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PhD Thesis

Controlling cell polarity in human pancreatic organoids significantly improves the resolution of *ex vivo* physiological modeling.

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1. PUBLICATION RELATED TO THE THESIS

I. Human pancreatic ductal organoids with controlled polarity provide a novel *ex vivo* tool to study epithelial cell physiology - **Árpád Varga,** Tamara Madácsy, Marietta Görög, Aletta Kiss, Petra Susánszki, Viktória Szabó, Boldizsár Jójárt, Krisztina Dudás, Gyula Farkas Jr., Edit Szederkényi, György Lázár, Attila Farkas, Ferhan Ayaydin, Petra Pallagi, József Maléth
Cellular and Molecular Life Sciences (**D1**); MTMT ID: 34024396
IF: 9.261

2. LIST OF PUBLICATIONS NOT RELATED TO THE THESIS

I. Orai1 calcium channel inhibition prevents progression of chronic pancreatitis - Viktória Szabó, Noémi Csákány-Papp, Marietta Görög, Tamara Madacsy, **Árpád Varga**, Aletta Kiss, Balint Tel, Boldizsár Jójárt, Tim Crul, Krisztina Dudás, Mária Bagyánszki, Nikolett Bódi, Ferhan Ayaydin, Shyam Jee, Laszlo Tiszlavicz, Kenneth A. Stauderman, Sudarshan Hebbar, Petra Pallagi, and József Maléth
JCI Insight (**D1**); DOI: https://doi.org/10.1172/jci.insight.167645
IF: 9.484

II. Confinement of Triple-Enzyme-Involved Antioxidant Cascade in Two-Dimensional Nanostructure - Adel Szerlauth*, **Árpád Varga***, Tamara Madácsy, Dániel Sebők, Sahra Bashiri, Mariusz Skwarczynski, Istvan Toth, József Maléth, Istvan Szilagyi
ACS - Materials letters (**D1**); DOI: https://doi.org/10.1021/acsmaterialslett.2c00580
IF: 11.17

III. Thiopurines impair the apical plasma membrane expression of CFTR in pancreatic ductal cells via RAC1 inhibition - Bálint Tél, Noémi Papp, **Árpád Varga**, Viktória Szabó, Marietta Görög, Petra Susánszki, Tim Crul, Aletta Kis, Ingrid H. Sendstad, Mária Bagyánszki, Nikolett Bódi, Péter Hegyi, József Maléth & Petra Pallagi
Cellular and Molecular Life Sciences (**D1**); DOI: https://doi.org/10.1007/s00018-022-04662-y
IF: 9.261

IV. Impaired regulation of PMCA activity by defective CFTR expression promotes epithelial cell damage in alcoholic pancreatitis and hepatitis - Tamara Madácsy, **Árpád Varga**, Noémi Papp, Bálint Tél, Petra Pallagi, Viktória Szabó, Aletta Kiss, Júlia Fanczal, Zoltan Rakonczay Jr., László Tiszlavicz, Zsolt Rázga, Meike Hohwieler, Alexander Kleger, Mike Gray, Péter Hegyi, József Maléth
Cellular and Molecular Life Sciences (**D1**); DOI: https://doi.org/10.1007/s00018-022-04287-1
IF: 9.261

V. Bile acid-and ethanol-mediated activation of Orai1 damages pancreatic ductal secretion in acute pancreatitis - Petra Pallagi, Marietta Görög, Noémi Papp, Tamara Madácsy, **Árpád Varga**, Tim Crul, Viktória Szabó, Melinda Molnár, Krisztina Dudás, Anna Grassalkovich, Edit Szederkényi, György Lázár, Viktória Venglovecz, Péter Hegyi, József Maléth
The Journal of Physiology (**D1**), DOI: https://doi.org/10.1113/JP282203
IF: 5.182

VI. Development of polymer-based multifunctional composite particles of protease and peroxidase activities - Szilárd Sáringer, Tamás Valtner, **Árpád Varga**, József Maléth, István Szilágyi
Journal of Materials Chemistry B (**Q1**); DOI: https://doi.org/10.1039/D1TB01861B
IF: 6.331

VII. Modelling Plasticity and Dysplasia of Pancreatic Ductal Organoids Derived from Human Pluripotent Stem Cells - MarkusBreunig, Jessica Merkle, Martin Wagner, Michael K. Melzer, Thomas F. E. Barth, Thomas Engleitner, Johannes Krumm, Sandra Wiedenmann, Christian M. Cohrs, Lukas Perkhofer, Gaurav Jain, Jana Krüger, Patrick C. Hermann, Maximilian Schmid, Tamara Madácsy, **Árpád Varga**, Joscha Griger, Ninel Azoitei, Martin Müller, Oliver Wessely, Pamela G. Robey, Sandra Heller, Zahra Dantes, Maximilian Reichert, Cagatay Günes, Christian Bolenz, Florian Kuhn, József Maléth, Stephan Speier, tefan Liebau, Bence Sipos, Bernhard Kuster, Thomas Seufferlein, Roland Rad, Matthias Meier, Meike Hohwieler, Alexander Kleger
Cell Stem Cell (**D1**); DOI: https://doi.org/10.1016/j.stem.2021.03.005
IF: 24.633

VIII. TRPM2-mediated extracellular Ca^{2+} entry promotes acinar cell necrosis in biliary acute pancreatitis - Júlia Fanczal, Petra Pallagi, Marietta Görög, Gyula Diszházi, János Almássy, Tamara Madácsy, **Árpád Varga**, Péter Csernay-Biró, Xénia Katona, Emese Tóth, Réka Molnár, Zoltán Rakonczay Jr, Péter Hegyi, József Maléth
The Journal of Physiology (**D1**); DOI: https://doi.org/10.1113/JP279553
IF: 5.182

IX. Intracellular Ca^{2+} Signalling in the Pathogenesis of Acute Pancreatitis: Recent Advances and Translational Perspectives - Petra Pallagi, Tamara Madácsy, **Árpád Varga**, József Maléth
International Journal of Molecular Sciences (**Q1**), DOI: https://doi.org/10.3390/ijms21114005
IF: 5.923

X. Mouse pancreatic ductal organoid culture as a relevant model to study exocrine pancreatic ion secretion – Réka Molnár, Tamara Madácsy, **Árpád Varga**, Margit Németh, Xénia Katona, Marietta Görög, Brigitta Molnár, Júlia Fanczal, Zoltán Rakonczay, Péter Hegyi
Laboratory Investigation (**D1**), DOI: 10.1038/s41374-019-0300-3
IF: 4.197

XI. Ca^{2+} Influx Channel Inhibitor SARAF Protects Mice From Acute Pancreatitis – Aran Son, Malini Ahuja, Daniella M Schwartz, **Árpád Varga**, William Swaim, Namju Kang, József Maléth, Dong Min Shin, Shmuel Muallem
Gastroenterology (**D1**), DOI: 10.1053/j.gastro.2019.08.042
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3. LIST OF ABBREVIATIONS

HCO_3^-	Bicarbonate ion
Cl^-	Chloride ion
ANO1	Anoctamin 1
ENaC	Epithelial Na^+ channel
PDAC	Pancreatic ductal adenocarcinoma
BMP	Bone morphogenetic protein
LGR5	Leucine-rich repeat-containing G-protein coupled receptor 5
OCs	Organoid cultures
ECM	Extracellular matrix
Ca^{2+}	Calcium ion
Na^+	Sodium ion
FBS	Fetal bovine serum
G418	Geneticin
DPBS	Dulbecco's phosphate-buffered saline
DMSO	Dimethyl sulfoxide
PFA	Paraformaldehyde
BCECF-AM	2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester
MQAE	N-(Ethoxycarbonylmethyl)-6-Methoxyquinolinium Bromide
hPOCs	Human pancreatic organois cultures
CFTR	Cystic fibrosis transmembrane conductance regulator
KRT19	Cytokeratin 19
OCLN	Occludin
SOX9	SRY-Box transcription factor 9
EPCAM	epithelial cell adhesion molecule
CDH1	E-cadherin
HES1	Hes family BHLH Transcription Factor 1
AMY1A/B/C	Amylase alpha 1A/B/C
PPY	Pancreatic polypeptide
INS	Insulin
CHGA/B	Chromogranin A/B
C*DH5*	Vascular endothelial cadherin
CaCC	Calcium activated chloride channel
PIEZO1	Piezo-Type Mechanosensitive Ion Channel Component 1
ORAI1	ORAI Ca^{2+} Release-Activated Calcium Modulator 1
SCNN1A	Sodium channel epithelial 1 subunit alpha
SCNN1D	Sodium channel epithelial 1 subunit delta
CBE	Chloride bicarbonate exchanger
HNF1B	Hepatocyte nuclear factor-1 beta
FOXA2	Forkhead Box A2
MYO7B	Myosin 7 B
CF	Cystic fibrosis

4. INTRODUCTION

4.1. Pancreatic ductal ion and fluid secretion

The pancreas is an upper abdominal organ of the digestive system consisting of two main distinct but essential parts, the endocrine and exocrine pancreas. As an endocrine gland, pancreas regulates blood sugar levels by secreting somatostatin, glucagon, pancreatic polypeptide and insulin, resulting in strictly regulated blood glucose concentrations. As the part of the digestive system, the exocrine pancreas consists of duct cells forming a tree-like brunching system, secreting around 2 L of HCO_3^- rich alkaline fluid daily in order to wash out bioactive molecules to the duodenum, included digestive enzymes produced by acinar cells which are the second major pancreatic exocrine cell type. The vectorial ion and fluid secretion of the ductal cells are essentially determined by the expression pattern and localization of ion channels and transporter proteins that display a strictly organized apical-to-basal polarity. However, due to the limitations of the currently available model systems, the details of human pancreatic HCO_3^- secretion, including the role of ENaC or other Cl^- channels such as ANO1, are still controversial and not fully understood.

4.2. *In vitro*/*ex vivo* model systems for investigating pancreatic ion and fluid secretion

The major bottleneck in the study of pancreatic secretory processes is the lack of human-relevant model systems that provide access to the apical membrane of ductal cells. Due to frequent species-specific differences, extrapolation of results from models or clinical translation of basic research findings remained a major unmet need.

One of the first *in vitro* models to explore secretion mechanisms was an adherent human PDAC cell line, the CAPAN-1 which recapitulate the basic properties of ductal cells. The initial results with PDAC cell lines were promising, but there were reasonable concerns about the implications of applying the conclusions drawn from tumor cell lines carrying various mutations (*KRAS*, *TP53* etc.) and are unable to develop a proper cell polarization to primary healthy cells.

They have therefore been almost completely overshadowed by the standardized isolation procedure of murine pancreatic ductal fragments allowing access to primary epithelial cells. The isolation of pancreatic ductal fragments is based on enzymatic digestion of pancreatic tissue by collagenase and manual microdissection of the ductal fragments by stereomicroscope. This technique is still extensively used to study the physiology and pathology of pancreatic ductal epithelium.

It has been reported previously that *ex vivo*, 3-dimensional epithelial organoid cultures can be established by maintaining WNT and BMP signal transduction pathways in LGR5 positive adult stem cells of the gastrointestinal tract. OCs which show high self-renewal capacity in general opened up new horizons for 3-dimensional model systems although their application possibilities are at an early stage. The recently developed patient-derived organoid cultures may provide a physiologically more relevant platform resolving previous difficulties. Due to the vectorial ion and fluid transport into the lumen of the organoids, the increasing intraluminal pressure alone may be sufficient to negatively regulate epithelial secretory or barrier functions via Piezo1 mechanosensitive receptor. One possible strategy to overcome this could be the manipulation of the cell polarity in organoids, which was successfully applied in intestinal and airway organoids by the removal of the ECM to investigate host-pathogen interactions and infectious diseases.

4.3. Basic principles of maintaining pancreatic ductal organoid cultures

Pancreatic organoids can be generated from isolated ductal fragments by embedding them in a so-called extracellular membrane matrix such as Matrigel and by using a special culturing medium. Essential components of organoid culturing media are a series of growth factors that include R-spondin and antagonists of BMP signaling such as Noggin. Therefore, OC feeding media is mainly produced now by L-WRN cell cultures which were originally derived from L-WNT3A cell line with co-expressing vectors of R-spondin 3 and Noggin. This technique has been also used successfully to establish and maintain patient-derived pancreatic organoids *in vitro*. The results so far are very promising; however, the manual isolation of ductal fragments makes standardization and even future automatization difficult. Moreover spherical-like structure of the organoids, thus the difficulty in accessing the apical side and therefore the apical channels and transporter playing role in secretory processes, still poses a challenge.

5. AIMS

We aimed to set up an optimized enzymatic digestion-based cell isolation protocol for establishing OCs which could be the next step in standardization and future automatization of the procedure.

We also aimed to establish an advanced culturing method for human pancreas derived organoids based on ECM-removal-induced apical-to-basal polarity switching in order to investigate ion and fluid secretion and to test well-known functional assays on polarity switched organoids.

Further goals include investigating the effect of polarity switching on the Ca^{2+} homeostasis of human pancreas organoids.

6. MATERIALS AND METHODS

6.1. Preparation of L-WRN conditioned media

L-WRN cell line was grown in selection medium containing 10% FBS and 0.5-0.5 mg/ml G418 and Hygromycin B in ATCC-formulated DMEM. The conditioned medium supplemented with 10% FBS and 1-1% kanamycin-sulfate and antibiotic-antimycotic solution.

6.2. Generation/maintenance and polarity-change induction of human pancreas OCs.

Human pancreatic tissue samples were collected from cadaver donors (Ethical approval No.: 37/2017-SZTE). Tissue samples were minced and enzymatic digestion at 37°C was performed in digestion media. The digested cell suspension was collected and washed 3 times in total and resuspended in Matrigel. 10 µl Matrigel was placed in each well of a 24-well cell culture plate. 500 µl feeding media was applied in each well. For culture splitting/subculturing, Matrigel removal and cell separation were performed simultaneously by using a 25V/V% TrypLE™ Express Enzyme in DPBS. Polarity change of fully grown organoids was induced by extracellular matrix removal with a 7-minute-long treatment of previously described digestion media

6.3. Cryopreservation of primary epithelial cells

Cryo medium is based on feeding media and supplemented with 40% FBS and 5% DMSO.

6.4. siRNA transfection of apical-out hPOCs

Apical-out hPOCs were transfected with 50 nM siRNA or siGLOGreen transfection indicator and Lipofectamine 2000 in feeding media for 48h.

6.5. Gene expression analysis by qRT-PCR method for validating siRNA-based knock-down efficiency

Apical-in and apical-out hPOCs were used for RNA purification by NucleoZOL reagent. In total, 1 µg purified mRNA was used for cDNA synthesis. Relative gene expression analysis was performed by ΔΔCq technique.

6.6. Gene expression analysis of hPOCs by RNA-Seq

RNA was extracted from collected and RNA-sequencing performed by Illumina NextSeq 500 instrument and data analyses process service were provided by DeltaBio2000 Ltd.

6.7. Immunofluorescent labelling and confocal microscopy

Immunofluorescent labelling on organoid cross sections was performed after fixation with 4% PFA-PBS. Antigen retrieval was performed by Sodium Citrate - Tween20 buffer at 94 °C followed by blocking step. Primary antibodies were applied overnight at 4°C while secondary antibodies were incubated for 2 hours at room temperature. Images were captured by a Zeiss LSM880 confocal microscope using a 40X oil immersion objective (Zeiss, NA: 1.4).

6.8. Scanning Electron Microscopy

Organoids were fixed with 2.5% (v/v) glutaraldehyde and 0.05 M cacodylate buffer (pH 7.2) in PBS at 4 ◦C. 8 µL samples spotted onto a silicon disc coated with 0.01% (w/v) poly-l-lysine. Discs were washed twice with PBS and dehydrated with a graded ethanol series (30%, 50%, 70%, 80%, 100% ethanol (v/v), for 2 h each at 4 ◦C and 100% EtOH, for overnight). Following dehydration, samples were immersed for 5 mins in pure hexamethyldisilazane and air dried. All specimens were mounted on aluminium stubs using double-sided carbon tape and then were sputter coated with 15 nm gold in a sputter coater and observed under a Zeiss Sigma 300 Field-Emission scanning electron microscope.

6.9. Fluorescent microscopy and reverse swelling assay

Apical-in and apical-out organoids were attached to poly-L-lysine coated cover glasses and were incubated in HEPES solution. BCECF-AM, Fura2-AM (5-5 µmol/L), MQAE (2 µmol/L) and SBFI-AM (10µmol/L) fluorescent indicators were applied for pH, Ca^{2+}, Cl^{-} and Na^{+} measurements, respectively.

7. RESULTS

7.1. Primary epithelial organoids established from human pancreatic tissue samples by enzymatic digestion retain ductal characteristics and polarity.

Pancreatic tissue samples were collected from 11 cadaver donors with no documented exocrine or endocrine pancreatic disease. After mechanical dissociation and enzymatic tissue digestion human pancreatic organoid cultures (hPOCs) were established in ECM (Matrigel) and grown in organoid feeding media. Conventional organoids with apical-in polarity were successfully generated from all 11 samples, which were subcultured and cryopreserved in large quantities

after the first passaging step for further applications such as creating polarity switched apical-out hPOCs. We demonstrated that manual isolation of human pancreatic ductal fragments is not necessary to generate organoids, and with the current method we describe, the initial organoid number does not depend on the number of ductal fragments, representing a more efficient methodology. RNA-sequencing showed the active expression of the adult stem cell marker *LGR5* and ductal markers such as *CFTR*, *KRT19*, OCLN, *SOX9*, *EPCAM*, *CDH1*, *HES1* was observed, while the absence of acinar- (*AMY1A-C*) endocrine- (*PPY, INS*, *CHGA-B*) and hematopoietic (C*DH5*) markers confirmed that the human pancreatic organoids containing ductal epithelial cells exclusively. Next, we also confirmed the expression and localization pattern of the key ductal markers on the protein level.

7.2. Extracellular matrix removal induces a polarity switch that reduces the epithelial cell tension in pancreatic organoids

To induce a polarity switch in human pancreatic organoids first we generated apical-in organoids in ECM, which then were placed into a suspension culture. We observed that after 48 h the morphology of the organoids was changed and the typical cystic form was replaced by a denser structure formed by a columnar cell layer. Immunofluorescence staining of the apical CFTR, ACTIN and OCLN revealed that the morphological changes were also followed by the redistribution of intracellular proteins. Our results showed that there were no significant differences in the expression of genes involved in F-actin bundling, membrane cytoskeleton crosslinking or intermicrovillar adhesion upon polarity switching. However, myosin 7B expression was significantly higher in polarity-switched organoids. These transcriptomic data suggest that the polarity change may increase the formation of microvilli and the brush border. Scanning electron microscopy revealed the formation of a dense brush border on the outer surface of the polarity-switched human pancreatic organoids.

We found that the diameter of the organoids significantly decreased, whereas the cell density increased after the polarity switch. We also compared the diameter of cells and nuclei in the apical-in and apical-out hPOCs, which revealed that the longitudinal diameter of the cells and nuclei significantly reduced in apical-out organoids leading to the formation of a columnal epithelial cell layer.

In epithelial cells, PIEZO1 function as a mechanosensor mediating extracellular Ca^{2+} influx upon increased intraluminal tension. Our results also showed that hPOCs express *PIEZO1* that can participate in the regulation of epithelial ion and fluid secretion. Finally, we showed that

the polarity switching decreases the gene expression of cyclins, which are major elements of cell division, suggesting a decrease in the cell proliferation.

7.3. The resting intracellular Ca^{2+} concentration is more consistent in apical-out hPOCs

We compared the Ca^{2+} signaling in apical-in and apical-out hPOCs. Evaluation of the resting intracellular Ca^{2+} levels revealed a significantly elevated basal Ca^{2+} level in apical-out hPOCs compared to apical-in organoids. Interestingly, if each organoid was plotted separately with the mean value of all ROIs, it is clearly shown that apical-in organoids have 2.8 times higher basal Ca^{2+} level deviation than their apical-in counterparts. No significant difference was found in either the Ca^{2+} efflux or Ca^{2+} influx rates suggesting that the observed deviation in the basal intracellular Ca^{2+} in the apical-in organoids may be a result of a pre-stimulated state (such as the activation of PIEZO1 by mechanical stress), which varies among different organoids.
To rule out that the changes observed in the intracellular Ca^{2+} homeostasis were caused by the altered expression of proteins involved in the Ca^{2+} signaling, we analyzed and compared the expression of several genes. However no biologically meaningful changes were observed between apical-in and apical-out organoids.

7.4. Functionally active Anoctamin 1 and ENaC are expressed in human pancreatic ductal cells

RNA-sequencing revealed that *Ano1* is the dominantly expressed CaCC channel in apical-in mouse pancreatic organoids which is 45.73 times higher than *Cftr*. Interestingly, transcriptome analyses showed a relatively high expression of *ANO1* and *SCNN1A* in the apical-in human pancreatic organoids. ANO1 and ENaC were detected by immunohistochemistry carried out on human pancreas tissue sections and by immunofluorescent labelling performed on apical-out hPOCs. ANO1 and ENaC are dominantly present in the apical membrane of the ductal epithelial cells.
Next, we used an intracellular Cl^- concentration ($[Cl^-]_i$) sensitive fluorescent indicator, MQAE to follow ANO1 driven Cl^- extrusion. 10 µM T16inhAO1 ANO1 inhibitor significantly reduced the intracellular Cl^- extrusion. In combination with 10 µM CFTR(inh)-172 the maximum response was further reduced. Direct application of siRNA to knock down gene expressions in organoids is hindered by the presence of Matrigel, which limits the cell permeation of siRNAs**.** However, this bottleneck was eliminated by culturing the organoids in suspension, which allowed the knock down of CFTR and ANO1 expression in apical-out organoids.

Gene silencing experiments demonstrated that both siCFTR and siANO1 significantly decreased the apical Cl^- extrusion, which was further impaired by the combination of the two siRNAs.

Among 4 subunits of ENaC, remarkable expression of *SCNN1A* and moderate expression of *SCNN1D* were detected in hPOCs**.** The subunit α (SCNN1A) was detected in the apical membrane of polarity shifted hPOCs by immunofluorescent labelling. The intracellular Na^+ indicator SBFI was used to directly measure the contribution of SCNN1A and SCNN1D to the channel activity, which further confirmed that both subunits are mandatory for the function of ENaC as both siRNA treatment resulted in significantly reduced Na^+ influx.

7.5. Polarity switching improve the performance of organoids in available functional assays

By capitalizing the switched polarity of apical-out organoids, we performed reverse FIS assay to demonstrate the activity of the wild type CFTR. 10 μM forskolin (FSK) decreased the relative volume of the apical-out organoids, which was abolished by the administration of 10 μM CFTR(inh)-172.

The Cl^- extrusion was significantly decreased by 10 μM CFTR(inh)-172 suggesting that the detected change is largely CFTR dependent in apical-in organoids. However, when the same protocol was applied on apical-out organoids, the response to Cl^- removal was higher than in conventional organoids. Due to the enhanced resolution of the apical-out model, even the administration of a 20 μM CFTR(inh)-172 inhibitor could not completely abolish the Cl^- efflux process.

We have investigated the effect of a known CFTR potentiator, VX-770 on the CFTR activity on the pancreatic ductal cells. The administration of 10 μM VX-770 significantly enhanced Cl^- efflux in apical-out organoids and kept the steady-state at an elevated $[Cl^-]_i$ proving proof-of-concept evidence for the applicability of apical-out hPOCs in *in vitro* drug testing assays.

The Cl^- and HCO_3^- exchanger, CBE activity was measured by using BCECF pH-sensitive dye with CFTR inhibition in HCO_3^-/CO_2 buffered solution. Cl^- withdrawal followed by direct apical perfusion of Cl^- containing HCO_3^- solution with simultaneous administration of 10 μM CFTR(inh)-172 decreased the slope of BCECF ratio (F495/F440) compared to the control group, which difference suggests indirect detection of CBE activity in hPOCs.

8. DISCUSSION

We generated human pancreatic organoids and advanced the culture technique further by manipulating the polarity of the epithelial cells. By switching apical-to-basal polarity the elongated cell morphology observed in the apical-in organoids changed to a cuboidal shape in the apical-out cultures, which was accompanied by a more consistent resting intracellular Ca^{2+} level. Capitalizing the improved accessibility of the apical plasma membrane, we identified the expression and function of ANO1 and ENaC in the human ductal epithelial cells. Finally, we demonstrated that functional assays (such as FIS, or CFTR activity measurements) display an improved dynamic range when performed using apical-out organoids.

In the recent years organoid cultures derived from tissue specific Lgr5+ adult stem cells emerged as novel models of organ development and disease. By maintaining the activity of Wnt/β-Catenin signal transduction cascade organoid cultures (OCs) can be grown *in vitro* for long-term in 3D extracellular matrix-based hydrogels; whereas, epithelial cells in the culture maintain the original cellular diversity and organization of the organ of origin. Our group previously provided morphological and functional comparison of primary epithelial cells in mouse isolated ductal fragments and pancreatic organoid cultures. We demonstrated that the apical-to-basal polarity of the epithelial cells, gene and protein expressions and ion transport activities in the mouse pancreatic organoids remarkably overlap with those observed in freshly isolated primary ductal fragments.

The previously published pancreatic organoid establishment protocol was based on manual picking of pancreatic ductal fragments after digesting the pancreatic tissue. This step eventually requires manual work of pancreatic ductal fragment isolation and experience. One significant innovation of the current study is the enzymatic digestion protocol of the whole pancreatic tissue, which improved the yield and success rate of organoid generation. The epithelial cells in the OCs expressed well-known ductal markers like CFTR, KRT19, SOX9, HNF1B or FOXA2, whereas non-ductal markers like amylase or insulin were completely absent. This suggests that our advanced technique is suitable to generate pure pancreatic ductal cell cultures with high efficiency without the presence of other cell types. Another potential model to study pancreatic diseases is the human-induced pluripotent stem cells (hiPSCs) based organoids provide a unique platform for developmental studies and regenerative medicine. Although the differentiation of PSCs towards endocrine pancreatic progeny has been published, the generation of ductal and exocrine-like cells has not yet been adequately achieved. In a recent study Hohwieler et al developed a novel differentiation protocol and successfully generated human pancreatic acinar/ductal organoids from controls and CF patients. The cells in this culture also expressed acinar cell markers, such as amylase suggesting that it was a mixture of

acinar and ductal cells. The advantage of the currently published protocol is that it can provide pure ductal organoid cultures within a relatively short timeframe.

Another well-known limitation of the use of organoids is the ECMs such as Matrigel. ECM can limit the diffusion of the test compounds and pharmacons due to the molecular size and charge specificity of the individual molecules, whereas it hinders the application of siRNAs and plasmids. In addition, in the conventional organoid cultures the apical membrane of the epithelial cells is not directly accessible for drugs. Moreover, due to the vectorial transport of ions and fluid the intraluminal pressure of the apical-in organoids is elevated without any pre-stimulation, which may affect the epithelial secretory processes. To overcome these limitations, we induced the switch of the apical-to-basal polarity of the organoids by replacing the established organoids into an ECM-free suspension culture. Recently, Co et al. developed a technique to reverse enteroid polarity to study host-pathogen interactions. In our study immunostaining of CFTR, actin and occluding and visualization of the brush border on the outer surface of the apical-out organoids by SEM revealed a complete switch of the polarity after 48 h. This switch also led to a change of the epithelial cell morphology from an elongated to a cuboidal shape, suggesting that the elimination of the intraluminal pressure also affects the cell homeostasis. We demonstrated that the resting intracellular Ca^{2+} levels in unstimulated apical-out organoids were more consistent, compared to the apical-in organoids. This finding may have a significant impact on the application of pancreatic organoids in different investigations, as the intracellular Ca^{2+} signaling determines the physiological secretion and pathological functions of the ductal cells. Notably, this property of the apical-out organoids may eliminate the interfere with the detectable effect of pharmacons acting on the intracellular Ca^{2+} signaling making this culture a preferential choice for such experiments. We also demonstrated that ductal epithelial cells express the mechanosensitive receptor Piezo1, which is a Ca^{2+} channel and senses the intraluminal pressure and stretch. PIEZO1 is also responsible for the induction of rapid epithelial cell division when it senses mechanical stretching thus regulating epithelial turnover, which appears to be attenuated by polarity switching. This was also confirmed by the decreased expression of the genes related to cell cycle in the apical-out organoids.

Notably, the polarity switch induced an increase in the expression of *MYO7*. Since *MYO7* is essentially responsible for the interaction between microvilli, its increased expression is presumably related to a decrease in luminal space and pressure, which could also account for the previously observed sparser microvilli density and size in mouse organoids compared to the patterns seen in the pressure draining tube-like primary isolated ducts. Thus, the presence of

intraluminal pressure in cystic organoids may therefore be a factor to be eliminated when comparing physiological and inflammatory conditions.

Next, we utilized the developed culture technique and the accessibility of apical membrane to investigate the ion transport of the polarized human pancreatic ductal cells. The secretion of the large amount of HCO_3^- depends on the interaction of the SLC26A6 Cl^-/HCO_3^- exchanger and the CFTR Cl^- channel. Using our novel tool, we identified two ion channels that are expressed on the plasma membrane of the ductal cells and were never considered in pancreatic ion secretion. Our results revealed that ductal cells express functionally active ANO1, which is a Ca^{2+} activated Cl^- channel. ANO1 expression was only reported on the apical membrane of pancreatic acinar cells, in which HCO_3^- secretion via ANO1 attenuated the pH shift during acute pancreatitis. In addition, we also demonstrated the expression and functional activity of ENaC on the apical membrane of the ductal cells. This was rather surprising, as researchers in the field agree, that ENaC does not participate in the pancreatic ductal functions, whereas it is widely expressed in many types of epithelial cells, mainly contributing to the reabsorption of luminal Na^+ and regulation of the volume and composition of the luminal fluid. The participation of these channels in the physiological secretory processes needs further evaluation. On the other hand, the introduction of two novel ion channels will require a detailed revision of the secretory process, that will remarkably improve the current model and should lead to a better understanding of the human exocrine pancreatic ion secretion as both ENaC and ANO1 are considered potential therapeutic targets in CF.

Finally, we provided examples, how this novel apical-out culture system could be used to improve the currently available functional assays. FIS assay on human rectal organoids is used to predict drug response of CF patients. The advantage of our approach is twofold, first, the elimination of the ECM could make the assay suitable for automatization and large-scale screening, second the improved dynamic range may lead to a better resolution of the results and higher precision of the clinical predictions.

Taken together polarity-switched human pancreatic organoids offer new options for regenerative therapies for diabetes, acute or chronic pancreatitis or for CF of the pancreas.

9. ABSTRACT

Introduction: Epithelial ion and fluid secretion determine the physiological functions of a broad range of organs, such as lung, liver, or pancreas. The molecular mechanism of pancreatic ion secretion is challenging to investigate due to the limited access to functional human ductal epithelia. Patient-derived organoids can overcome these limitations, but direct access to the

apical membrane has not been achieved previously. The previously used organoid generation technique requiring isolated pancreatic ducts, has limited the production of large quantities of organoids, which reduces their potential for high-throughput applications. Due to the vectorial transport of ions and fluid the intraluminal pressure in the organoids is elevated, which may hinder the study of physiological processes.

Aim: Therefore, we aimed to set up an optimized enzymatic digestion-based cell isolation protocol for establishing OCs and to adapt a culturing method based on apical to basal polarity switching induced by ECM removal in order to investigate ion and fluid secretion.

Methods: Pancreatic tissue samples were collected from 11 non-diabetic cadaver donors and used for generation of human pancreas organoid cultures (hPOCs). We induced the polarity switching of the cystic-shape organoids by removing the ECM. Apical-in and apical-out organoids were investigated by RNA-sequencing, immunostaining, fluorescent Cl^-, Ca^{2+} and fluid secretion measurements after siRNA silencing or compound treatment and also by scanning electron microscopy.

Results: We optimized the enzymatic digestion-based cell isolation protocol from tissue specimens. Polarity switching of conventional apical-in organoids by ECM removal was demonstrated with immunofluorescent labelling of CFTR, OCLN, ANO1 and ENaC. The presence the of microvilli formation was detected by SEM on the outer surface of the apical-out organoids. The cells in apical-out organoids had a cuboidal shape, whereas their resting intracellular Ca^{2+} concentration was more consistent compared to the cells in the apical-in organoids. Apical-out organoid method has been shown to be suitable for genetic perturbation techniques such as siRNA gene silencing. We demonstrated the expression and function of two novel ion channels, the Ca^{2+} activated Cl^- channel ANO1 and the epithelial Na^+ channel ENaC. Finally, we showed that the available functional assays, such as forskolin-induced swelling, or intracellular Cl^- measurement have improved dynamic range, when performed with apical-out organoids.

Conclusions: Taken together our data suggest that polarity-switched human pancreatic ductal organoids are suitable models to expand our toolset in basic and translational research.

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