Doctoral School of Multidisciplinary Medicine
University of Szeged

Investigation of esophageal ion transport mechanisms using mouse esophageal 3D organoid cultures

Ph.D. thesis

Marietta Margaréta Korsós

Supervisor:
Viktória Venglovecz Ph.D., D.Sc.

Gastroenterology Epithelial Cell Research Group
Department of Pharmacology and Pharmacotherapy
Albert Szent-Györgyi Medical School
University of Szeged

2023

PUBLICATIONS

List of scientific papers cited in the thesis

1. Marietta Margaréta Korsós, Tamás Bellák, Eszter Becskeházi, Eleonóra Gál, Zoltán Veréb, Péter Hegyi, Viktória Venglovecz. Mouse organoid culture is a suitable model to study esophageal ion transport mechanisms. AMERICAN JOURNAL OF PHYSIOLOGY – CELL PHYSIOLOGY; 321(5):C798-C811. doi: 10.1152/ajpcell.00295.2021. PMID: 34524930. (2021)

IF_{2021}: 5.282 (Q1)

2. Eszter Becskeházi, **Marietta Margaréta Korsós**, Eleonóra Gál, Tiszlavicz L, Hoyk Z, Deli MA, Zoltán Márton Köhler, Anikó Keller-Pintér, Attila Horváth, Kata Csekő, Zsuzsanna Helyes, Péter Hegyi, Viktória Venglovecz. Inhibition of NHE-1 Increases Smoke-Induced Proliferative Activity of Barrett's Esophageal Cell Line. INTERNATIONAL JOURNAL OF MOLECULAR SCIENCES; 22(19):10581. doi: 10.3390/ijms221910581. PMID: 34638919; PMCID: PMC8509038. (2021)

IF_{2021}: 6.208 (Q2)

List of other scientific papers related to the subject of the thesis

3. Eszter Becskeházi*****, **Marietta Margaréta Korsós***, Bálint Erőss, Péter Hegyi, Viktória Venglovecz. OEsophageal Ion Transport Mechanisms and Significance Under Pathological Conditions. FRONTIERS IN PHYSIOLOGY; 11:855. doi: 10.3389/fphys.2020.00855. PMID: 32765303; PMCID: PMC7379034. (2020)

IF_{2020}: 4.566 (Q2)

* contributed equally

Number of full publications: 3 (2 first author)

Cumulative impact factor: 16.056

ABBREVIATIONS

$[Ca^{2+}]_i$	intracellular calcium ion concentration
$[Cl^-]_i$	intracellular chloride ion concentration
2D	2-dimensional
3D	3-dimensional
BCECF-AM	2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester
BE	Barrett's esophagus
CBE	Cl^-/HCO_3^--exchanger
cDNA	complementary deoxyribonucleic acid
CFTR	cystic fibrosis transmembrane conductance regulator
CK14	cytokeratin 14
DRA	protein Down-regulated in adenoma protein (Slc26a3)
EAC	esophageal adenocarcinoma
EDTA	ethylene diamine tetra-acetic acid
EECs	esophageal epithelial cells
EOs	esophageal organoids
FACS	Fluorescence-activated Cell Sorting
GERD	gastroesophageal reflux disease
HEPES	(4-2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hepes	HEPES-buffered solution (generally used in our laboratory, for the detailed compositions, see Table 1)
HOE-642	cariporide, a selective Na^+/H^+ exchange inhibitor
IHC	immunohistochemistry
iPCS	induced pluripotent stem cells
LGR5	leucine-rich repeat-containing G-protein coupled receptor 5
MQAE	N-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide
mRNA	messenger ribonucleic acid
NBC	Na^+/HCO_3^- cotransporter
NHE	Na^+/H^+ exchanger
PAT1	putative anion transporter 1 (Slc26a6)
PCR	polymerase chain reaction
PFA	paraformaldehyde
pH_i	intracellular pH
ROIs	Region of Interests
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction

1. INTRODUCTION

The esophageal epithelium is made up of esophageal epithelial cells (EECs) arranged in layers. While the overall structure of the esophagus is similar in humans and rodents, the cellular composition of the esophageal epithelium varies by layer and species. EECs are essential in preventing damage to the esophagus from stomach acid reflux. The esophageal epithelial resistance, composed of physical barriers and functional components, is crucial in preventing reflux-induced injury. One of the most important components of the protective function of EECs is the ion transport system. They are found on the apical and basolateral membranes of EECs, where they regulate ion movements through the cell membrane and therefore help maintain cell homeostasis and contribute to defense mechanisms. To date, several studies have described some ion transporters on EECs with direct or indirect methods, such as the amiloride-sensitive Na^+/H^+ exchanger (NHE); the Na^+-dependent and Na^+-independent Cl^-/HCO_3^- exchanger (CBE); the Ca^{2+}-activated Cl^- channel (CaCC); and the $Na^+/K^+/2Cl^-$ cotransporter 1 (NKCC1).

The exact role of esophageal ion transport processes is not fully understood, especially in pathological conditions, mainly because of the lack of a suitable experimental model. Currently, there are different experimental models available for investigating ion transport processes in esophageal epithelium that may be suitable, but most of them have several limitations that may affect the experimental results.

Among them, three-dimensional (3D) culture techniques are the most advanced models that can be used to answer fundamental biological questions. Organoids are 3D models that can be derived *in vitro* from induced pluripotent stem cells (iPSC) or adult stem cells, both from human and animal tissues. There are successful examples for most tissues and organs, and a variety of techniques have been created. Organoids offer several advantages over traditional 2D cell culture models and animal models, and despite their limitations, they have become an important tool in basic science research, allowing for the study of complex organ systems in a controlled environment.

While epithelial organoids have been generated from many regions of the gastrointestinal tract, research on esophageal organoids (EOs) is still relatively new and limited. Although there have been some studies using EOs to investigate other aspects of esophageal biology and pathophysiology, no study has specifically used EOs to investigate ion transport processes in the esophageal epithelium.

2. AIMS OF THE STUDY

Publication No.1.:

Our objective was based on the hypothesis that EOs are suitable *in vitro* models for investigating the ion transport processes of the EECs from both a molecular and functional point of view. Our primary goal was to generate EOs from the esophagus of two, different strains of mice (wild type C57BL/6 and CD-1), and then to compare the organoids based on different aspects, with particular regard to ion transport processes. Our specific goals were:

I. Characterization of EOs both morphologically and by proving they are derived from stem cells of primary tissue.

II. Investigation of the mRNA and protein expression of the major acid-base transporters in EOs.

III. Examination of functional interactions between ion transporters.

Publication No.2.:

Primary cell cultures are another *in vitro* model that involves culturing cells directly from the esophageal epithelium. While EECs of guinea pig are excellent for conducting functional measurements *in vitro*, it is important to note that these cells exhibit limited growth potential in a two-dimensional (2D) environment and have a restricted lifespan. In this study, as part of our research, we used guinea pig EECs for functional measurements; therefore, our aim was:

I. To investigate how long guinea pig esophageal epithelial cells are viable *in vitro* after isolation.

3. MATERIALS AND METHODS

Animals

All animal experiments were performed according to national (1998. XXVIII; 40/2013) and European (2010/63/EU) animal welfare guidelines. The experimental protocols were approved by the National Scientific Ethical Committee on Animal Experimentation and by the Ethics Committee for Animal Research of the University of Szeged (approval ID: XIII./1667/2020.).

Primary esophageal epithelial cells (EECs)

8-12-week-old male mice were terminated by intraperitoneal injection of pentobarbital sodium (80-100 mg/kg). After removal and longitudinal opening of the esophagus, the intact tissue was digested with dispase II solution (PluriSTEM™ 1mg/ml) and the mucosa was peeled

from the submucosa. The mucosa was incubated at 37°C in a 1x trypsin–EDTA solution for 2x15 min, then the trypsin was inactivated with the soybean trypsin inhibitor (STI 250 μg/ml). EECs were suspended in 300 μl of complete organoid culture medium and counted using a Neubauer chamber. The isolation method was largely similar to that described above in the case of the guinea pig EECs. Adjusting the digestion time to the thickness of the esophageal tissue, the duration of the trypsin stage was enhanced to 20 minutes.

Evaluation of the viability of esophageal epithelial cells (EECs)

The freshly isolated cells were suspended in 300 μl KSFM medium and plated on poly-l-lizin treated (0.01 %) coverslips (Ø24 mm) and investigated by using Trypan Blue reagent (0.4 % solution, prepared in 0.85% NaCl; Lonza, Switzerland) hourly. After the incubation at 37°C, bright field images were taken under 10× magnification, and captures were analyzed using Fiji ImageJ software, where stained cells were counted and considered not viable.

Mouse esophageal organoid cultures (EOs)

The cell suspension was mixed with Matrigel® at a 40:60 ratio and portioned into the wells. The complete organoid culture medium was prepared according to the protocol of DeWard ((DeWard et al, *Cell Rep*. 2014). Wnt3A-conditioned medium was prepared by collecting the supernatant from L-Wnt3A cells (ATCC CRL-2647™) according to the manufacturer's protocol.

Flow cytometry analysis

The EECs were incubated for 30 min on ice while labeling with LGR5-PE (Origene, TA400001) and CK14-FITC (Novusbio, NBP2-47720F) fluorochrome-conjugated primary antibodies and their matching isotype controls (PE Mouse IgG1, κ Isotype Ctrl Antibody #400111 and FITC Mouse IgG3, κ Isotype Ctrl Antibody #401317). The expression of leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5) and cytokeratin 14 (CK14) proteins was measured by flow cytometry on a FACSCalibur flow cytometer. The data were analyzed using Flowing Software, and the percentage of positive cells was expressed as the mean ± SD.

Histology

Hematoxylin and eosin (HE) staining was performed according to the protocol of the Department of Anatomy, Histology and Embryology, Szeged. Microphotographs were taken using a DP-74 digital camera with a light microscope (BX-41) and CellSens software (V1.18). All images were further processed using the GNU Image Manipulation Program (GIMP 2.10.0) and NIH ImageJ analysis software.

Immunofluorescence staining

The recovered, intact EOs were fixed with 4% PFA, then cryoprotected in a 30% sucrose solution containing 0.01% sodium-azide. After embedding in Cryomatrix™, the 16-μm-thick multiple serial sections were cut using a cryostat (Leica CM 1850, Leica) and collected on gelatin-coated slides. Incubation with primary antibodies was performed at 4 °C overnight, and with isotype-specific secondary antibodies for 1 hour at room temperature. Sections were finally covered using Vectashield® mounting medium containing DAPI (1.5 μg/mL) and analyzed using a BX-41 epifluorescence microscope (Olympus) equipped with a DP-74 digital camera and CellSens software (V1.18, Olympus) or an Olympus Fv-10i-W compact confocal microscope system (Olympus) with Fluoview Fv10i software (V2.1, Olympus).

Gene expression analysis using RT-PCR

Total RNA was isolated from the recovered EOs using a NucleoSpin RNA Kit. Two micrograms of total RNA were reverse-transcribed using a High-Capacity cDNA Reverse Transcription Kit. PCR was performed using DreamTaq DNA polymerase enzyme (5U/ μl). The PCR products were separated by electrophoresis on a 2% agarose gel and visualized using an AlphaImager EC Gel Documentation System. As a positive control, mouse kidney cDNA was used in the cases of Slc9a1, Slc9a2, Slc26a6, Slc4a4, and CFTR, and mouse pancreatic cDNA was used in the cases of Slc26a3 and ANO-1.

Fluorescent measurements

For all microfluorimetry measurements, the recovered EOs were placed on glass coverslips (Ø24 mm) covered with poly-l-lizin (0,09%) for attachment. Measurements were performed using an inverted, fluorescence microscope (Olympus IX71), and the real-time alterations of the pH_i were measured using xCellence imaging software.

Determination of intracellular pH (pH_i)

EOs were loaded with the pH-sensitive fluorescent dye BCECF-AM (2 μM, 60 min, 37°C) in a standard Hepes solution. The fluorescence emission is measured ratiometrically at 535 nm when the sample is excited at 440 nm and 490 nm, respectively. The calibration of the fluorescence emission ratio to pH_i and determination of the initial pH of the EOs were performed using the high-K^+/nigericin technique.

Determination of buffering capacity and base efflux

The intrinsic buffering capacity (β_i) and base flux of the EOs were estimated according to the NH_4Cl-prepulse technique. The total buffering capacity (β_{total}) was calculated as follows:

$\beta_{total} = \beta_i + \beta_{HCO3-} = \beta_i + 2.3 \times [\ HCO_3^-]_i$, The β_i was estimated by the Henderson-Hasselbach equation. Converting the results of the pH_i changes obtained from the measurements into transmembrane base flux $J(B^-)$ the following equation was used: $J(B^-) = \Delta pH/\Delta t \times \beta_{total}$, where $\Delta pH/\Delta t$ was calculated by linear regression analysis. We denoted base influx as $J(B^-)$ and base efflux (secretion) as $-J(B^-)$.

Measurements of $[Cl^-]_i$

$[Cl^-]_i$ was estimated using the fluorescent, Cl^--sensitive dye MQAE (5 μM) and the CFTR activator forskolin (10 μM). Changes in $[Cl^-]_i$ were determined by exciting the EOs at 340 nm with emitted light monitored at 380 nm. Fluorescence signals were normalized to the initial fluorescence intensity (F/F_0) and expressed as relative fluorescence.

Measurement of the activity of the acid-base transporters

To estimate the activity of the Na^+/H^+ exchanger (NHE) and the Na^+/HCO_3^- cotransporter cotransporter (NBC), the NH_4Cl pre-pulse technique was used. In standard Hepes solution, the initial rate of pH_i ($\Delta pH/\Delta t$) recovery from the acid load (over the first 60 s) reflects the activities of NHEs, whereas, in HCO_3^-/CO_2-buffered solutions, this rate represents the activities of both NHEs and NBC. To block the activity of NHEs and NBC, a Na^+-free external solution was applied. A separate investigation of NHE isoforms was carried out with the NHE-specific inhibitor, cariporide (HOE-642). The NBC-dependent pH_i changes were detected in the presence of an NHE inhibitor, amiloride (0.2 mM).

Measurement of the activity of Cl^-/HCO_3^- exchanger (CBE)

Two independent methods were used to estimate CBE activity. Using the NH_4Cl pre-pulse technique, the initial rate of pH_i recovery (30 sec) from alkalosis in HCO_3^-/CO_2-buffered solutions was analyzed. In the Cl^- withdrawal technique, the initial rate of alkalization over the first 60 s reflects the activity of the exchanger.

Statistical analysis

Results are expressed as the mean ± SD. In the case of microfluorimetric measurements, statistical analyses were performed using the analysis of variance followed by the Bonferroni multiple comparison post hoc test. In the guinea pig EEC viability experiments, the cell viability was defined as the ratio of living cells to the total number of cells. A repeated measures ANOVA with Holm correction was used for multiple comparisons. $p \leq 0.05$ was accepted as significant.

4. RESULTS

Generation and characterization of mouse EOs

On the 3rd day after plating mouse EECs, organoid formation was observed, and the size of the organoids increased steadily in the following days, peaking between days 7 and 9. Organoids between 50 and 150 μm in size were used for our experiments. HE staining of the organoids illustrated that cells are arranged in several layers inside the organoids, and the centers of some organoids were empty or contained keratinized materials, formed by cornified epithelial cells.

Three-dimensional EOs generated from stem cells and epithelial cells in vitro

FACS analysis demonstrated that 42.70 ± 7.27% of the isolated C57BL/6 EECs and 46.46 ± 7.81% of the isolated CD-1 EECs were LGR5-positive. Furthermore, FACS analysis indicated that 45.29 ± 9.25% of the isolated C57BL/6 EECs and 55.32 ± 7.80% of the isolated CD-1 EECs were CK14-positive. Interestingly, there was a slight difference in the double-positive (LGR5 and CK14) fraction. The proportion of double-positive cells was higher in CD-1 mouse EECs (35.37 ± 1.24%) than in C57BL/6 EECs (19.34 ± 2.03%). The IHC labeling revealed strong LGR5 expression in both C57BL/6 and CD-1 organoids. The outer cell layer of the organoids was CK14-positive.

Evaluation of mRNA and protein expression of epithelial ion transporters in EOs

We revealed the presence of *Slc9A1* (NHE-1), *Slc9A2* (NHE-2), *Slc26a6* (PAT1), *CFTR*, *Slc4a4* (NBCe1B), and *ANO1* in both the C57BL/6 and CD-1 organoids. The presence of these transporters was also confirmed at the protein level using immunohistochemistry. Neither the mRNA nor the protein levels of Slc26a3 (DRA) anion exchanger were strong or specific in either EO. CFTR and Slc26a6 exhibited diffuse staining throughout cells without special localization to the apical or basal membrane. Interestingly, Slc26a6 staining was more detectable in cells on the periphery, whereas in the case of CFTR, central cells also displayed positive staining.

Resting pH_i of EOs and determination of buffering capacity

The resting pH_i of C57BL/6 organoids was 7.61 ± 0.03, whereas that of CD-1 organoids was 7.58 ± 0.03. The total buffering capacity of EOs was estimated using the NH_4Cl pre-pulse technique, as described in our previous research articles.

Activity of the NHE

The Na^+ removal induced a rapid and sharp decrease in pH_i, suggesting that EOs express functionally active NHE. There was no significant difference in the rate [$-J(B^-)$] and extent (ΔpH_{max}) of the pH_i-decrease between the two mouse strains. The presence of NHE was also confirmed using the NH_4Cl-pre-pulse technique. In the absence of Na^+, recovery from acidosis was negligible, indicating that in the absence of HCO_3^-, NHE is mainly responsible for the alkalization of cells. To identify the most active isoform on EOs, the NHE isoform selective inhibitor HOE-642 was used. The administration of 1 μM HOE-642 decreased pH_i recovery by 87.81 ± 1.17% in C57BL/6 EOs and 82.37 ± 7.32% in CD-1 EOs, whereas the administration of 50 μM HOE-642 resulted in further decreases (97.54 ± 0.52% in C57BL/6 EOs and 92.91 ± 3.76% in CD-1 EOs). These data indicate that although NHE-1 has higher activity, NHE-2 is also active on EOs.

Activity of the NBC

NBC activity was investigated by the NH_4Cl pre-pulse technique. Significant pH_i recovery was observed after initial acidification, suggesting the important role of HCO_3^- efflux into EOs through NBC. After the NH_4Cl pulse, recovery from alkalosis was more rapid than observed in the presence of standard Hepes solution, indicating that in addition to NHE, NBC is also active in the presence of HCO_3^-. To determine NBC activity, NHE function was blocked by the non-selective NHE inhibitor amiloride. The recovery from acidosis was decreased by 61.88 ± 5.3% in C57BL/6 organoids and 62.18 ± 7.3% in CD-1 organoids in the presence of amiloride, indicating that NHE is responsible for much of the recovery from acidosis. There was some remaining recovery from acidosis, for which the NBC is responsible. Interestingly, we found a significant difference in recovery following Na^+-withdrawal between the C57BL/6 and CD-1 EOs, suggesting greater NBC activity in C57BL/6 mice.

Activity of the CBE anion exchanger

Among the Slc26 exchangers, the presence of Slc26a6 (PAT1) was detected at both the mRNA and protein levels in the C57BL/6 and CD-1 EOs. To determine whether this Cl^-/HCO_3^- exchanger is functionally active on the organoids, the Cl^--withdrawal technique was used. Removal of Cl^- from standard HCO_3^-/CO_2-buffered solution induced strong alkalization because of the reverse mode of the exchanger, when it mediates HCO_3^- reabsorption rather than secretion. By contrast, in the absence of HCO_3^-, Cl^- removal caused minimal, reversible alkalization. The presence of functionally active CBE has also been confirmed by the NH_4Cl-prepulse technique.

Activity of the CFTR Cl^- channel

The presence of this ion channel has been detected at both the mRNA and protein levels in EOs; therefore, we also investigated its activity using pH microfluorimetry. The administration of 10 μM forskolin caused a small increase in the initial rate of Cl^- efflux (19.61 ± 4.52% in C57BL/6 organoids and 21.83 ± 9.72% in CD-1 organoids), and Cl^- loss reached a steady state after approximately 10 min. The effect of 5 μM forskolin was negligible. To investigate the presumed functional relationship between CFTR and the acid-base transporters, the activity of Slc26a6 was also examined in the presence of the CFTR inhibitor CFTRinh-172 (10 mM) while using the NH_4Cl prepulse technique. We found that the recovery from alkalosis, which reflects the activity of the Cl^-/HCO_3^- exchanger, was significantly decreased by CFTR inhibition (18.60 ± 3.34% in C57BL/6 organoids and 35.71 ± 11.77% in CD-1 organoids), whereas recovery from acidosis was only inhibited in C57BL/6 organoids.

Evaluation of the viability *in vitro* of primary EECs

After isolation of EECs of guinea pigs, the cell viability was 88.72 ± 3.41%, which we considered the control value. The cell viability significantly decreased until the third hours (62.96 ± 9.75% and 39.01 ± 6.87%). No significant difference was observed between the results measured in the third and fourth hour, then the cell viability decreased significantly. At the fifth hour, there were barely detectable living cells in the sample (7.41 ± 9.43%). We concluded that 4 hours is the maximum period during which the primary EECs can still be used for functional measurements after isolation.

5. DISCUSSION

The ion transport systems in EECs are crucial for maintaining the proper pH balance in the epithelial barrier. Alterations in pH_i have the potential to have a major influence on a wide variety of biological processes. Disruption of the ion transport systems in the esophagus can result in esophageal disorders such as gastroesophageal reflux disease and esophagitis. The exact role of esophageal ion transport processes is not fully understood, especially in pathological conditions, the main reason for which is the lack of a suitable experimental system reflecting the primary disease. The development of organoid cultures was a significant breakthrough in the examination of individual organs and tissues. Organoids are three-dimensional cell cultures that show some of the key features of the represented organ. They can be derived from adult, embryonic, or induced pluripotent stem cells, which can self-organize in three-dimensional culture and have a self-renewing capability. The stem cell population of the

esophageal epithelium is found in the basal layer of the epithelium, both in rodents and in the human esophagus. Under appropriate culturing conditions, organoids grown from stem cells develop a similar structure to the organ of origin, including the presence of several cell layers, so they can provide a suitable model for performing functional assays.

At first, we used two common laboratory mouse strains (C57BL/6 and CD-1) to follow the protocol of DeWard and colleagues for making EOs from mouse tissue and then optimized it. On the 3rd day after isolating and plating the mouse primer EECs, organoid formation was observed, and the size of the organoids increased steadily in the following days, peaking between days 7 and 9. Organoids between 50 and 150 μm in size were used for our upcoming experiments. To demonstrate that mature EOs are derived from epithelial stem cells, we determined the composition of the freshly isolated cell suspension based on cell surface markers. To prove that the organoids arise from epithelial stem cells, we used the stem cell marker Lgr5 and the epithelial marker CK14 on the freshly isolated cells. FACS analysis revealed that over half of the primary epithelial cells were Lgr5- and CK14-positive in both cases, indicating that the resultant organoids were produced from epithelial stem cells. Immunostaining showed similar results in both cases: a strong LGR5 expression was detected in both organoids, indicating that the organoids contain non-quiescent stem cells. Interestingly, CK14 expression shows the strongest signal in the outer layer of organoids, as can be seen in the basal cells of the primary esophageal tissue.

According to our current knowledge, no study has specifically used EOs to investigate ion transport processes. To investigate the ion transport mechanisms of EOs, we initially determined their resting pH and total buffering capacity. We discovered that the initial pH of the organoids was close to 7.6 for CD-1 organoids and marginally above 7.6 for C57BL/6 organoids.

Using functional and molecular biological techniques, we identified the major ion transporters on mouse EOs. Our findings revealed the presence of a functionally active Na^+-dependent H^+ efflux mechanism on EOs, most likely NHE. HOE-642 significantly decreased NHE function, implying that NHE-1 and NHE-2 account for more than 90% of functionally active NHEs in EOs. According to our assumption, other NHE isoforms or a proton pump are likely to be responsible for the residual activity. Immunolocalization of NHE-1 and NHE-2 showed that most NHE-1 expression was in the outermost, basal layer of the EOs, while most NHE-2 staining was in the inner cell layers. The fact that organoids are made up of epithelial cells with varied degrees of differentiation explains why NHE-1 and NHE-2 are found in distinct places.

The function of NBC in EECs is unknown, however, it is thought to be important in the regulation of pH_i and transcellular transfer of HCO_3^-. CO_2-induced acidosis in EOs was nearly fully reversed in our experiment, which can be explained by the input of HCO_3 via NBC. Furthermore, we discovered a substantial difference in acidosis recovery in the presence and absence of HCO_3^-, as well as a pretty considerable recovery in the presence of amiloride. These findings strongly suggest that EOs express functionally active NBC.

Because NBC mediates HCO_3^- uptake, its presence also presupposes the presence of CBEs on EOs, accordingly, we successfully detected a Cl^--dependent HCO_3^- efflux mechanism on EOs. Removal of Cl^- from the external solution in the presence of HCO_3^- induced strong alkalosis via the reverse mode of the CBE. In addition, the presence of HCO_3^- significantly increased the rate of recovery from alkalosis during NH_4Cl prepulse-technique. RT-PCR and immunohistochemistry revealed that the Slc26a6 CBE was significantly expressed in EOs, whereas Slc26a3 expression was moderate and non-specific.

An interesting result of our study is that the CFTR Cl^- channel is expressed on mouse EOs, which was demonstrated by immunohistochemistry, which showed that both peripheral and central cells strongly express CFTR. Costaining of CFTR and Slc26a6 revealed some colocalization, mainly in cells on the periphery of the EOs, indicating that the two transporters interact with each other in the basal layers. The microfluorimetric approach was utilized to study the functional relationship between CFTR and Slc26a6. The specific CFTR activator, forskolin, increased CFTR activity concentration-dependently, but the response to forskolin was relatively low, even at supramaximal concentrations, indicating that CFTR channel activity is lower than typically observed in secretory epithelia. The presence of the CFTR inhibitor, CFTRinh-172, decreased the rate of recovery from alkalosis in both C57BL/6 and CD-1 EOs, demonstrating that the channel interacts with the CBE. Interestingly, we found that CFTR inhibition also significantly reduced recovery from acidosis in C57BL/6 EOs. Because both NBC and NHE are involved in recovery from acidosis in the presence of HCO_3^-, CFTR may interact with one of these transporters, although this type of interaction has never been described before.

In addition, we also revealed the presence of the Ca^{2+}-activated Cl^- channel ANO1 or TMEM16A in EOs. So far, only one study has examined the presence of ANO1 in the esophageal epithelium.

In our research group, we work with several experimental models, including primary EECs isolated from different animal models. A large number of epithelial cells can be isolated from the esophageal epithelium of the guinea pig as well, which is excellent for conducting functional

measurements *in vitro*. To test how long a primary EEC is viable, we performed a cell viability test, and we found that 4 hours is the maximum period during which the primary EECs can still be used for functional measurements after isolation. Despite the fact that guinea pig EECs can be isolated easily and in large numbers, working with them is limited by their maximum viability *in vitro*.

Taken together, we successfully characterized for the first time the presence and activity of the main epithelial ion transporters using mouse EOs. This 3D cell culture model system can be used as a near-physiological experimental platform to study esophageal ion transport mechanisms not only under physiological but also under pathologic conditions. We can conclude that mouse EOs provide a relevant and suitable model system for studying the ion transport mechanisms of esophageal epithelial cells, and they can also be used as preclinical tools to examine the effects of different compounds on the ion transport system of the esophagus.

6. NOVEL FINDINGS

In summary, this is the first study to examine the ion transport system using mouse EOs, in which our major findings are:

I. We successfully generated EOs from two different, widely used laboratory mouse strains, and we described that mouse EOs are generated from tissue-resident epithelial stem cells. According to our results, there is no significant difference between the EOs from the two strains of mice in terms of culture conditions, ion transports, or usability.

II. We identified both the presence and function of the major acid-base transporters on EOs. These are the NHE, NBC, and CBE.

III. We first identified a functionally active CFTR chloride channel on mouse EOs, and we found that, CFTR may interact with CBE in the alkali recovery process and may interact with NBC or NHE in the acid recovery process.

IV. We found that the primary EECs of guinea pigs are viable for up to 4 hours after isolation, so their use *in vitro* is only possible for a limited time.

ÖSSZEFOGLALÓ

A nyelőcső epitélium iontranszport rendszere kulcsfontosságú a megfelelő intracelluláris pH-egyensúly fenntartásában. A nyelőcső epitélium iontranszport folyamatainak pontos szerepe még nem teljesen tisztázott, melynek fő oka feltételezhetően az élettanilag releváns kísérleti rendszer hiánya. Ezért fő célunk volt az organoid szövetkultúra modell létrehozása, amely *in vitro* mutatja a nyelőcső epitéliumának legfontosabb jellemzőit, másrészt alkalmas lehet funkcionális mérésekre. Az organoidok olyan háromdimenziós sejttenyésztő modellrendszerek, amelyek szöveti, embrionális vagy indukált pluripotens őssejtekből származhatnak, képesek önszerveződni háromdimenziós kultúrában, és önmegújító képességgel rendelkeznek. Az organoidok az eredeti szervhez hasonló szerkezetet mutatnak és a sejtek megőrzik életképességüket, így megfelelő modellt nyújthatnak *in vitro* morfológiai és funkcionális elemzések elvégzéséhez.

Ebben a tanulmányban optimalizáltuk a nyelőcső organoidok egérszövetből történő előállításának protokollját, majd átfogó elemzést végeztünk funkcionális és molekuláris biológiai technikák felhasználásával. Az egér nyelőcső epitélsejtekből előállított organoidok 250-300 μm átmérőjű sejttel teli szerkezetet alkotnak. Kimutattuk az Slc26a6 Cl^-/HCO_3^- kicserélő (CBE), Na^+/H^+ kicserélő (NHE), Na^+/HCO_3^- kotranszporter (NBC), cisztás fibrózis transzmembrán konduktancia szabályozó (CFTR) és anoktamin 1 (ANO1) jelenlétét az organoidokban konvencionális PCR és immunfestés alkalmazásával. A mikrofluorimetriás méréseink magas NHE, CBE és NBC aktivitást mutattak ki, míg a CFTR aktivitása viszonylag alacsony volt. Ezenkívül a CFTR gátlása funkcionális kölcsönhatást feltételez a fő sav-bázis transzporterek és a CFTR között. Továbbá megvizsgáltuk a primer tengerimalac nyelőcső epitélsejtek viabilitását, és azt találtuk, hogy 4 óra az a maximális időtartam, amely alatt a primer epitélsejtek izolálás után felhasználhatók funkcionális mérésekre.

Összefoglalva eredményeinket, elmondható, hogy első alkalommal jellemeztük sikeresen a fő epiteliális iontranszporterek jelenlétét és aktivitását egér nyelőcső organoidok használatával. Megállapíttottuk, hogy az egér nyelőcső organoidok fiziológiailag releváns és megfelelő modellrendszerek a nyelőcső epitélsejtek iontranszport mechanizmusainak tanulmányozására, valamint preklinikai eszközként is felhasználhatók különböző vegyületek nyelőcső iontranszport rendszerére gyakorolt hatásának vizsgálatára.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, **Dr. Viktória Venglovecz,** who allowed me to work in her research group. Her professional knowledge supported me throughout the years, and her proactive approach helped me overcome the scientific pitfalls that arose over the years. I am grateful to **Prof. Dr. István Baczkó** and **Prof. Dr. András Varró**, current and former head of the Department of Pharmacology and Pharmacotherapy, for allowing me to work in their department. I am also grateful to the **Multidisciplinary Medicine Doctoral School** for allowing me to do my research.

I owe a debt of gratitude to **Dr. Tamás Bellák** for his dedicated work in organoid immunohistochemistry. I also thank **Eleonóra Gál** and **Dr. Zoltán Veréb**, for their help in the FACS experiments. I would also like to thank **Gréta Elekes** and **Dr**. **Dóra Szarka**, current and former employees of the Ophthalmology Clinic, with whose help I was able to learn the work processes of the animal house. I especially thank **Tünde Pritz Horesnyiné** whose tireless work always established the proper conditions of our tissue culture laboratory. Thank you to **Anikó Kőrös** and **Rea Fritz** for supporting my work with their administrative backgrounds. I am grateful to all former members of our group: **Dr. Réka Ébert-Molnár**; **Dr. Eszter Becskeházi**, **Attila Ébert,** and **Kristóf Német.** I am also grateful to all members of the **Department of Pharmacology and Pharmacotherapy** and the **Laboratory of Pancreas in the First Department of the Medicine** for their support during my work.

In addition, I am very grateful to **Katalin Jósvay** and **Dr. Csaba Vizler,** the colleagues of the Institute of Biochemistry in the Biological Research Centre. Their scientific guidance and attitude contributed to my development in the field of molecular biology and biochemistry at the beginning of my research career. I truly appreciate the advice they gave me and their valuable insights about the academic career pathway. Last but not least, I owe a lot of thanks and gratitude to my partner **Dr. Szabolcs Zahorán,** and my friends: **Mátyás Bukva, Gréta Elekes, Dr. Lea Avarka Szentgyörgyi,** and **Dr. Krisztina Tóth**, without whom it wouldn't have been possible.

FUNDINGS

This study was supported by the National Research, Development and Innovation Office (NKFIH_FK123982 to Viktória Venglovecz and EFOP 3.6.2-16-2017-00006). This research was also funded by EFOP 3.6.3-VEKOP-16-2017-00009 (to Marietta Margaréta Korsós).

Co-author certification

I, myself as a corresponding author of the following publication(s) declare that the authors have no conflict of interest, and Marietta Margaréta Korsós Ph.D. candidate had significant contribution to the jointly published research(es). The results discussed in her thesis were not used and not intended to be used in any other qualification process for obtaining a PhD degree.

The publication(s) relevant to the applicant's thesis:

Publication No.1.:

Marietta Margaréta Korsós, Tamás Bellák, Eszter Becskeházi, Eleonóra Gál, Zoltán Veréb, Péter Hegyi, Viktória Venglovecz. Mouse organoid culture is a suitable model to study esophageal ion transport mechanisms. AMERICAN JOURNAL OF PHYSIOLOGY – CELL PHYSIOLOGY; 321(5):C798-C811. doi: 10.1152/ajpcell.00295.2021. PMID: 34524930. (2021)

Publication No.2.:

Eszter Becskeházi, Marietta Margaréta Korsós, Eleonóra Gál, Tiszlavicz L, Hoyk Z, Deli MA, Zoltán Márton Köhler, Anikó Keller-Pintér, Attila Horváth, Kata Csekő, Zsuzsanna Helyes, Péter Hegyi, Viktória Venglovecz. Inhibition of NHE-1 Increases Smoke-Induced Proliferative Activity of Barrett's Esophageal Cell Line. INTERNATIONAL JOURNAL OF MOLECULAR SCIENCES; 22(19):10581. doi: 10.3390/ijms221910581. PMID: 34638919; PMCID: PMC8509038. (2021)

Szeged, 2023.08.04.

Dr. Viktória Venglovecz
corresponding author, supervisor