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Human Enteroids as a Model of Upper Small Intestinal Ion Transport Physiology and Pathophysiology

Jennifer Foulke-Abel¹, Julie In¹, Jianyi Yin¹, Nicholas C. Zachos¹, Olga Kovbasnjuk¹, Mary K. Estes², Hugo de Jonge³, and Mark Donowitz¹

¹Department of Medicine, Division of Gastroenterology, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA ²Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, TX 77030, USA ³Department of Gastroenterology & Hepatology, Erasmus University Medical Center, Rotterdam, The Netherlands

Abstract

BACKGROUND & AIMS—Human intestinal crypt-derived enteroids are a model of intestinal ion transport that require validation by comparison with cell culture and animal models. We used human small intestinal enteroids to study neutral Na⁺ absorption and stimulated fluid and anion secretion under basal and regulated conditions in undifferentiated and differentiated cultures to demonstrate their functional relevance to ion transport physiology and pathophysiology.

METHODS—Human intestinal tissue specimens were obtained from endoscopic biopsy or surgical resections performed at Johns Hopkins Hospital. Crypts were isolated, enteroids were propagated in culture, induced to undergo differentiation, and transduced with lentiviral vectors. Crypt markers, surface cell enzymes, and membrane ion transporters were characterized using qRT-PCR, immunoblot, or immunofluorescence analyses. We used multiphoton and time-lapse confocal microscopy to monitor intracellular pH and luminal dilatation in enteroids under basal and regulated conditions.

RESULTS—Enteroids differentiated upon withdrawal of WNT3A, yielding decreased crypt markers and increased villus-like characteristics. NHE3 activity was similar in undifferentiated and differentiated enteroids, and was affected by known inhibitors, second messengers, and bacterial enterotoxins. Forskolin-induced swelling (FIS) was completely dependent on CFTR and partially dependent upon NHE3 and NKCC1 inhibition in undifferentiated and differentiated enteroids. Increases in cAMP with forskolin caused enteroid intracellular acidification in HCO₃⁻

Correspondence: Mark Donowitz, Johns Hopkins University School of Medicine, 720 Rutland Ave, Baltimore, MD 21205, USA; mdonowit@jhmi.edu; Phone: 410-955-9675.

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Author names in bold designate shared co-first authorship.

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free buffer. cAMP-induced enteroid pH_i acidification as part of duodenal HCO₃⁻ secretion appears to require CFTR and NBCE1.

CONCLUSIONS—Undifferentiated or crypt-like, and differentiated or villus-like, human enteroids represent distinct points along the crypt–villus axis; they can be used to characterize electrolyte transport processes along the vertical axis of the small intestine. The duodenal enteroid model revealed that NBCE1 might be a target in the intestinal mucosa for treatment of secretory diarrheas.

Keywords

intestinal organoids; NHE3 activity; Na⁺ absorption; stimulated secretion

INTRODUCTION

Human enteroids grown from isolated intestinal crypts^{1, 2} provide a model potentially closer to normal human physiology than other experimental preparations employed to date. The limitations of current cell culture or animal intestinal models have encouraged exploration of primary *ex vivo* enteroid cultures that not only retain the full complement of human epithelial transport function, but also maintain reproducibility among laboratories.^{3, 4} Enteroids originate from Lgr5⁺ intestinal stem cells¹ that can form “mini-intestines.” While human colon cancer-derived cell lines such as T84, Caco-2, HT29, and SK-CO15 have historically been valuable workhorses in the GI transport field, they lack specific intestinal segment representation and have altered transcriptional and metabolic profiles that support indefinite propagation. Retention of segment specificity in the enteroid model is essential for recapitulating diseases that afflict a particular region of the intestine, and this has been demonstrated via RNAseq comparison of enteroids and native tissue sections of the small intestine.⁵ Enteroids have great potential to address questions related to human GI physiology and pathophysiology and to serve as tools for personalized medicine, as clinical samples from single individuals retain genotypic and phenotypic characteristics.⁶

Epithelial transport including Na⁺ absorption and anion/fluid secretion are important to establish in the human enteroid model because dysregulation of these two processes contribute to both acute and chronic diarrheal diseases. NHE3, the brush border Na⁺/H⁺ exchanger, and CFTR, the primary channel for epithelial Cl⁻/HCO₃⁻ secretion, can be targeted pharmacologically or genetically to study their impact on neutral Na⁺ absorption, intracellular pH (pH_i) homeostasis and stimulated anion/fluid secretion. Comparison of ion transport in human enteroids and animal intestine can be made using microbial toxins or second messengers to mimic pathogenic infection and define signaling pathways that may shed light on potential differences in drug response between humans and rodents. The current experimental tools to monitor absorptive and secretory processes in animal intestine or cell lines are transferrable to enteroids despite three-dimensional morphology and indirect access to the apical surface. In this report, we describe the use of multiphoton microscopy, time-lapse confocal microscopy, immunodetection, and lentiviral transduction to evaluate the character of major ion transporters active in intestinal Na⁺ absorption and fluid/anion secretion and interrogate functional physiology in human small intestinal enteroids.

MATERIALS AND METHODS

Human Tissues

Tissues from human subjects were obtained under approval of the Johns Hopkins University School of Medicine Institutional Review Board (protocol NA_00038329). De-identified specimens determined to come from normal intestines were obtained from endoscopic biopsy or surgical resections performed at Johns Hopkins Hospital. Crypt isolation, enteroid propagation, differentiation, immunodetection, lentiviral transduction, and mRNA analysis are described in Supplementary Methods.

Reagents

Wnt3a conditioned medium was generated using the L-Wnt3a cell line (ATCC CRL-2647). R-spondin1 conditioned medium was produced using HEK293T cells stably expressing mouse Rspo1-Fc.⁷ Noggin conditioned medium was prepared from HEK293T cells stably expressing murine Noggin-Fc.⁸ Expansion medium (EM) is made in a base solution of Advanced DMEM/F12, 100 U penicillin/streptomycin, 10 mM HEPES, pH 7.4, 0.2 mM GlutaMAX with 50% v/v Wnt3a conditioned medium, 20% v/v R-spondin1 conditioned medium, 10% v/v murine Noggin conditioned medium, 1x B27 supplement, 1x N2 supplement, 1 mM *N*-acetylcysteine, 50 ng/mL murine EGF, 1 µg/mL [Leu-15]gastrin, 10 mM nicotinamide, 500 nM A83-01, 10 µM SB202190, and 100 µg/mL Primocin. Differentiation medium (DM) is EM lacking Wnt3a, nicotinamide, and SB202190. A comprehensive list of growth factors/small molecules and their respective sources is included in Supplementary Table 1.

Enteroid Imaging and Intracellular pH Measurement

Enteroid intracellular pH measurement was adapted from the procedure for murine tissues.^{9, 10} Enteroid fragments were plated in 50% Matrigel (20 µL) on a 35 mm dish with a bottom coverslip (P35G-1.5-10-C, MatTek), and then overlaid with 1.5 mL Expansion Medium (EM) for 3–5 d, followed by Differentiation Medium (DM) for 5 days. Enteroids were loaded with SNARF-4F-AM (5 µM) in 50 mM NH₄Cl-prepulse buffer (3 mL) for 30 min at 37 °C, 5% CO₂. Probenecid (1 mM) was present in buffers to inhibit SNARF-4F export. 35-mm dishes were mounted in a preheated chamber (37 °C, DH-35, Warner Instruments) and optical sections were collected at 10 µm intervals using a 25X/1.05 NA water immersion objective (XLPLN, Olympus) on a multiphoton laser scanning microscope (Fluoview FV1000-MPE, Olympus) at 780 nm excitation, 580/640 nm emission. For each condition, fluid was aspirated and replaced with 3 mL fresh buffer maintained at 37 °C. Additional images were collected after 15 min incubation in Na⁺-free tetramethylammonium chloride (TMA) buffer and at various intervals in Na⁺ buffer, both containing HOE-694 (50 µM). In some experiments, HCO₃⁻ buffer was also used (Na⁺ buffer but with 25 mM NaHCO₃, 98 mM NaCl). Intracellular pH was calibrated at the end of each experiment by exposure to 20 µM nigericin for 10 min in K⁺ clamp buffers to set the pH at 6.25, 6.70, and 7.50. Image analysis and pH calculations are outlined in Supplementary Methods.

Time-lapse confocal imaging used to capture enteroid cross-sections over time was based on the method previously described¹¹ and detailed in Supplementary Methods.

Statistics

Data are presented as the mean of at least 3 independent experiments $\pm$ SEM. Significance was determined using Student's t-tests or ANOVA analysis where appropriate. Values of $P < 0.05$ were considered significant; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

RESULTS

Enteroid differentiation allows separation of crypt and villus ion transporter profiles

We implemented the primary cell culture model based on three-dimensional propagation of human intestinal crypts^{2, 12} to compile a bank of enteroids derived from histologically normal duodenal, jejunal, and ileal endoscopic biopsies or surgical specimens. The enteroid model allows study of the undifferentiated and differentiated state separately, starting with the same passage of enteroids from the same subject. Induction of a differentiated phenotype in our enteroid cultures was documented by increased microvilli length, microvilli density, and glycocalyx (Figure 1A), markedly diminished proliferative potential indicated by a decrease in EdU incorporation and cells expressing Ki67, both which were undetectable by day 4 of Wnt3a removal (Figure 1B and Supplementary Figure 1A), Muc2-positive secretions in the enteroid lumen (Figure 1C), and surface expression of sucrase-isomaltase (SI) and alkaline phosphatase (Supplementary Figure 1B). mRNA analysis indicated downregulation of Ki67 and crypt base columnar cell markers LGR5, ASCL2, and OLFM4, and upregulation of the mature enterocyte brush border resident SI and goblet cell marker TFF3 in differentiated enteroids (Figure 1D). In addition to standard markers of intestinal epithelial maturation, the major plasma membrane ion transporters NHE3, DRA, CFTR, NKCC1, and NBCe1 were immunoblotted to evaluate quantitative changes in expression upon differentiation. Immunoblots of expressed protein in undifferentiated and 5-day differentiated duodenal enteroids from four individual donors indicated an increase in NHE3 ($26 \pm 5\%$), no significant change in DRA ($4 \pm 6\%$), CFTR ($5 \pm 6\%$), and NBCe1 ($3 \pm 8\%$), and a decrease ($89 \pm 2\%$) in NKCC1 (Figure 2A) following differentiation. All amounts were based on expression of the target protein relative to GAPDH expression, the latter of which is uniformly distributed in undifferentiated and differentiated enteroids (Supplementary Figure 2A). Immunofluorescence was used to confirm transporter localization in undifferentiated and differentiated duodenal enteroids by comparison to villus and crypt localization in human small intestinal tissue (Figure 2B–E and Supplementary Figure 2B). Apical NHE3, DRA, and CFTR were present in both crypt and villus of human duodenal tissue as well as in undifferentiated and differentiated enteroids, while NKCC1 exhibited basolateral localization that was largely limited to the crypt and undifferentiated enteroids. Key ion transporters were expressed and localized in human enteroids as in human intestine, providing the framework to study both basal and pathophysiological ion transport activities.

Human enteroids recapitulate the absorptive functions of native intestinal epithelium: NHE3 activity

Quantitative analysis of ion transport in enteroids thus far has focused on the secretory character of intestinal epithelia, namely via luminal dilatation or changes in membrane potential attributed to CFTR anion secretion.^{11, 13} For luminal dilatation studies, basolateral

exposure to buffers or pharmacological agents has proven to be acceptable to assess the effects on apical membrane-bound proteins in the closed structures.¹¹ While these approaches have been adequate for studying secretion, methods to assess absorptive processes under basal or pathophysiological conditions have not yet been described in enteroids. To measure Na^+ absorption, including that due to NHE3 activity, we monitored pH_i changes occurring in intact small intestinal enteroids immobilized in Matrigel via multiphoton microscopy and the pH-sensitive dye SNARF-4F. Basal NHE3 activity is proportional to the rate of Na^+ -dependent realkalinization in HCO_3^- -free HEPES-buffered medium that includes 50 μM HOE-694 to inhibit all plasma membrane NHEs except NHE3.¹⁴ Addition of Na^+ initiated realkalinization within 3 min and was linear for 10–12 min (Figure 3A and Supplementary Figure 3A). Basal NHE3 activity compared in undifferentiated and 5-day differentiated duodenal enteroids was found to be independent of differentiation status (Figure 3B). NHE3 activity was similar in spheroid (symmetrical, cyst-like units) or budding structures (asymmetric units with one or more protrusions from a main body), and was consistent in differentiated duodenal enteroids from a single subject studied over 11 weekly passages (Supplementary Figure 3B, C). Additionally, NHE3 activity was similar in differentiated duodenal, jejunal, and ileal enteroids each derived from three separate normal subjects (Supplementary Figure 3D).

NHE3 activity in differentiated enteroids was characterized in response to previously defined NHE inhibitors via basolateral exposure to the following: 1) HOE-694 (50 μM) to inhibit NHE1, NHE2, and NHE8, 2) the specific NHE3 inhibitor S3226 (20 μM), 3) the general NHE inhibitor 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA, 10 μM ; inhibits NHE1, NHE2, NHE3, and NHE8), or 4) a combination of S3226 (20 μM) and HOE-694 (50 μM) to block all plasma membrane NHE family members (Figure 3C, D). Each treatment that inhibited NHE3 (EIPA or S3226) resulted in Na^+ -dependent realkalinization rates that were substantially lower (approaching but greater than zero) than that observed under HOE-694 exposure (0.03 ± 0.005 pH/min). Total inhibitor-free experiments were not possible because cells failed to acid-load following NH_4Cl prepulse. NHE3 inhibition (S3226) was not statistically different from simultaneous inhibition of all NHEs, indicating that NHE3 is the major Na^+/H^+ exchanger in human enteroids.

To further demonstrate that Na^+/H^+ exchange observed in proximal small intestinal enteroids can be attributed to NHE3, knock-down in duodenal enteroids was accomplished via NHE3 shRNA lentiviral transduction (Figure 3E). Measurement of Na^+/H^+ exchange confirmed loss (>85%) of NHE3 activity in the presence of 50 μM HOE-694 (Figure 3F), an effect equal to that obtained by inhibition via S3226.

Second messengers and microbial enterotoxins affect Na^+ absorption in human enteroids

To demonstrate the ability of enteroids to respond to second messengers and bacterial enterotoxins acting through second messengers, NHE3 activity in enteroids was evaluated after basolateral exposure to cyclic nucleotides (Figure 4A) or enterotoxins associated with cholera or traveler's diarrhea (Figure 4B). Differentiated duodenal enteroids exposed to 8-Br-cAMP (100 μM) or forskolin (25 μM) decreased the rates of Na^+ -dependent realkalinization, as did incubation with 8-pCPT-cGMP (50 μM), a cell-permeable form of

cGMP that does not affect PKA activity.¹⁵ Differentiated jejunal enteroids treated with cholera toxin (CTX, 100 ng/mL, 5 h), which elicits activation of the cAMP pathway, or with heat-stable enterotoxin A (ST_a, 100 nM, 45 min), which targets brush border guanylate cyclase C to increase cGMP concentration, also inhibited NHE3. Because the same effects on NHE3 activity are observed in cell lines, enteroids should be explored as an *ex vivo* model to study changes in Na⁺ absorption related to pathogen exposure.

Human enteroids recapitulate the secretory functions of native intestinal epithelium: Anion/fluid secretion

The closed enteroid structure enables study of stimulated apical fluid secretion, which results in enteroid dilatation. We adapted the forskolin-induced swelling (FIS) assay described by Dekkers et al¹¹ to study the contribution of NHEs and CFTR to fluid secretion in both undifferentiated and differentiated duodenal enteroids (Figure 5A). Since some enteroid structures are highly irregular and not accurately represented by cross-sectional area measurements, only cysts or simple budding structures were studied. Enteroids from a single subject were preincubated in HCO₃⁻-buffered solution with inhibitors for 1 h prior to forskolin (5 μM) addition. There was no change in enteroid volume in the absence of forskolin. FIS occurred linearly for at least 60 min, and the rate of FIS in each condition tested was greater in differentiated than in undifferentiated enteroids. In agreement with findings reported by Dekkers et al, a combination of CFTR_{inh}-172 and GlyH-101 (50 μM each) completely blocked FIS in both undifferentiated and differentiated enteroids. Treatment with EIPA (10 μM) or S3226 (20 μM) each partially inhibited FIS and the magnitude was approximately equivalent in differentiated and undifferentiated enteroids (Figure 5B), indicating that the relative contribution of NHE3 inhibition to the rate of FIS does not change upon differentiation. However, bumetanide blockage of basolateral Cl⁻ uptake by NKCC1 decreased secretion (50 ± 8%) in undifferentiated enteroids, and a surprisingly even greater decrease (76 ± 5%) was observed in differentiated enteroids despite the marked decrease in NKCC1 protein upon differentiation (Figure 2A). Because NKCC1 inhibition does not completely abolish swelling, there must be additional contributing Cl⁻/HCO₃⁻ uptake sources that remain to be identified using this experimental approach.

Enterocyte pH_i regulation under elevated cAMP involves HCO₃⁻ transport via CFTR and NBCe1

In CFTR-null or wild type murine enteroids treated with CFTR_{inh}-172, epithelial cells lacking functional CFTR were reported to have a more alkaline pH_i than wild type enterocytes under basal conditions.¹³ The difference in pH_i is presumably due to intracellular HCO₃⁻ accumulation and suggests that HCO₃⁻ and CFTR are important elements of intestinal epithelial pH_i homeostasis. We further observed that addition of forskolin initiated acidification of the cytosol in differentiated human duodenal enteroids studied in the absence of HCO₃⁻ (HEPES-buffered) (Figure 6A, D). We hypothesized that forskolin-activated HCO₃⁻ secretion involving CFTR was the major apical component to account for this pH change. Duodenal enteroids preincubated with CFTR_{inh}-172 (15 μM) were not acidified by forskolin exposure, and the acidification process was reversed by administering CFTR_{inh}-172 after forskolin-induced acidification had already occurred.

CFTR activity is modulated by protein kinase A (PKA), and pretreatment with the PKA antagonist H-89 (10 μ M) also prevented intracellular acidification upon cAMP elevation. Taken together, these experiments indicate that the forskolin-induced acidification process is CFTR- and PKA-dependent. Under conditions of elevated cAMP, NHE3 is removed from the brush border, which would be expected to acidify the cells. However, forskolin-induced acidification in HEPES buffer does not primarily involve NHE3 because pre-blocking all NHEs with EIPA (10 μ M, 30 min) prior to adding forskolin elicited a similar decrease in pH_i , and the effect could be reversed by washing out forskolin while maintaining EIPA (Figure 6B).

When extracellular HCO_3^- was present in the system, forskolin did not cause acidification (Figure 6C, D). In the absence of extracellular HCO_3^- , duodenal enterocytes are limited to CO_2 diffusion and the products of carbonic anhydrases, whose role was verified via pretreatment of enteroids with acetazolamide (ATZ, 100 μ M) in HCO_3^- -free HEPES buffer, which prevented forskolin-induced pH_i acidification. Thus, a plausible explanation for the forskolin-induced pH_i acidification in HCO_3^- -free conditions is rapid, uncompensated efflux of HCO_3^- generated within the cell. In HCO_3^- buffer, ATZ treatment does not impact pH_i maintenance. These observations are consistent with a role for both intracellular and extracellular HCO_3^- in pH_i homeostasis under elevated cAMP concentrations with extracellular HCO_3^- being the dominant component.

With the role of CFTR in stimulated HCO_3^- secretion established, we next identified the major basolateral component of HCO_3^- uptake. Potential candidates for basolateral HCO_3^- exchange include the $\text{Na}^+/\text{HCO}_3^-$ electrogenic cotransporter NBCe1 and electroneutral NBCn1. In HCO_3^- buffer, forskolin elicited a decrease in pH_i when enteroids were pretreated with 4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS, 500 μ M), consistent with a role for NBCe1, which is inhibited by this concentration of DIDS, but rules out a role for DIDS-insensitive NBCn1 (Figure 6C, D).¹⁶ DIDS has been reported to affect multiple ion transporters,¹⁷ so the NBC-specific inhibitor S0859¹⁸ (20 μ M) was used to confirm specificity of the DIDS effect. S0859 pretreatment also caused intracellular acidification after forskolin exposure, supporting NBCe1 as necessary for basolateral HCO_3^- import in enterocytes. The putative role of NKCC1 in general CFTR function was assessed via bumetanide treatment, which did not significantly affect HCO_3^- expulsion (Figure 6C, D). Taken together, duodenal enteroids serve as a model for cAMP-stimulated duodenal HCO_3^- secretion and demonstrate that CFTR and NBCe1 are essential transporters for HCO_3^- transport (Figure 6E).

DISCUSSION

Our results provide one of the first functional ion transport studies in the human small intestinal enteroid model. We provide novel information concerning electrolyte transporter expression in enteroids and detailed description of upper small intestinal response to transporter regulation under physiologic and selected pathophysiologic conditions. The strengths and limitations of the enteroid model are discussed in comparison with established models of intestinal ion transport.

Several features make enteroids a valuable *ex vivo* model of normal human intestinal epithelia. Enteroids represent non-transformed epithelium, in that they can be established and propagated virtually indefinitely from normal, non-tumorigenic human specimens. Gut segment specificity⁵ and Na⁺/H⁺ exchange is retained in enteroid cultures across many passages, which allowed us to measure aspects of regulated duodenal ion transport on the same genetic background. Differentiation in enteroids is controlled primarily by Wnt3a availability, much the same as in native tissue.¹⁹ Removing Wnt3a from enteroids for 5 days halted cell proliferation, decreased message of crypt base columnar cell markers LGR5, ASCL2, and OLFM4, increased microvilli length, glycocalyx, and brush border density, and upregulated markers of differentiation including iron transporter SLC40A1,⁵ sucrase-isomaltase, alkaline phosphatase, and goblet cell marker TFF3. Though further characterization is warranted, our observations suggest enteroids should enable separate functional analyses of undifferentiated crypt/transit amplifying cells and more differentiated villus enterocytes.

Past studies have offered contradictory views on absorptive and secretory compartmentalization, in some instances arguing that the crypt is the primary Cl⁻ secretory locus and the villus/surface cell carries out most Na⁺ absorption,^{20–22} while in other situations determining that there is not a spatial distinction.^{23–28} It is tempting to suggest that these functions are implemented at the molecular level by crypt-specific upregulation of CFTR and downregulation of NHE3, and that the situation is reversed in the villus as shown for NHE3 expression in rodent models.²⁹ However, our data using human enteroids did not support this model since we demonstrated that fluid secretion and Na⁺/H⁺ exchange occurred in both the differentiated and undifferentiated enteroids. Our results indicate that differentiated (villus-like) and undifferentiated (crypt-like) compartments participate in both Na⁺ absorption and anion/fluid secretion, similar to observations made in animal models over several decades.^{23–28}

We further characterized NHE3-related Na⁺ absorption and fluid/anion secretion in small intestinal enteroids under both basal and select pathophysiological conditions. Individual enteroids were studied by multiphoton microscopy using the ratiometric pH-sensitive fluorophore SNARF-4F to monitor pH_i changes as a result of Na⁺-dependent realkalinization, an S3226 and EIPA-sensitive process defined as NHE3 activity.^{30, 31} Uniform NHE3 expression in human villi and crypts and in corresponding enteroids predicts that Na⁺ absorption in these compartments should be similar, and this aspect was confirmed. Inhibitors or agonists administered to the basolateral surface of enteroids appear to access the internal apical surface to drive Na⁺/H⁺ exchange, considering that: 1) NHE3 is on the apical surface of enteroids, yet rapid, S3226-sensitive Na⁺-dependent realkalinization occurred; 2) STa acts only via the receptor guanylate cyclase C on the apical surface of enterocytes, yet inhibited NHE3;^{32, 33} 3) EIPA prevented Na⁺-dependent realkalinization when added externally. NHE3 activity was stable across multiple passages of enteroids established from a single donor, and rates were similar in duodenal, jejunal, and ileal enteroids derived from multiple subjects. Endogenous NHE3 activity in Caco-2 cells (~0.03–0.06 pH/min)³⁴ is similar to that in enteroids, while SK-CO15 cells, which contain more NHE3 mRNA than Caco-2, are an order of magnitude faster (~0.2 pH/min).³⁵

However, it may not be meaningful to compare small intestinal enteroid 3-D cultures to Caco-2 or SK-CO15 cell monolayers that have increased apical access as well as being cancer-derived cell lines, so we propose to perform kinetic evaluation of NHE3 activity in 2-D enteroid monolayers. NHE3 shRNA-mediated knockdown in duodenal enteroids prohibited recovery from an acid load, demonstrating a functional effect following genetic manipulation in enteroids. These results, plus NHE3 inhibition by known ligands and second messengers similar to responses in intact murine intestine and Caco-2 cells supports use of human enteroids to characterize NHE3 regulation. Since NHE3 is linked to DRA for neutral NaCl absorption, these two transporters must be studied in parallel before concluding that crypts and villi contribute equally to intestinal neutral NaCl absorption.

Our studies uncovered novel molecular components of stimulated fluid secretion via the previously described FIS assay.¹¹ Similar to results in undifferentiated rectal organoids, we demonstrated that elevated cAMP caused significant intraluminal fluid increase in duodenal enteroids that was prevented by CFTR inhibition. Fluid accumulation in undifferentiated and differentiated enteroids demonstrates that secretion from both crypt-like and villus-like compartments can contribute to diarrhea. Unexpectedly, the net water accumulation in differentiated enteroids was significantly greater than in undifferentiated enteroids. This is likely a reflection of weaker cell-cell junctions in the latter that allow fluid/anions to leak out, since crypts are known to be more permeable than villi.³⁶ FIS assay caveats include fluid leakage through undetected breaks in the epithelium and inaccurate measurement of irregular/complicated structures.

Transporters other than CFTR contributed to apical fluid secretion. Inhibition of NHE3 or all NHE isoforms caused an approximately 30% decrease in cAMP-stimulated fluid/anion secretion in both undifferentiated and differentiated duodenal enteroids. This might be due to simultaneous changes in Na⁺ absorption and anion secretion, or may reflect the interdependence of CFTR and NHE3 activity that has been previously suggested as a systemic adaptation to balance fluid and ion fluxes.³⁷ The mechanism underlying their apparent ability to crosstalk remains unknown. Likewise, NKCC1 and CFTR activities are functionally linked, yet blocking NKCC1 does not account for all FIS, and suggests that residual stimulated fluid secretion may be supported by other Cl⁻ or HCO₃⁻ loaders. The increased NKCC1 dependence in the differentiated enteroids in spite of reduced NKCC1 expression supports that small amounts of trafficked NKCC1 load sufficient Cl⁻ to account for the above mechanism, or it implicates the participation of another basolateral Cl⁻ loader.^{38, 39} Further evaluation is required to parse out a complete model linking Cl⁻, HCO₃⁻, and fluid secretion in enteroids.

The enteroid model was applied to discern the role of HCO₃⁻ and specific transporters in pH_i homeostasis in differentiated duodenal enteroids. CFTR and NBCe1 were necessary for HCO₃⁻ transport under stimulated secretory conditions, while NKCC1 activity was not essential. Previous studies with NBCe1^{-/-} mice demonstrated that NBCe1 is the primarily HCO₃⁻ uptake mechanism under stimulated conditions in proximal colon.⁴⁰ The delineation between NBCs in our study was defined by the reported DIDS-sensitivity of NBCe1⁴¹ and DIDS-insensitivity of NBCn1.⁴² This may appear to be counter to murine duodenum in which NBCn1 was deemed a principle entry point for HCO₃⁻,⁴³ but functional evaluation

relied on acid-load recovery, the nature of which would make electrogenic transport unfavorable. During stimulated secretion, CFTR anion conductance would create an electrical gradient that supports electrogenic NBC activity. Most HCO_3^- enters the cell through transporters rather than being internally produced by carbonic anhydrases, consistent with results observed in CAII-deficient murine duodenocytes.⁴⁴ Stimulated HCO_3^- secretion may be an underappreciated aspect of certain secretory diarrheas. Cholera patients have stool that contains significant HCO_3^- (44 mM),⁴⁵ and serum levels of HCO_3^- often drop during cholera infection, which is at least one factor that contributes to acidemia.⁴⁶ These observations suggest that the cAMP-stimulated HCO_3^- secretory pathway involving basolateral entry via NBCe1 and apical exit that is dependent on CFTR should not be overlooked in a mechanistic understanding of secretory diarrhea caused by *V. cholerae* infection. Moreover, the model is consistent with that described for HCO_3^- secretion in pancreatic ducts.⁴⁷ The role of apical SLC26A family $\text{Cl}^-/\text{HCO}_3^-$ exchangers in this model of HCO_3^- secretion also awaits further definition.

These studies provide the background to use human enteroids to enhance understanding of human transport physiology during normal digestion and abnormal events in acute and chronic diarrheal diseases, as well as providing models to identify drug targets and anti-diarrheal drugs. Recent reports have employed human enteroid cultures to study rotavirus replication⁴⁸ and early events in enterohemorrhagic *E. coli* infection,⁴⁹ establishing the utility of enteroids as a model of different epithelial host-pathogen interactions in which altered ion transport is a common theme. The current study identifies NBCe1 as a putative target to treat cAMP-related secretory diarrheas. The 3-D enteroid model for study of fluid and electrolyte transport has some weaknesses, including: 1) The enclosed apical surface impedes normal access to nutrients as well as complicates study of host-pathogen interactions occurring in the lumen; 2) Enteroids contain epithelial cell types including Lgr5⁺ stem cells, enterocytes, and Paneth, goblet and enteroendocrine cells, but lack other cell types normally present in intestine, including myofibroblasts, enteric nerves, endothelial, inflammatory and immune cells which may contribute to ion transport regulation. Approaches to overcome these limitations are being considered using enteroids grown as monolayers^{49–51} and in enteroid co-cultures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

LGR5	leucine-rich repeat-containing G-protein coupled receptor 5
ASCL2	Achaete-scute complex homolog 2
OLFM4	olfactomedin 4
SI	sucrase-isomaltase
TFF3	trefoil factor 3
NHE3	Na ⁺ /H ⁺ exchanger 3
CFTR	cystic fibrosis transmembrane conductance regulator
NKCC1	Na ⁺ /K ⁺ /2Cl ⁻ cotransporter 1
DRA	down-regulated in adenoma
NBCe1	electrogenic Na ⁺ /HCO ₃ ⁻ cotransporter 1
NBCn1	electroneutral Na ⁺ /HCO ₃ ⁻ cotransporter 1
DIDS	4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid
ATZ	acetazolamide

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Figure 1.

Analysis of differentiation markers in human duodenal enteroids. (A) TEM images of the undifferentiated and differentiated enteroid brush border. Scale bar, 1 μ m. (B) EdU incorporation in whole-mount enteroids during each day of the differentiation time course. ND, undifferentiated; DF1, differentiation day 1, etc. Scale bar, 100 μ m. Note undetectable EdU incorporation at DF4 and DF5. (C) Luminal Muc2-positive secretions detected by immunostaining with Muc2 antibody in whole-mount enteroids. Muc2 is present only in differentiated enteroids. Scale bar, 50 μ m. (D) mRNA fold change analysis comparing markers of crypt base columnar cells (LGR5, ASCL2, OLFM4), proliferative potential (KI67), and lineage commitment (SI, TFF3) in undifferentiated and differentiated enteroids. Results are mean $\pm$ SEM; * P < 0.05, ** P < 0.01, *** P < 0.001; n = 5 different enteroid lines.

Figure 2.

Analysis of major ion transporters in human duodenal enteroids. (A) Representative immunoblots of NHE3, DRA, CFTR, NKCC1, and NBCe1 in undifferentiated (ND) and differentiated (DF) duodenal enteroids. The change in transporter expression after differentiation is normalized to GAPDH. Graph results are mean $\pm$ SEM; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; $n = 4$ different enteroid lines. (B–E) Immunofluorescence of NHE3, DRA, CFTR, and NKCC1 in intact human duodenal tissue and undifferentiated or differentiated duodenal enteroids. Similar results were obtained in 3 different enteroid lines. Scale bar, 20 μ m.

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Figure 3.

Na^+/H^+ exchange in human enteroids. A single donor was used for all experiments in Figure 3. (A) A typical NHE3 activity assay in enteroids as a function of Na^+ -dependent pH_i recovery. (B) NHE3 activity in undifferentiated and differentiated duodenal enteroids. $n = 3$ enteroids, each taken from a separate passage. (C) Representative traces show the effect of NHE family inhibitors in differentiated duodenal enteroids. (D) Average rate of realkalinization with inhibitors in (C). Results are mean $\pm$ SEM; $**P < 0.01$; $n = 3$ enteroids, each taken from a separate passage. (E) Representative immunoblot of NHE3 protein expression in scrambled shRNA control (Scr), wild-type (WT), and knock-down (KD) confirmed $>90\%$ knock-down of expressed NHE3 using this approach, and the knock-down

persisted for >10 passages. n=3 blots, each taken from a separate passage. (F) NHE3 activity in wild type (WT) and NHE3 knock-down (KD) differentiated duodenal enteroids. Results are mean $\pm$ SEM; ** P <0.01; n = 3 enteroids, each taken from a separate passage.

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Figure 4.

Microbial enterotoxins, cAMP, and cGMP inhibit NHE3 in differentiated enteroids. (A) Effect of cGMP elevation by 8-pCPT-cGMP or cAMP elevation via 8-Br-cAMP or forskolin in duodenal enteroids. Results are mean $\pm$ SEM; ** $P < 0.01$; $n = 3$ different enteroid lines. (B) Effect of STa or CTX in jejunal enteroids. Results are mean $\pm$ SEM; ** $P < 0.01$; $n = 3$ different enteroid lines.

Figure 5.

Ion transporters contribute to forskolin-induced apical fluid secretion. (A) Representative FIS time course in undifferentiated or differentiated duodenal enteroids. Results are mean $\pm$ SEM, $n = 10$ enteroids for each condition. (B) Contribution to the FIS rate by inhibition of each transporter calculated relative to the inhibitor-free forskolin-stimulated condition. Results are mean $\pm$ SEM; ** $P < 0.01$; $n = 3$ different enteroid lines.

Figure 6.

Role of HCO₃⁻ and ion transporters in forskolin-induced pH_i changes. (A–C) pH_i changes in differentiated duodenal enteroids after forskolin treatment in HEPES buffer with (A) CFTR_{inh}-172, H-89, ATZ, or (B) EIPA, or in HCO₃⁻ buffer with (C) DIDS, S0859, ATZ, CFTR_{inh}-172, or bumetanide. Representative traces from individual experiments are shown. (D) Average rate of pH_i under conditions in (A) and (C). n = 3 different enteroid lines for each condition. (E) Model of stimulated duodenal HCO₃⁻ transport based on studies in Figure 6. HCO₃⁻ secretion requires NBCe1 to replace HCO₃⁻ lost via CFTR. CA is not essential to maintain intracellular HCO₃⁻, and limiting Cl⁻ uptake by NKCC1 inhibition does not affect the ability of CFTR to conduct HCO₃⁻.