Scale bar denotes for 50 µm.

19. *Line 192: Is CFTR expressed similarly in the Type I PDLOs with apical side in and apical-out? Were all the PDLO II MUC13+?*

Answer: CFTR expression patterns were comparable to other apical markers such as acTUB or ARL13B and expression levels were similar in apical-in and apical-out regions of PDLOs (**new main Fig. 3, new Fig. 5a,b** and **new Supplementary Fig. 7a,b**). Yes, all stained cystic PDLOs were MUC13 positive. Please see the representative overview image in **new Fig. 5b** and the **reviewer Fig.** in our answer to question 12.

20. *Line 206: "In line with the trilineage differentiation potency of MPPs, these cells also produce endocrine cells without a clear evidence of an intermediate bipotent trunk domain" These experiments are not lineage tracing. They suggest a continuity between the endocrine cells and some populations with more progenitor hallmarks. The wording should be more careful. Whether there are bipotent progenitors in the culture should have been discussed earlier in the result section. From what we know in mouse (in the absence of human data) these cells should be NKX6.1+ but PTF1A-. PTF1A seems very low in the data. I am not sure it enables to make a strong statement here. I am sure some PTF1A- cells can be found but is it technical noise or do they cluster in UMAP?*

Answer: We rephrased the passage and discussion:

"Few endocrine cells emerged from the MPP cell cluster."

and

"We did not detect a PTF1A$^-$/NKX6.1$^+$-cell cluster in close proximity to endocrine and ductal cells that would represent an intermediate population giving rise to those two lineages. Possible explanations for

the absence of such a trunk domain as described in the mouse development(Schaffer et al., 2010) could be an early ductal priming of PDLOs or insufficient scRNA-seq sampling time intervals."

We also added the PTF1A expression profile as UMAP plot in **new Supplementary Fig. 6c**. *PTF1A* is primarily expressed in the cell population we assigned to 3D MPPs based on the *PTF1A, PDX1, NKX6-1* expression profile. Please also note that we revised the cluster description to avoid overstatements due to the lack of investigating the actual lineage potential by differentiation into the ductal lineage only.

Snapshots of scRNA-seq transcriptomes, however, allow to visualize the transitions of cell types. We did not detect a separate *PTF1A-/NKX6.1*+ intermediate cluster and did not find an intermediate stage giving rise to endocrine and ductal cells in paga trajectory analysis, which would have been expected for a trunk domain. Admittedly, closer scRNA-seq intervals and simultaneously directed differentiation into endocrine cells might be necessary to detect a putative trunk-population with high resolution. Accordingly, we rephrased the passage to avoid overstatements.

21. Line 200 and following: the authors should be careful not to over-interpret the velocity trajectories. The ductal populations seem to be linked to the other populations via multiple wide bridges made of few cells. The PAGA shows very well the uncertainty of trajectories.

Answer: Thanks a lot for raising your concern. We have carefully revised the text passage to not overstate.

"The corresponding RNA velocity streamlines indicate two differentiation routes from pancreatic progenitors towards duct-like cells: (i) duct-like 1 cells evolved from MPPs, and (ii) duct-like 2 cells mainly evolved from UDPs, which were already present at the PP 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 pancreas(Puri et al., 2015). Few endocrine cells emerged from the MPP cell cluster. Consistent with the velocity analysis, partition-based graph abstraction (PAGA) analysis showed a connectivity (edges) between clusters (dots) along the second ductal differentiation route (**Fig. 6c**)."

In addition, PAGA is a commonly used tool to visualize adequately scRNA-seq data and moreover used for assisting with the interpretation of datasets (Luecken and Theis, 2019). ScVelo as a tool for velocity analysis has been used before in different papers i.e. Bastidas-Ponce et al. (2019) used the velocity to predict the maturation of their endocrine progenitors towards the different islet cells.

22. Line 215 and thereafter: again one needs to be careful about these markers being true ductal markers and whether they are expressed in progenitors (see the comments above, notably for CFTR).

Answer: As indicated in previous answers (to comment 10/11), we have included head-to-head comparisons between PPs and PDLOs in **new Supplement Fig. 3**.

23. Line 239 and following: This is a very interesting analysis. The authors should however make it clear that a batch correction was done (here bbknn). This method will "force" the cells somewhere on the clusters detected in vivo. It by no means shows how close the cells grown in vivo are to the ductal cells but will find the most related. This should be made very clear in the results to the reader. The authors currently make claims that cannot be made with the data.

Answer: We have addressed this concern by preforming additional analysis without batch correction and data integration (**new Supplementary Fig. 10**) and have carefully revised the manuscript. Please see our detailed answer to the main concern query number 4.

24. In the discussion, it would be worth discussing the differences in media with the author's previous system which seemed to produce acinar cells.

Answer: The protocol development process was a study on its own and is currently under revision. We will add the reference upon acceptance of the work.

25. Lines 335-337: "Although the timing of particular marker expressions is slightly different, trunk/tip patterning is confirmed in human development15." It is not confirmed and was never shown. Marker expression suggests the presence of tip and trunk cells similar to those characterized functionally in mice.

Answer: Thanks a lot for rising this valid concern. We have carefully revised the text accordingly.

"Hitherto, it has been suggested that endocrine precursors delaminate from a common trunk domain arising at E12.5 in mice, which further undergoes tubular morphogenesis to subsequently form the ductal network (Nair and Hebrok, 2015). 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 (Jennings et al., 2013; Nair and Hebrok, 2015)."

26. Line 426: It would be useful to indicate the differentiation efficiency used as a cut-off to proceed further to PDLOs.

Answer: The cut off value is given in the method section under "hiPSC differentiation to PDLOs".

"Pancreas progenitors were only used when at least 70% of the cells were PDX1 and NKX6-1 double-positive."

27. Line 593: The data should be made available in a public repository

Answer: We submitted the data to the GEO repository and will make it available upon acceptance of the manuscript. Upon request we can make the data available for the review process.

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Answers to reviewer 3:

We thank the reviewer for appreciating our additional efforts and his/her comprehensive and constructive suggestions for the improvement of the manuscript. We have carefully addressed all issues accordingly.

Question 1: Though transplantation experiments illustrate the ductal commitment only 2 animals were analyzed. It is also unclear how rare cells would be identified in these experiments which do not appear to have a high number of engrafted tissue.

Answer: We have transplanted chip-derived PDLOs as a proof-of-concept add-on to further illustrate ductal commitment. For such analysis, we have only transplanted 2 mice, where we show overview stainings for both xenograft experiments in Fig.3b. Indeed, ductal cells proliferative less than pancreatic progenitors, presumably due to a more mature cell state, and explaining the rather low number of engrafted tissue. We hope that the reviewer appreciates the specificity of the engrafted tissue, albeit the number of engrafted tissue is not very high. We also want to highlight that we have only transplanted around 100.000 cells per mouse and that tube formation was observed over consecutive sections.

Question 2: In figure 3d, the Zo1 immuno does not suggest that apical is outside at day 28 and that there is no polarity in 3D PP aggregates. Most of the staining seems to be inside. Of note, these panels are very small. The small insets would be better used to show a magnified area.

Answer: We predominantly see apical outside staining in chip-derived PDLOs. As outlined in the text, apical outside polarity is not exclusive, but apical markers can be also found inside the organoid to some extent. We have revised the labelling in the subfigure to “mixed/apical outside”. For clarity we have additionally added overview images and higher magnifications of the depicted polarity stainings in Suppl.Fig.5a. We have also exchanged the stainings in Fig.3d.

Question3: In the title, the authors state: “we uncover molecular ductal differentiation mechanisms”. I would argue that the authors identify conditions conducive of molecular ductal differentiation but that they do not really uncover mechanisms (how do ductal cells differentiate? What is the medium component driving them? Which molecules do they rely on to trigger ductal differentiation?)

Answer: We thank the reviewer for pointing out this unclarity. Instead of referring to the mode of action of the instructed cytokines, we referred to differentiation mechanism in respect to time and marker resolution of different ductal subpopulations. We have revised the respective sentences accordingly:
“Here, we report the characterization of pancreatic duct-like organoid (PDLO) differentiation from human induced pluripotent stem cells (hiPSCs) with single-cell resolution.”

Question 4: Incorrect sentence line 143: “Stemness marker SRY-box 2 (SOX2) and octamer-binding transcription factor 4 (OCT4) were absent (Supplementary Fig. 3c) similar to non-ductal pancreas markers except for few endocrine cells located at the periphery of PDLOs (Supplementary Fig. 3d).” Suggestion: “Stemness markers SRY-box 2 (SOX2) and octamer-binding transcription factor 4 (OCT4) were absent (Supplementary Fig. 3c), as were non-ductal pancreas markers, except for few endocrine cells located at the periphery of PDLOs (Supplementary Fig. 3d).”

Answer: Corrected

Question 5: Line 333: Typo- “To reconfirmed” should read “To confirm”

Answer: Corrected

Question 6: Line 362: “Gene ontology (GO) term analysis for protein signatures and transcription factors revealed activation of both cell types as indicated by enrichment of pathways governing stellate cell activation45, energy metabolism, and cellular signaling (Supplementary Fig. 11c). Alongside, a constructed protein network in co-cultured PDLOs or HPaSteCs resembled a mitogenic pattern in further support of this notion (Supplementary Fig. 11d).” This sentence has a structural problem that prevents understanding. It is also not clear to this reviewer what these terms reveal about the association with mesenchyme.

Answer: We adjusted the sentences accordingly and highlighted the proof-of-concept character of our co-culture experiments better to avoid any overstatement.

“*Enrichment for similar gene ontology (GO) terms indicated reciprocal signalling between both cell types. Pathways involved in energy metabolism and cellular signalling were enriched in co-cultures when compared to single cultures (**Supplementary Fig. 11c**). Alongside, an in silico constructed protein network in co-cultured PDLOs and HPaSteCs resembled a mitogenic pattern in support of metabolic activation[4] (**Supplementary Fig. 11d**).”*