The investigation of HCO_3^- secretion in pancreatic ductal organoid cultures

Ph.D. Thesis

Réka Molnár

Supervisor: **József Maléth, M.D., Ph.D**

Theoretical Medicine Doctoral School

First Department of Medicine

University of Szeged

Szeged, Hungary

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LIST OF FULL PAPERS RELATED TO THE THESIS

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I. **Molnár R**, Madácsy T, Varga Á, Németh M, Katona X, Görög M, Molnár B, Fanczal J, Rakonczay Z Jr, Hegyi P, Pallagi P, Maléth J., Mouse pancreatic ductal organoid culture as a relevant model to study exocrine pancreatic ion secretion.
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1. INTRODUCTION

The exocrine pancreas consists of two main cell types: acinar and ductal cells and has a tree-like structure formed by pancreatic ductal epithelial cells and pancreatic acinar lobes. Acinar cells are responsible for the synthesis and secretion of pancreatic digestive enzymes. The pancreatic ductal system has three segment: the main duct, interlobular and intralobular ducts. The pancreatic ductal epithelia (PDE) secretes bicarbonate-rich fluid upon secretin stimulation, which washes out the digestive enzymes from the ductal system and neutralizes the acidic pH of the gastric juice in the duodenum. The alkaline pH of the pancreatic juice is determined by the secretion of HCO_3^-, which is exceptionally high in humans and reaches 140mM in the distal part parts of the ductal system, whereas in mice and rats the maximal HCO_3^- concentration is only ~50-70mM.

1.1. Physiology of ductal HCO_3^- secretion

HCO_3^- ions are accumulated in the cells by two different mechanisms: through the Na^+-HCO_3^- cotransporter (NBCe1-B), or with passive diffusion of CO_2 entering the cell. CO_2 is converted to carbonic acid by the carbonic anhydrase enzyme, which is then hydrolyzed to HCO_3^- and H^+ in the cytosol. The protons are secreted by the Na^+/H^+ exchanger (NHE) located on the basolateral side, whereas the HCO_3^- ions are secreted into the lumen. The NBCe1-B cotransporter facilitate the entry of HCO_3^- in the cytosol through the basolateral membrane, during this process Na^+ gradient is utilized. NHE supports the basolateral HCO_3^- accumulation in the cytosol by providing driving force to the Na^+ dependent HCO_3^- uptake. The transporters and channels which are producing the nearly 140mM HCO_3^- rich juice on the luminal side are the Cl^-/HCO_3^- exchangers (members of the SLC26 transporter family) and cystic fibrosis transmembrane conductance regulator (CFTR). Previously, CFTR was only considered as a Cl^- channel providing the luminal Cl^- for the Cl^-/HCO_3^- exchange. However, it is now generally accepted as a key contributor to the HCO_3^- secretion as well.

1.2. Pathophysiological role of ductal HCO_3^- secretion

Previously, our group investigated the changes of the ductal HCO_3^- secretion in different pathological AP models, such as biliary and alcohol-induced experimental AP. In biliary AP Venglovecz et al showed that the non-conjugated bile acid, chenodeoxycholate (CDC), dose-dependently affects the HCO_3^- secretion in ductal epithelial cells, in low concentration (100μM)

CDC stimulated, whereas in high concentration (1mM) inhibited the apical HCO_3^- efflux. The mechanism involved sustained intracellular Ca^{2+} overload and mitochondrial damage leading to intracellular ATP depletion. Excessive alcohol consumption is the other most common causes of AP, which is responsible for ~30-40% of all cases. The ethanol and non-oxidative ethanol metabolites can induce similar intracellular changes in ductal epithelial cells than bile acids. Ethanol and the non-oxidative ethanol metabolites, like fatty acid ethyl esthers and fatty acids can induce sustained intracellular Ca^{2+} elevation, which lead to ATP depletion and necrosis and inhibit the fluid and HCO_3^- secretion of the ductal epithelia. Moreover, ethanol reduces mRNA expression of the CFTR in pancreatic ductal cells, that contributes to the decreased HCO_3^- secretion. To understand the development and course of pancreatitis, it is essential to detect changes in the ductal cell and to detect changes in bicarbonate secretion.

1.3. Limitations of in vitro and in vivo models

2D cell cultures have been established from pancreatic ductal adenocarcinoma (such as CFPAC-1, Capan-1, or Capan-2). The human cystic fibrosis pancreatic ductal cell line allows to investigate the HCO_3^- secretion defect of CFTR channel. Although these cell lines are useful for the physiological and pathophysiological studies of ductal epithelial cells, due to their tissue of origin (usually adenocarcinoma) they fail to mimic the morphology of epithelial cells, especially apical-basal polarity, whereas the expression of functional proteins, such as ion channels and transporters are different as well. Standard isolation techniques of pancreatic ducts have been used extensively to study pancreatic ductal physiology and pathology and led to a better understanding of the ductal epithelia in health and disease. This technique made the study of primary PDE cell possible, including physiologically relevant measurements, such as forskolin induced swelling or fluorescent indicator-based intracellular pH measurements. Although animal models are more physiological and overcome this limitation, the differences of species-specific gene regulations are a huge drawback to use them for studying human disease. In addition, the generation of proper animal models is time consuming, it usually takes 6-12 months and have therefore extremely limited throughput. Therefore, to overcome current limitations of pancreatic research, we need novel models to study physiology, pathophysiology and possible drug treatments in translational pancreatic research. Organoids, a novel model in the gastrointestinal research fulfill these requirements and this model is an important bridge between *in vitro* and *in vivo* animal or human model.

1.4. Organoid cultures

Organoid cell cultures (OCs) can be established from embryonic stem cells and from induced pluripotent stem cells (iPSC), or utilizes tissue specific Leucine-rich repeat-containing G-protein coupled receptor 5 positive (Lgr5+) adult stem cells. Organoid cultures (OCs) derived from tissue specific Lgr5+ adult stem cells emerged recently as novel models of organ development and disease. By maintaining the activity of Wnt/β-Catenin signal transduction cascade 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. Clear advantages of OCs over conventional 2D cell cultures are that in OCs more relevant cell-to-cell contact is maintained, whereas in 2D cultures the cells are attached to the plastic surface and cell-to-cell contacts are limited to the edges. In pancreatic research currently OCs are studied as relevant human models of tissue development and carcinogenesis. The above described potential limitations of isolated ductal fragments might be overcome by the application of pancreatic OCs for both physiological and pathological studies. However, the physiological relevance of pancreatic OCs is currently not known.

2. AIM OF THE STUDY

Our aims in this project were

- to establish ductal epithelial organoid cell culture from mouse pancreatic ductal cells

- to compare the morphology and function of ductal epithelial cells in pancreatic OCs and in isolated pancreatic ductal fragments that is a standard model of exocrine pancreatic physiology

- to characterize the effect of bile acid on pancreatic ductal organoids and examine the Ca^{2+} release in TRPM2 konck-out (KO) ductal fragments compared to wild type (WT)

3. MATERIALS AND METHODS

3.1. Animals

10-12-week-old FVB/N mice were used to compare the morphology and function of epithelial cells in primary pancreatic ductal fragments and organoids. TRPM2 knockout mice were

generously provided by Yasuo Mori (Kyoto University; Kyoto, Japan). Experiments on live animals were carried out with adherence to the NIH guidelines and the EU directive 2010/63/EU for the protection of animals used for scientific purposes. The study was authorized by the National Scientific Ethical Committee on Animal Experimentation under licence number XXI./2522/2018 for the FVB/N mice and XXI./2523/2018 for the TRPM2 knockout mice.

3.2. Isolation of pancreatic ductal fragments and acinar cells

Pancreatic ductal cells. Mice were sacrificed by terminal anesthesia and the pancreatic tissue was digested with 100 U/ml purified collagenase, 0.1 mg/ml trypsin inhibitor and 1 mg/ml bovine serum albumin in DMEM at 37°C for 30 min. Small intra-/interlobular ducts were then isolated by microdissection under stereomicroscope and were used for measurements on the same day.

Pancreatic acinar cells. Mice were sacrificed using terminal anesthesia and pancreatic tissue was cut into small pieces in ice-cold HBSS solution. For acinar isolation, pancreatic sections were digested by 10 ml of ice-cold HBSS containing10 mM Hepes with 200 U/ml collagenase for 25-30 minutes at 37 ° C.

3.3. Pancreatic ductal organoid culture

To establish pancreatic organoid cultures (OCs), 8-12 weeks old FVB/N mice (20-25g bw) were used. The ductal fragments were isolated as described above and were cultured in Matrigel covered with 2 mL complex organoid Feeding Media in a standard 35 mm petri dish. The organoids were passaged every 7 days by gentle pipetting.

3.4. Production of conditioned media and cell culture

Conditioned media was produced by the L-Wnt-3A expressing cell line was purchased from ATCC (CRL-2647), Cultrex HA-R-Spondin1-Fc 293T cell line was from Trevigen and Noggin producing cell line was a kind gift from Hans Clevers (Hubrecht Institute, Utrecht, The Netherlands). All cell lines were grown in a selection media according to the manufacturer's instructions.

3.5. Gene expression analysis

The mRNA expression of OCs and primary isolated ductal fragments was carried out by conventional combining reverse-transcription (RT-PCR) and conventional polymerase chain reactions (PCR). Total mRNA was purified from 3 independent biological replicates of mouse whole brain tissue, mouse ductal fragments, or OCs.

3.6. Fluorescent measurements in ductal fragments and organoids

Pancreatic ductal fragments or organoids were attached to a poly-l-lysine coated coverglass and were incubated in standard HEPES solution with BCECF-AM (1.5 µmol/L), Fura2-AM (5 µmol/L), or MQAE (2 µmol/L) for 30 min. Cover glasses were then transferred to a perfusion chamber mounted on an Olympus IX71 inverted microscope. For confocal pH measurement with SNARF-, or SNARF-1 dextran organoids were attached to a poly-l-lysine coated coverglass and were incubated in standard HEPES solution with SNARF-1 (10 µmol/L) for 30 min at 37°C. SNARF-1 dextran was injected into the lumen of the organoids using a glass injection pipette. Images were captured by a Zeiss LSM880 confocal microscope.

3.7. Immunofluorescent labeling

Isolated pancreatic ductal fragments or organoids were frozen after the first passage in Shandon Cryomatrix and were cut with cryostat (Leica CM 1860 UV) at -20°C. Sections were fixed in 4% PFA-PBS and washed in 1x Tris buffered saline (TBS). Antigen retrieval was performed in Sodium Citrate - Tween20 buffer. Incubation with primary antibodies were performed overnight at 4°C. Sections were incubated with secondary antibody at room temperature. Nuclear staining was performed with 1 µg/ml Hoechst33342. Images were captured with a Zeiss LSM880 confocal microscope.

3.8. Electron microscopy

Sample preparation. Isolated pancreatic ductal fragments or organoids after the first passage were fixed in 3% glutaraldehyde at room temperature and washed in 0.3M cacodylate buffer. For contrasting samples were incubated in 3% potassium ferrocyanide and 2% osmium tetroxide. Sample dehydration was performed with 20%, 50%, 70%, 96% and absolute ethanol. For infiltration Epon 812 resin was used according to the manufacturer's instructions.

Sectioning and imaging. Before sectioning, indium tin oxide covered glasses were put into a Quorum carbon coater (Quorum Q150R ES Plus, Quorum Tech) for negative glow discharge.

The blocks were trimmed and 100 nm ultrathin sections were cut with 0.8 mm/sec cutting speed. Post contrasting was performed with 5% uranyl acetate and Reynolds solution. Sections were carbon-coated and placed into a Zeiss Sigma 300 scanning electron microscope (SEM).

3.9. Data Analysis

All statistical analysis was performed using GraphPad prism. Data distribution was tested by Shapiro-Wilk test of normality. T-test was applied to compare two groups, one-way analysis of variance (one-way ANOVA) was applied to compare multiple groups in parametric, Mann-Whitney U test (rank sum test) was applied to compare two groups, Kruskal-Wallis (one-way analysis of variance on ranks) was applied to compare multiple groups in non-parametric, p values < 0.05 considered significant.

4. RESULTS

4.1. Establishment of mouse ductal epithelial organoid cell culture

We established and optimized 3D epithelial organoid cultures (OCs) from mouse pancreatic ductal fragments in the Matrigel dome. By the end of the first week, the OCs reached maximal size, therefore OC passage was performed by mechanical disruption. The passage process resulted in small clusters and individual cells, which were embedded again in Matrigel. During the first passage conjunctive tissue and fibroblasts surrounding the basolateral side of the isolated ductal fragments was removed and pure epithelial cell OCs was established.

4.2. mRNA expression patterns of ion channels and transporters in OCs and ductal fragments

To confirm that OCs are suitable for studying pancreatic ductal secretion, similarities, and differences in gene expression patterns of ion channels and transporter proteins were investigated between isolated ducts and organoids. Gene expression of isolated ducts and organoids were investigated in 30 and 35 cycles. Intensity values were higher after 35 cycles, therefore we choosed to use these values to assess relative band densities. Expression of Slc26a6, CFTR, NHE1 and NBCe1 was confirmed in both isolated ductal fragments and in pancreatic OCs. Furthermore, the expression of non-gastric H^+/K^+ ATPase; Ca^{2+}-activated K^+

channel (BK channel); Slc26a3 Cl^-/anion exchanger and the basolateral $Na^+/K^+/Cl^-$ symporter (NKCC) was also demonstrated. The expression of K^+ channels in the pancreatic ductal epithelium is controversial and species dependent therefore, we selected four members of the voltage-gated subfamily Kcna1, Kcna2, Kcnd3 and Kcnh1, which were not yet described in the pancreatic ductal epithelium to further confirm the similarity of gene expression in primary ducts and OCs. Results show that all four members of the subfamily are expressed in both samples, further suggesting their potential uniformity. Interestingly, we also detected the expression of the genes encoding the epithelial sodium channel (ENaC), the Ca^{2+}-activated Cl^- channel Anoctamin1 (ANO1, or TMEM16A) and two members of the voltage-gated Cl^- channels (Clcn1 and Clcn3) in both isolated primary ductal fragments and pancreatic OCs.

4.3. The apical HCO_3^- secretion in ductal fragments and organoids

As the primary function of the ductal epithelia is ion (especially HCO_3^-) and fluid secretion, we used standard intracellular pH (pH_i) measurement based on the fluorescent pH indicator BCECF-AM to estimate the ion transporter activities to further confirm the functional similarity of OCs and isolated ducts. First organoids and ductal fragments were perfused with 20mM NH_4Cl in standard HEPES solution. As a result, the pH_i has markedly increased, since ammonia is able to diffuse trough the cell membrane, into the intracellular space, where it shifts the acid-base balance into an alkaline direction by proton depletion and the generation of HCO_3^-. After extracellular and intracellular ammonia concentrations are equalized, cells start to recover intracellular pH by secreting the generated HCO_3^-. This phase is defined as recovery from the alkali load. During this process HCO_3^- is secreted into the extracellular space trough the apical CFTR Cl^- channel and the SLC26A (3 and 6) anion exchanger. Removal of ammonia causes a rapid intracellular acidification due to the sudden increase of protons left behind by ammonia exiting the cells. For the regeneration from the acidosis, basolateral Na^+/H^+ antiporters are activated to remove protons, which increases the pH_i. HCO_3^- secretion was further investigated with the basolateral administration of 20mM NH_4Cl in HCO_3^-/CO_2-buffered solution. Similarly, to the previous experiments, the pH_i immediately increased referring to the rapid diffusion of ammonia through the cell membrane followed by a slower regeneration from the alkali load. After the removal of NH_4Cl a rapid drop in the pH_i was observed followed by the restoration of the resting pH_i. This recovery phase depends on activity of the basolateral NHE1, but due to the presence of the HCO_3^- under these conditions the basolateral Na^+/HCO_3^- cotransporter (NBCe1) is also active that facilitate HCO_3^- uptake. To quantify the recovery from

alkalosis and acidosis, pH has been measured (ΔpH/Δt) over the first 30 s and the base flux [J(B-)] was calculated as described in Maleth et al. 2015. With this approach, significant differences in the transport mechanisms in OC compared to isolated ductal fragments could not be observed. These results suggest that the activity of the apical Cl^-/ HCO_3^- exchanger and the basolateral HCO_3^- uptake is comparable. To assess the activity of CFTR and the contribution of the channel to HCO_3^- secretion in pancreatic ductal cells we treated isolated ducts and organoids with 10μM $CFTR_{(inh)}$-172, which is a specific inhibitor of the CFTR channel. The inhibitor was added during the NH_4Cl-pulse in HCO_3^-/CO_2 buffered solution. Apical Cl^- dependent HCO_3^- secretion was decreased both in OCs and ductal fragments, compared to the controls.

4.4. Indirect measurement of CFTR activity in pancreatic OCs using fluorescent Cl^- indicator

In this series of experiments, a Cl^- sensitive fluorescent indicator was used to measure $[Cl^-]_i$. The fluorescent signal of MQAE is quenched by Cl^- ions, therefore it is increased by the efflux of Cl^- from the intracellular matrix. Removal of extracellular Cl^- from the HCO_3^-/CO_2-buffered solution resulted in a decrease of $[Cl^-]_i$, most likely due to the Cl^- efflux from the cytosol via CFTR. Removal of extracellular Cl^- from the HCO_3^-/CO_2-buffered solution resulted in a decrease of $[Cl^-]_i$, most likely due to the Cl^- efflux from the cytosol via CFTR, which was significantly enhanced by Forskolin administration. In addition, 10μM $CFTR_{(inh)}$-172 completely abolished the Cl^- extrusion, whereas the protein kinase A (PKA) inhibitor H-89 significantly impaired it to the non-stimulated control level further indicating that the measured Cl^- were due to the activity of CFTR. These results are consistent with our current knowledge of CFTR activity and regulation and thus this technique may be a powerful toolkit for researchers studying CFTR activity in 3D cultures. We utilized this technique also to measure the activity of NKCC1 in isolated ducts and pancreatic organoids. The administration of 100μM Bumetanide (an NKCC1 inhibitor) decreased the $[Cl^-]_i$, suggesting an NKCC1-dependent basolateral Cl^- uptake in ductal fragments and pancreatic organoids.

4.5. Comparison of basolateral HCO3- uptake in isolated ducts and pancreatic organoids

To compare the activity of basolateral Na^+-dependent HCO_3^- uptake in primary pancreatic ductal fragments and pancreatic OCs, we applied the above described NH_4Cl administration in Na^+-free HCO_3^-/CO_2-buffered solution. Under these conditions the recovery from acidosis was almost completely abolished confirming that this process strongly depends on the extracellular Na^+ and suggesting the potential role of NHE1 and NBCe1 in the process. To characterize the contribution of each transporter in more details, we applied another protocol and specific inhibitors of NHE1 and NBCe1. During these series of experiments, the standard HEPES was switched to HCO_3^-/CO_2-buffered solution triggering a rapid drop in pH_i due to the influx and intracellular conversion of CO_2 to carbonic acid and its dissociation to HCO_3^- and H^+. In the presence of extracellular Na^+ the pH_i is restored to the resting level by NHE1 and NBCe1. As the average traces of individual experiments and the calculated base flux and $\Delta(pH_i)_{max}$ demonstrate, both primary ducts and pancreatic OCs showed similar responses to the specific inhibition of NHE1 (10μM EIPA) and NBCe1 (10μM S0859). In both cases the inhibition of NHE1 caused a higher decrease in the calculated base flux (79.01% in OC and 70.62% in ducts) compared to the inhibition of NBCe1 (60.82% in OC and 53.32% in ducts). The combined inhibition of NHE1 and NBCe1 did not decrease the basolateral Na^+ dependent HCO_3^- uptake further.

4.6. Measurement of intraluminal pH in pancreatic organoids

HCO_3^- secretion of the pancreatic ductal epithelia determines the intraluminal pH of the pancreas, therefore, to follow changes of intraluminal pH real time we developed a novel fluorescent technique. In the first step we optimized the pH measurement with SNARF-1, which has commercially available dextran-conjugated version as well, suitable for extracellular pH measurements. OCs were loaded with SNARF-1 and challenged with 20mM NH_4Cl in standard HEPES, or HCO_3^-/CO_2 buffered solution, which resulted in similar pH_i changes as observed previously with BCECF. The recovery from alkali load was then then inhibited by $CFTR_{(inh)}$-172. Next, SNARF1-dextrane was injected into organoids using a micropipette, and the changes of intraluminal pH was recorded during the administration of NH_4Cl. Administration of NH_4Cl in HEPES solution caused a moderate increase in the intraluminal pH which could be attributed to the diffusion of NH_3 into the organoid lumen. In contrast, NH_4Cl in HCO_3^-/CO_2-buffered solution triggered a rapid and notable elevation of intraluminal pH due to the efflux of HCO_3^- to the lumen. This elevation was completely abolished by 10μM $CFTR_{(inh)}$-172 administration suggesting the major role of CFTR in this process.

4.7. Morphological and functional polarity of pancreatic OCs

We wanted to compare the morphology of the cells using scanning electron microscope (SEM), the ductal cells show morphological polarity in both samples. In addition, epithelial cells in organoids form a polarized epithelial monolayer, whereas on the isolated ductal fragments several layers of connective tissue containing collagen fibers and fibroblasts can be observed. On the higher magnification SEM images the apical-basal polarity of primary ductal epithelial cells OC cells can be observed. On the apical membrane, we detected brush border in both samples, whereas mitochondria showed similar intracellular distribution around the lumen, forming a belt-like structure in the apical segment of the cells both in OCs and isolated ducts. To investigate functional polarity, immunofluorescent labeling of both the OCs and primary ducts was used. The confocal images show that NHE1 and NBCe1 are expressed on the basolateral membrane, whereas CFTR is expressed solely on the apical membrane of the epithelial cells in both samples. These results confirmed the morphological and functional polarity of OCs.

4.8. Use of pancreatic organoids in pathophysiological studies

As OCs could be utilized in pathophysiological studies as well, we wanted to compare pathophysiological Ca^{2+} signaling in response to chenodeoxycholate (CDC) – a non-conjugated bile acid known to trigger sustained intracellular Ca^{2+} elevation in ductal cells. OCs and isolated ductal fragments responded to CDC with sustained Ca^{2+} elevation. The elevation in OCs was higher, compared to ductal fragments, which might be explained by the lack of surrounding conjunctive tissue in case of OCs, which may allow CDC to reach the epithelial cells in higher concentration. These results suggest that organoids can be used in pathological studies as well. Our pervious experiments in pancreatic acinar cells suggested that the redox-sensitive non-selective cation channel TRPM2 plays an important role in the pathogenesis of bile acid induced cell injury. To study this in ductal epithelial cells, first we compared the bile acid induced Ca^{2+} elevations in wild type and TRPM2 knockout ducts. By contrast, to acinar cells, no significant difference was detected in isolated ductal fragments between the Ca^{2+} response of WT and TRPM2 KO ducts to 250μM CDC, suggesting that, in ductal cells, TRPM2 plays no role in bile-acid-induced cell injury. To provide mechanistic explanation for the different contribution of TRPM2 in bile acid generated Ca^{2+} response in acinar and ductal cells, we measured the

intracellular ROS using H2DCFDA. We showed that 250μM CDC increased the intracellular ROS level in pancreatic acini. Interestingly, the ROS production during bile acid treatment in ductal epithelial cells was significantly lower compared to acinar cells (13.6±2 vs 33.4±4 arbitrary unit). This observation can explain our observation that TRPM2 knockout ductal cells are not protected from bile acid induced sustained Ca^{2+} elevation. Based on these we didn't investigated this in further details in pancreatic OCs.

5. DISCUSSION

OCs have recently emerged as promising *ex vivo* models of tissue development, physiology and pathophysiology. Although organoids are used in an increasing number of studies, we only have limited experimental data about their physiological relevance, especially in case of pancreatic OCs. Therefore, we wanted to provide side-by-side comparison of gene expression, cell morphology and function of pancreatic ductal epithelial cells derived from primary isolated ductal fragments and of pancreatic OCs. Therefore, first we analyzed the expression of genes encoding these ion channels and transporters in isolated ducts and OCs. Our results confirmed the expression of these genes in primary mouse ductal fragments and also in pancreatic OCs. Moreover, the expression of *Atp12a* (encoding nongastric H^+/K^+ ATPase); *Kcnma1* (encoding BK channel), *Slc26a3* and *Nkcc1* overlapped in the two types of samples. We also found that four members of the voltage-gated potassium channel subfamily *Kcna1, Kcna2, Kcnd3* and *Kcnh1* are expressed in pancreatic epithelial cells, which have not been described earlier. In addition, we showed the expression of two voltage-gated Cl^- channels: *Clcn1* and *Clcn3* that were not suggested earlier. Our results showed that *Enac* and *Ano1* are expressed in both isolated primary ductal fragments as well as pancreatic OC. Measurements of apical HCO_3^- secretion and basolateral HCO_3^- uptake were highly comparable in OCs and primary ducts. In addition, the activities of other ion transport processes mediated by NHE1, or NKCC1 were similar in the two systems. Finally, the Ca^{2+} signaling, which is one of the major signal transduction pathways in the pancreatic ductal epithelia, showed similar characteristics in OCs further supporting the functional equality of pancreatic organoids with the primary ductal system. Earlier studies showed that protons are co-released during exocytosis of digestive enzymes thus causing significant intraluminal acidosis, therefore we developed a technique that utilizes dextran conjugated pH sensitive fluorescent dye SNARF1 to monitor pH changes in the lumen of the organoids. Using this technique, we were able to follow the pH elevation induced by NH_4Cl administration, which was completely blocked by CFTR inhibition. Forskolin-

induced swelling (FIS) is currently the state-of-the-art technique to measure CFTR-dependent ion and fluid secretion. FIS is relatively simple and robust method that correlates well with the individual response to cystic fibrosis treatment, it also might have some potential limitations. In wild type pancreatic organoids FIS leads to a relatively rapid rupture of the organoids (data not shown), therefore instead of measuring the relative luminal volume of the organoids, we utilized a $[Cl^-]_i$ sensitive fluorescent indicator to follow Cl^- movements in pancreatic OCs. Our results with this technique are consistent with the literature data since forskolin significantly enhanced, whereas CFTR, or PKA inhibition markedly decreased the increase of fluorescent signal. Therefore, this technique could be potentially capitalized in pancreatic physiology research and in drug screening studies to identify compounds that improve exocrine pancreatic ductal secretion. Finally, we compared the morphology of the isolated ducts and OCs with a special focus on apical-basal polarity. The ultrastructure of the epithelial cells in the two samples showed no major difference and the OCs epithelial cells represented the same features – increased apical density of mitochondria, brush border on the apical membrane – like primary ductal cells. Importantly, the distribution of proteins showed similar pattern in cross sections of organoids and isolated ducts. We confirmed that CFTR is expressed exclusively on the apical, whereas NHE1 and NBCe1 were found on the basolateral membrane, which is concordant to the current literature.

Taken together, our results demonstrated a complete overlap of gene expression and morphology of isolated ductal fragments and pancreatic OCs and more importantly the use of OCs can ensure that results are not derived from heterogeneous tissue fragments but from primary epithelial cell monolayers. As demonstrated above, pancreatic ductal OCs are suitable model to study ductal epithelial morphology and physiology.

Another important area in the pancreatic research field is focusing on the pathophysiology of acute pancreatitis (AP). Previous reports suggest that bile acids can trigger sustained intracellular Ca^{2+} elevation, increase intracellular and intramitochondrial ROS production and damage the mitochondrial network in both pancreatic acinar and ductal cells. In pancreatic acini, bile acids trigger dose-dependent intracellular Ca^{2+} elevation via the activation of IP_3 and ryanodine receptors. However, in pancreatic ductal cells, CDC dose-dependently elevated the intracellular Ca^{2+} level and inhibited HCO_3^- secretion. To establish the pathophysiological utility of pancreatic OCs we compared pathophysiological Ca^{2+} signaling in response to CDC. Our results showed that both OCs and isolated ductal fragments responded to CDC with sustained Ca^{2+} elevation. Notably, the elevation in OCs was higher, compared to ductal

fragments, which might be explained by the lack of surrounding conjunctive tissue in case of OCs, which may allow CDC to reach the epithelial cells in higher concentration. These results suggest that organoids can be used in pathological studies as well. Our experiments in pancreatic acinar cells suggested that the redox-sensitive non-selective cation channel TRPM2 plays an important role in the pathogenesis of bile acid induced cell injury (not included to the thesis). In our experiments on pancreatic acinar cells, CDC increased the $[Ca^{2+}]_I$ both in acinar and ductal cells, but genetic deletion of TRPM2 decreased Ca^{2+} elevation only in acinar cells. The results of this study show that the TRPM2 channel has a ~22% contribution to the bile-acid-generated Ca^{2+} signal in acinar cells. To study this in ductal epithelial cells, first we compared the bile acid induced Ca^{2+} elevations in wild type and TRPM2 knockout ducts. By contrast, to acinar cells, no significant difference was detected in isolated ductal fragments between the Ca^{2+} response of WT and TRPM2 KO ducts to 250μM CDC, suggesting that, in ductal cells, TRPM2 plays no role in bile-acid-induced cell injury. Interestingly, our results highlighted that the generation of intracellular ROS in response to bile acids is remarkably different in pancreatic acinar and ductal cells, which can provide mechanistic explanation for the different involvement of TRPM2 in bile acid generated Ca^{2+} response in these cell types.

Taken together, after thorough analysis, we have demonstrated that epithelial cells in OCs remarkably correspond with the primary ductal epithelia. Our results confirmed that pancreatic OCs could be a relevant, highly reproducible *ex vivo* model system with increased throughput to study pancreatic secretory physiology and pathology and thus could be a potential solution for an unmet need in pancreatic research.

6. NOVEL RESULTS AND OBSERVATIONS

- Epithelial cells in pancreatic organoid cultures form monolayers with apical-basal polarity
- The ultrastructure of epithelial cells in organoid cultures in isolated ductal fragments display remarkable similarity
- Gene expression and distribution of ion channels and proteins overlap in organoid cultures in isolated ductal fragments
- The ion transport activities in mouse OC are similar to those observed in freshly isolated primary tissue

- The changes of the intraluminal pH can be followed in organoid cultures using SNARF-1-dextrane
- Bile acids trigger sustained Ca^{2+} elevation in OCs and in isolated ductal fragments

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